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Laccases from *Actinobacteria*—What We Have and What to Expect

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Abstract

Laccases are blue multicopper enzymes, capable of oxidizing diverse aromatic and non-aromatic compounds of industrial interest, concomitantly with reduction of molecular oxygen to water. Tolerance to extreme conditions, such as high temperature, salinity or extreme pH, is required for practical industrial applications. Here we focus on bacterial laccases from the phylum *Actinobacteria*, notably the order *Actinomycetales*. Currently, less than 10 enzymes have been properly characterized, all belonging to genus *Streptomyces*, but it is noteworthy that all of them have exhibited industrially important properties. Furthermore, studies with enzymes from this phylum revealed a novel molecular structure of laccases, providing the basis for a distinct family, the two-domain laccases. The relevant traits of actinomycetes laccases emphasize the need for more studies involving the isolation of this bacterial group from lignin-rich environmental samples, detection of their laccase activity and thereafter, characterization of the proteins and related genes. The non-homogeneous responses of actinomycetes laccases to traditional inhibitors, substrates or metal ions have challenged the currently accepted "laccase concept". Finally, considering that distinguishing laccase activity *in vitro* from other ligninolytic enzymes becomes a difficult task due to overlaps in catalytical properties of the enzymes, we proposed a simple flow chart to help experimental assays.

Keywords

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1. Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are blue multicopper oxidases, capable of oxidizing a broad range of aromatic and non-aromatic compounds [1]. These enzymes were first discovered in plants [2], thereafter in fungi [3], and only one century later they were found in *Bacteria* [4]. Most studies about laccases are still conducted on fungi [5], and few are known about the physiological roles of laccases in bacteria, their potential for industrial application or their biochemical properties.

The role of laccase-producing microorganisms on the environment is not thoroughly elucidated, but it is speculated that these organisms are involved in the degradation of recalcitrant (poly) phenolic compounds [6]. Interestingly, the oxidation of large molecules by laccase is directly inhibited by steric hindrance, but the reaction can still occur through chemical mediators—small molecules oxidized by laccases that diffuse in surrounding medium and oxidize the substrate [7]. This laccase-mediator system (LMS) also allows indirect oxidation of non-phenolic compounds [8] or even molecules with higher redox potentials than laccase. Besides this intrinsic versatility and the large substrate variety, application of laccases has been suggested for diverse industrial processes; however, few have been accomplished so far, due to high costs of microbial cultivation or enzyme purification [5].

In fungi, laccases have been extensively reviewed in many aspects: occurrence, characterization of genes and proteins from different taxonomic divisions, functions and applications [9]-[14]. Whereas the literature provides some general reviews on bacterial laccases [15] [16], and some studies have dealt with lignocellulolytic potential of actinomycetes [17] [18], its current laccase *state-of-art* is still obscure. It is remarkable that actinomycete strains are generally detected in prospective bacterial studies, and laccases with unusual properties have been discovered [19]-[21]. Here, we present a view of this interesting microbial group, its characterized laccases and the effects of its properties on the concept of laccase enzymes.

2. Defining Laccase Enzymes

Laccase and Other Multicopper Oxidases

Laccases are generally defined as multi-copper oxidase enzymes (MCO) that catalyse oxidation of diverse phenolic and non-phenolic substances using dioxygen as final electron acceptor, producing water molecules as by-product, and that possess some conserved amino acid motifs responsible for binding to copper atoms [22]. However, advances in studies concerning the diverse group of multicopper oxidases (MCO) have raised a question about the classification of laccase, due to overlaps in amino acid signature and substrates are shared by many MCO; for instance, ascorbate oxidase, tyrosinase, polyphenol oxidase or bilirubin oxidase [23]. Thus, a narrow definition has not been achieved so far. Therefore, it has been proposed the use of the term "laccase-like multicopper oxidase" (LMCO) [24] for the formerly named laccase enzymes. This term has been adopted in this work for simplification.

3. Actinobacteria and Their LMCO

3.1. The Phylum and Its Biotechnological Potential

The phylum *Actinobacteria* represents one of the largest and most diverse groups within the *Bacteria* domain; it comprises Gram-positive bacteria with a high G + C mol% content in DNA [25]. It is a cosmopolitan group which was found inhabiting almost all known ecological niches. *Actinobacteria* are considered of great importance in the process of degradation of recalcitrant and relatively complex polymers that are naturally found in litter and soil, such as lignin and humic acid [6] [26]-[29]. Particularly, the order *Actinomycetales* has attracted industrial attention mainly due to its outstanding capacity to produce compounds with high biotechnological values, e.g., antibiotics. Indeed, actinomycetes are considered remarkable antibiotic-producing and since their first antibiotic discovery (streptomycin at late 40's), their participation in the antibiotic market continues to grow [30] [31]. Similarly to these unique antibiotic biosynthetic pathways, it has been stated that actinomycetes may also produce attractive enzymes for biotechnological applications [32].

3.2. Characterized LMCO from Actinomycetes

Studies on LMCO characterization from actinobacteria are scarce and it is an open field for exploration. To the

best of our knowledge, only LMCO from the following species have been characterized in gene and protein levels: *Streptomyces griseus* IFO 13350 [33], *Streptomyces lavendulae* REN-7 [34], *Streptomyces cyaneus* CECT 3335 [19], *Streptomyces coelicolor* M145 [35], *Streptomyces psammoticus* MTCC 7334 [20], *Streptomyces ipomoea* CECT 3341 [36], *Streptomyces sviceus* DMS 924 [21] and *Streptomyces* sp. C1 [37]. Their main properties are presented in **Table 1**. Many putative laccase genes are listed in data banks, and other LMCO have been characterized only in protein level; e.g., the thermotolerant LMCO from *Streptomyces* sp. SB086 [38].

3.3. Structural Properties—Two-Domain LMCO

LMCO belong to the group of blue multicopper proteins containing four copper atoms classified in types 1 to 3 (two atoms are type 3). All secondary structural elements typical of fungal LMCO are present in bacterial sequences [41]. The three Cu-binding domains that form the active site are composed by multiple histidine and one cysteine copper-binding residues, which are present in the type 1 (2 his + 1 cys), 2 e 3 sites (8 his), where Cu atoms are distributed [33] [42] [43]. This canonical structure is also found among the characterized actinobacterial LMCO (**Figure 1**). Furthermore, studies on actinobacterial LMCO have provided a very interesting finding concerning to their molecular structure. Instead of three structural domains, some actinobacterial LMCO present only two domains (wherein the three Cu-binding domains and the four Cu atoms are distributed). This peculiar two-domain structure, also named "small LMCO" was elucidated in *Streptomyces coelicolor* [35]. It lacks the second domain, which is composed by one loop at the end of domain one, two β-turns from domain three, and its own amino acid residues [42]. Two-domain LMCO has not been found in fungi so far, but was found in ten other phyla within *Bacteria*, and also in *Archaea*, by molecular analysis [44].

Table 1. Characterized laccases from *Actinobacteria* and their main properties.

Laccase, species	Secondary, quaternary structures	Molecular mass	Optimum pH, substrate	Kinetic parameters[*]	Optimum temperature	Tolerance/ Resistance	Reference
EpoA, *Streptomyces griseus*	2 domains, trimer	114 kDa, (monomer: 38 kDa)	6.5, DMPPDA	$K_m = 0.42$ mM, $V_{max} = 0.85$ nmol/min	40°C	High temperature	[33]
STSL, *Streptomyces lavendulae*	nd	73 kDa	4.5, catechol	$Km = 0.043$ mM, $k_{cat} = 10.9$ s^{-1}, $k_{cat}/K_m = 253$ mM^{-1}·s^{-1}	50°C	Alkaline medium, high temperature	[34]
Nd, *Streptomyces cyaneus*	nd	75 kDa	4.5, ABTS	$Km = 0.38$ mM	70°C	High temperature	[19]
SLAC, *Streptomyces coelicolor*	2 domains, trimer	69 kDa; (monomer: 32 kDa)	9.4, DMP 4.0, ABTS	$Km = 0.4$ mM, $k_{cat} = 4$ s^{-1}, $k_{cat}/K_m = 10$ mM^{-1}·s^{-1}	60°C	Alkaline medium, high temperature	[35] [39] [40]
Nd, *Streptomyces psammoticus*	nd	43 kDa	8.5, ABTS	$K_m = 0.39$ mM (ABTS), $K_m = 3.35$ (SGZ)	45°C	High salt concentration, alkaline medium	[20]
SilA, *Streptomyces ipomoea*	2 domains, dimer	79 kDa; (monomer: 44.7 kDa)	5.0, ABTS; 6.5, aromatic amine; 8.0, DMP	$K_m = 0.40$ mM, $V_{max} = 7.59$ mU/µg, $k_{cat} =, 9.99$ s^{-1}, $k_{cat}/K_m = 25$ mM^{-1}·s^{-1} (ABTS); $K_m = 4.27$ mM, $V_{max} = 3.19$ mU/µg, $k_{cat} = 4.20$ s^{-1}, $k_{cat}/K_m = 0.98$ mM^{-1}·s^{-1} (DMP)	60°C	High salt concentration	[36]
Ssl1, *Streptomyces sviceus*	2 domains, trimer	98.3 kDa; (monomer, 32.5 kDa)	4.0, ABTS; 8.0, SGZ; 9.0, DMP or guaiacol;	$K_m = 0.36$ mM, $k_{cat} = 7.38$ s^{-1} (ABTS); $K_m = 0.89$ mM, $k_{cat} = 0.32$ s^{-1} (DMP); $K_m = 15.8$ mM, $k_{cat} = 5.78 \times 10^{-2}$ s^{-1} (SGZ)	---	High temperature, alkaline medium, wide pH variation	[21]
SCLAC, *Streptomyces* sp.	2 domains	38 kDa	8.0, ABTS or guaiacol; 7.0, DMP	$K_m = 0.43$ mM, $V_{max} = 8.62$ mU/µg, $k_{cat} = 8.45$ s^{-1}, $k_{cat}/K_m = 17.6$ mM^{-1}·s^{-1} (ABTS); $K_m = 5.58$ mM, $V_{max} = 3.45$ mU/µg, $k_{cat} = 4.88$ s^{-1}, $k_{cat}/K_m = 0.83$ mM^{-1}·s^{-1} (SGZ); $K_m = 1.65$ mM, $V_{max} = 5.26$ mU/µg, $k_{cat} = 2.39$ s^{-1}, $k_{cat}/K_m = 1.45$ mM^{-1}·s^{-1} (guaiacol)	40°C	Alkaline medium, high temperature	[37]

Abbreviations: ABTS, 2,2'-*azino-bis*(3-ethylbenzothiazoline-6-sulphonic acid); DMP, 2,6-dimethoxyphenol; DMPPDA, dimethyl p-phenylenediamine sulphate; SGZ, syringaldazine; nd, no available data. Except where indicated, parameters were determined at optimum pH and cited substrate. For SLAC, parameters were determined with DMP, at pH 9.4.

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                             2 3              3 3             1 2 3             313      1
                             ↓ ↓              ↓ ↓             ↓ ↓ ↓             ↓↓↓      ↓
   S. araujoniae    99 VSLHVHGVD  153 WHYHDHVVG  228 EFYHTFHIHGHR  282 GAWMYHCHVQSHSDMGMA
   S. coelicolor    99 ASLHVHGLD  153 WHYHDHVVG  228 EYYHTFHMHGHR  282 GAWMYHCHVQSHSDMGMV
     S. ipomoeae    99 ASLHVHGLD  153 WHYHDHVVG  228 EYYHTFHMHGHR  282 GAWMYHCHVQSHSDMGMV
      S. griseus    99 ASLHVHGVD  153 WHYHDHVVG  228 EFYHTFHIHGHR  282 GAWMYHCHVQSHSDMGMA
      S. cyaneus   142 SVTHLHGAQ  182 WWYHDHAMN  505 PIVHPMHIHLAD  581 GRFMYHCHLLEHEDMGMM
   S. lavendulae   142 TVTHLHGAQ  182 WWYHDHAMN  508 PVVHPMHIHLAD  584 GRFMYHCHLLEHEDMGMM
      B. subtilis  102 TVVHLHGGV  150 LWYHDHAMA  416 RGTHPIHLHLVS  486 GRYVWHCHILEHEDYDMM
 M. mediterranea   195 TNFHTHGLW  238 FWYHAHVHG  580 APMHPYHIHVNP  651 GAFVLHCHILDHEDQGMM
T. thermophilus     92 TNLHWHGLP  132 FWYHPHLHG  390 DMDHPFHLHVHP  439 GRTVFHCHIVEHEDRGMM
    B. halodurans  102 TALHLHGFP  144 YWYHSHQDG  410 DFDHPMHLHGDF  460 GNWMFHCHEFHHASGGMV
      C. cinereus   91 TSIHWHGIF  137 YWYHSHFET  435 IGPHPFHLHGHA  490 GPWFFHCHIEFHLSGGLG
      P. radiata    82 TTIHWHGFF  127 FWYHSHLST  415 GGPHPFHLHGHT  468 GPWFLHCHIDWHLEAGFA
     A. nidulans    85 TTVHFHGTP  124 YFYHAHSRG  486 QPPHPIHKHSNK  557 GPFLLHCHIQIHQSGGMA
      N. crassa    141 TSIHWHGMH  186 SWYHSHFSA  474 SLPHPIHLHGHD  543 GSWLMHCHIAWHVSGGLS
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Figure 1. Alignment of Cu-binding domains of actinobacterial, bacterial and fungal laccases. Arrows and accompanying numbers indicate the amino acid residues for Cu-binding and the type of each ligand Cu. The position of the first residue of each fragment in the polypeptide chain is indicated. *Streptomyces* spp.: *S. araujoniae* (unpublished); *S. coelicolor* (CAB45586); *S. ipomoea* (ABH10611); *S. griseus* (BAB64332); *S. cyaneus* (ADX97492); *S. lavendulae* (BAC16804). Other bacteria: *Bacillus subtilis* (ADZ57284); *Marinomonas mediterranea* (AAF75831); *Thermus thermophilus* (BAE16261); *Bacillus halodurans* (AAP57087). Basidiomycota: *Coprinus cinereus* (ABP81837); *Phlebia radiata* (Q01679). Ascomycota: *Aspergillus nidulans* (Q96VT5); *Neurospora crassa* (AAA33592).

In silico studies have been performed by comparison of genomes with published gene sequences of characterized LMCO, generating thousands of putative LMCO genes that have broadened its distribution in the bacterial phyla. Searching within 2211 complete and draft bacterial genomes and four metagenomic data sets, Ausec *et al.* [44] have found 1240 genes for LMCO enzymes in 807 different microorganisms, or 36% of all organisms evaluated. This high frequency changes the idea that LMCO are proteins found only in few bacteria [39].

Two-domain LMCO characterized in actinomycetes are active as dimers (*S. ipomoea*) or trimers (*S. griseus*, *S. coelicolor* and *S. sviceus*). The oligomerization in actinobacterial LMCO (**Table 2**) contrasts with fungal, which are mainly monomeric enzymes.

4. Industrial Applications

Lignin is the most associated substrate for LMCO oxidation, in which the enzyme acts on its polymerization as well as on depolymerization [10]. Lignin tightly involves cellulose fibers in wood and is very resistant to breakdown. It is generally viewed as a side-product of lignocelluloses treatment and used as thermal resource after wood physicochemical pre-treatments. Lignin comprises approximately 10% to 30% of vegetal dry mass [27] and therefore, achieving an efficient method for lignin-rich biomass conversion into second generation ethanol production may be economically advantageous.

Lignin structure is very complex, composed by randomly linked subunits. Two consequences of this structure are: a) only a few enzymes are able to cause cleavages—mainly manganese peroxidase, lignin peroxidase and LMCO; and b) these enzymes are flexible for acting on many different covalent linkages. The versatility and eco-friendly mechanism (water is released as byproduct) of LMCO have long attracted industry interest and currently their application is desired in diverse industrial processes. For instance, application of LMCO has been proposed for activities from many sectors of industry [7] [10] [45]-[48]:

- textile: elimination or attenuation of dyes, improvement in whiteness in conventional bleaching of cotton, use in cleansing products for cloth washing;
- food: elimination of undesirable phenolic compounds in bread, juices, beer and wine for stabilization and improvement of their organoleptic properties; consumption of oxygen in packed food or derived from plants to avoid undesired oxidation;
- pulp and paper: cellulose bleaching, cross-linking in ligninaceous compounds, decolorization and deinking of printed paper;
- pharmaceutical: functional organic compounds synthesis (e.g. anesthetics, antibiotics and anti-inflammatory), biosensor for detection of target molecules in immunoassays;
- nanobiotechnology: polymers production, use in biosensors for detection of oxygen and phenolic compounds, use as biocatalyst for the electrode reactions in medical applications;

Table 2. Substrate specificity and inhibition profiles of characterized laccases from *Actinobacteria*.

Laccase, species	Substrate							Inhibition		References
	Syringal-dazine	Guaiacol	ABTS	DMP	DOPA	Catechol	Tyrosine	Sodium azide	EDTA	
EpoA, *Streptomyces griseus*	No	Low	---	No	Yes	No	No	Yes	Yes	[33]
STSL, *Streptomyces lavendulae*	Yes	Low	---	---	Yes	Yes	Very weak	Yes	Yes	[34]
Streptomyces cyaneus	Low	Very low	Yes	Yes	---	---	No	Yes	Yes	[19]
SLAC, *Streptomyces coelicolor*	Yes	---	Yes	Yes	Yes	Yes	---	No	No	[35] [39] [40]
Streptomyces psammoticus	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	[20]
SilA, *Streptomyces ipomoea*	Yes	---	Yes	Yes	---	Yes	No	No	Yes	[36]
Ssl1, *Streptomyces sviceus*	Yes	Yes	Yes	Yes	---	---	No	No	---	[21]
SCLAC, *Streptomyces* sp.	Yes	Yes	Yes	Yes	Yes	---	---	Yes	Yes	[37]

Abbreviations: ABTS, 2,2'-*azino-bis*(3-ethylbenzothiazoline-6-sulphonic acid); EDTA, ethylenediamine tetraacetic acid; DMP, 2,6-dimethoxy-phenol; DOPA, dihydroxyphenylalanine; ---, no available data.

- cosmetics: hair dye production, dermatological preparations for skin lightening, deodorants, toothpaste, mouthwash, detergent, soap, and diapers;
- bioremediation: degradation of phenolic compounds in wastewaters from olive oil, cellulose factories or food industry; decontamination of soils containing polycyclic aromatic carbohydrates or pesticides; dye bleaching of textile effluents; reduction of odor from garbage disposal sites; and, strikingly, microbial polyethylene and polystyrene degradation, a capacity attributed to LMCO of very few organisms, including the actinobacterium *Rhodococcus ruber* [49]-[51].

Until recently, commercial products based on LMCO were available only for food, paper and textile industries, due to the high cost of production, which impedes a broader application of this enzyme [52].

Each specific industrial application requires LMCO with specific properties, concerning optimum pH, temperature, resistance to metal inhibition, etc. For instance, wine stabilization occurs in an acid sulphite-rich environment, and therefore, stability in acid medium and reversible inhibition by sulphite are well desired properties for LMCO used in this process [53]. On the other hand, addition of LMCO to washing powders, decolourization of waste waters, or treatment of kraft pulps require enzymes with high activity at alkaline conditions [21]. Industrial processes may present such extreme conditions; thermostable enzymes are commonly more resistant to chemical denaturation, alkalinity or extreme acidity [54], and bacterial LMCO have proven to be more resistant than the average LMCO found in fungi [55]. In the last decades, although it has been reported thermotolerant LMCO in bacteria [35] [39] [56] they have not been industrially-explored.

The LMCO currently used by industries were obtained from fungi [52]. Some filamentous fungi secrete high amounts of LMCO into the medium, however, uncontrolled growth may also lead to an accumulation of polysaccharides or production of proteases that interfere in the LMCO yield [57]. Also, fungal LMCO generally present more acidic optimum pH when compared to bacterial enzymes [11] [24], whereas most of LMCO applications require activity in alkaline conditions [21]. Alkaline-tolerant LMCO have been found in bacteria [58] and many actinobacteria (**Table 1**).

Thus, the current LMCO biotechnological scenario urges for robust enzymes (pH and temperature tolerant) in high-yield producers. Obtaining such systems would render economic and more healthy or environment-friendly products, and bacteria seem to be a reliable alternative to overcome problems usually observed in LMCO from fungi.

Strikingly, all LMCO characterized from actinomycetes present one or more desirable properties for industrial application, although, up to date, they have been tested mainly for degradation of dyes used by textile industry. LMCO from *S. coelicolor* rapidly decolourised the common blue jeans dye Indigo carmine with syringaldehyde as mediator: over 85% decolourisation occurred within 20 minutes incubation at 45˚C [39]. Similarly, SCLAC, from *Streptomyces* sp., efficiently decolourized Indigo carmine and Diamond Black PV in presence of syringal-

dehyde [37]; dye Orange II was 90% degraded after 4h of incubation with the LMCO produced by *S. ipomoea* [36]. *S. psammoticus* LMCO was tested against 10 different dyes, but was effective only for RBBR (Remazol Brilliant Blue R) [59]. The only enzyme tested for pulp and paper industry was produced by *S. cyaneus*; this LMCO delignified kraft pulp in the presence of the mediator ABTS [19].

5. Catalytic Properties

5.1. Responses of Actinomycetes LMCO to Common Inhibitors

Ethylenediamine tetraacetic acid (EDTA) is a common LMCO inhibitor. Among characterized actinobacterial LMCO, EDTA inhibited SilA (*S. ipomoea*), but not SLAC (*S. coelicolor*) (**Table 2**). LMCO are also affected by metal ions, e.g. Ca, Mn, Co, Cu, Fe, Zn, Mg, Ni, Hg, but the profiles of inhibition or activation are very variable among enzymes. For example, Fe inhibited SilA and *S. cyaneu*s LMCO, Cu inhibited SilA and activated *S. cyaneus*, Zn inhibited *S. cyaneus*, whereas *S. psammoticus* LMCO was activated by all of these ions (**Table 2**). Furthermore, *S. cyaneus* CECT 3335 enzyme had 75.7% of its activity hindered by cinnamic acid. Sodium azide is considered as the only true LMCO inhibitor [60]. However, SLAC, SilA and Ssl1 (*S. sviceus*) did not present the typical complete inhibition in presence of 1 mM sodium azide: instead, SLAC was not affected at all at this concentration, SilA presented only 40% activity inhibition even at 10 mM sodium azide, whereas Ssl1 presented only 5% inhibition at 10 mM. An interesting observation concerning this resistance to sodium azide is that all of the three enzymes are 2-domains LMCO (**Table 1** and **Table 2**). Notwithstanding, although only few studies were performed, the obtained data strongly indicate the potential of actinobacterial LMCO for industrial purposes and raise the possibility that these enzymes may overcome fungal LMCO in industrial suitability.

5.2. LMCO and Ligninolytic Enzymes

LMCO, lignin peroxidase (LiP, E.C. 1.11.1.14) and manganese peroxidase (MnP, E.C. 1.11.1.13) are the three main ligninolytic enzymes. Unlike LMCO, MnP and LiP are heme-containing enzymes that use H_2O_2 as electron acceptor [61].

Some substances have long been cited as "specific laccase substrates", such as syringaldazine [34] [60] [62], or "model laccase substrates", such as guaiacol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), dihydroxyphenylalanine (DOPA) and catechol. Notably, concerning actinomycetes, syringaldazine is not a substrate for all LMCO: EpoA (*S. griseus*) does not oxidize it, while SLAC (*S. cyaneus*) exhibited very low affinity (**Table 2**). A *consensus* in oxidation was only obtained for the substrates ABTS and DOPA, but they still have to be tested for some of the enzymes. Both syringaldazine and ABTS can be oxidized by the ligninolytic enzymes MnP or LiP in presence of H_2O_2. Fortunately, the H_2O_2-dependent oxidation permits the differentiation between that enzymes and LMCO. Ahmad *et al*. (2011) tested oxidation of syringaldazine and ABTS in presence or absence of H_2O_2 to support the conclusion that the protein DypB from the actinobacterium *Rhodococcus jostii* RHA1 is a LiP [63]. LMCO can also be distinguished from MnP by an assay with the stain leucoberbelin blue, which specifically reacts with Mn compounds released by MnP and produces a blue color that absorbs at 620 nm [64]. Among characterized actinobacterial LMCO, this test was performed only for EpoA, from *S. griseus*, but has proved to be useful in determining classification of protein CotA, from *Bacillus subtilis* [65]. A third class of peroxidase that performs both MnP and LiP functions was discovered in *Pleurotus eryngii* and named versatile peroxidase [66]. Versatile peroxidases were not found in *Bacteria* so far.

Other steps are necessary in order to biochemically classify the enzyme, as tyrosine oxidation test and inhibition tests. Both LMCO and tyrosinases oxidize DOPA (dihydroxyphenylalanine), but only tyrosinases oxidize tyrosine to DOPA [9]. Then, tyrosine oxidation assay is often included in characterization studies. An exception to this pattern is the multipotent polyphenol oxidase from the melanogenic marine *Alteromonas* sp., which presents catalytic capabilities of both tyrosinases and LMCO [67].

However, as it is common that LMCO from actinomycetes are only biochemically-assayed, without gene sequencing, we have suggested a sequence of substrate tests for help in confirmation of which ligninolytic enzyme is present in extract, or if more than one is present. The flow chart (**Figure 2**) was built based on characterized actinomycetes LMCO (**Table 2**); besides the ligninolytic enzymes, we have included tyrosinase, a MCO, for its overlap with LMCO in DOPA oxidation. However, it seems likely that a similar scheme would be possible for distinguishing LMCO from other MCO as more proteins are isolated and characterized.
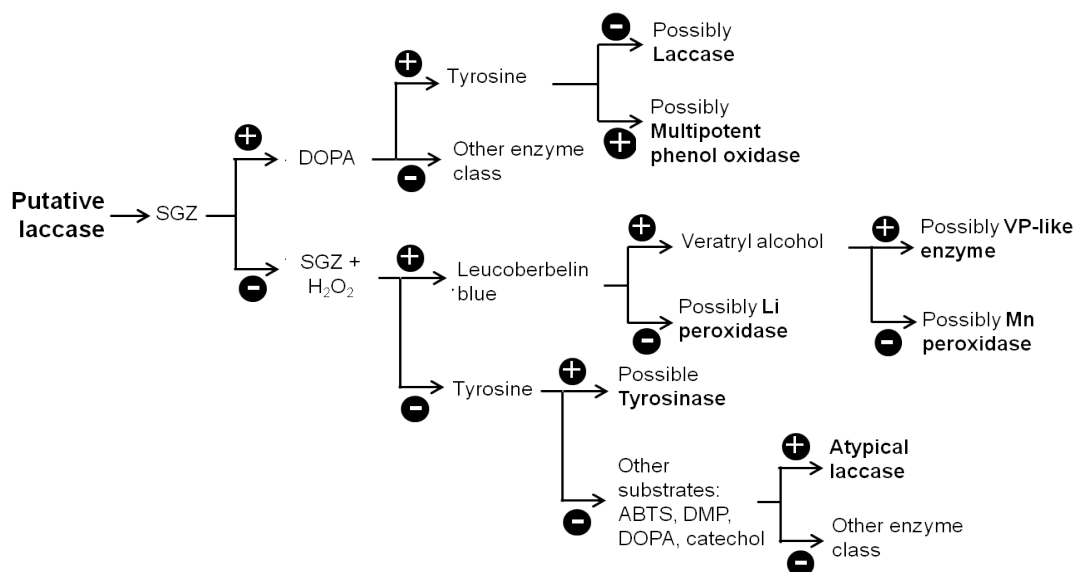
Figure 2. Suggested flow chart for differentiation of laccase and other ligninolytic enzymes. Plus (+) signals indicate positive reaction (e.g., colored reaction in leucoberbelin blue test; or higher enzyme activity in presence of H_2O_2, compared to in its absence); minus (-) signals indicate negative result (e.g., non-oxidation of tyrosine; or no higher activity in presence of H_2O_2). Abbreviations: Li, lignin; Mn, magnesium; SGZ, syringaldazine; ABTS, 2,2'-*azino-bis* (3-ethylbenzothiazoline-6-sulphonic acid); DMP, 2,6-dimethoxyphenol; DOPA, dihydroxyphenylalanine; VP, versatile peroxidase. Further assays can be performed after identification of the "possible enzyme", as affinity tests for common substrates and inhibitors.

6. Multigene LMCO Families

Fungal and plant LMCO genes occur as multigene families. For example, the fungus *Pleurotus ostreatus* harbors up to 12 LMCO genes [68] whereas *Coprinopsis cinerea* [69] and the plant model *Arabidopsis thaliana*, 17 genes [70]. Furthermore, the number of LMCO fungal genes seems to be linked to microbial lifestyle, as saprophytes (wide range of substrates) contain up to 8 genes per species, whereas mycorrhizal fungi (receives plant sugars) contain 1 to 3 per species [71]. Different isozymes develop different physiological roles and have different physico-chemical and catalytic characteristics within a single species [5]. The isoenzymes can be expressed in different growth stages, some are expressed in the initial colonization of the substrate, others in fruiting body formation [12].

In *Bacteria*, the existence of more than one LMCO gene per genome is rare and only recently multiple LMCO genes were found in the same organism. For instance, Ausec *et al*. [44] demonstrated that 252 out of 807 microorganisms were found to have more than one LMCO gene, located in chromosomes and/or in plasmids. Among *Actinobacteria*, 7 genes were detected in *Rhodococcus erythropolis*; 5 genes in *Rhodococcus opacus* and *Rhodococcus jostii*; 4 genes in *Arthrobacter* sp. FB24 and *Arthrobacter aurescens* TC1, and 3 genes in *Nocardia farcinica* IFM10152. One single gene was found only within the genus *Mycobacterium*.

Considering that multigene families have not been deeply studied in bacteria, so far, information on gene arrangement or coordinated expression is still to be investigated. As many physiological roles are currently assigned to bacterial LMCO proteins: pigmentation, oxidation of phenolic compounds, sporulation, UV and H_2O_2 resistance, Cu^{2+} resistance, efflux, and morphogenesis [16], it can be expected that the occurrence of multigene families may also contribute to the ecological importance and physiology of this group.

7. LMCO Classification into Superfamilies

7.1. LccED

In 2011, a database was built as a tool for a systematic sequence-based classification and analysis of the multi-copper oxidase protein family—LccED (Laccase and Multicopper Oxidase Engineering Database) [72]. The classification was based on 10 protein superfamilies (namely from A to J) formerly distinguished by Hoegger *et*

al. [73] based on DNA sequence similarity; and one more superfamily, K, was established for two-domain bacterial LMCO based on SLAC sequence from *Streptomyces coelicolor* [35]. So far, superfamily K is composed exclusively by *Actinobacteria* members, and contains the genera *Isoptericola*, *Saccharomonospora*, and *Streptomyces*, although there are evidences that other bacterial groups may also contain two-domains LMCO [44].

Besides superfamily K, actinobacterial LMCO enzymes are also found in superfamilies B, I and J. The characterized LMCO from *Actinobacteria* with available nucleotide sequences are classified into superfamilies I and K (**Figure 3**). All two-domain LMCO clustered together, as expected.

7.2. Primers for Detection of Bacterial LMCO Genes

Few primers for detection of bacterial LMCO genes can be found in literature. All of them were designed based on copper-binding regions (cbr), which have the same aminoacidic residues conserved in both bacterial and fungal LMCO. The oligonucleotide primer Cu2R [71] was first designed for detection of cbrII from basidiomycetes LMCO. Cu1AF primer [74] aligns to the sequence that codifies the two histidines from cbrI, and was designed by using sequences from diverse bacterial taxa (including *Actinobacteria*) as source; it presents a high degeneracy level (6 out of 20 bases) in order to detect LMCO genes in a variety of bacterial groups. Used together, the primers have shown specificity for bacterial LMCO genes, as they proved to be unable to amplify LMCO genes from DNA of basidiomycetes or ascomycetes [74].

Recently, another primer (Cu4R) was designed using the cbrIV region of three-domain LMCO sequences from five bacterial genera, including *Streptomyces* [75]. Along with Cu1AF, positive amplifications from many bacterial genera were obtained.

In our laboratory, we have for the first time designed primers for specific amplification of actinomycetes LMCO belonging to the LccED superfamilies I and K (unpublished). The designed primers have amplified LMCO fragments from actinobacteria isolates that were undetected by the above mentioned primers and the obtained fragments were correctly assigned to the predicted superfamily.

8. Cellular Location of Bacterial LMCO

Nowadays, fungal LMCO are considered to be extracellular enzymes [43], whereas bacterial LMCO are still assumed to be intracellular or spore-bound [16] [76]. However, this idea has long been contested, since Alexandre and Zhulin (2000), showed the presence of signal peptides in bacterial LMCO sequences. More recently, it has been revealed that 76% of 1200 putative genes for LMCO enzymes possessed signal peptides [44] which indicated an extracellular destination and corroborated the previous study. Currently, it is known [55] that both fungal and bacterial LMCO may be intracellular [77] [78] as well as extracellular [44] [59].

9. Conclusion

The phylum *Actinobacteria* is recognized by its intrinsic capacity to produce unconventional and biotechnologically important molecules for the most diverse sectors of industry. Currently, thousands of LMCO from *Actinobacteria* have been putatively assigned by molecular techniques, but only a few have been characterized. The
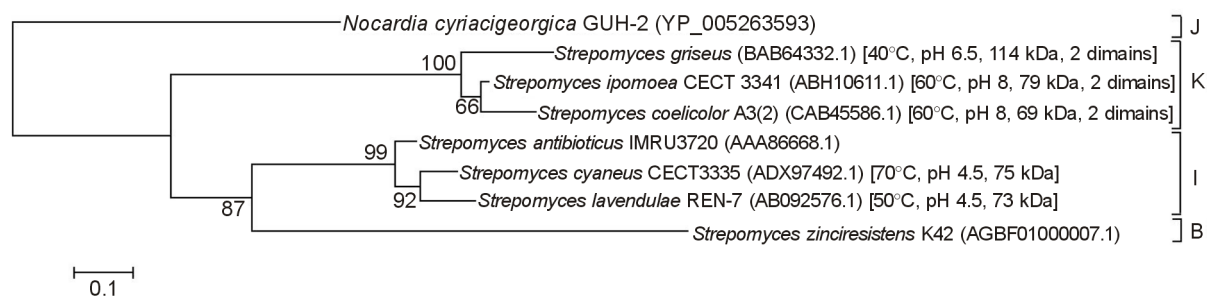

Figure 3. Neighbour-joining tree based on laccase gene sequences showing relationships among laccase superfamilies (according to The Laccase and Multicopper Oxidase Engineering Database—LccED). Only superfamilies containing actinobacteria laccases were used (indicated by letters B, I, J and K). Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets. Bar, 0.1 substitution per nucleotide position.

striking characteristics observed in these examples are not commonly found among fungal isolates, and reveal a huge potential within the enzymes from the bacterial phylum. All of the characterized LMCO presented some of the robust properties important for industrial application, *i.e.*, tolerance to high temperature, salt concentration, alkalinity/acidity and/or pH variation. The LMCO potential for industrial application is notoriously diverse, and the unusual properties of actinobacterial LMCO must be taken into account in order to strengthen efforts in its prospection and characterization. Nowadays, all LMCO used commercially are obtained from fungi, with many drawbacks related to the producer characteristics. Exploring actinobacterial LMCO may circumvent limitations and may make the processes more profitable, efficient and applicable to more industrial fields.

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Abbreviations

MCO: Multicopper Oxidase
LMCO: Laccase-Like Multicopper Oxidase