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Research Article

Improved laccase production in *Pleurotus djamor* RP by atmospheric and room temperature plasma (ARTP) mutagenesis

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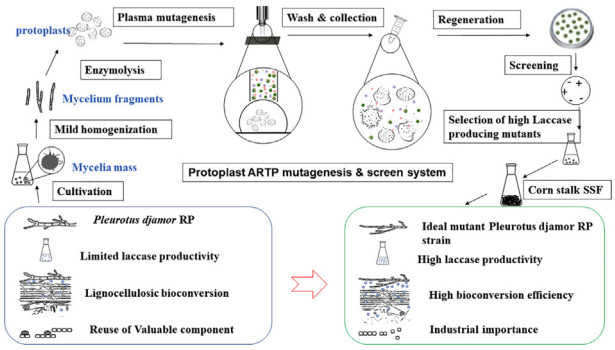
GRAPHICAL ABSTRACT



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ABSTRACT

Background: *Pleurotus djamor* is an edible mushroom with medicinal properties, and degrades lignocellulose by producing enzymes that have a variety of industrial applications. The aim of this study was to improve laccase producing potential of *P. djamor* that may easily convert lignocellulose waste into high-value products.

Results: In the present study, protoplast of the newly isolated fungus *P. djamor* RP was mutated by atmospheric pressure and room temperature plasma (ARTP) method. Guaiacol was used as an indicator for the rapid visual appearance of laccase positives in the screening process. From the results of preliminary screening, 10 promising positive mutants with a maximum laccase production were selected for further experimentation. Lac activity by the ABTS method showed that the mutant *P. djamor* strain 51-4 exhibited the maximum laccase production of 494.44 U/L in shake flask cultivation, which was 86.36% higher than wild strain. Notably, the other mutant *P. djamor* strain 62-27 exhibits a higher laccase production of 26.06 U/g which was 30.28% higher than wild strain when grown on solid-state fermentation. In addition to a higher yield of laccase, all mutants showed better growth and genetic stability than the wild strain.

Conclusions: *P. djamor* RP mutants with high laccase-producing potential were developed by ARTP Mutagenesis technique, and these isolates could be used as a possible candidate for lignocellulose conversion into high-value products.

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1. Introduction

Laccase enzymes (EC 1.10.3.2) are multicopper metallo-oxidases that oxidize a broad range of substrates that play a very important role in numerous areas of food biochemistry, pulp, and paper engineering, lignin degradation, bio bleaching, and bioremediation [1,2]. Valuable applications of Laccase inspired worldwide researchers to increase its production from various microbes [3]. Many well-known laccase producers belong to the basidiomycetes, among them, *Pleurotus* species, an edible white-rot fungi are highly involved in lignin metabolism [4]. Their capacity to utilize agricultural waste makes them beneficial, but their implementation in biotechnological processes have been limited due to high production costs caused by low enzyme activity. In recent years, researchers have shown increasing attention to laccase production tactics that boost activity while reducing production costs. Concurrently, mutagenesis strategies in appropriate host organisms would give a method for achieving high titers of laccase-producing strains [5].

The conventional mutation methods like ultraviolet radiation and alkylating agents do inevitable harm to organisms [6]. Recently, the developed atmospheric and room temperature plasma (ARTP) process provides a bright prospect for the genome mutation of the microorganisms with rapid mutation, high diversity of mutants, and simple and safe operations [7,8]. The mechanism underlying ARTP mutagenesis is thought to be connected to the effects of reactive oxygen species or reactive nitrogen species produced by the reaction of plasma with the aqueous environment of ARTP-treated cellular samples. Consequently, these reactive species damage cellular DNA and cause the activation of repair systems, such as the microbial SOS repair system, to be activated [9,10]. ARTP treatment caused structural alterations in the oligonucleotides, which could explain the wide and effective uses of ARTP-induced mutation breeding in many organisms [11]. There is still no clear understanding of how ARTP damages DNA. ARTP technology has already been applied in many microbial breeding studies like constructing a library of mutant strains resistant to low temperatures [12] to improve the fermentation efficiency of lycopene in *Blakeslea trispora* [13]. Conventional physical/chemical, multiple, and combinatorial mutagenesis has been reported to fungal laccase producing improvement [14,15]; however, plasma technology has yet to be widely adopted and applied to many microbial laccase enhancements.

P. djamor is a nutritionally rich edible mushroom with low cholesterol content, fat free, provide non-animal source vitamin-D, rich in protein, minerals, essential amino acids and other elements [16,17]. *P. djamor* has been found to have documented medicinal properties that include antineoplastic, antioxidant and anti-aging, hepatoprotective, antibacterial, and mosquito larvicidal activities [18,19]. Nowadays, as food-safety white-rot fungi, *P. djamor* was also employed in enzyme engineering and industrial biodegradation [20,22,21]. In our previous work, a laccase-producing strain *P. djamor* RP was isolated and evaluated for its selective delignification and bio-pretreatment properties [22]. In the present work, an attempt was done to improve laccase production in *P. djamor* RP by the atmospheric pressure and room temperature plasma (ARTP) technique. This is the first study to integrate the protoplast, plasma mutagenesis by ARTP technique as well as chemical screening for enhanced laccase productivity in the fungus *P. djamor*.

2. Materials and methods

2.1. Strains and culture media

2.1.1. Strains

Strain *Pleurotus djamor* RP was isolated from forest soil, identified and evaluated for laccase activity in our previous research work [22] and the same strain was also deposited in China General Microbiological Culture Collection Center, with an institutional number CGMCC No. 21074. The strains of *P. djamor* RP were maintained on agar slants of potato dextrose agar (PDA) supplemented with chloramphenicol (0.05 g/L) [23]. Protoplast regeneration media used were malt yeast extract glucose agar (MYGA) medium having composition malt extract (5 g/L), glucose (10 g/L), yeast extract (5 g/L), agar (18 g/L), pH 6.5, and malt yeast extract glucose sorbitol agar (MYGSA) medium having composition malt extract (5 g/L), glucose (10 g/L), yeast extract (5 g/L), sorbitol (osmotic stabilizer) (0.5 M), and agar (18 g/L), pH 6.5 [24]. Protoplast release buffer (PRB) for protoplast formation was 10 mM NaH_2PO_4-Na_2HPO_4 containing osmotic stabilizer 0.5 M D-Sorbitol with a final pH 6.5. For protoplast formation, PRB containing 5 mg/mL lysing enzymes from *Trichoderma harzianum* (Sigma L1412) and 0.3 μL/mL chitosanase from *Streptomyces sp.* (Sigma C0794) was used.

For screening Medium, tested fungi were initially cultivated on potato dextrose agar plates supplemented with 0.04% guaiacol as a visual indicator (G-PDA), at pH 6.0. For solid-state fermentation, corn stalks were collected from Zibo city, Shandong province. After air drying, the samples were crushed with pulverizer, then leached with a 20-mesh sieve to obtain straw powder substrates. 5 g of corn straw powder and 20 mL of medium containing KH_2PO_4 0.8 g/L, K_2HPO_4 0.2 g/L, $NaNO_3$ 3 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, yeast extract 2 g/L, were put into a 150-mL flask and autoclaved at 121°C for 30 min.

2.2. Preparation and formation of protoplast

Mycelium mat of *P. djamor* RP (5 mm in diameter) was cut from the margins of an actively growing culture (PDA agar plates) inoculated into PDB medium, that was incubated for 4 to 7 d at 28°C, with shaking at 150 rpm. The mycelia masses were collected by filtration, rinsed with sterile distilled water and protoplast release buffer (PRB). The 750 ± 25 mg of mycelia were transferred to a sterile homogenizing cup for mild homogenization and were centrifuged at 2000 rpm for 5 min, washed two times with PRB, then finally re-suspended in PRB containing lysing enzymes mixture, incubated at 28°C for 90–120 min with shaking 60 rpm to release protoplast.

2.3. Mutagenesis by ARTP

In the ARTP mutagenesis, argon (98% Ar + 2% O_2) was used as the plasma working gas. The atmospheric-pressure plasma jet (APPJ) design was based on atmospheric-pressure dielectric barrier discharge plasma source. The plasma generator used a 1.5 × 300 m m copper spring as the high-voltage electrode, and covered a 315 mm length quartz tube of copper mesh coat as the grounding electrode. The discharge length is 110 mm. The power was

a sinusoidal output with a peak voltage (Vp) of 2–50 kV and an alternating current frequency of 10.0 kHz. Oscilloscope was used to record discharge voltages and currents with a high-voltage probe and a current probe. The discharge voltage was 2.8 kV in APPJ treatment, the gas flow was 5 SLM (standard liters per minute); the distance between the plasma torch nozzle exit and the sample plate was 2 cm, the temperature of the sample surface was below 45°C. 100 μL of fresh protoplasmic suspension (1×10^6 protoplasts/mL) was at 4°C pre-cooling, transferred on a dry sterilized glass slide, and exposed sample under the argon plasma jet center for a treat. In plasma treatment, the sample stage was slightly moved in minor circles to change the APPJ center exposed to avoid temperature uniformity of sample center and edge. After treatment of the protoplasmic samples, each treated sample was soft eluted and pipetted with a 4°C pre-cooling sterilized osmotic stabilizer, short standing in 4°C dark storage, the eluent was properly diluted and spread on MYGAS medium.

The lethality rate of *P. djamor* RP protoplasts using ARTP mutagenesis was studied for ascertaining an optimum mutation time. To determine the optimal treatment period, 100 μL of fresh protoplasmic suspension (1×10^6 protoplasts/mL) was transferred on a sterilized glass slide and exposed to the argon plasma jet for treat time 15, 30, 45, 60, 90, 120, and 180 s, respectively. After treatment of the protoplasmic samples, each treated sample was gently spread on MYGSA medium. The regeneration plates were incubated at 28°C under dark conditions, the individual colonies on the control medium and each mutated medium were counted. Basidiospore (without protoplast formation) of *P. djamor* RP was also treated by the same method for comparative analysis with protoplasts for the lethal time. The lethality rate was evaluated as follows:

$$\text{Lethality rate (\%)} = (\text{control colonies - survival colonies}) / \text{control colonies} \times 100\ \%$$

where the number of control colonies is the total colony count of the sample without treatment, and the number of survival colonies is the total colony count after treatment by the ARTP treatment. All the colony numbers were obtained by the CFU (colony-forming units) method on PDA agar plates [25].

2.4. Regeneration of protoplast

The obtained protoplast was diluted with osmotic stabilizer to an appropriate concentration, 100 μL of suspension was gently spread on the MYGSA plates. The control was diluted to the same concentration with sterile water and was spread on the MYGA medium. The protoplasts and control plates were incubated under dark conditions at 28°C. The protoplast regeneration and development of colonies were observed in the plates after 5 days. Protoplast regeneration rates were calculated as the following equation:

$$\text{Protoplast regeneration rate}(\%) = (A - -B)/C \times 100\%$$

where A is the number of regenerated colonies counted on MYGSA medium containing osmotic stabilizer, B is the number of colonies counted on control MYGA medium (Non protoplast cells), and C is the total number of spread cells [26].

2.5. Screening and analytical methods of positive mutation

The survival colonies were randomly selected and sub-cultured on PDA plates for further analysis and morphological observation. For the screening process, purified colonies were further subcultured on G-PDA plates incubated at 28°C for 7 d. After the guaiacol oxidation, mutants were visually screened by determining the colony and dark zone diameter. Mutants with deep and large color-zone on the guaiacol plate indicate the presence of more phenol oxidases, specifically laccase [27,28]. After incubation, the diameter of colonies and color-zone were measured, then the description and calculation were as follows: The initial colony diameter: d0 = 5 mm; colony diameter: d1 = measured colony diameter - d0; color-zone diameter: d2 = measured color zone diameter - d0; d1/d2 < 1 represent ability on selective delignification of fungus, which were as preferable lac producers [29]. Laccase-dependent positive mutations were selected as increased guaiacol oxidative diameter as compared to that of the wild strain.

2.6. Verification of laccase activity

Selected laccase-positive strains were grown in a PDB medium by the shake flask method at 150 rpm for 6 d at 28°C, and the cultures were analyzed for laccase production. Laccase activity was measured using a reaction mixture containing 0.1 mL diluted culture supernatant, 0.2 mL 15 mM 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), and 50 mM sodium acetate buffer (pH 5.0) in a total volume of 3 mL. The reaction was monitored at a 420 nm wavelength. One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance at 420 nm required to oxidise 1 μmoL ABTS/min using an ε420 value for oxidated ABTS of molar absorption coefficients 36,000 M^{-1} cm^{-1} [30].

2.7. Genetic stability

Somatic incompatibility is a process of allorecognition and allorejection in the dikaryotic hyphae phase of basidiomycetes. To confirm that the mutants and wild strain had significant genetic differences, antagonist tests were conducted after mutants were active by sub-culturing 3–5 times. Mutants and wild strains were co-cultured into 20 mL of PDA in 90 mm Petri dishes at 28°C for 7 d. The results of antagonistic activity between the mutants and wild strain were observed. If mutant strains have strong antagonism resistance, a clear dividing line will appear at the junction of them. Antagonistic results indicated that the genetic background was different apparently. The mutants were also sub-cultured for at least 10 generations on PDA, and then evaluated for the laccase productivity with the same condition in 7 d PDB culture from each of 2 generations.

2.8. Cultivation characteristics and laccase production on corn straw

The mutagenesis strains regrew on PDA, mycelia plugs (5 mm) were transferred into a seed culture medium, incubated at 28°C for 7 d, each corn stalk medium was inoculated with 5 mL homogenized seeds and cultivated at 28°C for 6, 18, 30 d. Then substrates were measured to determine laccase concentration and all fermented substrates were comparatively analyzed for substrate consumption. The percent substrate consumption as a total organic matter in fungal-treated substrates was calculated as described below:

$$\text{Substrate consumption (\%)} = (\text{Mi} - \text{Mf}) / \text{Mi} \times 100\%$$

where Mi is the amount of the component in the control (non-fermented) substrate and Mf is the amount of the component in the mycosubstrates [14].

2.9. Statistical analysis

Experiments performed in this study were in triplicates and the data were presented as mean of triplicate values and analyzed using a one-way ANOVA followed by Duncan's multiple range test with SPSS.

3. Results and discussion

3.1. Protoplast formation and conditions

Protoplast preparation and isolation are the most important step for successful protoplast fusion and regeneration. In order to obtain protoplast with high quantity and good quality, significant factors such as mycelium age, enzyme concentration, and enzymolysis time were analyzed. As shown in Fig. 1, after 2 h of incubation of 2% lysing enzyme mixture hydrolysis, 5 d mycelium exhibited the maximum release of protoplasts. The effect of enzymatic hydrolysis is poor when the incubation time is too short or too long. The fungi in the different physiological states directly affect the cell structure, metabolism, and vitality of fungi.

To study the effect of enzyme concentration and enzymolysis time on the protoplast formation and regeneration, the wild strain was treated with different concentrations of enzymes-mixture. As shown in Table 1 a comparison of different concentrations showed that 2.0% lysing enzyme-chitinase mixture was more effective. The results of enzymolysis time showed that this concentration for 2 h resulted in the highest level of 4.05×10^7 protoplasts/mL formation and the regeneration ratio of protoplast formation was 1.08%.

The results obtained are higher than the protoplast preparation studies of *Pleurotus spp.* as previous research reported the amounts of protoplasts obtained in *P. ostreatus* and *P. djamor* were 4.59×10^6 and 3.36×10^6 protoplasts/mL, respectively [31]. Other studies obtained the maximum release of protoplasts with a mutant that yielded 4.0×10^6 protoplasts/mL [32]. He et al also reported the preparation of *P. eryngii* protoplast was 3.82×10^7 cells/mL [33]. Zhao's results showed that the protoplast formation of *P. ostreatus* was 5.01×10^8/mL [3]. However, the production of protoplast varies with the factors like species and age of fungal mycelia, type, and condition of the lytic enzyme [34]. Although enzymatic hydrolysis is an effective method in the protoplast preparation, filtering, elution, and low-speed centrifugation steps will bring the loss of upper suspended protoplasts and aggregation rupture against each other, which affected the final formation yield and regeneration ratio of protoplasts to a certain degree [35].

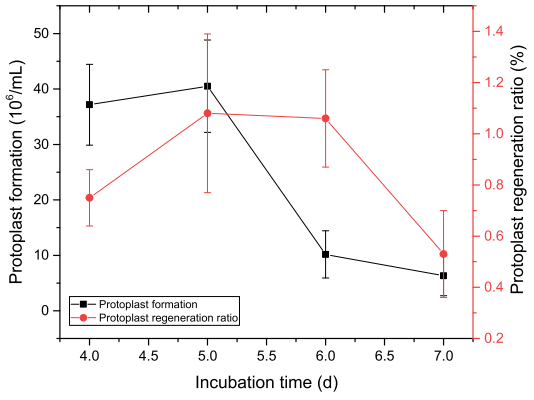

Fig. 1. Effect of incubation time on protoplast formation and regeneration ratio. Error bars represent standard deviations (n = 3).

3.2. Lethality rate of P. djamor RP by ARTP mutation

To achieve a satisfactory mutation rate, it is important to optimize mutation parameters like ARTP mutagenesis induced a dose-dependent lethality in *P. djamor* protoplasts with respect to treatment time. As data presented in (Fig. 2), when cells were treated by the argon-based ARTP jet for 15, 30, and 45 s, the lethality rate of the treated RP protoplasts increased to 96.90, 98.98 and 99.9%, respectively. When the fresh protoplast suspension was treated for more than 60 s under these conditions, the protoplasts could not survive, which indicates 60 s was the lethal time for RP protoplast. This method was also suitable for the determination of the lethal time of RP basidiospores. When basidiospores were treated under the same ARTP condition for 15, 30, 45, 60, 90 s, the lethality rate of RP basidiospores were 92.18 %, 97.55 %, 98.60 %, 99.88 % and 99.97 %, respectively. Lethal time of basidiospores was found to be 120 s. ARTP jets contain high linear energy transfer (LET) values, which has stronger biological effects than other low-LET physical methods [35,36], that can be used to generate diverse breakage in DNA and oligonucleotides by varying in the plasma dosage. ARTP system has been widely used to achieve mutation at a molecular level [5]. The minimum treated time set of 15 s leads to a more than 90% lethal rate on RP protoplasts and basidiospores. With lethality rate comparison analysis, it was also observed that the lethal time of RP protoplast under argon-ARTP was shorter than that of basidiospores, which indicated the argon-based ARTP mutagenesis method has stronger

Table 1
Effect of enzyme concentration, enzymolysis time on protoplast formation (PF), and protoplast regeneration ratio (PRR).

Enzymolysis time (h)	Enzyme concentrations (%)									
	0.5		1.0		1.5		2.0		2.5	
	PF (cell/mL)	PRR (%)	PF (cell/mL)	PRR (%)	PF (cell/mL)	PRR (%)	PF (cell/mL)	PRR (%)	PF (cell/mL)	PRR (%)
1	5.67×10^5	0.36	1.37×10^6	0.29	3.98×10^6	0.96	3.07×10^7	1.04	3.08×10^7	1.05
2	4.17×10^5	0.17	2.38×10^6	0.61	7.42×10^6	0.97	4.05×10^7	1.08	3.37×10^7	0.88
3	7.00×10^5	0.09	3.75×10^6	0.50	9.00×10^6	0.36	4.00×10^7	0.90	1.20×10^7	0.56
4	6.67×10^5	0.05	2.80×10^6	0.36	9.67×10^6	0.20	3.68×10^7	0.64	1.10×10^7	0.35

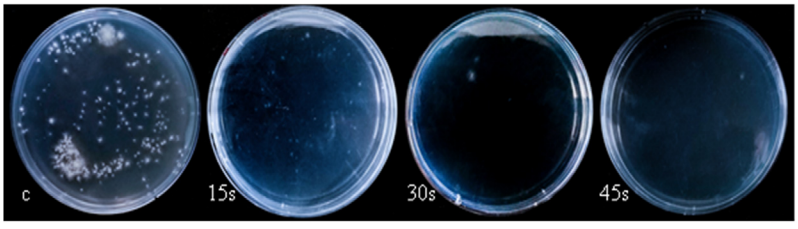

Fig. 2. Colony observation of regenerated protoplasts after ARTP mutagenesis. The lethality rate of *P. djamor* RP protoplasts using ARTP mutagenesis was studied for ascertaining an optimum mutation time. The regeneration plates were incubated at 28°C under dark conditions, the individual colonies of control and treated were observed.

biological effects on protoplasts than mycelium of basidiospores. Protoplast is the suitable biological mutagenic material in this method. Therefore, suitable protoplasts were prepared from fresh young mycelium for further experimentation. ARTP mutagenesis has been successfully developed and applied in industrial production to improve diverse traits in bacteria [37,38], fungi [39,40,41], and microalgae [8]. However, ARTP mutagenesis has not yet been used to generate mutants in the edible fungus *P. djamor*, and there are no reports of ARTP in the improvement of fungal laccase as far as literature survey is concerned. Therefore, the ARTP was applied to *P. djamor* as a novel microbial mutation tool to generate high laccase-producing mutants.

3.3. Mutation rate of P. djamor RP by ARTP method

Among various artificial mutagenesis methods, the ARTP method is an efficient tool for microbial breeding in improving mutation rates;, as earlier reported, ARTP has a higher positive genotoxic response and more effective mutation rate [10]. With the increase in the treatment time, plasma jets have been found to change DNA sequences [42], and sometimes also cause enzyme molecular structure alterations that lead to the increase in enzyme activity [9]. In present study, it was observed that mutation resulted in enhanced laccase yields while as growth rates were similar in the regenerated strains treated with ARTP for 15, 30 and 45 s. According to Fig. 3A, the total mutation rates assessed by laccase activity were 21.33%, 22.55% and 25.75% in 15, 30 and 45 s, respectively. The positive mutation rates were 13.35%, 14.94% and 17.62%, respectively. The total mutation rate and positive mutation rate increased slightly with the increase in plasma treatment time and reached the highest level at 45 s, but the negative mutation rate did not change significantly. As indicated in Fig. 3, the total mutation rates assessed by growth rate were 30.85%, 32.50% and 33.44%, the positive mutation rates were 21.29%, 23.75% and 25.31%, respectively. The total mutation rate and the positive mutation rate increase slightly with the increase in plasma treatment time. The negative mutation rate decreased slightly, indicating that ARTP treatment had a greater effect on the growth, and negative mutation affected the viability of mutant. This indicated that the positive mutation possibility of the strain surviving under the argon-ARTP treatment of 45 s was higher, and the screening efficiency was higher. The growth potential might affect the laccase production ability of strain. The regenerated mutants showed no significant differences in color and colony morphology from wild strains.

Previous literature on mutagenesis breeding suggested that the highest positive mutation rate is obtained when the lethality rate of the microorganism is between 90% and 99% [10]. The ARTP mutation method succeeds in mutants breeding various microorganisms [43]. Wang et al reported *Streptomyces avermitilis* under the lethal rate of 98.2% get about 21% positive mutates while total mutation rate was over 30% [44]. Fang et al. [8] reported *S. platensis* FACHB 904 positive/total mutant and the mutation rate reached from 25 to 45%. Cheng et al demonstrated in *C. glutamicum* positive mutation rates about 5% more than double the negative mutation rate when the lethality rate reached 92.8% [6]. When the irradiation time is too long, the protoplasts are hard to survive, so appropriate plasma mutagenesis time could be suitable for both desirable positive mutation and lethality rate. Based on our results and existing literature, an exposure time of 15 s was considered appropriate to obtain enough positive mutated strains with enhanced laccase activity.

3.4. Screening of mutants for their high laccase potential

To isolate laccase-enhanced mutants, the phenolic compound guaiacol was used as an indicator in this study. As per the literature survey, phenolic compounds like guaiacol are structurally related to lignin or lignin derivatives which activate a secondary metabolism responsible for improved lignolytic enzyme production. That is the reason such phenolic compounds are good inducers of laccase production and are generally used to detect the enhanced production of laccase [45]. A guaiacol-based screening strategy was employed to visually select potential mutants. Survival colonies of protoplast after mutation were selected and transferred on PDA medium. For pre-screening, the mutants were further inoculated on G-PDA plates for visual screening (Fig. 4).

During the screening process, 47 positive mutated colonies were selected and analyzed for their guaiacol oxidate zone on G-PDA plates. It was found that the guaiacol intensely suppressed the cell growth in both wild and mutants. Their antagonistic test results showed that most screened mutant strains have antagonism resistance with the original wild strain. Table 2 lists 10 laccase-enhanced mutants that passed laccase productivity, generation stability, and antagonism determination. With the comparison of the RP hyphal growth rate on PDA and G-PDA, it was found that most strains showed better ability on guaiacol oxidation, and grew faster on PDA. The growth rates of the mutant on PDA increase in a suitable range almost overgrowing the whole PDA plate in 5 days (except 61-34 strains in 6 d).

The guaiacol oxidation of all mutants was improved. Whereas not all mutants were improved in the mycelium growth on G-PDA, that indicates no correlation between the growth rate and oxidation speed on G-PDA medium. The wild strain and some

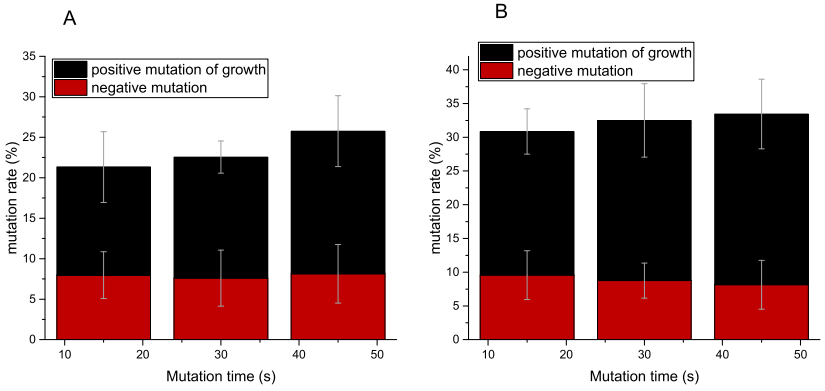

Fig. 3. The mutation rates of *P. djamor* RP protoplasts of different ARTP mutagenesis time (A) laccase-dependent mutation rates; (B) growth-dependent mutation rates. Error bars represent standard deviations ($n = 3$).

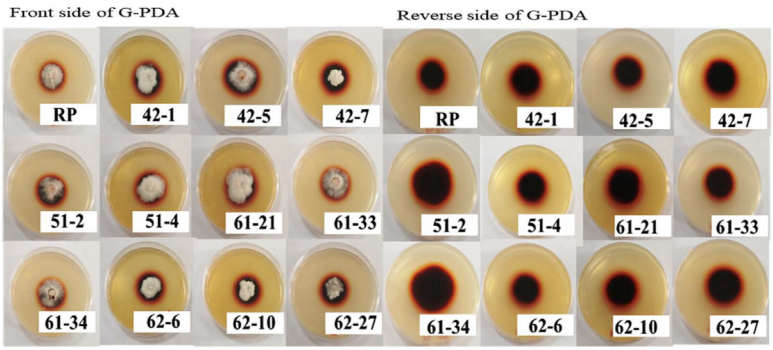

Fig. 4. Screening of wild strain and its mutants for Laccase activity on PDA agar plates supplemented with guaiacol.

Table 2
Preliminary screening of mutants. The diameters of colony and color-zone were recorded on PDA and G-PDA, respectively. Growth rate, Guaiacol oxidation speed, and colony/oxidated diameter (d1/d2) in 7 d of tested wild strain and potential mutants.

Strain(s)	Growth rate on PDA (cm/d)	Growth rate on G-PDA (cm/d)	Guaiacol oxidation speed (cm/d)	d1 (cm)	d2 (cm)	d1/d2
RP	1.24 ± 0.14	0.28 ± 0.06	0.42 ± 0.13	1.93 ± 0.45	2.93 ± 0.89	0.66 ± 0.12
42-1	1.35 ± 0.20	0.29 ± 0.05	0.46 ± 0.08	2.00 ± 0.35	3.19 ± 0.58	0.63 ± 0.15
42-5	1.43 ± 0.16	0.28 ± 0.03	0.50 ± 0.10	1.95 ± 0.23	3.48 ± 0.70	0.56 ± 0.05
42-7	1.42 ± 0.11	0.24 ± 0.01f	0.48 ± 0.13	1.67 ± 0.07	3.39 ± 0.91	0.49 ± 0.09
51-2	1.41 ± 0.15	0.29 ± 0.03	0.51 ± 0.11	2.00 ± 0.23	3.59 ± 0.75	0.56 ± 0.09
51-4	1.46 ± 0.17	0.29 ± 0.05	0.45 ± 0.07	2.01 ± 0.34	3.12 ± 0.50	0.64 ± 0.20
61-21	1.41 ± 0.10	0.26 ± 0.04	0.47 ± 0.11	1.82 ± 0.28	3.31 ± 0.80	0.55 ± 0.12
61-33	1.41 ± 0.09	0.26 ± 0.03	0.52 ± 0.20	1.81 ± 0.19	3.61 ± 1.39	0.50 ± 0.08
61-34	1.30 ± 0.15	0.24 ± 0.04	0.44 ± 0.12	1.71 ± 0.26	3.08 ± 0.81	0.55 ± 0.10
62-6	1.40 ± 0.18	0.27 ± 0.06	0.44 ± 0.08	1.87 ± 0.40	3.08 ± 0.56	0.61 ± 0.08
62-10	1.40 ± 0.11	0.26 ± 0.02	0.48 ± 0.11	1.79 ± 0.17	3.38 ± 0.75	0.53 ± 0.02
62-27	1.44 ± 0.11	0.27 ± 0.04	0.44 ± 0.10	1.92 ± 0.28	3.09 ± 0.70	0.62 ± 0.12

mutants were sensitive to guaiacol that resulted in suppressed growth of mycelium in these strains. The results showed guaiacol had a significant effect on the growth rate of mutants, indicating they are in different sensibility to the growing stress. The d1/d2 (colony/oxidated diameter) of listed positive mutants all have smaller value than that of wild strain after 7 d cultivation time, but a slight difference between these mutants means laccase produces associated both with growth ability and selective delignification capability. The mutants were generated which were both better on guaiacol oxidase and rapid growth. As it was reported, colored agar-plate effectively reflects positive mutants that can oxidize guaiacol reflecting laccase activity, a most effective fungus caused the highest color change from white to brown PDA [28].

3.5. Laccase productivity of mutants

Data presented in Table 3 show 10 mutants selected after initial screening were further studied for laccase production by shake flask fermentation and solid-state fermentation (SSF). Laccase assay analysis of shake flask fermentation showed that the laccase production of selected mutants was significantly improved after mutagenesis as compared to wild strain. Under shake flask cultivation in PDB, all mutants had shown different laccase productivity performance; however, the strain coded as 51-4 showed the highest laccase production of 494.44 ± 19.19 U /L, which was increased by 86.39% as compared to wild strain, followed by strains 62-10 of 405.00 U/L, 61-21 of 387.50 U/L, and 62-27 of 371.39 U/L.

Wild strain RP secreted laccase earlier, and the laccase activity reached the highest in 5–7 d. The mutant strain maintained the characteristic of rapid secretion laccase potential. As the lignin-olytic enzymes are produced as secondary metabolites in fungi, their production is delayed in most cases [46]. Sometimes laccase production optima are delayed on the 26th day under shake culture conditions [47]. However, early laccase production was also observed in some microorganisms mainly in fungi such as *B.*

Table 3
Laccase activity of mutants in PDB cultivation versus SSF cultivation.

Strain	PDB cultivation		SSF cultivation	
	Lac activity (U/L)	Increasing rate (%)	Lac activity (U/g)	Increasing rate (%)
RP	265.3 ± 25.8	–	20.0 ± 2.5	–
42-1	336.9 ± 32.9	27.0 ↑	21.0 ± 2.7	4.8 ↑
42-5	366.1 ± 20.4	38.0 ↑	21.0 ± 4.3	5.0 ↑
42-7	311.4 ± 35.0	17.4 ↑	20.9 ± 2.0	4.4 ↑
51-2	371.1 ± 22.5	39.9 ↑	24.3 ± 3.8	21.7↑
51-4	494.4 ± 19.2	86.9 ↑	25.2 ± 3.6	25.9↑
61-21	387.5 ± 54.6	46.1 ↑	20.6 ± 3.6	3.2 ↑
61-33	336.9 ± 44.6	27.0 ↑	24.9 ± 2.7	24.6↑
61-34	312.2 ± 16.4	17.7 ↑	21.0 ± 2.0	4.8 ↑
62-6	310.0 ± 17.2	16.9 ↑	21.2 ± 3.2	6.0 ↑
62-10	405.0 ± 48.0	52.7 ↑	21.3 ± 3.5	6.6 ↑
62-27	371.4 ± 33.8	40.0 ↑	26.1 ± 1.8	30.3 ↑

cinerea (5–7 d) [48], and some bacterial laccase from *Pseudomonas* [49] and *Bacillus* [50].

Under SSF cultivation, wastes produced by agriculture or agro-industrial are the natural medium and could be used to enhance laccase production in filamentous fungi [51]. Previous studies have shown that some fungal mutants showed strong tolerance to almost all inhibitors in lignocellulosic hydrolyzate and are flexible to diverse substrates [12]. Corn stalk is a familiar agro-waste, which is rich in cellulose, hemicellulose, lignin, etc. Biomass is a natural medium for edible mushroom cultivation, and SSF develops an economical and environmental way of laccase production. Therefore corn stalk mixture was used in this study for laccase production by RP mutants.

As shown in Table 3, the laccase productivity in SSF is very different from PDB fermentation liquid cultures. In SSF cultures, the highest productivity was observed in strain coded as 62-27 with laccase activity reaching 26.06 U/g, which significantly increased 30.28% than wild strain, followed by mutants coded as 51-4, 61-33, 51-2 having laccase activity 25.18, 24.92, 24.34 U/g respectively. Corn stalk is a lignocellulosic agricultural waste that accounts for 35% of the approximately 2.9 billion tons of straws produced globally each year. Corn stalks contain phenolic chemicals, which are similar to lignin and its degraded derivatives [52,53]. Phenolic compounds that have been identified in corn stalk include syringic acid, syringaldehyde, ferulic acid, vanillin, p-coumaric acid, p-hydroxybenzaldehyde, vanillic acid [54,55]. Wang et al. [56] found these phenolic substances to be probable laccase inducers in microbes. Several studies have indicated an increase in fungal-laccase production as a result of the addition or presence of phenolic chemicals in the fermentation substrate [52,53]. Another cause for enhanced laccase production in the SSF of *P. djamor* RP and its mutants could be the higher concentration of these phenolic compounds in the corn stalk.

Table 4 summarizes the total lignin consumption of wild and mutant strains after 6, 18, 30 d, and the results showed that biomass utilization rate of mutants were higher than that of wild strain. This phenomenon can be explained by an increase in laccase activity of mutants that resulted in the higher lignin decomposition and utilization of the substrate. Furthermore, it might also be linked to other ligninolytic enzymes like MnP, LiP CMCase, β-Glucosidase, and Avicelase that were also found to be slightly changed as compared to wild type. It was also observed that mutants showed faster and better adaptability to corn stalks resulting in an improved substrate consumption ratio in 6 d short fermentation period. Our results demonstrated that the laccase improved mutants showed high efficiency towards substrate in a shorter time in SSF cultivation than that of wild strain.

A better result was observed in mutant 62-27 with the highest laccase production of 26.06 U/g obtained on the 6th day under SSF cultivation resulting in 17.5% dry weight substrate loss and 23.28% lignin loss,. as the laccase production from agricultural and agro-industrial wastes is environmentally friendly in industrial bioconversion. Thus, our studies further encourage the investigation of reuses of both agro-wastes and lignin-based derivatives. Our results indicated that the ARTP biological breeding system was markedly effective to strengthen the growing capability of microbes to utilize an effectively wide range of substrates. As enzyme production at an industrial scale is produced mainly through submerged fermentation (SmF), the investigations have been more focused on the development of SmF rather than SSF [57]. However, recent studies are being conducted on the improvement of SSF due to the higher yield and cost-effective production in SSF rather than SmF [58,59]. In large-scale production, laccase from wastes is economic and environmentally friendly through the reuse of agro-wastes, and it is a strategic way of low-cost production.

3.6. Genetic stability of the mutant 51-4 strain

The mutant 51-4 strain that was found as a promising laccase producer was further evaluated for genetic stability via 10 rounds of generations. The 2, 4, 6, 8, 10 generations of 51-4 strain were measured with the same cultivation and laccase assay. After 10 generations, the production of laccase in PDB cultivation of the mutated strain maintained relative stability and the average production of laccase (492.04 ± 10.81 U/L). Thus, the results indicated that the mutant 51-4 strain was genetically stable and had a potential application value. In this study, it was also observed that isolated mutants have shown antagonism resistance with wild strain. Our study is in agreement with previous studies that showed ARTP mutagenesis is an effective method for genetic manipulation and bioproduct improvement in the breeding of human-safety fungi [51,60,61,62].

4. Conclusions

Pleurotus djamor is an edible mushroom known for its therapeutic capabilities. According to the findings of this study, the high laccase yield of *P. djamor* RP induced by ARTP mutagenesis consumes lignocellulose waste better than the wild strain. This study showed that the protoplast ARTP mutagenesis breeding strategy is a viable method for changing the functionalities of *P. djamor*; not only was the laccase level increased, but the growth rate was also raised. The ARTP mutagenesis and screen approach used in this study indi-

Table 4
Substrates consumption ratio in SSF cultivation. In SSF cultivation, corn straw was used as a nutrient source. The total weight (corn straw) and total lignin (a component of corn straw) were evaluated after the fungal treatment.

Strain(s)	lignin loss (%)			Corn weight loss (%)		
	6 d	18 d	30 d	6 d	18 d	30 d
RP	11.8 ± 2.1	42.3 ± 2.3	48.0 ± 5.1	8.2 ± 1.7	22.4 ± 1.6	31.6 ± 0.6
42-1	22.4 ± 2.2	44.0 ± 5.7	48.7 ± 1.9	15.4 ± 1.5	28.7 ± 1.3	31.9 ± 1.6
42-5	20.9 ± 0.5	44.6 ± 6.0	48.3 ± 4.1	15.7 ± 2.0	27.0 ± 1.4	32.5 ± 1.3
42-7	21.1 ± 2.4	41.3 ± 1.6	47.4 ± 3.8	14.8 ± 1.2	23.9 ± 2.0	33.0 ± 1.9
51-2	17.0 ± 1.5	42.6 ± 2.9	50.3 ± 1.4	11.0 ± 1.2	26.1 ± 2.0	32.0 ± 1.7
51-4	25.9 ± 4.5	45.1 ± 1.2	49.3 ± 4.9	19.2 ± 1.6	24.7 ± 1.9	33.3 ± 0.9
61-21	22.6 ± 5.1	44.0 ± 2.3	52.5 ± 4.1	15.2 ± 1.3	24.7 ± 2.5	34.5 ± 1.6
61-33	25.7 ± 2.6	42.1 ± 1.2	51.9 ± 4.3	19.4 ± 2.1	25.9 ± 1.9	35.1 ± 1.8
61-34	20.4 ± 7.0	44.8 ± 4.3	50.6 ± 3.0	15.9 ± 0.9	26.5 ± 2.1	33.7 ± 1.9
62-6	22.0 ± 4.7	41.5 ± 0.5	49.2 ± 2.9	11.5 ± 1.7	27.4 ± 2.0	33.7 ± 1.8
62-10	19.6 ± 5.2	43.4 ± 1.5	50.7 ± 1.3	14.7 ± 1.7	24.9 ± 1.4	33.0 ± 0.9
62-27	23.3 ± 4.7	45.7 ± 1.8	52.5 ± 1.0	17.5 ± 1.8	25.0 ± 1.0	32.0 ± 1.5

cated that the novel technology was simple to use, with high positive mutation rates and genetic stability of the mutants. This study would serve as foundation for the use of ARTP mutagenesis in the quality breeding of *P. djamor*, as well as laying the foundation for better exploitation and utilization of *P. djamor* for industrial applications.

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Conflict of interest

The authors declare no conflict of interest.

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