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Substrate Concentration as a Factor for Optimization of Direct Green Dye Biodegradation by Seven Fungal Strains

Wafaa M. Abd-El Rahim*, Hassan Moawad, Talaat El Sebai

Agricultural Microbiology Depart. National Research Centre, Dokki, Cairo, Egypt

Email address
wafaa10m@hotmail.com (W. M. Abd-El Rahim)

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Abstract
The ability to decolorize Direct green dye by seven fungal strains, previously isolated from industrial effluent discharge site, was evaluated at varying dye concentrations (200, 400, 500, 600 mg/l). All strains showed high decolorization capacity and were able to decolorize the green dye with at different degrees. The strains not only decolorized the dye, but also in parallel showed varied levels of laccase and Lignin peroxidase production through the course of decolorization. These two enzymes are known to play significant role in dye biodegradation. Three fungal strains were found to have capacity to decolorize as high as 600 mg/L concentration of this dye in the growth medium. The dye was not just only being decolorized but also was degraded as evident from the UV-Visible Spectra analysis that shows the ability of fungi to degrade this recalcitrant organic dye. The optimum Direct green dye concentration in growth media was in the range of 200 to 500 ppm. Also the result reflects that the fungus growth is affected by the tested dye concentrations.

1. Introduction

Environmental pollution has increased with increasing the industrial development (Osman et al. 2006). Contamination of water by wide range of pollutants is a serious environmental problem due to their potential human toxicity. The textile finishing generates large amount of dyes, pigments, dispersing agents, salts, leveling agents and heavy metals (Noroozi et al. 2007). The total world colorant production is estimated to be about 8,00,000 tons per year, and at least 10–15 % of the dyes/dyestuffs are released into the environment through the effluents (Revanker and Lele 2007). Owing to their chemical structures, dyes are resistant to fading on exposure to light, water and many chemicals and are difficult to be eliminated by conventional chemical and biological waste treatment methods, due to the effluents high degree of polarity. Decolorization of dye wastewater is a challenging process to the textile industry. The decolorizing of dye residues has emerged as effective tool for dye bioremoval. In the recent past there has been an intensive research on bioremediation of dyes, and the use of white rot fungi and ligninolytic fungi is turning into a promising alternative to replace or supplement present treatment processes (Dos- Santos et al. 2004; Asgher et al. 2006). The ability of white rot fungi in mineralization of xenobiotics to CO_2 and water through the highly oxidative and nonspecific ligninolytic system are well documented. These fungi were also responsible for decolorization and degradation of wide range of dyes (Boer et al. 2004; Patel et al. 2009) degradation of the recalcitrant dye structures by these fungi is linked to the production of extracellular enzymes including Laccase (E.C.1.10.3.2), Manganese peroxidase (MnP, E.C. 1.11.1.13), Lignin peroxidase (LiP, E.C. 1.11.1.14),

which are able to degrade various dyes of different chemical structures (Levin et al. 2004). This work aimed to study the substrate concentration as a factor for acceleration of Direct green dye bioremoval by seven fungal strains.

2. Materials and Methods

2.1. Preparation of Biomass for Decolorization Process

Five grams of 72 h mycelia of fungal strains were transferred aseptically into 250 ml Erlenmeyer flasks containing the sucrose media (100 ml) along with tested dye. The flasks were shaken at 150 rpm and incubated at 30oC 72h. Dye decolorization was determined spectrophotometrically by monitoring the absorbance of samples at k max of the respective dyes using a UV–Visible spectrophotometer (Shimadzu UV 1800, Japan). Results were reported as means of decolorization % of the three replicates. The decolorization or Relative Bioremoval Capacity (RBC) was expressed as % of the initial dye concentration and calculated as follows:

$$\% \text{ Decolorization} = 100 \text{ X } (A0 \_ At)/A0$$

where A0 is the absorbance value of the initial dye concentration and At is the absorbance value of the dye concentration in sample at time t.

2.2. Effect of Initial Dye (Direct Green) Concentrations on Fungal Dye Bioremoval Capacity and Linginolytic Enzyme Production

In order to examine the effect of initial dye concentrations on decolorization under shaking conditions, 200, 400, 600 mg l^{-1} of Direct green dye were added to the media and incubated at 30°C for 72h. The % decolorization and ligninolytic enzyme production were determined after 2th day of incubation. Abiotic control (without culture) was always included.

2.3. Spore Suspension Preparation

One square cm of fungal mycelium growth (4 to 5 days old culture) were transferred to 25 ml PD broth medium in a 250 ml conical flask and incubated at room temperature for 4 to 5 days to develop enough spores on rotary incubator shaker at 150 rpm at 28°C in growth medium. Spore suspension was used as inocula in dye removal experiments.

2.4. Preparation of Fungal Biomass Inoculum

Seven fungal strains were cultivated in 250 ml Erlenmeyer flasks containing 100 ml of basal mineral medium supplemented containing with 10g/l sucrose, 0.5g/l yeast extract. Flasks were shaken on rotary incubator shaker (150 rpm) at 28°C for 4-5 days. Fungal growth was separated by centrifugation at 8000 rpm for 20 min. under aseptic conditions and the pellet was re-suspended in sterile dye solution (500 mg/l). After re-suspension of fungal growth, homogenization of suspension was made using sterile magnetic bars on magnetic stirrer. Five mls were withdrawn by a wide mouth pipette and dried at 105°C to determine the fungal biomass. A proper volume of fungal biomass suspension containing 200-mg dry weight was used as inoculum (Wafaa, 2003).

2.5. Dry Weight of Pellets

Dry weight of pellets was obtained by filtering cultures through Watman filter paper No. 1 and drying it to constant weight at 65 °C. Results are means of at least three replicates.

2.6. Determination of the Highest Removal Value

The Michaelis constant (km) and maximum velocity (Vmax) for the decolorization by each fungus was studied from the Line weaver-Burk linear relationship as follows:

Four concentrations of the Direct-green dye (200, 300, 400, 600 ppm) in acetate buffer pH 5 were prepared and inoculated with a disk of each fungus. The decolorization was measured by spectrophotometer at 385 nm at intervals of 2, 4, 24, 48 and 120 hr. The initial velocity (V0) was calculated from the slope of the linear relationship of time (the X axes) versus the removed dye concentration (ppm) (the Y axes). The Line weaver-Burk linear relationship was drawn out from the plot of 1/dye concentration (1/S) versus (1/V). The line slope was the km/Vmax, while the Y axes intercept was 1/Vmax.

2.7. Assay of Laccase Enzyme

Laccase activity was determined according to a method described by Paszczynski *et al*. (1988). This method is based on the oxidation of Dimethoxy phenolic compound (DMP) by laccase enzyme. The Reagents used in this test are**:** 20 mM of 2, 6 DMP was obtained by dissolving 77.08 mg of DMP in 25ml distilled water, and sodium malonate buffer (pH 4.5). The sample to be assayed was added in a quantity of 600µl to a test tube in a water bath at 30ºC containing 200µl of 250 mM sodium malonate buffer (pH 4.5); 50 µl of 20 mM 2,6 DMP to make the total volume 850µl. The test tubes were incubated for 2 minutes after the addition of the enzyme source. The reaction was stopped by putting tubes in ice container. For each enzyme assay a calorimeter blank (without enzyme source) was included and incubated in the same way, except that enzyme source was replaced by distilled water. Changes in the absorbance at 468 nm were measured on UV visible spectrophotometer.

2.8. Assay of Lignin Peroxidase Enzyme

Lignin peroxidase was determined according to a method of Jadhav and Govindwar, (2006). This method is based on oxidation of n-propanol in the presence of H_2O_2 by lignin peroxidase enzyme with the formation of propanaldehyde. Reagents & Procedure of this method were as follows: 100 mM n-propanol was prepared by dissolving 1.5 ml of

n-propanol into 250ml distilled H_2O, 250mM tartaric acid was obtained by dissolving 9.38 g tartaric acid into 250 ml distilled H_2O and 10 mM H_2O_2 was obtained by dissolving 0.3505ml of H_2O_2 into 1L distilled H_2O. The enzyme source was added in a quantity of 0.5 ml to a test tube in a water bath at 30°C containing 1.0 ml of 10 mM H_2O_2; 0.5 ml of 250 mM tartaric acid and 0.5 ml of 100 mM n-propanol, to make the total volume 2.5 ml. All enzyme assays were run in triplicate and all tubes were incubated for 10 minutes after the addition of enzymes sources. The reaction was stopped by putting tubes in ice container. For each enzyme sample a calorimeter blank was prepared and incubated in the same way, except that the source enzyme was replaced by distilled water. One unit of enzyme activity was defined as a change in absorbance unit per min per ml of enzyme source.

3. Results and Discussion

Effect of Varying Dye (DG) Concentrations

One of the important factors for optimizing the enzyme activity is the substrate concentration. The concentrations of the residual dyes in dye house effluents were reported to be in the range from 60 to 300 mg l^{-1} (Wafaa et al., 2003 and Wafaa, 2006). In the present study different concentrations of the Direct green dye were studied in relation to the activities of laccase and LiP for optimization of enzyme activity and removal efficiency of the dye. The applied dye concentrations ranged from 200 ppm to 600 ppm. The results in Table 1 show that most of the fungal strains tolerated the dye concentrations up to 600 ppm in the growth media. Four of the strains; A2, A10, A11 and A12 continued to grow and accumulate the biomass even at the dye concentration of 500 ppm in the growth media. Four other strains A4, A6, A11 and A12 showed adaptation to the high concentration of dye (600 ppm) and accumulated fungal biomass in significant amounts. As regards the dye color removal the results (Fig. 1) show that at lower dye concentrations, the percent of decolorization was higher and reached up to 73 % at the concentration of 200 ppm. When concentration of the dye was increased from 200 to 500 ppm, the decolorization efficiency by fungal strains decreased to 56 % (Fig. 1).

In this study, three out of seven fungal strains showed the ability to decolorize up to 6 g/L, Direct green dye, which is much higher than the 2g/L reported by (Shazia and Safia, 2011) using *D.squalens*. On the contrary to that of Nyanhongo et al. (2002), who found that laccases from *Trametes hirsuta* and from *Trametes modesta*, required a longer time to be decolorized or could not be decolorized completely. In our study all seven strains of fungi decolorized Direct green dye to up 88% in 72 hours at dye concentration of 0.3 g/L. Five out of seven fungi tested have shown faster decolorization rates (more than 80% decolorization) within the first 48 hours, which is much faster than results reported elsewhere. Vasdev et al. 1995, reported decolorization time of 96 hours for various species of *Cyathus*, while Yesilada et al. (1995) reported 72 hours for decolorization of 62 % of Crystal violet by *Phanerochaete chrysosporium* ME446. Knapp et al. (1995) reported longer time 216 hours for decolorization by *Phanerochaete chrysosporium* NCIM1197. Malachite green was decolorized fastest, which is in accordance with the observations of Sani & Banerjee (1999) and Franciscon et al., (2009). Dye decolorization time required by fungi in this work did not show any marked effect on fungal growth at concentration up to 0.6 g/L of green dye tested.

As regards the ligninolytic enzymes activity, the results show that the increase in dye concentration up to 400 g ppm resulted in Laccase and LiP activity. Where in Laccase and LiP amount 0.35 and 1.62 U ml^{-1} at 200 ppm dye concentration, respectively whereas at 400 ppm dye concentration, Laccase and LiP activity were 0.36 and 0.87 U ml^{-1}, respectively (Fig. 2, 3). This suggests that the higher concentration of dye induces the ligninolytic enzyme production which likely to contribute the decolorization of Direct green dye. Vaithanomast et al., (2010) reported that the induction of different types of ligninolytic enzyme depends on the structure and concentration of dye. Laccase activity was much higher than LiP, which indicates that laccase could be more responsible for the dye decolorization degradation. Kashif et al. (2011) reported that 78 % decolorization of 0.5 g l^{-1} of solar golden yellow R dye was removed by *Pleurotous Ostreatus*. Vaithanomast et al. (2010) stated also that more than 90 % decolorization of 1.0 g l^{-1} reactive dyes (RBBR and RB5) was achieved by *Datronia* species; on the other hand Shazia and Safia (2011) stated that 0.2 g l^{-1} of AR151 dye was the maximum limit to be decolorized (60–70 %) by three indigenous *Aspergillus* species.

Whereas the responsible of the removal process is to the enzyme and the dye removal or residual of dye after removal process will be due mainly to the enzyme. Therefore has been applied to this equation on the results of the removal of Direct green dye using 7 fungal strains under study. To be achieve the highest degradation rate of the studied dye. In this study Table (2) shows the Km and Vmax values for the tested fungi. The lowest Km value (54.36 ppm) was obtained from the fungus A10. This reflects the high affinity of the fungus to the dye, however, it showed a low Vmax value (0.4 mg/hr). On the other hand, the highest Vmax value (34 mg/hr) was obtained from fungus A4, with a Km value of 281.1 ppm. Fungus A11 showed the highest Km value (4424.18 ppm), which reflects the lowest affinity of this fungus toward the dye during decolorization process. Negative values of Vmax were obtained from three fungal strains (A6, A2 and A12). This result reflects that the fungus growth is affected by the tested dye concentrations.

4. Conclusion

The optimization removal of green dye which is related to the legninolytic enzyme produced show that the optimum Direct green dye concentration in growth media to achieve the highest removal percent is in the range between 200 to 500 ppm. This is important for adjusting the contact of the media to help fungi and enzymes to remove the toxic dye from the

media.

***Table* (*1*).** *Fungal biomass accumulation (mg) on media amended with 200, 400, 500 and 600 ppm Direct green dye after 120 h.*

Fungal strains	fungus growth without dye	200 ppm	400 ppm	500 ppm	600 ppm
A 2	113.7	170	171	193	72
A 4	194	198.9	211	130	220
A 6	96.6	171	101.9	120	209
A 7	88.3	99.6	114.6	67.2	95
A 10	129.4	139.1	71.8	138.8	118
A 11	80.08	85.4	98	159.8	179
A 12	142.9	151	178.6	222.4	202

Table (2). *The Km and Vmax values for the dye decolorization by the tested fungi.*

Fungal strain	V_{max} (mg/hr)	Km
A 10	0.4	54.36
A 4	34.014	281.12
A 7	5.43	580.58
A11	8.76	4424.18
A 6	-5.39	--
A 2	-40.65	--
A 12	-3.33	--

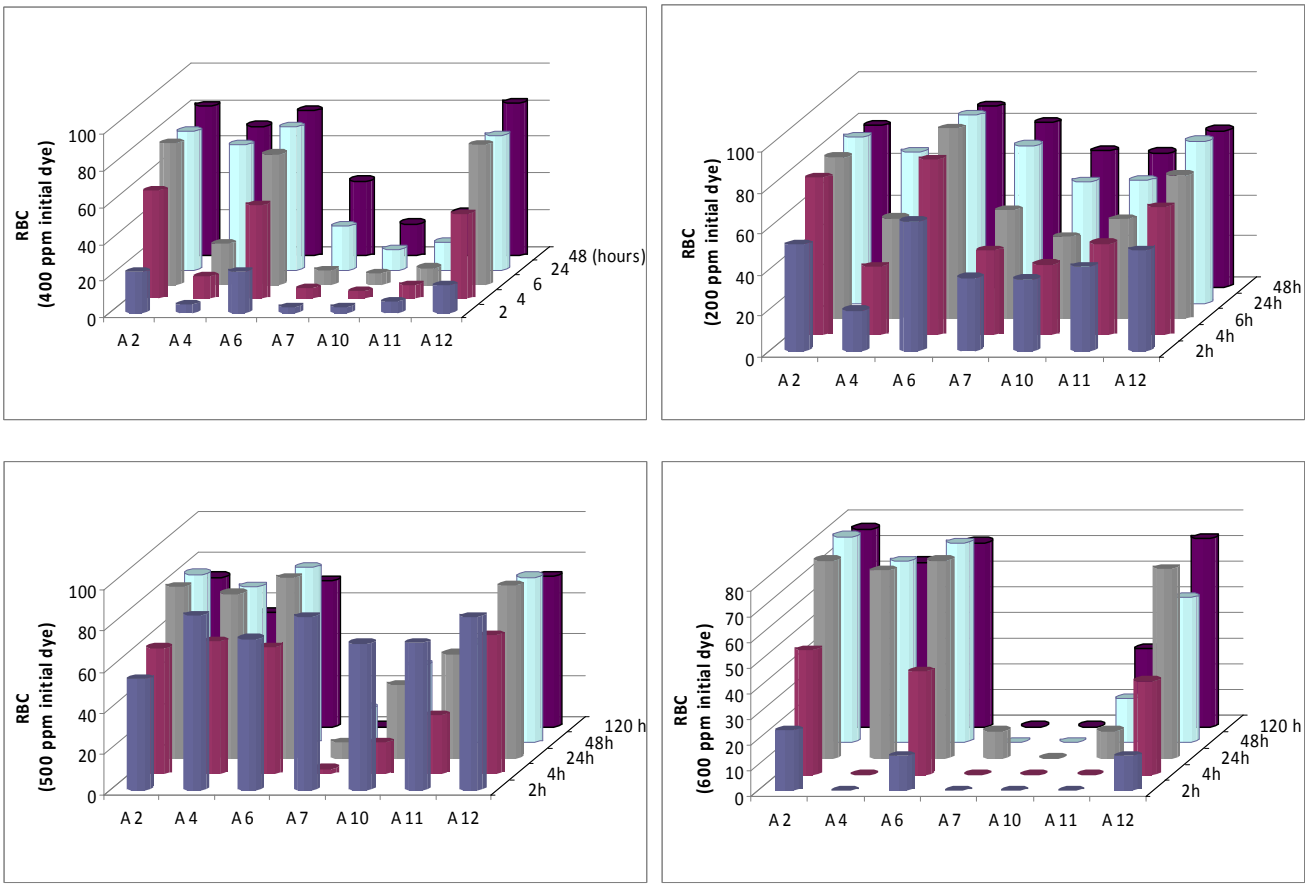

Fig. 1. *Direct green dye Relative Bioremoval Capacity (RBC) by seven fungal strains at different initial dye concentrations.*

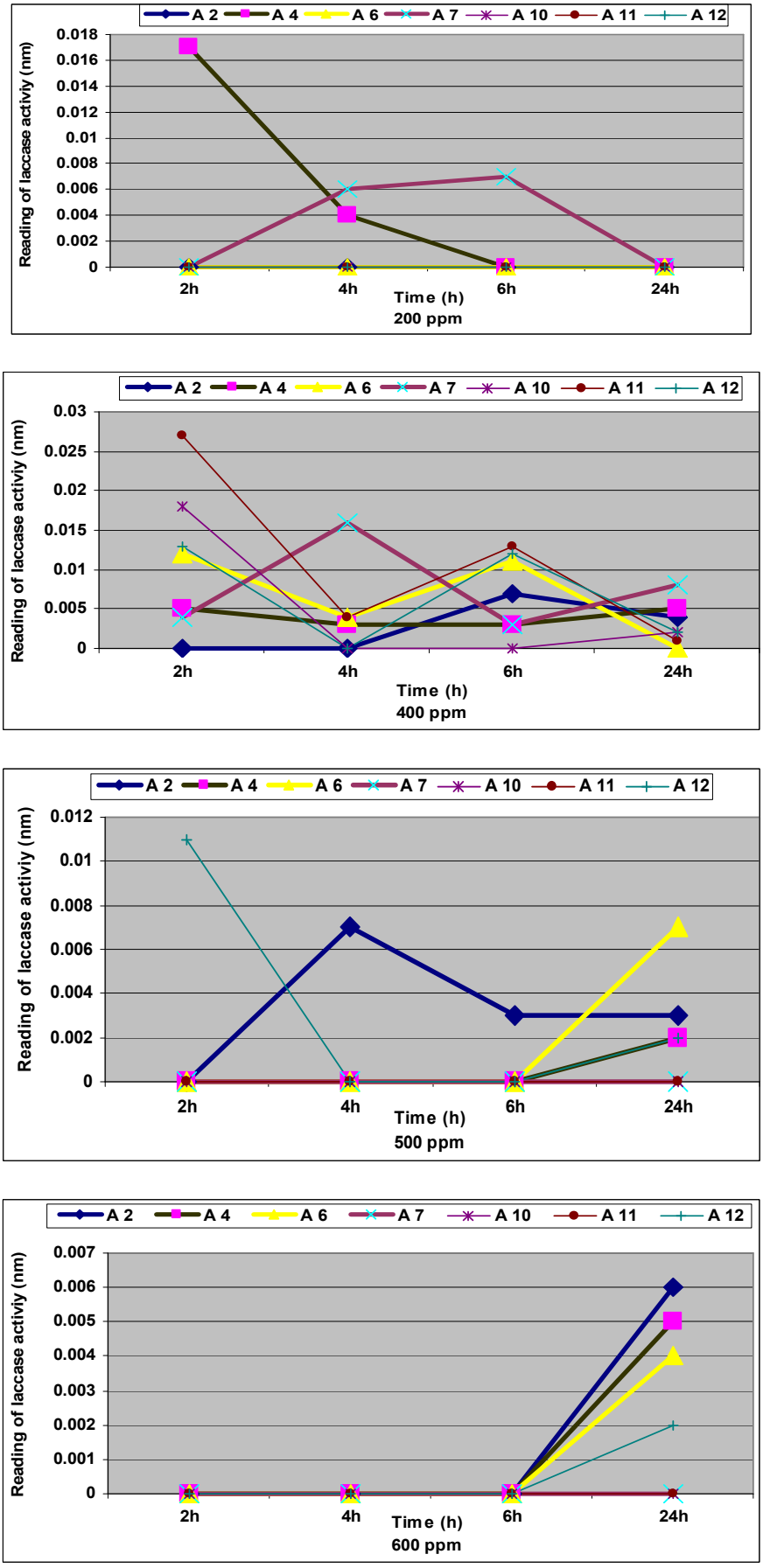

Fig. 2. *Changes in Laccase activity in liquid batch cultures of seven fungal strains grown at different dye concentrations.*

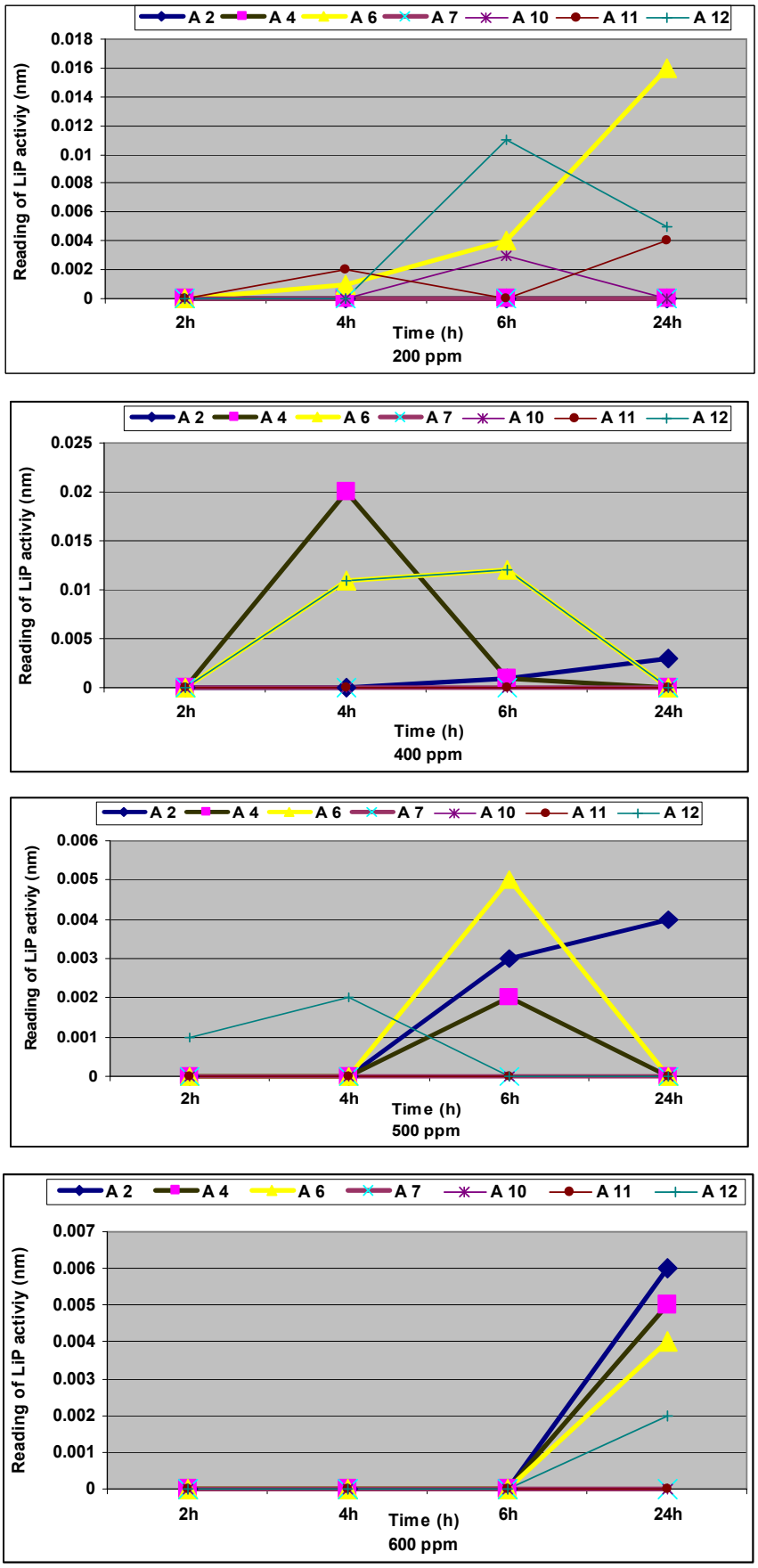

Fig. 3. *Changes in Lignin peroxidase activity in liquid batch cultures of seven fungal strains grown at different dye concentrations.*

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