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Decolourisation of Acid Violet 7 with complex pellets of white rot fungus and activated carbon

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Abstract Complex mycelium pellets of a white rot fungus, *Trametes versicolor*, with activated carbon powder were prepared and investigated for decolourisation of an azo dye, Acid Violet 7. The pellets had a black core of activated carbon powder that was surrounded by a layer of white fungal mycelium. Compared to the activated carbon powder, the mycelium pellets (activated carbon free), and the mycelium pellets plus the activated carbon powder that was added into a dye solution, the complex pellets showed the highest and the most stable activity of dye decolourisation in batch cultures. The high decolourisation rate of the complex pellets was attributed not only to dye adsorption by the activated carbon in the complex pellets, but also to adsorption of extracellular enzymes and other reagents involved in dye decolourisation as well as the closeness between the dye molecules and the fungal cells. The complex pellets were further evaluated in a fluidized-bed reactor in two operation modes: a continuous flow feeding and a repeated-batch feeding. The latter gave higher and more stable decolourisation efficiency than the former. Production of laccase in flask culture and the fluidized-bed bioreactor was also compared.

1 Introduction

Azo dyes, with the greatest variety of colours, constitute the largest group of synthetic dyes. There are at least 3000 azo dyes used in industries for various purposes [1]. Although they do not generally display acute toxicity, azo dyes are regarded as water pollutants because of their high colour intensity and possible mutagenesis in the environment [2, 3]. Up to 10–15% of the amount of synthetic dyes produced annually is discharged with aqueous effluents and most of them are resistant to degradation in conventional biological treatment process [4]. In the past two decades, several physicochemical decolourisation techniques were developed, but few of them have been accepted by the industries. Their lack of implementation is largely due to a high cost [2]. An effective and inexpensive biological alternative would be of great value.

As wood white-rotting fungi possess ligninolytic systems that are not specific in substrates, they constitute a promising group of microorganisms in biodegradation of recalcitrant xenobiotic compound [5]. The potential of white rot fungi for decolourisation of synthetic dyes was also investigated [6–9]. *Phanerochaete chrysosporium* and *Trametes* (*Coriolus*) *versicolor* are two representative white rot fungi in those studies; the former has the lignin peroxidase and the latter has the laccase as their key enzymes in dye decolourisation [10, 11]. Fungal decolourisation was usually investigated in flask cultures with different morphological mycelia formed under different experimental conditions such as mycelial mat in static cultures and mycelial pellets in shaken cultures. Few studies have been conducted in reactors. A fixed film reactor with immobilized *P. chrysosporium* was used for decolourisation of Red 533 dispersed dye [12]. Packed-bed, fed-batch fluidized-bed and fluidized-bed reactors were tested for decolourisation of Orange II by wood rotting fungal mycelia [13]. The decolourisation of Remazol Brilliant Blue R by white-rot fungus, *Pycnoporus cinnabarinus*, was demonstrated in a 200-l pilot packed-bed reactor [14]. We found that the fungal performance in the reactors was usually not as good as observed in flask cultures, particularly in the continuous flow reactors. It is known that in addition to the extracellular enzymes such as lignin peroxidase and laccase, extracellular fungal metabolites (e.g. veratryl alcohol) are also involved in dye degradation and play a mediation role between the dye molecules and the enzymes [10, 11]. These essential extracellular reagents are usually lost with discharged water in a continuous flow reactor, which may lead to the performance deterioration. How to retain the extracellular components in a continuously operated process is a challenging problem in process design and optimization.

Adsorption by activated carbon is an effective method for lowering the concentration of dissolved compounds in aqueous solutions. In this regard, activated carbon has been evaluated extensively for the treatment of different dyes [15]. While most dyes can be adsorbed on activated carbon, great difficulty was experienced with disperse dyes, vat dyes and pigments. Other major problems include the regeneration of activated carbon and the treatment of secondary effluent. A combined system of living microorganisms and activated carbon may offer a solution to the problems. Hutton et al. [16] combined activated carbon powder with activated sludge to improve dye

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removal from effluents. A co-immobilized system in which *P. chrysosporium* cells and activated carbon powder were entrapped in an alginate matrix was developed to enhance the degradation and mineralisation of pentachlorophenol [17].

We developed a complex system by combining *Trametes versicolor* mycelium pellets with activated carbon powder to improve the decolourisation efficiency. The complex pellets could be prepared in a simple way. This paper reports the preparation procedure and the effect of activated carbon dose on the formation of complex pellets. It also compares the performance of decolourisation of a synthetic azo dye (Acid Violet 7) with complex pellets, activated carbon and mycelium pellets (activated carbon free). The complex pellets were further used and evaluated in a fluidized-bed reactor in two operation modes: continuous feeding and repeated-batch feeding of the dye solution.

2
Materials and methods

2.1
Organism and chemicals

Subcultures of *T. versicolor* (ATCC48424) was maintained on a malt agar and stored at 4 °C. The malt agar contained (per liter of distilled water): 10 g glucose, 10 g malt extract, 2 g peptone, 2 g yeast extract, 2 g K_2HPO_4, 1 g L-asparagine, 1 g $MnSO_4$, 1 mg thiamin hydrochloride and 20 g agar. Neutralized activated carbon powder of 100–400 mesh and a malt extract (ME) medium as fungal growth medium were purchased from Sigma (St Louis, MO, USA). Acid Violet 7, an azo dye with a chemical structure as shown in Fig. 1 and other chemicals were purchased from Aldrich (Milwaukee, WI, USA).

2.2
Complex pellets and mycelium pellets

Trametes versicolor was cultivated in 20 ml ME medium (2% w/v) in a rotary shake bath at 28 °C and 100 rpm for 4–5 days to reach 5 g wet mycelium (ca. 0.05 g dry mass). The mycelium was aseptically homogenised in a sterile blender and stored at 4 °C as a fungal inoculum for pellet formation. Five milliliter inoculum was used to inoculate 200 ml ME medium in a 500 ml Erlenmeyer flask to which 0.6 g activated carbon powder was also added to give an initial concentration of 3 g/l. The flask cultures were incubated in a rotary shaker at 28 °C and 100 rpm for 3–4 days. Complex mycelium pellets with a black core of activated carbon were formed, harvested with a sterilized stainless steel wire mesh, and stored at 4 °C for the experiments of dye decolourisation. Mycelium pellets (activated carbon free) were also prepared in the same way except the activated carbon was omitted.

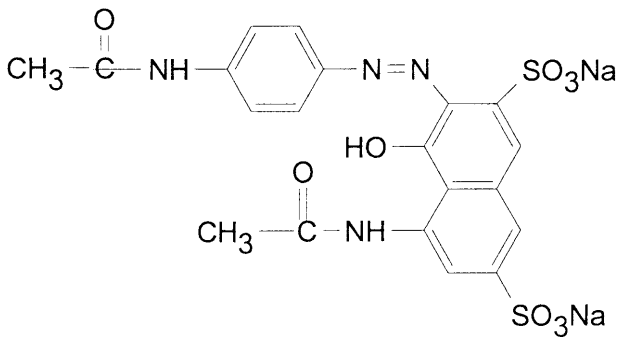

Fig. 1. The chemical structure of Acid Violet 7

2.3
Dye decolourisation in batch culture

The complex pellets or mycelial pellets were aseptically added into a dye-containing test solution in flasks. Unless otherwise indicated, about 5.0 g wet pellets and 50 ml test solution were added into a 250 ml flask in each test. The test solution was a dye-containing nitrogen limited (NL) medium (per liter of distilled water): 5 g glucose; 0.4 g malt extract; 3 mg $MnCl_2$; 4 mg $FeSO_4 \cdot 7H_2O$; 40 mg $MgSO_4 \cdot 7H_2O$; 0.25 g NH_4Cl and 10 mM 3,3-dimethylsuccinate. The pH was adjusted with 1 N NaOH to 5.0. The medium and dye solutions were autoclaved separately at 121 °C for 15 min, and then mixed aseptically as the test solution. The decolourisation tests were carried out under the same conditions as those for mycelial growth described above.

2.4
Fluidized-bed reactor

The schematic diagram of the fluidized-bed reactor is shown in Fig. 2. The complex pellets were fluidized in the test solution by air bubbles that was introduced at the bottom of the reactor and dispersed with a gas diffusing stone of fused crystalline alumina grains (average pore size 60 μm). The reactor system included a glass column of 1 l working volume (1.5 l in total) with a water jacket for temperature control, a water bath, a peristaltic pump for feeding the test solution at adjustable flow rates, and two 2 l flasks for medium and effluent storage. The reactor temperature was maintained at 28 °C for all the experiments. A continuous flow operation was conducted as follows. The reactor was first filled with 1000 ml fresh test solution and 50 g wet complex pellets and aerated with an air flow rate 0.5 l/min for 24 h. A fresh test solution was then continuously pumped into the reactor at a pre-determined rate for controlling the hydraulic retention time in the reactor. The dye concentrations of inlet and outlet as well as laccase activity were measured continuously to monitor the decolourisation performance of the complex pellets. A repeated-batch operation was also conducted in the same reactor. A fresh test solution (1000 ml) was first inoculated with 50 g wet pellets. After completion of solution decolourisation, the decolourised medium was discharged and replaced with 1000 ml fresh test solution for the next cycle of decolourisation by the same pellets. The batch operation was conducted repeatedly.

2.5
Assays

The dye concentration was measured spectrophotometrically at the maximum absorption wavelength and calculated from a calibration curve based on absorbance. The

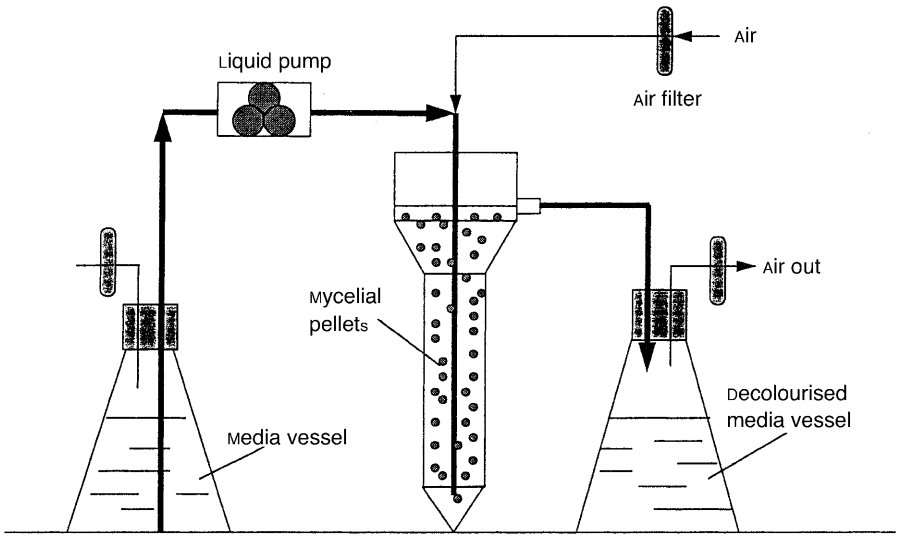

Fig. 2. Schematic diagram of a continuous flow fluidized-bed reactor

aqueous samples were first diluted with distilled water to an appropriate absorption range from 0 to 1. The wet fungal mass was determined after the biomass sample was dried on a stainless steel screen till no free water dropping under room conditions (23 °C, RH 60–70%). The dry mass was further determined after the wet biomass was dried in an oven at 80 °C to constant weight.

Laccase activity was determined by enzymatic oxidation of 2-2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) or ABTS [18]. ABTS oxidation was monitored with a spectrophotometer at 420 nm (molar extinction coefficient = 36,000 M^{-1} cm^{-1}). The enzyme assay solution contained 0.5 mM ABTS, 0.1 M sodium acetate (pH 5.0), and a suitable amount of enzyme. One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 μM of ABTS per min.

3 Results

3.1 Formation of complex pellets

The amount of activated carbon powder that was added into the fungal growth medium had a considerable effect on the formation of complex pellets. In order to prepare complex pellets of uniform size and shape with a black core of carbon powder, different amount of activated carbon was added into 100 ml ME medium at 1, 2, 3, 5 and 7 g/l. The results in Table 1 show the dose effect of activated carbon powder on formation of complex pellets. In the range of 1–3 g/l, the formed complex pellets had a diameter from 1 to 5 mm and a shape from short rod to sphere. All the pellets had a black core of activated carbon surrounded by a white layer of fungal mycelium. The immobilization efficiency of the carbon powder by fungal mycelium declined from 91% to around 76% with increase in carbon dose from 1 to 3 g/l. In contrast, the carbon content in the formed pellets was increased from 39 to 99%, a very high carbon content in the complex pellets. It implies that the carbon powder dose of 3 g/l was the maximum dose to form separated complex pellets under our experimental conditions. It also demonstrated that the carbon content in the complex pellets could be up to 99%. When the carbon dose was further increased, say to 5 g/l or above, a few large pieces of mycelial mat rather than separated mycelial pellets were formed. From the point of view of mass transfer limit and a good fluidization of the complex pellets, the large mycelium mat is not a desired form.

3.2 Decolourisation of Acid Violet 7 in different systems

In order to evaluate the effectiveness of the complex pellets in dye decolourisation, we compared the decolourisation of Acid Violet 7 in four systems. The systems were (A) complex pellets (5 g wet pellets in 100 ml test solution); (B) mycelial pellets of activated carbon free (5 g wet pellets in 100 ml test solution); (C) mycelial pellets plus activated carbon powder (5 g wet pellets and 0.05 g carbon powder

Table 1. Effect of activated carbon dose on the formation of complex pellets in 100 ml malt extract medium*

Dose level	A	B	C	D	E	F
Initial suspended carbon powder (g/l)	0	1	2	3	5	7
Wet complex pellets (g/l)	215 (±5.5)	230 (±19)	227 (±9)	233 (±13)	**	**
Residual suspended carbon powder (g/l)	0	0.09 (±0.02)	0.5	0.68 (±0.05)	N/A	N/A
Carbon powder entrapped (%)	0	91	75	77	N/A	N/A
Activated carbon in complex pellets (g C/g dry pellet)	0	0.39	0.66	0.99	N/A	N/A

* The experiment was conducted in duplicate (data in parentheses are the standard deviation)
** Large pieces of mycelium mats were formed

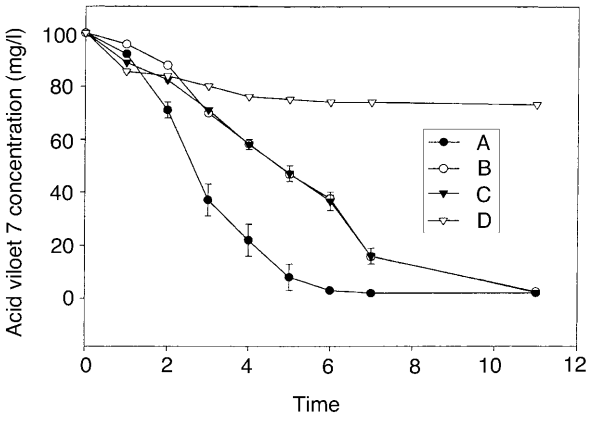

Fig. 3. Decolourisation of Acid Violet 7 in different systems: (A) complex pellets; (B) mycelial pellets (activated carbon free); (C) mycelial pellets plus suspended activated carbon powder and (D) activated carbon powder

in 100 ml test solution); and (D) activated carbon powder (0.05 g in 100 ml test solution). Figure 3 shows the color removal from the test solution in the four systems. The activated carbon had the fastest color removal in the first hour, but approached its maximum adsorption capacity quickly; the dye concentration declined from 100 to 78 mg/l. The mycelium pellets had the slowest color removal in the first 2 h because of their relatively poor dye adsorption compared to activated carbon. However, biological dye degradation by the fungal cells became important with time and finally led to almost complete dye removal in 11 h. Adding activated carbon into the activated-carbon-free mycelium pellets (system C) could speed up the initial dye removal, but gave the same dye removal efficiency as the mycelium pellets alone (system B) because of the same biological activity. The complex pellets (system A) showed the best dye removal efficiency in both rate and final residual dye concentration. The dye was almost completely removed in 6 h, almost a half time required by mycelium pellets. It is also very interesting to note the difference of systems A and C. Both systems had the mycelium pellets and activated carbon but different dye removal efficiency, which might be attributed to the location of activated carbon in the systems. The entrapped activated carbon inside the mycelium promoted the dye degradation compared to the carbon powder suspended in the test solution. The former might concentrate the dye molecules near the fungal cells for biological degradation. It seems that the complex pellets are not a simple addition of activated carbon and mycelium pellets like system C.

3.3 Decolourisation by complex and mycelium pellets in batch cultures

The complex pellets and mycelium pellets were further evaluated for their ability of decolourising Acid Violet 7 at different initial dye concentrations from 50–500 mg/l with 50 g wet pellets in 50 ml test solution. The mycelial pellets were re-used for three times with the same initial concentration of Acid Violet 7 in each batch. A quasi initial dye decolourisation rate was calculated from the color removal in the first 3 h in each batch and averaged over the first two batches. The effect of initial dye concentration on the decolourisation rate was shown in Fig. 4. The complex pellets showed a higher decolourisation activity than the mycelium pellets. The fact that the decolourisation rate increased with dye concentration suggests a Michaelis–Menten type kinetic model (Eq. (1)) in the decolourisation of Acid Violet 7 by the two types of pellets:

$$r = \frac{r_{\max}[S]}{K_s + [S]} \quad , \tag{1}$$

where r refers to the dye decolourisation rate (mg/l h) and $[S]$ the dye concentration (mg/l). The maximum decolourisation rate, r_{max}, and the half rate concentration, K_s, are estimated with the Lineweaver–Burke plots of Eq. (1) as shown in Fig. 5. For the complex pellets, r_{max} is 130.5 mg/l h or 1.3 mg/g wet pellet h with a half velocity concentration K_s around 345 mg/l. For the mycelium pellets, r_{max} is 67.6 mg/l h or 0.67 mg/g wet pellet h with a K_s around 156.7 mg/l.

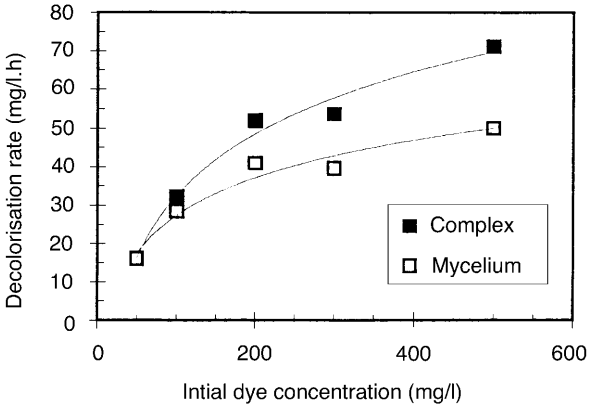

Fig. 4. Decolourisation of Acid Violet 7 at different initial dye concentrations with the complex pellets and mycelium pellets (activated carbon free). The initial rate was obtained from the decolourisation in the first 3 h in each batch and averaged with the first two repeated batches

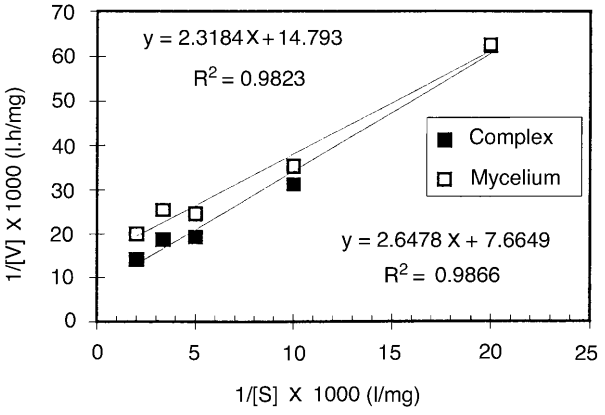

Fig. 5. Lineweaver–Burke plots of a Michealis–Menten type decolourisation model for the complex pellets and the mycelium pellets (activated carbon free)

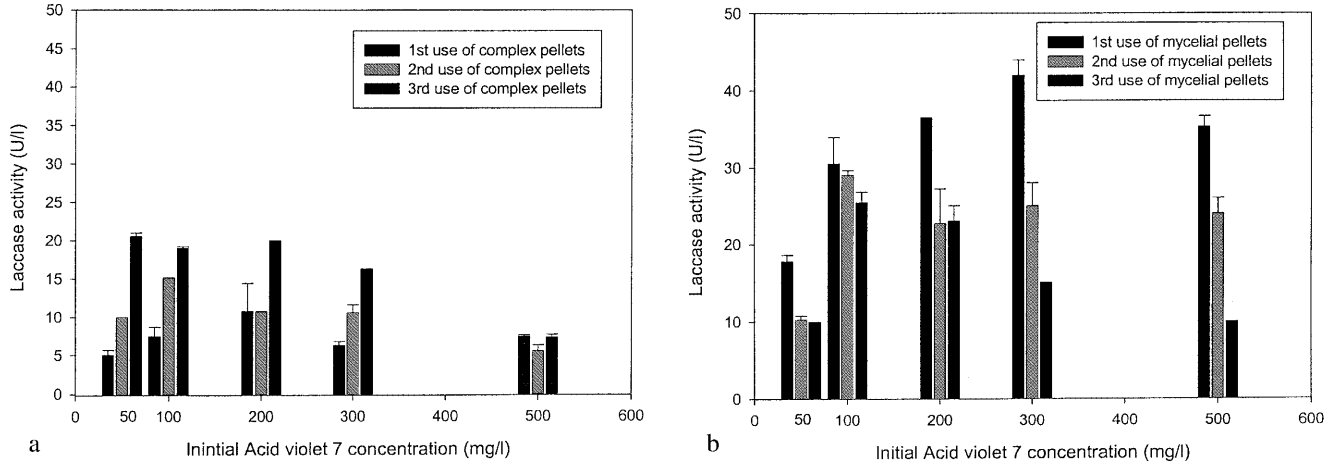

Fig. 6. **a** Laccase activity in the test solution of the complex pellets in three repeated batch decolourisation of Acid Violet 7. **b** Laccase activity in the test solution of the mycelium pellets (activated carbon free) in three repeated batch decolourisation of Acid Violet 7

The activity of laccase in the test solution was assayed at the end of each batch. The results in Fig. 6a and b indicate that the change of laccase activity was different in the two types of pellet systems. In the first batch of decolourisation, laccase activity was relatively high in the solution of mycelial pellets and low of complex pellets, an indication of enzyme adsorption on the activated carbon. In the following re-used batches, the laccase activity in the solution of mycelial pellets declined batch by batch. In contrast, the laccase activity in the solution of complex pellets increased batch by batch, an indication that the adsorbed laccase was released when less laccase was produced from fungal cells. Correspondingly, the complex pellets had a more stable performance in dye decolourisation than the mycelium pellets in the repeated batches. By comparing the general laccase activity in the solutions of complex pellets and mycelium pellets, the former had a relatively lower laccase activity in the solution than the latter. Laccase adsorption might lead to a low activity in the solution, but a high dye decolourisation rate of the complex pellets as shown above.

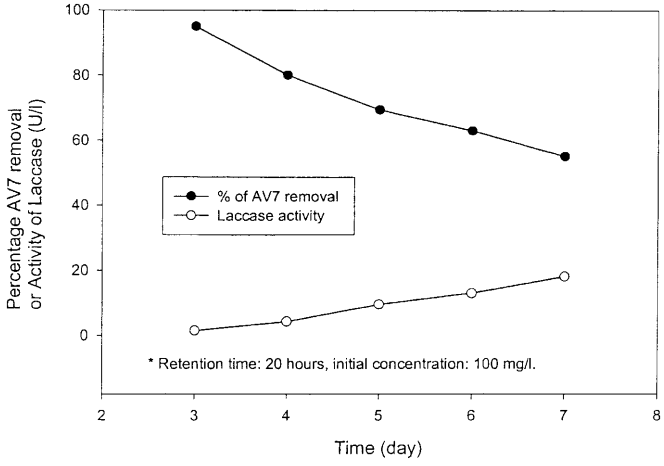

Fig. 7. Decolourisation of Acid Violet 7 with the complex pellets in a continuous flow fluidized-bed bioreactor and laccase activity in the solution

3.4 Decolourisation by complex pellets in a fluidized-bed reactor

The complex pellets were fluidized in the reactor and a test solution of 100 mg dye/l was fed continuously into the reactor at a retention time of 20 h. The system was operated for 7 days, giving a 95% or above decolourisation in the first 2 days and then declined gradually to 55% as shown in Fig. 7. In contrast to the decline in decolourisation, the laccase activity in the solution was increased from 1.4 to 18.1 U/l. Although laccase is the key enzyme of *T. versicolor* involved in the azo dye decolourisation, some small molecule metabolites are also essential as a mediator between the dye molecules and the enzyme because Acid Violet 7 itself is not a good substrate of laccase [11]. The complex pellets might not adsorb and retain the essential reagents enough in the continuous flow

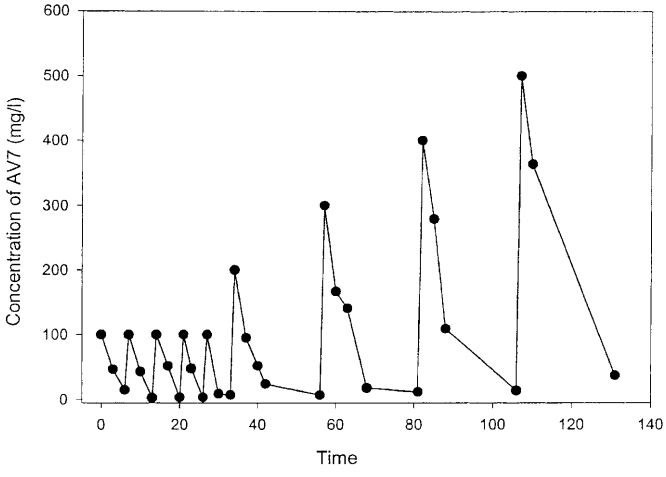

Fig. 8. Decolourisation of Acid Violet 7 with the complex pellets in a repeated-batch operation of the fluidized-bed bioreactor

reactor, typically under the conditions that the fungal cells produced a little essential reagents.

Since most fungi release their secondary metabolites at a stationary phase, a repeated batch experiment was designed to let *T. versicolor* produce the essential reagents in each batch. For that purpose, the same complex pellets were reused for 9 cycles of dye decolourisation. The test solution contained Acid Violet 7 at a concentration from 100 to 500 mg/l. As shown in Fig. 8, the complex pellets retained a high and stable decolourisation activity during the experiment and decolourised Acid Violet 7 by more than 95%. A high dye concentration up to 500 mg/l could also be decolourised satisfactorily in 20 h. Laccase activity was relatively low (6.7 U/l) in the first two batches and then increased to 19.2–68.9 U/l with the repeated use of the complex pellets.

4 Discussion

In the malt extract medium, white rot fungus *T. versicolor* was able to grow in the presence of activated carbon powder and formed the complex pellets in which activated carbon powder was entrapped by fungal mycelia. In a study on *P. chrysosporium,* Michel et al. (1990) observed that the mycelial pellets were hollow spheres. We also observed the empty core of the mycelial pellets of *T. versicolor* in this study. One explanation to the empty core is based on nutrient limitation. Once the pellet reaches a certain relatively large diameter, the diffusion of nutrients and oxygen into the center of mycelium mass is too slow to maintain the unrestricted growth of the entire mycelium. Thus, growth occurs mainly in the periphery of the pellet, and the hyphae in the center may even die leaving an empty core. In this study, we filled this empty space with activated carbon powder with an expectation of improved dye adsorption and degradation by the complex pellets.

The decolourisation performance of the complex pellets was evaluated in different systems including flask batch cultures, repeated-batch culture and continuous feeding cultures in the fluidized-bed reactor. The decolourisation ability and stability of complex pellets were obviously improved compared to the mycelial pellets (activated carbon free) when they were tested under the same conditions. The enhanced decolourisation ability of the complex pellets implies that the microenvironment around the fungal cells was changed in the presence of activated carbon and become more favorable for dye degradation. The activated carbon can adsorb and concentrate the chemicals from the environment, thereby enhancing the bio-availability of these compounds for microbial or enzymatic attack. Activated carbon can also retain the extracellular enzymes and mediators that are necessary for the degradation. In addition, transport of chemicals from the bulk solution into the mycelial pellets might be enhanced by the adsorption of activated carbon. Lin et al. (1988) reported that co-immobilized *P. chrysosporium* with activated carbon in alginate beads could rapidly remove PCP from solution and subsequently degrade the adsorbed PCP at a significantly higher rate in comparison with immobilized fungal cells or immobilized cells plus free activated carbon. In comparison with their co-immobilized fungal pellets, the complex pellets developed and investigated in this study did not need the immobilization process with alginate, but could be prepared in a simple growth culture and used as immobilized cells. The decolourisation performance of the complex pellets in the continuous feeding operation of fluidized-bed reactor was inferior to the repeated-batch operation of the same reactor. This might be attributed to a poor production of the extracellular reagents by the fungus at steady state developed in a continuous flow fluidized bed reactor.

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