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**Production and characterization of novel lignin-modifying
enzymes from actinomycetes and heterologous expression of
metagenome-source laccases**

**Produzione e caratterizzazione di enzimi ligninolitici in
attinomiceti e espressione eterologa di laccasi da metagenoma**

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1. Introduction

The microbial biodiversity

“Life would not long remain possible in the absence of microbes.”

Louis Pasteur

Microorganisms are the pillars of life on Earth. Over billions of years, they have evolved colonizing every conceivable niche on the planet. The total number of microbial cells in the Earth's biosphere has been estimated to be $4-6 \times 10^{30}$ including 10^6-10^8 individual genomes belonging to different species [1]. Microorganisms include archaea, bacteria, protozoans, and certain algae and fungi. They are essential for the life of other organisms as they are responsible for converting the key elements of life carbon, nitrogen, oxygen, and sulfur into accessible forms. Soil is a major reservoir of organic carbon on Earth and an important habitat for prokaryotes and fungi. One gram of soil may harbor up to 10 billion microorganisms of possibly thousands of different species [2]. Microbes are an essential component of the soil decomposition subsystem, in which plant and animal residues are degraded into organic matter. Also marine environments contain an enormous pool of as yet largely underexploited microbial biodiversity. Numerous estimates of cell density, volume, and carbon indicate that microbes are ubiquitous in marine and fresh water [3]. In addition, microorganisms are able to live in extremophile environments such as hot springs, acid mine waters, ocean trenches with depth of up to 11000 m, hydrothermal vents with temperatures of approximately 400 °C, as well as deep in Antarctic ice and kilometers below the Earth's surface. Microbial communities are closely associated with plants and animals making necessary nutrients, metals, and vitamins available to their hosts. For humans, the billions of gut microbes assist us to digest food, break down toxins, and fight off pathogens. Industry and medicine are increasingly reliant on microorganisms to generate chemicals, antibiotics, and enzymes that improve our world and save lives. Microbes are being recently domesticated with the tools of molecular biology for production of biodegradable plastics and all types of new materials. Biotechnology employs microorganisms in a number of ways, *i.e.* to

produce a vast array of antibiotics and drugs and diagnostic tools for clinical use, to remediate pollutants in soil and water, to produce biofuels, to enhance and protect agricultural crops, and to ferment human foods, and for gene therapy [4].

1.1 Exploring and exploiting the microbial diversity

Classical approach

The classical approach to explore microbial diversity is based on strain isolation and axenic cultivation. Isolation methods are usually applied to samples in a random way, without an *a priori* knowledge of the microbial composition of the source under investigation. In the past, various isolation methods were developed to isolate microorganisms which produce interesting bioactive molecules. Hundreds of microbial products were obtained after several decades of intensive empirical bioactivity-guided screening (more recently named also phenotype screening) mainly run by pharmaceutical companies. These screening campaigns involved millions of classically isolated microorganisms. Traditionally, the cultivation approach attempted to grow these strains and to produce microbial products by manipulating the macro-nutrients and micro-nutrients in the medium, and changing cultivation conditions. Many of the products currently used for human therapy and in agriculture are produced by microbial fermentation [5]. The success of this classical approach is undeniable, although the rate of discovery of novel microbial species it affords is low: just over 7000 valid species have been described to date [6]. In particular, the ability to produce a large number of different enzymes and bioactive molecules was found mostly associated with filamentous actinomycetes, pseudomonads, bacilli within the prokaryotic world, and with filamentous microscopical fungi among the eukaryotic microbes.

Genome mining

About a decade ago, the first released genome sequences of *Streptomyces coelicolor* A3(2) [7] and *Streptomyces avermitilis* [8] revealed that these soil-dwelling bacteria (filamentous actinomycetes) possess a vast array of genes devoted to the production and secretion of enzymes, due to the role they play in recycling organic material in the biosphere. *Streptomyces coelicolor* encodes 819 potentially secreted

proteins including hydrolases, proteases/peptidases, chitinases/chitosanases, cellulases/endoglucanases, amylases and pectate lyases. In addition, each streptomyces genome hosts twenty–thirty biosynthetic gene clusters (20–100 kbp each) devoted to the production of chemically diverse bioactive metabolites, the majority of which could not be linked to compounds discovered in the previous 30–50 years of investigation. This has raised the intriguing possibility that one needs not search far: new enzymes/metabolites could first be bio-informatically identified from the genomic sequences of available strains and, knowing the type of molecule to look for, a combination of targeted detection methods or simply screening different cultivation conditions would eventually lead to the identification of the predicted activity. These observations were subsequently expanded and facilitated by the diminishing cost of DNA sequencing and the development of bioinformatic tools for analyzing sequences and predicting the gene products encoded by each genome [9]. With the development of the genome mining approach, heterologous expression of the selected sequences become increasingly relevant and in some case the lack of appropriate tools for protein expression is limiting novel bioengineering (see below, the same potential bottleneck existing for the metagenomics).

Metagenomics

Starting from the original observation done in 1898 by Heinrich Winterberg [10] about the discrepancy between the number of culturable bacteria on nutrient media and the total bacteria counted by microscopy – the so-called “great plate count anomaly” –, microbial unculturability has long been recognized in microbiology [11, 12]. It is currently estimated that only 0.1 to 1% of the prokaryotes are culturable [11, 13]. Metagenomics refers to a non-culture based approach for collectively studying sets of genomes from a mixed population of microbes. A typical metagenomic study combines the potential of genomics, bioinformatics, and systems biology in exploring the collective microbial genomes isolated directly from environmental samples. In the last two decades, all sorts of natural environments, for example, soils, marine picoplankton, hot springs, surface water from rivers, glacier ice, Antarctic desert soil, and gut of ruminants, were targeted for metagenomic analysis [14].

Figure 1 briefly describes the steps for discovering novel enzymes through a metagenomic approach. More details are reported in the Appendix 1, in preparation as a Mini Review to FEMS Microbiology Letters.

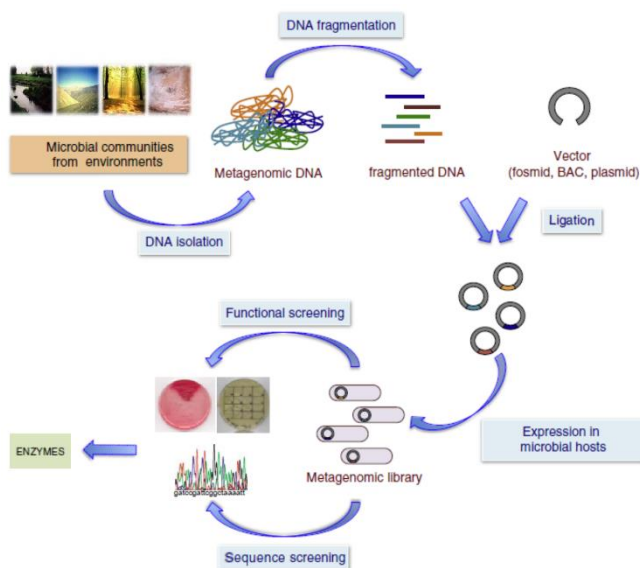


Figure 1. Schematic diagram describing the steps involved in the construction and screening of a metagenomic library.

The first step is the extraction of the so-called environmental DNA (eDNA). Two strategies can be applied: “direct extraction”, where the microbial community DNA is directly isolated from the sample, and “indirect extraction” where the microbial cells are first collected from the sample prior to cell lysis [15]. The extracted DNA should be representative of all cells present into the sample and sufficient amounts of high-quality nucleic acids should be obtained for subsequent library production and screening. Contamination of eDNA with substances that interfere with downstream processing, such as humic and fulvic acids occurring in the case of soil metagenome libraries [16] should be avoided. DNA isolation and purification steps are followed by the construction of DNA libraries in suitable cloning vectors and host strains. The classical metagenomic approach includes the construction of small insert libraries (< 10 kb) in a standard sequencing vector and in *Escherichia coli* as host strain. However, small insert libraries do not allow detection of large gene clusters or operons.

To circumvent this limitation, large insert libraries have been employed, such as cosmid DNA libraries with insert sizes ranging from 25–35 kb, fosmid libraries with inserts of 40 kb and/or bacterial artificial chromosome (BAC) libraries with insert sizes up to almost 200 kb [17]. One of the challenges of environmental cloning is the immense number of transformants that needs to be produced and then screened. It has been estimated that more than 10^7 plasmid clones (5 kb inserts) or 10^6 BAC clones (100 kb inserts) would be required in order to represent the collective genomes, *i.e.* the metagenome, of several thousand different species as typically present in a soil sample, assuming the idealized case of all species being equally abundant [18]. When expressing the metagenomic library in a host organism, two strategies can be applied: (i) single–host expression and (ii) multi–host expression. In recent years, a shift to multi–host gene expression is taking place. This is due to the idea that a substantial part of the transformed genes cannot be expressed in a single organism and that the use of multiple hosts either sequentially or in parallel offers great advantages. Single–host metagenomic expression systems rely on *E. coli*. This organism is readily used regarding gene expression based on many different vectors [19]. Indeed, *E. coli* is predicted to express 40% of environmental genes and this value drops to 7% for high–GC content actinomycete DNA [20]. In order to eliminate the limitation generated by using *E. coli* as a single host, shuttle vectors and non–*E. coli* host systems have been developed. Bacterial strains from genera like *Burkholderia*, *Bacillus*, *Sphingomonas*, *Streptomyces*, and *Pseudomonas* are currently used as alternative hosts [21]. Due to the complexity of metagenomic libraries, high–throughput and sensitive screening approaches need to be employed. Screens are based either on nucleotide sequence (sequence–based approach) or on metabolic activity (function–based approach). The application of sequence–based approaches involves the design of DNA probes or primers. In general, target genes are identified either by PCR–based or hybridization–based approaches employing primers and probes derived from conserved regions of known genes and gene products. Thus, only genes harboring regions with similarity to the sequences of the probes and primers can be recovered by this approach.

In this way, only novel variants of known functional classes of proteins can be generally identified. Notwithstanding this limitation, this strategy led to the successful identification of genes encoding novel enzymes [22]. The advantages of this screening strategy are the independence on gene expression and production of foreign genes in the library host. However, sequence-based screening is not selective for full-length genes and one possible risk is that partial genes would be cloned. The functional screening strategy has the potential to identify novel classes of genes encoding known or novel functions; it selects for full-length genes and functional gene products. Three different function driven approaches have been used to recover novel biomolecules: (i) phenotypic insert detection (PID), where the expression of a particular trait is used to identify positive clones, (ii) modulated detection (MD), a strategy that relies on the production of a gene product that is necessary for growth under selective conditions, (iii) substrate induction, a strategy that is based on the induced expression of cloned genes via a specific substrate [11]. In most of the cases, such screenings resemble those used by the classical approach applied to microbial isolates (see above): for searching novel enzyme, chemical dyes and/or insoluble or chromophore-bearing derivatives of enzyme substrates incorporated into the growth medium are used, where they register the specific metabolic capabilities of individual clones [23].

1.2 Novel enzymes from microbial diversity

Microbial enzymes find employment in almost all industrial sectors, from chemical, pharmaceutical and food industries, to the manufacturing of detergents, textiles, leather, pulp and paper [24]. Currently, the application of enzymes in industries generates a turnover of about \$ 5 billion, a value that is forecasted to rise in the next future, thanks to the continuously increasing demand for novel enzymatic biocatalysts with high process performances [12]. Food and beverage enzymes such as lipase, protease, isomerase, α -amylase and glucoamylase constitute the largest segment of industrial enzymes with revenues of nearly \$ 1.8 billion in 2016, at a composed annual growth rate of 10.4% [25]. Feed enzymes commercially available are phytases, proteases, α -galactosidases, glucanases, xylanases, α -amylases, and

polygalacturonases, and are mainly used for swine and poultry. Enzymes are used in the for preparing beta-lactam antibiotics such as semi-synthetic penicillins and cephalosporins (representing the 60%–65% of the total antibiotic market) and the top-selling statins that are cholesterol-lowering drugs. Bulk enzyme preparations are used in the detergent, textile, pulp and paper industries. In addition, introduction of enzymatic processes in the chemical industry replacing established chemical methods is today driven by the increasing need of “green” catalysts. Compared to industrial processes catalyzed by chemical reactions, processes based on enzymes are favored by reduced costs, increased efficiency, improved product recovery, and reduced use of toxic compounds. Biocatalysis is also achieving new advances in environmental fields from enzymatic bioremediation to the synthesis of renewable and clean energies and biochemical cleaning of “dirty” fossil fuels. Most of the in-use-today enzymes were discovered in past decades by the classical approach of strain isolation and cultivation; their production has progressively shifted from the homologous producers to the heterologous hosts, due to the ease of cloning procedures and to the reduction of molecular tool costs [24, 26]. Nowadays, the search for novel microbial enzymes benefits of the advances of genomics and metagenomics, as outlined in the above paragraphs (for a more comprehensive report on the metagenomics contribution to enzyme discovery, see the Mini Review reported as Appendix 1, in preparation to FEMS Microbiology Letters). Mains area of metagenomics contribution are those related to: (i) the conversion of recalcitrant biomasses such as starch, cellulose, hemicelluloses, lignin and chitin into biofuels or chemicals [27]; (ii) the use of lipases and esterases a in organic solvents for their exquisite chemo-, regio-, and stereo selectivities [28]; and (iii) the synthesis of carbonyl compounds, hydroxy acids, amino acids and chiral alcohols by oxidoreductases [29]. Interestingly, a novel β -galactosidase was recently identified by a functional-based screening of a metagenomic library constructed from a German soil sample, and it is widely used in the dairy industry for preparing lactose-free products for intolerant consumers [30]. An emerging field for novel enzyme applications is lignin valorization and the use of ligninolytic enzymes in different biotechnology-based processes, as reported below.

1.3 Lignocellulose and lignin

Lignocellulose is a renewable organic material and is the major structural component of all plants. Lignocellulosic wastes are produced in large amounts by many industries including those of forestry, pulp and paper, agriculture, and food. Lignocellulose consists of three major components: cellulose, hemicellulose and lignin. In general, dry plants comprise 40–50 % cellulose, 15–25 % hemicelluloses and 20–25 % lignin. Till now, large-scale biomass processing focused on the valorization of the carbohydrate part of lignocelluloses, since a variety of biofuels and chemicals compounds derived from the hydrolysis of celluloses and hemicelluloses. Annually, approximately 5×10^6 metric tons of lignin is produced industrially [31]. Lignin is a highly complex aromatic heteropolymer (Figure 2) whose role in plants is to increase cell wall integrity and resistance to pathogen attacks. It is made from the enzyme-mediated radical polymerization of three different phenolic subunits (coniferyl, *p*-coumaryl and sinapyl alcohols) that differ from each other by the number of methoxy substituents present on the aromatic ring, forming a complex three dimensional network linked by a variety of ether and carbon-carbon bonds. Among all the different linkages, the β -aryl ether motif is the most abundant comprising up to 70% [32]. The lignin content and monomeric unit distribution depend heavily on the type of plant and, within a single plant, rather broad variations are observed depending on the plant part.

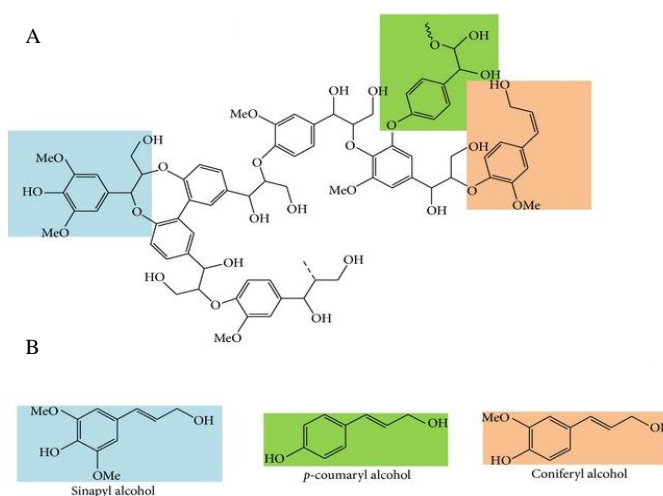


Figure 2. Schematic structures of lignin (A) and (B) three monolignol monomers.

1.4 Lignin modifying enzymes

In nature, lignin is primarily broken down via the action of oxidative enzymes from fungi and bacteria. The bio-degradation of lignin by lignin-degrading microbes was described as “enzymatic combustion”, where the oxidizing potential of hydrogen peroxide or molecular oxygen by ligninolytic peroxidase enzymes or laccase enzymes, respectively, is exploited to oxidize aromatic units. Ligninolytic microbes have developed a unique strategy to handle lignin degradation based on unspecific one-electron oxidation of the benzenic rings in the different lignin substructures by extracellular oxidative enzymes, such as laccases and peroxidases [33]. The most active microbes in lignin degradation identified to date are fungi, such as those belonging to the white-rot families (the model organism is *Phanerochaete chrysosporium*). In fungi, the onset of ligninolytic enzyme production is associated with secondary metabolism activation in response to nutrient depletion [34]. Lignin-degrading fungi rely on complex degradative machineries that generally catalyze two types of processes: first, direct enzymatic depolymerization, for example, by lignolytic enzymes and second, generation of oxidative species (e.g., radicals) that then act on the biomass. However, in typical white-rot degradation, the fungi employ a mode of attack that is primarily enzymatic. The enzymes employed by the white rot fungi generally include a complete suite of enzymes that can oxidize lignin components, including lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases, or a combination of these. Since lignin is composed of phenylpropanoid units, the oxidative breakdown of lignin could release low molecular weight products. Studies of lignin breakdown by *P. chrysosporium* identified 28 low molecular weight products, 10 of which were aromatic carboxyl acids. The aromatic acids were all benzoic acid derivatives, indicating that they arose from C_α-C_β oxidative cleavage of lignin components, and the compounds included a biphenyl dicarboxylic acid, and a diphenyl ether dicarboxylic acid, derived from the biphenyl and diphenyl ether components of lignin [35]. Although the microbial degradation of lignin was most intensively studied in white-rot fungi, there are a number of bacteria that can break down lignin. The soil bacteria that were identified as able to oxidize lignin are mostly members of Actinobacteria,

α -Proteobacteria or γ -Proteobacteria, as those found in termite guts and wood-boring insects. Particularly, actinomycetes were reported to react with lignin to both solubilize it and produce high molecular weight compounds termed acid-precipitable polymeric lignin (so-called APPL). Then, bacteria can produce smaller aromatics that can be imported into the cell for aromatic catabolism, which is also widespread in soil bacteria [36]. The first secreted bacterial heme-peroxidase was reported in the Gram-positive actinomycete, *Streptomyces viridosporus* T7A, indicating that bacteria may also possess a set of extracellular oxidative enzymes involved in lignin metabolism [37, 38]. Peroxidases were then characterized from the Gram-positive actinobacteria *Rhodococcus jostii* RHA1 and *Amycolatopsis* sp. 75iv2, and in the Gram-negative γ -proteobacterium *Pseudomonas fluorescens* Pf-5 [39–41]. Many laccases were identified in bacteria but they are involved in several physiological phenomena beyond possible lignin metabolism [42].

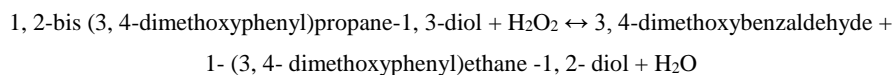
1. 5 Classification and source of ligninolytic enzymes

Peroxidases

Peroxidases involved in lignin degradation belong to class II superfamily of heme peroxidases and include lignin peroxidases (LiPs), manganese peroxidases (MnPs) and versatile peroxidases (VPs) [43]. Enzymes belonging to the peroxidase class II share a general tertiary folding and helical topography. They are globular proteins formed by 11–12 α -helices predominantly in two domains, with a central cavity containing the heme group. In order to stabilize the protein structure, LiPs contain four cysteine disulfide bridges, while MnPs contain a fifth bridge in its C-terminal region, and two Ca^{2+} binding sites. As reported above, the majority of the known peroxidase are produced by fungi belonging to white rot families. Their production is affected by different factors such as nutritional and growth state, carbon source, nitrogen concentration, presence of inductors (*i.e.*, copper, manganese and iron). Very often, obtaining ligninolytic enzymes from fungal native sources does not provide sufficient yield to meet industrial processes requirements. Indeed, fungi genetic manipulation is more difficult than with prokaryotic cells. Additionally, the fermentations of fungi are longer than the bacterial ones and this might imply

higher cultivation and purification costs. For these reasons, there is an increasing interest in searching bacterial peroxidases and/or in developing their heterologous expression.

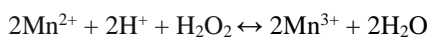
LiPs (EC 1.11.1.14) catalyze H₂O₂-dependent oxidative reaction. A model reaction is the following:



The fungal LiPs are glycoproteins of 38–46 kiloDalton (kDa), with isoelectric point (pI) values of 3.2–4.0 [44]. LiP activity was first discovered in *P. chrysosporium*, and various isoforms are produced by this fungus and by a number of other white-rot fungi. The catalytic process occurs through a multistep reaction. A 2e⁻ oxidation of the native ferric enzyme produces Compound I intermediate that exists as a ferryl iron porphyrin radical cation [Fe(IV)=O⁺], with the peroxide substrate (H₂O₂). This produces H₂O₂ reduction to water and the oxidation of the protein by two electrons. Next, two consecutive one-electron reduction steps complete the catalytic cycle. The first 1e⁻ reduction of Compound I by a reducing substrate yields Compound II and a substrate radical cation. Finally, a second 1e⁻ reduction returns the enzyme to the ferric oxidation (III) state, completing the catalytic cycle. In addition, in the presence of excess H₂O₂ and in the absence of a reducing substrate, Compound II can react with H₂O₂ to form a catalytic inactive form of the enzyme, known as Compound III. This Compound III can be converted to the resting state enzyme by spontaneous autoxidation or by oxidation with a veratryl alcohol (VA) radical cation through the displacement of superoxide from the active site [45]. LiPs are capable of oxidizing a wide variety of phenolic (guaiacol, vanillyl alcohol, catechol, syringic acid, acetosyringone) and non-phenolic compounds. LiPs are unique in their ability to oxidize substrates of high redox potentials [45]. This is in addition to the low pH optimum near pH 3.0 and sensitivity to excess H₂O₂ for activity. The Compound I stores a high redox potential (E'_o ~1.2 V at pH 3.0) that enables it to catalyze the oxidation of non-phenolic aromatic substrates not normally associated with other peroxidases. In a recent study, the production of LiP was improved in *P. chrysosporium* by the constitutive expression of the endogenous gene *lipH8*.

In this case, the production yield of LiP was increased to four times higher in comparison to previously published reports [46]. In another study, production of LiP was optimized in white rot fungus *Phlebia radiata* using semi-solid cultures and evaluating different concentrations of nitrogen and copper. The enzyme production was significantly promoted using wood as carbon source in high nitrogen complex medium supplemented with 1.5 mM copper [47]. In addition, LiP isoenzyme H2 from *P. chrysosporium* was successfully over expressed in *E. coli* host [48]. In this case, LiP was produced in an inactive and insoluble form into inclusion bodies, but the active enzyme was then obtained by refolding the protein in a buffer containing glutathione, urea, Ca²⁺ and heme. *P. chrysosporium* LiP was also expressed in *Pichia methanolica* cells under the control of the alcohol oxidase (AUG1) promoter of *P. chrysosporium*, with downstream either the lignin peroxidase leader peptide of *P. chrysosporium* or the *Saccharomyces cerevisiae* α -factor signal peptide. Both peptides efficiently directed the secretion of LiP from the recombinant yeast cell [49].

MnPs (EC 1.11.1.13) catalyze the Mn-dependent reaction. The model reaction is the following:



These heme-containing glycoproteins are often present in multiple isoforms with molecular mass in the range of ~ 40–50 kDa. The catalytic cycle of MnP resembles those of other heme peroxidases such as horseradish peroxidase (HRP) and LiPs, and includes the native ferric enzyme as well as the reactive intermediates Compound I and Compound II. In contrast to other peroxidases, MnPs use Mn²⁺, which is ubiquitous in all lignocelluloses and in soil, as the preferred substrate. MnPs oxidize Mn²⁺ to Mn³⁺, which in turn oxidizes a variety of phenolic compounds (such as 2,6-dimethoxyphenol (2,6 DMP), guaiacol, 4-methoxyphenol and phenolic lignin residues), whereas it is inactive on veratryl alcohol or on nonphenolic substrates. Mn³⁺ generated by MnPs is a strong oxidizer (1.54 V) but it is quite unstable in aqueous media. To overcome this drawback, white rot fungi secrete oxalic and other organic acids that form Mn³⁺ chelates acting as stable diffusing oxidizers of phenolic compounds [49].

The first extracellular MnP (MnP1) was purified from *P. chrysosporium*, where its expression and production was regulated by the presence of manganese (Mn^{2+}) into the culture medium. In fact, Mn^{2+} controls the *mnp* gene transcription that is growth- and Mn^{2+} concentration-dependent. In addition, MnP is also regulated at the level of gene transcription by heat shock and H_2O_2 . Expressing endogenous gene (*mnp1*) constitutively in *P. chrysosporium* increased the production yield of ligninolytic enzymes of four folds in comparison to previously published reports [46, 50]. *P. chrysosporium* possesses at least six different MnPs [35]. The MnP isoenzyme H4 was expressed in *E. coli* host. The inactive and insoluble protein was refolded in the presence of 2 M urea, $CaCl_2$, heme and oxidized glutathione [50]. *Mnp1* gene from *P. chrysosporium* was also successfully expressed in *Pichia pastoris* cells: its expression level in active form was increased by adding heme to the fermentation broth [51]. In another study, Mancilla and co-workers evaluated the effect of Mn^{2+} on *mnp* transcript levels in the basidiomycete *Ceriporiopsis subvermispora*: since no correlation between transcript levels and extracellular MnP activity was observed, a post-transcriptional role of manganese in controlling extracellular production of MnP was suggested in *C. subvermispora* [52].

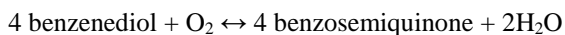
VPs (EC 1.11.1.16) were more recently characterized as enzymes sharing typical features of the MnP and LiP fungal peroxidases. VP activity was identified in the white rot fungal genera *Pleurotus* (i.e., *Pleurotus eryngii* and *Pleurotus ostreatus*) and *Bjerkandera* (i.e., *Bjerkandera adusta*). The catalytic cycle of VPs resembles the one reported for LiPs and other peroxidases: these enzymes catalyse electron transfer from the substrate to form Compound I and II intermediates. Indeed, this group of enzymes is not only specific for Mn^{2+} as in MnPs, but also oxidizes phenolic and non-phenolic substrates that are typical for LiPs, including veratryl alcohol, methoxybenzenes, and lignin model compounds, in the absence of manganese [45]. The interconversion of structure/function between LiP and MnP activity is evidenced by a series of site-directed mutagenesis to introduce MnP activity into LiP or vice versa [45]. Recently, *vpl2* gene from *P. eryngii* was successfully over expressed in *E. coli* cells as a chimeric protein fused to thioredoxin and by inducing protein expression by IPTG in the presence of 0.1 g/L

of heme. Over expressed VP thioredoxin was soluble and was produced with an overall productivity of 12.5 mg/L pure protein [53]. However, VP from *P. eryngii* was more efficiently produced as a soluble and active enzyme in *S. cerevisiae*, reaching a secretion level of 21 mg/L [53].

Laccases

Laccases belong to the blue multi-copper oxidases (MCOs) class of enzymes that are characterized by four copper atoms organized into three spectrally distinct sites. The Type 1 (T1) site copper atom absorbs intensely at 600 nanometers (nm) and imparts the blue color, the Type 2 (T2) site copper atom is invisible in the absorption spectrum whereas the Type 3 (T3) site has two copper atoms and absorbs markedly at 330 nm [54]. The mono-nuclear T2 and bi-nuclear T3 sites together form a tri-nuclear cluster.

Laccases (EC 1.10.3.2) are polyphenol oxidases that catalyzes the complete reaction:



The first laccase was discovered in the sap of the Japanese lacquer tree *Rhus vernicifera* [55]. Laccases have been found in nearly all wood-rotting fungi analyzed so far and are almost ubiquitary enzymes as they have been isolated from plants, from some kinds of bacteria, and from insects too [56], with fungal laccases being the most well characterized. Laccases catalyze the one–electron oxidation of four equivalents of a reducing substrate, with the concomitant four–electron reduction of dioxygen to water. The catalytic cycle is started at the T1 site where four substrate molecules are oxidized, producing four electrons. These electrons are transported to the tri–nuclear cluster (T2/T3 center) where the cycle is completed through the reduction of dioxygen to two molecules of water. Laccases possess moderately low redox potentials (0.5–0.8 V) and can oxidize phenolic compounds (*i.e.*, polyphenols, methoxy–substituted phenols and aromatic diamines) [57]. Substrates characterized by high redox potential (> 0.8 V) cannot be oxidized directly by laccases. However, the number and type of substrates oxidized by laccases can be extended by a mechanism involving the participation of redox mediators.

These mediators are low molecular weight compounds that can easily be oxidized by laccases, producing very reactive and unstable cationic radicals. At the same time, these cationic radicals can oxidize complex compounds before returning to their original state. Fungal laccases are extracellular and/or intracellular monomeric globular proteins of approximately 60–70 kDa with an acidic (pI) around pH 4.0. They are generally glycosylated [34]. Analysis of the essential sequence features of fungal laccases based on multiple sequence alignments of 100 laccases resulted in the identification of a set of four sequence-conserved regions [58]. All these laccases exhibit a similar molecular architecture organized in three sequentially arranged cupredoxin-like domains. Each of them has a greek key β -barrel topology, strictly related to that of small copper proteins common to all the members of the MCOs family. The T1 site is located in the third domain and the substrate binding site is located in a small cavity close to the T1 site, whereas the T2–T3 site is placed between the first and the third domain [44]. In fungi, laccases are widely distributed in ascomycetes, deuteromycetes, and basidiomycetes. These laccases producing fungi (called as wood-degrading fungi) include *Trametes versicolor*, *Cerrena maxima*, *Phlebia radiata*, *Coriolopsis polyzona*, *Lentinus tigrinus* and *Plreurotus eryngii* [59]. Most of these fungi, especially *Basidiomycetes* species, produce laccases in multiple isoforms. The high activity of secreted laccases in these wood-degrading fungi suggest that the main role of fungal laccases together with peroxidases is to depolymerize the complex cell wall constituents such as lignin. In recent years, the study of laccase gene expression regulatory mechanisms gained interest due to the need to understand the physiological role of different laccase isoforms produced in various organisms. Synthesis and secretion of laccases are highly influenced by nutrient levels, culture conditions, developmental state and the addition of inducing agents to culture medium. Laccase gene transcription is regulated by metal ions, various aromatic compounds related to lignin or lignin derivatives, nitrogen and carbon sources [60]. The *lcc1* gene from the white rot fungus *T. versicolor* was subcloned into *Pichia methanolica* and the production of active laccase was directly proportional to the concentration of copper in the growth medium [61].

The *lcc1* cDNA isolated from the white rot fungus *Trametes trogii* was successfully expressed and secreted in the methylotrophic yeast *Pichia pastoris*. Lcc1 showed high activity in the presence of organic solvents and a high decolorization capacity towards azo, triarylmethane, indigo carmine and anthraquinonic dyes [62]. A full-length *Ery3* cDNA encoding an extracellular laccase was isolated from the mycelia of *P. eryngii* and successfully expressed in immobilized *S. cerevisiae* cells [63]. Despite low sequence homology between fungal and bacterial laccases, the overall geometry of their active sites is similar, consisting of highly conserved features and an active site containing four specifically structured copper ions. The first bacterial laccase was discovered in 1993 in non-motile strains of *Azospirillum lipoferum* isolated from the rhizosphere of rice [64]. Nowadays, a large number of candidate MCO genes are being discovered in the genomes of a diverse set of bacteria, particularly in the streptomycetes, bacilli and pseudomonads genera [56]. The best studied bacterial laccase is the CotA, the endospore coat component of *Bacillus subtilis*. The *cotA* gene codes for a 65 kDa protein located into the outer spore coat. CotA participates in the biosynthesis of the brown spore pigment, which is a melanin-like product and seems to be responsible for most of the protection afforded by the spore coat against UV light and hydrogen peroxide [65]. The other well-known laccases such as CueO from *E. coli* [66] and CopA from *Pseudomonas syringae* and *Xanthomonas campestris* seem to be involved in copper resistance [67]. The above described laccases are three-domain laccases as the fungal ones. However, among bacteria and fungi a novel laccase family of small, two-domain laccases was discovered [68-70]. In two-domain laccases, domain 2 of their three-domain counterparts is lacking. This leads to a substantially different architecture as shown for the small laccase (SLAC) from *Streptomyces coelicolor* [68]. In order to build a functional tri-nuclear cluster, the small laccases oligomerize as homotrimers with the tri-nuclear cluster located at the interface of adjacent monomers. SLAC shows phenol-oxidizing activity at an unusually alkaline pH [68]. SLAC was expressed in *E. coli* host and the recombinant enzyme is stable at high temperature, retaining some activity after boiling and denaturing SDS treatments [68].

An extracellular laccase from *Streptomyces ipomoea* CECT 3341 was also cloned and over expressed in *E. coli* [71]. It showed a good stability at high pH, temperature and resistance to NaCl concentrations and to several laccase inhibitors: at alkaline pH, a wide range of phenolic compounds was oxidized, including the syringyl and guaiacil moieties derived from lignin [71].

1.6 Lignin modifying enzymes from metagenomics

Metagenomics can be a promising tool for the identification of novel ligninolytic enzymes otherwise encrypted in natural microbial communities. Naturally occurring suppressive soils and lignin-amended soils and sediments are thought to be particularly valuable resources for the construction of metagenomic libraries: in these environmental samples the fitness of lignin degrading bacteria and hence the proportion of genes related to lignin degradation in the metagenomic DNA are expected to be increased [72]. To date, only a few studies have employed metagenomics to identify novel putative ligninolytic sequences and even less works had led to the isolation and proper characterization of biologically active ligninolytic enzymes. As reported above, most of the know peroxidases and laccases were discovered by following the methods of traditional microbiology and phenotype-based screening. In the last two decades, genome mining contributed to identification of novel genes for bacterial laccases [73, 74] followed by their expression in heterologous hosts in few cases [75, 76]. To date, the genes *lbh1* from *Bacillus halodurans* [75] identified by homology sequence and *Ss11* from *Streptomyces sviveus* [76] identified in the laccase engineering database (LccED), were readily expressed in *E. coli*. More recently, also metagenomics is contributing to discovering few laccases through functional or sequence-based screening. These laccases showed interesting biochemical proprieties such as high chloride tolerance, alkaline-dependent activity and dye decolorization ability. *RL5*, a gene coding for a novel polyphenol oxidase with laccase activity, was identified through functional screening of a metagenome expression library from bovine rumen microflora [77]. *RL5* protein produced in *E. coli* was able to efficiently oxidize a wide range of substrates over an unusually broad range of pH from 3.5 to 9.0.

Bioinformatic analysis of the gene sequence and of the sequences of neighboring genes suggested a tentative phylogenetic assignment to the genus *Bacteroides* [77]. The laccase named Lac591 was isolated from the metagenomic library made from mangrove soil and identified through a functional-based screening in alkaline environment [78]. Subsequently, other two laccases, named Lac15 and Lac21 were isolated by a sequence screening strategy from a marine microbial metagenome library. These two laccases showed interesting biochemical proprieties such as chloride tolerance and dye decolorization ability [79, 80]. Finally, a metagenomic library of intestinal bacteria in giant panda gut was screened in both function- and sequence-based modes. The gene, designated as *lac51*, encoding for a laccase that oxidizes a variety of lignin-related phenolic compounds was identified. In the presence of the two lignin-derived phenolic compounds ferulic acid and syringic acid as the mediators, Lac51 showed to catalyze lignin effectively [81].

1.7 Possible applications of ligninolytic enzymes

Ligninolytic enzymes (peroxidase and laccases) are used in various applications such as processing pulp and paper, leather, and producing pharmaceuticals, chemical, food and beverages, biofuels, animal feed and for personal care. Peroxidases and laccases are known to catalyze a wide range of reactions, including enantioselective reduction of hydroperoxides to their corresponding alcohols, hydroxylation of arenes, oxidation of aromatic amines and phenols, epoxidation of olefins, halogenation, N-oxidation, and sulphoxidation [82]. Some examples of applications are given below:

Delignification of biomass. One of the major challenges to the conversion of cellulose to simple sugars and subsequent conversion of sugars to useable fuel is the presence of lignin. Removal of lignin before saccharification is recommended for optimal biological conversion of biomass to biofuel. Besides delignification, laccase and peroxidases can improve biofuels production through changes in the structure of the lignocellulose microfiber, which modify properties such as porosity, surface area, and hydrophobicity resulting in the reduction of unproductive binding of cellulases [27]. Separation of lignin from cellulose fibers is also an important step

in processing of wood for manufacturing of paper pulp. To produce paper pulp, lignin is actually eliminated either by mechanical or chemical methods, particularly for the production of the so-called Kraft pulp, which is eventually used to produce paper [83]. Extracellular LiPs, MnPs and VPs from *P. chrysosporium* are used for facilitating lignin depolymerization, and similar activities were described in streptomycetes and other actinobacteria [84].

Textile industry. Laccases are currently used in textile industry to improve the whiteness of cotton and as well as in biostoning. MnP and laccase activities from *T. versicolor* were involved in the sequential decoloration of azo dyes [85]. In recent years, the utilization of laccases in combination with mediators is replacing more expensive and less environmentally friendly chemical treatments of textile dye wastes. Kirby and colleagues reported that laccase from *Phlebia tremellosa* decolorized eight synthetic textile dyes added to culture under stationary growth conditions [86]. Dyes such as poly B-411, poly-R418 and remazol brilliant blue R (RBBR) are often used as substrates of streptomyces laccases. *Thermomonospora mesophila*, *S. badius* and *S. viridosporus* T7A are among the most active Poly R decolourizing actinobacterial strains [87].

Food industry. Many laccase substrates, such as carbohydrates, unsaturated fatty acids, phenols, and thiol-containing proteins, are important components of various foods and beverages and their modification by laccases may lead to new features, quality improvement, or cost reduction [88]. For example, color and taste of must and wine are dependent on particular phenolic compounds present in different kinds of wine [89]. All phenolics are subjected to various fates during shelf-life of wine and some problems can arise from their modifications, which are then involved in various chemical reactions. Many innovative treatments, such as enzyme inhibitors, complexing agents, and sulfate compounds, have been proposed for the removal of phenolics responsible for discoloration, haze, and flavor changes but the possibility of using enzymatic laccase treatments as a specific and mild technology for stabilizing beverages against discoloration and clouding represents an attractive alternative.

Bioremediation. Various aromatic xenobiotics and pollutants generated by coal conversion, petrochemical, alcohol distilleries, dyeing, and textile industries are responsible for imparting color to the wastewater and toxicity. Moreover, chemical and physical treatments methods, including ultrafiltration, ion exchange, and lime precipitation, are expensive, so alternative biotreatment processes are now being considered. Laccases and peroxidases oxidize phenolics to aryl-oxy radical insoluble complexes. Enzyme-mediated bioremediation processes include polymerization among pollutants themselves or copolymerization with other nontoxic substances such as humic materials, thus facilitating easy removal by such means as adsorption, sedimentation, or filtration [89].

Biosensor and immunochemical assay. Laccase-containing biosensors have been developed for detecting O₂, glucose, aromatic amines, phenolic compounds, and a wide variety of reducing substrates [90]. Laccases can be used to assay other enzymes. In these assays, either the enzyme of interest catalyzes the production of a compound whose subsequent oxidation by laccase generates detectable physical change or a product from laccase catalysis is quenched by the activity of the enzyme of interest. Moreover, laccases can be conjugated to an antibody or an antigen and can be used as marker enzymes for immunochemical assay. In this application, the binding of the antibody or antigen to its immunological counterpart is detected by localized laccase activity on a gel. Under certain conditions, the antibody-antigen binding impairs the function of the conjugated laccase, thus allowing immunochemical detection through modulation of laccase activity [89].

Medical applications. Laccases reported to synthesize products of pharmaceutical importance. Laccases oxidize iodide to produce iodine, a reagent widely used as disinfectant. Furthermore, Wang and co-workers carried out the assay for HIV reverse-transcriptase inhibitory activity using *Tricholoma giganteum* laccase purified from its fruiting body [89].

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2. Layout of my PhD thesis

Aim of the work

The aim of my PhD thesis is the identification, production and characterization of novel lignin-modifying enzymes from actinomycetes and, in the frame of the European project MetaExplore, from metagenomic libraries. Classical assay-based screening and PCR-based metagenomic approach have been applied to microbial culture collections and environmental libraries, respectively.

Outlines of the PhD thesis

The chapter 3 of this PhD thesis is focused on the identification of ligninolytic enzymes by classical approach. Ligninolytic microbes have developed a unique strategy to handle lignin degradation based on unspecific one-electron oxidation of the benzenic rings in the different lignin substructures by extracellular oxidative enzymes. In particular, I worked on the detection of laccases (EC 1.10.3.2), lignin peroxidases (LiPs) (EC 1.11.1.14), manganese peroxidases (MnPs) (EC 1.11.1.13) and versatile peroxidases (VPs) (EC 1.11.1.16) in actinomycetes such as *Nonomuraea gerenzanensis* and *Streptomyces coelicolor* A3 (2). 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 ligninolytic enzymes. In an earlier work, a screening campaign for lignin-degrading enzymes on forty three actinomycetes on minimal agar plates containing lignin as the only carbon source was conducted. Only *Nonomuraea gerenzanensis* (a rare actinomycete very recently classified) and *Streptomyces coelicolor* A3 (2) (the model strain for actinomycetes) formed a clear halo around the colony, and this might indicate the extracellular production of lignin-degrading enzymes. Given these results, I investigated the expression of lignin-degrading enzyme activities (i.e., MnPs, LiPs and laccases) by *Nonomuraea gerenzanensis* and *Streptomyces coelicolor* A3 (2) cultivated in different liquid media. Finally, in collaboration with Prof. Pollegioni's group of the Laboratory of Functional Post-Genomics and Protein Engineering, we worked on the production and biochemical characterization of a novel bacterial peroxidase activity in *Nonomuraea gerenzanensis*.

Compared to known peroxidases, the stability of *N. gerenzanensis* peroxidase at alkaline pHs and its thermostability are significantly higher. From a kinetic point of view, *N. gerenzanensis* peroxidase shows a K_m for H_2O_2 similar to that of *Phanerochaete chrysosporium* and *Bjerkandera* enzymes and a lower affinity for Mn^{2+} . 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 by classical approach, we may be able to discover and exploit alternative biological tools involved in lignin metabolism. This work was recently submitted to Biotechnology Reports entitled **Lignin degradation by actinomycetes: a valuable bacterial peroxidase activity from the novel species *Nonomuraea gerenzanensis*** (by Casciello C, Tonin F, Berini F, Fasoni E, Marinelli F, Pollegioni L, Rosini E). The chapter 4 of this PhD thesis was developed in the frame of the EC-funded project (MetaExplore: metagenomics for bioexploration - tools and application) on using a metagenomic approach to identify novel biocatalysts for recalcitrant biomass conversion into valuable products. In particular, I worked on the expression and characterization of a laccase isolated from a metagenomic library, prepared by the group of Prof. Ines Mandic-Mulec from the Department of Food Science and Technology, University of Ljubljana, Slovenia. The *metaLacc* gene was isolated from a metagenomic fosmid library made from the bog soil of Ljubljana marsh. In this case, the putative laccase gene was sorted out using a PCR-based screening approach. Sequence analysis showed that the putative laccase shares sequence identities lower than 30 % with well-known bacterial or fungal laccases and the full-length protein shows the highest sequence homology (66 %) with an uncharacterized laccase derived from an uncultured bacterium. MetaLacc is able to oxidize the substrates commonly used for laccase characterization such as the nonphenolic ABTS and the phenolic 2, 6-DMP. Additionally, MetaLacc also oxidizes other substrates such as L-DOPA, pyrogallol, pyrocatechol, vanillic acid and syringaldazine. Interesting, the highest enzyme activities were detectable on pyrogallol and pyrocatechol (phenolic substrates with three and two substituted hydroxyl groups, respectively). MetaLacc is not only thermostable in the range

from 40 to 60°C, but it displays a substantial heat activation, since after 2 hours at 40, 50, or 60°C, its enzymatic activity increased and it was *ca.* 1.2, 3.5, and 1.2-fold higher than the initial activity. Furthermore, compared to fungal laccases, MetaLacc shows a good chloride-tolerance and high resistance to the addition of solvents such as methanol. Finally, MetaLacc shows dye-decolorizing activity in the presence and absence of mediators. This work is being submitted to Bioresource Technology entitled **A novel bacterial laccase-like enzyme found with metagenomics** (by Ausec L, Berini F, Casciello C, Cretoiu S, van Elsas JD, Marinelli F, Mandic-Mulec I). In the chapter 5 of my PhD thesis, I describe the work done in collaboration with the group of Prof. Petra Patakova at the Department of Biotechnology of the University of Chemistry and Technology of Prague, where I spent a two-month stage (see Appendix 2). During that period, I followed the production of ligninolytic and cellulolytic enzymes in the model fungal strain (*Phanerochaete chrysosporium* CCM 8074) and in the two actinomycetes (*N. gerenzanensis*, *S. coelicolor* A3 (2)) cultivating them in media containing pre-treatment wheat straw as a carbon source. The three strains produced cellulolytic activity, although at different extent; surprisingly, no ligninolytic activities (i.e., laccases, MnPs and LiPs) were detected in the *P. chrysosporium* used strain, while *N. gerenzanensis* produced MnP and LiP activities (but no laccase one), and *S. coelicolor* A3 (2) produced only LiP activity. These experiments are preliminary for evaluating the feasibility of two-stage bioconversion process (TSBP). The first stage would be enzymatic digestion of pre-treated wheat straw by selected aerobic microorganisms (actinomycetes or fungi). A second stage of anaerobic fermentation to produce biofuels will eventually follow. Finally, I collaborated in writing the minireview on the impact of metagenomics for novel enzyme discovery and application that is reported as Appendix 1 to this PhD thesis entitled **Metagenomics, enzyme biotechnology from unculturable microorganisms** (by Berini F, Casciello C, Marinelli F). The revised version of this minireview will be submitted to FEMS Microbiology Letters.

3. Lignin degradation by actinomycetes: a valuable bacterial peroxidase activity from the novel species *Nonomuraea gerenzanensis*

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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 well characterized with respect to their lignin metabolism, although it is known that many soil bacteria, especially actinomycetes, attack and solubilize lignin. In this work, we screened 43 filamentous actinomycetes by assaying their ligninolytic activity on chemically different substrates, 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 osteratus* 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 involved in lignin metabolism.

Keywords

Lignin degradation; peroxidases; filamentous actinomycetes; *Nonomuraea gerenzanensis*.

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.

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, the lignin polymer is highly resistant to chemical and

biological degradation. Therefore, removing lignin constitutes a central issue for industrial exploitation of plant biomasses to produce second-generation biofuels, chemicals, and new bio-based materials [4]. Since most of the available chemical methods for 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, represents a great biotechnological challenge. Ligninolytic microbes have developed a unique strategy to circumvent the natural resistance of lignin and to degrade and mineralize the polymer by secreting 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) [1,5-7]. 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 H₂O₂ as the cosubstrate in addition to a mediator such as veratryl alcohol to degrade lignin 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 [5-7]. The best characterized lignin-degrading enzymes are those secreted by brown-rot and white-rot families, such as *Phanerochaete chrysosporium* [8].

Nevertheless, the industrial applications of fungal enzymes have been limited by the challenge of producing these post-translationally modified proteins in commercially viable amounts [9]. By contrast, bacterial lignin-active enzymes should be much easier to produce. Additionally, bacterial lignin-degrading 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 (DyP) [10-14]. Lignin-degrading activities were identified within members of different bacterial taxa, especially *Proteobacteria*, *Firmicutes*,

Acidobacteria, and *Actinobacteria* [1,10,12]. 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 ligninolytic enzymes [11,14]. Actinomycetes are aerobic, chemoorganotrophic, Gram-positive bacteria that play an important role in degrading organic polymers in nature, including lignin [15-17]. The first ligninolytic enzyme reported to be produced by a filamentous actinomycete was the extracellular LiP from *Streptomyces viridosporus* T7A [18]. Since then, it was reported that *Streptomyces* spp. produced a few laccases [19,20] and most recently peroxidase activity [21]. There are also reports of lignin degradation by other soil (not-filamentous) actinomycetes (i.e., *Nocardia* and *Rhodococcus*) [14,22]. 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 are actively involved in lignin degradation [23]. 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 [24,25]. 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* [26] that is involved in lignin degradation.

Material and methods

Plate assays for ligninolytic activity detection

Forty-three filamentous actinomycetes belonging to the culture collection of The Protein Factory research center [27] were screened for their ability to degrade lignin. *Escherichia coli* DH5 α (Invitrogen, Carlsbad, CA USA), cultivated according standard procedures [28], was used as a control. For primary screening, agar plates containing minimal salt medium and lignin (alkali, low sulfonate content, 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 lignin, 1.6 K₂HPO₄, 0.5 KH₂PO₄, 0.58 MgSO₄ · 7 H₂O, 0.25 NaCl, 0.013 CaCl₂ · 2 H₂O, 1.25 (NH₄)₂SO₄, 1 NH₄NO₃, 0.0025 FeCl₃ · 6 H₂O, 0.0025 CuCl₂, 0.0025 MnCl₂, 20 noble agar. For secondary screening, selected strains were then grown on mannitol agar medium (MAM) (in g/l: 20 mannitol, 2 KNO₃, 2 MgSO₄ · 7 H₂O, 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.

Growth conditions for actinomycetes

Nonomuraea gerenzanensis [26] and *Streptomyces coelicolor* A3(2) working cell banks (WCB) were prepared as previously described [24]. 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 lignin, 6 or 12 g/l yeast extract, 2 mM MnCl₂, 2 mM CuSO₄, 0.2 mM FeSO₄ · 7 H₂O, 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 x 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.

Enzyme assays

Enzyme activities were assayed spectrophotometrically at 25 °C as follows. Laccase and MnP activity was measured by monitoring the oxidation of ABTS ($\epsilon_{420 \text{ nm}} =$

36000 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 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 H₂O₂ 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 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 ($\epsilon_{510\text{ nm}} = 21647\text{ M}^{-1}\text{ cm}^{-1}$). 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-dimethoxy phenol (2,6-DMP) in 50 mM sodium acetate buffer, pH 5.0 (1 ml final assay volume, $\epsilon_{468\text{ nm}} = 49600\text{ M}^{-1}\text{ cm}^{-1}$).

Enzyme preparation

N. gerenzanensis was grown in 500-ml baffled Erlenmeyer flasks containing 100 ml VM medium supplemented with 0.8 g/l lignin and 2 mM CuSO₄. Cells were removed after 20 days by centrifugation at 10000 x 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 precipitate 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.

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 (2–10000 μM, $\epsilon_{410\text{ nm}} = 2211\text{ M}^{-1}\text{ cm}^{-1}$), 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 ($\epsilon_{310\text{ nm}} = 9300\text{ M}^{-1}\text{ cm}^{-1}$) 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 [29,30]. 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 [31]. The pH dependence of peroxidase activity was fitted using eq. 1, based on two ionizations:

$$y = [(a + b (10^{\text{pH}-\text{pKa}})/(1+10^{\text{pH}-\text{pKa}}))] + [(b + c (10^{\text{pH}-\text{pKa}2})/(1+10^{\text{pH}-\text{pKa}2}))] \quad (1)$$

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 to 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 (2–10000 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: $\epsilon_{270\text{ nm}} = 11590\text{ M}^{-1}\text{ cm}^{-1}$ for Mn³⁺-malonate complex, $\epsilon_{595\text{ nm}} = 8300\text{ M}^{-1}\text{ cm}^{-1}$ for RBBR.

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 H₂O₂ 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 H₂O₂ and 2 mM ABTS, at 25 °C.

Results

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 lignin as sole carbon source. Here, 33 strains grew in the presence of this polymer, 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 [20,32,33], whose role in degrading lignocellulosic biomass was recently demonstrated [11]. However, the genome of *N. gerenzanensis* is not available and, to our knowledge, this is the first report on its ability to use lignin for growing. *N. gerenzanensis* was therefore selected for further analyses and its ability to produce ligninolytic 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 DH5 α , 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 ligninolytic activity [34]. *N. gerenzanensis* was able to oxidize ABTS and decolorize both dyes, but lacked oxidative activity on guaiacol (Table 1 and Fig. 1). *E. coli* DH5 α 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).

Production of ligninolytic enzymes by N. gerenzanensis

N. gerenzanensis was cultivated in VM (lipid and rich medium usually employed for growing this microorganism) and MM-L (salt minimal medium containing lignin as sole 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 ligninolytic enzyme production since metal cations represent the main cofactors of these enzymes [35]. Indeed, the use of ethanol and aromatic compounds was previously reported to stimulate laccase activity [36]. Finally, different nitrogen sources and their concentrations can influence the production of ligninolytic enzymes in filamentous microorganisms and fungi [37]. 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 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 lignin and 2 mM CuSO₄. Since agitation rates can influence ligninolytic enzyme production in filamentous microorganisms and fungi [37], *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 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 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 the peroxidase-producing fungi [8,9].

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 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.

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 H_2O_2 , which showed a substrate inhibition effect ($K_i \approx 340 \mu\text{M}$). The highest activity was observed on catechol as substrate ($\approx 3.8 \text{ U/mg protein}$). *N. gerezanensis* peroxidase preparation also possessed a dye-decolorizing activity: notably, it showed a high affinity for RBBR ($K_m \approx 13 \mu\text{M}$, 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.

Effect of pH and temperature

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

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. gerezanensis* 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. gerezanensis* 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).

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 and that may be used for other biotechnology processes 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 ligninolytic peroxidase gene family, consisting of three VPs and six short-MnPs [38,39]. Notably, the production of MnP is apparently limited to certain basidiomycetous fungi [38], whereas *P. chrysosporium* wild type does not produce VPs [40]. Here we demonstrated that a novel bacterial species belonging to *Nonomuraea* genus produces 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) [40,41]. 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 [26], 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 ($\approx 30\text{-}55 \mu\text{M}$) and a lower affinity for Mn^{2+} ($K_m \approx 50\text{-}80 \mu\text{M}$) [40,42].

Our preparation also differs from *P. osteratus* MnPs: the six known MnP isoenzymes significantly differ in K_m for H_2O_2 ($23\text{-}530 \mu\text{M}$) and for Mn^{2+} ($\approx 7\text{-}101 \mu\text{M}$) [43]. 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 [14], 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 ligninolytic enzymes. Successful ingredients for such screening were (i) assaying enzyme activity on different compounds that act as preferential substrates for different families of ligninolytic activities (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 lignin-modifying 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*. Additionally, genome sequencing of this novel microbial species will contribute to the understanding of its ligninolytic system. Indeed, for biotechnological applications 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.

Appendix

Table A.1 List of strains from the culture collection of The Protein Factory research center screened for their ability to degrade lignin.

Acknowledgements

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Table 1 Screening for ligninolytic activities from *S. coelicolor* A3(2) and *N. gerezanensis* on agar plates. *E. coli* DH5 α did not produced any detectable ligninolytic 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	<i>S. coelicolor</i> A3(2)	<i>N. gerezanensis</i>
ABTS 10 mM	4.5	+++ (3)	+ (14)
	6.0	++ (3)	+ (14)
	8.0	++ (6)	+ (9)
Guaiacol 0.1% v/v	4.5	+++ (3)	-
	6.0	++ (14)	-
	8.0	+ (21)	-
RB5 20 mg/l	4.5	+++ (10)	-
	6.0	+++ (10)	-
	8.0	++ (10)	++ (10)
Azure B 25 mg/l	4.5	+++ (14)	+ (14)
	6.0	++ (14)	++ (14)
	8.0	+ (14)	++ (14)

Table 2 Peroxidase activity production by *N. gerezanensis* 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 lignin	3.1	9.3
	1.5 g/l lignin	0	7.3
	2 mM CuSO ₄ + 2 mM MnCl ₂ + 0.2 mM FeSO ₄	0	0
	0.8 g/l lignin + 2 mM CuSO ₄	65.9	13.8
	0.8 g/l lignin + 2 mM MnCl ₂	0	7.4
	0.8 g/l lignin + 0.2 mM FeSO ₄	0.6	7.9
	0.8 g/l lignin + 5 mM tryptophan	0	7.2
	0.8 g/l lignin + 3.5% v/v ethanol	5.8	0
	0.8 g/l lignin + 5.0% v/v ethanol	16.3	13.8
	0.8 g/l lignin + 3.5% v/v ethanol + 2 mM CuSO ₄	13.2	7.9

^aMM-L contains salts and 0.8 g/l lignin

^bVM contains complex nitrogen and carbon sources

Table 3 Partial purification of peroxidase activity from *N. gerezanensis* 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 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.

H₂O₂				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.

Mn²⁺				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	(5 ± 3) x 10 ⁻⁴	0.128 ± 0.007	13 ± 2	(9.8 ± 2.1) x 10 ⁻³

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).

Fig. 2 Fermentation of *N. gerezanensis* in VM medium supplemented with 0.8 g/l 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. gerezanensis* 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).

Fig. 3 Electrophoretic analysis of peroxidase from *N. gerezanensis* 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 (7 µl).

Fig. 4 Effect of pH and temperature on the activity and stability of *N. gerezanensis* peroxidase preparation. (A) pH effect on the enzymatic activity assayed on 2 mM 2,6-DMP and 0.125 mM H₂O₂ 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).

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 \pm standard error).

Figures

Figure 1

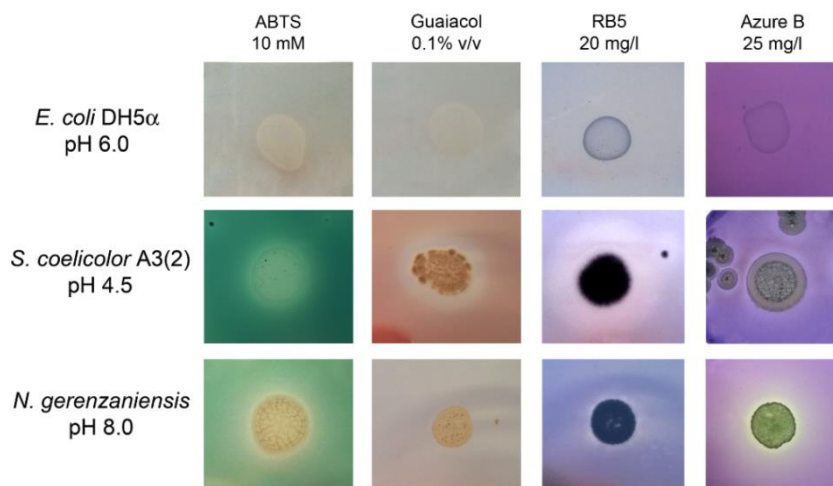


Figure 2

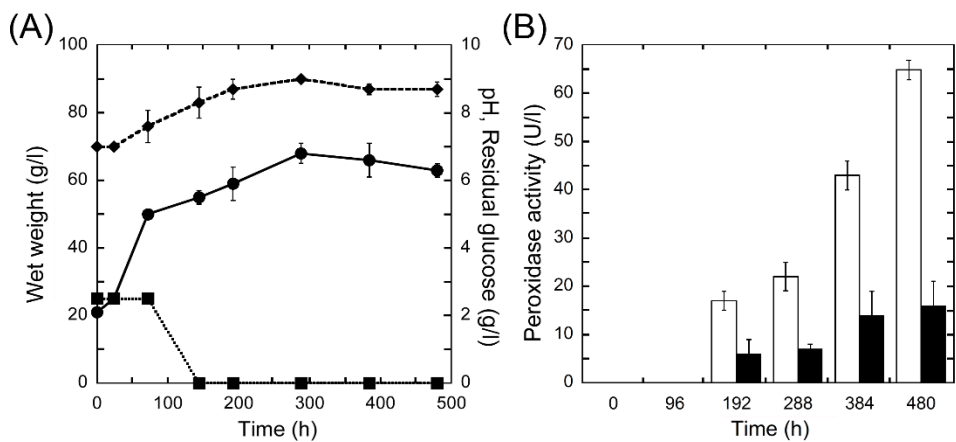


Figure 3

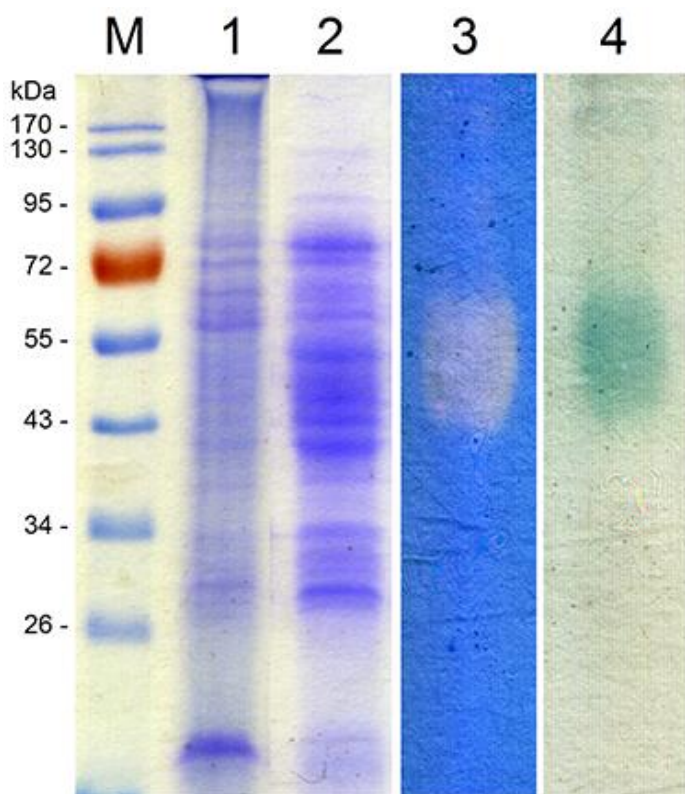


Figure 4

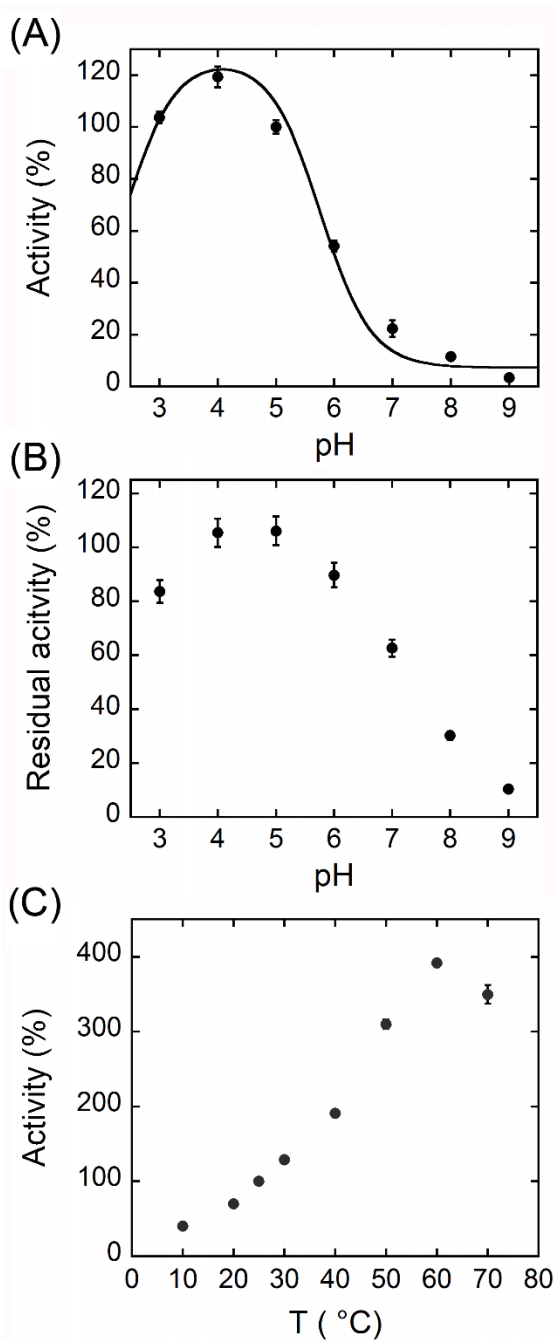
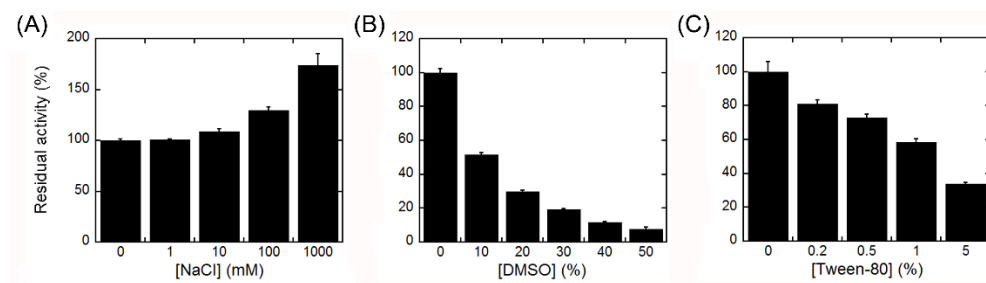


Figure 5



APPENDIX A. SUPPLEMENTARY DATA

Table A.1 List of strains from the culture collection of The Protein Factory research center [1] screened for their ability to degrade lignin. MIV code refers to a specific collection of filamentous actinomycetes within the same culture collection (for the generation and previous screening campaigns of this collection see [2-4]. Within MIV collection, uncommon actinomycetes (also named rare actinomycetes) are those environmental isolates not belonging to the mostly common *Streptomyces* genus, that have not being taxonomically classified yet (n.i. means not identified) [5].

Collection code	Strain	Family
ATCC 39727	<i>Nonomuraea gerenzanensis</i>	<i>Streptosporangiaceae</i>
NRRL 18924	<i>Planomonospora alba</i>	<i>Streptosporangiaceae</i>
NRRL 30420	<i>Microbispora corallina</i>	<i>Streptosporangiaceae</i>
ATCC 31121	<i>Actinoplanes teichomyceticus</i>	<i>Micromonosporaceae</i>
ATCC 14538	<i>Actinoplanes missouriensis</i>	<i>Micromonosporaceae</i>
ATCC 23342	<i>Actinoplanes missouriensis</i>	<i>Micromonosporaceae</i>
ATCC 14539	<i>Actinoplanes utahensis</i>	<i>Micromonosporaceae</i>
NRRL 3884	<i>Actinoplanes sp.</i>	<i>Micromonosporaceae</i>
A3(2)	<i>Streptomyces coelicolor</i>	<i>Streptomycetaceae</i>
ATCC 23899	<i>Streptomyces coelicolor</i> (Muller)	<i>Streptomycetaceae</i>
ATCC 10595	<i>Streptomyces venezuelae</i>	<i>Streptomycetaceae</i>
ATCC 29253	<i>Streptomyces hygroscopicus</i>	<i>Streptomycetaceae</i>
MIV 90842	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 90852	<i>Streptomyces violaceus</i>	<i>Streptomycetaceae</i>
MIV 90853	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 90892	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 90930	<i>Streptomyces violaceusniger</i>	<i>Streptomycetaceae</i>
MIV 90936	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 91003	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 91012	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 91020	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 91042	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 91043	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 91062	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 91081	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 91100	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 102622	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 105105	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>

MIV 105149	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 107599	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 107613	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 107678	<i>Streptomyces halstedii</i>	<i>Streptomycetaceae</i>
MIV 107721	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 109561	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 109574	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 110397	<i>Streptomyces sp.</i>	<i>Streptomycetaceae</i>
MIV 109942	Uncommon actinomycete	n. i.
MIV 110004	Uncommon actinomycete	n. i.
MIV 110023	Uncommon actinomycete	n. i.
MIV 110177	Uncommon actinomycete	n. i.
MIV 110320	Uncommon actinomycete	n. i.
MIV 110328	Uncommon actinomycete	n. i.
MIV 110339	Uncommon actinomycete	n. i.

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4. A novel bacterial laccase-like enzyme found with metagenomics

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Abstract

Laccase-like multicopper oxidases (LMCOs) are oxidoreductases that couple the oxidation of a substrate with the reduction of molecular oxygen to water. These enzymes have been mostly studied in fungi and some of them have been already applied in biotechnological applications at industrial scale. In the recent years, the attention has moved towards bacterial LMCOs, which proved to be more robust than the fungal counterparts thanks to their higher pH and temperature stability, and tolerance to chloride anions. In this paper, we describe the identification and characterization of a novel bacterial three-domain enzyme affiliated to the elusive *phylum* of *Acidobacteria*, named MetaLacc. The coding gene has been discovered by a PCR-based screening approach applied to a metagenomics library constructed from a natural wetland environment, and cloned and expressed in recombinant *Escherichia coli* cells. The paper contextualizes the biochemical properties of this

thermostable, halo- and solvent-tolerant enzyme, which furthermore possess the promising capability of decolorizing azo- and triphenylmethane dyes even in the absence of redox mediators.

Keywords: Metagenomics, laccase-like multicopper oxidase, heterologous expression, dye decolorization

Abbreviations: ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); CTAB: cetyl trimethylammonium bromide; 2,6-DMP: 2,6-dimethoxyphenol; DMSO: dimethyl sulfoxide; DNase: deoxyribonuclease; DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic acid; GO: glycine oxidase; IBs: inclusion bodies; IPTG: isopropyl β -D-thiogalactopyranoside; KPi: potassium phosphate buffer; LB: Luria Bertani broth; L-DOPA: L-3,4-dihydroxyphenylalanine; LMCO: laccase-like multicopper oxidase; MCO: multicopper oxidase; NLS: *N*-lauroylsarcosine; OD: optical density; O.N.: over night; PFGE: pulsed field gel electrophoresis; PMSF: phenylmethylsulfonylfluoride; PVP: polyvinylpyrrolidone; SDS-PAGE: sodium dodecyl sulphate - polyacrylamide gel electrophoresis; TBE: Tris-borate-ethylenediaminetetraacetic acid; TB: terrific broth;

1. Introduction

Laccases are oxidoreductases that couple the oxidation of a substrate with the reduction of molecular oxygen to water. Laccases can oxidase diverse substrates such as monophenolic and polyphenolic molecules as well as nonphenolic organic and some inorganic compounds. They are usually characterized as monomeric enzymes with three cupredoxine-like domains and with four characteristic histidine-rich copper-binding sites in the first and third domain. This description became insufficient with several recent discoveries, most notably with the thorough characterization of two types of two-domain laccases, which lack the second domain and are only active as homotrimer (or sometimes as homodimer) (Nakamura et al., 2003). Moreover, some enzymes were recently called laccases based on their ability to oxidase typical laccase substrates even though they were

structurally completely unrelated to multicopper oxidases (Beloqui et al., 2006). This paper describes a novel bacterial three-domain enzyme that is active as a monomer and is thus similar to “traditional” laccases initially described in fungi. However, we will refer to it with the term laccase-like multicopper oxidase (LMCO) rather than laccase to avoid ambiguity when referring to this extremely diverse group of enzymes (Reiss et al., 2013).

Fungal laccases have been most intensively studied and already applied in biotechnological applications at industrial scale (Rodriguez Couto and Herrera, 2006). However, recent discoveries indicated that bacteria encode a diverse array of genes showing high homology to three domain fungal laccases; some of them were also purified and their activity studied *in vitro* (Ausec et al., 2011b). While their substrate specificity varies widely, bacterial LMCOs usually prove to be robust in terms of their pH and temperature stability and often remain active at industrially relevant concentrations of chloride anions. These reasons justify further search for novel bacterial LMCOs. Several approaches to isolate new bacterial LMCOs have been successfully implemented, most notably (I) screening and direct isolation of the enzyme from LMCO-producing bacterial strains (Devasia and Nair, 2016), and (II) heterologous expression of LMCO-encoding genes from bacterial strains (Ausec et al., 2015). A radically different approach would be metagenomics, which aims at discovering the natural diversity that would otherwise be inaccessible due to poor cultivability of the vast majority of microorganisms. The way to achieve this is by direct extraction and cloning of total environmental DNA from a microbial community (Handelsman, 2004). In general, there are two ways of screening the metagenomic clones, each with its benefits and limitations (Schmeisser et al., 2007). Screening can be based on the functional traits that are expressed from the cloned metagenomic fragments. This can in principle yield completely unknown enzymes with desired properties; however low expression (or, more commonly, lack of expression) often limits the successful outcome of this approach. Alternatively, one can screen based on the fragment's DNA sequence, either by bioinformatic analysis of large amounts of sequencing data or by a PCR-based search-and-sequencing approach. While this approach may limit the scope of

novelty, it may be warranted especially when a heterogeneous group of enzymes such as bacterial LMCOs is explored.

Less than a handful of studies claim to have successfully implemented the metagenomic approach to find novel bacterial LMCOs (Beloqui et al., 2006; Ye et al., 2010; Fang et al., 2012a; Fang et al., 2012b). The reason for this low success lies in the difficulties brought about by the available screening methods, being those based on activity (metagenomic fragments are poorly expressed in *Escherichia coli* and similar hosts) or sequence (huge amount of manual work or sequencing). Still, the promise of metagenomics is too great to ignore, and we believe this is one of the very few papers that shows how metagenomics could yield interesting full-length laccase genes directly from an environment where fragments of such genes had previously been identified. In a previous study, we identified a natural wetland environment with a great diversity of bacterial LMCO genes that were in large part phylogenetically affiliated to *Acidobacteria*. This conclusion was based on the study of gene fragments identified through the traditional approach including PCR-based amplification, cloning and Sanger sequencing (Ausec et al., 2011a). This paper follows up on that story and demonstrates the use of metagenomics for finding full-length bacterial LMCO genes in the environment, previously recognized for high diversity of LMCO genes. A full-length gene for an acidobacteria-affiliated LMCO was cloned and expressed in recombinant *E. coli*, and the enzyme was characterized. The paper contextualizes the biochemical properties of the enzyme and discusses the difficulties in finding and purifying an enzyme derived from metagenomics.

2. Materials and methods

2.1 Metagenomics library construction

High molecular weight DNA was extracted from Ljubljana Marsh bog soil – Slovenia (soil described in Ausec et al., 2011a) using a modified protocol of (van Elsas et al., 2008). Briefly, 10 g of soil were suspended in 10 ml extraction buffer (100 mM Tris-HCl, 100 mM NaEDTA [Ethylenediaminetetraacetic acid sodium salt], 100 mM NaPO₄, 1.5% w/v NaCl, 1% w/v CTAB [cetyl trimethylammonium

bromide] pH 8.0) and sonicated (water bath sonicator) for 15 min. Proteinase K (100 μ l of 10 mg/ml) was then added and incubated for 2 h at 37°C with gentle shaking (200 rpm). DNA extraction was performed with phenol / chloroform / iso-amylalcohol (25:24:1) in a water bath at 60°C for 30 min. The metagenomic DNA was precipitated with 2-propanol and embedded in agarose plugs (1% w/v low melting point agarose). Removal of phenolic contaminants and separation of 30 - 40 kb size DNA fractions was performed by Pulsed Field Gel Electrophoresis (PFGE) on 1% w/v agarose gel supplemented in the upper part with 2% polyvinylpyrrolidone (PVP). Electrophoresis was run in 0.5X Tris-borate-ethylenediaminetetraacetic acid (TBE) at 14°C using a PFGE DRIII System (BioRad, Hercules, CA, USA) with the following parameters: gradient 6 V/cm, included angle 120°, initial switch time 0.5 s, final switch time 8.5, linear ramping factor, 20 h. The 2 cm agarose fragments containing DNA in the range of 30-40 kb were cut out of gel without staining and exposing to UV radiation. DNA was recovered using β -agarase (New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocol.

Metagenomic library was constructed using the CopyControl Fosmid Library Production Kit (Epicentre, Madison, WI, USA). The metagenomic DNA was blunt-ended, ligated into pCC1Fos fosmid vector and transformed into *E. coli* EPI300-T1^R (Epicentre, Madison, WI, USA). Positive selection of fosmid clones was done on LB (Luria Bertani) agar supplemented with 12.5 μ g/ml chloramphenicol. The metagenomic library was stored at -80°C according to manufacturer's recommendations). The clones were pooled into 27 pools with approximately 500 EPI300 colonies each. All the reagents were purchased from Sigma–Aldrich (St Louis, MO, USA) unless otherwise stated.

2.2 Screening the metagenomics library

Molecular PCR-based method was used to screen the 27 metagenomics library pools for potential novel laccase genes. In the initial step, PCR was used to amplify the putative metagenomics laccase genes using the primers Cu1AF and Cu4R and the protocol described in (Ausec et al., 2011a). These primers supported

amplification of three and two domain bacterial laccase genes with expected size of 1200-1500 bp or of ~ 600 bp for the three and two domain laccase genes, respectively. The pools giving rise to PCR amplicons of desired sizes were considered as potentially positive and further analyzed. First, the PCR amplicons of 1200-1500 bp and ~ 600 bp were gel purified and sequenced, and the sequences verified by BlastP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>). The pool that carried a clone with verified laccase gene was further analyzed to identify the specific clone carrying the LCMO gene. Hence, the pool was first diluted to 10^{-5} and cells plated on conventional LB agar supplemented with 12.5 µg/ml chloramphenicol. Approximately 800 clones per pool were picked randomly for further screening. At this step positive clones were verified by PCR using primers R1R and R1L, specific for metagenomics LCMO gene fragment identified during the initial screening of 27 pools. This laborious process allowed the identification of a specific individual clone that carried a putative LCMO gene referred to hereafter as *metaLacc*.

2.3 Sequence analysis of *metaLacc* gene

To obtain the whole gene of the putative laccase MetaLacc, the fosmid DNA was extracted from the identified clone and subjected to outward sequencing using primers R1FrC/R1RrC. MetaLacc sequence was compared with known protein sequences using BlastP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>). Multiple sequence alignment of MetaLacc with related sequences was performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Protein's module structure was predicted using SMART (Simple Modular Architecture Research Tool; <http://smart.embl-heidelberg.de/>). Presence and location of a putative signal peptide was estimated with the following bioinformatic tools, using default parameters: Phobius (<http://phobius.sbc.su.se/>), PRED-TAT (<http://www.compgen.org/tools/PRED-TAT/>), Signal Blast (<http://sigpep.services.came.sbg.ac.at/signalblast.html>), TatP 1.0 Server (<http://www.cbs.dtu.dk/services/TatP/>), SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). Neighbor-joining phylogenetic tree was constructed using Mega6 (Tamura et al., 2013) to compare MetaLacc with some

references retrieved from the public databases as well as genetic fragments obtained in our previous study (Ausec et al., 2011a).

2.4 Cloning and heterologous expression of MetaLacc

The *metaLacc* gene was amplified from a single metagenomic clone using a standard PCR protocol (as described in Ausec et al., 2015). Our goal was to clone the gene in two different plasmids, so it had to be amplified with two primer pairs to introduce different restriction sites for cloning. The two products of around 1400 bp were excised from 1% w/v agarose gel and purified using a PureLink Quick Gel Extraction Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). The gene was then cloned (I) into pET21c (Novagen, Merck Millipore, Kenilworth, NY, USA) between restriction sites *NdeI* and *XhoI*, which introduced His₆-Tag at C-terminal of the enzyme, and (II) into pET-DUET (Novagen, Merck Millipore, Kenilworth, NY, USA) between restriction sites *EcoRI* and *HindIII*, which introduced His₆-Tag at the N-terminal of the enzyme.

The protein was expressed using both vectors in *E. coli* BL21(DE3) pLysE expression host strain (Life Technologies, Carlsbad, CA, USA). In the Supplementary section, we describe in details the expression protocols that were tested with the aim of producing at least small amounts of active enzyme from recombinant *E. coli* strains. In brief, (I) conventional LB medium was compared with Terrific Broth (TB) medium, which is richer and does not contain sodium chloride, known as inhibitor of laccase activity (Xu, 1997; Baldrian, 2006). (II) Copper (0.25 or 0.5 mM CuSO₄) was added to the growth medium before cell inoculum or at the moment of induction to facilitate proper folding and full incorporation of copper into active sites of the enzyme (Durão et al., 2008). (III) Protein expression was induced by adding 0.1, 0.5 or 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to cells at the early exponential phase of growth (OD_{600nm} ~ 0.6 for LB or ~ 1 for TB) or at the late exponential phase of growth (OD_{600nm} ~ 4 for LB). (IV) Temperature after IPTG addition was kept at 37°C or reduced to 25 or 18°C. (V) Following IPTG addition, the cells were incubated for different time intervals (0, 2, 4 h or O.N.) before harvesting using different aeration

regimes (at 200 or 100 rpm or without shaking) since it has been reported that creating microaerobic conditions facilitates the production of fully copper-loaded laccases (Durão et al., 2008; Gunne et al., 2013). Total protein concentration in the fractions was estimated by the Biuret assay (Gornall et al., 1949), whereas MetaLacc production was estimated through sodium dodecyl sulfate polyacrylamide (10% w/v) gel electrophoresis (SDS-PAGE), Western Blot with Anti-HisTag antibody and laccase activity assay.

2.5 MetaLacc purification

The following protocol was used to produce the active enzyme for characterization. Overnight cultures of *E. coli* BL21(DE3) pLysE/pET-DUET::*metaLacc* were prepared by inoculating 2 ml of glycerol stocks into 80 ml of LB medium containing 100 µg/ml ampicillin and 20 µg/ml chloramphenicol. Cells (OD_{600nm} of 0.1) were inoculated in 2 l Erlenmeyer flasks containing 750 ml LB medium supplemented with 0.5 mM CuSO₄, 50 µg/ml ampicillin and 20 µg/ml chloramphenicol, and grown at 37°C and with shaking at 200 rpm. At the OD_{600nm} of 0.6, 1 mM IPTG was added and cells incubated for further 4 h. Cells were harvested by centrifugation at 8000 x g for 10 min. The cell pellets were resuspended in 20 mM phosphate buffer (KPi) pH 6.7, containing 10 µg/ml deoxyribonuclease I (DNaseI), 0.19 mg/ml phenylmethylsulfonylfluoride (PMSF), 0.7 µg/ml pepstatin, and then disrupted by sonication for 5 cycles of 30 s each (with a 30-s interval on ice) using a Branson Sonifier 250 (Danbury, CT, USA). After centrifugation at 34000 x g for 60 min at 4°C, N-terminus His₆-tagged MetaLacc was purified from the soluble cytoplasmic fraction by loading *E. coli* crude extracts, heated for 20 min at 50°C and centrifuged at 17000 x g for 30 min, onto a 5 ml Hitrap chelating affinity column (GE Healthcare Sciences, Little Chalfont, UK), loaded with 0.1 mM CuSO₄ and equilibrated with 20 mM KPi pH 6.7, 20 mM imidazole and 300 mM Na₂SO₄. The recombinant protein was eluted with increasing concentrations of 20 mM KPi pH 6.7, 300 mM Na₂SO₄ and 250 mM imidazole, and loaded onto a size-exclusion PD10 Sephadex G25 column (GE Healthcare Sciences, Little Chalfont, UK) equilibrated with 20 mM KPi pH 6.7.

Pure protein concentration was estimated by use of the theoretical extinction coefficient at 280 nm ($\epsilon_{280\text{ nm}} = 61.88\text{ mM}^{-1}\text{ cm}^{-1}$) based on aminoacid sequence.

2.6 SDS-PAGE electrophoresis, Western blot and zymogram

Protein purity was assessed by SDS-PAGE and staining with Coomassie Brilliant Blue (Schagger and van Jagow, 1987). For Western Blot analysis, the protein was identified by anti His-Tag antibody HRP conjugate (Novagen Inc., Madison, WI, USA) and detected by chemiluminescence (ECL Western Blotting Detection System, GE Healthcare Sciences, Little Chalfont, UK). Molecular weight markers were from the same supplier. For the zymogram (semi-denaturing SDS-PAGE), the purified enzyme was diluted in a sample buffer lacking any reducing agent and incubated for 10 min at room temperature before loading onto a 10% w/v SDS-PAGE gel. After electrophoresis running at 4°C, the gel was rinsed several times in distilled water to remove SDS and equilibrated for 1 h at 50°C in 100 mM sodium acetate buffer pH 4.0, containing 50 μM CuSO_4 and 10 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 2,6-dimethoxyphenol (2,6-DMP).

2.7 Standard ABTS assay

Laccase activity was routinely measured with a spectrophotometer (V460, Jasco, Easton, MD, USA) using ABTS ($\epsilon_{420\text{ nm}} = 36\text{ mM}^{-1}\text{ cm}^{-1}$) as a substrate: assay mixtures (1 ml) contained 1 mM ABTS, 10 μl of purified protein or 100 μl of crude extract, 50 μM CuSO_4 in a multi-component buffer at pH 4.0 (10 mM Trizma base, 15 mM sodium carbonate, 15 mM phosphoric acid and 250 mM potassium chloride) (Harris et al., 2001). Laccase from *Trametes versicolor* (Sigma-Aldrich) was used as positive control. All assays were performed in triplicate. The oxidation of the ABTS was followed as the change in absorbance at 420 nm and 25°C for 5 min. One activity unit (U) was defined as the amount of enzyme required for oxidizing 1 μmol of ABTS per min.

2.8 Substrate range

The ability of MetaLacc to oxidize phenolic and nonphenolic compounds was tested with a spectrophotometer using the following substrates at the designed final concentration and wavelength: 1 mM 2,6-DMP ($\epsilon_{468\text{nm}} = 49.6 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM pyrocatechol ($\epsilon_{450\text{nm}} = 2.21 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM pyrogallol ($\epsilon_{450\text{nm}} = 4.4 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM vanillic acid ($\epsilon_{316\text{nm}} = 2.34 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM $\text{K}_4\text{Fe}(\text{CN})_6$ ($\epsilon_{405\text{nm}} = 0.9 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM tyrosine ($\epsilon_{475\text{nm}} = 3.6 \text{ mM}^{-1} \text{ cm}^{-1}$), 0.5 mM L-3,4-dihydroxyphenylalanine (L-DOPA, $\epsilon_{475\text{nm}} = 3.7 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM guaiacol ($\epsilon_{468\text{nm}} = 12 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM syringic acid ($\epsilon_{300\text{nm}} = 8.5 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM ferulic acid ($\epsilon_{287\text{nm}} = 12.4 \text{ mM}^{-1} \text{ cm}^{-1}$), 10 μM syringaldazine ($\epsilon_{525\text{nm}} = 65 \text{ mM}^{-1} \text{ cm}^{-1}$), 1 mM syringaldehyde ($\epsilon_{320\text{nm}} = 8.5 \text{ mM}^{-1} \text{ cm}^{-1}$). All assays were performed in the range of pH from 2.0 to 8.0 in the multi-component buffer adjusted to appropriate pH with HCl or KOH.

2.9 Temperature optimum and thermostability

Enzyme activity at different temperatures was tested by following the oxidation of ABTS at pH 4.0 in the temperature range from 10 to 70°C. Enzyme stability was assessed by incubating MetaLacc for 30, 60, 90, or 120 min at 40, 50, 60, or 70°C; samples of the enzyme incubated at the different temperatures were withdrawn and residual activity on ABTS determined at pH 4.0 and at 25°C.

2.10 Enzyme kinetics

Michaelis-Menten curves for ABTS, 2,6-DMP and pyrogallol were obtained by using increasing concentrations of the substrates (0 to 20 mM), while maintaining constant the enzyme concentration. Assays were performed at 25°C and at pH 4.0 (for ABTS and pyrogallol) or 5.0 (for 2,6-DMP). Curve-fitting using Kaleidagraph (Synergy Software, Reading, PA, USA) was carried out to calculate K_m and k_{cat} value for MetaLacc.

2.11 Effect of inhibitory compounds

Effect of salts (up to 1 M final concentration), enzyme inhibitors (25-150 μM NaN_3 , 5% v/v dithiothreitol (DTT)), detergents (1-10% w/v Triton X-100, Nonidet P-40,

Tween-20, and N-lauryl sarcosine(NLS)), organic solvents (1-10% v/v ethanol, isopropanol, and methanol), and a chelating agent (20-100 μ M EDTA) on MetaLacc activity was investigated by adding each compound to the assay mixture and using the standard ABTS assay described above.

2.12 Dye decolorization

Decolorization experiments were conducted in 2-ml disposable cuvettes with 1 ml final reaction volume, over a panel of dyes: Remazol Brilliant Blue R (anthraquinone dye, maximum of absorbance at 495 nm), Direct Red 80 (azo dye, 528 nm), Reactive Black 5 (azo dye, 598 nm), Brilliant Green (triphenylmethane dye, 625 nm), Azure B (heterocyclic dye, 648 nm), Bromocresol Purple (triarylmethine dye, 719 nm). 3.5 μ g of purified MetaLacc were added to the reaction mixture containing the dye solution (previously prepared by dissolving the dye in the multi-component buffer at pH 4.0, at the final concentration of 100 μ g/ml), and 50 μ M CuSO₄. To evaluate if ABTS can serve as a redox mediator in dye oxidation, some reactions were supplemented with ABTS at final concentration of 10 μ M. Cuvettes were incubated at 200 rpm at 37°C for up to 48 h. Decolorization was evaluated with a spectrophotometer at constant time intervals at the maximum of absorbance for each dye in comparison with controls without MetaLacc. The percentage of decolorization was estimated by using the following equation (Zhuo et al., 2011): decolorization (%) = $[(A_i - A_t)/A_i] \times 100$, where A_i is the initial absorbance of the reaction mixture and A_t is the absorbance after incubation.

3. Results

3.1 Identification of the positive clone in metagenomics library by molecular approach

Molecular PCR-based method was used to screen the bog soil metagenomics library of 13,500 clones for the presence of LMCO genes. The library was divided into 27 pools, each with approximately 500 clones and was prepared from DNA extracted from an acidic soil, rich in SOM (soil organic matter), which has been previously

indicated for high diversity of bacterial laccases (Ausec et al., 2011a). We show here how metagenomics leads to the discovery of a novel bacterial laccase (laccase like multicopper oxidase - LMCO), which originates from a *phylum* dominating the soil that was used as a source of metagenomics DNA and whose representatives are still difficult to culture in the laboratory. First, by using the PCR amplification of LMCO genes by previously developed primers we identified two out of 27 pools to carry a putative laccase gene, which was first confirmed by sequencing of PCR fragments of appropriate length. The available sequence provided the needed information to design a new and more specific set of primers that helped us searching for a clone carrying the laccase gene. Only one out of the two pools finally gave rise to the clone carrying a 38 kbp long genomic fragment. The fragment was fully sequenced, affiliated through sequence comparison with *Acidobacteria* (data not shown) and proved to contain a gene coding for a three-domain laccase-like enzyme, which is referred to as MetaLacc.

3.2 Sequence analysis of *metaLacc* gene

metaLacc consists of 1359 nucleotides encoding a protein of 452 amino acid residues with a predicted molecular mass of 50.3 kDa and a theoretical isoelectric point of 6.54. *In silico* sequence analysis identified three conserved multicopper-oxidase (MCO) domains: a MCO type 3 domain (PFAM accession number PF07732) between amino acids 39 and 159, a MCO type 1 domain (PFAM accession number PF00394, residues 185-299) and a MCO type 2 domain (PFAM accession number PF07731, residues 326-451) (Figure 1a). Within these three domains, four copper binding regions were identified, corresponding to the highly conserved sequence motifs HxHG, HxH, HxxHxH and HCHxxxHxxxx[MLF] (Reiss et al., 2013). Histidine and cysteine residues involved in the coordination of the T1, T2 and T3 copper ions are highlighted in figure 1a. Additionally, a putative signal peptide, with a cleavage site between amino acids 29 and 30, was identified by four out of the five programs employed (Phobius, PRED-TAT, Signal Blast and TatP 1.0 Server). This signal peptide shows the typical features of TAT (Twin-Arginin Translocation) system-secreted proteins (Natale et al., 2008): a positively

charged n-region (residues 1-5), which includes the eponymous two-arginin motif, a hydrophobic h-region (residues 6-17) and a polar c-region (residues 18-29) (Figure 1a). MetaLacc shares amino acid sequence identities lower than 30% with well known bacterial or fungal laccases: this enzyme shows 23% identity with the outer spore coat copper-dependent laccase from *Bacillus subtilis* subsp. *subtilis* str. 168 (GenBank accession number CAB12449), 28% with the multicopper oxidase of *E. coli* str. K12 substr. W3110 (BAB96698), 24% and 25% with those from the fungi *Phlebia radiata* (CAA36379.2) and *Trametes versicolor* (CAA59161.1), respectively. The full length protein shows the highest sequence identity (66%) with a genetic fragment (ADV52204.1) identified in our previous study of bacterial laccases in bog soil (Ausec et al., 2011a). The phylogenetic tree (Figure 1b) indicates the novelty of our discovery, since the MetaLacc clusters with uncharacterized LMCOs from *Acidobacteria*.

3.3 Heterologous expression of MetaLacc

The gene for MetaLacc was cloned under the control of the IPTG inducible *T7* promoter into two plasmids, pET-DUET and pET21c, and expressed in the host *E. coli* BL21(DE3) pLysE either as a N-terminus or a C-terminus His₆-tagged protein, respectively. First expression trials following conventional procedures for heterologous protein production failed to produce any active laccase. For that reason, a panel of different cultivation and protein expression conditions were tested for both the recombinant strains as reported in detail in the Supplementary material section. In spite of these efforts, immunoblotting of soluble or insoluble cell extracts and of cell-free broths revealed that an intense protein band at the expected molecular mass for the His₆-tagged MetaLacc (51.2 kDa) was detectable only in insoluble fractions (Supplementary Material and Figure 2a). This band was absent in the control strains transformed with the empty vectors. The enzyme assay of these fractions revealed that the putative laccase was not active, probably due to its accumulation in inclusion bodies (IBs), as is often the case when *E. coli* is used to express enzymes from metagenomic sources (Sørensen and Mortensen, 2004; Hjort et al., 2014; Cretoiu et al., 2015).

From all the conditions tested, traces of active MetaLacc, however, were detected in the soluble cytoplasmic fractions only when *E. coli* BL21(DE3) pLysE/pET-DUET::*metaLacc* cells were grown at 37°C in LB medium supplemented with 0.5 mM CuSO₄ and 1 mM IPTG was added during early exponential growth phase (Figure 2a). Indeed, while approximately 95% of the MetaLacc accumulated into insoluble fractions using this approach, immunoblotting revealed faint bands of the enzyme in the cytoplasmic fractions that were collected after cell incubation for 4 or 24 h. These soluble fractions were shown to contain the active enzyme, approximately 0.10 U/g_{cell} corresponding to 0.25 U/l culture. This was sufficient for the initial enzymatic characterization presented in this paper.

MetaLacc was purified using affinity chromatography. *E. coli* cytoplasmic fractions were heated before being loaded onto the Hitrap chelating affinity column in order to facilitate the precipitation of most of *E. coli* proteins, as suggested for purification of other bacterial laccases (Tonin et al., 2016). Subsequent SDS-PAGE analysis showed MetaLacc to be an approximately 51 kDa protein (Figure 2b) that could be detected also with ABTS or DMP-based zymogram electrophoresis in semi-denaturing conditions (Figure 2c). Interestingly, when the Hitrap chelating affinity column was loaded with CuSO₄ instead of the most commonly employed NiCl₂, the activity of purified enzyme on ABTS tripled, indicating that copper may be additionally loaded into the enzyme at this stage of protein isolation. Finally, more than 50% of the activity initially detectable in soluble fraction was recovered as purified protein, with a purification yield of 47 µg/g_{cell} corresponding to 180 µg/l culture.

3.4 MetaLacc characterization

3.4.1 Addition of copper increased MetaLacc activity

As explained in the previous section, activity of the isolated enzyme was drastically improved by using a copper-loaded affinity column. Moreover, MetaLacc activity was increased if copper was added to the reaction mixture (Figure 3a): adding 10, 50 and 100 µM CuSO₄ to the ABTS assay mixture increased the activity of 5, 9 and 12-fold, respectively. This effect was confirmed also by zymogram electrophoresis

of the purified MetaLacc: when 50 μM CuSO_4 was added to the developing buffer (100 mM sodium acetate pH 4.0), the intensity of the band corresponding to the active laccase was significantly improved both using ABTS and 2,6-DMP as substrates (data not shown). For this reason, 50 μM CuSO_4 was consistently added to the reaction mixture of what we call the standard ABTS assay throughout this study.

3.4.2 Substrate range and pH optimum

The capability of MetaLacc to oxidize phenolic and nonphenolic compounds was tested on the thirteen substrates listed in the Material and Methods section. Since the activity of laccases on different substrates depends on pH, the enzyme activity of the recombinant protein was assayed for each substrate in the pH range between 2.0 and 8.0. The enzyme oxidized seven out of thirteen tested substrates: the nonphenolic ABTS (Figure 3b) and the phenolic 2,6-DMP, L-DOPA, pyrogallol, pyrocatechol, vanillic acid and syringaldazine (Figure 4a-f). For each compound, the pH-activity profile was bell-shaped, with optima at acidic pH. The highest enzyme activities were detectable on pyrogallol and pyrocatechol, *i.e.* phenolic substrates with three and two substituted hydroxyl groups respectively. MetaLacc optimally oxidized both these substrates at pH 4.0 with a specific activity of 57 U/mg on pyrogallol and 39.1 U/mg on pyrocatechol. Other substrates were oxidized much less efficiently. For ABTS, which is a reference nonphenolic substrate commonly used for laccase characterization, the maximum of activity was recorded at pH 4.0 (2.4 U/mg), while 2,6-DMP was better oxidized at pH 5.0 (2.1 U/mg): MetaLacc oxidized 2,6-DMP in a wide pH interval from 3.0 to 8.0 (Figure 4c). Finally, the highest activities on L-DOPA, syringaldazine and vanillic acid, at pH 4.0, were respectively 2.9, 2.4 and 1.0 U/mg. Other methoxy-substituted phenolic compounds like guaiacol, syringic acid, ferulic acid and syringaldehyde, as well as tyrosine and the non-phenolic $\text{K}_4\text{Fe}(\text{CN})_6$ were not oxidized by MetaLacc.

3.4.3 Kinetic constants

Kinetic parameters of MetaLacc were determined for two reference laccase substrates, ABTS and 2,6-DMP, and for pyrogallol, that was the preferred MetaLacc substrate. The initial reaction rates at various substrate concentrations were measured at the pH optimum for each substrate, as in Table 1. The results confirmed what described above: the lowest K_M value (0.13 mM) was found for pyrogallol; consistently, the turnover rate (kcat) and the catalytic efficiency (kcat/ k_M) values on this substrate were highest (22.4 s⁻¹ and 173.6 s⁻¹mM⁻¹, respectively). Catalytic efficiency for pyrogallol was 20.5 times and 27 times higher than that for ABTS and 2,6-DMP, respectively.

3.4.4 Temperature optimum and thermal stability

MetaLacc could oxidize ABTS in a range of temperatures (Figure 3c), with the highest oxidation rate recorded at 50°C (3.5-fold higher than the one recorded at standard conditions, *i.e.* 25°C). At 40 and 60°C, the enzyme maintains 65 and 56% of the maximum activity, respectively, while at 30°C and 70°C activity of MetaLacc was below 40%. Furthermore, activity of MetaLacc was severely reduced below 25°C. MetaLacc was not only thermostable in the range from 40 to 60°C (Figure 3d), but it displayed a substantial heat activation, since after 2 h at 40, 50, or 60°C, its enzymatic activity increased and it was approx. 1.2, 3.5, and 1.2-fold higher than the initial activity. However, after 2 h at 70°C MetaLacc retained only 28% of the initial activity.

3.4.5 Effect of salts and other compounds on Metalacc activity

The effect of several compounds on MetaLacc activity was then investigated. Strong inhibition by halide anions is a common feature of laccases (Xu, 1997; Baldrian, 2006); hence, the effect of chloride on MetaLacc activity was initially determined using NaCl as halide donor. Interestingly, between 95 and 85% of MetaLacc activity was preserved in the presence of salt concentrations up to 100 mM, and 34 and 10% residual activities were still detectable after enzyme incubation with 500 and 1000 mM NaCl (Figure 3e). Similarly, addition of 100 mM

KCl reduced MetaLacc activity by only 25% (Table 2). MgCl₂, CaCl₂ and ZnCl₂ were more powerful inhibitors than NaCl or KCl, probably because of the higher molar content of chloride anions. MgSO₄ and ZnSO₄ inhibited MetaLacc activity to a smaller extent than chloride salts, while K₂SO₄ and CoNO₃ even increased laccase activity at low concentrations. The chelating agent EDTA (at 0.1 mM) completely inhibited MetaLacc activity (Table 2), indicating the reversible nature of copper coordination within the active site of the enzyme. Known enzyme inhibitors as sodium azide and DTT strongly inhibited MetaLacc activity. The inhibitory effect caused by NaN₃ may be due to the binding of N₃⁻ to the metal ion sites of MetaLacc, which can disrupt the internal electron transfer of the catalysis process (Chen et al., 2015). DTT, instead, likely inhibits MetaLacc activity through the reduction of oxidized substrate by their sulfhydryl groups (Chen et al., 2015). Among the surfactants tested, Nonidet P-40, Tween-20 and *N*-lauryl sarcosine (NLS) significantly reduced MetaLacc activity only at 10% v/v final concentration, while with 1% v/v detergent more than 80% of the original activity was preserved. MetaLacc was more strongly affected by Triton X-100: at 1% v/v final concentration, the activity was reduced to approx. 40%, and at 10% v/v it was completely absent. Finally, in order to verify the potential for using MetaLacc in processes requiring organic solvents, for example for the solubilization of lignin, the effect of a panel of organic solvents on enzymatic activity was investigated (Table 2). At 1% v/v ethanol, isopropanol, and methanol activity of MetaLacc was increased. Interestingly, the positive effect of methanol was even higher at the final tested concentration of 10% v/v. In the presence of DMSO, the activity decreased, yet almost 50% of the initial activity was retained at 10% v/v DMSO.

3.4.6 Dye decolorization

Enzymatic decolorization of six synthetic dyes by the purified MetaLacc was assessed in the presence or absence of 10 μM ABTS as a redox mediator. The highest decolorization was found for the azo dye Reactive Black 5: after 48 h of incubation, the decolorization percentage was 92% and 43% in the presence or absence of ABTS, respectively (Figure 3f). The decolorization of the

triphenylmethane dye Brilliant Green was on the contrary higher in the absence of redox mediator, being 45 and 18% after 48h without and with ABTS, respectively (Figure 3f). The other dyes were not decolorized.

4. Discussion

This paper describes the discovery, production and characterization of MetaLacc, a novel bacterial laccase obtained using metagenomics. Based on the sequence similarity of the laccase gene, the new enzyme can be affiliated to the elusive, yet ubiquitous and diverse, bacterial *phylum* of *Acidobacteria*. The fact that the enzyme originates from metagenomics and *Acidobacteria* is at present certainly one of most interesting features of MetaLacc, and it will be discussed in more detail below.

Production of active MetaLacc in sufficient amount for its biochemical characterization was not straightforward. *E. coli* is generally predicted to readily express only 40% of environmental genes (Gabor et al., 2004). What is more, *E. coli* is not the most efficient microbe when it comes to secretion of proteins, since it tends to segregate heterologous proteins in inactive form in IBs (Hjort et al., 2014; Cretoiu et al., 2015). Consequently, relatively few enzymes from metagenomic sources could be purified in milligram amounts, hence usually allowing only for a limited biochemical and functional characterization. While massively produced in *E. coli*, the recombinant MetaLacc always precipitated in IBs as inactive. This was contrary to our expectations based on a preliminary *in silico* analysis of MetaLacc amino acid sequence using the model proposed by Diaz and co-workers (Diaz et al., 2010), which predicted a high chance of solubility (> 80%) if overexpressed in *E. coli*. Certainly MetaLacc was not the first problematic laccase in this respect, other examples of recombinant bacterial laccases accumulated in *E. coli* into IBs are reported by (Martins et al., 2002; Fang et al., 2011; Mollania et al., 2013; Ihssen et al., 2015). Different attempts for MetaLacc recovery from IBs were conducted (data not shown), including methods based either on strong denaturing agents like urea (Datta et al., 2013; Mollania et al., 2013), or on mild solubilizing conditions, such as the ones described in (Okumura et al., 2006; Tao et al., 2010; Singh et al., 2012). While we were able to partially dissolve IBs using these methods, MetaLacc was

never recovered in an active form. Alternative strategies tried for some other laccases might work for MetaLacc, e.g. removal of the TAT signal peptide (as in Fang et al., 2012a; Ihssen et al., 2015), the deletion of the His-Tag (as in Fang et al., 2014), or the application of random and site-directed mutagenesis (as in Koschorreck et al., 2009). These have thus far not been tried. However, after having tested many different expression conditions specifically aimed at increasing soluble laccase production (for instance, addition of CuSO₄, decrease of temperature after induction of gene expression, incubation in microaerobic environment), we could finally isolate sufficient amounts of the enzyme from the cytoplasmic fraction to allow for a biochemical characterization and comparison of MetaLacc with other bacterial laccases.

The substrate range of laccases varies significantly from one enzyme to another due to differences in shape and chemical composition of the substrate-binding site of the enzyme. In the case of phenolic substrates, the type, number, and position of chemical substitutions may influence the activity of laccases (Margot et al., 2013; Ihssen et al., 2015). MetaLacc was able to oxidize a variety of phenolic substrates with the highest specific activities recorded on pyrogallol and pyrocatechol, which are compounds with three and two hydroxyl groups, respectively. Among previously characterized bacterial laccases, high specificity towards these two products (that could be linked to lignin degradation) was reported only for a laccase from *Streptomyces lavendulae* (Suzuki et al., 2003). MetaLacc catalytic efficiency on pyrogallol was of 173.6 s⁻¹mM⁻¹, but we could not compare this result to the one from *S. lavendulae*, since the kinetic parameters for pyrogallol were not reported. MetaLacc was further able to oxidize 2,6-DMP (tri-substituted compound with two hydroxyl and one methoxyl group), but it was not active on guaiacol, a compound with one hydroxyl and one methoxyl group. Moreover, the recombinant enzyme oxidized L-DOPA and vanillic acid, but not syringic acid. The lack of activity on tyrosine indicated that MetaLacc was a laccase and not a tyrosinase, which is important since substrate specificities in these two groups of enzymes are known to overlap. Accordingly, MetaLacc could oxidize syringaldazine, which is a

compound commonly oxidized by laccases but not by tyrosinases or peroxidases (Fang et al., 2012b).

ABTS and 2,6-DMP are compounds most commonly used for laccase activity determination. For this reason, we analyzed the kinetic parameters of MetaLacc using these two substrates. Compared to metagenomic laccase Lac21 (Fang et al., 2012b) that could not oxidize ABTS, MetaLacc had a lower catalytic efficiency ($k_{\text{cat}}/k_{\text{M}}$ value of $6.42 \text{ s}^{-1}\text{mM}^{-1}$) on 2,6-DMP compared to Lac21 ($k_{\text{cat}}/k_{\text{M}}$ value of $82.5 \text{ s}^{-1}\text{mM}^{-1}$). If compared to the metagenomics-derived Lac591 (Ye et al., 2010), MetaLacc showed higher activity on the non-phenolic ABTS ($k_{\text{cat}}/k_{\text{M}} = 0.47 \text{ s}^{-1}\text{mM}^{-1}$ for Lac591, $8.45 \text{ s}^{-1}\text{mM}^{-1}$ for MetaLacc), but lower activity on the phenolic 2,6-DMP ($k_{\text{cat}}/k_{\text{M}} = 283 \text{ s}^{-1}\text{mM}^{-1}$ for Lac591). MetaLacc was less active on both ABTS and 2,6-DMP than CotA-like laccases from *Bacillus coagulans* (Ihssen et al., 2015) and *B. pumilus* (Reiss et al., 2011). It showed lower catalytic efficiency on ABTS than the CotA-like laccase from *B. clausii* (Ihssen et al., 2015) and SilA from *Streptomyces ipomoea* (Molina-Guijarro et al., 2009), but on 2,6-DMP its activity was comparable or even higher than the two last mentioned enzymes. MetaLacc was more active on both ABTS and 2,6-DMP than the recently described one from the alkaliphilic bacterium *Thioalkalivibrio* sp. (Ausec et al., 2015) and less active than RL5 from the bovine rumen microflora (Beloqui et al., 2006).

A general tendency of bacterial compared to fungal laccases is their activity in a wider pH range (Reiss et al., 2013; Brander et al., 2014). As was found for laccases originating from *Bacillus* spp. (Brander et al., 2014; Guan et al., 2014; Martins et al., 2002; Reiss et al., 2011) and *Streptomyces* spp. (Margot et al., 2013; Dubè et al., 2008; Reiss et al., 2013), MetaLacc was active on ABTS in the pH range 4.0-5.0. However, MetaLacc was active on phenolic substrates in a much wider range of pH, retaining 75% of activity on 2,6-DMP at pH 8.0. Similar behavior was observed on the two preferential substrates pyrogallol and pyrocatechol: at pH 8.0 the residual activity was higher than 50%. This feature of MetaLacc can be useful for future applications where neutral or slightly alkaline pH is desirable, including for instance oxidation of natural and synthetic dyes, bioremediation strategies at neutral pH, and organic synthesis reactions (Brander et al., 2014).

The thermo-stability of bacterial laccases is another key property making them attractive in diverse applications where protein lifetime is of major importance (Suzuki et al., 2003; Martins et al., 2002; Guan et al., 2014; Ihssen et al., 2015). MetaLacc proved to be a thermophilic enzyme with an optimum at 50°C, while maintaining a relatively high activity in a wide range of temperatures from the 25°C to the 70°C. Interestingly, MetaLacc was thermo-activated by incubating it for one or two hours at 50°C, and in this feature it differs from what had been observed for other thermostable bacterial laccases including CotA.

Additionally, MetaLacc exhibited a high tolerance towards salts, favoring its exploitation for industrial and environmental applications wherein high amounts of salts are used, such as treatment of industrial and municipal waste water or kraft pulp biobleaching. MetaLacc activity was even increased by adding MgSO₄, CoNO₃ and K₂SO₄, up to 10, 10 and 50 mM respectively. Enzyme activation by some of these ions had been reported in a few bacterial laccases (Molina-Guijarro et al., 2009; Fang et al., 2012a; Sondhi et al., 2014). Eukaryotic laccases are generally sensitive toward halides and most fungal laccases are inactivated by NaCl at concentrations higher than 100 mM (Guan et al., 2014; Jimenez-Juarez et al., 2005). Hence, bacterial laccases showing halide tolerance recently attracted much attention for their application in wastewater treatment (Molina-Guijarro et al., 2009; Jimenez-Juarez et al., 2005; Guan et al., 2014; Fang et al., 2011; Fang et al., 2012b). The high resistance of MetaLacc towards NaCl may be advantageous for its application in the treatment of both municipal waste water (where the NaCl concentrations is typically 2.5-5 mM), and industrial waste water or seawater (with NaCl concentrations up to 550 mM) (Margot et al., 2013).

Interestingly the addition of alcohols, in particular methanol, seemed to increase MetaLacc activity and that property might play a relevant role in applications that require getting access to insoluble substrates, such as in the detoxification of persistent organic pollutants. Fungal laccases are generally inactivated in organic solvents, whereas some bacterial solvent-resistant laccases are known. Less common is the activation by methanol seen in MetaLacc (Guan et al., 2014).

Considering relative robustness of MetaLacc, we tested its potential for the bio-bleaching of industrial textile dyes. Biological treatment of textile dyes in industrial waste water requires thermostable enzymes to remain active even under alkaline pH conditions or with high concentrations of organic solvents (Guan et al., 2014). After a preliminary screening of synthetic dyes, we discovered that MetaLacc might be further exploited for decolouring azo dyes and triphenylmethane dyes in the presence or absence of redox mediators.

To conclude, MetaLacc is a novel bacterial laccase-like enzyme. Apart from uncovering its interesting biochemical characteristics, the present study makes a case of metagenomics fulfilling its promise. In a follow-up from a previous study, metagenomics was used to obtain full length gene directly from an environment. While not identical to the genetic fragments we had identified earlier, the obtained gene still resembles them better than any other known laccase (or even database sequence). Only a few bacterial laccases have been identified through metagenomics: Lac15 and Lac21 from a marine microbial metagenomic library (Fang et al., 2011; Fang et al., 2012b), RL5 e Lac51 from a bovine rumen microflora (Beloqui et al., 2006), a laccase from a giant panda fecal microbiome (Fang et al., 2012a), and Lac591 from a mangrove soil metagenome (Ye et al., 2010). However, MetaLacc is the first one to be associated with acidobacteria. Members of the *phylum Acidobacteria* are among the most abundant microorganisms found in soil ecosystems and are also widely distributed in high-latitude terrestrial ecosystems (Mannisto et al., 2007; Pankratov et al., 2008; Campbell et al., 2010). Although ubiquitous in the environment, the ecological role of acidobacteria is poorly understood. Members of this *phylum* are relatively difficult to cultivate and thus only a few strains have been isolated and characterized (Mannisto et al., 2011; Janssen et al., 2006) or their genomes sequenced (Ward et al., 2009). Thus, MetaLacc is one of the very few enzymes of acidobacterial origin to have been extensively biochemically characterized.

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Figure captions

Figure 1 Analysis of MetaLacc protein sequence. **a)** MetaLacc protein sequence includes: a putative twin arginine translocation (TAT) system signal peptide (TATSP, residues 1-29), a multicopper oxidase type 3 domain (MCO-3, 39-159), a MCO type 1 domain (MCO-1, 185-299) and a MCO type 2 domain (MCO-2, 326-451). The amino acidic composition of the putative signal peptide (according to Phobius) is indicated in details. Amino acids corresponding to the four copper-binding regions are highlighted. Within them, residues involved in the coordination of the type 1 (T1 or blue) copper atom are indicated by asterisks (*), while residues responsible for binding the type 2 (T2 or normal) or the type 3 (T3 or coupled binuclear) coppers are indicated with the section sign (§) or with hash (#), respectively. **b)** Neighbor-joining phylogenetic tree of MetaLacc gene with reference sequences from GenBank and our previous work (Ausec et al., 2011) – *fen* and *bog* refer to two studied wetland environments. While different from previously identified sequences, MetaLacc clusters with other LMCO-like genes from *Acidobacteria*.

Figure 2 SDS-PAGE analysis of MetaLacc from *E. coli* BL21(DE3) pLysE/pET-DUET::*metaLacc* cells. **a)** Western Blot analysis of soluble and insoluble fractions of *E. coli* BL21(DE3) pLysE/pET-DUET::*metaLacc* cells grown in LB supplemented with 0.5 mM CuSO₄ at 37°C and 200 rpm; protein expression was induced by adding 1 mM IPTG during early exponential growth phase (OD_{600nm} of 0.6). The equivalent of 1 ml of culture was loaded in each lane. Protein concentration was calculated by densitometric analysis of SDS gel bands with the software Quantity One (Bio-Rad Laboratories, Hercules, USA) and His₆-glycyl oxidase (His₆-GO) from *Bacillus subtilis* (kindly provided by Loredano Pollegioni, University of Insubria) (Job et al., 2002) as a standard (Std). **b)** SDS-PAGE analysis of chromatography fractions. C.E.: crude extract; MetaLacc: purified protein. Std: reference standard protein, His₆-GO (5 µg, 42.66 kDa). LMW: molecular weight markers, from GE Healthcare Sciences, Little Chalfont, UK. **c)** Zymogram analysis in semi-native conditions of the purified MetaLacc with ABTS (1) or 2,6-DMP (2)

as substrates. In a) and b), LMW are molecular weight markers, from GE Healthcare Sciences, Little Chalfont, UK. From a) to c) MetaLacc is indicated by arrow.

Figure 3 MetaLacc Characterization. **a)** Effect of increasing concentrations of CuSO_4 on MetaLacc activity. Enzyme activities were measured on ABTS as substrate, at pH 4.0 and 25°C , and are reported as relative to the activity of 0.27 U/mg (set as 1) recorded in the absence of copper. **b)** Effect of pH on MetaLacc activity at 25°C on ABTS, in the presence of $50\ \mu\text{M}$ CuSO_4 . Activities are reported as relative to the activity of 2.4 U/mg protein (set as 100%) at pH 4.0. **c)** Effect of temperature on MetaLacc activity on ABTS at pH 4.0 in the presence of $50\ \mu\text{M}$ CuSO_4 . Enzyme activities are expressed as relative to the highest activity of 8.0 U/mg (set as 100%) recorded at 50°C . **d)** Stability on MetaLacc at different temperatures. The recombinant enzyme was incubated at 40°C (●, continuous line), 50°C (▲, continuous line), 60°C (■, dashed line) or 70°C (◆, dotted line) up to 120 min. At regular intervals, enzymatic fractions were cooled down and the residual activity on ABTS assayed at 25°C , pH 4.0 in the presence of $50\ \mu\text{M}$ CuSO_4 . The activity recorded at time 0 in standard assay condition (25°C , pH 4.0, $50\ \mu\text{M}$ CuSO_4) is set as 100% (2.4 U/mg). **e)** Effect of increasing concentrations of NaCl on MetaLacc activity on ABTS. The activity measured at pH 4.0 and 25°C , in the presence of $50\ \mu\text{M}$ CuSO_4 and without NaCl (2.4 U/mg) is set as 100%. **f)** MetaLacc decolorization of Reactive Black 5 (circles) or Brilliant Green (squares) in the presence (continuous line) or absence (dotted line) of $10\ \mu\text{M}$ ABTS. From a) to f), values represent the means of three independent experiments (mean \pm standard error).

Figure 4 MetaLacc activity versus pH on: **a)** vanillic acid, **b)** L-DOPA, **c)** 2,6-DMP, **d)** syringaldazine, **e)** pyrocatechol, **f)** pyrogallol. Enzyme activities were measured in a multi-component buffer in the pH range from 2.0 to 8.0, with $50\ \mu\text{M}$ CuSO_4 supplemented to the assay mixture. Values represent the means of three independent experiments (mean \pm standard error).

Figures

Figure 1

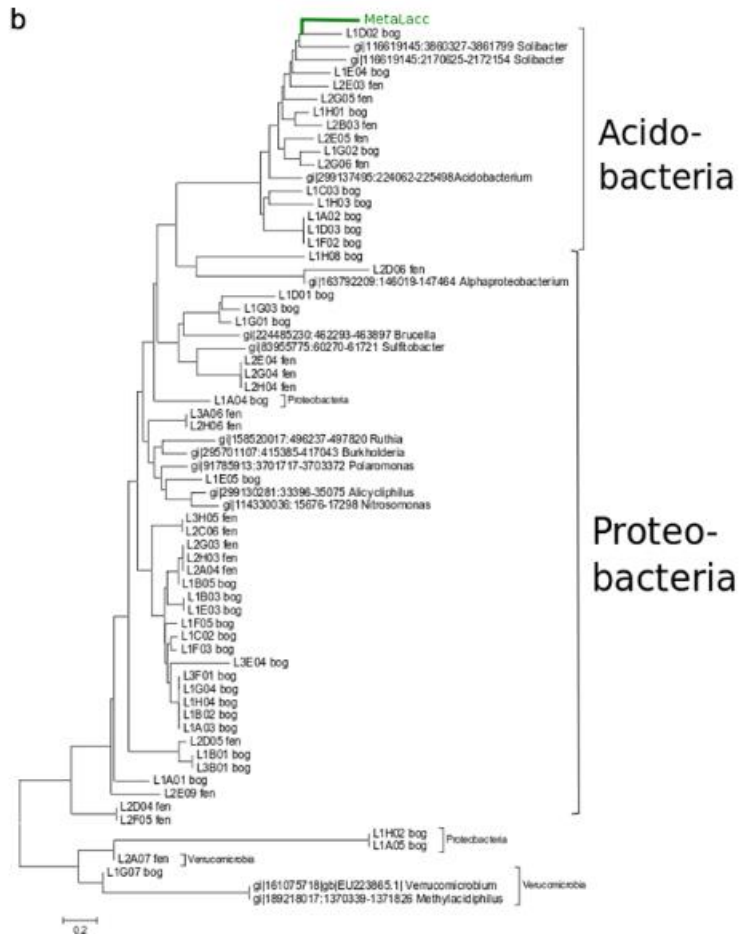
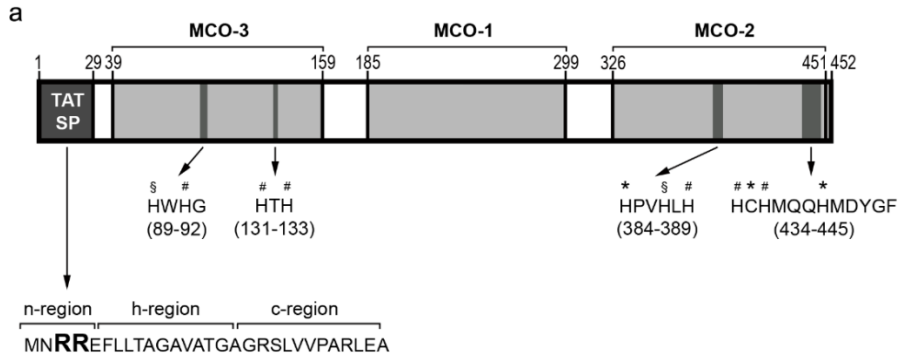


Figure 2

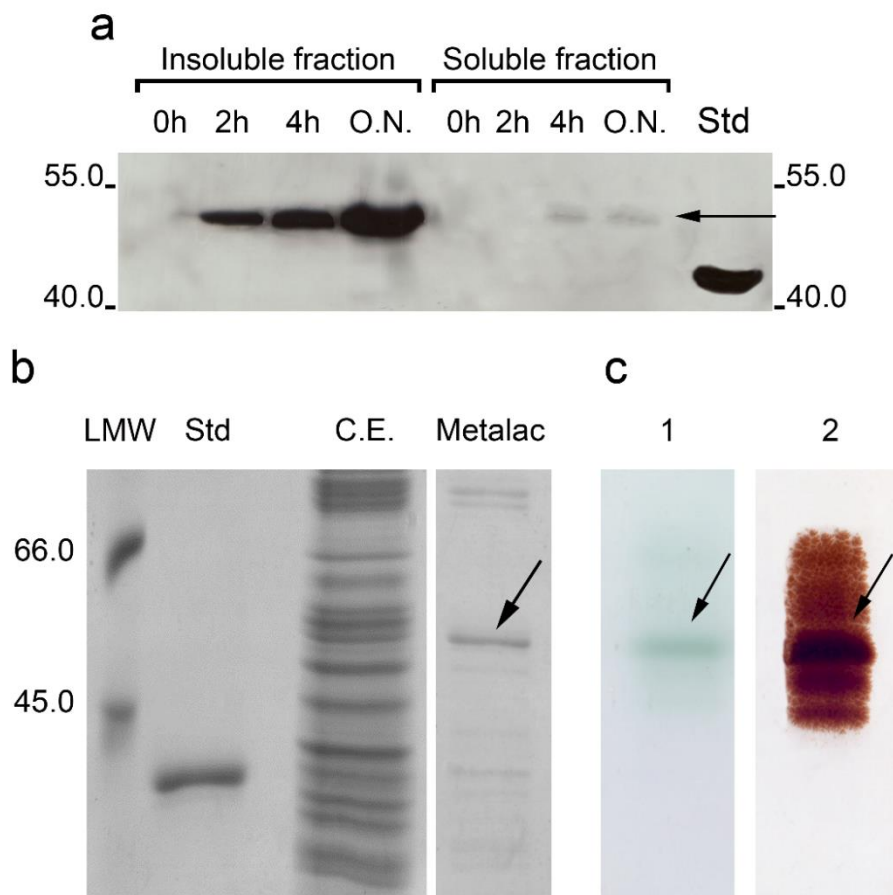


Figure 3

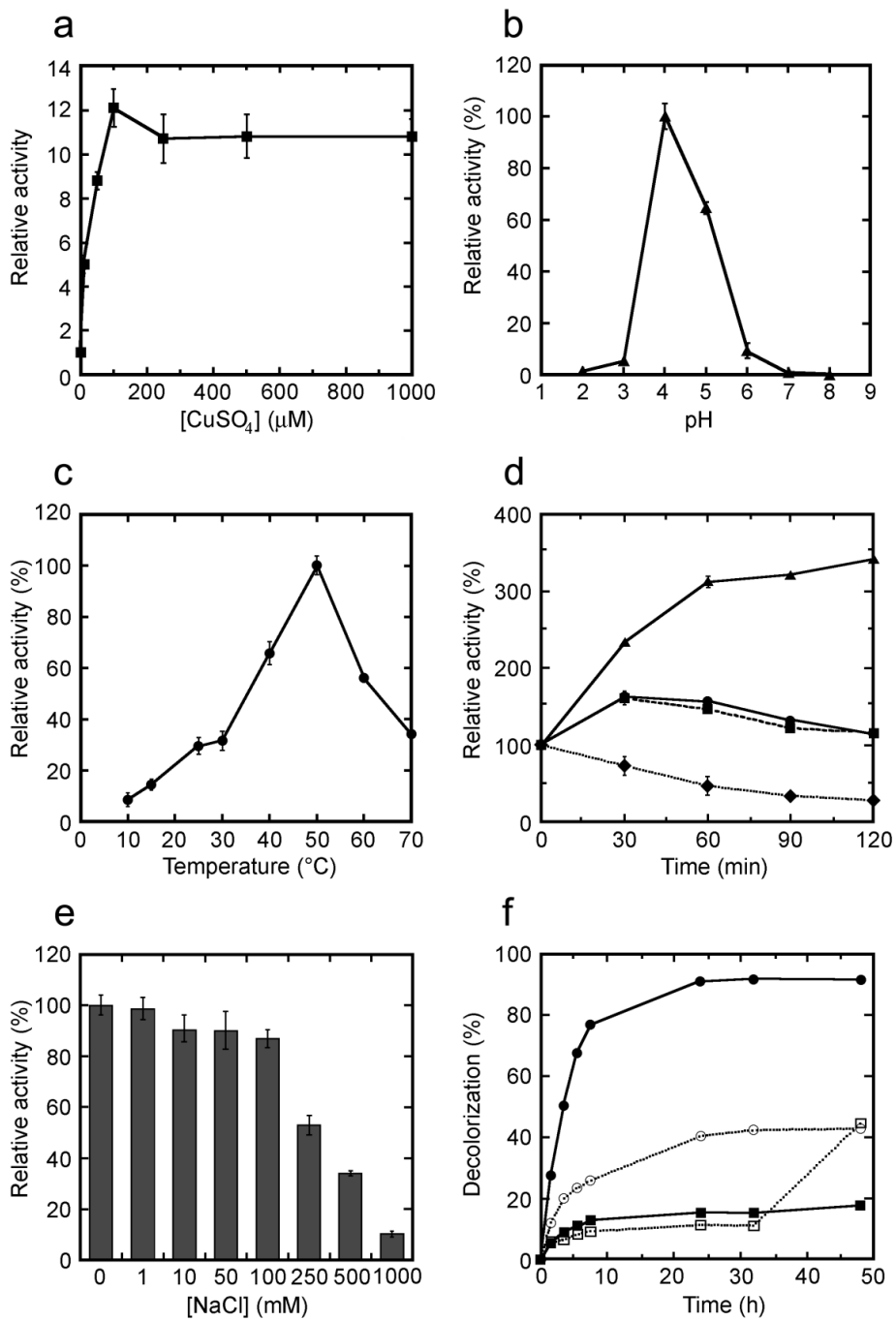


Figure 4

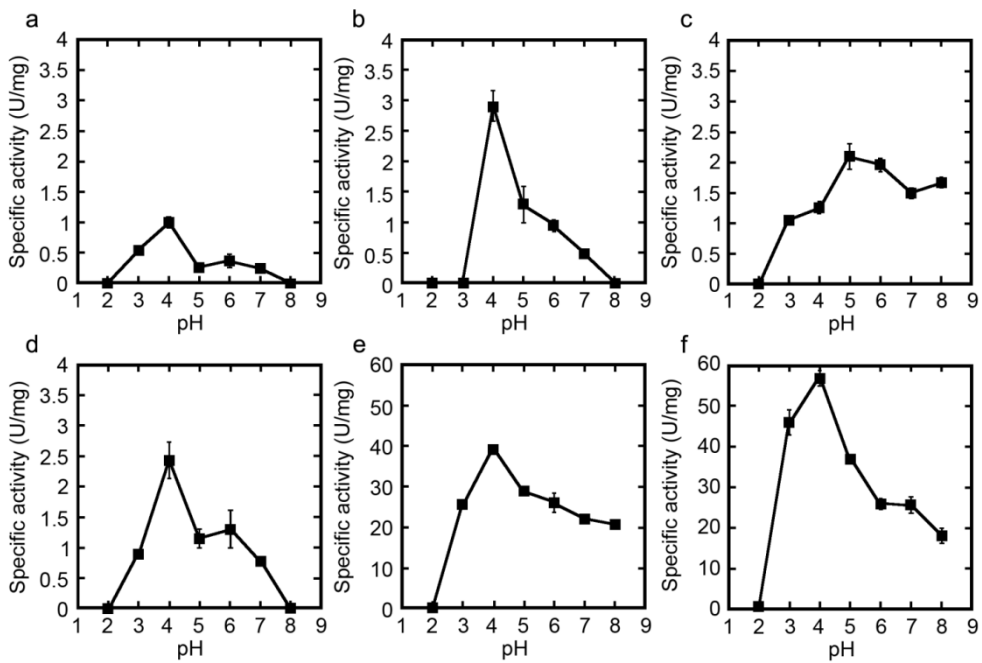


Table 1 Kinetic proprieties of MetaLacc on ABTS, 2,6-DMP and pyrogallol.

Substrate	k_M (mM)	k_{cat} (s^{-1})	k_{cat}/k_M ($s^{-1}mM^{-1}$)
ABTS	0.71	6.0	8.45
2,6-DMP	1.04	6.7	6.42
Pyrogallol	0.13	22.4	173.6

Table 2 Characteristics of MetaLacc. The enzyme activity is reported as relative to the activity of 2.4 U/mg protein (set as 100%), measured on ABTS at pH 4.0 and 25°C, with 50 μ M $CuSO_4$.

Compound	Final concentration	Relative activity (%)	Compound	Final concentration	Relative activity (%)
Control		100			
Salts (mM)					
CaCl ₂	10	98.1 ± 1.0	K ₂ SO ₄	10	169.0 ± 2.0
	50	66.9 ± 1.5		50	131.0 ± 1.0
	100	15.3 ± 8.6		100	71.0 ± 2.0
MgCl ₂	10	58.7 ± 2.3	ZnSO ₄	10	81.0 ± 1.5
	50	24.1 ± 1.3		50	<i>n.d</i>
	100	0.0 ± 0.0		100	57.0 ± 1.5
MgSO ₄	10	123.0 ± 1.0	ZnCl ₂	10	63.0 ± 2.0
	50	<i>n.d</i>		50	35.0 ± 5.5
	100	72.0 ± 1.5		100	26.0 ± 5.0
KCl	10	81.0 ± 10.5	CoNO ₃	10	141.0 ± 3.0
	50	<i>n.d</i>		50	71.0 ± 1.0
	100	75.0 ± 7.5		100	63.0 ± 6.5
EDTA (μ M)					
	20	96.0 ± 5.0			
	50	19.0 ± 4.5			
	80	9.0 ± 3.5			
	100	0.0 ± 0.0			

Enzyme inhibitors					
NaN ₃ (μM)	25	99.0 ± 4.5	DTT	(% 5	1.4 ± 0.8
	50	62.0 ± 4.5	(v/v)		
	100	38.0 ± 5.0			
	150	0.0 ± 0.0			
Organic solvents (% (v/v))					
Ethanol	1	117.0 ± 1.0	Methanol	1	143.0 ± 1.5
	10	95.0 ± 2.0		10	154.0 ± 3.0
Isopropanol	1	137.0 ± 1.5	DMSO	1	96.0 ± 1.5
	10	91.0 ± 4.0		10	48.0 ± 4.5
Detergents (% (v/v))					
Nonidet P-40	1	80.0 ± 7.0	Triton X-100	1	39.0 ± 0.0
	10	26.0 ± 6.0		10	0.0 ± 0.0
Tween-20	1	85.0 ± 1.7	NLS	1	88.0 ± 3.5
	10	0.0 ± 0.0		10	15.0 ± 5.5

n.d means not detected

Supplementary material

Heterologous expression of MetaLacc

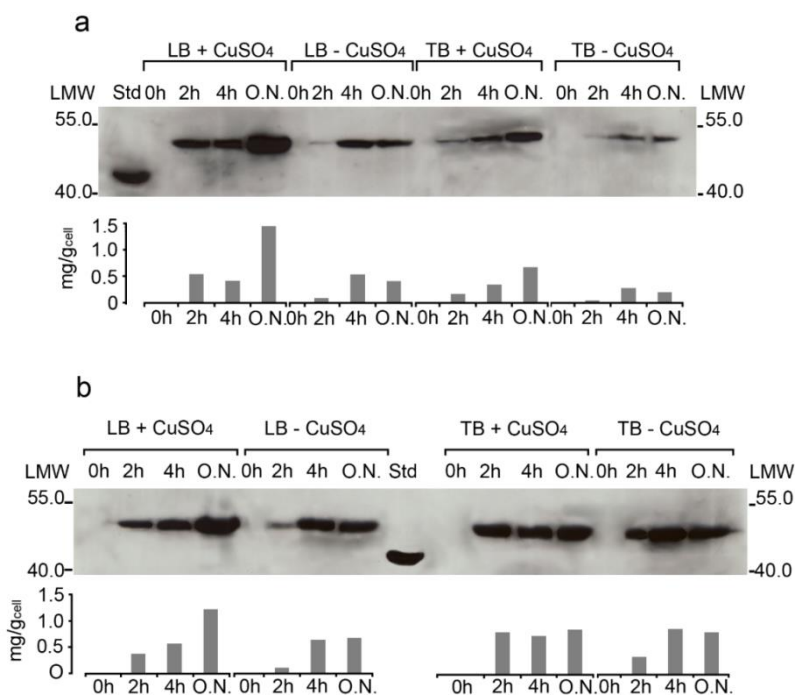
Protocols

2 l Erlenmeyer flasks containing 750 ml Luria Bertani (LB) or Terrific broth (TB) were inoculated with the starter culture (OD_{600nm} of 0.1), and incubated at 37°C and 200 rpm. When growing cells reached the early exponential phase of growth ($OD_{600nm} \sim 0.6$ for LB or ~ 1 for TB) or the late exponential phase ($OD_{600nm} \sim 4$ for LB), 0.1, 0.5 or 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) were added to induce the expression of recombinant MetaLacc. Into certain batches, 0.25 or 0.5 mM $CuSO_4$ was added to the cultivation media at the inoculum or at the moment of induction. Cells were then incubated at 37 or 25 or 18°C at 200 or 100 rpm or without shaking for 0, 2, 4 h or over night (O.N.). The cells were harvested by centrifugation at 8000 $\times g$ for 10 min. The supernatants (*i.e.* the extracellular broths) were collected and treated with 10% v/v trichoroacetic acid. The cell pellets were resuspended in 20 mM phosphate buffer (KPi) pH 6.7, containing 10 $\mu g/mL$ deoxyribonuclease I (DNaseI), 0.19 mg/mL phenylmethylsulfonylfluoride (PMSF), 0.7 $\mu g/mL$ pepstatin, and then disrupted by sonication for 5 cycles of 30 s each (with a 30-s interval on ice) using a Branson Sonifier 250 (Danbury, USA). After centrifugation at 34000 $\times g$ for 60 min at 4°C, soluble (cytoplasmic) and insoluble fractions (inclusion bodies and membrane debris) were collected. Total protein concentration in the fractions was estimated by the Biuret assay (Gornall et al., 1949), whereas MetaLacc production was estimated through sodium dodecyl sulfate polyacrylamide (10% w/v) gel electrophoresis (SDS-PAGE), Western Blot with Anti-HisTag antibody and laccase activity assay.

Results

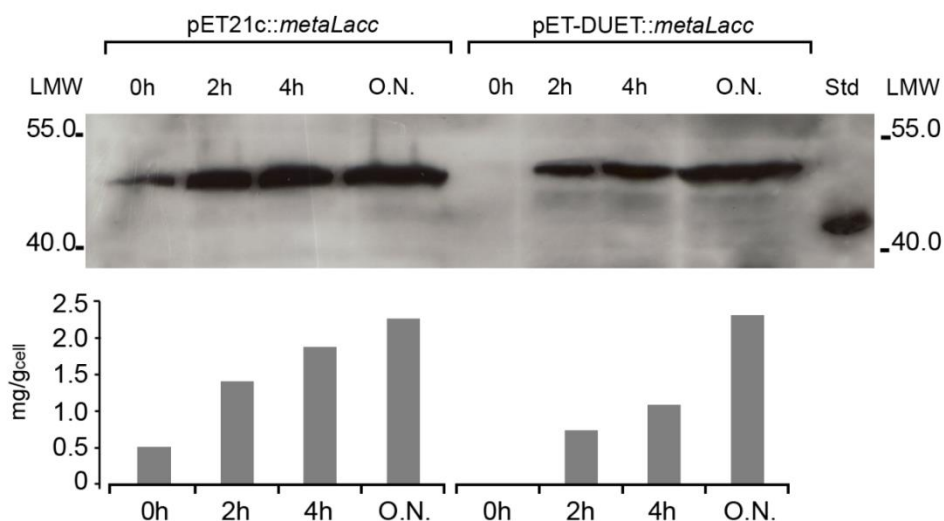
Supplementary Figure S1 shows the results of the experiments in which *E. coli* BL21(DE3) pLysE/pET-DUET::*metaLacc* (a) and *E. coli* BL21(DE3) pLysE/pET21c::*metaLacc* (b) (I) were grown in LB or in TB media; (II) protein expression was induced by adding 0.5 mM IPTG during the early exponential phase

of growth ($OD_{600} \sim 0.6$ for LB and ~ 1 for TB); (III) when stated, at the time of induction, 0.5 mM of $CuSO_4$ was added; (IV) cells were further incubated at $37^\circ C$ without shaking for 0, 2, 4 h or O.N..



Supplementary Figure S1. Western Blot analysis of insoluble fractions (equivalent to 1 ml of culture) from *E. coli* BL21(DE3) pLysE/pET-DUET::*metaLacc* (a) and *E. coli* BL21(DE3) pLysE/pET21c::*metaLacc* (b) grown at $37^\circ C$ in LB or TB medium. MetaLacc expression was induced by adding 0.5 mM IPTG to the cells in the early exponential growth phase ($OD_{600nm} \sim 0.6$ for LB, ~ 1 for TB). At the time of induction, 0.5 mM $CuSO_4$ was also added, when indicated. Over-expression of MetaLacc was allowed to proceed for 0 (control), 2, 4 h or O.N. at $37^\circ C$ without shaking. MetaLacc concentration was estimated by densitometric analysis of SDS gel bands using the Quantity One analysis software (Bio Rad, Hercules, CA, USA). Std: reference standard protein, Glycin oxidase from *Bacillus subtilis* (5 μg , 42.66 kDa), kindly provided by Loredano Pollegioni, University of Insubria (Job et al., 2002). LWM: molecular weight markers, from GE Healthcare Sciences, Little

Chalfont, UK. For both the recombinant strains, Western Blot analysis of insoluble fractions (Figure S1) revealed an intense protein band at the expected molecular mass for the His₆-tagged laccase (51.2 kDa). This protein band was undetectable in soluble (cytoplasmic) fractions and extracellular broths from the recombinant strains (data not shown) and, consistently, it was not found in the control strains transformed by the empty vectors (data not shown). As shown in Figure S1, MetaLacc presence in the insoluble fractions was nearly undetectable immediately after induction, and increased with the incubation time, reaching the highest production after O.N. incubation. Maximum MetaLacc specific productivity corresponding to 1.6 mg/g_{cell} for pET-DUET strain and 1.2 mg/g_{cell} for pET21c strain was obtained in LB medium supplemented with 0.5 mM CuSO₄ at the moment of induction. No laccase activity on ABTS was detected in either insoluble, soluble or extracellular fraction (data not shown). Supplementary Figure S2 shows the results of the experiments in which *E. coli* BL21(DE3) pLysE/pET21c::*metaLacc* and *E. coli* BL21(DE3) pLysE/pET-DUET::*metaLacc* were grown in LB medium; protein expression was induced by adding 0.5 mM IPTG during the early exponential growth (OD₆₀₀ ~ 0.6); at the time of induction, 0.5 mM of CuSO₄ was added; cells were further incubated at 18°C and 100 rpm for 0, 2, 4 h or O.N..



Supplementary Figure S2. Western Blot analysis of insoluble fractions (equivalent to 1 ml of culture) from the pET21c and pET-DUET strains grown in LB. MetaLacc expression was induced by adding 0.5 mM IPTG to the cells in the early exponential phase ($OD_{600nm} \sim 0.6$). At the moment of induction, 0.5 mM of $CuSO_4$ was also added. Over-expression of MetaLacc was allowed to proceed for 0 (control), 2, 4 h or O.N. at 18°C and 100 rpm. MetaLacc concentration was estimated by densitometric analysis of SDS gel bands using the Quantity One analysis software (Bio Rad, Hercules, CA, USA). Std: reference standard protein, Glycin oxidase from *B. subtilis*, 5 µg, 42.66 kDa. LWM: molecular weight markers, from GE Healthcare Sciences, Little Chalfont, UK. The results shown above (Figure S2) indicate that slowing down cell growth by reducing incubation temperature after induction and incubating cells in a moderate shaking regime did increase the amount of MetaLacc produced, but again it massively accumulated into inclusion bodies in both recombinant strains. Comparable maximum MetaLacc specific productivity (corresponding to *ca.* 2.3 mg/g_{cell}) was obtained in both strains. Again, no laccase activity (on ABTS) could be detected in the insoluble, soluble or extracellular fractions. Finally, we tried to produce MetaLacc by (I) inducing MetaLacc production in the two recombinant strains grown in LB medium by adding 0.5 mM IPTG in the late exponential growth phase ($OD_{600 nm} \sim 4$); or (II) by adding 0.1 or 0.5 mM IPTG and 0.25 mM $CuSO_4$ to early growing cells in LB medium ($OD_{600 nm} \sim 0.6$), followed by 4 h of growth at 25°C and 120 rpm, and a subsequent O.N. incubation at 25°C without shaking. In all these conditions except for the one described in the Methods section of the paper (Figure 2a), MetaLacc always accumulated into insoluble fractions and no enzymatic activity was detectable in cellular extracts.

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5. Ligninolytic and cellulolytic activities of selected microbes

In chapter 5 of this PhD thesis, I describe the preliminary work done in collaboration with the group of Prof. Petra Patakova at the Department of Biotechnology of the University of Chemistry and Technology of Prague as mentioned in letter described in Appendix 2 (two months-stage).

Introduction

Lignocellulose biomass is comprised of three main components, which are cellulose, hemicellulose, and lignin. They are packed closely in a crisscross network and glued with the help of a variety of non-covalent and covalent linkages [1]. The most active microbes in lignin degradation identified to date are fungi, such as those belonging to the white-rot families (notably *Phanerochaete chrysosporium*). Also bacteria as actinomycetes might play a role in lignin degradation in specific ecosystems and they are considered a yet-unexplored source of lignocellulose degrading enzymes [2]. The degradation of lignin occurs by an oxidative extracellular mechanism, involving the heme-containing peroxidases (i.e., lignin peroxidase (LiPs) (EC 1.11.1.14), manganese peroxidases (MnPs) (EC 1.11.1.13) and versatile peroxidases (VPs) (EC 1.11.1.16)) and laccases [3]. The most important enzymes involved in the decomposition of cellulose are classified according to their mechanism of action and include: endocellulases (cleaving internal bonds in the cellulose chain), exocellulases or cellobiohydrolases (acting on the reducing or non-reducing ends of cellulose chains) and β -glucosidases (converting cellobiose into glucose monomers) [4]. Finally, hemicellulases include a wide variety of hydrolytic enzymes, such as endoxylanases, endomannanases, xylosidases, glucosidases, arabinosidases, galactosidases, mannonidases and glucuronidases [4]. In the period spent at the University of Prague, I tested the ligninolytic and cellulolytic enzyme activities of the actinomycetes *Nonomuraea gerenzanensis* and *Streptomyces coelicolor* A3 (2) and of the fungus *Phanerochaete chrysosporium*, using culture media supplemented with lignin (as in [2]) or containing as carbon source wheat straw pretreated according to the procedure reported in [5].

Our ultimate goal is developing a two-step cultivation process, in which the first step is the cultivation of microbes attacking lignocellulosic material in aerobic conditions, followed by a second step consisting of anaerobic fermentations using cellulose degrading isolates (*Clostridia* consortia) to produce biofuels or chemical products.

Materials and methods

Microorganisms

- *Phanerochaete chrysosporium* CCM 8074, from Czech Collection of Microorganism, Masarykova Universita, Brno, Czech Republic.
- *Nonomuraea gerezanensis* ATCC 39727 [6].
- *Streptomyces coelicolor* A3 (2), gently provided by Prof. M. Bibb, John Innes Centre, UK.

Growth conditions of *Phanerochaete chrysosporium* CCM 8074

P. chrysosporium petri dishes were prepared using malt agar (see Table 1 for composition). The inoculum was performed by transferring the spores into new malt agar petri dishes using a sterile loop. The culture was grown at laboratory temperature until the appearance of white coat. The petri dishes with grown culture were washed with 2 mL of sterile water and transferred into the 500 mL baffled Erlenmayer flasks with 100 mL of different liquid media (Table 2) at which the following components could be added: 0.8 g/L lignin, 2 mM CuSO₄ and 6 g/L yeast extract. Flasks with cultures were incubated on a rotary shaker at 200 rpm at 28 °C for 20 days. Every 48 h a sample of 5 mL was taken from each culture and centrifuged at 3220 x g at 4 °C for 10 min to separate supernatant and pellets. On extracellular fractions, enzymatic activity (laccase, manganese and lignin peroxidases, cellulase) were tested and pH with “pH Test Strips 4.5-10.0” (Sigma-Aldrich) was measured. The pellet (containing mycelium and residual wheat straw) was weighted and corresponded to the “wet weight”.

Table 1. Agar media composition

CMC Agar		Avicel agar		Cellobiose agar		Malt agar	
g/L		g/L		g/L		g/L	
Carboxy methyl cellulose (CMC)	5.0	Avicel	5.0	Bacteriological peptone	10.0	Malt extract	30.0
NaNO ₃	1.0	NaNO ₃	1.0	Cellobiose	7.5	Agar	15.0
K ₂ HPO ₄	1.0	K ₂ HPO ₄	1.0	Agar	15.0	Bacteriological peptone	5.0
KCl	1.0	KCl	1.0	Bromothymol blue*	0.06		
MgSO ₄ *7H ₂ O	0.5	MgSO ₄ *7H ₂ O	0.5				
Yeast extract	0.5	Yeast extract	0.5				
Glucose	1.0	Glucose	1.0				
Agar	17.0	Agar	17.0				

*Added after autoclaving in sterile conditions

Table 2. Liquid media composition

Wheat straw medium (WSM)		Wheat straw water (WSW)		V medium (VM) §		MM-L ¥	
g/L		g/L		g/L		g/L	
(NH ₄) ₂ SO ₄	1.4	Pretreated wheat straw*	10.0	Soluble starch	24.0	Lignin	0.8
KH ₂ PO ₄	2.0			Glucose	24.0	KH ₂ PO ₄	0.5
Urea	0.3			Meat extract	3.0	K ₂ HPO ₄	1.6
CaCl ₂	0.3			Yeast extract	5.0	MgSO ₄ 7 H ₂ O	0.58
MgSO ₄ *7H ₂ O	0.6			Tryptose	5.0	NaCl	0.25
ZnSO ₄ *7H ₂ O	0.0014					CaCl ₂ *2 H ₂ O	0.013
FeSO ₄ *7H ₂ O	0.009					(NH ₄) ₂ SO ₄	1.25
MnSO ₄	1.4					NH ₄ NO ₃	1.0
Peptone	0.75					FeCl ₃ *6 H ₂ O	0.0025
Pretreated wheat straw*	10.0					CuCl ₂	0.0025
						MnCl ₂	0.0025

*The composition of the alkali-pretreated wheat straw (determined at the University of Chemistry and Technology of Prague) is as follows (% in dry matter): cellulose 54.63, xylose 4.45, arabinose 0.78, lignin 16.16, ash 0.82.

§ For fermentation in 500 mL flasks, the medium was supplemented with 0.8 g/L lignin and 2 mM CuSO₄.

¥ For fermentation in 500 mL flasks, the medium was supplemented with 6 g/L yeast extract and 2 mM CuSO₄.

Growth conditions of *Nonomuraea gerenzanensis* and *Streptomyces coelicolor* A3 (2)

N. gerenzanensis and *S. coelicolor* A3 (2) working cell bank (WCB) were prepared as previously described in Marcone *et al.* [7] and stored at -80 °C. Pre-inoculum cultures were set up transferring 0.75 mL of the WCB into 15 mL of VM in 100 mL Erlenmeyer flasks. The cultures were grown at 28 °C at 200 rpm for 72 hours. Then, 3.6 mL of these cultures were transferred into 500 mL baffled Erlenmeyer flasks containing 100 mL of different liquid media (Table 2). The cultures were grown at 28 °C at 200 rpm. Growth parameters and enzymatic activities were measured as described in the previous subsection.

Enzymatic assays for ligninolytic enzymes

Laccase, MnP and LiP activities were measured as described in [2], using as substrate 2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) for laccase and MnP detection, or 2,4-dichlorophenol (2,4-DCP) for LiP.

Enzymatic assay for cellulolytic enzymes

The enzymatic activity of cellulases was monitored by measuring the release of reducing sugar moieties and using as substrate the cellulose-containing Whatman qualitative filter paper, Grade 1 (Sigma-Aldrich). The employed method is based on the protocol described in Cretoiu *et al.* [8], adapted to cellulose activity detection. Briefly, for each sample 50 mg of the cellulose Whatman filter were equilibrated for 15 min at 50 °C in 1.5 mL Eppendorf tubes with 1 mL of 50 mM sodium citrate pH 4.5. Then, 500 µL of protein sample were added and the mixture incubated at 55 °C for 1 h. After centrifugation for 5 min at 20000 x g at 25 °C, 200 µL of the supernatant were mixed with 200 µL of 0.5 M NaOH. Subsequently, MBTH (3-methyl-2-benzothiazolinone hydrazine) solution at concentration of 3 mg/mL and DTT (dithiothreitol) at concentration of 1 mg/mL were mixed in 1:1 ratio and 200

μL of this mixture were added to the tube. After incubation at $80\text{ }^{\circ}\text{C}$ for 15 min, $400\text{ }\mu\text{L}$ of a solution composed of $0.5\text{ }\%$ w/v $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{ H}_2\text{O}$, $0.5\text{ }\%$ w/v sulfamic acid, 0.25 M HCl were added, allowing the mix to cool to room temperature. Absorbance at 620 nm was then measured. Released reducing sugars were estimated by comparison to a standard curve prepared with increasing concentrations ($0\text{-}100\text{ }\mu\text{M}$) of glucose. Positive and negative controls corresponded to the commercial enzyme Cellic CTec3 (Novozymes) and to the medium (without inoculums) respectively. One unit of enzyme activity was defined as the amount of enzyme that release $1\text{ }\mu\text{mol}$ glucose in 60 min at $50\text{ }^{\circ}\text{C}$.

Agar test for endo/exoglucanase and β -glucosidase activities

$25\text{ }\mu\text{L}$ of microbial WCB were deposited at the center of petri dishes containing CMC agar, Avicel agar or cellobiose agar for testing endoglucanase, exoglucanase or β -glucosidase activities, respectively (Table 1). The plates of the two actinomycetes were incubated at $28\text{ }^{\circ}\text{C}$, whereas the plates of *P. chrysosporium* were stored at room temperature. For the endo/exoglucanase activities, at regular intervals (after 5, 10 and 15 days of incubation), the surface of the petri dishes was treated with 1 mg/mL Congo red and incubated at room temperature for 30 minutes. The buffer was the poured out and replaced by a destaining solution composed of 1 M NaCl and $2\text{ }\%$ v/v acetic acid in distilled water. If the microorganism degraded CMC or Avicel, a clear halo around the colony appeared. In the case of β -glucosidase activity, change in the color around the colony was monitored: in the presence of activity, the bromothymol blue embedded in the plate changed from blue to yellow.

Results

Ligninolytic and cellulolytic activities of *Phanerochaete chrysosporium*

P. chrysosporium was grown in different liquid media (listed in Table 2): the salt minimal MM-L or the lipid and rich VM, both supplemented with copper ions and lignin (conditions known to induce the production of ligninolytic activities in actinomycetes [2]); WSW, wherein wheat straw was the only nutrient source or WSM where wheat straw was added with salts and organic components, even if at lower extent (Table 2). In none of them, ligninolytic activities (laccase, MnP and LiP) were detected. The only condition in which cellulolytic activities were detectable, was in the presence of wheat straw (Table 3). Figures 1 A-D show the cultivation time courses and the cellulolytic activity profiles of the fungus in WSM and WSW. In both media, the pH of the culture was almost constant during the entire fermentation, and corresponded to ca. 7.0-7.5 in WSW and 5.0-5.5 in WSM. In both cases, fungal mycelium was observable at optical microscope as growing on the surface of the wheat straw fiber (see for example Figure 1 E). In both media, cellulolytic activities appeared after 8 days of cultivation, increased during the fermentation up to three weeks and were overall higher in WSM (Figure 1 B) than in WSW (Figure 1 D). When the fungus was grown in WSW (Figure 1 D), the highest cellulolytic activity was measured at 20 days and corresponded to 1.95 U/g_{cells}. In WSM the highest value (34 U/g_{cells}) was recorded after 18 days from the inoculum (Figure 1 B).

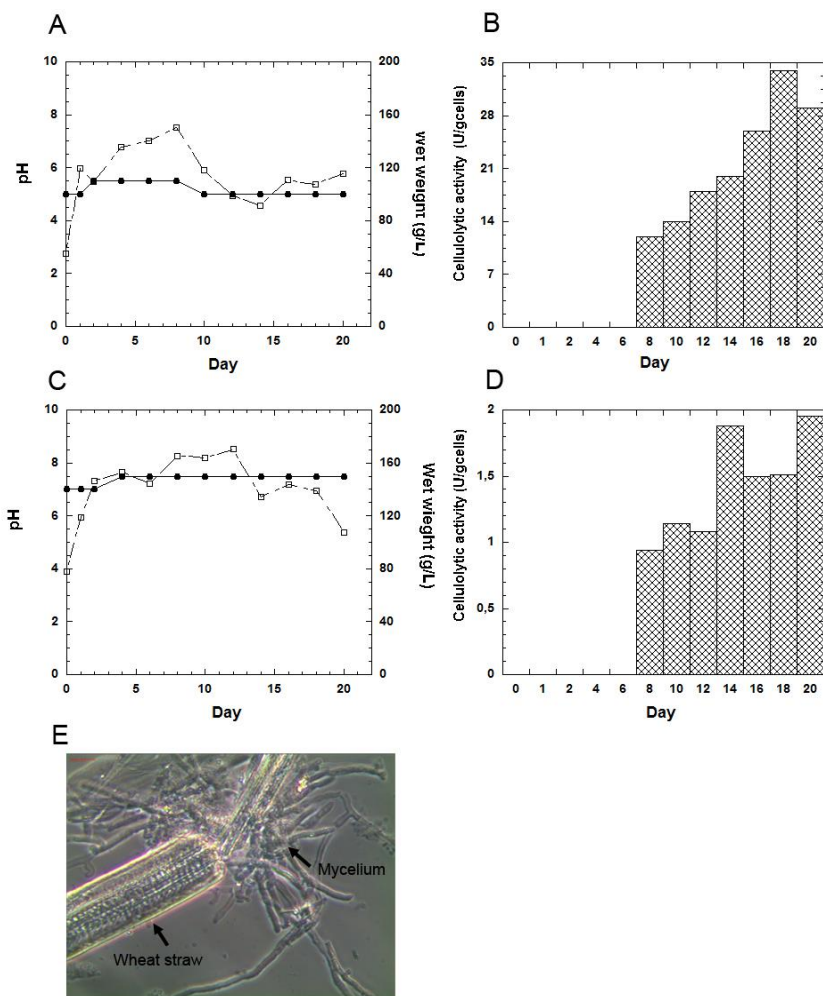


Figure 1. (A, C) pH (•, continuous line) and “wet weight” (□, dashed line) and (B, D) cellulolytic activity of *P. chrysosporium* in WSM (A, B) or WSW (C, D). E: mycelium of the fungus grown on wheat straw in WSM (photo at optical microscope with 400X enlargement).

Ligninolytic and cellulolytic activities of *Nonomuraea gerenzanensis*

As previously described in Casciello *et al.* [2], *N. gerenzanensis* produced MnP and LiP activities both in VM medium supplemented with 0.8 g/L lignin and 2 mM copper sulfate, and in MM-L medium added with 6 g/L yeast extract and 2 mM copper sulfate. In these growth conditions, no laccase activity was detected (see also Table 3).

Here we report the cultivation parameters and the enzyme activity profiles of *N. gerenzanensis* in WSM (Figure 2 A-C). The pH was around 6.0 until the end of the fermentation (Figure 2 A). As seen for *P. chrysosporium*, also the mycelium of the actinomycete grew on the surface of the straw fiber, as a scaffold supporting bacterial growth (Figure 2 D). In WSM, MnP and LiP (but no laccase) activities were detected, both the activities showing the same temporal profile, increasing after 7 days from the inoculums and maintaining stable levels along the three weeks of analyses (Figure 2 B shows, as example, the profile of MnP activities; LiP profile is almost overlapping). *N. gerenzanensis* showed in WSM also cellulolytic activity (Figure 2 C), starting after 7 days of growth. The highest value (ca. 6 U/g_{cells}) was measured after 19 days.

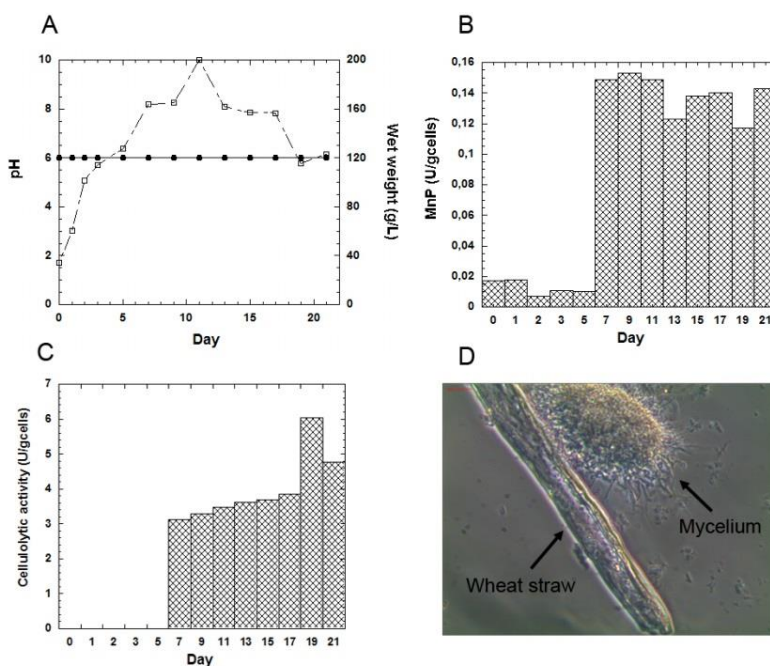


Figure 2. (A) pH (•, continuous line) and “wet weight” (□, dashed line), (B) MnP activity and (C) cellulolytic activity of *N. gerenzanensis* in WSM. D: mycelium of the *N. gerenzanensis* grown on wheat straw (photo at optical microscope with 400X enlargement).

Ligninolytic and cellulolytic activities of *Streptomyces coelicolor* A3 (2)

S. coelicolor A3 (2) grew in VM and WSM media, but not in MM-L. Figure 3 A and B show the cultivation parameters in WSM: the pH was around 6.0-6.5 for the entire fermentation. In VM, only traces of LiP activity were detected (data not shown). In WSW medium, both LiP and cellulolytic activities were found, whereas no MnP and laccase were observed. Figure 3 B shows the cellulolytic activity profile of *S. coelicolor* A3 (2). Activities were undetectable in the first 7 days of growth, whereas at this time a high value of activity (2.8 U/g_{cells}) was measured. After a decrease after the ninth day, at 19 and 21 days of cultivation an again increasing cellulolytic activity was recorded.

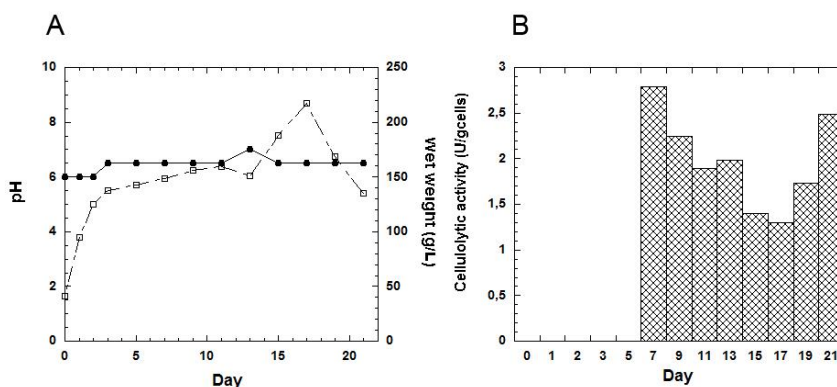


Figure 3. (A) pH (•, continuous line) and “wet weight” (□, dashed line) and (B) cellulolytic activity of *S. coelicolor* A3 (2) in WSM.

Table 3 summarizes the ligninolytic and cellulolytic activities recorded for the three used strains in four different cultivation media.

	<i>P. chrysosporium</i>				<i>N. gerezanensis</i>				<i>S. coelicolor</i> A3 (2)			
	cellulase	Li-P	Mn-P	laccase	cellulase	Li-P	Mn-P	laccase	cellulase	Li-P	Mn-P	laccase
WSM	Yes	No	No	No	Yes	Yes	Yes	No	Yes	Yes	No	No
WSW	Yes	No	No	No	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
VM	No	No	No	No	No	Yes*	Yes*	No*	No	Yes	No	No
MM-L	No	No	No	No	Yes	Yes	Yes	No	n.m.	n.m.	n.m.	n.m.

*results taken from [2]

n.m. means not measured

Screening for cellulolytic enzymes in agar media

The production of cellulolytic enzymes was investigated also in solid media, by growing the three strains on agar plates supplemented with CMC (for detecting endoglucanase activity), Avicel (for exoglucanase activity) or cellobiose (β -glucosidase activity). As summarized in figure 4, *P. chrysosporium* attacked on all the tested substrates: endoglucanase and β -glucosidase activities were detected starting from the fifth and the third day of growth, respectively, whereas Avicel started to be used only after prolonged incubation (15 days). Also *N. gerenzanensis* showed activity on the tested substrates, with a profile similar to the fungal one: endoglucanase and β -glucosidase activities were clearly detectable after 5 days of growth and the diameter of the degradation/colored halos around the colony increased prolonging incubations. Activity on Avicel was less intense and was observed only after 10-15 days from inoculums. Finally, *S. coelicolor* A3 (2) showed activity on CMC and Avicel agar (starting from the fifth day of incubation), but not on cellobiose.

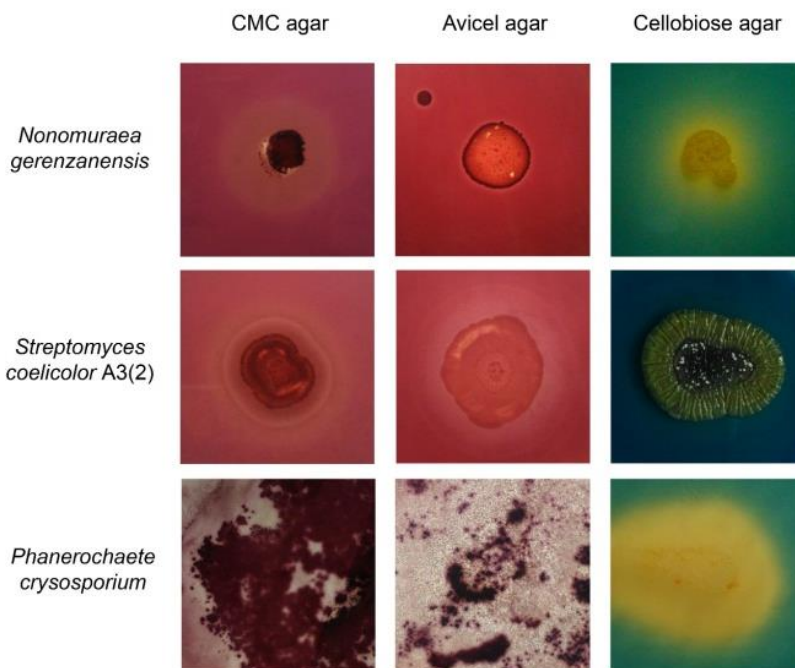


Figure 4. Plates assays for cellulolytic activities.

Discussion and future perspectives

The work here reported represents a preliminary analysis of the lignocellulosic system of two actinomycetes, *N. gerenzanensis* and *S.coelicolor* A3 (2), and of the white rot fungus *P. chrysosporium* done in collaboration with Prof. Patakova at the Department of Biotechnology of the University of Chemistry and Technology of Prague.

Traditionally, the degradation of lignocellulose is attributed to fungi, especially to white-rot and brown-rot families. Accordingly, the sequencing of *P. chrysosporium*'s genome [9] revealed a plethora of genes putatively involved in the degradation of this complex biomass. In particular, 10 genes coding for LiPs and 5 genes for MnPs were identified, together with 5 copper radical oxidases, 1 glyoxal oxidase and 4 multicopper oxidases distantly related to laccases. The complement of cellulases in *P. chrysosporium*'s genome includes at least 40 putative endoglucanases, 7 exocellobiohydrolases and 9 β -glucosidases, along with other hemicellulases [9]. Additionally, a few studies dealing with the production of biologically active ligninases and cellulases by the fungus were published. For instance, Singh and Chen [10] and Coconi-Linares and co-workers [11], reported on the secretion by this fungus of MnPs and LiPs, whereas the work of Westereng and co-workers [12] focuses on the characterization of a metal-dependent endoglucanase. The preliminary results described in this section of my PhD thesis confirm the ability of *P. chrysosporium* to secrete cellulolytic enzymes. Indeed, the agar plate screening revealed the presence of endo/exoglucanase and β -glucosidase activities, and cellulolytic activity was recorded also in liquid culture by spectrophotometric measurement. In liquid culture, the secretion of cellulases seems to be induced by the presence of cellulose in the medium: in fact, high cellulolytic activity was detected in WSW and WSM, i.e. in media containing wheat straw, but it did not occur in VM and MM-L, which are only supplemented with lignin. Further investigation are needed to better understanding why in the fermentation conditions hereby evaluated, surprisingly no ligninolytic activity was detectable. Even though the majority of studies on lignocellulose degradation in nature is focused on fungi, recently different bacterial strains capable of degrading this complex biomass were also isolated.

Most of these strains belong to the phyla *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*. [1, 4, 13]. A detailed analysis of the ligninases secreted by *S. coelicolor* A3(2) and *N. gerezanensis*, the two actinomycetes employed in this study, is reported in chapter 3 of this PhD thesis [2]. Hence, here I focus only on the production of cellulases in these two bacteria. The genome sequence of *S. coelicolor* A3(2) revealed the presence of 8 genes coding for putative cellulases and hemicellulases [14]. Among them, only one cellobiosidase [15] and one xylanase [16] were so far characterized. To our knowledge, nothing is known about the production of cellulases and hemicellulases in *N. gerezanensis*, whose genome sequence is not yet publicly available. The preliminary results thus far obtained highlighted the production of cellulases in both the bacteria: according to the plate assays, endo- and exoglucanases are secreted both by *N. gerezanensis* and *S. coelicolor*, whereas β -glucosidase activities seems to be produced only by the first microorganism. Spectrophotometric assays in liquid culture, moreover, confirmed the secretion of cellulases in the broths of the two bacteria grown on wheat straw. An interesting result is the production of cellulolytic activities by *N. gerezanensis* also in MM-L medium, where lignin (but not cellulose) is the nutrient present. On the contrary, no cellulases seem to be produced in VM medium. A possible explanation is that, in the latter medium, the production of cellulases is inhibited or at least slowed-down by the presence of a more-readily carbon source such as dextrose.

In conclusion, even though the results here reported are preliminary, they are useful to shed light on the complex enzymatic systems involved in the degradation of lignocellulosic biomasses by both fungi and actinomycetes. Future studies will be aimed not only at completing the characterization of these hydrolytic systems, but also at evaluating the possible exploitation of these microbes in the two-stage fermentation processes aimed at biofuel production.

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6. Conclusion and future perspectives

The main drive of this PhD thesis was discovering and producing novel enzymes involved in the biodegradation of lignocellulose biomass. Novel ligninolytic enzymes have the potential to be applied in many different industrial sectors and to be employed in biorefineries [1]. Additionally, this thesis was part of the follow-up research from the MetaExplore EU Consortium that focused on the metagenomics approach for converting recalcitrant biopolymers such as lignin and chitin. During the early phase of this work, I used the classical approach of screening microbial isolates for discovering novel ligninolytic enzymes. 43 filamentous actinomycetes belonging to different genera/families were screened using an array of validated tests for ligninolytic enzyme detection [2]. Through this approach, a novel peroxidase activity was found. Interestingly, it was produced by a novel species, very recently classified, belonging to the uncommon *Nonomuraea* genus [3]. *Nonomuraea gerenzanensis* peroxidase showed features that favorably compare with the ones of the more widely explored fungal peroxidases [2]. Peroxidases represent one of the main components of the ligninolytic system and comprise several members, namely lignin peroxidases (LiPs), versatile peroxidases (VPs), and manganese peroxidases (MnPs). The white-rot fungus *Phanerochaete chrysosporium* secretes an exceptional array of peroxidases, which act synergistically during ligninolysis and that may be used for other biotechnology processes including transformation of environmental pollutants and biobleaching of pulp water. Indeed, when compared to *P. chrysosporium* MnP, our *N. gerenzanensis* peroxidase preparation showed a significantly higher stability at pH > 6.5 and a higher thermostability [4, 5]. Furthermore, *N. gerenzanensis* peroxidase showed 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 [2]. It would be extremely interesting sequencing the genome of *N. gerenzanensis* to know more about the role of this enzyme in the producing microbe. Since we proved that *N. gerenzanensis* is able to grow in a minimal medium containing only lignin as carbon source, it is likely that genome mining would reveal other enzymes involved in the

process of ligninolysis, and that would be, to our knowledge, the first report on such a system in a soil bacterium.

Although approaching uncommon bacteria (as *N. gerenzanensis*) as a source of novel biocatalysts might be successful, the unease of producing and characterizing the discovered enzymes due to the poor growth of the producing organisms under standard laboratory conditions, might represent a serious bottleneck to industrial application. In fact, almost three weeks of cultivation were needed to produce peroxidase activity in *N. gerenzanensis* and scaling up in fermenters proved unsuccessful (unpublished results). Identification of the encoding sequence in the genome of *N. gerenzanensis* and its heterologous expression might represent a rational solution for the future enzyme production. Metagenomics allows to transcend cultivation-dependent limitation, directly analyzing genomes' potential in environmental samples [6, 7]. In the second paper of this PhD thesis, I collaborated to the discovering of a novel bacterial laccase (MetaLacc) using the metagenomic approach. Based on sequence similarity, this new enzyme could be affiliated to the elusive, yet ubiquitous and diverse, bacterial *phylum* of *Acidobacteria*. As reported in chapter 4, MetaLacc was able to oxidize a variety of phenolic substrates with the highest specific activities recorded on pyrogallol and pyrocatechol in a wide range of pHs. MetaLacc was also able to decolor azo dyes and triphenylmethane dyes in the presence or absence of redox mediators. These features of MetaLacc can be useful for future applications where neutral or slightly alkaline pH is desirable, including for instance oxidation of natural and synthetic dyes, bioremediation strategies at neutral pH, and organic synthesis reactions [8]. Interestingly, MetaLacc was thermo-activated by incubating it for one or two hours at 50°C, and in this feature it differs from what had been observed for other thermostable bacterial laccases. Additionally, MetaLacc showed a high tolerance towards salts, favoring its exploitation for industrial and environmental applications wherein high amounts of salts are used, such as treatment of industrial and municipal waste water or kraft pulp biobleaching [9]. Finally, MetaLacc activity was increased in the presence of alcohols, in particular methanol, and that property might play a relevant role in applications that require getting access to insoluble substrates, such as in the

detoxification of persistent organic pollutants. Biological treatment of textile dyes in industrial waste water requires thermostable enzymes to remain active even under alkaline pH conditions or with high concentrations of organic solvents [10]. In conclusion, the metagenome-sourced MetaLacc thus represents a novel three-domain bacterial laccase with interesting biochemical characteristics and it is one of the very few enzymes of acidobacterial origin to have been extensively biochemically characterized to date. Although in the case of *N. gerenzanensis* peroxidase the bottleneck was the homologous expression, with MetaLacc I experimented the unease to produce the recombinant protein by the heterologous expression. Only few micrograms of the pure enzyme in the soluble active form could be produced, since most of the recombinant protein accumulated into inclusion bodies. Future exploitation of this promising enzyme urgently needs the development of an adequate production and purification process and probably other heterologous systems beside *Escherichia coli* could be investigated.

Finally, in the last part of this PhD thesis I evaluated the production of ligninolytic and cellulolytic enzymes in liquid media containing lignin or pretreated wheat straw as unique carbon sources. This work is part of a collaboration with Prof. Patakova at the Department of Biotechnology of the University of Chemistry and Technology of Prague and it is preliminary for eventually developing a two-step cultivation process (TSCP), where the aerobic microorganisms attack lignocellulosic material and then biofuels are produced by anaerobic consortia. The most relevant result from these first investigations was the detection of cellulolytic activities in actinomycetes. Production of cellulolytic activities was recently described in streptomycetes [11, 12], but much less is known on other actinomycetes. Here I reported that *N. gerenzanensis* produced secreted endo- and exoglucanases and β -glucosidase activities. The production of cellulolytic activities by *N. gerenzanensis* appeared associated to the secondary phase of growth but it was detectable also in the absence of cellulose (or wheat straw) and in the presence of lignin as the only carbon source. These data confirm that it would be interesting shedding light on the complex enzymatic systems involved in the degradation of lignocellulosic biomasses by this novel actinomycete.

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Appendix 1

Draft of the review to be prepared for FEMS Microbiology Letters

Metagenomics, enzyme biotechnology from unculturable microorganisms

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Abstract Microorganisms encompass the largest resource of metabolic and genetic diversity encountered on Earth. Human civilisation has largely improved by the development of technologies that have their source in microbes, and microbially-produced enzymes find application in almost every industrial sector. Unfortunately, a major part of the microbiota in natural ecosystems (up to 99 – 99.9%) is unculturable by traditional methods and therefore inaccessible for biotechnology research. Among the culture-independent approaches recently developed, metagenomics represents a promising and innovative tool for the exploitation of the biotechnological potential encrypted in natural microbial communities. Herein, we review the different methods required for constructing and screening metagenomic libraries, with a particular attention to the potential for metagenomics to uncover novel enzymes with multiple biotechnological applications.

Keywords: metagenomics, biocatalysts.

Introduction

Industrial (white) biotechnology is a central feature of the sustainable economic future of modern societies (Lorenz and Eck, 2005). A central role in this frame is covered by microbial products,

such as proteins, nucleic acids, polymers, anti-infective and antitumor agents, which are currently exploited in various industries and commercial applications (Demain and Adrio, 2008). Microbial enzymes, in particular, find employment in almost all industrial sectors, from chemical, pharmaceutical and food industries, to the manufacturing of detergents, textiles, leather, pulp and paper (Demain and Adrio, 2008). Currently, the application of enzymes in industries generates a turnover of about 5 billion USD, a value that is forecasted to rise in the next future, thanks to the continuously increasing demand for novel enzymatic biocatalysts with high process performances (Ferrer et al., 2016). Compared to industrial processes catalysed by chemical reactions, processes based on enzymes are favoured by reduced costs, increased efficiency, improved product recovery, and reduced use of toxic compounds (Demain and Adrio, 2008). The extreme versatility of microbial enzymes is not surprising, if the following factors are taken into consideration: (i) microorganisms (archaea, bacteria, protozoans, and certain algae and fungi) represent by far the largest proportion of individual organisms present on Earth, with a total number of microbial cells estimated to be $4-6 \times 10^{30}$ (Bunge et al., 2014); (ii) prokaryotic microorganisms – archaea and bacteria – make up most of the Earth’s biomass and constitute the oldest form of life, living on this planet since more than three billions of years (Bunge et al., 2014); (iii) moreover, microorganisms inhabit the widest variety of ecosystems, from nutrient-rich environments as soils, lakes, oceans or inside other organisms, to the less-hospital habitats on Earth, such as hot springs, nearly saturated salt brines, acid mine waters at pHs near zero, deep-sea hydrothermal vents, as well as deep in Antarctic ice and kilometres below the Earth’s surface (Mirete et al., 2016). For all these reasons, prokaryotes have evolved and accumulated remarkable physiological and functional heterogeneity, thereby constituting the world’s major reserve of genetic diversity (Lorenz and Eck, 2005; Ekkers et al., 2012). The microbial world, however, is enormous also from the perspective of the current limitations of human knowledge. Starting from the original observation done in 1898 by Heinrich Winterberg about the discrepancy between the number of culturable bacteria on nutrient media and the total bacteria counted by microscopy, microbial unculturability - the so-called “great plate count

anomaly” – has long been recognised in microbiology (Ekkers et al., 2012; Ferrer et al., 2016). Unculturable microorganisms can be found in nearly every group within the bacteria and archaea. It has been estimated that only 0.1 to 1% of the prokaryotes are culturable by traditional cultivation and isolation methods, being therefore accessible for biotechnology or basic research (Culligan et al., 2014; Ekkers et al., 2012); even among culturable prokaryotes, four phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*) are overwhelming (Culligan et al., 2014). There are several reasons for microbial unculturability under laboratory conditions: for instance, extremely high substrate concentrations, the lack of specific nutrients required for growth or in general the inability to recreate *in vitro* the complex ecological niche in which the microorganism lives. Some authors suggested that “unculturable” bacteria should be more specifically called “not-yet-culturable”, as the ongoing development of techniques for isolation and culturing could in the future allow the successful cultivation of recalcitrant microbes, which may simply be in a physiological state that eludes our actual ability to culture them (van Elsas et al., 2008).

To overcome the difficulties and limitations associated with cultivation approaches, several culture-independent methods have been developed, including phospholipid fatty acid analysis (PLFA) or PCR methods based on the analysis of the “molecular clock” 16S rRNA gene (18S rRNA for eukaryotes). However, data thus generated usually do not cover the complexity of prokaryotic diversity and provide only little if any information about the functional role of the different microbes within the community and the genetic information they contain (Randle-Boggis et al., 2016). Therefore, alternative technologies, the so-called Meta-omics (metagenomics, metatranscriptomics, metaproteomics and metabolomics), have lately gained more and more success. These innovative methods utilise genomic, proteomic, metabolomic and transcriptomic toolsets to transcend cultivation limitation by studying the collective material of organisms from environmental samples. Hence, meta-omic technologies can enable the identification of novel natural products, new enzymatic activities and metabolic pathways as well as a better understanding of relationships between microorganisms (Schofield and Scherman, 2013).

Metagenomics

The term ‘Metagenomics’ was first coined by Handelsman and co-workers in 1998 and could be defined as the analysis of the genetic complement of an entire habitat by direct extraction and subsequent cloning of DNA from an assemblage of microorganisms (Handelsman et al., 1998). Metagenomics combines the potential of genomics, bioinformatics and system biology, and it has been applied for the analysis of a broad range of environments, in order to access the genetic potential of the microbial communities therein present. These studies include terrestrial, marine and freshwater habitats, as well as wastewater treatment sludges, compost and eukaryotic-associated microbiomes (Ferrer et al., 2016; Ekkers et al., 2012). More recently, also extreme environments such as the Arctic, glacial ice and soils, acidic and hypersaline environments, as well as solfataric hot springs and hyperthermal ponds have been addressed by metagenomics-based studies (Mirete et al., 2016). The first step in a metagenomic approach is the extraction of the environmental DNA (eDNA) from the selected sample (Figure 1). DNA extraction can be achieved by two general strategies: direct extraction, the most commonly used and the fastest, which consists of cell lysis directly within the sample matrix, followed by separation of DNA from the matrix and cell debris; and indirect extraction, where cells are first removed from the matrix and subsequently lysed, which is more time-consuming but allows achieving higher purity (Delmont et al., 2011). When choosing the extraction method, three major problems have to be taken into consideration: (i) the DNA should be extracted from as broad a range of microorganisms as possible to be representative of the original microbial population; (ii) DNA shearing has to be avoided; and (iii) DNA must be free from contaminating substances that interfere with downstream processing, such as humic and fulvic acids often co-extracted during soil metagenomics library construction (Lombard et al., 2011). A way to improve the quality of the eDNA and to enhance the screening hit rate, is the ecological enhancement, also called habitat biasing or targeted metagenomics. The microbial community is manipulated generally prior to the DNA extraction, in order to increase *in situ* the prevalence of target functions (Ekkers et al., 2012). For example, the use of DNA isolated from enriched cultures

grown on chitin as their major carbon source increases the isolation of chitinases, compared with isolates from libraries made directly from total eDNA (Cretoiu et al., 2013). Other methods for ecological enhancement allow separating prokaryotic cells from eukaryotic ones (Hallam et al., 2004), microorganisms with high G+C content DNA from bacteria with low G+C DNA (Nusslein and Tiedje, 1998), as well as metabolically active organisms from inactive/dead ones (Dumont and Murrell, 2005).

After eDNA extraction, the subsequent step is the construction of the metagenomic library, attained by DNA fragmentation by enzyme digestion or mechanical shearing, followed by cloning into an appropriate host-vector system (Figure 1). Libraries can be classified into two groups with respect to average insert size: small-insert libraries in plasmid vectors (less than 15 kb) and large-insert libraries in cosmids (15-40 kb), fosmids (25-45 kb) or bacterial artificial chromosomes (BAC, >100 kb). The choice of the vector system depends on the quality of the isolated DNA, the desired average insert size of the library, the required vector copy number, the host and the screening strategy that will be used. Small-insert libraries are useful for the isolation of single genes or small operons; large-insert libraries are more appropriate to recover complex pathways encoded by large gene clusters or large DNA fragments for the characterisation of genomes of uncultured soil microorganisms (Ekkers et al., 2012; van Elsas et al., 2008; Lombard et al., 2011). In most metagenomics studies performed thus far, *Escherichia coli* has been used as the preferential cloning host. Because of its status as the most well-known model host, in fact, there is ample knowledge about different useful gene expression strategies and an extended genetic toolkit is available for this microorganism. However, significant differences in the levels of expression can occur depending on the taxonomic groups present within the metagenomic DNA sample, a problem that needs to be considered especially when a functional screening is planned. It has been estimated that on average only 30-40% of bacterial genes could be efficiently expressed in *E. coli*, a value dropping to 7% for high G+C DNA, indicating that *E. coli* is at best a suboptimal host for the heterologous expression of genes from many non-enteric bacteria (Gabor et al., 2004a). One way to enhance the possibility

of a successful expression of genes may be to engineer *E. coli* expression machinery on the basis of the expected prevalence of genes from source hosts, for instance by co-expressing the metagenome-sourced protein with a chaperone to promote the correct folding (Ferrer et al., 2004). An alternative is the development of other prokaryotic non-*E. coli* hosts. Bacteria belonging to the genera *Agrobacterium*, *Burkholderia*, *Bacillus*, *Pseudomonas*, just to cite a few, have been employed as alternative expression system, along with few archeal genera (*Methanococcus*, *Pyrococcus*, *Sulfolobus* and *Thermococcus*) (Ekkers et al., 2012; van Elsas et al., 2008). Among the different proposed hosts, *Streptomyces* spp. appear to be one of the most promising for metagenome libraries construction, also because of the well-developed methods of genetic transfer from *E. coli* to *Streptomyces* (McMahon et al., 2012). In addition to single-host expression, also multi-host expression strategies have been studied, thanks to the exploitation of broad-host-range vectors (Craig et al., 2010; Aakvik et al., 2009; Biver and Vandebol, 2013).

Screens of metagenomic libraries could be based either on metabolic activity (function-based or activity-based approach) or on nucleotide sequence (sequence-based or molecular approach) (Figure 1). The first one depends on the successful expression of target gene(s) in the metagenomic host; instead, molecular screening is based on the detection via hybridisation or PCR approaches of conserved DNA regions (Ekkers et al., 2012; van Elsas et al., 2008; Coughlan et al., 2015). Three major function-driven approaches have been used to recover novel biomolecules: (i) phenotypical detection, which is based on the identification of specific phenotypic traits associated with the activity of interest (for instance, colony pigmentation, degradation- or inhibition-halo formation); (ii) heterologous complementation, which relies on selection of clones that have acquired capability to grow under selective conditions, as in the presence of antibiotics or of a specific substrate given as sole carbon source; and (iii) induced gene expression (Ekkers et al., 2012; Coughlan et al., 2015). Substrate-induced gene expression screening (SIGEX) was first introduced in 2005 by Uchiyama and co-workers (Uchiyama et al., 2005) and is based on the use of an operon-trap GFP (green fluorescent protein) expression vector, where the metagenomic DNA is cloned upstream of the *gfp*

gene. Positive clones, co-expressing the GFP upon substrate-induced expression, could be isolated by fluorescence-assisted cell sorting (FACS). Similar approaches include METREX (metabolite-regulated expression) (Williamson et al., 2005) and PIGEX (product-induced gene expression) (Uchiyama and Miyazaki, 2010). As sequence information is not required, functional-based methods are the only strategy with the potential to identify new classes of genes encoding either known or new functions and lacking homologies to known sequences. Another advantage is the possibility to recover full-length genes and therefore functional gene products (Coughlan et al., 2015). However, the major drawback of this approach is its dependence on the eDNA expression in the surrogate host, which, as stated previously, is not always feasible. This limitation is particularly relevant when the expression of an entire biosynthetic gene cluster, which requires the coordinated production of multiple proteins, is needed (Katz et al., 2016). On the other hand, sequence-based approaches have the advantage of being expression-independent. However, being based on the identification of conserved nucleotide sequences, with this type of screening it is possible to identify only members of already known gene families. Besides, the detection of sequences of interest does not guarantee a functional and efficient expression of the target gene. Molecular screening is generally achieved by PCR techniques or by hybridisation studies with primers and probes specific for conserved regions of the genes being targeted, which for enzymes are usually the catalytic domains (Ekkers et al., 2012; Coughlan et al., 2015).

In the last few years, the rapid advancement of next generation sequencing (NGS) technologies, coupled with a significant reduction in sequencing costs, have given birth to the so-called ‘shotgun metagenomics’, *i.e.* the direct sequencing of isolated eDNA, bypassing the laborious steps of library construction and screening (Figure 1). Compared to the classical gene-focused sequence-based metagenomics screening, this approach has the advantage to potentially uncover genes that are more divergent and more interesting than the consensus genes with known sequences. Additionally, the detection of very low abundant members of complex populations is facilitated (Culligan et al., 2014; Kumar et al., 2015). Through the development of a number of sequencing platforms, such as

GS-FLX 454 pyrosequencer (Roche), MiSeq, HiSeq and Genome Analyzer II (Illumina), Ion Torrent and Ion Proton (Life Technologies), and the PacBio RS II (Pacific Biosciences) (Kumar et al., 2015; Escobar-Zepeda et al., 2015), shotgun metagenomics has become the method of choice for varied applications. NGS platforms allow massive parallel sequencing where hundreds of thousands to hundreds of millions of sequencing reactions are performed and detected simultaneously (Kumar et al., 2015). Such approach has been exploited for instance in the Human Microbiome Project (Turnbaugh et al., 2007), in the Earth Microbiome Project (Gilbert et al., 2010) and for the study of the complex microbial communities associated with coral reefs (Wegley et al., 2007). The popularity of this approach is expected to rise significantly as advancing sequencing technology and better bioinformatics analysis pipelines will improve effectiveness and throughput. From its outset, metagenome-based approaches have led to the accumulation of an extraordinary number of DNA sequences. In the last decade, metagenomic approaches have provided comprehensive data on microbial diversity and population dynamics in a large variety of ecosystems such as soil, global ocean and extreme environments. More recently, these technologies have been applied to medical and forensic investigations, for example for the identification of new viruses (Li et al., 2016) and for the reconstruction of the human gut and skin microbiome (Bashan et al., 2016), as well as for the analysis of extinct species and the study of microbial communities in food and animal feed (Santiago-Rodriguez et al., 2016). Metagenomics is currently thought to be one of the most likely technologies to provide new biotechnological products and processes. As described in details in the next section, metagenomics approaches have led to the discovery and characterization of a significant number of novel genes encoding biocatalysts (Fernandez-Arrojo et al., 2010). In addition to novel enzymes, also genes encoding novel therapeutic molecules and pharmacologically active secondary metabolites (such as antibiotics, antitumor agents, immunosuppressants and pigments) have been detected in metagenomics libraries (Schofield and Scherman, 2013; Coughlan et al., 2015; Katz et al., 2016). Natural products identified by such approaches include for instance turbomycin A and B (Gillespie et al., 2002), indirubin (Lim et al.,

2005), terragines (Wang et al., 2000), glycopeptide antibiotics (Owen et al., 2013), as well as fluostatins (Feng et al., 2011) and plantaricins (Pal and Srivastava, 2014). Furthermore, with metagenomic approaches lots of information has been gained about the diversity of natural antibiotic resistance mechanisms (DeCastro et al., 2014; Berman and Riley, 2013).

Metagenomics as tool for the identification of new biocatalysts

Since its establishment, metagenomics has been considered as the most likely and promising methodology for the identification of new and innovative biocatalysts. As stated by Lorenz and Eck in 2005, metagenome-sourced enzymes might function as backbones to be manipulated with protein engineering and *in vitro* evolution technologies for the production of the so-called ‘ideal biocatalysts’, *i.e.* improved tailored enzymes that optimally fit specific process requirements (Lorenz and Eck, 2005).

Metagenomic analyses in the last decade, conducted with educated function- or sequence-based screenings, have led to the identification of a series of putative genes, coding for potentially interesting and valuable biocatalysts. However, the subsequent characterization and exploitation of these enzymes have been limited by the persistent bottleneck of metagenomics analysis, which is the unsatisfactory heterologous expression of genes of unknown origin in the commonly used microbial hosts. For this reason, only a fraction of the identified putative genes has been successfully brought to expression and biochemically/structurally/functionally characterized (Fernandez-Arrojo et al., 2010). Common targets in metagenomics investigations include glycosyl hydrolases (cellulases, hemicellulases, amylases, chitinases, pectinases), lipases/esterases, and proteases; but also other industrially important enzymes, such as acylases, phosphatases, oxidoreductases and nitrilases, to cite a few, have been examined by metagenomics approaches (Table 1) (Fernandez-Arrojo et al., 2010; Ferrer et al., 2016). In this subsection, examples of enzymes with useful industrial application that have been unlocked by metagenomics are reported.

Cellulases/hemicellulases

Cellulases and hemicellulases (for instance xylanases, β -glucanases, mannanases, arabinases) collaborate to the saccharification of lignocellulose, the most abundant biopolymer on Earth, and find application in the production of a wide range of high-value-added products, first of all bioethanol. Several papers deal with the identification and characterization of (hemi)cellulases from metagenomics libraries: a comprehensive list of metagenome-sourced enzymes for lignocellulose conversion can be found in (Montella et al., 2015), whereas a general overview of glycosyl hydrolases from metagenomes is present in (Sathya and Khan, 2014). To these, some recent papers can be added. For example, Maruthamuthu and co-workers (Maruthamuthu et al., 2016) analysed by function-based screening two fosmid metagenomic libraries from wheat straw-degrading microbial consortia, which resulted in the discovery of novel thermo-alkaline β -galactosidase and β -xylosidase (Table 1). The NGS screening of a library from enriched anaerobic beer lees converting consortium allowed the identification of three novel acidophilic endo- β -1,4-glucanases, one of them with an interesting tolerance to high salt concentrations and to ionic liquids used in cellulose pre-treatments (Yang et al., 2016) (Table 1). Another versatile halotolerant and thermal-stable β -glucanase was identified by screening a library from paddy soil (Zhou et al., 2016), whereas two novel xylanases were discovered in libraries constructed from sheep's or goat's rumen (Wang et al., 2015; Cheng et al., 2016) (Table 1).

Laccases

Another class of enzymes involved in the degradation of lignocellulose biomass is the one of laccases, multi-copper oxidases with broad range of activity on both phenolic and non-phenolic substrates. Laccases are exploited at industrial level for the production of bioethanol, as well as in bioremediation strategies, and in paper, textile, chemical and food industries. To our knowledge, only five bacterial laccases have been characterized so far through metagenomics approaches (Table 1): two dye decolorizing, halotolerant laccases from marine microbial metagenomics libraries (Fang

et al., 2011; Fang et al., 2012a), one alkaline laccase from mangrove soil metagenome (Ye et al., 2010) and two laccases from bovine rumen microflora (Beloqui et al., 2006) and panda fecal microbiome (Fang et al., 2012b), respectively.

Chitinases

Chitinases are glycosyl hydrolases active on chitin, the second most abundant biopolymer after cellulose. These enzymes are attracting attention thanks to their various industrial and agricultural applications, especially as biocontrol agents in integrated pest management strategies, and for the production of chitin-derivatives (chitosan and chitooligosaccharides) with high pharmaceutical and nutritional potential. In the past decade, different research groups have applied metagenomics approaches for the identification of genes coding chitinolytic enzymes both in terrestrial and aquatic environments, but only in few cases a characterization of the encoded enzyme has been conducted (Table 1). In 2014, a chitinase with an interesting antifungal activity has been identified by a combination of sequence- and activity-based screenings applied to a suppressive soil metagenome and proposed as biocontrol agent (Hjort et al., 2014). The PCR-based analysis of a library from a chitin-amended agricultural soil, led to the discovery of a novel halophilic chitinase, with the potential to be exploited for the treatment and valorisation of seafood wastes (Cretoiu et al., 2015).

Lipases/esterases

Lipolytic enzymes catalyse the hydrolysis or the synthesis of ester bonds and are attractive biocatalysts for a variety of applications, including detergent, food, pulp and paper industries, diagnostics and therapeutics, as well as biodiesel production and biopolymer synthesis. Dozens of environmental DNA libraries have been screened for identifying novel lipases/esterases; particular attention has been directed to extreme environments, as reviewed in Lòpez-Lòpez et al., 2014. In Table 1 a few examples of metagenome-sourced enzymes with lipolytic activity are reported. Among the more recent papers on metagenome-sourced esterases, we can cite, for instance, a salt-

tolerant esterase from permafrost (Petrovskaya et al., 2016), an enzyme resistant to organic solvent and detergents from a soil contaminated with petroleum hydrocarbons (Pereira et al., 2015) and an alkaline, thermostable and solvent-tolerant esterase from marine mud (Gao et al., 2016). Peng et al. reported on an alkaline-stable lipase from marine sediments with a potential application in milk flat flavour production (Peng et al., 2014), whereas Su et al. and Kim et al. identified two novel lipases from marine sponge-associated microbiome and oil polluted mud flats, respectively, with potential application in detergent industry and organic synthesis (Su et al., 2015; Kim et al., 2015).

Proteases

Proteases represent one of the main groups of industrial enzymes due to their wide range of applications, spanning from detergents, leather and food processing, to peptide and pharmaceutical synthesis, brewing and wastewater treatment. Several proteases have been discovered by screening environmental DNA libraries; some of them are summarized in Table 1. For example, novel alkaline serine proteases were identified from a tannery activated sludge library (Devi et al., 2016), a saline habitat (Purhoit and Sing, 2013) and a forest soil (Biver and Vandenbol, 2013).

The potential of metagenomic analysis for the identification of innovative biocatalysts is testified by the high number of metagenome-sourced products that have been patented. Table 2 collects some representatives of patented enzymes from metagenomes, belonging to various protein families, from glycosyl hydrolases to lipases/esterases and oxidoreductases.

Additionally, a few enzymes from metagenomes have been already translated to the market. This limited number is not surprising, taking into consideration that several years are always required for the development of new enzyme-based production processes. In particular, it has been estimated that at least 5-7 years span from the identification of a putative biocatalyst to the final implementation of the catalytic process, with the more time- and cost-consuming steps being (i) the development of an optimised expression system allowing the production of sufficient amounts of enzyme, and (ii) the optimisation of its biochemical properties to meet the industrial requirements

(Ferrer et al., 2016; Kumar et al., 2016). Different companies, including BASF, DSM, Syngenta, Genencor International, and BRAIN AG have collaborated with various research groups for the commercialisation of technologies and natural products that evolved out of metagenomics (Lorenz and Eck, 2005; Kumar et al., 2016; Cowan et al., 2005). In particular, Diversa Corp., merged in 2007 with Celunol Corp to create Verenum, subsequently acquired in 2013 by BASF, has developed proprietary technologies to prepare and screen expression libraries from a variety of environmental DNAs (Mathur et al., 2005). The high-throughput screening of billions of samples, resulted in the identification of some lead enzymes, successively commercialized in the past few years (Table 3).

Conclusions

Metagenomics represents an innovative and promising tool for the exploration and exploitation of the microbial diversity encrypted in natural samples. Almost unheard-of only ten/fifteen years back, nowadays this area of research is under rapid development. Technical challenges still exist, connected for instance to screening procedures and heterologous expression of metagenome-derived enzymes, but clearly metagenomic approaches have the high potential to substantially impact industrial production.

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Table 1 Examples of industrially relevant enzymes and biocatalysts from metagenomic libraries

Target	Origin	Metagenomic library type	Number of screened clones	Screening approach	Reference
Acylase	Sediment	Cosmid	7.0×10^3	Function-based	Zhang et al. 2014
Agarase	Soil	Cosmid	1.5×10^3	Function-based	Voget et al. 2003
Alcohol dehydrogenase	Soil	Plasmid	2.8×10^3	Sequence- and function-based	Itoh et al. 2014
	Digester	Cosmid	2.01×10^5	Function-based	Wexler et al. 2005
Amidase	Soil	Fosmid	3.0×10^4	Function-based	Tannières et al. 2013
	Soil	Plasmid	na	Function-based	Gabor et al. 2004b
Amylase	Faeces	Fosmid	5.0×10^4	Function-based	Xu et al. 2014
	Digester	Phagemid	2.0×10^3	Function-based	Jabbour et al. 2013
Cellulase/hemicellulase	Wheat straw	Fosmid	1.4×10^5	Function-based	Maruthamutu et al. 2016
	Beer lees	/	/	Sequence-based*	Yang et al. 2016
	Paddy soil	Fosmid	2.5×10^4	Function-based	Zhou et al. 2016
	Sheep rumen	Fosmid	1.2×10^4	Function-based	Wang et al. 2015
	Goat rumen	BAC	1.2×10^4	Function-based	Cheng et al. 2016
Chitinase	Soil	Fosmid	7.8×10^3	Sequence- and function-based	Hjort et al. 2014
	Soil	Fosmid	1.45×10^5	Sequence-based	Cretoiou et al. 2015
Laccase	Sea	BAC	2.0×10^4	Sequence-based	Fang et al. 2011
	Sea	BAC	2.0×10^4	Sequence-based	Fang et al. 2012a
	Bovine rumen	Phagemid	2.0×10^5	Function-based	Beloqui et al. 2006
	Faeces	BAC	3.1×10^4	Sequence- and function-based	Fang et al. 2012b
	Mangrove soil	Plasmid	8.0×10^3	Function-based	Ye et al. 2010
Lipase/esterase	Permafrost	Fosmid	5.0×10^3	Function-based	Petrovskaya et al. 2016
	Soil	Fosmid	4.2×10^3	Function-based	Pereira et al. 2015
	Marine mud	Fosmid	4.0×10^4	Function-based	Gao et al. 2016
	Sediment	Plasmid	1.18×10^5	Function-based	Peng et al. 2014
	Marine sponges	Plasmid	6.5×10^3	Function-based	Su et al. 2015
	Mud flats	Plasmid	3.0×10^3	Function-based	Kim et al. 2015
Nitrilase	Soil	Plasmid	3.79×10^6	Function-based	Bayer et al. 2011
	Soil and water	Phagemide	$1.0 \times 10^6 - 1.0 \times 10^9$	Function-based	Robertson et al. 2004
Pectinase	Soil	Plasmid	2.0×10^3	Function-based	Sathya et al. 2014
	Hot spring	/	/	Sequence-based#	Singh et al. 2012
Phosphatase/phytase	Groundwater	na	na	Sequence-based§	Tan et al. 2015
	Fungus garten	na	na	Sequence-based§	Tan et al. 2016
	Sediment	Fosmid	8.0×10^4	Function-based	Lee et al. 2015
Protease	Tannery sludge	Plasmid	1.0×10^4	Function-based	Devi et al. 2016
	Soil	Plasmid	na	Sequence- and function-based	Purhoit and Singh 2013
	Soil	Plasmid	7.0×10^4	Function-based	Biver and Vandenbol 2013
Racemase	Soil	Plasmid	na	Function-based	Chen et al. 2009
Rhamnosidase	Faeces	Fosmid	2.0×10^4	Sequence- and function-based	Rabausch et al. 2014
Tannase	Soil	Plasmid	9.2×10^4	Function-based	Yao et al. 2011

*= shotgun metagenomic analysis; §=bioinformatic analysis of the metagenomes available in the IMG/M database;

#=PCR amplification directly on eDNA; na = not available

Table 2 Examples of patented enzymes from metagenomes and their suggested applications.

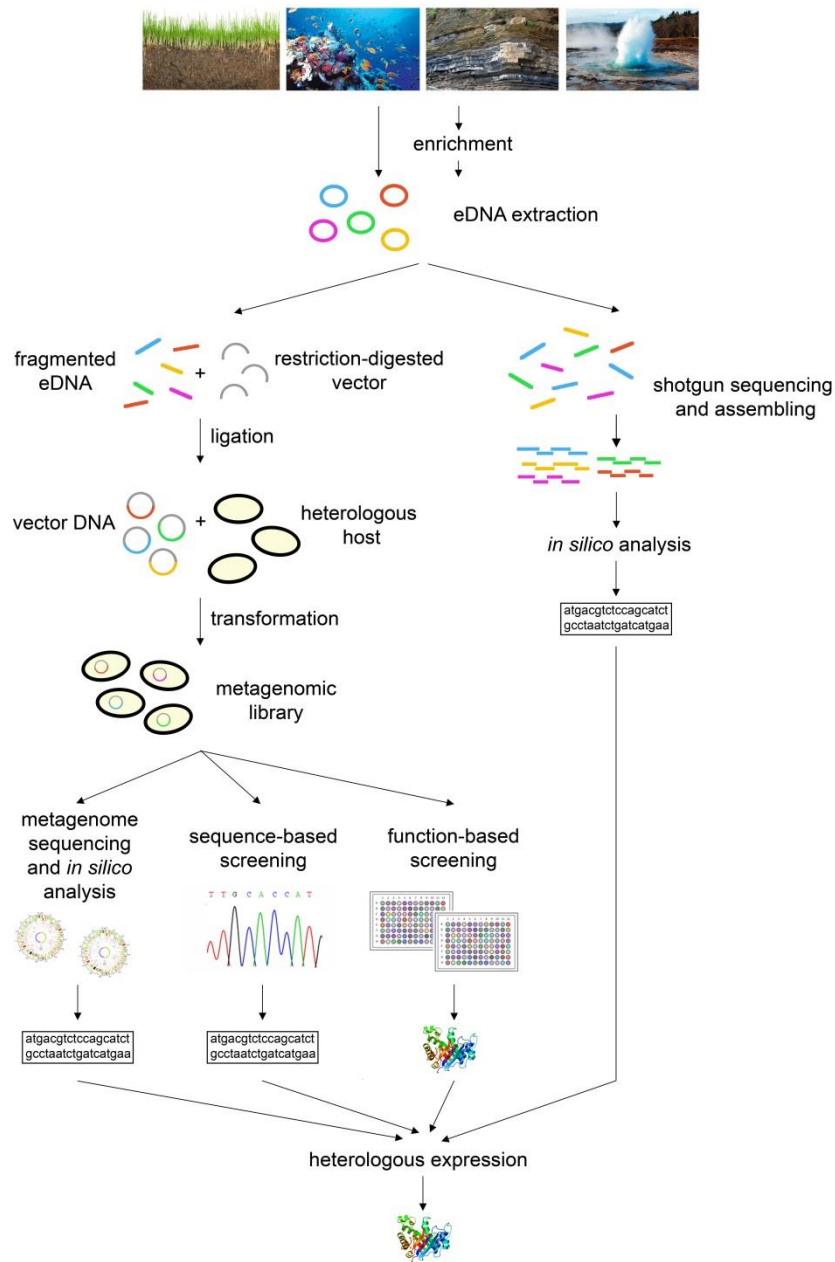
Enzyme	Source	Patent number	Suggested applications
Amylase	na	EP1463810 A2	Washing and cleaning products
	Animal manure	CN103290039 B	Animal feed, starch processing industries
Cellulase/hemicellulase	Hot spring	EP2980212 A1	Biofuel production from lignocellulosic biomasses
	Bovine rumen	WO2014142529 A1	Biofuel production from lignocellulosic biomasses, fibers, detergents, feed, food, pulp, paper production
	Hot spring	EP2982751 A1	Biofuel production from lignocellulosic biomasses
	Hot spring	EP2990482 A1	Biofuel production from lignocellulosic biomasses
DNA polymerase	Viruses of halophilic archebacteria	WO2012173905 A1	Nucleic acid amplification
β -galactosidase	na	EP2530148 A1	Food processing (lactose depletion)
Laccase	Bovine rumen	US20090305339 A1	Kraft pulp biobleaching, food processing; bioremediation
Lipase/esterase	Atlantis II Red Sea brine pool	US20160053239 A1	Industrial processes for lipases/esterases (for instance, leather manufacture, oil biodegradation, synthesis of pharmaceuticals and chemicals) under harsh conditions
	Fermented food	CN105368802 A	Food and cosmetic production
	Mangrove soil	CN103834626 A	Industrial applications which require high temperature resistant lipases
	Tidal flat sediment	EP2784160 A1	Oil and fat purification and conversion, biomedicine, fine chemistry
L-methionine γ -lyase	Deep sea sediment	CN101962651 B	Clinical detection, food flavour production, cancer therapy
Muramidase (Lysozyme)	Soil	CN101892252 B	Antibiosis, bacteriolysis
Phosphatase	na	US8647854 B2	Genetic cloning, enzyme immunoassays
Peptidase	Compost soil	CN103409443 A	Food processes, life science research, protein waste treatment
Sucrase	Waste contaminated soil	CN101407820 A	Sugar cane degradation

na = not available

Table 3 Examples of enzymes commercialised by BASF Enzymes LLC and identified by screening of environmental samples.

Commercial name	Enzyme class	Applications
Luminase™	Xylanase	Pulp biobleaching in paper production
Fuelzyme®	α -Amylase	Fuels and industrial-use alcohols production
Pyrolase™ 160, Pyrolase™ 200	Cellulase	Secondary oil and gas recovery
Phyzyme™ XP	Phytase	Additive for livestock feed

Fig. 1 Schematic view of metagenomics strategies for the identification of novel biocatalysts from environmental DNA.



Appendix 2



In Prague, August 19, 2016

To whom may concern:

With this letter, I state that Carmine Casciello has spent two months (from 11 April 2016 to 13 June 2016) in the laboratories of Microbial Biotechnology at the University of Chemistry and Technology Prague, under my supervision. He worked on the enzyme characterization and production of ligninolytic and cellulolytic enzymes in actinomycetes and fungi, in the frame of a collaborative program with the laboratories of Microbial Biotechnology at the University the Insubria, where he is completing his PhD under the supervision of prof. Flavia Marinelli.

On the basis of the two months Carmine has been working in Prague, I can say that he is a helpful and hard-working student who fitted well into our international laboratory team. He collaborated with both my Czech students and an ERASMUS student from Portugal. I also appreciate his good humour and ability to work precisely and correctly.

Sincerely yours,

Petra Pataková

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