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Enhanced delignification of lignocellulosic substrates by *Pichia* GS115 expressed recombinant laccase

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Utilization of energy-rich crop residues by ruminants is restricted by the presence of lignin, which is recalcitrant to digestion. Application of lignin degrading enzymes on the lignocellulosic biomass exposes the cellulose for easy digestion by ruminants. Laccases have been found to be considerably effective in improving the digestibility by way of delignification. However, laccase yields from natural hosts are not sufficient for industrial scale applications, which restricts their use. A viable option would be to express the laccase gene in compatible hosts to achieve higher production yields. A codon-optimized synthetic variant of *Schizophyllum commune* laccase gene was cloned into a pPIC9K vector and expressed in *P. pastoris* GS115 (his4) under the control of an alcohol oxidase promoter. Colonies were screened for G418 resistance and the methanol utilization phenotype was established. The transformant yielded a laccase activity of 344 U·mL^{-1} after 5 days of growth at 30°C (0.019 g·mL^{-1} wet cell weight). The laccase protein produced by the recombinant *Pichia* clone was detected as two bands with apparent molecular weights of 55 kDa and 70 kDa on SDS-PAGE. Activity staining on native PAGE confirmed the presence of bioactive laccase. Treatment of five common crop residues with recombinant laccase recorded a lignin loss ranging between 1.64% in sorghum stover, to 4.83% in finger millet, with an enhancement in digestibility ranging between 8.71% in maize straw to 24.61% in finger millet straw. Treatment with recombinant laccase was effective in enhancing the digestibility of lignocellulosic biomass for ruminant feeding through delignification. To date, a number of hosts have been adventured to produce laccase in large quantities, but, to our knowledge, there are no reports of the expression of laccase protein from *Schizophyllum commune* in *Pichia pastoris*, and also on the treatment of crop residues using recombinant laccase for ruminant feeding.

Key Words: Laccase gene; lignocellulose; *Pichia pastoris*; recombinant; synthetic codon

Introduction

Lignocellulosic biomass obtained from agricultural crops represents an enormous energy resource for feeding ruminants, but the presence of lignin decreases their digestibility. Of the wide range of pretreatments reported for the deconstruction of lignin (Saritha et al., 2012), bioconversion with fungal enzymes from white rot fungi (WRF) is safe, with a low environmental impact. Biological pretreatment, however, is constrained by low enzyme production efficiency, long residence times, and considerable loss of the carbohydrates. Direct application of lignin-degrading enzymes to the lignocellulosic biomass enhances accessibility to the underlying cellulose, thus rendering a greater digestibility (Sridhar et al., 2015).

Laccases (benzenediol/oxygen oxidoreductases, EC.1.10.3.2) are enzymes that catalyze the one-electron oxidation of a wide variety of organic and inorganic substrates including mono-, di- and polyphenols, amino

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phenols, methoxy phenols, aromatic amines and ascorbate with a concomitant four-electron reduction of molecular oxygen to water (Galhaup et al., 2002). On account of this exemplary capability to oxidize polyphenols, fungal laccases have a widespread application in a number of biotechnological processes, including the biodegradation of lignin.

Low yields of purified laccase (Kumar et al., 2015) in a native state limits their practical use in the deconstruction of lignocellulosic biomass for feeding ruminants. Enzymes in bulk quantities are required to treat biomass which have a greater product consistency with less lot to lot variations. *S. commune* NI-07 strain, isolated in our work (Kumar et al., 2015), is a potent laccase producer, the genome sequence of which recommends it as having a unique potential to degrade lignin (Ohm et al., 2012). Moreover, fungi are also known to utilize FOLymes to degrade lignin (Levasseur et al., 2008), and *S. commune* contains 16 genes encoding FOLymes (Ohm et al., 2012). Two laccase genes (Madhavan et al., 2014), one CDH gene, and 13 LDA genes, including four genes encoding glucose oxidases and benzoquinone reductases, are well conserved in *S. commune* (Ohm et al., 2012). Because of the diversity of laccase genes from *S. commune*, and the ambiguity in isolating these genes using laccase specific universal primers, the expression of recombinant protein using a *P. pastoris* host system through codon optimization (Xiong et al., 2006) was considered for further study.

Objectives

To investigate the potential of recombinant laccase in upgrading various varieties of lignocellulosic biomass for feeding ruminants, a codon-optimized laccase gene was expressed in *Pichia* GS115 strain, given the diversity of laccase genes from *S. commune* and the ambiguity in isolating laccase genes using laccase specific universal primers.

Materials and Methods

Codon optimization and construction of an expression vector. Gene sequences coding for laccase could not be isolated from mRNA transcripts of *S. commune* NI-07 strain (Kumar et al., 2015) using laccase specific degenerate primers, as well as primers designed to isolate the laccase gene from *S. commune*. Considering the ambiguity in the use of laccase specific primer pairs to isolate *S. commune* laccase, a full length laccase gene from *S. commune* (Hatamoto et al., 1999) was chemically synthesized and optimized according to the codon usage bias of *P. pastoris* using a GeneArt Gene optimizer tool (Graf et al., 2000). The nucleotide sequence of the full length cDNA from *S. commune* containing a 1554 bp open reading frame that encoded a polypeptide of 518 amino acid residues, including a putative signal peptide, was adopted (Hatamoto et al., 1999). This sequence was modified with the excision of the signal pro peptide with correct reading frame, and the gene was synthesized (Life Technologies) using self-annealing oligos. The full length gene sequence was split into overlapping contigs and these were synthesized from scratch by annealing the oligos. The assembly was carried out by a finishing PCR reaction with a proof reading enzyme that filled the gaps. The final sequence was verified by sequencing and cloned directly into the production vector. The insert was released by standard restriction enzyme digestion using *Eco*R1 and *Not*1 and directionally sub-cloned into the pPIC9K vector. The complete codon-optimized *S. commune* laccase gene has been submitted to GenBank (GenBank accession number KX147091).

Propagation of a pPIC9K/Scom-Lac vector. The expression cassette was transformed into competent *E. coli* DH5α cells as per the standard protocol and glycerol stocks of the clones were prepared. Single colonies from the LB/Ampicillin plate were inoculated into tubes containing 50 mL of LB/Ampicillin broth and the cells were harvested for plasmid isolation after incubation. To check if the insert was in frame with the secretion signal, α-factor (5′-TACTATTGCCAGCATTGCTGC-3′) and 3′AOX1 (5′-GCAAATGGCATTCTGACATCC-3′) primer sequences were used.

Transformation into Pichia pastoris GS115 (his4). Clean up of the linearized (*Bgl*II (Cat. No. 15213-010, Invitrogen)) plasmid was performed using the Wizard SV gel PCR clean up system (Promega). Electro competent cells for *Pichia* transformation were prepared as per the standard protocol outlined by the company (Invitrogen). The pPIC9K/*Scom*-Lac linearized DNA was transformed into competent *P. pastoris* GS115 (his4) strain by electroporation. The various parameters of the pulse generator were set up with the following program: Charging voltage of 1500 V, capacitance of 25 μF, resistance of 200 ohm, and pulse length of 5 ms (Gene Pulser Xcell Electroporation system, Biorad, USA). A cuvette was placed in the electroporation chamber, connected to the pulse generator and was subjected to a single pulse. Five microlitres of *Bgl*II linearized pPIC9K/*Scom*-Lac DNA (188 ng/μL) was added to a 40 μL aliquot of competent *P. pastoris* and the same was transferred into a 2 mm ice-cold electroporation cuvette and placed on ice. The above step was repeated with a *Bgl*II linearized pPIC9K vector DNA without insert. The vector pPIC9K without insert served as the control. Immediately, 0.5 mL of ice cold 1 M sorbitol and 0.5 mL of ice cold YPD were added and the cuvette contents transferred to a sterile micro centrifuge tube and incubated in a shaking incubator for 4 h (30°C, 100 rpm). The reaction mixture was transferred onto Minimal Dextrose (MD) agar plates (10X Dextrose, 10XYNB, 500X Biotin) deficient in histidine, incubated at 30°C and monitored regularly for the development of colonies. Transformed clones were subjected to antibiotic selection on YPD medium containing varying concentrations of the antibiotic G418 which confers resistance to kanamycin in *Pichia*.

Direct PCR screening of Pichia clones. The presence of laccase gene was confirmed by PCR using gene specific primers GS1F/R (F-ACTACCATTCACTGGCACGG, R-GGCAAGGATGGTGGGATGAA) and GS2F/R (F-TTCATCCCACCATCCTTGCC, R-GCCAAACCA-GTGTTGACGAC) designed using NCBI Primer Blast.

Pichia cells were lysed by using a combination of enzyme freezing and heat treatment. A single colony of transformed *Pichia* clone was suspended in 10 μL of water in a clean 1.5 mL tube. Five microlitres of lyticase enzyme (5 U/μl) was added and incubated at 30°C for 10 min. The cell lysate was then frozen at –80°C for 10 min before using for a PCR assay. Fifty microlitres of PCR reaction mix contained 10X reaction buffer (5 μL), forward and reverse primer (1 μL each), $MgCl_2$ (25 mM, 5 μL), dNTPs (25 mM, 1 μL), nuclease free water (27 μL) and cell lysate (5 μL). The solution was incubated at 95°C for 5 min. To this, 5 μL of Taq polymerase (0.8 U) was added. The PCR reaction was set using 30 cycles of denaturation (95°C, 1 min), annealing (54°C, 1 min) and elongation (72°C, 1 min) and a final extension for 7 min at 72°C. After PCR, 10 μL of PCR products were analysed on agarose gel. Transformed colonies which grew on the highest concentration of Geneticin (G418) were further considered for their methanol utilization phenotype by growing them on Minimal Methanol (MM) plates.

Expression studies. Clones which grew on higher concentrations of geneticin antibiotic (G418) were selected for expression studies. Positive clones were spotted onto Buffered Minimal Methanol (BMM) plates supplemented with 0.5 mM 2,2′azino bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 0.1 mM $CuSO_4$ and incubated at 30°C to identify laccase-producing clones. Extracellular laccase activity (liquid cultures) was monitored by measuring the oxidation of 1.6 mM ABTS using a sodium acetate buffer (pH 5.2) at A_{420}. GS115/Sec-HSA/Mut^s (HSA), which secretes albumin into the media extracellularly was used as a positive control and GS115/pPIC9k without insert, was used as a negative control. One clone, which produced a green halo more efficiently than the others, was further analyzed in a liquid culture along with an HSA control devoid of insert control. Colonies from minimal plates, corresponding to each culture, were used for induction studies. A single colony was inoculated into 10 mL of Buffered Minimal Glycerol (BMG) medium and grown at 30°C (250 rpm) for 18 h. The cells were harvested by centrifugation (2,000 × *g* for 5 min), the supernatant was decanted, and the cell pellet was resuspended in BMM medium. To this, 0.1 mM $CuSO_4$ and 0.5 mM ABTS were added and incubated at 30°C (250 rpm). 100% methanol was added to a final concentration of 0.5% (v/v) every 24 h to maintain induction.

A single colony of the transformed *Pichia* clone was grown in BMM broth at 30°C for 6 days and the media was subjected to 70% ammonium sulphate precipitation. The protein precipitate was harvested by centrifugation for 30 min (10,000 × *g*, 4°C), resuspended in 0.4 M sodium acetate buffer (pH 5.2) and dialysed against the same buffer at 4°C. After dialysis, the sample was centrifuged for 10 min (16,000 × *g*) and the obtained pellet was dissolved in 100 μL Phosphate-Buffered Saline (PBS). Aliquots of 10 μL were stored at –20°C.

For SDS-PAGE, a 10 μL sample buffer was added to equal volumes of sample and boiled for 10 min. The sample was centrifuged at 8,000 × *g* (10 min) and 15 μL was used for gel loading. Native PAGE was run (Biorad Mini protein Tetra system) without SDS in stacking as well as separating gel and stained with ABTS solution (15 min) for the development of colour and imaging. The gel was then washed three times with distilled water for 2 min, and fixed for 30 min in a fixing solution (50% methanol and 10% glacial acetic acid). After fixation, the gel was rewashed for 10 min with distilled water and stained with Biosafe Coomassie G-250 stain (Biorad cat# 161-0786) for 45 min. The stained gel image was then captured.

LC-MS/MS. The obtained protein band was excised from the Coomassie stained gel using a clean scalpel for in-gel digestion (Shevchenko et al., 2006) and subsequently used for LC-MS/MS analysis (Centre for cellular and molecular platforms (C-CAMP), Bengaluru, Karnataka, India). An ITMS mass analyser with ESI-TRAP was used to analyse the peptides with ESI (nano-spray) as the ion source. Collision-induced dissociation (CID) with a positive polarity scanned for the peptides. Generated data was used to check for laccase identity on a MASCOT search engine using Swiss-prot, TrEMBL and Basidiomycetes databases.

Efficacy of recombinant laccase in the deconstruction of lignocellulosic biomass. Paddy straw, finger millet straw, wheat straw, maize straw, and sorghum stover were procured from the local market, manually chaffed into 2-cm-length pieces and treated (Sridhar et al., 2015) with both the non-recombinant and recombinant laccases. Individual crop residues were sprayed with a laccase enzyme in the case of treated straws before being considered for analysis. The enzyme and straw were allowed to react for 24 h at 28 ± 2°C (room temperature), dried overnight at 60 ± 2°C, ground to pass through a 1-mm screen and analyzed for changes in the proximate composition and deconstruction of lignin. *Pichia* clone yielded 344 $U \cdot mL^{-1}$ of laccase activity. An activity of 100 $U \cdot mL^{-1}$ was used for each treatment, and each experiment was performed in three replicates. The enzyme to straw ratio adopted was 1:2.5 (1 mL enzyme (100 $U \cdot mL^{-1}$) added to every 2.5 g of straw), based on earlier work (Sridhar et al., 2015). Laccase enzyme obtained using native wild type *S. commune* culture under submerged cultivation conditions was considered as the non-recombinant enzyme, while enzyme produced from a positive clone of *Pichia* GS115 strain containing the expression cassette was considered as the recombinant enzyme. Straws treated with buffered culture media (without enzyme) served as the control (1 mL culture media added to every 2.5 g of straw).

Dry matter of the straw and stover samples was determined by placing them in a hot air oven at 100 ± 5°C for 8 h. Nitrogen (N) content was determined by the standard Kjeldhal method (AOAC, 2012) and the crude protein (CP) content was calculated (N × 6.25). The ash content was determined using a muffle furnace. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) content of treated and untreated lignocellulosic biomass was determined by the method of Van Soest et al. (1991). The procedure of Tilley and Terry (1963) was adopted for evaluating *in vitro* dry matter digestibility (IVDMD) of the straw and stover samples. All experiments were carried out in three replicates.

Statistical analysis. Distribution of the data was studied in terms of the mean and standard deviations. Proc-GLM

(SAS 9.3 software) was used to obtain Mean ± SD and box whisker plots for analysis and to study the extent of delignification. A correlation coefficient between two parameters was established using proc CORR of SAS (9.3).

Results

Codon optimization and construction of the vector

The full length cDNA from *S. commune* considered for this study had 1554 nucleotides encoding a protein comprised of 518 aa which was modified before chemical synthesis. All non-coding sequences were excised from 5′ and 3′ends. The 48 nucleotides signal sequence was also removed. An *Eco*R1 site was incorporated at the 5′end upstream to the initiation codon at 193 bp distance. A *Not*1 site was inserted after the terminator (TAG) codon. Removal of complex secondary structures, repeated sequences in the genes, and early termination of translation were the factors considered for codon optimization.

The length of the optimized coding sequence was 1520 bp and the gene encoding this protein was named *Scom*-Lac. The first ATG codon of the open reading frame occurred at position 166 bp downstream from the 5′end after the *Eco*R1 restriction site. The gene of *P. pastoris* commonly has A/T ended codons. The GC content was adjusted to prolong the mRNA half life. The codon exchange resulted in a GC content of 48% where regions of very high (>80%), or very low (<30%), GC content were avoided. The laccase gene was redesigned by replacing 282 non-preferred codons of the native gene by the *P. pastoris* preferred codons in the synthetic gene and cloned into a pPIC9K vector.

Comparison of the codon-optimized *Scom*-Lac gene with the native gene (Fig. 1) showed that the amino acid sequence of the laccase gene contained five potential N-linked glycosylation sites (Asn-X-Ser/Thr). The deduced aa sequence showed a 74% homology with *Polyporus burmalis* Lac2 mRNA and *Polyporus ciliates* lcc3 mRNA, and a 73% homology with *Cerrena* sp. WR1 lcc2 mRNA. Amino acid residue located 10 amino acids downstream of the conserved cysteine, which affects the redox potential of type 1 copper, in the active site, when methionine is present as class1, leucine is present as class II and phenyl alanine is present as class III (Eggert et al., 1998). The laccase from *S. commune* was classified as a Class II enzyme with leucine at that site.

PCR amplification of the fragment containing the gene of interest was carried out in such a way that the compatible restriction ends formed for ligation into the appropriate vector. The fragment was cloned into pPIC9K generating *Eco*R1 and *Not*1 restriction sites, respectively. The vector pPIC9K is a 9276 fusion vector with four unique restriction sites for cloning in frame with the α-factor secretion signal. The laccase gene was cloned into pPIC9K, downstream of the native *S. cerevisiae* α-factor secretion signal. The vector contained a kanamycin resistant gene for *in vivo* screening and secretes a recombinant protein into the medium. The AOX1 promoter/*Scom*-Lac gene expression cassette was incorporated into the plasmid (pPIC9K). The physical structure of the plasmid with laccase gene (Fig. 2b) had His4 and Kan^r selectable markers. The AOX1 promoter was fused to α-MF prepro signal sequence.

Propagation of the pPIC9K/Scom-Lac vector

The pPIC9K is a shuttle vector and was transformed into competent *E. coli* DH5α cells. Colony PCR was performed to confirm the presence of pPIC9K/*Scom*-Lac using α-factor and 3′AOX1 primers after growing the transformants on LB plates/Ampicillin plates for 24 h. Clones were grown in liquid cultures (LB broth/Ampicillin) for the isolation of the plasmid. The presence of the correct open reading frame was verified by restriction and sequencing of the recombinant plasmid, recovered from *E. coli* DH5α cells (Invitrogen, USA).

Transformation into Pichia pastoris GS115 (his4)

The laccase expression construct was linearized using *Bgl*II and transformed into *P. pastoris* GS115 (his4) strain by the electroporation method. The pPIC9K vector with the laccase gene inserted in frame with the alpha factor signal sequence had two restriction sites for *Bgl*II which linearized the vector into two fragments (Fig. 2a). The fragment containing sites for propagation in *E. coli* was removed and the one with the laccase gene cloned in frame with the alpha factor secretion signal HIS4 region, and the region for kanamycin resistance which confers resistance to geneticin in *Pichia* (Fig. 2b), was inserted into the genome of *Pichia* by recombination between the homologous regions. Proper insertion of the fragment results in the deletion of the entire AOX1 gene.

Following electroporation, GS115/pPIC9K/*Scom*-Lac was plated onto YNB minimal plates for colonies to appear. Clones appeared after six days of incubation (Fig. 3a). Around forty-five colonies appeared which were considered for G418 screening. To test for the upshot of the codon-optimized laccase gene on expression, colonies were screened for multi-copy inserts by increasing the concentration of G418 from 0.25 to 4 mg/mL. After 7 days at 30°C, there were no colonies on plates containing 2–4 mg/mL geneticin. Out of 45 clones, only 10 clones exhibited growth on 0.75 mg/ml G418 containing medium, while only three clones were able to grow on 1.75 mg/mL G418 containing medium.

Direct PCR screening of Pichia clones

Integration of a laccase expression cassette in *Pichia* was validated using primers (GS1F/R and GS2F/R) specific for a codon-optimized laccase gene. Colony PCR of amplified product visualised a band size of 246 bp on agarose gel confirming stable integration (Fig. 3b). The transformants were further grown on minimal methanol plates to screen for methanol utilization (Mut$^+$/Mut^s) phenotype and their ability to utilize methanol (Mut±). In the present study, all the clones exhibited a methanol utilisation slow (Mut^s) phenotype.

Expression studies

The pPIC9K/*Scom*-Lac expression construct was introduced into the *Pichia* GS115 (his4) genome and extracellular expression of laccase was confirmed under methanol inducible conditions by ABTS assay on BMM agar

Fig. 1. Sequence comparison between the native *S. commune* laccase gene and the codon-optimized laccase gene with their corresponding single letter aminoacid code.

The nucleotide bases of the codon-optimized open reading frame laccase gene is compared with the native *S. commune* gene which codes for 446 aminoacids. aa corresponds to aminoacids, co is the codon-optimized synthetic gene and wt is the wild type native gene. A star indicates aminoacids involved in binding copper (10 his and 1cys).

plates. A green halo around the colony with a laccase gene insert was observed after 8 days (Fig. 4a). A time course analysis was performed by analysing the samples at 0, 24, 48, 72, 96, 120 and 144 h for extracellular laccase production in BMM medium (Fig. 4d). Laccase activities increased in the extracellular broth after 3 days. The maximum activity (344 $U \cdot mL^{-1}$) was recorded (0.019 $g \cdot mL^{-1}$ wet cell weight) five days after induction with 0.5% methanol at 30°C.

SDS-PAGE and Zymogram analysis revealed extracellular production of laccase. Cell free extract of GS115/pPIC9K/*Scom*-Lac was subjected to ammonium sulphate fractionation/dialysis and separated on polyacrylamide gel electrophoresis. The laccase protein produced by the recombinant *Pichia* clone was detected as two bands with an apparent molecular weights of 55 kDa and 70 kDa on SDS-PAGE (Fig. 4b). The native PAGE showed extracellular production of two laccase isozymes by recombinant *Pichia* clone (Fig. 4c). Activity staining on native PAGE was performed to confirm the presence of bioactive laccase. Laccase activity was confirmed by zymogram analysis using ABTS as substrate, the oxidised radical of which was visualized as a green colour band (Fig. 4c). Activity staining confirmed that the *Pichia* clone produced laccase extracellularly and was biologically active. These bands were not observed in the control strain (*Pichia* transformed with pPIC9K alone).

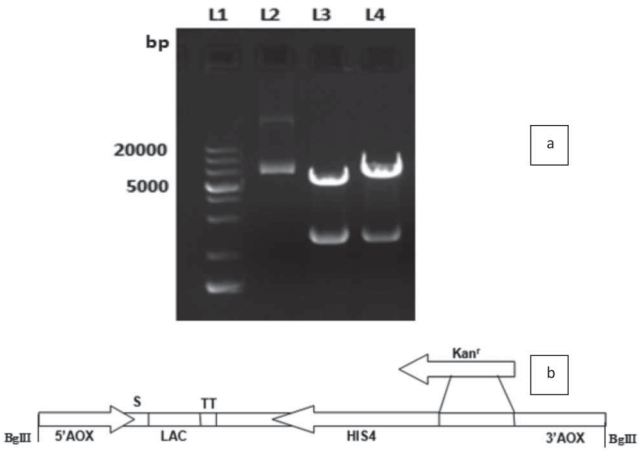

Fig. 2. Construction of expression casette.

a. Linearization of a propagated pPIC9K/*Scom*-Lac vector in *E. coli* DH5α cells. Plasmid was isolated and linearized using the *Bgl*II restriction enzyme. Two microlitre samples were electrophoresed on agarose gel (1%). L1-Gene ruler^{TM} 1 kb plus DNA Ladder., L2-Plasmid as such without linearization, L3-Linearized pPIC9K vector alone plasmid, L4-Linearized plasmid pPIC9K vector with a laccase gene insert. b. Diagrammatic representation of the *Bgl*II linearized fragment containing the laccase construct cloned in frame with the alpha factor secretion signal in pPIC9K vector.

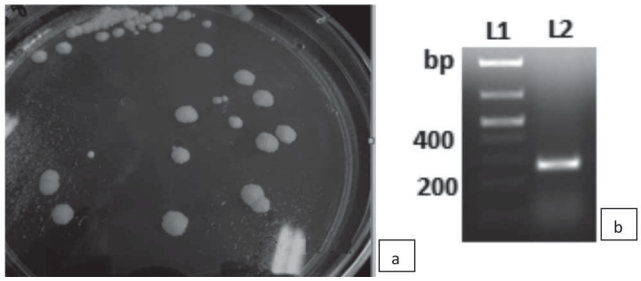

Fig. 3. Transformation into *Pichia* pastoris.

a. Plates showing the transformed *Pichia* GS115/pPIC9K/*Scom*-Lac clones on Histidine deficient YNB plates. b. *Pichia* clone confirming the laccase gene insert using gene specific primers GS1F/R and GS2F/R.

LC/MS-MS

Tryptic *in-gel* digestion of the commassie stained bands (Fig. 4b) was performed to identify recombinant protein produced extracellularly in the culture medium of *P. pastoris*. LC-MS/MS analysis of the bands A and B on SDS-PAGE confirmed laccase as the recombinant protein produced. Analysis of both bands showed fungal laccase amino acid signature sequences corresponding to Lac 1, confirming YSFVLEANQAVDNYWIR to be the major peptide (*m/z* of 1044.5138). Protein blast matched them to the cupredoxin domain of fungal laccases. The masses of tryptic fragments determined by MS analysis for sample A and B corresponded to Lac 1 and the spectra are shown in Fig. 5.

In the present study, LC-MS/MS with Mascot search was used to identify proteins secreted from the recombinant strain of *P. pastoris*. Signature sequences corresponding to Lac 1 were obtained. A comparison of the protein sequence with other laccases indicated a high similarity to basidiomycete laccases with a high affinity for the copper binding regions, and with all 'his' and 'cys' residues conserved. The mass spectrometry analysis enabled a more accurate estimation of the average molecular mass of around 55.6987 kDa.

Efficacy of recombinant laccase in the deconstruction of lignocellulosic biomass

Five common crop residues were treated with laccase obtained from recombinant and non-recombinant strains and evaluated for their proximate composition and lignin degrading capability. The *in vitro* dry matter digestibility of the straws was also assessed. Recombinant strain expressed 344 U·mL^{-1} (specific activity-163.80) laccase activity in the buffered medium while the non-recombinant strain produced 236 U·mL^{-1} (specific activity-56) laccase activity in the crude culture filtrate. The changes obtained in the cell wall constituents of five different crop residues treated with both the non-recombinant laccase and the recombinant laccase versus the control are depicted in Fig. 6a. Untreated control straw (A) recorded a minimal increase in protein content of 0.38%, 0.44%, 0.57%, 1.1% and 2.2% for wheat straw, maize straw, sorghum stover, paddy, and finger millet straws, respectively compared to B and C. A marginal decrease in DM content of straws ranging between 1.5 to 3% was observed in B and C as compared with A the control untreated straws. Treatment with both the non-recombinant laccase and the recombinant laccase recorded a decrease in NDF, ADF and ADL contents for all five crop residues compared with untreated straw which served as a control, proving their efficacy in the delignification of the straws. The significant loss in lignin upon treatment with recombinant laccase ranged from the least loss of 1.64% in sorghum stover to the greatest loss of 4.83% obtained in finger millet straw. The increase obtained *in vitro* dry matter digestibility upon treatment with recombinant laccase ranged from 8.71% in maize straw to the greatest increase of 24.61% obtained in finger millet straw.

The relationship between the increase in digestibility and lignin degradation was established by plotting a correlation graph using proc CORR of SAS (9.3) (Fig. 6b). A decrease in fibre content with enhancement in digestibility in straws treated with laccase obtained from *S. commune* was earlier reported (Kumar et al., 2015). In the current study, ADL and IVDMD data from different crop residues (Fig. 6b) demonstrated successful degradation of lignin with an increase in digestibility upon treatment with recombinant laccase in comparison with both the untreated crop residues as well as those treated with the non-recombinant laccase. The ADL and IVDMD values obtained by treating different crop residues were taken into consideration and correlation was established in terms of lignin degradation and *in vitro* dry matter digestibility. There was a strong negative correlation (correlation coefficient $r = -0.99$) (F value 213.8) between enhancement of *in vitro* digestibility and lignin degradation for finger millet straw treated with recombinant laccase. A strong negative correlation was also observed in all other straws treated with recombinant laccase with F-values of 32.19 for maize, 89.19 for paddy, 7.406 for sorghum stover and

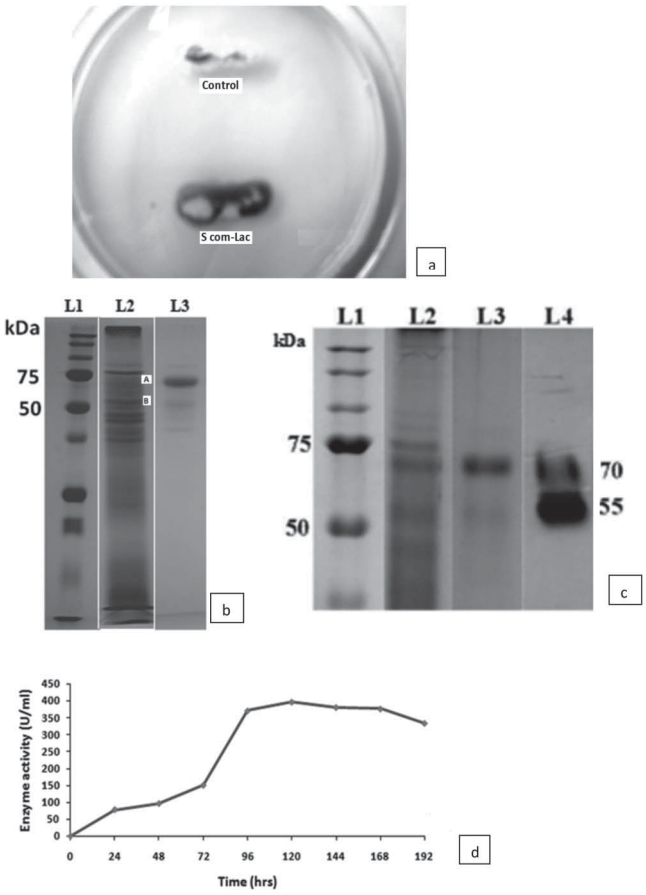

Fig. 4. Detection of laccase activity on BMM agar plates with 0.1 mM $CuSO_4$ and 0.5 mM ABTS for transformant with lac gene from *S. commune*.

a. Green halo around the positive *Pichia* pPIC9K/*Scom*-Lac clone after 8 days of methanol induction, and absence of green colour around the control GS115/pPIC9K without insert. b. SDS PAGE and Zymogram analysis of recombinant laccase from GS115/pPIC9K/*Scom*-Lac. c. Native PAGE L1 - Protein standard marker (Biorad) L2-Zymogram of 10 μL of culture filtrate in loading buffer not containing SDS/β-mercaptoethanol L3: commercial laccaseL4-Activity staining of laccase on Native PAGE using ABTS as substrate. d. Time course of extracellular laccase production in Buffered Minimal Methanol (BMM) liquid medium.

22.47 for wheat being obtained with a more significantly pronounced increase in IVDMD for finger millet, maize, paddy, and wheat ($p < 0.05$). The increase in digestibility of finger millet straw was 24.61% with a loss of 4.83% lignin with a confidence interval of 99.9% and was highly significant. Maize straw recorded an increase in digestibility of 8.71% with a 1.86% decrease in lignin content. Paddy straw and sorghum stover confirmed a 1.69% and 1.64% loss in lignin with corresponding increases in digestibility of 16.6% and 9.86%, respectively. In the case of wheat straw, a 16.48% increase in digestibility was recorded with a concomitant 1.92% decrease in lignin. A significant difference with and without enzyme treatment ($p < 0.0001$) at a 99% confidence interval was observed in all straws. IVDMD was found to increase significantly ($p < 0.05$) and the highest and best *in vitro* digestibility was observed in the case of finger millet straw where an increase of 24.61% in digestibility showed the maximum degradation and enhanced digestibility.

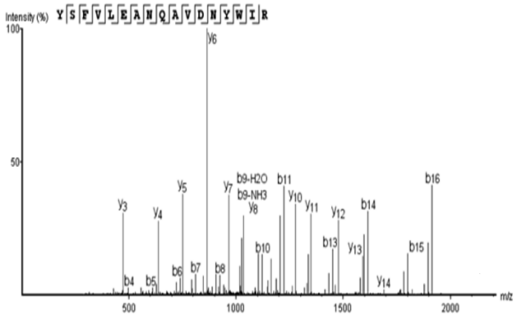

Fig. 5. Mass spectra of peptide fragments of three types of laccase: Mass fingerprint analysis of peptides arising from SDS-PAGE separated, and *in situ*, trypsin digested bands of recombinant laccase.

The *x*-axis represents mass (*m*/*z*) and the *y*-axis represents % intensity Sample A and B for Lac 1.

Discussion

One of the limitations to the large-scale application of laccases is the lack of capacity to produce large volumes of these highly active enzymes, and lignolytic enzymes such as laccases are notoriously difficult to express heterologously (Ergün and Çalık, 2016). The production of laccases in the native state cannot meet the requirements of commercial applications. Expression in heterologous hosts would be a viable option to consider as large volumes of the enzyme can be obtained with low production costs. There are several reports of laccase gene expression in heterologous hosts such as yeasts, filamentous fungi, bacteria, etc. An attempt has been made in the present study to express the laccase gene from *S. commune* in *Pichia*, whilst lowering application costs, which otherwise is a bottleneck in scaling up and sustainably use laccases for industrial applications. There is a continuous quest for isolating and screening laccase producers with desirable yields and properties (Olajuyigbe and Fatokun, 2017). Hatamoto et al. (1999) expressed *S. commune* laccase in *Aspergillus sajae* strain 1860 (with low protease levels) suggesting protein engineering strategies for improved production (Upadhyay et al., 2016). In the present study, the laccase gene was codon optimized as per the codon bias of *P. pastoris* (Mellitzer et al., 2012; Wang et al., 2012). The codon-optimized laccase gene was cloned into a pPIC9K vector and linearized for transformation. A *Bgl*II linearized construct is integrated into the genome of *P. pastoris* (Cregg and Madden, 1987), where the entire AOX1 structural gene is deleted and replaced with a construct composed of the AOX1 promoter, the laccase gene expression cassette, His4 sequence and kanamycin resistance gene. Transformed clones were selected by histidine phototrophy (His+) and antibiotic resistance. His+ transformants grew slowly on methanol as a second alcohol oxidase gene; AOX2 is expressed (Cregg and Madden, 1987; Cregg et al., 1985) with a frequency of <0.1% when the total length of the targeting fragments is <500 bp (Higgins and Cregg, 1998) with a frequency of 10–20% (Li et al., 2007), or up to 30% (Higgins and Cregg, 1998) when extensive ~1 kb regions of homology are used.

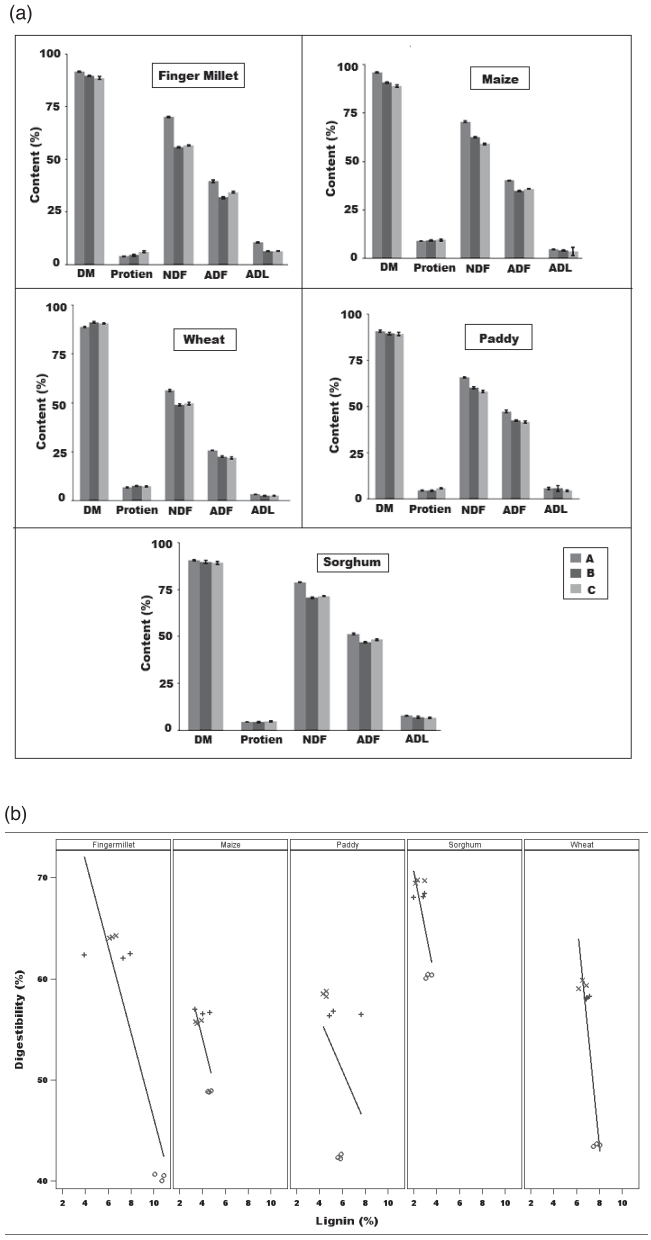

Fig. 6. Delignification of crop residues.

a. Treatment of five different straws with (A) control: straw without enzyme treatment, (B) straw treated with non-recombinant laccase, and (C) straw treated with recombinant laccase. The *y*-axis represents '*%*' conversion and the *x*-axis represents the 'nutritional parameters' analyzed. DM-dry matter, Protein-crude protein, NDF-neutral detergent fiber, ADF-acid detergent fiber, ADL-acid detergent lignin, IVDMD-*in vitro* dry matter digestibility; values are: Means ± SE. b. Correlation between *in vitro* dry matter digestibility (%) and acid detergent lignin (%) for different crop residues treated with laccase. Symbols (○, +, ×) represent untreated straw (A), straw treated with non-recombinant laccase (B), and straw treated with recombinant laccase (C).

The Tn903 kanamycin resistance (Kan) gene in the construct confers resistance to G418 in *Pichia*, and is reported to have a strong correlation with copy number which increases expression levels (Cregg et al., 1993). *Pichia* clones which grew on the higher concentrations of geneticin were chosen for production studies.

Laccases are metallo-proteins and, hence, addition of copper allows the excreted protein to fold correctly in the culture filtrate, as is shown in previous studies (Giardina et al., 2007; Lorenzo et al., 2006). Over expressing lignolytic enzymes in an active form is difficult (Leif et al., 1997) as media physical conditions, such as pH and temperature, play an important role (Gelo-Pujic et al., 1999; Klibanov, 2001). During experimentation, protease inhibitors were used in the production phase to curb the activity of acid proteases (if produced) at lower pH levels so that the stability of the recombinant protein produced is not compromised. It is also known that *P. pastoris* secretes no endogenous lignolytic enzymes (Cregg et al., 1993) which confirms the purity of heterologous preparations without requirements for extensive downstream processing procedures.

The occurrence of signature peptides of laccase at different positions within the gels is indicative of post-translational modifications or homo/heterodimerization of enzymes. Many WRF exhibit multiple isozymes/isoforms of laccase. Five isoforms of *Schizophyllum* laccase ranged in molecular weight from 36,000 to 48,000 (De Vries et al., 1986). Bands visualized after SDS-PAGE analysis showed two bands with apparent molecular weights of 55 kDa and 70 kDa, respectively. Moreover, the theoretically calculated molecular weight is around 55 kDa for 506 amino acids. The molecular weight of the laccase from *S. commune* was reported to be 62 KDa by SDS-PAGE (De Vries et al., 1986), suggesting the carbohydrate content to be around 13.7%. The variations in the experimental findings could be due to the presence of many laccase isoforms with different characteristics (Dong et al., 2005; Giardina et al., 2007; Lisova et al., 2010). Slight variations in the expressed peptide sequences are attributed to differences in patterns of glycosylation in *P. pastoris* contributing to the differences observed. Comparison of the peptide mass fingerprint data indicated the presence of signature peptides matching Lac1 of known basidiomycetes.

Expansion of agro industrial activity accumulates large quantities of lignocellulosic residues which comprise 80% DM, and the quality of feeds in their natural state is low in protein content (2.5 to 6%) associated with high lignin (7 to 14%). The energy in the form of cellulose and hemicelluloses is trapped within the lignin matrix owing to the poor digestibility by ruminants. Solid state fermentation using WRF is being continuously employed by many workers for the degradation of crop residues to be successfully used as animal feed (Misra et al., 2007; Sharma and Arora, 2010; Shrivastava et al., 2012; Tripathi et al., 2008). Many observed an increase in the *in vitro* DM digestibility of spent straw (Zadražil, 1997). However, the majority of the experiments carried out using WRF in SSF resulted in organic matter losses after 5–7 days of fermentation, and, hence, to compensate for the losses an increased organic matter digestibility is needed. Therefore, SSF for a maximum period of only 6–8 days has been recommended as the most beneficial in order to reduce dry matter losses (Owen et al., 2012; Sridhar et al., 2014). The problems associated with organic matter losses in SSF are circumvented when cell-free enzyme-rich media is applied directly for treatment. The FAO (2011) perspective was to isolate and identify potent lignolytic fungi in nature to cultivate it for the commercial production of lignolytic enzymes. Biological treatment methods employed an enzymatic system of WRF to upgrade the qual-

ity of fibres avoiding consumption of polysaccharides (Zadražil, 1997). Kumar et al (2015) observed an increase in the digestibility of finger millet straw with a notable decrease in the lignin content upon treatment with the enzyme-rich media. Improvement in the nutritional quality and digestibility of the feed material is not only dependent upon the fungal strain used, but also on the type of substrate employed for the purpose (Chalamcherla et al., 2009). The dry matter losses were minimal between 1.5 to 3% in all the straws evaluated in the present study. This trivial loss in dry matter is circumvented as the enzyme is sprayed onto the straws during treatment. Unlike SSF, which results in a significant protein increase on account of a multiplication in the microbial biomass, only a marginal increase witnessed in the protein content of the treated straws could be attributed to the addition of the enzyme a protein by nature. The linear increase observed in IVDMD of treated straws is in agreement with earlier work (Karunanandaa and Verga, 1996). A decrease in the cell wall components advocates that vegetal cell wall components of the straws were degraded on account of the laccase which is capable of oxidising lignin and facilitating enhanced digestibility of the crop residues.

In this study, a successful deconstruction of lignin in the straws was observed using both recombinant and non-recombinant laccase. The percentage of lignin and the type of lignin monomers vary from straw to straw, and, hence, an observed variation in digestibility. Digestibility is generally inversely correlated to the amount of lignin in the substrate (Han, 1975). A reported digestibility value for different types of straws is between 40–55% (Van Soest, 2006). Treatment of crop residues using recombinant laccase was exemplified by more lignin degradation and a greater digestibility compared with the treatment carried out using non-recombinant laccase. This is because the *Pichia* recombinant clone produces only recombinant protein into the extracellular environment with very negligible amounts of native protein. However, laccase produced from the non-recombinant strain into the culture medium is interspersed by the media components and other metabolites released along with laccase, which may bring down the degradation potential to some extent when diluted. This is also evident from the specific activity values in the ratio of 3:1 for recombinant and non-recombinant laccases, respectively, demonstrating the availability of more laccase for treatment in the former as compared with the latter. The current research facilitated the successful degradation of lignin using laccase treated straw as compared with untreated straw. An overall enhancement in digestibility of 26.33% was obtained. The work carried out successfully proves the hypothesis that laccases from white rot fungi are effective in biodelignification and enhancing the digestibility of crop residues for ruminants. Also, the recombinant laccase obtained in the present study was biologically active and equally effective in enhancing the digestibility of all the evaluated straws.

Summary

Laccases are considered to be environmentally important enzymes, and emphasis must be brought to bear on boosting up the yields for industrial applications in general, and delignification of crop residues in particular. The limitations of the large-scale application of the laccase enzyme in terms of efficiency and economic feasibility was addressed to a great extent by treating the lignocellulosic biomass using a produced recombinant laccase. In conclusion, the full length optimized gene sequence of laccase from *S. commune* was successfully expressed under the control of an AOX1 promoter and laccase protein was secreted into the culture medium of *P. pastoris* GS115. This study has highlighted the potential use of the laccase enzyme in the successful degradation of lignin in various crop residues, enables their efficient use as animal feed. The method described previous an effective, simple, and economically viable technology which would improve the nutritive quality and digestibility of straw. *In vivo* pilot studies in ruminants are, however, warranted to confirm the results obtained *in vitro*.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest.

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