Optimization Production Xylanase Thermostable by *Bacillus licheniformis* TS10 Using Substrate Oil Palm Empty Fruit Bunches

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ABSTRAK

Optimasi produksi xilanase termostabil oleh *Bacillus licheniformis* TS10 menggunakan substrat tandan kosong kelapa sawit telah dilakukan di Laboratorium Genetika dan Bioteknologi, Jurusan Biologi Fakultas Matematika dan Ilmu Pengetahuan Alam, Universitas Sriwijaya, Indralaya. Penelitian dilakukan sejak November 2015 hingga Januari 2016. Penelitian ini bertujuan untuk mengetahui potensi tandan kosong kelapa sawit (TKKS) sebagai substrat untuk produksi xilanase termostabil oleh *Bacillus licheniformis* TS10 serta kondisi optimum suhu, pH dan konsentrasi substrat pada proses fermentasi. Metode penelitian adalah dengan membuat kurva pertumbuhan dan kurva produksi enzim dari *Bacillus licheniformis* TS10 pada substrat TKKS. Jumlah sel bakteri ditentukan dengan menggunakan spektrophotometer UV-VIS dan *total plate count* (TPC) melalui kurva standar. Optimasi dilakukan pada variasi pH (5, 6, 7, 8, 9), suhu (50 °C, 60 °C, 70 °C, 80 °C) dan substrat (1%, 2%, 3%, 4%). Uji aktivitas pada masing-masing pH, suhu dan substrat menggunakan metode DNS dengan mengukur aktivitas enzim berdasarkan hasil gula reduksi yang dilepaskan oleh substrat dengan menggunakan dinitrosalicylic acid (DNS). Berdasarkan hasil penelitian dapat diketahui bahwa tandan kosong kelapa sawit (TKKS) memiliki potensi sebagai substrat untuk produksi xilanase termostabil oleh *Bacillus licheniformis* TS10, produksi xilanase termostabil dari *Bacillus licheniformis* TS10 pada substrat TKKS memiliki kondisi optimum pada pH 6, suhu 80 °C dan konsentrasi substrat sebesar 4%.

Kata kunci : Optimasi, Xilanase Termostabil, *Bacillus licheniformis* TS10, Tandan Kosong Kelapa Sawit.

ABSTRACT

Optimization of thermostable xylanase production by *Bacillus licheniformis* TS10 using substrate oil palm empty fruit bunches has been conducted from November 2015 to January 2016 in the Laboratory of Genetics and Biotechnology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Sriwijaya, Indralaya. The aims to determine the potential of oil palm empty fruit bunches (EFB) as a substrate for the production of thermostable xylanase by *Bacillus licheniformis* TS10 and the optimum conditions of temperature, pH and substrate concentration in the fermentation process. The research method are to make the growth curve and the curve of *Bacillus licheniformis* TS10 enzyme production on EFB substrate. The number of bacterial cells was determined using a UV-VIS spectrophotometer and total plate count (TPC) through a standard curve. The optimization performed of pH (5, 6, 7, 8, 9), temperature (50 °C, 60 °C, 70 °C, 80 °C) and substrate (1%, 2%, 3%, 4%). The test activity in various pH, temperature and substrate using methods DNS by measuring enzyme activity based on the reducing sugar released by the substrate by using Dinitro Salicylic Acid (DNS). The results showed that oil palm empty fruit bunches (EFB) has a potential as a substrate for the production of thermostable

xylanase by *Bacillus licheniformis* TS10, thermostable xylanase production of Bacillus licheniformis TS10 on the substrate EFB has an optimum condition at pH 6, 80 °C and the substrate concentration of 4%.

Keywords: Optimization, thermostable xylanase, *Bacillus licheniformis* TS10, oil palm empty fruit bunches (EFB).

INTRODUCTION

An enzyme is a protein that has a biochemical activity as a reaction catalyst (Safaria, 2013). As a catalyst, an enzyme is an option that is expected to reduce the impact of environmental pollution and waste of energy because the reaction does not require high energy, as well as specific and non-toxic. Xylanase is an enzyme that hydrolyzes xylan (hemicellulose) into xylose. Hydrolysis of xylan by xylanase produce simple sugars monomer form xylooligosakarida, xylobiose, and xylose (Richana *et al*., 2002).

Xylanase can be applied in various industrial fields. In the papermaking industry, xylanase act as substitutes for the use of chlorine to bleach paper has provided opportunities for biotechnological applications. Utilization of xylanase in the pharmaceutical industry as tablet coating material and low-calorie sweetener (Kulkarni *et al*., 1999). In the food and beverage industry to work to improve the quality of bread and syrup purification. It also serves as an animal feed mixes as well as the manufacture of sugar xylose (Putra *et al*., 2012).

Utilization of xylanase in the industrial field generally requires suitable environmental factors, one of which is a high temperature. Therefore, it needs to be thermostable xylanase, due to the environmental factors most damaging enzyme is temperature. According to Trismilah and Waltam (2009), thermostable xylanase is stable at high temperatures. This enzyme has some advantages in their use, which increases the speed of the reaction so as to save time, labor, and operating costs; reduce the possibility of contamination; and more stable during longer storage.

Xylanase can be produced by several organisms such as bacteria, algae, fungi, actinomycetes (Beg *et al*., 2001), yeast, protozoa, gastropods and arthropods (Kulkarni *et al*., 1999). Some types of bacteria are known to produce extracellular xylanase to hydrolyze hemicellulose into xylose (Septiningrum and Chandra, 2009). Bacteria were reported to have activity xylanolytic was like, *Bacillus halorudans* CM1 (Wibowo, 2014), *Pseudomonas* sp. (Susilowati *et al*., 2012), *Streptomyces* sp. S27 (Li *et al*., 2009) and *Actinomadura* sp. S14 (Sriyapai, 2011). Thermophilic bacteria can survive at high temperatures 50-65 °C (Soeka *et al*., 2011). *Bacillus licheniformis* TS10 is one of the thermophilic bacteria isolated from Hot Springs Tanjung Sakti Lahat which has the highest xylanolytic index of 0.63 (Muharni *et al*., 2013) so that it can be used as a superior strain for the production of enzymes.

Enzyme production process using microorganisms, have stages that need to be considered include the selection of strains and setting the process conditions. Setting process conditions include the composition of the media, inducers, repressors, pH, temperature, aeration and agitation (Murni *et al.*, 2011). Determination of the temperature and pH of cultivation is an important factor for the growth of microorganisms and their metabolites product formation (Septiningrum and Chandra, 2011). pH is too high or low can inhibit enzyme activity and allows the structure to be damaged (Supryanto, 2009). Temperature also affects the action of the enzyme, since the enzymes consist of protein. The enzyme can perform its activities in a certain temperature range. The higher the temperature of the chemical reaction will be faster, but the enzyme will undergo

denaturation if the temperature is too high (Septiningrum and Chandra, 2011). The substrate serves as an inducer for the growth of microbes in producing xylanase.

Economically pure xylan use on an industrial scale is too expensive, therefore it should attempt to find the source of carbon is relatively cheaper. Agricultural wastes which have the main components of lignocellulose (hemicellulose, cellulose, and lignin) such as bagasse and oil palm empty fruit bunches (EFB) is expected to be used as a carbon source. Based on research Yuniar (2013), hemicellulose contained in EFB amounted to 22.84%. According to Puspaningsih *et al*. (2007), that the hemicellulose content high enough is approximately 30%.

Production of *Bacillus licheniformis* TS10 thermostable xylanase uses EFB substrate needs to be optimized. This optimization includes pH, temperature, and substrate concentration it aims to increase xylanase production so as xylanase produced optimally. Therefore, in this research, the optimization of production of *Bacillus licheniformis* TS10 thermostable xylanase using oil palm empty fruit bunches (EFB).

MATERIALS AND METHODS

This research was conducted in November 2015 to January 2016. The research was conducted at the Laboratory of Microbiology, Laboratory of Genetics and Biotechnology, Department of Biology, Faculty of Mathematics and Natural Sciences, Sriwijaya University, Indralaya.

Preparation

Rejuvenation Bacterium *Bacillus licheniformis* TS10

Bacteria which will be used first in the media rejuvenated NA slant and incubated at 55 °C for 48 hours.

Culture *Bacillus licheniformis* TS10

One isolate of *Bacillus licheniformis* TS10 ose who has rejuvenated inoculated into 10 ml NB medium, then incubated in an incubator shaker at a speed of 150 rpm and a temperature of 55 °C for 24 hours. These cultures were then transferred to 75 ml NB new medium and incubated for 48 hours.

Curves Standard

Liquid cultures of *Bacillus licheniformis* TS10 dilution from 10^{-1} to 10^{-7} each 1.5 ml taken into eppendorf tube and its absorbance determined using UVmini-1240 UV-VIS spectrophotometers at a wave length of 550 nm. Then 1 mL is taken from each dilution and grown in petri dishes containing medium NA with techniques so poor (pour plate), incubated for 24 hours at 55 °C and then the number of colonies that grow calculated by standard plate count method.

The results of OD value used to create a standard curve. The number of colonies is plotted on the x-axis and data OD values were plotted on the y-axis. Furthermore, the linear regression equation determined.

Linear regression equation: $y = a + bx$

Information :

y = absorbance (OD)

a = point of intersection with the y-axis

x = number of colonies

b = regression coefficient

The standard curve that has been created is used to calculate the number of bacterial cells.

Preparation Hemicellulose

Agricultural waste oil palm empty fruit bunches to first cut into small pieces and then dried and then oven-dried, after dried and then milled. Delignification oil palm empty fruit bunches (EFB) conducted by the sample was washed with water and heated. The residue is then soaked 2% NaOH for 2 hours, then washed to pH neutral. Delignification samples dried in an oven at 80 °C (Li *et al*., 2010).

The Growth Curve

NB sterile media containing oil palm empty fruit bunches 1% inoculated with 10% (v/v) inoculum of bacteria. The cultures were incubated at a temperature of 55 °C with a speed of 150 rpm. The culture samples were taken periodically every 4 hours for 48 hours, and bacterial growth is determined using UVmini-1240 UV-VIS spectrophotometers at a wavelength of 600 nm.

The Effect Of Temperature On Xylanase Production

The effect of temperature on xylanase production was determined by culturing the bacteria in media NB (Nutrient Broth) of 50 mL in the respective substrates 1% pure xylan, and 1% of empty oil palm bunches at varying temperatures at 50 °C, 60 °C, 70 °C and 80 °C. Incubation was performed in accordance with the time of optimum growth of bacteria in the manufacture of a growth curve in the incubator shaker with a speed of 150 rpm. Production of the enzyme was determined by measuring the activity of xylanase. The pure xylan used is birchwood

Effect Of pH On Xylanase Production

The optimum pH for the activity of production is determined by culturing the bacteria in media NB (Nutrient Broth) of 20 mL in the respective substrates 1% pure xylan, and 1% of empty fruit bunches of oil palm on the pH value varied at 5, 6, 7, 8, 9 using acetate buffer pH 5, phosphate buffer pH 6-8, and NaOH-glycine buffer pH 9, at a temperature that is obtained after optimization of temperature. Incubation was performed in accordance with the time of optimum growth of bacteria in the manufacture of a growth curve in the incubator shaker with a speed of 150 rpm. Production of the enzyme was determined by measuring the activity of xylanase.

Effect Of Substrate Concentration On The Production Of Xylanase

Effect of substrate concentration on the production of xylanase was determined by culturing the bacteria in media NB (Nutrient Broth) of 20 mL in the substrate concentration is varied at 1%, 2%, 3%, and 4% at a temperature and pH were obtained after optimization of temperature and pH. Incubation was performed in accordance with the time of optimum growth of bacteria on a growth curve in the manufacture incubator shaker with a speed of 150 rpm. Production of the enzyme was determined by measuring the activity of xylanase.

Observation of Cells Number

Bacterial cell growth determined using UVmini-1240 UV-VIS spectrophotometers at a wavelength of 600 nm. The number of cells is calculated using the standard curve created.

Xylanase Activity (Bailey, 1992)

Bacterial culture is taken as 1.5 mL in NB medium containing xylan substrate into eppendorf tubes. The bacterial culture containing xylan substrate was then centrifuged at a speed of 5000 rpm for 15 minutes 4 °C. The supernatant is at the top was taken as a crude extract enzyme. The xylanase activity was measured by mix enzyme crude extract 70 mL, 630 mL of 1% xylan substrate, plus 1 ml of phosphate buffer. Solution mixture is then incubated for 5 min at 55 °C. Incubation was stopped by addition of 750 mL of DNS reagent, then mix the solution is heated for 5 minutes then cooled to room temperature. Absorbance xylanase activity was measured using UVmini-1240 UV-VIS spectrophotometers at a wavelength of 485 nm xylose. As a control xylanase activity (to zero), to use the reaction mixture as above without using enzymes.

The xylanase activity was determined by measuring the levels of reducing sugars liberated during the hydrolysis reaction of xylan by xylanase. One unit of xylanase activity is defined as the amount of enzyme which can produce 1 mol of xylose/minute/mL in certain circumstances. Levels of xylose contained in each sample and control are calculated based on the standard curve xylose.

Data Presentation

Data obtained from the results of the research presented in the form of images and tables.

RESULTS AND DISCUSSION

Growth and Production of Xylanase from *Bacillus licheniformis* TS10 On Substrates Oil Palm Empty Fruit Bunches (EFB) and Pure Xylan

Growth and production of xylanase from *Bacillus licheniformis* TS10 can be seen in figure 1.

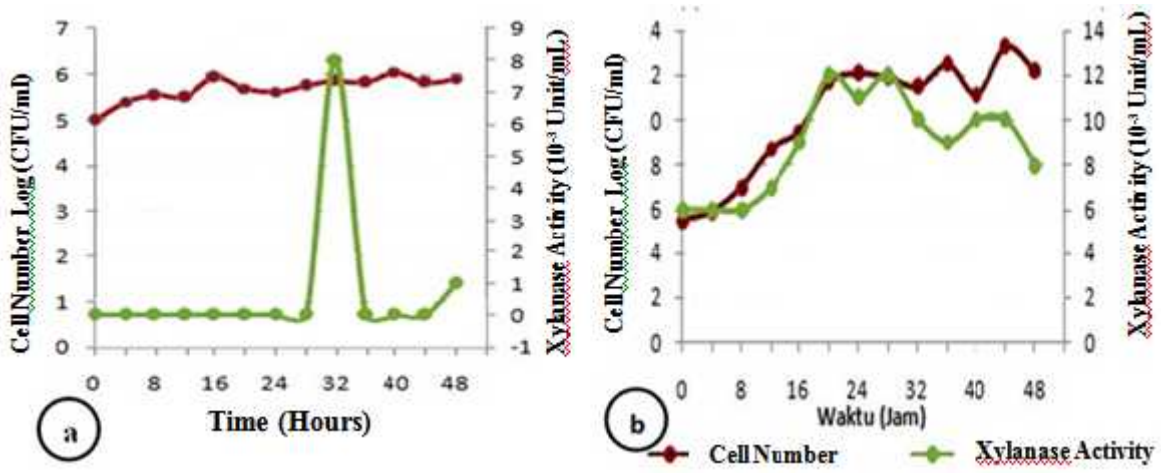

Figure 1. Growth and Production of Xylanase from *Bacillus licheniformis* TS10 On substrate EFB (a) and Pure Xylan (b).

The growth of cells on substrates oil palm empty fruit bunches (EFB) are lower when compared to pure xylan (Fig 1a.). The highest number of cells on substrates EFB an 7.10×10^5 CFU/mL at 32 hour. Differences in growth and the number of cells of *Bacillus licheniformis* TS10 on each substrate caused by different substrates used. Different substrates have different nutrient content, therefore the growth of *Bacillus licheniformis* TS10 on each substrate is also different. Nutrients needed in growth such as

carbon which can be found on the substrate for cell formation. According to Safaria *et al*. (2013), that the carbon source is obtained from the substrates used. Carbon serves as a key element in the formation of cells.

The growth of *Bacillus licheniformis* TS10 on the substrate EFB through a phase of adaptation on the hour to 0-4 and 20-24 (Fig 1a.). In this phase, there was a period in which the culture are incorporated to adjust to the new environment (Judoamidjojo *et al*., 1989). Upon entering the adaptation phase, *Bacillus licheniformis* TS10 entering log phase, in this phase the bacteria split rapidly. Log phase in the growth of *Bacillus licheniformis* TS10 substrate EFB has two phases log (Fig.1a.) With different growth rate. Growth occurs in an EFB diauxic growth. According to Saragih (2013), diauxic growth at isolates had two logarithmic phase of growth at different speeds.

Diauxic growth on substrate EFB, allegedly caused by *B. licheniformis* TS10 using NB (Nutrien Broth) as a simpler source of nutrition in the early log phase that occurs at 16 hour. After the NB runs out, *B. licheniformis* TS10 immediately enters the adaptation phase at 20-24 hour on the EFB to break the hemicellulose (xylan) into a simple carbon. Next enter the second log phase at the 40 hour. According to Baker *et al*. (2011) *in* Saragih (2013), diauxic growth are due to the utilization of the availability of different nutrients in the media resulting changes in the growth rate of the isolates.

Optimization Of pH

Based on the research, showed that the optimum pH for growth and xylanase production on various substrates with a predetermined pH range (Figure 2). Figure 2 shows that the optimum pH EFB growth is obtained at pH 5 and pH optimum for the xylanase production was at pH 6.

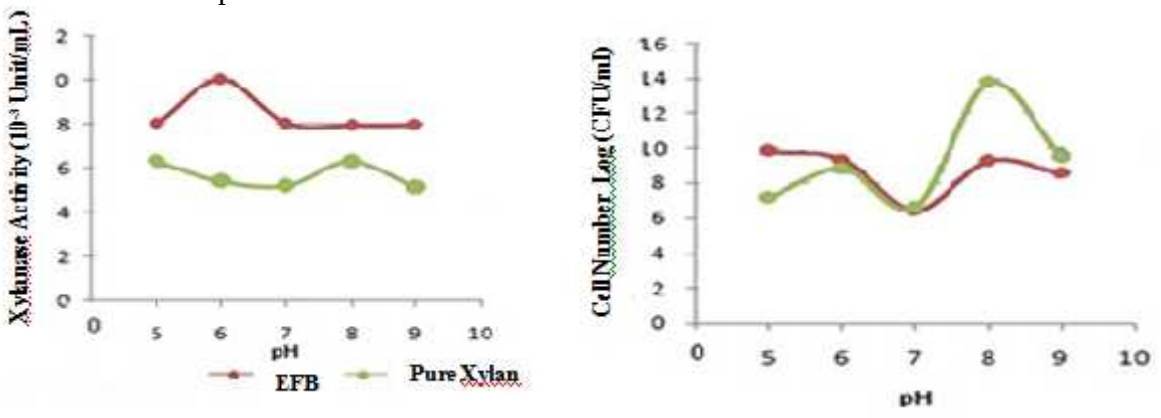

Figure 2. Number of Cells and xylanase activity at Various pH

Unlike the case with the EFB, the pure xylan optimum pH for growth at the optimum pH for production xylanase at pH 8 (Figure 2). According to Ilmi *et al*. (2013), that the optimum pH of the enzyme is not necessarily the same as the normal environmental pH, the pH may slightly be above or below the optimum pH. The catalytic activity of the enzyme inside the cell may be governed in part by changes in the pH of the medium environment. It is also endorsed by Augustine (2005) *in* Nareswari (2007), *Bacillus licheniformis* AQ1 are bacteria that can produce xylanase enzyme with optimum conditions of pH 8. Meanwhile, according to Nareswari (2007), xylanase produced from *Bacillus licheniformis* AQ1 have a pH range of 7- 9 after incubation for 2 hours.

Temperature Optimization

Based on temperature optimization that has been implemented in this research, showed that the substrate oil palm empty fruit bunches (EFB) have an optimal temperature of growth of *Bacillus licheniformis* TS10 similar to pure xylan are 50 °C (Figure 3). In EFB substrate has a cell count of 9.95×10^{13} CFU/mL while on a pure xylan substrate has a cell count of 1.40×10^{13} CFU/mL.

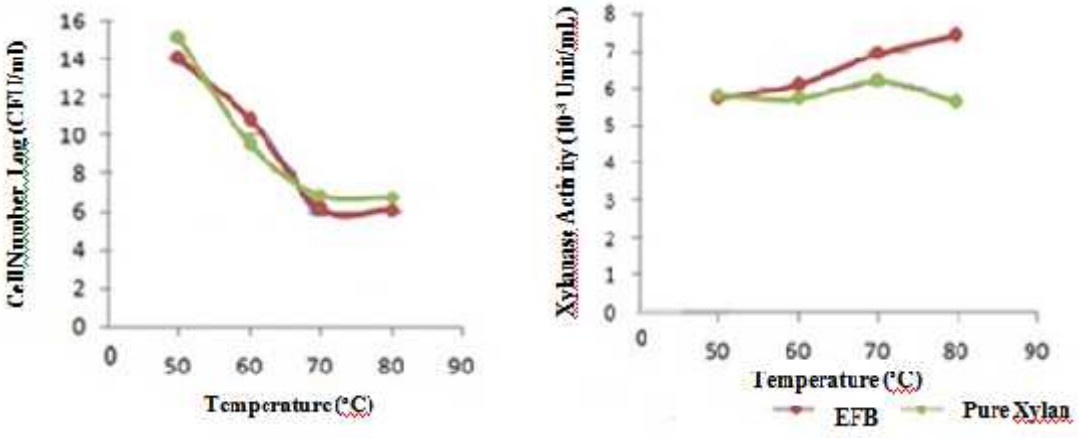

Figure 3. Number of Cells and xylanase activity on Temperature Variations

The production of xylanase from *Bacillus licheniformis* TS10 on a substrate EFB different from pure xylan (Figure 3). In EFB substrate, obtained the optimal temperature for the production of xylanase from *Bacillus licheniformis* TS10 is 80 °C of 7.4×10^{-3} Unit/mL pure xylan while at the optimal temperature for xylanase production was 70 °C at 6.1×10^{-3} units/mL. In this research, using a temperature range of 50-80 °C. Xylanase activity produced at any temperature increased so that the optimal temperature for the production of xylanase obtained from the highest xylanase activity.

Optimization Substrate

The results showed that the optimum substrate concentration EFB substrate for the growth and production of xylanase that is 4% with a xylanase activity of 5.3×10^{-3} Unit/mL and a cell count of 1.01×10^{10} CFU/mL, at pH 6 and fermentation conditions temperature of 80 ºC for 32 hours at 150 rpm agitation. In pure xylan, optimum substrate concentration on the growth and production of xylanase that is 4% with a xylanase activity of 4.1×10^{-3} Unit/mL and a cell count of 1.40×10^{9} CFU/mL, on condition of fermentation pH 8 and temperature 70 ºC for 20 hours at 150 rpm agitation.

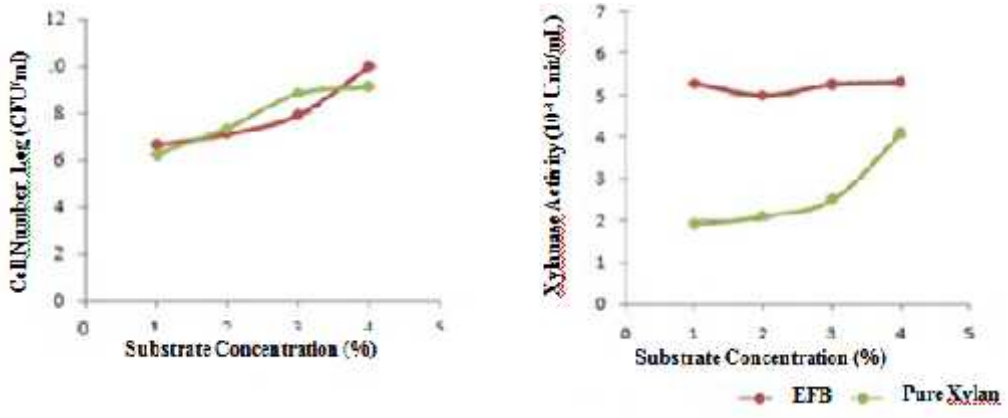

Figure 4. Number of Cells and xylanase activity on Substrate Variation (%)

In Figure 4 it can be seen that in each substrate has a xylanase production different. The substrate used in the fermentation media affect the production of xylanase. This is consistent with the statement Hastari *et al*. (2014), that the type of substrate affects the production of xylanase.

CONCLUSION

Based on the research was concluded that the production of a thermostable xylanase from *Bacillus licheniformis* TS10 substrate EFB has optimum at pH 6, a temperature of 80 °C and the substrate concentration of 4%.

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