

RESEARCH ARTICLE

Detoxification and Bioremediation of Sulfa Drugs and Synthetic Dyes by *Streptomyces mutabilis* A17 Laccase Produced in Solid State Fermentation

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Abstract

Laccase enzyme has many important applications as bioremediation and removal of environmental wastes and also in industrial processes. Therefore, large-scale of novel producers of laccase is demanded to use cheap and low cost substrates. Hence, solid state fermentation is the best strategy to achieve this proposes. The present study is designed to optimize laccase production by *Streptomyces mutabilis* A17 using agro-wastes including rice bran, castor seed cake, wheat bran, wheat straw, soybeans cake, peanut cake, cotton seed cake and chicken feathers. Cotton seed cake significantly enhanced *S. mutabilis* A17 laccase production when it was incubated for 6 days at 37°C and 70% moisture content in presence of yeast extract and glucose as the best nitrogen and carbon sources, respectively. After purification steps, the purified laccase showed maximum activity at 40°C and pH 8.0 within stability pH range of 7.0 to 9.0. Laccase activity was highly increased to 195, 180 and 166% by the addition of Ba⁺², Cu⁺² and Mn⁺², respectively. Sulfa drugs and synthetic dyes cause various pollutants due to their toxic effects in different environments. Therefore, a purified laccase was utilized for removal of sulfa drugs as sulfadiazine (SDZ) and sulfathiazole (STZ) and also synthetic dyes. The results showed that the maximal enzymatic removal of SDZ and STZ was attained at 50°C and pH 6.0 for 1 h at presence of 1mM HBT(1-hydroxybenzotriazole) as a laccase mediator by removal percentage of 73% and 90%, respectively. Detoxification effects of laccase-treated sulfa drugs and two synthetic dyes; congo red and malachite green solutions, were investigated using microbial toxicity test. Our results indicated that the toxicity of these laccase-treated samples against tested bacterial strains were significantly decreased. It was concluded from this study that SSF laccase is playing an important role for decreasing the toxic effects of pharmaceutical wastes and organic pollutants.

Keywords: Solid state fermentation, Laccase, Bioremediation, Sulfa drugs, Synthetic dyes.

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INTRODUCTION

Laccase is a multi-copper oxidase enzyme produced from fungi, plants or few bacteria. This enzyme can catalyze the oxidation of numerous substrates by removal of one electron coupled with four-electron reduction of oxygen to water¹. Most bacterial laccases are highly thermotolerant and maintain their activity in neutral to alkaline conditions as in bacterial strain *Brevibacterium halotolerans* N11², whereas fungal laccases can usually reduce their activities rapidly at wide ranges of pH and temperatures³.

Laccases have numerous potential in bioremediation, biofuel production, removal of water and soil wastes, and modification of biopolymers⁴ and also synthetic dyes decolorization². The presence of synthetic dyes in dye manufacturing, sewages of textile and leather tanning industries causes an environmental pollutions due to their toxic effect on plants, animals and also on human health⁵. The toxicity degree of degradation products depends on their structure which determines the structure of degraded dyes and enzymes used for their degradation⁶. Due to low yields and high production costs of laccases from host microorganisms, it is important to improve the techniques used for enhancement of laccases yield and reduction of the production cost by utilization of cheap substrates. Therefore, SSF is the most effective technique to promise a higher laccase yield using cheap and waste substances as substrates and low energy input⁷. Solid-state fermentation is the fermentation type in which microorganisms are inoculated into moist solid substrates without free water content⁷. The utilization of such solid wastes, in addition to the presence of substitutional substrates, assist to resolve environmental problems, which are caused by their elimination in the open environment⁸. Agro-industrial residues that commonly used for SSF are wheat bran, cereal grains, wood shavings, sawdust and various other animal and plant materials⁹.

Laccases production is influenced by several factors such as time, medium composition, temperature, pH and carbon and nitrogen sources¹⁰. The optimal temperature and pH are mainly dependent on the substrate for high yield of laccase¹¹. The stability of laccase enzymes at different pH, temperature, and different substrates

has to be estimated for its improvement in industrial applications².

High cost, limited degradation ability and hazardous by-products are disadvantages of some traditional physical and chemical methods for removal of pollutants¹². Therefore, the removal and degradation of pharmaceuticals (sulfa drugs) using effective and eco-friendly methods as enzymatic reaction is necessary to apply¹³. The degradation of organic pollutants by laccases have received huge interest because of their ability to oxidize a broad range of these wastes and their higher efficiency and lower toxicity of produced metabolites¹⁴.

The objective of the current work is to utilize solid state fermentation technique for production *S. mutabilis* A17 laccase, using different agro-industrial wastes as substrates. Optimization, purification and characterization of laccase were also accomplished even to study their potential towards industrial applications. This research also aimed to study the removal of sulfa drugs as sulfadiazine (SDZ), sulfathiazole (STZ) and synthetic dyes. In addition, the toxicity of laccase-treated sulfa drug and synthetic dye samples was investigated against different pathogenic bacterial strains (Gram +ve and Gram –ve).

MATERIALS AND METHODS

Actinomycete strain and Solid state fermentation conditions

Streptomyces mutabilis A17 (KY883987) was selected as the most potent laccase producer from our previously study. Laccase production from the selected strain was studied under SSF, using cheap raw natural agro-industrial residues. These agro-wastes as rice bran, castor seed cake, wheat bran, wheat straw, soybeans cake, peanut cake, cotton seed cake and chicken feathers were obtained from local Egyptian Markets, and used as solid substrates for screening the laccase production by *S. mutabilis* A17.

The chemical constitutions of these natural agro-industrial by-products were outlined in Table 1¹⁵⁻²⁴. Five grams of each collected substrate was dispensed in 250 ml Erlenmeyer conical flask, moistened with 10 ml of Starch - nitrate solution containing (g/l): soluble starch, 20; NaNO₃, 2; KCl, 0.5; K₂HPO₄, 1; MgSO₄·7H₂O, 0.5; CaCO₃, 1; FeSO₄·5H₂O, 0.01 and distilled water up

Table 1. Chemical structure of the experimental natural solid substrate

By products	Protein %	Fiber %	Arginine %	Lysine %	Alanine %	Ash %	Fat %	References
Peanut cake	2.5	5	1.54	0.54	0.8	8.3	45.6	[15], [16]
Wheatbran	14.5-5	6.8-10.4	1.01	0.61	0.74	4.0-6.5	2.9-4.3	[17], [18]
Rice bran	15.6	26.4	1.32	0.69	0.91	21.0	23.4	[19]
Cottonseed cake	41.0	13.6	5.6	2.19	2.04	7.0	20	[20]
Soy beans cake	5.12	37.08	2.45-3.1	2.5-2.66	1.5-2.03	4.86	18.38	[21]
Castorseed cake	31.06	11.90	8.00	4.11	3.96	11.10	19.40	[22]
Feathers	91	0.57	4.30	—	3.44	5.41	1.53	[23]
Wheat straw	21.00	7.35	2.35	1.89	2.83	6.35	2.60	[24]

to 1000 ml pH 7.0. After autoclaving, the medium was inoculated by 1 ml spore suspension (6 day age culture) then mixed and incubated for 6 days at 35°C.

Optimization of solid state fermentation parameters

Different conditions including inoculum size and initial moisture content of substrate were optimized to increase the production *S. mutabilis* A17 laccase at different temperatures (25- 50°C) under SSF. The effect of supplementation of different carbon sources (1% W/V) as glucose, fructose, arabinose, sucrose, maltose, and lactose) and nitrogen sources (asparagine, glycine, tyrosine, glutamine and cysteine, urea, yeast extract, beef extract, tryptone, peptone and casein) on laccase production was investigated. Each nitrogen source was supplemented to the basal SSF medium according to its equivalent molecular weights, regarding to the maximum concentration of NaNO₃.

Extraction of enzyme from the solid actinomycete culture

The crude enzyme was extracted according to the method recorded by El-Sayed²⁵, using potassium phosphate buffer (pH 7.0). The mixture was agitated for 1h at 200 rpm, the culture was filtrated by Whatman No.1 filter paper, and filtrate was centrifuged at 4000 rpm for 15 min. The filtrate was utilized as a crude enzyme. The activity of enzyme and its protein content were assayed as described later.

Laccase assay and protein determination

Laccase assay has been examined by

oxidation of guaiacol according to the method of Kalra et al.²⁶. The oxidation of guaiacol by laccase to develop dense reddish brown color was utilized to assay enzyme activity at 450 nm. The reaction mixture can be designed as follows: 1mL of Guaiacol (2 mM), sodium acetate buffer (10mM) 3mL and enzyme source 1mL (bacterial supernatant). Distilled water (1mL) was used instead of enzyme as a blank. The mixture was incubated for 15 min at 30°C and the absorbance was estimated at 450 nm using UV spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of guaiacol per min under optimal conditions.

Protein content of free enzyme was estimated on the crude enzyme using technique of Lowry et al.²⁷.

Enzyme purification and molecular weight determination

The proteins in the crude enzyme extracted from solid state culture of *S. mutabilis* A17 were precipitated by 70% ammonium sulfate with stirring at 4°C and then centrifugated at 10,000 rpm for 15 min. The collected pellets were dissolved in 10mM phosphate buffer (pH 6.5). The precipitated protein was dialyzed by the same buffer using dialysis membrane-60, then the dialyzed product was kept at 4°C²⁸. The dialyzed product was fractionated by ionexchange chromatography followed by gel-filtration chromatography (Sephadex G100). The most active fractions were pooled and used for subsequent studies. The homogeneity and molecular mass of the purified enzyme was evaluated by using SDS-PEAGE

analysis according to the method of Laemmli²⁹.

Characterization of laccase

Optimal pH and pH stability of purified enzyme was studied according to a protocol described by El-Batal et al.³⁰. The pure enzyme was separately incubated for 30 min with citrate phosphate buffer for pH (5–8) and Glycine-NaOH for pH (9–11). The pH stability of enzyme was studied when its incubated at different pH values (5-11) for 2h at 35°C. The residual activity was calculated at each pH-value. Laccase activity was investigated at different temperature by incubating the enzyme at 25°C up to 60°C for 15 min. The reaction mixture was incubated for 10, 20, 30, 40, 50 & 60 min and assessment of laccase activity was performed as previously mentioned.

Moreover, the influence of various metal ions such as Cu²⁺, Mg²⁺, Zn²⁺, Ca²⁺, Cd²⁺, Co²⁺, Fe²⁺, Mn²⁺, Na²⁺, K²⁺, Ba²⁺ and EDTA with the concentration of 10mM was estimated. The reaction assay mixture of laccase was pre-incubated with each activator or inhibitor ion for 30 min before adding guaiacol. The relative enzyme activity was examined after incubation at 37°C for 30 min.

Substrate specificity

The affinity of laccase towards various substrates including 2,6 dimethoxy phenol, catechol, guaiacol, hydroquinone and tyrosine was investigated using different concentrations of each substrate (2-10 mM).

Application of SSF laccase

Removal of sulfa drugs using purified laccase

In this experiment, the removal of certain sulfa drugs (SDZ and STZ) using purified *S. mutabilis* A17 laccase was investigated. Sample solutions were intended by dissolving each sulfa drug in a citrate-phosphate buffer (0.1M, pH 5.0). After which, the reaction mixture was incubated for 60 min at 40°C and 50 rpm. Removal experiments were designed using a protocol described by Rahmani et al.³¹ with slight modifications. To each sulfa drug solution (final concentration of 50 mg L⁻¹), laccase (81.3 Umg⁻¹) and 1mM HBT were added. Treated and untreated sample solutions were taken every 5 min and filtered through 0.45µm Millipore filters and resolved for residual concentration of sulfa drugs. Removal of sulfa drug solutions were recorded by using UV-VIS spectrophotometer at 450 nm.

Removal percentage (%) = $C_0 - C_t / C_0 \times 100$

where C₀ is the initial concentrations of sulfa drug solution and C_t is the residual concentrations of sulfa drug solution after *t* minutes of treatment at experimental conditions. Then, the reaction mixture was further incubated overnight to affirm any a detectable change in the removal percentage. Heat-inactivated laccase was added to the reaction mixture to determine a negative control.

Factors affecting removal of sulfa drugs using purified laccase

To evaluate the effect of incubation temperature on enzymatic removal technique, the purified laccase was incorporated into each sulfa drug solution containing 1mM HBT and incubated at different temperatures (30-60°C) for 60 min.

The influence of different pH values on removal percentage of SDZ and STZ by laccase was determined after adjusting the initial pH of each sulfa drug solution using 0.1M citrate-phosphate buffer to pH ranged from 3 to 8. Then, the purified laccase (81.3 Umg⁻¹) and 1mM HBT were supplemented to the reaction mixture and incubated for 60 min at 40°C and 50 rpm.

The effect of HBT on the removal percentage of each sulfa drug was investigated by insertion of different HBT concentrations (0.25 up to 1.5 mM) to the SDZ and STZ solutions. The removal percentage of both SDZ and STZ using laccase was determined as mentioned above.

Sulfa drug and dye toxicity test

The toxicity effect of untreated and laccase-treated SDZ and STZ solutions and dyes (congo red and malachite green dye solutions, 100 mgL⁻¹) on *Bacillus cereus* LC314797 and *Staphylococcus aureus* KT337489 (as Gram-positive bacteria), and *Klebsiella pneumoniae* KF771031 and *Salmonella enterica* MK127926 (*Salmonella*_invA. Edited.sqn *Salmonella*-Anatum-Alb118-strain) (as Gram-negative bacteria) was estimated. In this experiment, each tested bacterial strain was cultured in the Mueller-Hinton broth to reach the OD₆₀₀ of 0.1. Each sulfa drug and dye solution treated and untreated with laccase (previously prepared) was inserted separately into bacterial culture media and incubated at 37°C for 10 h. Changes in the OD₆₀₀ of each bacterial strain were then recorded every 2 h. Bacterial cultured in the absence of sulfa drug and dye was performed

as a negative control for each experiment. The percentage of bacterial growth inhibition was defined as $[(1 - OD_{600S} / OD_{600C}) \times 100]$, where OD_{600C} = OD of the control, OD_{600S} = OD of the sample³². Well-diffusion method was also used to investigate dye toxicity.

RESULT AND DISCUSSION

Selection of best substrate for laccase production

Since the high cost of the enzyme is a major limitation in using laccase in an industrial scale; using agricultural wastes not only decreases the cost but also solves an environmental problem³³. Therefore, the current study elucidated the potential economical advantages of different solid substrates for *S. mutabilis* A17 laccase production under SSF. Table 2 showed a significant variation on laccase production regarding to the chemical structure of the substrates. The maximum production of *S. mutabilis* A17 laccase was detected using cotton seed cake (6.3 Umg^{-1}) followed by peanut cake (5.5 Umg^{-1}) and castor seed cake (5.0 Umg^{-1}). The other solid substrates such as chicken feather, wheat straw, wheat bran, soybean and rice bran showed decreasing in the productivity of laccase. It was observed that, the detectable variation on laccase production by

S. mutabilis A17 with the experimental natural substrates may be due to the fluctuation of the chemical composition of these natural substrates as observed in Table 1. The highest productivity of laccase by tested strain using cotton seed cake may be closely related to its high content of amino acids, including arginine, lysine and alanine compared to the other substrates. Thus, seed cotton was selected as the best solid substrate for maximum laccase production for further studied. The maximum *Neurospora sitophila* laccase production was achieved when using corn cobs as substrate after 96 hours of fermentation period³⁴.

Optimal conditions of solid state fermentation

The effect of initial moisture content of cotton seed cake substrate and inoculums size of *S. mutabilis* A17 on laccase production was studied. The maximum enzyme production was attained at 70% moisture content of cotton seed and 3 ml of inoculums size (data not shown). Similarly, maximum activity of *N. sitophila* laccase was attained at 70% moisture content for corn cobs and rice straw and 60% for sugarcane bagasse³⁴. The higher moisture content can affect enzyme production, promote contamination and reduce heat and oxygen transfer, while low moisture content can disturb nutrients availability and

Table 2. Laccase production by *S. mutabilis* A17 using solid natural substrates

Substrate (5 g)	Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mgProtein)
Chicken feather	3.21±0.08d	0.749±0.02c	4.3±0.11d
Cotton seed cake	5.84±0.15a	0.931±0.03a	6.3±0.17a
Peanut cake	5.23±0.14b	0.957±0.03a	5.5±0.15b
Wheat straw	3.25±0.08d	0.855±0.02b	3.8±0.11e
Castor seed cake	4.32±0.11c	0.864±0.03b	5.0±0.13c
Rice bran	2.42±0.06f	0.732±0.01c	3.3±0.09f
Wheat bran	2.8±0.07e	0.728±0.02c	3.8±0.11e
Soybean	2.5±0.07ef	0.701±0.01c	3.6±0.09ef

Table 3. Effect of different metal ions on activity of the purified SSF laccase produced by *S. mutabilis* A17

Metal ions	Relative activity %
Control	100±2.66fg
Zn ²⁺	92±2.43gh
Cu ²⁺	180±4.76b
Ca ²⁺	128±3.38d
Mg ²⁺	89±2.35h
EDTA ²⁺	101±2.67fg
Ba ²⁺	195±5.16a
CO ²⁺	109±2.88ef
Fe ²⁺	70±1.85j
Mn ²⁺	166±4.39c
K ²⁺	112±2.96e
Na ²⁺	102±2.69fg
Cd ²⁺	25±0.66k

The enzyme activity was determined in the absence and presence of different metal ions at concentration 10 mM after a 30 min exposure to each ion. The relative activity was expressed as the percentage ratio of the activity of the enzyme incubated with the effectors to that of the untreated enzyme.

growth⁷. The highest *T. versicolor* laccase activity was produced in SSF at 28, pH5 and moisture content 75%³⁵.

The supplementation of basal solid medium of *S. mutabilis* A17 with extra carbon sources exerts significant effect on enzyme production (Fig. 1A). Out of these carbon sources, glucose (1% w/v) exhibited maximal enzyme production (7.6 Uml⁻¹) comparing to the basal solid medium without addition of external carbon sources. The over production of *S. mutabilis* A17 laccase under SSF using addition of glucose as carbon sources may be due to the compatible effect of glucose that begins biomass production. Vantamuri and Kaliwal³⁶ reported that the maximum *Marasmius* sp. BBKAV79 laccase production was estimated after addition of ammonium sulphate and starch into the growth medium.

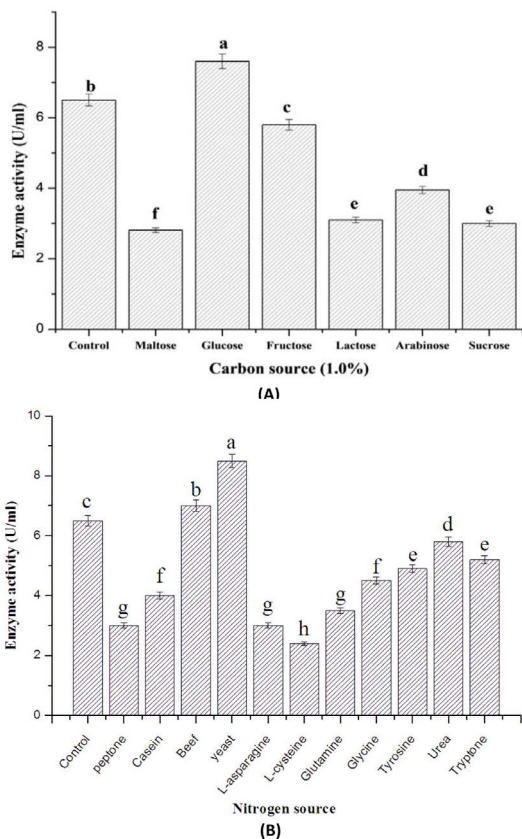


Fig. 1. Effect of supplementation of different carbon sources (A) and nitrogen sources (B) on laccase production by *S. mutabilis* A17 strain under solid state fermentation.

The incorporation of various nitrogen sources of basal solid medium of tested strain exhibited a significant effect on enzyme production (Fig. 1B). It was observed that, the maximal enzyme production was obtained by insertion of yeast extract (8.5 Uml⁻¹) followed by beef extract (7.0 Uml⁻¹), meanwhile L-cysteine had repressor effect on enzyme production. El-Batal et al.³⁰ revealed that the highest *Pleurotus ostreatus* laccase activity was attained when malt extract was added into SSF medium. Production of *Y. lipolytica* YL4 recombinant laccase was significantly increased after optimization of ammonium chloride, thiamine, yeast extract and sucrose levels in the medium³⁷.

Purification of laccase

The crude enzyme was precipitated by using 70% ammonium sulfate followed by anion exchange and gel filtration. The final specific activity and purity of *S. mutabilis* A17 laccase were raised to 81.3 Umg⁻¹ and 19.4 fold, respectively, with 52.8 % yield and its molecular weight was 115 kDa (Data not shown). *Streptomyces psammoticus* laccase precipitation by ammonium sulfate (30–60% saturation) increased the specific activity of laccase to 7.6 Umg⁻¹, followed by DEAE cellulose and gel filtration to increase the purification fold to 12.1, specific activity of 21.6 Umg⁻¹ with final yield of 22.1%³⁸. Gunne and Urlacher³⁹ reported that the molecular weights of *Streptomyces griseus* and *Streptomyces aviceus* laccases were 114 and 98 kDa respectively.

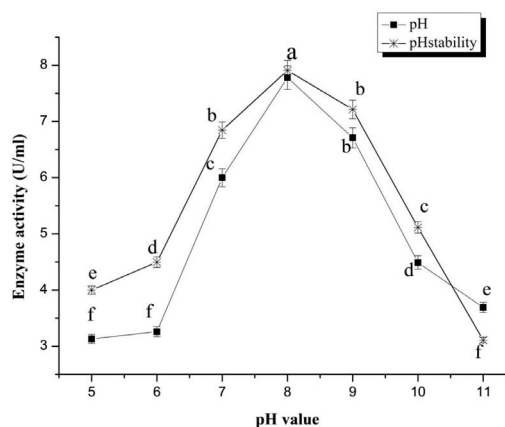


Fig. 2. Effect of pH and pH stability on the activity of purified SSF laccase

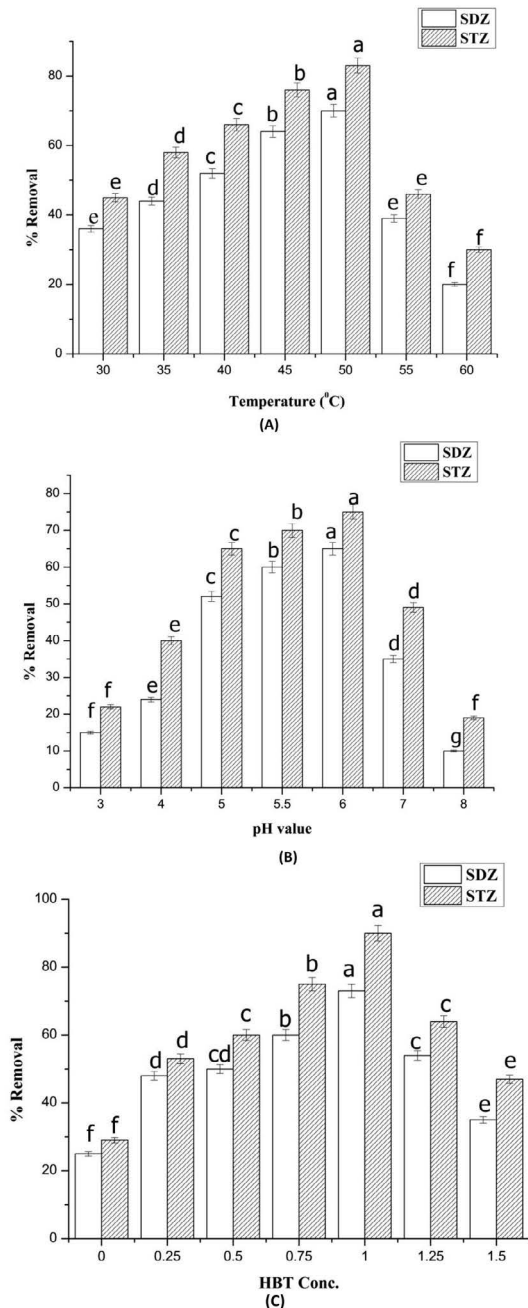


Fig. 3. Optimization of removal conditions of sulfadiazine (SDZ) and sulfathiazole (STZ) using purified SSF laccase. The sulfa drug (SDZ and STZ) solutions were treated with laccase (81.3 U/mg) and incubated at different incubation-temperatures (A), initial pH-values (B) and HBT concentrations (C).

Characterization of the purified laccase

Optimal pH and pH stability

From the profile of pH stability, the purified *S. mutabilis* A17 laccase had maximum structural and catalytic stability at pH range from 7.0–9.0 with optimum at pH 8.0 (Fig. 2). The optimum temperature achieved maximum SSF laccase activity was 40°C (Data not shown). Kuddus et al.⁴⁰ reported that *P. putida* laccase exhibited maximum activity at pH 9. Optimum pH for *Neurospora sitophila* laccase activity through solid state fermentation was attained at pH 5³⁷.

Effect of different metal ions

S. mutabilis A17 laccase activity was highly increased by the addition of Ba²⁺, Cu²⁺ and Mn²⁺ by 195, 180 and 166%, respectively. It could be observed that, there was significant reduction in the activity of enzyme in presence of Cd²⁺ by 25% (Table 3). The presence of EDTA as chelating agent exerted no inhibitory effect on enzyme activity. Telke et al.⁴¹ reported that *Pseudomonas* sp. LBC1 laccase showed increased activity by insertion of CuSO₄. Activators as Cu²⁺, Mg²⁺, Ca²⁺ and Zn²⁺ had stimulating effect on activity of *Pleurotus ostreatus* laccase. Meanwhile, inhibitors as Cd²⁺ and Ba²⁺ caused inhibition of laccase activity, and

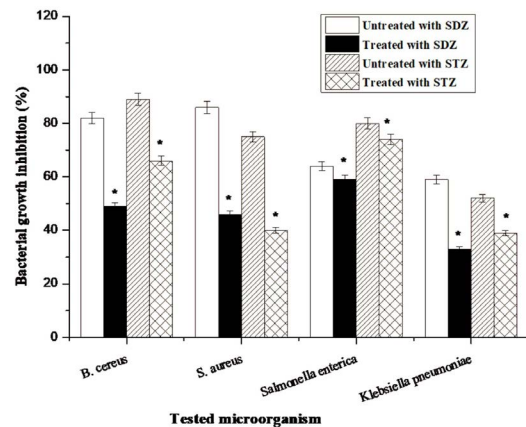


Fig. 4. Growth inhibition percentage of untreated and laccase-treated sulfa drugs (sulfadiazine (SDZ), sulfathiazole (STZ)) against tested bacterial strains. Where, Untreated: sulfa drug solution without SSF laccase; Treated: sulfa drug solution treated with SSF laccase. The columns followed by asterisks are significantly different according to paired-samples t test. Values are the mean of three replicates. The bars represent the standard error of the mean.

Co²⁺ giving highest inhibition reaching 70%. On the other hand, EDTA did not have any effects on laccase activity³⁰

Substrate specificity

The affinity of purified *S. mutabilis* A17 laccase towards various substrates was summarized in Table 4. It was observed that the enzyme preparation showed broad substrate specificity with higher affinity for 2,6 dimethoxy phenol and guaiacol followed by hydroquinone. A low affinity of enzyme towards tyrosine was exhibited. Liu et al.⁴² reported that the oxidation of phenolic substrates by laccases were dependent on pH profiles. The redox potential difference between 2,6 dimethoxy phenol and the type I copper of laccase increased with increasing pH value⁴³. Moreover, *Klebsiella pneumoniae* laccase exhibited high activity toward 2,6 dimethoxy phenol in the pH 7.5–8.5.

Removal of sulfa drugs using SSF laccase

In the current study, removal of SDZ and STZ by laccase in the presence of 1mM HBT was investigated. Both sulfa drugs were efficiently removed after incubation with laccase (81.3 Umg⁻¹) for 60 min by removal percentages; 60% of SDZ

and 70% of STZ (Data not shown). Liang et al.⁴⁴ reported that the removal of sulfadimethoxine at 1 ppm and 10 ppm concentrations were 73.3% and 65.6%, respectively, when using *Trametes versicolor* assisted-laccase. The removal percentage of sulfamethazine, sulfadiazine and sulfamethoxazole by *Pleurotus ostreatus* HAUCC 162 mediated-laccases were 97.5%, 98.1% and 97.8%, respectively, after 60 min⁴⁵.

Factors affecting sulfonamide removal

The maximal removal percentages of SDZ and STZ solutions treated with laccase were attained at 50 and pH 6 in presence of 1mM HBT, represents 73 and 90% removal percentage, respectively (Fig. 3). Margot et al.⁴⁶

Table 4. Affinity of the purified SSF laccase towards various substrates with different concentrations

Substrate	Concentration (mM)	Enzyme activity (U/mL)
2,6 dimethoxy phenol	2	6.97±0.18d
	4	9.15±0.24c
	6	10.43±0.27b
	8	11.52±0.31a
	10	11.97±0.32a
Catechol	2	2.72±0.07c
	4	4.55±0.12b
	6	5.89±0.16a
	8	5.98±0.16a
	10	6.13±0.16a
Guaiacol	2	4.50±0.12e
	4	5.98±0.16d
	6	6.63±0.18c
	8	7.37±0.19b
	10	8.97±0.24a
Hydroquinone	2	3.41±0.09d
	4	5.49±0.15c
	6	6.28±0.17b
	8	6.43±0.17ab
	10	6.87±0.18a
Tyrosine	2	2.91±0.08d
	4	3.80±0.11c
	6	4.50±0.12b
	8	5.04±0.13a
	10	5.10±0.14a

Affinity of SSF laccase towards various substrates was determined by incubation of the enzyme (81.3 U/mg protein) in 10 mM phosphate buffer (pH 6.5) with various concentrations of substrate (2-10 mM) under the standard assay conditions.

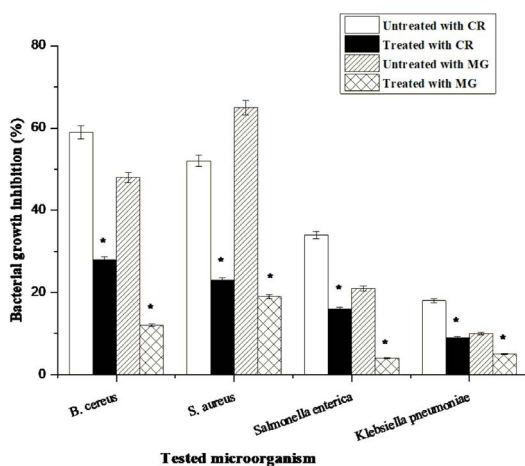


Fig. 5. Growth inhibition percentage of untreated and laccase-treated synthetic dyes (congo red (CR) and malachite green (MG)) against tested bacterial strains. Where, Untreated: dye solution without SSF laccase; Treated: dye solution treated with SSF laccase. The columns followed by asterisks are significantly different according to paired-samples t test. Values are the mean of three replicates. The bars represent the standard error of the mean.

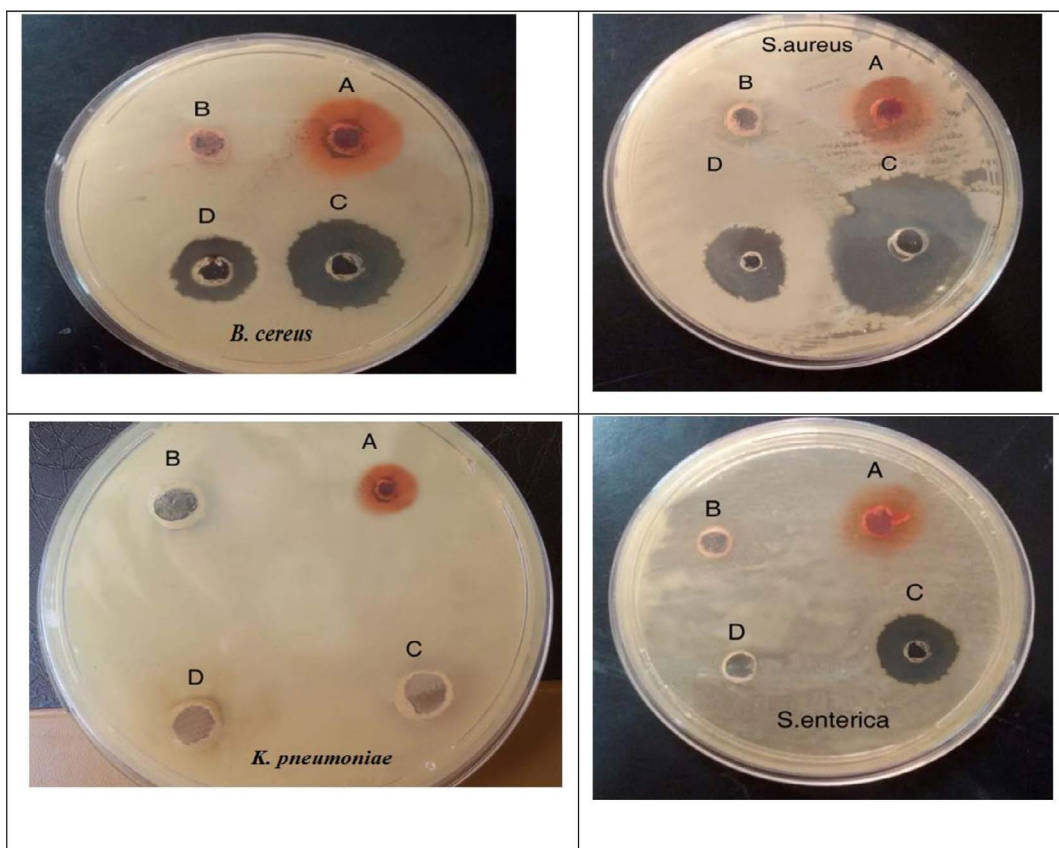


Plate 1. The toxicity effect of untreated and laccase-treated dyes (congo red and malachite green solutions, 100 mgL^{-1}) on *B. cereus* LC314797, *S. aureus* KT337489, *S. enterica* MK127926 and *K. pneumoniae* KF771031. This figure shows the growth inhibition of the tested strains treated as the following; (A) $50 \mu\text{L}$ congo red dye and $50 \mu\text{L}$ of 0.1 M sodium acetate (B) $50 \mu\text{L}$ of laccase enzyme mixed with $50 \mu\text{L}$ congo red dye, (C) $50 \mu\text{L}$ malachite green and $50 \mu\text{L}$ of 0.1 M sodium acetate (D) $50 \mu\text{L}$ of laccase enzyme mixed with $50 \mu\text{L}$ malachite green dye. The change in diameter of clear zone around the well was compared after addition of both dye solutions treated with laccase against bacterial strains.

showed that acidic pH values ranged from 5-6 caused fast oxidation of sulfamethoxazole and isoproturon compounds. Fabbrini et al.⁴⁷ reported that the mediators are considered as electron-shuttles between the substrate and laccase, with stimulating activity of the mediator. Ostadhadi-Dehkordi et al.⁴⁸ indicated that HBT is a non-phenolic laccase mediator and it considered one of the most efficient mediators, due to its high redox potential and catalytic role of N-OH group. This laccase mediator has been widely utilized for removal of synthetic dyes, polycyclic aromatic hydrocarbons, and pharmaceutical agents.

Sulfa drug and dye toxicity studies

Different bacterial strains including *B. cereus* LC314797, *S. aureus* KT337489, *S. enterica*

MK127926 and *K. pneumoniae* KF771031 were used for the evaluation of toxicity of sulfa drugs (SDZ and STZ) and also the synthetic dyes (congo red and malachite green) after laccase treatment. The growth inhibition percentage of all bacterial strains was significantly decreased after addition of both sulfa drugs and dye solutions treated with laccase to culture media. From the results in Fig. 4, it was observed that the tested sulfa drug alone have the capability to suppress the growth of all tested pathogenic bacterial strains. Meanwhile, the growth inhibition of these strains was decreased after treated with laccase-sulfonamide solutions (Fig. 4). These results proved that the antimicrobial activity and toxicity effect of SDZ and STZ was significantly inhibited by addition of

enzymatic laccase solution. The results are shown in Fig. 5 and illustrated in Plate 1 indicated that the growth inhibition of *B.cereus*, *S. aureus*, *S.enterica* and *K. pneumoniae*, treated with laccase-congo red solutions was significantly decreased by 28, 23, 16 and 9%, respectively, and 12, 19, 5 and 4% respectively in the case of laccase-malachite green.

The microbial growth of *P. syringae*, *M. luteus*, *B. subtilis*, *E. coli* and *P. aeruginosa* strains, using orange 2 dye treated with *Trametes versicolor* laccase, was increased higher than that without laccase added⁴⁹. Also, Ashrafi et al.³² proved that the growth of *M. luteus*, *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. typhi* strains was significantly increased in the presence of laccase-treated Direct Blue 71.

CONCLUSION

SSF is a very promising cultivation technique for the production of industrially-relevant laccases, especially utilizing agro-wastes as support-substrates. From our results, it could be concluded that the maximum laccase production by *S. mutabilis* A17 has been attained when cotton seed cake was used as natural substrate. Using the purified SSF *S. mutabilis* A17 laccase in the biological oxidation and detoxification of sulfonamides, and synthetic dyes including congo red and malachite green as potentially useful agent to remove environmental pollutants. The toxicity study using certain Gram positive and Gram negative bacterial strains showed a detectible decrease in the percentage of growth inhibition of the tested bacterial strains in presence of laccase-treated dyes and sulfonamides antibiotics.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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