Studies on a better laccase-producing mutant of *Fusarium incarnatum* LD-3 under solid substrate tray fermentation

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Received: 4 July 2018 / Accepted: 1 February 2019 / Published online: 21 February 2019
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Abstract

Forty-seven (47) mutant strains were generated from the wild-type fungus, *Fusarium incarnatum* strain LD-3 after exposure to ultraviolet radiation (UV) and a further seventeen (17) mutants were generated after exposure to ethyl methane sulfonate (EMS). Amongst these, the mutant strain, identified as UC-14, was the most promising laccase producer and produced threefold more laccase than the wild strain LD-3. Solid substrate tray fermentation using wheat straw and rice bran showed a twofold increase in laccase productivity and a fivefold loss of total organic matter (TOM) by mutant UC-14 over the wild strain LD-3. The mutant strain UC-14 also showed 25% and 54% weight loss of TOM after 36 days of fermentation which was 10% higher than the wild-type LD-3. Scanning electron microscopy suggested that the delayed condidiation in mutant strain UC-14 may be responsible for better laccase production.

Keywords Litter-dwelling fungi · Laccase · Mutagenesis · Solid substrate fermentation · Total organic matter (TOM)

Introduction

Phylogenetically old enzyme laccase (benzenediol:oxygen: oxidoreductases, (EC. 1.10.3.2) is a major secretome of many of the white rot fungi and capable of oxidizing diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, benzenethiols, polyphenols and even some inorganic compounds (Bakkiyaraj et al. 2013). Laccase has wide applications in various fields including paper manufacturing (Flory et al. 2013), wood processing (Fackler et al. 2008), environmental bioremediation (Asharfi et al. 2013), food industry (Dhillon et al. 2012), as well as in textile engineering (Basto et al. 2007) and removal of crude oil through petroleum degradation (Azin et al. 2018). Due to increase in the demand for specific industrial applications, its production has been customized. The high-quality laccase production process has been improved over the last few decades but due to the lack of long-term efficient strategy for production,

industrial applications of laccase were hampered (Quingqing et al. 2014).

A host organism only produced a relatively low quantity of the enzyme and improvements using inducers which are toxic and expensive is not a satisfactory alternative. Improvement of production of ligninolytic enzymes from fungi by medium optimization is a useful tool to search for alternative nutrient sources. However, there are several rate limiting steps in the biosynthetic pathways which cannot be overcome by simply using medium optimization. Hence, emphasis is being continuously placed on other production strategies like cloning and heterologous expression of laccase genes (Gianfreda et al. 1999).

Ligninolytic enzyme production has been increased in basidiomycetes by the genetic crossing of monokaryotic strains derived from spores, followed by screening of the resultant dikaryotic strains for improved enzyme production. Eichlerová and Homolka (1999) achieved tenfold increase in the laccase production from *Pleurotus ostreatus* using this methodology.

Improvement in the laccase production can also be achieved by mutating wild-type fungal strains followed by selection of the strain with improved character (Ramzan et al. 2013). Application of UV radiation as a physical mutagen has been reported as one of the best methods of strain improvement for better productivity in mycelia cell

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and sporophore production in *P. florida*, *P. pulmonarius* (Adegoke et al. 2012) and *P. cinnabarinus* (Khanam and Gyanprasuna 2014). Studies on laccase production have been extensively investigated in model white rot fungi like *Trametes versicolor*, *Pleurotus ostreatus* and a few others. However, there are very few reports regarding the role of litter-dwelling fungi (LDF), inhabiting the natural environment of soil and decaying litter (Chhaya and Gupte 2013).

Enzyme production has been successfully carried out by submerged fermentation (SmF) techniques worldwide. Solid-state fermentation (SSF) has marked advantages over SmF in terms of productivity, concentration of the product and effluent generation (Mathur et al. 2013). Despite having difficulty in agitation of substrate bed, fermentation control and mass transfer, etc., this technique has successfully proved its potential for the production of a large number of enzymes (Singhania et al. 2010).

The present work attempts to come up with a better laccase-producing strain of litter-dwelling fungus *Fusarium incarnatum* LD-3 by random mutagenesis using ultraviolet radiation (UV radiation) and ethyl methanesulphonate (EMS) followed by rationale selection of the better producing strain. The performance of the better laccase-producing mutant was evaluated under solid substrate tray fermentation using wheat straw and rice bran as lignocellulosic substrates.

Materials and methods

Chemicals

2,2-Azino-bis (3ethylbenzthiozoline-6-sulphonic acid) (ABTS) was purchased from Sigma (St. Louis M.O., U.S.A.). Sabaroud dextrose agar and malt extract agar were procured from Hi-Media Labs (Mumbai, India). Ortho-dianisidine and ethyl methanesulphonate (EMS) were procured from Central Drug House (P) Ltd. (CDH fine chemicals, Mumbai, India). All other chemicals used were of analytical grade and procured from Qualigens, (Mumbai, India). Wheat straw and rice bran were collected locally and used as lignocellulosic substrates.

Microorganisms used

Wild-type fungal strain *Fusarium incarnatum* LD-3 (GenBank Accession No. EU 426,883) was used in the present study (Chhaya and Gupte 2013). The fungal isolate was maintained on malt extract agar at 28 ± 2 °C and stored at 4 °C. The cultures were transferred once in a month.

Mutagenesis of *Fusarium incarnatum* LD-3 by UV radiation

The suspension of *Fusarium incarnatum* LD-3 was prepared by transferring four agar plugs (diameter, 8 mm), from an actively growing fungus on malt extract agar in the solution of sterilized Tween 80 (1:1000 diluted)-containing glass beads. The suspension was vortexed thoroughly and diluted serially in the sterile distilled water and Sabouraud Dextrose Agar plate supplemented with 0.01% (w/v) of ortho-dianisidine, 200 ppm of anthracene and 300 ppm of naphthalene were plated with 0.1 ml culture suspension and exposed for the UV mutagenesis.

The mutants were obtained from *Fusarium incarnatum* LD-3 by treating these plates with UV rays (Mulligan et al. 1991). During UV mutagenesis, *Fusarium incarnatum* LD-3 was exposed to short wavelength UV rays (210 nm) from a distance of 30 cm (Philips 30 W germicidal UV lamp) for various time intervals (0,3,5,7,10,15 and 20 min) under dark conditions and incubated at 28 ± 2 °C for 48 h. Mutants were isolated from 0.1% survivors at minimum lethal dose (MLD).

Mutagenesis of UV mutant UC-36 by EMS

The suspension of UV mutant *Fusarium incarnatum* UC-36 was prepared as mentioned in the previous section. After washing the suspension twice with sterile 0.1 M potassium phosphate buffer (pH 7.0), it was resuspended in an equal volume of the same buffer. After spinning for the third time, the cells were resuspended in half the original volume of the buffer. An aliquot of 1.0 ml of the washed cells were withdrawn in 1.5 ml micro-centrifuge tubes and kept on ice and treated with 0.015 ml of EMS corresponding to the final concentration of 50 µM and were placed in a shaker water bath at 37 °C for 5, 15, 30, 45 and 60 min. After incubation, the cells were washed in 0.1 M phosphate buffer of pH 7.0 and resuspended in 1 ml of the same buffer. The survivors were cultured and evaluated as mentioned earlier in this section.

The screening of the mutants was performed by observing the intensity of brown coloration around the colonies after 24, 48 and 72 h of incubation. The colonies with higher colour intensity were subcultured on malt extract agar plates and evaluated in the previously optimized wheat straw-based medium for the laccase production at flask level under solid substrate fermentation.

Stability analysis and production profile of selected mutants

The mutants which were shortlisted in the above two experiments were subcultured up to five generations on malt extract agar medium and evaluated for the laccase production from

each generation. The final selection of the mutant was based upon the value of coefficient of variance. The comparison of production profile of control isolate LD-3 and mutant isolate UC-14 isolate was performed for 14 days in preoptimized medium using wheat straw and rice bran as substrates.

Bioreactor configuration and culture conditions

A tray bioreactor consisted of an enamel flat tray with an area of 20 cm X15.7 cm and a height of 7 cm, where a layer of solid substrate of about 1.0 cm of thickness (50 g of solid substrate in 200 ml of optimized production medium) was prepared. The bioreactor was sterilized by autoclaving at 121 °C for 20 min.

The wild-type isolate LD-3 and a mutant isolate UC-14 were grown at 28 ± 2 °C in malt extract broth inoculated with four fungal discs (8 mm in diameter each) from a 8 day old colony growth on malt extract agar medium, pH 5.0. The culture pellets were harvested and transferred into fresh 200 ml of production medium in 1000 ml Erlenmeyer flasks. Each sterilized tray was inoculated with $0.25 \text{ g} \pm 0.016$ of fungal dry mass that corresponded to 0.5% (w/w) on dry weight basis per gram of dry substrate. The tray bioreactors were then incubated in humidity controller incubator (Electroquip) at 28 ± 2 °C with 60% relative humidity for 40 days. The samples (mycosubstrates) were harvested periodically, washed thoroughly and analysed for laccase production, fungal biomass and percentage weight loss.

Analytical determinations

Determination of laccase production

Laccase activity during the course of tray fermentation was measured spectrophotometrically using 2,2'-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS) as a substrate using 20 mM Na-acetate buffer pH-5.0. One activity unit (U) was defined as the amount of enzyme that oxidised 1 µmol of substrate per minute. The laccase production was expressed in terms of unit activity (units per ml), volumetric yield (units) and specific productivity (units per gram of dry substrate).

Biomass estimation

The estimation of fungal biomass during the tray fermentation was estimated as per Rodríguez Couto et al. (2009). It was necessary to determine the amount of substrate degraded by the action of the fungal enzymes as mycelium was bound to the solid substrate. Determination of this variable was conducted by labelling 25% of the solid substrates (SS) applying small holes in different locations to monitor the cultivation process. Solid substrates were dried and

weighed to determine the original dry weight (W_0) at different time intervals. The complex substrate mycelium was collected and dried until constant weight (W_c) was obtained after cultivation. Labelled solid substrates without fungus were then separated and weighed for calculating the amount of degraded substrate. The amount of degraded substrate (Ds) that corresponded to the mean difference between original and final dry weight of labelled SS without fungus was determined from:

$$Ds = \frac{1}{N} \sum_{n=1}^N (W_0 - W_f)n$$

where W_0 and W_f are the original and the final dry weight of each labelled SS (n), respectively, in grams N is the total labelled SS used in the test. The ratio between DS and the original dry weight of the labelled SS without fungus corresponded to the mean amount of degraded SS (RDS) as indicated in the following equation:

$$RDS = \frac{Ds}{\sum_{n=1}^N (W_0)n}$$

On the other hand, fungal biomass (FB) was calculated as the difference between W_c , W_0 plus RDS multiplied by W_0 as given in,

$$FB = W_c - W_0 \cdot (1 - RDS).$$

Fungal biomass production correlated very well with the dry weight loss of this system, therefore, higher losses of dry weight (%) of the solid substrate were assumed as higher biomass production.

Determination of percent weight loss

The percent weight loss as a total organic matter (TOM) in fungal-treated substrates was calculated as per the following equation,

$$\text{Loss (\%)} = \frac{M_i - M_f}{M_i} \times 100$$

where M_i is the amount of component in the control (non-fermented) substrate and M_f is the amount of the component left in the mycosubstrates.

Scanning electron microscopy (SEM)

A small quantity of solid substrate fermented for 8 days with *Fusarium incarnatum* LD-3 and UC-14 was air dried. A very thin particle from the fermented substrate was selected for observation under scanning electron microscopy (SEM with emergency dispersive analysis of X-rays) (Philips, Netherlands, XL 30 ESEM with EDAX). Unfermented solid

substrate was also observed under SEM and treated as the control.

Statistical analysis

Experiments performed in this study were in triplicates and the presented data is the mean value of the triplicates. The standard deviation remained within the range of $\pm 10\%$.

Results and discussion

UV mutagenesis and selection of better laccase-producing mutant

UV mutagenesis of *Fusarium incarnatum* LD-3 was carried out at different time intervals (0, 3, 5, 7, 10, 15 and 20 min). The survivors ranged between 7% and 0.004%. UV mutagenesis was further performed at 0.1% survivors, i.e., for 10 min to obtain laccase overproducing mutants. A total of 47 mutants were selected depending upon the brown colouration surrounding the colonies (Bavendam's reaction) and the time period. Out of 47 UV mutants, 27 mutants (UC-1 to UC-27) were selected randomly, while 12 mutants (UC-28 to UC-39) were selected from 200 ppm anthracene containing SDA plates and 8 mutants (UC-40 to UC-47) were selected from 300 ppm naphthalene containing SDA plates and analysed quantitatively for the laccase production at flask level under solid substrate fermentation using wheat straw as a substrate and the medium optimized earlier (Chhaya and Gupte 2010). Two mutants UC-11 and UC-14 showed two- and threefold increase in the laccase production as compared to control isolate LD-3, with 1453 ± 165 U/g and 1688 ± 109 U/g of laccase production, respectively, out of the 27 mutants tested.

After screening 12 mutants resistant to 200 ppm anthracene, UC-36 and UC-38 showed a threefold increase in the laccase production as compared to control isolate LD-3, with 1925 ± 343 U/g and 1899 ± 446 U/g of laccase production, respectively. After screening, eight mutants were resistant to 300 ppm naphthalene, but none of the mutants was found to be a better laccase producer than control isolate LD-3 (Table 1).

EMS mutagenesis and selection of better laccase-producing mutant

The survivors of 200 ppm anthracene-resistant UV mutant *Fusarium incarnatum* UC-36 after EMS treatment ranged between 11 and 0.001%.

Selection of EMS mutants was carried out at 0.1% survival at treatment time of 30 min which resulted in emergence of 16 mutants picked up from SDA plates incorporated

with 0.01% of ortho-dianisidine depending upon the intensity of brown colour and time period. All the mutants were tested quantitatively for the laccase production and out of which EMS mutants UC-59 and UC-60 exhibited 29% and 40% increase in the laccase production as compared to control isolate UC-36, with 1835 ± 75 U/g and 1981 ± 346 U/g of laccase production, respectively (Table 2).

Stability analysis and production profile of selected mutants

Shortlisted mutants were analysed for stable and consistent increase in the laccase production by successive subcultures on malt extract agar plates for five consecutive times. After each subculture, the mutant was subjected to solid substrate fermentation at a flask level in an optimized medium supplemented with wheat straw and rice bran and was monitored for laccase production (Table 3). Out of the six different short-listed mutants (UC-11, UC-14, UC-36, UC-38, UC-59 and UC-60), UC-14 was found to show a consistently high laccase production of 1721 ± 139 U/g and 2639 ± 311 U/g on wheat straw and rice bran-based medium, respectively, with lowest coefficient of variance, i.e., 8 and 12 among all the mutant isolates tested and hence was selected for further experiments.

Dynamics of laccase production was compared for control isolate LD-3 and mutant isolate UC-14 under optimized culture conditions and medium with wheat straw and rice bran as substrates under solid substrate fermentation at flask level. Control isolate LD-3 and mutant isolate UC-14 exhibited maximum laccase production of 984 ± 97 U/g and 1805 ± 155 U/g on the 8th day of fermentation, respectively, and hence showed a twofold increase in laccase production in wheat straw-based medium. Similarly, in rice bran-based medium on the 8th day of fermentation, laccase production from control isolate LD-3 and mutant isolate UC-14 was found to be 1353 ± 143 U/g and 2415 ± 290 U/g, respectively, corresponding to the twofold increase in the laccase production (Fig. 1). However, on further incubation, reduction in laccase production was more prominent in control isolate LD-3 which was found to be 47% and 49% within 48 h for wheat straw and rice bran-based medium, respectively. In mutant isolate reduction was found to be 27% and 18%, respectively, within 48 h of incubation. Moreover, in rice bran-based medium, the laccase production in the mutant isolate UC-14 sustained up to 14 days. Hence, the mutant isolate UC-14 was not only the better laccase producer but also sustained it for a longer period of time and hence can be commercially more viable than control isolate LD-3.

Different approaches have been used to optimize the production of extracellular proteins over the last 10 years. Optimization of cultivation conditions and medium components

Table 1 Laccase production in the wild-type isolate LD-3 and mutant isolates after UV mutagenesis

Mutant code	Brown coloration on ortho-dianisidine containing SDA plates	Laccase production (U/g)
UC-1	+	501 ± 57
UC-2	—	ND
UC-3	+	691 ± 70
UC-4	+	600 ± 57
UC-5	++	769 ± 77
UC-6	—	ND
UC-7	+	665 ± 64
UC-8	—	ND
UC-9	+	656 ± 57
UC-10	—	ND
UC-11	+++	1453 ± 165
UC-12	++	778 ± 75
UC-13	+	646 ± 67
UC-14	+++	1688 ± 109
UC-15	++	1187 ± 189
UC-16	+	608 ± 61
UC-17	—	ND
UC-18	+	540 ± 51
UC-19	++	838 ± 84
UC-20	—	ND
UC-21	—	ND
UC-22	+	579 ± 59
UC-23	+	614 ± 61
UC-24	++	946 ± 113
UC-25	+	527 ± 42
UC-26	—	ND
UC-27	+	459 ± 47
UC-28	+	669 ± 73
UC-29	+	423 ± 45
UC-30	+	625 ± 59
UC-31	+	538 ± 50
UC-32	++	819 ± 89
UC-33	+	565 ± 53
UC-34	++	801 ± 90
UC-35	+	687 ± 74
UC-36	+++	1925 ± 343
UC-37	++	964 ± 101
UC-38	+++	1900 ± 446
UC-39	++	987 ± 99
UC-40	+	494 ± 56
UC-41	+	582 ± 61
UC-42	—	ND
UC-43	—	ND
UC-44	+	588 ± 60
UC-45	—	ND
UC-46	+	469 ± 44
UC-47	+	628 ± 67
LD-3	+	627 ± 60

Bold indicates hyper laccase production than wild type/control

Brown coloration after + – 48 h, ++ – 36 h, +++ – 24 h of incubation at 28 ± 2 °C on ortho–dianisidine containing SDA plates

Table 1 (continued)

ND not detected

Table 2 Laccase production in the parent isolate UC-36 and mutants after EMS mutagenesis

Mutant code	Brown coloration on ortho-dianisidine containing SDA plates	Laccase production (U/g)
UC-48	+	510 ± 55
UC-49	—	ND
UC-50	+	594 ± 72
UC-51	+	616 ± 67
UC-52	+	669 ± 79
UC-53	+	490 ± 50
UC-54	++	888 ± 99
UC-55	—	ND
UC-56	++	872 ± 98
UC-57	—	ND
UC-58	+	600 ± 62
UC-59	++	1835 ± 75
UC-60	+	1981 ± 346
UC-61	++	821 ± 90
UC-62	+	487 ± 59
UC-63	—	ND
UC-36	+++	1412 ± 260

Bold indicates hyper laccase production than wild type/control

Brown coloration after + – 48 h, ++ – 36 h, +++ – 24 h of incubation at 28 ± 2 °C on ortho–dianisidine containing SDA plates

ND not detected

cannot overcome certain rate limiting steps involved in the biosynthetic pathway of the aimed protein. Hence, many researchers have adopted a mutation-selection strategy to obtain better producing mutants. Weenink et al. (2005) performed UV mutagenesis of *Aspergillus niger* strain XWA5.2 for 1 min and obtained better secretion mutants of laccase detected by ABTS overlay assay method. A seven-fold increase in the laccase production from strain XWA5.2 No.64 was reported as compared to the parental strain XWA5.2 due to the alteration in a posttranscriptional step that results in a higher yield of the glucoamylase–laccase fusion protein. Dhawan et al. (2003) successfully demonstrated mutation-selection strategy and achieved a sixfold increase in the production of laccase from *Cyathus bulleri* after mutation with ethidium bromide, possibly due to the respiratory stress induced in the cells. When the mutants of *Mucor mucedo* MMM1 and MMM2 were treated with UV radiation and EMS, respectively, when subjected to protoplast fusion, the fusant MMFu3 showed 84% and 95% decolorisation of malachite green and crystal violet, respectively, after 10 days of incubation due to the twofold increase in the

Table 3 Stability analysis of different mutants

Mutant code	Average laccase production of five sets (U/g)		Standard deviation		Coefficient of variance	
	Wheat straw	Rice bran	Wheat straw	Rice bran	Wheat straw	Rice bran
UC-11	1522	3451	152	1066	10	31
UC-14	1721	2639	139	311	8	12
UC-36	1823	4551	523	1258	29	28
UC-38	1488	3075	414	1026	28	33
UC-59	1825	3922	565	699	31	18
UC-60	1751	3509	552	584	32	17
LD-3	605	1026	60	117	14	11

Fig. 1 Comparison of production profile of laccase from control LD-3 and mutant UC-14 under optimized culture conditions and medium using wheat straw (WS) and rice bran (RB) as solid substrates

laccase activity than parent strain (Moturi and Charya 2010). The higher laccase activity of 2.5 U/ml was observed from *P. pulmonarius* LAU 90 (mutant) as compared to wild-type *P. pulmonarius* LAU 09 (1.75 U/ml) when exposed to UV radiation at 210 nm for 90 min. Khanam and Prasuna (2014) reported a 15% increase in the laccase production from *P. cinnabarinus* after mutagenesis with UV radiation, X-ray, ethidium bromide and colchicine. Chemical mutagen ethyl methanesulfonate was used to obtain better Mn peroxidase-producing mutant of *T. versicolor* IBL-04 and EMS-90 mutant showed a 3.26-fold increase in the Mn peroxidase production (Ramzan et al. 2013).

Scale-up for laccase production from control isolate LD-3 and mutant isolate UC-14

Scale-up in tray bioreactor using lignocellulosic substrates like wheat straw and rice bran was conducted under previously optimized conditions of pH, temperature, particle size, moisture content and medium constituents at flask level. The study revealed that laccase production and percentage weight loss were found to be comparatively more at each

stage of fermentation in mutant isolate UC-14 than control isolate LD-3.

Tray fermentation in wheat straw-based medium

Tray fermentation was performed for 40 days and samples were withdrawn at an interval of 4 days to determine laccase production, biomass and % weight loss in terms of total organic matter (TOM) and microscopic observation as described in material and methods.

Good growth and laccase production was supported by wheat straw, after 2–3 days of incubation, fungus growth started colonizing the substrates and complete colonization was observed within 8 days of incubation.

Laccase productivity was found to be maximum in terms of volumetric yield (units), specific productivity (U/g of substrate), production rate (U/ml/day) and specific production rate (U/g/day) on the 8th day of fermentation for both control isolate LD-3 and mutant isolate UC-14 (Table 4). On the 12th day of fermentation biomass for control isolate LD-3 and mutant isolate UC-14 were found to be 23g% and 18g%, respectively.

However, in case of control isolate increment in the biomass was found to be 16% at every 96 h of incubation and reached up to 30 g% on 28th day of fermentation, while in case of mutant isolate UC-14 increment in the biomass was found to be at a lesser extent, i.e., 5% increase at every 96 h of incubation and reached up to 24 g% on the 36th day of incubation. Weight loss in terms of total organic matter (TOM) was found to be 21% in case of control isolate LD-3 and 25% in case of mutant isolate UC-14 on the 8th day of fermentation. However, percent weight loss was found to be consistent with both control and mutant isolate and on the 36th day of fermentation, it was found to be 27% for control LD-3 and 30% for mutant UC-14 (Fig. 2).

Tray fermentation in rice bran-based medium

Tray fermentation using rice bran was performed for 40 days and it supported better colonization of both control isolate

Table 4 Comparison of laccase production by mutant UC-14 on wheat straw-based tray fermentation at 8 days

Mutant code	Volumetric yield (units)	Specific productivity (U/g)	Production rate (U/ml/day)	Specific production rate (U/g/day)
Wheat straw-based tray fermentation				
UC-14	11,198	433	33	75
LD-3	6585	274	20	51
Rice bran-based tray fermentation				
UC-14	105,538	3673	739	208
LD-3	20,352	812	163	39

LD-3 and mutant isolate UC-14 after 4th day of fermentation. Laccase productivity in terms of volumetric yield (Units), specific productivity (U/g), production rate (U/ml/day) and specific production rate (U/g/day) was observed on 8th day of fermentation and was found to be better than wheat straw-based medium (Table 4).

The biomass and percent weight loss in terms of total organic matter (TOM) for control LD-3 and mutant isolate UC-14 was found to be 39 g % and 37 g % and 28% and 33% respectively. The maximum weight loss was observed at the end of fermentation cycle which corresponded to 43% for control LD-3 and 54% for mutant UC-14 (Fig. 3). This difference in the percent weight loss can be directly correlated with the higher and sustainable laccase productivity of mutant UC-14 as compared to control LD-3. However, increase in the weight loss at the later stages of fermentation when the laccase production was decreased also indicated that reduction in total organic matter could be due to the cumulative effect of both laccase production and biomass development. Hence, it is difficult to establish a correlation between the production profile of enzyme and percent weight loss. This might be attributed to the degradation of substrate not only depending upon the production of enzyme

but also a variety of other physiological factors (Li et al. 2008; Sharma and Arora 2010). Progressive degradation of wheat straw by *Pleurotus ostreatus* and *Tremetes versicolor* was observed and concluded that several enzyme complexes (glyoxal oxidase, cellobiose oxidase, cellobiose oxidase:quinine reductase) working in consortia might get activated at different stages of incubation and contribute to the weight loss (Shrivastava et al. 2011). Gupta et al. (2012) reported that loss of TOM with respect to lignocellulosic substrates *L. camara* and *P.juliflora* by *Pleurotus cinnabarinus* was found to be 18.87% and 15.40%, respectively, on the 15th day of tray fermentation which might be attributed to the CO₂ evolution due to the metabolic activity of the fungus.

Starch or lignocellulose-based agroindustrial substrates which can play a dual role, as a support as well as a substrate have been employed extensively in tray bioreactor-based SSF (Pandey 1992). In addition, the utilization of this kind of support helps in solving both the economic and the environmental problems caused by their disposal. Lignocellulosic residue wheat straw stimulated laccase production (3518 U/g) on 10th day of fermentation (Shrivastava et al. 2011). Lignin peroxidase production was favoured by the utilization of organic wastes rich in lignin (Rodriguez Couto et al. 2003), whereas organic wastes stimulated laccase production and the use of rice bran having a high starch content led to the production of laccase and amylases (Lorenzo et al. 2002). Furthermore, there are many other reports of using lignocellulosic substrate for SSF. Higher production of laccase from *Phlebia radiata* was observed under SSF using wheat straw as a substrate (Vares et al. 1995). Maximum laccase production was reported from *Lentinus edodes* from peanut meal-supplemented wheat straw amongst the five different organic supplements tested (Kapoor et al. 2009). Laccase production level increased up to 9100 mU/mg after 13 days of fermentation with *Fusarium proliferatum* when starch-free wheat bran was used as a substrate (Hernández

Fig. 2 Comparison of control LD-3 and mutant UC-14 under solid substrate tray fermentation using wheat straw as a solid substrate **Fig. 3** Comparison of control isolate LD-3 and mutant isolate UC-14 under solid substrate tray fermentation using rice bran as a solid substrate

Fernaund et al. 2006). Balaraju et al. (2010) employed rice bran for the cellulase and laccase production under SSF and obtained 367 U/ml/min of cellulase and 1.476 U/ml/min of laccase on 14th day of fermentation from *Oudemansiella radicata*. Boran and Yesilada (2011) used wheat bran moistened with olive oil mill waste water, alcohol factory waste water (Vinasse) for the laccase production from *F. trogii* and *T. versicolor*. Bakkiyaraj et al. (2013) obtained 9300 U/L laccase production from *T. hirsuta* using wheat bran as a lignocellulosic substrate. Laccase production of 10.73 U/g under solid substrate fermentation from *Trichoderma longibracheatum* using rice bran as a lignocellulosic substrate was achieved and it was found to be tenfold higher than submerge fermentation using synthetic medium (Mathur et al. 2013). Solid substrate fermentation also proved to be successful for other substrates. Akpınar and Urek (2017) obtained optimum laccase production of $43,761.33 \pm 845$ U/L using peach waste from *Pleurotus eryngii* under solid-state fermentation. The highest laccase production of 6708.3 ± 75 U/L was obtained using potato peel waste on 23rd day of fermentation from *Pleurotus ostreatus* as compared to other agroindustrial wastes tested earlier. (Ergun and Urek 2017). In mutant *Bacillus subtilis* MTCC 2414, 183.32 ± 0.29 U/g of laccase production was observed under solid-state fermentation using sesame oil cake (Venkatanagaraju et al. 2018).

The immersion tray bioreactor was employed operating with wood shavings as a lignocellulosic support. MnP and LiP activities of 803 and 509 U/L were obtained, respectively, by *Trametes versicolor* (Rodríguez Couto et al. 2003).

Laccase production was also observed to be stimulated again on the 24th and 28th day of fermentation in our study with wheat straw and rice bran-based tray fermentation, respectively. Mycelial autolysis and release of bound enzyme or nitrogen starvation might be responsible for the second peak observed during the production profile of enzyme (Arora et al. 2002; Dhawan et al. 2005; Gupte et al. 2007). Moreover, the fermentation operational problems were not detected during the course. However, experiments performed by other research groups showed that the tray configuration was the most suitable process to produce

ligninolytic enzymes under solid-state conditions (Osma 2009).

Morphological aspects during tray fermentation

Microscopic observations revealed from the tray fermentation that control LD-3 entered in the condidiation stage on the 8th day of fermentation process. The increase in the condidiation led to decrease in laccase productivity with both rice bran and wheat straw-based medium. On the other hand, condidiation in the mutant UC-14 was delayed and initiated on the 16th day of fermentation which correlated well with higher and sustainable laccase productivity (Fig. 4).

In the present study, laccase production was inversely proportional to the rate of condidiation in laccase-producing fungus. Moreover, due to the faster growth rate of control LD-3, oxygen depletion occurred rapidly than mutant UC-14 which might hamper laccase production and favour condidiation in control LD-3. Moturi and Charya (2010) reported that the mutants that were exposed to UV radiation for a extensive period of time such as 12 and 15 min showed reduced growth rate as well as reduction in extension and branching of hyphae and hence reduction in the laccase production, while EMS mutants inhibited the elongation of germ tube and hyphae and also spore germination and showed 20.88% of reduction in the laccase production relative to control. Better laccase-producing X-ray mutant of *P. cinnabarinus* KX-8 exhibited yellowish, cottony, thicker and less fuzzy appearance as compared to its wild-type counterpart which showed completely white, cottony and fuzzy appearance on potato dextrose agar (Khanam and Gyanaprasuna 2014).

Peberdy (1994) correlated enhanced secretory capacity of filamentous fungi with the increase in the number of hyphal tips. A three to fivefold increase of extracellular protein was observed in hyper-branching mutants with increased apical surface (Lee et al. 1998) and highly branched mutants of *Aspergillus oryzae* showed increased glucoamylase production in stirred batch cultures (Bocking et al. 1995). We have observed in the present study that the pellet morphology

Fig. 4 Scanning electron microscopy of **a** control isolate LD-3 and **b** mutant isolate UC-14 under 800X magnification

for control LD-3 and mutant UC-14 was different. The pellets of control LD-3 were pigmented and without spikes, while the pellets of mutants UC-14 were found to be non-pigmented with spikes. The penetration and better hold of the mutant UC-14 to the substrate may be attributed to the presence of spikes as compared to that of control LD-3. The pellet morphology and ligninolytic enzyme production have been investigated in wood-degrading fungus *Trichophyton rubrum* LSK-27 and difference in the pellet morphology was observed under oxygen-limiting conditions. It was observed that pellets generated in baffled flask were rigid and with spikes, while pellets generated in non-baffled flasks were loose, fluffy and without spikes (Bermek et al. 2004). Inoculation of fungal pellets is advantageous over filamentous inoculum to obtain decreased viscosity, desirable mixing, better mass and oxygen transfer into the biomass and lower energy consumption for aeration and agitation (Liao et al. 2007). Thus, the use of fungal pellet inoculum would increase utilization of lignocellulosic substrate and eventually improve laccase production. Recent approaches like microparticle-enhanced cultivation (MPEC) have increased laccase activity by 3.5-fold in *C. unicolor* and *P.sapidus* (Antecka et al. 2016).

Conclusion

Mutation and rational selection were found to be significant approaches for the isolation of a better laccase-producing mutant. In our investigation, random mutagenesis using UV radiation was found to be more successful than EMS, as it led to a higher number of better laccase-producing stable mutants including UC-14 of *Fusarium incarnatum* LD-3 which was found to be a better and sustainable producer of laccase. A two- and fivefold increase was exhibited in the laccase productivity with wheat straw and rice bran-based medium under solid substrate tray fermentation. However, rice bran was found to be a better substrate at flask as well as tray level solid substrate fermentation. Due to the morphological changes observed at inoculation level and production level, it is inferred that better laccase production in UC-14 might be because of delayed condensation process due to which laccase production can be extended further. When better laccase production was correlated with the weight loss of total organic matter (TOM) of lignocellulosic substrates, it was observed that laccase production and biomass development cumulatively contribute to the reduction in the TOM. This investigation paves the way to apply such better laccase-producing mutant more efficiently than the wild-type strain for various biotechnological applications.

Acknowledgements Financial assistance provided by University Grants Commission (UGC), New Delhi to carry out the aforementioned

research work is acknowledged by the authors. (Sanction no: F.47-052/06.)

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