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Remediation of Textile Dye Waste Water Using a White-Rot Fungus *Bjerkandera adusta* Through Solid-state Fermentation (SSF)

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Abstract A strict screening strategy for microorganism selection was followed employing a number of white-rot fungi for the bioremediation of textile effluent, which was generated from one Ireland-based American textile industry. Finally, one fungus *Bjerkandera adusta* has been investigated in depth for its ability to simultaneously degrade and enrich the nutritional quality of highly coloured textile effluent-adsorbed barley husks through solid-state fermentation (SSF). Certain important parameters such as media requirements, moisture content, protein/biomass production and enzyme activities were examined in detail. A previously optimised method of dye desorption was employed to measure the extent of dye remediation through effluent decolorisation achieved as a result of fungal activity in SSF. *B. adusta* was capable of decolourising a considerable concentration of the synthetic dye effluent (up to 53%) with a moisture content of 80–85%. Protein enrichment of the fermented mass was achieved to the extent of 229 g/kg dry weight initial substrate used. Lignin peroxidase and laccase were found to be the two main enzymes produced during SSF of the dye-adsorbed lignocellulosic waste residue.

Keywords Barley husks · Textile dyes · *Bjerkandera adusta* · Laccase · Lignin peroxidase · Manganese peroxidase · Decolourisation · Solid-state fermentation (SSF)

Introduction

White-rot fungi, such as *Phanerochaete chrysosporium*, *Hirschioporus larincinus*, *Inonotus hispidus*, *Phlebia tremellosa* and *Coriolus versicolor* have been well documented for their ability to degrade various textile dyes under submerged fermentation (SmF) conditions [1–3]. The use of white-rot fungi for the purpose of dye remediation in conditions similar to their natural environment, i.e. in SSF, was investigated by Nigam et al. [4].

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Solid-state fermentation (SSF) may be characterised by a fermentation process carried out on a solid medium with a low moisture content (water activity, a_w), typically 0.40–0.90, which occurs in a non-aseptic and natural state [5]. Many of the solid materials used as the main substrates for SSF are unrefined and are of agricultural origin [6, 7]. Their complex and unrefined composition makes the complete characterisation and exact reproducibility of such fermentation processes very difficult [8].

SSF has been successfully exploited for the production of food [9,10] animal feed [11–13], production of biofuel [14–16], and for the biosynthesis of various microbial enzymes [17, 18]. This same SSF process has also been experimented to be exploited in environmental biotechnology by authors of this paper for the bioremediation of textile dye for the purpose of decolourisation [4, 19].

The low moisture content of the solid substrate cultivation systems means that such fermentation can only be carried out by a limited number of microorganisms, for example, white-rot fungi are ideal to grow under lower water activity [20, 21]. This also provides extra benefit that if SSF is carried out as a non-aseptic fermentation, contamination by unwanted bacteria is reduced due to the low water activity level of the system. Due to the fact that low water activity inhibits the growth of most of bacterial cultures, SSF at lower water activity eliminates the chances of contaminations [22]. Besides that, if such a process is to be considered for large-scale bioremediation of textile dyes, the absence of free flowing water required in SSF, would mean smaller size fermenters could be used for the purpose. Since in textile industry the volume of waste water generated is vast and is not practical to apply submerged fermentation to treat this large effluent.

This study assessed the capabilities of two white-rot fungi to grow on dye-adsorbed barley husks on a variety of SSF salts media at a moisture content of 75%. From this the best medium for SSF was selected and the effect of moisture content on the colonisation and dye degrading capabilities of the fungi were assessed. Our previous studies concluded that barley husks were the better sorbent of colour for the purpose of dye removal by the physical process of adsorption and also proved the better substrate for dye desorption for analytical work [23, 24].

Studies in liquid-submerged fermentations have illustrated that *B. adusta* and *C. gallica* were the better fungi at degrading the dyes present in the synthetic textile effluent [25, 26]. The use of white-rot fungi for the purpose of dye remediation in conditions similar to their natural environment, i.e. SSF, has not yet been investigated. Hence, the present study was undertaken to establish an economically viable process for the remediation of textile waste water using white-rot fungus in their natural environment by providing SSF conditions.

Materials and Methods

Preparation of Synthetic Textile-dye Effluent

The reactive dyes used in this study were Cibacron Yellow C-2R, Cibacron Red C-2G, Cibacron Blue C-R, Remazol Black B, and Remazol Red RB. The dyes were a gift from Textile Company, Fruit of the Loom, in Buncrana, Republic of Ireland. The synthetic effluent was prepared by dissolving the above five dyes, in equal amounts, in deionised water to produce a stock solution of 1,000 mg/l. This stock solution was stored at 4 °C for use in various studies after it was diluted to produce an initial dye concentration (*C*o) of 100 mg/l. In our previous experiments dye concentrations ranging from 50 to 500 mg/l have been tested. In current SSF experiments 100 mg/l was selected due to the fact the

actual dye effluent produced in textile industry contains dye concentration lower than 100 mg/l.

Adsorption of Synthetic Textile-dye Effluent onto Barley Husks

Barley husks (average particle size of 1 mm×2 mm) were washed in distilled water to remove any colour of husk particles, dried and stored in airtight containers. To prepare dye-adsorbed substrate for SSF, husk particles were soaked in the synthetic textile-dye effluent, at a *C*o of 100 mg/l, and left for dye adsorption at the room temperature (25 °C) up to the level of dye-saturation. Effluent samples were then filtered and the remaining colour in solution was determined using a spectrophotometer at λ_{max}. On knowing the residual colour concentration of textile-dye effluent in the solution, the amount of dyes bound to the sorbent material (barley husk particles) could be determined.

The amount of the dyes adsorbed to the barley husks would then be taken as the initial concentration (as 100%) dyes present onto barley substrate in SSF. The dye-adsorbed barley husks were then dried at 50 °C in order to remove any free moisture. Solid dye-adsorbed substrate in doses of 5.0 g, d.w. for each day of the fermentation was used and experiments were carried out in triplicate in 200 ml flasks. Control solid substrates used in SSF were not soaked in the synthetic textile-dye effluent and were normal barley husks of same particle size.

White-rot Fungi Inoculum

Bjerkandera adusta and *Coriolopsis gallica* were selected for SSF as they displayed the highest percentage decolourisation of dyes at *C*o 100 mg/l in submerged liquid fermentation (SmF) as previously reported by Robinson et al. [25, 26]. Fungal plugs (10 mm, diameter), were cut from freshly prepared culture plates of *B. adusta* and *C. gallica* and inoculated into Malt Extract Broth (using one fungal-plug per 10 ml of MEB). Cultures were then incubated at their optimal temperatures and shaken gently (100 rpm) for 3–5 days. After this period excess MEB medium was drained and the recovered fungal mycelia were gently macerated using a small sterilised homogeniser Kenwood mini chopper CH100, UK.

Solid-State Fermentation Media

Four different SSF salts media were investigated in order to select a most suitable medium which allowed better fungal biomass production. The first medium (Medium I) used for SSF was the one described by Gupte and Madamwar, [27]. It had the following composition: urea (0.30 g/l), $(NH_4)SO_4$ (1.40 g/l), KH_2PO_4 (2.00 g/l), $CaCl_2$ (0.30 g/l), $MgSO_4.7H_2O$, (0.30 g/l), peptone (1.00 g/l), $FeSO_4{\cdot}7H_2O$ (5.00 mg/l), $MnSO_4{\cdot}7H_2O$ (1.60 mg/l), $ZnSO_4{\cdot}7H_2O$ (1.40 m g/l), $CoCl_2$ (2.00 g/l), Tween 80 (1 ml/25 ml). The second medium (Medium II) was a very simple medium only with three ingredients used by Nigam [28]. It consisted of $(NH_2)_4HPO_4$ (6.6 g/100 g d.w. substrate), $MgSO_4{\cdot}7H_2O$ (1.5 g/100 g d.w.), $CaCl_2{\cdot}H_2O$ (1.5 g/100 g d.w.).

A third medium (Medium III) described by Brand et al. [29] was used, consisting of: KH_2PO_4 (1 g/l), $CaCl_2{\cdot}H_2O$ (0.70 g/l), $MgSO_4{\cdot}7H_2O$ (3.5 g/l), $FeSO_4{\cdot}7H_2O$ (0.35 g/l), $ZnSO_4{\cdot}7H_2O$ (0.25 g/l), $MnSO_4{\cdot}7H_2O$ (0.17 g/l), $CuSO_4{\cdot}5H_2O$ (0.35 g/l). Finally, a fourth medium (Medium IV) also described by Brand et al. [29], was tried; containing: KH_2PO_4 (2 g/l), Na_2HPO_4 (0.2 g/l), $MnSO_4{\cdot}7H_2O$ (0.5 g/l).

Desorption of Synthetic Textile-dye Effluent from Barley Husks

A modified method for dye desorption from soil samples, as described by Bhatt et al. [30], was adopted. A mixture of methanol, chloroform and water in equal *v*/*v* ratio (1:1:1) was used for the desorption of effluent colour from the barley husks post SS-fermentation in order to estimate the amount of dyes degraded, this method of desorption was previously optimised by us…. Samples of 5 g wet weight were taken from SSF flasks and dried at 50 °C in order to remove any moisture. From this dried SSF-sample, three 0.5-g dry weight samples were used in analysis. Samples were placed in small glass universal bottles and treated with solvent mixtures. The desorption method was optimised by us particularly for such SSF work [24].

Moisture Content

The moisture content of 75% was set in SSF-medium in the experiments of medium selection using four different media. The required moisture content was achieved by using equal volume of two components, i.e. 1.5 ml of fungal inoculum and 1.5 ml of salts media. After selecting the best medium, effect of variation in moisture contents was studied in SSF. Three different levels of 75%, 80% and 85% were investigated in order to determine if moisture content was directly related to fungal biomass production using dye-adsorbed solid substrate, and more importantly to dye degradation in SSF when the dyes were adsorbed on solid substrate particles and not present freely in liquid as in submerged fermentation.

Protein/biomass Estimation

The Biuret method [31] was used for protein estimation of the fermented barley husks. SSF samples of 5 g wet weight were placed in boiling test tubes with 5 ml, 1 N NaOH solution. This was heated to 100 °C for 15 min then allowed to cool, and 5 ml of distilled H_2O were added in each tube. The digested barley husks were then vortexed and centrifuged at 3,000 rpm for 10 min. The supernatant volume was then collected in graduated tubes. The residual husks in centrifuge tubes were washed with distilled water and washing were added to alkaline supernatant in graduated tubes. The distilled water was added to make up to a total working volume of 25 ml in each tube.

Using appropriately diluted samples from this stock protein digested solution, all protein analysis [4, 19], were carried out in duplicate. Biomass was calculated using protein values as *B. adusta* mycelium was found to contain about 40% protein from SmF experiments (results not shown), as it was not possible to accurately determine biomass under SSF conditions. The results presented are within standard deviation of 3–5%.

Enzyme Activity

SSF samples of 5.0 g wet weight were placed in 15-ml centrifuge tubes along with 10 ml of 50 μM sodium acetate (pH 6.0) buffer [32]. The tubes were placed in a shaker to mix the contents vigorously for 1 h in order to wash all extracellular enzymes out of the fermented material enriched with fungal mycelia into the buffer. This was then centrifuged at 3,000 rpm for 10 min, the supernatant was collected and a working volume of 10 ml was produced by adding the required amount of sodium acetate buffer solution. This freshly prepared solution was used to assay for enzymes activities of Lignin peroxidase (LiP), Manganese peroxidase (MnP) and laccase, following the method as described by Tien and Kirk [33].

Results and Discussion

Media Optimisation

The amount of dyes left in solution after the soaking process for dye adsorption was 19.4 mg/l; so the quantity of dyes adsorbed to the barley husks was calculated as 80.6 mg/l. This amount of 80.6 mg/ml was taken as the initial 100% dye present on solid substrate before SSF for the calculation of actual dye decolourisation in SSF. *B. adusta* and *C. gallica* were initially grown on four different media at 75% moisture content. *C. gallica* was unsuccessful in colonising in appropriate amount in SSF of both the control and dye-adsorbed barley husks, a poor scanty growth was seen in all four media types.

The experiment was then repeated at 85% moisture content but *C. gallica* again failed to colonise the barley husks successfully, the growth was very scanty and inadequate for further analysis. A visible thick fungal growth in SSF of *B. adusta* was achieved using the medium described by Gupte and Madamwar [27]. Due to the fact that *C. gallica* was unable to grow in the presence of any of the SSF media; the effects of various moisture contents on fungal colonisation (protein/biomass production), enzyme production in SSF and dye degradation capabilities were investigated employing *B. adusta* only.

B. adusta was capable of colonising and increasing the protein content of the fermented barley husks. There was a gradual but steady increase in protein content throughout the fermentation period and it could be seen that the presence of dyes, once adsorbed to the barley husks, did not have a detrimental effect on the performance of *B. adusta*. Figure 1 illustrates the higher protein enrichment achieved through the use of *B. adusta* on both the control- and dye-adsorbed barley husks.

The protein content in SSF of control barley husks was slightly better than in SSF of dye-adsorbed barley husk. The final protein content of 192 g/kg dry weight and 149.3 g/kg dry weight were recorded for the control and dye-adsorbed barley husks, respectively. Up

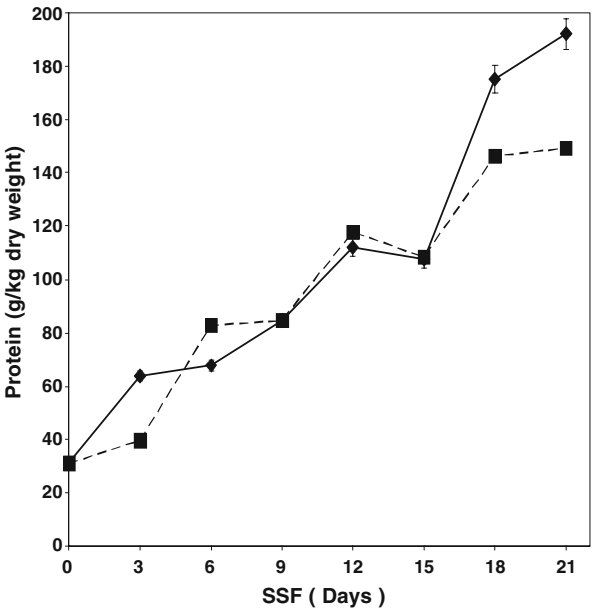

Fig. 1 Growth of *B. adusta* in SSF of best performing medium (no. I) Control *empty diamond*, dye-adsorbed barley husks *filled square*

until day 15, protein enrichment on both substrate conditions were very similar, with *B. adusta* ultimately enriching the control solid substrate to a greater extent. From this experiment it is clear that *B. adusta* was best suited to Media I and its successful growth on the dye-adsorbed barley husks was not significantly compromised due to the synthetic effluent.

These results can be compared to those found by Nigam et al. [4] using *P. chrysosporium* and *C. versicolor*. These two white-rot fungi were successfully used to enrich the protein content of dye-adsorbed (synthetic textile-dye effluent consisting of eight different textile dyes) wheat straw by four-fold. A lesser degree of protein enrichment was achieved when SSF was performed using different substrates, wood chips and corncobs.

Moisture Content

On determining the optimal medium for *B. adusta*, the effects of moisture content on biomass and protein production were then investigated. Moisture ratios of 75%, 80% and 85% moisture were used to prepare SSF material. Figure 2 shows both biomass and protein estimation for *B. adusta* on the control and dye-adsorbed barley husks in SSF of 75% moisture. It can be explained that biomass gain is closely correlated to the subsequent protein enrichment of the barley husks due to the successful colonisation and adequate growth of *B. adusta* (Fig. 2). As with protein production, *B. adusta* seemed to prefer the control barley husks, as after the 21-day fermentation period, 480 g/kg and 372.3 g/kg d.w. initial substrate of biomass were produced.

Figure 3 shows the effect of increasing the moisture content to 80%. Protein gain throughout the fermentation occurred in a similar fashion as that was seen at 75% (Fig. 2). At 80% moisture content, protein enrichment occurred at a more rapid rate on the dye-adsorbed barley husks in comparison to the control and also that produced under 75%. Between days 3 and 15, protein enrichment increased from 39.8 g/kg to 108.5 g/kg and 55.3 g/kg to 129.9 g/kg for dye-adsorbed barley husks at 75% and 80% moisture levels, respectively.

There was a sharp rise in the protein content of the barley husks from day 15 to the end of the fermentation, with similar final values achieved for both conditions; 210.3 g/kg dry

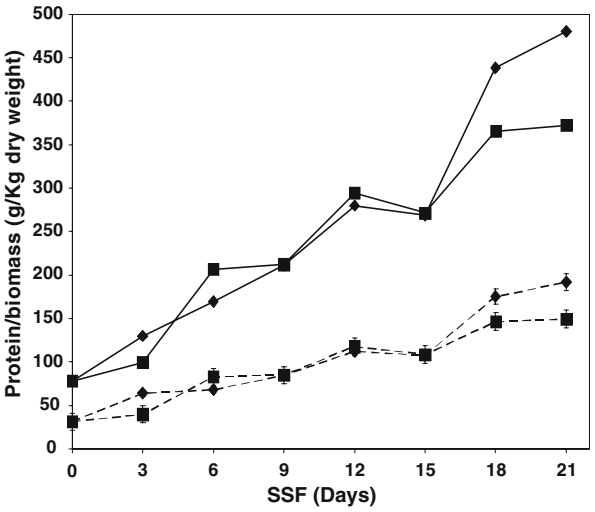

Fig. 2 SSF of *B. adusta* at 75% moisture content. Protein control *empty diamond with broken lines*, protein dye-adsorbed barley husks *filled square with broken lines*. Biomass control *empty diamond*, biomass dye-adsorbed barley husks *filled square*

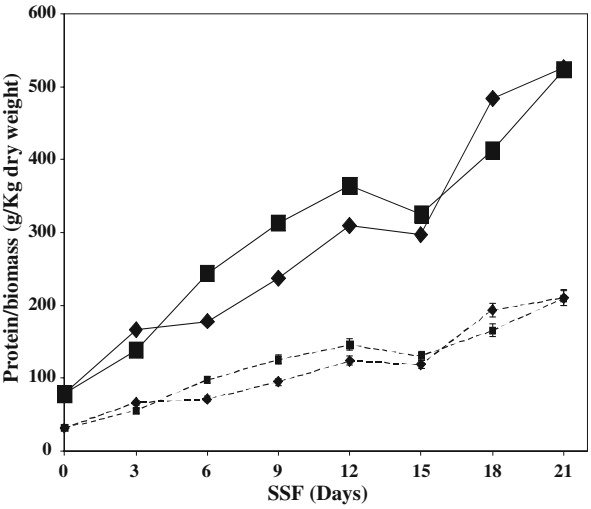

Fig. 3 SSF of *B. adusta* at 80% moisture content. Protein control *empty diamond with broken lines*, protein dye-adsorbed barley husks *filled square with broken lines*. Biomass control *empty diamond*, biomass dye-adsorbed barley husks *filled square*

weight and 209.4 g/kg dry weight for the control and dye-adsorbed barley husks, respectively. A similar pattern, as regards to biomass production, can be seen also. Final biomass values of 525.8 g/kg dry weight and 523.6 g/kg dry weight for control and dye-adsorbed, respectively, were achieved. As with protein content, biomass production was higher when the fungus was grown with slightly higher moisture content. The higher moisture content also reduced the difference in biomass/protein production that existed between the control and dye-adsorbed samples at a lower moisture level.

Figure 4 illustrates the effects of increasing the moisture content to 85%. It is apparent that both the protein and biomass values are much higher at the end of the fermentation, in comparison to those at 75% and 80% moisture. For both biomass and protein production, *B. adusta* appears to grow slightly better on the dye-adsorbed barley husks in comparison to

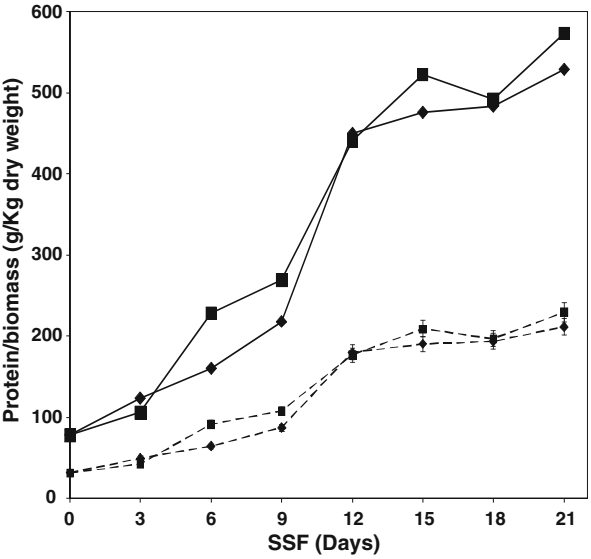

Fig. 4 Protein and biomass estimation for *B. adusta* at 85% moisture content. Protein control *empty diamond with broken lines*, protein dye-adsorbed barley husks *filled square with broken lines*. Biomass control *filled square*, biomass dye-adsorbed barley husks *empty diamond*

Table 1 Protein enrichment of control (natural substrate) and dye-adsorbed barley husks at various moisture levels in 21 days SSF.

Moisture level	Protein enrichment g/kg initial d.w. substrate	
	Control substrate	Dye-adsorbed substrate
75 (%)	192	144.3
80 (%)	210.3	209.4
85 (%)	211.6	229.4

the control samples. At 85% moisture content, *B. adusta* is capable of producing 229.4 g/kg protein dry weight, on dye-adsorbed barley husks compared with 211.6 g/kg protein dry weight on the control barley husks.

It seemed that by making conditions more favourable for *B. adusta*, in relation to moisture content, fungus was able to proliferate better on the dye-adsorbed substrate. At 75% the control substrate produced a higher quantity of protein (g/kg dry weight) than the dye-adsorbed samples and by increasing moisture content to 80% the difference in protein production on each of the treated types of substrate was reduced. A moisture content of 85% allowed the *B. adusta*, grown on the dye-adsorbed barley husks, to enrich the protein content of the fermented mass to a greater extent than the control (Table 1).

Enzyme Activities

Figure 5 shows the enzyme activities of *B. adusta* when grown under SSF conditions (moisture content 85%) on dye-adsorbed barley husks. It was observed that a lag period existed before any LiP activity was detected, although after this period, activity rose sharply producing a peak value of 17,000 U/kg dry weight. LiP activity then decreased gradually over the next 9 days, before rising again at the end of the fermentation period. LiP still had the highest enzyme activity of all the assayed enzymes, with MnP increasing (days 9 to 15) when LiP was decreasing, indicating its involvement in dye degradation during this period. Similarly laccase activity was not apparent until after day 6. This delay in enzyme production by both enzymes can be correlated with the slow biomass and protein production of *B. adusta* during this time period (Fig. 4). A period of time may have been

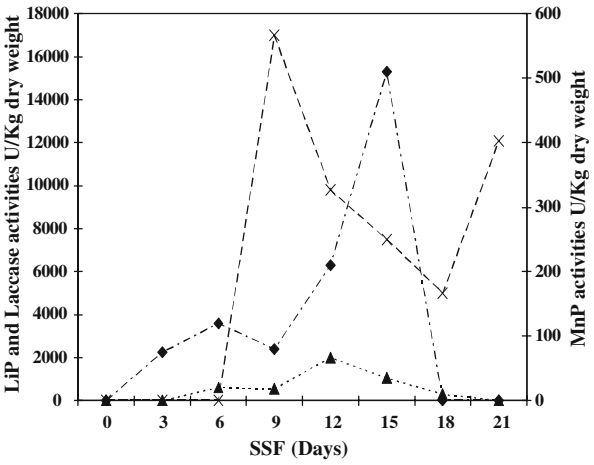

Fig. 5 Enzyme activities for *B. adusta* on dye-adsorbed barley husks at 85% moisture content. MnP *empty diamond*, laccase *filled upright triangle*, LiP x

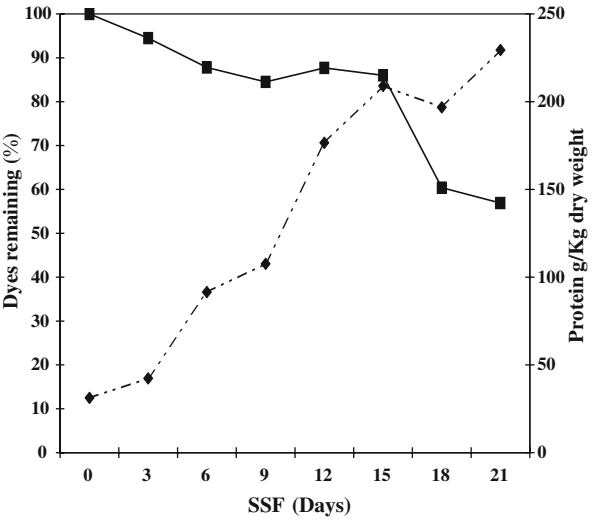

Fig. 6 Simultaneous dye degradation and protein enrichment by *B. adusta* on effluent-adsorbed barley husks at 85% moisture content. Dyes remaining *filled square*, protein enrichment *empty diamond*

required in order for the fungus to become established and proliferate successfully on the solid substrate. From day 6 onwards, LiP, laccase activity and biomass/protein production increased at a more rapid rate. Laccase activity peaked on day 12 of the fermentation before decreasing on day 21.

MnP activity was dramatically lower than the other two assayed enzymes, although MnP production began as early as day 3. MnP activity increased until day 15 when it peaked at 510 U/kg dry weight. After this period enzyme activity dropped dramatically until the fermentation ended. All peak enzyme activities were produced when biomass and protein production were at the stage of most rapid increase. The main enzymes that appear to be involved in the degradation of the synthetic effluent under SSF conditions are LiP and laccase. It should be noted that other non-assayed enzymes may also have been responsible for the remediation of the adsorbed synthetic effluent, although the three assayed ligninolytic enzymes are widely reported to be responsible for lignin and xenobiotic compounds degradation. Peak fluctuations in SSF as well as in SmF may be due to various parameters such as variation in sampling times and the difficulties involved in the assay procedure.

Dye Degradation and Protein Enrichment

As mentioned previously, 80.6 mg/l of the total amount of dyes in solution was adsorbed onto the barley husks; this value was then regarded as 100% of the dyes adsorbed onto the sorbent. Figure 6 illustrates how *B. adusta* was capable of degrading these dyes through SSF. A gradual rate of degradation can be seen to have occurred from the onset of the fermentation, until day 15. In this period a total of 14% of the dyes was degraded by the fungus and it was not until close to the end that the concentration of dyes adsorbed to the solid substrate was broken down to a greater extent. On day 21, 53.1% of the dyes adsorbed were successfully degraded.

A continuous increase in protein content of the fermented mass and also a subsequent decrease in dyes remaining adsorbed to the barley husks can be seen. Although only half of the total percentage of dyes were degraded, it is clear that *B. adusta* was capable of, not

only degrading the five dyes present in the synthetic effluent, but could also increase the protein content of this lignocellulosic residue.

The increase in protein content and decrease in recovered effluent colour can be linked with the enzyme assay findings (Fig. 5). In the first few days (days 0–6) of the fermentation, very little of the recovered dyes have been degraded by *B. adusta* (10%). This is supported by the lack of LiP and laccase activity in the early stages of SSF (days 0–6). MnP activity was recorded on day 3, and although it was only detected in small amounts, it could still have been implicated with early degradation. The amount of laccase and MnP present at this time was relative to the little degradation that occurred. Lignin is a model compound, if enzymes are present in system to degrade lignin, they can also act on all similar compounds such as dyes.

The majority of effluent degradation does not begin until day 15, yet enzyme activities had been seen to be consistently high before this period. Even though these ligninolytic enzymes were produced but did not appear to be involved in dye degradation until a later point in the fermentation. So, initially, these may have been employed for the use in the breakdown of the lignocellulose component of the barley husks to support a good fungal growth on husk particles and then later in dye degradation.

Conclusions

Lots of work is being done by other workers to decolourise dyes in liquid fermentations, which is not feasible economically and time wise for the treatment of thousands of litres of actual effluent produced in textile companies. Our approach was to remove colour from textile effluent by adsorbing the colour on waste agricultural residues, which is the only economical and time-wise feasible approach for the large-scale treatment of effluents. We could have disposed dye-adsorbed material by simple burning, but we have recycled it by SSF. The fermented material rich in ligninolytic enzymes and fungal biomass could be used as soil conditioner. There is no need of analysing dye degradation. This process may be considered for large-scale bioremediation of textile dyes, the absence of free flowing water required in SSF, would mean smaller size fermenters could be used for the purpose. Since in the textile industry, the volume of waste water generated is vast and it is not practical to stock for a longer time to apply submerged fermentation to treat this large effluent.

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