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Competition strategies for the incubation of white rot fungi under non-sterile conditions

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ABSTRACT

White rot fungi is capable of oxidizing many persistent organic pollutants including dyes. The application of the fungi has been limited for wastewater treatment under non-sterile conditions due to the contamination by bacteria and other micro-organisms. We developed a treatment approach by using immobilized white rot fungi *Phanerochaete chrysosporium* to degrade reactive dye K-2BP under non-sterile condition. Four different inert carriers were tested for immobilization of the white rot fungi with orthogonal experiments in comparison with suspension culture. The activity of manganese peroxidase (MnP) was used for the evaluation of oxidization performance in order to understand whether contamination of bacteria and other micro-organisms was suppressed. Under non-sterile conditions, the immobilized fungal cultures successfully restrained the growth of microzymes, coccies, and bacillus but suspension culture was highly contaminated with poor MnP activity. Under non-sterile conditions, higher MnP enzymatic activity (690 U/L vs. 125 U/L), higher decolorization efficiency (93.5% vs. 15%) and shorter reaction period (3 days vs. 6 days) were achieved in immobilized cultures in comparison with suspension culture. With the immobilized fungal cultures, no difference was observed under non-sterile and sterile conditions for the degradation of reactive dye K-2BP.

1. Introduction

The degradation of persistent organic pollutants by white rot fungi *Phanerochaete chrysosporium* was reported in 1985 [1]. Afterwards, Kirk [2] found out the nonspecificity of the lignin peroxidase system produced by white rot fungi at the secondary metabolism stage. White rot fungi can degrade lignin and many organic pollutants, such as polycyclic aromatic hydrocarbons, chloro-aromatics, dyes, dynamites and pesticides [3–5]. Shim and Kawamoto reported the immobilization of *P. chrysosporium* in bioreactor to degrade PCP [6]. The immobilized *P. chrysosporium* on polyurethane foam and rotating biological contactor (RBC) with polyethylene discs degraded more than 90% 4-chlorophenol in three repeated batches. Couto et al. [7] studied the decoloration of different synthetic dyes with white rot fungi *Trametes hirsuta* in an expanded-bed reactor and reported the high decoloration rate with hydraulic retention time (HRT) of 3 days.

Almost all the studies using white rot fungi for the treatment of dye wastewater are of laboratory-scale and they performed well under strict sterile conditions [8–11]. There was no successful report with white rot fungi for various organic dye under non-sterile conditions. The main reason is that white rot fungi were low-grade eukaryotic micro-organisms and grow slowly compared with bacteria. Once bacteria which grow and reproduce much faster than white rot fungi invaded into the reaction system, they could compete for nutrients with the white rot fungi and predominate in the reaction system rapidly. As a result, white rot fungi would stop growing further and lose the degradation ability for dyestuffs in the entire processing system and the invading bacteria exerted much impact on the secretion of extracellular degradation enzymes [12]. Furthermore, it is obviously unpractical to use the sterilization means (which are used in laboratory) to prevent a full-scale reactor system from bacterial contamination due to operational cost. Therefore, to study the methods of using white rot fungi under non-sterile conditions has important significance and extensive value.

The application of white rot fungi for dye wastewater treatment requires no or less bacterial contamination. In recent years, researchers had already realized that the bacterial contamination was a bottle-neck problem for the development

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of technology of degrade dyes by white rot fungi, and they tried to work on it [13,18]. Libra et al. [13] and our group's previous research [14] indicated that a nitrogen-limited medium could effectively suppress bacterial growth in different white rot fungi systems during dyestuff degradation. Bacterial growth can be effectively suppressed by providing a low pH favorable for white rot fungi [15], incubation method [9], injecting time of dyes [16], and the levels of trace element Fe [17] in order to enhance the degradation efficiency by white rot fungi. Most of the above research was performed with the growth of fungi first under sterile conditions for 4–5 days, and then using it for the decolorization of dyestuff under non-sterile conditions. Leidig et al. used PVAL hydrogel to embed the mycelia of *Trametes versicolor* in order to avoid the fungi and extracellular enzyme being attacked by bacteria under non-sterile conditions [18]. Leidig et al. also found out white rot fungi broken out of the PVAL hydrogel beads easily. Therefore, selection of proper carriers for the attached growth may help in the suppression of bacterial growth and improvement of degradation efficiency of persistent organic pollutants.

Based upon the literature review, immobilization may help the white rot fungi growth and enhance enzyme production with less contamination by other micro-organism. In order to figure out a better way to apply white rot fungi for dye wastewater treatment, in this study, *P. chrysosporium* was incubated under non-sterile conditions in comparison with sterile conditions. The aim of the study was to screen out an optimal condition for immobilization of white fungi under which bacterial growth is suppressed and decolorization of dyes is enhanced. Four inert carriers were tested and orthogonal experimental approach was employed.

2. Materials and methods

2.1. Micro-organism

P. chrysosporium BKM-F-1767 was obtained from ESPC State Key Joint Laboratory, Department of Environment Science and Engineering, Tsinghua University, Beijing, China. The culture was maintained on potato–glucose–agar slants at 4 °C.

2.2. Culture media

2.2.1. Solid medium

(1) PDA medium: lixivium of potato 200 g/L, glucose 20 g/L, and agar 20 g/L.
(2) Beef lotion and peptone medium: beef lotion 5.0 g/L, peptone 10.0 g/L, Na_2Cl 5.0 g/L, and agar 15 g/L.

2.2.2. Liquid medium

The medium used for the growth of *P. chrysosporium* and decolorization was prepared according to Tien and Kirk [19] with modification. The medium contained glucose, 10 g/L; ammonium tartrate, 0.8 g/L [20]; KH_2PO_4, 2 g/L; $MgSO_4$, 0.5 g/L; $CaCl_2$, 0.1 g/L; veratryl alcohol, 0.22 mL [21]. Vitamin B1 was added under sterile condition to obtain a concentration of 1 mg/L.

2.3. Carriers

Four inert carriers having different physics structure and nature were used in the experiments, i.e. stainless steel net (30 mesh, aperture 0.61 mm), polyamide fiber net (30 mesh, aperture 0.4 mm), fiberglass net (14 mesh, aperture 1.3 mm), and polyurethane foam (aperture 0.031 cm, density 0.039 g/cm^3). These carriers were washed three times with distilled water, then dried at 60 °C and autoclaved at 121 °C for 20 min prior to use.

2.4. Dyestuff

The dye used in this research was Reactive brilliant red K-2BP, a disperse dye which belongs to the class of diazo dyes. The initial concentration of the dye added to the medium was 30 mg/L.

2.5. Culture condition

Inoculum was obtained on PDA slants at 37 °C for 6 days. The growth of cultures (suspended cells and immobilized cells) was carried out in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium in a reciprocating shaker (model TZ-2EH) at 160 rpm and under 37 °C. The inoculum level was 2.5×10^6 spores.

2.6. Experiment scheme design

2.6.1. Control experiments

The control experiments were conducted without carriers under three process conditions: (a) incubation of *P. chrysosporium* and decolorization under sterile conditions, (b) incubation under sterile conditions but decolorization under non-sterile conditions, and (c) incubation and decolorization under non-sterile conditions.

2.6.2. Immobilization experiments

The immobilization experiments were conducted with multifactors to optimize treatment performance by the white fungi. In order to select the key factors for optimization of the immobilized cultures incubation, an orthogonal experimental approach was used for screening the growth conditions. The orthogonal experiments were mainly composed of the evaluation index, the test factor, the level of the test factor and the orthogonal table.

The test factor was interpreted as reasons or elements, which might influence the characteristic value of the evaluation indexes. From relevant results [22], the material, weight, quantity, size, and shape of the carriers were the main factors influencing immobilized white rot fungi to produce enzyme. To combine the physical structure characteristics of the inert carriers selected in this research, the test factors were confirmed as carrier material, size, quantity, and shape.

The orthogonal table of L_9 (3^4) was used according to the number of factors and corresponding level. The experiment was completed with two orthogonal tables, because there were great differences in structure characteristics between reticular carrier and polyurethane foam. The list of factors and levels were reported in Table 1.

All experiments were carried out in triplicate, and the results were expressed as the mean values.

2.7. Analytical methods

Manganese peroxidase (MnP) activity was measured as described by Paszczynski et al. [23]. One unit (U) of MnP was defined as the amount of enzyme necessary to oxidize 1.0 μmol Mn(II) to Mn(III) per minute.

Laccase (Lac) activity was measured as described by Bourbonnais and Paice [24]. One unit (U) of Lac was defined as the amount of enzyme necessary to oxidize 1.0 μmol ABTS per minute.

Glucose concentration in the liquid medium was determined enzymatically using a commercially available assay kit (Infinity Glucose Reagent, Sigma).

Table 1
Factors and levels of carrier for orthogonal experiment

Carrier	Factors	1	2	3
Reticular carrier	A: carrier material	Stainless steel net	Polyamide fiber net	Fiberglass net
	B: carrier size (cm^2)	1.5 × 1.5	3.0 × 3.0	6.0 × 6.0
	C: carrier quantity	10	5	3
	D: carrier shape	Plane	Abnormity	Cylinder
Polyurethane foam	A: carrier size (cm^3)	1.5 × 1.5 × 1.5	1.0 × 1.0 × 1.0	0.5 × 0.5 × 0.5
	B: carrier weight (g)	0.3	0.6	1.2
	C: carrier shape	Three prismatoid	Cube	Six prismatoid
	D: empty column			

Table 2
Analysis of orthogonal experiment data of reticular carrier

Test number	Factors				MnP (U/L)		
	A, carrier material	B, carrier size	C, carrier quantity	D, carrier shape	Incubation and decolorization were under sterile conditions	Incubation was under sterile conditions and whereafter decolorization was under non-sterile conditions	Incubation and decolorization were under non-sterile conditions
1	1	1	1	1	18.46 ± 0.72	11.08 ± 0.64	11.08 ± 0.87
2	1	2	2	2	140.3 ± 10.44	77.53 ± 10.44	70.15 ± 2.61
3	1	3	3	3	195.68 ± 3.61	103.38 ± 1.34	95.99 ± 2.34
4	2	1	2	3	62.76 ± 4.51	70.15 ± 0.85	18.46 ± 1.90
5	2	2	3	1	494.73 ± 11.65	487.34 ± 19.60	472.58 ± 14.28
6	2	3	1	2	66.46 ± 2.62	55.38 ± 5.22	59.07 ± 5.22
7	3	1	3	2	11.08 ± 2.62	92.30 ± 2.64	51.69 ± 2.05
8	3	2	1	3	22.15 ± 0.00	14.77 ± 2.61	147.68 ± 60.05
9	3	3	2	1	335.97 ± 14.22	48.80 ± 21.45	310.13 ± 14.46
Control test	No carrier, suspended incubation				324.90 ± 6.39	247.36 ± 3.05	125.53 ± 1.89

Total nitrogen in the liquid medium was measured according to standard methods [25].

The H_2O_2 was determined by the method of Pick colorimetry [26].

The dye concentration was measured spectrophotometrically at 533 nm (SHIMADZU UVmini 1240, Japan). Decolorization is reported as % decolorization = $((A_0 - A_t)/A_0) \times 100$, where A_0 is the initial dye absorbance and A_t is the dye absorbance at time t.

The microscopic examination of liquid media was conducted daily using a microscope (Motic B SERIES, Japan).

All samples were centrifuged at 9000 rpm for 10 min at 4 °C, and the supernatant was analyzed.

3. Results and discussion

3.1. The optimal incubation strategy for immobilization

3.1.1. The results of orthogonal experiments for optimal incubation strategy

According to Libra et al. [13], the enzyme productivity of white rot fungi was correlated with the bacteria infection, and decreased evidently when the system was infected by bacteria under non-sterile condition. The peroxidase enzyme can be used as an evaluation index to screen the optimal strategy. As mentioned previously, the limited nitrogen liquid culture (C/N = 56/8.7 mM) was used in this study. Therefore, only MnP produced was detected while LiP was under detection limits as reported by other researchers [27,28]. The result of MnP was shown in Tables 2 and 3.

Using the data in Tables 2 and 3, the optimal incubation strategies of reticular carrier and polyurethane foam were screened out from the three process conditions, i.e. (a) sterilized incubation and sterilized degradation, (b) sterilized incubation and non-sterilized degradation, and (c) non-sterilized incubation and non-sterilized degradation. The results through orthogonal experiments were $A_2B_2C_3D_1$ and $A_2B_3C_1$, respectively. Subsequently, it was found that under the three process conditions, comparing the results from single factor contrasting experiments (Fig. 1), the MnP levels of the two groups optimal incubation strategies were evidently higher than suspended culture strategy of control test obviously. In addition, the MnP of strategy $A_2B_3C_1$ was also clearly higher than strategy $A_2B_2C_3D_1$ clearly. The best optimal incubation strategy $A_2B_3C_1$ was carrier material, polyurethane foam; the size of carrier, 1.0 cm × 1.0 cm × 1.0 cm; the weight of carrier, 1.2 g; the shape of carrier, three prismatoid.

3.1.2. Suppression of bacterial growth

The test showed that the optimal incubation strategy had suppressed bacterial growth better than suspended culture strategy under non-sterile condition. On the third day, the liquid medium of suspended culture system was turbid (OD_{650} = 0.502) under non-sterile condition, and the white rot fungi grew poorly. The liquid medium of the optimal incubation system was still clear (OD_{650} = 0.032), and the white rot fungi grew very well. In order to further evaluate the suppressing bacteria effect of the optimal system, the liquid media of suspended culture system and the optimal system under non-sterile condition were observed by microscope. A lot of elliptic germ colonies were found in the suspended culture. These organisms had slippery edge, had some buds on surface, could not be discolored with alcohol after dyeing by crystal violet, and the size of these colonies was (2.5 μm × 7.5 μm)–(2.5 μm × 12.5 μm). Then the cultivated test

Table 3
Analysis of orthogonal experiment data of polyurethane foam

Test number	Factors				MnP (U/L)		
	A, carrier size	B, carrier weight	C, carrier shape	D, empty	Incubation and decolorization were under sterile conditions	Incubation was under sterile conditions and whereafter decolorization was under non-sterile conditions	Incubation and decolorization were under non-sterile conditions
1	1	1	1	1	7.38 ± 2.61	14.77 ± 0.64	59.07 ± 1.56
2	1	2	2	2	62.76 ± 1.85	7.38 ± 2.62	33.23 ± 7.83
3	1	3	3	3	310.13 ± 15.66	594.41 ± 60.04	491.04 ± 5.23
4	2	1	2	3	14.77 ± 5.23	40.61 ± 3.91	66.46 ± 18.28
5	2	2	3	1	0 ± 0.00	0 ± 0.00	18.46 ± 2.61
6	2	3	1	2	915.62 ± 5.57	934.08 ± 13.29	897.16 ± 28.14
7	3	1	3	2	22.15 ± 2.61	33.23 ± 1.87	22.15 ± 2.61
8	3	2	1	3	29.54 ± 2.62	40.61 ± 2.85	51.69 ± 1.29
9	3	3	2	1	36.92 ± 1.46	51.69 ± 2.07	297.21 ± 3.92
Control test	No carrier, suspended incubation				324.90 ± 6.39	247.36 ± 3.05	125.53 ± 1.89

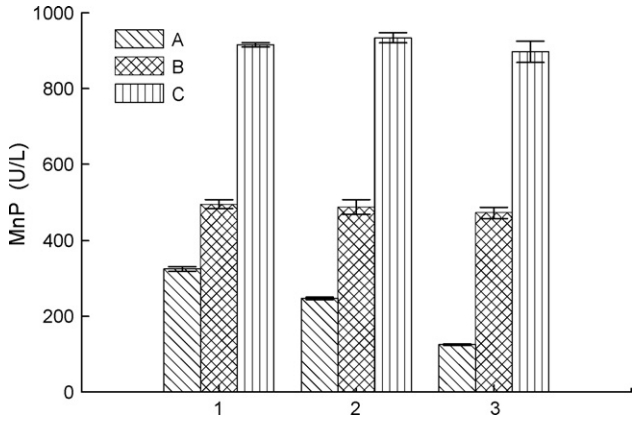

Fig. 1. MnP excreted by white rot fungi of suspended (A) and best immobilized culture with reticulation (B) and polyurethane foam (C). 1, decolorization under sterile condition after incubation under sterile condition; 2, decolorization under non-sterile condition after incubation under sterile condition; 3, decolorization under non-sterile condition after incubation under non-sterile condition.

for the liquid medium was made with solid medium of beef lotion and peptone. The result showed that the flat cultivated germ colony was ivory-white, approximated butter, slippery and humid. The shape and size were equal to the germ colony in the turbid liquid medium. The microscope observation and flat cultivated test could prove that the germ colony infecting was microzyme, otherwise, some bacteria such as coccies and bacillus were found by microscope observation in the system after 6 days. On the contrary, there was few microzyme and other bacteria were observed in the medium of the optimal system under the non-sterile condition.

3.2. The mechanism of suppressing bacterial growth

3.2.1. Effect of pH

White rot fungi grew well slightly under acidic pH environment. The pH fluctuates and trends to increase eventually when the white rot fungi culture system was infected by bacteria under non-sterile condition. The extent of pH change was influenced by the variety and extent of infecting bacteria [20]. The variation of pH had negative influence on the growth and reproduction of white rot fungi.

The optimal incubation strategy maintained a stable pH range of 4.0–5.0 in 10 days by restraining bacteria growth under non-sterile condition (Fig. 2). Thereby this ensured white rot fungi to grow and reproduce well. However, under the non-sterile condition, the pH of suspended culture system fluctuated acutely because of the contamination by microzyme. Eventually, the pH rose to 7.0 from 4.8 after the system was influenced by coccies and

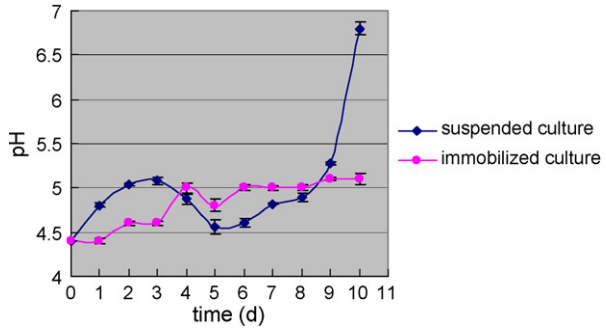

Fig. 2. Variation of pH during incubating *P. chrysosporium* of suspended and immobilized cultures with polyurethane foam under non-sterile conditions.

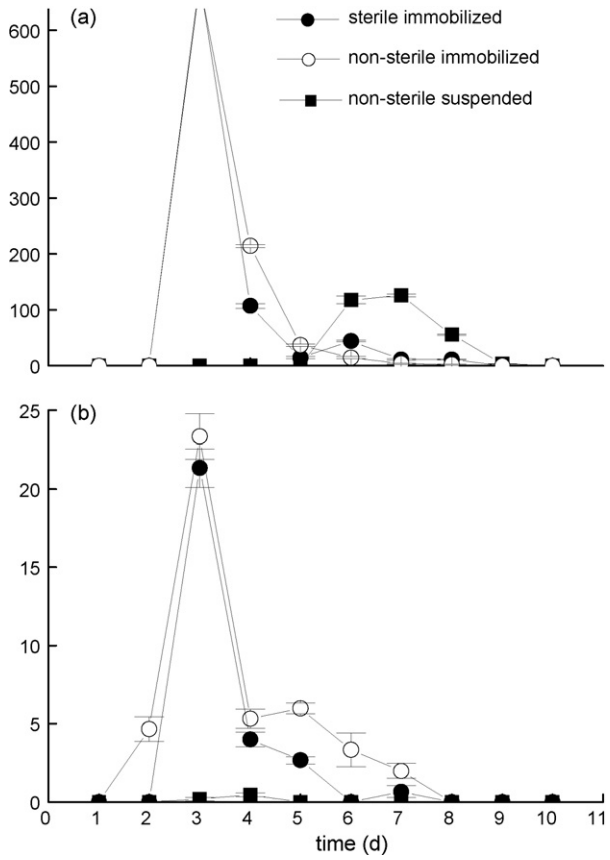

Fig. 3. MnP and Lac excreted by *P. chrysosporium* under different conditions: (●) immobilized with polyurethane foam under sterile condition; (○) immobilized with polyurethane foam under non-sterile condition; (■) suspended under non-sterile condition.

bacillus at anaphase. This result showed that the optimal incubation strategy could protect the system from bacterial contamination and facilitated the growth of white rot fungi effectively.

3.2.2. MnP and Lac production

The white rot fungi degrade lignin, and many persistent organic pollutants [2,29,30] due to their lignin degrading enzyme systems. Previous studies [9,14] have demonstrated that bacterial contamination is a bottle-neck problem in application because the enzyme production was inhibited in the presence of bacterial contamination. In this study, we tested enzymatic activities of LiP, MnP, and Lac in both immobilized and suspended cultures under sterile and non-sterile conditions (Fig. 3). Under non-sterile condition, the immobilized white rot fungi grown with the optimal strategy showed much better performance in comparison with the control (suspended culture strategy). First, the enzyme activities were enhanced. The maximum level of MnP and Lac in the suspended culture was 125 U/L and 0.5 U/L, respectively, while the maximum activities of MnP and Lac in the immobilized cultures were up to 690 U/L and 24 U/L, respectively. The levels in the immobilized cultures were approximately 5 and 48 times than that in the suspended culture as observed previously [31]. Secondly, the enzymatic activity reached peak earlier. The peak levels of MnP and Lac in the suspended culture appeared on the 7 day and 4 day, respectively. The peak levels of MnP and Lac in the immobilized cultures were observed on the third day. The appearance of the peak levels was accordant to the time of complete consumption of total nitrogen. The increased enzyme

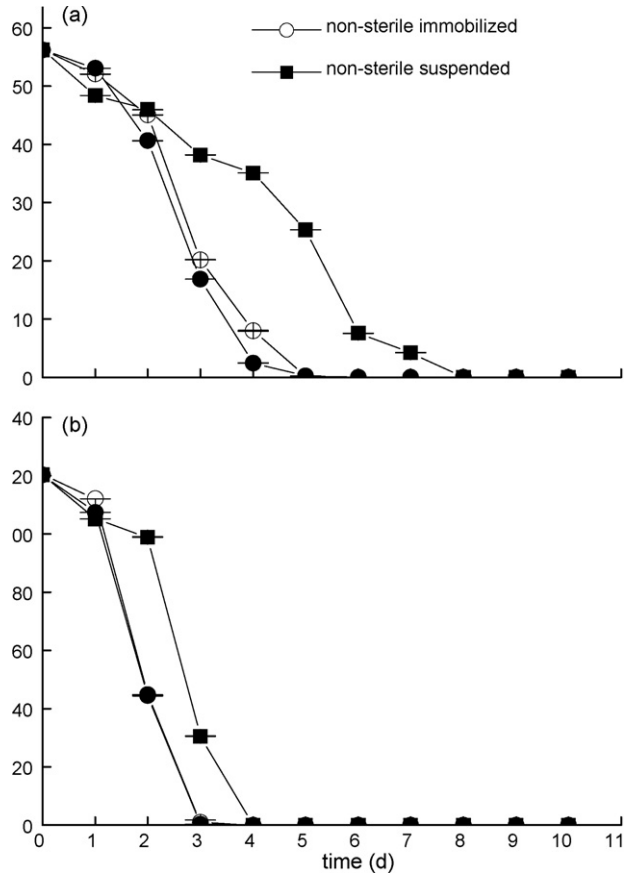

Fig. 4. Nutrient consumption by *P. chrysosporium* under different conditions: (●) immobilized with polyurethane foam under sterile condition; (○) immobilized with polyurethane foam under non-sterile condition; (■)suspended under non-sterile condition.

activities and early peak level of enzymatic activity are likely due to the predominance of white rot fungi in the immobilized cultures using the optimal incubation strategy selected in this study. Using the optimal strategy, though the white rot fungi were cultivated under non-sterile condition, the enzyme activities and peak levels of MnP and Lac were the same as under sterile condition. It was obvious that the optimal strategy had suppressed bacterial growth.

In addition, the limited nitrogen liquid culture (C/N = 56/8.7 mM) medium was used in this study. Therefore, no LiP was detected as reported [32,33]. The reason of no LiP in the limited nitrogen condition might be that the mass transfer of oxygen was counterchecked due to white rot fungi excreting overmuch extracellular amylose in the superfluous carbon condition, thereby the composing of LiP was influenced. On the contrary, the high enzyme activities LiP could be obtained in the limited carbon condition [32]. According to characteristic that the different rates of C/N influenced the immobilized white rot fungi producing enzymes of LiP and MnP in air, we could control the corresponding enzyme production effectively.

3.2.3. Carbon and nitrogen consumption processes

Fig. 4 showed that white rot fungi consumed the nutrients of carbon and nitrogen under different conditions. As shown in Fig. 4(a), there were three phases in glucose consumption under non-sterile condition. The consumption rate of glucose was low on the initial stage (in 0–2 days), then glucose was consumed rapidly by 5 days or 8 days. Afterward the cultivation process was in no carbon state. This observation was similar to other reports [33]. The consumption of total nitrogen was basically the same as that of glucose (Fig. 4(b)), but consumption rate of total nitrogen was faster than that of glucose. The total nitrogen in both immobilized and suspended cultures dropped below detection limits on third and fourth days, respectively. At that time the production of the lignin degrading enzymes increased rapidly. This observation may correlate to adopt the limited nitrogen culture (C/N = 56/8.7) in the experiment. When the total nitrogen was exhausted, white rot fungi came into the secondary metabolism stage and began excreting enzyme. As result, the enzyme peak appeared. In addition, the consumption of glucose and total nitrogen in the immobilized cultures were faster than the suspended culture (Fig. 4). This may be due to that the polyurethane foam in the immobilized cultures system had high surface area, therefore, the fungi mycelium could extend to much large space, and grew and reproduced better than that in suspended culture. This result was also in accordance with the production of the MnP in the immobilized cultures. The more rapid consumption of carbon and nitrogen were, the more early white rot fungi came into the secondary metabolism stage and began to excrete enzyme.

As shown in Fig. 4, under both sterile condition and non-sterile condition, the patterns of consumption of glucose and total nitrogen under immobilized conditions were basically the same. This was likely because the optimal incubation strategy suppressed bacterial growth effectively, thereby white rot fungi grew predominantly even under non-sterile condition. Therefore, the patterns of enzymatic activities were also similar under both sterile and non-sterile conditions.

3.2.4. H_2O_2 excretion

White rot fungi have special ability to degrade lignin but the mechanism of degradation is complicated. Under carbon and nitrogen-limited conditions, white rot fungi excrete peroxidases. Under oxygenous condition, the Fe^{3+} in these peroxidases was oxidized after being activated by H_2O_2 excreted by white rot fungi itself, thereby the catalysis reactions of these peroxidases were activated, and then the lignin was degraded and oxidized to CO_2 and H_2O [34]. Therefore, the production of H_2O_2 is important in the biodegradation process by white rot fungi [19].

Decolorization of the dyes with the lignin peroxide enzyme requires extra H_2O_2. Lopez et al. [35] demonstrated that joined different amounts of H_2O_2 influenced the decolorization effect. However, how much H_2O_2 the fungi excreted and what the rules of the fungi excreting H_2O_2 were during incubating white rot fungi,

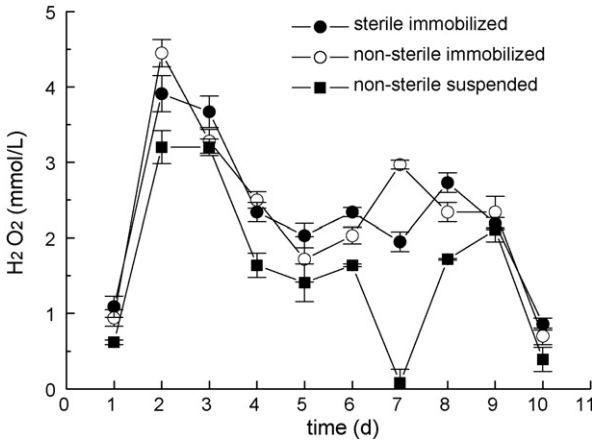

Fig. 5. H_2O_2 excreted by *P. chrysosporium* under different conditions: (●) immobilized with polyurethane foam under sterile condition; (○) immobilized with polyurethane foam under non-sterile condition; (■) suspended under non-sterile condition.

were few reported. Fig. 5 shows the white rot fungi excreted H_2O_2 under various culture conditions. H_2O_2 was detected steadily during the whole incubating process in the immobilized and suspended cultures, under sterile or non-sterile conditions. There was a similar pattern that the peak appeared at the early stage of incubation (by 2–3 days), where the levels reached up to 3–4 mM. Subsequently, the levels descended slightly and then tended stable, at about 2 mM all the time, and finally dropped on 9 days. As shown in Fig. 5, H_2O_2 levels with the optimal incubation strategy were slightly higher than that in the suspended culture strategy under non-sterile condition. The immobilized cultures strategy was favorable for the growth of white rot fungi and stimulated the fungi to excrete H_2O_2. With the optimal strategy, the patterns of H_2O_2 production under both sterile and non-sterile conditions were almost the same (Fig. 5). The optimal strategy restrained the bacteria growth under non-sterile condition effectively, and consequently reduced the influence of bacteria on excreting H_2O_2.

Our previous research indicated that the H_2O_2 level was a limiting factor of influencing decolorization of dyes by white rot fungi because H_2O_2 was used as an electron acceptor or oxidant for the decolorization. Based on the results in this study and previous results by addition of H_2O_2 for decolorization, it was estimated that if in a treatment system dyes were degraded by LiP, a high decolorization rate could be obtained when H_2O_2 amount reached 4–5 mol; if dyes were degraded using MnP, an appropriate H_2O_2 level would be 3–4 mol. Because of the instability of the H_2O_2, the optimal H_2O_2 level for the degradation of dyes may vary depending on the process applied and environmental conditions [36,37].

3.3. Decolorization of dye

The experiments of dye decolorization by white rot fungi under different conditions were carried out in order to understand the performance of suppressing bacteria and operational parameters with the optimal incubation strategy screened out. As described previously, the optimal incubation strategy provided a favorable condition for the growth and reproduction of the fungi and enhanced enzymatic activities. We found that this strategy also enhanced the performance of decolorization.

3.3.1. Decolorization efficiency

Previous study indicated that the decolorization performance by the fungi was poor if the dye was added to the treatment systems after MnP and H_2O_2 passed their peak levels [15]. Based on the results of the peak level of MnP and H_2O_2 observed, we decided to add the dye before the peak was reached to maximize the decolorization efficiency, i.e. the dye was added to the immobilized fungal cultures after incubation for 2 days. The performance of decolorization by the immobilized *P. chrysosporium* was compared with the suspended cultures to which dye was added after incubation for 5 days. Three test conditions were used: (a) sterilized culture and sterilized degradation, (b) sterilized culture and non-sterilized degradation, and (c) non-sterilized culture and non-sterilized degradation.

As shown in Fig. 6, the decolorization efficiency by the suspended culture strategy was more than 80% when the cultures grew under sterile conditions but less than 20% when the culture grew under non-sterile condition. The decolorization efficiencies of immobilized cultures under three conditions were identical, reaching greater than 90%. The incubation time to reach the peak activity was only 2 days for the immobilized cultures in comparison with 5 days for the suspended cultures. The short incubation time would reduce the operational cost for a wastewater treatment system.

3.3.2. Decolorization and restraining contamination

The suspended culture maintained under sterile conditions decolorized dye effectively with up to 89% efficiency 1 day after adding dye (Fig. 6(a)). When the white rot fungi grew under sterile condition but degraded the dye under non-sterile condition, the decolorization efficiency was slightly lower (Fig. 6(b), up to 80% on the third day. A few microzymes and bacteria grew in the culture after the incubation was switched from sterile to non-sterile condition. The suspended culture grew under non-sterile condition and maintained under non-sterile condition for the dye degradation showed poor decolorization performance (Fig. 6(c)). Only 18% of dye removal efficiency was obtained during initial 8 h and then remained unchanged. This low removal efficiency of dye may be mainly caused by the adsorption to fungi cells rather than degradation. The poor performance was likely caused by the growth of microzymes, coccies, and bacillus in the system under non-sterile condition culture. They grew faster than *P. chrysosporium* and competed for nutrients and substrates, and *P. chrysosporium* was inhibited for growth and enzyme production, resulting in poor decolorization efficiency.

The immobilized cultures grew with the optimal incubation strategy showed high decolorizartion efficiency regardless under sterile or non-sterile conditions. As shown in Fig. 6(a), under sterile culture condition and sterile degradation condition, the decolorization efficiency by the immobilized cultures reached to 73% and 95% in 1 and 3 days, respectively, even slightly higher than suspended culture. Under sterile culture condition and non-sterile degradation condition, the immobilized cultures also achieved high decolorization efficiency which was 72.9% on day 1 and 94% on day 3. These efficiencies were higher than that of the suspended culture. Although *P. chrysosporium* was incubated only for 2 days

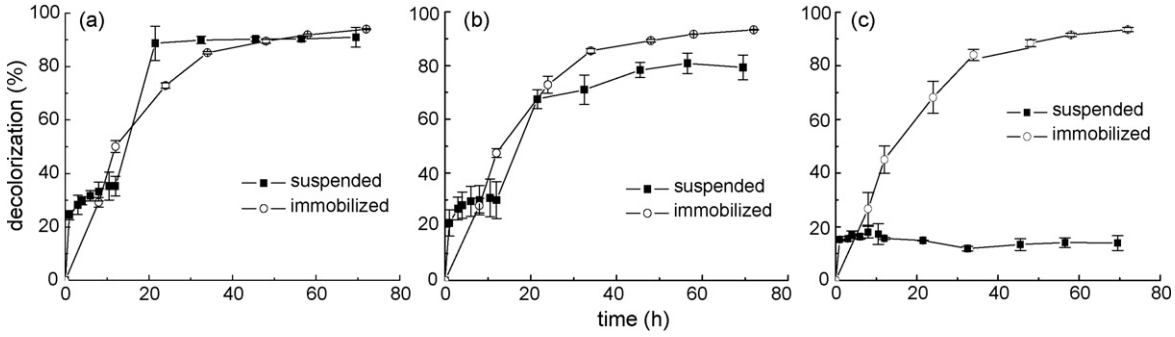

Fig. 6. Decolorization of reactive dye after incubation of *P. chrysosporium* for 5 days of suspended (■) and after incubation of *P. chrysosporium* for 2 days of immobilized cultures with polyurethane foam (○). (a) Decolorization of reactive dye under sterile condition after incubation of *P. chrysosporium* under sterile condition. (b) Decolorization of reactive dye under non-sterile condition after incubation of *P. chrysosporium* under sterile condition. (c) Decolorization of reactive dye under non-sterile condition after incubation of *P. chrysosporium* under non-sterile condition.

under sterile condition, then adding dye under non-sterile condition, *P. chrysosporium* had been preponderant strain in the decolorization system in the 2 days with high decolorization performance. On the contrary, the decolorization efficiency of the suspended culture descended obviously due to interferences by a few bacteria. The immobilized cultures, which was cultivated under sterile condition and then added with dye under non-sterile condition, had not only shorter sterile incubation time, but also effective decolorization of dye.

The immobilized *P. chrysosporium* cultures grown and subsequently decolorized dye under non-sterile conditions showed high decolorization efficiency of 69% on day 1 and 93.5% on day 3. The efficiency was the same as those of the immobilized cultures grown under sterile conditions but much better than those of the suspended cultures. Also, the total operational period was reduced to 3 days. Microscopic examination indicated the suspended culture under non-sterile condition for both the cultivated phase and the dye degradation phase, the dominate species were microzymes, coccies, and bacillus. In contrast, the bacterial infection in the immobilized cultures under non-sterile condition was limited and the white rot fungi remained preponderant strain all the time. Only at the end of dye degradation, a small number of coccies and bacillus was observed.

In summary, under non-sterile condition, the immobilized cultures showed high decolorization efficiency with short operational time compared with the suspended culture. This demonstrated that immobilized white rot fungi could be used for dye degradation under non-sterile condition. More studies are still needed to develop and scale-up the process with the immobilized white rot fungi for real wastewater.

4. Conclusions

(1) The optimal incubation strategy for immobilized fungal cultures was screened out from the three processes, namely (a) sterilized culture and sterilized degradation, (b) sterilized culture and non-sterilized degradation, and (c) non-sterilized culture and non-sterilized degradation. This was achieved using multifactor orthogonal experiments and single factor contrasting experiments with MnP as the evaluation indicator. The strategy included use of polyurethane foam as carrier, the size of carrier of 1.0 cm × 1.0 cm × 1.0 cm, the weight of carrier of 1.2 g, and three prismatoid shaped carrier.
(2) Under non-sterile condition, the immobilized cultures suppressed the bacterial growth effectively. But suspended culture was infected by microzymes, coccies, and bacillus in 3 days.
(3) Under non-sterile condition, the immobilized cultures maintained stable pH of 4.0–5.0 in 10 days but the suspended culture did not.
(4) Under non-sterile condition, the immobilized cultures had high enzymatic activity and short incubation time to reach peak level of the enzymes. MnP was up to 683 U/L, being five times of that in suspended culture. The peak level of the enzyme was attained earlier by 4 days. The consumption of carbon and total organic nitrogen was higher than that in suspended culture.
(5) Under non-sterilized culture and non-sterilized degradation, because of being infected by microzymes and bacteria, the decolorization efficiency of the suspended culture was as poor as 15%. In contrast, the immobilized cultures had high decolorization efficiency, which reached 69% in 1 day and 93.5% in 3 days. The decolorization efficiency of the immobilized cultures was the same under both sterile and non-sterile conditions. The incubation time for the decolorization was reduced by 3 days when compared to the suspended culture.

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