Biotechnol Lett (2020) 42:2189–2210
https://doi.org/10.1007/s10529-020-02925-x

ORIGINAL RESEARCH PAPER

Proteomic analysis reveals the damaging role of low redox laccase from *Yersinia enterocolitica* strain 8081 in the midgut of *Helicoverpa armigera*

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Received: 21 January 2020 / Accepted: 25 May 2020 / Published online: 29 May 2020

Abstract
Objective Earlier, we have found that the entero-pathogenic *Yersinia enterocolitica* have evolved the survival mechanisms that regulate the expression of laccase-encoding genes in the gut. The present study aims to characterize the purified recombinant laccase from *Y. enterocolitica* strain 8081 biovar 1B and understand its effect on the midgut of cotton boll-worm, *Helicoverpa armigera* (Hübner) larvae.
Results The recombinant laccase protein showed high purity fold and low molecular mass ($\sim$ 43 kDa). *H. armigera* larvae fed with laccase protein showed a significant decrease in body weight and damage in the midgut. Further, transmission electron microscopy (TEM) studies revealed the negative effect of laccase protein on trachea, malpighian tubules, and villi of the insect. The proteome comparison between control and laccase-fed larvae of cotton bollworm showed signif-icant expression of proteolytic enzymes, oxidoreduc-tases, cytoskeletal proteins, ribosomal proteins; and proteins for citrate (TCA cycle) cycle, glycolysis, stress response, cell redox homeostasis, xenobiotic degradation, and insect defence. Moreover, it also resulted in the reduction of antioxidants, increased melanization (insect innate immune response), and enhanced free radical generation.
Conclusions All these data collectively suggest that *H. armigera* (Hübner) larvae can be used to study the effect of microbes and their metabolites on the host physiology, anatomy, and survival.

Shruti Ahlawat and Deepti Singh have contributed equally.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10529-020-02925-x) contains supplementary material, which is available to authorized users.

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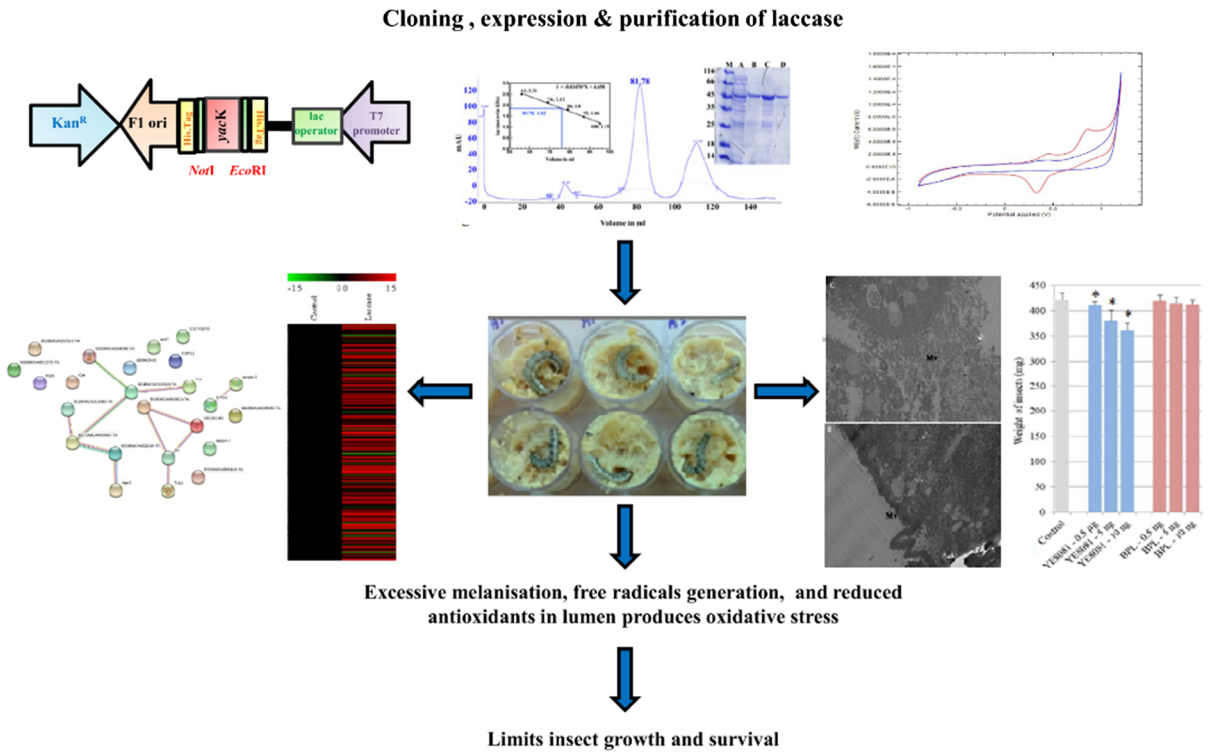
Graphic abstract



Keywords Laccase · Cloning · Protein purification · *Yersinia enterocolitica* · *Helicoverpa armigera* · Proteome · Reactive oxygen species

Introduction

Yersinia enterocolitica is a potential human pathogen with extensive serotype diversity, which is further differentiated into six biovars based on their biochemical and physiological characteristics (Howard et al. 2006; Virdi et al. 2012). *Y. enterocolitica* biovar 1B is a food- and water-borne pathogen, but high mortality has also been reported due to the blood-transfusion associated septicemia (Leclercq et al. 2005). The enteropathogenic *Y. enterocolitica* penetrates the intestinal epithelium and proliferates in the lymphatic system (Fallman et al. 2001). The virulence factor type-III secretion system (T3SS) of the pathogen inhibits its phagocytosis in the host (Barison et al. 2013). *Y. enterocolitica* biovar 1A strains were also found to survive phagocytosis (Dhar and Virdi 2013) in the absence of virulent genes and plasmid (Burnens et al. 1996); further, causing nosocomial infection (Greenwood and Hooper 1990). One of the factors which facilitate the pathogen to survive phagocytosis in the host is reported to be the free radical scavenging characteristic of multicopper oxidases (MCOs) (Singh et al. 2016). Laccases (EC 1.10.3.2), the member of the MCOs family, catalyzes the oxidation of various aromatic compounds (Sharma and Kuhad 2009). Interestingly, the antioxidative property of laccase is well known in the virulence of a basidiomycetes yeast *Cryptococcus neoformans* (Zhu and Williamson 2004; Sharma et al. 2018). Laccases are also prevalent in several pathogenic bacteria, e.g., *Salmonella enterica*, and are reported to be involved in their pathogenicity (Achard et al. 2010). *Y. enterocolitica*, which is considered as a mammalian pathogen, also confers toxicity to insects (Heermann and Fuchs 2008). It is orally toxic to *Manduca sexta* (Bresolin et al. 2006) and colonizes the intestine of *Caenorhabditis elegans*, expanding its intestinal lumen, thus, killing the nematode (Spanier et al. 2010).

Helicoverpa armigera or cotton bollworm classified in family Noctuidae and order Lepidoptera, is a polyphagous insect with broad host spectra. Its polyphagy feeding habit is due to the highly complex and diverse environment of the insect gut (Shinde et al. 2019). The proteins carry out almost all the functions of a cell collectively forming the proteome. It is a highly diverse '-omics' approach, concerned with protein identification and quantification from high-throughput data. In general, proteomics is a large-scale analysis of proteins used to reveal the biological functions in the quantitative and qualitative terms (Tsigaridas et al. 2017). The proteome is studied mostly with mass spectrometry (MS)-based approaches. It involves the biological interpretation of their subcellular localizations, concentration changes, post-translational modifications (PTMs), and interactions (Chen et al. 2017; Sinitcyn et al. 2018; Jain et al. 2019).

Previous reports suggested greater wax moth (*Galleria mellonella*) as a rapid, cost-effective model to assess the virulence for a range of microorganisms and for the rapid evaluation of antimicrobial drug effectiveness (Tsai et al. 2016). In an earlier work, silkworm (*Bombyx mori*) was used as a model animal to test the toxicity of neonicotinic pesticides and its biodegradation metabolites (Phugare et al. 2013). Therefore, the present study was undertaken to characterize the recombinant laccase from *Y. enterocolitica* strain 8081 biovar 1B and to study its effect on the midgut of *H. armigera* (Hübner) larvae. Finally, the gut proteome profile was also studied.

Materials and methods

Cloning, expression, and purification of laccase

The laccase (*yac*K) gene amplified from the genomic DNA of *Y. enterocolitica* strain 8081 (Accession No. YP_001005057.1) (Singh et al. 2014) using forward primer 5′ AT*GGATCCGAATTC*ATGCCATGCA TCGCCGTGATTTTAT 3′ with restriction sites *Bam*HI, *Eco*RI and reverse primer 5′ AA*GCGGCC GCCTCGAG*CTAAGCACTGACAGTAAGCC 3′ with restriction site *Not*I, *Xho*I was cloned in pTZ57R/T (TA cloning vector). Further, the amplified *yac*K gene was digested with *Eco*RI/*Not*I, cloned in expression vector, pET28a (+), and transformed in host cells, *Escherichia coli* BL21 (DE3).

The 1% (v/v) 12 h old transformed culture was used to inoculate LB medium containing kanamycin (50 µg/mL) and were grown at 37 °C, 200 rpm until attains an optical density (OD) of 0.5–0.6 at wavelength 600 nm. Further, isopropyl-β-D-1-thiogalactopyranoside (IPTG; 1 mM) was added to induce the laccase expression under different incubation conditions of temperature (16, 25, 30, or 37 °C), aeration (100, 150, or 200 rpm), incubation temperature after induction (4, 16, 25, or 30 °C), incubation time after induction (2, 3, 4, 5, or 16 h), and copper ($CuCl_2$) concentration (0.25, 0.5, 1.0, 1.5, or 2.0 mM) (Supplementary Fig. 1). The laccase from inclusion bodies (IBs) was solubilized by the modified method of Sambrook and Russell (2001). The cell pellet obtained by harvesting cells at 5000×*g*, 15 min, 4 °C was washed and suspended in lysis buffer [Tris–HCl (50 mM, pH 8.0); EDTA (1 mM, pH 8.0); NaCl (100 mM)] followed by addition of protease inhibitor (phenylmethylsulfonyl fluoride), lysozyme, and deoxycholic acid. The laccase was solubilized using urea (8 M) and β-mercaptoethanol (β-ME; 0.4 mM) in potassium-phosphate buffer (50 mM, pH 8.0) and refolded using refolding buffer [potassium-phosphate buffer (50 mM, pH 8.0); glutathione reduced (5 mM); glutathione oxidized (1 mM); $CuCl_2$ (1 mM)] by pulsatile dilution method (Kamen and Woody 2002). Thereafter, the protein was purified via nickel–nitrilotriacetic acid (Ni–NTA) affinity column chromatography (GE Health care), pre-equilibrated with 10 bed volume of suspension buffer for affinity purification, washed with 10 bed volume of wash buffer [NaCl (150 mM), Tris–HCl (50 mM), β-ME (5 mM)] to remove nonspecific binding, and eluted by elution buffer [NaCl (150 mM), imidazole (250 mM), Tris–HCl (50 mM), β-ME (5 mM)]. The purity of recombinant protein was analysed on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The positive fractions were concentrated using 10 kDa cutoff Centricon (Millipore) and loaded on a pre-equilibrated [Tris–HCl (50 mM, pH 8.0); NaCl (50 mM); β-ME (5 mM)] Superdex 200 HiLoad 16/60 column (GE Healthcare, New Jersey, USA). The active protein was confirmed as laccase using zymogram with [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] ABTS as substrate followed by biochemical and electrochemical characterization.

As reported earlier, the cloning, expression, and purification of laccase from *Bacillus pumilus* strain DSKK1 (Ahlawat et al. 2019) was done similarly.

Biochemical characterization of laccase

Guaiacol was used for the determination of optimal catalysis conditions. The enzyme was incubated with guaiacol (25 mM) in Tris–HCl buffer (0.2 M, pH 9.0) at 70 °C for 30 min. One unit laccase was defined as the absorbance change of 0.01/mL/min at wavelength 470 nm (Singh et al. 2016). Various buffers such as citrate–phosphate buffer (pH 3.0–7.0), potassium-phosphate buffer (pH 8.0), Tris–HCl buffer (pH 9.0), and glycine–NaOH buffer (pH 10.0) were used to determine the effect of pH (3.0–10.0) on purified laccase. While, the effect of temperature on enzyme activity was studied in the range from 20 to 90 °C with a difference of 10 °C. Guaiacol (0.5 mM to 4.0 mM) was used to determine the Michaelis–Menten constant (K_m) and the maximum rate of reaction (V_{max}) from the Eadie–Hofstee plot. The effect of various metal ions (0.00625–0.5 mM), surfactants (0.025–1.0 mM), and inhibitors (0.025–1.0 mM) on laccase activity was determined by measuring the residual activity under standard assay conditions.

Electrochemical characterization of laccase

Yersinia enterocolitica strain 8081 recombinant laccase was electrochemically characterized by cyclic voltammetry. Cyclic voltammetric (CV) experiments were carried out with a computer-controlled potentiostat (Autolab Potentiostat/Galvanostat PGSTAT204, Metrohm, USA) using software Nova version 1.6. Cyclic voltammograms were performed on screen printed carbon electrode (SPCE; Dropsens) having surface diameter of 4.0 mm; where working, counter, and reference electrodes were made of carbon, carbon, and silver (Ag), respectively.

Before experiment, the electrode was washed with milli Q water and dried at RT. Thereafter, the reactive ester groups were generated on electrode after incubation for 2–3 h with 6 µl mixture of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC; 10 mM) and *N*-hydroxysuccinimide (NHS; 10 mM) (1:1) followed by washing and drying. Now, the modified electrode was incubated overnight at RT with 6 µL of *Y. enterocolitica* strain 8081 or *B. pumilus* strain DSKK1 recombinant laccase, followed by repeated washing. Cyclic voltammetry was done at three pH's i.e., 5.0 (citrate–phosphate buffer), 7.0 (Tris–HCl buffer), and 10.0 (carbonate-bicarbonate buffer) with and without 1 mM ABTS at a scan rate of 20 mV/s between − 0.9 and + 1.2 V v/s Ag|AgCl|KCl.

Effect of laccase on insect gut

The effect of purified laccase was studied on the growth of *H. armigera* larvae at different concentrations (0.5, 5.0, and 10.0 µg/g insect diet). The laccase was added to the insect diet (Supplementary Table 1) followed by their growth till third larval stage. The experiments were done in five replicates with initial weight, final weight, and mortality in insect observed and recorded. The data of insect weights was calculated as mean values [± standard deviation (SD)]. Statistical analysis was done by one-way analysis of variance (ANOVA) using Microcal Origin Version 6.0. The mean values obtained from each sample were compared using an unpaired *t* test. Probability values ≤ 0.05 were considered as statistical significant. Thereafter, the midguts of the insects were excised aseptically to study the biochemical changes. Catalase (CAT) assay was performed as per the procedure described by Beers and Sizer (1952). Lipid peroxidation (LPO) was assayed by the modified method of Wills (1966). Glutathione (GSH) was estimated as described by Kumar et al. (2008). Further, transmission electron microscopy (TEM) was done commercially at AIRF, Jawaharlal Nehru University (JNU), New Delhi, India. For TEM, the aseptically excised gut tissues were fixed in 2% paraformaldehyde and 2% glutaraldehyde in potassium-phosphate buffer (0.1 M, pH 8.0). Ultra-thin sections were sliced using Leica EM UC 6 ultramicrotome and examined using JEOL-2100F TEM at 120 kV. The samples were studied at various magnifications of × 1000–6000.

Proteomic analysis of laccase-fed insect gut

The excised gut were lysed in extraction buffer containing 125 mM NaCl, 0.5 M Tris, and 1 M EDTA with protease inhibitors for 4 min and, then centrifuged for 20 min at 10,000 rpm, 4 °C. The extracted proteins were precipitated using TCA: acetone (1:8) at − 20 °C for overnight and, then centrifuged at 15,000 rpm, 4 °C for 40 min. The obtained pellets

were purified by repeatedly washing with 5 mM dithiothreitol (DTT)-acetone. Thereafter, the total protein was solubilized in guanidine hydrochloride (GdmHCl; 6 M) at 90 °C for 30 min, reduced using 10 mM DTT for 45 min at 56 °C, and alkylated with 55 mM indole acetic acid at RT for 30 min in dark. Then, the samples were diluted up to 1 M of GdmHCl using 25 mM ABC followed by digestion with trypsin (MS grade) in ratio 1:50 at 37 °C. After that, 2% formic acid (FA) was added to stop the digestion and desalting was performed by the manufacturer's protocol (Thermo Fisher Scientific). Finally, the samples were dried by speed vacuum, reconstituted in 0.1% FA, and loaded onto a C_{18} reverse phase column. Digested peptides were analyzed using nano-LC–MS/MS. Peptides were loaded onto a PepMap RSLC C_{18} 2 µmx50 cm pre-column (Thermo Scientific Easy-nLC 1200 column). The column was eluted using gradient of solvent A (95% water, 5% acetonitrile, and 0.1% FA) and solvent B (90% acetonitrile, 10% water, and 0.1% FA) at a flow-rate of 300 nL/min and total run time of 123 min. For precursor mass tolerance (MS1) and fragment mass tolerance (MS2) maximum ion transfer time was kept 60 ms and 120 ms, respectively. Full-scan MS spectra with mass scan range of m/z 350–2000 were acquired on a Q Exactive Orbitrap (Thermo Scientific) in a positive ion mode with 27% normalized collision energy (NCE).

Raw MS data files were analyzed in proteome discoverer software (ver. 2.2, Thermo Scientific) against the constructed database of *Helicoverpa* available in UniProt (https://www.uniprot.org/). Trypsin was set as the digestion enzyme with two missed cleavage. The peptides and proteins were inferred using peptide spectrum matches (PSMs) and were validated at false discovery rate (FDR) $\leq$ 1% estimated using the decoy hit distribution. The results were filtered by Xcorr (< 1.5), ΔCn (< 0.01) for peptides and q-value (≤ 0.05), PSMs (≥ 1) for proteins with high confidence. Protein quantification was conducted using the total spectrum count of identified proteins as described earlier (Kamal et al. 2018). Function assignment and annotation by InterPro terms, gene ontology (GO) terms, enzyme classification (EC) codes, and metabolic pathways (KEGG, Kyoto Encyclopaedia of Gene and Genomes) were determined using Blast2GO (https://www.blast2go.com/) and OmicsBox (https://www.biobam.com/omicsbox/) software suite. Heatmap cluster was generated by MeV software (ver. 4.9; https://www.tm4.org/) and protein–protein interactions were studied by String software (ver. 11.0; https://string-db.org/).

Results

Cloning, expression, and purification of laccase

The size of amplified laccase (*yacK*) gene was 1.6 Kb (Fig. 1a) which was similar to previously studied laccase genes from *Y. enterocolitica* (Singh et al. 2014, 2016). *Yersinia* laccase gene (*yacK*) was cloned in pET28a (+) (Fig. 1b) and over-expressed in *E. coli* BL21 (DE3) for the hyperproduction of recombinant protein under varied conditions (Supplementary Fig. 1). The maximum expression was observed after incubation at 30 °C, 200 rpm for 4 h (Supplementary Fig. 2I); however, most of the protein was in the form of IBs. Therefore, the incubation temperature was reduced to 25 °C and 16 °C, and the agitation was changed to 100 rpm. The maximum protein was obtained at 25 °C and 100 rpm, but in the form of IBs. Further, the formation of IBs was avoided by lowering the post-induction temperature, such that the incubation time was increased to 16 h at 30 °C (Supplementary Fig. 2II) and 4 °C under static conditions after incubation at different temperatures (30, 25, or 16 °C) for 4 h at 100 rpm (Papaneophytou and Kontopidis 2014). Then the amount of expressed protein in IBs increased with increasing incubation time, but the yield of refolded protein was reduced drastically. The optimum conditions chosen for induction was incubation for 4 h at 25 °C, 100 rpm, thereafter, under static condition for 16 h at 4 °C (Supplementary Fig. 1, Supplementary Fig. 2III).

Solubilization of the IBs in urea and β-ME showed protein recovery in supernatant on SDS-PAGE (Supplementary Fig. 3A). The protein was refolded using oxidized and reduced glutathione in sodium-phosphate buffer (pH 8.0) having chloride salt of copper; with activity of 36.71 U/mL and specific activity of 48.94 U/mg. The protein was purified using Ni–NTA affinity chromatography column (43.11 µg/mg) (Supplementary Fig. 3B) with an activity and specific activity of 355.13 µg/mL and 8230 U/mg, respectively. The concentrated protein was loaded on Superdex 200 HiLoad 16/60 column for further

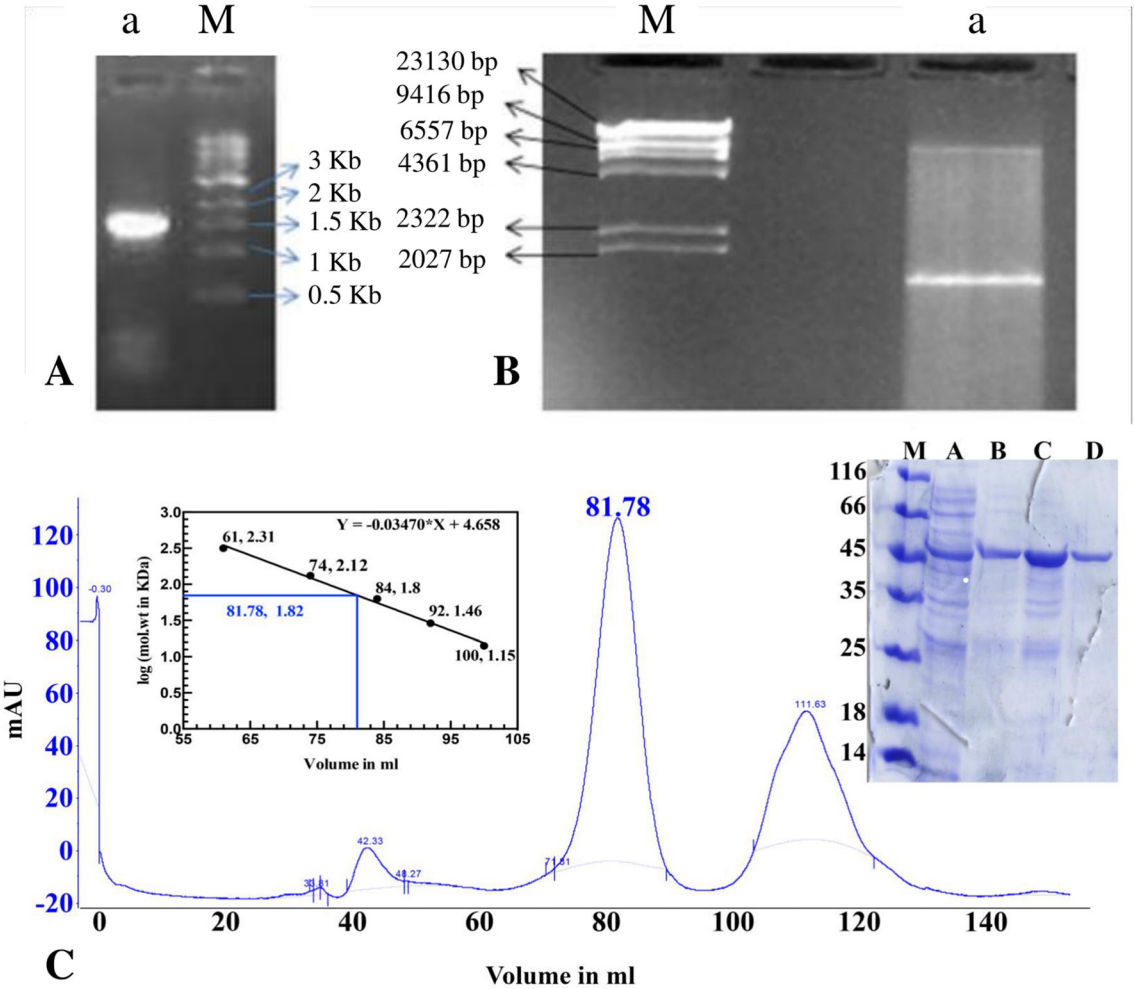


Fig. 1 **a** Agarose gel electrophoresis showing band of [a] laccase gene (*yac*K) of *Y. enterocolitica* strain 8081 [M] 1 Kb Marker **b** [a] TA cloning vector (pTZ57R/T) with *yac*K and [M] Lambda DNA *Hin*dIII digest marker **c** Laccase purification profile using gel filtration chromatography. Inset: Lane M: molecular marker; Lane A: crude lysate; Lane B and C: Ni–NTA purified protein; Lane D: gel filtration purified protein (Molecular mass ~ 43 kDa)

purification. The eluted fraction of laccase peak was validated by securing a single band on SDS-PAGE. Finally, the purified laccase was of 82 kDa dimer in a single peak (Fig. 1c) and was further confirmed by zymogram study with [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS) as a chromogenic substrate (Supplementary Fig. 3C).

Biochemical characterization of laccase

The maximum activity of the purified laccase was observed at pH 8.0 and 70 °C (Fig. 2a) with residual activity of 91% and 47% after 2 h and 12 h, respectively. At 80 °C, the residual activity of laccase was 73% for 10 min of incubation, but after 30 min drastic loss in enzyme activity (41%) was observed (Fig. 2b). The K_m and V_{max} for guaiacol were found to be 0.68 mM and 60.5 mM/min, respectively. Lower is the K_m value; higher is the affinity towards the substrate (Robinson 2015). Metal ions can stabilize or destabilize the protein conformation by binding to the enzyme and thus, can change its activity. The recombinant laccase was highly stable in the presence of all the metal ions studied (Fig. 2d). The inhibitory effect of sodium azide (NaN_3) was also negligible on the activity of laccase. The ability of calcium, copper, magnesium, and manganese to induce the laccase from different bacteria had been reported earlier (Kim et al.

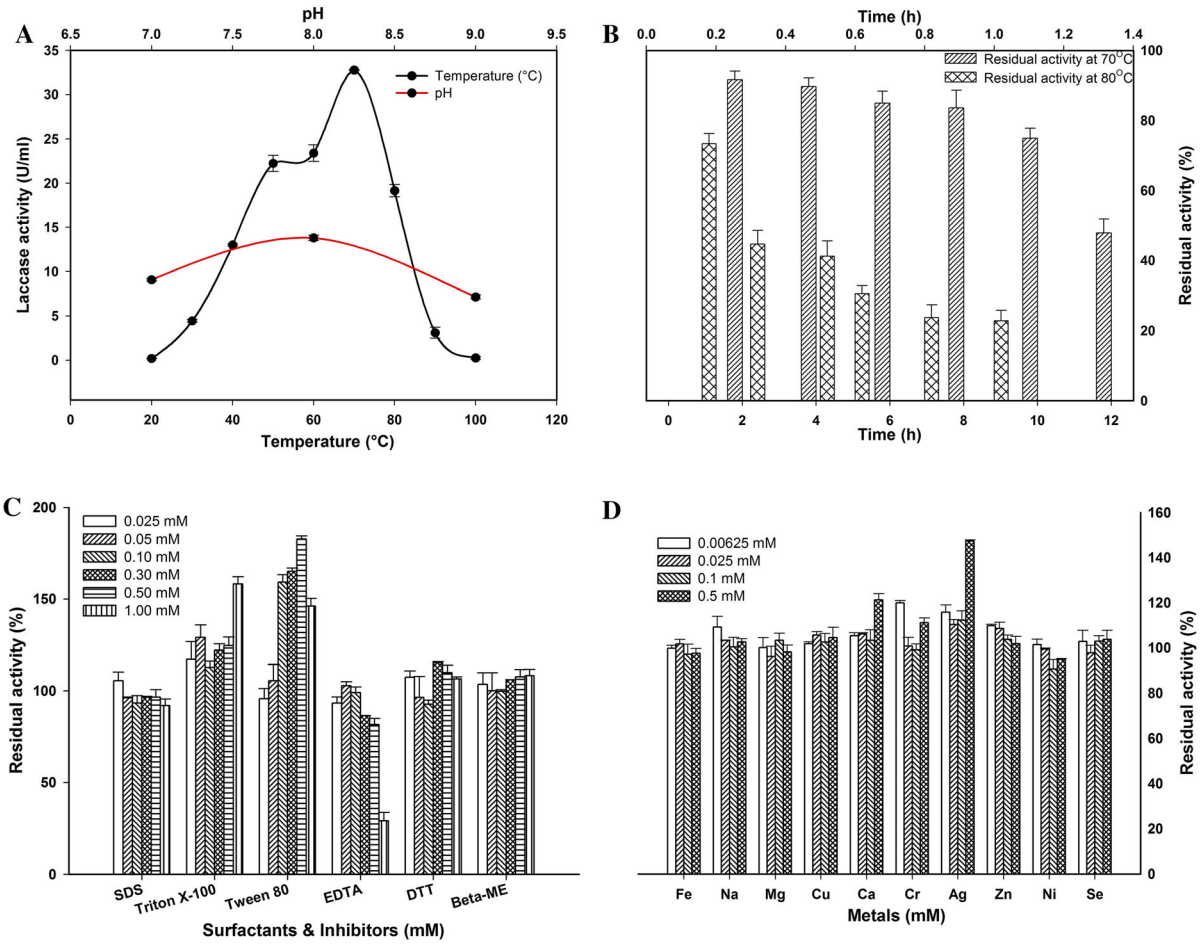


Fig. 2 **a** Effect of different pH and temperature on the activity of purified laccase **b** Thermostability of purified laccase **c** Effect of different surfactants and inhibitors on laccase activity at different concentrations (mM) **d** Effect of different metal ions on laccase activity at different concentrations (mM)

2001; Martins et al. 2002). The non-ionic surfactants Triton X-100 and Tween 80 showed high residual activity. Anionic surfactant (SDS) and the inhibitors of fungal laccase (Kumar et al. 2015), β-ME, DTT, and EDTA showed no significant inhibitory effect on laccase activity (Fig. 2c). However, a higher concentration (1%) of EDTA was inhibitory.

Electrochemical characterization of laccase

Cyclic voltammograms obtained for the reaction of ABTS and *Y. enterocolitica* strain 8081 recombinant laccase at pH 5.0 and pH 7.0 exhibited a pair of cathodic and anodic peaks I_a/I_c having $E^{o'}$ of 0.309 V (at pH 5.0) and 0.381 V (at pH 7.0) (vs Ag|AgCl) with an additional anodic peak (II_a) at 0.790 V (at pH 5.0) (Supplementary Fig. 4A) and 0.845 V (at pH 7.0) (Supplementary Fig. 4B). Where, I_a, II_a, and I_c corresponds to ABTS/$ABTS^+$ oxidation, $ABTS^+$/$ABTS^{+2}$ oxidation, and $ABTS^+$/ABTS reduction, respectively (Ley et al. 2013). Reduction peak (I_c) was found to be increased strongly in size when compared to the corresponding oxidation peaks which can be explained by the comproportionation reaction between ABTS and its dication $ABTS^{+2}$, yielding two molecules of $ABTS^+$. It causes the disappearance of $ABTS^{+2}$/$ABTS^+$ reduction peak (Fernández-Sánchez et al. 2002). No prominent cathodic or anodic peaks were obtained in cyclic voltammograms obtained for the reaction of ABTS and *Y. enterocolitica* strain 8081 recombinant laccase at pH 10.0 (Supplementary Fig. 4C), suggesting its maximum activity at pH 7.0,

followed by pH 5.0 but not at pH 10.0. On contrast, cyclic voltammograms obtained for the reaction of ABTS and non-pathogenic *B. pumilus* strain DSKK1 recombinant laccase at all three pH's, exhibited a pair of cathodic and anodic peaks I_a/I_c with $E^{o'}$ of 0.348 V (at pH 5.0), 0.349 V (at pH 7.0) and 0.354 V (at pH 10.0) (vs Ag|AgCl) with an additional anodic peak (II_a) at 0.828 V (at pH 5.0), 0.803 V (at pH 7.0), and 0.808 V (at pH 10.0) (Supplementary Fig. 4D–F).

The comparison of the cyclic voltammograms between the recombinant laccases revealed three important differences. First, *Y. enterocolitica* strain 8081 recombinant laccase was active at pH 5.0 and pH 7.0 but not at pH 10.0, whereas *B. pumilus* strain DSKK1 recombinant laccase was active at all three pH's (i.e. 5.0, 7.0, and 10.0) (Ahlawat et al. 2019). Second, the E^o of *Y. enterocolitica* strain 8081 recombinant laccase varied at pH 5.0 (0.309 V) and pH 7.0 (0.381 V), whereas *B. pumilus* strain DSKK1 recombinant laccase had almost same $E^{o'}$ at all three pH's 5.0 (0.348 V), 7.0 (0.349 V), and 10.0 (0.354 V). Finally, at pH 7.0, $E^{o'}$ for recombinant laccase from pathogenic *Y. enterocolitica* strain 8081 (0.381 V) was higher than non-pathogenic *B. pumilus* strain DSSK1 (0.349 V). Further, $E^{o'}$ for bacterial laccases (both pathogenic and non-pathogenic) was lower than previously studied fungal laccases (Table 1).

Effect of laccase on insect gut

It was observed that the insect body weight decreased significantly with gradual increase in the concentration of *Yersinia* laccase in the artificial diet of an insect (Fig. 3). However, no mortality was reported. The gut of third instar larvae was dissected and homogenized for further analysis such that 2.71 fold decreased CAT activity and 1.57 fold decreased GSH was observed in laccase-treated gut (Table 2). The 7.19 fold higher malondialdehyde (MDA) levels in laccase-treated gut showed increased LPO as compared to untreated gut. GSH detoxifies the potentially deleterious substances like oxygen free radicals and modulates the redox state of the cells. The higher MDA levels usually results in the loss of membrane integrity (Ayobola 2012). The increased lipid peroxidation and altered antioxidant enzymes leads to oxidative stress in cells and thus, contributes to the deleterious effects on the growth of *H. armigera* (Akbar et al. 2012).

To understand the effect of laccase on the midgut of *H. armigera*, TEM was done, which showed distorted trachea (Tr), damaged malpighian tubules (MT), and reduced villi (Mv) in laccase-treated insects (Fig. 4).

Proteomic analysis of laccase-fed insect gut

A total of 1105 (387 exclusively in control insect gut, 66 exclusively in laccase-fed insect gut, and 652 shared between both groups) unique protein

Table 1 List of $E^{o'}$ obtained for laccases from different organisms at pH 5.0, 7.0, and 10.0

S. No.	Organism	Accession No.	Source of origin	Country of origin	$E^{o'}$ (pH 5.0) (in V)	$E^{o'}$ (pH 7.0) (in V)	$E^{o'}$ (pH 10.0) (in V)	References
1	*Yersinia enterocolitica* strain 8081 recombinant laccase	YP_001005057.1	Human	USA	0.309	0.381	–	From present study
2	*Bacillus pumilus* DSKK1 recombinant laccase (non-pathogenic)	*Bacillus pumilus* strain DSKK1: JQ639010	Earthworm cast	India	0.348	0.349	0.354	From present study
3	*Yersinia enterocolitica* strain 7 recombinant laccase	KC113580	Human stools	India	0.3	0.39	–	Ahlawat et al. (2019)
4	Fungal laccase from *Trametes villosa* (*Polyporus pinsitus*)	–	Novo Nordisk	Denmark	0.444	–	–	Fernández-Sánchez et al. (2002)
5	Fungal laccase from *Myceliophthora thermophila*	–	Novo Nordisk	–	0.486	–	–	Solís-Oba et al. (2005)

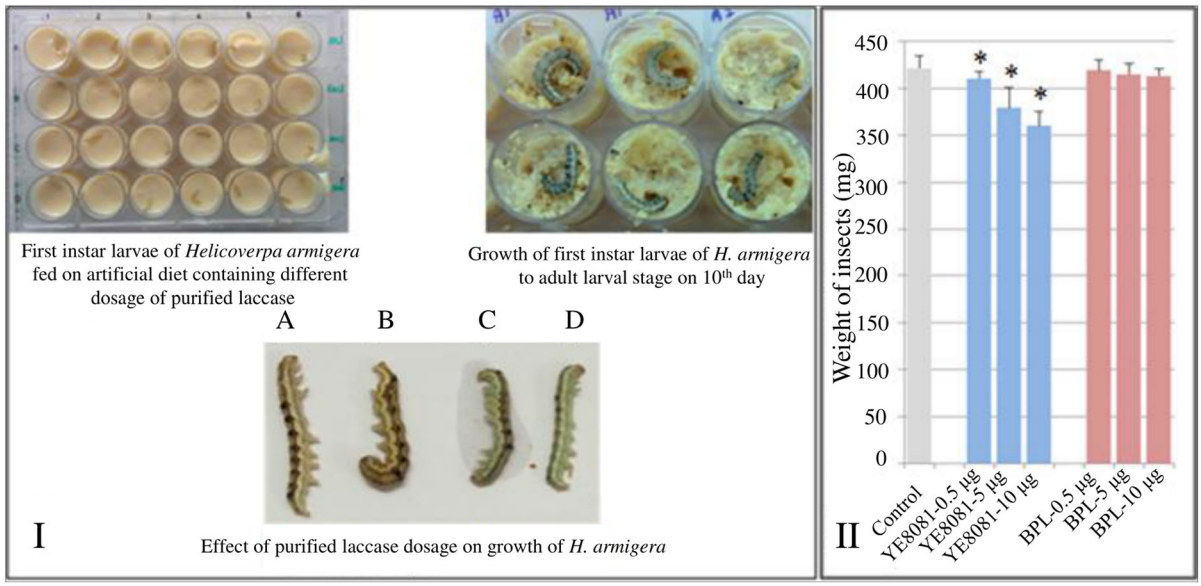


Fig. 3 **I** Effect of laccase on *H. armigera* larvae: A. Control B–D. Treated with different concentrations of laccase from *Y. enterocolitica* strain 8081; **II** Reduction in weight of *H. armigera* larvae after treatment with different concentration of laccases from *Y. enterocolitica* strain 8081 and *Bacillus pumilus* DSKK1 (BPL) [control]. *Represent significant differences between the control (artificial diet) and laccase treated larvae (unpaired Student's *t* test $p < 0.05$)

Table 2 Effect of laccase on catalase (CAT), glutathione (GSH), and MDA levels of *H. armigera*

Parameter and unit	Control insects	Laccase-fed insects	Fold change
CAT (nmol of H_2O_2 decomposed/min/mg protein)	50.14	18.46	2.71
GSH (µg glutathione/mg protein)	0.134	0.211	1.57
MDA (nmol MDA/mg protein)	3.62	26.05	7.19

accessions (with $\geq$ 1 PSM) were identified and quantified with high confidence (Fig. 5a). Proteins were classified depending on biological process (BP), cellular component (CC), and molecular function (MF) categories in gene ontology (GO) annotations by score distribution. GO analysis of control insect gut proteins revealed 37.6% BP, 19.97% CC, and 42.42% MF (Supplementary Table 2, Fig. 6). Upon strict filtering of the dataset using criterion of $\geq$ 5 PSMs, 453 proteins were stringently determined. Out of 453 proteins, 221 were commonly shared between both groups and 232 (225 in control, 7 in laccase-fed insect gut) were exclusively identified in single group (Fig. 5b). The differentially expressed proteins were displayed in a heatmap format using normalized PSMs (Fig. 5d, e). The heatmap data clearly demonstrate diverse intensities (81% upregulated, 19% downregulated) of proteins between the control and laccase-fed insect gut (Fig. 5f). Further, the identified proteins (453 proteins, $\geq$ 5 PSMs) were classified depending on BP, CC, and MF categories in GO annotations by score distribution. GO analysis of control insect gut proteins revealed 29.3% BP, 22.46% CC, and 48.23% MF proteins (Supplementary Table 2, Fig. 7). According to the metabolic pathway analysis, 106 assigned EC codes were identified for the provided protein accessions (Fig. 5c). Further, distribution of differentially regulated proteins (with PSMs $\geq$ 5) into various enzyme classes showed increased oxidoreductases and reduced hydrolases in response to laccase-feeding (Supplementary Table 3). Among the reported enzymes, majority of them belongs to the families:

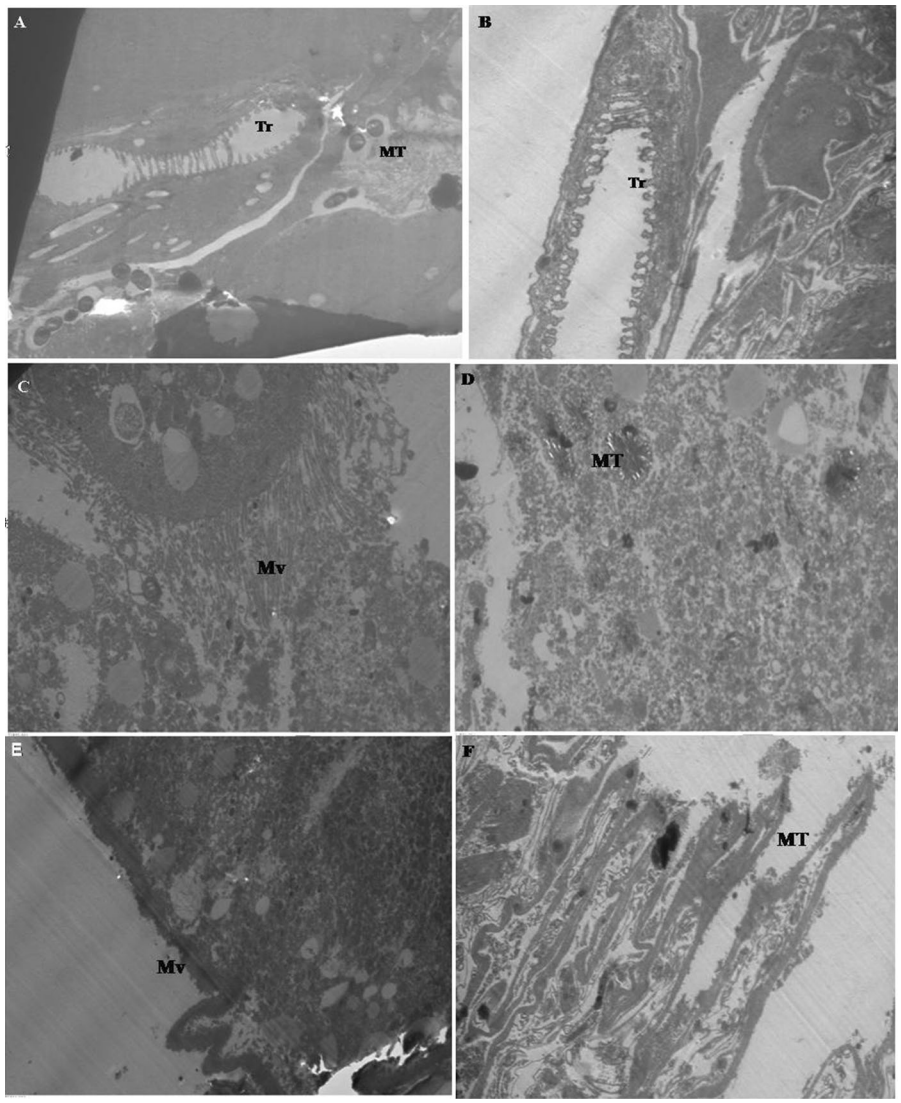


Fig. 4 Transmission electron microscopy (TEM) of midgut of *H. armigera* larvae treated with laccase. **a** Control **b**, **e** treated with 5 µg laccase **c**, **d** and f 10 µg laccase. Tr-Trachea, MT-Malpighian tubules, and Mv-Microvilli

type-B carboxylesterase/lipase family, peptidase (C1/M1/S1/T1A/S9B) family, thiolase family, thiolase-like superfamily, and glycosyl hydrolase 31 family. Around 72 KEGG pathways were represented by 453 protein accessions (with PSMs $\geq$ 5) including purine metabolism (KO00230: 11 and 2 EC codes in control and laccase-fed gut, respectively), citrate (TCA cycle) cycle (KO00020: 10 and 3 EC codes in control and laccase-fed gut, respectively), pyruvate metabolism (KO00240: 9 and 1 EC codes in control and laccase-fed gut, respectively), carbon fixation pathways in prokaryotes (KO00720: 8 and 3 EC codes in control and laccase-fed gut, respectively), and amino sugar and nucleotide sugar metabolism (KO00520: 7 and 1 EC codes in control and laccase-fed gut, respectively) pathways.

The proteome comparison between control and laccase-fed larvae of cotton bollworm showed significant expression of enzymes, cytoskeletal proteins, ribosomal proteins, citrate (TCA cycle) cycle & glycolysis proteins, and cell redox homeostasis & stress response proteins. All the identified proteins can be differentiated under three broad categories: (i) proteins for xenobiotic degradation, immune response,

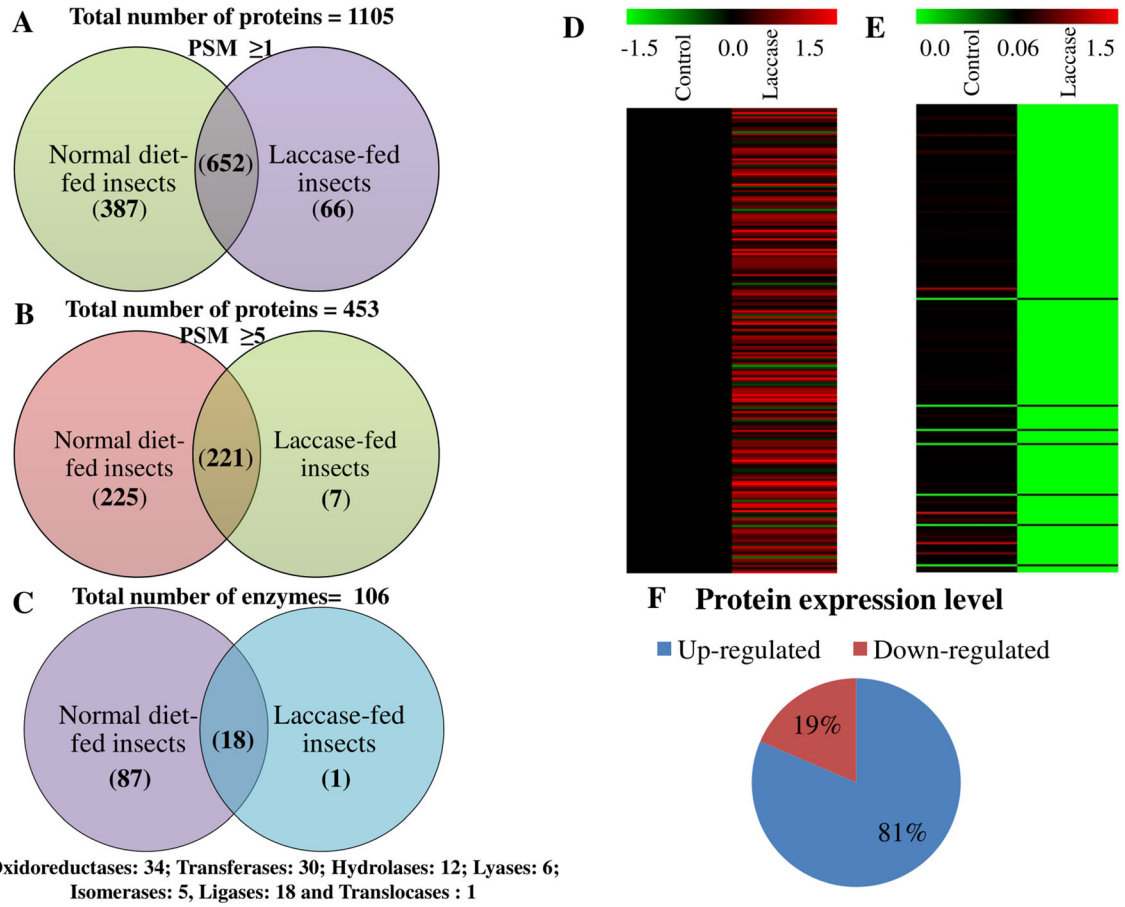


Fig. 5 **a** Distribution of the identified proteins (PSMs ≥ 1), **b** Distribution of identified proteins (PSMs ≥ 5), **c** Distribution of enzymes (PSMs ≥ 5), **d** Heatmap depicting the changes in protein expression in *H. armigera* larvae with and without laccase-feeding using proteins common to both conditions, **e** Heatmap depicting the changes in protein expression in *H. armigera* larvae with and without laccase-feeding using proteins present in either group, **f** Distribution of the identified proteins (PSMs ≥ 5) based on expression levels

and insect defence; (ii) reactive oxygen species (ROS) and antioxidants; and (iii) proteins involved in cellular metabolism. The expression of antioxidant proteins like catalase (CAT; EC 1.11.1.6; Uniprot ID-H9BEW3) and thioredoxin reductase (TrxR; EC 1.8.1.9; Uniprot ID-A0A0A7RB97) along with prophenoloxidase (PPO; EC 1.14.18.1; Uniprot ID-Q2VIY6), NADPH–cytochrome P450 reductase (CPR; EC 1.6.2.4; Uniprot ID-E0A3A7), serpin (Uniprot ID-F5B4G8), and serpin-9 (Uniprot ID-A0A290U612) were found to be significantly reduced in laccase-fed larvae. Further, proteins related to cytoskeleton like Uniprot ID-A0A2W1BVZ9 for motor activity (GO: 0003774) was downregulated and Uniprot ID-A0A2W1BIB1 for actin filament depolymerisation (GO: 0030042) was upregulated. Malic enzyme (Uniprot ID-A0A2W1BVZ1), aminopeptidase (Uniprot ID-Q6R3M5), aminopeptidase N1 (Uniprot ID-A0A1L5JK78), carboxyl/choline esterase (Uniprot ID-D5G3E6), aspartate aminotransferase (Uniprot ID-A0A2W1B2Y8), UDP-glucuronosyltransferase (Uniprot ID-A0A2W1BAL9), and peptidyl-prolyl cis–trans isomerase (Uniprot ID-A0A2W1BDP4) were identified as the significantly downregulated proteins in response to laccase treatment (Table 3). However, significantly upregulated proteins identified were: NADH dehydrogenase (ubiquinone) activity (EC 1.6.99.3; Uniprot ID-A0A2W1BUD9), succinate dehydrogenase (ubiquinone) flavoprotein subunit, mitochondrial (EC 1.3.5.1;

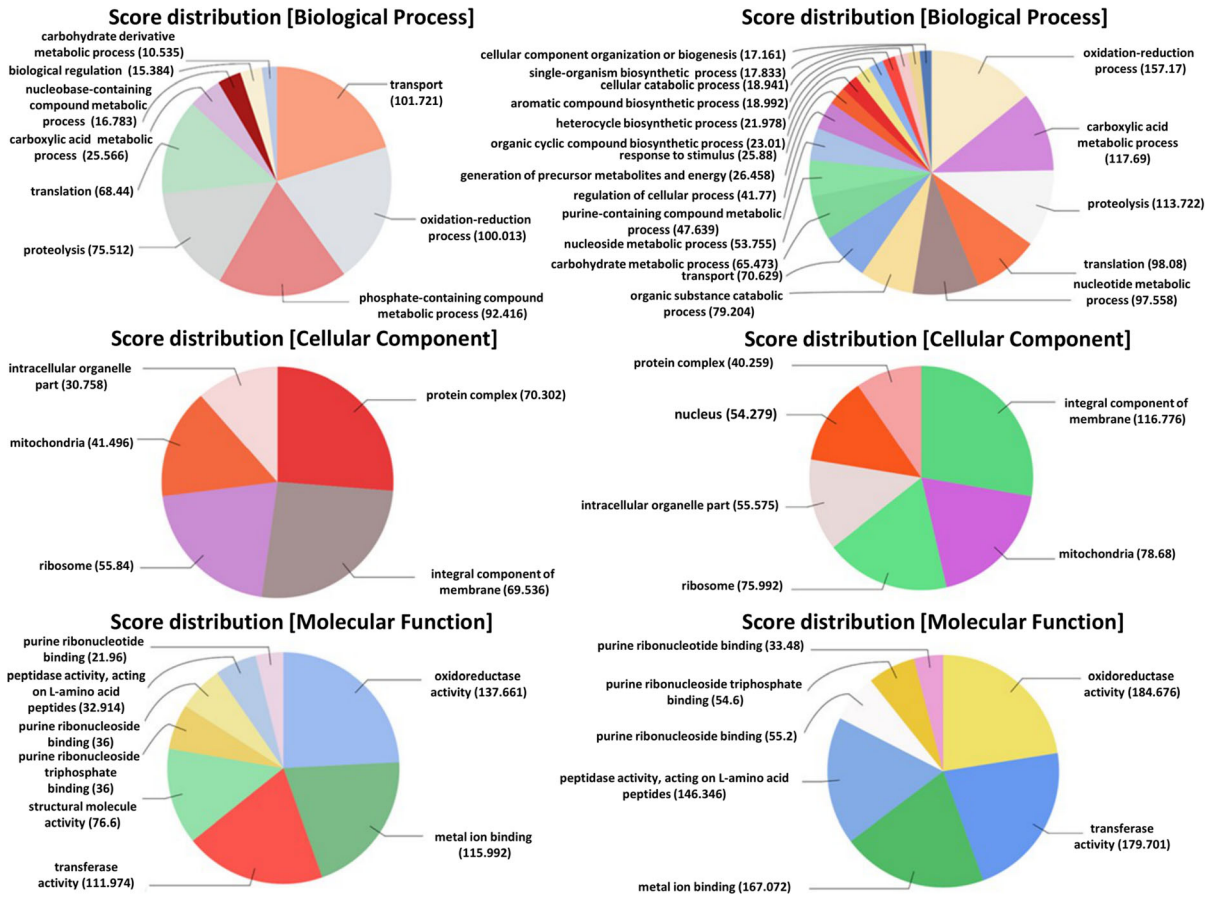


Fig. 6 Gene ontology (GO) analysis of differentially regulated proteins (with PSMs $\geq$ 1) from normal diet-fed *H. armigera* larve gut (on left) and recombinant laccase-fed *H. armigera* larve gut (on right)

Uniprot ID-A0A2W1BZK9), carboxylic ester hydrolases [EC 3.1.1.- (Uniprot ID-D5KXB9; Uniprot ID-D5G3G4; Uniprot ID-D5KX87; Uniprot ID-H9ZVH4; Uniprot ID-S4WMC9)], aminopeptidase (Uniprot ID-A0A2W1BLC5), chemosensory protein (CSP; Uniprot ID-A0A0A0VG76), tubulin beta chain (Uniprot ID-A0A2W1BMR7), actin (Uniprot ID-E2IV62), and glutamine synthetase [GS; EC 6.3.1.2; Uniprot ID-A0A2W1BMY2) (Table 3).

To establish the known and predicted protein interactions, 221 differentially expressed proteins ($\geq$ 5 PSMs) and 30 selected proteins (related to stress and gut damage) were investigated via STRING database (ver. 11.0), using Lepidoptera as a reference organism. *Bombyx mori*, *Danaus plexippus*, and *Heliconius melpomene* match our reference organism Lepidoptera. Maximum match of input proteins was with proteins from *B. mori*; thus, it was selected for further analysis. On analyzing the interactome of 221 proteins; 186 nodes, 999 edges were found with average node degree of 10.7, average local clustering coefficient of 0.419, and enrichment p-value of < 1.0 e$-$16 PPI (Fig. 8). The resulting network contains 44 proteins with no associations to other proteins. Analysis of the interactome data generated for 30 selected proteins revealed 24 nodes, 11 edges with average node degree of 0.917, average local clustering coefficient of 0.389, and PPI enrichment p-value of < 2 e$-$06 (Fig. 8). Interestingly, the analysis revealed interaction of prophenoloxidase subunit 2 (PPO2) with serpin (serpin 1). Further, actin (A1) was found to interact with proteins for actin filament depolymerisation (100101180; Uniprot ID-A0A2W1BIB1), motor activity (BGIBMGA000613-TA; Uniprot ID: A0A2W1BVZ9), and tubulin beta chain (Tub1). Major cluster involves: succinate dehydrogenase

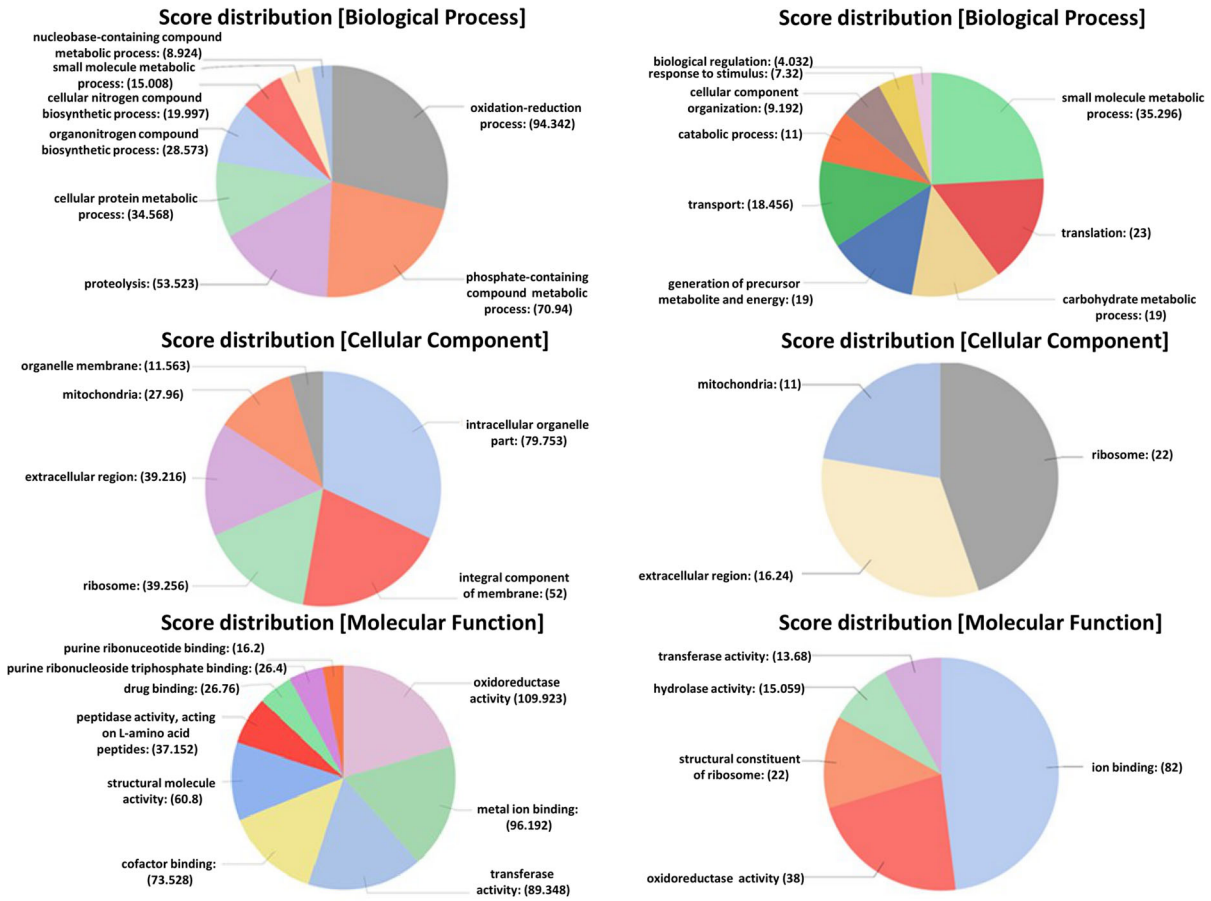


Fig. 7 Gene ontology (GO) analysis of differentially regulated proteins (with PSMs $\geq$ 5) from normal diet-fed *H. armigera* larve gut (on left) and recombinant laccase-fed *H. armigera* larve gut (on right)

(ubiquinone) flavoprotein subunit, mitochondrial (BGIBMGA009000-TA), aspartate aminotransferase (BGIBMGA003319-TA), NADH dehydrogenase (ubiquinone) activity (BGIBMGA014483-TA; Uniprot ID-A0A2W1BUD9), aminopeptidase (Apn1), methylenetetrahydrofolate dehydrogenase ($NADP^+$) activity (BGIBMGA004950-TA; Uniprot ID-A0A2W1BQ31), thioredoxin reductase (BGIBMGA002218-TA), and catalase (Cat).

Discussion and conclusion

In the present work, *H. armigera* was used as a model organism to study the effect of microbial metabolite, i.e., *Yersinia* laccase (polyphenol oxidase) on host survival, anatomy, and physiology. The *yacK* gene from *Y. enterocolitica* strain 8081 was amplified, cloned, and expressed to overproduce the laccase protein. The IBs were formed as a result of aggregation of the overexpressed protein due to the metabolic burden on the host (Hunke and Betton 2003; Sørensen and Mortensen 2005) and can be partially controlled by slowing down the rate of protein synthesis by reducing incubation temperature and agitation (Vera et al. 2007). Previous studies suggests that the IBs formed at low temperatures solubilize faster, improving the efficiency of the refolding strategies (de Groot and Ventura 2006). The IBs were further solubilized in urea and β-ME in corroboration with previous studies which also showed the highest solubilization of IBs using urea and β-ME (Mollania et al. 2013). Besides, refolding was done using glutathione with $CuCl_2$. As reported previously, the addition of prosthetic groups or cofactors essential for proper folding or stability into culture medium prevents IBs formation

Table 3 List of most upregulated and downregulated proteins in laccase-fed larvae

Accession number	Name	Expression
B6A8H5	Acyl-CoA binding protein	1.784
D5KXB9	**Carboxylic ester hydrolase**	**1.604**
A0A2W1BA87	Histone H2A	1.592
A0A2W1BGN7	Uncharacterized protein	1.592
A0A2W1BIB1	**Uncharacterized protein**	**1.369**
A0A2W1BTI5	Uncharacterized protein	1.369
A0A2W1BUD9	**Uncharacterized protein**	**1.369**
A0A0A0VG76	**Chemosensory protein**	**1.369**
A0A2W1BHB1	Uncharacterized protein	1.369
A0A2W1BMY2	**Glutamine synthetase**	**1.369**
A0A2W1BSE8	Delta-1-pyrroline-5-carboxylate synthase	1.369
D5G3G4	**Carboxylic ester hydrolase (fragment)**	**1.369**
A0A2W1B7S2	Polyadenylate-binding protein	1.27
B6CMG0	Fatty acid-binding protein 3	1.236
A0A2W1BM92	40S ribosomal protein S4	1.177
A0A2W1BZK9	**Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial**	**1.177**
B6A8G0	Ribosomal protein L22 (fragment)	1.177
D5KX87	**Carboxylic ester hydrolase**	**1.158**
A0A2H4LI83	Heat shock cognate protein 90	1.147
A0A2W1BMR7	**Tubulin beta chain**	**1.147**
A0A2W1BVE6	**Uncharacterized protein**	**1.147**
S4WMC9	**Carboxylic ester hydrolase**	**1.147**
E2IV62	**Actin**	**1.13**
H9ZVH4	**Carboxylic ester hydrolase**	**1.118**
A0A2W1BDC2	Isocitrate dehydrogenase [NAD] subunit, mitochondrial	1.106
A0A2W1BWS0	Uncharacterized protein	1.106
A0A2W1BLC5	**Aminopeptidase**	**1.080**
A0A2W1BNE3	ATP synthase subunit alpha	1.053
F5B4G8	**Serpin**	**− 0.745**
A0A2W1BED4	Uncharacterized protein (fragment)	− 0.689
Q2VIY6	**Prophenoloxidase subunit 2**	**− 0.669**
A0A2W1BK92	Uncharacterized protein	− 0.577
D5G3E6	**Carboxyl/choline esterase**	**− 0.568**
A0A2W1BUU3	Uncharacterized protein	− 0.504
E0A3A7	**NADPH–cytochrome P450 reductase**	**− 0.456**
A0A2W1BMM7	Uncharacterized protein	− 0.453
A0A2W1BVZ1	**Malic enzyme**	**− 0.407**
A0A2W1BAL9	**UDP-glucuronosyltransferase**	**− 0.395**
A0A2W1BSJ9	Uncharacterized protein	− 0.391
A0A2W1BR38	**Uncharacterized protein**	**− 0.367**
A0A2W1B2Y8	**Aspartate aminotransferase**	**− 0.251**
A0A290U612	**Serpin-9**	**− 0.215**
A0A2W1BXA3	Uncharacterized protein	− 0.215
A0A0A7RB97	**Thioredoxin reductase**	**− 0.215**
A0A2W1C4D9	Uncharacterized protein	− 0.215

Table 3 continued

Accession number	Name	Expression
H9BEW3	**Catalase**	**− 0.215**
A0A2W1BMR8	Uncharacterized protein	− 0.215
A0A2W1BSI4	Uncharacterized protein	− 0.215
A0A2W1C146	Uncharacterized protein	− 0.215
D5LTC5	Translational controlled tumor protein	− 0.215
A0A2W1BQ31	**Uncharacterized protein**	**− 0.17**
A0A2W1BDP4	**Peptidyl-prolyl cis–trans isomerase**	**− 0.153**
A0A2W1B9W8	ATP-citrate synthase	− 0.132
Q6R3M5	**Aminopeptidase (fragment)**	**− 0.124**
A0A2W1BNG9	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1	− 0.115
A0A2W1BBG8	Uncharacterized protein	− 0.904
A0A2W1BVZ9	**Uncharacterized protein**	**− 0.082**
A0A1L5JK78	**Aminopeptidase N1 (fragment)**	**− 0.077**

The bold rows in the table represents the proteins used for constructing the interactome (via STRING database)

(Papaneophytou and Kontopidis 2014). The recombinant metallo-oxidase from *Aquifex aeolicus* was also found to require copper for its maximum catalytic efficiency (Fernandes et al. 2007). After solubilization, the recombinant laccase protein was purified sequentially with the help of Ni-affinity and gel filtration chromatography, which showed high purity fold of 184 and low molecular mass ($\sim$ 43 kDa) with activity and specific activity of 355.13 µg/mL and 8230 U/mg, respectively. Recent studies found that 71.5 kDa acid-tolerant recombinant laccase from *Setosphaeria turnica* had a maximum activity of 127.78 U/mg (Ma et al. 2018). Similarly, recombinant laccases from *Y. enterocolitica* strain 7 and *B. pumilus* strain DSKK1 showed the enzyme activity (µg/mL) of 338.48 and 355.15, specific activity (U/mg) of 4630 and 6450, with purity fold of 108 and 148, respectively (Ahlawat et al. 2019).

On characterizing biochemically, the maximum activity was observed at pH 8.0 and 70 °C. This data supports the previous studies, where the optimum pH for bacterial laccases with ABTS was reported to be in the range of 3.0–7.5 (Singh et al. 2016). The results of kinetic constants (K_m and V_{max}) also corroborate with the earlier studies, which showed lower K_m values for laccases from pathogenic bacteria (Hall et al. 2008) as compared to the laccases from non-pathogenic bacteria (Martins et al. 2002). Recombinant laccase was found to be stable in the presence of metal ions and inhibitors. Vertebrates limit pathogens by reducing essential metal availability while at the same time it enriches the infection site with other metals (Palmer and Skaar 2016). Transition metals are essential in trace amount; however, in excess are toxic as they disrupt the normal metabolic processes (Becker and Skaar 2014). The stability of recombinant laccase toward all metal ions studied suggests that the laccase might be helping *Y. enterocolitica* strain 8081 against the metal toxicity. On characterizing electrochemically, it was evident that at pH 7.0, redox potential ($E^{o'}$) for recombinant laccase from pathogenic *Y. enterocolitica* strain 8081 was higher than non-pathogenic *B. pumilus* strain DSSK1 but lower than previously studied fungal laccases. Interestingly, immune activation reduces the bacterial load and metabolic activity, which eventually enhances the gut redox potential, under which Enterobacteriaceae and disease-causing species survive (Reese et al. 2018). Redox enzyme laccase contributes to the growth retardation of *H. armigera* larvae and supports the previous study, showing the toxicity of *Y. enterocolitica* strain W22703 towards *C. elegans* by colonizing the nematode gut (Spanier et al. 2010). Also, there is an isolated report on the role of class 1 laccase (MLAC1) from insect pathogenic fungus *Metarhizium anisopliae* in the virulence to caterpillars at the last instar stage of *Galleria mellonella* larvae (Fang et al. 2010).

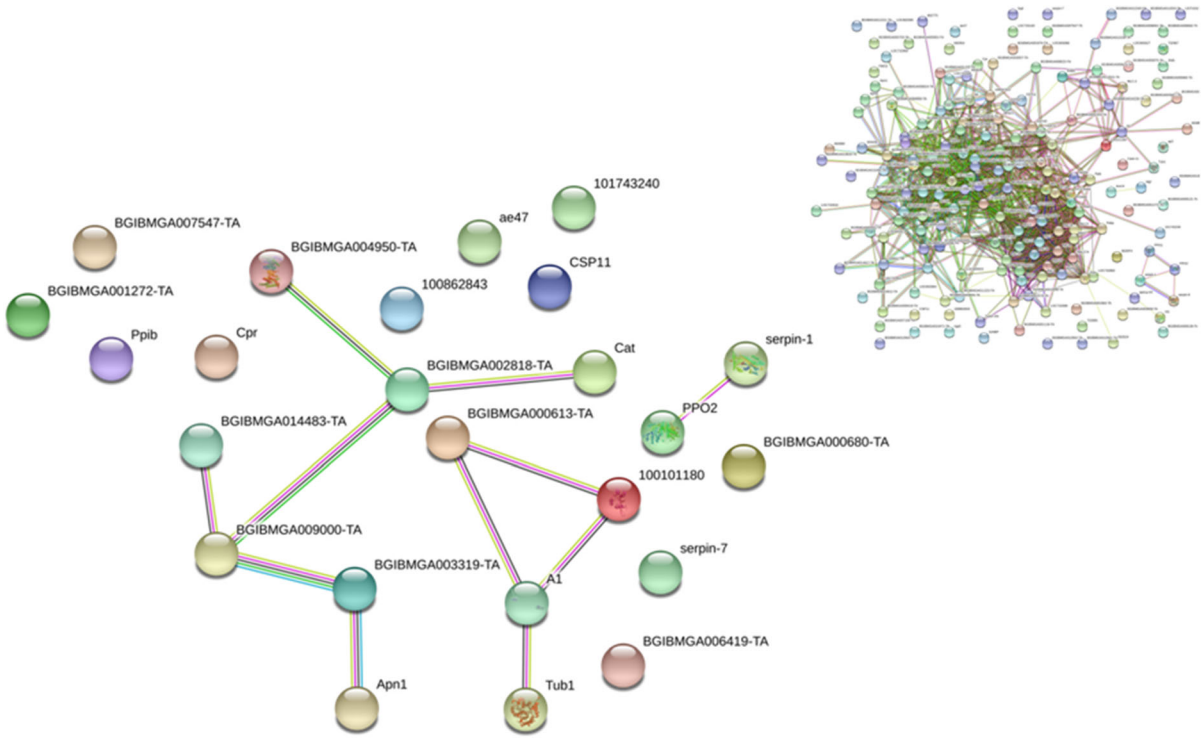


Fig. 8 Protein interaction network of identified differentially expressed (of interest) proteins generated with STRING (ver. 11.0). Nodes represent proteins and edges represent protein–protein interactions. Empty nodes represent proteins of unknown 3-D structure and filled nodes represents some 3-D structure is known or predicted. Known interactions: from curated database, experimentally determined. Predicted interactions: gene neighborhood, gene fusions, gene co-occurrence. Others: textmining, co-expression and protein homology. Inset: Protein interaction network of all the identified 221 differentially expressed proteins generated with STRING (ver. 11.0)

Biochemical assays (LPO, CAT, and GSH) revealed a higher concentration of MDA and reduced level of antioxidant enzymes in laccase-fed larval gut. Earlier, boric acid was reported to increase LPO in the midgut and fat body of *G. mellonella* (Büyükgüzel et al. 2013). Similarly, thermal stress also increases LPO in *Bactrocera dorsalis* (Jia et al. 2011). Another study reported decreased CAT activity in larvae of *Bombyx mori* in response to tryptophan (Priya Bhaskaran et al. 2015). The declined CAT activity causes cell damage due to the accumulation of hydrogen peroxide (H_2O_2). Catalase (CAT; EC 1.11.1.6) is a first-line defence antioxidant which catalyses the breakdown of H_2O_2 to less toxic products (Ighodaro and Akinloye 2018). In corroboration to our catalase assay data, the proteomic analysis further suggests significant reduction in catalase (CAT; EC 1.11.1.6; Uniprot ID-H9BEW3) protein in laccase-fed larvae of *H. armigera*. Similarly, an antioxidant, thioredoxin reductase (TrxR; EC 1.8.1.9; Uniprot ID-A0A0A7RB97) was downregulated in laccase-fed larval gut. It is already known that one-electron reduction of molecular oxygen produces free radicals or reactive species, which in excess are destructive to living organisms. In contrast, antioxidants (enzymatic and non-enzymatic) are the molecules that together act against the free radical attack. Various potential sites for reactive oxygen (H_2O_2 and $O_2^{\cdot-}$) production have been identified in Kreb's cycle and electron transport chain (ETC) of mitochondria. In an earlier report, complex II of ETC was suggested as the site for $O_2^{\cdot-}$ or H_2O_2 generation at rates approaching or exceeding the maximum rates achieved by complex I (NADH dehydrogenase; EC 1.6.99.3) or complex III (coenzyme Q: cytochrome c-oxidoreductase; EC 1.10.2.2). Respiratory complex II (succinate dehydrogenase; SDH; EC 1.3.5.1) reduces ubiquinone in ETC and oxidizes succinate to fumarate as a part of Kreb's cycle

(Quinlan et al. 2012). H_2O_2 inactivates several enzymes, oxidizes keto-acids, degrades haem protein, damages DNA via oxo-copper complexes (Gutteridge 1986), and causes direct irreversible damage to epithelial cells due to its ability to induce Fenton reaction. Further, thioredoxin reductases (TrxRs) belonging to the family of selenium-containing pyridine nucleotide-disulphide oxidoreductases contains a conserved -Cys-Val-Asn-Val-Gly-Cys- redox catalytic site along with another redox-active site, i.e., a C-terminal -Cys-SeCys- (SeCys: selenocysteine). TrxRs protect against oxidant injury and play an essential role in the recycling of ascorbate from its oxidized form, cell growth, and transformation (Mustacich and Powis 2000).

Moreover, downregulated prophenoloxidase subunit 2 (PPO; EC 1.14.18.1; Uniprot ID-Q2VIY6), as reported previously, is detrimental for the defence against bacteria (Cerenius and Söderhäll 2012). Insect PPO, belonging to type-3 copper protein group is an essential innate immunity protein. Its activation occurs via a cascade of pattern recognition proteins, serine proteases, and serine protease inhibitors or serpins after initial pathogen detection. Activated phenoloxidase (PO) oxidizes phenolic molecules to produce melanin around invading pathogens and wounds. During the process of melanization, various toxic molecules like reactive oxygen and cytotoxic quinones are produced. As a result, uncontrolled melanization is lethal to insects; therefore, insects have evolved the mechanisms to regulate melanization. Further, serpins limit the activity of corresponding proteinases; thereby, avoiding excessive melanization (Lu et al. 2014). The present study revealed downregulated serpin (Uniprot ID-F5B4G8) and serpin-9 (Uniprot ID-A0A290U612) proteins in laccase-fed larvae of cotton bollworm; hence, it suggests extensive melanization and reactive oxygen species generation. Whereas, Uniprot ID-A0A2W1BVE6 protein with serine-type endopeptidase activity (GO: 0008236) was upregulated in laccase-fed larvae. Earlier, serine-type endopeptidase has been reported to play a vital role in coagulation, melanization, and antimicrobial peptides generation via protease cascades (Hu et al. 2012).

Moreover, malic enzyme (EC 1.1.1.38; Uniprot ID-A0A2W1BVZ1) which oxidizes malate to pyruvate was underexpressed in the lumen of laccase-fed larvae. According to a previous report, overexpressed malic enzyme extends the lifespan of *Drosophila melanogaster* by enhancing the free radical production as well as the expression of ROS-scavenging enzymes (Wang et al. 2018). Another protein (Uniprot ID-A0A2W1BQ31) with methylenetetrahydrofolate dehydrogenase ($NADP^+$) activity (GO: 0004488) was underexpressed in the laccase-treated lumen. 5,10-methylenetetrahydrofolate dehydrogenase (MTHFD; EC 1.5.1.5) is essential for the production of specific nucleic acids (purine) and amino acids (alanine, glycine, methionine, and serine) (Haque et al. 2019). Further, downregulated aminopeptidase (Uniprot ID-Q6R3M5), aminopeptidase N (Uniprot ID-A0A1L5JK78), and metallopeptidase (Uniprot ID-A0A2W1BR38) were found in laccase-fed larval gut. Aminopeptidase N (APN) is a well-known protein-digesting enzyme, abundantly found in brush border membrane of the intestine (Hu et al. 2012). In a previous study of the midgut lumen, various digestive enzymes involved in the digestion of substances including carbohydrates, proteins, and lipids were revealed. Further, many non-digestive enzymes including multidomain lipocalin, arginine kinase, and others with unknown functions were also identified (Pauchet et al. 2008).

Insects lack an adaptive immune response, such that they solely depend on innate immune system to fight infections (Li et al. 2019). They have evolved a detoxification system that includes carboxylesterases (CarEs), cytochrome P450 monooxygenases (P450s), UDP-glucuronosyltransferases (UGTs), and glutathione S-transferases (GSTs) (Sun et al. 2019). In laccase-fed larvae, carboxylic ester hydrolases [EC 3.1.1.- (Uniprot ID-D5KXB9; Uniprot ID-D5G3G4; Uniprot ID-D5KX87; Uniprot ID-H9ZVH4; Uniprot ID-S4WMC9)] were upregulated. Carboxylesterase, a multifunctional superfamily found in all living organisms, plays crucial roles in the process of xenobiotic detoxification, neurogenesis, pheromone degradation, and developmental regulation (Hu et al. 2012). Furthermore, chemosensory protein (CSP; Uniprot ID-A0A0A0VG76) with a role in xenobiotic degradation and insect defence was upregulated in laccase-fed larval gut. In an earlier report, CSPs genes in silkworm moth were significantly upregulated in response to an insecticide. Also, 2 CSPs genes were upregulated during bacterial infection in *Drosophila.* Besides, CSPs has a role in both innate as well as adaptive immunity against a specific infectious agent

such as highly toxic host plant chemical molecule (Liu et al. 2016). Overexpressed glutamine synthetase (GS; EC 6.3.1.2; Uniprot ID-A0A2W1BMY2) and under-expressed NADPH–cytochrome P450 reductase (CPR; EC 1.6.2.4; Uniprot ID-E0A3A7) and carboxyl/choline esterase (CCE; Uniprot ID-D5G3E6) were reported in the lumen of cotton bollworm larvae in response to the feeding of recombinant laccase. Interestingly, P450s along with CCE is involved in conferring resistance against insecticides (Demaeght 2015). Where, GS is an essential detoxification enzyme in stress and immune responses (Wei et al. 2019). While, CPR transfers electrons from $NADPH + H^+$ to ferrous cytochrome P450 monooxygenase (P450), enabling the P450 redox reaction to metabolize insecticides; thus, it leads to detoxification and resistance in insects (Suwanchaichinda et al. 2014). In the present study, UDP-glucuronosyltransferase (Uniprot ID-A0A2W1BAL9), a multi-functional detoxification enzyme with a role in the biotransformation of compounds was found to be significantly downregulated in response to laccase feeding. Other proteins, aspartate aminotransferase (AST; EC 2.6.1.1; Uniprot ID-A0A2W1B2Y8) and peptidyl-prolyl cis–trans isomerase (EC 5.2.1.8; Uniprot ID-A0A2W1BDP4) were found to be significantly suppressed in response to laccase treatment. The previous reports on cotton leafworm suggest the inhibitory effect of studied insecticides on AST (Assar et al. 2016). Another study on destruxin A (DA): a major secondary metabolite and mycotoxin secreted by an entomopathogenic fungus *Metarhizium anisopliae* reported the interaction between DA and peptidyl-prolyl cis–trans isomerase (BmPPI). DA has various bioactivities including antifeedant, insecticidal, and growth-retarding effects with inhibition of immunity to insects (Wang et al. 2019).

Finally, protein–protein interaction was studied using the STRING database. Three interaction clusters were generated: one between PPO and serpin; another between cytoskeleton proteins; and third among antioxidants (Cat, Trx), digestive enzyme (aminopeptidase), other enzymes (AST, MTHFD), and Kreb's cycle proteins (succinate dehydrogenase, NADH dehydrogenase). In an earlier report on molecular mechanism of resistance in *Drosophila* fruit fly, the protein interaction network for a total of 528 proteins constructed by STRING and Cytoscape identified 13,514 protein–protein interactions, in which endopeptidase (Prosα5, Prosα6, Prosβ4, Prosβ6), and ribosomal protein (RPL40) were the central nodes, which if removed, will crash the network (Zhang and Zhang 2018).

In conclusion, increased free radical generation and reduced antioxidants level in the lumen of laccase-fed larvae creates redox imbalance due to altered redox signalling, leading to oxidative stress that deteriorates the biomolecules in the living system. The TEM results reveal the damage in the malpighian tubules, which excretes nitrogenous waste products from the insect body. Also, tracheae are vital for respiration in *H. armigera* and villi helps in the absorption of digested food. Thus, damaged malpighian tubules and tracheae can result in increased toxicity, and reduced villi leads to decreased absorption, thereby, inhibiting the insect growth. Further, the oxidatively damaged intestinal cells affect functions such as food utilization and nutrients uptake, thus, limiting the insect growth and survival. Hence, we conclude that the cotton bollworm can be used as a model organism to study the effect of microbes and the microbial metabolites.

Acknowledgements The authors thank Prof. Deepak Pental, CGMCP, University of Delhi South Campus, New Delhi, India for the insect trial facility.

Supplementary information Supplementary Figure 1—Optimization of recombinant laccase production from *Yersinia enterocolitica* strain 8081.

Supplementary Figure 2—**I** Coomassie-stained SDS-PAGE gel of induced *E. coli* BL21 cells harboring expression plasmid vector pET28a with laccase gene from *Y. enterocolitica* strain 8081. Induction was done at 200 rpm with 1 mM IPTG. Lanes: **a** Control (without induction) **b** Induction for 2 h at 25 °C; **c** Induction for 3 h at 25 °C; **d** Induction for 4 h at 25 °C; **e** Induction for 2 h at 30 °C; **f** Induction for 3 h at 30 °C; **g** Induction for 4 h at 30 °C; **h** Induction for 2 h at 37 °C; **i** Induction for 3 h at 37 °C; **j** Induction for 4 h at 37 °C. **II** Expression at 100 rpm for 4 h and then kept at static for 16 h at 30 °C a–d: from **a** Induction at 30 °C; **b** Induction at 25 °C; **c** Induction at 16 °C and **d** uninduced **III** Expression at 100 rpm for 4 h and then kept at static for 16 h at 4 °C. **a** Induction at 25 °C; **b**. Induction at 16 °C.

Supplementary Figure 3—**A** Coomassie-stained SDS-PAGE gel of solubilized inclusion bodies: **I** Supernatant **II** Pellet **B** & **C**. Purification of laccase protein using **B**. Ni–NTA affinity chromatography: **I** Wash (I) **II** Wash (II) and **III** Eluted laccase **C** Zymogram of purified laccase using ABTS as substrate

Supplementary Figure 4—Cyclic voltammograms obtained for *Yersinia enterocolitica* strain 8081 (A, B, C) and *Bacillus pumilis* strain DSKK1 (D, E, F) recombinant laccases with 1mM ABTS (in red) and without ABTS (in blue) **A**, **D** at pH 5 using citrate-phosphate buffer; **B**, **E** at pH 7 using Tris buffer,

and **C**, **F** at pH 10 using carbonate-bicarbonate buffer.

Supplementary Table 1—Composition of artificial diet

Supplementary Table 2—Gene ontology (GO) analysis of identified proteins (≥ 1 PSMs in black and ≥ 5 PSMs in blue) from *H. armigera* larvae with and without recombinant laccase feeding

Supplementary Table 3—Distribution of differentially regulated proteins (with PSMs ≥ 5) from normal diet-fed and recombinant laccase-fed *H. armigera* larvae gut into various enzyme classes

Funding This work is supported by grants from the UGC-New Delhi [Grant No. 39-204/1010(SR)]. The FIST-DST grant to the Department of Microbiology is sincerely acknowledged.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Research involving animal and/or human rights This article does not contain any studies with animals performed by any of the authors. *Helicoverpa armigera* has not been notified under any act or laws and rules thereof of the Government of India as an endangered or threatened species restricting or regulating its collection and observation. Therefore, no permits were required.

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