Journal of Hazardous Materials 209–210 (2012) 199–203

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Removal of chlorophenolic derivatives by soil isolated ascomycete of *Paraconiothyrium variabile* and studying the role of its extracellular laccase

Hamid Forootanfar[a,b], Mohammad Mehdi Movahednia[a,c], Soheila Yaghmaei[c], Minoosadat Tabatabaei-Sameni[a], Hossein Rastegar[d], Armin Sadighi[a], Mohammad Ali Faramarzi[a,*]

[a] *Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, P.O. Box 14155–6451, Tehran 14174, Iran*
[b] *Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Pharmaceutics Research Center, Herbal and Traditional Medicine Research Center, Kerman University of Medical Sciences, Kerman, Iran*
[c] *Department of Chemical and Petroleum Engineering, Biotechnology Research Center, Sharif University of Technology, Tehran, Iran*
[d] *Food & Drug Laboratory Research Center, Ministry of Health & Medical Education, Tehran, Iran*

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form
25 December 2011
Accepted 4 January 2012
Available online 11 January 2012

Keywords:
Paraconiothyrium variabile
Removal
Laccase
Chlorophenol
Pollutant

ABSTRACT

The ability of *Paraconiothyrium variabile*, a laccase producing ascomycete recently isolated from soil, was studied to eliminate chlorophenol derivatives in submerged culture medium. Among the tested compounds, ρ-chlorophenol (ρ-CP) and pentachlorophenol (PCP) were found to have minimum and maximum toxic effects, respectively, on the growth of the microorganism and at the same time high and low bioelimination percentages. The fungal strain was able to remove 86% of ρ-CP (with initial concentration of 40 mg l^{-1}) and 56% of 2,4-dichlorophenol (2,4-DCP; with same concentration as ρ-CP) after 9 days of incubation while no elimination was observed in the presence of 2,4,6-trichlorophenol (2,4,6-TCP) and PCP. Monitoring of laccase production level in the fermentation broth together with pollutant removal confirmed the key role of this copper-containing oxidase in chlorophenol derivatives elimination. The type of laccase inducer (guaiacol) and its final concentration (250 μM) and also initial pH of the fermentation broth (pH = 5.5) in the elimination of ρ-CP increased the final removal yield from 86% to 94.3%.

1. Introduction

Among xenobiotics, chlorophenols are considered one of the most hazardous classes of organic compounds for living organisms [1]. The entrance of such highly carcinogenic, mutagenic, and toxic pollutants into the environment due to the activities of chemical, plastic, paper, oil refinery, and agricultural industries as well as their extensive applications as insecticides, herbicides, preservatives, antiseptics, and disinfectants have persuaded researchers to study methods for eliminating these harmful chloroaromatics [2–4]. The disadvantages of physicochemical techniques such as incomplete degradation of pollutants and the need for further treatments could be overcome by biological methods supplying lower cost, higher efficiency, and less toxicity [3].

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a multi-copper oxidase catalyzing the oxidation of a wide range of aromatic substrates including benzenethiols, phenol derivatives, polyphenols [5], and polycyclic aromatic hydrocarbons (PAHs) [6]. In recent decades, laccase-producing microorganisms, especially white-rot fungi [6,7] and also the purified enzyme both in free and immobilized forms, have been employed for biological treatment of different pollutants [8,9]. *Phanerochaete chrysosporium* was the first basidiomycete used for degradation of a wide spectrum of organopollutants like polychlorinated biphenyls and chlorinated phenols and has been applied as a model for xenobiotic biodegradations [10,11]. Biodegradation of brominated phenols using cultures and laccase of *Trametes versicolor* was investigated by Uhnakova et al. [7]. The ability of other white-rot fungi such as *Anthracophyllum discolor* [12] and *Ganoderma lucidum* [6] to degrade PAHs was also recently reported. Zhang et al. [5] studied the degradation of 2,4-dichlorophenol (2,4-DCP), 4-chlorophenol (ρ-CP), and 2-chlorophenol catalyzed by laccase from *Coriolus versicolor*. In addition to basidiomycetes, the ability of other laccase producing fungal families e.g. ascomycetes for degradating chlorophenolic pollutants have been also studied [13]. Recently, a newly isolated laccase producing ascomycete, identified as *Paraconiothyrium variabile*, was isolated from soil and its extracellular laccase was

* Corresponding author.
E-mail address: faramarz@tums.ac.ir (M.A. Faramarzi).

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doi:10.1016/j.jhazmat.2012.01.012

successfully applied for decolorization of some synthetic dyes [14,15]. The aim of the present study was to investigate the ability of the mentioned fungus for elimination of chlorophenolic compounds. Removal studies in the presence of laccase inhibitors and inducers were also investigated.

2. Materials and methods

2.1. Chemicals

Chlorophenols including ρ-CP, 2,4-DCP, 2,4,6-trichlorophenol (2,4,6-TCP), and pentachlorophenol (PCP) as well as 2,5-dimethylaniline (2,5-xylidine), 2-methoxyphenol (guaiacol), sabouraud-2%-dextrose broth (SDB), and sabouraud-4%-dextrose agar (SDA) were purchased from Merck (Darmstadt, Germany). 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Fungal strain and toxic effects of monoaromatics

The ascomycete used in the present study was isolated from soil and identified as *P. variabile* based on18S rDNA analysis [14]. The fungal strain was maintained on an SDA plate containing 0.1% guaiacol as a laccase inducer at 4 °C and subcultured every 2 weeks. The toxic effects of chlorophenolic pollutants on the fungal strain were investigated by estimation of biomass production in the absence and presence of each monoaromatic (with initial concentration of 20 mg l^{-1}). Briefly, one plug (3 mm in diameter) of fresh fungal colony was transferred from the SDA plate into each 250-ml Erlenmeyer flask (one flask for each day) containing 50 ml of SDB medium and incubated at 30 °C and 150 rpm for 14 days. At the third day of incubation, each chlorophenolic compound (ρ-CP, 2,4-DCP, 2,4,6-TCP, and PCP) was added to the fermentation broth to reach a final concentration of 20 mg l^{-1}. Contents of the flasks were filtered daily through filter papers (Whatman No. 1) using a vacuum pump, and the produced biomass was weighed after drying of the filter paper at 70 °C. These experiments were carried out three times, and the biomass averages were used to draw a growth curve.

2.3. Elimination studies on ρ-CP and 2,4-DCP

Separate cultivations were done in order to investigate the degradation of ρ-CP and 2,4-DCP. 250-ml Erlenmeyer flasks each containing 50 ml of SDB medium designed for each concentration of ρ-CP and 2,4-DCP (10, 20, 40, 50, 60, and 100 mg l^{-1} of each pollutant) were inoculated with one fresh plug (3 mm in diameter) of fungal colony and incubated at 30 °C and 150 rpm for 14 days. Each monoaromatic was added to culture broth to reach the pollutant concentrations of 10, 20, 40, 50, 60 and 100 mg l^{-1} at the third day of incubation. 2,5-Xylidine (100 μM) and $CuSO_4{\cdot}5H_2O$ (250 μM) were also introduced to fermentation media to induce laccase production in the same day. Simultaneously, the fungal strain was cultivated in the absence of chlorophenolic compounds under similar conditions. Control abiotic experiments were carried out under the same conditions in uninoculated media. Daily samples of 1 ml were taken from each culture broth and subjected to high performance liquid chromatography (HPLC) after removal of biomass by centrifugation (12,000 × *g* for 5 min) and filtration. The remained biomass was extracted by 1 ml methanol. The supernatant, after centrifugation at 12,000 × *g* for 5 min, was studied by HPLC analysis in order to investigate any possible chlorophenol bioaccumulation.

2.4. Removal of ρ-CP and 2,4-DCP using purified laccase of P. variabile

In order to investigate the influence of laccase from *P. variabile* on the studied monoaromatics, the culture filtrate (produced in presence of ρ-CP) was subjected to laccase purification using the method previously described [14]. Each chlorophenolic pollutants (ρ-CP and 2,4-DCP dissolved in methanol) was added to the reaction mixture (final volume of 5 ml; citrate buffer 0.1 M, pH 5) containing the purified laccase (25 U, dissolved in the same buffer) to reach final concentration of 100 mg l^{-1} followed by incubating of prepared mixture at 30 °C with mild shaking. At the end of treatment (5 h), the content of each glass tube was analyzed for remained chlorophenolic pollutants using HPLC. Same experiments without purified laccase were also designed as negative controls separately for ρ-CP and 2,4-DCP. Removal study was performed in triplicate.

2.5. HPLC analysis

HPLC apparatus consisted of a Smartline HPLC Pump 1000, a PDA Detector 2800, and a Degasser 5000, all from Knauer (Berlin, Germany). After filtering the samples through 0.45-μM PTFE filters from (Schleicher & Schull, Germany), each sample (20 μl) was injected using a Smartline Autosampler 3950 with a sample loop of 100 μl. The data were acquired and processed by means of ChromGate software (version 3.3.1) from Knauer (Berlin, Germany). Chromatographic separation was achieved on a Lichrospher 100 RP & EC C_8 reverse phase column (C8, 25 cm × 0.46 cm id, 5 μM particle size) from Teknokroma (Barcelona, Spain). The applied mobile phases and flow rates for each chlorophenolic compound are summarized in Table 1. The method of Petroutsos et al. [16] was used in the case of ρ-CP, and in other cases some modifications were performed.

2.6. Determination of laccase activity

Oxidation of ABTS as a laccase substrate was used to determine the laccase activity of the *P. variabile* submerged culture during the incubation period [17–19]. An assay was carried out after adding 0.5 ml of diluted culture broth to 0.5 ml of 5 mM laccase substrate (ABTS dissolved in 0.1 M citrate buffer, pH 4.5) followed by incubation at 37 °C and 120 rpm for 10 min. Change in absorbance at 420 nm was monitored by a UV/vis spectrophotometer (UVD 2950, Labomed, Culver City, USA) and the laccase activity was calculated using the molar extinction coefficient of ABTS (ε_{420} = 36,000 M^{-1} cm^{-1}). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute [14].

2.7. Effect of laccase inducers on the removal of ρ-CP

The effect of laccase inducers including 2,5-xylidine, guaiacol, and veratryl alcohol on the removal of ρ-CP (with the initial concentration of 40 mg l^{-1}) was studied by adding each induction agent to the cultivation media (prepared as previously mentioned) in a sterile condition to reach the final concentrations of 125, 250, and 500 μM. After 9 days of incubation, samples were taken from the media and analyzed for laccase activity and remained ρ-CP concentration. In order to determine the sole effect of laccase inducers on the enzyme productivity, the laccase activity of cultivated fungus in absence of ρ-CP was also evaluated.

2.8. Effect of pH on the removal of ρ-CP

To investigate the effect of pH value on the removal of ρ-CP by *P. variabile*, the initial pH of SDB media was adjusted from 4 to

Table 1
Mobile phases and flow rates applied for HPLC analysis of chlorophenolic pollutants.

Chlorinated phenol	Mobile phase (methanol/water/acetic acid)	Flow rate ($ml\,min^{-1}$)	Maximum absorbance (nm)	Retention time (min)
p-CP	50/49/1	1	280	14
2,4-DCP	50/49/1	1.5	280	19.5
2,4,6-TCP	60/39/1	1.5	280	16.5
PCP	75/24/1	1.5	280	14.5

8 with NaOH or HCl in steps of 0.5 followed by culturing of the fungal strain as previously mentioned. ρ-CP was added to culture media aseptically to reach a final concentration of 40 $mg\,l^{-1}$ at the third day of incubation. After nine days of incubation, the residual pollutant in the culture supernatant was determined using HPLC.

3. Results

3.1. Toxic effects of monoaromatics on P. variabile

To evaluate the toxicity of the four tested monoaromatics, the biomass productivity in the presence of each chlorophenol (with the initial concentration of 20 $mg\,l^{-1}$) was determined. As shown in Fig. 1, the growth of *P. variabile* was significantly decreased by 2,4,6-TCP and PCP, while ρ-CP and 2,4-DCP represented a less inhibitory effect. Therefore, the less-toxic pollutants (ρ-CP and 2,4-DCP) were subjected for more studies.

3.2. Elimination of ρ-CP and 2,4-DCP by P. variabile

The growth curves of *P. variabile* in the presence of ρ-CP and 2,4-DCP at different initial concentrations are illustrated in Fig. 2a and b, respectively. As shown in Fig. 2, increasing the ρ-CP and 2,4-DCP concentrations decreased biomass productivities. Fig. 3a and b represent the profile of removal percentages for ρ-CP and 2,4-DCP, respectively. Based on obtained data, the production of fungal biomass was more suppressed in the presence of 2,4-DCP compared to ρ-CP. At concentrations above 40 $mg\,l^{-1}$, the fungal growth in presence of both chlorophenols was completely suppressed. The elimination percentages decreased from 86% to 15% for ρ-CP and from 56% to 5.4% for 2,4-DCP. Determination of laccase activity in the supernatant together with pollutant removal measurement (Fig. 4) showed the same profiles during incubation time. As shown in Fig. 4, inhibitory effect of ρ-CP on the fungal growth decreased both laccase activity and consequently ρ-CP elimination. Same results were achieved in presence of 2,4-DCP (Fig. 4). In uninoculated flasks, no decrease in concentration of tested phenolic compounds was acquired.

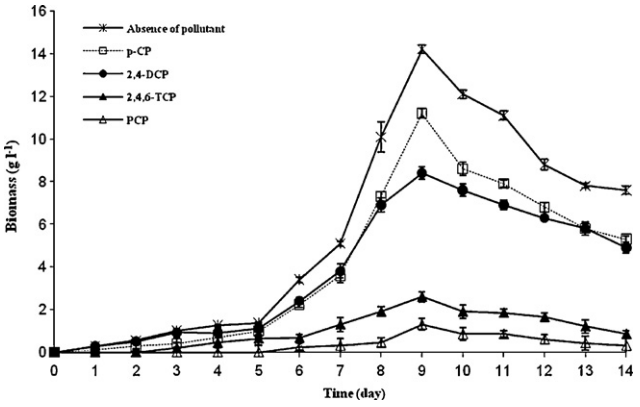

Fig. 1. Time courses of biomass production of *P. variabile* in the absence and presence of different chlorophenolic derivatives (with the initial concentration of 20 $mg\,l^{-1}$).

3.3. Removal of ρ-CP and 2,4-DCP using purified laccase

Elimination studies in presence of the purified laccase revealed that the mentioned phenol oxidase was able to remove 94% of ρ-CP (initial concentration of 100 $mg\,l^{-1}$) and 72% of 2,4-DCP after 5 h of incubation.

3.4. Effect of laccase inducers on the removal of ρ-CP

The study showed that addition of the laccase inducers into the fungal cultivation media in absence of ρ-CP increased the enzyme activity from 512 $U\,l^{-1}$ (basal medium) to 1430 $U\,l^{-1}$ (250 μM of guaiacol), 825 $U\,l^{-1}$ (250 μM of xylidine) and 576 $U\,l^{-1}$ (250 μM of veratryl alcohol). However, in presence of pollutant, guaiacol (250 μM) was the most potent inducer and increased laccase activity and removal percentage to 835 $U\,l^{-1}$ and 94%, respectively. In the case of xylidine (250 μM), 88% of ρ-CP was removed from culture broth and the supernatant showed 650 $U\,l^{-1}$ of laccase activity. With veratryl alcohol (250 μM), laccase activity and pollutant elimination percent were 483 $U\,l^{-1}$ and 82.6%, respectively. For all of the phenolic inducers, maximum elimination of ρ-CP was observed at a concentration of 250 μM; below and higher this critical concentration, both the removal percentage and laccase production decreased.

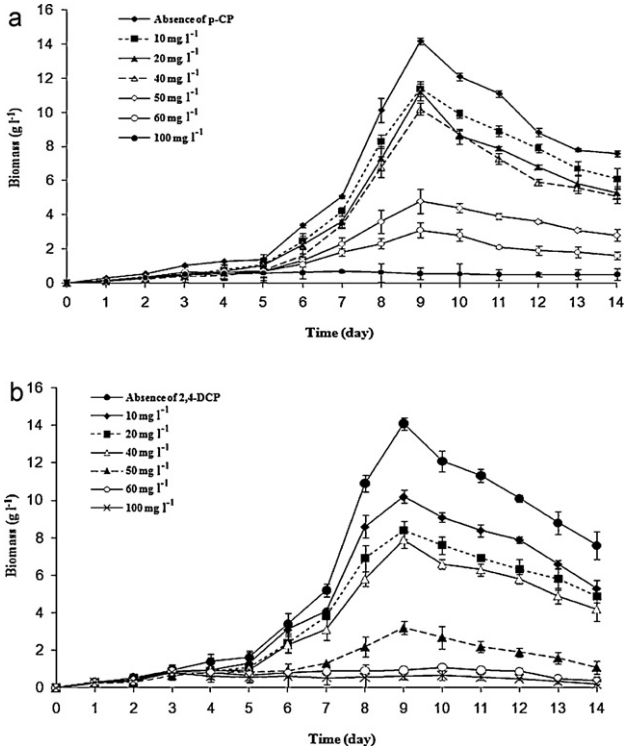

Fig. 2. Growth curves of *P. variabile* in the presence of different concentrations of (a) ρ-CP and (b) 2,4-DCP.

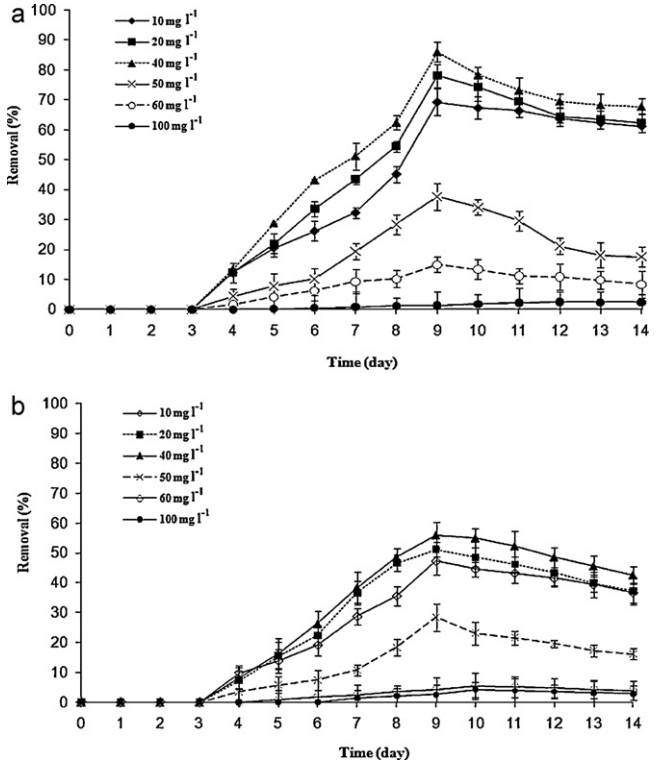

Fig. 3. Profiles of (a) ρ-CP and (b) 2,4-DCP eliminations by *P. variabile* during 14 days of incubation.

3.5. Effect of different pH on the removal of ρ-CP

Table 2 summarizes the removal percentage of ρ-CP (with initial concentration of 40 mg l^{-1}) after 9 days of incubation at the initial pH range of 4–9. As is shown, the initial pH of 5 was suitable in which 86% of ρ-CP elimination was achieved. However, both decreasing and increasing the pH value of fermentation broth greater than 5 led to a decrease in the removal of ρ-CP.

4. Discussion

During the last decades, the release of chlorophenolic derivatives into the environment due to large-scale applications such as pesticides and herbicides in the agricultural industry as well as their use for manufacturing of disinfectants, pharmaceuticals, dyes, aromatic compounds, and other organic materials has made them one of the most important subjects in xenobiotic degradation studies [20–22]. Recently, the biodegradation of monoaromatics using biological resources and enzymes has received more attention because physicochemical methods degrade such pollutants incompletely and the formed byproducts need more treatments [23].

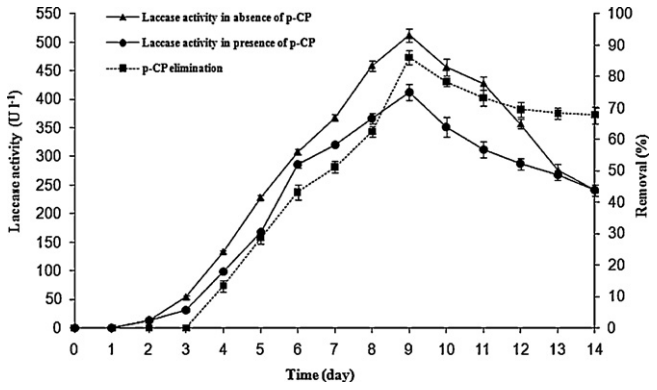

Fig. 4. Profiles of laccase activity and pollutant removal in broth culture of *P. variabile* in the presence of ρ-CP and 2,4-DCP at the initial concentration of 40 mg l^{-1}.

Table 2
Effect of initial pH of broth culture on the elimination of ρ-CP by *P. variabile*.

pH	Elimination percentage[a]
4	48.6 ± 0.6
4.5	74.4 ± 1.5
5	86 ± 1.3
5.5	75.8 ± 2.4
6	74.3 ± 1.8
6.5	69.3 ± 0.9
7	62.3 ± 2.7
7.5	55.9 ± 1.8
8	48.2 ± 1.1

[a] Elimination percent of ρ-CP (with the initial concentration of 40 mg l^{-1}) was determined after 9 days fungal incubation.

The present study focused on the ability of a newly isolated laccase-producing ascomycete, *P. variabile*, for elimination of chlorophenolic compounds. ρ-CP and 2,4-DCP decreased biomass production of *P. variabile* to some extent, but 2,4,6-TCP and PCP were able to inhibit the growth of the fungal strain completely. This finding is in agreement with Petroutsos et al. [16] indicating the toxicity of phenols depends on the presence, number, and position of halogen on a phenolic ring. Increasing the concentration of ρ-CP and 2,4-DCP from 10 mg l^{-1} to 100 mg l^{-1} reasonably decreased *P. variabile* growth, and in concentrations above 60 mg l^{-1} the growth was completely inhibited.

In contrast to peroxidase which inhibited by phenolic compounds, phenol and its derivatives are laccase substrates [11], and the ability of laccase and other related lignin-degrading enzymes (lignin peroxidase and manganese peroxidase) to eliminate mono- and polyaromatic hydrocarbons is a general concept reported in many studies [5,6,24]. In our study, based on the profile of laccase production by the fungus and also the removal curves of ρ-CP and 2,4-DCP (Fig. 4), the key role of such copper-containing benzenediol oxidase was confirmed in the biodegradation of these two monoaromatics. Additional studies using purified laccase of *P. variabile* revealed the similar results.

Compare to basal medium (SDB), addition of each of the three phenolic inducers (in absence of pollutant) into the cultivation media increased laccase activity to some extent. This finding is in agreement with the report of Ryan et al. [3] determined that supplementing the culture of *T. versicolor* by 1 mM guaiacol elevated the laccase activity to 780%. However, presence of toxic pollutant together with inducer in the cultivation medium led to a negligible decrease in laccase activity and consequently reducing of pollutant removal while only the laccase inducer was presented in fungal culture broth. A similar result was observed by Ting et al. [6], who indicated that more laccase activity in the presence of laccase inducers enhances the biodegradation of phenanthrene and pyrene by *G. lucidum*.

The alteration of the initial cultivation pH from 5 decreases both biomass production and pollutant removal percentage. In a recent study, the optimum pH of purified laccase from *P. variabile* was found to be 4.8 [14] where the maximum of ρ-CP removal was determined. A similar result was reported by Zhang et al. [5], who showed more than 80% degradation of 2,4-DCP achieved in the pH range of 5–6 and very low elimination at acidic pH.

Acknowledgment

This work was supported financially by the grant Number 89-02-90-11015 from Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran.

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