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Characterization of Azo Dye-degrading *Coriolopsis* sp. strain arf5

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Abstract

Sixty-three local white-rot fungi were isolated from soil and wood samples on potato dextrose agar (PDA). All these isolates were screened for their ability to degrade four textile azo dyes; Ponceau 2R (C.I. 16450), Orange G (C.I. 16230), Direct Blue 71(C.I. 34140) and Biebrich Scarlet (C.I. 26905). Out of forty isolates that gave positive results, one promising isolate which can completely degrade all four dyes in the minimum amount of time was selected for further studies. This isolate was from Universiti Putra Malaysia (UPM) Serdang campus. The isolate was tentatively identified to the species level as *Coriolopsis* sp. strainarf5 based on restriction fragment length polymorphisms of the internal transcribed spacer region. Nutritional studies on defined solid medium showed that this isolate was only able to degrade the four azo dyes under nitrogen-limiting conditions and an additional carbon source (glucose) need to be added to provide sufficient energy for the degradation to occur. Various parameters such as initial pH and incubation temperature were optimized.

INTRODUCTION

The modern world uses synthetic dyes to replace natural dyes, especially in the textile industries. However, they present their own new set of problems. The most obvious problem is aesthetic pollution of water ways caused by the presence of dyes leached from textile factories since they are visible even at low concentrations [1]. In addition, the presence of dyes could also potentially reduce the amount of sunlight reaching the bottom of rivers and lakes and thus affects the ability of aquatic plants to carry out photosynthesis [1-3]. This will have net effect of reducing the availability of oxygen in the water to other aquatic animals [4]. They also produce potentially carcinogenic aromatic compound from partial cleavage of azoic dye groups. Current azo dye removal methods usually involve physical and/or chemical treatments.

These methods have many disadvantages. Chemical treatment produces large amounts of chemical sludge with the attendant disposal problems [5]. Physical treatments are also very expensive due to the high operating expenses to produce and regenerate activated carbon. For these reasons, biological treatments such as utilizing the biodegradative ability of bacteria and lignolytic fungi are being investigated as a viable and cost-effective alternative. Fungi such as *Phanerochaete chrysoporium* and *Tinctporia* sp., both belonging to the ligninolytic white-rot group, are among the first to have been shown to have the ability to degrade azo dyes [6]. Until recently however, most published research, including those that have been done in Malaysia have focused on these temperate species [6, 7] while ignoring the rich biodiversity available in our tropical country.

MATERIALS AND METHODOLOGY

Sampling

Sixty-three samples were collected from soil and wood from various locations in Selangor, Kelantan, Terengganu and Perak in Peninsula Malaysia. Samples from soil had soft and fleshy fruiting bodies (mushrooms). Samplings were carried out by scooping out the fruiting bodies with some of the surrounding soil underneath it to obtain the whitish mycelia that are growing in the soil. Wood samples were rotten wood underneath and surrounding the fruiting bodies, or pieces of rotten tree bark that were covered with white rot's mycelia.

Isolation

Collected samples were first washed with tap water, and cut into smaller pieces. For soil samples, only stipeses of fruiting bodies and mycelia in soil were used. Cleaned and cut samples were immersed in a commercial solution (5.25 % sodium hypochlorite Clorox ™ in sterile distilled water) for approximately 15 min for disinfection purpose. Samples were gently shaken to remove excess disinfectant solution before being placed on potato Dextrose Agar (PDA). Once white-rot mycelia (whitish or yellow-brown, flat or cotton-like mycelia radiate out of the original sample, no spore-forming structure) were detected, they were cut and subcultured on

new PDA plates. This process was repeated until a pure culture (homologous mycelial morphology) was obtained.

Preparation of inoculum

Isolates were grown on PDA plate for seven days. For inoculation, a sterile metal cork-borer was used to punch out 1 cm diameter mycelia disks from the edges of 7 days old PDA cultures that were then aseptically placed, face down onto the centre of the solid medium.

Screening

Screening of isolated white-rot cultures for azo dye degrading abilities were carried out in solid, nitrogen deficient defined medium containing a modified Kirk's Basal Medium (KBM) [8, 9] with added azo dye. The full composition of the modified KBM is as follows; 10.1 g/L glucose monohydrate, 2 g/L L-Asparagine, 0.2 g/L KH_2PO_4, 0.05 g/L $MgSO_4.7H_2O$, 0.01 g/L $CaCl_2.2H_2O$ and 0.1 ml/L of stock trace mineral solution, all dissolved in distilled water. The stock trace elements (TE) solution contains 30 g/L $MgSO_4.7H_2O$, 10 g/L NaCl, 5 g/L $MnSO_4.H_2O$, 1 g/L $FeSO_4.7H_2O$, 1 g/L $ZnSO_4.7H_2O$, 0.82 g/L $CaCl_2$, 0.1 g/L $CuSO_4.5H_2O$, 0.1 g/L $NaMoO_4.2H_2O$, 0.1 g/L H_3BO_3 and 1 g/L EDTA dissolved in deionized water.

Bacteriological agar was added at 30 g/L as a solidifying agent. For screening purposes, the L-asparagine was omitted to obtain nitrogen deficient condition and replaced with 0.2 g/L azo dyes. Four different azo dyes were used for screening purposes which are Ponceau 2R (C.I. 16450; Acid Red 26), Orange G (C.I. 16230; Acid orange 10), Direct Blue 71 (C.I. 34140) and Biebrich Scarlet (C.I. 26905; Acid Red 66). The screening medium was adjusted to pH 5.0 and prepared into Petri dishes. The screening culture plate were observed qualitatively for 21 days for growth and the formation of translucent decolourized zones around the growing mycelia that indicates the presence of azo dye degrading ability.

ITS region sequencing

DNA was extracted from fresh mycelia grown in Potato Dextrose Broth after 7 days on orbital rotary shaker. The mycelia were filtered using sterile filter paper via Buchner funnel and then ground using pestle and mortar with sufficient liquid nitrogen forming fine white powder. DNA extraction was done using Masterpure™ Yeast DNA Purification Kit according to the Epicentre Biotechnologies manufacturers in strategies. PCR amplification was performed using a thermal cycler (Biometra, Gottingen, Germany). The PCR mixture contained 1.5 µM of each primer, 200 µM of each deoxynucleotide triphosphate,10x reaction buffer, 2.5 U of Taq DNA polymerase (Promega) to achieve a final volume of 50 µL. The internal transcribe spacer (ITS) region from the genomic DNA was amplified by PCR using the following primers; ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') which is specific for higher fungi [10] and the universal primer ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') [11].

PCR was performed under the following conditions: initial denaturation at 94 °C for 1 min 25s; 35 cycles of 95 °C for 35s, 55 °C for 55s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. Cycle sequencing was subsequently performed with the Big Dye terminator kit (Perkin-Elmer Applied Biosystems) as recommended by the manufacturer. Sequence data were initially recorded and edited using CHROMAS Version 1.45. The resultant 772 bases were compared with the GenBank database using the Blast server at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).The ITS region sequences for this isolate have been deposited in GenBank under the following accession number EU863194.

Phylogenetic analysis

A multiple alignment of 20 ITS region sequences which closely matches strain arf5 was retrieved from GenBank and was aligned using ClustalW [12] with the PHYLIP output option. Alignment positions with gaps were excluded from the calculations. A phylogenetic tree was constructed by using PHYLIP, version 3.573 [J. Q. Felsenstein, PHYLIP—phylogeny inference package, version 3.573, Department of Genetics, University of Washington, Seattle, WA(http://evolution.genetics.washington.edu/phylip.html)], with *Rhizopus oryzae* strain FSU 6022 EU484218 as the outgroup in the cladogram. Evolutionary distance matrices for the neighbor-joining/UPGMA method were computed using the DNADIST algorithm program.

The program reads in nucleotide sequences and writes an output file containing the distance matrix. The model of nucleotide substitution is from Jukes and Cantor (1969) [13]. Phylogenetic tree (Fig. 1) was inferred by using the neighbor-joining method [14]. With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1,000 bootstraps [15] by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed for the topologies found using a family of consensus tree methods called the Ml methods [16] using the CONSENSE program, and the tree was viewed using Tree View [17].

Effects of carbon limitation and nitrogen limitation on azo dye degradation

A solid media was prepared based on the modified KBM [9]. The chemical composition was as mentioned in screening but modified to suit this study as shown in **Table 1**. Four azo dyes were used which were Ponceau 2R, Orange G, Direct Blue 71 and Biebrich Scarlet. Glucose monohydrate was omitted in the media used for the study on the degradation of azo dyes in carbon-limiting conditions. L-asparagine was used as the original nitrogen source. However, amino acids were known to be a carbon source for fungi, therefore, an inorganic nitrogen source, NH_4Cl was also used in place of L-asparagine. No nitrogen source was added in the medium used study nitrogen limiting conditions.

RESULTS AND DISCUSSION

Isolation and Screening

Sixty-three local white-rot fungi were successfully isolated from one hundred and eighteen samples from various Peninsular Malaysia locations in Selangor, Kelantan, Perak and Terengganu were cultured on PDA. In screening for the ability to degrades azo dyes (Ponceau 2R, Orange G, Direct Blue 71 and Biebrich Scarlet) degradation, only forty isolates showed positive result with varying degree of degradation.

Degradation can be seen when decolorized zone was forming, radiated outward from the inoculum. This can be done by naked eyes. The best isolate was *Coriolopsis* sp. strain arf5 which can completely degraded Ponceau 2R and Basal Scarlet in nine days, and Orange G and Direct Blue 71 in eight days. This isolate was from Universiti Putra Malaysia, Serdang Campus, Selangor.

Table 1. Modified KBM compositions for investigating the effects of carbon and nitrogen limitation on azo dye degradation by *Coriolopsis* sp. Strain arf5.

	carbon limitation	carbon limitation	nitrogen limitation
Components	Concentration (g/L)		
glucose.H_2O	0.0	0.0	10.1
KH_2PO_4	0.2	0.2	0.2
L-Asparagine	2.0	0.0	0.0
NH_4Cl	0.0	2.0	0.0
$MgSO_4.7H_2O$	0.05	0.05	0.05
$CaCl_2.2H_2O$	0.01	0.01	0.01
TE solution	0.1 mL/L	0.1 mL/L	0.1 mL/L
azo dye	0.2	0.2	0.2
bacteriological agar	30	30	30

Identification

The isolate is assigned as *Coriolopsis* sp. strain arf5. A high bootstrap value (86.6 %) is obtained when *Coriolopsis* sp. strain arf5 is associated to *Coriolopsis polyzona* strain Dai9495 and strain Dai9468 indicating that the phylogenetic relationship between the species is strong (**Fig. 1**). Most of the reported dye degrading fungus are white rot fungi [18-20]. This feature is probably due to the ability of white rot fungi to degrade a wide range of ligninolytic compounds [21].

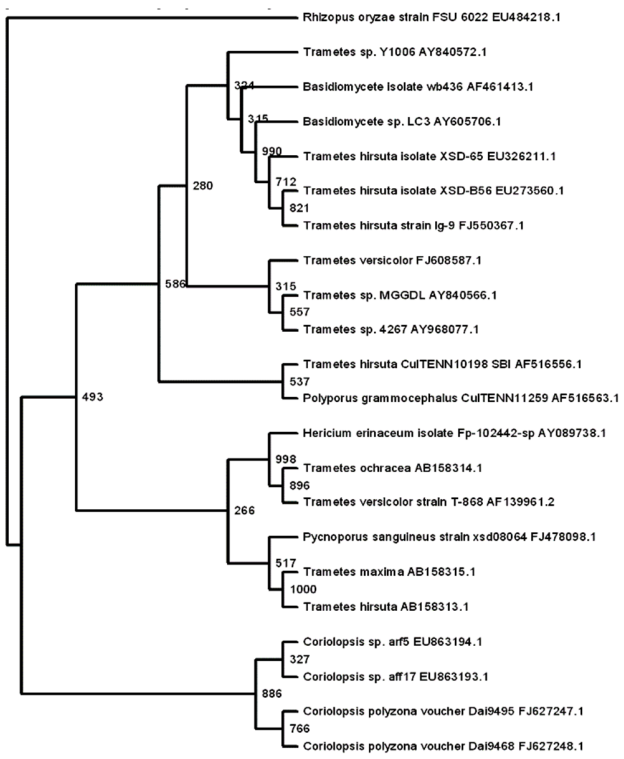

Fig. 1. A phylogram (neighbor-joining method) showing genetic relationship between Strain *Coriolopsis* sp. strain arf5 and other related reference fungi based on the ITS region sequence analysis. Species name is followed by the accession numbers of their ITS region sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1,000 re-samplings. *Rhizopus oryzae* strain FSU 6022 is the outgroup.

Effects of carbon limitation and nitrogen limitation on azo dye degradation

The degrees of degradation and the days required to achieve it are summarized in **Tables 2** and **3**.

Table 2. Degradation by *Coriolopsis* sp. strain arf5 in carbon-limiting conditions.

Carbon Source	Nitrogen Source	Days
Ponceau 2R	L-Asparagine	-
	NH_4Cl	-
Orange G	L-Asparagine	-
	NH_4Cl	-
Direct Blue 71	L-Asparagine	> 21 (incomplete)
	NH_4Cl	> 21 (incomplete)
Biebrich Scarlet	L-Asparagine	-
	NH_4Cl	-

Table 3. Degradation by *Coriolopsis* sp. strain arf5 in nitrogen-limiting conditions.

carbon source	nitrogen source	days
Glucose	Ponceau 2R	10
	Orange G	7
	Direct Blue 71	12
	Biebrich Scarlet	12

Based on the results in **Tables 2** and **3**, no translucent decolourized zones were detected in *Coriolopsis* sp. strain arf5 cultures that were incubated in carbon-limiting conditions with L-asparagine or NH_4Cl as the nitrogen source. The presence of additional nitrogen sources, whether organic or inorganic is known to repress the ligninolytic system of the white-rot fungi [22, 3]. Direct Blue 71 was the azo dye that easiest to degrade even under nitrogen sufficient conditions, with degradation being faster when NH_4Cl was used as the nitrogen source instead of L-asparagine. In contrast, complete or almost complete degradation occurred under nitrogen-limiting conditions.

These observations are in agreement with the results of many published studies. Máximo et al. demonstrated that *P. chryso*s*porium*, *Ganoderma* sp., *I. lacteus*, *P. magnoliea*, *Rigidoporus* sp., *T. versicolor* and *Geotrichum* sp. carried out azo dye degradation under nitrogen-limiting conditions while Kapdan et al. showed that nitrogen concentrations determined the decolourization performance of *Coriolus versicolor* [19,20]. Nitrogen-limiting conditions are required to trigger the production of ligninolyic enzymes in secondary metabolism [3]. Under this condition, the azo dyes might also serve as the source of nitrogen for the fungus. However, the requirement for nitrogen-limiting conditions is not obligatory for all white-rot fungi. Toh et al. showed that an unidentified white-rot fungus isolated from Singapore was able to degrade azo dyes in nitrogen-rich medium with the degradation rates comparable to those of *P. chryso*s*porium* [18].

Visual observations on *Coriolopsis* sp. strain arf5 mycelia growth showed that growth was better in complete cultures than culture that utilized azo dyes as nitrogen source. These meant that

the four azo dyes served as poor sources of carbon and nitrogen for growth and *Coriolopsis* sp. strain arf5 requires additional sources of carbon to degrade the azo dyes. This observation adds further evidence to the claim that azo dye degradation occurs during secondary metabolism and that the ligninolytic enzyme system functions optimally when other more readily metabolized carbon sources are available [22]. In conclusion, azo dye degradation by *Coriolopsis* sp. strain arf5 occurs best under nitrogen-limiting conditions where the azo dyes served as the nitrogen source and in the presence of a co-substrate.

Effects of Incubation Temperatures on Azo Dye Degradation

Analysis of data from 48 hours-old cultures (96 h for Biebrich Scarlet) (**Figs. 2 to 5**) using one-way ANOVA shows that the degradation rates of all four azo dyes were significantly affected by the different incubation temperatures ($P < 0.05$). *Coriolopsis* sp. strain arf5have a general azo dye degradation optimum temperature of between 35 ºC to 40ºC which is similar with the optimum temperature of 35 °C for *Schizophyllum commune* IBL-06 when it was degrading Solar golden yellow R [23]. This relatively high degradation temperature optimum is potentially advantageous when it is to be used to treat dye effluents because less effort will be required to remove the heat produced by fungi metabolism. Degradation rate rises as the temperature increases, peaking at 40 ºC but then rapidly slows down at higher temperatures.

Results from one-way ANOVA shows that Ponceau 2R degradation was only weakly affected by the tested temperatures ($P < 0.05$). Cultures incubated at room temperature, 30, 35 and 40 °C (**Fig. 2**) have similar degradation rates. However, when temperature was increased until 45 ºC, only 18.70 % of final degradation percentage was achieved. This pattern of degradation also applies to the three other dyes (**Figs. 3 to 5**). Although the effects of different incubation temperatures were more statistically significant ($P < 0.05$). The degradation rates are also relatively higher at 40 °C for Orange G, Direct Blue 71 and Biebrich Scarlet.

Based on DT_{50} (the time (hours) required to degrade 50% of azo dyes) values of this study (**Table 4**), Orange G was the easiest azo dye to degrade, with the smallest DT_{50} values at 30, 35 and 40 °C followed by Direct Blue 71, Ponceau 2R and finally Biebrich Scarlet using *Coriolopsis* sp. strain arf5. Biebrich Scarlet was the only dye that was degraded less than 90% after 120 h of incubation at all temperatures, indicating that it is more resistant to degradation by *Coriolopsis*sp strain arf5. Biebrich Scarlet's structure consist more than one single aromatic ring compare to others three dyes (**Fig. 6**). This single aromatic ring might cause the degradation becomes difficult. This might probably due to the structure of the Biebrich Scarlet. The killed-mycelia culture served as biotic control and uninoculated culture served as abiotic control.

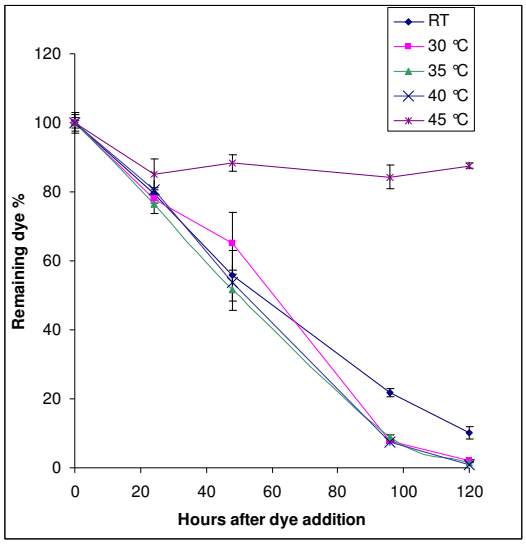

Fig. 2. Effects of incubation temperatures on Ponceau 2R (0.2 g/L) degradation in static cultures. Data collection begun after the replacement of the spent growth medium with the azo dye-containing degradation medium. Data represent means ± SEM, n=3.

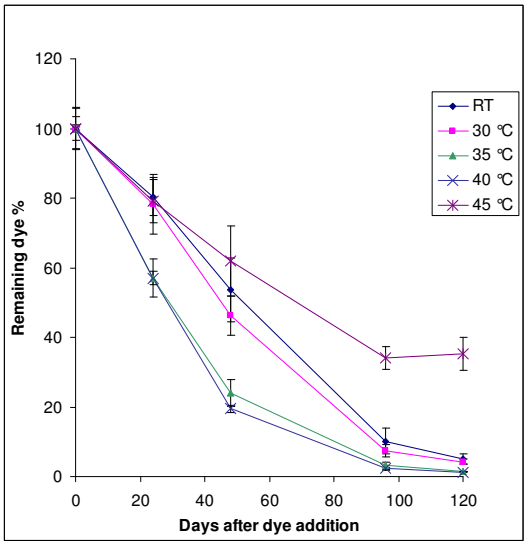

Fig. 3. Effects of incubation temperatures on Orange G (0.2 g/L) degradation in static cultures. Data collection begun after the replacement of the spent growth medium with the azo dye-containing degradation medium. Data represent means ± SEM, n=3.

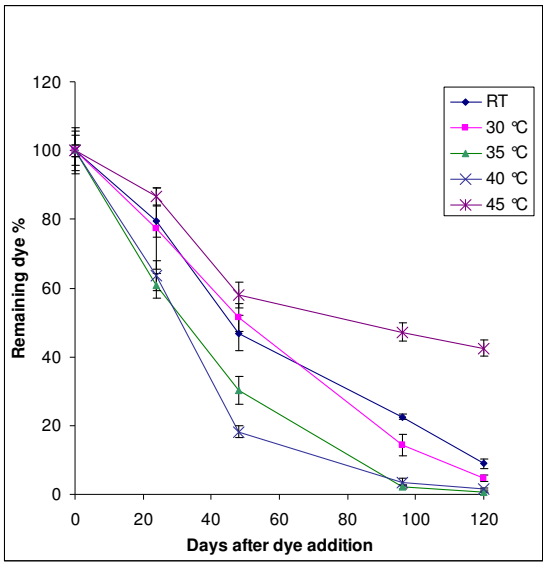

Fig. 4. Effects of incubation temperatures on Direct Blue 71 (0.2 g/L) degradation in static cultures. Data collection begun after the replacement of the spent growth medium with the azo dye-containing degradation medium. Data represent means ± SEM, n=3.

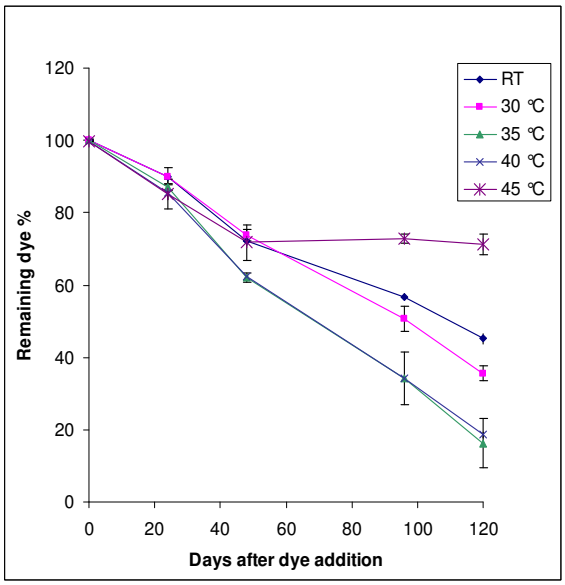

Fig. 5. Effects of incubation temperatures on Biebrich Scarlet (0.2 g/L) degradation in static cultures. Data collection begun after the replacement of the spent growth medium with the azo dye-containing degradation medium. Data represent means ± SEM, n=3.

Effects of different initial degradation medium ph on azo dye degradation

The effects of different initial degradation medium pH on azo dye degradation by *Coriolopsis* sp. strain arf5 were investigated. The results are shown in **Figs. 7** to **10**. Using one-way ANOVA, the different initial pH of the degradation medium does not have any statistically significant effects on azo dye degradation after 48 hours of incubation except on Ponceau 2R ($P < 0.05$) although it was only weakly significant. Degradation of Ponceau 2R was slightly faster at the initial pH of 4.5 while the slowest degradation rate was at pH 5.0. However, degradation still occurred at the other initial pH indicating that *Coriolopsis* sp. strain arf5 was able to degrade the four azo dyes within a wide range of pH. In comparison, Tekere et al. found that *T. versicolor*, *Trametes pocas*, *Trametes cingulata*, *Pycnoporus sanguineus* and *D. concentrica* were significantly affected by the initial pH when degrading Poly R478 [24]. Their results indicated a general trend of low optimum pH of around pH 3.0 to 5.0 for dye degradation. Kapdan et al. also demonstrated that *C. versicolor* had a pH optimum of pH 4.5 when degrading Everzol Turquoise Blue [20].

The final pH for the medium are listed in **Table 5**. From the table, the final values are in the region pH 4.0. It appears as that *Coriolopsis* sp. strain arf5 modified the pH of the degradation medium during the incubation period. This was also observed by Tekere et al. where six white-rots that they used modified the pH of the medium near to the optimum pH [24]. They suggested that the fungi produced organic acids during secondary metabolism which would have caused a gradual lowering of the pH. Another possible explanation is that the possibility of the degradation medium buffer being metabolized by the fungi. Swamy and Ramsay found that citrate-phosphate and tartrate buffers were consumed by their fungi as carbon sources during azo dye degradation, where sodium acetate buffer was not metabolized [25]. Since sodium acetate buffer is the buffer used in this study, it is probably not metabolized by *Coriolopsis* sp. strain arf5. Based on the DT_{50} values of **Table 6**, Direct Blue 71 was the dye that was degraded fastestwith smallest values for all of the initial pH, followed by Ponceau 2R, Orange G and Biebrich Scarlet. The uninoculated abiotic controls and killed-mycelia controls did not show any significant azo dye degradation.

Table 4. DT_{50} (hours) values of azo dye cultures incubated at different temperatures.

Temperature (ºC)	DT_{50} (Hours) P2R	OG	DB71	BS
RT (27.5)	57.5	56.0	48.0	112.0
30	61.3	46.7	48.0	98.6
35	51.3	29.3	34.6	69.3
40	52.5	29.3	32.0	69.3
45	>120	70.6	88.0	>120

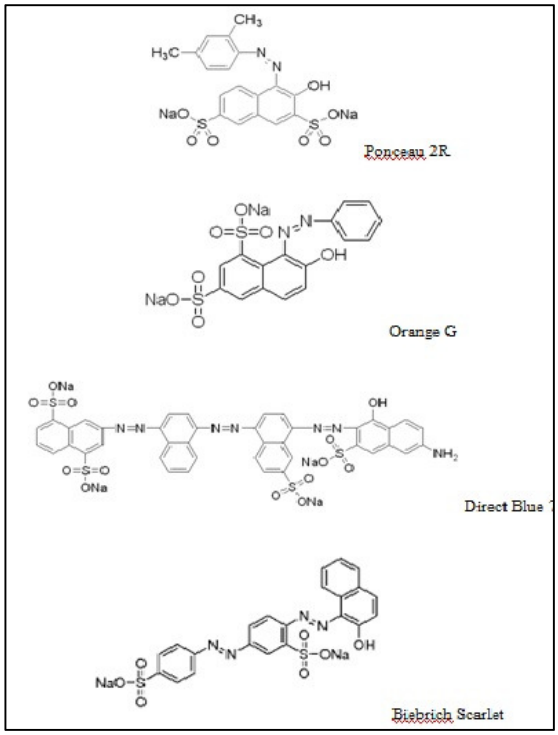

Fig. 6. Structures of different azo dyes.

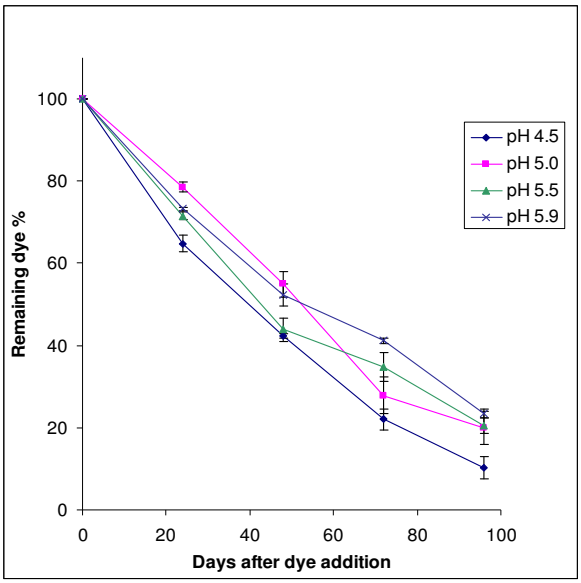

Fig. 7. Effects of initial degradation medium pH on Ponceau 2R (0.2 g/L) degradation. Data collection begun after the replacement of the spent growth medium with the azo dye-containing degradation medium. Data represent means ± SEM, n=3.

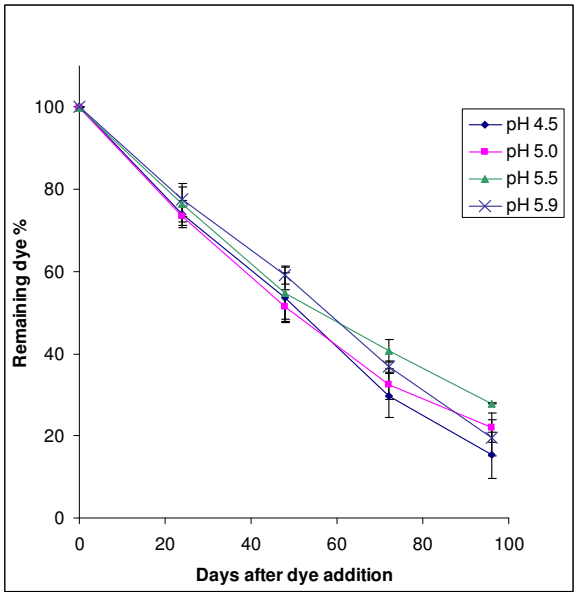

Fig. 8. Effects of initial degradation medium pH on Orange G (0.2 g/L) degradation. Data collection begun after the replacement of the spent growth medium with the azo dye-containing degradation medium. Data represent means ± SEM, n=3.

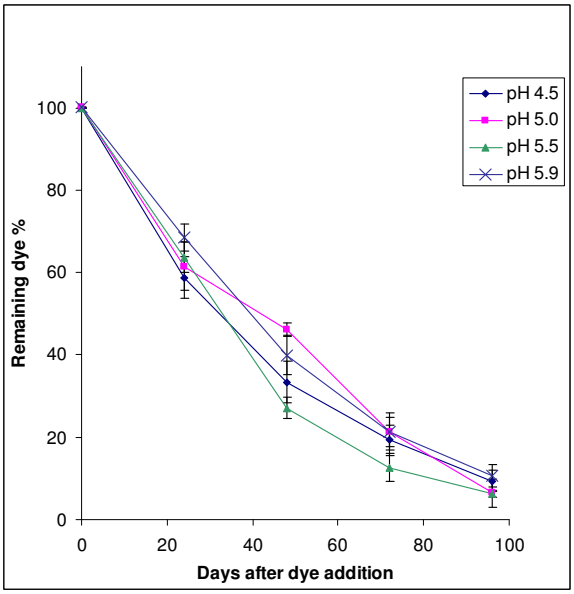

Fig. 9. Effects of initial degradation medium pH on Direct Blue 71 (0.2 g/L) degradation. Data collection begun after the replacement of the spent growth medium with the azo dye-containing degradation medium. Data represent means ± SEM, n=3.

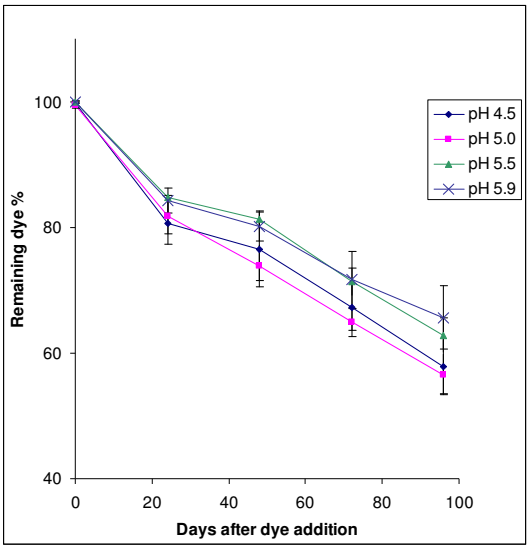

Fig. 10. Effects of initial degradation medium pH on Biebrich Scarlet (0.2 g/L) degradation. Data collection begun after the replacement of the spent growth medium with the azo dye-containing degradation medium. Data represent means ± SEM, n=3.

CONCLUSION

Sixty three white-rot fungi cultures were successfully isolated from various locations in the Malaysia Peninsular and were screened for the ability to degrade the azo dyes Ponceau 2R, Orange G, Direct Blue 71 and Biebrich Scarlet on solid media. Based on results, *Coriolopsis* sp. *strain*arf5, which was sampled from Universiti Putra Malaysia Serdang was selected as the best isolate for further investigations. Experiments on the effects of different nutritional conditions on azo dyes degradation showed that *Coriolopsis* sp. strain arf5 only degraded azo dyes in nitrogen-limiting conditions. Investigations into effects of different environmental conditions showed that the incubation temperatures for the degradation cultures were found to have a significant effect on azo dyes degradation rates although the initial degradation medium pH did not have a significant effect, except in Ponceau 2R cultures. In conclusion, further research needs to be carried before *Coriolopsis* sp. arf5 could be applied in any large-scale applications.

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