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Biodecolorization and Biodegradation of Textile Dyes by the Newly Isolated Saline-pH Tolerant Fungus *Pestalotiopsis* sp.

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ABSTRACT

Wastewater from textile industry effluents contains high amounts of colored and toxic compounds that can interrupt aquatic life systems when they are discharged to the environment without being treated. The physicochemical characteristics of effluents typically have a wide range of pHs and salinities, which are difficult for conventional techniques to remove. In addition, a limited number of microorganisms with the ability to grow and produce degradative enzyme systems can survive under those condition. Therefore, identifying microorganisms that are capable of decolorizing and degrading textile dyes under various pHs and salinities is needed. Among the fifteen strains tested in this study, *Pestalotiopsis* sp. NG007 exhibited the strongest ability to grow and decolorize Reactive Red 4 under saline conditions at pH 8. The ability of this strain to decolorize three textile dyes: Reactive Green 19, Reactive Orange 64 and Reactive Red 4, was investigated in a liquid medium and bioreactor system using immobilized mycelia. The fungus displayed a high decolorization capacity (20-98%) over 3 days in a wide range of pHs (pH 3-12) and salinities (0-10% w/v). In the bioreactor system, immobilized mycelia of the strain exhibited the ability to decolorize textile dyes by both adsorption (6-53%) and degradation (34-41%) mechanisms. This study demonstrated that *Pestalotiopsis* sp. has the potential to decolorize textile dye effluents containing a wide range of pHs and salinities.

Key words: Biodecolorization, bioreactor, pH-saline tolerant fungus, *Pestalotiopsis* sp., textile dyes

INTRODUCTION

Textile dye effluents from industries typically contain high amounts of colored and toxic compounds with a wide range of pHs and salinities (Chatzisymeon *et al*., 2006; D'Souza *et al*., 2006). Due to inefficient techniques, approximately 10-15% of the dyes are lost during the dyeing process and 2-20% are directly discharged as aqueous effluent to the environment (Jin *et al*., 2007), in which they may persist and accumulate to toxic levels for aquatic life systems (Pointing *et al*., 2000). Azo dyes are the largest chemical class of synthetic dyes and are widely used as colorants in textile dyeing, leather, plastic, food, cosmetics, paper printing, with the textile industry being the largest consumer. Azo dyes were originally designed to be recalcitrant for long-term use and thus,

resistant to aerobic wastewater treatment. Due to their low biodegradability, conventional physical or chemical wastewater treatment systems are typically inefficient in treating dye wastewater and are costly and not very adaptable to a wide range of dye wastewaters. These methods are commonly effective in decolorizing textile dyes only if the effluent volume is small (Robinson *et al*., 2001), but often fail to reduce the toxicity of effluents (Saparrat and Hammer, 2006).

Biological treatments based on fungal decolorization offer a potential strategy to not only remove the color of dyes, but also reduce their toxicities. These methods are cost-effective and can be applied to a large volume of effluents. In addition, most xenobiotic compounds contained in effluents were easily degraded by the ligninolytic system of the fungus (Dhanjal *et al*., 2013; Hadibarata *et al*., 2012; Sari *et al*., 2012), resulting in less toxic compounds. However, the physicochemical characteristics of textile dye effluents typically have a wide range of pHs and salinities generates a constraint for some fungi since a limited number of fungi are active under these conditions. Therefore, identifying microorganisms that can adapt to various pHs and salinities is an important strategy for the successful decolorization of textile dye effluents.

Screening for fungi capable of decolorizing dyes under salinity and pH 8 was conducted in this study. The potential fungus was selected to investigate its ability to decolorize and degrade three textile dyes under various pHs and salinities in liquid medium. Finally, the application of a vertical bioreactor system to decolorize textile dyes was employed using immobilized mycelia. This is a new low-cost method to decolorize textile dye effluents that can be applied to a wide range of pHs and salinities.

MATERIALS AND METHODS

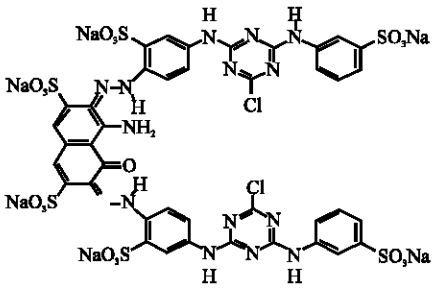
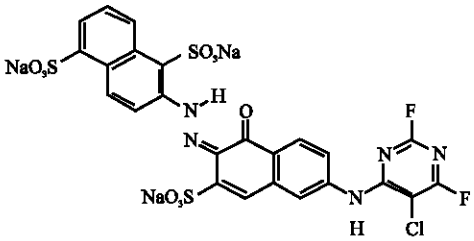
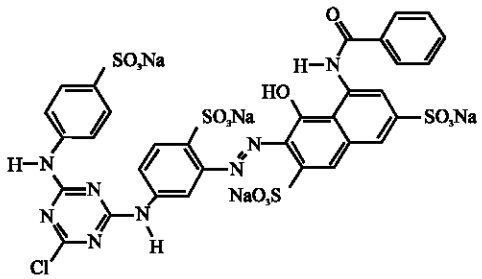
Chemicals and microorganisms: Fifteen fungi were isolated and selected from several location in Ehime Prefecture, Japan. Three textile dyes: Reactive Green 19 (RG19), Reactive Orange 64 (RO64) and Reactive Red 4 (RR4), were used as substrates. The characteristics of these substrates are shown in Table 1. Malt extract, glucose, polypeptone, sodium alginate, calcium chloride and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Japan).

Screening of textile dye decolorizing fungi: Fifteen isolates were used to investigate their ability to grow and decolorize RR4 (100 mg L^{-1}) under saline conditions (3.5% w/v) and pH 8. Their growth abilities were tested in Malt Extract Agar (MEA) containing RR4 and decolorization was conducted in liquid medium.

Decolorization of textile dyes in liquid medium: The ability of the most active fungus (strain NG007) to decolorize three textile dyes: RG19, RO64 and RR4, was investigated in Malt Extract (ME) liquid medium under various pHs (3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) and salinities (0, 1, 2, 2.5, 3.5, 5, 7 and 10% w/v). In a parallel experiment, the fungal biomass was measured by the gravimetric method.

Preparation of immobilized mycelia: The immobilization of mycelia was prepared by mixing homogenized mycelia and alginate (1.5% w/v). After the pre-incubation of strain NG007, mycelia was homogenized at 10000 rpm for 10 min. Sodium alginate (1.5% w/v) was added to the homogenized mycelia and stirred until homogeny. The mixture was dropped into the calcium chloride solution (0.1 M) and stirred until the formation of calcium alginate was complete. Immobilized mycelia were washed with distilled water until they were free of chloride ions and were then stored in a refrigerator at 4°C for 1 day before being used for the decolorization study.

Table 1: Characteristics of the textile dyes used in this study

Dye (C.I No.)	Molecular structure	Molecular weight	λmax (nm)
Reactive green 19 (205075)		1418.92	631
Reactive orange 64 (179095)		767.99	488
Reactive red 4 (18105)		995.21	523

Biodecolorization in a bioreactor system: Experiments were conducted using the bioreactor system MasterFlex®L/S® (Cole-Parmer Instrument Company) with two easy-loaders (model 7518-10) and tubing size No. 16. Immobilized mycelia were placed into the bioreactor column (Ø = 2.5 cm, h = 10 cm, v = 49 cm^3) and the dye-simulating textile effluent (100 mg L^{-1}) was flowed from an Erlenmeyer flask (100 mL) into the beads at a flow rate of 1.5 mL min^{-1}. The experimental design of the vertical bioreactor system is shown in Fig. 1.

Decolorization analysis: Dye decolorization was determined by monitoring the decrease in absorbance at the wavelength of the maximum absorbance of each dye: RG19 (λ_{max} = 631 nm), RO64 (λ_{max} = 488 nm) and RR4 (λ_{max} = 523 nm). The absorbance of the dye solution was monitored for 1, 2 and 3 day reactions for treatments in liquid medium or for 1, 2, 3, 6, 12, 24 h for treatments in the bioreactor. The decolorization efficiency (R, %) was calculated as follows:

$$R = \frac{(1-(Afinal)}{(Ainitial)} \times 100\%$$

where, $A_{initial}$ is the initial absorbance and A_{final} is the observed absorbance.

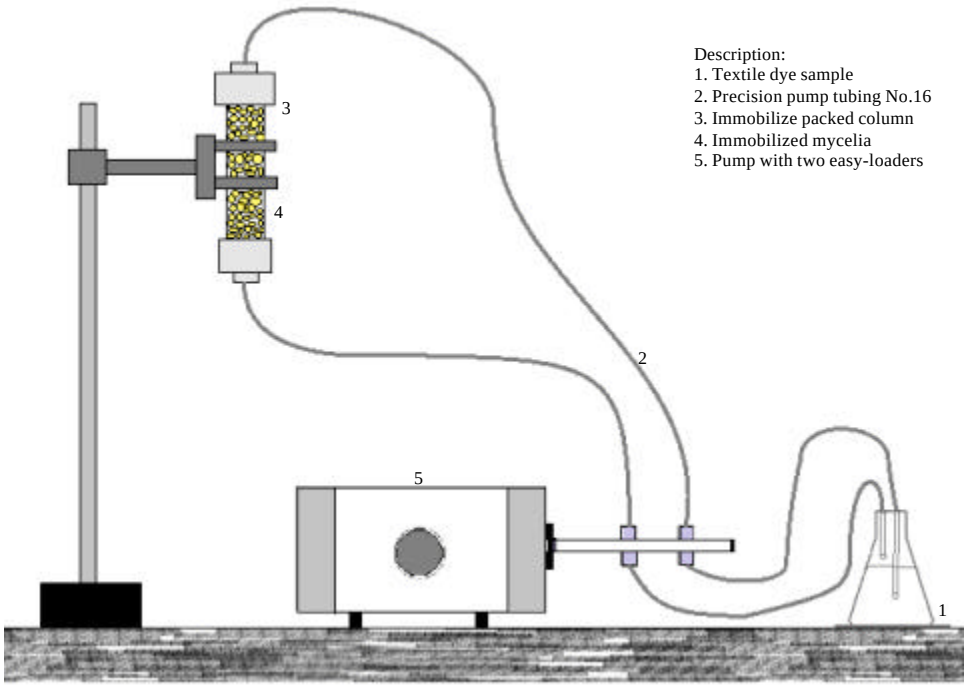


Fig. 1: Vertical bioreactor system used in this study

RESULTS

Screening pH-saline tolerant fungi: The characteristics of textile dye effluents with a wide range of pHs and salinities makes a screening method to identify fungi with the ability to decolorize textile dyes under these conditions is important to investigate. The abilities of fifteen fungi on RR4 under saline (3.5% w/v) at pH 8 are shown in Fig. 2. All fungi decolorized RR4 with varying abilities and strain NG007 had the highest abiliy, followed by D413, IG05, IG03, D17, F092 and F094, respectively. Analysis by DNA sequencing reveal that NG007 was similar (100%) to *Pestalotiopsis* sp., a deuteromycete fungus (Yanto and Tachibana, 2013). The ability of genera *Pestalotiopsis* to decolorize dyes has been reported previously by Saparrat and Hammer (2006). However, its ability to decolorize textile dyes under saline conditions and pH 8 has not. Therefore, strain NG007 was further examined to investigate its ability to decolorize three textile dyes (RG19, RO64 and RR4) under various pHs and salinities.

Effect of pH: The ability of *Pestalotiopsis* sp. NG007 to decolorize textile dyes was investigated in various pHs. The pH of the textile dye solution affected the ability of the fungus to decolorize and grow on it (Fig. 3). Decolorization was optimum for all textile dyes at pH 3. At pH 3, free cells of NG007 decolorized 98% of RG19, 95% of RO64 and 91% of RR4 over 3 days, respectively. The decolorization ability of NG007 decreased with an increase in the pH of the medium. In contrast, the growth ability of NG007 increased by increasing the pH to pH 10 and then markedly decreased at pH 11 and 12. A significant difference was observed in the fungal biomass between alive NG007 and the control (autoclaved mycelia) and NG007 showed the ability to grow and decolorize the dyes. The fungal biomass of *Pestalotiopsis* sp. was optimum for all three dyes at pH 10 at 0.48, 0.36 and

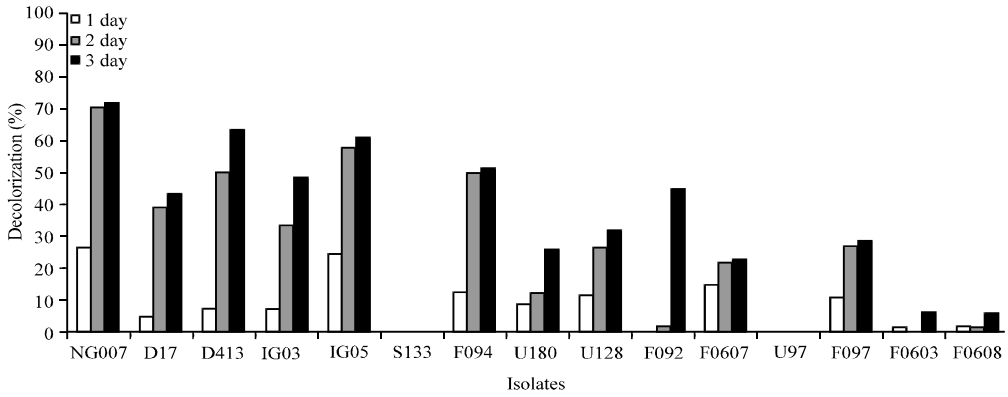


Fig. 2: Screening of pH-saline tolerant fungi with the ability to decolorize RR4 under saline conditions at pH 8

0.25 g 20 mL^{-1} for RG19, RO64 and RR4, respectively. At pH 3, no significant difference was observed in biomass between autoclaved and alive strains, however, the alive strain had the highest decolorization efficiency. During decolorization, the strain produced optimum ligninolytic activities. Manganese peroxidase, laccase and lignin peroxidase at pH 3 were 30, 46 and 2.5 U L^{-1}, respectively. All these enzymes have been suggested to play an important role in decolorizing textile dyes (Moldes *et al*., 2012; Iqbal and Asgher, 2013; Kalpan *et al*., 2012).

Effect of salinity: Experiments were conducted in the present study at various salinities (0-10%). The results showed that the salinity of the dye solution had no negative impact on the ability of *Pestalotiopsis* sp. to decolorize three textile dyes or its fungal biomass (Fig. 4). The decolorization ability of the strain in 1% salinity was higher than that of the control (0% salinity), but showed no significant difference in ability up to 10% salinity. The decolorization of RG19 by *Pestalotiopsis* sp. NG007 was optimum at 1% salinity (96%) and was 37% higher than that of the control (70%). The optimal decolorization of RO64 and RR4 was achieved at 2.5 and 5% salinity, respectively, with the decolorization yields being 89 and 87%, respectively. These results indicated that decolorization increased by approximately 29 and 58%, respectively. Fungal biomass was also similar to the decolorization ability. In general, fungal biomass increased with an increase in salinity. However, the optimal fungal biomass was shown at 2.5% salinity for RG19 (0.92 g 20 mL^{-1}) and at 3.5% salinity for RO64 (0.76 g 20 mL^{-1}) and RR4 (0.42 g 20 mL^{-1}), respectively and subsequently remained steady until 10% salinity. The high decolorization efficiency and growth rate of this strain under saline conditions demonstrated *Pestalotiopsis* sp. NG007 to be a halotolerant fungus. This study was in accordance with other studies showing the effects of salinity on the growth and enzymatic activities of *Pestalotiopsis* sp. in highly saline medium (Chen *et al*., 2011; Arfi *et al*., 2013).

Decolorization in the bioreactor: Dye decolorization of RG19, RO64 and RR4 was evaluated using autoclaved and alive immobilized mycelia of *Pestalotiopsis* sp. NG007 in a bioreactor system. The flow rate of 1.5 mL min^{-1} was used in this study because it showed optimum decolorization relative to other flow rates. In a previous experiment using the bioreactor, we found that the faster the flow rate, the lower the biodecolorization rate by the immobilized enzymes. In this study, the

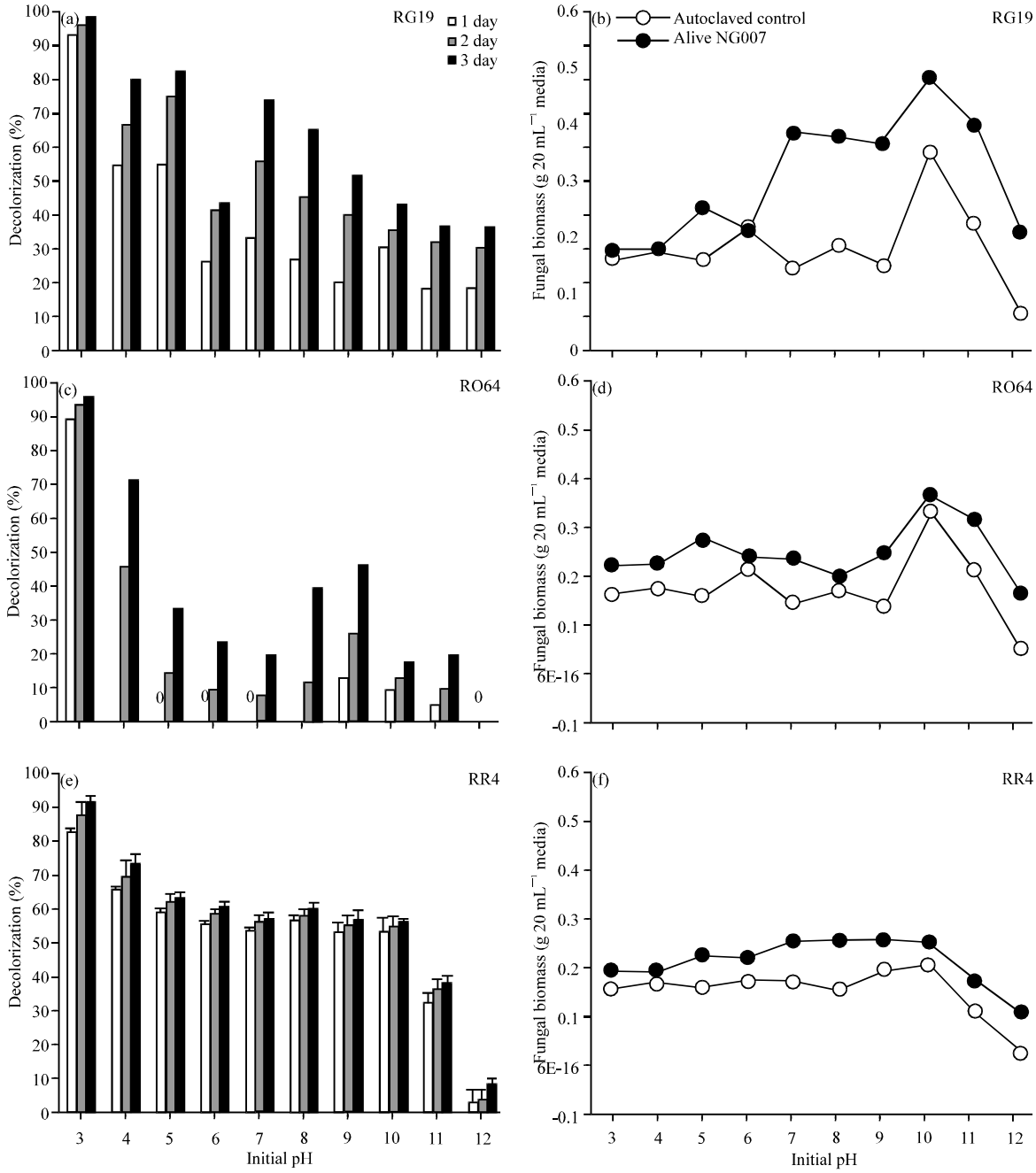


Fig. 3(a-f): Effect of pH on decolorization and the fungal biomass, (a, c,e) Decolorization vs initial pH and (b, d, f) Fungal biomass vs initial pH

decolorization rates of RG19, RO64 and RR4 for 24 h by the immobilized mycelia of NG007 in the bioreactor were 94, 54 and 47%, respectively. However, the decolorization rates of RG19, RO64 and RR4 by autoclaved mycelia were 53, 17 and 7%, respectively, due to an adsorption mechanism by the dead mycelia. Some dead microorganisms including algae (Khataee *et al*., 2013), macrophytes (Pathomsiriwong and Reanprayoon, 2012) and fungi (Assadi and Jahangiri, 2001; Seyis and Subasioglu, 2008) have been shown to retain the ability to decolorize dyes by an adsorption mechanism. This study showed that decolorization occurred by bioadsoprtion at the initial stage and

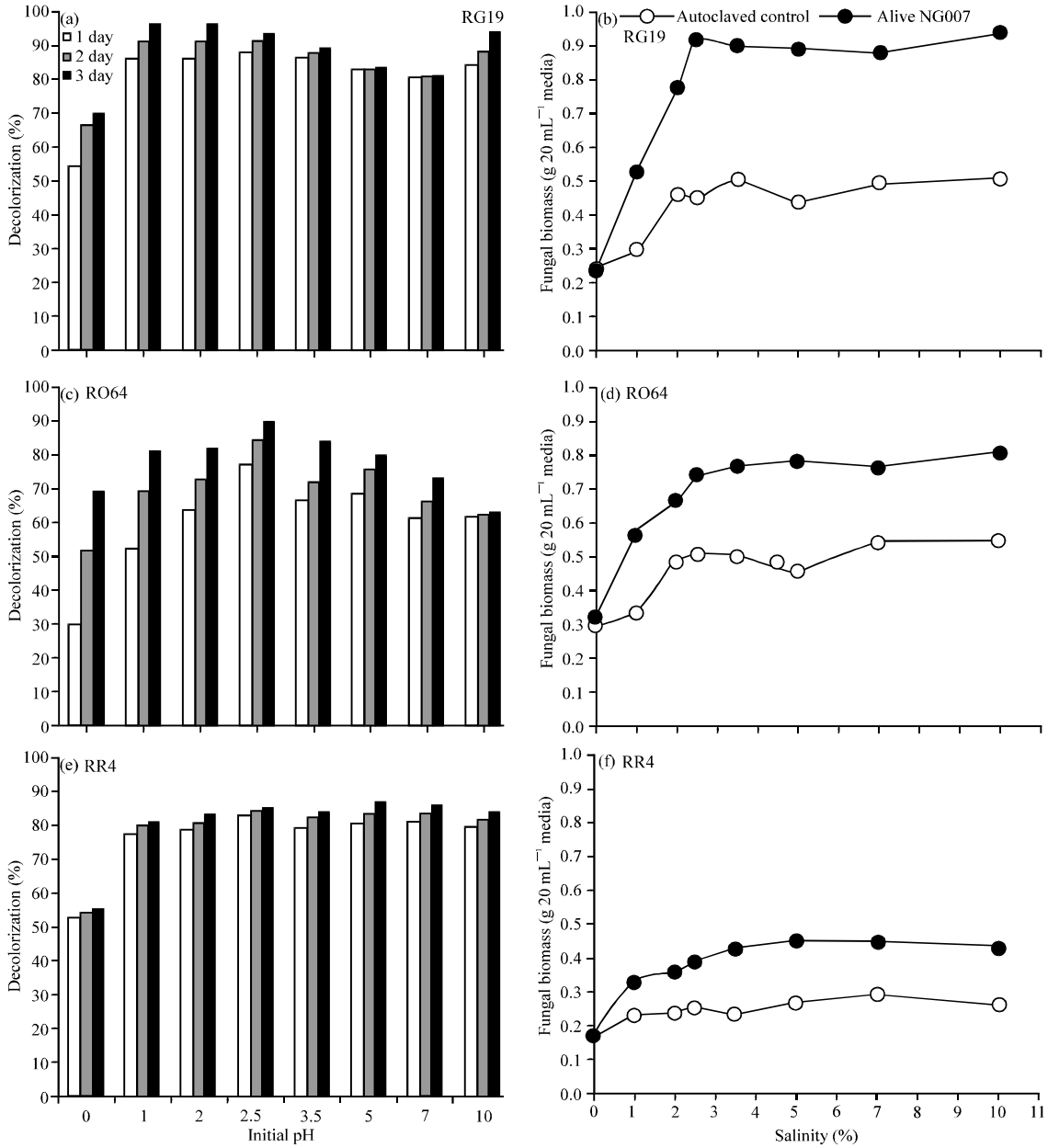


Fig. 4(a-f): Effect of salinity on decolorization and the fungal biomass, (a, c, e) Decolorization vs salinity and (b, d, f) Fungal biomass vs salinity

then by biodegradation. The filamentous fungus *Acremonium kiliense* showed the ability to decolorized dyes using these two mechanisms (Youssef *et al.*, 2008). The application of the bioreactor system allowed a higher efficiency for the biodecolorization of textile dyes because it facilitates a continuous drop from the dyes to the immobilized mycelia. The interaction a small concentration of dyes with mycelia from a periodically dropped solution lead to a more effective decolorization than using liquid medium, due to overload capacity of the free cells.

DISCUSSION

Mycelia growth rates and decolorization zone evaluations on dye-containing media have been extensively used in most research on screening and isolating dye-decolorizing fungi (Borokhov and

Rothenburger, 2000). However, it is better to conduct screening under conditions as similar as possible to those to which they will be applied (Dos Santos *et al*., 2004). Textile dye effluents from industries typically contain a wide range of pHs and salinities. Therefore, in the present study, screening for textile dye-decolorizing fungi was conducted under saline conditions (3.5% w/v) at pH 8 using RR4 as a model compound. All fungi tested in this study decolorized RR4 with varying yields. Among them, genera *Pestalotiopsis* showed the most potential for decolorization under these conditions. Other fungi belonging to basidiomycetes had difficulties in growing and decolorizing RR4 due because most require specific growth conditions. Genera *Pestalotiopsis* sp. has been shown to have the potential to remove substituted phenylurea herbicides (Vroumsia *et al*., 1996), lignin (Osono, 2007; Osono *et al*., 2011; Homma *et al*., 2007), other lignocellulosic materials (Hao *et al*., 2006), resin (Dorado *et al*., 2000) and dyes (Saparrat and Hammer, 2006; Hao *et al*., 2007). Genera *Pestalotiopsis* has also been found in extra heavy crude oil (EHCO)-polluted soil and showed the potential to upgrade Venezuelan EHCO (Naranjo *et al*., 2007), used motor oil contaminated soil (Husaini *et al*., 2008) and discarded creosote-treated wood (Kim *et al*., 2010). All these activities may be positively correlated to the growth and decolorization ability of the strain in textile dyes containing media under saline conditions at pH 8.

Since the pHs of textile dye industry effluents vary, the decolorization potential of *Pestalotiopsis* sp. was evaluated at various pHs. The pH of raw textile mill effluents was previously shown to be 2.5 or 8.9 (Verma *et al*., 2010), whereas the average pH of dye industry effluent was approximately 7-8 (Jolly *et al*., 2009), which is slightly alkaline in nature. The pHs of solutions typically affected the metabolism and enzymatic activities of fungi, which influenced their growth and decolorization abilities. In the present study, experiments were conducted at acidic pH values of 3-6, a neutral pH of 7 and alkaline pH values of 8-12. The ability of *Pestalotiopsis* sp. to decolorize textile dye effluents differed significantly under various pHs. Optimal decolorization for all dyes occurred at pH 3 and decreased with an increase in pH. In contrast with the decolorization rate, the fungal biomass of NG007 increased with an increase in pH, which indicated the absence of a positive correlation between decolorization and fungal biomass of this strain. This result was in accordance with Hsueh *et al*. (2009) who reported that azo dye decolorization was non-growth associated, but was maximum when cell growth was repressed. We suggest that the high decolorization rate was due to strong enzymatic activities under these conditions in spite of adsorption by mycelia. At pH 3, ligninolytic activities were optimal in strain NG007, which may be involved in decolorization. Manganese peroxidase, laccase and lignin peroxidase activities at pH 3 were 30, 46 and 2.5 U L^{-1}, respectively.

In addition to pH, the wastewater from textile dye industry effluents contain substantial amounts of salts. Salt concentrations up to 15-20% have been detected in released wastewater (EPA, 1997). The presence of a high amount of salt is one of the major limiting factors for developing of an effective biodecolorization methods for dyes from textile effluents (Khalid *et al*., 2012). Although the majority of fungi are sensitive to high salinity, certain fungi species had previously shown the ability to decolorize dyes under saline conditions (Li *et al*., 2003; Hao *et al*., 2007; Verma *et al*., 2010). Ligninolytic enzymes, particularly laccase and MnP from some marine or marine-derived fungi, have been shown to be produced even under saline conditions (Li *et al*., 2003; D'Souza *et al*., 2006). In this study, *Pestalotiopsis* sp. a marine ascomycete, exhibited the ability to decolorize textile azo dyes under saline conditions. Moreover, its growth rate and decolorization capability was higher in saline than in non-saline conditions. *Pestalotiopsis* sp. is a

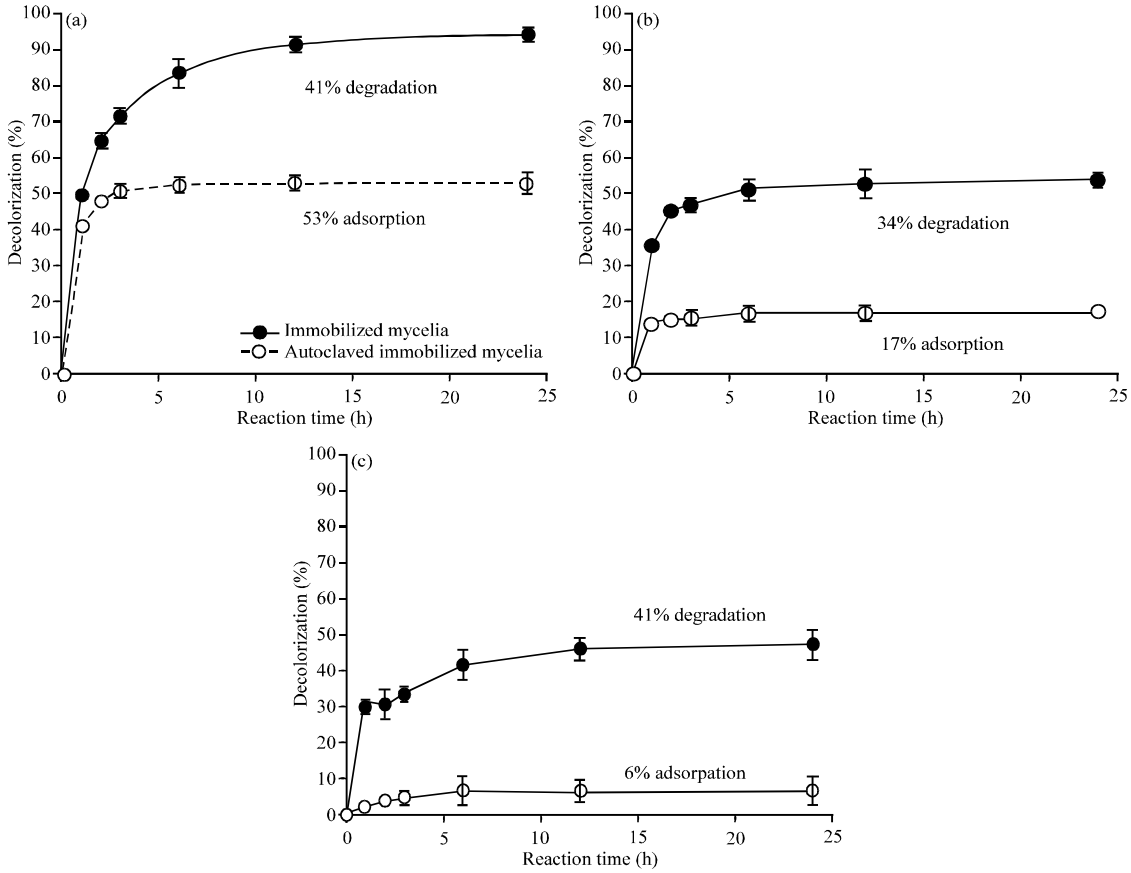


Fig. 5(a-c): Decolorization of three textile dyes using the bioreactor system

halo-tolerant fungus that has also been demonstrated to produce ligninolytic enzymes under saline conditions (Chen *et al*., 2011; Arfi *et al*., 2013) and has the ability to decolorize textile dyes due to these enzymes (Hao *et al*., 2007; Verma *et al*., 2010).

Regarding chemical structures, the decolorization rate differed significantly among the dyes tested and was in the order of RG19>RO64>RR4, respectively, which is in accordance with Pasti-Grigsby *et al*. (1992). Hsueh *et al*. (2009) reported that the chemical structures of dyes affected the color-removing ability of *Aeromonas hydrophila*. Both the positions of substituents on the aromatic ring and electronic characteristics of substituents in azo dyes significantly affected biodecolorization by *A. hydrophila*. However, we found that the removal of color from dyes in this study was also influenced by the adsorption characteristics of the dyes to the mycelia of the strain.

Fungal treatment by both dead and alive free mycelia displayed the potential to remove the color of textile dye effluents in a batch system only (Robinson *et al*., 2001) because they need many days to completely remove the color. However, most textile dye industry effluents with a continuous system need a more effective technique to rapidly remove the color. The application of the bioreactor system as shown in Fig. 1 was established in order to improve the biodecolorization rate by immobilized mycelia. The results showed that color removal was markedly increased in the first 6 h after reactions and subsequently remained almost stable. The decolorization of RG19, RO64 and RR4 after 6 h was 83, 51 and 41%, respectively. In the first 3 h, decolorization mainly occurred via adsorption by the mycelia. Color was removed by autoclaved immobilized mycelia (Fig. 5)

(dead mycelia) in the first 3 h. The dead mycelia decolorized 51% RG19, 15% RO64 and 5% RR4, respectively. However, the decolorization rate by alive immobilized mycelia was significantly higher than that of dead mycelia, which indicated that alive mycelia used degradation by enzymatic activities. The decolorization rate by immobilized mycelia in the bioreactor system was more efficient than that in liquid medium. The high decolorization rate by immobilized mycelia was due to the efficient reaction of the dyes to the beads and the transfer of oxygen during the reaction. This technique allows the dropping of dyes to react with the beads (containing mycelia) at a low concentration, leading to higher ability of the beads to degrade compounds in the dyes. In addition, the bioreactor system allowed the higher transfer of oxygen to the mycelia than that of the batch system, leading to the higher supporting condition for the enzymatic reaction involved in the decolorization process. Therefore, this technique offers a potential strategy to decolorize textile dyes from industry effluents with a continuous system.

CONCLUSION

The present study revealed that *Pestalotiopsis* sp. NG007 has the ability to decolorize three textile dyes (RG19, RR4 and RO64) with a wide range of pHs (3-12) and salinities (0-10%). The decolorizing ability of this strain was optimum at pH 3 and decreased with an increase in pH in the solution. Strain NG007 had a better decolorization rate under saline conditions than under non-saline conditions. An investigation of the bioreactor system using both autoclaved and non-autoclaved strains revealed that NG007 decolorized the three textile dyes by two mechanisms: adsorption and degradation due to enzymatic activities. This results indicated that the dead biomass of *Pestalotiopsis* sp. could also be used as a biosorbent to remove the three textile dyes from aqueous solution. The decolorization ability of the strain for the three dyes was found to be in the following order: RG19>RO64>RR4, respectively. The bioreactor system used in the present study has the potential to be used in treating textile dye effluents from industries that contain varying pHs and salinities.

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