Fungal Biology and Biotechnology

RESEARCH Open Access

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

Background

Laccases are categorized as multi-copper oxidases (MCO) belonging to the Auxiliary Activities from family 1 (AA1) in the Carbohydrate-Active Enzymes (CAZy) database [1]. They play a dual role in catalyzing the oxidation of a wide range of both phenolic and non-phenolic compounds, including lignin [2, 3]. 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 [4, 5]. Lignin primarily consists of phenolic and some non-phenolic compounds [6]. 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.

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From the CAZy database, it is clear that laccases have been identified in diverse organisms, including fungi and bacteria [1]. Fungal laccases are widely represented explored in many basidiomycete and ascomycete fungi, while research on bacterial laccase is mainly focused on *Streptomyces*, *Bacillus*, *Pseudomonas* [3, 7].

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 [8–10]. 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) [11]. *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 [11–17] 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 [18, 19]. The CBM domain exhibits a high affinity for carbohydrate structures, hence enhancing the proximity of the enzyme and its substrate, thereby promoting substrate degradation [20–22]. 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 [23]. In *Aspergillus*, the CBM from family 1 (CBM1) is the sole CBM family identified in combination with cellulases [24]. However, CBM1 has not been identified in laccases from *Aspergillus* [1, 25]. 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 [26–29]. For instance, laccase expressed in *Trichoderma reesei* resulted in improved lignocellulosic biomass saccharification from corn cob residue [28]. Moreover, expression of chimeric fungal laccase in *A. niger* enhanced lignocellulose bioconversion [23].

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 [30], making it an ideal host for this investigation. Furthermore, *A. niger* has been frequently used as a host for three-domain laccase production [23, 31–33] 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.

Materials and methods

Microbial strains, plasmid and culture medium

A.niger strain MGG029 *pyrE*$^{-}$ [34] was used as the transformation host for heterologous expression of SLAC genes, while *Escherichia coli* DH5α was employed for plasmid propagation [35]. *E. coli* DH5α was grown on Luria–Bertani medium supplemented with ampicillin at 100 µg/mL. Plasmid pMA351 containing the *gpdA* promoter and *trpC* terminator from *Aspergillus nidulans*, was derived from pAN52-1Not [36, 37] 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.

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) [38]. Signal sequence was predicted using SignalP 6.0 [39]. 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′-GCTACATCCATACTC CA) and MBL858-reverse (5′-ATATCCAGATTCGTC AAGCTG). These primers amplify the *gpdA* promoter sequence, the inserted SLAC open reading frame, and part of the *trpC* terminator.

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 [40]. *A. niger pyrE*$^{+}$ transformants were selected on minimal media containing 0.95 M sucrose as an osmotic stabiliser [40]. 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 $CuSO_4{\cdot}7H_2O$, inoculated with 1×10^8 *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 [15].

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 [15]. 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 min. 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.

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 [41], 1 endo-glucanase unit (EGU) of cellulase (*Trichoderma reesei*, ATCC 26921, Sigma-Aldrich C2730), 100 µL culture medium from the *A. niger* co-transformant that was 5 times concentrated using a spin filter (Vivaspin, 10000 MWCO, VS0102) and 400 µL 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 [41]. 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 µL) were taken after 8 h, 24 h and 48 h of incubation. Subsequently, the amount of reducing sugars released was determined with the DNS assay, as essentially described in [42]. To remove remaining rice straw, 50 µL of each time point sample was centrifuged and 25 µL of supernatant was mixed with 25 µL 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 µL of sodium-potassium tetrahydrate solution (40% w/v). The absorbance was measured at 540 nm (TECAN, Spark 10 M) 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.

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 [35]. 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 µL 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 h at room temperature. Afterwards, the SDS-PAGE gel staining process was conducted as addressed in [35] 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 [15]. 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 h while shaking gently using a rocking shaker (VWR®) at room temperature. The buffer was refreshed every 20 min. Afterwards, the gel was incubated at 37 °C for 15 min using 100 mM NaPi pH 6.8 buffer. The buffer solution was discarded and the gel was overlayed with 50 mL of DMPPDA-solution and incubated for 15 min 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.

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* [11]. 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 min 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.

Cellulose binding analysis

Cellulose binding was carried out based on the method described in [35, 43]. 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.

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 [44].

Results and discussion

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.

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 [45]. 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 mononuclear center containing type-1 (T1) copper ion in the Cu-oxidase_2 domain [9, 10]. 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 [45, 46]. Moreover, several amino acid residues close to the T1 copper center have been implicated in substrate binding [8–10]. Additionally, the middle Cu-oxidase domain of three-domain laccase plays a role in bridging the other two domains, facilitating the proper 3-dimentional structure for the formation of the tri-nuclear cluster [10, 45].

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

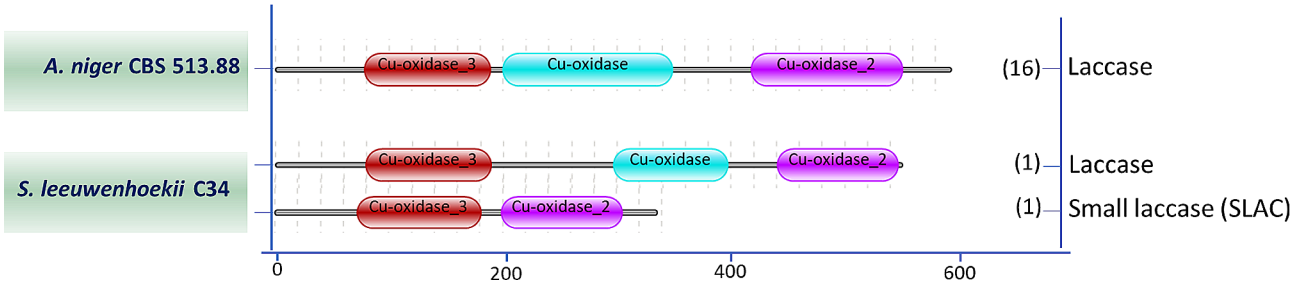

Fig. 1 Domain organization of laccases in the genome of *A. niger* CBS 513.88, and *S. leeuwenhoekii* C34. The number of identified laccase sequences from *A. niger* CBS 513.88 and *S. leeuwenhoekii* C34 are presented in brackets. The domain annotation was predicted based on Pfam domains using the HMMER program [38]. 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. leuwenhoekii* C34 are included in Additional file 1

centers, contributing to their catalytic activity [47–49]. 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 [10, 50]. 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 [10, 50]. 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 [51]. 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 [11–13, 17, 52, 53].

Both *Aspergillus* and *Streptomyces* have been well documented as laccase-producing organism [17, 31, 54]. 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 [19]. The C-terminal CBM attachment to fungal laccase has been effective in improving pulp bio-bleaching [23]. 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.

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. leeuwenhoekii* 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 [31] (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. leeuwenhoekii* 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.

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 1: 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

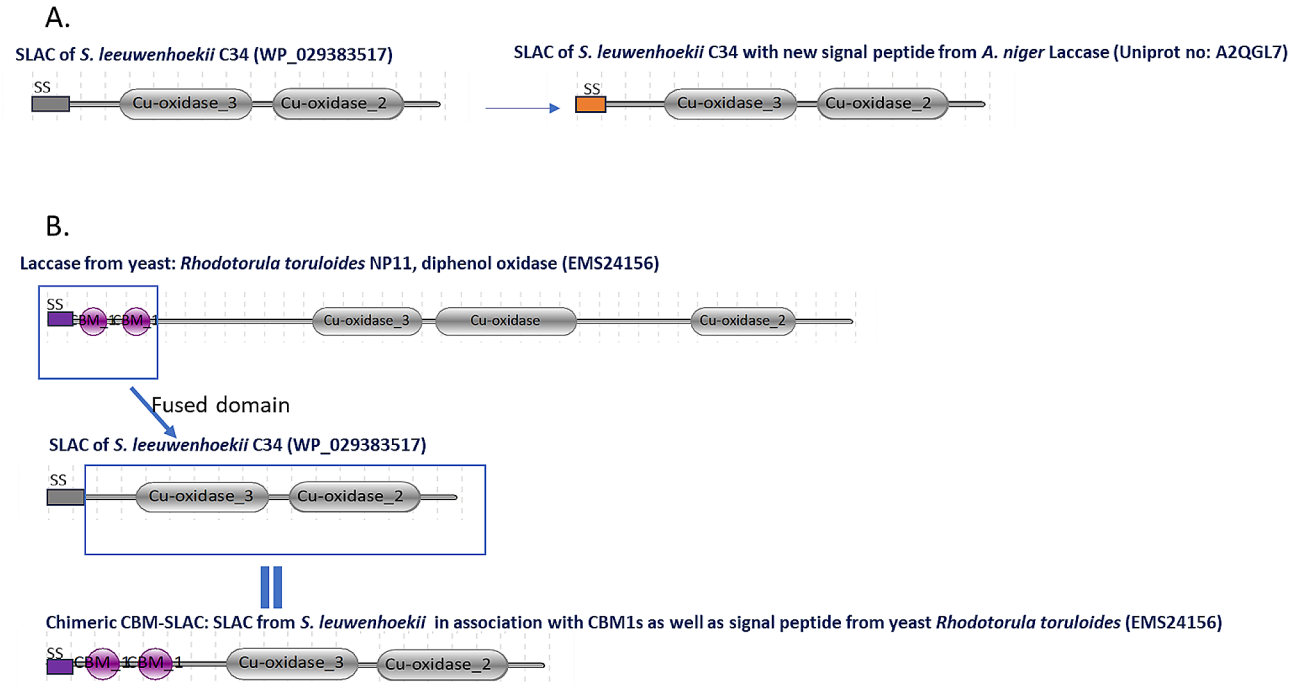

Fig. 2 Modular design of engineered SLAC variants. (A) SLAC variant without CBM domain (SLAC). (B) Chimeric SLAC assembled with two CBM1 domains at the N-terminus (CBM-SLAC)

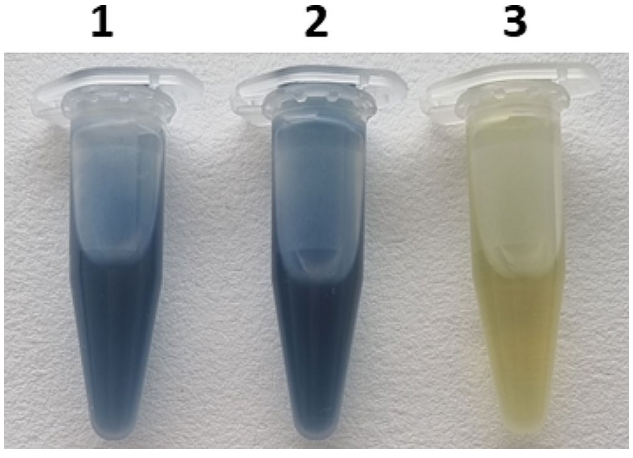

Fig. 3 Activity of SLAC variants based on DMPPDA assay using the culture medium from the cultivation of selected putative co-transformants. 1: CBM-SLAC#22 co-transformant, 2: SLAC#14 co-transformant, 3: negative control (parental strain *A. niger* MGG029 with empty vector)

introduced in *A. niger* transformants (Additional file 2: Fig. S3).

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 [11, 17]. Therefore, Zymogram analysis was carried out to further confirm and explore if the proteins observed on SDS-PAGE corresponded to active SLAC protein.

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 [10, 50]. 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

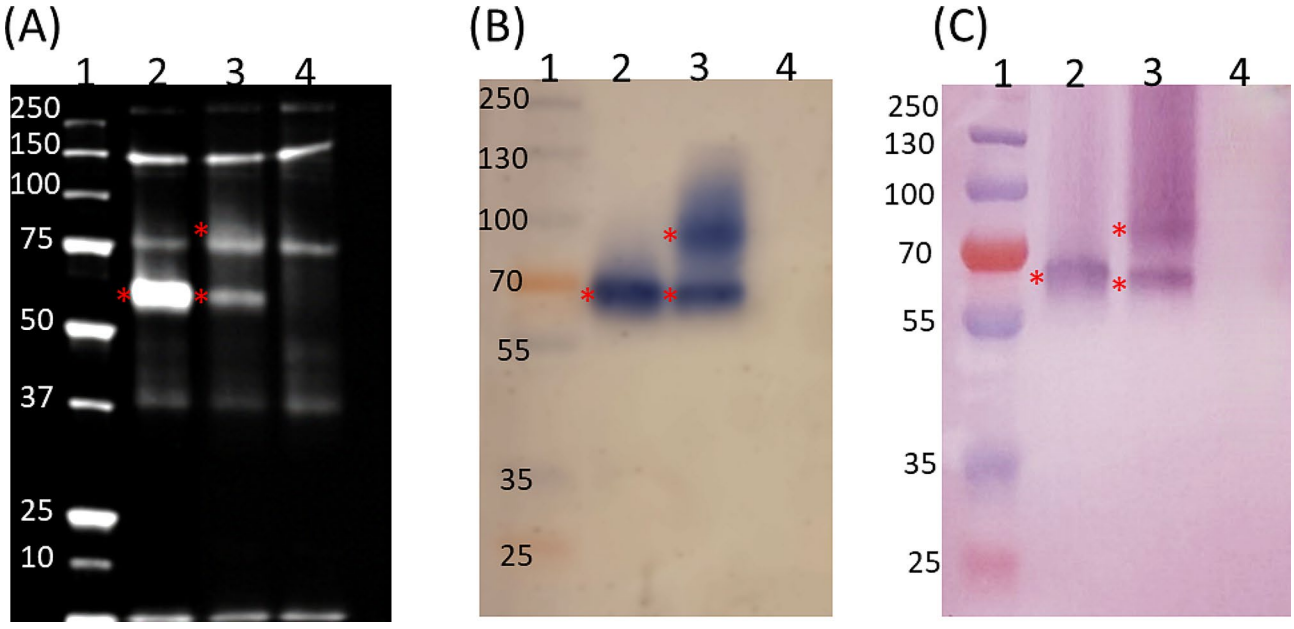

Fig. 4 Detection of SLAC variants from the culture medium of *A. niger* transformants. (A) SDS-PAGE of culture medium of selected co-transformants stained with Sypro Ruby staining solution. (B) Zymogram analysis of culture medium from selected co-transformants showing activity of SLAC variants on the gel overlayed 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

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 [51, 55–57]. 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* [11, 49, 53], while the trimeric structures have been reported for *EpoA* from *Streptomyces griseus* and *Ssl1* from *Streptomyces aviceus* [15, 16], all expressed in *Escherichia coli*. Moreover, an active SLAC from *S. coelicolor* exhibited trimeric and dimeric conformation when expressed in different hosts [11, 50, 57]. 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 [58].

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.

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

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SLAC design       ------------------------------------------------------------  0
CBM-SLAC design   SSAHHVAGYYQQCGGHGWHGATKCPSKWACAKQSDWFSLCKPILDNHGCTNDEYAQCGGI  60

SLAC design       -------------------------ATAALRTAPAGGEVRHLKLYAEKLPDGQMGYGL  33
CBM-SLAC design   GFKGEKCCKVGTTCKKDSDWWSACAPVATAALRTAPAGGEVRHLKLYAEKLPDGQMGYGL  120
                                            ********************************

SLAC design       EKGKATIPGPLIELNEGDTLHIELENTMDVAASLHVHGLDYEISSDGTKLSRSDVEPGGT  93
CBM-SLAC design   EKGKATIPGPLIELNEGDTLHIELENTMDVAASLHVHGLDYEISSDGTKLSRSDVEPGGT  180
                  ************************************************************

SLAC design       RTYTWRTHAPGRRKDGTWRAGSAGYWHYHDHVVGTEHGTGGIRKGLYGAVIVRRKGDVLP  153
CBM-SLAC design   RTYTWRTHAPGRRKDGTWRAGSAGYWHYHDHVVGTEHGTGGIRKGLYGAVIVRRKGDVLP  240
                  ************************************************************

SLAC design       DRTHTVVFNDMLINNRPPHTGPDFEATVGDRVEFVVITHGEYYHTFHLHGHRWADNRTGL  213
CBM-SLAC design   DRTHTVVFNDMLINNRPPHTGPDFEATVGDRVEFVVITHGEYYHTFHLHGHRWADNRTGL  300
                  ************************************************************

SLAC design       LTGPEDPSQVVDNKIVGPADSFGFQVIAGEGVGAGAWMYHCHVQSHSDMGMVGLFLVKKT  273
CBM-SLAC design   LTGPEDPSQVVDNKIVGPADSFGFQVIAGEGVGAGAWMYHCHVQSHSDMGMVGLFLVKKT  360
                  ************************************************************

SLAC design       DGTIPGYDPHDHAHTAQPSPGPEAESRHEH      303
CBM-SLAC design   DGTIPGYDPHDHAHTAQPSPGPEAESRHEH      390
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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

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 [59] 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 [25].

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 [35, 43]. 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

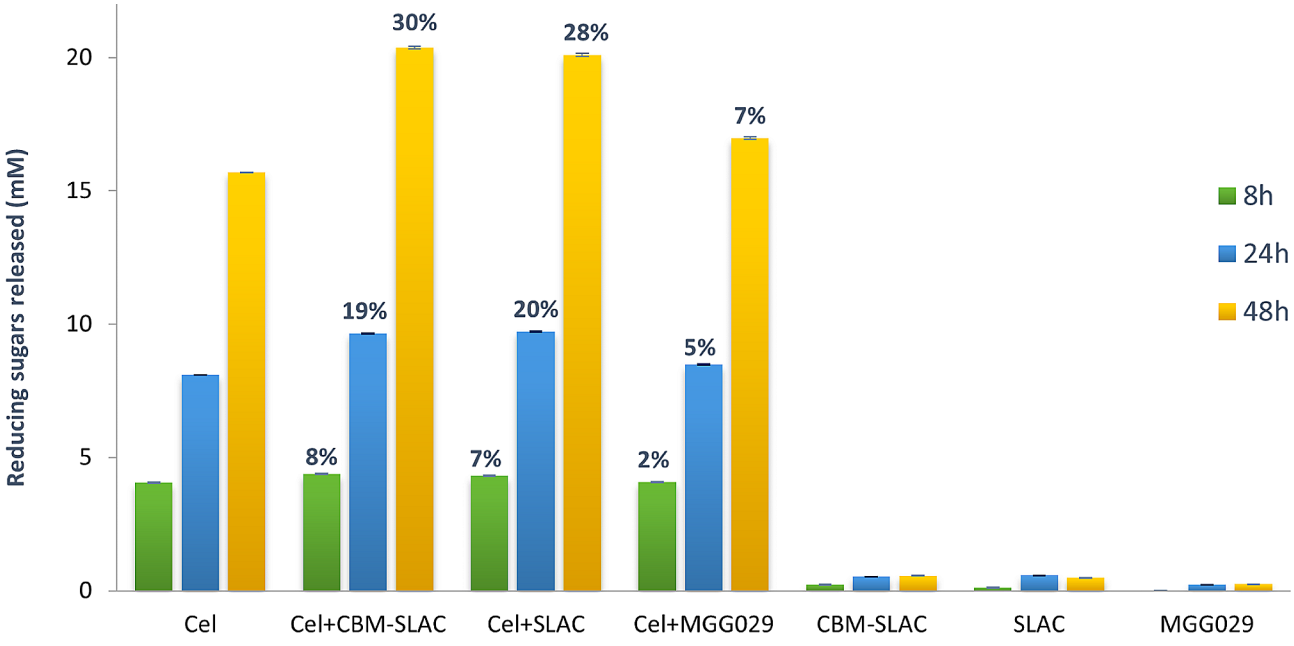

Fig. 6 Effect of SLAC on lignocellulose rice straw degradation. The amount of reducing sugar is measured after incubation of rice straw with 1 endoglucanase unit (EGU) of commercial cellulase (Cel) and crude protein samples containing SLAC variants CBM-SLAC and SLAC. MGG029 is the crude protein sample 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 ($n = 3$)

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 [13, 60].

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 [61, 62]. In these studies laccase was employed to degrade lignin in wheat straw [61] and ginseng [62] 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.

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 h, 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 h, 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 [18, 63, 64]. 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 [65–68].

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.

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

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40694-024-00181-6.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Acknowledgements

We thank Prof. Dr. Arthur F.J. Ram (Fungal Genetics and Biotechnology, Institute Biology Leiden University) for the insight during work discussion. Additionally, we greatly appreciate Ing. Mark Arentshorst (Fungal Genetics and Biotechnology, Institute Biology Leiden University) for providing the plasmid containing pyrE gene and some laboratory technical assistance. Moreover, we thank Dr. Erica D. Albuquerque for the CBM-SLAC construct.

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.

Funding

This study was supported by a scholarship of the Indonesia Endowment Fund for Education (LPDP) from the Ministry of Finance, Indonesia (20160422026103) to AS and by a grant of the Dutch National Organization for Scientific Research NWO, in the framework of an ERA-IB project FilaZyme (053.80.721/EIB.14.021) to EV, GV, and PP.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 17 April 2024 / Accepted: 6 August 2024
Published online: 02 September 2024

References

1. Drula E, Garron ML, Dogan S, Lombard V, Henrissat B, Terrapon N. The carbohydrate-active enzyme database: functions and literature. Nucleic Acids Res. 2022;50(D1):D571–7.
2. Khan SI, Sahinkaya M, Colak DN, Zada NS, Uzuner U, Belduz AO, et al. Production and characterization of novel thermostable CotA-laccase from Bacillus altitudinis SL7 and its application for lignin degradation. Enzyme Microb Technol. 2024;172:110329.
3. Zhang W, Wang W, Wang J, Shen G, Yuan Y, Yan L, et al. Isolation and characterization of a Novel Laccase for Lignin Degradation, LacZ1. Appl Environ Microbiol. 2021;87(23):e01355–21.
4. Rangelov S, Nicell JA. Modelling the transient kinetics of laccase-catalyzed oxidation of four aqueous phenolic substrates at low concentrations. Biochem Eng J. 2018;132:233–43.
5. d'Acunzo F, Galli C, Masci B. Oxidation of phenols by laccase and laccase-mediator systems. Eur J Biochem. 2002;269(21):5330–5.
6. Vasile C, Baican M. Lignins as promising renewable biopolymers and bioactive compounds for high-performance materials. Polym (Basel). 2023;15(15):3177.
7. Guan ZB, Luo Q, Wang HR, Chen Y, Liao XR. Bacterial laccases: promising biological green tools for industrial applications. Cell Mol Life Sci. 2018;75(19):3569–92.
8. Camarero S, Pardo I, Cañas AI, Molina P, Record E, Martínez AT, et al. Engineering platforms for Directed Evolution of Laccase from Pycnoporus Cinnabarinus. Appl Environ Microbiol. 2012;78(5):1370–84.
9. Mehra R, Muschiol J, Meyer AS, Kepp KP. A structural-chemical explanation of fungal laccase activity. Sci Rep. 2018;8:17285.
10. Hakulinen N, Rouvinen J. Three-dimensional structures of laccases. Cell Mol Life Sci. 2015;72(5):857–68.
11. Machczynski MC, Vijgenboom E, Samyn B, Canters GW. Characterization of SLAC: a small laccase from Streptomyces coelicolor with unprecedented activity. Protein Sci. 2004;13(9):2388–97.
12. Blánquez A, Ball AS, González-Pérez JA, Jiménez-Morillo NT, González-Vila F, Arias ME, et al. Laccase SilA from Streptomyces ipomoeae CECT 3341, a key enzyme for the degradation of lignin from agricultural residues? PLoS ONE. 2017;12(11):e0187649.
13. Majumdar S, Lukk T, Solbiati JO, Bauer S, Nair SK, Cronan JE, et al. Roles of small laccases from Streptomyces in lignin degradation. Biochemistry. 2014;53(24):4047–58.
14. Lu L, Zeng G, Fan C, Ren X, Wang C, Zhao Q, et al. Characterization of a laccase-like multicopper oxidase from newly isolated Streptomyces sp. C1

in agricultural waste compost and enzymatic decolorization of azo dyes. Biochem Eng J. 2013;72:70–6.
15. Endo K, Hayashi Y, Hibi T, Hosono K, Beppu T, Ueda K. Enzymological characterization of EpoA, a Laccase-Like Phenol Oxidase produced by Streptomyces griseus. J Biochem. 2003;133(5):671–7.
16. Gunne M, Urlacher VB. Characterization of the Alkaline Laccase Ssl1 from Streptomyces sviceus with unusual properties discovered by Genome Mining. PLoS ONE. 2012;7(12):e52360.
17. Kaur R, Salwan R, Sharma V. Structural properties, genomic distribution of laccases from Streptomyces and their potential applications. Process Biochem. 2022;118:133–44.
18. Boraston AB, Bolam DN, Gilbert HJ, Davies GJ. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J. 2004;382(Pt 3):769–81.
19. Bernardes A, Pellegrini VOA, Curtolo F, Camilo CM, Mello BL, Johns MA, et al. Carbohydrate binding modules enhance cellulose enzymatic hydrolysis by increasing access of cellulases to the substrate. Carbohydr Polym. 2019;211:57–68.
20. Telke AA, Ghatge SS, Kang SH, Thangapandian S, Lee KW, Shin HD, et al. Construction and characterization of chimeric cellulases with enhanced catalytic activity towards insoluble cellulosic substrates. Bioresour Technol. 2012;112:10–7.
21. Duan CJ, Huang MY, Pang H, Zhao J, Wu CX, Feng JX. Characterization of a novel theme C glycoside hydrolase family 9 cellulase and its CBM-chimeric enzymes. Appl Microbiol Biotechnol. 2017;101(14):5723–37.
22. Monica P, Ranjan R, Kapoor M. Family 3 CBM improves the biochemical properties, substrate hydrolysis and coconut oil extraction by hemicellulolytic and holocellulolytic chimeras. Enzyme Microb Technol. 2023;174:110375.
23. Ravalason H, Herpoël-Gimbert I, Record E, Bertaud F, Grisel S, de Weert S, et al. Fusion of a family 1 carbohydrate binding module of Aspergillus Niger to the Pycnoporus cinnabarinus laccase for efficient softwood kraft pulp biobleaching. J Biotechnol. 2009;142(3):220–6.
24. Sidar A, Albuquerque ED, Voshol GP, Ram AFJ, Vijgenboom E, Punt PJ. Carbohydrate binding modules: diversity of Domain Architecture in Amylases and Cellulases from Filamentous microorganisms. Front Bioeng Biotechnol. 2020;8:871.
25. The UniProt Consortium. UniProt: the Universal protein knowledgebase in 2023. Nucleic Acids Res. 2023;51(D1):D523–31.
26. dos Santos TC, dos Santos Reis N, Silva TP, Pereira Machado F, de P, Ferereira Bonomo RC, Franco M. Prickly palm cactus husk as a raw material for production of ligninolytic enzymes by Aspergillus Niger. Food Sci Biotechnol. 2016;25(1):205–11.
27. Huang J, Wang J, Chen L, zhi, He J ling, Wu Ypeng, Cui X, peng et al. Improved expression of *Cerrena unicolor* Laccase in *Aspergillus niger* via combined strategies and its applications. Biochemical Engineering Journal. 2024;209:109371.
28. Zhang J, Qu Y, Xiao P, Wang X, Wang T, He F. Improved biomass saccharification by *Trichoderma reesei* through heterologous expression of *lacA* gene from *Trametes* sp. AH28-2. J Biosci Bioeng. 2012;113(6):697–703.
29. Gomaa OM, Momtaz OA. Copper induction and differential expression of laccase in aspergillus flavus. Braz J Microbiol. 2015;46(1):285–92.
30. de Vries RP, Visser J. Aspergillus enzymes involved in degradation of Plant Cell Wall Polysaccharides. Microbiol Mol Biol Rev. 2001;65(4):497–522.
31. Ramos JAT, Barends S, Verhaert RMD, de Graaff LH. The aspergillus Niger multicopper oxidase family: analysis and overexpression of laccase-like encoding genes. Microb Cell Fact. 2011;10:78.
32. Bohlin C, Jönsson LJ, Roth R, van Zyl WH. Heterologous expression of Trametes Versicolor laccase in Pichia pastoris and aspergillus Niger. Appl Biochem Biotechnol. 2006;129–132:195–214.
33. Piscitelli A, Pezzella C, Giardina P, Faraco V, Sannia G. Heterologous laccase production and its role in industrial applications. Bioeng Bugs. 2010;1(4):252–62.
34. Weenink XO, Punt PJ, van den Hondel CAMJJ, Ram AFJ. A new method for screening and isolation of hypersecretion mutants in Aspergillus Niger. Appl Microbiol Biotechnol. 2006;69(6):711–7.
35. Sidar A, Voshol GP, Vijgenboom E, Punt PJ. Novel Design of an α-Amylase with an N-Terminal CBM20 in Aspergillus Niger improves binding and Processing of a broad range of starches. Molecules. 2023;28(13):5033.
36. Van den hondel CAMJJ, Punt PJ, Van gorcom RFM. 18 - Heterologous Gene Expression in Filamentous Fungi. In: Bennett JW, Lasure LL, editors. More Gene Manipulations in Fungi. San Diego: Academic Press; 1991 [cited 2023 Feb 24]. pp. 396–428. https://www.sciencedirect.com/science/article/pii/B9780120886425500259
37. Punt PJ, van den Hondel CAMJJ. [39] Transformation of filamentous fungi based on hygromycin b and phleomycin resistance markers. In: Methods in Enzymology. Academic Press; 1992 [cited 2024 Apr 11]. pp. 447–57. (Recombinant DNA Part G; vol. 216). https://www.sciencedirect.com/science/article/pii/007668799216041H
38. Potter SC, Luciani A, Eddy SR, Park Y, Lopez R, Finn RD. HMMER web server: 2018 update. Nucleic Acids Res. 2018;46(W1):W200–4.
39. Teufel F, Almagro Armenteros JJ, Johansen AR, Gíslason MH, Pihl SI, Tsirigos KD, et al. SignalP 6.0 predicts all five types of signal peptides using protein language models. Nat Biotechnol. 2022;40(7):1023–5.
40. Arentshorst M, Ram AFJ, Meyer V. Using Non-homologous End-Joining-Deficient Strains for Functional Gene Analyses in Filamentous Fungi. In: Bolton MD, Thomma BPHJ, editors. Plant Fungal Pathogens. Totowa, NJ: Humana Press; 2012 [cited 2023 Feb 24]. pp. 133–50. (Methods in Molecular Biology; vol. 835). https://link.springer.com/https://doi.org/10.1007/978-1-61779-501-5_9
41. Sidar A, Voshol GP, El-Masoudi A, Vijgenboom E, Punt PJ. Highly variable domain architecture in carbohydrate-active enzymes highlights *Streptomyces* as promising resource for rice straw bioconversion. Bioresource Technol Rep. 2024;25:101775.
42. Miller GL. Use of Dinitrosalicylic Acid Reagent for determination of reducing Sugar. Anal Chem. 1959;31(3):426–8.
43. van der Kaaij RM, Yuan XL, Franken A, Ram AFJ, Punt PJ, van der Maarel MJEC, et al. Two novel, putatively cell wall-associated and glycosylphosphatidylinositol-anchored alpha-glucanotransferase enzymes of Aspergillus Niger. Eukaryot Cell. 2007;6(7):1178–88.
44. Craig R, Beavis RC. TANDEM: matching proteins with tandem mass spectra. Bioinformatics. 2004;20(9):1466–7.
45. Arregui L, Ayala M, Gómez-Gil X, Gutiérrez-Soto G, Hernández-Luna CE et al. Herrera de los Santos M,. Laccases: structure, function, and potential application in water bioremediation. Microbial Cell Factories. 2019;18(1):200.
46. Solomon EI, Chen P, Metz M, Lee SK, Palmer AE. Oxygen binding, activation, and reduction to Water by Copper proteins. Angew Chem Int Ed Engl. 2001;40(24):4570–90.
47. Gabdulkhakov A, Kolyadenko I, Kostareva O, Mikhaylina A, Oliveira P, Tamagnini P, et al. Investigations of accessibility of T2/T3 copper Center of two-domain laccase from Streptomyces griseoflavus Ac-993. Int J Mol Sci. 2019;20(13):3184.
48. Worrall JAR, Vijgenboom E. Copper mining in Streptomyces: enzymes, natural products and development. Nat Prod Rep. 2010;27(5):742.
49. Trubitsina LI, Tishchenko SV, Gabdulkhakov AG, Lisov AV, Zakharova MV, Leontievsky AA. Structural and functional characterization of two-domain laccase from Streptomyces viridochromogenes. Biochimie. 2015;112:151–9.
50. Skálová T, Dohnálek J, Østergaard LH, Østergaard PR, Kolenko P, Dušková J, et al. The structure of the small laccase from Streptomyces coelicolor reveals a link between Laccases and Nitrite Reductases. J Mol Biol. 2009;385(4):1165–78.
51. Komori H, Higuchi Y. Structure and molecular evolution of multicopper blue proteins. Biomol Concepts. 2010;1(1):31–40.
52. Dubé E, Shareck F, Hurtubise Y, Daneault C, Beauregard M. Homologous cloning, expression, and characterisation of a laccase from Streptomyces coelicolor and enzymatic decolourisation of an indigo dye. Appl Microbiol Biotechnol. 2008;79(4):597–603.
53. Molina-Guijarro JM, Pérez J, Muñoz-Dorado J, Guillén F, Moya R, Hernández M, et al. Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from Streptomyces Ipomoea. Int Microbiol. 2009;12(1):13–21.
54. Välimets S, Pedetti P, Virginia LJ, Hoang MN, Sauer M, Peterbauer C. Secretory expression of recombinant small laccase genes in Gram-positive bacteria. Microb Cell Fact. 2023;22(1):72.
55. Komori H, Miyazaki K, Higuchi Y. X-ray structure of a two-domain type laccase: a missing link in the evolution of multi-copper proteins. FEBS Lett. 2009;583(7):1189–95.
56. Lawton TJ, Sayavedra-Soto LA, Arp DJ, Rosenzweig AC. Crystal structure of a two-domain Multicopper Oxidase *. J Biol Chem. 2009;284(15):10174–80.
57. Skálová T, Dohnálek J, Ostergaard LH, Ostergaard PR, Kolenko P, Dusková J, et al. Crystallization and preliminary X-ray diffraction analysis of the small laccase from Streptomyces coelicolor. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2007;63(Pt 12):1077–9.
58. Janusz G, Pawlik A, Świderska-Burek U, Polak J, Sulej J, Jarosz-Wilkołazka A, et al. Laccase properties, physiological functions, and evolution. Int J Mol Sci. 2020;21(3):966.

59. Mormann M, Eble J, Schwöppe C, Mesters RM, Berdel WE, Peter-Katalinić J, et al. Fragmentation of intra-peptide and inter-peptide disulfide bonds of proteolytic peptides by nanoESI collision-induced dissociation. Anal Bioanal Chem. 2008;392(5):831–8.
60. Rashid GMM, Sodré V, Luo J, Bugg TDH. Overexpression of endogenous multi-copper oxidases mcoA and mcoC in Rhodococcus jostii RHA1 enhances lignin bioconversion to 2,4-pyridine-dicarboxylic acid. Biotechnol Bioeng. 2024;121(4):1366–70.
61. Deng Z, Xia A, Liao Q, Zhu X, Huang Y, Fu Q. Laccase pretreatment of wheat straw: effects of the physicochemical characteristics and the kinetics of enzymatic hydrolysis. Biotechnol Biofuels. 2019;12(1):159.
62. Zhang R, Lv C, Lu J. Studies on laccase mediated conversion of lignin from ginseng residues for the production of sugars. Bioresour Technol. 2020;317:123945.
63. Carli S, Parras Meleiro L, Salgado JCS, Ward RJ. Synthetic carbohydrate-binding module-endogalacturonase chimeras increase catalytic efficiency and saccharification of lignocellulose residues. Biomass Conv Bioref. 2022 May 3 [cited 2024 Jan 31]; https://doi.org/10.1007/s13399-022-02716-6
64. Pan R, Hu Y, Long L, Wang J, Ding S. Extra carbohydrate binding module contributes to the processivity and catalytic activity of a non-modular hydrolase family 5 endoglucanase from Fomitiporia mediterranea MF3/22. Enzym Microb Technol. 2016;91:42–51.
65. Rajasekaran N, Kaiser CM. Co-translational folding of Multi-domain proteins. Front Mol Biosci. 2022;9:869027.
66. Monterrey DT, Ayuso-Fernández I, Oroz-Guinea I, García-Junceda E. Design and biocatalytic applications of genetically fused multifunctional enzymes. Biotechnol Adv. 2022;60:108016.
67. Elleuche S. Bringing functions together with fusion enzymes—from nature's inventions to biotechnological applications. Appl Microbiol Biotechnol. 2015;99(4):1545–56.
68. Yu K, Liu C, Kim BG, Lee DY. Synthetic fusion protein design and applications. Biotechnol Adv. 2015;33(1):155–64.

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