

DECOLORISATION OF AQUEOUS SOLUTIONS OF SYNTHETIC DYES BY *Lentinus polychrous* Lév. CULTIVATED ON CASSAVA RHIZOME

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ABSTRACT

Cassava rhizomes are left in fields after harvesting. This agricultural waste is rich in lignocellulosic material which is a substrate for white rot fungi. Disposal of synthetic dyes poses a problem to the environment and it needs to be addressed. The ability of *Lentinus polychrous* Lév., a white rot fungus, grown on the cassava rhizome chips, to decolorise three kinds of synthetic dye was studied. The effects of the initial moisture content of cassava rhizome used for fungal cultivation, the temperature during the decolorisation, and the pH of synthetic dye solution on the extent of decolorisation were investigated. The decolorisations of Reactive blue 49, Navy blue and Acid blue 62 were affected by the initial moisture content of cassava rhizome. The highest extents of decolorisation of these dyestuffs were observed when the fungus was cultivated at 70% initial moisture content. Temperatures of 30, 37 and 45°C did not alter the extent of decolorisation of the dyestuffs. The most extensive decolorisations of Reactive blue 49 and Acid blue 62 (anthraquinone dyes) were at pH 3.0 while that of Navy blue (azo dye) was at pH 7.0. Adsorption was the main mechanism of decolorisation of Navy blue. However, both enzymic degradation and adsorption were involved in the decolorisations of Reactive blue 49 and Acid blue 62.

Keywords: Agricultural waste, cassava, decolorisation, dye, *Lentinus polychrous*

INTRODUCTION

Cassava is a plant with large tuberous roots and it is a major source of dietary carbohydrate in tropical and subtropical regions. The rhizome and the lower part of the stalk 15cm above ground are normally left in the fields and burned after the harvest. In this work, these are termed cassava rhizome. Cassava plantations worldwide can be estimated [using the estimates and reports of FAOSTAT (FAO, 2014)] to have occupied 117 million ha in 2013. If 9,000 – 10,000 cassava plants are cultivated per ha, over 1 trillion cassava plants are grown annually. Pattiya *et al.* (2010) reported that cassava rhizome contained 27.82% cellulose, 39.67% hemicelluloses, and 21.71% lignin. Given this composition, cassava rhizome has the potential of being used as a substrate for the growth of white rot fungi. The potential use of cassava rhizome for gasification (Sornkade *et al.*, 2014; 2013) and pyrolysis (Homchat *et al.*, 2012; Sirijanusrorn *et al.*, 2013; Suttibak *et al.*, 2012) has been suggested.

Synthetic dyes are widely used in textile industries and are discarded in wastewater from the dyeing process. The discharge of these colored wastewaters into rivers and lakes leads to reduced sunlight penetration, which in turn causes a reduction of photosynthetic activity and dissolved oxygen concentration (Sukumar *et al.*, 2007). Biological treatment of wastewater contaminated with synthetic dyes has been studied extensively. One group of microorganisms used for these treatments is the white rot fungi. These fungi are capable of degrading recalcitrant compounds including synthetic dyes by the action of their ligninolytic enzymes, mainly lignin peroxidase, manganese peroxidase and laccase. Some white rot fungi produce all three enzymes, others produce at least one (Wesenberg *et al.*, 2003).

Lentinus polychrous Lév. is a white rot fungus that is grown commercially for mushrooms in the northeast and north of Thailand. It has been reported that *L. polychrous* Lév. produced laccase as the main lignolytic enzyme involved in dye decolorisation (Budda *et al.*, 2012; Khammuang and Sarnthima, 2007; Sarnthima *et al.*, 2009). The cultivation of *L. polychrous* Lév. on solid substrates is a common practice and lignocellulosic agricultural waste is commonly used.

In this work *L. polychrous* Lév. was cultivated on cassava rhizome and its capacity to decolorise aqueous solutions of dyes was studied. The effects of the initial moisture content of cassava rhizomes used for fungal cultivation, the temperature during decolorisation, and the pH of solutions of three dyes were investigated.

MATERIAL AND METHODS

Materials

Reactive blue 49, Acid blue 62, and Cibacron Navy blue WBT Crude (Navy blue) were supplied by Huntsman (Thailand), Dystar (Thailand), and Ciba Specialty Chemicals Industries, respectively. Cassava rhizomes were collected from Buriram Province, Thailand; they were sun-dried and chopped into small pieces of 0.85 – 1.4 cm in length. The chopped rhizomes were rinsed with tap water and oven dried at 105°C overnight before use. *Lentinus polychrous* Lév. was obtained from the Mushroom Collection Center, Clearing House Mechanism of the Department of Agriculture, Thailand. The fungus was maintained on Potato Dextrose Agar (PDA) at 30°C and transferred to Mushroom Complete Medium (MCM: 1 liter contained 20g glucose, 2g yeast extract, 0.5g MgSO₄, 1g K₂HPO₄, 0.46g KH₂PO₄, and 15g agar; its pH was 6.0) and incubation was for 5 days at 30°C.

Fungal cultivation

The moisture content of the cassava rhizome was varied to investigate its effects on decolorisation. The moisture contents were adjusted by the addition of calculated quantities of distilled water and the mixtures were sterilised at 121°C for 30min. Single circular plugs (1cm diameter) excised from the inoculated and incubated MCM plates were added to 500ml Erlenmeyer flasks that contained 8g dry weight of cassava rhizome. The moisture content of the cassava rhizome was varied to investigate its effect on decolorisation. When the other effects were studied, the moisture content of cassava rhizome was adjusted to 70%. The cultures (in triplicate) were incubated at 37°C for 3 weeks. The evaporation of water during incubation was minimized by adding sterilized distilled water to replace the total weight loss of the cultures every second day. During the growth of fungi on solid substrates, loss of dry weight has been used as an index of the consumption of organic material in several earlier publications (Eichlerová *et al.*, 2000; Nicolini *et al.*, 1987; Zadrzil and Brunnert, 1980). The authors of these reports have shown that dry weight loss is positively correlated with the growth of fungi (Eichlerová *et al.*, 2000; Nicolini *et al.*, 1987). In this work, we have used dry weight loss as a rough measure of the extent of growth of *L. polychrous*. The dry weight loss of the culture was measured by oven drying at 105°C for 12h.

Determination of concentrations of synthetic dyes

The concentrations of synthetic dyes in aqueous solutions were determined spectrophotometrically at the λ_{\max} (visible) of the dyes using a Helios Gamma 9423 spectrophotometer (Thermospectronic, UK). The λ_{\max} of Reactive blue 49, Acid blue 62, and Navy blue were 580, 640, and 600nm, respectively. Calibration employed solutions of the dyes in the concentration range 0-100mg/L and plots of absorbance against concentration were linear.

Decolorisation by *L. polychrous* Lév. cultivated on cassava rhizome

To three week-old fungal cultures were added 250ml of the dye solutions (50mg of dye/L in distilled water) which had been filter-sterilized (0.45 μ m cellulose nitrate filter, Whatman). The mixtures were incubated at 37°C with agitation (rotary incubator, 200rpm). The concentrations of the dyes remaining in the liquid phases were determined at various times throughout the period of incubation. The oven dried (105°C, 12h) three-week old fungal cultures were used as the control. When the effects of temperature during decolorisation were studied, the mixtures were incubated at 30, 37 and 45°C. When the effects of pH on the extent of the decolorisation of the dyes were studied, the filter-sterilised dye solutions prepared in citrate-phosphate buffer (0.1M citric acid and 0.2M Na₂HPO₄) adjusted to pH 3, 5, and 7 were used. In these experiments concerning the effects of temperature and pH, the concentration of Navy blue in the solution used was 50mg/L but those of Reactive blue 49 and Acid blue 62 were increased to 300mg/L.

Determination of laccase activity at various pHs

The extracts for the determination of activity were prepared by adding 250ml of distilled water to the cultures and agitating (200rpm) the mixtures for 1 hour at 30°C. The liquid phases were separated by filtration (Whatman No. 4 paper). The activity of laccase in the liquid phases extracted from the 3 week-old cultures of *L. polychrous* Lév. after cultivation was measured by using the method of **Johannes and Majcherzyk (2000)**. The reaction mixtures contained (final concentrations) 5 mM ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], 0.1M citrate-phosphate buffer of various pHs in the range 3-7, and 50 μ l of liquid phase. The concentration of oxidized ABTS produced after 5min was determined from the increase in absorbance at 420nm. ($\epsilon = 36,000\text{M}^{-1}\text{cm}^{-1}$).

Determination of the stability of laccase activity at various pHs

The extracts of the enzyme were prepared by adding 100ml of water to the cultures. After agitation and filtration as described above, 10ml of liquid phase were added to 15ml of 0.167M citrate-phosphate buffer of a pH within the range of 3-7. The mixtures were incubated at 37°C for various times. At these times, laccase activity at pH 3 was determined with 50 μ l portions of the diluted liquid phases as described above.

RESULTS AND DISCUSSION

The effects on decolorisation of the initial moisture content of cassava rhizome used for fungal cultivation

The greatest extent of decolorisation was observed when *L. polychrous* Lév. was cultivated on cassava rhizome with 70% initial moisture content (figure 1). Moisture content is the main characteristic of solid substrate cultivation processes that controls growth and the metabolic systems of microorganisms (**Pérez-Guerra et al., 2003**). Reactive Blue 49 was extensively decolorised by cultures cultivated at 50% moisture content and only a slight increase in the extent of decolorisation was observed when the moisture content of the substrate was 70% (figure 1A). These results are correlated with the dry weight loss of the substrate at 70 and 50% moisture content (figure 2). On the other hand, very different extents of decolorisation of solutions of Navy Blue were observed when cassava rhizome with 70% and 50% moisture contents were used (figure 1C). However, the results of figure 2 merely indicate the extent of consumption of organic material by the growth of the fungus. During solid state cultivation, microorganisms usually require more water to produce metabolites and enzymes than to promote growth (**Pérez-Guerra et al., 2003**). The results of figures 1A-D show clearly that the two dyestuffs which were decolorised mainly by enzymic degradation were decolorised more extensively when the moisture content of the cassava rhizomes used for fungal cultivation was increased. This phenomenon is due perhaps to the larger amounts of fungal degradative enzymes that are produced at less restricting moisture contents. Decolorisation by *L. polychrous* Lév. grown on cassava rhizome occurred via two mechanisms: enzymic degradation and adsorption onto both the cassava rhizome and the fungal mycelium. In this study, inactivated cultures were used to estimate the extent of adsorption of dyestuffs onto cassava rhizomes and the fungal mycelium (figure 1B, 1D, and 1F). Acid blue 62 was adsorbed most extensively onto cassava

rhizome and fungal mycelium (figure 1F). Approximately 80% of Acid blue 62 was removed from the liquid phase while only 40-50% and 15% of Reactive blue 49 and Navy blue, respectively, were adsorbed. Acid blue 62 is an acidic sulphonated dye of relatively low molecular weight while Reactive blue 49 and Navy blue are reactive dyes (**Zollinger, 2013**). Several factors, e.g. chemical structure, substrate affinity, pH, temperature, ions present in solution, affect adsorption and the mechanisms of adsorption of these dyestuffs onto the mixture of cassava rhizome and the fungal mycelium require further investigation.

The effects of temperature on decolorisation

Because Reactive blue 49 and Acid blue 62 were very extensively decolorised when the concentration of the dyes used was 50mg/L, it was decided to increase the concentration of these dyes to 300mg/L in order to ensure that there was sufficient dye to act as substrate for ligninolytic enzymes to reveal clearly if there were any effects caused by variation of temperature and pH.

The temperature at which the solutions of dyes were treated might affect adsorption capacity and the amounts and activities of the enzymes produced. The optimum temperature of crude laccase, the predominant enzyme produced by *L. polychrous*, is 55°C and its activity at 30°C is approximately 45% of that at the optimum temperature (**Budda et al., 2012**). The results shown in figure 3 demonstrate clearly that temperatures between 30 – 45°C had no significant effect on the extent of decolorisation of the dyestuffs examined. Even when the concentrations of Reactive blue 49 and Acid blue 62 were increased from 50 to 300 mg/L, more than 93% decolorisation of all of the dyes was achieved. Presumably, during cultivation of *L. polychrous* Lév. on cassava rhizome with 70% initial moisture content there was sufficient water not to restrict the production of the degradative enzymes involved in the decolorisation of the dyestuffs.

The effects of pH of the solutions of dyes on decolorisation

Enzyme activity varies with pH and citrate-phosphate buffer was selected to control the pH of dye solutions during decolorisation because it provides buffering capacity over a wide range. It was suspected that the activity of fungal laccase was inhibited by this buffer in the pH range of 3-7 because of the delay of the onset of decolorisation of the dyes (cf the results in figures 3 and 4). Moreover, laccase activities of extracts prepared with water were always higher than those of extracts prepared with citrate-phosphate buffer of any pH. pH 3 was the optimum pH for the decolorisation of Reactive 49 and Acid blue 62 (figure 4A and 4E) and it is interesting to note that the optimum pH of laccase produced by *L. polychrous* is 3.0 (**Budda et al. 2012**). The results of figure 5A show that maximum laccase activity also was observed at this pH under these experimental conditions.

The suspected inhibition of the ligninolytic enzyme by the components of citrate-phosphate buffer might have had a significant effect on the degradation of the recalcitrant dyestuff; Navy blue did not undergo degradation (Figure 4C). Even although the laccase produced by *L. polychrous* Lév. in this study had its maximum activity at pH 3 (figure 5A), it was unstable on storage at 37°C in citrate-phosphate buffer of pH 3 (figure 5B). This observation might explain why decolorisation of Reactive blue 49 was delayed during the first 12 hours of incubation at pH 3 (see figures 4A and 3A).

The effects of pH on adsorption of dyestuffs onto the mixture of cassava rhizome and fungal mycelium are illustrated in figures 4B, 4D, and 4F. Various effects were observed, presumably caused by the different chemical structures and affinities of the dyes for the solid substrate. Generally, the most effective dyeing conditions for acid dyes are at acidic pH (**Zollinger, 2003**). Acid blue 62, an acid dye, was adsorbed most extensively at pH 3. However, it was adsorbed almost as extensively at pH 5 and 7 (figure 4F). Dyeing cellulose fiber with reactive dyes demands alkaline conditions in order to promote covalent bonding (**Zollinger, 2003**). Although Reactive blue 49 is a reactive dye, it also is a sulphonated anionic species. Therefore, it might be expected to be adsorbed most extensively at pH 3, as was Acid blue 62. Moreover, citrate and phosphate in the buffer might also affect the extent of adsorption.

Acid blue 62 was decolorised most effectively, followed by Reactive blue 49 and then Navy blue (figure 4). The chemical structure of the dye also profoundly influences the degradative capability of white rot fungi. **Jarosz-Wilkolazka et al. (2002)** found that the azo dye, Acid Red 183, was more resistant to degradation by some white rot fungal strains than the anthraquinone Basis blue 22. In addition, **Eichlerová et al. (2006)** showed that anthraquinones were biodegraded by *Dichomitus squalens* much more rapidly than azo dyes. **Eichlerová et al. (2006)** also suggested that azo dyes were more recalcitrant because azo compounds are rarely encountered in nature.

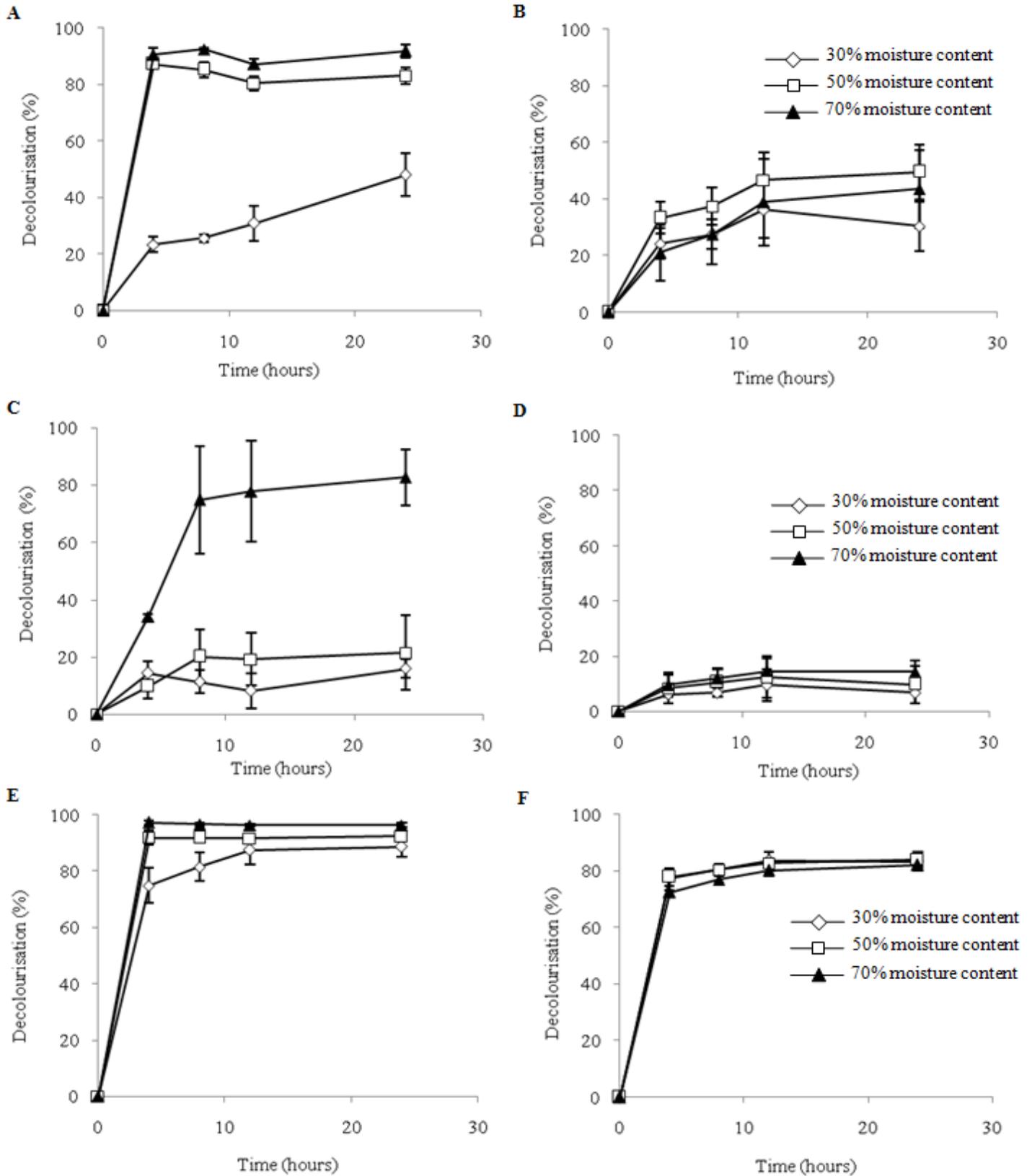


Figure 1 Decolourisation by *L. polychrous* Lév. cultivated on cassava rhizome at different initial moisture contents. (A) active culture, Reactive blue 49, (B) inactivated culture, Reactive blue 49, (C) active culture, Navy blue, (D) inactivated culture, Navy blue, (E) active culture, Acid blue 62, and (F) inactivated culture, Acid blue 62

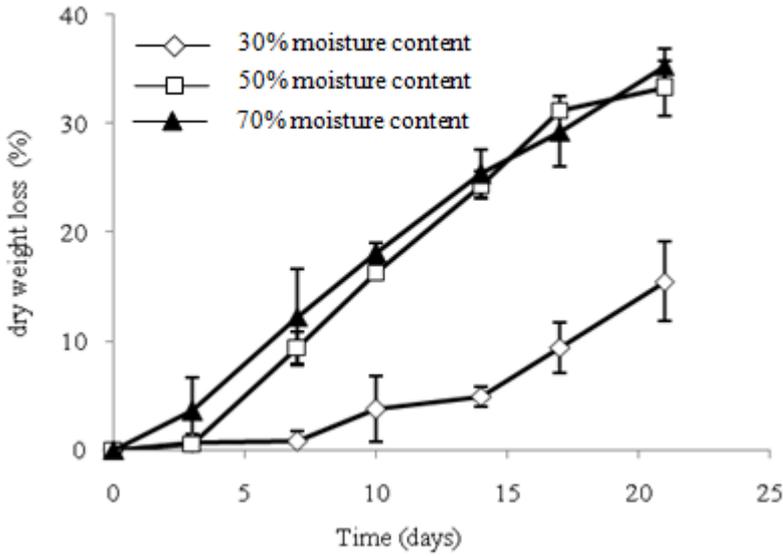


Figure 2 Dry weight loss of cultures of *L. polychrous* Lév. grown on cassava rhizome at different initial moisture contents

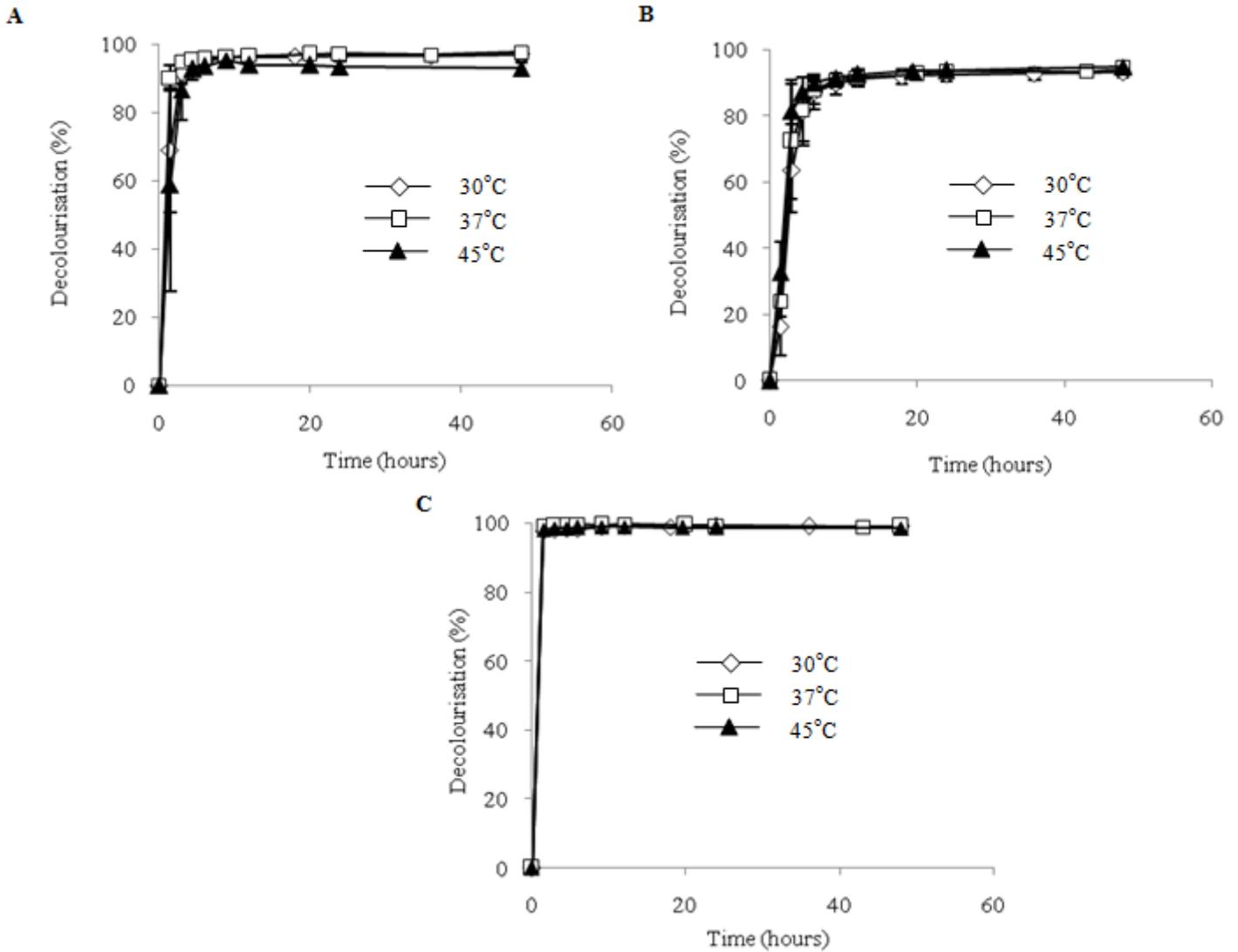


Figure 3 Extents of decolorisation at different temperatures by *L. polychrous* Lév. grown on cassava rhizome. (A) Reactive blue 49, (B) Navy blue and (C) Acid blue 62
The solutions of dyes were prepared with distilled water (see the Materials and Methods).

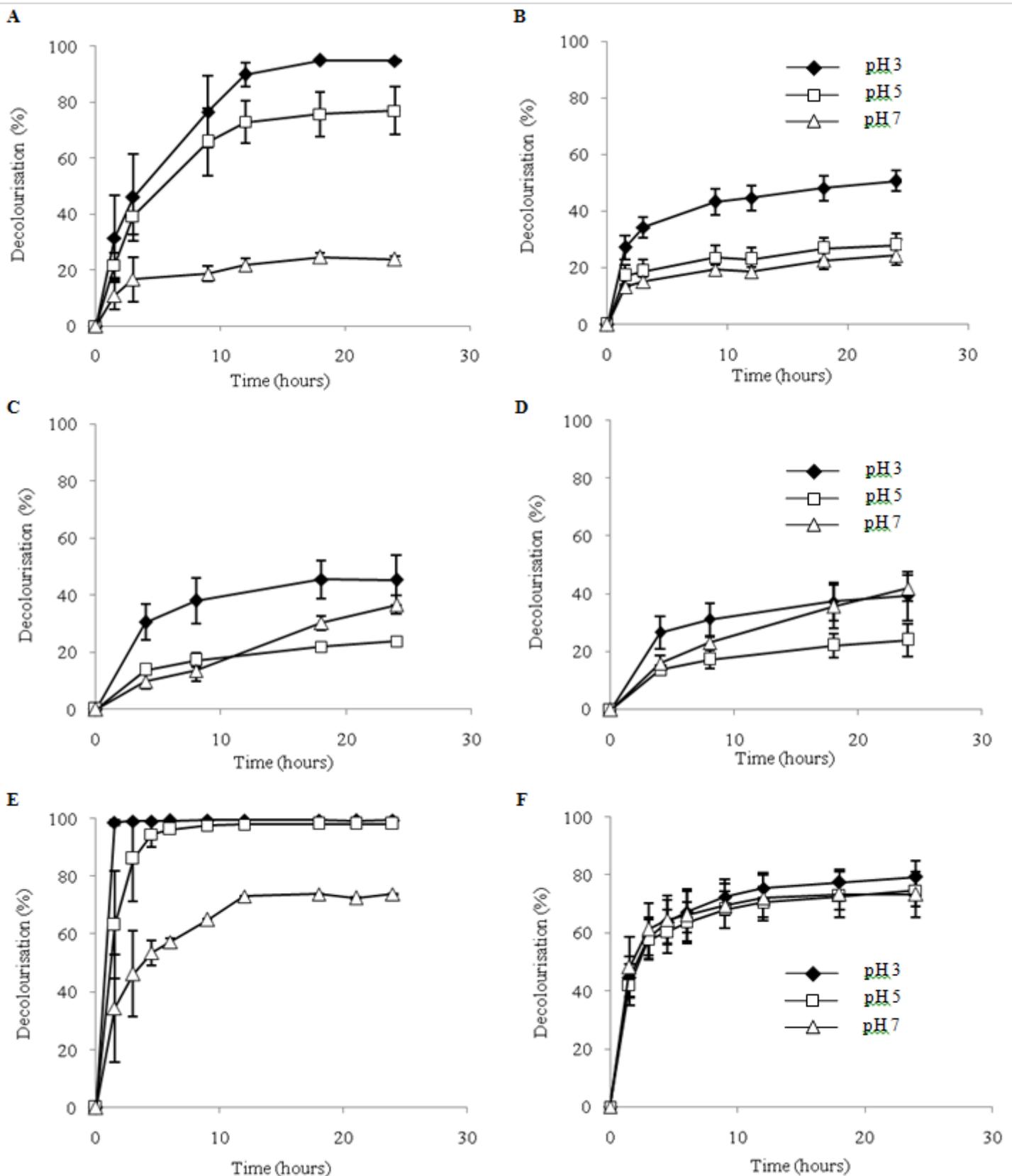


Figure 4 Decolourisation of solutions of dyes at various pHs by *L. polychrous* Lév. cultivated on cassava rhizome. (A) active culture, Reactive blue 49, (B) inactivated culture, Reactive blue 49, (C) active culture, Navy blue, (D) inactivated culture, Navy blue, (E) active culture, Acid blue 62, (F) inactivated culture, Acid blue 62. The solutions of dyes were prepared with citrate-phosphate buffers (see the Materials and Methods)

The effects of pH on adsorption of dyestuffs onto the mixture of cassava rhizome and fungal mycelium are illustrated in figures 4B, 4D, and 4F. Various effects were observed, presumably caused by the different chemical structures and affinities of the dyes for the solid substrate. Generally, the most effective dyeing conditions for acid dyes are at acidic pH (Zollinger, 2003). Acid blue 62, an acid dye, was adsorbed most extensively at pH 3. However, it was adsorbed almost as

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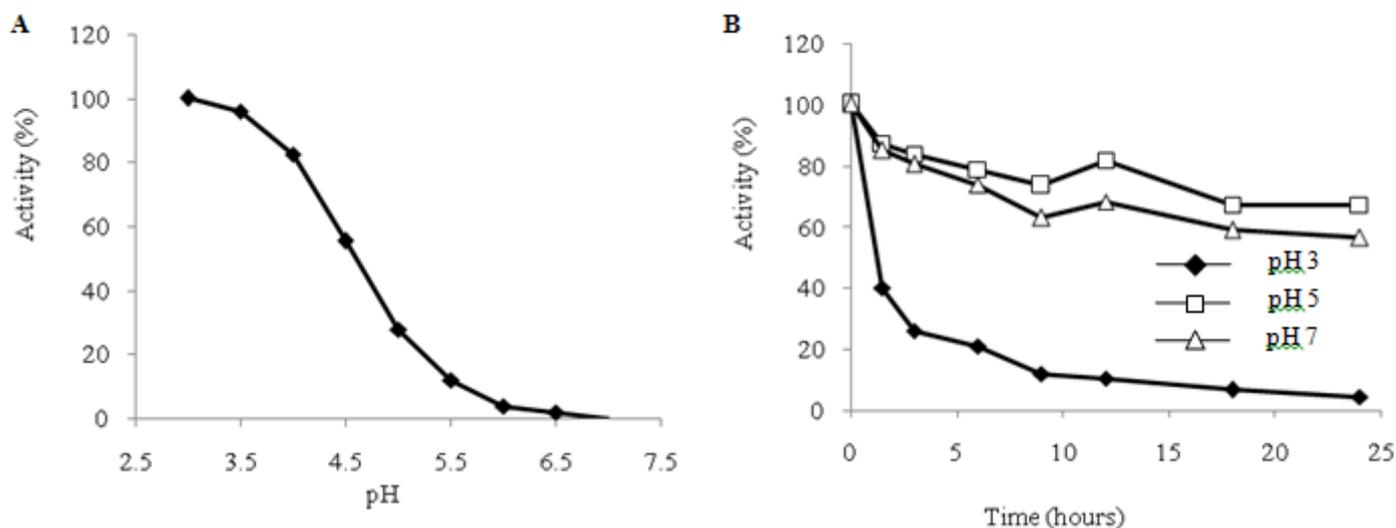


Figure 5 Activities (A) and stability at 37°C (B) at various pHs of laccase produced by *L. polychrous* Lév. grown on cassava rhizome. (A) Activity (Y-axis) is the percentage of the activity observed at pH 3 (B) Activity (Y-axis) is the percentage of the activity observed at zero hours of storage at 37°C. See the Materials and Methods.

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CONCLUSION

The moisture content of cassava rhizome used for fungal cultivation had a significant impact on the extent of decolorisation of aqueous solutions of synthetic dyes when enzymic degradation was the main mechanism of decolorisation. The highest extent of decolorisation was observed when the moisture content of the cassava rhizome used for fungal cultivation was 70%. Temperatures between 30°C – 45°C did not affect the extent of decolorisation. The optimum pH for enzymic degradation by laccase was 3.0. The effect of pH on the adsorption of dyestuffs onto the mixture of cassava rhizome and fungal mycelium depended on the molecular structure of the dyestuff. The anthraquinone reactive dye (Reactive blue 49) was adsorbed most at pH 3, while pHs from 3.0 – 7.0 did not influence the adsorption of the acidic anthraquinone (Acid blue 62). The reactive azo dye Navy blue was adsorbed extensively at pH 3.0 and 7.0. It was suspected that the components of the buffer used to control pH (citrate-phosphate) inhibited the degradative enzymes produced by *L. polychrous* Lév.

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