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Research Article

Decolorizing an anthraquinone dye by *Phlebia brevispora*: Intra-species characterization

Remazol brilliant blue R (RBBR) is an anthraquinone dye derived from anthracene that is decolorized by a white rot fungus, *Phlebia brevispora*. Interestingly, *P. brevispora* produces two phenomena of yellowish and pinkish colors during the degradation of RBBR. Here, we characterized the decolorization of RBBR by *P. brevispora*. The fungus was significantly different between the two colors via UV spectrophotometry, and the morphology of the hyphae observed in the respective color culture was also entirely different. Moreover, both of the two ligninolytic enzymes, laccase and manganese-dependent peroxidase (MnP), were remarkably stimulated in the yellowish culture at the beginning of the decolorization. It is possible that the RBBR decolorizing mechanism might be primarily related to the amount of laccase and MnP produced in the yellowish culture. Thus, the decolorized color may be rapidly estimated at initial period of incubation. In addition, GeneFishing technology revealed that two genes were differentially expressed in yellowish culture.

Keywords: Differentially expressed gene / Ligninolytic enzyme / *Phlebia brevispora* KUC9045 / Remazol brilliant blue R / White rot fungi

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Received: February 24, 2016; revised: June 17, 2016; accepted: July 4, 2016

DOI: 10.1002/elsc.201600059

1 Introduction

Synthetic dyes are extensively used for textile dyeing and other industrial applications, such as in the paper printing, cosmetic, pharmaceutical, food, and leather industries [1, 2]. The textile industry is one of the greatest producers of liquid effluent pollutants due to the high quantities of water used in the dyeing process [3]. More than 10 000 dyes are commercially available, and at least 10% of the used dyestuff enters the environment through waste [4]. Synthetic dyes, classified by their chromophores, have different and stable chemical structures to encounter a variety of coloring requirements and often are not degraded and/or removed by conventional physical and chemical processes [5].

Remazol brilliant blue R (RBBR) is a typical anthraquinone dye used in the textile industry and it represents an important class of toxic and recalcitrant organic pollutants due to an anthracene derivative [2]. Anthraquinonic dyes have a wide range of colors spanning almost the entire visible spectrum, but they are most commonly used for violet, blue, and green colors [6]. Among them, RBBR is frequently used as a starting material in the production of polymeric dyes [7]. In addition, RBBR has been used to distinguish whether fungi are white rot or brown rot, and RBBR is also used to screen ligninolytic microorganisms [8].

White rot fungi (WRF) attract increasing attention for use in bioremediation processes due to their degrading capability of various environmental pollutants, including highly recalcitrant synthetic dyes [9]. WRF have the ability to produce extracellular oxidative enzymes, such as laccase and peroxidases, in order to obtain a carbon source from the pollutants [10]. There is a significant number of recent reports on the decolorization and degradation of synthetic dyes by WRF [2, 11].

The ability of WRF to decolorize synthetic dyes has been widely reported, and although the involvement of ligninolytic enzymes in these processes has been studied in vitro, the in vivo decolorization mechanisms are still not clearly understood

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Abbreviations: ACP, annealing control primers; LiP, lignin peroxidase; ME, malt extract; MnP, manganese-dependent peroxidase; RBBR, Remazol brilliant blue R; WRF, white rot fungi

[9]. It has been reported that the ligninolytic enzymes seem to play an important role in the decolorization capacity of fungal cultures and the dye degradation and sorption to the fungal biomass [12]. Little information is available concerning their specificities toward dye decolorization, and the molecular basis of decolorization reactions is still partially unknown.

In terms of determining the mechanism of RBBR decolorization, *Phlebia brevispora* KUC9045 has been found to be one of the most effective fungi. In previous studies, the species demonstrated the oxidation of gallic acid with a dark brown color within two weeks, and the fungus effectively degraded recalcitrant xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) [10]. Interestingly, two phenomena were observed in the decolorization of RBBR by the fungus, with yellowish and pinkish colors resulting. Hence, the objective of the present study was to investigate the characteristics of *Phlebia brevispora* KUC9045-mediated decolorization of RBBR using a variety of methods, including ligninolytic enzymes and differentially expressed genes (DEGs) in the fungus. GeneFishing, a technique that uses annealing control primers (ACP), was especially highlighted, and it might provide more information on the degradation of anthraquinonic dyes by WRF [13]. Therefore, the mechanism of decolorizing RBBR was determined with several characteristics of the fungus.

2 Materials and methods

2.1 Fungal species and medium

Phlebia brevispora KUC9045 was isolated in Incheon, Korea. The fungus was identified and preserved in the Korea University Culture Collection (KUC) and was deposited in the Korean Agricultural Culture Collection (WDCM806, Suwon, Korea) as KACC93149P. The fungus was maintained as pure cultures. For the decolorization of RBBR, ten fungal agar plugs (dia. 5 mm) were inoculated in a 250-mL Erlenmeyer flask containing 100 mL of 2% malt extract (ME) liquid medium including of 200 mg/L of RBBR (Sigma Chemical Co., St. Louis, USA). An uninoculated flask was used as an abiotic negative control and inoculated flask without RBBR was used as a positive control. The cultures were incubated on a rotary shaker at 150 rpm (27°C) for 16 days.

2.2 Decolorization of RBBR

To investigate the decolorized color of the synthetic dye in the liquid medium, mycelium was removed in each sample from the individual flask with a syringe filter (0.45 μ m) on the 16th day of the incubation, and the percentage of color removal was estimated by measuring the absorbance from 400 to 700 nm using a UV-Vis spectrophotometer (Optizen 2120UV, Mecasys Co. Ltd., Korea).

2.3 Measurement of protein content and pH

The determination of protein by the Bradford method was carried out by UV spectrophotometry [14]. After the filtration of mycelium from each sample, 0.5 mL of the protein solution and 0.5 mL of the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) were mixed thoroughly and kept in a dark place for 5 min. Absorbance was then measured at 595 nm using a UV-Vis spectrophotometer in triplicate. In addition, pH was measured in the medium of the respective color at the end of the incubation ($n = 3$).

2.4 Ligninolytic enzymes assay

During the degradation of RBBR to the two colors, three ligninolytic enzymes, laccase, manganese-dependent peroxidase (MnP), and LiP were assessed in the medium of the respective color using a UV-Vis spectrophotometer ($n = 3$). After the filtration of the mycelium via a syringe filter (0.45 μ m), the enzymatic activity of the crude supernatant was measured. For laccase, 0.1 M sodium acetate (pH 4.5) and 1.5 mM 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were used [15]. And 150 μ L supernatant was added and mixed into buffer for 1 min. For MnP, 0.5 M sodium malonate (pH 4.5), 5 mM MnSO_4 , 1 mM 2,6-dimethoxyphenol, and 1 mM H_2O_2 were used [16]. For LiP, 0.25 M sodium tartrate (pH 2.5), 10 mM veratryl alcohol and 5 mM H_2O_2 were used [17]. One unit of the ligninolytic enzyme activity produced 1 μ mol of reaction product per minute at room temperature; activity was expressed in U/mL [18, 19].

2.5 Isolation of total RNA

At the end of the incubation, RBBR was decolorized in the liquid medium with two color patterns, which were yellowish and pinkish. Total RNA was extracted from the medium of the respective color using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of the isolated RNA were assessed using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.6 ACP-based RT-PCR

Total RNA extracted from the fungal hyphae was used to synthesize cDNA. To detect the differentially expressed genes (DEGs), we used the GeneFishing DEG 101 & 102 Premix Kit (Seegene, Seoul, Korea). First-strand cDNA was synthesized using the dT-ACP1 primer with MMLV reverse transcriptase (Beams Biotechnology, Kyounggi-do, Korea) and an RNase inhibitor (INTRON Biotechnology, Kyounggi-do, Korea) according to the manufacturer's instructions. The second-strand cDNA synthesis and the second-stage PCR amplification were performed using dT-ACP2 and one of 20 arbitrary ACPs according to the provided instructions. The PCR products were separated on a 2% agarose gel using electrophoresis, stained with ethidium bromide and detected under UV light. The differentially expressed bands were

obtained from the gel and purified using an Accuprep Gel Purification Kit (Bioneer, Seoul, Korea).

2.7 Cloning and analysis of the DEGs

Purified DNA fragments were cloned using the T&A Cloning Vector Kit (RBC, Kyounggi-do, Korea) and transformed into competent DH5 α *Escherichia coli* cells. The colonies were grown at 37°C for 12–16 h on Luria broth agar containing ampicillin and X-gal for blue/white selection. The plasmids from the selected colonies were extracted using an Accuprep Plasmid Mini Extraction Kit (Bioneer), and the inserts were sequenced (Macrogen, Seoul, Korea). The resulting sequences were compared and identified using the GenBank database and the BLASTX program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

2.8 Data analysis

The degradation data were analyzed using the Statistical Analysis Systems (SAS 9.3, SAS Institute, Inc., Cary, NC, USA) package. A one-way analysis of variance (ANOVA), followed by Tukey's test, was used to assess the significance of the differences in pH, protein concentration, and the production of ligninolytic enzymes in the fungus. The mean values were compared using a significance level of $p < 0.05$.

3 Results and discussion

3.1 Characterization of *Phlebia brevispora* KUC9045 during degradation of RBBR

RBBR has been used as an indicator substrate to distinguish whether basidiomycetes are white rot or brown rot fungi [20]. The decolorization test was performed in 2% ME solid medium using basidiomycetes to select an appropriate species [10, 19]. In the previous study, RBBR was generally decolorized to two types, yellowish and pinkish colors, by WRF in 2% ME solid medium [21], with some species decolorizing RBBR to the yellowish and others to the pinkish color. Interestingly, RBBR was decolorized to two colors in the same strain of *P. brevispora* KUC9045 that is intrastrain variation after 15 days of incubation in the liquid medium. The fungus demonstrated successful decolorizing ability of RBBR, while two color patterns were consistently maintained after the decolorization (Supporting Information Fig. 1). In the current study, *P. brevispora* KUC9045 was characterized by its decolorizing color types in terms of absorbance, protein contents, pH, ligninolytic enzyme activities, mycelial morphology, and DEGs in order to understand the mechanism responsible for the two decolorized colors of RBBR in *P. brevispora* KUC9045.

The degradation of the anthraquinonic dye RBBR by *Phlebia brevispora* KUC9045 was evaluated in the liquid media by observing several characteristics of the fungus. RBBR was fully decolorized within 16 days of incubation, and the fungus demonstrated its ability to decolorize 200 mg/L of RBBR; however, two colors from the decolorized RBBR were examined in the media.

Figure 1. UV-vis spectrum of RBBR solution (200 mg/L, initial concentration) and the two medium that decolorized RBBR to pinkish and yellowish by *P. brevispora* KUC9045. The inset indicates the different absorbance between the two colors. The asterisk indicates that significantly differ between pinkish and yellowish in decolorization rates of RBBR at the specific wavelength ranges from 400 to 500 nm ($p < 0.05$).

One was a pinkish color, and another was yellowish (Supporting Information Fig. 1). On the 16th day of the incubation, the absorbance of the two colors in the respective medium was determined in visible light wavelengths (400–700 nm) (Fig. 1). The absorbance of the yellowish medium was lower than that of the pinkish one throughout the range of wavelengths, and the absorbance of the yellowish and pinkish media were 0 and 0.007, respectively, at 595 nm, which corresponds to the absorbances at the maximum visible wavelength of the dye [20]. It indicated the species is capable of decolorizing RBBR thoroughly, while one strain fungus exhibited the two different colors during degradation. In addition, the different characteristics of the fungus were evaluated from the different colors of the medium. *P. brevispora* KUC9045 showed higher protein concentration in the yellowish medium than in the pinkish one (Fig. 2), and the protein contents produced in both media were higher than in the positive control. Meanwhile, there was no significant difference in the pH of the media among the samples.

The white rot fungus, *P. brevispora* KUC9045, did not demonstrate interchangeable color types in the decolorization of RBBR. Some of media inoculated with the species certainly decolorized RBBR to the yellowish color, and the other media were examined with the pinkish color at the end of incubation. These phenomena indicated certain characters were probably be involved in the mechanism of the degradation of RBBR. In order to define the characteristics of *P. brevispora* KUC9045 in the decolorized phenomena of RBBR, absorbance was first determined to evaluate the difference between the two color types. As shown in Fig. 1, the absorbance was significantly different between the yellowish and pinkish colors at the specific wavelength range of 400–500 nm ($p < 0.05$), although there was no significant difference at 595 nm, which is the maximum wavelength of RBBR [20]. The different color types were verified by absorbance, and the individual color samples were characterized via several methods. The two color

Figure 2. Protein concentration and pH after decolorization of RBBR. The asterisk indicates means that significantly differ among the samples ($p < 0.05$) ($n = 3$).

samples and the positive control showed a similar pH range of 3.5–4.0, and there was no significant difference among the samples (Fig. 2). Similarly, as Vyas and Molitoris reported [20], the optimal pH range was 3.5–4.0 in the liquid culture during the decolorization of RBBR by the white rot fungus, *Pleurotus ostreatus*, and the optimal pH range was related to peroxidases because the intermediates of RBBR were H_2O_2 -dependent. It was also determined that *P. brevispora* KUC9045 maintained an optimal pH range to effectively produce ligninolytic enzymes. On the contrary, there were different protein contents among the samples. The highest protein contents were produced in the yellowish sample, suggesting that RBBR was more degraded in the yellowish sample than in the pinkish sample.

Moreover, after 16 days of incubation, electron microscopy showed substantial morphological changes in the mycelia of the respective samples growing in the media containing RBBR. Morphological evaluation of mycelia was carried out using a microscope (Olympus BX51) with magnification of $20\times$ (Supporting Information Fig. 2). In the decolorized samples, deformation of the hyphae was observed microscopically when compared with the hyphae of the positive control. The formation of the spherical swollen structures was observed in the pinkish sample. This is caused by the interference of the dyes and the products of their decolorization with the fungal cytoskeleton [22]. Synthetic dyes and the products of their decolorization influence apical growth in a similar way as Cytochalasin A, which causes the polymerization of actin fibers and the formation of swollen apical structures [23]. In future studies, the characterization of individual products derived from the decolorization of RBBR and their influence on morphological changes of the fungal hyphae should be determined.

3.2 Ligninolytic enzyme activities during degradation of RBBR

The ligninolytic enzymes of *P. brevispora* KUC9045 cultures that turned one of the two colors during the degradation of RBBR

Figure 3. Ligninolytic enzyme activities during decolorization of RBBR. (A) Laccase; (B) MnP; and (C) LiP ($n = 3$).

were assessed. As shown in Fig. 3, three ligninolytic enzymes were induced as the addition of RBBR into the medium, compared with the positive control, which the fungus was inoculated in the absence of RBBR. After 4 days of incubation, it was decreased in the production of the three enzymes from the fungus. In terms of the production of both laccase and MnP, *P. brevispora*

KUC9045 significantly secreted the enzymes in the beginning of the decolorization when RBBR was being decolorized to yellow color (Fig. 3A and B); however, laccase and MnP activities were more rapidly reduced in the yellowish culture than in the pinkish culture. Finally, there were little amounts of laccase and MnP on the 13th and 16th day of incubation in the yellowish medium, respectively, than in the beginning of the decolorization. On the contrary, laccase and MnP were still produced in the pinkish medium at the end of incubation. LiP was secreted with high efficiency in both cultures during the decolorization of RBBR compared with the positive control (Fig. 3C). And it was observed higher efficiency in the pinkish medium than the yellowish one for the whole period of the decolorization of RBBR.

WRF are capable of decolorizing RBBR by ligninolytic enzymes, laccase, MnP, LiP, and other peroxidases [24]. Likewise, the production of ligninolytic enzymes may also be related to the differences in decolorization ability between the samples of the two color types. *P. brevispora* KUC9045 statistically produced laccase with the highest efficiency (95.7 U/mL) in the beginning of decolorizing RBBR to the yellowish color (Fig. 3A). But after 10 days of incubation, laccase production was more rapidly decreased in the yellowish sample than in the pinkish sample. Arora and Sharma [25] reported that *P. brevispora* KUC9045 remarkably produced laccase to digest wheat straw, and when the wheat straw was entirely consumed at 30 days, the amount of laccase was reduced. This mechanism of laccase production was characterized by a sequential entry of the reducing and oxidizing of RBBR and the immediate release of the respective products [26]. RBBR was fully decolorized within the 16 days of incubation by *P. brevispora* KUC9045. Thus, the products derived from the degradation of RBBR no longer existed, and the species was not needed to produce laccase from the fungus in the yellowish medium. On the contrary, laccase was still produced in the pinkish sample at the end of the incubation. Similar to laccase production in the yellow sample, MnP activity was continuously decreased during the degradation of RBBR in the yellowish sample (Fig. 3B), however, less amounts of MnP were produced in the pinkish sample than in the yellowish sample, and the enzyme activity was not reduced in the pinkish sample by 16 days of incubation. It was demonstrated a similar tendency of both laccase and MnP in the yellowish sample during decolorization. The fungus in the pinkish sample produced LiP with higher efficiency during decolorization than in the yellowish sample (Fig. 3C). LiP isozymes of *Phaenerochaete chrysosporium* decolorize various dyes in the presence of veratryl alcohol, and the decolorizing ability significantly decreased when the alcohol was omitted from the reaction culture [24]. This peroxidase is known as an indirect enzyme, and the mechanism of production was different with MnP [26]. Many other papers have declared key roles of laccase and MnP in the decolorization of anthraquinone dye [22]. It has been suggested that different intermediates of RBBR may be involved in the production of two colors during the decolorization of RBBR by the same strain of the species [7]. Thus, it is inferred that RBBR can be decolorized to the yellowish color because the ligninolytic enzymes, such as laccase and MnP were produced with high efficiency in the beginning of incubation.

Figure 4. Agarose gel electrophoresis of annealing control primers (ACP)-based RT-PCR products. Differentially expressed genes are indicated by arrows (L, DNA marker (Bioneer 100 bp plus DNA Ladder); C, control; P, pinkish; Y, yellowish).

3.3 Differentially expressed gene involved in decolorization of RBBR by *Phlebia brevispora* KUC9045

To determine the genetic mechanism responsible for the production of the decolorized color of RBBR by *P. brevispora* KUC9045, changes in gene expression were investigated in the fungus using ACP-based GeneFishing with a combination of 20 arbitrary primers. A total of two differentially expressed bands were detected from ACPs 8, and 16 (Fig. 4). The extracted DNA was used for cloning and sequencing in order to identify the differentially expressed genes (DEGs). The sequences were similar to the hypothetical proteins (Table 1), including transglutaminase and retrovirus polypeptide. The DEGs were identified previously in functional proteins-related colorization in several dyes and pharmaceutical industries [27, 28].

Two genes were differentially expressed in the samples during the phenomena of RBBR being decolorized into yellow color types (Fig. 4). The two genes were matched using a BLAST search as shown in Table 1. Gene1 detected in the yellowish sample via ACP8 primers, was determined to belong to the transglutaminase family in the coagulation factor XIII A chain (Accession No. XP 004694857). The addition of transglutaminases catalyzes the formation of an isopeptide bond and a hydroxamate group, and upon the addition of a concentrated ferric chloride solution, results in the development of a yellow color [27]. Thus, it is possible that this gene is involved in the production of the yellowish color. Gene2 was detected in the yellowish sample via ACP16 primers and was determined to be a retrovirus polypeptide from the Chromatin organization modifier domain (Accession No. XP 002145641). Golovnin et al. [28] reported that the effects of the retrotransposon involved in the Chromatin organization modifier domain can be attributed to the yellow pigmentation. Taken together, the evidence suggests these two genes might affect the colorization of the culture during the decolorization of RBBR. Consequently, the RBBR decolorizing mechanism was constructed over a wide range of methods in the same species, *P. brevispora* KUC9045. Above all, the mechanism of the color phenomena in the degradation of RBBR by the fungus was specifically related to laccase and MnP expression in the beginning of the incubation. The cause of different phenomena in the degradation of RBBR should be further investigated with WRF in future studies.

Table 1. Oligonucleotide primers used in this study

Gene	ACPs	GenBank accession No.	Length (bp)	Putative identity ^{a)}	BLAST hit accession No.	Score	Query cover	E value	Identity
Gene1	ACP8	KJ690387	402	Transglutaminase family	XP_004694857	37	52%	2.6	34%
Gene2	ACP16	KJ690388	516	Retrovirus polyprotein	XP_002145641	57	36%	9E-07	45%

^{a)} The putative identity of each gene was searched by Basic Local Alignment Search (BLASTX) at GenBank, NCBI.

4 Concluding remarks

One of the most effective fungi, *Phlebia brevispora* KUC9045, which has been reported as a great degrader of recalcitrant xenobiotics, such as PAHs demonstrated interesting two types of phenomena during the decolorization of RBBR along to yellowish and pinkish colors. In the yellowish culture, both laccase and MnP were significantly secreted in the beginning of the degradation, subsequently laccase and MnP activities were more rapidly reduced in the yellowish culture than in the pinkish culture. However, less amount of both enzymes were still produced at the end of the incubation in the pinkish culture due to residue of degraded RBBR. These two phenomena were assessed by several characteristics, such as protein, pH, microscopic observation, and differentially expressed genes. With the results, there are significantly different between the phenomena in protein and mycelia transformation via microscopic observation. Especially, two genes were differentially expressed in the yellowish culture, which indicates that the fungal species differentially decolorizes RBBR by the strain variation via genetic expression. This study provided that one strain probably has potential for different ways to decolorize RBBR dye. And mechanisms of degrading RBBR by the fungal strain should be discovered in detail for further studies.

Practical application

RBBR was decolorized by *Phlebia brevispora* KUC9045, which has been reported as a great degrader of recalcitrant xenobiotics. And the species demonstrated interesting two types of phenomena during the decolorization of RBBR along to yellowish and pinkish colors. In the yellowish culture, both laccase and MnP were significantly secreted in the beginning of the degradation, subsequently laccase and MnP activities were more rapidly reduced in the yellowish culture than in the pinkish culture. Thus, the color patterns might be related with the ligninolytic enzymes in the secretion time during the decolorization of RBBR. Additionally, there were significantly different between the phenomena in protein and mycelia transformation via microscopic observation. Especially, two genes were differentially expressed in the yellowish culture via GeneFishing technology, which indicates that the fungal species differentially decolorizes RBBR by the strain variation via genetic expression. These phenomena indicate intrastrain variation in the identical species.

This work was supported by the National Research Foundation of Korea(NRF) grant funded by the Korea government(MSIP) (NRF-2013R1A2A2A01068649); the “Oil Spill Environmental Impact Assessment and Environmental Restoration” (PM57431) funded by the Ministry of Oceans and Fisheries of Korea; and a Korea University Grant.

The authors have declared no conflicts of interest.

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