ISOLATION, SCREENING AND OPTIMIZATION OF SUBMERGED CULTURE CONDITIONS FOR MYCELIAL BIOMASS PRODUCTION WITH ENHANCED ANTIBACTERIAL ACTIVITY OF THE FUNGUS MYROTHECIIUM SPP. MRP001 AGAINST MULTI DRUG RESISTANT PATHOGENS

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ABSTRACT

Fungi are interestingly; produce the bioactive natural products thus being screened for the production of valuable antibacterial product. In the present study the fungus was isolated from soil and identified as Myrothecium spp. MRP001 based on morphological characteristics and it was reinforced by 16S rRNA gene sequence analysis. The submerged culture conditions and nutritional requirements for the production of mycelial biomass with improved antibacterial activity were studied in shaker flask culture using OFAT (one factor at time) in six different media. After 120 h of growth, Tryptone Soya Broth (TSB) and Potato Dextrose broth (PDB) shows maximum spore account (2×10^10 CFU/ml) of Myrothecium spp MRP001. Among the all different culture media, the maximum biomass production of 2.14 g/L and 2.23 g/L were observed in Tryptone Soya Broth (TSB) and Potato dextrose broth (PDB) respectively, over a period of 120 h of incubation at pH of 6.5 in 25-30 °C in Myrothecium spp. Strain MRP001. Among the carbon sources, glucose supplemented basal medium showed an effective biomass production of 2.24 g/L. Whereas in nitrogen sources, yeast extract had significant biomass production of 2.12 g/L. The optimized ethyl acetate extract of cultural filtrate showed a maximum inhibition zone of 28.4 mm and 26.3 mm against multi-drug resistant P. aeruginosa strain 1 and S. aureus strain 5, respectively. These results are useful for further investigation of the fungus in the future for microbial disease treatment for multi drug resistant human pathogens.

Keywords: Fermentation parameter, improved bioactivity, Myrothecium spp., multi drug resistant human bacterial pathogens

INTRODUCTION

Natural products play a major role in the discovery of leads for the development of drugs in the treatment of human diseases. Natural products are an unsurpassed source of bioactive compounds and constitute a relevant economic resource for the pharmaceutical, cosmetic and food industry. Since fungi live in a biologically competitive environment with unique physical and chemical parameters, such as pH, temperature, pressure, oxygen, light, nutrients and salinity, the chemical diversity of the secondary metabolites and their range of applications from marine fungi is high (Debbah et al., 2010; Jiang et al., 2013; Bhadury et al., 2013; Wu et al., 2013; Sun et al., 2012). Biopharmaceutical approach provide a unique environment for fungi and have been recognized as a repository of fungi with novel metabolites of pharmaceutical importance (Tan & Zou, 2001; Strobel et al., 2004). Fungi are important resources for discovery of natural products. Fungi are producing important components of microbial diversity. Fungi represent an important and quantifiable component of fungal diversity, and are known to affect community diversity and structure (Kring et al., 2007). Fungi isolated from soil are more likely exhibit pharmaceutical potentials. Fungi have been found in soil sample examined, and it is estimated that there are over one million fungi existed in the nature (Petrini, 1991). Fungi provide a wide variety of structurally unique, bioactive natural products. These plentiful natural products represent a huge reservoir which offers an enormous potential for exploitation for medicinal, agricultural and industrial uses (Tan & Zou, 2001, Zhang et al., 2006). There has been a great interest in fungi as potential producers of novel, biologically active products (Schulz et al., 2002, Strobel & Daisy, 2003). The secondary metabolites produced by endophytes associated with medicinal plants can be exploited for curing diseases (Tejesvi et al., 2007). More exciting possibilities exist in the wild and unexplored part of the world for discovery of novel fungi, their biology, and their potential usefulness. The potential application of the fungal species of Myrothecium spp., particularly in the prospect of bioactive compounds production, makes it more noteworthy to study the different cultural conditions that affect the growth of these microorganisms. In the present study, attempt was made to investigate the cultural conditions Myrothecium spp. for improved biomass production and antibacterial activity and fermentation process for high level production of bioactive compounds.

MATERIAL AND METHODS

Source of bioactive compound producing fungus

A soil sample was collected from local area at Akola, Maharashtra, India. Strain MRP001 was isolated from the sandy soil sample on starch-casein agar18 adjusted to pH 8.5 (1 M NaOH) and was preserved as a mixture of spores and mycelium fragments in glycerol (20% v/v) at 28 °C. The strain is deposited in the Microbial culture collection of the Department of Biotechnology at SGB Amravati University, Maharashtra, India. It was examined for chemotaxonomic and morphological properties known to be of value in Myrothecium systematic and by partial 16S rRNA gene sequence analysis in previous study (Rizwan et al., 2012).

Cultivation and identification of fungal isolate

The screening of microorganisms was done against various human pathogenic bacteria and antimicrobial activity found in isolated fungal strain was further confirmed to be Myrothecium spp. MRP001. The strain Myrothecium spp
MRP001 which was used for this study was isolated from soil of PDKV region of Akola District. The stock culture of strain was maintained on a potato dextrose agar (PDA) slants. Slants were inoculated, incubated at 28 °C for 7 days and then stored at 4 °C. The fungal isolate was identified to genus level at Molecular Biology Lab, Department of Biotechnology, SGB Amravati University, Amravati, Maharashtra, India and the bioactive compound producer MRP001 was identified as Myrothecium spp. using 16S rRNA sequence analysis (Rizwan et al., 2012).

Effect of culture media

The media requirements of fungus were determined by mycelial dry weight method (Jonathan 2002). Myrothecium spp. For effect of culture media on biomass production studies, MRP001 growth and antimicrobial production were evaluated in six different synthetic media after 96 h of incubation. Two millilitres of Myrothecium spp. MRP001 overnight culture were inoculated in 100 ml of following synthetic media: Mueller Hilton (MH), Plate count broth (PCB), Tryptone Soya Broth (TSB), Rogosa and Sharpe (MRS), Potato dextrose broth (PDB) and Fred Waksman Basic 77 broth (WFB). The cultures were incubated for 96 h at 30 °C on rotary shaker (120 rpm). Total counts, constituted by vegetative cells and spores, and were assessed after 96 h of incubation using Haemocytometer by the standard serial dilution method (Kobayashi et al., 1996 and Petriini et al., 1992). The experiments were done in triplicate.

Effect of temperature and pH

The temperature and pH requirements of fungus were determined by mycelial dry weight method (Jonathan 2002). For temperature, potato dextrose broth (PDB) medium was dispensed into 500 ml Erlenmeyer flasks (200 ml per flask). The flasks were autoclaved at 121°C for 20 min. After cooling, each Erlenmeyer flask was inoculated with mycelia disc (5.0 mm diameter) from 6 day old culture and incubated at different temperatures (20–40 °C) for 120 h. All the experiments were done in triplicate. The mycelia were harvested using the method of (Jonathan 2002). For pH, the same potato dextrose broth medium was employed but the medium was adjusted to pH values of 4.5–7.0. 200 ml of each treatment was dispensed into 500 ml Erlenmeyer flask and replicated three times.

Effect of carbon and nitrogen sources

The fungus was grown in basal liquid medium that was described by (Jin-zhong et al., 2003). The basal medium was supplemented separately with carbon sources (15 g/L) such as glucose, fructose, maltose, cellulose, starch & xylan. In the case of nitrogen (10 g/L), various nitrogen sources such as ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate, calcium nitrate and yeast extract and the media were autoclaved. After cooling, each flask was inoculated with 5 agar discs (5.0 mm size) of 6 day old mycelium and incubated at 30°C for 120 h on rotary shaker (120 rpm). The mycelial biomass were harvested and dried at 40 °C and weighed. Assessment of mycelial weight was carried out using the procedure described by (Kadiri & Fasilid, 1994).

Extraction of bioactive compounds

Discs were cut from the edge of an actively growing colony on PDA with a flamed cork borer (5 mm diameter) and transferred aseptically into 500 ml flasks containing optimized basal media containing glucose as carbon source (15 g/L) and yeast extract as nitrogen source (10 g/L). The culture was incubated for 120 h on rotary shaker (120 rpm). The mycelial biomass were harvested and dried at 40 °C and stored at 4 °C for further study.

Antimicrobial activity

Test microorganisms

The clinical strains of Staphylococcus aureus were obtained from skin infections and wounds of some patients, whereas strains of Pseudomonas aeruginosa were obtained from lung infections of cystic fibrosis patients from Government Clinical Laboratory, Main Hospital (District Akola, Maharashtra, India). All the clinical strains were stored in screw cap tube at -20 °C in deep freezer. Staphylococcus aureus and Pseudomonas aeruginosa strains were identified by standard biochemical methods (Essers & Radebold, 1980; Pourshadi & Klaas, 1984).

Susceptibility test

The Kirby–Bauer disk diffusion test was used to determine the antibiotic resistance of S. aureus strains (1–10) and P. aeruginosa strains (1–8). The isolated colonies of the above strains from mother culture were inoculated into nutrient broth. The broth was incubated at 37 °C until it equaled 0.5 McFarland standards. A McFarland 0.5 turbidity standard corresponds to inoculums of 1 × 10⁶ CFU/ml (Acar & Goldstein, 1991). The antibiotic disks of methicillin (5 μg/disk), penicillin (10 units/disk), and vancomycin (30 μg/disk) were used for clinical strains of S. aureus. Ciprofloxacin (5 μg/disk), cefotaxime (30 μg/disk), ofloxacin (5 μg/disk), and amikacin (30 μg/disk) were used for clinical strains of P. aeruginosa. Inoculated plates were inverted and incubated at 37 °C for 18 h. After the incubation period, the diameter of zone of inhibition was measured, and results were interpreted according to the standards of Clinical and Laboratory Standards Institute (NCCLS 2008).

Disk diffusion method

The paper disk diffusion method was used to determine the antibacterial activity of isolated fungal extract (Acar & Goldstein, 1996). Sterile disks (6 mm) were impregnated with 10 ml of extract at a concentration of 1mg/ml. For bacteria, microorganism swabs were swabbed on the surface of Muller Hinton agar was used. Paper disks treated with 10% DMSO were used as negative controls. The plates were incubated at 37 °C for 18 h for bacteria. The diameter of the inhibition zone around each disk was measured at the end of the incubation time. Experiments were performed in triplicate and the antimicrobial activity was expressed as the average of inhibition zone diameters (in mm) produced by the fungal extract.

Statistical analysis

The triplicate data are expressed as the mean value ± standard error and presented in the form of figures. The error bars are depicted at 5% limit. The overlapping and non-overlapping bars show non-significant and significant respectively differences among different treatments.

RESULTS AND DISCUSSION

Morphological characteristics of Myrothecium spp. MRP001

The morphological characteristic of isolated fungi was observed on PDA after 2 weeks of growth at 30 °C. Colonies on PDA was circular, raised, at first white, later it was brown to reddish colour. Hyphae were thin walled and branched. Morphological characters of the fungus including sporodia, conidiophores, conidigenous cells and conidia, were observed with a Nomarski differential interference contrast optical system and a scanning electron microscope (SEM). For SEM, specimens were placed in fixative (3% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.2) at 4 °C for 24 h. The specimens were washed three times for 20 min each in 0.1 M cacodylate buffer, pH 7.2, and then fixed 24 h with 1% osmium tetroxide in 0.1 M cacodylate buffer. After three 20 min deionized water washes the samples were dehydrated in an ethanol series (30%, 50%, 70%, 95%, and 100%, 20 min each, except 100% had three changes, two for 20 min and one overnight). The dehydrated samples were critical point dried (Polaron E3000). All specimens were attached to aluminum mounts on carbon tape, sputter coated with AuPd (Polaron ES100) and observed in the FESEM (Zeiss DSM982 Gemini Field Emission Scanning Electron Microscope). All of these properties are consistent with the classification of the strain in the genus Myrothecium spp., an assignment supported by the partial sequencing of the 16S rRNA gene.
Tryptone Soya Broth (TSB) and Potato dextrose broth (PDB) respectively, over a period of 120 h in Myrothecium spp. Strain MRP001 (Figure 2).

Effect of temperature and pH on biomass production

The mycelia biomass of Myrothecium spp. Strain MRP001 was investigated at various temperatures (15 °C to 40 °C) in Potato dextrose broth at pH 6.5. The influences of temperature on the mycelia biomass of fungus was presented in Figure 3. The maximum biomass of 2.23 g/L was observed at 25 °C and pH 6.5, whereas moderate mycelial biomass of 2.14 g/L was observed at temperature 30 °C in pH 6.5. At 15 °C there was no fungal growth was observed. The results indicated that the optimum temperature for maximal biomass production was 25 °C to 30 °C and the pH was 6.5 in Figure 4.

Effect of carbon and nitrogen sources on production of fungal biomass

Myrothecium spp. Strain MRP001 was grown in basal growth medium of pH 6.5 at 30 °C for 120 h to find out the suitable carbon sources for the maximum mycelial biomass production, various carbon sources were separately provided instead of glucose (15 g/L) in the basal medium. Among the various carbon sources, glucose supplemented medium produced maximum biomass of 2.3 g/L. The other carbon sources produced the moderate mycelial biomass Figure 5.

Antibacterial Activity

The clinical strains of S. aureus (1–10) were found to be positive for various biochemical tests such as the coagulase test, mannitol utilization test, DNase test, and catalase activity. The antibiotic resistance profile of S. aureus strains (1–10) was determined using commercial antibiotics such as methicillin, penicillin, and
vancomycin. In contrast, P. aeruginosa (1–8) were identified by colony morphology, growth on cephaloridine cruciferin centrimide agar, a positive oxidase test, and growth at 42 °C. Antibiotics such as ciprofloxacin, cefotaxime, ofloxacain, and amikacin were used to determine the resistance profile of P. aeruginosa strains (1–8). The optimized ethyl acetate extract of cultural filtrate showed a maximum inhibition zone of 28.4 mm and 26.3 mm against multidrug resistant P. aeruginosa strain 1, and S. aureus strain 5, respectively (Table 1).

**Table 1** Antibacterial activity of optimized cultural filtrate extract of Myrothecium spp. against multidrug resistant S. aureus strains (1–10) and P. aeruginosa strains (1–8)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Penicillin (10 unit/ml)</th>
<th>Methicillin (10 µg/ml)</th>
<th>Vancomycin (30 µg/ml)</th>
<th>Ethyl Acetate Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>CF (5 µg/ml)</td>
<td>CE (30 µg/ml)</td>
<td>OF (5 µg/ml)</td>
<td></td>
</tr>
<tr>
<td>Strain 1</td>
<td>12.5 ± 0.15 (R)</td>
<td>10.8 ± 0.14 (R)</td>
<td>16.5 ± 0.08 (S)</td>
<td>20.8 ± 0.15</td>
</tr>
<tr>
<td>Strain 2</td>
<td>13.5 ± 0.4 (R)</td>
<td>12.4 ± 0.4 (R)</td>
<td>12.5 ± 0.35 (R)</td>
<td>27.5 ± 0.45</td>
</tr>
<tr>
<td>Strain 3</td>
<td>12.8 ± 0.14 (R)</td>
<td>11.2 ± 0.31 (R)</td>
<td>15.4 ± 0.28 (S)</td>
<td>24.6 ± 0.44</td>
</tr>
<tr>
<td>Strain 4</td>
<td>20.6 ± 0.1 (S)</td>
<td>22.5 ± 0.14 (S)</td>
<td>19.3 ± 0.28 (S)</td>
<td>13.5 ± 0.15</td>
</tr>
<tr>
<td>Strain 5</td>
<td>14.2 ± 0.28 (R)</td>
<td>12.1 ± 0.16 (R)</td>
<td>16.0 ± 0.22 (S)</td>
<td>25.2 ± 0.22</td>
</tr>
<tr>
<td>Strain 6</td>
<td>15.4 ± 0.28 (R)</td>
<td>14.9 ± 0.14 (R)</td>
<td>13.5 ± 0.4 (R)</td>
<td>27.6 ± 0.33</td>
</tr>
<tr>
<td>Strain 7</td>
<td>11.5 ± 0.35 (R)</td>
<td>10.2 ± 0.31 (R)</td>
<td>17.2 ± 0.14 (S)</td>
<td>21.1 ± 0.11</td>
</tr>
<tr>
<td>Strain 8</td>
<td>23.7 ± 0.23 (S)</td>
<td>16.8 ± 0.1 (R)</td>
<td>18.6 ± 0.31 (S)</td>
<td>22.7 ± 0.30</td>
</tr>
<tr>
<td>Strain 9</td>
<td>11.8 ± 0.14 (R)</td>
<td>8.9 ± 0.22 (R)</td>
<td>18.5 ± 0.28 (S)</td>
<td>26.3 ± 0.01</td>
</tr>
<tr>
<td>Strain 10</td>
<td>23.3 ± 0.04 (S)</td>
<td>24.8 ± 0.15 (S)</td>
<td>19.5 ± 0.14 (S)</td>
<td>22.4 ± 0.22</td>
</tr>
<tr>
<td>Strain 11</td>
<td>10.3 ± 0.15 (R)</td>
<td>9.5 ± 0.35 (R)</td>
<td>12.4 ± 0.35 (R)</td>
<td>21.0 ± 0.21</td>
</tr>
<tr>
<td>Strain 12</td>
<td>22.3 ± 0.15 (S)</td>
<td>25.4 ± 0.07 (S)</td>
<td>18.4 ± 0.28 (S)</td>
<td>19.3 ± 0.31</td>
</tr>
<tr>
<td>Strain 13</td>
<td>13.8 ± 0.16 (R)</td>
<td>15.5 ± 0.07 (S)</td>
<td>16.9 ± 0.14 (S)</td>
<td>23.4 ± 0.10</td>
</tr>
<tr>
<td>Strain 14</td>
<td>17.5 ± 0.22 (R)</td>
<td>23.7 ± 0.18 (S)</td>
<td>13.9 ± 0.4 (R)</td>
<td>23.0 ± 0.17</td>
</tr>
<tr>
<td>Strain 15</td>
<td>10.5 ± 0.23 (R)</td>
<td>8.8 ± 0.14 (R)</td>
<td>13.5 ± 0.21 (R)</td>
<td>22.7 ± 0.21</td>
</tr>
<tr>
<td>Strain 16</td>
<td>18.7 ± 0.14 (R)</td>
<td>16.5 ± 0.35 (R)</td>
<td>16.5 ± 0.14 (S)</td>
<td>22.8 ± 0.15</td>
</tr>
<tr>
<td>Strain 17</td>
<td>15.0 ± 0.21 (R)</td>
<td>12.2 ± 0.10 (R)</td>
<td>14.8 ± 0.22 (R)</td>
<td>20.4 ± 0.13</td>
</tr>
<tr>
<td>Strain 18</td>
<td>13.8 ± 0.1 (R)</td>
<td>8.7 ± 0.18 (R)</td>
<td>18.5 ± 0.35 (S)</td>
<td>22.6 ± 0.22</td>
</tr>
</tbody>
</table>

Legend: R – Resistant and S – Sensitive, CF – Ciprofloxacin, CE – Cefotaxime, OF – Ofloxacain

**DISCUSSION**

Identification fungal species of Myrothecium spp. Strain MRP001

The fungal isolate of Myrothecium spp. Strain MRP001 could not be identified to a species level using available sequence data in Myrothecium database (BLASTN) and morphological characteristics. However, based on the morphological and sequence analysis, these isolates are found to be different species of Myrothecium, hence we have named as Myrothecium spp. Strain MRP001.

Optimal culture conditions

The growth profile of Myrothecium spp. Strain MRP001 included studies on mycelial biomass. The maximum growth in terms of mycelia production occurred on Potato Dextrose Broth (PDB) followed by Tryptone Soya Broth (TSB), while it was lowest on Fred Waksman Basic 77 broth (FWB). On the contrary, (Bilay et al., 2000) reported that Ganoderma lucidum had a slow growth rate in PDB medium, whereas there was a significant fungal biomass production in Mueller Hilton Broth (MHB). Similarly, the maximum fungal biomass production of Ganoderma species was observed in basal medium (Roberts 2004). The study of mycelial characteristics showed white, thick mat of fluffy growth on Potato dextrose broth while it was white thick mat of strand growth on Tryptone Soya Broth (TSB). At the same time, slow growth of slightly strandy mycelium was observed on Mueller Hilton Broth (MHB). The initial medium pH is a critical factor associated with the growth of fungi because it will affect the cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis (Qing & Jian, 2002). In this study, Myrothecium spp. Strain MRP001 was cultivated in the PDB and Tryptone Soya Broth (TSB) with different initial pH 4-7 and different initial temperatures 20-40 °C, in culture conditions to investigate the effects of pH and temperature on mycelial biomass and antibacterial activity.

The results indicated that the optimum temperature and pH for maximal biomass production were 6.5 and 25 °C to 30 °C respectively. Earlier reports revealed that many kinds of fungi grow at acidic pH optimia (Kim et al., 2003; Lee et al., 1999; Shu & Lung, 2004). Similarly, (Lee et al., 2004) reported that the optimum pH was 5.5 for the maximum production of mycelial biomass of the fungus Grifola frondosa. The effect of temperature on mycelial production is comparable to the growth of many kinds of fungal species (Bae et al., 2000; Kim et al., 2003). Similarly, present results seem to be consistent with other reports in which the optimum temperature for fungal growth was 20 °C to 30 °C (Boddy, 1985; Lee et al., 2004; Lai et al., 2012).

**Optimal nutrient sources**

In general, mycelial cells of fungi grow over a broad range of carbon source (Burns et al., 1994). To determine the suitable carbon source for the production mycelial biomass with enhanced antibacterial compound in Myrothecium spp. Strain MRP001, six carbon sources were separately provided at the concentration of 15 g/l instead of glucose employed in the basal medium. Among the carbon sources tested, glucose yielded the highest mycelial production. Medium containing glucose was significant in yielding the highest mycelia growth when compared to the other carbon sources. Similarly, (Xiao et al., 2006) reported that glucose, sucrose, yeast and peptone were supported more fungal biomass production in Xylaria sp. 2508. Glucose was found to be the best source for maximum biomass production in Lentinus edodes (Song & Cho, 1987). Nitrogen plays an important role in fungal growth and metabolite production (Kim et al., 2005). Thus, the effects of nitrogen on mycelial biomass production of Myrothecium spp. Strain MRP001 in the form of ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate, calcium nitrate and yeast extract were studied. Among the six nitrogen sources that have been tested in this study, yeast extract was most suitable for the growth of mycelial biomass (1.3 g/l). However, current findings are contrary to those of (Shih et al., 2006) that suggested most basidiomycetes prefer complex organic nitrogen sources for their favorable submerged cultures. The final pH of the medium remains in slightly acidic range. It is generally known that the byproduct of fungal metabolic process was acidic in nature (Fang & Zhong, 2002).

**Antibacterial Activity**

Many fungi have a great potential for the production of useful bioactive metabolites and they are a prolific resource for drugs. The spectrum of pharmacological activities of fungi is fascinating. Discovery and evaluation of new bioactive compounds from various fungi as new safe compounds for the treatment of various diseases has become a hot research spot.

In present study, the antibacterial activity of the optimized ethyl acetate extract of culture filtrate was investigated against drug resistant pathogens. The antibiotic resistant profiles of S. aureus and P. aeruginosa strains were reported earlier (Ramesh et al., 2012). Maximum inhibition zone of 28.4 mm and 26.3 mm was observed against multidrug resistant P. aeruginosa strain 1, and S. aureus strain 5, respectively. Similarly, (Iwalokun et al., 2007) reported that the petroleum ether and acetone extract of fungal species of Pleurotus ostreatus showed significant antibacterial activity against multidrug resistant P. aeruginosa.

**CONCLUSIONS**

Therefore, from the above results it can be concluded that it was possible to develop an optimized medium with regard to cultural conditions and nutritional
souces to produce biomass of *Myrothecium* spp. Strain MRPP01 with enhanced antibacterial activity against drug resistant human bacterial pathogens. High biomass was obtained in culture filtrate using the optimized medium with 15 g/L of glucose and 10 g/L of yeast extract as carbon and nitrogen sources respectively. To the best of our knowledge, this is the first report concerning the highest biomass production obtained in submerged fermentation by the same species.

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