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ENHANCING LACCASE ENZYME PRODUCTION BY CO-CULTURE OF ENDOPHYTIC FUNGI WITH POTENTIAL APPLICATION IN BIOREMEDIATION OF DYES

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ABSTRACT

In the search for safe and economical methods for laccase enzyme production with high levels, the co-cultivation of two endophytic fungi *Alternaria tenuissima* MG975633 and *Alternaria arborescens* MK629314 was examined. *In-vitro* antagonistic characteristics indicated the ability of both *Alternaria spp.* to grow together without affecting each other. Maximum laccase production obtained from the co-culture fermentation reached 49.5 $U.ml^{-1}$ using rice bran medium under shaking conditions, that increased about 2.44-fold than the synthetic medium (20.31 $U.ml^{-1}$), and increased 1.50 & 1.21-folds compared to *Alternaria tenuissima* MG975633 and *Alternaria arborescens* MK629314 monocultures, respectively after incubation for 12 days. The fungal isolates exhibited degradative and decolourization potential against the different dyes tested at varying degree. On solid medium the highest decolorization zones were obtained by *A. arborescens* MK629314 for Trepan blue (90 mm), followed by Brilliant green (85 mm), Rimazol brilliant blue R and Methyl red (80mm). Application of the crude laccase enzyme exhibited the highest decolorization rate in Rimazol brilliant blue R (15.57%) and Trepan blue (13.71%), followed by Methyl red (11.12) and Congo red (8.83%). The potential performance of *A. tenuissima* MG975633 and *A. arborescens* MK629314 in improving laccase production and biodegradation of different textile dyes encourage the use of laccase based production for environmental decontamination in future.

Keywords: Laccase enzyme, Co-culture, *Alternaria spp.*, Applications, Decolorization. Dyes

INTRODUCTION

Laccases (EC 1.10.3.2) are biotechnologically important enzymes. They have low substrate specificity and could be used in various biotechnological applications such as pulp and paper industry, bioremediation, textile applications, food processing, pharmaceutical applications, biodegradation of xenobiotic compounds and bio-bleaching of synthetic dyes **(Birhanlı and Yeşilada, 2013; Abd El Aty *et al*., 2016).** In addition, Laccase mediated enzymatic treatment has been considered as, an effective process for real industrial decolourization of dye-contaminated wastewater in affordable cost, less-time consuming without environmental risks **(Singh and Gupta, 2020; Sannino *et al*., 2023).**

Laccase are widely distributed in nature ranging from prokaryotes to lower eukaryotes and fungi to plants. The majority of laccases are often found in white rot fungi, which able to produce several laccase isozymes **(Janusz *et al*., 2006; Giardina *et al*., 2010; Bucchieri, *et al*., 2024; Abd El Aty, 2024).** The diverse sources of fungal laccase also have been obtained from the a*scomycetous, basidiomycetous* and *deuteromycetous* **(Giardina *et al*., 2010; Sun *et al*., 2021).**

Currently, the search for safe and economical methods for laccase enzyme production with high levels is considered one of the most interesting research areas **(Saparrat *et al*., 2014; Abd El Aty, 2023).** In this regard, laccase production on a large scale is hampered by several technical, including the use of chemical inducers, but they are expensive and, in some cases, toxic and ineffective **(Saparrat *et al*., 2010).** One of the new sustainable techniques for laccases induction from fungal sources is the co-cultivation with other fungi **(Ma and Ruan, 2015).** Although this mechanism has not been fully elucidated, it mainly related to their role in biological interactions **(Crowe and Olsson, 2001).** Also the combination of ligninolytic fungal strains showed dramatic dynamic effects on the production of lignocellulosic enzymes **(Gao *et al*., 2018).** In this context, other studies of **(Savoie *et al*., 2001; Mata *et al*., 2005; Flores *et al*. 2009, 2010; Chan-Cupul *et al*., 2014)** have shown that co-cultivation of white-rot fungi, such as *Trametes versicolor*, *Lentinula edodes*, *Pleurotus ostreatus*, and *Phanerochaete chrysosporium*, with filamentous fungi, such as *Trichoderma spp*. and *Paecilomyces carneus*, increases laccase production.

The production of laccase by filamentous endophytic fungi in co-culture with other fungi has not been documented, to date, there are no studies of *Alternaria spp.* laccase production in co-culture. This study was therefore undertaken in order to (a) improve the production of laccase by co-culture of the isolated endophytic fungi (*Alternaria spp.*) to obtain high levels of laccase activity available for use in various applications; (b) evaluation of the endophytic fungal strains to decolorize six chemically different synthetic dyes by agar –plate technique and by applying the extracellular crude laccase enzyme.

MATERIALS AND METHODS

Chemicals

The enzyme substrate 2,2′Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) was obtained from MP Biomedicals, LLC. 29525 Fountain Pkwy, Solon,OH 44139, USA. The dyes Congo red, Rimazol brilliant blue R (RBBR), Trepan blue, Brilliant green, Orange G and Methyl red were purchased from Sigma Aldrich (St. Louis, MO, USA), and were prepared as stock solutions (final concentration of 100ppm) by dissolving them in sodium acetate buffer (0.1 M, pH 5.0). All other chemicals used were of analytical grade.

Microorganisms and maintenance

The endophytic fungi *Alternaria arborescens* MK629314 and *Alternaria tenuissima* MG975633 were isolated from medicinal plants of Wadi Abu Matir, Saint-Catherine-Protectorate, South Sinai-Egypt **(Shaheen and Abd El Aty, 2018; Abd El Aty *et al*., 2022).** The pure isolates were maintained at 4 °C on PDA slants **(Abd El Aty *et al*., 2015).**

Evaluation of antagonistic potential.

Direct confrontation method was used to examine the antagonistic potential between the two selected endophytic fungi according to **(Zohair *et al*., 2018).** In this method, one agar disc (10mm diameter) of each isolate was placed on the same Petri dish containing 15 ml of PDA medium. They were both placed along a diametrical axis 3 cm away. Plates incubated at 28 °C for 4, 6 and 10 days in the dark conditions. The experiment was conducted in duplicates. At the end of different incubation periods, radial growth of each strain was measured in (mm) and contact zone between them was evaluated.

Co-culture fermentations.

Two different fermentation media were screened for laccase enzyme production using two isolates.

Synthetic fermentation medium.

The synthetic yeast extract peptone dextrose medium (YPD) was evaluated for laccase enzyme production. YPD medium containing (in g/L), glucose 10, peptone 2, yeast extracts 1 **(Abd El Aty and Ammar, 2016).** Each 250 ml flask contained 50 ml medium.

Agricultural residues fermentation medium.

The agriculture waste rice bran was used as natural culture medium (RBM) for laccase production. About 2 g of rice bran was added in 250 ml flask with 50 ml distilled water without any additives **(Abd El Aty *et al.*, 2016; Alharbi *et al.*, 2023).**

Culture conditions

After media sterilization**,** mono-culture fermentation flask (Control) was inoculated with about 1.0 ml suspension containing (5×10^7spores.ml^{-1}) of 7 days old monoculture. Co-culture flasks were inoculated with (0.5 ml) of each fungal suspension. The inoculated flasks were incubated for 5 days at temperature 28°C under static or shaking (150 rpm) conditions. The laccase inducer copper sulphate (1 mM) was added on the5th day to each culture flask. Finally, the culture growth was filtered on days (12, 14 and 17) to separate the mycelium from the filtrate in which the enzyme activity was evaluated. All the experiments were carried out in duplicate, and the average enzyme activity are reported as mean±SD using MS Excel.

Laccase assay

According to **(Abd El Aty *et al.*, 2017)** laccase activity was measured based on the oxidation of the substrate 2, 2′-azino–bis (3-ethylbenzothiazoline) 6-sulphonic acid (ABTS). One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmol of ABTS per minute.

Applications of laccase enzyme in bioremediation of synthetic dyes

Decolorization of dyes on solid medium

Decolorization of six chemically different synthetic dyes using the endophytic fungi *A. arborescens* and *A. tenuissima* was investigated according to **(Abd El Aty *et al.*, 2016).** About 100 ppm of the dyes, Congo red, Rimazol brilliant blue R (RBBR), Orange G, Trepan blue, Brilliant green, and Methyl red were added to Potato Dextrose Agar (PDA) solid medium, contained (g/L) Potato 200, Dextrose 20, Agar 15. Petri dishes were inoculated with mycelial plugs (10 mm diameter) cut from actively growing mycelia and incubated at 28 ºC in dark for 7 and 14 days. The diameter of the fungal growth and decolorization zones were recorded in millimeters (mm) at three different points and the average values are reported as Mean ± SD using MS Excel.

Decolorization of dyes using laccase enzyme

The optimized laccase enzyme was used as a biological system for dyes decolorization. About 950.23 U of laccase enzyme was added into 2ml sodium acetate buffer (0.1 M, pH 5.0) and mixed with 1ml of each dye at (100ppm) concentration. The reaction mixture incubated at 35 ºC for 6, 12 and 24 hrs under shaking conditions (150rpm).
Decolorization was determined by investigating the absorbance changes at the maximum absorbance wavelength λ_{max} of each dye, Congo red (540), Rimazol brilliant blue R (RBBR) (595), Trepan blue (580), Brilliant green (610), Orange G (485), Methyl red (525) and was expressed in terms of percentage.
Percent of dye decolorization was calculated as the formula: Decolorization (%) = [(Ai-At)/Ai] × 100
Where, Ai: initial absorbance of the dye, At: absorbance of the dye at any time interval **(Abd El Aty and Mostafa, 2013).** The reaction mixtures without enzyme were designed as negative controls. Each decolorization experiment was performed in triplicate and mean of decolorization percentage were reported.

RESULTS AND DISCUSSION

***In-vitro* antagonistic characteristics of laccase producing endophytic fungi**

The most promising endophytic fungi (*A. tenuissima* and *A. arborescens*) for laccase enzyme production were examined for their antagonistic potential. The direct confrontation assay of the two selected *Alternaria spp.* (Fungal disc=10mm diameter) at different incubation periods 4, 6 and 10 days indicated that, the fungal growth rate increased directly with increasing the incubation period without inhibiting each other, until the growth of both fungi completely full the plate after 10 days. At the same time the contact zone between the two strains decreased from 5mm after 4 days incubation to zero after 10 days incubation, indicated that the tested fungi *A.tenuissima* and *A. arborescens* can grow together without affecting each other as a co-culture **Figure 1.** The fungal strains *Pleurotus ostreatus* and *Trichoderm aviridae* were found to be compatible for their co-culture for laccase activity as reported by **Singh *et al.*, (2017).** Also, **Uzar *et al.*, (2017)** showed a successful relationship between *L. tigrinus* and *Postreatus* strains for laccase production.

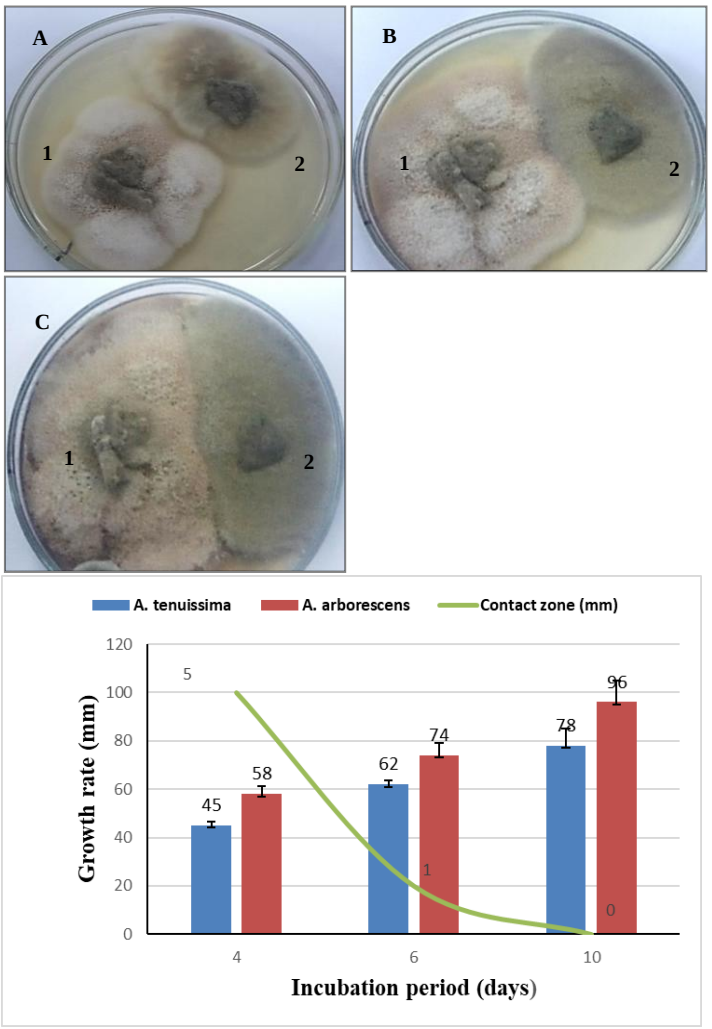

Figure 1 The antagonistic potential between the endophytic fungi *A. tenuissima* and *A. arborescens* at different incubation periods, 4days (A), 6 days (B) and 10 days (C). The experiment was conducted in duplicates and values calculated as Mean±SD. **1**, *A. arborescens.* **2**, *A. tenuissima***.**

Laccase production by co-culture of *A.tenuissima* and *A. arborescens*

The co-culture of endophytic *A. tenuissima* and *A. arborescens* were quantitatively assayed for laccase enzyme production using two different media in comparison with monoculture (Control). The synthetic (YPD) and natural rice bran (RBM) media were used for fungal fermentation with addition of 1 mM copper sulphate, under different incubation periods 12, 14 and 17 days.
In co-culture fermentation with synthetic YPD medium, potential enhancement was observed in laccase enzyme production as 9.41 U.ml^{-1} after 17 days incubation under static conditions and improved to 20.31 U.ml^{-1} after 14 days incubation under shaking conditions **Figure 2.** The results obtained revealed that laccase enzyme production was 1.65 and 1.81 times higher than that of *A. tenuissima* and *A. arborescens* monocultures under static conditions, respectively. At 150 rpm incubation condition the enzyme production also improved about 1.49 and 1.15-fold higher than pure cultures, respectively. Our results agreed with that reported by some authors for laccase production improvement by co-culture technique **(Hu *et al.*, 2011; Uzar *et al.*, 2017).** Also, **Peláez *et al.*, (2023)** investigates the effects of the co-culture between the filamentous fungus *Panus lecomtei* and the yeast *Sporidiobolus pararoseus* in the production of laccases.
On the other hand, growing the two fungal cultures together in natural rice bran media as co-culture improved the enzyme production in both static and shaking conditions to be 28.89 and 49.5 U.ml^{-1}, respectively, which was distinctly higher than monocultures. The results obtained with co-culture shaking fermentation represented about 1.71-fold improvement in laccase production than static conditions **Figure 2**. It was clearly seen that the application of *A. tenuissima* and *A. arborescens* as co-culture appeared to be more successful for enhancing laccase enzyme production (49.5 U.ml^{-1}). The results of the present co-culture study were distinctly higher than **Flores *et al.*, (2010)** who showed an increasing in the activity

of laccase enzyme (6.21 U.ml^{-1}) by *Trametes sp.* AH 28-2 when co-cultured with *Trichoderma sp.* ZH1.

The optimum enzyme production obtained from co-culture fermentation of rice bran medium (49.5 U.ml^{-1}) under shaking conditions increased about 2.44-fold than the synthetic medium (20.31 U.ml^{-1}), also the time required for high enzyme production was shorter and decreased from 14 to 12 days.

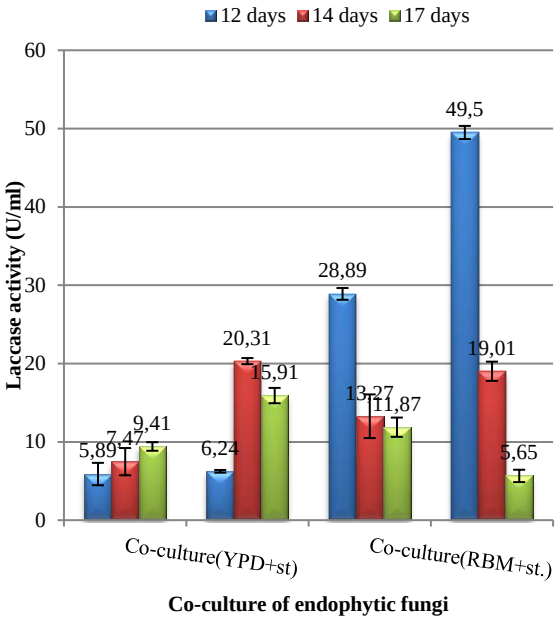

Figure 2 Laccase production from co-cultures of *A. tenuissima* and *A. arborescens* using synthetic yeast extract peptone dextrose (YPD) medium and the agricultural residue rice bran medium (RBM) at different incubation periods (12, 14 and 17 days) under static (st) or shaking (sh.) conditions.

Agar-Plate technique for synthetic dyes Decolorization

The ability to decolorize six different synthetic dyes by the endophytic fungi *A. arborescens* and *A. tenuissim* were evaluated on potato dextrose agar medium. Decolorization zones were determined on agar plates containing 100 ppm concentration of each dye after 7 and 14-day incubation period. Results obtained in **Table 1** showed that both tested strains were able to grow on solid media in the presence of the synthetic dyes and causes decolorization with different degree **Figures 3&4**. Out of the tested dyes, it is found that, the endophytic strains *A. arborescens* and *A. tenuissim* showed maximum growth rate with 90 and 65 mm decolorization zones after incubation for 14 days on medium supplemented with Trepan blue **(Figures 4 g& h)**. Other decolorization zones by *A. arborescens* were in range from 80-85 mm with Brilliant green, Rimazol brilliant blue R and Methyl red. But the dyes Congo red and Orange G showed 60 mm decolorization zones after 14days incubation. **Olufunke *et al.*, (2016)** reported considerable degradation ability of another filamentous fungus, *Aspergillus niger* on Malachite green and Methylene blue solid medium by 47mm zone diameter, and showed significant decolorization of Malachite green by *Cryptococcus spp*. On the other hand, Malachite green and Metyhlene blue were recalcitrant to *Aspergillus fumigatus* and showed low decolorization ability on carbon fuchsine by creating zone of clearance of just 5mm diameter.

Table 1 Decolorization of six chemically different synthetic dyes on Potato Dextrose Agar (PDA) solid medium

Synthetic dyes (100 ppm)	Decolorization zone diameter* (mm)			
	A. arborescens		*A. tenuissima*	
	7 days	14 days	7 days	14 days
Congo red	29±4.24	60±0.77	19±1.40	45±3.51
Rimazol brilliant blue R	40±3.55	80±1.53	00±0.00	00±0.00
Orange G	32±1.41	60±2.13	00±0.00	00±0.00
Trepan blue	45±1.42	90±3.14	27±3.50	65±3.10
Brilliant green	30±1.41	85±1.41	20±3.50	55±1.11
Methyl red	27±2.12	80±2.91	00±0.00	42±1.10

*Plates incubated for 7 and 14 days with 10 mm mycelial plugs of *A. arborescens* and *A. tenuissima.*

Results demonstrated that, the fungus *A. tenuissima* did not show any clearance zones with Rimazol brilliant blue R and Orange G synthetic dyes after 7 and 14-day incubation **(Figures 3 d& f)**. In same time period, the decolorization zones for this fungus were seen on a good level as 55, 45, 42 mm in medium supplemented with Brilliant green, Congo red and Methyl red respectively after 14 days incubation. **Abd El Aty *et al*, (2016)** reported the same observation about the good decolorization activity of the azo dye, Congo red used in textile industries, by the marine-derived *A. tenuissima* KM651985 after 14 days incubation.

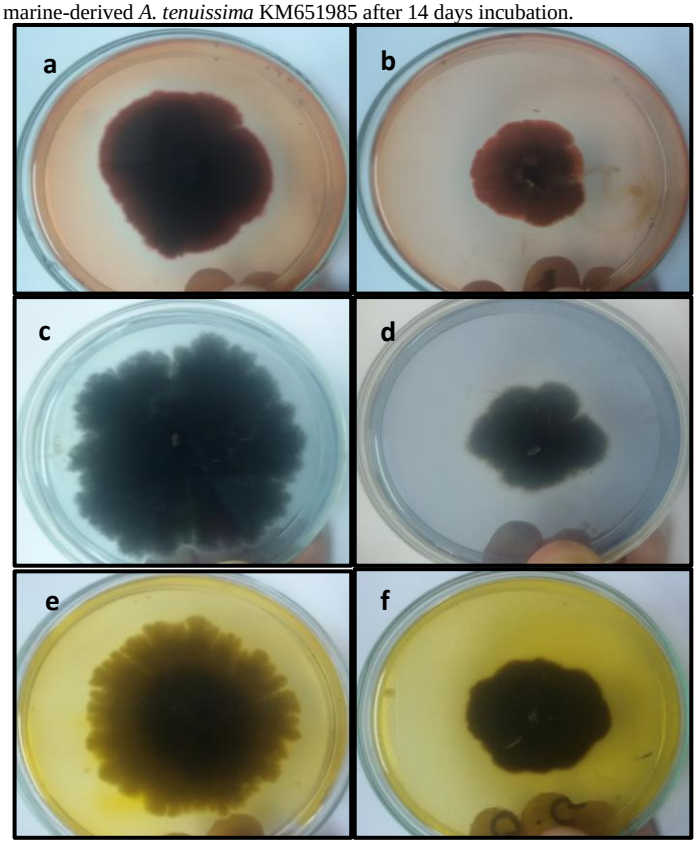

Figure 3 Photos of synthetic dyes decolorization, Congo red (a,b), Rimazol brilliant blue R. (c,d), Orange G (e,f) using the endophytic fungi *A. arborescens* and *A. tenuissima* after 14 days respectively.

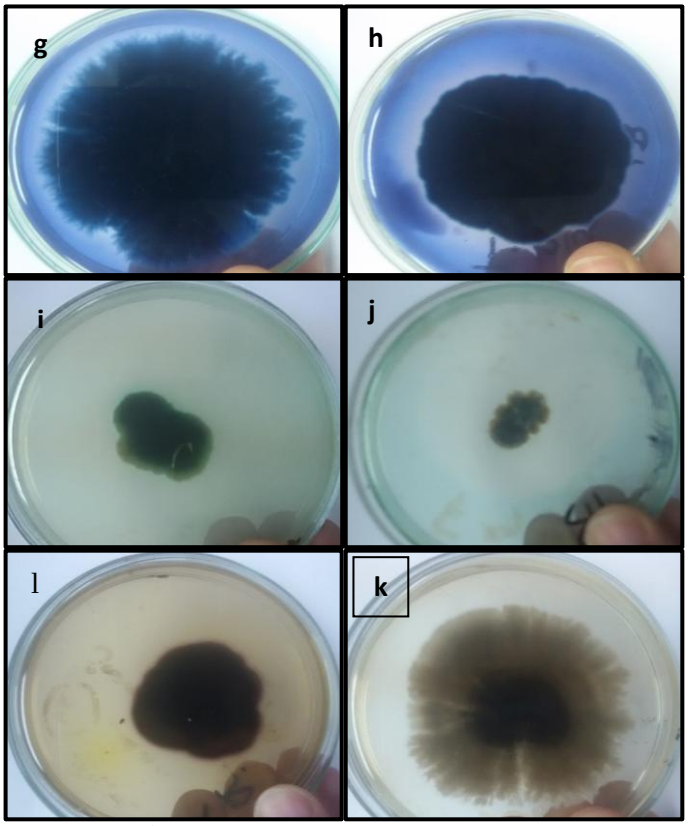

Figure 4 Photos of synthetic dyes decolorization, Trepan blue (g,h), Brilliant green (i,j), Methyl red (k,l) using the endophytic fungi *A. arborescens* and *A. tenuissima* after 14 days, respectively.

Enzymatic decolorization of synthetic dyes

Dye decolorization using the crude laccase enzyme exhibited different tendency as that of agar-plate technique. Results in **Figure 5** exhibited that, incubation time and chemical structure of synthetic dyes were important factors affecting the decolorization percentage **(Rodriguez-Couto, 2007; Michniewicz *et al*., 2008).** The highest decolorization was observed in Rimazol brilliant blue R (15.57%) and Trepan blue (13.71%), followed by Methyl red (11.12) and Congo red (8.83%). And least by Orange G and Brilliant green, 5.48%, 2.44% respectively. Similar results were obtained by **Yeşilada *et al*., (2014)** who indicated the decolorization activity (12%) of *Funalia trogii* ATCC 200800 crude laccase, against the synthetic dye Reactive Black 5 at pH 4.5and 30 °C**. Mostafa and Abd El Aty (2018)** reported the ability of *A. tenuissima* KM651985 free laccase enzyme to decolorize Remazol Brilliant Blue R (RBBR) and Malachite Green (MG) with 22% and 13% respectively**. Zampolli *et al*., (2023)** indicated the Oxidative degradation of polyethylene by two novel laccase-like multicopper oxidases from *Rhodococcus opacus* R7.

Some studies reported that laccase could not decolorize some textile dyes without mediators, as the crude laccase obtained from solid-state culture of *G. lucidum* could decolorize RB 5 only in the presence of a mediator **(Tavares *et al*., 2008; Zeng *et al*., 2011).** In the present study, the degradation obtained depends on using the biological systems without mediators which could be an ecofriendly solution to avoid pollution.

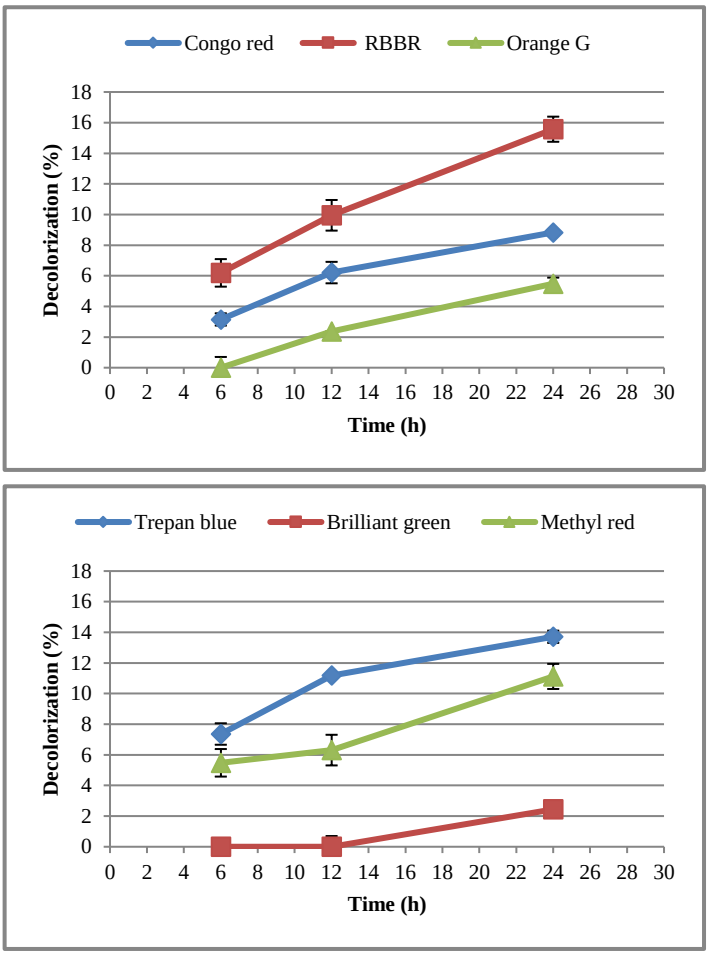

Figure 5 Decolonization capacity (%) of synthetic dyes using optimized laccase enzyme.

CONCLUSION

The increase in laccases produced by *Alternaria spp*. in a co-culture system could be an attractive alternative to those required in a monoculture system using chemical inductors. Moreover, both endophytic fungi showed good efficiency in degrading different synthetic dyes with different degree, using agar- plate technique and the crude laccase enzyme. Obtained results demonstrated the ability of applying *A. arborescens and A. tenuissima* in solving the pollution problem as an alternative environmentally friendly technique.

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Conflict of interests : The author declares that there is no conflict of interests regarding the publication of this paper.

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