UNIVERSIDADE FEDERAL DE VIÇOSA

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LACASES DE ACTINOBACTÉRIAS: CARACTERIZAÇÃO, OBTENÇÃO DE NOVOS PRIMERS E EXPRESSÃO HETERÓLOGA NA LEVEDURA *Kluyveromyces marxianus* UFV-3

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Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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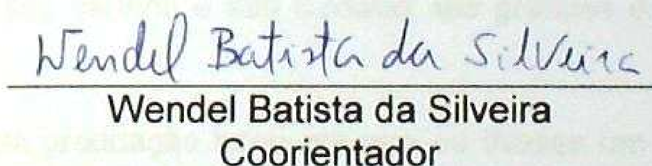

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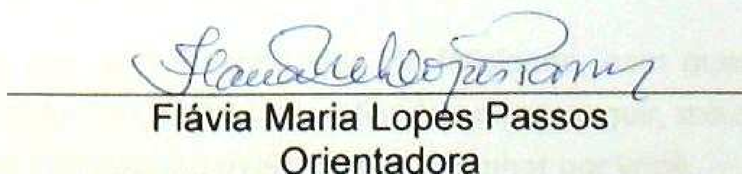

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RESUMO

FERNANDES, Tatiana Alves Rigamonte, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Lacases de actinobatérias: caracterização, obtenção de novos primers e expressão heteróloga na levedura *Kluyveromyces marxianus* UFV-3.** Orientadora: Flávia Maria Lopes Passos. Coorientadores: Wendel Batista da Silveira e Tiago Domingues Zucchi.

Dentre as enzimas multicobre oxidases, as lacases, por sua grande variedade de substratos, tem atraído atenção tanto para pesquisa básica quanto aplicada a atividades industriais e ambientais. Com relação às lacases de origem microbiana, pouco se conhece sobre aquelas produzidas por bactérias. Neste trabalho, destaca-se o filo *Actinobacteria*, ordem Actinomycetales, devido à sua reconhecida importância biotecnológica e sua habilidade para degradação de compostos recalcitrantes, sendo muitos deles substratos de lacases. Tendo em vista a demanda por grande volume de enzima por parte das diversas indústrias, e a dificuldade de manipulação dos produtores nativos, é sugerida aqui a expressão heteróloga de lacases de actinobactérias na levedura *Kluyveromyces marxianus*, em virtude das propriedades industriais que a mesma apresenta, tais como alta velocidade do crescimento, termotolerância e *status Generally Regarded as Safe* (GRAS). O objetivo deste trabalho foi obter uma linhagem de *Kluyveromyces marxianus* portando gene de lacase de actinobactéria, capaz de expressá-lo e secretar a enzima ativa; buscando-se, para isso, isolar actinobactérias produtoras de lacase a partir de solo, caracterizar a enzima, isolar um gene de lacase, clonar em vetor de expressão e, por fim, transformar *K. marxianus* UFV-3. A partir de dados da literatura, realizou-se uma revisão crítica relacionada ao tema, e as sequências gênicas que codificam proteínas caracterizadas foram identificadas e utilizadas na síntese de oligonucleotídeos iniciadores que se revelaram adequados para detecção e isolamento do gene neste grupo, havendo distinção de genes correspondentes a proteínas com duas estruturas distintas. Havendo o isolamento de um dos genes, o mesmo foi clonado em cassete de expressão para integração no genoma da *K. marxinus* UFV-3; os dados sugerem que houve sucesso na integração.

ABSTRACT

FERNANDES, Tatiana Alves Rigamonte, D.Sc., Universidade Federal de Viçosa, February, 2014. **Laccases from actinobacteria: characterization, designing of new primers and heterologous expression in the yeast *Kluyveromyces marxianus* UFV-3.** Adviser: Flávia Maria Lopes Passos. Co-advisers: Wendel Batista da Silveira and Tiago Domingues Zucchi.

The great diversity in substrate range oxidized by laccase enzymes has attracted attention in both areas of research: basic, and applied to industrial and environmental activities. Among microbial laccases, little is known about the ones produced by bacteria. In this work, it is highlighted the phylum *Actinobacteria*, order Actinomycetales, due to its recognized biotechnological importance and capacity of degrading recalcitrant compounds. Owing to the large amounts of enzyme required for industrial application, summed to the difficulty in manipulation of native producers, heterologous expression is here suggested using the yeast *Kluyveromyces marxianus* as host, which presents the industrial-relevant properties such as fast growth, thermotolerance and status generally regarded as safe (GRAS). The objective of this work was to obtain a strain of *Kluyveromyces marxianus* expressing and secreting a laccase from actinobacteria; and, in order to accomplish the main objective, we searched for isolating laccase-producing actinobacteria from soil, characterizing the enzyme, isolating a laccase gene, cloning into expression vector and, finally, transforming *K. marxianus* UFV-3. In this work, an isolation from soil samples provided laccase-producer colonies, having one of the selected been characterized. From literature data, a critical review was produced, and available gene sequences that codify characterized proteins were employed for designing oligonucleotide primers. The new primers proved to be effective for detection of laccase genes in actinobacteria, and, furthermore, allowed distinction of genes that corresponded to two structurally different laccases. After selection of a complete laccase gene, it was cloned into an expression cassette for integration into *K. marxianus* UFV-3 genome, and data suggest successful integration.

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INTRODUÇÃO GERAL

Lacases (benzenodiol:oxigênio oxidorredutases, EC 1.10.3.2) são enzimas pertencentes ao grupo das multicobre oxidases, recebendo destaque das demais pela grande diversidade de moléculas, aromáticas ou não aromáticas, naturais ou sintéticas, que podem por elas serem oxidadas. O mecanismo de ação envolve três tipos de centros de cobre com diferentes funções: o tipo 1 (que confere a cor azul característica da enzima) catalisa a retirada de elétrons do substrato, enquanto os tipos 2 e 3 formam um grupo responsável por ativar o oxigênio molecular, havendo liberação de moléculas de água.

A enzima foi primeiramente descoberta na seiva da planta *Rhus vernicifera*, sendo o elemento que, na presença de oxigênio, causava polimerização e endurecimento do látex. Pouco tempo após, proteínas similares foram descobertas em fungos, e por quase um século acreditou-se que apenas estes organismos – plantas e fungos – produziam a enzima, até a descoberta em bactérias e insetos.

Em fungos, sabe-se que lacases desempenham variados papéis fisiológicos, atuando na morfogênese, interação planta-fungo, defesa a estresses e degradação de lignina. Em geral, lacases fúngicas são proteínas globulares monoméricas de aproximadamente 60 a 80 kDa com ponto isoelétrico próximo de pH 4,0, embora muitas exceções sejam conhecidas. A maioria das lacases fúngicas são extracelulares e glicosiladas.

A grande diversidade de substratos oxidados por lacases tem elevado o interesse em sua aplicação em diferentes setores da indústria; por exemplo, na eliminação de compostos fenólicos indesejáveis em sucos de fruta, cerveja e vinho; no clareamento da celulose; na síntese de compostos orgânicos funcionais, como fármacos e corantes; na produção de polímeros; tinturas para cabelo; e produção de combustíveis. Há ainda um campo relevante de aplicação ambiental, sendo úteis para descoloração de efluentes têxteis, eliminação do odor emitido por reservatórios de lixo ou fábricas de celulose, remoção de compostos fenólicos de águas residuárias de fábricas, descontaminação de solos contendo hidrocarbonetos policíclicos aromáticos, degradação de pesticidas, polietileno e poliestireno.

Atualmente, a maior parte das lacases estudadas são produzidas por fungos, das quais muitos aspectos têm sido consideravelmente abordados, dentre eles, a ocorrência em basidiomicetos e ascomicetos, caracterização dos genes e proteínas,

elucidação de funções e potenciais aplicações. Em contraste, pouco se conhece sobre as funções em bactérias, o potencial para aplicação industrial e suas propriedades bioquímicas. Dentre o domínio *Bacteria*, um grupo destaca-se pela reconhecida capacidade de degradar macromoléculas complexas e recalcitrantes do solo, como lignina e ácidos húmicos – o filo *Actinobacteria*.

Actinobactérias têm sido intensamente exploradas, revelando-se fontes de inseticidas, fungicidas, antibióticos, agentes anti-câncer e diversas outras enzimas de interesse biotecnológico, como, por exemplo, de biorremediadores, atuando, por exemplo, na biodegradação de pesticidas. É notável que membros deste filo, especialmente da ordem Actinomycetales, são geralmente detectados em estudos de prospecção de produtores de lacase, e, embora poucas sejam suas lacases já caracterizadas, todas apresentaram propriedades relevantes para exploração industrial, como, por exemplo, tolerância/resistência a altas temperaturas, alta salinidade, presença de inibidores e extremos de pH. A busca por actinomicetos produtores de lacase pode resultar na descoberta de enzimas com propriedades únicas, e mesmo levar à descrição de novas espécies e/ou até mesmo gêneros, por conseguinte, expandindo nosso conhecimento sobre a biodiversidade de actinomicetos do Brasil.

Destaca-se, contudo, que o maior desafio para a aplicação industrial é a obtenção de um sistema biológico capaz de sintetizar e secretar a enzima ativa de forma eficiente e abundante. Lacases secretadas a partir de fontes nativas geralmente não são adequadas para produção em larga escala, principalmente devido à baixa produtividade e alto custo dos procedimentos de preparação e purificação da enzima. Como alternativa, a expressão heteróloga pode prover maior produtividade e permitir a produção de lacases com propriedades melhoradas e interessantes a diversos setores da indústria.

As leveduras são potenciais hospedeiras para expressão heteróloga visando aplicação industrial, em virtude da facilidade de manipulação genética, crescimento disperso das células em meio líquido, baixo custo do substrato de cultivo. A expressão de lacases tem sido relatada, por exemplo, em *Saccharomyces cerevisiae*, *Pichia pastoris*, *Pichia methalonica*, *Yarrowia lipolytica* e *Kluyveromyces lactis*. Especificamente quanto ao gênero *Kluyveromyces*, somente a espécie *K. lactis* foi empregada, para expressão de lacases dos fungos *Trametes trogii* e *Pleurotus ostreatus*. No entanto, dados da literatura têm revelado o potencial de *Kluyveromyces marxianus*, sendo indistinguível de *K. lactis* quanto à capacidade de promover correto dobramento e secreção de proteínas heterólogas testadas e *status* GRAS, havendo ainda a vantagem do

crescimento a temperaturas mais altas que *K. lactis*. É interessante ressaltar que *K. marxianus* tem sido considerado o organismo eucarioto de menor tempo de geração já visto, e que a expressão heteróloga em *Kluyveromyces* tem produzido melhores resultados que em *Saccharomyces*, em virtude do metabolismo mais oxidativo de *Kluyveromyces*, havendo maior produção de biomassa, e menor tendência de hiperglicosilação de proteínas heterólogas.

Ao longo dos experimentos que compõem tese, buscou-se na natureza, a partir de amostras de solo, actinobactérias produtoras de lacase. Um isolado foi selecionado (*Streptomyces* sp. SB086), pela mais intensa oxidação do substrato indicador, havendo caracterização tanto da enzima quanto da linhagem produtora. No entanto, tal caracterização foi apenas parcial, uma vez que, após alguns ciclos de repicagem em placa, não se obteve mais atividade de lacase a partir de extratos de cultivo. Embora seja possível que o gene esteja ainda presente no micro-organismo, sendo necessária apenas uma melhor condição de indução para expressão da enzima, tal ocorrido deve nos levar a atentar para a necessidade de imediata preservação dos isolados, antes que ocorram ciclos de repicagem. É conhecido que repicagens sucessivas podem causar perda de fenótipos de interesse, devido a, por exemplo, cura de plasmídeos (lacases, por exemplo, podem estar presentes tanto no genoma quanto em plasmídeos bacterianos). O uso do gene de lacase deste isolado para transformação de *Kluyveromyces marxianus* não foi possível, uma vez que não houve sucesso na amplificação com primers disponíveis na literatura para lacases de bactérias, nem com novos primers desenhados. No entanto, havendo indicações que actinobactérias possuem lacases também das superfamílias B e J, a ausência de produto de amplificação com os primers desenhados não é suficiente para indicar perda do gene pelo isolado, uma vez que tais primers são específicos para enzimas das superfamílias I e K, de acordo com classificação do The Laccase and Multicopper Oxidase Engineering Database (LccED).

Alternativamente, utilizou-se como fonte do gene a actinobactéria *Streptomyces araujoniae* ASBV-1^{T}, uma vez que seu genoma havia sido recentemente sequenciado em nosso laboratório, e foi detectada a presença de um gene de lacase da superfamília K. Como genoma disponível, houve maior facilidade para desenho de primers para amplificação do gene completo. Os dados obtidos após a transformação da levedura indicam que houve sucesso na integração em seu genoma, como cópia única nas colônias positivas. A otimização da expressão da enzima em *K. marxianus* prossegue como objeto de futuras pesquisas.

CAPÍTULO 1

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 exhibit industrially important properties. Furthermore, studies with enzymes from this phylum revealed a novel laccase molecular structure, providing the basis for a distinct family, the two-domain laccases. The relevant traits of actinobacteria 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 actinobacteria laccases to traditional laccase 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 in experimental assays.

KEYWORDS: actinobacteria, lignin degradation, multicopper oxidase, industrial applications

ABBREVIATIONS: MCO multicopper oxidase; LMCO: laccase-like multicopper oxidase

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 (Claus, 2004). These enzymes were first discovered in plants (Yoshida 1883), thereafter in fungi (Bertrand 1896), and only one century later they were found in *Bacteria* (Givaudan 1993). Most studies on laccases are still conducted on fungi (Piscitelli et al. 2010), and few is known about 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 (Bugg et al. 2011). 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 (Strong & Claus 2011). This laccase-mediator system (LMS) also allows indirect oxidation of non-phenolic compounds (Bourbonnais & Paice 1990) 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 (Piscitelli et al., 2010).

In fungi, laccases have been extensively reviewed in many aspects: occurrence, characterization of genes and proteins from different taxonomic divisions, functions and applications (Thurston 1994; Gianfreda et al. 1999; Baldrian 2005; Giardina et al. 2010; Galli et al. 2011; Janusz et al. 2013). Whereas the literature provides some general reviews on bacterial laccases (Claus 2003; Sharma et al. 2006), and some studies have dealt with lignocellulolytic potential of actinobacteria (Ball et al. 1989; Kirby 2005), its current laccase *state-of-art* is still obscure. It is remarkable that actinobacteria strains are generally detected in prospective bacterial studies, and laccases with unusual properties have been discovered (Arias et al., 2003; Niladevi et al., 2008; Gunne and Urlacher, 2012). 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.

1 **DEFINING LACCASE ENZYMES**

2 *Laccase and other multicopper oxidases*

3 Laccases are generally defined as multi-copper oxidase enzymes (MCO) that catalyse
4 oxidation of diverse phenolic and non-phenolic substances using dioxygen as final
5 electron acceptor, producing water molecules as byproduct, and that possess some
6 conserved amino acid motifs responsible for binding to copper atoms (Kumar et al.,
7 2003) However, advances in studies concerning the diverse group of multicopper
8 oxidases (MCO) have raised a question about the classification of laccase enzymes,
9 considering that the amino acid signature and substrates are shared by many MCO; for
10 instance, ascorbate oxidase, tyrosinase, polyphenol oxidase or bilirubin oxidase
11 (Sakurai and Kataoka, 2007). Thus, a narrow definition has not been achieved so far
12 and therefore, it has been proposed the use of the term "laccase-like multicopper
13 oxidase" (LMCO; Reiss et al., 2013) for the formerly named laccase enzymes. This
14 term has been adopted in this review for simplification.

15

16 ***ACTINOBACTERIA* AND THEIR LMCO**

17 *The phylum and its biotechnological potential*

18 The phylum *Actinobacteria* represents one of the largest and most diverse groups
19 within the *Bacteria* domain; it comprises Gram-positive bacteria with a high G+C %mol
20 content in DNA (Whitman et al. 2012). It is a cosmopolitan bacterial group which was
21 found inhabiting almost all known ecological niches. *Actinobacteria* are considered of
22 great importance in the process of degradation of recalcitrant and relatively complex
23 macromolecules that are naturally found in litter and soil, such as lignin and humic acid
24 (Goodfellow & Williams 1983; Lee 1997; Godden et al. 1992; Bugg et al. 2011; Taylor
25 et al. 2012). Particularly, the order *Actinomycetales* has attracted industrial attention
26 mainly due to its outstanding capacity to produce compounds with high
27 biotechnological values, e.g., antibiotics. Indeed, actinobacteria are considered
28 remarkable antibiotic-producing and since their first antibiotic discovery (streptomycin
29 at late 40's), their participation in the antibiotic market continues to grow (Bérdy, 2005;
30 Goodfellow and Fiedler, 2010). Similarly to these unique antibiotic biosynthetic
31 pathways, it has been stated that actinobacteria may also produce attractive enzymes
32 for biotechnological applications (Prakash et al. 2013).

33

1 *Characterized LMCO from* actinobacteria

2 Studies on LMCO characterization from actinobacteria are scarce and it is an open
3 field for exploration. At the time of writing, only LMCO from the following species have
4 been characterized: *Streptomyces griseus* IFO 13350 (Endo et al. 2003), *Streptomyces*
5 *lavendulae* REN-7 (Suzuki et al. 2003), *Streptomyces cyaneus* CECT 3335 (Arias et al.
6 2003), *Streptomyces coelicolor* M145 (Machczynski et al. 2004), *Streptomyces*
7 *psammoticus* MTCC 7334 (Niladevi et al. 2008), *Streptomyces ipomoea* CECT 3341
8 (Molina-Guijarro et al. 2009), Str*eptomyces sviceus* DMS 924 (Gunne & Urlacher
9 2012), *Streptomyces* sp. C1 (Lu et al. 2013) and *Streptomyces* sp. SB086 (Fernandes
10 et al., 2013). Their main properties are presented in Table 1.

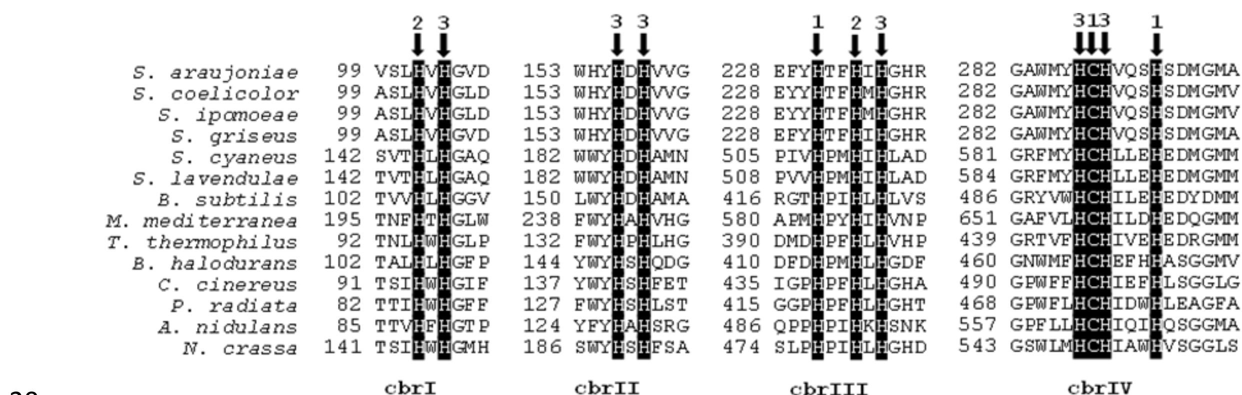
1 **Table 1.** Characterized laccases from *Actinobacteria* and their main properties

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	Endo et al. 2003
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	Suzuki et al. 2003
Nd, *Streptomyces cyaneus*	nd	75 kDa	4.5, ABTS	Km = 0.38 mM	70°C	High temperature	Arias et al. 2003
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	Machczynski et al 2004; Dubé et al 2008; Skálová et al 2009
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	Niladevi et al. 2008
SilA, *Streptomyces ipomoea*	2 domains, dimer	79 kDa; (monomer:4 4.7 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	Molina-Guijarro et al. 2009
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=0.36mM, k_{cat}=7.38 s^{-1} (ABTS); K_m=0.89mM, k_{cat}=0.32 s^{-1} (DMP); K_m=15.8mM, k_{cat}=5.78x10^{-2} s^{-1} (SGZ)	---	High temperature, alkaline medium, wide pH variation	Gunne & Urlacher 2012
SCLAC, *Streptomyces* sp.	2 domains	38 kDa	8.0, ABTS or guaiacol; 7.0, DMP	K_m=0.43mM, 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=5.58mM, 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=1.65mM, 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	Lu et al. 2013
Nd, *Streptomyces sp.*	nd	nd	4.0, ABTS	nd	50°C	High temperature	Fernandes et al. 2013

2 **Abbreviations:** ABTS, 2,2'-*azino-bis*(3-ethylbenzothiazoline-6-sulphonic acid); DMP, 2,6-dimethoxyphenol; DMPPDA, dimethyl p-phenylenediamine sulphate; SGZ, syringaldazine;
3 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.

1 *Structural properties – two-domain LMCO*

2 As stated before, LMCO belong to the group of blue multicopper proteins containing
3 four Cu atoms classified in types 1 to 3 (two atoms are type 3). All secondary structural
4 elements typical of fungal LMCO are present in bacterial sequences (Alexandre and
5 Zhulin 2000). The three Cu-binding domains that form the active site are composed by
6 multiple histidine and one cysteine copper-binding residues, which are present in the
7 type 1 (2 his + 1 cys), 2 e 3 sites (8 his), where copper atoms are distributed
8 (Hakulinen et al. 2002; Endo et al. 2003; Valderrama et al. 2003). This canonical
9 structure is also found among the characterized actinobacterial LMCO (Figure 1).
10 Furthermore, studies on actinobacterial LMCO have provided a very interesting finding
11 concerning to their molecular structure. Instead of three structural domains, some
12 actinobacterial LMCO present only two domains (wherein the three Cu-binding
13 domains and the four Cu atoms are distributed). This peculiar two-domain structure,
14 also named “small LMCO” was elucidated in *Streptomyces coelicolor* (Machczynski et
15 al. 2004). It lacks the second domain, which is composed by one loop at the end of
16 domain one, two β-turns from domain three, and its own amino acid residues
17 (Hakulinen et al. 2002). Two-domain LMCO have not been found in fungi so far, but
18 were found in ten other phyla within *Bacteria*, and also in *Archaea*, by molecular
19 analysis (Ausec et al. 2011).



20

21 **Figure 1.** Alignment of Cu-binding regions of actinobacterial and other bacterial and
22 fungal laccases. Arrows and accompanying numbers indicate the amino acid residues
23 for Cu-binding and the type of each ligand Cu. The position of the first residue of each
24 fragment in the polypeptide chain is indicated. *Streptomyces* spp.: *S. araujoniae*
25 (unpublished); *S. coelicolor* (CAB45586); *S. ipomoea* (ABH10611); *S. griseus*
26 (BAB64332); *S. cyaneus* (ADX97492); *S. lavendulae* (BAC16804). Other bacteria:
27 *Bacillus subtilis* (ADZ57284); *Marinomonas mediterranea* (AAF75831); *Thermus*

1 *thermophilus* (BAE16261); *Bacillus halodurans* (AAP57087). Basidiomycota: *Coprinus*
2 *cinereus* (ABP81837); *Phlebia radiata* (Q01679). Ascomycota: *Aspergillus nidulans*
3 (Q96VT5); *Neurospora crassa* (AAA33592).

4

5 *In silico* studies have been performed by comparison of genomes with published
6 sequences of characterized LMCO, generating thousands of putative LMCO genes,
7 which have broadened the distribution of the genes into the bacterial phyla. Searching
8 within 2211 complete and draft bacterial genomes and four metagenomic data sets,
9 Ausec et al. (2011) have found 1240 genes for LMCO enzymes in 807 different
10 microorganisms, or 36% from all organisms evaluated. This high frequence changes
11 the idea that LMCO are proteins found only in few bacteria (Dubé et al. 2008).

12 Two-domain LMCO characterized in actinobacteria are active under quaternary
13 structure as dimer (*S. ipomoea*) or trimer (*S. griseus, S. coelicolor* and *S. sviceus*). The
14 oligomerization in actinobacterial enzymes (Table 2) contrasts with fungal, considering
15 that from more than 100 different characterized fungal LMCO there is a predominance
16 of monomeric proteins.

17

1 **Table 2.** Substrate specificity and inhibition profiles of characterized laccases from
2 *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	Endo et al. 2003
STSL, *Streptomyces lavendulae*	Yes	Low	---	---	Yes	Yes	Very weak	Yes	Yes	Suzuki et al. 2003
Streptomyces cyaneus	Low	Very low	Yes	Yes	---	---	No	Yes	Yes	Arias et al. 2003
SLAC, *Streptomyces coelicolor*	Yes	---	Yes	Yes	Yes	Yes	---	No	No	Machczynski et al. 2004; Dubé et al. 2008; Skálová et al. 2009
Streptomyces psammoticus	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Niladevi et al. 2008
SilA, *Streptomyces ipomoea*	Yes	---	Yes	Yes	---	Yes	No	No	Yes	Molina-Guijarro et al. 2009
Ssl1, *Streptomyces sviceus*	Yes	Yes	Yes	Yes	---	---	No	No	---	Gunne and Urlacher 2012
SCLAC, *Streptomyces* sp.	Yes	Yes	Yes	Yes	Yes	---	---	Yes	Yes	Lu et al. 2013
Streptomyces sp. SB086	---	Yes	Yes	---	---	---	---	---	---	Fernandes et al., 2013

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

7

8 **INDUSTRIAL APPLICATIONS**

9 Lignin is the most associated substrate for LMCO oxidation in which the enzyme acts
10 on its polymerization as well as on depolymerization (Gianfreda et al. 1999). Lignin
11 tightly involves cellulose fibers in wood and is very resistant to breakdown. It is
12 generally viewed as a side-product of lignocelluloses treatment and used as thermal
13 resource after wood physicochemical pre-treatments. Lignin comprises approximately
14 10 to 30% of vegetal dry mass (Lee 1997) and therefore, achieving an efficient method
15 for lignin-rich biomass conversion into second generation ethanol production may be
16 economically advantageous.

Lignin structure is very complex, composed by randomly linked subunits. Two consequences of this structure are: a) only few enzymes are able to cause cleavages – e.g. 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, LMCO has been proposed for activities from many sectors of industry (Couto and Herrera, 2006; Gianfreda et al. 1999; Kunamneni et al., 2008; Widsten and Kandelbauer 2008, Shradda et al., 2011; Strong and Claus 2011, Gilan et al., 2004; Sivan et al., 2004; Mor and Sivan 2004):

- 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; addition to dish-washing products; 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;
- 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.*

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 (Osma et al., 2010).

Each specific industrial application requires LMCO with specific properties, e.g., optimum pH, temperature and resistance to metal inhibition. 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 (Madhavi & Lele 2009). 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 (Gunne & Urlacher 2012). Industrial processes may present such extreme conditions; thermostable enzymes are commonly more resistant to chemical denaturation, alkalinity or extreme acidity (Haki & Rakshit 2003), and bacterial LMCO have proven more resistant than the average LMCO found in fungi (Hildén et al. 2009). In the last decades, although it has been reported thermotolerant LMCO in bacteria (Machczynski et al. 2004; Miyazaki 2005; Dubé et al. 2008) they have not been industrially-explored.

The LMCO currently used by industries were obtained from fungi (Osma et al., 2010). 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 (Couto & Toca-Herrera 2007). Also, fungal LMCO generally present more acidic optimum pH when compared to bacterial enzymes (Baldrian 2005, Reiss et al., 2013), whereas most of LMCO applications require activity in alkaline conditions (Gunne & Urlacher 2012). Some alkaline-tolerant LMCO have been found in bacteria (Niladevi et al. 2008; Gunne & Urlacher 2012; Yang Li et al. 2012).

Thus, it has become increasingly clear that 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 health- 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 actinobacteria 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 (Dubé et al. 2008). Similarly, LMCO SCLAC, from *Streptomyces* sp., efficiently decolourized Indigo carmine and Diamond Black PV in presence of syringaldehyde (Lu et al. 2013); dye Orange II was 90% degraded after 4h of incubation with the LMCO

produced by *S. ipomoea* (Molina-Guijarro et al. 2009). *S. psammoticus* LMCO was tested against 10 different dyes, but was effective only for RBBR (Remazol Brilliant Blue R; K. N. Niladevi & P. Prema 2008). 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 (Arias et al. 2003).

CATALYTIC PROPERTIES

Responses of actinobacterial LMCO to common inhibitors

A common LMCO inhibitor is ethylenediamine tetraacetic acid (EDTA). Among characterized actinobacterial LMCO, EDTA inhibited SilA (*S. ipomoea*), but not SLAC (*S. coelicolor*). 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. cyaneus* LMCO, Cu inhibited SilA and activated *S. cyaneus*, Zn inhibited *S. cyaneus*, whereas *S. psammoticus* LMCO was activated by all of these ions (references in Table 2). Furthermore, *Streptomyces cyaneus* CECT 3335 enzyme had 75.7% of its activity hindered by cinnamic acid. Sodium azide is considered as the only true LMCO inhibitor (Johannes & Majcherczyk 2000). However, SLAC, SilA and Ssl1 (*S.* sviceus) did not present the typical complete inhibition in presence of 1 mM sodium azide (Table 2): 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 (Tables 1 and 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.

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, which use H_2O_2 as electron acceptor (Ruiz-Dueñas & Martínez 2009).

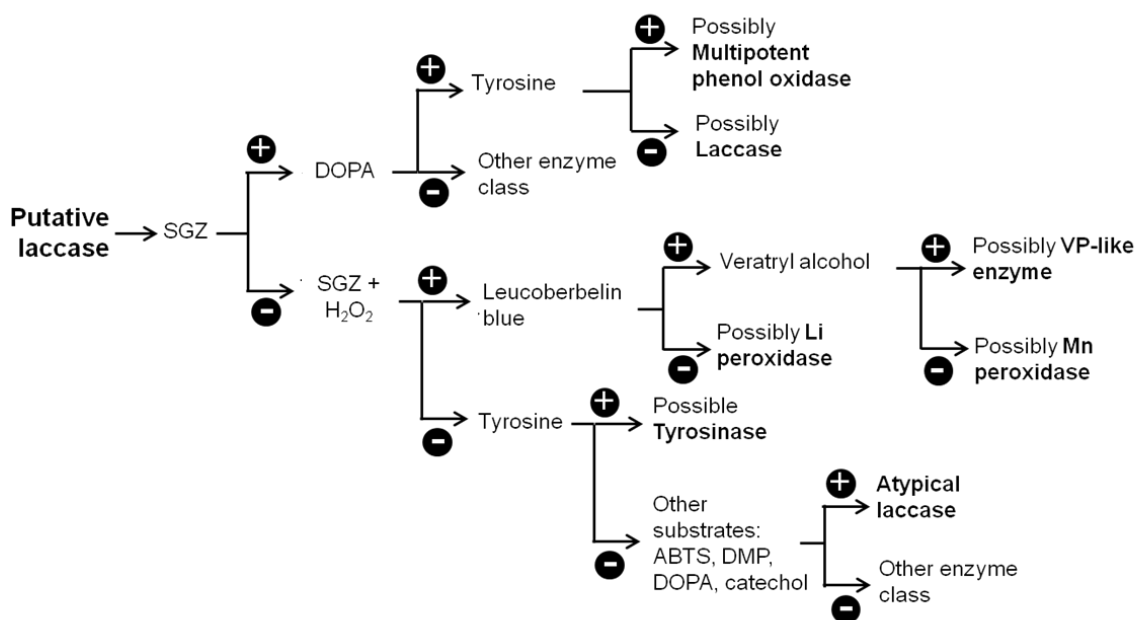
Some substances have long been cited as "specific laccase substrates", such as syringaldazine (Harkin & Obst 1973; Johannes & Majcherczyk 2000; Suzuki, Takashido et al. 2003), or "model laccase substrates", such as guaiacol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), dihydroxyphenylalanine (DOPA) and catechol. Notably, concerning actinobacteria, 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. 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 (Anderson et al. 2009). 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* (Hullo et al. 2001). A third class of peroxidase that performs both MnP and LiP functions was discovered in *Pleurotus eryngii* and named versatile peroxidase (Camarero et al. 1999). 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 (Thurston 1994). 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 (Sanchez-Amat & Solano 1997).

However, as very often LMCO from actinobacteria 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 (Figure 2). The flow chart was built based on characterized actinobacteria 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

1 similar scheme would be possible for distinguishing LMCO from other MCO as more
2 proteins from actinobacteria are isolated and characterized.

3



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5 **Figure 2.** Proposed flow chart for classification of laccase and other ligninolytic
6 enzymes. Plus (+) signals indicate positive reaction (e.g., colored reaction in
7 leucoberbelin blue test; or higher enzyme activity in presence of H_2O_2, compared to its
8 absence); minus (-) signals indicate negative result (e.g., non-oxidation of tyrosine; or
9 no higher activity in presence of H_2O_2). Abbreviations: Li, lignin; Mn, magnesium; SGZ,
10 syringaldazine; ABTS, 2,2'-*azino-bis*(3-ethylbenzothiazoline-6-sulphonic acid); DMP,
11 2,6-dimethoxyphenol; DOPA, dihydroxyphenylalanine; VP, versatile peroxidase.
12 Further assays can be performed after identification of the “possible enzyme”, as
13 affinity tests for common substrates and inhibitors.

14

15

16

17

18 **MULTIGENE LMCO FAMILIES**

19 Fungal and plant LMCO genes occur as multigene families. For example, the fungus
20 *Pleurotus ostreatus* harbors up to 12 LMCO genes (Castanera et al. 2012) whereas
21 *Coprinopsis cinerea* (Kilaru et al. 2006) and the plant model *Arabidopsis thaliana*, 17
22 genes (Mccaig et al. 2005). Furthermore, the number of LMCO fungal genes seems to
23 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 (Luis et al. 2004). Nevertheless, different isozymes develop different physiological roles and have different physico-chemical and catalytic characteristics within a single species (Piscitelli et al. 2010). The isoenzymes can be expressed in different growth stages, some are expressed in the initial colonization of the substrate, others in fruiting body formation (Giardina et al. 2010).

In *Bacteria*, the existence of more than one LMCO gene per genome is rare and only recently it was found multiple LMCO genes in the same organism. For instance, Ausec et al. (2011) 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. However, many physiological roles are currently assigned to bacterial LMCO/LMCO proteins: for example, pigmentation, oxidation of phenolic compounds, sporulation, UV and H_2O_2 resistance, Cu^{2+} resistance, efflux, and morphogenesis (Sharma et al. 2006); therefore, the occurrence of multigene families may also indicate their importance in the ecology and physiology of this group.

LMCO CLASSIFICATION INTO SUPERFAMILIES

LccED

In 2011, a database was built as a tool for a systematic sequence-based classification and analysis of the multicopper oxidase protein family – LccED (Laccase and Multicopper Oxidase Engineering Database) (Sirim et al. 2011). The classification was based on 10 protein superfamilies (namely from A to J) formerly distinguished by Hoegger et al. (2006) based on DNA sequence similarity; and one more family, K, was established for two-domain bacterial LMCO based on SLAC sequence from *Streptomyces coelicolor* (Machczynski et al. 2004). 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 (Ausec al. 2011).

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

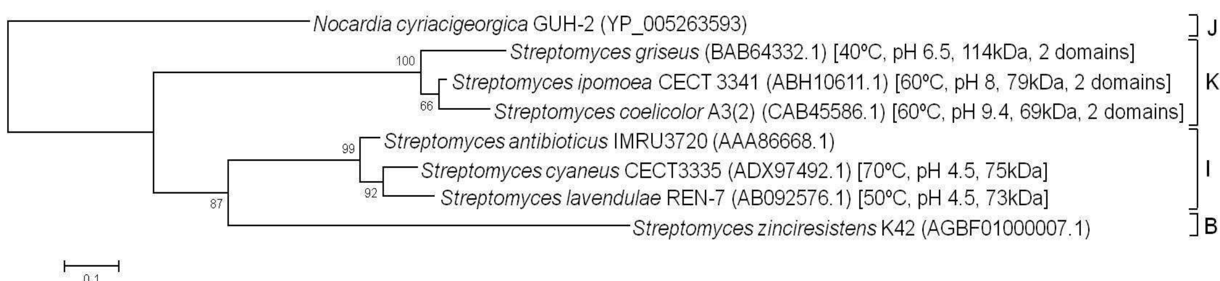

Figure 3. Neighbour-joining tree based on laccase gene sequences showing relationships among laccase superfamilies (according to The Laccase and Multicopper Oxidase Engineering Database – LccED). Only superfamilies containing actinobacteria laccases were used (indicated by letters). Numbers at nodes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

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 using copper-binding regions (cbr), which have the same aminoacidic residues conserved in both bacterial and fungal LMCO. The oligonucleotide primer Cu2R (Luis et al., 2004) was first designed for detection of cbrII from basidiomycetes LMCO and presents degenerations in 3 out of 22 bases. Cu1AF primer (Kellner et al., 2008) aligns to the sequence that codifies the two histidines from cbrI, and was designed by using sequences from diverse bacterial taxa (including *Actinobacteria*); 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 (Kellner et al 2008).

Recently, another primer (Cu4R) was designed using the cbrIV region of three-domain LMCO sequences from five bacterial genera, including *Streptomyces* (Ausec et al., 2011). 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 actinobacteria LMCO belonging to the LccED superfamilies I and K (Fernandes et al., *unpubl.*). 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.

CELLULAR LOCATION OF BACTERIAL LMCO

Nowadays, fungal LMCO are considered to be extracellular enzymes (Valderrama et al. 2003), whereas bacterial LMCO are assumed to be intracellular or spore-bound (Sharma et al. 2006; Dwivedi et al. 2011). 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 1,200 putative genes for LMCO enzymes possessed signal peptides (Ausec, Zakrzewski, et al. 2011) which indicated an extracellular destination and corroborated the previous study. Currently, it is common sense (Hildén et al. 2009) that both fungal and bacterial LMCO may be intracellular (Schlosser & Grey 1997; Diamantidis et al. 2000) as well as extracellular (K. N. Niladevi & P. Prema 2008; Ausec, Zakrzewski, et al. 2011).

CONCLUSIONS

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 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. The unusual properties of actinobacterial LMCO must

be taken into account in order to strengthen efforts in prospection and characterization of LMCO from this bacterial group. 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 other industrial fields. Considering that LMCO production is usually expensive, it is also noteworthy that natural and economically-feasible mediators need to be investigated.

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6 **Characterization of a thermotolerant laccase produced by *Streptomyces* sp.**
7 **SB086**

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20 **KEY WORDS:** actinobacteria, laccase, lignin degradation, multicopper oxidase

1 **ABSTRACT**

2 Laccases have become desirable enzymes for application in many industrial
3 processes. Nowadays, most of these enzymes were obtained from fungi. Among the
4 prospective studies for bacterial laccase genes, some have included actinobacteria, but
5 only a few have characterized the produced enzyme. Thus, we have isolated a
6 laccase-producing actinobacteria inomycete from forest soil under restoration process
7 and we further aimed to characterize its produced enzyme. The isolate SB086 was
8 placed into the *Streptomyces* genus by a combination of phenotypical, chemical and
9 phylogenetic properties. Our data indicate that the bacterium produces a
10 thermotolerant laccase. The maximum activity was obtained in a pH range from 4.0 to
11 5.0 and at 50°C in reaction mixture containing 5 mM $CuSO_4$; thermal stability was
12 noted at 60°C and 70°C – a well-desired characteristic for industry. The active enzyme
13 presented a high molecular mass (over 100 kDa) and was less sensitive to inhibition by
14 metal ions than generally described for bacterial laccases. Our findings support *in silico*
15 data of bacterial laccase secretion, and reinforce that actinobacteria may be a rich
16 laccase source for industrial application.
17

INTRODUCTION

The search for alternative energy supplies has received increasingly efforts, and bioethanol production has been highlighted as a promising option, mainly because this fuel is obtained from microbial fermentation of an abundant renewable source: lignocellulose biomass. For the fermentation to occur, in the biological pre-treatment the complex structure of the polymer has to be degraded by a complex of enzymes, in which laccases play a crucial role (Hatakka 1994). Laccases (EC 1.10.3.2) are multicopper oxidases capable of taking electrons from a broad range of organic and inorganic compounds, even non-aromatic ones, concomitantly with molecular oxygen reduction, producing water (Claus 2004). The broad substrate range of laccase makes the enzyme attractive for industrial application in numerous fields, besides energy supply: in pulp and paper industry for pulp delignification; in food industry for elimination of undesirable phenolic components in fruit juices, beer and wine; in cosmetics and pharmaceutical industry for polymers, hair dyes and drug production; and also in bioremediation, for bleaching of textile effluents, phenols removal from residuary water of factories, pesticide degradation and decontamination of aromatic polycyclic aromatic hydrocarbons (PAHs) from soil (Schroeder et al. 2008; Piscitelli et al. 2010).

Physiological roles of microbial laccases are known to involve a variety of processes, such as endospore pigmentation, morphogenesis, stress response and copper homeostasis (Martins et al. 2002; Sharma et al. 2007; Piscitelli et al. 2010). The molecular structure of the enzyme generally presents four domains in the active site, containing three types of copper centers: type 1, which confers blue color to the purified enzyme, catalyzes the removal of electrons from substrate; and types 2 and 3, which form a group that activates molecular oxygen (Claus 2004). Furthermore, the nucleotide sequence conservation of the domains makes possible the designing of specific primers for taxonomic groups (Luis et al. 2004; Kellner et al. 2008; Ausec et al. 2011).

Since laccase discovery in 1883, for one century it was thought that only plants and fungi synthesized this enzyme. Although it is nowadays known that bacteria are also producers, most laccases recently studied are still obtained from fungi (Piscitelli et al. 2010). It is possible that this difference is due to the rooted idea that bacterial laccases occur mainly intracellularly or bound to spores (Sharma et al. 2007), then, this retention

of the enzyme would, *a priori*, make bacterial laccases less adequate for industrial applications. However, this idea has long been contested, since Alexandre and Zhulin (2000), showed the presence of signal peptides in bacterial laccase sequences. More recently, it has been revealed that 76% of 1,200 putative genes for laccase-like enzymes possessed signal peptides (Ausec et al., 2011), indicating extracellular destination and corroborating the previous study.

Among the prospective studies for bacterial laccase genes, some have included actinobacteria (for example, *Streptomyces griseus,* Endo et al. 2002; *S. lavendulae,* Suzuki et al. 2003; and *S. cyaneus,* Arias et al. 2003), but only a few proceed to enzyme characterization. Besides *Streptomyces*, hypothetical laccase-like genes can be found in over 20 actinobacteria genera in NCBI (National Center for Biotechnology Information) database. Since *Actinomycetales* has been recognized as a producer of unique biomolecules and the predicted proteins vary enormously in chain length, this group has also been considered as a reliable source for enzyme prospection with new biochemical properties. Therefore, our main aim was to characterize laccase produced by an actinobacteria isolated from soil in order to investigate its potential for industrial applications.

MATERIAL AND METHODS

Isolation, selection and maintenance of laccase positive actinobacteria

Actinobacteria isolates were recovered from humic acid agar plates (HVA; Hayakawa and Nonomura 1987) which had been inoculated with a suspension of reforestation soil collected in the city of Santa Bárbara d'Oeste, State of São Paulo, Brazil, and incubated at 28°C for 21 days. The isolates were maintained on oatmeal agar plates (ISP medium 3; Shirling and Gottlieb 1966), and glucose-yeast extract agar plates (GYEA; Gordon and Mihm 1962) at 28°C. Mycelial fragments were maintained in glycerol solution (20% v/v) at -80°C. Laccase production was screened by inoculating the isolates on oatmeal agar amended with 0.01% guaiacol, which acquires a reddish-brown coloration after oxidative polymerization by laccase (Coll et al. 1993). The positive isolates were pre-inoculated in liquid GY medium for 3 days at 28°C. The pre-inoculum (1 ml) was then used to inoculate basal mineral medium supplemented with

0.025% vitamin-free casaminoacids (Sigma) (Crawford 1978) and 0.5% wheat bran. After four days incubation at 28°C, the culture was centrifuged (15,000 x g; 10 min at 4°C) and the supernatant was tested for laccase activity.

Enzyme assay

Laccase activity was further assayed by determining the oxidation of 5 mM ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; Sigma-Aldrich] in 0.1 M McIlvaine buffer (pH 5.0). The increase in absorbance at 436 nm was monitored with a Shimadzu UV-1601 PC spectrophotometer, and the temperature of the reaction mixture was controlled in water bath. Laccase activity in the crude or partially purified extracts was assessed in a 990 µL reaction mixture containing 5 mM ABTS as a substrate, 330 µL of 0.1 M McIlvaine buffer, and 330 µL enzyme extract. The progress of the reaction was monitored at 436 nm for 20 minutes. The activity was expressed as units of activity (one unit of laccase activity was defined as one unit change in the absorbance reading at 436 nm per 1 minute). An initial assay at 30°C was conducted with positive results from guaiacol test; the supernatant from the previously selected isolate, namely SB086, was chosen for enzyme characterization.

Characterization of laccase

Estimate of the best condition for laccase activity was obtained assaying the culture supernatant within the temperature range from 20 to 70°C and pH range from 3 to 8 with 10 mM McIlvaine buffer. Temperature effect on reaction was evaluated by pre-treating the reaction mixture at 60°C or 70°C for intervals from 20 to 180 minutes, then transferring to 50°C bath for the reads of ABTS oxidation. The effects of metal ions ($CuSO_4$, $MnSO_4$, $MgSO_4$, $ZnSO_4$, $CaCl_2$, $FeSO_4$ and K_2SO_4) at concentrations ranging from 0 to 20 mM were also determined, with 0.1 M McIlvaine buffer (at 50°C, optimum pH 4.0).

Purification with acetone gradient and molecular weight estimation

Different volumes of cold acetone (−20°C), providing the saturation ranges of 0–20%, 20–40%, 40–60%, and 60–80% acetone were added to the culture supernatant for sequential protein precipitation. At each acetone addition, samples were homogenized by inversion, incubated at 4°C for 5 min, and the protein was harvested by centrifugation (13,000 g, 15 min, 4°C). The supernatant was transferred to a new tube for the next acetone addition, and the pellet was completely dried, at room temperature. The pellet was, then, resuspended in distilled water to the initial sample volume. For laccase molecular weight estimation, Amicon® Ultra Centrifugal Filter Devices (30, 50 and 100 kDa) for the Concentration and Purification of Biological Samples (Millipore) were used following manufacturer's protocol.

Taxonomy analysis of isolate SB086

Phylogeny

Genomic DNA was extracted from the selected SB086 isolate using PureLink™ Genomic DNA kit (Invitrogen) following manufacturer's protocol. PCR amplification and 16S rRNA gene sequencing were achieved following the methods of Zucchi et al. (2011). The almost complete 16S rRNA gene sequence [1,371 nucleotides (nt)] was aligned manually using MEGA version 5 software (Tamura et al. 2011) against corresponding sequences of closely related type strains of *Streptomyces* species retrieved from the GenBank database using the EzTaxon-e server (Kim et al. 2012). Phylogenetic trees and bootstrap analysis were inferred following the procedures described by Zucchi et al. (2012). The root position of the neighbour-joining tree was inferred by using *Streptomyces albus* subsp. *albus* (GenBank Accession n° J6216022) and *Streptacidiphilus albus* (GenBank accession n° AF074415) as outgroups.

Chemotaxonomy

Isolate SB086 was examined to establish whether it had a chemotaxonomic profile typical of the actinobacteria genus addressed by the phylogenetic analysis. Cell mass for fatty acid analysis was harvested from Trypticase Soy Broth (TSB; Difco) which had been incubated at 28°C for 3 days. Fatty acids of the isolate were methylated,

determined by gas chromatography (Hewlett Packard 6890) and analyzed using the standard Sherlock Microbial Identification (MIDI) system and the TSB version 6 database (Sasser 1990). The DNA base composition of the isolate was determined following the methods of Gonzalez and Saiz-Jimenez (2002).

Cultural and morphological properties

Cultural properties were determined using standard ISP media (Shirling and Gottlieb 1966) after incubation of plates at 28°C for 14 days. Isolate SB086 was examined for phenotypic properties known to be valuable in actinobacteria systematics, and additional tests (pH and NaCl) were carried out on the isolate using GYEA as the basal medium, as described by Williams et al. (1983).

RESULTS

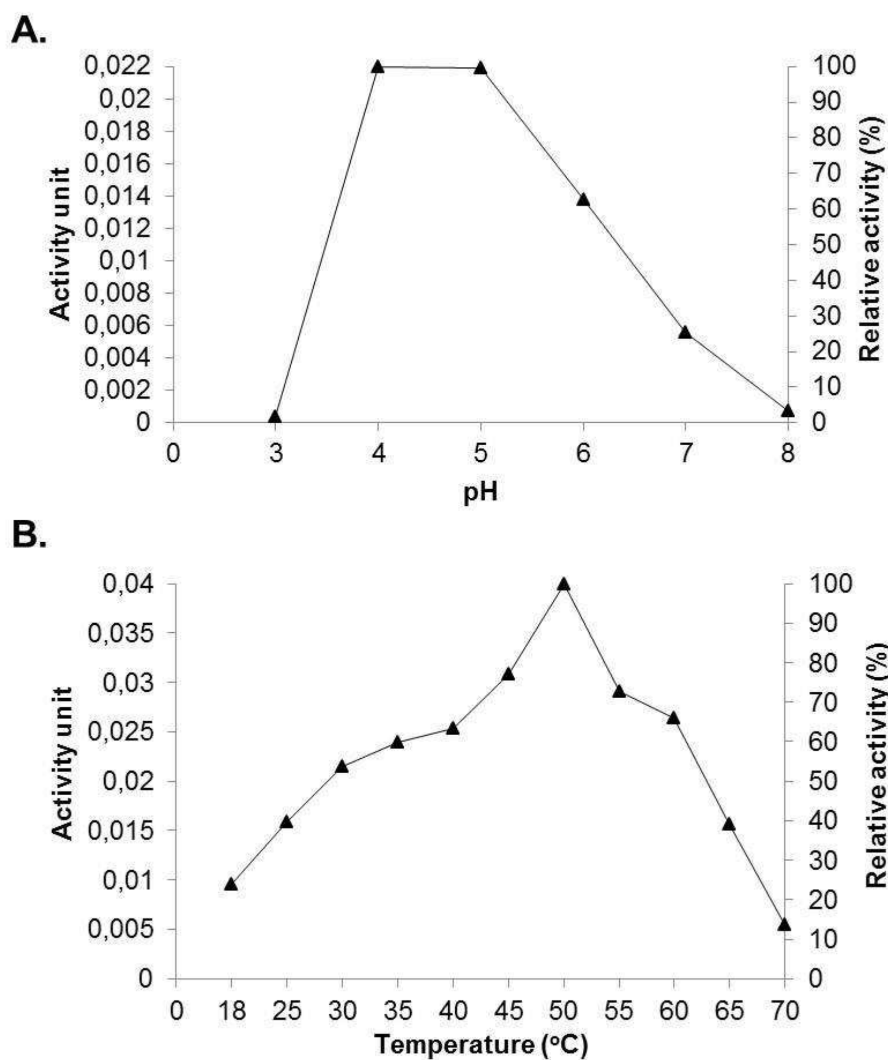
Selection of laccase producer actinobacteria

A total of 25 strains isolated from forest soils under restoration process presented morphological features typical to members of actinobacteria. Only four isolates demonstrated ability to oxidize guaiacol on oatmeal agar. Some of them, such as isolate SB086, naturally pigmented the growth medium (data not shown) making more difficult the detection of laccase production. All positive and doubtful positive isolates were further evaluated for laccase activity using ABTS as substrate.

Laccase characterization in crude extract

Isolate SB086 supernatant provided the most intense ABTS oxidation, indicating the highest laccase activity among the isolates (data not shown) and therefore, this isolate was chosen for further laccase characterization. The laccase produced by SB086 was active between pH 4.0 and 7.0 (Fig. 1a) and was completely inactivated at pH 3.0 and 8.0. The optimum activity for this enzyme was observed both at pH 4.0 and 5.0, and enzyme activity decreased to 60% at pH 6.0. The optimum temperature for SB086

1 laccase was observed at 50°C (Fig. 1b). At lower (45°C) and higher (55°C)
2 temperatures, the enzyme activity was reduced to around 75%. At the highest
3 temperature assayed, 70°C, 14% of activity was still detected. Considering the
4 optimum of 50°C, the thermal stability was evaluated at higher temperatures: 60°C and
5 70°C. The enzyme retained about 50% of its activity after 20 min of pre-treatment at
6 60°C or 70°C, and about 15% after 180 min at 60°C or 60 min at 70°C (Table 1).



7

8 **Figure 1.** Effect of pH **(a)** and temperature **(b)** on laccase activity from crude extract of
9 *Streptomyces* sp. SB086, with McIlvaine buffer 0.1M. Optimum pH was estimated at
10 50°C, and optimum temperature was estimated at pH 4.0.
11
12

Table 1. Thermal stability of laccase against pre-treatments at 60 and 70°C

	Relative activity (%)	
Time (min)	Pre-treatment at 60°C	Pre-treatment at 70°C
0	100	100
20	46,4	46,7
30	44,4	20,6
60	26,4	14,3
90	28,7	nt
120	nt	1,6
180	15,7	nt

nt: not tested

Laccase activity from SB086 was enhanced in the presence of the metals Cu, Ca, K, Mg, Mn and Zn, and inhibited by Fe. The highest effect on laccase activity was observed at 5 mM $CuSO_4$, which triggered a relative activity of 212.57%. $MgSO_4$ and K_2SO_4 progressively enhanced enzyme activity, producing 186.39% and 133.51% of relative activity, respectively, at 20 mM. $ZnSO_4$ at 5 or 10 mM induced around 159% of relative activity. Laccase activity was also enhanced with 1 mM $CaCl_2$ or $MnSO_4$, less enhanced with 5 mM $MnSO_4$ and inhibited by 5 mM $CaCl_2$. $FeSO_4$ intensely inhibited laccase activity even at the lowest tested concentration (Table 2).

Table 2. Effect of metal ions on laccase activity.

Metal ion	1 mM	5 mM	10 mM	20 mM
$CuSO_4$	130,4	212,6	12,57	nt
$CaCl_2$	124,1	86,4	nt	nt
K_2SO_4	97,4	103,1	125,1	133,5
$MgSO_4$	111,5	122	178	186,4
$MnSO_4$	138,2	112,6	nt	nt
$ZnSO_4$	108,4	158,1	159,7	nt
$FeSO_4$	8,4	8,9	nt	nt

Note: The values represent the relative activity (activity of control, without any metal ion added to the reaction mixture, was considered 100%). The values are averages of two repetitions. Nt, not tested.

Purification with acetone gradient and molecular weight estimation

After precipitation with cold acetone, laccase activity was only observed in the 60% acetone saturation fraction. Laccase size was estimated to be above 100 KDa with Amicon® Ultra Centrifugal Filter Devices.

Characterization of isolate SB086

Isolate SB086 forms a distinct phyletic line that is associated with the *Streptomyces xanthophaeus* 16S rRNA subclade, a result supported by all of the tree-making algorithms and by a bootstrap value of 58% (Figure 2). The organism shares a 16S rRNA gene similarity of 99.3% with the type strains of *Streptomyces xanthophaeus*, *Streptomyces spororaveus*, *Streptomyces avidinii*, *Streptomyces nojiriensis* and *Streptomyces subrutilus*, a value which corresponds to 9 nt difference out of 1,373 sites. The isolate was found to contain major amounts of *anteiso*-$C_{15:0}$ (23.34%), *iso*-$C_{16:0}$ (15.65%), $C_{16:0}$ (14.07%) and *iso*-$C_{15:0}$ (12.33%) (online resource, Supplementary Table 1). The G + C contents of the DNA preparations of isolate SB086 was determined to be 69.8%. All of these characteristics are consistent with the classification of the strain in the genus *Streptomyces* (Williams et al. 1983; Manfio et al. 1995; Anderson & Wellington 2001; Kämpfer 2012).

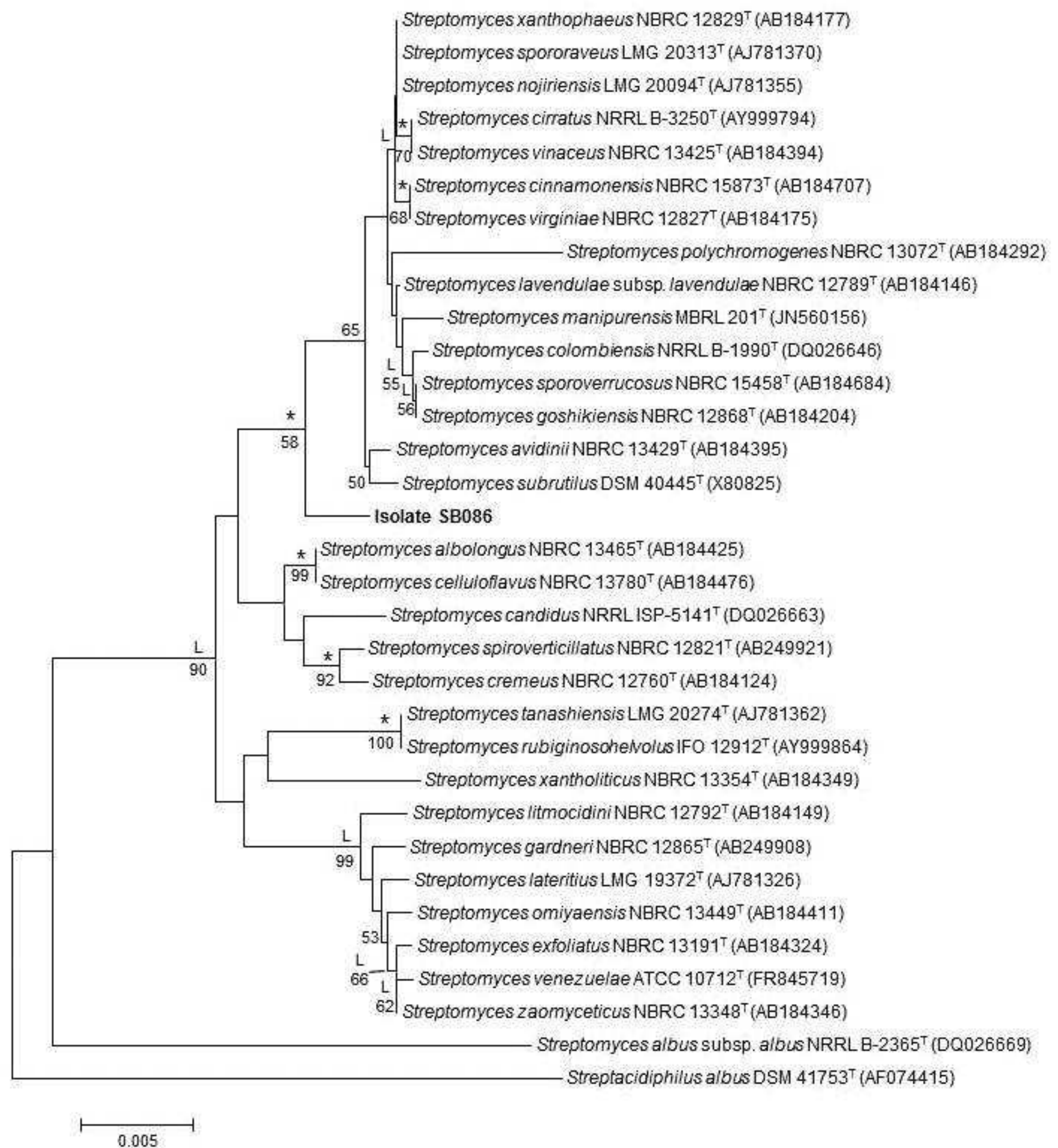


Figure 2. Neighbour-joining tree based on nearly-complete 16S rRNA gene sequences (~1400 bp) showing relationships between isolate SB086 and the type strains of phylogenetically close *Streptomyces* species. Asterisks indicate branches of the tree that were recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms; L and P stand for branches which were also recovered using the maximum-likelihood or maximum-parsimony tree-making algorithms, respectively. Numbers at the nodes are percentage bootstrap values based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50% are given. Bar, 0.005 substitutions per nucleotide position.

1 **Supplementary Table 1.** Fatty acid composition of isolate SB086

Fatty acids	Content (%)
Iso-$C_{14:0}$	2.41
$C_{14:0}$	0.75
Iso-$C_{15:0}$	12.33
Anteiso-$C_{15:0}$	23.34
$C_{15:0}$	---
Iso-$C_{16:1}$ H	1.39
Iso-$C_{16:0}$	15.65
$C_{16:0}$	14.07
Anteiso-$C_{17:1}$ ω9c	1.90
Iso-$C_{17:0}$	7.55
Anteiso-$C_{17:0}$	10.79
$C_{16:1}$ ω6c / $C_{16:1}$ ω7c	6.49
$C_{17:1}$ ω9c / $C_{16:0}$ 10-methyl	3.33

2

3

4 Isolate SB086 was observed to form an extensively branched brownish substrate
5 mycelium which bore an abundant light pink aerial mycelium on oatmeal agar. With a
6 single exception (ISP 7), the isolate was observed to grow well on ISP and GYEA
7 media (online resource, Supplementary Table 2). Isolate SB086 can be readily
8 distinguished from its nearest phylogenetic neighbors using a broad range of
9 phenotypic properties, notably by its ability to grow in a broader range of different
10 sugars (Table 3). Additional phenotypic properties of *Streptomyces* sp. SB086 are
11 presented in online resource, Supplementary Table 3.
12

1 **Supplementary Table 2.** Growth and cultural characteristics of isolate *Streptomyces*
2 sp. SB086 after incubation at 28°C for 14 days

Medium	*Streptomyces* sp. SB086
Tryptic-yeast extract-iron agar (ISP medium 1)	
Growth	++
Aerial mycelium	White
Color of substrate mycelium	Pale yellow
Diffusible pigment	Light brown
Yeast extract-malt extract agar (ISP medium 2)	
Growth	+++
Aerial mycelium	White
Colour of substrate mycelium	Reddish brown
Diffusible pigment	Reddish brown
Oatmeal agar (ISP medium 3)	
Growth	+++
Aerial mycelium	Light pink
Colour of substrate mycelium	Light brown
Diffusible pigment	Pink
Inorganic salts–starch agar (ISP medium 4)	
Growth	+++
Aerial mycelium	Light grey
Color of substrate mycelium	Undeterminated
Diffusible pigment	Purple
Glucose-asparagine agar (ISP medium 5)	
Growth	++
Aerial mycelium	White
Colour of substrate mycelium	Pale yellow
Diffusible pigment	Ligh pink
Tyrosine agar (ISP medium 7)	
Growth	-
Aerial mycelium	-
Colour of substrate mycelium	-
Diffusible pigment	-
Glucose-yeast extract (GYE medium)	
Growth	++
Aerial mycelium	White
Colour of substrate mycelium	Brown
Diffusible pigment	Dark brown

3 **Note:** +++, abundant growth; ++, moderate growth; -, no growth

4

1 **Table 3.** Cultural characteristics of *Streptomyces* sp. SB086 and the phylogenetically

2 closest species

Characteristic	SB086	*S. xanthophaeus*	*S. spororaveus*	*S. avidinii*	*S. subrutilus*
Appearance in organic salt-starch agar (ISP-4)					
Spore-mass colour	Light gray	Grayish yellowish pink to light grayish reddish brown	nd	Grayish yellowish pink; light grayish reddish brown	Grayish yellowish pink
Substrate mycelium color	nd	Very dark reddish gray to near black	nd	No distinctive pigments	
Soluble pigment	Purple	Traces of yellow	nd	No pigments	No distinctive pigments
Melanin production on tyrosine agar (ISP-7)	No growth	nd	nd	No pigment production	Only weakly or not at all
Growth on sole carbon sources					
D-fructose	+	nd	-	+	+
Maltose	+	nd	+	nd	nd
Galactose	+	nd	-	nd	nd
D-glucose	+	nd	+	+	+
L-Arabinose	+	-	-	-	-
D-Xylose	+	-	-	-	-
D-Manitol	+	-	nd	-	+/-
Myo-Inositol	+	-	nd	-	-

3 nd: information not determined in literature, to our knowledge

4

1 **Supplementary Table 3.** Phenotypic properties of *Streptomyces* sp. SB086

Test	*Streptomyces* sp. SB086
pH:	
4,0	-
5,0	-
9,0	+
10,0	+
NaCl (%):	
1,0	+
3,0	+
5,0	-
7,0	-
Antibiotics (μg.ml^{-1}):	
Nalidixic Acid (10)	+
Kanamycin (10)	-
Novobiocin(10)	-
Penicillin (20)	+
Streptomycin (20)	-
Degradation of:	
Starch	+
Uric acid	-
Casein	+
Cellulose	+
Gelatin	+
Guanine	-
Hypoxanthine	-
Pectin	-
Tyrosine	-
Tributyrin	-
Tween 20	-
Tween 80	-
Xanthine	-
Xylan	+

2 **Note:** +, growth; -, no growth.

3

4

5 **DISCUSSION**

6 Bacterial isolates with known ability to degrade lignin belong to three classes:
7 *Actinobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria*, including the isolates
8 obtained from guts of termites and wood-boring beetles (Bugg et al. 2011). This
9 information reinforces the potential of bacteria as source for novel laccase enzymes,
10 and also strengthens the need for extending knowledge in this research field. In this
11 work, we have focused on *Actinobacteria*, a group with still a small number of

characterized laccases, and recognized for being a copious producer of unique biomolecules.

The wide range of substrates of laccase makes the enzyme attractive for many biotechnological applications in industry. In order to be adequate for an industrial purpose, one of the desirable properties of enzymes is thermotolerance. *Streptomyces* sp. SB086 laccase presented an optimum temperature of 50°C, close to the average for microbial laccases of 55°C (Strong & Claus 2011). The same 50°C-optimum was observed for the fungal *Pycnoporus coccineus* laccase (Suzuki et al. 2003), but its half-life at 70°C was 100 min, while SB086 laccase half-life at 60°C or 70°C (pre-incubations) was about 20 min. In contrast, the most studied bacterial laccase, CotA, from *Bacillus subtilis* endospores, is an exception to this pattern, as its optimum activity is at 75°C (Martins et al. 2002). The relatively high optimum temperature and thermal stability observed for the laccase produced by isolate SB086 and its optimum pH range is advantageous for industrial application, considering that cellulolytic enzymes generally require similar condition (temperature range from 40 to 50°C, and pH 4 to 5; reviewed by Howard et al. 2003; Taherzadeh and Karimi 2007).

The optimum pH of SB086 laccase (between pH 4,0 and 5,0) was similar to laccases of *Streptomyces cyaneus* (pH 4.5; Arias et al. 2003), *S. lavendulae* (pH 4.5; Suzuki et al. 2003) and most microbial laccases described, which have an acidic optimum pH. Considering that high activity at alkaline pH is desirable for many industrial applications (Strong and Claus 2011), this feature limits the range of possible applications of SB086 laccase to the ones where stability at acidic pH is required: for example, in wine industry for polyphenol elimination at pH around 2.5-4.0 (Madhavi & Lele 2009). Surprisingly, the small laccase from *Streptomyces coelicolor* presented highest activity at pH 9.4 with 2.6-dimethoxyphenol (2,6-DMP) as substrate (Machczynski et al. 2004).

SB086 laccase activity presented a new pattern of response to metal ions. It was stimulated by many metal ions and was inhibited only by iron. Differently, laccase produced by *S. cyaneus* was inhibited by calcium and zinc (Arias et al. 2003), whereas *S. lavendulae* laccase was stimulated by 0.2 mM calcium or manganese ions, but was not affected by copper, zinc, magnesium and potassium ions (Suzuki et al. 2003). Copper and manganese inhibited *Sinorhizobium meliloti* laccase, while potassium ion stimulated it (Strong and Claus 2011). *Daedalea quercina* laccase was stimulated by

copper, but inhibited by manganese ion (Baldrian 2004). Similarly to SB086, iron inhibited *S. cyaneus* and *Sinorhizobium meliloti* laccases, and both ferric and ferrous ions are recognizedly inhibitors of cellulolysis reactions (Tejirian and Xu 2010). After comparison, it can be noted that SB086 laccase was less sensitive to metal inhibition. Furthermore, the variable response to the metal ions among laccases indicates that these are very heterogeneous enzymes in microbial sources.

The molecular weight of the active protein (≥ 100 kDa) indicates a non-monomeric organization and it is in line with several reported streptomycetes laccases which need to be at least dimeric to be active. For instance, *Streptomyces griseus* and *Streptomyces aviceus* produce trimerical laccases with 114 and 98 kDa, respectively (Endo et al. 2003; Gunne and Urlacher 2012) and *Streptomyces ipomoea* produces a dimerical laccase with 79 kDa (Molina-Guijarro et al. 2009).

CONCLUSION

In the presented *Actinobacteria* isolation from forest soil under restoration process, the isolate with highest laccase activity was found to belong to genus *Streptomyces*. Our data suggest that the isolate SB086 might be the nucleus of a new taxon. This isolate was found to synthesize and secrete a laccase-like enzyme with industrially interesting properties. Since most bacterial laccases are only hypothetically assigned, our findings are relevant for supporting *in silico* data of bacterial laccase secretion, and for reinforcing that *Bacteria* are a valuable source of laccases for industrial application.

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11

CAPÍTULO 3

Design of primers for specific detection of actinobacterial laccases from superfamilies I and K

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ABSTRACT

Few laccases from *Actinobacteria* have been characterized so far; however, the properties with industrial relevance presented by this small number of enzymes and the capability to degrade recalcitrant soil polymers has evoked attention to this phylum. In this work, we have designed and tested primers that were specific for detection of sub-groups of laccase-like gene within *Actinobacteria*, which coincided with the superfamilies I and K from the classification proposed by the Laccase and Multicopper Oxidase Engineering Database. The designed primers have amplified laccase-like gene fragments from actinobacteria isolates that were undetectable by primers available in literature. Furthermore, phylogenetic alignments suggest that some of these fragments may belong to new laccases.

KEYWORDS:

actinobacteria, multicopper oxidase, laccase primers, lignin degradation, LMCO

ABBREVIATIONS:

cbr: copper-binding regions

LccED: Laccase and Multicopper Oxidase Engineering Database

LMCO: laccase-like multicopper oxidase

PCR: polymerase chain reaction

1 **INTRODUCTION**

2 Laccases (EC 1.10.3.2) are multicopper oxidases capable of capturing electrons from a
3 broad range of organic and inorganic compounds, even non-aromatic ones,
4 concomitantly with reduction of molecular oxygen, producing water (Claus, 2004). The
5 extensive substrate range of laccase makes the enzyme very attractive for application
6 in several fields of industry, e.g., pulp and paper, bioethanol, food, pharmaceutical and
7 cosmetics. In addition, laccases are also required for environmental applications, such
8 as bioremediation of textile effluents, phenol degradation from residuary water of
9 factories, pesticide degradation and decontamination of aromatic polycyclic aromatic
10 hydrocarbons (PAHs) from soil (Piscitelli et al., 2010; Schroeder et al., 2008).

11 Among *Actinobacteria*, laccase-like multicopper oxidase genes (LMCO) can be found
12 in over 20 genera in National Center for Biotechnology Information (NCBI) database.
13 However, to our knowledge, only nine LMCO have been completely or partially
14 characterized, and, surprisingly, each of them has presented desired properties for
15 industrial application, i.e., tolerance/resistance to high temperatures, alkalinity, high pH
16 variation, salinity or common industrial inhibitors. This peculiarity, summed to the fact
17 that actinobacteria in degradation of recalcitrant and complex polymers found in litter
18 and soil (Bugg et al., 2011; Godden et al., 1992; Goodfellow & Williams, 1983; Lee,
19 1997), highlights the need for more studies concerning its lignocellulolytic enzymes.
20 Furthermore, considering that native LMCO producers generally present low
21 expression of these enzymes (Piscitelli et al., 2010) and their location may be
22 intracellular in some bacteria (Ausec et al., 2011b), it seems suitable to use molecular
23 approaches for detection of the gene along with biochemical analysis using culture
24 extracts.

25 LMCO enzymes contain conserved regions dedicated to bind copper atoms in active
26 protein (Alexandre and Zhulin, 2000; Claus, 2004; Hoegger et al., 2006; Kumar et al.,
27 2003). These copper-binding regions (cbr) are usual targets for degenerate primer
28 designing and have been frequently used for LMCO detection from fungi (D'Souza et
29 al., 1996; Eggert et al., 1998; Luis et al., 2004; Taprab et al., 2005) and bacteria (Ausec
30 et al., 2011a; Kellner et al., 2008).

Previous attempts demonstrated that the degenerate bacterial primers available in literature were ineffective to amplify LMCO genes from actinobacteira. Thus, main aim of this work was to design primers for this finality.

Even though an exclusive definition of the “laccase” is complicated by the overlaps in substrate range and the sequence similarity of cbr among laccase and other multicopper oxidases enzymes, alignment of hundreds of gene sequences have provided a profile of a comprehensive clustering of enzymes from different sources: basidiomycetes, ascomycetes, insect, plants, for instance, suggesting a putative functional distribution of sequences. (Hoegger et al., 2006). The combination of information on sequences, alignments, annotations and structures of MCOs is provided by the Laccase and Multicopper Oxidase Engineering Database (LccED). LccED distributes MCO into 11 superfamilies; actinobacteria laccases can be found in 4 of them: B, I, J and K, but the already characterized enzymes from this group belong to superfamilies I and K. Superfamily I contains the more traditionally known enzymes with three structural domains, including LMCO from over 10 actinobacteria genera; superfamily K contains homologues to the small laccase from *Streptomyces coelicolor*, which lacks the second domain (Machczynski et al., 2004); only LMCO from three genera, all from actinobacteria, are so far assigned to superfamily K.

MATERIAL AND METHODS

Isolates and maintenance conditions

Actinobacteria were isolated from soil of Atlantic Forest fragments under restoration process (Fernandes et al., 2013; Zucchi et al., *unpubl.*) and were deposited in the EMBRAPA Collection of Microorganisms of Agricultural and Environmental Importance (CMAA). The isolates were maintained in GYEA (glucose 2% w/v, yeast extract 2% w/v, agar 1.5% w/v) plates at 4°C and as mycelial fragments in 20% (w/v) glycerol at -80°C.

Design of primers

Six characterized actinobacteria laccases with available gene sequences were used for primer designing, all belonging to genus *Streptomyces*: *S. cyaneus*, *S. lavendulae*, *S. antibioticus*, *S. coelicolor*, *S. ipomoea* and *S. griseus.* Laccases from the first three species were classified into laccase superfamily I, and the three others into superfamily K. The online PriFi software (Fredslund et al., 2005) was used to provide primers with few or no degenerations (Table 1). The type strain of *Streptomyces araujoniae* ASBV-1^T (da Silva et al., 2013) was used as positive control due to the presence of laccase-like gene in its genome.

Table 1. Primers used in this study

		Primer	5'-3' Sequence	Expected bands (bp)	Reference
		Cu1AF	ACMWCBGTYCAYTGGCAYGG	142	Kellner et al. 2008
		Cu2R	GRCTGTGGTACCAGAANGTNCC		
		Cu4R[a]	TGCTCVAGBAKRTGGCAGTG	600-1500	Ausec et al. 2011
Sets of primers	*1I*	LacI11F	CCAGTGGTGGTACCACGACCA	1012	This study
		LacI22R	AGRTGGATGTGCATGGGGTG		
	2I	LacI65F	AGTAYCCGAACGACCACCAGG	1037 bp	
		LacI22R	AGRTGGATGTGCATGGGGTG		
	3I	LacI11F	CCAGTGGTGGTACCACGACCA	1264 bp	
		LacI92R	ATCATGCCCATGTCCTCGTG		
	4I	LacI65F	AGTAYCCGAACGACCACCAGG	1289	
		LacI92R	ATCATGCCCATGTCCTCGTG		
	1K	LacK105F	GATGTACGCCGAGAAGCTG	736	
		LacK120R	CCCATGTCGGAGTGGCTC		
	2K	LacK155F	GGCTACGGCYTCGAGAAG	702	
		LacK120R	CCCATGTCGGAGTGGCTC		
	3K	LacK105F	GATGTACGCCGAGAAGCTG	667	
		LacK161R	ATGATCTGGARGCCGWAGGAGT		
	4K	LacK188F	TACGGCYTCGAGAAGGGC	488	
		LacK199R	CCGTGCGTGATCATGACG		

[a], primer was used with Cu1AF

DNA extraction, laccase-like putative genes amplification and sequencing

The isolated actinomycetes were incubated in GY broth at 28°C for 7 days; cells were harvested by centrifugation (6,000 x *g*, 5 min) and the genomic DNA was extracted by

using the PureLink® Genomic DNA mini kit (Life Technologies). Polymerase chain reaction (PCR) mixture contained final concentrations of 0.6 μM of each primer, 0.2 μM dNTP, 3.2% DMSO, 3 μM $MgCl_2$, 2.5 μl PCR buffer, 1 U Taq DNA polymerase (or Platinum® Taq DNA Polymerase for primers Cu1AF-Cu4R) (Life Technologies), 1μl DNA extract and sterile MilliQ water to final 25 μl volume. PCR condition for the primers pairs Cu1AF-Cu2R and Cu1AF-Cu4R were performed as described by Kellner et al. (2008) and Ausec et al. (2011a), respectively. For Cu1AF-Cu2R, positive amplification was only obtained when annealing time was elevated from 30 to 90 seconds. The PCR conditions for the designed primers consisted of an initial denaturation at 94°C for 4 min; a touchdown on annealing temperature decreasing 0.5°C per cycle, for 10 cycles, initiating at 67°C, for 45 s, 72°C for 1 min; 25 cycles of 94°C for 45 s, 62°C for 45 s and 72 for 1 min, followed by a final 5 min extension at 72°C. PCR products were visualized by electrophoresis in a 1.0% (w/v) agarose gel. PCR products were purified using a PCR Product Purification Kit (Qiagen, USA), according to the manufacturer's instructions. Direct sequencing of the PCR products was performed using an ABI PRISM Big Dye™ Terminator Cycle Sequencing Kit (Applied Biosystems). Sequences were compared with entries in the updated GenBank and EMBL databases by means of the BLAST program.

The designed primers were tested for amplification of laccase gene from *Bacillus subtilis*, *Bacillus megaterium* and *Pseudomonas putida* using the same conditions previoulsly described.

Phylogenetic analysis

The laccase sequences obtained with the newly designed primers were manually aligned using MEGA version 6 software (Tamura et al., 2013) against corresponding sequences of closely related laccases and charaterized laccases from *Streptomyces* species retrieved from the GenBank database. Phylogenetic trees and bootstrap analysis were inferred following the procedures described by Zucchi et al. (2013).

16S rDNA identification

1 Genomic DNA of isolates with positive laccase amplifications was extracted and 16S
2 rDNA PCR amplification and sequencing were achieved following the same conditions
3 of Zucchi et al. (2011). The nucleotide sequences of 16S rRNA gene sequences of the
4 isolates have been deposited within the GenBank database under accession numbers
5 KJ149453-KJ149462.

6

7 **RESULTS**

8 **Laccase gene amplification with Cu1AF-Cu2R and Cu1AF-Cu4R primers**

9 Around one hundred actinomycetes isolates were analysed. The Cu1AF-Cu2R primer
10 pair was designed to amplify the DNA between copper-binding regions I and II, with an
11 expected product size of 142 bp (Kellner et al., 2008). Three positive isolates (SBB001,
12 SBB018 and SBB018A) were only obtained by using a longer annealing time (90
13 instead of 30 seconds).

14 These isolates were further tested with the primer Cu4R, which, along with the forward
15 primer Cu1AF provides fragments from 600 bp (2-domains laccases) to 1500 bp (3-
16 domains; Ausec et al., 2011a). Isolates SBB001, SBB018 and SBB018A presented
17 bands around 1200 pb. The BLAST search of these sequences in the GenBank
18 database retrieved 23S rRNA sequences instead of multicopper-oxidase proteins
19 (Table 2).

20

21 **Table 2.** Nearest matches of Cu1AF/Cu4R amplicons. Similarities compared with
22 entries from GenBank and EMBL databases

Isolate	Nearest match	Similarity (%)	*E*-value
SBB001	23S rRNA of *Amycolatopsis mediterranei* RB (CP003777)	93%	0.0
SBB018	23S rRNA of *Amycolatopsis mediterranei* U32 (NR_103002)	94%	0.0
SBB018A	23S rRNA of *Amycolatopsis mediterranei* U32 (NR_103002)	94%	0.0

23 **Note:** sequences can be found in the Supplementary Material

24

25

26 **Laccase gene amplification with the designed primers and specificity test**

Eight sets of primers were used to survey the actinomycetes for laccase-like genes from superfamilies I and K (Table 1). Positive amplifications were found for 11 isolates and the amplicons sequencing revealed that the set of primers were effective in placing the laccase-like genes into the corresponding laccase superfamily (Table 3). The characterization of 16S rRNA of the isolates indicated that laccase-like genes from superfamily I were found among members of *Amycolatopsis*, *Nonomureae* and *Streptomyces* whereas superfamily K genes were found only in *Streptomyces* spp. Furthermore, only the sets of primers 3I and 4I were able to amplify laccases genes from superfamily I and the primer set 3I provided 80% of the positive results. On the other hand, all sets of primer contributed to the detection of laccase from superfamily K; but there was a slight predominance for sets 1K and 2K which were present in 33.3% and 41.7% of all positive amplifications, respectively. Finally, isolate SB003 was found to contain genes from both laccase superfamilies.

Alignment of cbrIII of the obtained sequences revealed an unexpected divergence in aminoacid conservation, which is rare even among different phylogenetic groups (Reiss et al., 2013): genes from superfamily I presented the standard proline residue after the first histidine of the cbr; however, threonine was present at this position in superfamily K sequences (Figure 1).

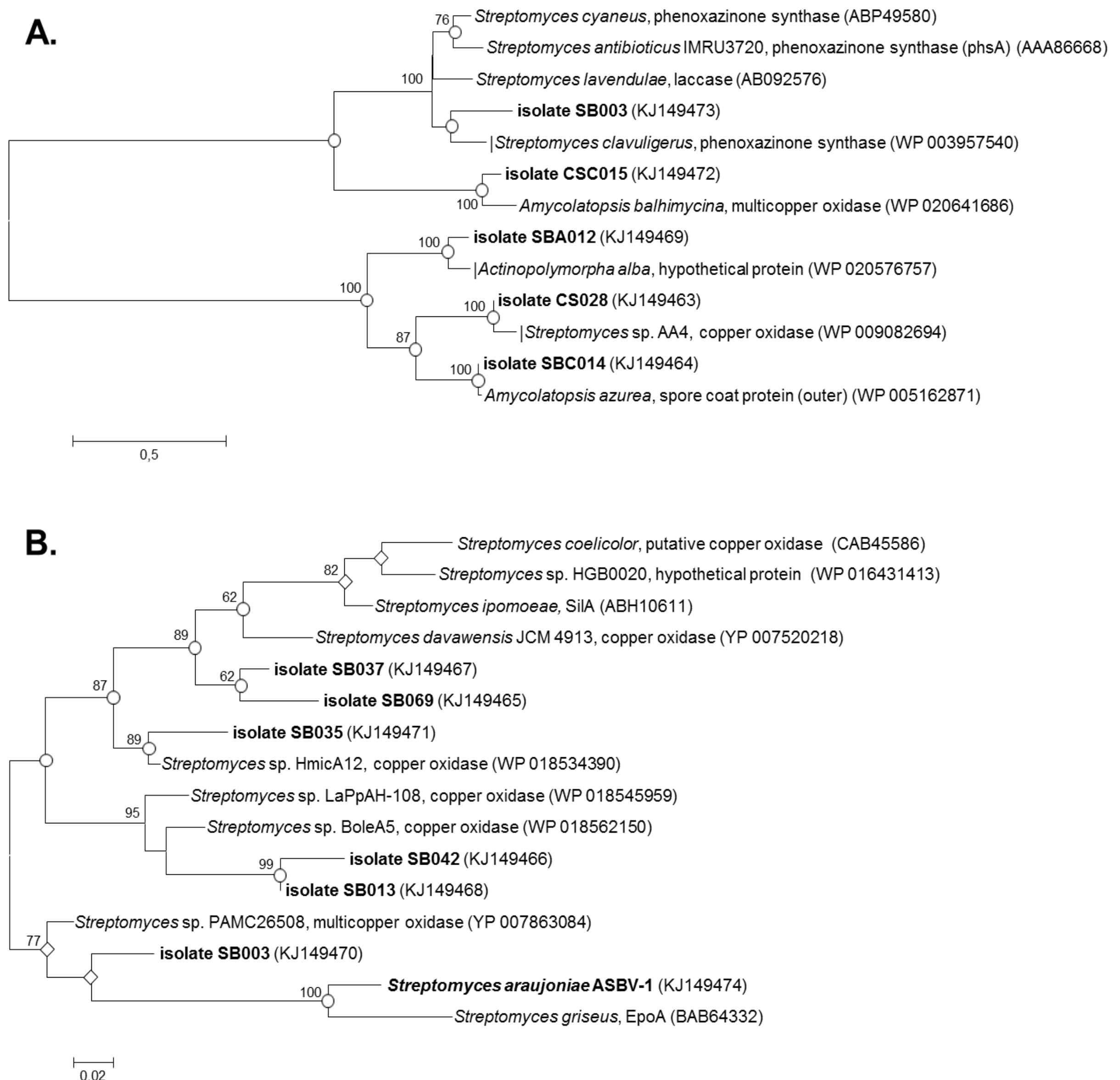
1 **Table 3.** Nearest matches of 16S rRNA sequences and laccase-like genes of actinomycetes from soil of Atlantic Forest fragments under restoration
2 process

LccED super family	16S rRNA similarity with type-strains			Laccase nearest matches			Primer set
	Isolate	**16S identification**	**Similarity (%)**	**Nearest match**	**Identity (%)**	**E-value**	
I	CS028	*Amycolatopsis albidoflavus* IMSNU 22139^T (AJ252832)	99.1	Copper oxidase (WP_020669126)	94	5e-178	3I
	CSC015	*Amycolatopsis lexingtonensis* NRRL B-24131^T (AY183358)	99.0	Multicopper oxidase (WP_020641686)	88	2e-111	3I
	SB003	*Streptomyces gelaticus* NRRL B-2928^T (DQ026636)	100.0	Phenoxazinone synthase (WP_003957540)	70	4e-104	4I
	SBA012	*Nonomuraea bangladeshensis* 13651M ^{T} (EU741170)	99.0	Multicopper oxidase (WP_020576757)	89	4e-160	3I
	SBC014	*Amycolatopsis lurida* DSM 43134^T (AJ577997)	99.3	Spore coat protein (WP_005162871)	98	8e-180	3I
K	ASBV-1	*Streptomyces araujoniae* ASBV-1^T (EU792889)	Type-strain	Copper oxidase (WP_018491986)	98	0.0	1K
	SB003	*Streptomyces gelaticus* NRRL B-2928^T (DQ026636)	100.0	Multicopper oxidase (YP_007863084)	93	3e-156	1K
	SB013	*Streptomyces scopuliridis* RB72^T (EF657884)	99.1	Copper oxidase (WP_018562150)	93	3e-112	1K, 2K
	SB035	*Streptomyces avermitilis* MA-4680^T (BA000030)	98.7	Copper oxidase (WP_018534390)	96	1e-126	2K, 4K
	SB037	*Streptomyces aureus* NBRC 100912^T (AB249976)	99.8	Copper oxidase (YP_007520218)	92	7e-133	2K, 4K
	SB042	*Streptomyces sannanensis* NBRC 14239^T (AB184579)	99.2	Copper oxidase (WP_018562150)	92	1e-107	1K, 2K, 3K
	SB069	*Streptomyces sp.* PL28 ^{T} (KC789714)	99.8	Multicopper oxidase (WP_016431413)	91	8e-124	2K, 4K

```
Superfamily I
S. antibioticus  519 PILHPMHIHLAD
      S. cyaneus 505 PIVHPMHIHLAD
  S. lavendulae  508 PVVHPMHIHLAD
  Isolate CS028      DHLHPIHLHLVH
 Isolate CSC015      GPAHPIHIHLTE
  Isolate SB003      GPTHPMHIHLAD
 Isolate SBA012      DVHHPIHLHLVG
 Isolate SBC014      DIHHPVHLHLVG
                         *
Superfamily K
  S. coelicolor  228 EYYHTFHMHGHR
    S. ipomoeae  228 EYYHTFHMHGHR
     S. griseus  228 EFYHTFHIHGHR
  Isolate SB003      EYYHTFHIHGHR
  Isolate SB013      EFYHTFHMHGHR
  Isolate SB035      EYYHTFHMHGHR
  Isolate SB037      EYYHTFHIHGHR
  Isolate SB042      EFYHTFHMHGHR
  Isolate SB069      EYYHTFHMHGHR
  S. araujoniae  228 EFYHTFHIHGHR
                         *
```

Figure 1. The third copper-binding region (cbrIII) of LMCO from actinomycetes used in this work, emphasizing with an asterisk the presence of proline residue in protein sequences from superfamily I, and its absence in superfamily K.

Phylogenetic analysis showed that each of the laccase sequences formed distinct phyletic lines within the laccase superfamilies I and K (Figure 2). Sequences that fall into laccase superfamily I subclade shared similarity values of 70-90% with their closest laccase-like neighbours whereas sequences placed into superfamily K were 91-98% similar to their closest matches (Table 3). Furthermore, isolates SB037 and SB069 formed a group strongly associated with the putative copper oxidases from *Streptomyces coelicolor* subclade (Figure 2B); this phylogenetic position was supported by all tree-making algorithms and a 89% bootstrap value. Similarly, a subclade supported by all tree-making algorithms and a high bootstrap value was formed by isolates SB042 and SB013, albeit it was loosely associated with copper oxidase from *Streptomyces* sp. BoleA5.



1

2 **Figure 2.** Maximum likelihood trees based on laccase amino acid sequences showing
3 relationship between the isolates and representatives of the closest related laccases
4 genes from superfamily I (A) and K (B). Empty circle indicate branches of the tree that
5 were recovered with the neighbour-joining and maximum-parsimony tree-making
6 algorithms; white diamonds stand for branches which were also recovered using the
7 maximum-parsimony tree-making algorithms. Numbers at the nodes are percentage
8 bootstrap values based on a neighbor-joining analysis of 1,000 resampled datasets;
9 only values above 50% are given. Bar, 0.02-0.5 substitutions per nucleotide position.

10

11

12

Primer specificity analysis

The new primers have been tested with genomic DNA from *Bacillus subtilis, Bacillus megaterium* and *Pseudomonas putida*, and no amplification was obtained (data not shown).

DISCUSSION

Aiming to prospect LMCO genes from soil actinomycetes, degenerate oligonucleotide primers available in literature and indicated for diverse bacterial taxa have been employed (Table 1). However, for our specific study, their PCR amplifications were unsuccessful, considering that, after selecting positive isolates with Cu1AF-Cu2R and proceeding to amplification of longer products with Cu1AF-Cu4R, 23S rRNA was obtained when final sequences were blasted (Table 2). It is possible that modification of the original protocol may have decreased specificity of the primers, which could not be as suitable for our LCMO source – *Actinobacteria* – as to the other microorganisms assayed by the authors (Ausec et al., 2011a; Kellner et al., 2008).

Alternatively, designing of specific actinomycetes primers was accomplished by alignment of genes from six characterized LMCO, clustered into two different sets, which coincided with classification in superfamilies I or K from LccED. Therefore, instead of designing degenerate primers for annealing in all the 6 sequences, we have looked for obtaining olinucleotide sequences with the best pairing to each of the two 3-sequences groups (Table 1). *A posteriori*, the primers have proved to amplify LMCO from actinomycetes isolates that were undetectable by using the set of primers Cu1AF-Cu2R (Table 3).

Although a *consensus* on strict "laccase" definition is still unavailable (Reiss et al., 2013; Sirim et al., 2011), in the current sequence-based classification, the designed primers were suffice to discriminate actinomycetes LMCO genes fragments into the cited superfamilies I and K (Figure 1). Interestingly, the superfamily-specific profile of the sequences details the observation of Reiss et al. (2013) that, in a rare fashion, primary structure of some tested *Streptomyces* LMCO did not possess the standard proline residue after the first histidine of cbrIII, present in the sequences from other bacteria and plants: this proline seems to be really absent in superfamily K enzymes, but, in contrast, it is present in all sequences from superfamily I we have analyzed

1 (Figure 1). This fact may emphasizes the value of superfamily- or subgroup-selective
2 prospection of LMCO gene sequences.

3 Furthermore, our results enrich the content list of organisms with superfamily I LMCO,
4 due to its detection in a genus not yet cited in LccED, i.e. *Nonomureae*. Interestingly,
5 superfamily I contains almost 10 times more LMCO proteins than superfamily K, and
6 bioinformatic analysis suggests that less than 10% actinobacteria laccases present
7 only 2 domains (Ausec et al., 2011b). Indeed, it may highlights either a primers
8 inefficiency or an underestimation on the size of superfamily K. The performed
9 specificity tests indicated that the primers can amplify laccases from *Actinobacteria*, but
10 it seems unsuitable for other other bacterial groups known to house LMCO genes.
11 Hence, they seem to be superfamily-specific but not *Streptomyces*-specific, as
12 fragments from the genera *Amycolatopsis* and *Nonomuraea* were also detected. To the
13 best of our knowledge, the design and use of superfamily-specific primers has not been
14 applied for detection of laccase-like genes so far. Therefore, according to our best PCR
15 results, we would recommend that studies addressing superfamily I laccases employ
16 the primer pairs LacI11F-LacI92R (3I) and LacI65F-LacI92R (4I); and, addressing
17 superfamily K, employ LacK105F-LacK120R (1K) and LacK155F-LacK120R (2K).

18 A benefit of using superfamily- or group-specific primers in prospection studies is the
19 possibility of obtaining a more substantial detection of LMCO genes, whereas distantly-
20 related sequences could, disadvantageoulsly, generate primers with bias for one or a
21 few of the superfamilies. Moreover, alignment of the fragments in the phylogenetic
22 trees suggests that many of obtained fragments – i.e., *Streptomyces* sp. SB003 from
23 superfamily I and most of those from superfamily K – may belong to new LMCO, since
24 no close clustering was observed with nearest sequences retrieved from NCBI (Figure
25 2). Furthermore, some of these closest sequences belong to non-characterized
26 laccases and further enzyme characterization may reveal unique properties.

27 Finally, our results reinforce the need for further characterization of laccases from
28 actinomycetes. In order to explore the potential for industrial application of the newly
29 detected LMCO, further biochemical characterization of the enzymes have to
30 accomplished, e.g., optimal conditions and substrate range. Notwithstanding, the
31 demand of large amounts of enzymes for industrial applications – partly impaired by
32 the low expression of laccases in the native organisms (Piscitelli et al., 2010) – could
33 be provided by heterologous expression of selected genes in strains with simple
34 genetic engineering or growth manipulation, providing efficient systems for large scale

1 production. The primers designed herein may premilinar information for a more suitable
2 exploitation of actinomycetes laccase-like genes.

3

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10

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SUPPLEMENTARY MATERIAL

Sequences obtained after amplification with primer pair Cu1AF-Cu4R using actinomycetes genomic DNA as templates.

>SBB001

GGGCATTTTGCCGAGTTCCTTAACCACAGTTCACCCGATGAAACAGAATTCTCTACCTGACCACCTGTGTTGGTTTG
GGGTAMRGGCCGTGCATGCACTCACTAGAGGCTTTTCTCGACAGCATAGGATCACCCACTTCACCTCAAACGGCT
ACGCATCACGTCTCAGCCTATTGCCATGCGGATTTGCCTACATGACGGCCTACACGCTTACACCAGTACAACCACT
CACTGGCGGAGCTACCTTCCTGCGTCACCCCATCGCTTGACTACTACGGAATCAGATCCCACGCTCCACACACACC
ACTCCGTCCGAAGACATCACAGCGCAGCTTCAGGTGGTTAGTATCAACCGCCTCGCCATGGGCGCACATGCTCGG
GTACGGGAATATCAACCCGTTGTCCATCGACTACGCCTGTCGGCCTCGCCTTAGGTCCCGACTTACCCTGGGCGG
ATTAGCCKGGCCCAGGAACCCTTGGTCATCCGGCGGCAGAGTTTCTCACTCTGCATTCGCTACTCATGCCTGSATT
CTCACTCCCACACCCTCCACGACTGGCTTACGCCGCCGCTTCWCCCGGARGCAGGAWGCTCCCCTAYCCA

>SBB018

ACGGGCCGTGCATGCACTCACTAGAGGCTTTTCTCGACAGCATAGGATCACCCACTTCACCTCAAACGGCTACGCA
TCACGTCTCAGCCTATTGCCATGCGGATTTGCCTACATGACGGCCTACACGCTTACACCAGTACAACCACTCACTG
GCGGAGCTACCTTCCTGCGTCACCCCATCGCTTGACTACTACGGAATCAGATCCCACGCTCCACACACACCACTCC
GTCCGAAGACATCACAGCGCAGCTTCAGGTGGTTAGTATCAACCGCCTCGCCATGGGCGCACATGCTCGGGTACG
GGAATATCAACCCGTTGTCCATCGACTACGCCTGTCGGCCTCGCCTTAGGTCCCGACTTACCCTGGGCGGATTAG
CCTGGCCCAGGAACCCTTGGTCATCCGGCGGCAGAGTTTCTCACTCTGCATTCGCTACTCATGCCTGCATTCTCAC
TCCCACACCCTCCACGRCTGGCTTACGCCGCCGCTTCACCGGATGCAGGACGCTCCCCTACCCACCCACACCACT
AGACACAACCCCGAAAGGCTGAGCCGATGTATCGTGTGAGTGACACAGCTTCGGCGGTGTGCTTAAGCCCCGCTA
CATTGTCGGCGCAGGACCACTTGACCAGTGAGCTATTACGCACTCTTTCAAGGGTGGCTGCTTCTAAGCCAACCTC
CTGGTTGTCTGGGCAATCCCG

>SBB018A

GGGAACAGCCCAGAACACCAGCTAAGGCCCCTAAGTGTGTGCTCAGTGGGAAAGGATGTGGGATTGCCCAGACAA
CCAGGAGGTTGSCTTARAARCAGCCACCCTTGAAAGAGTGCGTAATAGCTCACTGGTCAAGTGGTCCTGCGCCGA
CAATGTAGCGGGGCTTAAGCACACCGCCGAAGCTGTGTCACTCACACGATACATCGGCTCAGCCTTTCGGGGTTG
TGTCTAGTGGTGTGGGTGGGTAGGGGAGCGTCCTGCATCCGGTGAAGCGGCGGCGKAAGCCAGYCGTGGAGGG
TGTGGGAGTGAGAATGCAGGCATGAGTAGCGAATGCAGAGTGAGAAACTCTGCCGCCGGATGACCAAGGGTTCCT
GGGCCAGGCTAATCCGCCCAGGGTAAGTCGGGACCTAAGGCGAGGCCGACAGGCGTAGTCGATGGACAACGGGT
TGATATTCCCGTACCCGAGCATGTGCGCCCATGGCGAGGCGGTTGATACTAACCACCTGAAGCTGCGCTGTGATG
TCTTCGGACGGAGTGGTGTGTGTGGAGCGTGGGATCTGATTCCGTAGTAGTCAAGCGATGGGGTGACGCAGGAA
GGTAGCTCCGCCAGTGAGTGGTTGTACTGGTGTAAGCGTGTAGGCCGTCATGTAGGCAAATCCGCATGGCAATAG
GCTGAGACGTGATGCGTAGCCGTTTGAGGTGAAGTGGGTGATCCTATGCTGTCGAGAAAAGCCTCTAGTGAGTGC
ATGCACGGCCCGTACCCCAAACCAACMCAGGTGGTCAGGTAGAGAATACTGAGGCATCGGGTGAACTGTGGTTAA
GGAACTCGGCAAAATGCCCCCGTAACTTCGGGA

CAPÍTULO 4

Construction of a *Kluyveromyces marxianus* strain expressing a laccase-like gene from the actinomycete *Streptomyces araujoniae*

INTRODUCTION

Diverse industrial and environmental applications are associated to this single group of enzymes, the laccase-like multicopper oxidases (LMCO), which presents an ample substrate range and tolerance/resistance to some extreme conditions common in industrial preparations, and is capable of oxidizing aromatic and non-aromatic substances present in highly recalcitrant environmental pollutants (Couto and Herrera, 2006), with simple production of water molecules as byproduct. However, this great potential is not even close to be fully explored: the high cost of production has impaired a broader application of this enzyme. To the best of our knowledge, commercial LMCO-based products are nowadays available only for food, paper and textile industries (Osma et al., 2010). Furthermore, the large-scale production of one unique LMCO could not satisfy the diverse demands: the various applications require enzymes with different biochemical properties – e.g., activity at low or at high pH, affinity for a specific group of substrates – hence, prospective studies are also made necessary.

Considering that native producer organisms generally present low yield and productivity of the enzyme (Piscitelli et al., 2010) or may have high-cost needs for adequate nutrition or provision of conditions for optimum growth, a possible strategy to allow large-scale synthesis of interesting enzymes, and ease of production, is heterologous expression (Kunamneni et al., 2008; Reiss et al 2013).

Among microbial LMCO, enzymes with peculiar properties have been found among actinomycetes. LMCO genes within this group can be found in over 20 genera in National Center for Biotechnology Information (NCBI) database, although few have so far been biochemically characterized, completely or partially. Despite the few enzymes – only nine LMCO to the best of our knowledge – surprisingly, all of them have presented one or more desired properties for industrial application: tolerance/resistance to high temperatures, alkalinity, high pH variation, salinity or common industrial inhibitors. This peculiarity, associated to the fact that *Actinobacteria* is a phylum with recognized biotechnological importance and capable of degrading

recalcitrant and complex polymers found in litter and soil (Goodfellow & Williams 1983; Lee 1997; Godden et al. 1992; Bugg et al. 2011; Taylor et al. 2012), highlights the need for more studies concerning its lignocellulolytic enzymes.

Heterologous expression of LMCO has been reported in yeast hosts, e.g. *Saccharomyces cerevisiae, Pichia pastoris, Pichia methalonica, Yarrowia lipolytica* e *Kluyveromyces lactis* (Kunamneni et al., 2008). Concerning *Kluyveromyces* genus, only *K. lactis* has been employed, for expression of LMCO from *Trametes trogii* and *Pleurotus ostreatus* (Piscitelli et al., 2010). However, data in literature reveal the potential of *K. marxianus* as host, once it was not distinguishable from *K. lactis* concerning correct folding and secretion of enzymes from molds and from the Gram-negative bacterium *Thermus thermophilus* (Rocha et al. 2010, 2011). Furthermore, *K. marxianus* is thermotolerant and presents *status* GRAS (Generally Regarded as Safe), interesting properties for growth in industrial bioreactors and application in food industry, respectively. Therefore, in this work, we intended to obtain a strain of *Kluyveromyces marxianus* UFV-3 (Silveira et al., 2005) expressing a LMCO gene from a new actinomycete named *Streptomyces araujoniae* (da Silva et al., 2013), whose genome has recently been sequenced.

MATERIAL AND METHODS

In silico analysis of LMCO gene and predicted protein from *Streptomyces araujoniae*

LMCO genes were searched in *S. araujoniae* genome; one sequence was found, and correctly blasted. InterProScan4 (Quevillon et al., 2005) was used for search of protein domains; Phyre2 server (Kelley and Sternberg, 2009) was used for prediction of LMCO protein structure; 3DLigandSite server (Wass et al., 2010) was used for prediction of metal ligands.

Strains, media and growth conditions

Kluyveromyces marxianus UFV-3 was used as host for heterologous expression. The yeast strain was routinely pre-cultured in liquid YP [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose] overnight at 30°C under agitation and it was maintained on YPD agar plates [YP + 2% (w/v) agar]. Solid YPD containing 200 µg/mL geneticin (G418, Sigma®) was used to select transformants. Induction of expression cassette was obtained on YPGal medium [YP + 2% (w/v) galactose]. Stock cultures were maintained in YP medium with 20% (v/v) glycerol and stored at -80°C. *Streptomyces araujoniae* ASBV-1^T was maintained in GYEA plates [2% (w/v) glucose, 2% (w/v) yeast extract and 1.5% (w/v) agar] at 4°C and as mycelial fragments in 20% (v/v) glycerol at -80°C. Actinomycetes isolates were incubated in GY broth at 28°C for 7 days; cells were harvested by centrifugation (6,000 xg, 5 min) and genomic DNA was extracted by using the PureLink® Genomic DNA mini kit (Life Technologies) according to manufacturer's manual. *E. coli* JM109 was used for maintenance and amplification of plasmid DNA. The *E. coli* cells were grown in Luria–Bertani (LB) medium supplemented with 50 µg/mL ampicillin and incubated at 37°C under agitation. Competent cells were maintained at -80 °C.

LMCO gene amplification

Polymerase chain reaction (PCR) mixture contained final concentrations 0.6 µM of each primer, 0.2 µM dNTP, 3.2% DMSO, 3 µM $MgCl_2$, 2.5 µl PCR buffer, 1 U polymerase (Taq DNA polymerase, Life Technologies; GoTaq® Flexi DNA Polymerase, Promega; or Phusion® High-Fidelity DNA Polymerase, New England Biolabs, when indicated), 1µl DNA extract and sterile MilliQ water to final 25 µl volume. All primers used in this work are listed in Table 1.

Table 1. Primers used in this work

Identification	Restriction enzyme	Sequence (5'→3')	Ref
LacK105F	---	GATGTACGCCGAGAAGCTG	Unpubl.
LacK155F	---	GGCTACGGCYTCGAGAAG	Unpubl.
LacK120R	---	CCCATGTCGGAGTGGCTC	Unpubl.
PSarLac4F	BamHI	TTC GGA TCC ATG GAC CGA AGG ACC TTC AG	
PSarLac5F	NdeI	TG TGC CTC CAT ATG ATG GAC CGA AGG ACC TTC AG	
PSarLac4R	StuI	TTT **AGG CCT** TTA ATG ATG ATG ATG ATG ATG GTG CTG GTG CCC CGC CGC	This study
PSarLac5R	BamHI	TTT **GGA TCC** TTA ATG ATG ATG ATG ATG ATG GTG CTG GTG CCC CGC CGC	
PKmLacF	---	AAC CAT TAG TGT GGT TGC AGA AGG CGG	
PKmLacR	---	TTT TTG CCA ATG GTT CCT GTG CCC GTG C	
Integration Primer 1		ACACACGTAAACGCGCTCGGT	NEB
Integration Primer 2		ATCATCCTTGTCAGCGAAAGC	NEB
Integration Primer 3		ACCTGAAGATAGAGCTTCTAA	NEB

Restriction sites are in bold; 6-histidines sequences for future enzyme purification are underlined. Last letters F or R indicate forward or reverse primers, respectively. NEB, New England Biolabs Inc.

PCR for fragment of the gene was performed with primers for LMCO from superfamily K – LacK105F, LacK155F and LacK120R, using Taq DNA polymerase. PCR touchdown conditions consisted of an initial denaturation at 94°C for 4 min; annealing temperature decreasing 0.5°C per cycle for 10 cycles, initiating at 67°C, for 45 s; extension at 72°C for 1 min; 25 cycles of 94°C for 45 s, 62°C for 45 s and 72 for 1 min, followed by a final 5 min extension at 72°C.

Amplification of the complete ORF with PSarLac primers (see Table 1) was performed with GoTaq® Flexi, with an initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 45 s, 58 or 67°C for 1 min and 72°C for 80 s; last cycle followed by a final extension at 72°C for 5 min.

Amplification of the expression cassette from the pKMCL vector was performed with Phusion® High-Fidelity with an initial denaturation at 98°C for 30 s; 30 cycles of 98°C

1 for 10 s, 60°C for 45 s and 72°C for 1min45s; last cycle followed by a final 72°C
2 extension for 5 min.

3 PCR products were visualized by electrophoresis in a 1% (w/v) agarose gel.

4

5 **Construction of expression cassette and *K. marxianus* transformation**

6 Plasmids maps used for construction of the vector pKMCL are shown on Figure 1.
7 pKMCL is derived from pKLAC2 expression vector (New England Biolabs, Inc.,
8 Ipswich, MA, USA) having replaced its *amdS* (acetamidase) selection mark by KanMX,
9 which confers dominant resistance to geneticin in yeast. Acetamidase selection, unlike
10 *K. lactis*, is inadequate for *K. marxianus*, due to its growth capacity using acetamide as
11 sole nitrogen source. pKLJC/GME (Rosa et al., 2013) was used as source of the LoxP-
12 KanMX-LoxP cassete which was subcloned into pKLAC2 *BsrG*I and *Xma*I sites
13 generating the plasmid pKMCL.

14 The fragment corresponding to the LMCO gene was amplified by PCR with the forward
15 primer PSarlac5F and either the reverse PSarLac4R or 5R (Table 1). Products were
16 then digested with NdeI and StuI (for primer pair PSarlac5F-PSarLac4R) or NdeI and
17 BamHI (primer pair PSarlac5F-PSarLac5R) and cloned into pKMCL, generating,
18 respectively, pKMCL-Lacc1 and pKMCL-Lacc2. All DNA manipulations were performed
19 according to Sambrook et al.

20 Plasmid DNA was extracted using a Wizard Plus SV Minipreps DNA Purification
21 System (Promega, Madison, USA). As the two enzymes commonly used for
22 linearization of pKLAC2 – *Sac*II and *Bst*XI – presented sites also in the cassette
23 sequence, primers PKmLacF and PKmLacR were used for amplification of the
24 complete expression cassette in the linearized form, from the plasmids pKMCL-Lacc1
25 and pKMCL-Lacc2. Yeast transformation was performed according to Kooistra et al.,
26 2004.

27 The cassette integration into the *LAC4* promoter locus of *K. marxianus* UFV-3
28 chromosome was analyzed by PCR using Integration Primer 1 (forward) and
29 Integration Primer 2 (reverse) (Table 1).

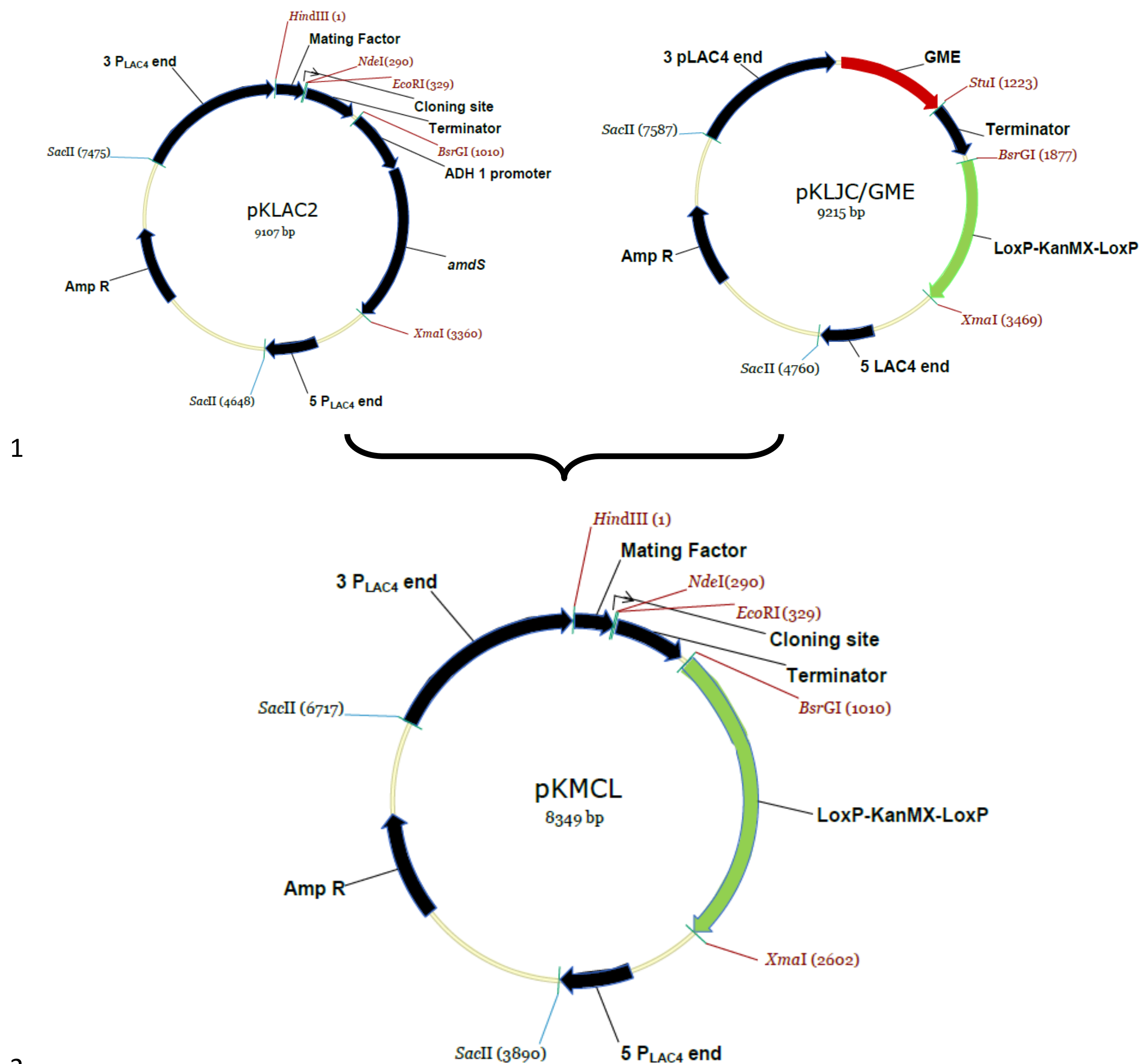


Figure 1. Scheme of pKMCL construction from replacement of *amdS* from pKLAC2 by LoxP-KanMX-LoxP from pKLJC/GME.

LMCO expression assays

K. marxianus UFV-3 transformant colonies were streaked onto YPGal agar plates (salt composition as in Olga et al., 1998) containing either guaiacol (0.02% v/v) or ABTS (2mM) as indicators for LMCO activity. Guaiacol acquires a reddish-brown coloration and ABTS becomes dark blue under oxidative polymerization by LMCO. *K. marxianus* wild-type was used as negative control. Plates were incubated at 30 ºC.

1 **RESULTS AND DISCUSSION**

2

3 ***Streptomyces araujoniae* ASBV-1^T LMCO: gene and protein sequences**

4 *Streptomyces araujoniae* ASBV-1^T LMCO gene presents 88% identity with EpoA (from
5 *Streptomyces griseus*) and 75% with SilA (from *Streptomyces ipomoeae*). It belongs to
6 superfamily K from The Laccase and Multicopper Oxidase Engineering Database
7 (LccED; Sirim et al., 2011), which contains, exclusively, two-domain LMCO from
8 actinomycetes.

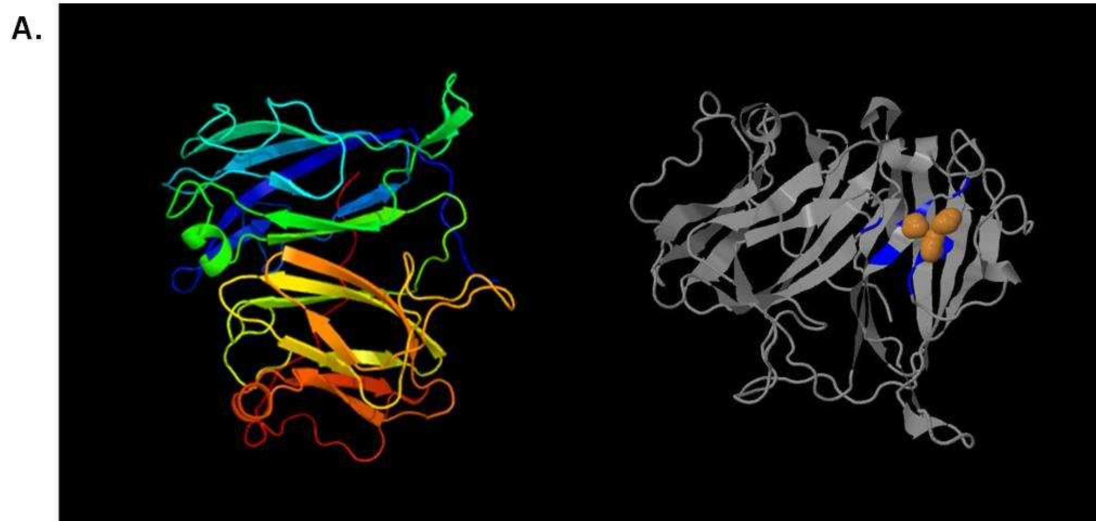
9 InterProScan4 analysis has shown that the putative protein contains the sites of the
10 twin arginine translocation (TAT) system, used for exportation of proteins from
11 cytoplasm across cytoplasmic membrane (Figure 2). Two cupredoxin domains were
12 found in the amino acids intervals 39-179 and 197–310.

```
   1   ATGGACCGAAGGACCTTCAGCCGGCGGATGCTGGTCGGCGGCGCGGCCGCGGCGGCCACC
   1    M  D  R  R  T  F  S  R  R  M  L  V  G  G  A  A  A  A  A  T 
  61   GGTGTGACATCGTTGTCGCTCGGTGCTGTGCAGGCAAGCTCCGCGGAGAGCGGACCGCGT
  21    G  V  T  S  L  S  L  G  A  V  Q  A  S  S  A  E  S  G  P  R 
 121   ACGGCCCCGGCCGGGGGAGCGGTGCGCCGCATCAAGATGTACGCCGAGAAGCTGCCCAAC
  41    T  A  P  A  G  G  A  V  R  R  I  K  M  Y  A  E  K  L  P  N 
 181   GGCGAACTGGGCTACGGCTTCGAGAAGGGCAAGGCGTCGATCCCCGGCCCCCTCATCGAG
  61    G  E  L  G  Y  G  F  E  K  G  K  A  S  I  P  G  P  L  I  E 
 241   CTGAACGAGGGCGACACCCTCCACATCGACTTCGAGAACCTCACCGACGGCGACGTCAGC
  81    L  N  E  G  D  T  L  H  I  D  F  E  N  L  T  D  G  D  V  S 
 301   CTCCATGTCCACGGCGTCGACTACGACATCGCCAACGACGGCACCCGGATGAACAAGAGC
 101    L  H  V  H  G  V  D  Y  D  I  A  N  D  G  T  R  M  N  K  S 
           T2    T3
 361   CACGTCGAGGCCGGCGGCACCCGCACGTACACCTGGCGCACCCACAAGCCGGGCACGCGC
 121    H  V  E  A  G  G  T  R  T  Y  T  W  R  T  H  K  P  G  T  R 
 421   AAGGACGGTACGTACGAGCCGGGCAGCGCGGGCTACTGGCACTACCACGACCACGTCGTC
 141    K  D  G  T  Y  E  P  G  S  A  G  Y  W  H  Y  H  D  H  V  V 
                                                     T3    T3
 481   GGCACCGACCACGGCACCGGAGGCATCCGCAAGGGGCTGTACGGGCCGCTCGTGGTGCGC
 161    G  T  D  H  G  T  G  G  I  R  K  G  L  Y  G  P  L  V  V  R 
 541   AGGAAGGGCGATCTGCTGCCCGACCAGACGTGCACGGTCGTCTTCAACGACATGATGATC
 181    R  K  G  D  L  L  P  D  Q  T  C  T  V  V  F  N  D  M  M  I 
 601   AACAACAAGACGGCCCACAACAGCGTCAACTTCGAGGCCACCGTGGGGGACCGGCTCGAA
 201    N  N  K  T  A  H  N  S  V  N  F  E  A  T  V  G  D  R  L  E 
 661   TTCGTGATGATCACGCACGGCGAGTTCTACCACACCTTCCACATCCACGGTCACCGCTGG
 221    F  V  M  I  T  H  G  E  F  Y  H  T  F  H  I  H  G  H  R  W 
                                      T1       T2    T3
 721   GCGGACAACCGGACCGGCATCCTCACCGGCCCCGACGACCCGAGCCGGGTCATCGACAAC
 241    A  D  N  R  T  G  I  L  T  G  P  D  D  P  S  R  V  I  D  N 
 781   AAGATCTGCGGCCCCGCCGACTCCTTCGGCCTCCAGATCATCGCGGGCGAACGGGTGGGC
 261    K  I  C  G  P  A  D  S  F  G  L  Q  I  I  A  G  E  R  V  G 
 841   GCGGGCGCCTGGATGTACCACTGCCATGTGCAGAGCCACTCCGACATGGGCATGGCCGGC
 281    A  G  A  W  M  Y  H  C  H  V  Q  S  H  S  D  M  G  M  A  G 
                          T3 T1 T3          T1
 901   CTGCTGCTGATCAAGAAGCCGGACGGCACCATCCCGGGGTACGAGCCGCACCACCACGGG
 301    L  L  L  I  K  K  P  D  G  T  I  P  G  Y  E  P  H  H  H  G 
 961   GCGGGCGGAGCAGAGAAGAAGTCCGCCCAGGACAAGGACAAGGGCGAGGGCAAGGGGGCG
 321    A  G  G  A  E  K  K  S  A  Q  D  K  D  K  G  E  G  K  G  A 
1021   GACGAGGGAGCGGCGGGGCACCAGCACTGA
 341    D  E  G  A  A  G  H  Q  H ···
```

Figure 2. Gene and protein sequences from *Streptomyces araujoniae* ASBV-1^T LMCO. Twin arginines from TAT system are shown underlined and in bold. Histidine or cysteine residues responsible for binding to copper atoms are indicated with the type of copper linkage (T_1 to T_3).

Phyre2 and 3DLigandSite provided predictions on 3D structure and ligation to copper atoms (Figure 3).

A.



B.

Predicted Binding Site

Residue	Amino acid	contact	av distance	JS divergence
102	HIS	49	0.27	
103	VAL	20	0.43	
104	HIS	40	0.00	
154	HIS	19	0.08	
156	HIS	19	0.00	
158	HIS	19	0.00	
266	ALA	18	0.52	

Heterogens present in Predicted Binding Site

Heterogen	Count	source structures
CU	59	3cg8_B,3fu7_A,3fu9_A,3dkh_A,3fpx_A, 1gyc_A,1gw0_B,2qt6_A,2ih9_A,2ih8_A, 1kya_C,2hzh_A,2hrg_A,2zwn_A,2hrh_A, 1v10_A,2vds_A,2q9o_B,2h5u_A,3fu8_A, 3div_A

Figure 3. Prediction of three-dimensional structure of *Streptomyces araujoniae* ASBV-1^T LMCO. **A, left.** Image coloured by rainbow N → C terminus of predicted 3D structure of *S. araujoniae* ASBV-1^T LMCO; **right**, prediction of linkage to copper atoms (orange spheres). **B.** Predicted binding sites and indication of linkage to copper.

LMCO gene amplification and yeast transformation

The expression vector pKLAC2, although originally constructed for *Kluyveromyces lactis,* has been adapted and employed for successful heterologous expression of cellulolytic enzymes *K. marxianus*, with high transformation efficiency and accuracy (Chang et al., 2012, 2013). The requirement of homologous recombination for transcription of the ORF represents a first control to avoid selection of transformants with ectopic integration of the gene in genome sites that could reduce cell fitness.

Amplification of LMCO gene from *S. araujoniae* ASBV-1^{T} genome was obtained with forward primer PSarLac5F, either with the reverse PSarLac4R or PSarLac5R. The fragments were separately used for cloning into pKMCL and *E. coli* transformation. However, the cleavage of plasmidial *E. coli* DNA with enzymes corresponding to restriction sites from primers PSarLac5F, PSarLac4R and PSarLac5R (see Table 1) did not release the expected 1.1 Kb fragment (data not shown). Then, a linear cassette was obtained from pKMCL-Lacc1 and from pKMCL-Lacc2 by *in vitro* amplification using the oligonucleotide primers PKmLacF and PKmLacR. The fragment comprises the LMCO gene, the geneticin resistance gene (LoxP-KanMX-LoxP) and also the sequences 3'P_{LAC4} (1.578 bp) and 5'P_{LAC4} (525 bp) which drive the cassette integration into the *LAC4* promoter region by homologous recombination.

Four *K. marxianus* UFV-3 transformant colonies were obtained on YPGal+geneticin agar medium, and tested with Integration Primers as cited by pKLAC2 manual. The expected 2.4 Kb band, indicative of single-copy integration at locus LAC4, was obtained with Primers 1 (P1) and 2 (P2), although along with many unexpected bands (Figure 4, columns 3 to 6). Primers 3 (P3) and P2 would amplify a 2.3 Kb band if multiple-copies had integrated, which seems not to have occurred in our assay (Figure 4, last four columns).

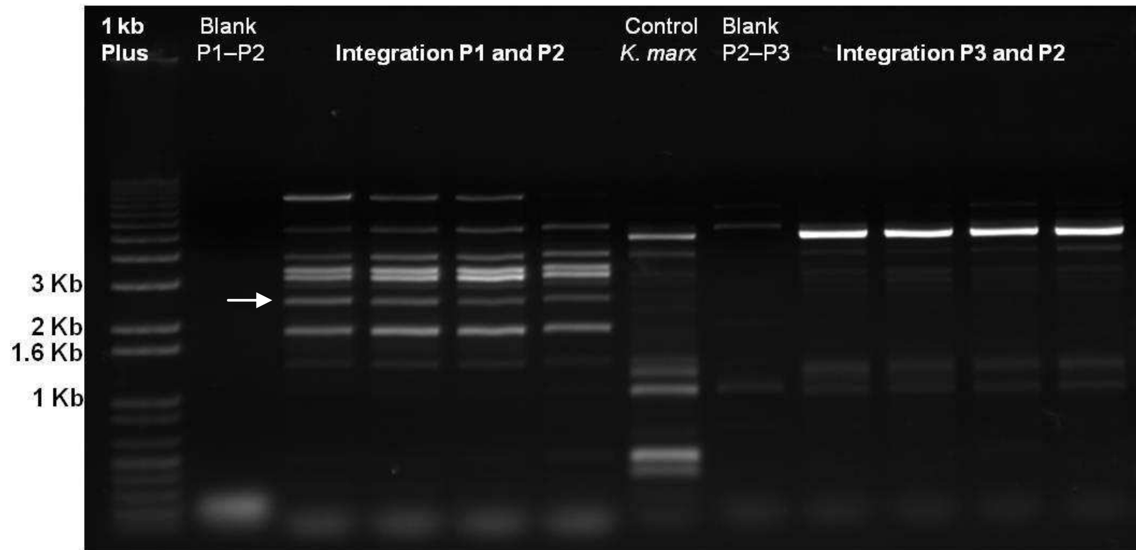


Figure 4. Gel electrophoresis with Integration Primers 1, 2 and 3 (P1, P2, P3) for analysis of *K. marxianus* UFV-3 transformants and wild-type control. Indicated by an arrow, the expected fragment indicating single-copy integration.

We consider the unspecific bands may be caused by need of optimization of PCR conditions, to allow specific annealing and extension of the expected fragment. However, absence of the indicative band in *K. marxianus* wild-type strain (negative control) indicates presence of the cassette in the transformants.

Expression of LMCO in *K. marxianus* UFV-3 strain

Analysis of the gene sequence of *S. araujoniae* ASBV-1^T LMCO suggests that the protein is exported through plasma membrane, and, therefore, adapted to be active out of the intracellular or membrane environment. This fact, along with to the addition of the signal peptide (codified in pKMCL) to the translated LMCO, provides a good scenario for secretion of active LMCO from *K. marxianus.*

Longer incubation time has been necessary for evaluation of plate assays here suggested and performed. Therefore, integration of the cassettes is suggested by PCR amplification, but more conclusive data will be obtained in the following days.

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7

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6

CONCLUSÕES GERAIS

O filo *Actinobacteria* abriga micro-organismos com abundantes peculiaridades, que se destacam quanto à aplicabilidade de suas biomoléculas a diversas atividades socio-econômicas e ambientais – da diversa produção de antibióticos, à degradação de compostos altamente recalcitrantes naturais ou sintéticos, como a lignina e o polietileno, por exemplo. As características das lacases de actinomicetos surpreende quanto à diversidade de propriedades de relevância para aplicações industriais, e sugerem grande potencial de exploração. Em um procedimento convencional de isolamento a partir de amostras de solo, obteve-se um isolado, possivelmente uma nova espécie do gênero *Streptomyces*, apresentando alta atividade de LMCO, dentre outras colônias produtoras. Embora poucos genes e enzimas tenham sido bem caracterizadas até o momento, tais sequências permitiram a síntese de primers específicos para LMCO das superfamílias às quais pertencem – I e K, tendo esta última sido criada exclusivamente para comportar a nova estrutura de LMCO descoberta em actinomicetos. Finalmente, a expressão heteróloga de LMCO de *S. araujoniae* ASBV-1^T em *Kluyveromyces marxianus* aguarda confirmação dos testes realizados em placa para, posteriormente, haver caracterização da enzima secretada e otimização da produção.