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Correspondence and requests for materials should be addressed to R.C.K. (kuhad85@gmail.com)

Middle-redox potential laccase from *Ganoderma* sp.: its application in improvement of feed for monogastric animals

Krishna Kant Sharma[1,2], Bhuvnesh Shrivastava[1], V. R. B. Sastry[3], Neeta Sehgal[4] & Ramesh Chander Kuhad[1]

[1]Lignocellulose Biotechnology Laboratory Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India, [2]Department of Microbiology, Maharshi Dayanand University, Rohtak-124001 India, [3]Ganesh Scientific Research Foundation, Kirti Nagar, New Delhi -110 015, India, [4]Department of Zoology, University of Delhi, Delhi – 110007 India.

The variables influencing laccase production by white-rot fungus *Ganoderma* sp. rckk-02 were optimized employing response surface methodology. Malt extract (6.0% w/v), lignin (0.5% w/v) and pH (5.5) were found to be the most significant factors for enhanced laccase production by 7 fold (226.0 U/ml) as compared to unoptimized growth conditions (32.0 U/ml). The N-terminal sequence of laccase revealed its distinct amino acid profile (S- I- R- N- S- G), which suggested it as a novel enzyme. The Far-UV CD spectrum of the laccase showed single broad negative trough at around 213 nm, a typical signature of all β proteins. The laccase was found to fall in the range of middle redox potential laccases. Purified laccase at dosage of 2.5 Ug^{-1} body weight when supplemented with pelleted diet of rats, a significant improvement ($p < 0.05$) in nutrients digestibility without causing any elevation of blood stress enzymes was observed.

White rot fungi are known to be the potential producer of ligninolytic enzymes i.e. mangnese peroxidase, lignin peroxidase and laccases. Among these, laccases are one of the widely studied oxidoreductases and majority of them contain four copper ions per molecule. They catalyze single-electron oxidation of a wide variety of organic and inorganic substrates including mono-, di-, polyphenols, methoxyphenols, aromatic amines and ascorbate with concomitant four-electron reduction of oxygen to water[1]. This makes laccases useful for their varied applications in several biotechnological processes such as phenolics elimination for stabilization and browning of fruit juices, beer/wine, in bleaching of paper pulp, decolourization of dyes and textile effluents, oxidation-reduction reaction in biosensors development, PAHs and other xenobiotics degradation for soil bioremediation, production of complex polymers in organic synthesis and mild oxidizing agent in cosmetics development[2]. In addition, its being an efficient lignin degrading biocatalyst, has been applied in biopulping but scarcely been tried for its possible application in improving the bioavailability of nutrients and digestibility of animal feed, especially for monogastric diets.

Laccase can either act on feed components prior to or after consumption i.e. within the gastrointestinal tract[3]. However, to make use of laccase commercially viable in feed improvement, its production level should be high as well as it should be nontoxic to animals. The critical safety evaluation of the enzymes used for improving animal feed is must and tests involving rat models have been found essential to provide some preliminary indication of possible cellular mutagenicity, potential carcinogenicity and teratogenecity during the developmental stages[3–5]. Furthermore, random addition of enzymes to diets without consideration for specific situations, toxicity and substrate targets will only discourage or delay on- farm adoption of enzyme technology[6]. Therefore, we have also selected the rat model for evaluation of efficiency of laccase in improvement of pelleted diets specifically for monogastric animals. Thus for producing higher amount of such enzymes researchers have gained interest in minimizing the cost of enzyme preparation to be used as ruminants diet supplement[7]. Thereby, designing and optimization of fermentation media for the most economical production is one of the most important task.

The objectives of this work were to optimize production of laccase with middle redox potential from an indigenous white rot fungus, *Ganoderma* sp. rckk-02 using response surface methodology under liquid static cultivation conditions, its characterization and its evaluation for possible application as animal feed supplement.

Table 1 | Experimental range and levels of the independent variables used in RSM in terms of actual and coded values

	Range of levels				
Variables	$-\alpha$	−1	0	+1	$+\alpha$
A. Malt extract (% w/v)	4.32	5	6	7	7.68
B. Lignin (% w/v)	0.33	0.4	0.5	0.6	0.67
C. pH	4.66	5	5.5	6	6.34

Results

Laccase production from *Ganoderma* sp. rckk-02, when grown under static cultivation conditions, started after 48 h of incubation (3.8 U/ml) and reached maximum (32 U/ml) after 240 h and declined thereafter (Graph not shown).

Response surface methodology (RSM). For the selection of the key factors like carbon source, nitrogen source and trace metal one factor at a time strategy was adopted followed by Plackett-Burman Design (PBD). Thereafter, RSM employing CCRD was used to optimize individual concentrations of selected variables viz. malt extract, lignin and pH, identified to possess maximum influence on laccase production from *Ganoderma* sp. rckk-02. Table 2 depicts the CCRD analysis along with experimental and predicted responses obtained in each individual experiment. The regression equations obtained after the analysis of variance (ANOVA) gave the level of laccase produced as a function of the initial values of malt extract, pH and lignin (Table 3). The final response equation that represented a suitable model for laccase production is given below: $Y = 224.97 + 10.12A - 2.33B - 2.08C - 34.35A^2 - 17.79B^2 - 7.41C^2 - 5.25AB + 1AC + 1.13A^3 + 0.083B^3 + 0.083C^3$, where Y is laccase production; A is malt extract; B is lignin; and C is pH.

The coefficient of determination (R^2) was calculated to be 0.9998 for laccase production and thus the model could explain 99.98% variability in the response. The R^2 value provided a measure of variability in the observed response values, which could be explained by the experimental factors and their interactions. The closer the R^2 value is to 1.00, stronger the model is and the better it predicts[8]. Predicted R^2 (0.9931) was found to be very close to the adjusted R^2 (0.9993) for laccase yield, which shows number of terms in the model relative to the number of squares of points in the design. The adjusted R^2 corrects the R^2 value for the sample size and number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than predicted R^2. The purpose of statistical analysis is to determine the experimental factors, which generate signals that are large in comparison to noise. The adequate precision measuring the signal to noise ratio was found to be 131.839 for laccase production. A signal to noise ratio greater than 4 is desirable. The model is thus fit and can be used to navigate the design space. The model *F*-value of 1981.84 and values of Prob > F (<0.05) indicated that model terms are significant (Table 3).

Three-dimensional response surface curves were plotted to study the interaction among different physiochemical parameters to find out optimum laccase production from *Ganoderma* sp. rckk-02. The plots were generated by plotting the response (Laccase production) using the Z-axis against two independent variables while keeping the other independent variables at their O-level.

An interaction between the two parameters (pH and lignin) gave a higher enzyme yield at lignin concentration of 0.5% (w/v) and pH 5.5 (Fig. 1a, d). The response between pH and malt extract at O-level of lignin indicated that a moderately acidic pH (5.5) was desirable, since laccase production from *Ganoderma* sp. rckk-02 is sensitive to pH (optimum range pH 5.0–5.8) (Fig. 1b). Response surface plot of the interaction between malt extract and lignin, while keeping pH at O-level is depicted in Figure 1c where, a linear increase in laccase production was observed when malt extract concentration increased up to 6.0% (w/v) and thereafter it declined steadily. Moreover, a linear increase in the laccase production with increase in lignin level from 0.4 to 0.5% (w/v) was recorded.

To validate and confirm these predictions, experiments were designed with random levels of factors within and outside the design space. Validation was carried out under conditions predicted by the model (varying levels of pH, lignin and malt extract). The experimental values were found to be very close to the predicted values, and hence, the model was successfully validated.

Purification of laccase. Extracellular laccase from *Ganoderma* sp. rckk-02 was purified to homogeneity. Crude enzyme was precipitated and concentrated using ammonium sulphate and ultrafiltration column (10 kDa), respectively, resulting into 2.42 fold

Table 2 | Results of central composite rotatable design (CCRD) using three independent variables showing observed and predicted response

	Malt extract (A)		Lignin (B)		pH (C)		Laccase Yield (U/ml)	
Run order	Actual	Coded	Actual	Coded	Actual	Coded	Observed	Predicted
1	5.00	−1	0.4	−1	5.0	−1	154.00	152.87
2	7.00	1	0.4	−1	5.0	−1	185.00	183.91
3	5.00	−1	0.6	1	5.0	−1	160.00	158.76
4	7.00	1	0.6	1	5.0	−1	170.00	168.80
5	5.00	−1	0.4	−1	6.0	+1	148.00	146.80
6	7.00	1	0.4	−1	6.0	+1	183.00	181.84
7	5.00	−1	0.6	1	6.0	+1	154.00	152.69
8	7.00	1	0.6	1	6.0	+1	168.00	166.73
9	4.32	$-\alpha$	0.5	0	5.5	0	129.00	130.74
10	7.68	$+\alpha$	0.5	0	5.5	0	167.00	168.65
11	6.00	0	0.33	$-\alpha$	5.5	0	158.00	159.57
12	6.00	0	0.67	$+\alpha$	5.5	0	150.00	151.82
13	6.00	0	0.5	0	4.66	$-\alpha$	199.00	200.62
14	6.00	0	0.5	0	6.34	$+\alpha$	192.00	193.78
15	6.00	0	0.5	0	5.5	0	225.00	224.90
16	6.00	0	0.5	0	5.5	0	225.00	224.90
17	6.00	0	0.5	0	5.5	0	224.00	224.90
18	6.00	0	0.5	0	5.5	0	226.00	224.90
19	6.00	0	0.5	0	5.5	0	226.00	224.90
20	6.00	0	0.5	0	5.5	0	224.00	224.90

Table 3 | Analysis of variance (ANOVA) for central composite rotatable design (CCRD)

Form	Laccase yield
F− value †	1981.84
P > F*	0.0001
Mean	183.34
R^2	0.9998
Adjusted R^2	0.9993
Predicted R^2	0.9931
Coefficient of variance	0.48
Adequate precision	131.839

*Value of P > F less than 0.0500 indicate model terms are significant
†The model F-value of 1981.84 for laccase implies that model is significant. There is only 0.01% chance that a 'Model F value' this large could occur due to noise.

purification. The final fold purification of laccase was estimated as 9.3, with a yield of 8.7%. Conventional multi-step procedures have generally been followed for complete purification of laccase that follow almost similar protocol involving concentration step (ammonium sulphate or acetone precipitation) followed by dialysis, ion-exchange chromatography and gel-filtration[9].

The purity of the enzyme was checked by native PAGE, SDS-PAGE, and zymogram analysis (Fig. 2). On the native PAGE, the purified enzyme showed a single band of 68 kDa. The zymogram analysis of laccase using guaiacol revealed colored band within 30 min of incubation, coinciding with Coomassie stained protein band on Native PAGE. The purified enzyme showed a single band of approximately 62 kDa molecular weight on 10% SDS-PAGE following Coomassie staining. In order to accurately determine the molecular mass of laccase protein, the enzyme was analysed on MALDI-ToF mass spectra.

Characterization of laccase. *Effect of pH and temperature on laccase activity.* Purified laccase was active over acidic pH range (3.0–5.5) with maximum activity at pH 4.5. Laccase was highly stable at pH ranging from 3.0 to 5.0. The enzymes retained almost 50% activity even after 6 h incubation at pH 6, whereas, at pH 5 it showed almost 95% residual activity.

Laccase showed maximum temperature stability at 30°C and was observed to retain 100% residual activity even after 150 min. However, the laccase stability started to decline at temperatures 60°C and above with 30–77% residual activity (Fig. 3a). Purified laccase had temperature optima at 50°C hence the subsequent

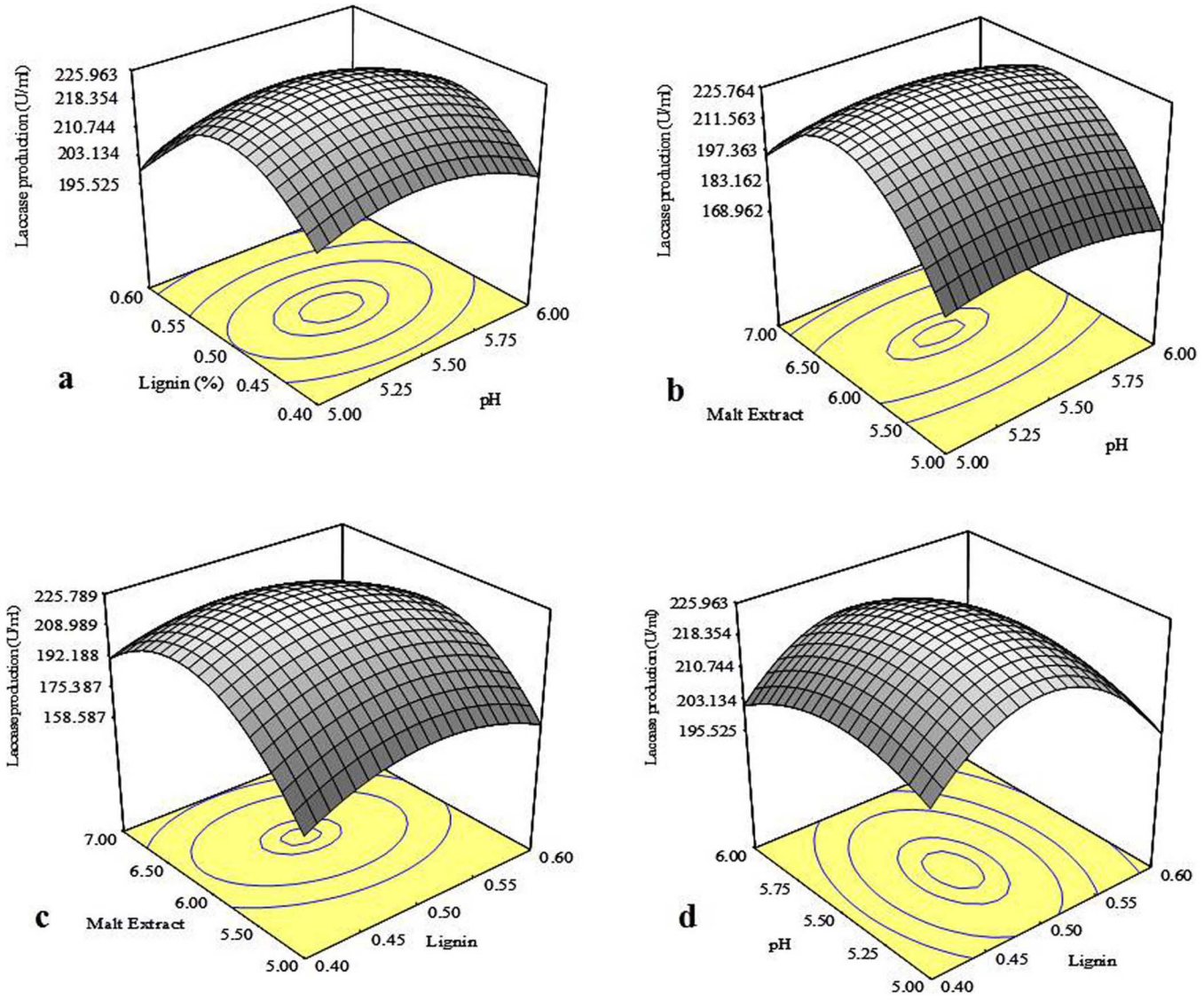

Figure 1 | Response surface curve showing the various interaction effect. (a) lignin and pH (b) Malt extract and pH (c) Malt extract and lignin and (d) pH and lignin on laccase production from *Ganoderma* sp. rckk-02.

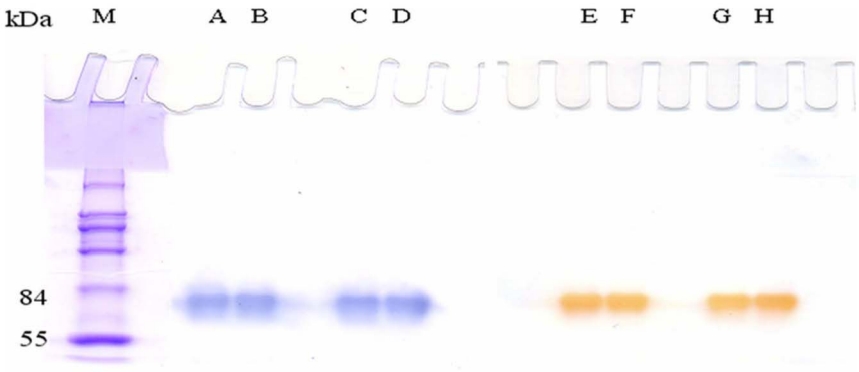

Figure 2 | **SDS-PAGE analysis of purified laccase.** (A–D) Coomassie and (E–H) Zymogram with Guaiacol as substrate. M-Molecular weight marker (kDa).

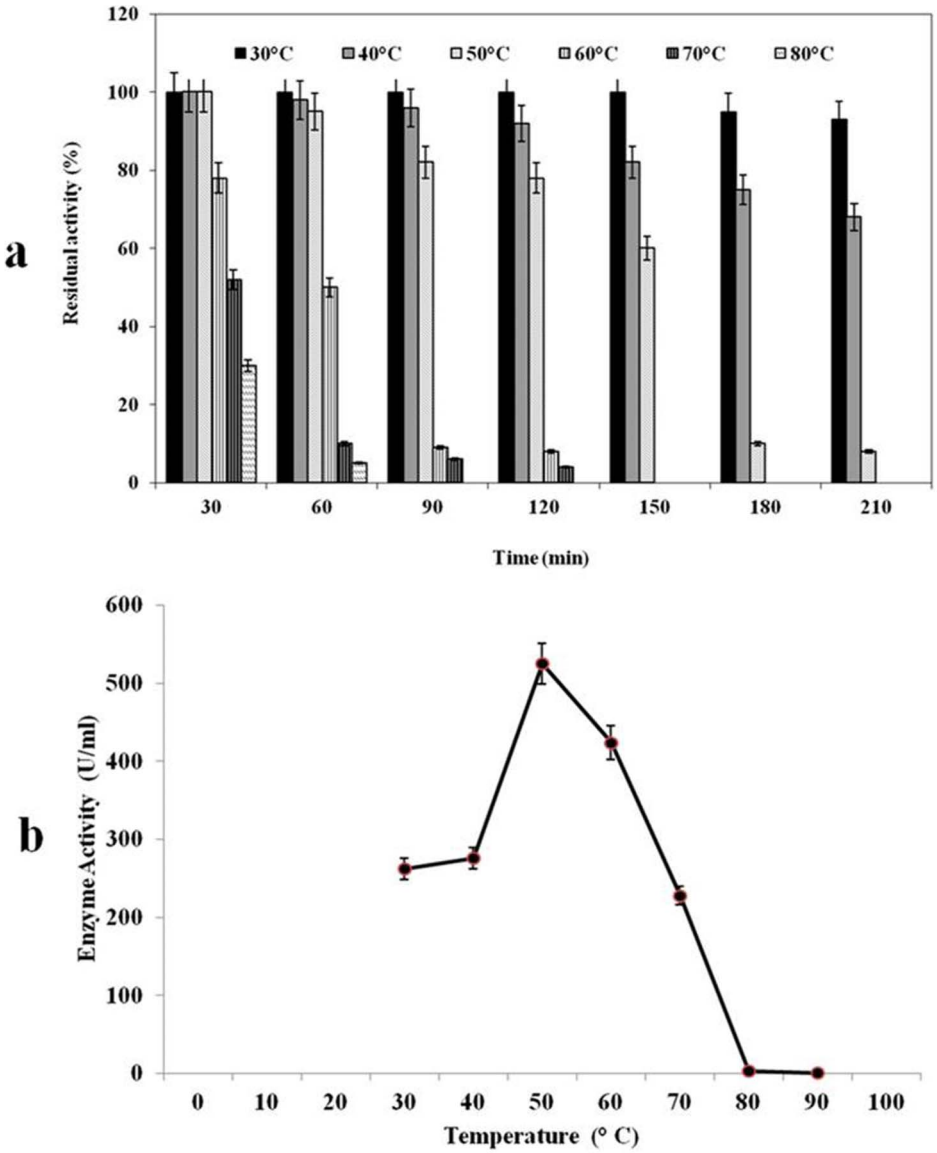

Figure 3 | (a)Thermostability of purified laccase from *Ganoderma* sp. rckk-02 at the temperature range 30°C–80°C. (b) Effect of temperature on purified laccase from *Ganoderma* sp. rckk-02. The optimum temperature for laccase activity was determined by performing assay at pH 5.4 at temperature ranging from 30°C to 90°C.

Table 4 | Comparison of N-terminal sequence of laccase from *Ganoderma* sp. rckk-02 with other laccases.

S. No.	Fungal Cultures	Sequences
1.	*Cantharellus cibarius* laccase	G C C N C G H A
2.	*Coriolus versicolor* laccase	G I G T K··A
3.	*Coriolus versicolor* laccase	A I G P T··A
4.	*Agaricus bisporus* laccase I	K T R T F D F D
5.	*Agaricus bisporus* laccase II	D T K T F N F D
6.	Basidiomycete PM1 laccase	S I G P V··A
7.	*Ceriporiopsis subvermispora* laccase I	A I G P V T D L
8.	*Pleurotus eryngii* laccase I	A X K K L D F H
9.	*Pleurotus eryngii* laccase II	A T K K L D F H
10.	*Phlebia radiata* laccase	S I G P V T D F
11.	*Pycnoporus cinnabarinus* laccase	A I G P V··A
12.	*Trametes versicolor* laccase I	A I G P V··A
13.	*Trametes versicolor* laccase II	G I G P V··A
14.	*Trametes versicolor* laccase III	G I G P V··A
15.	*Ganoderma* sp. rckk-02 laccase I	S I R N S G

*All the colored amino acids are partially conserved.
Reference cited from: S. NO. 2–3Bourbonnais et al. (1995)[15]. S. NO. 4–7, 10–14Eggert et al. (1996)[10]. S. NO. 1, 8–9Ng and Wang (2004)[36].

experiments during enzyme characterization were performed at 50°C (Fig. 3b).

Effect of substrate concentration. Laccase activity increased with increasing substrate concentration and maximum value was observed with 4.5 mM guaiacol and 4 mM ABTS, thereafter enzyme activity gradually declined. Laccase had a Km of 217 µM and 77 µM and Vmax of 92.59 µmoles/ml/min and 250 µmoles/ml/min for guaiacol and ABTS, respectively.

Effect of metal ions. Metal ions are known to bind to the enzyme and alter its activity by stabilization or destabilization of the protein conformation. The laccase from *Ganoderma* sp. rckk-02 was observed to be affected differently with majority of metal ions in a concentration dependent manner. Maximum inhibition of laccase was observed with $CoCl_2$ and $HgCl_2$ inhibited laccase maximally. The enzyme was comparatively stable in presence of $CaCl_2$, $MnSO_4$, $FeCl_3$, $ZnSO_4$ and $BaCl_2$ (0.5–3 mM) with a significant inhibition only at very high concentrations i.e. 10 mM of the metal ions. Exceptionally, $CuSO_4$ and $CuCl_2$ showed 100% stability at 0.5 to 10 mM.

Effect of surfactants and detergents. Laccase from *Ganoderma* sp. rckk-02 was found to be moderately stable in both ionic as well as non-ionic surfactants. The enzyme retained almost 52% relative laccase activity at 0.5 mM concentration of Triton X-100 and all tweens except Tween 20, where it had 39% residual activity. Detergents such as SDS and CTAB did not show any noticeable effect on enzyme activity at 5 and 10 mM concentration.

N-Terminal sequencing. The N-terminal sequence of laccase from *Ganoderma* sp. rckk-02 was determined (S I R N S G) and compared with various fungi reported elsewhere[10] (Table 4). N-terminal sequencing of *Ganoderma* sp. rckk-02, through MALDI-ToF had resulted into very distinct amino acid profile. From the results, it is suggested that the laccase from *Ganoderma* sp. rckk-02 may be a novel laccase and encoded by different laccase genes.

Far-UV CD spectrum of laccase. The Far-UV CD spectrum of the purified laccase showed single, broad negative trough at around 213 nm, which is a typical signature of all β-proteins (Fig. 4). The spectrum depicted different intensities of secondary structural element. The content of the secondary structure was estimated using K2D software, which fit to β-structure with 8% α-helix and has higher proportion of β-sheet i.e. 44%. The information generated was similar to the known structure of several laccases. These enzymes (with the full complement of copper atoms) consist mainly of antiparallel β-barrels as observed in the *Trametes versicolor*[11].

Redox potential of laccase. Laccases can be divided in to three groups as a function of potential: low, middle and high potential laccases, having redox potential of 430, 430–710 and 780 mV, respectively[12]. Laccase from *Ganoderma* sp. rckk-02 was observed to have redox potential of 630 mV, hence belongs to middle redox potential laccases.

***In vivo* rat trial of laccase supplemented diets.** *Dry matter and Nutrient digestibility*. Except for the digestibility of crude protein which was comparable among rats of all the groups, the rats supplemented with laccase at a dose level of 2.5 units g^{-1} body weight (T2) digested significantly ($p < 0.01$) more dry matter (DM), organic matter (OM), ether extract (EE), crude fibre (CF), nitrogen free extract (NFE) and total carbohydrate (T-CHO) than those without laccase supplementation (T1) or those supplemented with 5 units of laccase g^{-1} body weight (T3) (Table 5). However, at 5% level of significance ($p < 0.05$), the digestibility of DM, OM and T-CHO was higher in the rats on 5.0 units laccase supplementation per g body weight (T3) in comparison to those devoid of laccase supplementation.

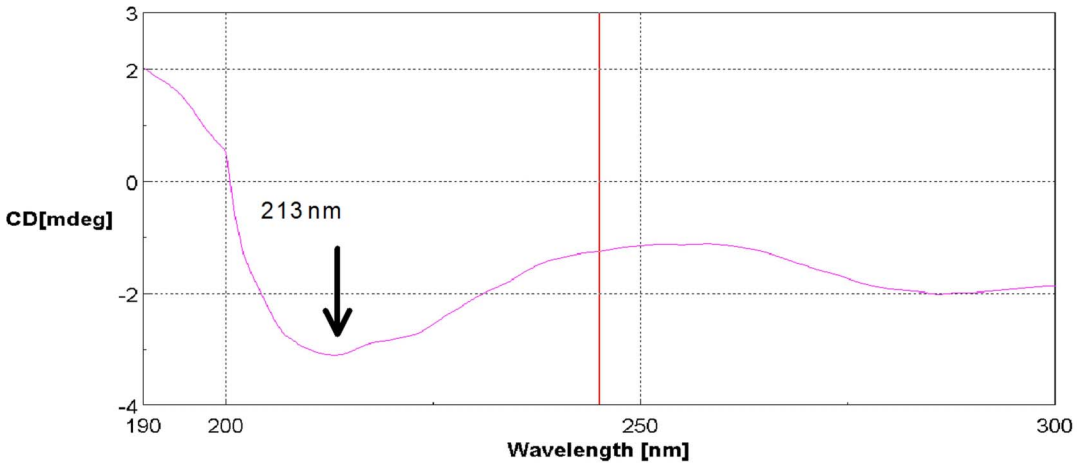

Figure 4 | Far-UV CD spectrum of purified laccase from *Ganoderma* sp. rckk-02 with negative trough at 213 nm.

Table 5 | Dry matter and nutrient digestibility among rats fed with different laccase dose

Attribute	T1 (Control)	T2 (2.5 units g^{-1} B.Wt.)	T3 (5.0 units g^{-1} B.Wt.)	SEM
Dry matter	53.56^{Bc}	66.75^{Aa}	57.52^{Bb}	0.773
Organic matter	53.07^{Bc}	66.45^{Aa}	57.37^{Bb}	1.284
Crude protein	62.69	65.83	65.94	1.020
Ether extract	70.09^{B}	76.33^{A}	71.92^{AB}	0.505
Crude fibre	47.83^{B}	58.58^{A}	46.84^{B}	0.908
Nitrogen free extract	51.28^{C}	69.15^{A}	58.01^{B}	0.837
Total carbohydrate	50.34^{Bc}	66.26^{Aa}	54.96^{Bb}	0.845
NDF	46.10^{Bc}	57.55^{Aa}	50.88^{Bb}	0.901
ADF	21.22^{Bc}	42.68^{Aa}	27.45^{Bb}	1.315
Cellulose	27.82^{Bc}	46.03^{Aa}	34.99^{Bb}	1.198
Hemicellulose	49.41^{Bc}	66.59^{Aa}	54.58^{Bb}	0.833

Means bearing different superscripts (^{ABC} $p < 0.01$; ^{abc} $p < 0.05$) in a row differ significantly.

The digestibility of neutral detergent fibre (NDF), acid detergent fibre (ADF), cellulose and hemicellulose was significantly ($p < 0.01$) higher in rats supplemented with 2.5 units laccase enzyme g^{-1} body weight (T2) (Table 5) as compared to the digestibility of these fibre fractions in rats on diets without (T1) or with 5 units g^{-1} body weight laccase supplementation. Though it was comparable at 1% level of significance, the digestibility of all the fibre fractions was superior ($p < 0.05$) in rats of T3 group, which received 5 units laccase g^{-1} body weight when compared to those on control diet (T1) having no laccase supplementation. Moderate level of enzyme supplementation was found superior in improving animal performance in the present study and similar observation was reported by Beauchemin et al. (2003) where, high level of enzyme addition can be less effective than low levels and the optimum level may depend on the diet[6]. Nsereko et al. (2002) has also observed similar results and speculated that application of a moderate level of enzyme to the ruminant feeds caused some beneficial disruption of the surface structure of the feed either before or after ingestion[24]. Application of ligninolytic enzymes as feed supplements promises to be the simplest method for digestibility improvement. However, mechanistic model for ruminal or gut activity of exogenous enzyme supplementation is not very well studied (Graminha et al. 2008)[25]. Beauchemin et al. (2003) extensively discussed the application of exogenous enzymes on animal feed and the mechanisms by which nutrient utilization is improved[6]. Moreover, ruminant animals (e.g., cattle and sheep) have the advantage of alloenzymatic digestion provided by rumen microflora, which enables ruminants to obtain nutrients from complex feed matrices that are not made available through autoenzymatic digestion. Accordingly, ruminants are able to subsist on diets comprised, for example, entirely of forages. Pigs, chickens and other monogastric animals lack the alloenzymes from rumen microflora, so for these species to derive optimal nutrient benefit from complex feed matrices it is necessary to provide added enzyme supplementation not available from resident intestinal microflora (Pariza and Cook, 2010)[3].

Blood enzyme activity. The activity (U/L) of SGPT (ALT) and SGOT (AST) did not vary significantly ($p > 0.05$) due to dietary variations (Table 6). The activity of SGPT (ALT) was found to be within the normal physiological range (35.1 ± 13.3 U/L) but that of SGOT (AST) was marginally higher than the normal range (42.9 ± 10.1 U/L) on all the diets as reported for rats earlier[13]. The LDH activity was significantly ($p < 0.05$) higher in rats supplemented with 5 units of laccase g^{-1} body weight (T3) but comparable between rats fed control diet (T1) and diet supplemented with 2.5 units of laccase g^{-1} body weight (T2) and was almost within the normal range (46.6 ± 22.0) as has been observed by Kaneko et al[13].

Table 6 | Activity (U/L) of blood stress enzymes among rats fed with different laccase dose

Enzyme	T1 (Control)	T2 (2.5 units g^{-1} B.Wt.)	T3 (5 units g^{-1} B.Wt.)	SEM
SGPT (ALT)	39.50	40.97	47.78	3.469
SGOT (AST)	60.65	60.36	60.85	1.463
LDH	57.39^{b}	57.10^{b}	73.83^{a}	2.119

Means bearing different superscripts (^{ab} $p < 0.05$) in a row differ significantly.

Discussion

The increase in laccase yield from *Ganoderma* sp. rckk-02 with increase in lignin concentration might be due to (a) synthesis of inducible laccase isozymes[14] and (b) combinatorial effect of laccase and HOBT (a heterocyclic compound with N-OH moiety which can be oxidized by laccase to its nitroxide radical). These redicals act as proximal oxidant of lignin which depolymerizes lignin and give rise to phenolics (ferulic acid, vanillic acid, 3–4 dihydroxy benzoic acid) as well as non phenolic structural polymers[15,16]. The phenolics produced would have served the dual purpose of acting as both substrate and inducer for laccase biosynthesis. This observation is well supported by existing reports in literature regarding laccase induction. Mansur et al.[14] have reported addition of veratryl alcohol enhanced laccase production by 10-fold from a lignin-degrading basidiomycete. 2,5-xylidine was reported to be the most effective inducer for production of laccase from *Cyathus bulleri* and *Pycnoporus cinnabarinus*[10,17]. Fungal laccases typically exhibit pH optima in the acidic range. While the pH optima for the oxidation of ABTS are generally lower than 4.0, phenolic compounds like DMP, guaiacol and syringaldazine exhibit higher values i.e. 4.0–7.0[18]. The enzyme activity at higher pH is decreased by the binding of a hydroxide anion to the T2/T3 coppers of laccase, which blocks the internal electron transfer from T1 to T2/T3 centres[19]. Not only the rate of oxidation but also the reaction products can differ according to pH. This may be due to abiotic follow-up reactions of primary radicals formed by laccase. The stability of fungal laccases is generally higher at acidic pH[18].

Temperature profiles of laccase activity are almost similar with other extracellular ligninolytic enzymes with optima between 50°C and 70°C[18]. On the other hand laccase from *Ganoderma lucidum* has been reported with highest activity at 25°C[20]. Laccase from *Ganoderma* sp. rckk-02 exhibited higher temperature optima compared to other fungi studied earlier but especially when compared to *Ganoderma lucidum*[20,21]. Thus the higher temperature optima make the enzyme of wider utility in various biotechnological applications. The kinetic parameters obtained for ABTS and guaiacol fall well within the ranges reported for other laccases[9]. Earlier fungal laccase from *G. lucidum* has been reported to be more stable on different concentration (0.5–5.0 mM) of heavy metals i.e. Mn^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+} and Mg^{2+}[21]. Contrary to that Baldrian[18] has discussed the

sensitivity of laccases toward heavy metal ions. The stability of this enzyme in presence of various heavy metals makes it an efficient agent in the treatment of wastewater containing heavy metals. While, the detergent could not change enzyme considerable which could be due to the reduction of the unproductive enzyme adsorption to the substrate[22]. Stability of laccase from *Ganoderma* sp. rckk-02 in both ionic and nonionic surfactants makes it robust enzyme for diverse applications.

To the best of our knowledge, the N-terminal sequencing of laccase from *Ganoderma* sp. rckk-02 is not identical to many known laccases but is closer to various wood degrading fungi. As reported previously, the N-terminal amino acid sequence of most fungal laccases (A/G-I-G-P-V/T) appears to be conserved and quite homologus in terms of primary sequences/conserved sequences[10]. Whereas, earlier reports of the CD spectrum of the laccase protein from *C. bulleri* indicated 37% α-helix and 26% β-sheet, which differed significantly from our findings[23].

Moderate level of enzyme supplementation was found superior in improving animal performance in the present study and similar observation was reported by Beauchemin et al.[6] where, high level of enzyme addition can be less effective than low levels and the optimum level may depend on the diet. Nsereko et al.[24] has also observed similar results and speculated that application of a moderate level of enzyme to the ruminant feeds caused some beneficial disruption of the surface structure of the feed either before or after ingestion. Application of ligninolytic enzymes as feed supplements promises to be the simplest method for digestibility improvement. However, mechanistic model for ruminal or gut activity of exogenous enzyme supplementation is not very well studied[25]. Beauchemin et al.[6] extensively discussed the application of exogenous enzymes on animal feed and the mechanisms by which nutrient utilization is improved. Moreover, ruminant animals (e.g., cattle and sheep) have the advantage of alloenzymatic digestion provided by rumen microflora, which enables ruminants to obtain nutrients from complex feed matrices that are not made available through autoenzymatic digestion. Accordingly, ruminants are able to subsist on diets comprised, for example, entirely of forages. Pigs, chickens and other monogastric animals lack the alloenzymes from rumen microflora, so for these species to derive optimal nutrient benefit from complex feed matrices it is necessary to provide added enzyme supplementation not available from resident intestinal microflora[3].

The enzyme ALT and AST are widely reported in the case of hepatitis, cirrhosis, metastatic carcinoma and myocardial infarction[26,27]. LDH, an oxidoreductase, catalyzes the inter-conversion of lactate and pyruvate. LDH is most often measured to evaluate the presence of tissue or cell damage[28]. The laccase from *Ganoderma* sp. rckk-02 appears to have different properties compared to other laccases and it holds potential to be a suitable candidate for animal feed supplement.

Methods

All animal experiments were performed at Department of Zoology, University of Delhi, in accordance with current guidelines of the Organization for Economic Cooperation and Development (OECD)[29] and Igmoris Draft, March 2008. All animal experiments were performed in strict compliance with Indian laws in an ethical way dually approved by Institutional Ethical committee.

Chemicals and Feed materials. Guaiacol and protein markers were purchased from Sigma - Aldrich Corporation, USA. All other media components and chemicals used were of highest purity grade available commercially. All the kits for biochemical assays were purchased from Teco Diagnostics, Anaheim, CA 92807. Wheat straw and other feed ingredients were purchased locally.

Microorganism and culture conditions. Laccase producing basidiomycetous fungus *Ganoderma* sp. rckk-02 was isolated from stump of *Euclyptica lanceolata*, Delhi ridge forest, New Delhi, India (Accession No. GenBank: AJ749970.1). The culture was grown and maintained on malt extract agar (MEA) containing (g/l): Malt extract 20.0, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $Ca(NO_3)_2 \cdot 4H_2O$ 0.5, pH 5.4 at 30°C [17]. Laccase production under RSM was carried out in 50 ml malt extract broth (MEB) in 250 ml Erlenmeyer flasks. Each flask was inoculated with 8 fungal discs (7 mm each) from the periphery of 5-day-old cultures of *Ganoderma* sp. rckk-02 and incubated for 11 days at 30°C under static cultivation conditions. HOBT (1- Hydroxybenzotriazole) was filter sterilized through 0.22 μm membrane (Millipore, Germany) and added to each flask on day 5 at a final concentration of 1.0 mM for induction of laccase production. The fungal mass was separated from fermentation broth by centrifugation at 4000× g for 20 min at 4°C and the supernatant was used for estimating laccase activity.

Statistical analysis and modeling. *Response surface methodology (RSM).* For the selection of the key factors like carbon source, nitrogen source and trace metal one factor at a time was adopted followed by Plackett-Burman Design (PBD) (data not shown). Thereafter, RSM employing CCRD was carried out to optimize the levels of variables identified to possess maximum influence on laccase production from *Ganoderma* sp. rckk-02. The level of three independent variables viz. malt extract (A), lignin (B) and pH (C), chosen for this study were optimized by the statistical software package 'DESIGN-EXPERT® 6.0, (Stat-Ease, Inc., USA). Each factor in the design was studied at five different levels (Table 1). A set of 20 experiments was carried out and all variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables used and the full experimental plan with respect to their values in actual and coded form is also given in Table 2. Upon completion of experiments, the average maximum laccase production was taken as response (Y). A second order polynomial equation was fitted to the data by a multiple regression method. The quadratic model for predicting the optimal point was expressed according to the following quadratic equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC \quad (1)$$

Where Y is predicted response; β_0 is intercept; β_1, β_2, β_3 are linear coefficients, and β_{12}, β_{23}, β_{13} are interaction coefficients. Eq. (1) was analyzed using DESIGN EXPERT software to estimate the responses of the dependent variables and regression analysis of experimental data. The quality of the fit of the quadratic model equation was expressed by the coefficient of determination (R^2) and its statistical significance was checked by Fishers test value (F-value).

Analytical procedure. Laccase activity was determined using Guaiacol as substrate following the method described elsewhere[17]. Guaiacol (10 mM) prepared in 100 mM citrate phosphate buffer (pH 5.0) was used as substrate. One unit (U) of laccase was defined as the change in absorbance of 0.01/ml/min at 470 nm. The protein content was estimated by standard Lowry's method using bovine serum albumin as the standard.

Purification of laccase. The culture broth (950 ml) was filtered through Whatman filter no. 1 and centrifuged at 13,000× g for15 min at 10°C. The protein extract was concentrated using an Amicon Ultra-15 membrane filter (Millipore, Germany). Partial purification of laccase from the culture filtrate was carried out by addition of finely ground ammonium sulfate at three different saturation levels, i.e., 0–20%, 20–40% and 40–80%. After overnight incubation at 4°C, the culture filtrate was centrifuged at 9,000× g for 20 min. Precipitates were dissolved in 20 mM citrate-phosphate buffer (pH 5.4) and dialysed overnight against same buffer at 4°C.

The gel filtration column HiPrep 16/60 Sephacryl S 100 HR (GE Healthcare, USA) was equilibrated and washed with 20 mM citrate-phosphate buffer (pH 5.5). The column was loaded with concentrated protein sample containing laccase activity and were eluted with the same buffer at the flow rate of 0.5 ml/min using a Pump P-920 (ÄKTA basic 100, GE Healthcare, USA). Fractions containing laccase activity were pooled and loaded on to a DEAE cellulose anion exchange column (10 cm × 2.8 cm), which was equilibrated and washed with 50 mM sodium phosphate buffer, pH 6.0. The enzyme was eluted with a linear gradient of NaCl (0–1.0 M) at the flow rate of 0.5 mL/min and was monitored for absorbance at 280 nm.

Enzyme characterization. *Molecular mass determination.* Fractions containing laccase activity were pooled and applied on a Resource-Q (GE Healthcare, USA) column and eluted with a linear gradient from 0 to 0.5 M NaCl. Peak fractions were pooled and concentrated by ultrafiltration using an Amicon Ultra-15 membrane. The enzyme purity and molecular weight of laccase from *Ganoderma* sp. rckk-02 were determined by SDS-PAGE as per the standard procedure. The molecular weight of the purified native laccase was confirmed by gel filtration on Sephadex G-100.

Effect of pH and temperature on laccase activity. The enzyme activity was assayed in the pH range of 3.0–7.5 using buffers of varying pH (citrate-phosphate, pH 3.0–6.5; phosphate buffer, pH 6.0–7.5). Activity was expressed as percentage relative activity with respect to maximum activity, which was considered as 100%. The optimum pH was determined by incubating the enzyme in buffers of varying pH (5.0 and 7.0) for 4 h at room temperature (25 ± 1°C). Effect of temperature on laccase activity was determined by incubating the reaction mixture at different temperatures varying from 30 to 90°C under standard assay conditions.

Thermostability of laccase. The temperature stability of laccase was determined by incubating the enzyme sample at various temperatures ranging from 30 to 80°C for 210 h and the residual activity was determined.

Effect of various inhibitors and metal ions. The effect of various inhibitors *viz.* Ethylenediamine tetraacetate (EDTA), β-mercaptoethanol, Dithiothreitol (DTT)

and urea on laccase activity was determined by incubating the enzyme at a final concentration ranging from 0.5–10.0 mM for 30 min at room temperature and then measuring the residual activity following the standard assay conditions. The effect of various metal ions on laccase activity was studied by incubating the enzyme with various metal ions at concentrations ranging from 0.5–10 mM at room temperature for 30 min.

Effect of surfactants. The effect of surfactants (SDS, tweens, CTAB, triton-x-100) on purified laccase activity was determined by incubating the enzyme with surfactant of concentrations ranging from 0.5 –10 mM for 30 min at room temperature.

Effect of substrate concentration on enzyme activity and determination of Km and Vmax for different substrates. Michaelis-Menten constant (K_m- substrate concentration at which rate of reaction is half the maximum rate of reaction) and maximum rate of reaction (V_{max}) were determined by using guaiacol and ABTS as substrates at concentration ranging from 0.5–5 mM in 20 mM citrate-phosphate buffer (pH 5.0). The values of K_m and V_{max} were calculated from Lineweaver-Burk plot.

N-terminal amino acid sequence analysis. Purified laccase from *Ganoderma* sp. rckk-02 was separated by native PAGE, blotted to polyvinylidene difluoride (PVDF) membranes and stained with Coomassie Brillient Blue. The PVDF membranes containing the purified protein blot were processed for N-terminal amino acid sequence analysis by Trypsin degradation on a MALDI-ToF (Bruker Daltonik, GmbH, Germany).

Far-UV CD spectrum of laccase. Far-UV CD spectrum of purified laccase was determined in 10 mM Sodium Cacodylate buffer (pH 5.2) in a Jacob J-810 Spectropolarimeter (Jasco Corporation, Japan). Laccase was properly dialyzed in the sodium cacodylate buffer at a protein concentration of 0.5 mg/ml. The parameters used for the measurement were: sensitivity, 100 mdeg; wavelength, 200–250 nm; data pitch, 0.2 nm; in continuous scanning mode. The scan speed was set at 50 nm/min with a response of 1 s and b and width at 1 nm[23].

Redox potential. The redox potential of laccase was determined using cyclic voltammetry (CV) following the method described by Dutton (1978)[30].

***In vivo* animal trial.** *Animals housing and laccase feeding*. Thirty six weaned male rats (Holtzman strain, n = 36) of uniform body weight and 30–32 day old were procured from Department of Zoology,University of Delhi, Delhi and divided into three (T1, T2 and T3) experimental groups as described earlier[5]. Each group consisted of three replicates of four rats each in a completely randomized design. The animals were acclimatized for 5 days on normal pelleted diet (concentrate and wheat straw; 50 : 50) before the start of feeding trial. They were housed in cages fitted with feeders, waterers and with facilities for the collection of feaces. Tap water was used for drinking and supplied *ad libitum* and the temperature and relative humidity of the room was maintained at 26°C ± 5°C and 50–55%, respectively.

The rats of group T2 and T3 were daily offered partially purified laccase enzyme (2.5 and 5.0 units g^{-1} body weight) from *Ganoderma* sp. rckk-02 in drinking water through out the experimental period. T1 group was offered pure tap water devoid of laccase.

Determinations of Body weights, feed efficiency, dry matter (DM) utilization and nutrient digestibility studies. Measured quantity of the pelleted composite diets was offered daily morning in a way to meet *ad libitum* and had access to it at all times. Individual body weights were recorded at weekly intervals before offering feed and water. The efficiency of feed and DM utilization was calculated as unit (g) intake per unit (g) gain. A digestibility trial of 4 d duration was conducted on all the experimental rats, involving total collection method to assess. Feed and faecal samples were analyzed for various proximate principles and fibre fractions as per the standard methods of Association of Official Agricultural Chemist (AOAC)[31–33]. Nitrogen in wet faeces was estimated with the help of CHNS analyzer (Model Elementar – Vario EL III).

Determination of blood enzyme activity. Blood was collected from rats of all the groups through ocular puncture with the help of heparinised capillary tubes into sterile stoppard glass tubes for the estimation of the activity of target enzymes. Thereafter, plasma was obtained and collected by centrifuging the blood at 4000 rpm for 5 min. Blood was analysed for the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) as per the methods described by Henry (1974)[26] and Young et al. (1975)[27] and lactate dehydrogenase (LDH)) was analyzed as per the method of Babson and Babson (1973)[34]. The data were statistically analyzed using one way analysis of variance (ANOVA) as per the method of Snedecor and Cochran (1989)[35].

1. Thurston, C. F. The structure and function of fungal laccases. *Microbiol.* **140**, 19–26 (1994).
2. Couto, S. R. & Herrera, J. L. T. Industrial and biotechnological applications of laccases: A review. *Biotechnol. Adv.* **24**, 500–513 (2006).
3. Pariza, M. W. & Cook, M. Determining the safety of enzymes used in animal feed. *Regulat. Toxicol. Pharmacol.* **56**, 332–342 (2010).
4. Sinskey, A. J. & Batt, C. A. Fungi as a source of protein. In: Food and Beverage. Ed. Benchat, L. R. Von Nostrand Reinhold, New York. pp. 435–471 (1987).
5. Sharma, K. K. *et al*. Nutritional and Toxicological Assessment of White-Rot Fermented Animal Feed. *Ind. J. Microbiol.* **52**, 185–190 (2012).
6. Beauchemin, K. A., Colombatto, D., Morgavi, D. P. & Yang, W. Z. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *J. Anim. Sci.* **81**, 37–47 (2003).
7. Colombatto, D., Mould, F. L., Bhat, M. K., Morgavi, D. P., Beauchemin, K. A. & Owen, E. Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal microorganisms in vitro. *J. Anim. Sci.* **81**, 1040–1050 (2003).
8. Haaland, P. D. Statistical problem solving. In: Experimental Design in Biotechnology. Eds. Haaland, P. D., Marcel Dekker, Inc., New York and Basel. pp. 1–18 (1989).
9. Litthauer, D., Vuuren, M. J., Tonder, A. & Wolfaart, F. W. Purification and Kinetics of a thermostable laccase from *Pycnoporus sanguineus* (SCC 108). *Enzyme Microb. Technol.* **40**, 563–568 (2007).
10. Eggert, C., Temp, U. & Eriksson, K.-E. L. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Appl. Environ. Microbiol.* **62**, 1151–1158 (1996).
11. Piontek, K., Antorini, M. & Choinowski, T. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.9 Å resolution containing a full complement of copper. *J. Biol. Chem.* **277**, 37663–37669 (2002).
12. Sadhasivam, S., Savitha, S., Swaminathan, K. & Lin, Feng-Huei. Production, purification and characterization of mid redox potential laccase from a newly isolated Trichoderma harzianum WL1. *Process Biochem.* **43**, 736–742 (2008).
13. Kaneko, J. J., Harvey, J. W. & Bruss, M. L. Clinical biochemistry of domestic animals. (5th Edn.), pp. 895–899 (1997).
14. Mansur, M., Suárez, T., Fernández-Larrea, J. B., Brizuela, M. A. & González, A. E. Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT20197. *Appl. Environ. Microbiol.* **63**, 2637–2646 (1997).
15. Bourbonnais, R., Paice, M. G., Freiermuth, B., Bodie, E. & Borneman, S. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Appl. Environ. Microbiol.* **63**(**12**), 4627–4632 (1997).
16. Srebotnik, E. & Hammel, K. E. Degradation of nonphenolic lignin by the laccase/1-hydroxybenzotriazole system. *J. Biotechnol.* **81**, 179–188 (2000).
17. Vasdev, K. & Kuhad, R. C. Induction of laccase production in *Cyathus bulleri* under shaking and static culture conditions. *Folia Microbiol.* **39**(**4**), 326–330 (1994).
18. Baldrian, P. Fungal laccases-occurrence and properties. *FEMS Microbiol. Rev.* **30**, 215–242 (2006).
19. Munoz, C., Guillen, F., Martinez, A. T. & Martinez, M. J. Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn21 oxidation. *Appl. Environ. Microbiol.* **63**, 2166–2174 (1997).
20. Ko, E.-M., Leem, Y.-E. & Choi, H. T. Purificatin and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*. *Appl. Microbiol. Biotechnol.* **57**, 98–102 (2001).
21. Kumari, H. L. & Sirsi, M. Purification and properties of laccase from Ganoderma lucidum. *Arch. Mikrobiol.* **84**, 350–357 (1972).
22. Eriksson, T., Brjesson, J. & Tjerneld, F. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme Microb. Technol.* **31**, 353–364 (2002).
23. Salony Garg, N., Baranwal, R., Chhabra, M., Mishra, S., Chaudhri, T. K. & Bisaria, V. S. Laccase of *Cyathus bulleri*: structural, catalytic characterization and expression in *Escherichia coli*. *Biochim. et Biophy. acta.* **1784**, 259–268(2008).
24. Nsereko, V. L. *et al*. Effect of fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. *Can. J. Microbiol.* **48**, 14–20 (2002).
25. Graminha, E. B. N., Goncalves, A. Z. L., Pirota, R. D. P. B. & Balasalobre, M. A. A., DA Silva, R. & Gomes, E. Enzyme production by solid- state fermentation: Application to animal nutrition. *Anim. Feed Sci. Technol.* **144**, 1–22 (2008).
26. Henry, J. B. Clinical Diagnosis and Management by Laboratory Method. W. B. Saunders Co., Philadelphia. pp. 332–335 (1974).
27. Young, D. S., Pestaner, L. C. & Gibberman, V. Effects of Drugs on Clinical Laboratory Tests. *Clin. Chem.* **21**, 5 (1975).
28. Coley, H. M., Lewandowicz, G., Sargent, J. M. & Verrill, M. W. Chemosensitivity testing of fresh and continuous tumor cell cultures using lactate dehydrogenase. *Anticancer Res.* **17**, 231–236 (1997).
29. OECD. OECD guidelines for the testing of chemicals, No. 425. Acute oral toxicity—modified up and down procedure. Organisation for economic cooperation and development, Paris (2001).
30. Dutton, P. L. Redox potentiometry: determination of midpoint potential of oxidation- reduction components of biological electron transfer systems. *Methods Enzymol.* **54**, 411–435 (1978).
31. AOAC. Association of official analytical chemists (AOAC), official methods of analysis, 17th edn. AOAC, Arlington (2000).
32. Goering, H. K. & Van Soest, P. J. Forage fibre analysis (apparatus, reagents, procedures and some applications). Agriculture Hand book 379, ARS, USDA, Washington, DC (1970).

33. Van Soest, P. J., Robertson, J. B. & Lewis, B. A. Methods of dietary fibre, neutral detergent fibre and non-starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **74**, 3583–3597 (1991).
34. Babson, A. L. & Babson, S. R. Kinetic colorimetric measurement of serum lactate dehydrogenase activity. *Clin. Chem.* **19**, 766–769 (1973).
35. Snedecor, G. W. & Cochran, W. G. Statistical methods, 8th edn. Iowa State University Press, Ames(1989).
36. Ng, T. B. & Wang, H. X. A homodimeric laccase with unique characteristics from the yellow mushroom *Cantharellus cibarius*. *Biochem. Biophys. Res. Commun.* **313**, 37–41 (2004).

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Author contributions

Conceived and designed the experiments: KKS, BS, VRBS and RCK. Performed the experiments: KKS, BS, VRBS and NS. Analyzed the data: KKS, BS,VRBS, NS and RCK. Contributed reagents/materials/analysis tools/Animal Facility: NS. Wrote the manuscript: KKS, BS and RCK. All authors prepared the manuscript.

Additional information

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