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Metabolic coupling in the co-cultured fungal-yeast suite of *Trametes ljubarskyi* and *Rhodotorula mucilaginosa* leads to hypersecretion of laccase isozymes

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ABSTRACT

Trametes ljubarskyi produces multiple laccase isozymes under various physicochemical conditions. During co-cultivation condition *Rhodotorula mucilaginosa* showed inter-specific interactions with *T. ljubarskyi* and hypersecretion of laccases; however, the underlying molecular mechanism is less-known. The analysis of proteomics data of co-cultivated cultures revealed the mechanism of metabolic coupling during fungal-yeast interactions. The results suggested high score GO terms related to stimulus-response, protein binding, membrane components, transport channels, oxidoreductases, and antioxidants. The SEM studies confirmed the cellular communication and their inter-specific interactions. This study allows us to deepen and refine our understanding of fungal-yeast symbiotic interaction; further, it also establishes a mutual relation by metabolic coupling for 10-fold higher laccase isozyme secretion (6532 U/ml). The purified laccase isozymes showed acidic pH optima (pH 3–4), higher thermo-stability (60 °C), and broad enzyme kinetics (K_m) values. Our study also provides an in-depth understanding of laccase isozymes and their potential to degrade synthetic dyes, which may help the fungi to survive in an adverse environment.

1. Introduction

In nature, fungi live in the complex environment of competition where inter-specific interactions are the critical component to support fungal community development (Arfi et al., 2013; Zhong et al., 2019). These interactions represent the physiological fusion between two independent organisms, in which essential metabolites are traded between interacting partners (Shitut et al., 2017). Also, these interactions develop competition for their survival and nutritional uptake, which in response trigger secretion of stress-related component (Zhong et al., 2019). Reactive oxygen species (ROS) are released during fungal interactions, which favor the induction and secretion of multiple oxidative enzymes (Arfi et al., 2013; Jain et al., 2019). Inter-specific interactions also result in the secretion of enzymes (laccase, chitinase, phosphatase), which function to maintain pH, nutrient uptake, stress defense, and are responsible for host cell wall hydrolysis (Arfi et al., 2013; Lindahl and Finlay, 2006; Zhong et al., 2019). Laccases (EC 1.10.3.2) are an oxidative enzyme having wide substrate (phenolics and amines) specificities and play an anti-oxidative role during stress condition (Jain et al., 2019; Kumar et al., 2015). Laccases are mostly glycoproteins, secreted extracellularly or intracellularly, depending upon their source of production (Kumar et al., 2017; Singh et al., 2014b). Various physiological roles are attributed to this enzyme, some of them are: fungi-mediated delignification of lignocellulosic substrates, cell wall formation, and lignification by plant laccase in PAL pathway, cuticle sclerotization in insects, spore formation in *Bacillus* species, a virulence factor in *Cryptococcus neoformans* (Mayer and Staples, 2002; Sharma and Kuhad, 2008; Zhu and Williamson, 2004). Apart from their physiological roles; fungal laccases have immense industrial potential and are widely studied for their applicability in green chemistry, polymer synthesis, food, and pharmaceutical industries (Pezzella et al., 2015; Riva, 2006; Sharma et al., 2013).

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White-rot basidiomycete fungi are an excellent source of laccases (Piscitelli et al., 2011; Sharma et al., 2005). Under *in-vitro* conditions, laccase production can be enhanced in the presence of certain aromatic compounds and metal salts (Kumar et al., 2015; Piscitelli et al., 2011). Laccases are mainly secreted in multiple isoforms and are regulated differentially during various developmental stages of fungi. It has evolved under diverse environmental conditions, that is, developmental stages, change in pH, humidity, and temperature (Dong et al., 2005; Fonseca et al., 2013; Kumar et al., 2017). Recently, the specific role of laccase isozymes has been reported, for example, oxidative stress tolerance, detoxification of lignocellulosic hydrolysates, and in decolorization of industrial dyes (Fang et al., 2015; He et al., 2015; Kumar et al., 2015). Ligninolytic enzyme production by white-rot fungi has been carried out under solid-state fermentation conditions (SSF) and submerged fermentation (Kumar et al., 2015; Sharma et al., 2005). However, SSF at large scale makes down-streaming of the enzyme more complex; moreover, the aromatic compounds and metal salts have been reported to cause toxicity in the culture media, which ultimately leads to restrictive cell growth or cell death (Baldrian, 2003).

Apart from incorporating production media with aromatic compounds and divalent metal salts, another effective strategy employed for higher production of laccases is by co-culturing of microorganisms (basidiomycetes and ascomycetes) (Dong et al., 2012; Li et al., 2011; Wang et al., 2015). Interestingly, fungi secrete laccases in higher amount during co-culture conditions. The prominent sector for utilizing fungal laccase is the dye decolorization. Synthetic dyes are widely used in various industrial applications, that is, textile, paper, food, cosmetics, and pharmaceutical industries (Singh et al., 2014b). Physico-chemical methods have higher efficiency in achieving better decolorization but are generally costly and of limited applicability (Bhatia et al., 2017). Some studies showed that laccase isozymes with different biochemical properties play a different role. These catalytic activity-based studies show high potential for dye decolorization (He et al., 2015; Jiang et al., 2013; Moldes and Sanromán, 2006; Rühl et al., 2013).

Many studies have shown the induction and higher production of laccases by using metal salts, xenobiotic compounds, and co-culturing of micro-organism; however, the underlying molecular mechanism of laccase induction in co-culture condition is still lacking. Therefore, in this investigation, we have tried to emulate laccase production from *Trametes ljubarskyi* under various physicochemical conditions. Also, the co-culture approach with yeast *Rhodotorula mucilaginosa* was explored to study its effect on laccase production and increased isozyme secretion. Moreover, the outcome of proteome profiling of co-cultivated *T. ljubarskyi* and *R. mucilaginosa* will provide an in-depth understanding of the fungal-yeast symbiotic interaction for co-adaptation and co-survival strategies. Finally, this work is also focused on the purification and characterization of laccase isozymes, and their ability to decolorize the synthetic dyes and industrial effluents.

2. Materials and methods

2.1. Chemicals

The substrates for laccase assay (e.g., guaiacol and *o*-tolidine), chitinase assay (e.g., chitin, 3,5-Dinitrosalicylic acid), and other chemicals, that is, phenolic inducers (e.g., D-quinic acid, 3,5-Dihydroxytoluene, orcinol, 3,4-Dihydroxybenzoic acid, catechol, *o*-toluidine tannic acid), 1-Hydroxybenzotriazole, and gel staining dye CBB-R250 were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other culture media and chemicals were of the highest purity grade, purchased from Hi-media (Mumbai, India); whereas, the synthetic dyes were purchased locally. The industrial effluent was procured from a local cotton dyeing industry.

2.2. Isolation of fungus and yeast

The fungal culture was isolated from the delignified biomass collected from hilly regions of India, Haryana (latitude, and longitude of 28.6924° N, 76.9240° E), whereas, basidiomycetous yeast was an air-borne environmental isolate. Both the cultures were isolated by following standard isolation protocol (Diwaniyan et al., 2012). The basidiomycetous fungus was maintained on malt extract agar (MEA) media containing (g l^{-1}): malt extract 20.0, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.5, $Ca(NO_3)_2.4H_2O$ 0.5, Agar 20.0 (pH 5.2) at 4 °C (Kumar et al., 2015). Whereas, yeasts were maintained on slants with medium containing (g l^{-1}): peptone 20.0, yeast extract 10.0, glucose 20.0, and agar 20.0 at 4 °C.

2.3. DNA extraction, PCR, and sequencing

Genomic DNA of both the fungi and yeast were isolated as described earlier (Kuhad et al., 2004). The fungus was inoculated in 50 ml of malt extract broth (MEB) and allowed to grow in 250 ml Erlenmeyer flask for 5 d at 30 °C. Whereas, yeast was inoculated in yeast extract potato dextrose broth (YEPD) and allowed to grow at 30 °C for 24 h for DNA isolation.

Primer sequences (internal transcribed spacer region from rDNA) ITS1 – 5′-TCC GTA GGT GAA CCT GCG G-3′ (forward) and ITS4 – 5′-TCC TCC GCT TAT TGA TAT-3′ (reverse) were used to identify laccase producing basidiomycetous fungus and yeast (Sharma et al., 2014). The amplified products were run on 1 % agarose electrophoresis gel and the desired band was purified using HiYield™ Gel/PCR DNA Mini Kit (Real Genomics, RBC). The PCR-purified products were sequenced commercially. The sequences were further analyzed using the NCBI BLAST and submitted to GenBank. The phylogenetic trees were constructed to locate the taxonomic position of the fungus using the MEGA analysis tool v. 6 with the bootstrap value calculated from 1000 runs.

2.4. Analytical procedure

2.4.1. Laccase assay

Guaiacol was used as a substrate for assaying laccase activity following the method as described earlier (Sharma et al., 2005). One unit (U) of laccase was defined as the change in absorbance of 0.01 ml^{-1} min^{-1} at 470 nm wavelength.

2.4.2. Chitinase assay

The reaction mixture containing 0.5 ml colloidal chitin (0.1 %) in 100 mM acetate buffer (pH 5.0) and 0.5 ml of the appropriately diluted enzyme was kept at 50 °C for 30 min (Tanaka et al., 1999). The reducing sugars liberated were determined using 1 ml DNSA reagent. One unit of chitinase is defined as the amount of enzyme required to liberate 1 nmol of reducing sugars, as N-acetylglucosamine from colloidal chitin per second under the reaction conditions.

2.5. Inocula preparation for co-cultivation

Mycelial mat formation was carried out in 25 ml MEB in 250 ml Erlenmeyer flask. Each flask was inoculated with four fungal discs (8 mm dia each) from the periphery of 5-d-old culture of *T. ljubarskyi* MDU-01. The 4-d-old mycelia were crushed with the help of sterile mortar and pestle, thereafter, 1 ml of crushed inocula

(2 %) was added in 50 ml of MEB and 0.06 mM $CuSO_4$ (v/v) in 250 ml Erlenmeyer flask under sterilized conditions.

R. mucilaginosa MDU-02 was cultured in a medium containing (g l^{-1}): peptone 20.0, yeast extract 10.0, and glucose 20.0 for 24 h at 30 °C on a rotatory shaker at 125 rpm. The secondary inocula used as a co-culture was prepared from 24-h-old primary culture medium grown at 30 °C and 125 rpm. *Saccharomyces cerevisiae* was used as a negative control and grown on media with similar composition.

2.6. Fungal pellet optimization

Under sterilized conditions, 1 ml of crushed inocula (2 %) was added in 50 ml of MEB in 250 ml Erlenmeyer flask. Thereafter, the flasks were incubated at 30 °C for pellet formation at different rpm varied from 75 rpm to 125 rpm.

2.7. Effect of different pH and temperature conditions on laccase isozymes production

Production of laccase isozymes was studied from *T. ljubarskyi* MDU-01 at different pH (2.5–5.0) and incubation temperature (25–35 °C) in shake-flask conditions (125 rpm). Crude enzyme samples were harvested from 2nd day onward for zymogram analysis of laccase isozymes. The culture was induced separately on the 2nd day with ethanol (3 % v/v), $CuSO_4$, and different aromatic compounds (1 mM).

2.8. Effect of co-cultivation on laccase isozymes secretion and metabolic coupling

Different dosage (2 % and 4 % v/v) of the *R. mucilaginosa* MDU-02 and *S. cerevisiae* yeast culture broth were inoculated into the *T. ljubarskyi* MDU-01 growing pellets at 48 h, 25 °C, and at 125 rpm. Crude enzyme samples were harvested from the 3rd day onward for the estimation of laccase activity and zymogram analysis of laccase isozymes. All experiments were performed in triplicates and their mean value and standard deviation represented the results.

2.8.1. Protein preparation for LC–MS/MS

The co-cultured fungal mycelia and yeast cells (three flasks were pooled) were harvested for total proteome (intra and extracellular) analysis on the 9th day of incubation at 25 °C and 125 rpm condition. The intracellular as well as extracellular soups were precipitated using trichloroacetic acid (TCA):Acetone; 1:4 and kept overnight at 4 °C, after that, it was centrifuged at 11 000×g for 30 min at 4 °C. Further, the obtained pellets were washed repeatedly to remove impurities using acetone/5 mM dithiothreitol (DTT)-acetone. Finally, total protein was directly solubilized in 6 M Guanidine hydrochloride (GdmHcl, Sigma) in 50 mM ammonium bicarbonate (ABC, pH ~ 7) and heat for 5 min at 95 °C for complete solubilization. The samples were cooled, and 100 μg of protein was reduced with 5 mM (tris(2-carboxyethyl)phosphine) (TCEP, Sigma) in 25 mM ABC (pH ~ 7) at 37 °C for 30 min. Samples were then alkylated using 55 mM Indole acetic acid (IAA) (Sigma) in 25 mM ABC (pH ~ 7) at RT in the dark for 30 min. Then the samples were diluted with 25 mM ABC so that final GdmHcl concentration becomes 1 M. Trypsin (Promega) was added in a ratio of 1:50 for overnight digestion at 37 °C. Desalting of samples was done by the manufacturer's protocol (Thermo Fisher Scientific). Samples were SpeedVac till dry and finally reconstituted in 0.1 % formic acid. Approximately, 2 μg of each sample was loaded on a C18 reverse-phase column. Lastly, raw data obtained were analyzed in Proteome Discoverer 2.1 (Thermo Scientific).

2.8.2. LC–MS/MS analysis of proteome from co-cultivated fungi

Peptide mixtures from enzymatic digestions were dissolved in 5 μl solution A (0.1 % formic acid in 2 % acetonitrile), and analyzed by nanoLC–MS/MS in a nanoEasy-HPLC (Thermo Scientific) coupled with nano-electrospray ion source (Thermo Scientific). Peptides were loaded onto a PepMap 100 C18-nano viper, 75 μm × 2 cm pre-column (Thermo Scientific EASY-Column) and then eluted onto a PepMap RSLC C18, 75 μm × 50 cm (Thermo Scientific), 2 μm particle size of 100 Å, at a flow-rate of 300 nl/min using the gradient of the solution A (95 % water + 5 % Acetonitrile + 0.1 % Formic acid) and solution B (10 % water + 90 % Acetonitrile + 0.1 % Formic acid), for a total run time of 123 min. Technical replicates were run to obtain the best chromatogram. For precursor mass tolerance (MS1) maximum ion transfer time was kept 60 ms; whereas, for fragment mass tolerance (MS2), normalized collision energy and maximum ion transfer time were kept 27 and 120 ms, respectively. Full-scan MS spectra (m/z 300–1700) were acquired on an LTQ-OrbitrapVelos (Thermo Scientific) in the positive ion mode. The 10 most intense ions were selected for collision-induced dissociation (CID) fragmentation in the LTQ Velos.

2.8.3. Proteome analysis of co-cultivated fungi

Raw MS data files obtained from the mass spectrometer were processed by using Proteome Discoverer 2.1 (Thermo Scientific). Mass spectra files were searched against the constructed fungal database (database available in UniProt) related to fungi, yeast, *Trametes*, and *Rhodotorula*, from UniProt (http://www.uniprot.org/) using the MASCOT search engines through Proteome Discoverer version 2.1 (Thermo Scientific). Peptides and proteins were inferred from the spectrum identification results using Proteome Discoverer Peptide Spectrum Matches (PSMs), and were validated at a 1.0 % False Discovery Rate (FDR) estimated using the decoy hit distribution. Function assignment and annotation by gene ontology terms (GO; www.geneontology.org), InterPro terms (InterProScan, EBI), enzyme classification codes (EC), and metabolic pathways (KEGG, Kyoto Encyclopedia of Genes and Genomes) were determined using the Blast2GO software suite (Götz et al., 2008).

2.8.4. Scanning electron microscopy of co-cultivated fungi

Scanning electron microscopy (SEM) was used to observe the surface morphology of *T. ljubarskyi* MDU-01 mycelia and *R. mucilaginosa* MDU-02 cells in both monocultures and co-culture. The samples for SEM were collected from the *T. ljubarskyi* MDU-01 culture and the co-culture on the 9th day and from *R. mucilaginosa* MDU-02 culture on the 2nd day. The culture samples were fixed by 2 % (v/v) glutaraldehyde for 1 h, washed 3 times with 100 mM sodium-phosphate buffer (pH 7.2), fixed by 1 % (w/v) aqueous osmium tetroxide for 3 h, and dehydrated by alcohol gradient (30 %, 50 %, 70 %, 90 %, and 100 %) and amyl acetate. The dehydrated samples were subjected to critical point drying and palladium sputter coating, prior to SEM studies.

2.9. Purification and identification of laccase isozymes produced from T. ljubarskyi MDU-01

The culture broth was filtered through Whatman filter paper no. 1 and centrifuged at 13 000×g for 15 min at 4 °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 gradual addition of finely grounded ammonium sulfate at three different saturation levels, that is, 0–20 %, 20–40 %, and 40–80 %. After overnight incubation at 4 °C, the culture filtrate was centrifuged at 9000×g for 20 min. Precipitates were dissolved in 20 mM citrate-phosphate buffer (pH 4.0) and dialyzed overnight against the same buffer at 4 °C. Further,

laccase isozymes were purified from native-PAGE (12 %) as reported earlier by our group (Kumar et al., 2017). After native-PAGE, the gel was stained with 0.1 M citrate-phosphate buffer (pH 4.0) containing 2 mM *o*-tolidine and 10 mM guaiacol, respectively, and incubated at 30 °C in dark conditions (Kumar et al., 2015). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was used to determine the molecular mass and purity of laccase isozymes.

2.9.1. Peptide mass fingerprinting using MALDI-TOF mass spectrometry

The identification of all laccase isozymes was done by peptide mass fingerprinting. The desired bands obtained by SDS–PAGE analysis were cut and trypsin digested. The resulted samples were spotted on a MALDI target plate to obtain peptide spectra using ABI SCIEX MALDI-ToF/ToF 5800. The peptide sequences were then analyzed by comparing mass spectrometry data in the National Center for Biotechnology Information (NCBInr) protein database using the Mascot search algorithm (Cottrell and London, 1999).

2.10. Biochemical characterization of purified laccase isozyme

The enzyme activity was assayed in the pH range of 3.0–9.0 using suitable buffers. The substrate (guaiacol) was prepared in 50 mM of citrate-phosphate buffer (pH 3.0–6.0), phosphate buffer (pH 7.0), and Tris–HCl (pH 8.0–9.0). Effect of temperature on laccase activity was determined by incubating the reaction mixture at a different temperature varying from 30 °C to 65 °C under standard assay conditions.

The pH stability was determined by incubating the isozymes in different buffers; like, 50 mM of citrate-phosphate buffer (pH 3.0–6.0), phosphate buffer (pH 7.0), and Tris–HCl (pH 8.0–9.0), for 30 min at 30 °C. Whereas, the temperature stability was determined by incubating the isozymes samples at various temperatures (30–60 °C) for different time intervals (1–8 h).

Michaelis–Menten constant (K_m) and the maximum rate of reaction (V_{max}) were determined by using guaiacol (470 nm) and *o*-tolidine (627 nm) as a substrate, at different concentrations, ranging from 0.5 mM to 3.0 mM and 0.1 mM to 0.8 mM, respectively, in citrate-phosphate buffer (pH 4.0). The values of K_m were calculated from Eadie-Hofstee plot.

2.11. Application of laccase isozymes in dye decolorization

Decolorization of standard dye, like, malachite green (100 mg l^{-1}), procion blue (200 mg l^{-1}), procion green (2000 mg l^{-1}), procion red (200 mg l^{-1}), and industrial effluent (from dyeing industry) by laccase isozymes, that is, lac 3, lac 4, and lac 5 were studied. The reaction mixture contained laccase isozymes (100 U/ml), dye solution, and citrate-phosphate buffer (50 mM; pH 4.6) in a total volume of 2 ml. The decolorization was carried out in citrate-phosphate buffer (pH 4.6) at 30 °C for up to 10 h. The time course of decolorization was studied by measuring the absorbance at 618 nm for Malachite green, 584 nm for Procion blue, 618 nm for Procion green, 497 nm for Procion red, and 560 nm for industrial effluent (Singh et al., 2014b).

Dye decolorization was expressed as:

$$\text{Dye decolorization } (\%) = [(A_i - A_t) / A_i] \times 100$$

where A_i is the initial absorbance of the dye, A_t is the absorbance of the dye along the time.

To study the effect of redox mediator on decolorization of industrial effluent by different isozymes, Hydroxybenzotriazole (HoBT) was added into the reaction mixture at a final concentration of 1 mmol l^{-1}.

Decolorization was monitored by UV–VIS spectroscopic analysis. The spectral study was carried out using Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) and changes in the absorption spectrum (200–800) of all the dyes were recorded and analyzed.

3. Results

3.1. Isolation and identification of fungi

Using ITS taxonomic marker, the basidiomycetous fungus was identified as *T. ljubarskyi* MDU-01 (accession no. KP069022); whereas, basidiomycetous yeast was identified as *R. mucilaginosa* MDU-02 (accession no. KT000654). The neighbor-joining phylogenetic tree of ITS conserved sequence of *T. ljubarskyi* MDU-01 showed similarity with *T. ljubarskyi* TNAU CBE 10 and shared a clade with other *T. ljubarskyi* strains. Their bootstrap analysis suggests a close phylogenetic relationship with a score value of 70–88 % (Fig. 1A). Whereas, *R. mucilaginosa* MDU-02 showed phylogenetic similarity with *R. mucilaginosa* ANT12-058 (bootstrap value 63 %) (Fig. 1B).

3.2. Effect of agitation, pH, and temperature on laccase isozymes production

3.2.1. Fungal pellet optimization

T. ljubarskyi was grown at different shaking condition, that is, 75–125 rpm for fungal mycelial pellet formation. The pellet formation was found to be optimum at 125 rpm with more number of fungal pellets of similar shape and size (Fig. 1C). Agitation speed was found to have a higher impact on pellet formation than the size of the flask, media volume, and inocula conditions (e.g., age and quantity).

3.2.2. Temperature optimization

T. ljubarskyi showed maximum laccase yield (495 U/ml) on the 6th day at 25 °C (Fig. 1D). It secrets almost similar pattern of laccase isozymes, except one additional laccase isozyme produced at high temperature (35 °C) at later stages of fermentation conditions (Fig. 1E). Insignificant effect of this additional isozyme was observed on the laccase yield. Therefore, 25 °C temperature and 125 rpm agitations were selected for further isozyme studies.

3.2.3. pH optimization

T. ljubarskyi produces maximum laccase enzyme (703 U/ml) on the 6th day onward at pH 4.0, 25 °C temperature, and 125 rpm (Fig. 1F). Laccase activity was found to be significantly less on the 2nd day of fermentation in culture broth (Fig. 1F). However, high laccase activity at pH 5.0 was observed on later stages of fermentation conditions. The reason behind this may be highly viscous polysaccharide formation in the culture broth, which leads to the high concentration and stability of the isozymes and, thus, the higher laccase activity. Further, pH 4.0 was selected for laccase production to ease the extraction of laccase isozymes. A comparative study of laccase isozymes showed unique pattern of isozyme secretion at pH 2.5 and pH 5.0, while laccase isozyme secretion was somewhat similar at all others pH conditions (Fig. 1G). The pellet size and morphology of *T. ljubarskyi* were unchanged at pH 5.0 and laccase activity was also found to be significantly high (1200 U/ml) on the 8th day (Fig. 1F).

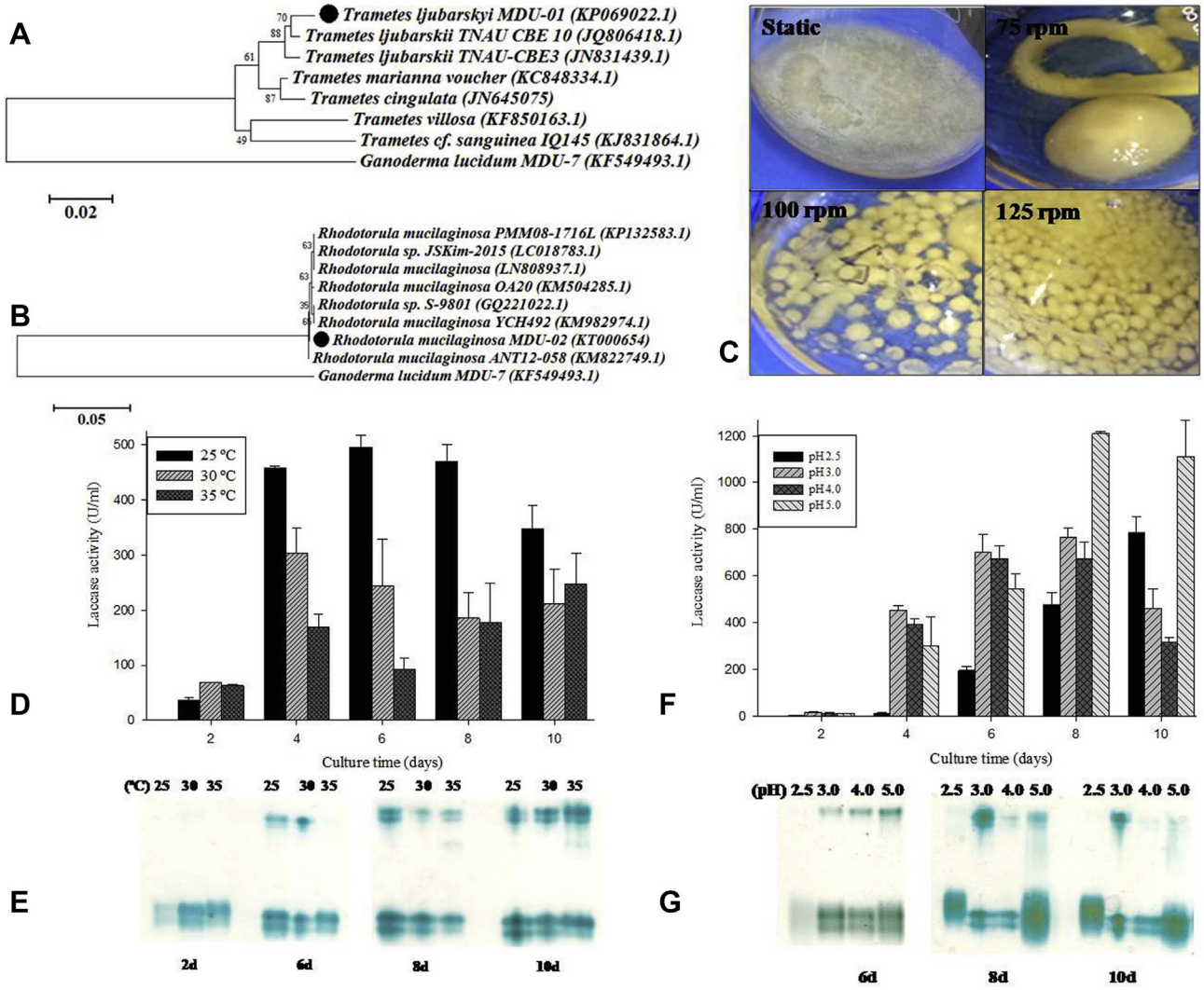

Fig. 1. Phylogenetic tree using internal transcribed spacer (ITS) regions of (A) *T. ljubarskyi* MDU-01 and (B) *R. mucilaginosa* MDU-02. (C) Mycelial pellet formation of *T. ljubarskyi* MDU-01. Effect of different pH and temperature conditions on (D, F) laccase production and (E, G) isozymes secretion from *T. ljubarskyi* MDU-01.

3.3. Effect of copper sulfate on the production of laccase isozymes

Laccase activity in copper sulfate-induced fermentation conditions of *T. ljubarskyi* was found to be the highest on the 5th day (1660 U/ml) (Fig. 2A). Activity staining of native-PAGE with *o*-tolidine showed four laccase isozymes from *T. ljubarskyi* (Fig. 2B). A comparative study of copper-induced laccase isozyme pattern between *Ganoderma lucidum* MDU-7, *Ganoderma* sp. kk-02, and *T. ljubarskyi* is depicted in Fig. 2C. A very diverse range of high- and low-molecular-mass laccase isozyme was observed in *T. ljubarskyi*.

3.4. Effect of phenolic compounds on laccase isozymes production

Majority of the phenolic compound (e.g., anisaldehyde, caffeic acid, catechol, 3,4-dihydroxybenzoic acid, and 3,4-dihydroxytoluene) used in this study suppressed laccase production (data not shown). Interestingly, tannic acid completely inhibited laccase production; whereas, *o*-toluidine and 2,5-xylidine showed insignificant effect on laccase production. A comparative study for laccase isozyme secretion pattern from *T. ljubarskyi* in the presence of different phenolic compounds, that is, 2,5-xylidine, quinic acid, and anisaldehyde, showed a different secretion pattern of laccase isozymes (Supplementary Fig. 1).

3.5. Effect of co-cultivation on laccase isozymes secretion and metabolic coupling

T. ljubarskyi yielded the highest laccase activity (6532 U/ml) when co-cultured with 4 % dosage (v/v) of *R. mucilaginosa*, which was 10-fold higher than the *T. ljubarskyi* monoculture (Fig. 3A). Besides, *T. ljubarskyi* was found to secrete laccase isozymes of different molecular mass during co-culture with *R. mucilaginosa* (Fig. 3B). A similar experiment performed with *S. cerevisiae* as a yeast partner in co-culture suggests insignificant effect on laccase isozymes secretion from *T. ljubarskyi* (data not shown).

3.5.1. Protein profiling of co-cultivated T. ljubarskyi and R. mucilaginosa

The proteomic analysis was done using LC–MS/MS to explore its feasibility for identification of the key regulatory protein responsible for the fungal-yeast interaction. The initial review of the LC–MS/MS data using 'UniProt' database led to the identification of a significant amount of fungal (234) and yeast (282) proteins.

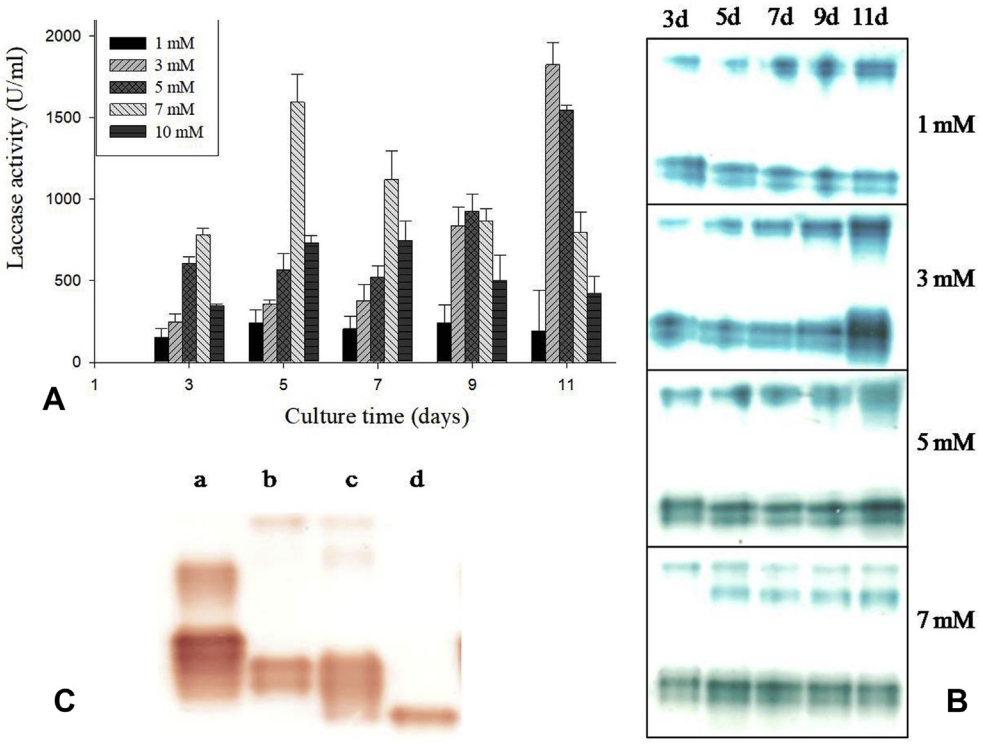

Fig. 2. Effect of $CuSO_4$ on (A) laccase production and (B) isozymes secretion from *T. ljubarskyi* MDU-01 (activity staining with *o*-tolidine). (C) A comparative study of laccase isozymes produced in presence of $CuSO_4$ from I, *G. lucidum* MDU-7; II, III, *T. ljubarskyi* MDU-01 (3d and 7d, respectively) and IV, *Ganoderma* sp. kk-02 (activity staining with guaiacol).

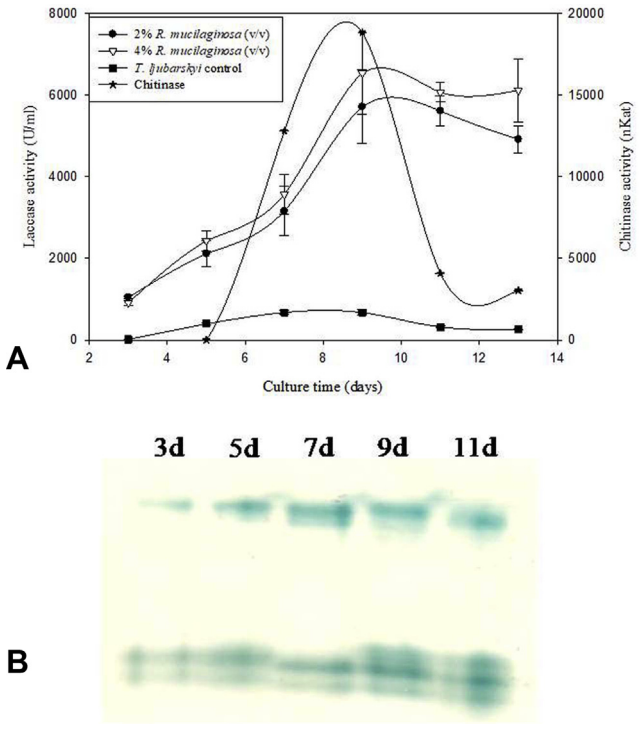

Fig. 3. Effect of *R. mucilaginosa* MDU-02 yeast cells on (A) laccase and chitinase production and (B) isozymes secretion from *T. ljubarskyi* MDU-01 in co-culture conditions.

Thereafter, the database of *Trametes* and *Rhodotorula* was used and significant amount of fungal proteins (289) and few yeast proteins (43) were identified from co-cultivated cultures.

We used Blast2GO suite to assign Gene Ontology (GO) terms to study functions of total protein obtained from co-cultivated *T. ljubarskyi* and *R. mucilaginosa* (Supplementary 1, Supplementary 2). Among the total protein identified from mycelial fungi, 10 categories of cellular components, 9 categories of molecular functions, and 15 categories of biological processes were the enriched GO terms (Fig. 4). Also, 9 categories of cellular components, 7 categories of molecular functions, and 16 categories of biological processes were the enriched GO terms among all yeast identified proteins (Fig. 4). The GO terms supposed to be responsible for the inter-specific interactions are: (i) integral component of membrane under cellular component category (score distribution 34.423 for *T. ljubarskyi* and 31.178 for *R. mucilaginosa*), (ii) Protein binding under molecular function category (score distribution 105.925 for *T. ljubarskyi* and 85.254 for *R. mucilaginosa*), and (iii) response to stimulus under biological process category (score distribution 142.522 for *T. ljubarskyi* and 34.748 for *R. mucilaginosa*).

3.5.2. Scanning electron microscopy to study the surface attachment and interaction between T. ljubarskyi *and* R. mucilaginosa

To further validate the surface attachment of *T. ljubarskyi* and *R. mucilaginosa*, SEM analysis was performed. Studies showed that the fungal mycelia in monoculture were uniform and undamaged (Fig. 5A), whereas the mycelia of *T. ljubarskyi* in co-cultivation (Fig. 5C) revealed the damaged surface. It was also observed that the cells of *R. mucilaginosa* adhere and colonize to the surface of *T. ljubarskyi* mycelia. The SEM results have also revealed that the yeast cells in co-culture were morphologically different (smooth) than the yeast cells grown in monoculture conditions, see Fig. 5C, B, respectively.

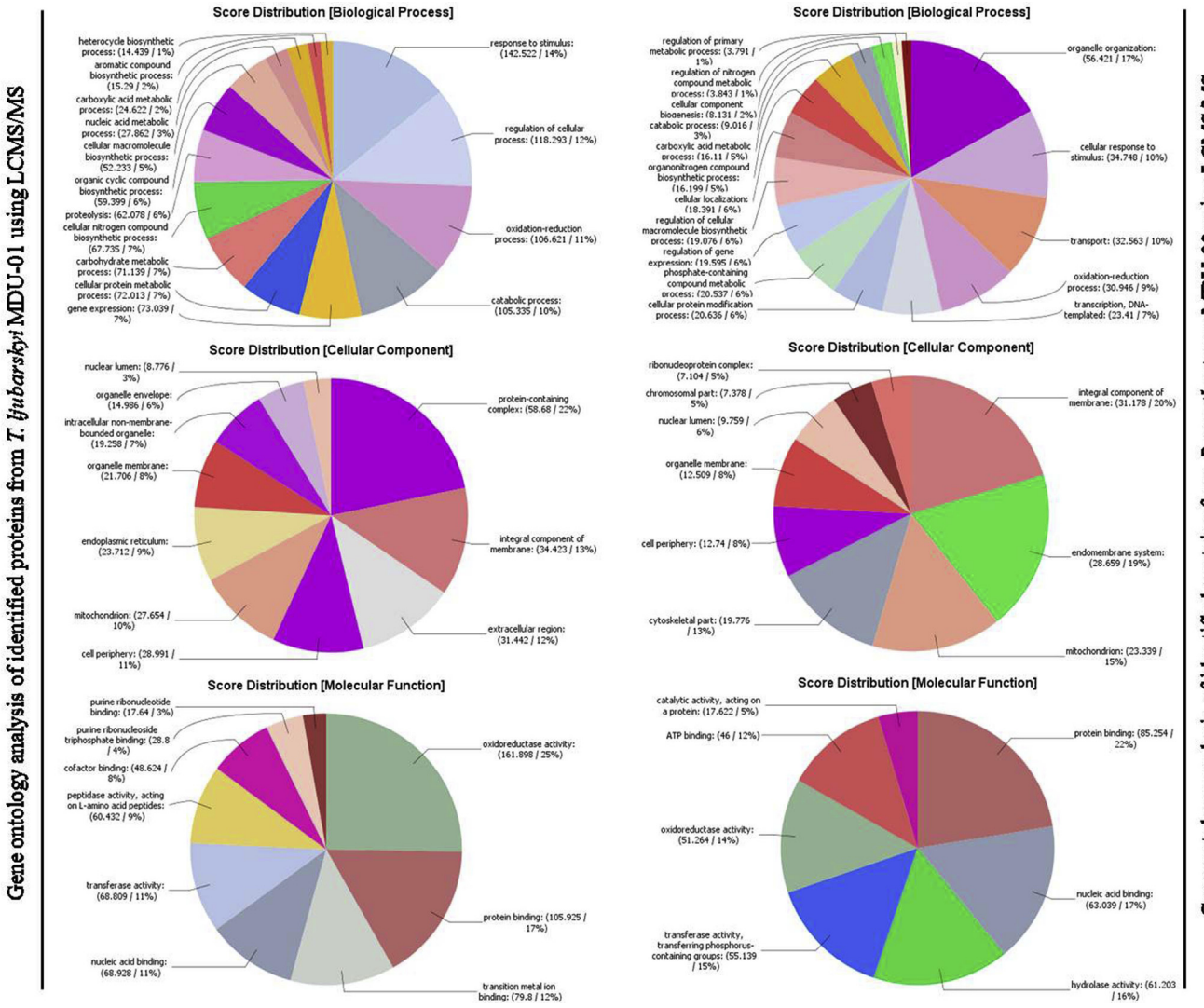

Fig. 4. Gene ontology analysis of total proteome from co-cultivated *T. ljubarskyi* MDU-01 and *R. mucilaginosa* MDU-02 cultures.

3.6. Purification of laccase isozymes

Among different biotic and abiotic compounds studied in this investigation, co-culture with *R. mucilaginosa* has shown a significant effect on the high laccase isozymes production from *T. ljubarskyi*. Furthermore, five laccase isozymes (lac 1–lac 5) of high and low molecular masses, secreted during co-cultivation conditions were purified from the native-PAGE method.

All purified laccase isozymes showed a single band in native gel electrophoresis followed by activity staining (Fig. 6A). The SDS–PAGE analysis of purified laccase isozymes also showed a single band after coomassie staining (Fig. 6B). MALDI-TOF peptide fingerprint were compared from the NCBI protein database, and the fingerprints were finally identified as the laccase isozymes. The peptide sequence of purified lac 3 (score 101) and lac 5 (score 96) confer the laccase isozymes. Earlier, six purified laccase isozymes have been reported from *G. lucidum* MDU-7 by using native-PAGE purification method (Kumar et al., 2017).

3.7. Biochemical characterization and the kinetic parameters of purified laccase isozymes

The purified laccase isozymes (lac 1–lac 5) were active over acidic pH, with optimum activity at pH 3.0 (lac 1 and lac 2) and pH 4.0 (lac 3–lac 5) (Fig. 7A). Also, all the laccase isozymes were found to be stable over a wide pH range, that is, 3.0–9.0 (Fig. 7C). The laccase isozymes lac 1, lac 2, lac 3, lac 4, and lac 5, showed a wide range of temperature optima, that is, 50 °C, 60 °C, 40–45 °C, 60 °C, and 60 °C, respectively (Fig. 7B). The temperature stability for isozymes varies such as lac 1 and lac 2 lost 66–70 % of residual activity after 2–4 h at 50 °C, respectively, whereas, lac 3, lac 4, and lac 5 lost 50 % of residual activity after 2 h at 60 °C (Fig. 8). The laccase isozymes showed substrate-specific and differential catalytic (K_m) properties (Table 1). Laccase isozymes, that is, lac 2 and lac 5 have high substrate affinity toward guaiacol, whereas, lac 1, lac 3, and lac 4 have a high affinity toward *o*-tolidine.

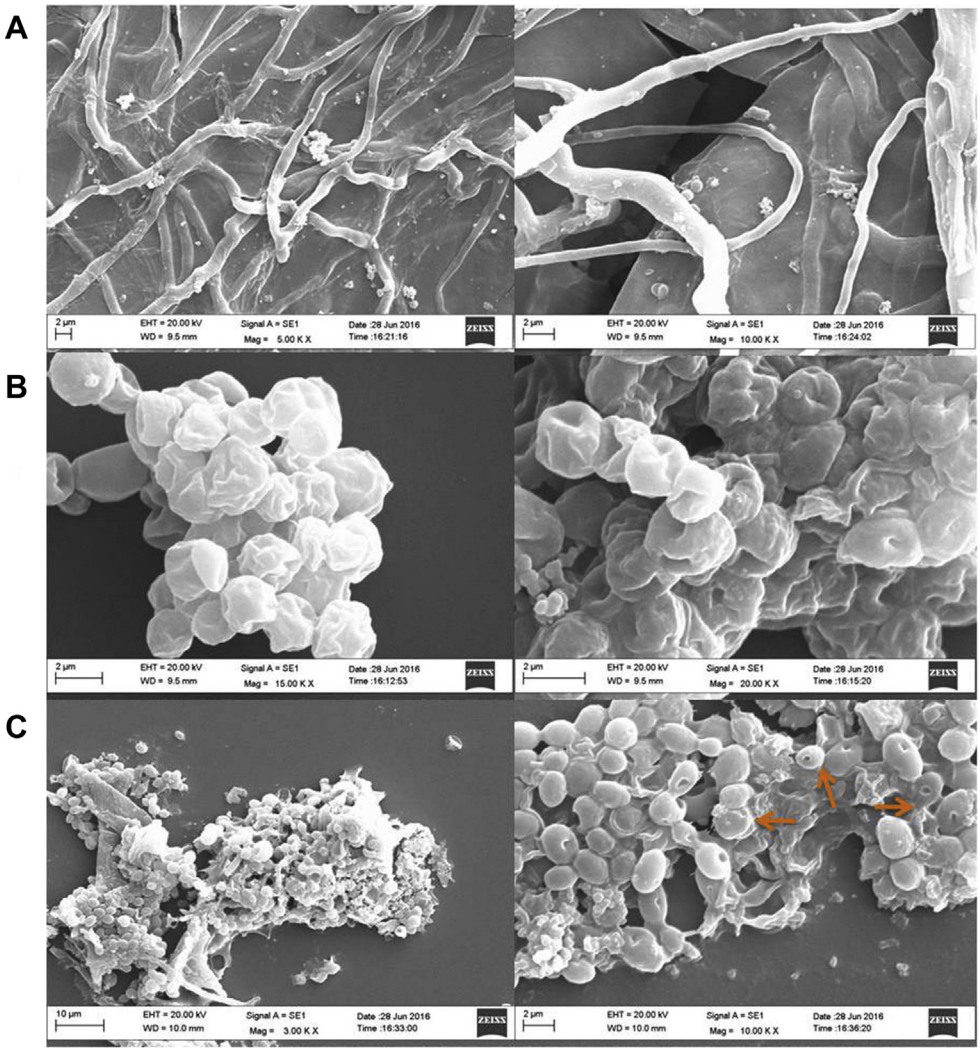

Fig. 5. SEM images of the basidiomycetous fungi (A) *T. ljubarskyi* MDU-01 monoculture, (B) *R. mucilaginosa* MDU-02 monoculture and (C) co-culture (arrows indicate the surface attachment of yeast with mycelial fungus).

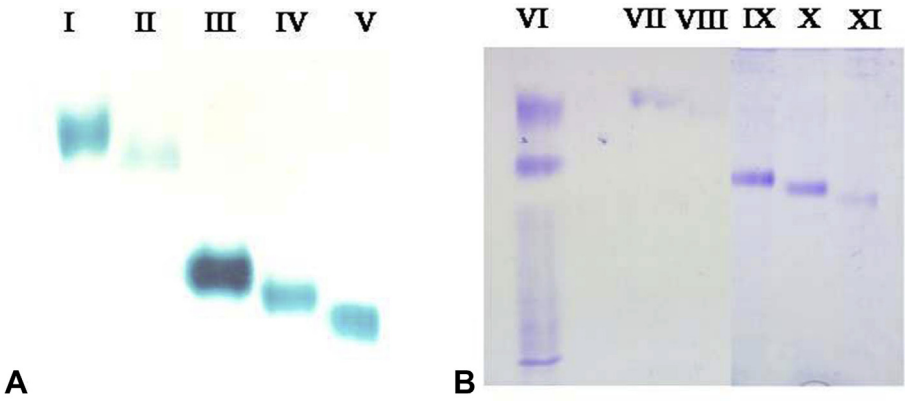

Fig. 6. Zymogram (A) and SDS–PAGE (B) of purified laccase isozymes. I–V, activity staining with *o*-tolidine and VI–XI, Coomassie staining. I, VII, lac 1; II, VIII, lac 2; III, IX, lac 3; IV, X, lac 4; V, XI, lac 5; VI, partially purified protein.

3.8. Application of laccase isozymes in dye decolorization

In this work, we have studied the effect of different laccase isozymes on dye decolorization. After 10 h of incubation, malachite green was decolorized up to 91 % with lac 4 and lac 5; whereas, 86 % decolorization was achieved with lac 3 (Fig. 9). Procion blue was decolorized up to 70 % by lac 3 and lac 4, whereas, 75 % decolorization was achieved with lac 5 after 10 h of incubation. Procion

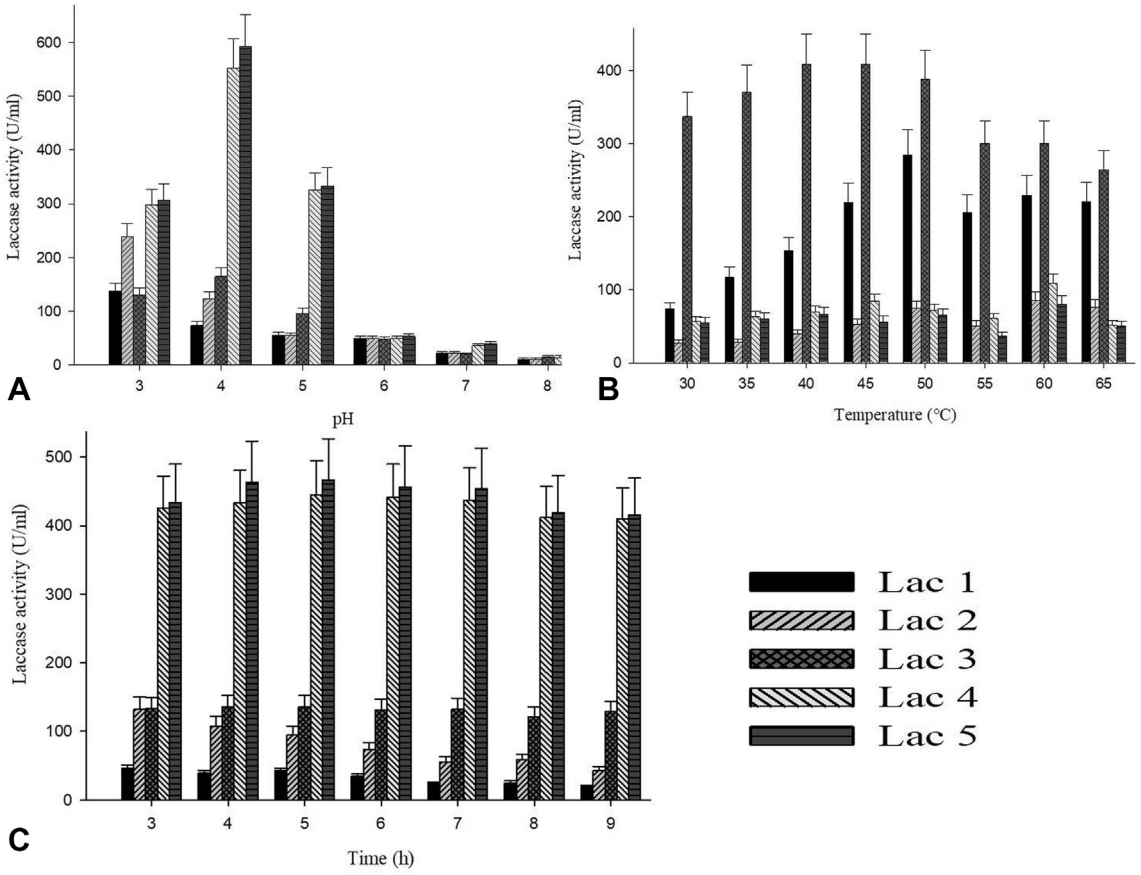

Fig. 7. The optimal pH, optimal temperature and the pH stability of purified laccase isozymes (lac 1–lac 5) from *T. ljubarskyi* MDU-01 were determined. (A) optimal pH; (B) optimal temperature; (C) the pH stability of laccase isozymes.

green was decolorized up to 63 %, whereas, procion red was decolorized between 47 % and 51 % by all the laccase isozymes (Fig. 9). The Industrial effluent was decolorized (41 %) uniformly by all the laccase isozymes. Interestingly, in the presence of redox mediator lac 4 and lac 5 decolorized industrial effluent by 57–59 %, and 46 % by lac 3 (Fig. 9). Thereafter, UV–VIS spectrum studies of the dye decolorization were performed and compared with the control group, after 10 h of incubation with laccase isozymes. The relative absorption of the laccase isozymes-treated dyes indicated that the major peak had disappeared in each case (Supplementary Fig. 2). These results showed that the dyes were enzymatically degraded.

4. Discussion

In this study, the potential of *T. ljubarskyi* for high laccase production was identified; either by varying the fermentation conditions (both physical and chemical) or by co-culturing with *R. mucilaginosa*. We know that fungal morphology plays an important role in the secretion of extracellular proteins. Therefore, agitation is considered to be an important parameter for fungal pellet morphology. Earlier, many researchers have reported significant effect of agitation on pellet formation, depending upon the cell type and microorganism cultivated (Jiménez-Tobon et al., 1997; Johannes et al., 1996; Kim and Song, 2009). Apart from fungal morphology, pH and temperature of the fermentation condition are a few crucial parameters, which regulate the secretion of extracellular laccase (Kumar et al., 2015). Similar to our results, other white-rot fungi, such as *Pleurotus ostreatus* and *Trametes versicolor* have been reported with higher laccase activity at low-temperature conditions (Snajdr and Baldrian, 2007). Furthermore, the secretion of laccase isozymes has been reported to be varied at different temperature by different basidiomycetous fungi, like, *Coriolus versicolor*, *Phlebia brevispora*, *Ganoderma applanatum*, *Pycnoporus sanguineus*, and *G. lucidum* (Fonseca et al., 2013; Kumar et al., 2017). It is well known that the fungal laccases are acidic with copper as a central metal atom (Sharma et al., 2013; Thurston, 1994). In general, the production of laccases by basidiomycete fungi is favored at acidic pH (Kumar et al., 2015, 2017; Sharma et al., 2013). However, the secretion of isozymes is under the influence of broad pH range, which indicates the specific function of each isozyme during the continuous metabolic processes.

Palmieri et al. (2000) identified that the laccase production is under the influence of copper ions. In due course of time, the research on copper-mediated laccase gene regulation in white-rot fungi increased significantly (Jain et al., 2019; Kumar et al., 2015; Piscitelli et al., 2011).

Interestingly, laccase isozymes secretion pattern can also be used for the evolutionary studies of different fungal species and to solve phylogenetic or taxonomic conflicts. In *C. neoformans* and *Yersinia enterocolitica*, the laccases gene sequences have already been used as a taxonomic marker (Singh et al., 2014a; Zhu and Williamson, 2004). Further, the ability of white-rot fungi in the biodegradation of many xenobiotic compounds led the quest for the identification of potential oxido-reductases. The role of laccases (an oxido-reductase) was found to be crucial for the biodegradation of many xenobiotics, which encouraged researchers to identify the specific laccase isozymes, with high catalytic activity and stability.

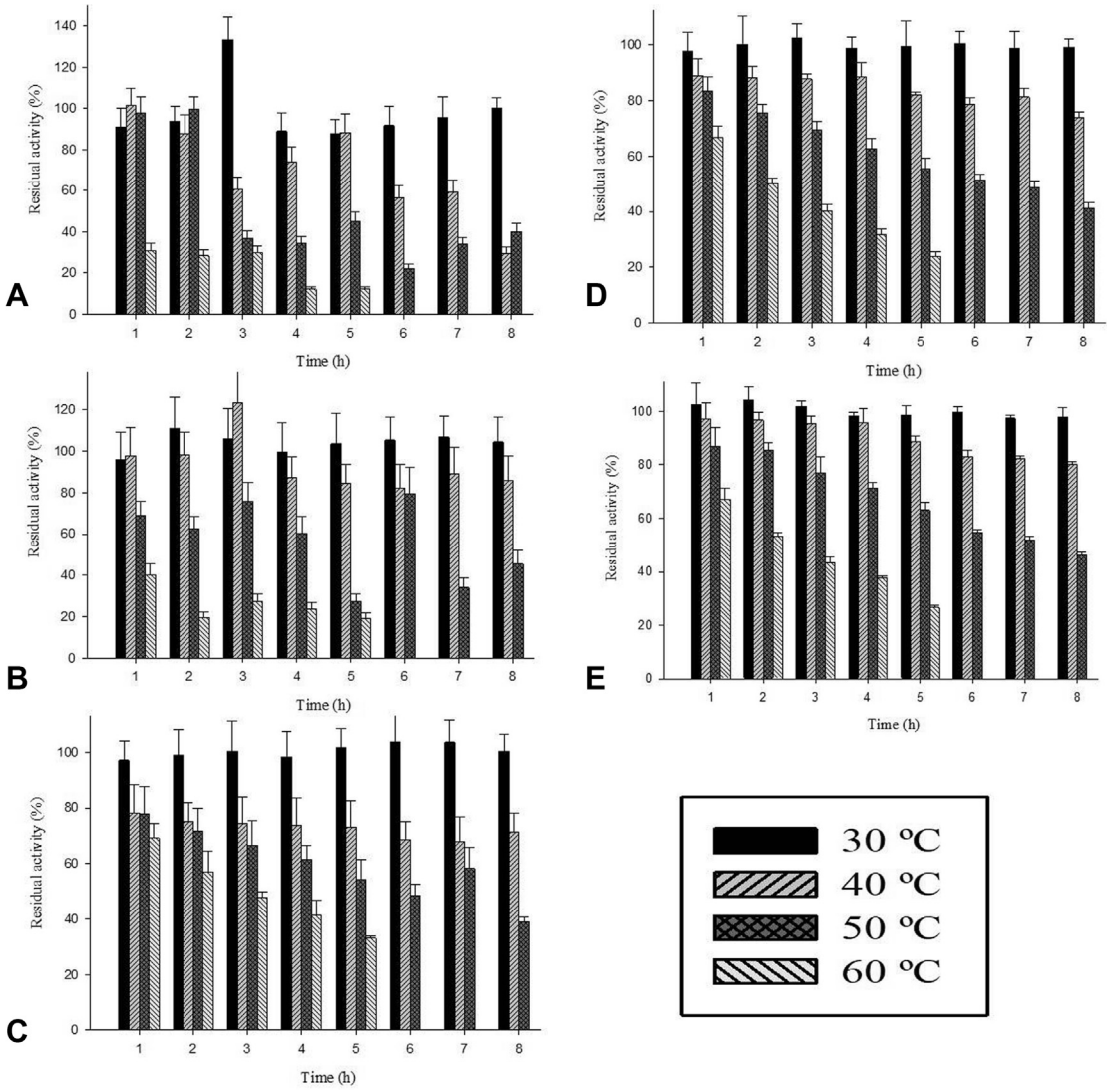

Fig. 8. Thermostability of laccase isozymes from *T. ljubarskyi* MDU-01 was determined. (A) lac 1; (B) lac 2; (C) lac 3; (D) lac 4; (E) lac 5.

Table 1
Michaelis–Menten kinetic constant of laccase isozymes from *T. ljubarskyi* MDU-01.

Substrate		lac 1	lac 2	lac 3	lac 4	lac 5
o-tolidine	K_m (μM)	24.5	313	143	275	855
	R^2	0.91	0.99	0.94	0.91	0.95
Guaiacol	K_m (μM)	480	210	360	440	170
	R^2	0.96	0.93	0.97	0.95	0.93

Previous studies have also shown that 2,5-xylidine stimulates laccase production in different white-rot fungi, for example, *Fomes annosus*, *Pholiota mutabilis*, *G. lucidum*, *P. ostreatus*, *T. versicolor*, and *Trametes modesta* (Kumar et al., 2017; Nyanhongo et al., 2002; Piscitelli et al., 2011). Caffeic acid (3,4-dihydroxy-cinnamic acid), a polyphenol has been reported to enhance laccase production from *T. modesta* (Nyanhongo et al., 2002). Other studies have also shown the secretion of laccase isozymes in the presence of other phenolic compounds, namely, vanillin, ferulic acid, and 2,5-xylidine (Giatti Marques De Souza et al., 2004; Yaver et al., 1996).

The laccase production increased by implying the methods mentioned in the manuscript; however, the yield, media toxicity, and time duration have also been addressed. Earlier, co-culturing of two different species to enhance the laccase production has been reported, that is, *Phlebia radiata* with *Dichomitus squalens* (Dong et al., 2012), *G. lucidum* with *Candida* sp. (Li et al., 2011), and *Pleurotus ferulae* JM301 with *R. mucilaginosa* (Wang et al., 2015). Besides, efforts have been made to understand the mechanism of high laccase production in co-culturing, but till date, no conclusive evidence has been found. In this study, an insight into the total proteome of a co-cultured *T. ljubarskyi* and *R. mucilaginosa* provides the significant number of identified proteins for mycelial fungus (522) and yeast (325). Previously, secretome studies from *G. lucidum* showed a lesser number (40) of identified proteins (Manavalan et al., 2012). The recent secretome studies from *G. lucidum* also revealed a moderate amount (271) of cellular proteins (Jain et al., 2019). However, proteome identification with *in-silico*-translated data generated from whole genome or transcriptome have predicted a higher number of proteins (Gao et al., 2017; Jain et al., 2019).

Interestingly, the proteome analysis reveals essential section of information for the mutual interaction between *T. ljubarskyi* and *R. mucilaginosa* (Supplementary 1 and Supplementary 2). It is evident from the previous reports that chitinase is present in the periplasmic membrane of yeast, and it plays a vital role during cell

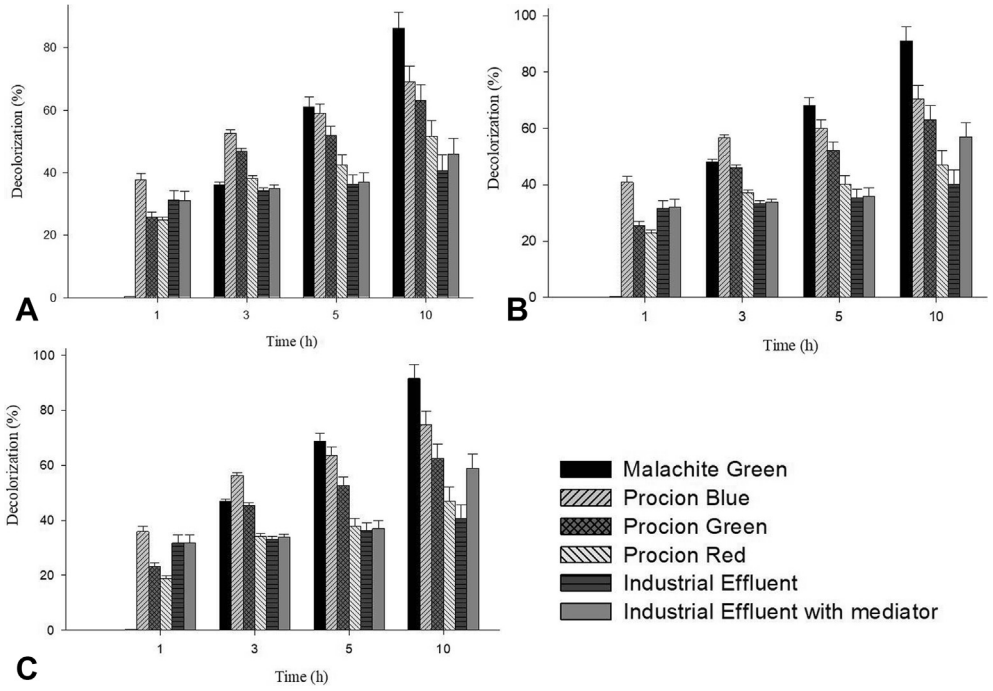

Fig. 9. Decolorization of dyes with laccase isozymes from *T. ljubarskyi* MDU-01. (A) lac 3; (B) lac 4; (C) lac 5.

separation (Santos and Snyder, 1997). In this study, chitinase was found to be secreted in the culture medium during yeast cell division (Fig. 3A). Our studies revealed the presence of chitin synthase in yeast, which plays an essential role in cell wall repair during cell division (Supplementary 2). However, in the *T. ljubarskyi*, the low-chitin synthase activity facilitates the cell wall damage, which eventually helps in the secretion of high amount of laccase isozymes in the culture broth (Fig. 3). The proteome study suggests that in *R. mucilaginosa*, cell wall attachment protein IFF4, high-affinity glucose transporter, glucose starvation modulation protein 1 (GSMP1) transcription factor, and chitin synthase are major regulatory proteins for fungal-yeast interaction (Supplementary 2). In *Candida albicans*, IFF4 protein has been reported to be responsible for cell adherence (Kempf et al., 2007), which might also be responsible for mycelial fungus–yeast surface attachment during co-cultivation. Further, glucose starvation leads to the activation of the transcription factors, which are further associated with the activation of multiple stress-related proteins (Zhang and Cao, 2017).

In *T. ljubarskyi*, mechanosensitive ion channel, sterigmatocystin biosynthesis regulatory protein (SRP), GPI-anchored wall transfer protein 1 (GWT1), and numerous antioxidant enzymes are key regulatory proteins responsible for fungal-yeast interaction (Supplementary 1). The mechanosensitive ion channel plays a vital role by sensing the physical environment of micro-organism and helps to maintain turgor pressure to avoid cell lysis (Kumamoto, 2008). Whereas, GWT1 has a role in the reconstruction of the cell wall (Bowman et al., 2006), which was found to be damaged by the action of chitinase during co-cultivation (Figs. 4 and 5). Furthermore, *T. ljubarskyi* also secretes molecules for self-defense; therefore, mycotoxin and other secondary metabolite production are regulated by SRP in response to cellular stress (Yu et al., 1996). In our study, protein chondroitin lyase was found to be present in the proteome of mycelial fungus (Supplementary 1). Chondroitin lyase, cleaves (1–4)-β-galactosaminic bonds between N-acetylgalactosamine and D-glucuronic acid/L-iduronic acid, which shows its potential to degrade the peptidoglycans (Lombard et al., 2014). Similarly, another recent study has also reported chondroitin lyase from *T. versicolor* during the algicidal process (Gao et al., 2017). A recent transcriptome and proteome study by our research group has suggested that under copper stress conditions, *G. lucidum* produces several enzymes from CAZy family and FOLzyme (e.g., glycosyl hydrolases, laccases, chitinase, and antioxidant enzymes), which help to combat damaging ROS (Jain et al., 2019). Similarly, our current proteome studies revealed multiple enzymes against ROS that were produced during oxidative stress in response to fungal-yeast interaction. Therefore, it can be hypothesized that few key metabolite secretions under co-cultivation condition might have played an important role during *T. ljubarskyi–R. mucilaginosa* interaction (Fig. 10).

The current proteome study has also revealed a surface protein IFF4 that is responsible for cellular attachments (Fig. 10, Supplementary 2). Furthermore, the proteins related to stimulus-response and membrane-related proteins play a significant role in inter-specific interaction during co-cultivation (Fig. 4, Supplementary 1 and 2). Interestingly, chitinase activity was also detected in co-culture conditions (Fig. 3A) and may have resulted in deconstructing the cell wall of *T. ljubarskyi*, which eventually increased the secretion of laccase isozymes. A recent study has suggested that the removal of metabolites from the donor's cytoplasm to the recipient may increase the production levels of the cellular metabolites (Shitut et al., 2017). The auxotrophic bacterial cells establish intercellular nanotubes to derive the amino acids they require for growth from other cells (Shitut et al., 2017). Interactions of laccase producing fungi with co-cultured microorganisms differ widely in accordance with the type of microorganisms and culture conditions (Savoie et al., 1998; Wang et al., 2015). Recently, β-carotene was reported to be responsible for the higher laccase production from *P. ferulae* during co-culture conditions (Guo et al., 2017). However, the actual mechanism of enhancement of laccase in co-culture strategy is not clear, but some studies suggest that the cell–cell communications (Wang et al., 2015) and other carbon sources secreted in the presence of co-

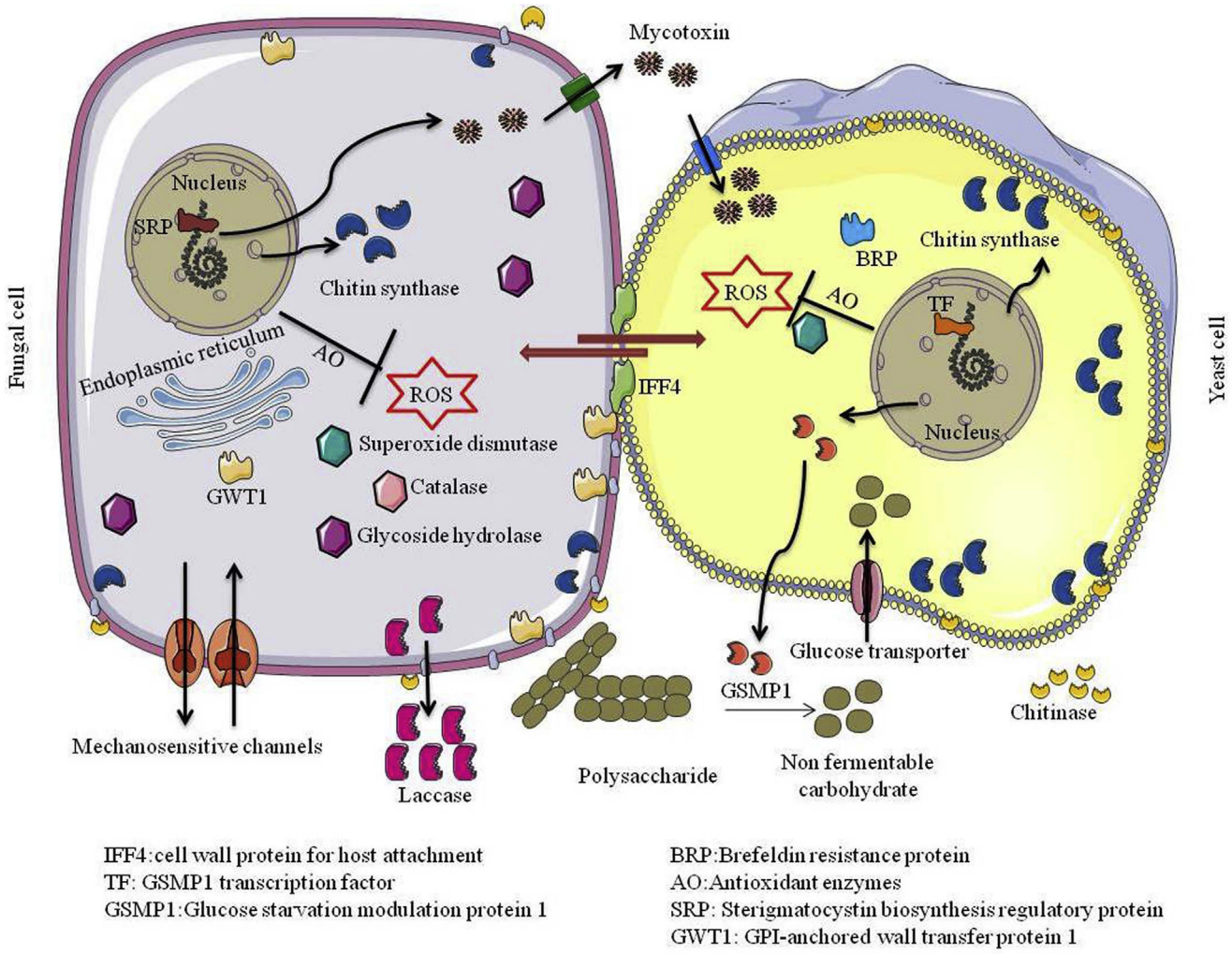

Fig. 10. Model represents the metabolic coupling between *T. ljubarskyi* MDU-01 and *R. mucilaginosa* MDU-02.

culture conditions are responsible for enhanced laccase production (Li et al., 2011). Here, we propose that the probable mechanism of high laccase production may be due to the metabolic coupling during co-cultivation between *R. mucilaginosa* and *T. ljubarskyi*, which may lead to alteration of *T. ljubarskyi* morphology and eventually higher protein secretion (Fig. 10). Inter-specific interactions lead to selective advantages resulting from a mutual division of labor, which enhances the emergence of these associations (Mariscal and Flores, 2010). By interacting with individuals that feature novel traits, microorganisms could significantly extend their metabolic repertoire (Harcombe, 2010; Mariscal and Flores, 2010; Phelan et al., 2011; Shitut et al., 2017). In this way, through metabolic coupling benefits, ecological and evolutionary opportunities became available to the newly emerged pathways consortium that otherwise would be inaccessible to individual fungal species.

Moreover, the laccase isozymes produced from the co-culturing suite of *T. ljubarskyi* and *R. mucilaginosa* were purified, biochemically characterized, and assessed for their industrial applicability of bioremediation. The laccase isozymes showed a wide range of pH and temperature stability. The results are in agreement with previous reports, where laccase activity declined after pH 5.0, and the optimum temperature for laccase activity varied according to their source (fungal species) of production, that is, 35 °C (Abd El Monssef et al., 2016), 40–55 °C (Kumar et al., 2015, 2017; Sharma et al., 2013). In recent reports, laccase isozymes PsLacI and PsLacII from *P. sanguineus* showed 60 °C and 50 °C thermal stability (Orlikowska et al., 2018). Therefore, the higher temperature stability of laccase isozymes qualifies them to be used in many novel biotechnological applications. Moreover, the kinetic properties of isozymes can be used in specific catalytic biotransformation in several biotechnological applications. Recently, our group has reported different biochemical properties (enzyme kinetics) of Glac (H1), and Glac (L1-L5) from *G. lucidum* MDU-7 (Kumar et al., 2015, 2017). Thus, these findings suggest that the evolution of diverse laccase isozymes from different white-rot fungi may be due to complex ecological niche and variable environmental conditions.

Finally, based on biochemical and kinetic properties of laccase isozyme produced from *T. ljubarskyi*, their potential for dye degradation was evaluated. Earlier studies on dye decolorization with laccase isozymes purified from *Ganoderma* sp. En3 (He et al., 2015), *Coprinus comatus* (Jiang et al., 2013), *Cerrena unicolor* (Michniewicz et al., 2008), and *T. versicolor* (Moldes and Sanromán, 2006) have shown higher specificities of one isozyme over others. Similarly, in our studies, we have found diverse specificities of isozymes according to the type and nature of dyes. Furthermore, lac 4 and lac 5 showed higher decolorization of industrial effluent in the presence of redox mediator. In this proteome study, DyP

peroxidase was also observed in addition to laccases (Supplementary 1). DyP peroxidase was found to be present in other white-rot fungi and has excellent potential in dye-decolorization (Jain et al., 2019; Sugano et al., 2007). Therefore, in future, its role in dye-decolorization in combination with laccases can be exploited to achieve better results.

5. Conclusion

The pre-wiring for necessary signals exists in the genome of the microorganisms, which facilitates their survival either by conflict, trade-off, or mutual support, during environmental challenges. This study demonstrates that co-cultivation supports synergistic growth and development, under oxidative stress with the production of poly-phenol multi-copper oxidases (e.g., laccases, DyP peroxidases, and cytochrome c peroxidase). The proteome studies further suggest that inter-specific interactions alter the functioning of both mycelial fungus and yeast, which helps them in the re-allocation of nutrients and space. The arsenal of oxidoreductases, antioxidants, membrane-related proteins, and transporter proteins helps in the co-survival of both mycelial fungus and yeast. Further, their SEM study confirms cellular attachments and inter-specific interactions. Therefore, the co-culture condition may be helpful in the survival of both the organisms under hazardous or unfavorable condition, by the secretion of several xenobiotic degrading enzymes, like isoforms of laccases and DyP peroxidases. Finally, these microorganisms and their enzymes can also be exploited to meet the industrial need of bioremediation.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.09.013.

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