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RESEARCH ARTICLE

Dyes decolorization by spore-bound laccase from local isolate of *Bacillus subtilis*

Ahmed Majeed Abd Zaid*, Abdulkareem J. Hashim, Ziad M. Alkhozai

College of science, Baghdad University | College of science, Baghdad University | College of biotechnology, AL-Qadisyia University

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Corresponding Author

Ahmed Majeed Abd Zaid

Abstract

B. subtilis growth on production medium resulted in spores-bound laccase activity (439.23) U/ml.The optimum pH of spore-bound laccase activity was 6.8 and retained its initial activity at pH 6.8 after incubation for 1 hour at 37˚C. The optimum temperature was at 40 ˚C and retained its initial activity after 3 hours when incubated at temperature ranged (10-50)°C.The spore-bound laccase retained 100% of its activity after 4 days.Dyes decolorization on solid medium tested by a halo formed around the growth area for all dyes used except for Methyl orange.Dyes decolorization by spore-bound laccase showed that the dyes (eriochrome black T, crystal violet, azure B) were completely decolorized in all concentrations within 10 minutes while Textile (blue), Methyl violet and Methyl orange dyes were decolorized in different percentage.

INTRODUCTION

Laccase (benzenediol:oxygenoxidoreductase; EC 1.10.3.2.) is a multi-copper blue oxidase capable of oxidizing ortho- and para-diphenols an aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical (Coll*et al*.,1993). In most cases laccases are monomeric glycoproteins contain around 500 amino acids with molecular weights in the range 60–85 kDa, depending on the carbohydrate content (Lyashenko*et al*., 2006). Laccases were widely distributed among plants, fungi (Morozova*et al*., 2007) and bacteria (Claus, 2003). CotA, which is the endospore coat component of *Bacillus subtilis*, was the most-studied bacterial laccase (Hullo*et al*., 2001). Since spores allow microorganisms to survive under drastic conditions, spore coat enzymes might also withstand high temperatures or extreme pH values. Since most fungal laccases are unstable at pH values higher than 7.0, their detoxification efficiencies for pollutants often decrease under alkaline conditions. This limits the industrial potential of fungal laccase as many processes are performed in alkaline conditions. Alternatively, spore laccases which were active in the alkaline pH range could be used for bioremediation or application in membrane reactors (Held*et al*.,2005). Approximately 10,000 different dyes and pigments were produced annually worldwide and used extensively in the dyeing and printing industries. The total world colorant production is estimated to be 800,000 tons per year, and at least 10 % of the used dyestuff enters the environment through wastes (Palmieri*et al*., 2005). The decolorization of dyes by white rot fungi was first reported by Glenn and Gold (1983), who developed a method tomeasure the lignin lytic activity of *P. chrysosporium* based upon the decolorization of sulphonated polymeric dyes. White rot fungi offers significant advantages for decomposition of recalcitrant compounds. Ligninolytic enzymes produced by white rot fungi are substrate-nonspecific, and therefore they can degrade wide variety of recalcitrant compounds.

This study aimed to examine the ability of *B.subtilis* spores-bound laccase for decolorization of 6 type of dyes.

Material and methods

B. subtilis isolatewas obtained from Department of Biotechnology, Baghdad University, Iraq. Nutrient agar, (Syringaldazine, SGZ), and all other reagent grand chemicals were purchased from Hi – Media and Sigma-Aldrich, India. Textile (blue) dye was provided from Al Diwaniyah textile factory south of Baghdad

Laccase production from *B.subtilis*

The isolate was inoculated on production medium for laccase production containing 0.8% nutrient broth, 0.2 mM $Cuso_4$, 3% glucose, 0.2% tryptone, 1 mMKcl and 1mM pyrogallol.The pH of the medium was adjusted to 7 and incubated at 37 ˚C for 3 days.The spores were collected from the tubes by centrifuging for 20 min at 4000g and then washed with 0.5 mol/L NaCl, and suspended in 0.1 M potassium phosphate buffer (pH 6.8). Finally, 1 ml spores suspension contained 100 mg wet cell. (Shukur, 2015).

Spore-bound laccase activity

Laccase activity of the spores suspension was determined using syringaldazine as the substrate. The oxidation of syringaldazine was detected by measuring the absorbance increase at 525 nm (ε525 = 65,000 L/(mol·cm)) using a spectrophotometer (Biotech Engineering Management Co. Ltd). The reaction mixture (3 ml) contained 100 µl of spores suspension sample, 2.4 ml of potassium phosphate buffer (0.1 M, pH 6.5) and 500 µl of 0.5 mmol/L syringaldazine. Spore sedimentation was not observed during incubation. The enzyme activity was assayed by the method described by Annua*et al.*, (2009) as follows:

$$\text{Laccase activity (U/L)} = \frac{\Delta Abs}{\Delta t \varepsilon l} \times \frac{\text{Total assay volume}}{\text{Enzyme sample volume}}$$

Where ΔAbs is the change in absorbance, Δt is the time of incubation (min), ε is the extinction coefficient of syringaldazine at 525 nm (65,000 M−1 cm−1), and l is the cuvette diameter (1 cm). One unit of enzyme activities was defined as the amount of enzyme required to oxidize 1µmol of substrate per minute.

Characterizations spore-bound laccase

Optimum pH for spore-bound laccase activity

The effect of pH on the activity of the laccase was determined in 0.1 M citrate-phosphate buffer (pH 3.0−5.6) , 0.1 M potassium phosphate buffer (pH 5.8 – 8) and 0.1 M Tris-HCl buffer (pH 9.0).The spore-bound laccase activity was determined by using bacterial spores suspension and the activity was measured with different buffers

Effect of pH on spore-bound laccase stability

Spores suspension (0.1 ml) was added to tube contain 2.4 ml of buffer with different range of pH included: 0.1 M citrate-phosphate buffer (pH 3.0−5.6), 0.1 M potassium phosphate buffer (pH 5.8 – 8) and 0.1 M Tris-HCl buffer (pH 8.0−9.0), then tubes incubated in 37 ◦ C for 1 hr. Laccase activity was estimated, then the relationship between pH and the remaining activity % of spore-boundlaccase was plotted.

Optimum temperature for spore-bound laccase activity

Spore-bound laccase activity was determined by incubation the spore-bound laccase in different range of temperature (10-90◦C) with optimum pH for 1 hour and then the spores-bound laccase activity was determined.

Effect of temperature on spore-bound laccase stability

Spores suspension (0.1 ml) was added to a tube and incubated at (10-90 ◦C) for 3 hr, then the laccase activity was determined. The relationship between temperature and the remaining activity % of spore-bound laccase was plotted.

Effect of operational time in spore-bound laccase stability:

Spores suspension (0.1 ml) was added to a tubes , then incubated at 10 ◦ C with optimum pH (6.8) using different time range (10) days, then the spore-bound laccase activity was detemined. The relationship between operational time and the remaining activity % of enzyme was plotted.

Dyes decolorization

Dyes decolorization by *B. subtilis* isolate on solid-state medium

The dyes eriochrome black T, azur B, crystal violet, methyl violet, methyl orange and textile (blue) dye were prepared at concentration 150 mg/l in nutrient agar plates . The *B. subtilis* isolate were inoculated in the center of the plate and incubated at 37℃ for 3 days.

Dyes decolorization by *B.subtilis* spore-bound laccase

For decolorization experiments, the dyes eriochrome black T , azur B, crystal violet, methyl violet, methyl orange and textile(blue) dye were used. Dyes were prepared in 100 ml distilled water at different concentrations (25, 50, 75, 100, 125 ppm) containing 10 ml of spore suspension (100 mg / ml) ,dye samples without spores

suspension were given the same treatment as control, dyes was shook at 120 rpm by water bath at 40 °C. Samples were withdrawn every 10 min and centrifuged at 4000 rpm for 20 min and analyzed by UV/VIS spectroscopy using a (Biotech Engineering Management Co. Ltd) spectrophotometer in the spectral range of 400–800 nm.Percent of decolorization was calculated as follows (Telke *et al*., 2010).

$$\text{Decolorization (\%)} = \frac{\text{Initialabsorbance} - \text{finalabsorbance}}{\text{Initialabsorbance}} \times 100\%$$

Results and discussion

Laccase production from *Bacillus subtilis*

B.subtilis growth on production medium resulted in spore-bound laccase activity (439.23) U/ml.

Characterizations spore-bound laccase

Optimum pH for spore-bound laccase activity

The optimum pH of spore-bound laccase activity was 6.8 with activity (452.48) U/ml, with a decrease in spores laccase activity at the pH value moved towards alkaline range (8.0-90), it was also noticed a decrease in the activity of the enzyme at acidic values (3.0-5.0). However, spores laccase activity was highest at natural pH (6-7) fig. (1).This finding was agreed with Wang *et al*.,(2011) who reported that "The pH profile for laccase activity showed a peak of maximum activity at pH 6.8".

Effect of pH on spore-bound laccase stability

Spore-bound laccase was retained its initial activity at pH 6.8 after incubation for 1 hr at 37˚C, and the spore-bound laccase maintained 94-98 % of its initial activity at pH (6-6.5), while maintained about 76% of its activity at pH 9 fig. (2).Wang *et al*., (2011) observed that, the laccase activity of strain WD23 showed higher stability over a broad pH range. Within a pH range from 5.0 to 7.0, the half-life was more than 240 h. In contrast to fungal laccases, spore-bound laccase activity of *B. subtilis* WD23 showed a very high stability at alkaline pH values (t1/2 = 15 d at pH 9.0).

Optimum temperature for spore-bound laccase activity

The result showed an increase in the activity at 40°C laccase activity reached to (468.2) U/ml then the activity was declined with increasing temperature up to 40°C, and minimum activity observed at 90°C was (146.75) U/ml. However, laccase activity was decreased under 40°C too fig. (3).Wang *et al*.,(2011) reported that, "The optimum temperature of the spore-bound laccase was determined at pH 6.8, and the maximum activity was observed at 60°C. It showed higher activity within a temperature range from 40 to 70°C".

Effect of temperature on spore-bound laccase stability

Results in figure (4) showed that *B.subtilis* spore-bound laccase retained its initial activity after 3 hours when incubated at temperature ranged (10-50) °C. Then the activity decreased with increasing temperature at 90˚C, the enzyme retained 27% of the initial activity.Wang *et al*.,(2011) reported that ," the laccase had a high stability at the optimum temperature (t1/2 = 68 h at 60 °C).

Effect of operational time in spore-bound laccase stability

To determination the operational time activity of *B. subtilis* spore-bound laccase, spores suspension were incubated for 10 days at 10 C˚, pH 6.8. Enzyme activity started to decrease after 5 days of incubation period. The enzyme retained 100% of its activity after 4 days, 72% after 5 day and 23% after 10 days of incubation fig. (5).Wang *et al*.,(2011) observed that, the spore-bound laccase could be reused and the residual activity remained about 50% after 5 consecutive cycles.

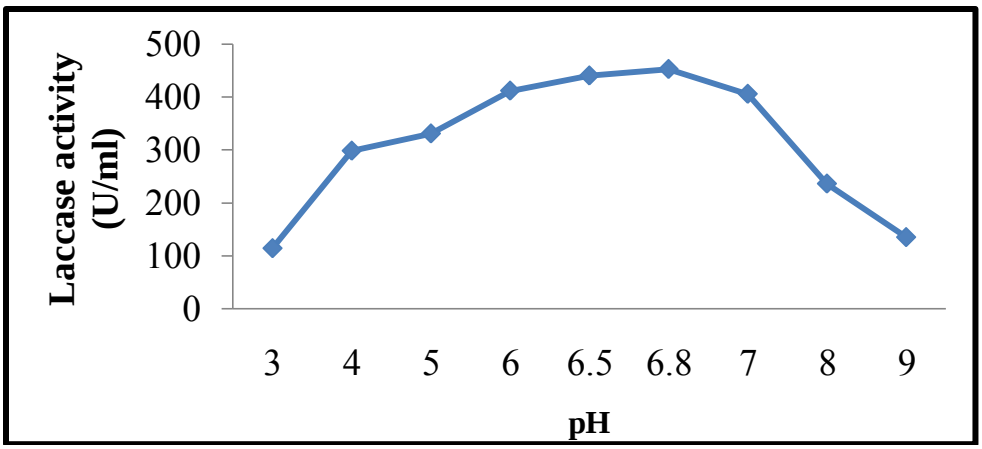

Figure (1): Effect of pH on laccase activity

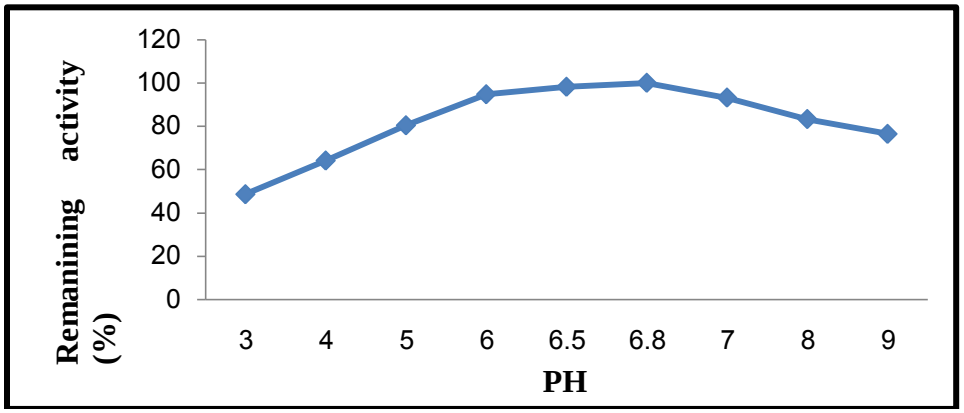

Figure (2) : Effect of pH on laccase stability after incubation for 1 hour in 37 ℃

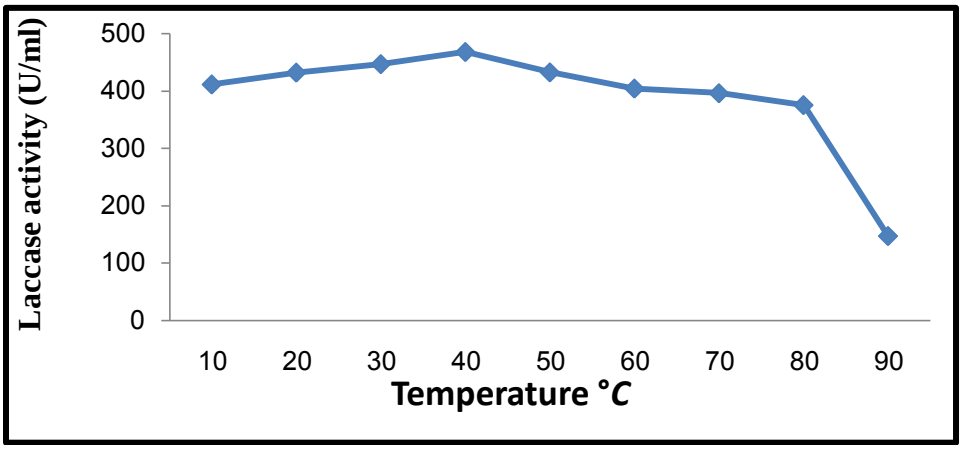

Figure (3) : Effect of temperature on laccase activity after incubation for 1 hour at pH 6.8

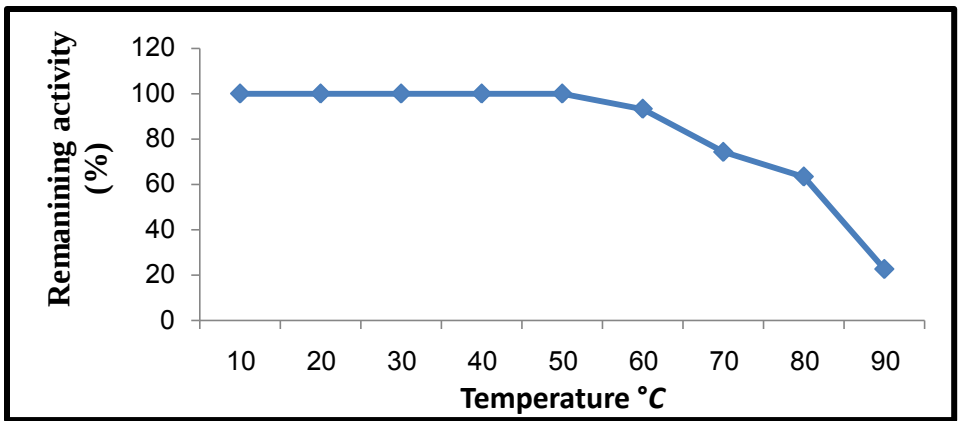

Figure (4) : Effect of temperature on laccase stability after 3 hours of incubation at pH 6.8

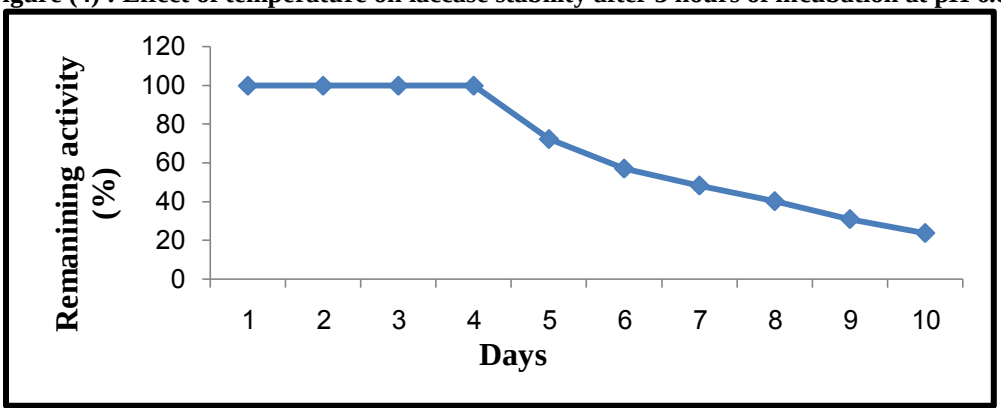

Figure (5) : Effect of operational time on laccase stability and incubation at 10 ℃ at pH 6.8

Dyesdecolorization

Dyes decolorization by *B.subtilis* isolate on solid-state medium

B.subtilis growth after 3 days at 37℃ on nutrient agar plates containing dyes at concentration 150 mg/l caused clear halo around the growth area for (Crystal violet, Eriochrome Black T, Azur B and Methyl violet) dyes. For textile (blue) dye, the halo was formed, but was not clear. Finally, no halo was appearedaround the growth area for methyl orange, this may be due to the chemical structure of this dye fig. (6).This finding is also in consistent with findings of Montira and Sukallaya, (2012) who reported Bacterial decolorization ability was confirmed by the clear halo formed around each colony by plate assay within 48 h.

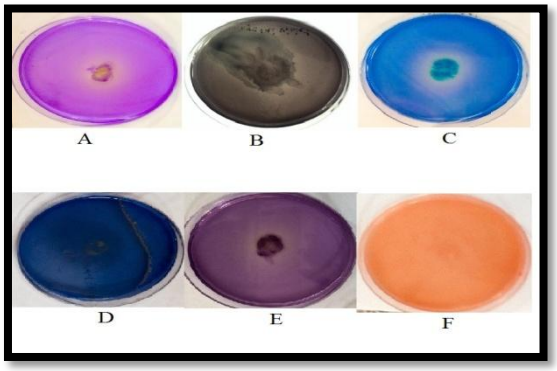

Figure (6) :Decolorization of six dyes by *B. subtilis* isolate after incubation for 3 days at 37 C˚ using nutrient agar supplement with each dye at concentration 150 mg/L. (A) Crystal Violet , (B) Eriochrome Black T, (C) Azur B, (D) Textile (blue), (E) Methyl Violet, (F) Methyl orange

Dyes decolorization by *B.subtilis* spore-bound laccase

Dyes decolorization by *bacillus subtilis* spore has been investigated using spores with laccase activity (468.2) U/ml in order to prove their potential application in the treatment of dyestuff wastewater. The reaction

mixture contains 10 ml of spore suspension and 100 ml of dyes (25, 50, 75, 100, 125 ppm) in distilled water, the dyes decolorization was studied at 40◦C. The results showed that the dyes (eriochrome black T, crystal violet, azure B) was completely decolorized in all concentrations within 10 minutes ,the same for textile (blue) dye in concentrations (25, 50, 75 ppm) which was completely decolorized within 10 minutes , while in concentrations (100, 125 ppm) was completely decolorized after 20 minute. Methyl violet and Methyl orange dyes were decolorized in different percentage, table (2) fig. (7).Increasing the concentration of methyl violet and the methyl orange lead to decrease percentage of dye decolorization by spore laccase. This may be interrelated to molar extinction coefficients and purity of each particular dye. Moreover, it is known that nature and position of the dye substituent group strongly affect the decolorization extent (Couto, 2006).

Wang *et al*., (2010) showed that the ability of spore laccase from *B. subtilis* to decolorize dyes (methyl orange and methyl violet), the methyl orange was maximally decolorized (≈ 70 %), followed by methyl violet (≈ 50 %) at 5 days treatment.

Table (2): Dyes decolorization (%) by spore-bound laccase.

Dyes concentration (ppm)	**Decolorization of Textile dye (blue) %**			**Decolorization of Methyl Violet %**			**Decolorization of Methyl Orange %**		
Incubation Times (min.)	**10**	**20**	**30**	**10**	**20**	**30**	**10**	**20**	**30**
25	**100**	**-**	**-**	**77.4**	**83.4**	**98.7**	**24.2**	**27.6**	**29.5**
50	**100**	**-**	**-**	**64.6**	**69.3**	**73.5**	**13.2**	**16.3**	**20**
75	**100**	**-**	**-**	**52.1**	**56.8**	**60.5**	**10.8**	**12.2**	**13.8**
100	**90.3**	**100**	**-**	**37**	**39.3**	**41.3**	**2.2**	**3.4**	**4.1**
125	**87.6**	**100**	**-**	**14.3**	**15.2**	**17.4**	**1.7**	**2.3**	**2.6**

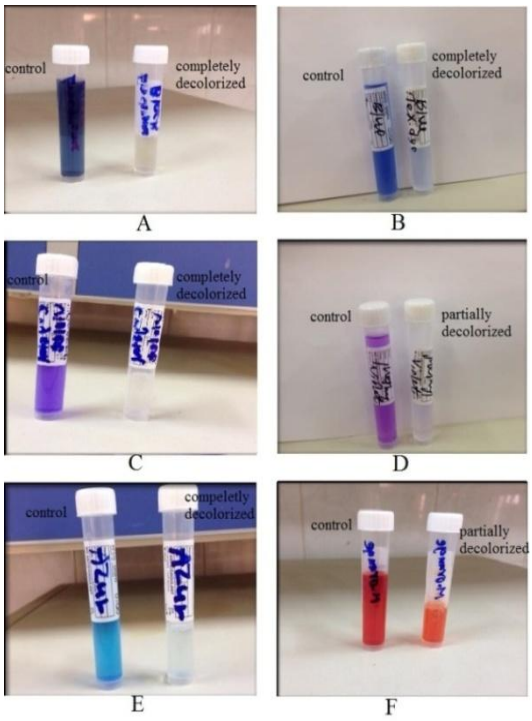

Figure (7) : Dyes decolorization by spores-bound laccase, dyes with concentration 25 ppm:
A :Eroichrome Black T was completely decolorized within 10 minute.
B: Textile dye (blue) was completely decolorized within 10 minute.
C: Crystal Violet was completely decolorized within 10 minute.
D: Methyl Violet was partially decolorized within 30 minute.
E Azure B was completely decolorized within 10 minute.
F: Methyl Orange was partially decolorized within 30 minute.

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