**RIZIANE FERREIRA GOMES**

# STUDY AND EXPRESSION OF A THERMOSTABLE LACCASE FROM *Chrysoporthe cubensis* IN *Komagataella phaffii* AND APPLICATION IN DYE DECOLORIZATION

Thesis submitted to the Applied Biochemistry Postgraduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Valéria Monteze Guimarães

Co-adviser: Gabriela Piccolo Maitan-Alfenas

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## ABSTRACT

GOMES, Riziane Ferreira, D.Sc., Universidade Federal de Viçosa, August, 2024. **Study and expression of a thermostable laccase from *Chrysoporthe cubensis* in *Komagataella phaffii* and application in dye decolorization**. Adviser: Valéria Monteze Guimarães. Co-adviser: Gabriela Piccolo Maitan-Alfenas.

Faced with the need to identify new and more robust laccases, with unique characteristics, which may result in different substrate specificities, the phytopathogenic fungus *Chrysoporthe cubensis* was grown on wheat bran and coffee husks to evaluate the production of laccases. Coffee husks induced specific activity 8.4 times greater than the extract obtained from wheat bran. The application of two purification steps resulted in a 12-fold purification of the laccase-containing extract, achieving a yield of 95.42 %. To identify the induced and purified laccase, the purification-related bands from SDS-PAGE were analyzed using mass spectrometry (LC-MS/MS). This analysis revealed a single protein band corresponding to laccase, identified as MCO12. Characterization of this laccase demonstrated optimal activity within a pH range of 2.2 to 5.0, indicating an acidic nature. The enzyme exhibited higher activity at 55 °C and maintained over 80 % of its activity between 45 °C and 60 °C. In terms of thermostability, laccase MCO12 had a half-life of 290 minutes at 45 °C, 22 minutes at 55°C, and 3 minutes at 65 °C. After applying laccase to decolorize various dyes using 0.005 U of enzyme, the most significant result was observed with Coomassie Brilliant Blue, achieving a 40 % discoloration. This laccase also acted on the dyes Reactive Blue 4 and Malachite Green, with discolorations of 23 and 20 %, respectively. The MCO12 laccase from *C. cubensis* had its gene sequence optimized and overexpressed in *Komagataella phaffii*. Biochemical characterization tests demonstrated that the recombinant MCO12 laccase exhibited improved properties compared to its native form. The recombinant laccase retained over 80 % of its activity within the temperature from 55 to 70 °C, with the optimum temperature increased to 60 °C. The recombinant laccase also displayed significant thermostability, with half-life values of 11 h, 45 h, and 142 h at 70 °C, 60 °C, and 50 °C, respectively. Homology modeling and molecular docking of the native and recombinant laccases were conducted using the acidic substrate 2,6-dimethoxyphenol (ABTS). The results indicated that the recombinant MCO12 laccase has a higher binding energy of -6.2 compared to -5.4 for the native MCO12 laccase. Additionally, the recombinant enzyme formed a greater number of hydrogen bonds with ABTS, suggesting increased stabilization of the complex and enhanced specificity in substrate recognition. Given its potential for industrial applications, the recombinant MCO12 laccase was tested for dye

decolorization. Among the tested dyes, the MCO12 laccase achieved 99 % decolorization of Malachite Green and 71 % decolorization of Congo Red, after 72 h of incubation at 50 °C using 0.1 U of enzyme. These findings underscore the potential of recombinant MCO12 laccase as a promising enzyme for industrial applications, particularly for the bioremediation of dyes used in the textile industry, which require enzymatic activity at elevated temperatures.

Keywords: Laccase, Dye decolorization, *Chrysoporthe cubensis*, *Komagataella phaffii,* Thermostability.

## RESUMO

GOMES, Riziane Ferreira, D.Sc., Universidade Federal de Viçosa, agosto, 2024. **Estudo e expressão de uma lacase termoestável de *Chrysoporthe cubensis* em *Komagataella phaffii* e aplicação na descoloração de corantes**. Orientadora: Valéria Monteze Guimarães. Coorientara: Gabriela Piccolo Maitan-Alfenas.

Diante da necessidade de identificar novas lacases mais robustas, com características únicas, que podem resultar em diferentes especificidades de substrato, o fungo fitopatogênico *Chrysoporthe cubensis* foi cultivado em farelo de trigo e casca de café para avaliar a indução de lacases. A casca de café promoveu atividade específica 8,4 vezes maior que o extrato obtido do farelo de trigo. A enzima foi purificada 12 vezes com a aplicação de duas etapas de purificação, atingindo um rendimento de 95,42 %. Para identificação da lacase induzida e purificada, as bandas proteicas visualizadas em SDS-PAGE, relacionadas ao extrato purificado, foram analisadas por espectrometria de massas (LC-MS/MS). Essa análise revelou uma única banda proteica correspondente à lacase, identificada como MCO12. A caracterização desta lacase demonstrou atividade ótima dentro de uma faixa de pH de 2,2 a 5,0, indicando uma natureza ácida. A enzima exibiu atividade máxima a 55 °C e manteve mais de 80 % de sua atividade entre 45 °C e 60 °C. Em termos de termoestabilidade, a lacase MCO12 teve uma meia-vida de 290 minutos a 45 °C, 22 minutos a 55 °C e 3 minutos a 65 °C. Após a aplicação da lacase para descolorir vários corantes usando 0,005 U de enzima, o resultado mais significativo foi observado com Coomassie Brilliant Blue, atingindo uma descoloração de 40 %. Esta lacase também atuou nos corantes Reactive Blue 4 e Malachite Green, com descolorações de 23 e 20 %, respectivamente. A lacase MCO12 de *Chrysoporthe cubensis* teve sua sequência genética otimizada e foi superexpressa em *Komagataella phaffii*. Testes de caracterização bioquímica demonstraram que a lacase MCO12 recombinante exibiu propriedades melhoradas em comparação com sua forma nativa. A lacase recombinante reteve mais de 80 % de sua atividade na faixa de 55-70 °C, com a temperatura ótima aumentada para 60 °C. A lacase recombinante também apresentou termoestabilidade significativa, com meias-vidas de 11h, 45h e 142 h a 70 °C, 60 °C e 50 °C, respectivamente. A modelagem por homologia e o encaixe molecular das lacases nativas e recombinantes foram conduzidos usando o substrato ácido 2,6-dimetoxifenol (ABTS). Os resultados indicaram que a lacase MCO12 recombinante possui uma energia de ligação maior de -6,2 em comparação com -5,4 para a lacase MCO12 nativa. Além disso, a enzima recombinante formou um número maior de ligações de hidrogênio com ABTS, sugerindo maior estabilização do complexo e especificidade aprimorada no reconhecimento do substrato. Dado seu potencial para aplicações industriais, a lacase MCO12

recombinante foi testada para descoloração de corantes. Entre os corantes testados, a lacase MCO12 atingiu 99 % de descoloração do Verde Malaquita e 71 % de descoloração do Vermelho Congo, após 72 h de incubação a 50 °C usando 0,1 U de enzima. Esses resultados consolidam o potencial da lacase MCO12 recombinante como uma enzima promissora para aplicações industriais, particularmente para a biorremediação de corantes usados na indústria têxtil, que requerem atividade enzimática em temperaturas elevadas.

Palavras-chave: Lacase, Descoloração de Corantes, *Chrysoporthe cubensis*, *Komagataella phaffii,* Termoestabilidade.

**SUMMARY**

## 1. INTRODUÇÃO

A poluição ambiental aumentou drasticamente devido à rápida urbanização e industrialização, levando a efeitos adversos à saúde humana e a todo o ecossistema (Jeyabalan et al., 2023). A indústria têxtil, que tem um valor de mercado estimado em cerca de 2 bilhões de dólares, influencia significativamente a economia mundial, mas também é responsável por 54 % dos efluentes de corantes (Sahu and Poler, 2024; Samsami et al., 2020). Anualmente, cerca de $7x10^7$ toneladas de corantes sintéticos são produzidas, com mais de 10.000 toneladas desses corantes utilizados pelas indústrias têxteis (Al-Tohamy et al., 2022). O uso generalizado desses corantes representa um risco tanto para a saúde humana quanto para o meio ambiente, pois até 50 % dos corantes aplicados na indústria têxtil não são absorvidos pelas fibras, sendo liberados em águas residuais (Herath et al., 2024). Consequentemente, a indústria têxtil é responsável por liberar cerca de 10 a 15 % das águas residuais industriais coloridas no mundo (Rodrigues et al., 2023).

Os corantes apresentam uma estrutura estável, projetada para resistir à luz, ao calor e aos agentes químicos, principalmente devido à presença de grupos azo, antraquinona e trifenilmetano em sua estrutura, o que lhes confere alta recalcitrância e os torna persistentes no ambiente (Al-Tohamy et al., 2022). O uso de métodos físico-químicos para remover corantes, como coagulação, floculação, adsorção, precipitação e fotodegradação, apresenta limitações, dentre elas o alto custo e a produção de lodo, o que resulta em poluição secundária (El-Bendary et al., 2023). Essas desvantagens impulsionam a busca por alternativas biológicas, que são de baixo custo, ecologicamente corretas e que produzem menos lodo (Samsami et al., 2020).

Lacases (*p*-difenol oxidases) são multicobre oxidases (MCOs) oxidam uma ampla gama de substâncias fenólicas, como orto e para -difenóis, ácidos fenólicos, aminas aromáticas, e não fenólicas, que geralmente são ricas em elétrons (Alizadeh Sani et al., 2024; Rahman et al., 2024). Essa classe de enzimas acoplam a redução de uma única molécula de oxigênio ($O_2$) a duas moléculas de água ($H_2O$), após a oxidação de quatro moléculas de substrato, o que as torna um “biocatalisador verde” (Kaur et al., 2022). Em função dessas características, essa enzima apresenta o potencial de ser aplicada em vários processos industriais, incluindo branqueamento de papel e celulose, produção de biocombustíveis e descoloração de corantes têxteis (Aghaee et al., 2024).

Lacases podem ser encontradas em uma grande variedade de organismos, como fungos, bactérias, plantas, insetos e arqueias, no entanto, as lacases mais estudadas são as de origem fúngica ou bacteriana (Martin et al., 2024). As lacases bacterianas são reconhecidas por suportarem condições mais extremas de pH e de temperatura, mas apresentam um baixo

potencial de oxirredução, o que diminui a diversidade de substratos aos quais podem atuar (Rahman et al., 2024). Já as lacases fúngicas, apresentam um alto potencial redox, o que resulta em uma alta versatilidade de substratos, mas geralmente são restritas a condições de pH ácido e temperaturas inferiores a 55 °C (Jeyabalan et al., 2023). No entanto, os estudos de lacases fúngicas se concentram em torno de fungos basidiomicetos, principalmente os conhecidos como fungos da podridão branca (Herath et al., 2024). Dessa forma, diante da escassez de estudos envolvendo lacases de ascomicetos, ainda existe um potencial inexplorado de multicobre oxidases que podem apresentar propriedades de interesse industrial.

O fungo ascomiceto fitopatogênico *Chrysoporthe cubensis*, conhecido por causar lesões profundas em espécies de *Eucalyptus* spp., secreta um conjunto de enzimas que possibilita a hidrólise eficiente da biomassa lignocelulósica (Falkoski et al., 2013). A eficiência do extrato enzimático desse fungo, comparado a coquetéis comerciais, Multifect® CL, Multifect® XL e Accellerase® 1500, que apresentaram rendimentos inferiores de glicose e xilose, pode estar associado à presença de lacase, já que essa enzima não foi identificada nesses coquetéis (Tavares et al., 2024; Maitan-Alfenas et al., 2015). A atuação de lacases nos compostos fenólicos, reduziu os seus efeitos inibitórios, o que permitiu ativar xilanases e outras hemicelulases (Ladeira Ázar et al., 2018). Além disso, estudos *in silico* do secretoma de *C. cubensis*, seguidos de análises proteômicas, possibilitaram a identificação de vários genes que codificam MCOs, incluindo novas lacases, que podem apresentar propriedades únicas e diferentes especificidades ao substrato (Tavares et al., 2021).

As lacases nativas fúngicas, embora promissoras para aplicações biotecnológicas, apresentam limitações na sua utilização em alguns setores industriais, como o têxtil. Uma das principais limitações é a baixa expressão dessas enzimas em organismos nativos, o que dificulta a obtenção de quantidades suficientes para atender a demanda em larga escala (Huang et al., 2024). A limitação de expressão, aliada à baixa atividade em condições extremas de pH e temperatura, diminui a atratividade de lacases nativas fúngicas, ao passo que impulsiona o desenvolvimento de variantes recombinantes que podem além da superexpressão, resultar em propriedades melhoradas em função de alterações no padrão de processamento pós-traducional e mudanças no peptídeo sinal (Garg et al., 2012).

A partir do apresentado, os objetivos desse estudo foram identificar e caracterizar uma lacase de *Chrysoporthe cubensis*, produzida em casca de café, verificar o seu potencial na descoloração de corantes e expressá-la na levedura *Komagataella phaffii*. A comparação das propriedades da lacase recombinante e nativa mostraram que a enzima recombinante apresentou propriedades melhoradas, que promoveram uma descoloração eficiente de corantes. Esse

estudo possibilitou a identificação e produção heteróloga de uma lacase com propriedades requeridas para a indústria têxtil, especialmente para o tratamento eficiente de corantes presentes em efluentes, minimizando os efeitos negativos desses poluentes.

# 2. OBJETIVOS

## 2.1 Objetivo Geral:

Identificar e caracterizar uma lacase produzida por *Chrysoporthe cubensis*, cultivado em casca de café, verificar o seu potencial na descoloração de corantes e expressá-la na levedura *Komagataella phaffii,* visando à obtenção de uma lacase com propriedades melhoradas, destinada à descoloração eficiente de corantes utilizados na indústria têxtil.

## 2.2 Objetivos Específicos:

Capítulo I: Realizar uma revisão das principais espécies de ascomicetos produtores de lacases, com potencial aplicação em processos industriais, relacionando sua diversidade, métodos de produção e purificação, e diferenciais em relação a propriedades bioquímicas e funcionais.

Capítulo II: Induzir a produção de lacase por *Chrysoporthe cubensis*, cultivado em casca de café, purificar parcialmente a lacase, identificar, caracterizar e avaliar o seu potencial na descoloração de corantes utilizados na indústria têxtil.

Capítulo III: Clonar e expressar a lacase de *Chrysoporthe cubensis* em *Komagataella phaffii*, avaliar as propriedades da lacase recombinante e demonstrar o seu potencial na descoloração de corantes têxteis.

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## CAPÍTULO I – Potenciais Aplicações Industriais de Lacases de Ascomicetos: uma revisão dos últimos 10 anos

ARTIGO I – **Potenciais Aplicações Industriais de Lacases de Ascomicetos: uma revisão dos últimos 10 anos**

Gomes, R.F.; Carmo, M.R.A.; Ventorim, R.Z.; Maitan-Alfenas, G.P.; Guimarães, V.M.

### RESUMO

A rápida industrialização também resulta em consequências ambientais, como a contaminação de ambientes aquáticos e terrestres por produtos da indústria têxtil e da atividade agrícola. As lacases, enzimas oxidativas de multi-cobre, destacam-se pela versatilidade de substratos e propriedades catalíticas diferenciadas, encontrando aplicações em diversos setores industriais, incluindo têxtil, papel e celulose, alimentício, tratamento de efluentes e biocombustíveis. Entretanto, a aplicação industrial dessas enzimas ainda é limitada, principalmente em decorrência de condições extremas necessárias em muitos dos processos, o que justifica novos estudos para suprir a demanda existente. Embora as lacases fúngicas mais estudas e aplicadas em processos industriais sejam aquelas produzidas por basidiomicetos, as lacases de ascomicetos, em particular, são de grande interesse devido ao seu alto potencial redox e diversidade genética. No entanto, existe uma escassez de estudos evidenciando o potencial e diversidade de lacases de ascomicetos. Portanto, o objetivo deste trabalho foi demonstrar as principais espécies de ascomicetos produtores de lacases com potencial para aplicações industriais nos últimos 10 anos, destacando sua diversidade, propriedades, métodos de produção e purificação, bem como seus diferenciais. A partir dessa revisão foi possível demonstrar coquetéis baseados em lacases de ascomicetos já disponíveis comercialmente, além de lacases produzidas por ascomicetos com aplicações promissoras nos setores de biorremediação, têxtil, biocombustíveis, alimentos, biossíntese e biossensores.

## 1- INTRODUÇÃO

A crescente industrialização resulta em consequências ambientais, como a contaminação extensiva de ambientes aquáticos e terrestres com produtos oriundos da indústria têxtil e da atividade agrícola (Herath et al., 2024; Joseph et al., 2024). As enzimas oxidativas, em especial as lacases, encontram aplicações em todos esses setores devido à sua versatilidade de substratos e propriedades catalíticas diferenciadas (Dong et al., 2023). A busca por enzimas adaptadas às condições industriais, e processos economicamente sustentáveis, impulsiona estudos em torno do tema.

As lacases (EC.1.10.3.2) pertencem à classe de enzimas oxidases de multi-cobre (MCOs), que são consideradas enzimas versáteis devido à sua baixa especificidade de substrato, o que permite oxidar um grande número de moléculas fenólicas e não fenólicas (Arregui et al., 2019; Rahman et al., 2024). Essa classe de enzimas é conhecida como "catalisador verde", pois requer apenas oxigênio molecular e libera moléculas de água como subproduto (Wang et al., 2024). Elas são encontradas em bactérias, plantas, insetos e fungos, sendo que cada espécie exibe características e propriedades únicas (Mate and Alcalde, 2015).

Essa enzima é amplamente utilizada em diversos setores da indústria, como têxtil, papel e celulose, alimentícia, tratamento de efluentes e de biocombustíveis, devido às suas propriedades catalíticas e físico-químicas (Leynaud Kieffer Curran et al., 2022) (Alizadeh Sani et al., 2024). Na indústria têxtil, as lacases são utilizadas para clarificar diferentes tipos de corantes, como o verde malaquita, que são contaminantes de águas residuais (Song et al., 2024). Outra aplicação importante é no branqueamento da celulose na indústria de papel e celulose. Na indústria alimentícia, são utilizadas para a clarificação de extratos de sucos e vinhos (Danait-Nabar and Singhal, 2022). Além disso, essas enzimas são empregadas na biorremediação de solos contaminados por herbicidas, resultantes da atividade agrícola (Dong et al., 2023; Bebić et al., 2020). A lacase também apresenta o potencial para ser aplicada na indústria de biocombustíveis por meio da sua atuação na lignina, de forma a aumentar os rendimentos da sacarificação (Leynaud Kieffer Curran et al., 2022; Tavares et al., 2022).A aplicação de lacases em processos industriais depende fortemente da origem da enzima, sendo as lacases fúngicas, especialmente de ascomicetos, de particular interesse devido ao alto potencial redox e diversidade genética.

A aplicação industrial de lacases ainda é limitada devido às condições extremas necessárias em muitos dos processos em que essas enzimas são aplicadas (Aghaee et al., 2024). As lacases que se destacam na aplicação em setores industriais são oriundas de bactérias e fungos. Lacases oriundas de plantas, envolvidas nas respostas a ferimentos e na polimerização

da lignina, exibem um potencial redox menor que a de bactérias, e já as enzimas com atividade de lacase descobertas insetos participam da esclerotização, com poucos relatos de aplicação industrial (Martin et al., 2024; Mate and Alcalde, 2015). Lacases bacterianas, apesar de exibirem propriedades adaptadas às condições extremas, tanto de temperatura como de pH, frequentemente requerem mediadores caros, devido ao seu baixo potencial redox, tornando desafiador seu uso em ambientes industriais (Rahman et al., 2024). As funções de lacases fúngicas nativas, que evolvem processos de defesa contra estresse, patogênese de plantas e degradação de lignina, potencializam essas enzimas para uso em aplicações no setor industrial (Arregui et al., 2019). Lacases de fungos basidiomicetos da podridão branca, são extensamente estudadas, devido aos seu alto potencial redox e consequentemente, alta versatilidade de substratos, e por tradicionalmente serem conhecidos degradadores eficientes da lignina (Herath et al., 2024). Por outro lado, estudos evidenciando o potencial de lacases de ascomicetos são escassos (Mtibaà et al., 2018). Portanto, o objetivo dessa revisão é levantar as principais lacases oriundas de ascomicetos com aplicações potenciais em processos industriais, exibindo a sua diversidade, métodos de produção/purificação e propriedades.

## 2- Sítio ativo e mecanismo catalítico de lacases

Lacases (*p*-difenol: oxidorredutase de oxigênio) são glicoproteínas monoméricas, diméricas ou triméricas com peso molecular de 50–130 kDa (Martin et al., 2024). Assim como as outras oxidases de multi-cobre, as lacases fúngicas possuem três domínios semelhantes de ligação ao cobre (denominados D1, D2 e D3) arranjados sequencialmente e quatro átomos de cobre (Cu) distribuídos em três centros de ligação ao metal: T1, T2 e T3 (Deska and Kończak, 2019). O centro T1 é mononuclear, localizado mais na superfície da enzima, caracterizado por um íon cobre, o T2, conhecido como sítio normal, e o T3, sítio binuclear, organizados em dois sítios metálicos ativos, T1 e T2/T3 (Rodrigues et al., 2023).

Lacases caracterizam-se por conter dez resíduos de histidinas e uma cisteína conservados como ligantes de cobre (Figura 1). Duas histidinas e uma cisteína servem como ligantes para o Cu tipo 1, às vezes, um quarto ligante (Met) pode estar presente em algumas lacases na posição axial (Avelar et al., 2018). Os oito resíduos de histidinas restantes são distribuídos entre o Cu tipo 2 e tipo 3. Esses resíduos conservados são distribuídos por quatro regiões de aminoácidos conservadas, que são designadas como sequências de assinatura L1–L4 que podem ser usadas para identificar as lacases (Fan et al., 2014).

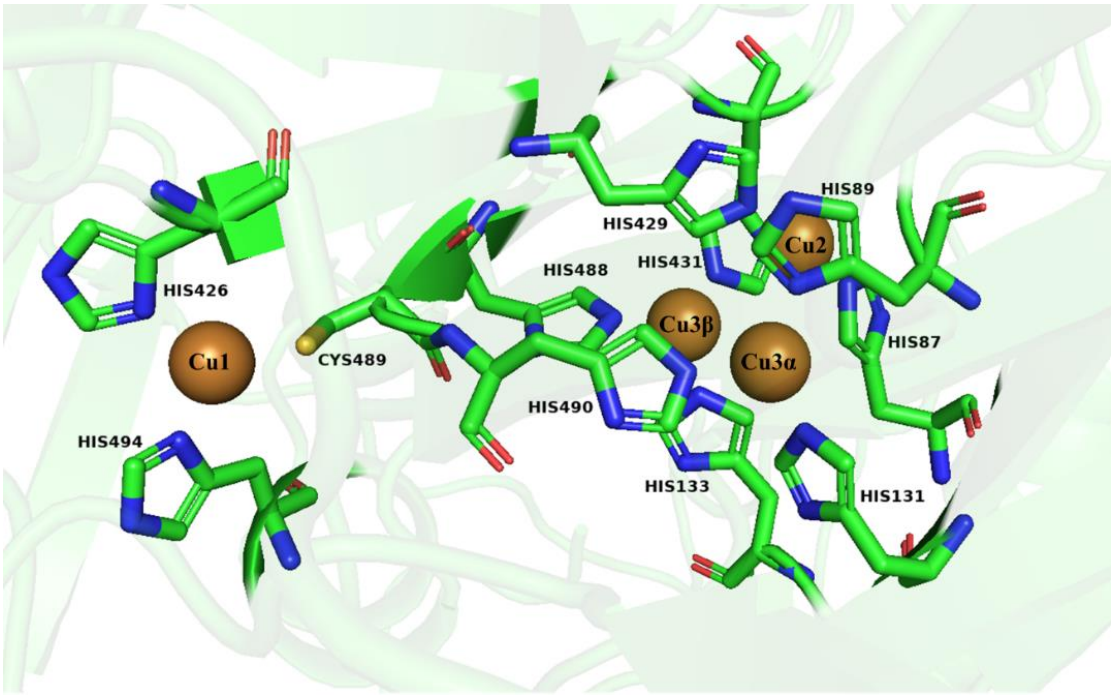


Figura 1 – Representação das 10 histidinas e 1 cisteína que coordenam os cobres catalíticos no sítio ativo da lacase do fungo ascomiceto *Botrytis aclada,* visualizados no software PyMOL versão 2.5.5 (Código PDB:3SQR). Fonte: o autor.

O mecanismo catalítico das lacases envolve a oxidação do substrato no sítio T1 e a redução do sítio T1, convertendo o estado de oxidação do $Cu^{2+}$ para $Cu^{+}$ (Figura 2). Dessa forma, o sítio T1 possui uma relação direta com o potencial redox ($E^0$) apresentado pela enzima, uma vez que ele está relacionado com a energia necessária para remover um elétron do substrato redutor (Rivera-Hoyos et al., 2013). Para reduzir completamente o oxigênio, são necessárias quatro moléculas de substrato redutor, que utilizam da sequência tripeptídica His-Cys-His do sítio T1, para transferir sequencialmente os elétrons extraídos para o sítio T2/T3, onde finalmente ocorre a redução do oxigênio molecular a água (Wang et al., 2024; Alizadeh Sani et al., 2024).

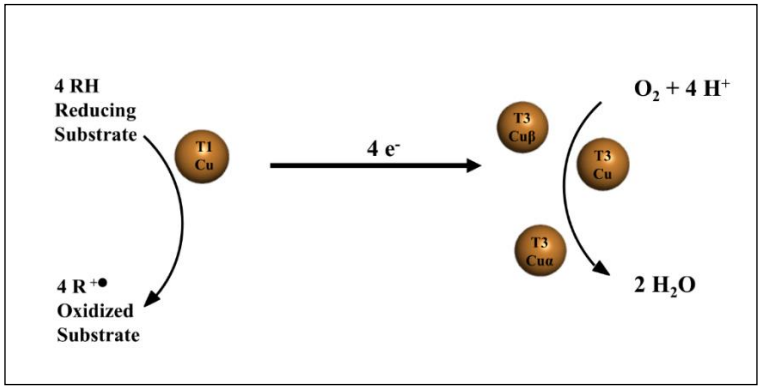


Figura 2 – Representação do mecanismo de transferência de elétrons da lacase de *Botrytis aclada.* Fonte: o autor.

### 3- Diversidade de lacases produzidas por ascomicetos

A diversidade genética das lacases produzidas por ascomicetos tem atraído crescente interesse devido ao seu potencial para aplicação em diferentes setores industriais. Estudos filogenéticos, que destacam as principais lacases de ascomicetos aplicadas em processos industriais, Figura 3, revelam uma notável variação evolutiva desses microrganismos, que refletem à adaptabilidade e especialização dessas enzimas em diferentes habitats e condições ambientais. Essas características, contribuem para explicar a ampla gama de substratos utilizados por essas enzimas e a consequente diversidade de aplicações dessas lacases.

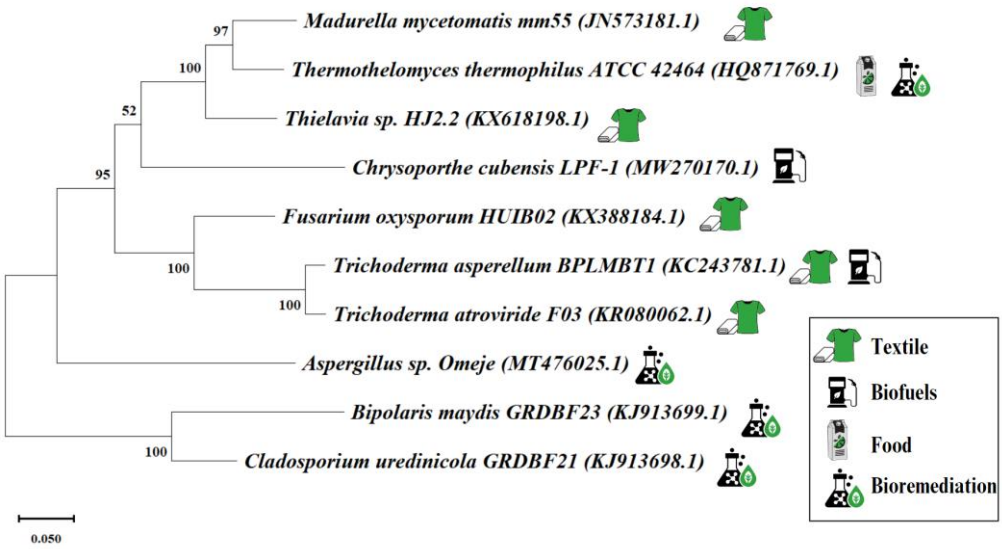


Figura 3 – Árvore filogenética de espécies de ascomicetos produtoras de lacases com aplicações potenciais em processos industriais, construída usando o método Neighbor-Joining, conduzidas no MEGA11. A porcentagem de árvores replicadas nas quais os táxons associados se agruparam no teste bootstrap (1000 replicatas) é mostrada ao lado dos ramos. Fonte: o autor.

Estudos de diversidade de lacases de ascomicetos *stricto sensu*, como o realizado por Tavares et al. (2022), que avaliou 13 sequências de multicopper oxidases (MCOs) preditas no genoma do fungo ascomiceto fitopatogênico *Chrysophorte cubensis*, organizaram a maioria dessas enzimas em cluster em um chamado de II, distinto das ferroxidases, enzimas pigmentares/ascorbato oxidases, e lacases de basidiomicetos, que se agrupam nos clusters I, III e IV, respectivamente (Figura 4). Dessa forma, foi demonstrado que as enzimas desse grupo apresentam assinaturas típicas das lacases de ascomicetos *stricto sensu*, incluindo domínios L1-L4, DSGL e SDS-gate, Figura 5, reforçando a importância dos critérios de similaridade de sequência na classificação funcional dessas enzimas (Tavares et al., 2022)(Shanmugam et al., 2018). O domínio DSGL (Asp-Ser-Gly-Leu) é uma sequência de aminoácidos encontrada na região C-terminal das lacases de ascomicetos, crucial para a clivagem pós-traducional, e que

facilita a transferência de prótons, contribuindo para sua eficiência catalítica. O trabalho de Shanmugam et al. (2018) demonstrou, por meio do alinhamento de sequenciais parciais homólogas da lacase de *Trichoderma asperellum* com outras sequencias de lacases de ascomicetos, que o domínio DSGL é altamente conservado. Outra característica que permite caracterizar lacases de ascomicetos é o SDS-gate, constituído por dois resíduos de serina e um aspartato na assinatura DSGL, o qual auxilia na transferência de prótons do sítio T1 para o centro trinuclear (Tavares et al., 2022; Moreno et al., 2017).

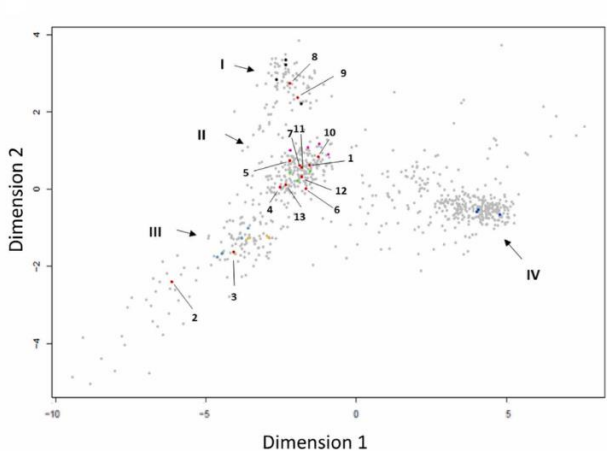


Figura 4 –Análise de Escala Multidimensional (MDS) de 845 MCOs de 78 fungos com diferentes estilos de vida, classificadas em ferroxidases, lacases de ascomicetos, enzimas pigmentares/ascorbato oxidases, e lacases de basidiomicetos, que se agrupam nos clusters I, III e IV, respectivamente. Os pontos vermelhos correspondem aos 13 MCOs previstos para o fungo *C. cubensis* (1–13). Adaptada de Tavares et al. (2022).

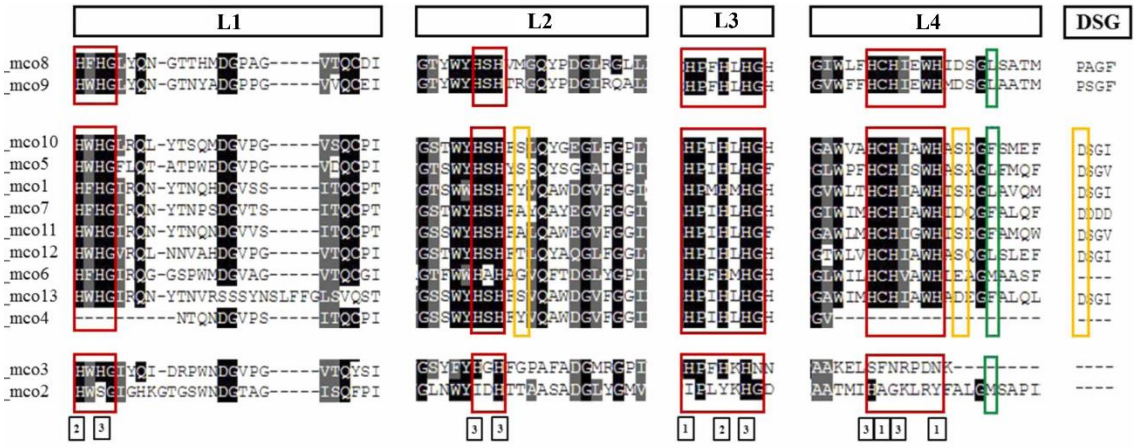


Figura 5 – Assinaturas conservadas provenientes do alinhamento de sequências múltiplas de MCOs *de C. cubensis* (Adaptado de Tavares et al., 2022). As regiões L1-L4 e a região contendo a assinatura DSGL foram destacadas dos alinhamentos e organizadas em blocos. Os resíduos conservados envolvidos na ligação com os átomos de Cu são indicados em retângulos vermelhos. Os números 1–3 na parte inferior da figura mostram os centros de ligação de Cu onde cada resíduo pertence. Os retângulos amarelos indicam os resíduos de aminoácidos que compõem a assinatura SDS-gate.

## 4- Lacases comercias de ascomicetos

Apesar de poucas representantes, algumas lacases de ascomicetos já estão disponíveis comercialmente, como as lacases de *Aspergillus sp.* e *Thermothelomyces thermophilus,* anteriormente chamado de *Myceliophthora thermophila* (Tabela 1). Bhatt et al. (2023) demonstraram que a lacase purificada de *Aspergillus sp.,* produzida pela Sigma-Aldrich (Shanghai), foi eficiente para atuar na degradação de quatro xenobióticos diferentes: glifosato, polímero de lignina (siringol, álcool di-hidroconiferílico, guaiacol), isoproturon e paration. Uma lacase também de *Aspergillus sp.* é utilizada para compor o coquetel DeniLite® da Novozymes (Dinamarca), que possui a principal finalidade de atuar no branqueamento a frio do denim (Martin et al., 2024).

Tabela 1: Lacases de ascomicetos disponíveis comercialmente.

| Fonte de lacase | Coquetel | Companhia | Referência |
|---|---|---|---|
| *Aspergillus sp.* | | Sigma-Aldrich | (Bhatt et al., 2023)(Mate and Alcalde, 2017) |
| *Myceliophthora thermophila* expressa em *Aspergillus oryzae* | Suberase® | Novozymes | (Karaki et al., 2017) |
| *Myceliophthora thermophila* expressa em *Aspergillus oryzae* | Novozym® 51003 | Novozymes | (Bebić et al., 2020) |
| *Myceliophthora thermophila* expressa em *Aspergillus oryzae* | Flavourstar® | Novozymes | (Mate and Alcalde, 2017) |
| *Aspergillus oryzae* | Denilite® II Base | Novozymes | (Decarli et al., 2022) |

As lacases oriundas de ascomicetos apresentam um grande potencial em várias áreas industriais, no entanto, assim como outras enzimas nativas de fungos, a produção de lacase a partir de fontes não recombinantes enfrenta problemas de baixo rendimento, o que torna inviável para produção em escala industrial (Martin et al., 2024). Lacase de *Myceliophthora thermophila*, expressa heterologamente em *Aspergillus oryzae*, compõe coquetéis com diferentes funcionalidades produzidos pela Novozymes (Dinamarca), como o Suberase®, Novozym® 51003 e Flavourstar® (Tabela 1). O Suberase® é comercializado para o tratamento de rolhas de cortiça para garrafas de vinho e Flavourstar® é utilizado para oxidar polifenóis na cerveja (Mate and Alcalde, 2017). Já o Novozym ® 51003, é utilizado na deslignificação de polpa de papel (Martin et al., 2024). Esse pequeno número de representantes de lacases de ascomicetos disponíveis comercialmente, diante do amplo espectro de aplicações industriais

existentes para essa enzima, demonstra a importância de novos estudos para diminuir essa divergência.

## 5- Lacases de ascomicetos com aplicações potenciais em processos industriais

### 5.1 Indústria têxtil

Lacases produzidas por ascomicetos exibem um grande potencial de aplicação em diversos setores industriais, como pode ser observado na Tabela 2. Uma das aplicações potenciais dessa classe de enzimas é na indústria têxtil, de forma atuar na otimização do branqueamento de tecidos e na descoloração de corantes. Tülek et al. (2021) aplicaram uma lacase de *Madurella mycetomatis*, expressa em *Pichia pastoris,* imobilizada em nanocompósitos revestidos de sílica, no branqueamento de algodão ecologicamente correto. Essa aplicação resultou em um melhor desempenho, em relação à enzima livre, sendo uma alternativa aos métodos tradicionais de branqueamento, de forma a diminuir os custos e as preocupações ambientais nesse setor (Tülek et al., 2021).

A principal aplicação de lacases na indústria têxtil é na descoloração de corantes, o que também faz parte do setor de biorremediação, por serem aplicadas no tratamento de águas residuais. As classes de corantes Azo, Antraquinona e Triarilmetano são as mais comumentes encontradas como contaminantes (Figura 6) (Dassanayake et al., 2021). O gênero *Trichoderma* possui algumas lacases com potencial aplicação nesse setor. O fungo *Trichoderma atroviride* F03, cultivado em meio suplementado com glicose e extrato de levedura, produziu um extrato enzimático bruto capaz de biodegradar 91,1 % do corante preto reativo 5 (50 mg/L), por meio da mediação com uma lacase secretada por esse fungo, com atividade máxima de 5,8 U/ml (Adnan et al., 2015). Já Shanmugam et al. (2017) alcançaram 97,18 % de biodegradação do verde malaquita (122,66 mg/L) por do uso de uma lacase de *Trichoderma asperellum* (1,50 U/mL), após otimização do processo por meio metodologia de superfície de resposta.

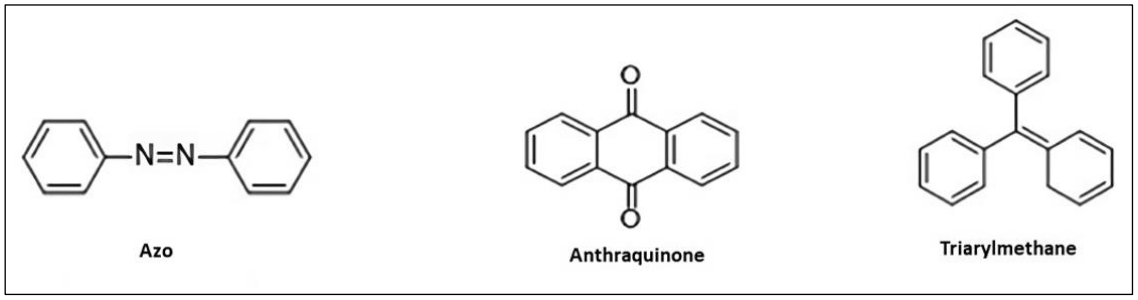


Figura 6 – Estruturas moleculares das principais classes de corantes têxteis (Adaptado de Dassanayake et al., 2021).

Uma lacase de *Fusarium oxysporum* HUIB02, expressa em *Pichia pastoris*, foi capaz de atuar em um conjunto de corantes sintéticos (10 mg/L), com aproximadamente 10 U de lacase por reação, com taxas de degradação máximas para o azul de anilina (90,87 %), laranja de metila (82,80 %), índigo carmim (73,44 %) e azul brilhante de remazol (79,61), após 24 h de tratamento e sem fazer uso de mediadores (Huy et al., 2021). A lacase secretada e purificada de *Thielavia sp.* (3 U/mL) também foi capaz de descolorir eficientemente o corante recalcitrante azul brilhante de remazol R (50 mg/L), sem uso de mediador redox, atingindo 90 % de descoloração após 24 h (Mtibaà et al., 2018). Esses resultados apresentados sugerem que as lacases de ascomicetos, que constituem recursos relativamente inexplorados, são consideradas candidatas promissoras para a biodegradação de corantes (Shanmugam et al., 2017).

### 5.2 - Biorremediação

Nas últimas décadas, houve um aumento significativo no número de poluentes nocivos liberados no meio ambiente, devido à atividade humana, industrialização e rápido desenvolvimento. Ao mesmo tempo, estudos têm relatado a capacidade de lacases de ascomicetos atuarem na biorremediação de alguns desses poluentes. Uma das aplicações de lacases nesse setor é na biorremediação de pesticidas. Estudos envolvendo os fungos produtores de lacases, *Cladosporium uredinicola* GRDBF21 e *Bipolaris maydis* GRDBF23 (Tabela 2), com atividades máximas de 290,14 e 164,91 U/L, respectivamente, demostraram uma biodegradação eficiente do inseticida organofosforado clorpirifós estruturalmente complexo (100 mg/L), após 20 dias de incubação com a lacase, avaliada por cromatografia gasosa/espectrometria de massas (Joseph et al., 2024). Ao utilizar 3 U de lacase comercial de *Myceliophthora thermophila,* expressa em *Aspergillus oryzae* (Novozym 51003®), imobilizada em nanopartículas de sílica, na degradação do pesticida organoclorado lindano (30 µmol/L), Bebić et al. (2020) demonstraram uma redução de 49 % na concentração desse poluente, após 6 dias de incubação.

Outra aplicação de lacases é na biorremediação de corantes contaminantes de água residuais. Ali et al. (2023) demonstraram que os sobrenadantes das cepas de *Aspergillus niger* e *Trichoderma viride*, com atividade de lacase, atuaram na degradação de águas residuais contaminadas com os corantes azo, vermelho synozol, azul synozol e amarelo synozol, possibilitando o desenvolvimento de sementes de feijão. Objetivando também potencial aplicação em processos de biorremediação, uma lacase de *Aspergillus sp.* Omeje, produzida por fermentação submersa em resíduos agrícolas e parcialmente purificada, com atividade

específica de 76,75 U/mg, promoveu uma descoloração máxima para o corantes azul valt 5 (82 %) e roxo azo (73 %), após 120 minutos de incubação a 37 °C (Omeje et al., 2020).

Outra potencial aplicação de lacases é sobre resíduos da produção de papel e celulose, presente em águas residuais, como o licor Kraft preto, rico em lignina e fenóis. A utilização do extrato de *Aspergillus uvarum*, possibilitou a degradação de 67 % dos fenólicos e descoloração de 81 % desse resíduo, após 96 h de incubação, eficiência essa associada a alta atividade de lacase (Díaz et al., 2022). Esse resultado é especialmente interessante, uma vez que não existe relatos de aplicação de lacases oriundas desse fungo no tratamento de efluentes da indústria de papel. Além disso, nesse mesmo estudo, foi avaliada a aplicação do extrato do fungo basidiomiceto *Phanerochaete chrysosporium*, que apresentou resultados inferiores, com 56 % de degradação de fenólicos e 37 % de descoloração (Díaz et al., 2022). Isso destaca o potencial das lacases de ascomicetos, especialmente quando comparado à enzima produzida por um fungo basidiomiceto, já conhecido por suas aplicações nesse setor.

Contaminantes oriundos da indústria farmacêutica também são alvos da aplicação de lacases de ascomicetos. Poluentes derivados do antibiótico tetraciclina, presentes na água do mar, foram totalmente degradados pela lacase comercial de *Aspergillus sp.* (200 U/L), fazendo uso do mediador siringaldeído, na proporção 1U:1 μmol, após 2 h de incubação (Wang et al., 2023). A combinação de lacase/mediadores-redox, utilizando a lacase de *Xylaria polymorpha*, também foi utilizada para degradar o antibiótico amoxicilina (1,0 mg/L), resultando em 97 % de oxidação após 2 h, ao utilizar o ácido violúrico como mediador. Essas aplicações bem-sucedidas de lacases de ascomicetos, demonstram o potencial dessa enzima na eliminação de micropoluentes em ambientes de águas residuais.

### 5.3 - Biocombustíveis

A atuação de lacases na lignina, componente da biomassa lignocelulósica e substrato natural da enzima, impulsiona estudos de aplicação dessa classe de oxidases no setor de biocombustíveis. Em geral, lacases de ascomicetos são aplicadas nas etapas de pré-tratamento da biomassa ou na hidrólise enzimática, sendo uma alternativa aos métodos físicos e químicos, que liberam subprodutos, como furfurais, ácido fórmico e ácido levulínico, inibidores da fermentação subsequente (Shanmugam et al., 2018).

Visando atuação na etapa de pré-tratamento, Shanmugam et al. (2018) identificou uma lacase extracelular de *Trichoderma asperellum*, capaz de remover 76,93 % de lignina da palha do sorgo doce, após 8h de tratamento a 60 ºC, utilizando 3 U/mL de lacase e 30 % de carga de

sólidos. Esse processo de deslignificação, resultou em um aumento de 3,26 vezes na produção de biohidrogênio em relação à biomassa não tratada. A aplicação de lacase de *Aspergillus fumigatus,* cultivado sob fermentação submersa, em palha de arroz pré-tratada com 1,0 % de NaOH, no processo de hidrólise enzimática, foi demonstrada por Jin et al. (2020). A aplicação desse extrato (200 FPU/mL), com atividade de lacase, na hidrólise enzimática por 20 h, seguido do processo de fermentação, foi responsável por um rendimento de 83,5 % do bioetanol.

Como demonstrado, a presença de lacase favorece a eficiência e o rendimento da sacarificação, uma vez que essa enzima atua nos compostos fenólicos, de forma a despolimerizar a lignina e diminuir o efeito de inibidores fenólicos. Apesar do potencial de aplicação, as lacases ainda não são utilizadas como parte do conjunto de enzimas lignocelulolíticas presentes nos coquetéis comerciais, como o Cellic ® CTec2 (Novozymes), que visam à despolimerização da biomassa vegetal na etapa de sacarificação (Ladeira Ázar et al., 2018). A co-incubação de lacases com as mono-oxigenases líticas de polissacarídeos (LPMOs), presentes nos coquetéis comerciais de celulases, pode gerar uma competição pelo substrato oxigênio molecular, inibindo as LPMOs (Brenelli et al., 2018; Zhang, 2020). Entretanto, foi demonstrado por Ladeira Ázar et al. (2018) que a presença de lacases, resultantes da combinação de extratos 1:1 de *Chrysoporthe cubensis* e *Penicillium pinophilum,* atua de forma aumentar a atividade de um conjunto de enzimas hemicelulolíticas, o que resulta em um aumento do rendimento da sacarificação. Assim, dependendo da composição da biomassa vegetal e do tipo de pré-tratamento utilizado, a suplementação de coquetéis comerciais com a lacase, em concentrações que não promovam competição com LPMOs, pode ser vantajosa, de forma a aumentar o rendimento do processo.

### 5.4 - Outras aplicações de lacases de ascomicetos

Como mencionado anteriormente, o alto potencial redox exibido pelas lacases de ascomicetos, favorece a aplicação dessas enzimas em diversos outros setores. Existem estudos de aplicação da lacase comercial de *Myceliophthora thermophila* no setor de alimentos, biossíntese e de biossensores. A lacase de *Myceliophthora thermophila* foi aplicada como biocatalisador da reação de funcionalização da pectina cítrica com produtos de oxidação do ácido ferúlico, com a finalidade de melhorar suas propriedades funcionais e ampliar o campo de suas potenciais aplicações (Karaki et al., 2017). Essa mesma lacase atuou na catálise da funcionalização da quitosana, sob condições ácidas, com 4-hexiloxifenol, melhorando

propriedades antioxidantes e hidrofóbicas, com potencial para ser aplicado em embalagem de alimentos ou como agente de revestimento (Liu et al., 2018).

O coquetel Suberase®, lacase de *Myceliophthora thermophila* expressa em *Aspergillus oryzae*, foi utilizado como catalisador na síntese de coumestans, composto natural com atividade anticancerígena, resultando em inibição moderada a potente do crescimento das linhas celulares de câncer renal TK10, melanoma UACC62 e mama MCF7 (Qwebani-Ogunleye et al., 2017). Já a utilização da lacase de *Aspergillus oryzae*, coquetel Denilite® II BASE, como base de um biossensor voltamétrico, possibilitou a determinação bem sucedida de dopamina em amostras de formulação farmacêutica (Decarli et al., 2022).

Tabela 2 – Lacases de ascomicetos aplicadas em processos industriais nos últimos 10 anos.

| Organismo | Setor Industrial | Aplicação | Referencias |
|---|---|---|---|
| *Aspergillus flavus* | Têxtil | Degradação do corante azul de bromofenol/ | (Kumar et al., 2016) |
| *Trichoderma asperellum* | Têxtil/ Biocombustíveis | Biodegradação e desintoxicação do corante verde malaquita/ Deslignificação da biomassa lignocelulósica | (Shanmugam et al., 2017) (Shanmugam et al., 2018) |
| *Thielavia sp.* | Têxtil | Descoloração do corante azul brilhante de remazol R | (Mtibaà et al., 2018) |
| *Madurella mycetomatis* | Têxtil | Branqueamento de algodão ecologicamente correto | (Tülek et al., 2021) |
| *Fusarium oxysporum* HUIB02 | Têxtil | Degradação de vários corantes sintéticos: azul de bromotimol, azul de metileno, laranja de metila, remazol Brilliante Blue R, azul de anilina, azul de Evans, índigo carmim e laranja II | (Huy et al., 2021) |
| *Trichoderma atroviride F03* | Têxtil | Biodegradação do corante preto reativo 5 | (Adnan et al., 2015) |
| *Aspergillus uvarum* | Biorremediação | Tratamento do efluente Kraft Black Liquor | (Díaz et al., 2022) |
| *Aspergillus niger* | Biorremediação | Degradação de corantes azo: vermelho synozol, azul synozol e amarelo synozol | (Ali et al., 2023) |
| *Trichoderma viride* | Biorremediação | Degradação de corantes azo: vermelho synozol, azul synozol e amarelo synozol | (Ali et al., 2023) |

| | | | |
|---|---|---|---|
| *Aspergillus sp.* | Biorremediação | Desintoxicação do polímero de lignina, glifosato, isoproturon e paration/ Biorremediação de tetraciclinas na água do mar/ Descoloração de corantes sintéticos: violeta básico 2, amarelo azo 6, vermelho ácido 337, azul básico 22, roxo azo, amarelo azo, preto brilhante azo, azul valt 5 e azul ácido 74 | (Bhatt et al., 2023)(Wang et al., 2023) (Omeje et al., 2020) |
| *Xylaria polymorpha* | Biorremediação | Desintoxicação do fármaco amoxicilina | (Bankole et al., 2022) |
| *Bipolaris maydis GRDBF23* | Biorremediação | Biodegradação do pesticida organofosforado clorpirifós | (Joseph et al., 2024) |
| *Cladosporium uredinicola GRDBF21* | Biorremediação | Biodegradação do pesticida organofosforado clorpirifós | (Joseph et al., 2024) |
| *Myceliophthora thermophila* | Biorremediação/ Alimentícia/ Biossíntese/ Biossensores | Bioegradação do lindano/ Modificação da pectina cítrica com produtos de oxidação do ácido ferúlico/ Funcionalização da quitosana com 4-hexiloxifenol/ Biossíntese de cumestanos/ Componente de biossensor aplicado na determinação de dopamina | (Bebić et al., 2020) (Karaki et al., 2017) (Liu et al., 2018) (Qwebani-Ogunleye et al., 2017) (Decarli et al., 2022) |
| *Chrysoporthe cubensis* | Biocombustíveis | Aumento na sacarificação do bagaço de cana-de-açúcar | (Tavares et al., 2022) (Tavares et al., 2024) |
| *Aspergillus fumigatus* | Biocombustíveis | Aumento na sacarificação da palha de arroz | (Jin et al., 2020) |

## 6- CONCLUSÃO

Foram demonstradas aplicações de lacases oriundas de ascomicetos nos setores de biorremediação, têxtil, biocombustíveis, alimentos, biossíntese e biossensores. Com a ascensão da química verde e problemas ambientais emergentes, a utilização de enzimas versáteis, com propriedades catalíticas diferenciadas, como as lacases de ascomicetos, surgem com uma alternativa às demandas industriais existentes. No entanto, a baixa produção de lacases a partir de organismos nativos, condições industriais extremas e escassez de estudos envolvendo lacases de ascomicetos, surgem como desafios significativos a serem superados para possibilitar as aplicações industriais dessas enzimas. Diante do demonstrado, é necessário ampliar os estudos e explorar o potencial de novas cepas de ascomicetos produtores de lacases, que apresentem propriedades únicas, necessárias para contornar os desafios existentes. Com a ampliação dos estudos, espera-se que as lacases de ascomicetos possam ter a sua aplicação consolidada em vários setores indústrias, de forma a promover processos mais sustentáveis e eficientes, alinhados com as necessidades de um mundo cada vez mais preocupado com a sustentabilidade ambiental.

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## CHAPTER II - Dyes decolorization by a laccase from *Chrysoporthe cubensis* grown in coffee husks

PAPER II - **Dyes decolorization by a laccase from *Chrysoporthe cubensis* grown in coffee husks**- Submitted in the International Biodeterioration & Biodegradation.

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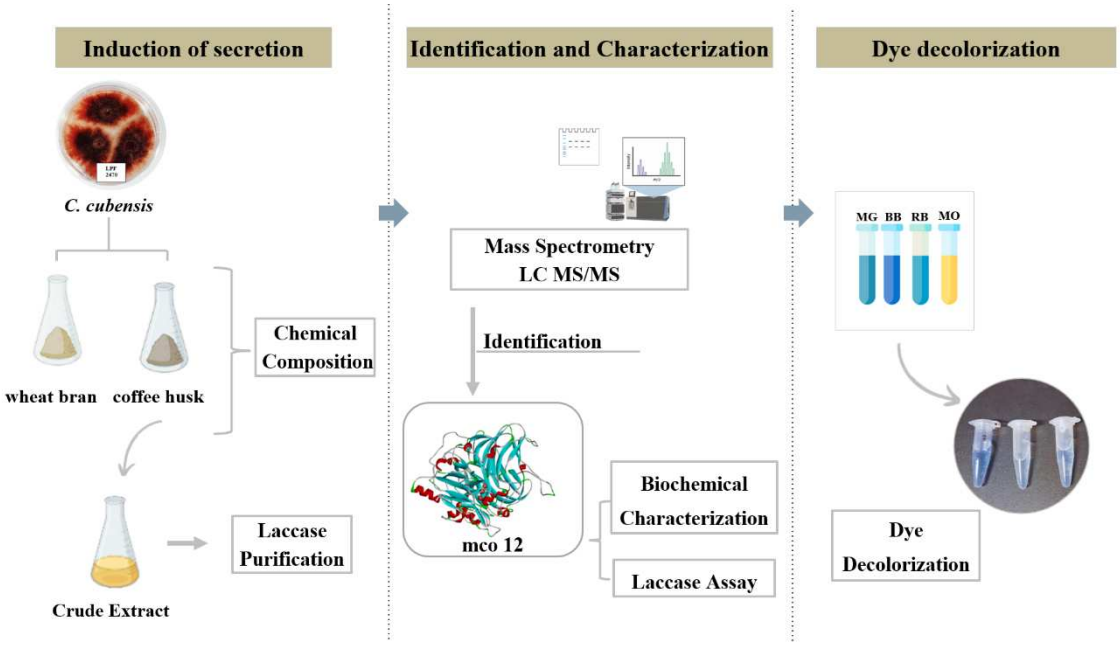
### ABSTRACT

Faced with the need to identify new and more robust laccases, with unique characteristics, which may result in different substrate specificities, the phytopathogenic fungus *Chrysoporthe cubensis* was grown on wheat bran and coffee husks to evaluate the induction of laccases. The coffee husk, which had a higher lignin content in its composition, proved to be more efficient in inducing the expression of laccase, since the crude extract showed 8.4 times more specific activity than the extract obtained from wheat bran. The application of two purification steps resulted in a 12-fold purification of the laccase-containing extract, achieving a yield of 95.42 %. To determine which laccase was induced and purified, the purification-related bands were analyzed using mass spectrometry (LC-MS/MS). This analysis revealed a single protein band corresponding to laccase, identified as MCO12. Characterization of this laccase demonstrated optimal activity within a pH range of 2.2 to 5.0, indicating an acidic nature. The enzyme exhibited peak activity at 55 °C and maintained over 80 % of its activity between 45 °C and 60 °C. In terms of thermostability, laccase MCO12 had a half-life of 290 minutes at 45 °C, 22 minutes at 55°C, and 3 minutes at 65 °C. After applying laccase to decolorize various dyes, the most significant result was observed with Coomassie Brilliant Blue, achieving a 40 % discoloration. This laccase also acted on the dyes Reactive Blue 4 and Malachite Green, with discolorations of 23 and 20 %, respectively. This study demonstrates the potential of laccase MCO12 from *C. cubensis* for bioremediation of dyes used in the textile industry, particularly triphenylmethane class dyes.

**Keywords:** Laccase, dye decolorization, coffee husk, *Chrysoporthe cubensis*.

## GRAPHICAL ABSTRACT



## 1- INTRODUCTION

The widespread use of synthetic dyes in the textile industry's dyeing process, where up to 50 % of the applied dyes are not absorbed by the fibers, poses a risk to human health and the entire aquatic ecosystem (Herath et al., 2024; Abu-Hussien et al., 2022). The dyes have a stable structure, designed to resist light, heat, and chemical agents, mainly due to the presence of azo, anthraquinone, and triphenylmethane groups in their structure, which gives them high recalcitrance and makes them persistent in the environment (El-Bendary et al., 2023; Al-Tohamy et al., 2022). The use of physical and chemical methods to remove dyes has limitations, including the use of toxic oxidizing reagents, the accumulation of concentrated toxic sludge after treatment, and high costs (Shanmugam et al., 2017; Ardila-Leal et al., 2021). These challenges drive the search for innovative and more efficient methods, such as those involving the application of oxidative enzymes like laccases.

Laccases (EC.1.10.3.2) are enzymes widely studied due to their versatility and potential industrial applications. They are multicopper oxidases (MCOs), belonging to the Auxiliary Activity Family 1 (AA1), and are found in organisms such as fungi, bacteria, and plants (Zerva et al., 2019; Tavares et al., 2022). The ability of these enzymes to catalyze the oxidation of a wide range of organic compounds, including phenols, dyes, and environmental pollutants, has aroused great interest in their study (Jeyabalan et al., 2023). Laccases require only molecular oxygen and release only water molecules as a by-product. Due to this characteristic, this class of enzymes is called “green catalysts”(Alvarado-Ramírez et al., 2021). Structurally, laccases are characterized by their complex architecture and require the presence of metal ions, primarily copper. They contain three conserved cupredoxin domains (D1, D2, and D3) and feature four copper ions that constitute the catalytic core: a T1 copper ion that accepts an electron from the reducing substrate, and a trinuclear cluster formed by one T2 copper ion and two T3 copper ions, which is responsible for transferring four electrons to reduce $O_2$ to $H_2O$ (Jeyabalan et al., 2023; Pardo et al., 2018; Olmeda et al., 2021). This reaction results in the formation of highly reactive free radicals, performing a wide range of biological functions. A high redox potential of the laccases is required for the oxidation of complex substrates. The redox potential of laccases found in plants and bacteria is generally low when compared to fungal laccases (Kolomytseva et al., 2017). Due to this characteristic, the most promising laccases for industrial applications are those of fungal origin, particularly from white-rot basidiomycete fungi. Laccases from *Phanerochaete chrysosporium*, *Trametes versicolor*, *Phlebia radiata*, and *Pleurotus* species are the most extensively studied (González et al., 2021). Although studies with laccases from ascomycete fungi are scarcer, some laccases

of this phylum are already commercially available, such as laccases from *Aspergillus sp.* and *Myceliophthora thermophila* (Mate and Alcalde, 2017).

The phytopathogenic ascomycete fungus *Chrysoporthe cubensis*, when grown on wheat bran or sugar cane bagasse as a carbon source, demonstrates significant potential for producing enzymes that efficiently hydrolyze lignocellulosic biomass, surpassing existing commercial enzyme cocktails (Falkoski et al., 2013; Dutra et al., 2017). *In silico* studies of the *C. cubensis* secretome, followed by proteomic analyses, have confirmed the wide range of lignocellulose-degrading enzymes secreted by this fungus and enabled the identification of various genes encoding MCOs, including new laccases (Tavares et al., 2021). Furthermore, it was demonstrated that the presence of *C. cubensis* laccase increased the efficiency and yield of sugar cane bagasse saccharification. This enzyme acted on phenolic compounds, enhancing the tolerance of xylanase and other hemicellulases to phenolic inhibitors and deactivating compounds (Ladeira Ázar et al., 2018). The high efficiency of the *C. cubensis* enzyme cocktail could be, in part, related to the presence of laccase, since the activity of this enzyme was not identified in commercial cocktails, such as Multifect® CL, Multifect® XL and Accellerase® 1500 (Tavares et al., 2024; Maitan-Alfenas et al., 2015).

Given the potential displayed by the enzymatic extract of the fungus *C. cubensis* to act on phenolic substrates, combined with the identification of different genes that could encode new laccases in this fungus, this study aimed to induce the production of laccase by *C. cubensis*, in medium containing coffee husks as carbon source, partially purify, identify and characterize the induced laccase. In pursuit of simple, sustainable and environmentally friendly alternatives to mitigate the impact caused by dyes discarded in effluents, we evaluated the potential of this laccase in the decolorization of dyes used in the textile industry.

## 2- MATERIALS AND METHODS

### 2.1- Microorganism and fermentation in semi-solid state

The fungus *Chrysoporthe cubensis* (LPF 2470) (Taxonomy ID: 305400), used in this study, was obtained from the mycological collection of the Laboratory of Forest Pathology, Federal University of Viçosa, MG, Brazil. The fungus was maintained on potato dextrose agar (PDA) medium plates at 28 °C and periodically subcultured (Falkoski et al., 2013).

The inoculum of *C. cubensis* was prepared by adding 10 disks, each containing 5-day-old colonies of the fungus grown on PDA plates, into 250 mL Erlenmeyer flasks containing 100 mL of previously sterilized medium with the following composition (in g/L): glucose, 10.0; $NH_4NO_3$, 1.0; $KH_2PO_4$, 1.0; $MgSO_4$, 0.5 and yeast extract, 2.0. Subsequently, the submerged

cultures were maintained in an automatic shaker for 5 days, at 150 rpm and 28 ºC. The obtained cultures were aseptically homogenized and inoculated in the semi-solid medium.

The cultivation of *C. cubensis* for enzyme production under semi-solid-state fermentation (SSF) was conducted using two types of biomasses: coffee hulls and wheat bran. In 250 mL Erlenmeyer flasks, 12.5 g of biomass and 18.75 mL of culture medium (60 % final moisture) were added. The culture medium contained, in g/L: $NH_4NO_3$, 1.0; $KH_2PO_4$, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25; and yeast extract, 2.0. Trace elements were also added to the medium, in mg/L: $MnCl_2$ 0.1; $H_3BO_3$ 0.075; $Na_2MoO_4$ 0.02; $FeCl_3$ 1.0; and $ZnSO_4$, 3.5. The flasks were autoclaved at 121 ºC for 20 minutes, and then inoculated with approximately 10 mL of the previously prepared inoculum. The flasks were kept at 28 °C in a temperature-controlled incubator for 7 days. Enzymes secreted during SSF were extracted using 50 mM sodium acetate buffer at pH 5, in a 10:1 ratio (buffer to dry substrate), under agitation at 150 rpm for 60 minutes at room temperature. Then, solids and mycelium were separated from the supernatant by filtration through a nylon membrane, followed by centrifugation at 15000 x *g* for 10 min at 4 °C.

### 2.2- Chemical composition of coffee husk and wheat bran

The cellulose, hemicellulose, lignin, protein, ash and extractive contents of coffee husk and wheat bran were evaluated as described by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008; Pimentel et al., 2024). The composition determination began with the removal of extractives from the biomass, which had been previously dried in an oven at 45 °C, through exhaustive extraction with petroleum ether, according to NREL (Sluiter et al., 2008). The extractives were determined by the difference in samples weight before, and after extraction. Extractive-free biomasses were used to determine total carbohydrates, soluble and insoluble lignin. A threefold dilution of the filtered acid hydrolysis product was performed for the determination of carbohydrates and soluble lignin, while the solid fraction was used for quantifying insoluble lignin (Sluiter et al., 2006). Sugar monomers were quantified by High-Performance Liquid Chromatography in a CBM-20A/20Alite (Shimadzu) chromatograph coupled with an RID - 20A (Shimadzu) refractive index detector (Pimentel et al., 2024). Soluble lignin was determined by spectrophotometry through absorbance values at 280 nm. The insoluble lignin was determined by the weight of the insoluble residue retained, and the ash content was determined after burning the material in the muffle for 2 hours at 300 °C followed by 4 hours at 575 °C. Finally, protein determination was performed by the Kjeldahl method and

the protein content was estimated using the Nitrogen Factor (NF) of 6.25 (Mossé, 1990; Bradstreet, 1954).

**2.3- Enzyme assay and quantification of total proteins**

To quantify the activity of *C. cubensis* native laccase, the enzymatic assay was performed in 100 mM sodium acetate buffer, pH 4.0, at 55 ºC. Laccase activity was determined by monitoring the oxidation of the substrate 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Tavares et al., 2022). The reaction medium consisted of 50 µL of enzymatic extract, 850 µL of buffer, and 100 µL of 10 mM ABTS. This mixture was incubated for 15 min and the absorbance was measured at 420 nm. The laccase activity was calculated by the Lambert-Beer principle, using a molar extinction coefficient of 3.6 x 104 $M^{-1}$ $cm^{-1.}$ One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 µmol of oxidized ABTS per minute of reaction. The concentration of proteins was determined by the Bradford colorimetric method using a standard curve of bovine serum albumin (Bradford, 1976).

**2.4- Laccase partial purification**

Initially, 1.5 mL of the crude extract containing the native *C. cubensis* laccase was carefully applied to a Sephadex G-25 HiTrap Desalting gel filtration column, in a fast protein liquid chromatography (FPLC), with the ÄKTA system, using potassium phosphate buffer 50 mM, pH 6.0. Fractions of 1.0 mL were collected at a flow rate of 2.5 $mLmin^{-1}$. Laccase activity and protein concentration were determined in the fractions, as mentioned in item 2.1.3. The active fractions (5 mL) were pooled and applied to a HiTrap DEAE Fast Flow column, which had been previously equilibrated with 50 mM potassium phosphate buffer at pH 6, in an FPLC system. The proteins were eluted with 25 mL of the same buffer, followed by the linear gradient of 0-1M NaCl in potassium phosphate buffer 50 mM, pH 6.0. Fractions of 1 mL were collected at a flow rate of 2.5 mL $min^{-1}$ and analyzed for laccase activity and protein concentration, as described in item 2.3.

**2.5- One-dimensional electrophoresis (SDS-PAGE)**

SDS-PAGE was performed in the Mini-Protean II System Cell (BioRad), using the stacking and resolving gels at concentrations of 5 % (m/v) and 12 % (m/v), respectively, according to the Laemmli protocol (Laemmli, 1970). The protein bands were stained with Commassie Brilliant Blue G-250 (Neuhoff et al., 1988).

### 2.6- Tryptic Digestion, Liquid Chromatography and Mass Spectrometry (LC-MS/MS)

The protein bands from the unidirectional SDS-PAGE were excised, crushed, and submitted to enzymatic digestion (Shevchenko et al., 2007). The analysis of tryptic peptides was performed in an LC-MS/MS system, composed of a nanoAcquity UPLC (Waters, USA) and an Amazon Ion Trap® model mass spectrometer (Bruker Daltonics, Germany). Ions were scanned in positive mode for MS1 spectra in the mass range between 300 and 1500m/z and MS2 between 70 and 3000 m/z. The generated peak lists were compared with protein sequences from the *C. cubensis* database, for protein identification. The comparison was performed using the PEAKS application, version 7.0 (Bioinformatics Solutions Inc., Canada)(Ma et al., 2003).

### 2.7- Biochemical Characterization of laccase

To evaluate the effect of pH on the activity of partially purified laccase from *C. cubensis*, the assay was conducted in the pH range of 2.0-8.0, with McIlvaine buffer (citric acid and sodium phosphate) at 50 ºC, for 15 min. The effect of temperature was evaluated in the range of 30-70 ºC, using 100 mM McIlvaine buffer, at pH 4.0. The native laccase thermostability was evaluated at 45, 55, and 65 °C using 100 mM McIlvaine buffer, pH 4.0. Aliquots were incubated in these temperatures during different time intervals and enzyme activities were measured using standard enzyme assay, as mentioned in item 2.3. The data obtained were expressed in relative activity, with the highest value for each enzymatic activity being considered 100 %.

### 2.8- Dye decolorization

Methyl Orange, Malachite Green, Reactive Blue 4, and Brilliant Blue G-250 were dissolved in 100 mM sodium acetate buffer (pH 4.0) to achieve a final concentration of 100 mg/L. The reaction mixture was prepared in 100 mM sodium acetate buffer, pH 4.0, at 45 °C, and contained a final concentration of 0.01 mg of dye and 0.005 U of laccase. The UV-VIS spectrum of each dye was analyzed at specific wavelengths - 595 nm for Brilliant Blue and Reactive Blue 4, 617 nm for Malachite Green, and 464 nm for Methyl Orange - after 1, 6, 12, 24, 48 and 72 hours. Two types of controls were processed in parallel: enzyme-substituted buffer and dye-substituted buffer. Decolorization was evaluated as follows (equation 1):

$$\text{Decolorization (\%)} = \frac{(initial\ absorbance - final\ absorbance) \times 100}{initial\ absorbance} \quad (1)$$

The initial absorbance and final absorbance refer to the absorbance measurements of the dye sample solutions before (0 hours) and after treatment with laccase, respectively.

## 3- RESULTS AND DISCUSSION

### 3.1- Induction of laccase production by *Chrysoporthe cubensis*

*C. cubensis* was cultured under SSF using coffee husks and wheat bran as carbon sources to induce laccase production. Coffee husks proved to be more efficient in inducing laccase expression in *C. cubensis*, as the laccase specific activity, 0.238 U/mg, was 8.4 times higher than the specific activity detected in the crude extract produced by this fungus grown in a medium containing wheat bran, which was 0.028 U/mg (Table S1). Lignin is a natural substrate for laccase; therefore, it is expected that the higher lignin content in the biomass used in the culture medium can more effectively induce laccase production by the fungus (Rahman et al., 2024). The lignin content in coffee husks, which includes the outer skin, pulp, and parchment (Nguyen et al., 2023), was 31.57 %, while wheat bran contained 16.24 % lignin (Table 1). Wheat bran is widely used as a substrate in fermentation processes for enzyme production by fungi (Li et al., 2022), while the use of coffee husks for this purpose remains limited (de Almeida et al., 2023). The significant laccase activity detected in the extract of *C. cubensis* grown on coffee husks indicates the potential of this residue for laccase production (Andrade et al., 2012).

Table 1. Chemical composition of coffee husks and wheat bran.

| Components (%) | Coffee Husk | Wheat Bran |
|---|---|---|
| **Lignin** | 31.57 ± 1.66 | 16.24 ± 0.21 |
| **Cellulose** | 29.25 ± 1.06 | 21.78 ± 0.56 |
| **Hemicellulose** | 24.82 ± 1.48 | 17.29 ± 0.60 |
| **Ashes** | 6.0 ± 0.33 | 4.93 ± 0.10 |

### 3.2- Partial purification and identification of laccase secreted by *C. cubensis*

The laccase from the crude extract produced by *C. cubensis* after growth on coffee husks was partially purified. Initially, the crude extract underwent gel filtration chromatography using a Sephadex G-25 HiTrap Desalting column, followed by ion exchange chromatography with a HiTrap DEAE column (Table 2). The laccase was purified approximately 12-fold with a yield of 95.42 %.

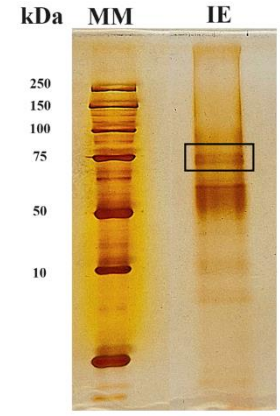
Table 2. Purification steps of the laccase secreted by *C. cubensis.*

| Purification Step | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg) | Yield (%) | Purification (X) |
|---|---|---|---|---|---|
| **Crude Extract** | 0,255 | 0,032 | 0,13 | 100 | 1 |
| **Filtration Gel Chromatography** | 0,109 | 0,031 | 0,28 | 96.06 | 2.25 |
| **Ion Exchange Chromatography** | 0,020 | 0,030 | 1,53 | 95.42 | 12.22 |

After the partial purification of *C. cubensis* laccase, four major protein bands were visualized in SDS-PAGE (Figure 1A). To identify the protein band corresponding to laccase, the bands were excised from the gel and analyzed using mass spectrometry (LC-MS/MS). This analysis indicated that only one protein band was relative to laccase, which was confirmed by the coverage of 2 unique peptides, as shown in Figure 1B. This protein was identified as a multicopper oxidase (MCO), referred to as MCO12 by TAVARES et al. (2022). The identified peptides from the other protein bands did not match with any other predicted sequences belonging to *C. cubensis* MCOs (Table S2).

*In silico* studies of *C. cubensis* MCOs identified thirteen enzymes predicted to belong to the AA1 family in the genome of this fungus (MCOs 1-13) (Tavares et al., 2022). This study revealed that the MCO12 enzyme contained the conserved laccase signatures, including the Asp-Ser-Gly-Leu (DSGL) consensus sequence. Additionally, the SDS-gate exhibited a substitution, with the first serine replaced by threonine. Since SDS-gate is involved in transferring protons from the T1 site to the trinuclear center during catalysis, it was suggested that the substitution of serine with threonine could confer an advantage by facilitating proton flow. Additionally, these predictive studies indicated that the MCO12 laccase had a molecular mass of 64.64 kDa and contained a signal peptide sequence, suggesting that this enzyme would be an efficient laccase and could be secreted by *C. cubensis* (Tavares et al., 2022). Indeed, our results demonstrated that *C. cubensis* grown on coffee husks was capable of producing and secreting an active MCO12 laccase (Fig. 1B and 1C) with a molecular mass of about 75 kDa (Fig 1A), which is consistent with the predicted molecular mass 64.64 kDa, considering that it has two predicted N-glycosylation sites.

A)




B)

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  1  MRLLLQYLTL VTGSLALSLP QGDHLHHLQT KAESGLKPRQ SCDNTATSRD CWGDYSIDTN YYDVIPDTGV NREYWLTVDE
 81  VACAPDGYNT TCLTFNGTVP GPTLYADWGD TMTVHVTNNV PNNGTTIHWH GVRQLNNVAH DGVPGVTQCP IAPGQSLTYE
161  FQVSQYGSTW YHSHFTLQYA QGLFGGLVFN GPATSNYDED LGVLFLGDWH HTDIFELWLT ARLGAPPTMQ SGIINGTNTY
241  NCTASGDMTC TGTVGQKFET VFQSGTKYRI RLINVATDGH FQFSIDGHSL TVIATDFVPI VPYATDSVLV SIGQRVDVIV

321  EANATAGDYW LRGGWVSACN NIDNIDDITG IVRYDASSTA TPTSNSTVIA STSCGDEPYD SIVPYLSLDV TDIAVETDQS
401  LGLNRSTLVT WTLNNSSLWL NWSDPTLGQV IAGDTIFPTD YNVVAVDGPA TSANATNSTS EWALLVIEDD TGIGLTHPIH
481  LHGHDFWVLS QSTAAYEKGV TTLNTANPPR RDVASLPGNG HLAIAFKMDN PGTWLVHCHI AWHASQGLSL EFVESESQIA

561  SASLTDSDKT TLSSQCAAWD SYILTDEYDG QDDSGI
```

C)

| Peptide | Uniq | -10lgP | Mass | ppm | m/z | z | RT | Scan | #Spec | Start | End | PTM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| K.FETVFQSGTK.Y | Y | 72.72 | 1142.5608 | 89.1 | 572.3386 | 2 | 50.41 | 2695 | 1 | 258 | 267 | |
| K.GVTTLNTANPPR.R | Y | 66.73 | 1239.6571 | 1.7 | 620.8369 | 2 | 38.62 | 2050 | 3 | 499 | 510 | |
| **total 2 peptides** | | | | | | | | | | | | |

Figure 1 – Identification of native *C. cubensis* laccase expressed in the medium containing coffee husks. A) SDS-PAGE of laccase partially purified by ion exchange (IE) chromatography using a diethylaminoethyl cellulose anion exchange column (DEAE) at pH 6.0; B) Coverage of the two peptides identified in the protein sequence of the mco12 laccase; C) Information about the identified peptides.

### 3.3- Characterization of the laccase from *Chrysoporthe cubensis*

The laccase from *C. cubensis* exhibited the highest activity in the pH range of 2.5 to 3.0. However, it retained more than 60 % of its activity within the pH range of 2.2 to 5.0, demonstrating the acidic nature of this enzyme (Figure 2A). At pH 6.0, the laccase activity was null.

This enzyme showed optimal activity at 55 °C and maintained more than 80 % of its activity between 45 and 60 °C (Figure 2B). Additionally, thermostability assays revealed that the laccase had half-lives of 290, 22, and 3 minutes at 45, 55, and 65 °C, respectively (Figure 1C). Thermostability is one of the main characteristics of an enzyme from the point of view of industrial application. The properties exhibited by the laccase from *C. cubensis* indicate its potential for biotechnological applications, particularly due to its thermostability at 45 °C. Temperatures around 45-50 °C are commonly used in bioremediation of textile dyes (Si et al., 2021), enzymatic hydrolysis of lignocellulosic materials (Martins et al., 2020), and in the food industry for the clarification of fruit juices and the removal of undesirable phenolic compounds that can affect the color and flavor of products (Bezerra et al., 2015; Bertrand et al., 2016; Wang et al., 2018).

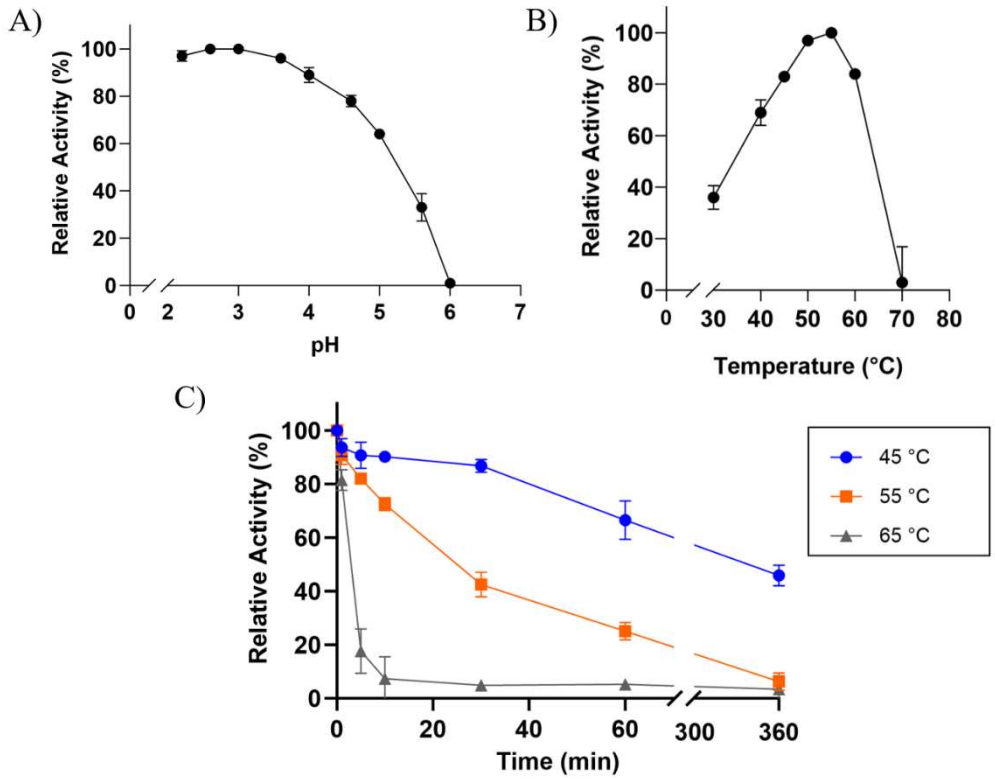


Figure 2 - Partial characterization of the purified mco12 laccase. A) Effect of pH (2.2-8.0); B) Effect of temperature (30-70 °C) and C) Thermostability of laccase at temperatures of (•) 45, (■) 55 and (▲) 65 °C.

### 3.4- Dye Decolorization

The potential of *C. cubensis* laccase MCO12 to promote dye discoloration was investigated. Partially purified laccase MCO12 was utilized in decolorization assays with representatives from the main chemical classes of dyes. Methyl Orange for the Azo class, Reactive Blue 4 for the Anthraquinone class, Coomassie Brilliant Blue G-250 and Malachite Green for the Triphenylmethane class. The decolorization assays were carried out during 72 h, at 45 °C and pH 4.0, using a final concentration of 0.01 mg of the dye and only 0.005 U of laccase (Figure 3). Decolorization control assays were conducted under identical conditions to exclude any background decolorization. This involved substituting the enzyme with buffer in the no-enzyme control and replacing the dye with buffer in the no-dye control. Despite the reduced enzyme load, it was observed that laccase MCO12 was capable of decolorizing the dyes, albeit with varying effectiveness. The most significant result was observed with the Coomassie Brilliant Blue dye. The decolorization of this dye increased with the duration of treatment, reaching 40 % decolorization after 72 hours. After 12 hours, Reactive Blue 4 exhibited a higher percentage of decolorization by laccase, approximately 15 %. After 72 hours, Reactive Blue 4 and Malachite Green showed 23 and 20 % decolorization, respectively. Methyl Orange achieved 6 % decolorization after 72 hours of laccase application. It is important to note that the dye decolorization assays were conducted solely to assess the potential of laccase for this purpose.

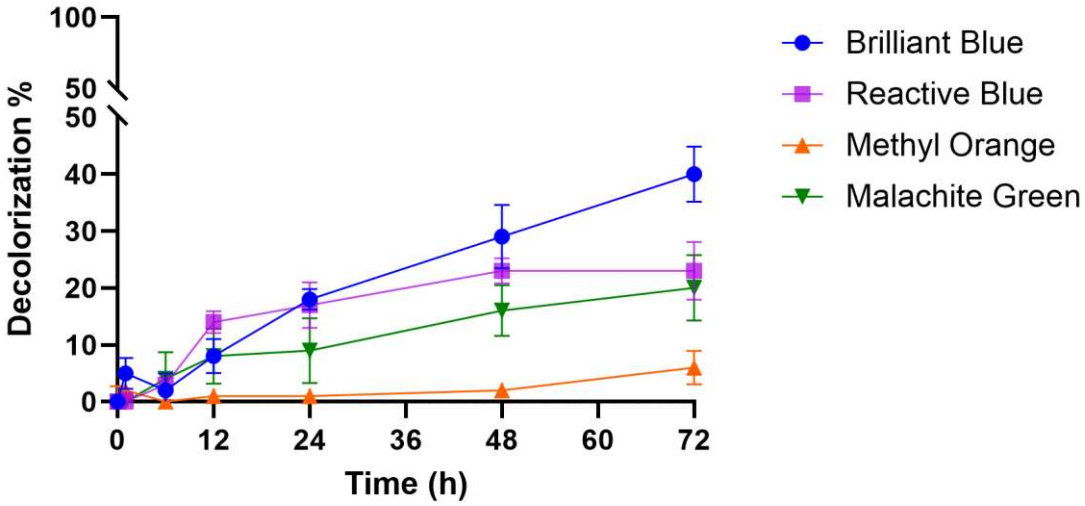


Figure 3 – Decolorization of synthetic dyes by semi purified laccase mco12 from *C. cubensis*. Brilliant blue (•); Reactive Blue 4 (■); Methyl Orange (▲); Malachite Green (▼).

Reactive Blue 4 represents the class of anthraquinone dyes, which are characterized by the presence of a chromophore group that comprises two carbonyl groups (=C=O) on both sides of a benzene ring (Espina et al., 2021). The dyes of this class are considered the most toxic and are still widely used on an industrial scale (Ardila-Leal et al., 2021). The dye Coomassie Brilliant Blue G-250, previously used in the textile industry and currently used to color proteins in biochemistry assays, can contaminate aquatic environments for a long period due to its high stability (Paz et al., 2017). This class of dyes, which includes Malachite Green, is among the three main classes of aquatic contaminants. These dyes are characterized by aromatic rings containing a chromophore with three phenyl groups linked by a central carbon atom, which can have various substituents (Espina et al., 2021). Methyl Orange, belonging to the azo dye class, features a functional group (-N=N-) connecting two alkyl or aryl radicals and is commonly used due to its low cost, high intensity, and color fastness (Ardila-Leal et al., 2021). The demonstrated ability of laccase MCO12 to act on these dyes, even at low enzyme concentrations and under non-optimized conditions, underscores the potential of this enzyme for biotechnological applications, particularly in dye decolorization.

Laccases from basidiomycetes, especially from white-rot fungi, have been extensively studied (Herath et al., 2024). A laccase from the white rot fungus *Cotylidia pannosa*, was applied by Sharma et. al. (2015) in the discoloration of Coomassie Brilliant Blue, and resulted in 40 % discoloration after 96 h at 30 ºC, using 2.5 U of enzyme load (Sharma et al., 2015). The laccase MCO12 from *C. cubensis* promoted a similar degree of discoloration of this dye, after 72 h of treatment, using only 0.005 U of enzyme. Furthermore, the MCO12 laccase identified and used in this study was applied without the use of mediators, which makes it even more interesting for applications in industrial processes, since these chemical mediators can be toxic and make the process more expensive (Mani et al., 2018). The demand for more efficient laccases underscores the need to investigate new fungal laccases from ascomycetes, such as *C. cubensis*. This study highlights the potential of laccase MCO12 from *C. cubensis* as a biological catalyst for the bioremediation of dyes in effluents, particularly those belonging to the triphenylmethane class.

The search for new laccases with potential applications in dye decolorization has attracted great interest in the industry. Some bacterial laccases have been studied, however, even with high activity and thermal stability, they have a reduced redox potential, compared to fungal laccases, which limits the spectrum of varied substrates on which they can be catalyzed (Rodrigues et al., 2023). Regarding fungal laccases, there are few studies that characterized MCO laccases from ascomycetes, despite having representatives reported as laccase producers.

Ascomycete laccases, such as MCO12 from *C. cubensis*, present promising potential due to their wide distribution and unique characteristics, which can result in different substrate specificities and more flexible operating conditions (Tavares et al., 2022).

## 4- CONCLUSION

The laccase MCO12, produced by the ascomycete fungus *C. cubensis* grown in coffee husks was partially purified and characterized. This enzyme exhibited notable properties, particularly significant thermostability at 45-55 °C, a temperature range commonly employed in the bioremediation processes of textile dyes. The potential of this laccase to promote the decolorization of different dyes was evaluated in tests using low enzymatic load compared to those normally used in dye decolorization. Laccase MCO12 successfully decolorized representatives from the main chemical classes of dyes used in the textile industry. These findings underscore the potential of MCO12 laccase from *C. cubensis* for biotechnological applications, particularly in the bioremediation of dyes.

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## SUPPLEMENTARY MATERIAL

Table S1. Laccase activities (U/mg) produced by *C. cubensis* grown on coffee husks and wheat bran.

| **Biomass** | **Enzymatic Activity (U/mL)** | **Protein Concentration (mg/mL)** | **Specific Activity (U/mg)** |
|---|---|---|---|
| Coffee husk | 0.043 ±0.015 | 0.181±0.003 | 0.238±0.02 |
| Wheat bran | 0.007 ±0.000 | 0.228±0.002 | 0.028±0.05 |

Table S2. Peptides identified by LC – MS/MS mass spectrometry.

| Band | Accession | Coverage (%) | #Unique | Avg. Mass | Description | Peptide sequence | | | Accession | Description/Organism |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | g4632.t1 | 4 | 3 | 71263 | g4632.t1 | R | ALVEGATLASALGK | S | PSR93690 | glycoside hydrolase family 15 protein/ *Coniella lustricola* |
| | | | | | | R | DSALTFK | E | | |
| | | | | | | K | VVTDSFR | S | | |
| **1** | g7214.t1 | 7 | 4 | 74908 | g7214.t1 | K | HDDIGVYMVNR | M | PSR76994 | hypothetical protein BD289DRAFT_378234/ *Coniella lustricola* |
| | | | | | | R | LTNWPIFSMK | H | | |
| | | | | | | R | IRPSDLDTLASWQTNINTR | L | | |
| | | | | | | K | QIGLWTSSR | F | | |
| **1** | g2605.t1 | 8 | 5 | 76398 | g2605.t1 | K | VNIVTGIGWNK | G | KUI61591 | Beta-glucosidase cel3A/ *Valsa mali* var. pyri |
| | | | | | | K | GCGVHVLLGPVAGPLGK | N | | |
| | | | | | | K | LPYTIAK | A | | |
| | | | | | | R | DTMSSNLDDR | T | | |
| | | | | | | K | HYILNEQELNR | D | | |
| **1** | g594.t1 | 4 | 2 | 64635 | g594.t1 | K | FETVFQSGTK | Y | PSR81428 | laccase precursor/ *Coniella lustricola* |
| | | | | | | K | GVTTLNTANPPR | R | | |
| **1** | g7853.t1 | 3 | 2 | 58836 | g7853.t1 | R | VNVFSFPNAK | G | XP_040774150 | alpha/beta-hydrolase/ *Cryphonectria parasitica* EP155 |
| | | | | | | R | HVPYPR | I | | |
| **2** | g2236.t1 | 5 | 2 | 48231 | g2236.t1 | K | DFYGSGMTVDTSQK | F | XP_040774804 | family 7 glycoside hydrolase/ *Cryphonectria parasitica* EP155 |
| | | | | | | R | TYLMESSTK | Y | | |

## CHAPTER III - Expression of the thermostable laccase MCO12 from *Chrysoporthe cubensis* in *Komagataella phaffi*i and application in dye decolorization

PAPER III - **Expression of the thermostable laccase MCO12 from *Chrysoporthe cubensis* in *Komagataella phaffii* and application in dye decolorization**

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### ABSTRACT

The MCO12 laccase from *Chrysoporthe cubensis* had its gene sequence optimized and overexpressed in *Kamagataella phaffii*. Biochemical characterization tests demonstrated that the recombinant MCO12 laccase exhibited improved properties compared to its native form. The recombinant laccase retained over 80 % of its activity within the temperature range of 55 to 70 °C, with its optimal activity temperature increasing from 55 °C in the native form to 60 °C in the recombinant form. The recombinant laccase also displayed significant thermostability, with half-lives of 11 h, 45 h, and 142 h at 70 ºC, 60 °C, and 50 °C, respectively. Homology modeling and molecular docking of the native and recombinant laccases were conducted using the acidic substrate 2,6-dimethoxyphenol (ABTS). The results indicated that the recombinant MCO12 laccase has a higher binding energy of -6.2 compared to -5.4 for the native MCO12 laccase. Additionally, the recombinant enzyme formed a greater number of hydrogen bonds with ABTS, suggesting increased stabilization of the complex and enhanced specificity in substrate recognition. Given its potential for industrial applications, the recombinant MCO12 laccase was tested for dye decolorization. Among the dyes tested, the MCO12 laccase achieved 99 % decolorization of Malachite Green and 71 % decolorization of Congo Red, after 72 h of incubation at 50 °C. These findings underscore the potential of recombinant MCO12 laccase as a promising enzyme for industrial applications, particularly for the bioremediation of dyes used in the textile industry, which require enzymatic activity at elevated temperatures.

**Keywords:** Laccase, dye decolorization, *Kamagataella phaffii, Chrysoporthe cubensis.*

## 1. INTRODUCTION

Laccases (EC.1.10.3.2) are multicopper oxidases (MCOs), members of the CAZy auxiliary activity family 1 (AA1), found in organisms such as fungi, bacteria and higher plants (Kyomuhimbo and Brink, 2023; Singh et al., 2023). This enzyme reacts with monophenols and diphenols, but can also oxidize polyphenols and polyamines with varying redox potentials (Dong et al., 2023). The high redox potential of laccases, especially those of fungal origin, confers a wide range of biotechnological applications, including their use in biodegradation processes, kraft bleaching, dye decolorization, and industrial wastewater treatment (Song et al., 2024). Laccases require only oxygen molecules as reactants, thus producing only water molecules as by-products. Due to this characteristic, this class of enzymes is called “green catalysts” (Wang et al., 2024).

Since laccases show ability to act on a diverse range of substrates, these enzymes demonstrate significant potential for application in various industrial sectors. The textile industry stands out as one of the most extensively studied and, consequently, one of the most promising for laccase application (Brugnari et al., 2021). Estimates indicate that approximately 10 to 15 % of the synthetic dyes used in the textile industry end up in wastewater, raising significant environmental concerns due to the difficulty of degrading these effluents, which are designed to be resistant to natural degradation processes (Martin et al., 2024). With approximately 10.000 synthetic dyes available, most of which are not absorbed during the dyeing process, environmentally friendly solutions, such as the use of laccases, emerge as effective means to mitigate the environmental impact associated with this sector (Jeyabalan et al., 2023).

The yield of laccase from native sources, as with other enzymes, does not meet industrial demands. In addition to being produced in limited quantities, natural hosts often express multiple isoenzymes, making it challenging to isolate the laccase of interest (Arregui et al., 2019). Native fungal laccases, especially from basidiomycetes, which are the most studied, have their industrial application hampered due to the long cultivation time, and low stability at high temperature and alkaline pH (Chauhan et al., 2017). Many industrial processes are carried out under extreme conditions, such as pulp bleaching and wastewater treatment (Mehandia et al., 2020). Thus, there is a great demand for new fungal laccases that are more suitable for the process conditions, or for improved laccases that present higher stability.

The current market size for laccases was of USD 3 million in 2021 and is set to increase in the coming years with a projection of USD 4 million by 2028, reflecting the increased demand for these enzymes in various industrial applications (Martin et al., 2024). To meet the

growing demand, especially in the textile industry, it is crucial to investigate new fungal laccases, such as those from ascomycetes, which are poorly studied and could have advantageous properties. In addition, heterologous expression of laccases in a system that increases production levels becomes necessary to meet the industrial demand for these enzymes. The ascomycete fungus *Chrysoporthe cubensis* cultivated on coffee husks was able to produce the MCO12 laccase, which exhibited the ability to decolorize dyes belonging to different classes (Gomes et al., 2024 – submitted). Although this laccase has shown potential for biotechnological applications, its production by the fungus is reduced. Therefore, this work aimed to express the MCO12 laccase from *C. cubensis* in *Kamagataella phaffii*, previously called *Pichia pastoris*, to make the comparative study of the properties of native and recombinant laccases and to evaluate its potential in the decolorization of dyes.

## 2. MATERIALS AND METHODS

### 2.1 Strains and vectors

The pPICZαB vector containing the selected laccase gene of *C. cubensis* was synthesized by GenScript (Piscataway, NJ, USA). Competent *Escherichia coli* DH5α cells, supplied by Stratagene (La Jolla, CA, USA), were used for plasmid multiplication and manipulation. The *Komagataella phaffii* GS115 strain, provided by Invitrogen (Carlsbad, CA, USA), was used for heterologous expression. Restriction enzymes and DNA ligases were purchased from Thermo Fisher (Waltham, MA, USA).

### 2.2 *Chrysoporthe cubensis* laccase codon optimization for heterologous expression in *K. phaffii* GS115

The fungal secretion signal peptide sequence was removed from the original coding sequence of the selected *C. cubensis* laccase using the TargetP 2.0 prediction tool (http://www.cbs.dtu.dk/services/TargetP-2.0) (Armenteros et al., 2019), to obtain only of the coding sequence for expression in *K. phaffii*. The coding sequence for the secretion signal peptide, *K. phaffii* α-mating factor, was added to the pPICZαB expression vector, in order to ensure that the gene was in frame after subcloning into the pPICZαB vector.

After removing the signal peptide, the sequence of the selected laccase was optimized for *K. phaffii* expression using the OPTIMIZER online software (http://genomes.urv.es/OPTIMIZER/). The optimized nucleotide sequence was translated into the corresponding amino acid sequence using the Translate tool (https://web.expasy.org/translate/) and the protein sequence with optimized codons was

compared with the original sequence, using the Mafft version 7 program (https://mafft.cbrc.jp/alignment/server/).

The Gene Designer software (DNA 2.0) was used to change some codons, in order to reduce the content of long sequences rich in AT or CG and repetitive codons, which could lead to errors during expression. Two stop codons were inserted at the end of the gene sequence and restriction sites for PstI and NotI were added at the 5' and 3' ends, respectively, to allow the in frame cloning of the coding sequence with the sequence of the peptide signal. The final laccase coding sequence was synthesized in pPICZαBTEV vector by Genscript (Piscataway, NJ, USA).

### 2.3 Transformation into *Escherichia coli* DH5α and plasmid DNA extraction

Competent *E. coli* DH5α cells were transformed by heat shock with the pPICZαB vector, containing the optimized laccase gene, and plated on medium containing 25 µg/mL of the antibiotic zeocin, according to transformation method described in EasySelectTM *Pichia* expression kit user manual (Invitrogen). Transforming colonies were selected and the plasmid DNA was purified according to the protocol described by GREEN & SAMBROCK. The quality of the plasmid DNA was evaluated electrophoretically, in 0.8 % (w/v) agarose gel.

### 2.4 Induction of laccase expression in *Komagataella phaffii*

A set of four different media were used in the laccase expression assays: BMGH and BMMH minimal media (100 mmol $L^{-1}$ potassium phosphate buffer pH 6.0, 1.34 % yeast nitrogen base, $4\times10^{-5}$ % biotin, $4x10^{-4}$ % histidine and 1 % glycerol for BMGH or 0.5 % methanol for BMMH); BMGY and BMMY complex media (100 mmol $L^{-1}$ potassium phosphate buffer pH 6.0, 1.34 % yeast nitrogen, $4x10^{-5}$ % biotin, $4x10^{-4}$ % histidine, 1 % yeast extract, 2 % peptone, 1 % glycerol for BMGY or 0.5 % methanol for BMMY). The expression assay was conducted with an untransformed GS115 strain as a negative control. Transformed colonies were individually inoculated into 25 ml of BMGH or BMGY medium in 125 ml Erlenmeyer flasks and maintained at 28 °C under 250 rpm shaking until the culture reached $OD_{600}$ 2–6. The cultures were centrifuged at 3000 x *g* and the cells were resuspended in 50 mL of BMMH or BMMY medium, at final $OD_{600}$ of 1, to induce laccase expression. At 24 h intervals, methanol was added to the cultures at a final concentration of 0.5 % in order to maintain the induction of laccase expression. Aliquots of 1 ml were withdrawn at 0, 24, 48, 72 and 96 h. Additional laccase expression assays were carried out by supplementing BMMH and BMMY media with 0.25 mM $CuSO_4$.

### 2.5 Enzyme assay and quantification of total proteins

To quantify the activity of recombinant laccase MCO12, the standard enzymatic assay was performed in 100 mM sodium acetate buffer, pH 4, at 60 ºC. Laccase activity was determined by monitoring the oxidation of the substrate 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Tavares et al., 2022). The reaction medium consisted of 50 µL of enzymatic extract, 850 µL of buffer and 100 µL of 10 mM ABTS. This mixture was incubated for 15 min and the absorbance was measured at 420 nm. The laccase activity was calculated by the Lambert-Beer principle, using a molar extinction coefficient of $3.6 \times 10^4$ $M^{-1}$ $cm^{-1}$. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 µmol of oxidized ABTS per minute of reaction. The concentration of proteins was determined by the Bradford colorimetric method using a standard curve of bovine serum albumin (Bradford, 1976).

### 2.6 Biochemical characterization of recombinant laccase

To evaluate the effect of pH on the activity of recombinant laccase MCO12, the assay was conducted in the pH range of 2.0-8.0, with McIlvaine buffer (citric acid and sodium phosphate) at 60 ºC, for 15 min. The effect of temperature was evaluated in the range of 30-70 ºC, using 100 mM McIlvaine buffer, at pH 4.0. The thermostability of the recombinant laccase was evaluated in 100 mM sodium acetate buffer, pH 4.0, at 50, 60 and 65 °C. Aliquots were pre-incubated in these temperatures during different time intervals and enzyme activities were measured using standard enzyme assay. The data obtained were expressed in relative activity, with the highest value for each enzymatic activity being considered 100 %. The effect of potential inhibitors and metal ions was monitored by adding concentrations of 10 mM $CuSO_4$, $CaCl_2$, $MnCl_2$, SDS, $ZnSO_4$, $CoCl_2$, EDTA, Tween 80, $FeSO_4$ and NaF to the standard laccase assays with ABTS as substrate.

### 2.7 Molecular modeling of native and recombinant laccase

The sequence from recombinant laccase MCO12 was subjected to molecular homology modeling using the Phyre2 tool with the intensive analysis mode (Kelley et al., 2015). To compose the three-dimensional structures of the laccases, four $Cu^{2+}$ ions were added by superimposing them with the crystallographic structure of the laccase from *Melanocarpus albomyces* (PDB code: 2Q9O) in the PyMOL software version 2.5.5 (Tavares et al., 2022). Energy minimization was performed on the YASARA server (Krieger et al., 2009). The

structures defined after homology modeling and energy minimization were validated using a set of software as described by TAVARES et al. (2022).

### 2.8 Molecular docking with ABTS

The recombinant and native laccase protein sequences obtained from the molecular modeling step (described in item 2.7) were subjected to molecular docking analysis, carried out with the AutoDock Vina tool of the PyRx software version 0.8 (Dallakyan and Olson, 2015). The search space was determined based on the location of the T1 copper-binding site in both sequences, delimited using the AutoDock Tolls software version 1.5.7 (Hakulinen et al., 2008). For the native laccase the parameters were center_x = -2.002000, center_y = -6.680000, center_z = -9.751000, size_x = 25.0000 Å, size_y = 25.0000 Å, size_z = 25.0000 Å; for recombinant laccase the parameters were center_x = -1.645000, center_y = -4.785000, center_z = -7.479000, size_x = 25.0000 Å, size_y = 25.0000 Å, size_z = 25.0000 Å. Both sequences were prepared on the CHARMM-GUI platform, the protonation state was kept neutral, in accordance with the standard state. Docking was performed with exhaustiveness of 8 and was configured to return conformations in order of lowest energy.

The substrate for docking was 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (CID: 5464076), with its 3-D structures obtained from the PubChem database. AutoDock vina's OpenBabel tool was used to convert sdf format to pdbqt format and Autodock Vina automatically added charges to the substrate using the Gasteiger method. After docking, the highest binding affinity compounds were imported to BioVia discovery studio client 2021 software version 21.1.0.0 for compilation and visualization of docked results.

### 2.9 Dye decolorization

Congo Red, Methyl Orange, Malachite Green, Reactive Blue 4, and Brilliant Blue G-250 were dissolved in 100 mM sodium acetate buffer (pH 4.0) to achieve a final concentration of 100 mg/L. The dye decolorization reaction mixture was prepared in 100 mM sodium acetate buffer, pH 4.0, at 50 °C, and contained 0.01 mg of dye and 0.1 U of laccase. The UV-VIS spectrum of each dye was analyzed after 1, 6, 12, 24, 48, and 72 hours of reaction, at specific wavelengths: 490 nm for Congo Red, 495 nm for Brilliant Blue and Reactive Blue 4, 617 nm for Malachite Green, and 464 nm for Methyl Orange. Two types of reaction controls were processed in parallel, in which the volume of enzyme was replaced by buffer and the volume of dye solution was replaced by buffer. Decolorization was evaluated as follows (equation 1):

$$\text{Decolorization (\%)} = \frac{(initial\ absorbance - final\ absorbance) \times 100}{initial\ absorbance} \quad (1)$$

The initial absorbance and final absorbance refer to the absorbance measurements of the dye sample solutions before and after treatment with laccase, respectively.

## 3. RESULTS AND DISCUSSION

### 3.1 Heterologous expression of *C. cubensis* laccase MCO12 in *K. phaffii*

The gene of *C. cubensis* laccase, referred to as mco12 (Tavares et al., 2022) was cloned into *K. phaffii* GS115 and the transformants were selected by zeocin resistance (Figure S1). The expression of laccase in *K. phaffii* was initially performed by selecting transformant colonies grown in high concentration of zeocin, (250 µg/mL), followed by cultivation in minimal media (BMGH) during 96 h, using methanol as an inducer of laccase expression. However, in this condition, the expression of the *C. cubensis* laccase in *K. phaffii* was extremely reduced and there was no detection of the protein band related to laccase in SDS-PAGE, although there was significant cell growth (data not shown). Then, in order to increase the expression of laccase, *K. phaffii* cells were grown on the complex media (BMGY) supplemented with 0.25 mM $CuSO_4$. With these changes in the cultivation medium, laccase overexpression reached $2.46.10^{-1}$ U/mL after 96 h of induction with methanol (Table S1). Laccase is a multicopper enzyme, which requires copper ions for correct structural conformation and stability. The supplementation of the medium with $CuSO_4$ likely contributed to positive post-translational regulation, facilitating proper protein folding, resulting in a stable and active conformation, and preventing the activation of degradation pathways for misfolded proteins (Akpinar and Ozturk Urek, 2017). In accordance, it was showed that the presence of copper is also responsible for regulating the production of laccases, demonstrating the importance of this cofactor for this enzyme (Durán-Sequeda et al., 2021).

The SDS-PAGE analysis of the active samples from each *K. phaffii* laccase induction time, followed by purification of the recombinant laccase (Figure S2), showed an approximately 65 kDa protein band, which matches with the predicted molecular mass of the MCO12 laccase, that was 64.64 kDa. These results confirm that the MCO12 laccase from *C. cubensis* was successfully cloned and expressed in *K. phaffii.*

### 3.2 Characterization of the laccase MCO12 from *C. cubensis* laccase expressed in *K. phaffii*

The recombinant laccase showed the highest activity in the pH range between 4.0 and 5.0, in which it retained more than 80 % of activity. In pH below 3.0 and above 6.5, its activity was practically null (Figure 1A). The analysis of pH stability showed that this recombinant enzyme had considerable activity in the pH values from 3.0-8.0, indicating that this laccase was able to renature and regain activity even after 1 hour of pre-incubation in these pH values (Figure 1A).

The recombinant laccase maintained more than 80 % of its activity in the temperature range of 55 to 70 °C, showing highest activity at 60 °C (Figure 1B). Interestingly, the recombinant laccase showed an increase in the temperature of greatest activity compared to the native one, going from 55 to 60 °C (Gomes et al., 2024, submitted). Furthermore, this recombinant laccase was significantly thermostable and showed half-life values of 142, 45 and 11 h at 50, 60 and 70 °C, respectively (Figure 1C). On the other hand, the native MCO12 laccase showed half-lifes of 4.83 h, 22 min and 3 min at 45, 55 and 65 °C, respectively and highest activity in the pH range between 2.5 and 3.0 (Gomes et al., 2024, submitted). The thermostability of an enzyme is one of the main characteristics that determine its potential from the point of view of industrial applications. Enzymes more thermostable are extremely valuable in applications that require long-term incubation, such as saccharification and dye decolorization (Maitan-Alfenas et al., 2015; Yang et al., 2020; Zhang et al., 2021).

The results of the recombinant MCO12 laccase, compared with those obtained for the native form, demonstrate that the heterologous expression resulted in an increase in enzyme expression and also in an improvement in its biochemical properties, with emphasis on stability at neutral/alkaline pH and thermostability. The difference in stability between native and recombinant enzymes can be attributed to post-translational modifications, caused by changes in the expression organism, changes in the signal peptide and also by the exchange of some amino acids (Garg et al., 2012). In addition to post-translational modifications that may occur due to signal peptide exchange and alterations in the glycosylation pattern, specific changes were made to the recombinant enzyme. Two arginines were replaced with lysines to eliminate the Kex2 protease cleavage site, involved in the removal of the α-mating factor signal peptide in *K. phaffii*, and a codon for glycine was added at the beginning of the sequence to ensure that the amino acid residues of the MCO12 laccase were in phase with the signal peptide (Khlebodarova et al., 2024). Given that arginine has a longer side chain, making it more flexible, the substitution of arginine with lysine, which has a shorter and more rigid side chain,

may reduce local flexibility in the protein, potentially leading to a more thermostable structure (Nadar and Rathod, 2019; Ryan and Ó'Fágáin, 2007).

The most promising sources of laccases for industrial applications are fungal and bacterial. Fungal laccases are notable for their higher redox potential, while some bacterial laccases tend to have greater tolerance to the extreme conditions found in industrial environments, such as those in the textile industry (Rodrigues et al., 2023)(Jeyabalan et al., 2023). Jeyabalan et al. (2023) highlight that one limitation of fungal laccases is their optimal temperature range, which typically varies from 30 to 55 °C. This limitation is overcome by the MCO12 laccase from *C. cubensis* expressed in *K. phaffii*, since this enzyme maintained more than 80 % of its activity from 55 to 70 °C. Additionally, the remarkable thermostability demonstrated at 50, 60, and 70 °C reinforces this laccase as a strong candidate for applications in extreme industrial conditions.

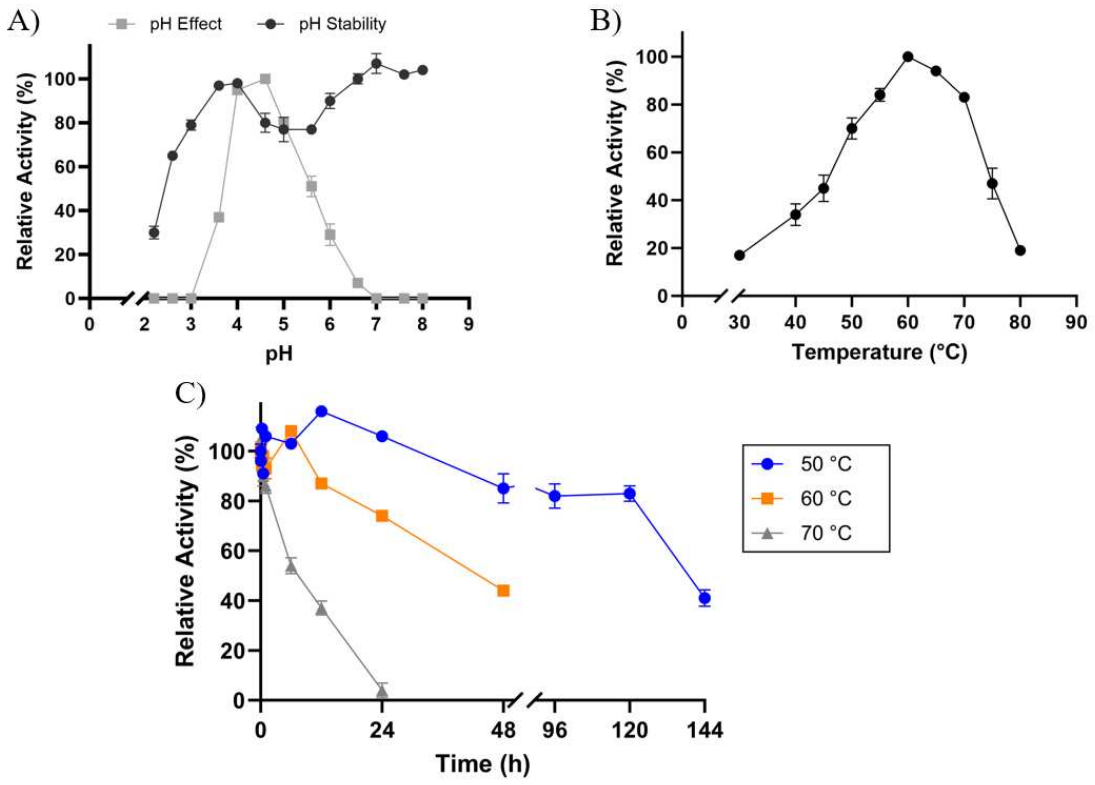



Figure 1 - Partial characterization of the recombinant MCO12 laccase. A) Effect of pH in the laccase activity and pH stability (2.2-8); B) Effect of temperature in the laccase activity (30-80 °C); and C) Thermostability at (●) 50 °C, (■) 60 °C and (▲) 70 °C.

### 3.3 Effect of ions and surfactants on recombinant MCO12 laccase activity

The analysis of the effect of ions and other compounds on recombinant laccase activity (Figure 2) showed that, at the concentrations tested, EDTA, SDS and $Fe^{2+}$ were able to completely inhibit the enzyme activity. Since laccase is a copper-dependent metalloenzyme,

the inhibition by EDTA was probably due to its chelating effect, which promoted the removal of the copper from the laccase. The SDS probably interacted with laccase to form a negatively charged SDS-laccase complex, leading to enzyme denaturation and loss of activity. The enzyme inhibition by EDTA and SDS was also reported for laccases from *Trichoderma harzianum* S7113 (Elsayed et al., 2023).

It is suggested that $Fe^{2+}$ interferes with the oxidation of the classical organic laccase substrates. In accordance with our results, the enzyme inhibition by $Fe^{2+}$ was reported for several laccases, such as laccase from *Morchella importuna* (Zhang et al., 2019)*;* laccase from *Laccaria bicolor* expressed in *K. phaffii* (Wang et al., 2016)*;* and laccase from *Trametes versicolor* (Asgher et al., 2012).

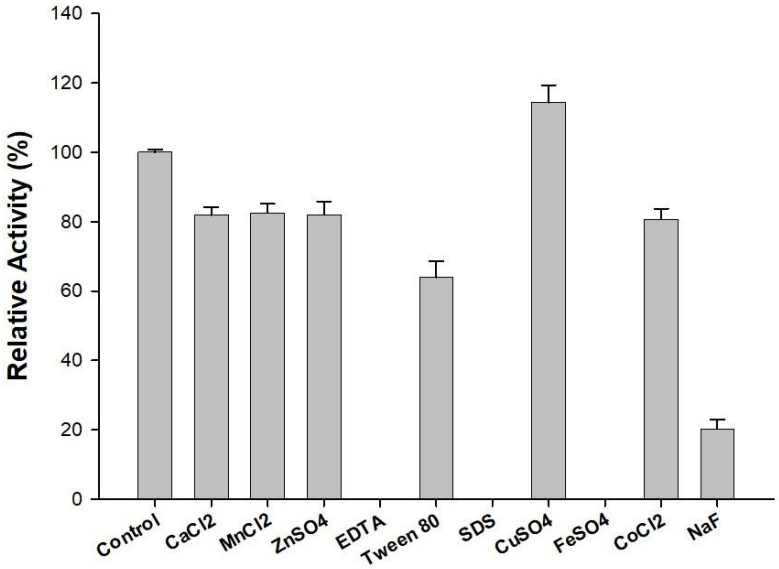



Figure 2 - Effect of ions and surfactants (10 mM) on recombinant MCO12 laccase activity.

Furthermore, the addition of NaF promoted a considerable impact by inhibiting 80 % of the recombinant MCO12 laccase activity, corroborating previous findings for laccases from *Thermus thermophilus* (Liu et al., 2015). Tween 80, $CaCl_2$, $MnCl_2$, $ZnSO_4$ and $CoCl_2$ showed a certain effect on laccase activity, suggesting a possible interference with the enzyme's access to the substrate.

Copper ($CuSO_4$) was the only reagent that exhibited a positive effect on the recombinant MCO12 laccase activity, resulting in an approximately 15 % increase enzyme activity. A similar increase in enzyme activity, around 15 %, was displayed by the laccase from the ascomycete fungus *Fusarium oxysporum* HUIB02 expressed in *K. phaffii*, also using a final

concentration of 10 mM $Cu^{2+}$ (Huy et al., 2021). These results indicate the importance of copper as an essential cofactor in the active site of the MCO12 laccase.

### 3.4 Molecular Modeling and Molecular Docking

The three-dimensional models of the native and recombinant MCO12 laccases, obtained by homology (Figure 3), were used in molecular docking with the substrate ABTS (2,6-dimethoxyphenol acid) to evaluate their interactions with copper in the T1 site. Among the interactions found, hydrogen bonds are the most significant (Figure 4), which allows for adequate positioning of the molecules during the oxidation process. Thus, the recombinant laccase presented a greater number of amino acids carrying out this type of interaction, demonstrating a more stabilized positioning with the substrate. The binding affinity of native and recombinant laccases with ABTS were −5.4 and −6.2 Kcal/mol, respectively (Table 1). These data indicate that the recombinant enzyme, in addition to having increased expression, exhibits greater binding affinity to ABTS than the native enzyme, resulting in higher substrate specificity.

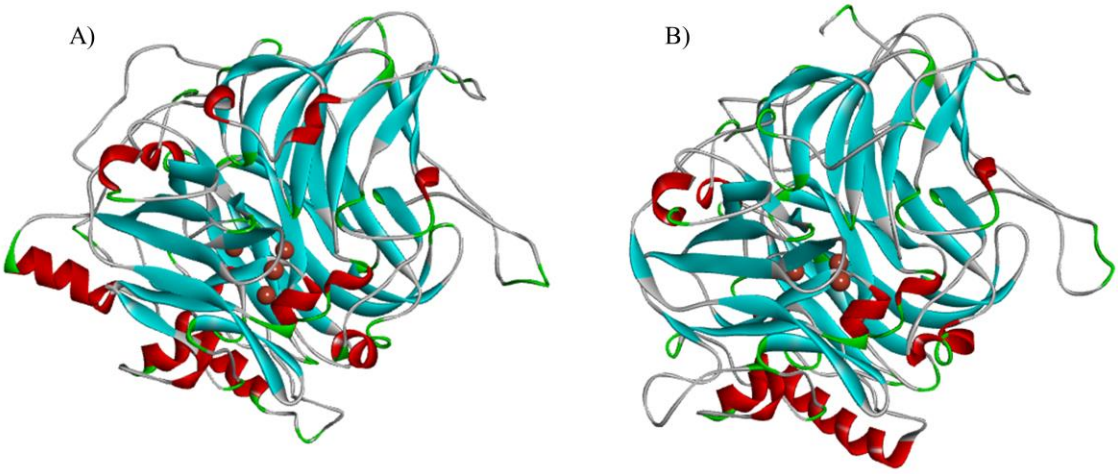



Figure 3 – Homology models of laccase protein sequences. A) Native MCO12 lacase; B) Recombinant MCO12 lacase.

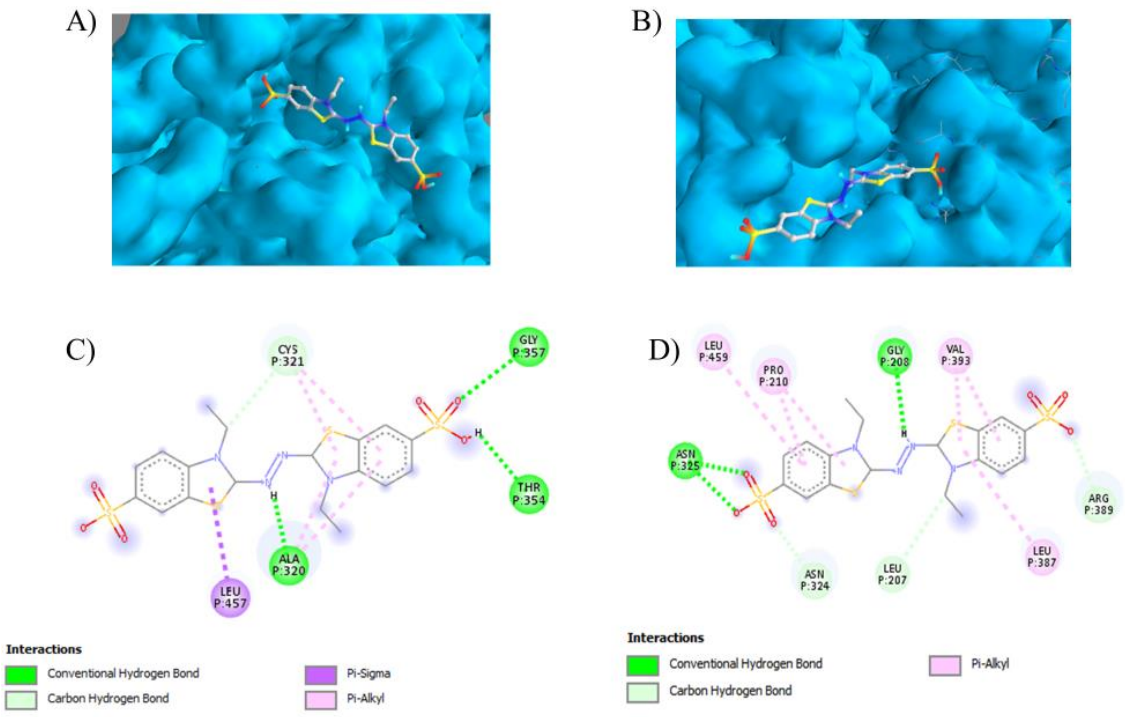
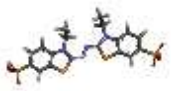



Figure 4 - Molecular docking. A) Surface diagram of docking between native lacase and ABTS; B) Surface diagram of docking between recombinant lacase and ABTS; C) 2D diagram of ligand-residue interactions between native lacase and ABTS using Discovery Studio Visualizer; D) 2D diagram of ligand-residue interactions between recombinant lacase and ABTS using Discovery Studio Visualizer.

Tabel 1 – Binding energy, number of hydrogen and amino acids involved in interaction between lacases and ABTS substrate.

| Compound | Laccase | Binding energy (Kcal/MOL) | N° of H bonds | Amino acids involved in interaction |
|---|---|---|---|---|
| ABTS  | Native | -5.4 | 4 | Ala 320, Cys 321, Thr 354, Gly 357 |
| | Recombinant | -6,2 | 6 | Leu 207, Gly 208, Asn 324, Asn 325 (2), Arg 389 |

### 3.5 Dye Decolorization

The potential of recombinant laccase MCO12 to promote dye discoloration was investigated, with representatives from the main chemical classes of dyes. Methyl Orange and Congo Red for the Azo class, Reactive Blue 4 for the Anthraquinone class, Coomassie Brilliant Blue G-250 and Malachite Green for the Triphenylmethane class. The decolorization assays were carried out during 96 h, at 50 °C and pH 4.0, using 0.01 mg of the dye and only 0.12 U of laccase. Control assays were carried out by replace the enzyme or the dye volumes by buffer. Considering the different classes of dyes tested, the MCO12 laccase expressed in *K. phaffii* demonstrated significant activity against malachite green and Congo red (Figure 5). After just

6 h of dyes treatment with laccase, malachite green and Congo red showed 34 and 39 % of decolorization, respectively. For malachite green, the laccase was able to decolorize nearly all the dye, achieving 99 % of decolorization after 72 h. For Congo red, the action of the enzyme resulted in 71 % of decolorization 72 h. The percentage of decolorization of the dyes did not increase after 72 h of treatment with laccase, and the values were maintained with 96 h of treatment.

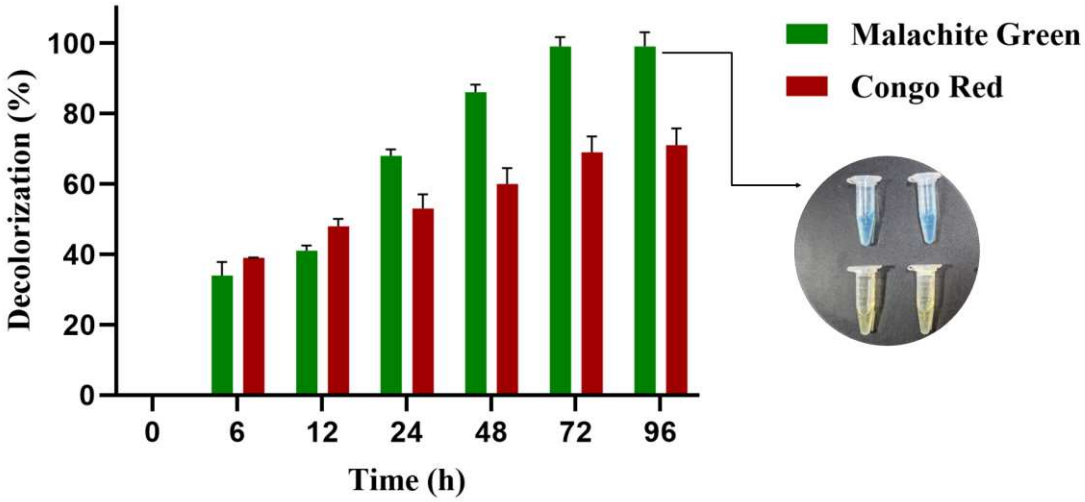



Figure 5 – Dye decolorization of Malachite Green and Congo Red by MCO12 recombinant laccase.

Malachite green is a synthetic dye belonging to the triphenylmethane class, widely used in various industrial sectors, including textiles, paper manufacturing, and aquaculture, due to its antimicrobial properties and low cost (Bian et al., 2024). The widespread use of malachite green in the dyeing industry, and the consequent contamination of aquatic environments, results in mutagenic, carcinogenic, and teratogenic effects in humans (Pandey et al., 2022; Himanshu et al., 2023). Therefore, the efficient degradation of this dye demonstrated by the recombinant MCO12 laccase offers a promising approach to remediate malachite green contamination, given the environmentally friendly nature of using the generally recognized as safe (GRAS) yeast *K. phaffii*.

Congo red is an azo dye, a class that represents up to 70 % of all textile dyes used, characterized by nitrogen-to-nitrogen double bonds (-N=N-) that are connected to at least one aromatic group, such as benzene or naphthalene (Herath et al., 2024). The aromatic structure of this dye imparts strong physico-chemical stability, making it difficult to degrade (Li et al., 2023). This dye is commonly found in industrial dyeing effluents of paper, textile, wood, and

cosmetics industries (Yan et al., 2022). Given the necessity of degrading this dye and the significant decolorization results achieved with the recombinant MCO12 laccase, the potential of this enzyme for application in this sector is evident.

It is important to highlight that the decolorization results achieved, particularly for Malachite Green, which reached 99 % after 72 h of incubation with recombinant MCO12 laccase at 50 °C, are noteworthy compared to existing literature. For example, the lcc1 laccase from *Trametes trogii* expressed in *Saccharomyces cerevisiae* decolorized 95 % of Malachite Green after 10 h of incubation at 30 °C (Song et al., 2024). Although expressive, this result was obtained at 30 °C, which is lower than the typical temperature of 50-60 °C at which dye treatment effluents are released (Das et al., 2023). Another study reported that an extracellular laccase from *Fusarium oxysporum* HUIB02 achieved about 90 % decolorization of Malachite Green after 24 h at 40 °C, using 1-3 mM of 1-hydroxybenzotriazole as a mediator and 1 mM of $Cu^{2+}$ as a cofactor (Thoa et al., 2022). While significant, the additional use of mediators and cofactors, increasing the cost of the process for industrial applications and these compounds can be toxic (Mani et al., 2018). Notably, MCO12 laccase, without the use of mediators, achieved 68 % of Malachite Green decolorization after 24 h at 50 °C, demonstrating its advantage for dye decolorization processes.

## 4- CONCLUSION

Overexpression of 246 U/L of the MCO12 laccase from *Chrysoporthe cubensis* expressed by *Komagataella phaffii* overcomes the challenge of low yields of laccases produced from native sources, meeting the demands of existing industrial processes. This recombinant expression not only resulted in higher yields but also enhanced the enzyme's temperature-related properties. The recombinant laccase retained 80 % of its activity across a temperature range of 55-70 °C, with an optimal activity temperature of 60 °C, higher than that of the native enzyme. Additionally, its thermostability was significantly improved, with half-lives of 11, 45, and 142 h at 70, 60, and 50 °C, respectively. These enhancements overcome the limitations of activity under extreme conditions commonly associated with fungal-derived enzymes. The heterologous enzyme also exhibited greater substrate specificity, with higher binding affinity and a greater number of hydrogen bonds to ABTS compared to the native form. With these improved properties, the recombinant laccase demonstrated high efficiency in the degradation of textile dyes, achieving 99 % decolorization for Malachite Green and 71 % for Congo Red after 72 h of incubation at 50 °C. These results underscore the potential of the MCO12 laccase

for industrial applications, particularly in environments requiring high thermal resistance, such as dye treatment in the textile industry.

## 5 – ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

Table S1. Enzymatic activity (U/mL) of the expression assay of *Komagataella phaffii* clone 4 transformed with MCO12 laccase, selected in the plate containing 250 µg/mL of zeocin, at different times of induction with methanol (0-96h).

| | **Time (hours)** | | | | |
|---|---|---|---|---|---|
| | **0** | **24** | **48** | **72** | **96** |
| **Enzymatic activity (U/mL)** | 0 | $2.87.10^{-4}$ | $1.04.10^{-3}$ | $1.18.10^{-1}$ | $2.46.10^{-1}$ |

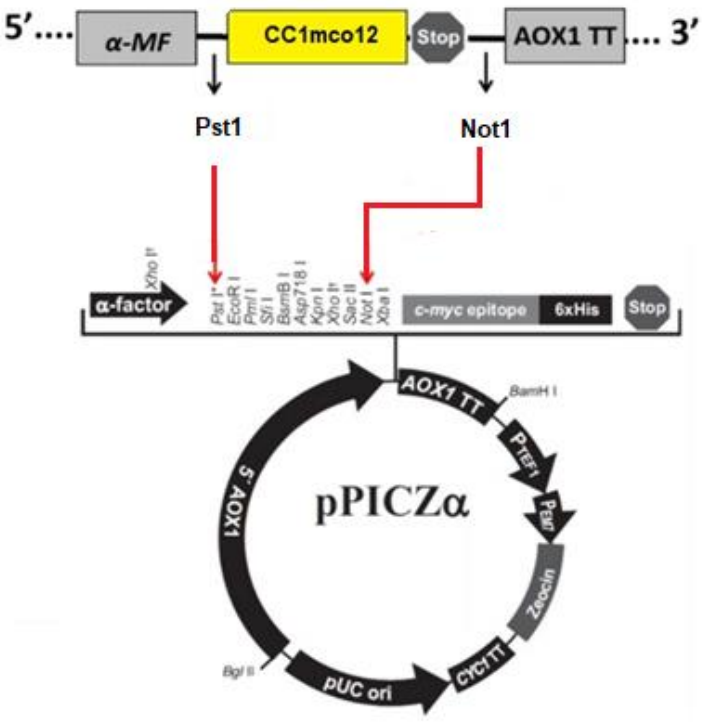


Figure S1. pPICZαB vector containing the mco12 laccase gene and Pst1 and Not1 restriction sites.

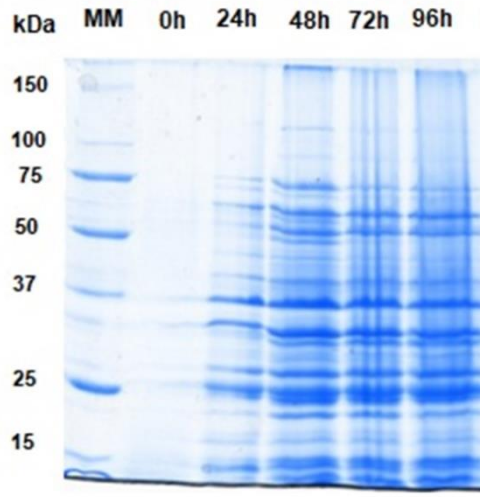


Figure S2. SDS-PAGE of samples obtained after the MCO12 laccase expression assay in *Komagataella phaffii*, clone 6, selected from the plate containing 250 µg/mL of zeocin. MM- molecular weight marker; 0 - 96h of induction.

## 4. CONSIDERAÇÕES FINAIS

O presente trabalho demonstrou que estudos envolvendo lacases de fungos ascomicetos aplicadas em processos industriais ainda são bastante limitados. Apesar da escassez desses estudos, lacases de ascomicetos demonstraram ser enzimas versáteis, com representantes sendo aplicados nos setores de biorremediação, têxtil, biocombustíveis, alimentos, biossíntese e biossensores. Entretanto, ainda existe uma demanda por lacases que exibam propriedades que atendam às demandas e condições adversas de vários processos industriais, como a descoloração de corantes têxteis.

Na busca por novas lacases, o fungo ascomiceto *Chrysoporthe cubensis* foi cultivado em casca de café, o que resultou na indução e produção da lacase MCO12, uma das 13 multicobre oxidases preditas no genoma desse fungo. Após purificação parcial, caracterização e identificação dessa enzima, a lacase exibiu propriedades relevantes, como uma termoestabilidade significativa a 45-55 °C, faixa de temperatura comumente empregada nos processos de biorremediação de corantes têxteis. O potencial desta lacase para promover a descoloração de diferentes corantes foi avaliado em testes usando baixa carga enzimática em comparação com aquelas normalmente usadas na descoloração de corantes. A lacase MCO12 descoloriu com sucesso representantes das principais classes químicas de corantes usados na indústria têxtil. Estes resultados permitiram confirmar o potencial da lacase MCO12 de *C. cubensis* para aplicações biotecnológicas, especialmente na biorremediação de corantes.

Buscando solucionar o problema da baixa expressão da lacase no organismo nativo, a lacase MCO12 de *C. cubensis* foi expressa em *Komagataella phaffii,* atingiu níveis de atividade enzimática de 246 U/L. Essa expressão heteróloga não apenas resultou em rendimentos mais altos, mas também propiciou a melhora das propriedades da lacase MCO12 relacionadas à estabilidade. A lacase recombinante reteve 80 % de sua atividade em uma faixa de temperatura de 55-70 °C, aumentando a temperatura de atividade ótima de 55 ºC na forma nativa para 60 °C. Além disso, sua termoestabilidade foi significativamente melhorada, com tempos de meia-vida de 11, 45 e 142 h a 70, 60 e 50 °C, respectivamente. Essas melhorias contribuem para superar as limitações de atividade da enzima sob condições extremas, o que constitui um desafio comumente associado às enzimas derivadas de fungos. A enzima heteróloga também exibiu maior especificidade pelo substrato ABTS, com maior afinidade de ligação e um maior número de ligações de hidrogênio, em comparação com a forma nativa. A lacase recombinante demonstrou alta eficiência na degradação de corantes têxteis, alcançando 99 % de descoloração para Verde Malaquita e 71 % para o Vermelho Congo, após 72 h de incubação a 50 °C. Esses resultados ressaltam o potencial da lacase MCO12 para aplicações

industriais, particularmente em ambientes que exijam alta estabilidade térmica, como tratamento de corantes na indústria têxtil.