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ORIGINAL PAPER

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Biodecolorization of azo, anthraquinonic and triphenylmethane dyes by white-rot fungi and a laccase-secreting engineered strain

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Abstract One laccase-secreting engineered strain and four white-rot fungi were tested for their capacity to decolorize nine dyes that could be classified as azo, anthraquinonic and triphenylmethane dyes. *Trametes versicolor* was the most efficient of the tested strains under these experimental conditions. Anthraquinonic dyes were decolorized more easily than the other two types. Small structural differences among the dyes could significantly affect decolorization. None of the strains showed lignin peroxidase or veratryl alcohol oxidase activity. None of the dyes were decolorized completely by laccase alone. It is likely that other phenoloxidases, such as Mn-dependent and versatile peroxidase, were also involved in decolorization of the dyes.

Keywords Biodecolorization · Dyes · Laccase · Mn-dependent peroxidase · Versatile peroxidase

Introduction

White-rot fungi have a significant environmental role in the recycling of wood and related materials [2]. It is their non-specific enzymes, such as lignin peroxidase, veratryl alcohol oxidase, Mn-dependent peroxidase, versatile peroxidase and laccase, which provide them with the potential to colonize wood at all stages of decomposition [9, 18]. During the degradation of lignin, these enzymes produce many kinds of highly reactive intermediates. The ability to generate these intermediates allows white-rot fungi to be used in degrading a variety of persistent environmental pollutants [3]. Among such compounds are many synthetic dyes characterized by high stability against light- and heat-treatment.

Synthetic dyes are used extensively in a number of industries, such as textile dyeing, paper printing, color photography and food processing and as additives in petroleum products. More than 10,000 dyes with an annual production of over 7×10^5 t worldwide are commercially available; and 10–15% of the dyestuffs may be found in industrial effluents [21]. Based on the chemical structure of the chromophoric group, dyes can be classified as azo dyes, anthraquinonic dyes, triphenylmethane dyes and heterocyclic dyes [23]. Most of these dyes are mutagenic and carcinogenic and cannot be completely removed by conventional wastewater treatment systems. Some anaerobic bacteria can decolorize several azo dyes. However, under anaerobic conditions, dyes are usually reduced to aromatic amines that are also carcinogenic [8]. In recent years, increasing numbers of experiments have been conducted on the decolorization of dyestuffs by fungi. Several white-rot fungal strains (e.g. *Phanerochaete chrysosporium*, *Pleurotus sajorcaju*, *P. ostreatus*) and the extracellular enzymes of these fungi have shown a capacity to decolorize a broad spectrum of structurally diverse dyes [4, 5, 6, 16].

In this study, four fungal strains (*Trametes versicolor*, *T. hispida*, *Fome lignosus*, *Coriolus hirsutus*) and one laccase-secreting engineered strain were tested for their decolorization of nine dyes that could be classified as azo, anthraquinonic and triphenylmethane dyes. Moreover, the possible correlation between decolorization of dyes and extracellular enzymes was estimated.

Materials and methods

Organisms

The fungal strains *T. versicolor*, *T. hispida*, *F. lignosus* and *C. hirsutus* were obtained from the China General Microbiological Culture Collection Center, Beijing,

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China. The laccase-secreting engineered strain (*Lse*) was constructed and screened by our laboratory. *Lse* was a modified *Pichia pastoris* GS115 that was transformed with the recombinant expressional vector pGAPZA-*lcc* (laccase cDNA; GenBank accession number AY365228). The *lcc* was cloned from the total RNA of the fungus *F. lignosus* [12].

The fungal strains were maintained on malt agar slants, and *Lse* was maintained on a YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose) agar plate.

Dyes

The dyes used in this study are shown in Table 1.

Cultivation of strains and decolorization of dyes

All of the fungal strains were precultured on PDA (1 l potato extract with 20 g dextrose, 3 g KH_2PO_4, 1.5 g $MgSO_4{\cdot}7H_2O$) plates at 28 °C for 1 week. After the strains were subcultured three times, mycelia were used to detect the decolorizing capacity of these fungi.

The selection of fungal strains for decolorizing dyes was performed using Kirk's medium [10] plates with dyes supplemented separately; and the decolorizing capacity of the strains tested was assessed by the disappearance of color within 2 weeks.

The decolorization of dyes was also evaluated using Kirk's liquid medium. Flasks containing 50 ml liquid medium were each inoculated with five plugs (5 mm diameter) obtained from the edge of actively growing mycelia of selected fungi and incubated at 28 °C in a shaking incubator (180–200 rpm) for 10 days. Dyes were added either initially or after 5 days cultivation at concentrations shown in Table 1.

For the engineered strain, a single colony was inoculated into 5 ml YPD liquid medium and incubated at 28 °C in a shaking incubator (250 rpm) overnight. Then, 0.5 ml overnight culture was used to inoculate 50 ml YPD^{-} (containing 1.25 g l^{-1} yeast extract, 2.5 g l^{-1} peptone, 2.5 g l^{-1} dextrose) supplemented with 60 mM $CuSO_4$. The cultures grew at 20 °C in a shaking incubator (250 rpm). Dyes were added initially or after 24 h cultivation.

At appropriate time intervals, samples (1 ml) were taken from the cultures and centrifuged. The supernatants were analyzed for the absorbance (A) due to residual dyes and for ligninolytic enzyme activity. The decolorization of dyes was deduced from the formula: decolorization (%) = $(A_0-A) \times 100/A_0$, in which A_0 indicates the initial absorbance of the dye.

For cultures of *T. versicolor* and *F. lignosus* with the anthraquinonic dyes added, samples were taken and centrifuged. The supernatants were then analyzed for their extracellular ligninolytic enzyme activity, while the mycelia were washed, suspended in different buffers (to detect the activity of different enzymes; see Enzyme activity, below), and disrupted with an ultrasonic oscillator at 19 kHz (model JY92-II; Science Institute of Biotechnology, China). After centrifugation, the supernatants were assayed for ligninolytic enzyme activity in vivo. The decolorizing capacity of crude enzymes produced by the two fungi was also assayed. The crude enzymes were prepared from 8-day-old cultures of the two fungi. The cultures were harvested and centrifuged; and the supernatants were used as crude enzymes.

In order to avoid the effect of residual dyes, the analysis of enzyme activity was compared with control cultures. In control cultures, the solutions were heated at 100 °C for 5 min to inactivate of the enzymes.

Experiments were performed in triplicate with two controls (without dyes or strains added). Each value shown in this study was the average of three experiments.

Enzyme activity

Laccase activity was determined spectrophotometrically as ΔA at 420 nm for 0.5 mM 2′2-azinobis-(3-ethylbenzthiaoline-6-sufonic acid) (Sigma) in 100 mM sodium acetate buffer, pH 4.5 ($\epsilon_{420} = 36{,}000$ mol^{-1} cm^{-1}) [19]. Manganese peroxidase activity was assayed by oxidation of 1 mM 2,6-dimethoxyphenol (DMP) in 100 mM sodium tartrate buffer (pH 4.5) in the presence of 0.1 mM H_2O_2 and 1 mM $MnSO_4$ [13]. Versatile peroxidase activity was determined by oxidation of DMP in the absence of Mn^{2+} at pH 4.5 ($\epsilon_{469} = 27{,}500$ mol^{-1} cm^{-1}) [17]. Lignin peroxidase activity was determined with veratryl alcohol as substrate in 10 mM sodium tartrate

Table 1 Structures, concentrations and maximum absorbance wavelengths of dyes

Chemical structure of dye	Dye	Concentrations of dye (mg l^{-1})	Maximum absorbance wavelength (nm)
Azo dyes	Acid red B_{2GL} (Beijing, China)	100	518
	Reactive red X-3B (Beijing, China)	100	585
	Directed blue B_{2RL} (Beijing, China)	100	530
Anthraquinonic dyes	Reactive blue K-GR (Beijing, China)	100	600
	Remazol brilliant blue R (Sigma)	100	590
Triphenylmethane dyes	Coomassie brilliant blue G-250 (Fluka)	100	585
	Cresol red (Sigma)	100	435
	Crystal violet (Fluka)	25	590
	Malachite green (Fluka)	15	615

buffer (pH 3.0) supplemented with 0.1 mM H_2O_2 [20]. Veratryl alcohol oxidase activity ($\epsilon_{310}=9{,}300\ mol^{-1}\ cm^{-1}$) [14] was detected without hydrogen peroxide.

All enzyme activities were determined immediately after an initial 3 min reaction at 25 °C. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol substrate min^{-1}.

Results

Capacity of different strains to decolorize dyes

Agar-plate screening showed that *T. hispida* and *C. hirsutus* did not decolorize the dyes efficiently within 2 weeks. Therefore, *T. versicolor*, *F. lignosus* and *Lse* were tested for their capacity to decolorize dyes in liquid media. The results in Table 2 show that *T. versicolor* was more efficient than the other strains under these experimental conditions. It decolorized all dyes almost completely, except Cresol red, a triphenylmethane dye. Anthraquinonic dyes were the most easily decolorized among the three structurally different kinds of dyes. The two selected fungi were able to decolorize the anthraquinonic dyes within 36 h, if they were added to the cultures initially. When dyes were added to the cultures after 5 days cultivation, they could be decolorized within 12 h. Compared with the other two azo dyes, Directed blue B_{2RL} was more easily decolorized. The time at which dyes were added significantly influenced the decolorization of three triphenylmethane dyes: Coomassie brilliant blue G250, Crystal violet and Malachite green. This was especially true in *F. lignosus* cultures. *Lse* decolorized the dyes somewhat, but not completely.

The relationship between enzyme activity and decolorization

In order to study the potential relationship between decolorization of dyes and extracellular enzymes, liquid cultures with three dyes added separately [Acid red B_{2GL}, Remazol brilliant blue R (RBBR), Crystal violet] were analyzed for ligninolytic enzyme activity and decolorization. However, neither lignin peroxidase nor veratryl alcohol oxidase activity was detected in any culture supernatant of the three strains.

The relationship between enzyme activity and decolorization of Acid red B_{2GL}

In three cultures tested with Acid red B_{2GL} added initially, high levels of laccase were detected (Fig. 1A). A broad peak of accumulation of this enzyme was detected over days 6–9 in cultures of two fungal strains. The laccase produced by *Lse* reached a maximum activity on day 3. The activity of laccase in cultures of *T. versicolor* and *Lse* was significantly higher than in cultures of *F. lignosus*. The trends of Mn-dependent and versatile peroxidase activity in the fungal cultures tested were similar to that of laccase (Fig. 1B, C). In cultures of *Lse*, neither Mn-dependent nor versatile peroxidase activity was detected.

Decolorization of Acid red B_{2GL} by three strains is shown in Fig. 2. Compared with the two fungi tested, *Lse* could not efficiently decolorize the azo dye, which suggested that Mn-dependent and versatile peroxidase might also be involved in decolorization of the dye. A positive correlation was found between enzyme activity and the decolorization of this azo dye.

Table 2 Maximum decolorization (%) of dyes and days required by different strains. *When added*: *A* strains were inoculated in medium already supplemented with dyes, *B* dyes were added into fungal cultures after 5 days, or into *Lse* cultures after 24 h. Strain GS115 (*Lse*) was also studied for its ability to decolorize dyes, but no positive result was found

Dye	When added	Maximum decolorization (%) of dye and days required					
		Lse		*F. lignosus*		*T. versicolor*	
		%	Days required	%	Days required	%	Days required
Acid red B_{2GL}	A	69.8	5	80.9	10.0	98.6	8.0
	B	70.2	5	81.3	10.0	97.5	10.0
Reactive red X-3B	A	61.5	4	85.7	10.0	98.4	10.0
	B	61.3	5	85.0	10.0	96.0	10.0
Directed blue B_{2RL}	A	78.6	4	98.0	7.0	98.0	4.0
	B	76.0	4	98.5	7.0	98.0	6.0
Reactive blue K-GR	A	77.2	2	98.6	1.5	99.0	1.5
	B	78.4	3	98.2	5.5	98.5	5.5
Remazol brilliant blue R	A	80.2	2	98.9	1.5	99.1	1.5
	B	76.7	3	99.0	5.5	98.7	5.5
Coomassie brilliant blue G250	A	54.7	5	75.0	10.0	87.0	10.0
	B	55.0	7	97.0	10.0	92.4	10.0
Cresol red	A	45.7	5	72.8	10.0	79.2	10.0
	B	43.6	7	74.2	10.0	79.4	10.0
Crystal violet	A	52.6	5	72.0	10.0	99.1	8.0
	B	70.0	7	99.1	10.0	99.0	7.0
Malachite green	A	75.1	5	66.5	10.0	99.5	10.0
	B	69.1	7	99.2	10.0	99.5	8.0

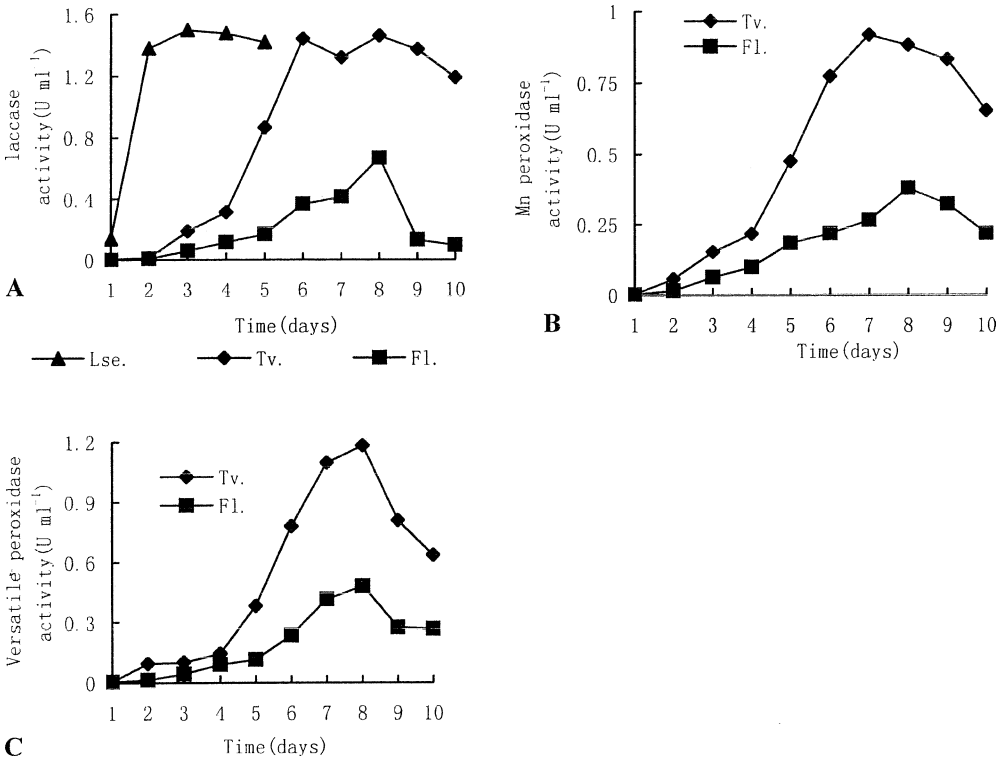

Fig. 1 Enzyme activity in three cultures with Acid red B_{2GL} added initially: laccase (**A**), Mn-dependent peroxidase (**B**) and versatile peroxidase (**C**). *Fl. Fome lignosus*, *Lse* laccase-secreting engineered strain (see Materials and methods), *Tv. Trametes versicolor*

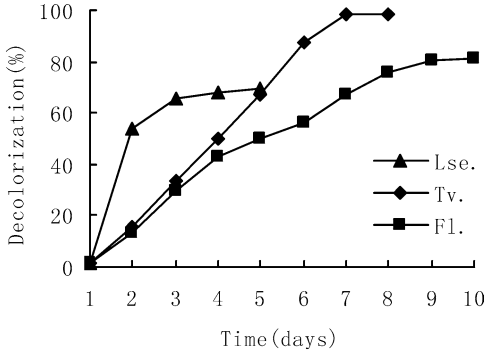

Fig. 2 Decolorization of Acid red B_{2GL} in three cultures

The relation between enzyme activity and decolorization of Crystal violet

No significant difference was found in the trends of enzyme activity (Table 3) and decolorization (Fig. 3) when Crystal violet was added initially instead of Acid red B_{2GL}. However, the enzyme activity in cultures of *F. lignosus* was much lower than in cultures of *T. versicolor*. Accordingly, the decolorizing capacity of *F. lignosus* was also lower than that of *T. versicolor*. However, the decolorizing capacity of *Lse* was the poorest among the three tested strains.

Factors related with the decolorizing of RBBR

In *Lse* cultures, a positive correlation was found between laccase activity and decolorization of RBBR (Fig. 4A,

Table 3 Enzyme activity (units ml^{-1}) in different strain cultures with 25 mg l^{-1} Crystal violet added.*Lcc* Laccase, *MnP* Mn-dependent peroxidase, *VP* versatile peroxidasem, – not assayed

Time (days)	*Lse*	*F. lignosus*			*T. versicolor*		
	Lcc	Lcc	MnP	VP	Lcc	MnP	VP
1	0.12	0	0.001	0	0.001	0.005	0.002
2	1.31	0.009	0.005	0.004	0.009	0.047	0.067
3	1.35	0.030	0.019	0.012	0.093	0.175	0.193
4	1.41	0.051	0.068	0.063	0.217	0.201	0.152
5	1.32	0.079	0.102	0.091	0.714	0.316	0.423
6	–	0.092	0.163	0.132	0.965	0.682	0.658
7	–	0.107	0.185	0.319	0.984	0.941	0.947
8	–	0.191	0.271	0.338	0.991	1.327	0.926
9	–	0.294	0.317	0.372	1.024	1.204	1.027
10	–	0.184	0.194	0.276	0.796	1.072	0.935

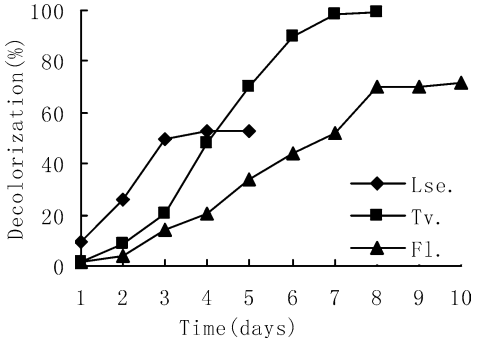

Fig. 3 Decolorization of Crystal violet in three cultures

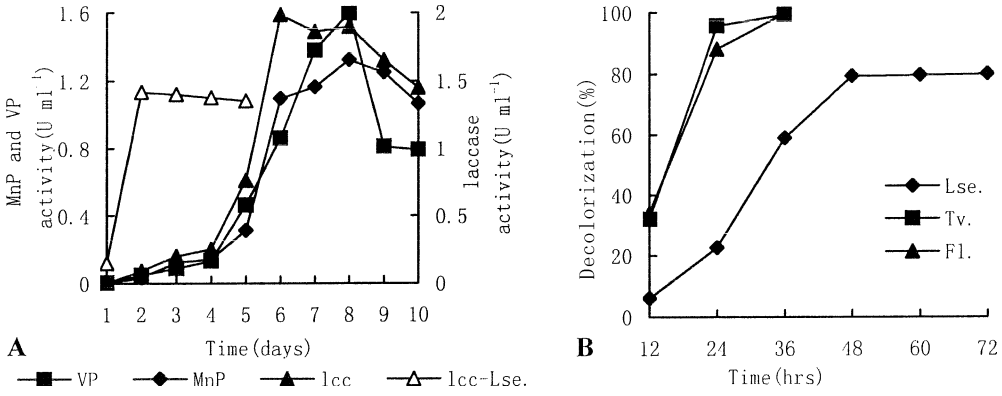

Fig. 4A, B Enzyme activities. **A** Enzyme activity produced by *T. versicolor* and laccase activity secreted by *Lse*. **B** Decolorization of Remazol brilliant blue R in three cultures

Table 4 Comparison of decolorization of two anthraquinonic dyes by two fungi in Kirk's medium and by crude enzymes produced by the fungi. + Dye was decolorized completely within 36 h, + + dye was decolorized completely within 24 h. Crude enzymes were prepared from 8-day-old cultures

Dye (100 mg l^{-1})	Strain		Crude enzyme	
	F. lignosus	*T. versicolor*	*F. lignosus*	*T. versicolor*
Reactive blue K-GR	+	+	+ +	+ +
Remazol brilliant blue R	+	+	+ +	+ +

B). But the dye was not decolorized completely. This suggested that laccase might play an important role in the decolorization of this dye, but complete decolorization depended on other factors. Figure 4B shows the dye can be completely decolorized by the two fungal strains within 36 h. But the ligninolytic enzyme activity in the *T. versicolor* cultures was very low, even after 48 h cultivation (Fig. 4A). The activity of these ligninolytic enzymes in vivo was also negligible at that time (data not shown). The trends of enzyme activity in *F. lignosus* cultures were similar to those in *T. versicolor* cultures. The same phenomena were also found in cultures with Reactive blue K-GR added. The decolorizing capacity of the crude enzymes produced by the two fungi was also assayed. The activity of these extracellular ligninolytic enzymes as crude enzymes was significantly higher than that in the fungal cultures at 36 h (data not shown). The results are shown in Table 4. This study showed that the crude enzymes secreted by the fungi could decolorize the dyes within 24 h. It can be inferred that there might be other factors besides these ligninolytic enzymes involved in completely decolorizing the anthraquinonic dyes that were tested.

Discussion

In this study, we examined the decolorization of azo, anthraquinonic and triphenylmethane dyes by four fungal strains and one laccase-secreting engineered strain. Agar-plate screening showed that the fungi *T. hispida* and *C. hirsutus* could not efficiently decolorize all the dyes tested within 2 weeks. *T. versicolor* was the most efficient strain in decolorizing the dyes tested. This was supported by subsequent experiments using liquid cultures (Table 2).

Lignin peroxidase is involved in dye decolorization in many fungal cultures [15, 22]. However, under this experimental condition, none of the strains produced detectable lignin peroxidase or veratryl alcohol oxidase, which suggested that these enzymes might not be involved in decolorization of the dyes tested. A comparison between the decolorizing capacity of the laccase-secreting engineered strain and that of the two selected fungi indicated that laccase might play an important role in decolorizing all the dyes, but could not completely decolorize any tested dyes by itself. It is likely that additional phenoloxidases, such as Mn-dependent and versatile peroxidases, were also involved in decolorization of the dyes. The difference in the dye-decolorizing capacities of *T. versicolor* and *F. lignosus* showed that the activity of phenoloxidases, including laccase, Mn-dependent and versatile peroxidases, correlated positively with decolorization of the dyes tested.

Among the three structurally different kinds of dye, the anthraquinonic dyes were the most easily decolorized. Although each is an azo dye, Directed blue B_{2RL} was more easily decolorized than with the other two. Thus, small structural differences between the dyes could significantly affect their decolorization. This might be due to differences in electron distribution, charge density or steric factors [11]. Therefore, the results obtained using model dyes cannot be extrapolated to other related compounds.

The time at which the dyes were added had some effect on the decolorization of triphenylmethane dyes, such as Coomassie brilliant blue G250, Crystal violet, and Malachite green, especially in *F. lignosus* cultures (Table 2). This might result from the initial addition of dyes that influenced the accumulation of extracellular enzymes in the fungal cultures. In order to avoid this phenomenon and gain efficient decolorization, it was more feasible to add the dyes to the cultures after 5 days cultivation.

Blue anthraquinones are often used to screen for ligninolytic activity [7]. Data shown in Table 4 show

that the crude fungal enzymes could decolorize two anthraquinonic dyes efficiently. However, the dyes could be completely decolorized by the two fungi, even when the ligninolytic enzyme activity was negligible. Similar results were also shown in research by Anna et al. [1]. Therefore, the screening method might not be applicable in all cases.

In conclusion, *T. versicolor* was the most efficient among the strains tested. Anthraquinonic dyes were more easily decolorized than the other two structural kinds. A small structural difference in dyes can significantly affect their decolorization. None of the strains showed lignin peroxidase or veratryl alcohol oxidase activity. No dye could be decolorized completely by laccase alone. It is likely that other phenoloxidases, such as Mn-dependent and versatile peroxidases, were also involved in the decolorization of the dyes.

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