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Decolorization of Reactive Dyes by Spore-bound Laccase from Local Isolate of *Bacillus* sp.

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Abstract

Utilization of bacterial activity for decolorization of coloured products is one the most promising industrial strategy, as an eco-sustainable and cost-competitive alternative to physicochemical methods. Laccase production from *Bacillus* sp. was studied for its decolorization influences on different dyes (Indian ink, Brilliant green, Bromothymol blue, Crystal violet, Safranin, Bromophenol blue, Methelen blue, Giemsa stain, Nigrosin, Toluidin blue, Neutral red, Phenol red, Hanna, Blood, Bengal rose B, Bromkresol green, 4-Bromoaniline, Aniline blue, 2,6-Dichlorophenol invophenol, Curcumin, Acridine orange, Indigo carmine, Xylene cynol FF,10G, Alizarine yellow GG and Kongorose). After 5 days of incubation of the spore-bound laccase with different dyes, the maximum decolorization have seen in toluidin blue (89%), neutral red (84%), and indigo carmine (83%), brilliant green (82%) and acridine orange (82%), while the final percentage of degradation of other dyes were less than 65%. However, no additional redox mediator was added to the reaction, which suggests that this enzyme could be used in industries for effluents treatment. In addition, our results indicate that the percentage of decolonization have decreased as the dyes concentrations were increased.

Keywords: Spore-bound laccase; *Bacillus* sp.; Decolonization

الإزالة اللونية للأصباغ الفعالة بواسطة انزيم اللاكييز المرتبط بالسبور المنتج من العزلة المحلية *Bacillus* sp.

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الخلاصة

الاستفادة من النشاط البكتيري لإزالة اللون من المنتجات الملونة هو احد الاستراتيجيات الصناعية الواعدة، كبديل بيئة مستدامة وتنافسية من حيث التكلفة للطرق الفيزيائية. تمت دراسة التأثيرات المختلفة لأنزيم اللاكييز المرتبط بالسبور المنتج من بكتيريا *Bacillus* sp. على إزالة الألوان للأصباغ المختلفة Indian ink, Brilliant green, Bromothymol blue, Crystal violet, Safranin, Bromophenol) blue, Methelen blue, Giemsa stain, Nigrosin, Toluidin blue, Neutral red, Phenol red, Hanna, Blood, Bengal rose B, Bromkresol green, 4−Bromoaniline, Aniline blue, 2,6−Dichlorophenol invophenol, Curcumin, Acridine orange, Indigo carmine, Xylene cynol FF,10G, Alizarine yellow GG and (Kongorose. بعد خمس أيام من حضن انزيم اللاكييز البوغي مع الاصباغ المختلفة، فأن الحد الاعلى لإزالة اللون كان في toluidin الأزرق (89٪) و neutral red (84%) و indigo carmine (83٪) و brilliant green (82٪) و acridine orange (82٪) ، في حين كانت النسبة النهائية لتحطم الأصباغ الأخرى أقل من 65٪. لم يتم اضافه أي وسيط لعملية الأكسدة والاختزال ، وهذا يشير إلى أن هذا الإنزيم يمكن أن يستخدم في الصناعات لمعالجة النفايات السائلة. بالإضافة إلى ذلك، اشارت النتائج إلى انخفاض نسبة الإزالة عند زيادة تراكيز هذه الأصباغ.

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Introduction:

Laccases (EC 1.10.3.2) are a group of highly unspecific enzymes containing up to 4 copper atoms within their catalytic sites. It's a multicopper blue oxidase capable of oxidizing orthodiphenols, paradiphenols and aromatic amines by removing an electron and proton from a hydroxyl group to form a free radical. Laccases oxidize their substrates by a one-electron oxidation mechanism, and they use molecular oxygen as an electron acceptor [1]. Among laccases all the primary sequence, induction mechanism, physico-chemical (e.g., isoelectric point and carbohydrate content), and biochemical characteristics are variable. However copper binding sites are strictly conserved [2]. In general, the laccase molecule as an active holoenzyme form is a dimeric or tetradimeric glycoprotein exhibiting four neighbor copper atoms, which are distributed among different binding sites. Laccases from fungal and plant sources have been extensively isolated and studied [3]. On the other hand, laccase activity was reported in relatively few bacteria such as *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Streptomyces griseus*, *Streptomyces cinnamomensis*, *Bacillus subtilis* and *Ralstonia solanacearum* [4, 5, 6, 7]. Nevertheless, databank searches have increasingly providing evidence for laccase genes distribution in genomes of many other bacterial species. The most-studied bacterial laccase is the one isolated from endospore coat component of *B. subtilis*, which coded by *CotA* gene [8]. Bacterial spores known for their abilities to survive drastic conditions, therefore spore coat enzymes are assumed to withstand high temperatures and extreme pH values. In contrast most fungal laccases are rendering at alkaline pH and that often lead to reduction in their pollutants detoxification efficiency. Consequently, this limits the industrial potency of fungal laccase as many processes are performed in alkaline conditions. Alternatively, spore laccases active in broad alkaline pH range and could be used for bioremediation or applied in membrane reactors [9]. Furthermore, laccases have many biotechnological applications due to their oxidation ability towards a wide spectrum of phenolic and non-phenolic compounds. Other applications of laccase include the cleaning the industrial effluents, mostly from paper, pulp, textile and petrochemical industries. Also it is used in medical diagnostics and for cleaning herbicides, pesticides and some explosives in soil. Laccase has many applications in agricultural, medicinal and industrial areas. Additionally Laccases are used to clean water in purification systems. It has enormous ability to remove xenobiotic substances and produce polymeric products, and hence used for many bioremediation purposes [10]. Therefore, we aimed in this study to measure the decolorization of different dyes in variable concentration by laccase.

Media and chemicals:

Microorganism and culture conditions

Bacillus sp. isolate was obtained from Department of Biology, Baghdad University, Iraq. The culture of *Bacillus* sp. was maintained on nutrient agar slants, and subcultured on nutrient agar containing 0.4 mM of $CuSO_4$ after adjusting the pH to 7 as described in [11]. The plats were incubated at 37 °C for 5 days. Microbial growth were harvested in 3 ml of 1 M KCl and used in the preparation of spore suspension.

Preparation of spore suspension:

Bacterial suspension centrifuged, the precipitated cells then washed with 0.5 M NaCl, and suspended in 0.1 M sodium phosphate buffer (pH 6.8) [12]. Spore laccase activity was determined using spore suspension contained 100 mg wet cell per ml.

Spore laccase activity:

Spore-bound laccase activity was determined at 40°C using syringaldazine (dissolved in absolute ethanol) as the substrate. The oxidation of syringaldazine was detected by measuring the absorbance increase at 530 nm after 3 min using a spectrophotometer. The reaction mixture (3 ml) contained 100 µl of spore suspension, 2.4 ml of 0.1 M phosphate buffer, (pH 6.8) and 0.5 ml of 0.216 mM/L syringaldazine in methanol (0.3 ml) [13].

Dye decolorization by spore-bound laccase:

Stock solutions of 25 dyes (Indian ink, Brilliant green, Bromothymol blue, Crystal violet, Safranin, Bromophenol blue, Methelen blue, Giemsa stain, Nigrosin, Toluidin blue, Neutral red, Phenol red, Hanna, Blood, Bengal rose B, Bromkresol green, 4-Bromoaniline, Aniline blue, 2,6-Dichlorophenol invophenol, Curcumin, Acridine orange, Indigo carmine, Xylene cynol FF,10G, Alizarine yellowGG and Kongorose) were prepared in sterilized distilled water and diluted to 25 mg/L. The optical density of each dye was measured depending on its λ-max using spectrophotometer. The reaction mixture was prepared by adding volume 25mg/ml spores (0.5 ml) to volume dye solution (9.5 ml) and incubated at 37 C° for 5 days. Control sample was prepared for each dye without spores and treated under the same

condition. Decolorization efficiency of spore laccase was assessed by monitoring the decrease in absorbance under maximum wavelength of the dye and expressed in terms of percentage [14]. Decolorization activity was calculated as follows [15]:

$$\text{Decolorization } \% = \frac{\text{Control sample } - Ab\ of\ dye\ treatment}{Control\ sample} \times 100\%$$

Effect of various concentrations of dyes on decolorization efficiency:

The dyes which showed more than 80 % decolorization by spore laccase were prepared at different concentrations 25, 50, 100, 200, 400 and 800 mg/L, then treated with 25 mg/ml spores suspension and incubated at 37 °C for 5 days. The percentage of decolorization was measured, while dye solutions without spores were used as a control for each sample.

Results and Discussions:

Dye decolorization experiments:

The spore-bound laccase was used for the decolorization of Indian ink, Brilliant green, Bromothymol blue, Crystal violet, Safranin, Bromophenol blue, Methelen blue, Giemsa stain, Nigrosin, Toluidin blue, Neutral red, Phenol red, Hanna, Blood, Bengal rose B, Bromkresol green, 4-Bromoaniline, Aniline blue, 2,6-Dichlorophenol invophenol, Curcumin, Acridine orange, Indigo carmine, Xylene cynol FF,10G, Alizarine yellowGG and Kongorose in order to demonstrate their potential in the treatment of dyestuff wastewater. The results show that 89, 84, 83, 82 and 82, % of the Toluidin blue, Neutral red, Indigo carmine, Brilliant green and Acridine orange respectively, were removed in five days, while the final percentage of degradation of other dyes were less than 65% , Figure-1. The spore-bound laccase could efficiently decolorize the dyes without additional redox mediators.

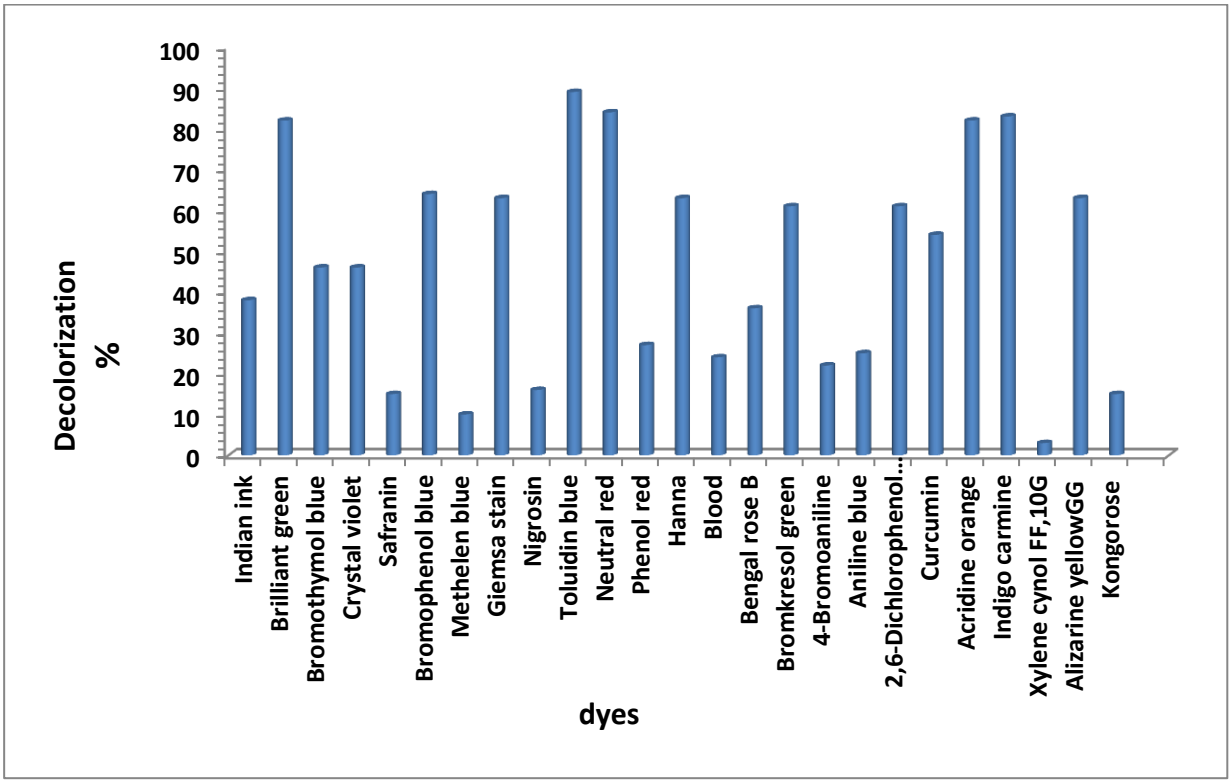

Figure 1- Decolorization of dyes with spore-bound laccase from *Bacillus* sp. incubated at 37 C° for 5 days.

Wang, *et al.*, [16], found that 90% of the remazol brilliant blue R and alizarin red were removed by spore laccase from *B. subtilis* when incubated at 37 C° for 5 days, while 50 - 70% in the treatments observed for other dyes (congo red, methyl orange and methyl violet). Saratale, *et al.*, [15], found that 97% of the malachite green and cotton blue were removed in the first day by laccase from mycelium of *Aspergillus ochraceus* when incubated at 30 C°, while the final percentage of degradation of other dyes (crystal violet and methyl violet) was less than 61%. Ratanapongleka and Phetsom [17], found that laccase from *Lentinus polychrous* was removed 85% of the anthraquinonic acid blue 80 (20 mg/l) in 2 houres, while 20 % was removed of indigo carmine and less than 10% decolorization of methyl

orange in the same period of time. These results indicate that spore laccase from *Bacillus* spp. decolorizes toluidin blue, brilliant green, neutral red, acridine orange and indigo dyes more efficiently than other dye groups. Ciullini *et al.*, [18], explained that laccases show substrate specificities and the chemical structures of the dyes due to the differences in electron distribution, charge density and steric hindrances.

Effect of various concentrations of dyes on decolorization pattern:

The results of different concentrations of dyes which gave more than 80% of decolorization like Brilliant green, Toluidin blue, Neutral red, Acridin orange and Indico carmine, showed that increment in concentration of theses dyes leads to decrease in decolorization activity Table-1.

Table 1- Effect of various concentrations of dyes to decolorization performance (%)

Conc. mg/L / Dye	25	50	100	200	400	800
Toluidin blue	89%	63%	44%	31%	25%	18%
Neutral red	84%	59%	35%	26%	22%	17%
Indigo carmine	83%	59%	37%	28%	23%	17%
Brilliant green	82%	58%	36%	25%	18%	13%
Acridine orange	82%	56%	34%	24%	19%	16%

Ratanapongleka and Phetsom [17], found that the effect of dyes concentration on decolorization was reduced by increasing the dye concentration from 20 to 140 mg/L. Furthermore, it was noticed that the enzyme decolorized dye up to 85% of the day in 3 hours when the day concentration was 50 mg/l, while up to 85% in 2 hours when its concentration was 20 mg/l. The percent of decolorization decreased at higher dye concentrations. The correlation between decolorization rate and dye concentration follow Michaelis-Menten behavior. Michniewicz *et al.* [19] reported that the Km values for dye oxidation varied depending on dyes and Km values of purified laccase, as it become lower than Km of the crude laccase, which indicate higher affinity of the purified enzyme to the dye.

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