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Decolorization of Orange II by Immobilized Thermotolerant White Rot Fungus *Coriolus versicolor* RC3 in Packed-Bed Bioreactor

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Abstract: Immobilized *Coriolus versicolor* RC3, a thermotolerant white rot fungus, was investigated for Orange II decolorized capability both in batch and continuous system. In batch study, immobilized fungal cell was tried on 20 ppm Orange II dissolved in synthetic liquid medium using various carbon and nitrogen sources. One gram per liter of glucose without nitrogen source was a minimal carbon source level for providing the high decolorization rate. Optimal initial pH and temperature were 6.5 and 30-37°C, respectively. In continuous decolorization, packed bed bioreactor was designed to use with 2 g of immobilized fungal cells filled with synthetic liquid medium obtained from batch study. At 8 h of Hydraulic Retention Time (HRT), more than 90% of the decolorization was maintained over 120 h. Considering for the size of polyurethane foam (PUF), 1.5 cm³ PUF with 8 h of HRT could maintain a high efficiency and the decolorization duration increased to 2 weeks. After fungal decolorization, the toxicity of wastewater evaluated with *Daphnia* sp., normal and cancer cell lines were reduced completely.

Key words: Decolorization, Orange II, *Coriolus versicolor*, packed-bed bioreactor, immobilization

INTRODUCTION

During the dyeing, 5-20% of the used dyestuffs are released into the process water (Soares *et al.*, 2001; Wang and Yu, 1999). The azo dye is the most frequently used synthetic dye and particular required to be cleaned up to reduce the impact on the environment since itself derived metabolic intermediates are potentially carcinogenic or mutagenic (Chen *et al.*, 2005). The current existing techniques for the treatment of wastewater containing dyes have high cost, formation of hazardous by-products or intensive energy requirement (Stolz, 2001). In addition, the conventional biological treatments such as activated sludge systems can not give a satisfaction of dye removal because the complex structure of dyes is hard to degrade by general microorganism (Jarosz-Wilkolazka *et al.*, 2002). If anaerobic treatments were used, they can only remove the azo dyes by activity of azo reductase and generate toxic by-product, aromatic amine, which is included in carcinogenic substance group (Novotny *et al.*, 2001; Kapdan and Kargi, 2002).

In nature, white rot fungi produce some ligninolytic enzymes for lignin degradation for utilizing as their carbon

and nitrogen sources (Swamy and Ramsay, 1999) and some dyes can be degraded by these enzymes resulting in the decreasing of toxic substances (Chen, 2002). In addition, extracellular enzymes system enables white rot fungi to tolerate high concentration of pollutants (Kapdan *et al.*, 2000). These characteristics of fungi are interested to use as an alternative choice in textile dye treatment and possible to be developed in large scale. Immobilization of microbial cells has received increasing interest in the field of wastewater treatment due to some advantages including long retention time of biomass in the system, manipulation of growth rate independent from washout effect, necessary protection from high concentrations of recalcitrant organics that are toxic to free cells, have the potential to degrade toxic chemicals faster than conventional wastewater treatment systems, ease of use in a continuous reactor and their ability for scale up (Christopher *et al.*, 2002).

There are few reports in which organisms are grown and used for the decolorization in stirred tanks bioreactors presumably because of concern about the shear forces effect on enzymes or mycelial structure. Most authors prefer to use aeration to provide mixing as well as

aeration. Packed bed bioreactors are the one in a group of fixed bed bioreactors that cells are immobilized on large particles and the effluent was pass through a fixed material (Nicoletta *et al.*, 2000). The reactors have the advantages of simplicity of construction and operation and they can give a high reaction rate. Continuous packed bed bioreactors are the most widely used reactors for immobilized enzymes and immobilized microbial cell and have also been successfully tested for various organic pollutants (Zilouei *et al.*, 2006).

In this study, the optimal condition on the azo dye decolorization in batch decolorization was studied and the Orange II was used for the azo dye model substances in the experiments. In addition, the effect of hydraulic retention times, polyurethane foam sizes and the dye concentration on the continuous decolorization in packed bed bioreactor were also investigated.

MATERIALS AND METHODS

Microorganism and cell immobilization: The thermotolerant white rot fungus *Coriolus versicolor* RC3 isolated from Chiang Mai, Thailand by Khanongnuch *et al.* (2004) was used through all experiments. The fungal strain was maintained on Potato Dextrose Agar (PDA) and stored at 4°C. For immobilization, PUF was cut into the cubic shape about 1 cm³ in size, boiling for 10 min, washing thoroughly three times with distilled water and dried overnight at room temperature (26-34°C). One agar plug of *C. versicolor* RC3 (Ø = 1 cm) was inoculated to 1 g of PUF soaked in 50 mL Malt Extract Medium (ME) within the Erlenmeyer flasks and incubated on a rotating shaker (Lab-Therm; Kühner AG, Switzerland) at 120 rpm and 37°C for 4 days, then, the fungal colonized PUF was washed by sterile tap water for removing of mobilized cells and used in continuous decolorization.

Batch decolorization experiments: For studying the effect of carbon and nitrogen concentration on dye decolorization, the modified Kirk's medium supplemented with 20 ppm of Orange II was used as the main composition. Glucose quantity was varied as the carbon source at 0, 0.5, 1.0, 5.0, 10.0 and 20.0 g L⁻¹, while ammonium oxalate used as nitrogen source was also varied at 0, 0.1, 0.2, 0.5, 1.0 and 2.0 g L⁻¹. Experiments were designed in triplicates of the 80 mL dye supplemented medium in 250 Erlenmeyer flasks. One agar plug (Ø = 1 cm) of *C. versicolor* RC3 from an actively growing mycelium on malt extract agar (MA) was used as inoculum. The cultures were incubated on a rotating shaker at 120 rpm at 37°C for 4 days. The treated dye solutions were sampling

at 0, 24, 48, 60, 72 and 84 h and determined for the decolorization (%), pH and laccase activity. The decolorization of Orange II was measured by the decrease of absorbance value at 483 nm by UV-VIS spectrophotometer (JASCO V-530) comparing to the initial value. After the optimal concentration of glucose and nitrogen sources were obtained, effect of the initial pH was studied. The initial pH of the medium was varied between 3.5-8.5. In addition, the effect of temperature was also determined varying in 25, 30, 35, 37, 40 and 45°C, respectively.

Continuous decolorization: Packed-bed bioreactor design was modified from Feijoo *et al.* (1995). The main component is a glass column with 30 cm height and 4 cm of internal diameter. With 16 cm height of working area caused a working volume of 201 mL covered by porous Teflon lid connected to rubber cork with glass rod. It was autoclaved and then packed with fungal immobilized PUF overgrown. A medium with the same composition as investigated batch experiment was prepared, autoclaved and cooled to room temperature. The medium was then pumped into the reactor. The air-phase of the reactor was renewed by the aid of aquarium pumps connected with tubes at the bottom of the reactors. The air was led into and out from the reactors through sterile filters (0.45 µm) in order to avoid contamination of the reactors with 5 vvm of aeration rate. The dye concentration in the medium introduced to reactor was 20 ppm. The reactor was incubated at 37°C for 5 h before the continuous feeding and HRT were varied as 5, 6, 8 and 12 h, respectively. The size and amount of PUF used in the reactor were varied as 1, 1.5 and 2 cm³ and 2, 1.75 and 1.5 g, respectively. The HRT used was 8 h. After the optimal HRT, size and amount of PUF were determined the Orange II concentration in the medium was varied up to 50 and 100 ppm. Samples were taken from the over flow of feed outlet and determined decolorization (%), pH and laccase activity.

Enzyme assay and protein determination: Laccase activity was determined by oxidation of 2,6-Dimethoxyphenol (DMP) and the increasing in absorbance at 470 nm was measured (Khanongnuch *et al.*, 2004). One unit of laccase activity was defined as the amount of enzyme that oxidized 1 µmole of DMP per minute. The digital pH meter was used to measure the pH of the dye solution.

Toxicity test: Dye solution before and after decolorization was prepared to study the toxicity using *Daphnia magna* compare with *ex vivo* assay for cytotoxic activity with Sulphorhodamine B (SRB) assay (Skehan *et al.*, 1990). Ten

neonates of *Daphnia magna* were transferred to glass Petri dish containing the various concentrations of test solutions. The toxicity results were evaluated on the basis of immobilization percentage obtained by dividing the number of immobilized animals by total animals. The toxicity of wastewater samples was explained as toxic when the immobilization percent is higher than 50% (Selçuk, 2005).

RESULTS AND DISCUSSION

Batch decolorization experiments

Effect of glucose and nitrogen concentration on dye decolorization: When glucose in the culture broth was supplemented to the culture in various concentrations, we found that 1 g L⁻¹ of glucose was a minimum level for maintaining a satisfying the decolorization. In addition, maximum decolorization was obtained at 72 and 60 h under nitrogen limited (0.1 g L⁻¹) and nitrogen rich (2.0 g L⁻¹) condition, respectively (Fig. 1a and 1b). However, the best minimum glucose concentration giving the highest decolorization rate was 5 g L⁻¹, which

corresponding with the previous report by Fu and Viraraghavan (2001) who reviewed that good carbon sources for white rot fungi in decolorization purpose were glucose, starch and maltose, but 5 g L⁻¹ of glucose was the most suitable concentration. In the control, no addition of glucose, gave a poor decolorization rate in both nitrogen limited and nitrogen rich condition.

Effect of nitrogen concentrations on dye decolorization are demonstrated in Fig. 2. Without addition of glucose (Fig. 2a), all of the treatments showed a poor decolorization rate while the addition of 1 g L⁻¹ glucose gave the active decolorization (Fig. 2b). The treatment giving a fastest decolorization rate was 0.2 g L⁻¹ ammonium oxalate, while the slowest one was 2 g L⁻¹ ammonium oxalate. However, in the control experiment, no addition of any ammonium salts, the maximum of decolorization was occurred at 72 h. The comparison of Orange II solution before and after the fungal decolorization was shown in Fig. 3. Nitrogen is generally required by microorganisms and azo dyes can be utilized as the nitrogen source and the colors will be disappeared after microbial degradation of azo-chromophore. Too rich of nitrogen concentration in the culture broth made the fungus grew easily due to the easy obtaining of nitrogen for metabolism and no need to find another nitrogen source from the dye molecule causing the slow decolorization rate. Wang and Yu (1998) studied the synthetic dye degradation by white rot fungi *Trametes versicolor* and reported that the adsorbed dye molecules could be degraded by the extracellular and/or intracellular enzymes which were produced by a 10 day old fungal mycelium after the essential nitrogen nutrient (NH₄⁺) had been consumed completely. However from our results, even the treatment of no ammonium salt supplementation gave the slower decolorization than that of 0.2 g L⁻¹ ammonium oxalate supplementation, but the difference in the duration time used for reaching the maximum level of decolorization was only 12 h. As high accumulation of fungal mycelia may cause the problem when continuous decolorization mode was operated, then the condition of nitrogen limitation is attractive choice and it was selected to perform in next experiment.

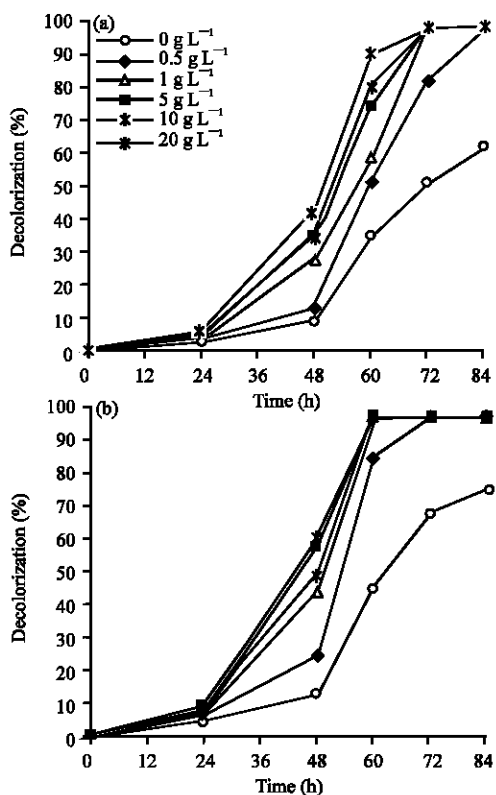


Fig. 1: Decolorization of Orange II by free cell of *C. versicolor* RC3 in various glucose concentrations. (a) Addition of 0.1 and 2.0 g L⁻¹ of ammonium oxalate are limited nitrogen and (b) nitrogen rich condition, respectively

Effect of initial pH and temperature on dye decolorization:

Effect of initial pH, Fig. 4 shows the decolorization in the various initial pH values 6.5 at the 60 h, a fastest decolorization rate had been observed while at the 72 h maximum decolorization occurred in initial pH 6.0 and 6.5 indicating that initial pH 6.5 was the best one.

Decolorization of the Orange II and cotton bleaching effluent has been shown to depend on the initial pH (Knapp *et al.*, 2001). Optimal decolorization could occur in the pH range between 5.5 to 7.0. The higher and the lower initial pH values will cause neither no decolorization nor

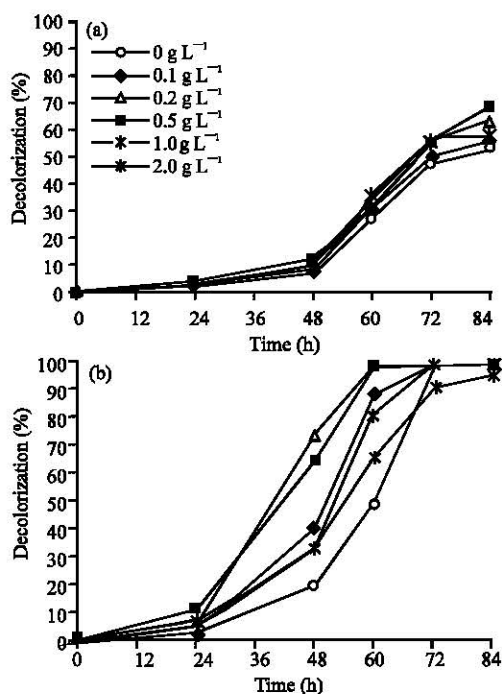


Fig. 2: Decolorization of the Orange II by free cell of *C. versicolor* RC3 in various nitrogen concentrations. (a) Without glucose (b) With glucose concentration of 1 g L⁻¹

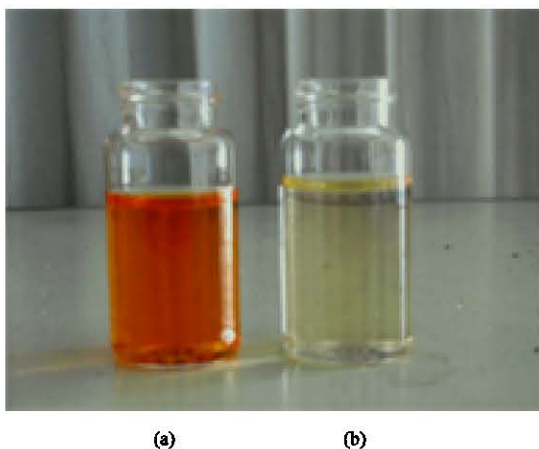


Fig. 3: Comparison between the Orange II solution before (a) and after (b) decolorization (~80%) by *C. versicolor* RC3

slow decolorization. The result of temperature effect on the Orange II decolorization was shown in Fig. 5. The decolorization in various temperatures monitored at the 48, 60 and 72 h were found that 30°C gave the fastest decolorization rate. However, at the 84 h of cultivation, the decolorization of all treatments including 30, 35 and

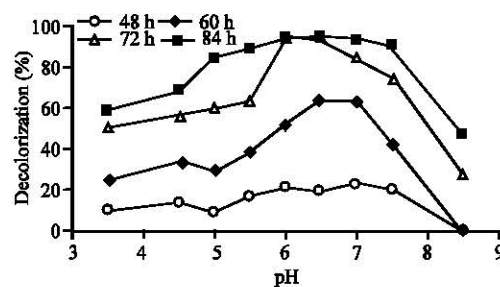


Fig. 4: Decolorization of the Orange II by free cell of *C. versicolor* RC3 in various initial pH values

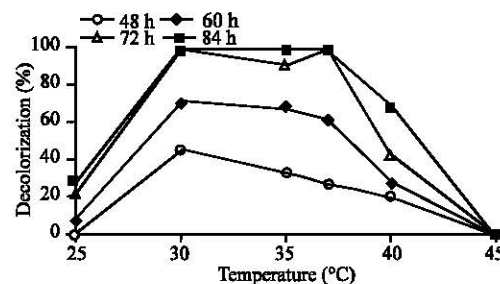


Fig. 5: Effect of the temperature on decolorization of the Orange 2 by free cell of *C. versicolor* RC3 at the different hours

37°C gave almost the same level of decolorization. This indicated that temperature in the range of 30-37°C gave the same decolorization rate when the incubation time was at least 84 h. Moreover, at 40°C still gave a decolorization activity, but there was no decolorization obtained at 45°C. This might be caused from the temperature was too high over the metabolism limit of the fungus. Previously report was also concluded the optimal temperature of decolorization by *Coriolus* sp. was 35°C (Fu and Viraraghavan, 2001). The main potential advantage of microorganisms having high optimal temperature does not lie in more rapid catalysis but in the fact that in large scale bioreactors removal of excess metabolic heat will present less of a problem at a higher temperature, cooling costs will be less (Knapp *et al.*, 2001).

Continuous decolorization: The results in Fig. 6 demonstrated the continuous decolorization of Orange II when different HRT was operated. More than 90% decolorization could be obtained about 36, 72 and 120 h of duration from 5, 6 and 8 h of HRT, respectively. However, if we considered at the level of more than 80% decolorization, 8 h of HRT gave a highest quantity of treated broth. In addition, at 12 h of the HRT maximum decolorization could be achieved for over a week. This indicated that the increase of HRT was also increased the duration of decolorization. At the low HRT, ligninolytic

enzymes, a key enzyme produced by the fungal strain and involved in azo dye decolorization could be washed out easily. In the other hand, the high HRT could maintain those enzymes for the longer period and the longer decolorization ability of the system was obtained. However, we found that the reactor was clogged by growth of the mycelia and caused a decreasing of aerated space in the reactor. Even there were ligninolytic enzymes present in the reactor, but oxygen for using in electron acceptor was lacked result a poor decolorization subsequently.

Most size of the PUF used by many researched was 1 cm³ (Feijoo *et al.*, 1995; Kasinath *et al.*, 2003) and the others used 1.2 cm³ (Fujita *et al.*, 2000) and 1.5 cm³ (Zouari *et al.*, 2002). There was no report about reason for using of any specified size of the PUF. The profiles of decolorization and laccase activity are shown in Fig. 7. The decolorization results obtained from both 1.5 and 2.0 cm³ were clearly higher than that of 1.0 cm³ and the decolorization capability was corresponding with the quantity of laccase found in the treated solution. The relationship between decolorization capability and laccase activity confirmed the function of laccase in dye oxidation as reported previously in *Funalia trogii* (Unyayar *et al.*, 2005; Devenci *et al.*, 2004). From our results, 1.5 cm³ of the PUF showed the most suitable compare with other treatments as at 1.5 cm³ of the PUF, the mycelium could be well retaining in the reactor as that obtained from 1 cm³ of the PUF and there was no clogging problem at the effluent

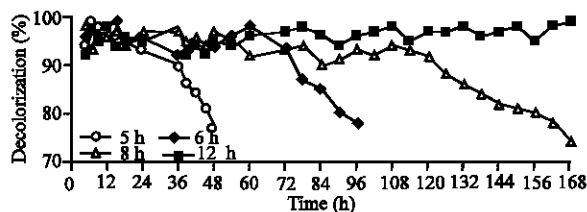


Fig. 6: Continuous decolorization of the Orange II by the PUF immobilized *C. versicolor* RC3 in various HRTs

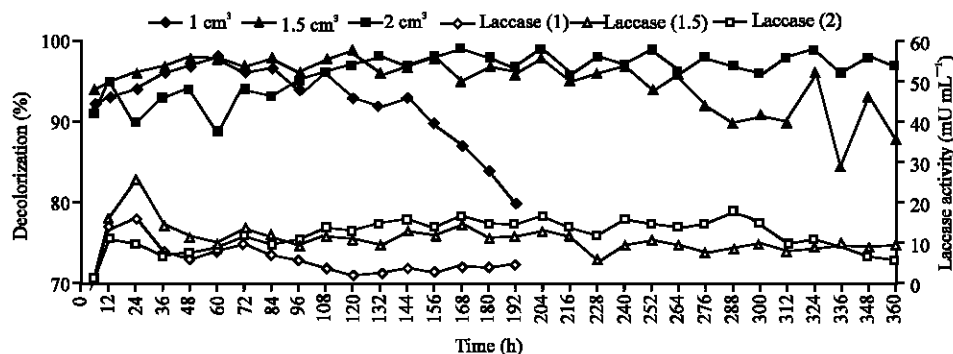


Fig. 7: Profile of the Orange II decolorization and laccase activities found with various PUF sizes during the decolorization

port and tube like the 2 cm³ PUF. Initial decolorization, between 24-72 h, showed a same good result as 1 cm³ of the PUF and better than the result obtained from the 2 cm³ PUF. In addition, it could be run in longer time than 1 cm³ PUF.

It is necessary to find suitable dye concentration applied to the reactor. The 20 ppm Orange II was used in previous experiments. In this study, the dye concentration was varied up to 50 and 100 ppm compared with previous study using the condition of 8 h HRT and 1.5 cm³ PUF. The result of the decolorization was shown in Fig. 8. Decolorization percentages were decreased when increased the dye concentration. It was concluded that 50 and 100 ppm of the dye loading were over a capacity level of the system. However, 8 h of the HRT was a very fast loading rate. Many researchers used 12-24 h of the HRT (Knapp *et al.*, 2001; Mielgo *et al.*, 2001). It is possible that 50 and 100 ppm could be completely decolorized if the HRT of the system was increased. From our result, the amount of dye removal was increased along with the increase of dye concentration; however, the remaining dye in the effluence was also increased.

Toxicity test: Toxicity experiments with water fleas, *Daphnia* sp., were carried out at different dilutions to evaluate of the toxicity of the Orange II dye before and after the decolorization (Fig. 9). The decolorization process significantly reduced toxicity in the Orange II dye after the decolorization was not toxic, while the untreated wastewater was found to be toxic at 70% concentration. *Daphnia* sp. is a small freshwater crustacean that is excellent organisms to use in bioassays because they are sensitive to the changes in water chemistry and are simple and inexpensive to cultivate in an aquarium. Navarro *et al.* (2001) used daphnids as bioassays to determine the LC₅₀ values of textile wastewater sample taken from different stages of the finishing textile industries. All effluents from the five company samples were toxic in terms of LC₅₀ and exhibited very high toxicity with Acute Toxicity Unit (ATU) levels between 2.2 and 960.

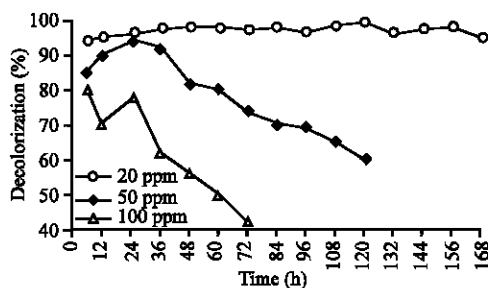


Fig. 8: Continuous decolorization of the Orange II by immobilized *C. versicolor* RC3 with different dye concentrations in packed-bed bioreactor

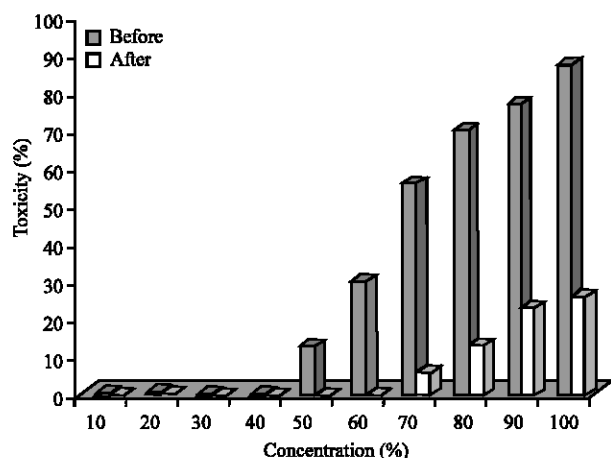


Fig. 9: Toxicity of the Orange II before and after degradation by immobilized *C. versicolor* RC3 on *Daphnia* sp. according to the concentration level. 100% concentration is non-diluted sample of treated dye solution

Villarroel *et al.* (2003) also tested for the acute and chronic toxicity of propanil on *D. magna* and the effect of the toxicant on filtration and ingestion rates was determined. It was found that feeding rates of *D. magna* declined with increasing propanil concentrations. The toxicity test using *D. magna* was also used efficiently in toxic evaluation of textile wastewater after ozonation treatment (Selcuk, 2005). In addition, 2 animal cell lines including the COR-L23 large cell lung carcinoma and the 3T3 Albino Swiss mouse embryo fibroblast were also tested for toxicity of treated dye solution. The Orange II dye solution after decolorization was also reduced in toxic in both cell lines. Our results demonstrated the successful in toxicity reduction of the Orange II by this biodegradation process.

CONCLUSIONS

The Orange II decolorization by immobilized *C. versicolor* RC3 on the PUF with the 1 g L^{-1} of glucose 0.2 g L^{-1} of ammonium oxalate provided a fastest decolorization rate, however, a satisfying decolorization could be also obtained in nitrogen limited condition. Optimal initial pH and temperature were 6.5 and $30\text{-}37^\circ\text{C}$, respectively. Major lininolytic enzyme produced was laccase and it's activity could be found up to 6 mU mL^{-1} . A simple construction of packed-bed bioreactor can be used in decolorization. At 8 h of the HRT, 90 and 80% decolorization could be obtained within 120 and 156 h, respectively and the duration of decolorization was increased when HRT increased. Suitable size of the PUF was 1.5 cm^3 , which gave almost 2 weeks of decolorization duration. After decolorization the toxicity of wastewater with *Daphnia* sp., normal and cancer cell lines were reduced toxic completely.

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