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Unraveling modular architecture and domain engineering of carbohydrate-active enzymes: key insights for sustainable bio-based processes

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Chapter 5

Streptomyces Small Laccase Expressed in *Aspergillus niger* as a New Addition for the Lignocellulose Bioconversion Toolbox

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Abstract

Laccases are multi-copper oxidases that are usually composed of three Cu-oxidase domains. Domains one and three house the copper binding sites, and the second domain is involved in forming a substrate-binding cleft. However, *Streptomyces* species are found to have small laccases (SLAC) that lack one of the three Cu-oxidase domains. This type of SLAC with interesting lignocellulose bioconversion activities has not been reported in *Aspergillus niger*. In our research, we explored the expression and engineering of the SLAC from *Streptomyces leeuwenhoekii* C34 in *A. niger*. Genes encoding two versions of the SLAC were expressed. One encoding the SLAC in its native form and a second encoding the SLAC fused to two N-terminal CBM1 domains. The latter is a configuration also known for specific yeast laccases. Both SLAC variants were functionally expressed in *A. niger* as shown by *in vitro* activity assays and proteome analysis. Laccase activity was also analyzed toward bioconversion of lignocellulosic rice straw. From this analysis it was clear that the SLAC activity improved the efficiency of saccharification of lignocellulosic biomass by cellulase enzyme cocktails.

Keywords: Small laccase, *Streptomyces*, *Aspergillus niger*, protein-domain engineering, lignocellulose degradation, carbohydrate binding module

5.1 Introduction

Laccases are categorized as multi-copper oxidases (MCO) belonging to the Auxiliary Activities from family 1 (AA1) in the Carbohydrate-Active Enzymes (CAZy) database (Drula et al., 2022). They play a dual role in catalyzing the oxidation of a wide range of both phenolic and non-phenolic compounds, including lignin (Khan et al., 2024; Zhang et al., 2021). Phenolic compounds are oxidized through direct electron transfer from the enzyme to these phenolic substrates, while nonphenolic substrates can also be oxidized with the assistance of oxidation mediators which facilitate the electrons transfer to non-phenolic substrates, thereby enabling their degradation (d'Acunzo et al., 2002; Rangelov and Nicell, 2018). Lignin primarily consists of phenolic and some non-phenolic compounds (Vasile and Baican, 2023). Since lignin is embedded in lignocellulose, the intricate structure of lignin acts as a barrier for cellulolytic enzymes attempting to access the (hemi)-cellulose chains within lignocellulose. Therefore, laccases are pivotal players in the intricate process of lignocellulose breakdown, holding a significant interest in various industrial applications, including the production of biofuels and other value-added biobased products.

From the CAZy database, it is clear that laccases have been identified in diverse organisms, including fungi and bacteria (Drula et al., 2022). Fungal laccases are widely represented explored in many basidiomycete and ascomycete fungi, while research on bacterial laccase is mainly focused on *Streptomyces*, *Bacillus*, *Pseudomonas* (Guan et al., 2018; Zhang et al., 2021).

According to the molecular architecture, a typical laccase consists of three Cu-oxidase domains holding a total of 4 copper ions. In three-domain laccase, domain 1 and 3 harbor the four copper ions, whereas domain 2 is involved in substrate binding (Camarero et al., 2012; Hakulinen and Rouvinen, 2015; Mehra et al., 2018). In contrast to this domain arrangement, the genomes of various *Streptomyces* encode smaller laccases which lack one of the Cu-oxidase domains (typically domain 2 in the three-domain laccase), known as small laccase (SLAC) (Machczynski et al., 2004). *Streptomyces* two-domain laccases exhibit unique properties, such as high stability at a wide range of temperatures and pH-values. Furthermore they demonstrated activity on a broad range of substrates including lignin (Blánquez et al., 2017; Endo et al., 2003; Gunne and Urlacher, 2012; Kaur et al., 2022; Lu et al., 2013; Machczynski et al., 2004; Majumdar et al., 2014) which makes them interesting enzymes for lignocellulose bioconversion.

Many CAZY enzymes in particular of the GH families have a modular domain architecture, with the enzyme domains linked to an accessory domain involved in carbohydrate binding such as Carbohydrate Binding Modules (CBM). CBMs are non-catalytic domains that are commonly associated with enzymes participating in the degradation of complex insoluble substrates (Bernardes et al., 2019b; Boraston et al., 2004). The CBM domain exhibits a high affinity for carbohydrate structures, hence enhancing the proximity of the enzyme and its substrate, thereby promoting substrate degradation (Duan et al., 2017; Monica et al., 2023; Telke et al., 2012). The fusion of CBM1 at the C-terminus of the three-domain laccase from *Pycnoporus cinnabarinus* expressed in *A. niger* was less thermostable than the native laccase but improved its efficiency for softwood kraft pulp biobleaching (Ravalason et al., 2009). In *Aspergillus*, the CBM from family 1 (CBM1) is the sole CBM family identified in combination with cellulases (Sidar et al., 2020). However, CBM1 has not been identified in laccases from *Aspergillus* (Drula et al., 2022; The UniProt Consortium, 2023). Recent studies have increasingly focused on the use of filamentous fungi for the functional characterization of laccase enzymes and their application in biomass conversion. These studies have demonstrated the potential of filamentous fungi such as *Aspergillus* and *Trichoderma* species to express laccase with high efficiency and stability, which are crucial for various industrial applications including biomass conversion and bioremediation (dos Santos et al., 2016; Gomaa and Momtaz, 2015; Huang et al., 2024; Zhang et al., 2012). For instance, laccase expressed in *Trichoderma reesei* resulted in improved lignocellulosic biomass saccharification from corn cob residue (Zhang et al., 2012). Moreover, expression of chimeric fungal laccase in *A. niger* enhanced lignocellulose bioconversion (Ravalason et al., 2009).

Given the unique domain arrangement and characteristics of the SLAC from *Streptomyces*, especially considering its absence in *A. niger* genome, it is of great interest to investigate the expression of engineered SLAC in *A. niger* for an improved fungal enzyme cocktail in lignocellulose degradation. *A. niger* is well-known as an enzyme producer, including cellulases (de Vries and Visser, 2001), making it an ideal host for this investigation. Furthermore, *A. niger* has been frequently used as a host for three-domain laccase production (Bohlin et al., 2006; Piscitelli et al., 2010; Ramos et al., 2011; Ravalason et al., 2009) but no reports have been published on the utilization of *A. niger* as host for bacterial SLAC production. Hence, this study presents the novel heterologous expression of engineered SLAC variants in *A. niger*. The activity of the SLAC variants toward lignocellulose was investigated using rice straw biomass as lignocellulosic substrate.

5.2 Materials and Methods

5.2.1 Microbial strains, plasmid and culture medium.

A.niger strain MGG029 *pyrE* (Weenink et al., 2006) was used as the transformation host for heterologous expression of SLAC genes, while *Escherichia coli* DH5 α was employed for plasmid propagation (Sidar et al., 2023). *E. coli* DH5 α was grown on Luria–Bertani medium supplemented with ampicillin at 100 μ g/mL. Plasmid pMA351 containing the *gpdA* promoter and *trpC* terminator from *Aspergillus nidulans*, was derived from pAN52-1Not (28, 29) and was used for SLAC gene construction. Additionally, a plasmid containing the *A. niger pyrE* gene (An04g08330) including promoter and terminator region was amplified from the genomic DNA of *A. niger* N402 as a template using primers pyrEP13f (5'-GACGTGTCGTTCCGGTATCC) and pyrEP14r (5'ACTGTGCCGCATCCCAAT), for selection marker in fungal transformation. Minimal medium (MM) and complete medium (CM) were utilized for culturing the *A. niger* strains.

5.2.2 Gene design and expression vector construction

The SLAC gene from *S. leeuwenhoekii* C34 (WP_029383517) was used as the basis to generate two versions of engineered SLAC, including native SLAC (SLAC) and a chimeric variant with two N-terminal CBM1 domains (CBM-SLAC), respectively. In CBM-SLAC, the sequence of the two CBM1 domains including the signal peptide was retrieved from the yeast laccase of *Rhodotorula toruloides* NP11 (EMS24156), which is naturally connected to two CBM1s. While, for the native SLAC, the signal sequence from *A. niger* laccase (Uniprot accession: A2QGL7) was used for secretion. Protein domain analysis to demarcate the various domains was performed using HMMER program (Version 3.3.2) (Potter et al., 2018). Signal sequence was predicted using SignalP 6.0 (Teufel et al., 2022). SLAC with and without CBM1 were codon optimized for *A. niger* expression and synthesized by Invitrogen GeneArt and BaseClear, respectively. For generating the expression vectors, each SLAC variant was cloned into NcoI-BamHI-linearized pMA351 plasmid. The final constructs with SLAC variants were verified by sequencing a PCR product (Macrogen Europe B.V), from a pair of primers MBL852-forward (5'-GCTACATCCATACTCCA) and MBL858-reverse (5'-ATATCCAGATTTCGTC AAGCTG). These primers amplify the *gpdA* promoter sequence, the inserted SLAC open reading frame, and part of the *trpC* terminator.

5.2.3 Fungal transformations and cultivation

For fungal transformation, the expression vectors of SLAC variants and selection marker vector of *A. niger pyrE* gene were co-transformed into *A. niger* MGG029 *pyrE*⁻, following the protocol outlined in (Arentshorst et al., 2012). *A. niger pyrE*⁺ transformants were selected on minimal media containing 0.95 M sucrose as an osmotic stabiliser (Arentshorst et al., 2012). Twenty *pyrE*⁺ transformants were purified by single colony streaks of conidial spores on minimal medium and incubated for 3 days at 30°C. Subsequently, these *A. niger* transformants were grown on complete medium agar for 3 days at 30°C for spore production followed by spore isolation with the addition of 10 mL of physiological saline solution (0.9% NaCl dissolved in milliQ water) to the plate as well as gently scraping with a sterile cotton stick. The spore suspension was then transferred to a 15 mL screw-cap tube and stored at 4°C. The spore suspension was then used for cultivation and screening of the putative laccase transformants. Cultivation was carried out by submerged fermentation using 300 mL Erlenmeyer flasks with a 100 mL working volume of complete medium supplemented with 0.01 mM CuSO₄·7H₂O, inoculated with 1 × 10⁸ *A. niger* transformant spores, and incubated at 180 rpm, 30°C for 3 days. The culture medium was collected by filtration. The culture medium were tested for laccase activity with N,N-Dimethyl-p-phenylenediamine sulfate salt (DMPPDA) as a substrate (Endo et al., 2003).

5.2.4 DMPPDA enzymatic assay

For the screening of the laccase-producing transformants, 200 µL of the culture medium were mixed with 800 µL DMPPDA solution in 1.5 mL Eppendorf tube, as essentially described (Endo et al., 2003). DMPPDA solution contained 0.125 mg/mL DMPPDA and 0.125 mg/mL 1-naphthol in 100 mM NaPi pH 6.8 buffer. The assay solution was incubated at 30°C for 30 minutes. The presence of laccase activity was visualized by the formation of a blue precipitate. As a negative control the culture medium from the parental strain, *A. niger* MGG029 transformed with an empty expression vector was used.

5.2.5 Effect of SLAC on lignocellulosic rice straw degradation

To measure the effect of the presence of SLAC on the degradation of rice straw the following reaction mixture was prepared: 5 mg of pre-treated rice straw prepared according to (Sidar et al., 2024), 1 endo-glucanase unit (EGU) of cellulase (*Trichoderma reesei*, ATCC 26921, Sigma-Aldrich C2730), 100 μ L culture medium from the *A. niger* co-transformant that was 5 times concentrated using a spin filter (Vivaspin, 10000 MWCO, VS0102) and 400 μ L of 100 mM NaPi buffer (pH 6.8) in a 1.5 mL Eppendorf tube. For the pretreatment of rice straw, alkaline-based method was conducted using 2% NaOH solution at a 1:10 ratio (1 g rice straw fibers were soaked in 10 mL of 2% NaOH). Subsequently, the rice straw fibers were washed with distilled water to neutralize the pH and then dried overnight in an incubator at 60°C until they reached a constant weight, following the detailed pretreatment method described by (Sidar et al., 2024). The control samples had no culture medium added or an aliquot of culture medium of the transformed with the empty expression vector. The reaction mix was briefly vortexed and incubated at 30°C. Samples (200 μ L) were taken after 8 hours, 24 hours and 48 hours of incubation. Subsequently, the amount of reducing sugars released was determined with the DNS assay, as essentially described in (Miller, 1959). To remove remaining rice straw 50 μ L of each time point sample was centrifuged and 25 μ L of supernatant was mixed with 25 μ L of DNS reagent. The DNS reagent comprises 1% 3,5-dinitrosalicylic acid, 0.05% sodium sulfite, and 1% sodium hydroxide. The samples were incubated at 100°C for 10 min. The reaction was stopped by adding 30 μ L of sodium-potassium tetrahydrate solution (40% w/v). The absorbance was measured at 540 nm (TECAN, Spark 10M) and plotted against a standard curve prepared with 0 – 10 mM glucose to quantify the amount of reducing sugar released (mM). The measurements were done in triplicate.

5.2.6 SDS-PAGE and Zymogram analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% precast polyacrylamide gels (Bio-Rad, Mini-PROTEAN® TGX™, #4561033) as described in (Sidar et al., 2023). The *A. niger* transformants culture medium sample was mixed with SDS-PAGE loading buffer containing 50 mM Tris-HCL pH 6.8, 25% glycerol, 0.05% bromophenol blue, 1% SDS, in a 3:1 ratio, without boiling and then loaded into the precast polyacrylamide gel. As a molecular weight standard, 5 μ L unstained protein

marker (Bio-Rad, precision plus protein unstained standard #161-0363) was loaded as well. The electrophoresis was run at 120 V for 1 hour at room temperature. Afterwards, the SDS-PAGE gel staining process was conducted as addressed in (Sidar et al., 2023) using Sypro Ruby staining solution (Bio-Rad, #1703125). The Gel imaging was performed with the BioRad GelDoc™ EZ Imager.

SLAC zymogram analysis was carried out based on activity staining using DMPPDA substrate as essentially described in (Endo et al., 2003). Sample preparation and PAGE electrophoresis was conducted as addressed in SDS-PAGE procedure above. For a molecular weight standard, the prestained protein ladder ranging from 10 to 250 kDa was used (Thermo Scientific™, #26619). Upon electrophoresis, the gel was incubated in 100 mM NaPi pH 6.8 buffer for 1 hour while shaking gently using a rocking shaker (VWR®) at room temperature. The buffer was refreshed every 20 minutes. Afterwards, the gel was incubated at 37°C for 15 minutes using 100 mM NaPi pH 6.8 buffer. The buffer solution was discarded and the gel was overlaid with 50 mL of DMPPDA-solution and incubated for 15 minutes at 37°C. The SLAC activity was visible directly on the gel from the formation of a blue precipitate at the position of the laccase protein bands.

5.2.7 Western-blot of SLAC

The presence of SLAC was confirmed using a western-blot as essentially described according to the instruction manual (Bio-Rad, Mini-Trans-Blot Electrophoretic Transfer Cell), using polyvinylidene difluoride (PVDF) membrane and polyclonal SLAC antiserum. Polyclonal antibodies against the small laccase were raised in rabbits using purified SLAC of *S. coelicolor* as antigen (Vijgenboom., unpublished). SLAC was purified following overproduction in *E. coli* (Machczynski et al., 2004). Rabbit serum containing the SLAC antibodies was used in a 1:5000 dilution. The small laccase in Western blots was detected using an anti-rabbit IgG alkaline phosphatase secondary antibody (sigma, A8025), diluted 1:5000 and BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium) as substrates. Furthermore, protein samples were run on SDS-PAGE as mentioned in SDS-PAGE method section above (without boiling and with 5 minutes boiling for the denatured sample), and transferred onto PVDF membrane. For blocking, 5% non-fat milk was used and as secondary antibodies, goat-anti-rabbit-alkaline phosphatase (SIGMA A3812) was used.

5.2.8 Cellulose binding analysis

Cellulose binding was carried out based on the method described in (Sidar et al., 2023; van der Kaaij et al., 2007). The evaluation of binding was confirmed by western blot and testing the activity in the culture medium, the unbound fraction, and the eluted protein fraction using DMPPDA assay and Zymogram.

5.2.9 Proteomic analysis

Samples for proteomic analysis were prepared from the culture medium of SLAC and CBM-SLAC transformants grown for 3 days. The culture medium was collected by filtration to remove *A. niger* biomass. Subsequently, 2 mL of culture medium was 10x concentrated through ultrafiltration using a spin column with 3 kDa molecular cut-off membrane (Amicon® Ultra-15 Centrifugal Filter Units, MWCO 3 kDa). In total 200 µL of the concentrated culture medium was dissolved in 800 µL of 20 mM phosphate buffer. The MS/MS analysis was carried out by Alphalyse (Alphalyse A/S, Odense, Denmark). The tandem mass spectrometry (MS/MS) data converted into mgf format were analyzed using X! Tandem version 17-02-01-4 (Craig and Beavis, 2004).

5.3 Results and Discussion

5.3.1 Modular architecture of laccases from *Streptomyces* and *Aspergillus* genera

For the exploration of the laccase modular architecture, sequence datasets of laccases from *Streptomyces* and *Aspergillus* were retrieved from Uniprot database, accessed on December 2023 (Additional file 1). In *Aspergillus*, to date a comprehensive analysis revealed 708 sequences identified as three-domains of multicopper oxidases / laccases. A parallel investigation in *Streptomyces* identified 748 sequences predicted to be three-domain laccase as well. In addition, for *Streptomyces*, 871 sequences were specifically classified as two domain multicopper oxidases, also called small-laccases, which are absent from *Aspergillus* and any other filamentous fungi. The modular-domain architecture represented by the different laccases found in the genome of *A. niger* CBS 513.88 and *S. leeuwenhoekii* C34 is shown in Fig. 1.

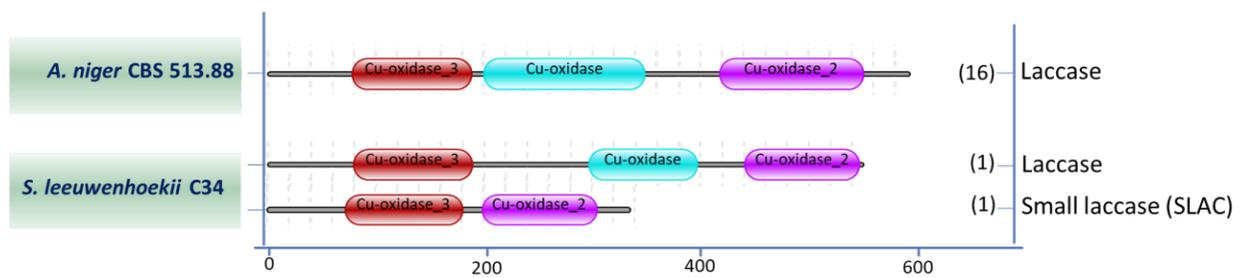


Fig. 1. Domain organization of laccases in the genome of *A. niger* CBS 513.88, and *S. leuvenhoekii* C34. The number of identified laccase sequences from *A. niger* CBS 513.88 and *S. leuvenhoekii* C34 are presented in brackets. The domain annotation was predicted based on Pfam domains using the HMMER program (Potter et al., 2018). According to Pfam database, in three-domain laccase, the three domains were defined as Cu-oxidase_3 (in red), Cu-oxidase (in blue), and Cu-oxidase_2 (in purple), respectively. Whereas, in two-domain laccase (SLAC), one of Cu-oxidase domains (domain highlighted in blue in laccase) is absent. The Uniprot accession number of these 16 laccases of *A. niger* CBS 513.88 as well as 1 laccase and 1 SLAC for *S. leuvenhoekii* C34 are included in Additional file 1.

Three-domain laccases typically consist of three cupredoxin domains, of which according to Pfam protein family database are defined as Cu-oxidase_3, Cu-oxidase, and Cu-oxidase_2, respectively (Fig. 1). Based on the crystal structure the Cu-oxidase_3 and Cu-oxidase_2 domains are responsible for the catalytic function (Arregui et al., 2019). Within these domains, two catalytic copper sites have been identified, a tri-nuclear cluster consisting of a single type-2 (T2) and a pair of type-3 (T3) copper ions located at the interface of Cu-oxidase_3 and Cu-oxidase_2 and a mono-nuclear center containing type-1 (T1) copper ion in the Cu-oxidase_2 domain (Hakulinen and Rouvinen, 2015; Mehra et al., 2018). Previous studies have indicated that the T1 copper ion plays a role in the initial substrate oxidation process, hence receiving electrons from the reducing substrate. Subsequently, these electrons are transferred from T1 copper ion to the tri-nuclear copper cluster, facilitating dioxygen reduction into water (Arregui et al., 2019; Solomon et al., 2001). Moreover, several amino acid residues close to the T1 copper center have been implicated in substrate binding (Camarero et al., 2012; Hakulinen and Rouvinen, 2015; Mehra et al., 2018). Additionally, the middle Cu-oxidase domain of three-domain laccase plays a role in bridging the other two domains, facilitating the proper 3-dimensional structure for the formation of the tri-nuclear cluster (Arregui et al., 2019; Hakulinen and Rouvinen, 2015).

On the other hand, SLAC is characterized by its reduced size lacking one of Cu-oxidase domains compared to the typical three-domain laccases (Fig.1). Despite their smaller size, the SLAC still maintains the essential cupredoxin-like domains and copper centers, contributing to their catalytic activity (Gabdulkhakov et al., 2019; Trubitsina et al., 2015; Worrall and Vijgenboom, 2010). The mono-nuclear copper site of SLAC from *S. coelicolor* resides in the Cu-oxidase_2 domain as is the case in the three-domain laccases (Hakulinen and Rouvinen, 2015; Skálová et al., 2009). To form a catalytically active structure with a tri-nuclear site, SLAC needs to form a dimeric or trimeric structure where this catalytic site is at the interface between the N-terminal domain of one monomer and the C-terminal domain of another monomer (Hakulinen and Rouvinen, 2015; Skálová et al., 2009). This configuration compensates for the absence of one Cu-oxidase domain, which is the second cupredoxin domain in three-domain laccase.

Moreover, the T1 copper site of the Cu-oxidase_2 of SLAC is important for substrate binding, suggesting different substrate interaction compared to that of three-domain laccases. The smaller size and enzymatic characteristics of the SLAC such as broad pH and temperature range, wide substrate acceptance, and inhibitor resistance, make them interesting enzymes for biotechnological applications such as lignin degradation (Blánquez et al., 2017; Dubé et al., 2008; Kaur et al., 2022; Machczynski et al., 2004; Majumdar et al., 2014; Molina-Guijarro et al., 2009).

Both *Aspergillus* and *Streptomyces* have been well documented as laccase-producing organism (Kaur et al., 2022; Ramos et al., 2011; Välimets et al., 2023). However, as SLAC-encoding genes were absent in the *A. niger* genome (Fig. 1), we explored *S. leeuwenhoekii* SLAC expression in *A. niger*, whereby we also engineered a SLAC variant containing CBM1 domain. CBM1 has been known for its ability to bind specifically to crystalline cellulose, a component found alongside lignin in complex lignocellulosic substrates (Bernardes et al., 2019b). The C-terminal CBM attachment to fungal laccase has been effective in improving pulp bio-bleaching (Ravalason et al., 2009). Incorporating CBM1 into SLAC was expected to guide the SLAC to anchor more effectively towards complex lignocellulosic substrate, thereby increasing SLAC proximity to lignin which facilitating lignin degradation.

5.3.2 The design of SLAC variants and screening of co-transformants

Two variants of SLAC-encoding genes were generated, one with and one without CBM1-encoding domains, defined as CBM-SLAC and SLAC, respectively (Fig. 2). For the design of SLAC minus CBM1 (Fig. 2A), the SLAC sequence originated from *S. leuwenhoekii* C34 (WP_029383517) and the native signal peptide was substituted with that from *A. niger* CBS 513.88 laccase (Uniprot accession: A2QGL7) that is known to be secreted (Ramos et al., 2011) (Additional file 2: Fig.S1A, for the sequence). For the chimeric SLAC-encoding gene with two CBM1s (Fig 2B), the design was inspired by the modular domain of a yeast laccase carrying two CBM1 domains at the N-terminus (EMS24156) (Additional file 2: Fig.S1B, for the sequence). The yeast signal peptide along with its two CBM1s encoding sequence was retrieved from *Rhodotorula toruloides* NP11 (EMS24156), while the gene encoding the SLAC was obtained from *S. leuwenhoekii* C34 (WP_029383517). The coding sequences for both SLAC variants were obtained by gene synthesis and cloned in plasmid pMA351 in between the constitutive *gpdA* promoter and *trpC* terminator as described in the Methods section.

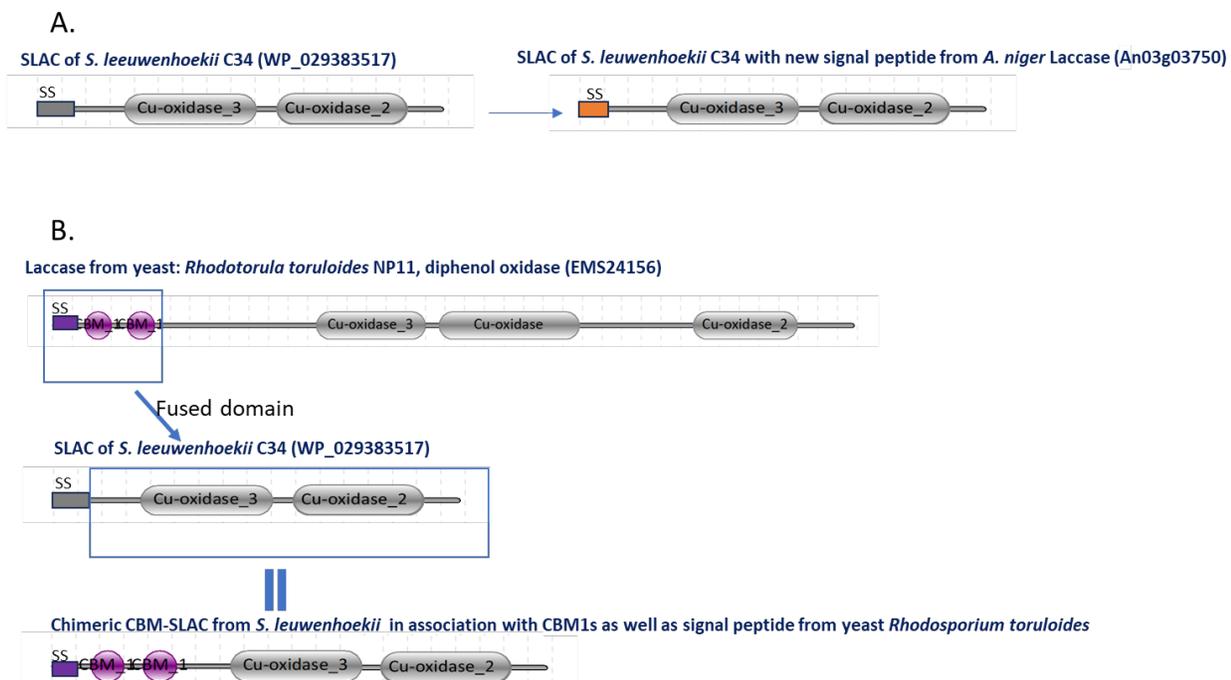


Fig. 2. Modular design of engineered SLAC variants. (A) SLAC variant without CBM domain (SLAC). (B) Chimeric SLAC assembled with two CBM1 domains at the N-terminus (CBM-SLAC).

Subsequently, each SLAC gene variant was transformed into *A. niger* MGG029. To identify the best performing transformants, an activity-based screen was conducted on around twenty putative co-transformants of SLAC (Additional file 2: Fig. S2A) and CBM-SLAC (Additional file 2: Fig. S2B). The activity screen used DMPPDA as substrate and culture medium collected from submerged fermentation of the co-transformants as enzyme source. Several transformants showed laccase activity (Additional file 2: Fig. S2). One Co-transformant from each laccase variant showing the most intense activity, CBM-SLAC#22 and SLAC#14, was selected for further research (Fig. 3). Moreover, colony PCR was carried out to confirm the presence of the SLAC gene variants introduced in *A. niger* transformants (Additional file 2: Fig. S3).

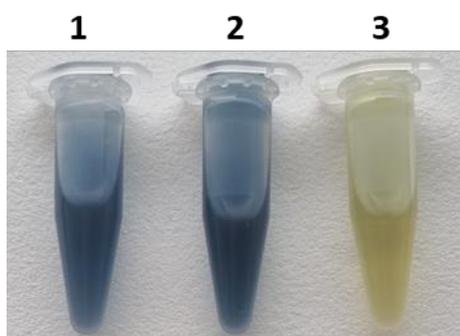


Fig. 3. Activity of SLAC variants based on DMPPDA assay using the culture medium from the cultivation of selected putative co-transformants. 1: CBM-SLAC#22 co-transformant, 2: SLAC#14 co-transformant, 3: negative control (parental strain *A. niger* MGG029 with empty vector).

5.3.3 SDS-PAGE, Zymogram, and Western blot analysis

The presence of heterologous SLAC variants in the culture medium of PCR- and DMPPDA activity-validated co-transformants were further investigated through a series of rigorous protein gel analysis (Fig. 4). Two protein bands were observed from SLAC, while only a single band appeared from CBM-SLAC sample (Fig. 4A). As expected, these bands were not present in the culture medium of transformants with empty vector, *A. niger* MGG029. Notably, these bands appeared at approximately two and three times the size of the monomeric size of CBM-SLAC and SLAC, which is 45 kDa and 35 kDa, respectively. This suggested that these SLAC proteins as produced in *Aspergillus* remained non-denatured in un-boiled samples when run on SDS-PAGE. Previous studies confirmed the resilience of the SLAC to denaturation by SDS thus retaining laccase

activity under these conditions (Kaur et al., 2022; Machczynski et al., 2004). Therefore, Zymogram analysis was carried out to further confirm and explore if the proteins observed on SDS-PAGE corresponded to active SLAC protein.

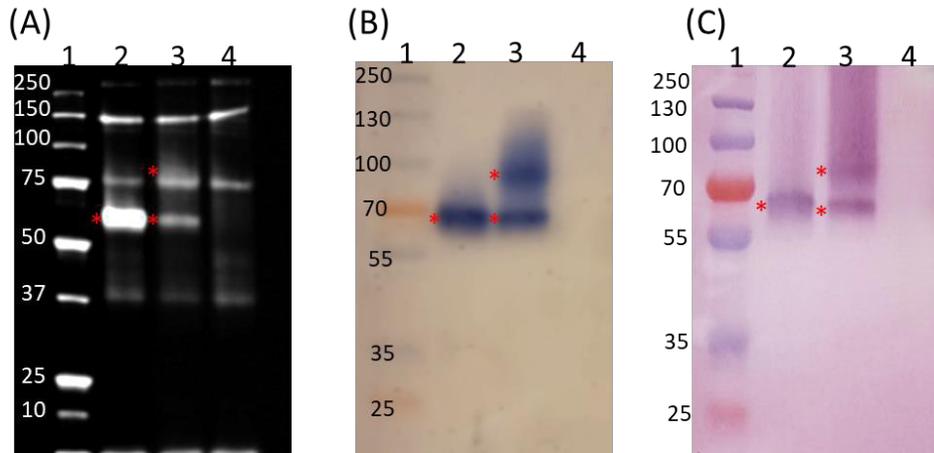


Fig. 4. Detection of SLAC variants from the culture medium of *A. niger* transformants. (A) SDS-PAGE of culture medium of selected co-transformants stained with Sypro Ruby staining solution. (B) Zymogram analysis of culture medium from selected co-transformants showing activity of SLAC variants on the gel overlaid with DMPPDA substrate. (C) Western blot analysis of culture medium from selected co-transformants using SLAC-antibody. 1: protein marker, 2: CBM-SLAC, 3: SLAC, 4: *A. niger* MGG029 with empty vector (negative control). The same amount of protein samples was loaded on the gel.

The Zymogram analysis using DMPPDA substrate was conducted in the presence of SDS without boiling the samples similar to that performed on SDS-PAGE in panel A and the Western blot in panel C (Fig. 4B), as detailed in the methods section. The results revealed enzymatic activity corresponding to the protein bands identified in the SDS-PAGE gel. Specifically, the active CBM-SLAC displayed a single band, while the active SLAC exhibited double bands, indicative of the typical di- and tri-meric state of active SLAC, in accordance with previous reports (Hakulinen and Rouvinen, 2015; Skálová et al., 2009). Our result suggests that CBM-SLAC is present as an active dimer, and not capable of forming a trimer which could possibly be due to structural hindrance attributed to the presence of two CBM's at the N-terminus. For further confirmation, a Western blot using specific SLAC antibody was conducted (Fig. 4C). As expected, the western blot analysis

showed a consistent result with those of SDS-PAGE and Zymogram (Fig. 4C). CBM-SLAC exhibited a single band, while SLAC displayed double bands (Fig. 4C).

The fact that we detected protein bands in the gel analysis corresponding to putative dimeric and trimeric conformations of SLAC is in line with previous crystal structure analysis and the conclusion that functional SLAC requires multimerization (Komori et al., 2009; Komori and Higuchi, 2010; Lawton et al., 2009; Skálová et al., 2007). The dimeric forms of active *Streptomyces* SLACs have been documented for the *SilA* from *Streptomyces ipomoea*, *SvSL* from *Streptomyces viridochromogene*, and SLAC from *Streptomyces coelicolor* (Machczynski et al., 2004; Molina-Guijarro et al., 2009; Trubitsina et al., 2015), while the trimeric structures have been reported for *EpoA* from *Streptomyces griseus* and *Ssl1* from *Streptomyces aviceus* (Endo et al., 2003; Gunne and Urlacher, 2012), all expressed in *Escherichia coli*. Moreover, an active SLAC from *S. coelicolor* exhibited trimeric and dimeric conformation when expressed in different hosts (Machczynski et al., 2004; Skálová et al., 2009, 2007). In our study, SLAC expressed in *A. niger* displayed both the putative dimeric and trimeric conformation in a Zymogram with DMPPDA as substrate. The difference in reported multimeric forms of SLAC could be attributed to the use of different substrates (Janusz et al., 2020).

Overall, the protein gel analysis conducted through above electrophoresis techniques (Fig. 4) provided confirmation that these SLAC variants were expressed and exhibited activity. On SDS-PAGE, the band corresponding to CBM-SLAC was more intense compared to that of SLAC, suggesting a higher expression level of CBM-SLAC. However, on the Zymogram, the levels of active bands were comparable between CBM-SLAC and SLAC, while the same amount of sample was loaded into the gel. This suggested that SLAC was more active than CBM-SLAC toward the DMPPDA substrate, which may be related to conformational differences between the multimeric form of the two proteins, as was also mentioned above.

5.3.4 Protein analysis for identification of SLAC variants

In an attempt to further characterize the SLAC variants as secreted in *A. niger* culture medium, mass-spectrometry (LC-MS/MS)-based proteomics analysis was conducted with the two protein samples obtained for SLAC and CBM-SLAC. Through meticulous peptide matching, peptide fragments corresponding to most of the SLAC protein variants were successfully identified with high reliability (Additional file 3). A total of 228 and 121

amino acids (aa) were identified based on the peptide fragments referred to the sequence from SLAC and CBM-SLAC, respectively (Fig. 5). These numbers represent a protein coverage of 75% for SLAC and 31% for CBM-SLAC sequences (Fig. 5).

The identified peptides are in agreement with the effective secretion of SLAC from *S. leeuwenhoekii* in *A. niger*, highlighting the robustness of the heterologous expression system. However, the absence of peptide fragments corresponding to the N-terminal region containing the CBM1 domain as depicted in Fig. 5, precluded conclusions on the presence of this N-terminal domain of the CBM1-SLAC protein. The specific interactions or structural properties of the domain could hinder CBM detection in (LC-MS/MS)-based proteomics analysis. This could be attributed to the challenges associated with the trypsin fragmentation and identification of peptides containing disulfide bonds (Mormann et al., 2008) and the fact that the CBM1 region is rich in Cysteine residues (Additional file 2: Fig. S4) which are involved in forming disulfide bonds in the CBM1 domains (The UniProt Consortium, 2023).

As a further method to confirm the presence of a functional CBM domain, the SLAC variants were analyzed for their affinity toward cellulose using the method as previously described (Sidar et al., 2023; van der Kaaij et al., 2007). This analysis, however did not show any significant difference between SLAC and CBM-SLAC (data not shown), further suggesting that the CBM1 is no longer present in the CBM-SLAC protein present in the *A. niger* culture supernatant or not functionally contributing to cellulose binding due to incorrect folding of the CBM-SLAC protein.

The most plausible explanation for the observed results is that the CBM domain is proteolytically degraded once secreted in the medium, while it was still present during the folding in the endoplasmic reticulum (ER) or elsewhere in the secretion pathway. We speculate that folding in its trimeric, but not in its dimeric conformation, was hindered by the presence of CBM1 domain when the N-terminally attached CBM1 to the SLAC still occurred. After folding and secretion, the CBM domain was subsequently proteolytically cleaved, resulting in only one protein band for CBM-SLAC, while two bands were found for SLAC (Fig. 4). This corresponds with our results when both SLAC variants were run in SDS-PAGE gel after boiling, showing protein bands with a similar position representing the SLAC monomeric size (Additional file 2: Fig. S5). Notwithstanding this somewhat confusing result, we conclude that SLAC can be successfully produced in *Aspergillus*, laying the groundwork for further investigation into its impact on the

degradation of lignocellulosic material, as laccases are known to play a role in the efficient degradation of lignin (Majumdar et al., 2014; Rashid et al., 2024).

SLAC design	-----	0
CBM-SLAC design	SSAHHVAGYYQQCGGHGWHGATKCPKSWACAKQSDWFLCKPILDNHGCTNDEYAQCQGGI	60
SLAC design	-----ATAALRTAPAGGEVRHLKLYAEKLPDGMGYGL	33
CBM-SLAC design	GFKGEKCKVGTTCCKDSDWWSACAPVATAALRTAPAGGEVRHLKLYAEKLPDGMGYGL	120

SLAC design	EKGKATIPGPLIELNEGDTLHIELENTMDVAASLHVHGLDYEISSDGTKLSRSDVEPGGT	93
CBM-SLAC design	EKGKATIPGPLIELNEGDTLHIELENTMDVAASLHVHGLDYEISSDGTKLSRSDVEPGGT	180

SLAC design	RTYTWRTTHAPGRRKDGTRWAGSAGYWHYHDHVVGTEHGTGGIRKGLYGAVIVRRKGDVLP	153
CBM-SLAC design	RTYTWRTTHAPGRRKDGTRWAGSAGYWHYHDHVVGTEHGTGGIRKGLYGAVIVRRKGDVLP	240

SLAC design	DRTHTVVFNMDLINNRPPHTGPDFEATVGDRVEFVVITHGEYYHTFHLHGHRWADNRTGL	213
CBM-SLAC design	DRTHTVVFNMDLINNRPPHTGPDFEATVGDRVEFVVITHGEYYHTFHLHGHRWADNRTGL	300

SLAC design	LTGPEDPSQVVDNKIVGPADSFQVIAGEGVGAGAWMYHCHVQSHSDMGVGLFLVKKT	273
CBM-SLAC design	LTGPEDPSQVVDNKIVGPADSFQVIAGEGVGAGAWMYHCHVQSHSDMGVGLFLVKKT	360

SLAC design	DGTIPGYDPHDAHTAQPSGPEAESRHEH	303
CBM-SLAC design	DGTIPGYDPHDAHTAQPSGPEAESRHEH	390

Variants	Total amino acid in sequence	Total amino acid from identified peptides	Coverage (%)
SLAC	301	228	75 %
CBM-SLAC	390	121	31 %

Fig. 5. Matching sequence-based peptide fragments found in SLAC and CBM-SLAC sample. Sequences from identified peptide fragments of secreted SLAC protein are highlighted in yellow, while sequences from identified peptide fragments of secreted CBM-SLAC protein are highlighted in blue.

5.3.5 Effect of SLAC on lignocellulose degradation

The functional significance of the SLAC variants in lignocellulose degradation was explored using lignocellulosic biomass derived from rice straw. Crude enzyme samples containing recombinant SLAC were supplemented with commercial cellulases to evaluate the impact of SLAC on enhancing cellulase access and subsequent release of

reducing sugars from rice straw. Previous studies have reported that the degradation of lignin by laccase in lignocellulose substrates enhances the accessibility of cellulase onto the cellulose chain to release reducing sugars (Deng et al., 2019; Zhang et al., 2020). In these studies laccase was employed to degrade lignin in wheat straw (Deng et al., 2019) and ginseng (Zhang et al., 2020) for subsequent enhancement of enzymatic saccharification by cellulases to release sugars. In our analysis, the amount of reducing sugars released was quantified using the DNS assay as described in method section and reflects the effect of SLAC activity on the lignocellulose degradation.

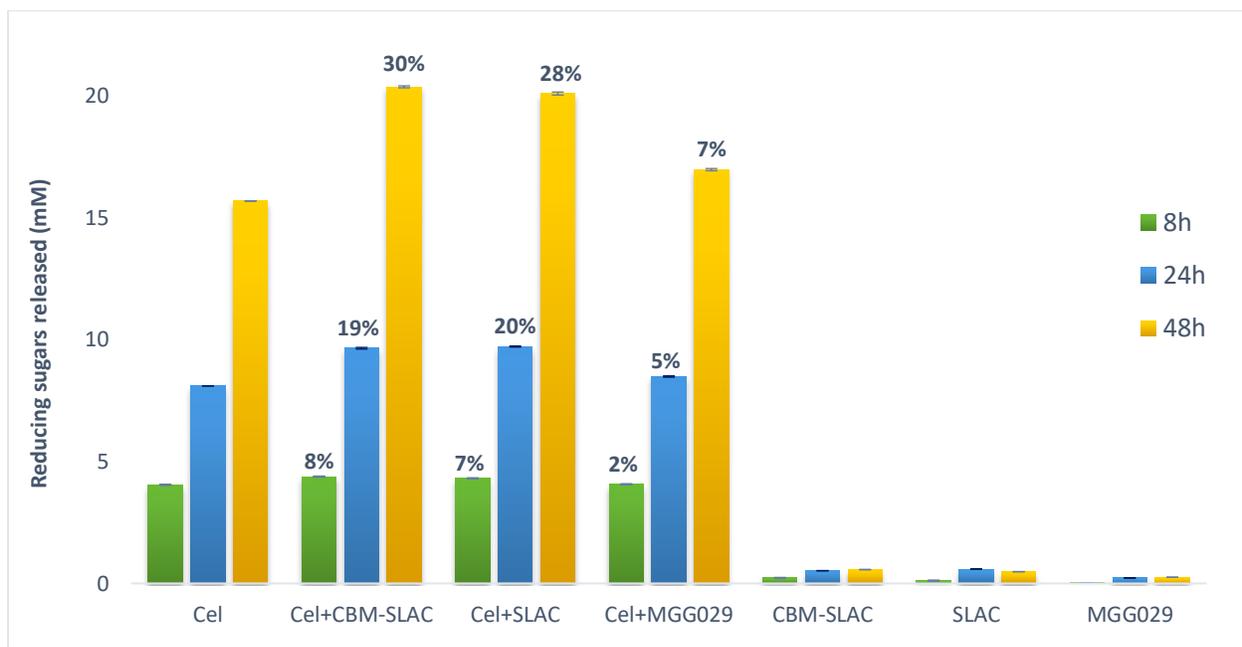


Fig. 6. Effect of SLAC in lignocellulose rice straw degradation. The amount of reducing sugar is measured after incubation of rice straw with 1 endo-glucanase unit (EGU) of commercial cellulase (Cel) and crude protein samples containing SLAC variants CBM-SLAC and SLAC. MGG029 is the crude protein sample from the parental strain lacking SLAC. Controls includes rice straw substrate and crude protein samples without commercial cellulases. Percentage (%) indicates the increase in released reducing sugars when laccase variants were added to the commercial cellulase compared to the reactions containing only commercial cellulase at the same time point. Error bars represent standard deviation of each experimental point (n=3).

A notable increase in reducing sugar concentration was observed following 24- and 48-hours incubation of rice straw with crude enzyme containing SLAC alongside with cellulase (Fig. 6). At 24 hours, the sample treated solely with commercial cellulase displayed 8 mM of reducing sugars, whereas with SLAC variants added, the amount rose

to 10 mM, marking a 20% increase in released reducing sugars. Moreover, a substantial enhancement in reducing sugar release was detected after 48 hours, with approximately a 30% rise observed in samples containing each SLAC variant alongside commercial cellulase compared to cellulase treatment alone (Fig. 6). This suggests that cellulase gains better access to cellulose degradation, likely due to reduced hindrance from lignin post degradation by laccase, implying SLAC addition enhances rice straw degradation into reducing sugars.

Furthermore, a similar increase in release of reducing sugars from the rice straw was observed for the reactions with the addition of both SLAC variants in line with what we concluded in protein analysis section above. Whereas, CBM domains augment enzymatic hydrolysis by facilitating recognition and adsorption of the enzyme onto the substrate surface (Boraston et al., 2004; Carli et al., 2022; Pan et al., 2016). The data presented suggest that in this case the addition of CBM could also affect intermolecular interactions between CBM-SLAC monomers, which may affect folding of a multi-domain protein like CBM-SLAC, similar as observed for catalytic activity and enzyme-substrate interactions of other multi-domain proteins (Elleuche, 2015; Monterrey et al., 2022; Rajasekaran and Kaiser, 2022; Yu et al., 2015).

5.4 Conclusion

In conclusion, the engineered SLAC genes from *Streptomyces leeuwenhoekii* were successfully expressed in *A. niger*. The addition of CBM1 may have resulted in conformational changes of the CBM-SLAC fusion, resulting in the inhibition of its trimerization, but not its dimerization into its active form. Both SLAC variants exhibited activity towards DMPPDA as substrate. When combined with a commercial cellulase, they showed a considerable increase in the release of reducing sugars, highlighting its potential synergistic role for SLAC in lignocellulosic biomass degradation. These results allow further exploration of targeted strategies in enzyme domain engineering to produce heterologous modular enzymes as a constituent of improved fungal enzyme cocktails in lignocellulose degradation. In summary, this study represents the first report of the expression of *Streptomyces* SLAC in *A. niger* and its impact on lignocellulosic substrate degradation.

Supplementary materials

Supplementary data to this chapter can be found online at this following link.
<https://doi.org/10.1186/s40694-024-00181-6>.

List of abbreviations

SLAC: Small laccase

CBM: Carbohydrate Binding Modules

CBM1: Carbohydrate Binding Modules family 1

AA1: Auxiliary Activities from family 1

CAZy: Carbohydrate-Active Enzymes

DMPPDA: N,N-Dimethyl-p-phenylenediamine sulfate salt

NaPi: Sodium Phosphate

EGU: endo-glucanase unit

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

T1, T1, T3: type-1,-2, -3 copper ion in respectively

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Author contributions

AS wrote the original draft, generated figures, performed the experiment, data and formal analysis. GV interpreted the MS/MS analysis data and involved in software and resources. AEM involved in conducting formal analysis. EV and PJP were involved in the conceptualization, presents ideas, planning, supervision, validation, manuscript outline, writing, review and editing. All authors reviewed and approved the final version of manuscript.

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