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Zymomonas mobilis immobilized on loofa sponge: levan and ethanol production in semi-continuous fermentation

Vidiany Aparecida Queiroz Santos[1*], Jesus Vega- Estrada[2], Maria Del Carmen Montes Horcasitas[2] and Crispin Humberto Garcia-Cruz[1]

*[1]Departamento de Engenharia e Ciências de Alimentos, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Cristóvão Colombo, 2265, 15054-000, São José do Rio Preto, São Paulo, Brazil. [2]Departamento de Biotecnologia e Bioengenharia, Centro de Pesquisa e Estudos e Avançados do Instituto Politécnico Nacional, San Pedro Zancatenco, México, DF. *Author for correspondence. E-mail: vidianyqueiroz@yahoo.com.br*

ABSTRACT. *Zymomonas mobilis* is a promising microorganism in the biotechnological production of ethanol and levan due to its low biomass and high tolerance to ethanol concentrations. Ethanol and levan production by *Z. mobilis* CDBB-603 immobilized on loofa sponge using semi-continuous fermentation is evaluated. The first experiment, conducted with initial volume 50 mL and incubated for 24 hours, produced maximum ethanol and levan production, respectively 13.56 g L^{-1} and 23.94 g L^{-1}, with 250 g L^{-1} sucrose and without agitation, at 30°C. The second experiment was based on the best condition obtained in the first, using fermenter with production scale of 200 mL and by semi-continuous process. The second experiment also assessed immobilized biomass reuse during 10 days (240 hours) and produced higher ethanol (34.64 g L^{-1}) and levan (26.40 g L^{-1}) production rates than in the first experiment. Experiment 2 also verified that the microorganism remained viable and produced ethanol and levan until the last (10^{th}) recycle day.

Keywords: Luffa cylindrical, attachment, exopolysaccharide, alcoholic fermentation.

Zymomonas mobilis imobilizada em bucha vegetal: produção de etanol e levana por sistema semi-contínuo

RESUMO. *Zymomonas mobilis* é um microrganismo com alto potencial tecnológico para a produção de etanol e levana, devido à baixa formação de biomassa e alta tolerância a elevadas concentrações de etanol. O objetivo deste estudo foi avaliar a produção de etanol e levana por *Z. mobilis* imobilizada em bucha vegetal, utilizando fermentação semi-contínua. No primeiro experimento, conduzido com volume inicial de 50 mL de meio de cultivo, obteve-se máxima produção de etanol (13.56 g L^{-1}) e levana (23.94 g L^{-1}), utilizando 250 g L^{-1} de sacarose como substrato, ausência de agitação e temperatura de 30°C durante 24h. O segundo experimento, foi realizado com base nas melhores condições de fermentação obtidas no primeiro, utilizando processo semi-contínuo em reator de bancada e 200 mL de volume final. No segundo experimento, foi avaliada a reutilização do suporte contendo o microrganismo imobilizado durante dez dias (240h) de fermentação. O experimento 2 apresentou maior produção de etanol (34.64 g L^{-1}) e levana (26.40 g L^{-1}) em relação ao primeiro experimento e o microrganismo imobilizado permaneceu viável e produziu etanol e levana até o último dia da fermentação semicontínua (10^{o} reciclo).

Palavras-chave: Luffa cylindrica, adsorção, exopolissacarídeo, fermentação alcoólica.

Introduction

The use of microorganisms in the production of important industrial biocompounds, such as ethanol and levan, is object of intense research worldwide, due to exhausting exploration of conventional sources (Bandaru, Somalanka, Mendu, Medicherla, & Chityla, 2006; Behera, Ray, & Monhanty, 2010; Cazzeta, Celligoi, Buzato, & Scarmino, 2007; Nikolic´, Mojovic´, Rakin, & Pejin, 2009).

Zymomonas mobilis has potential to ethanol and levan production. However, its efficiency may be impaired due to free cells that remain largely exposed to adverse conditions during fermentation (Behera et al., 2010; Fu, Peiris, Markham, & Bavor, 2009). In fact, this bacterium is a high biotechnological potential microorganism due to its tolerance to high ethanol and sugar concentrations, low biomass production and different metabolic pathway use. *Z. mobilis* is able to produce different compounds by changing growing conditions and has cell growth independent of carbon consumption (Kalnenieks, 2006).

The main issue problem encountered in traditional fermentation techniques that use free

cells is the high cost of raw materials and low production yield (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). Owing to these difficulties, immobilized cells have been intensively analyzed lately for ethanol and levan production since they have advantages over conventional cell suspension. Among advantages can be highlighted the higher cell concentration in the immobilization support, repeated recycle operations and improvement in efficiency and productivity (Genisheva, Mussatto, Oliveira, & Teixeira, 2011; Yu, Yue, Zhong, Zhang, & Tan, 2010).

Several supports for cell immobilization, such as alginate, carrageen, rice husk, sugarcane or sorghum bagasse, wheat; corn cobs and loofa sponge, have been evaluated and showed interesting results (Kandylis, Drouza, Bekatorou, & Koutinas, 2010; Behera et al., 2010; Yao, Wu, Zhu, Sun, Zhang, & Miller, 2011; Yu et al., 2010).

Loofa sponge is a natural and fibrous material basically composed of cellulose, hemicellulose and lignin forming an interlaced structure with high porosity. This support shows many advantages such as good stability to different chemical and physical treatments, biodegradability, non-toxicity, low cost and a large surface for cell attachment (Marques, Buzato, & Celligoi, 2006; Behera et al., 2010). In front of these characteristics, loofa sponge has been studied as immobilization support for different compounds production (Fujii, Oki, Sakurai, Suya, & Sakakibara, 2001; Roble, Ogbonna, & Tanaka, 2002; Ogbonna, Liu, Liu, & Tanaka, 1994; Ogbonna, Tomiyama, & Tanaka, 1996; Ogbonna, Mashima, & Tanaka, 2001; Liu, Dong, Zhong, Ryu, & Bao, 2010; Phisalaphong, Budiraharj, Bangrak, Mongkolkajit, & Limtong, 2007).

Therefore, the aim of this study was to evaluate the ethanol and levan production by *Zymomonas mobilis* CDBB-603 immobilized on loofa sponge using semi-continuous fermentation.

Material and methods

Microorganism and culture conditions

Zymomonas mobilis CDBB-603, obtained from the National Collection of Microbial Cultures CINVESTAV-IPN-MEXICO, was grown and maintained in a liquid medium g L^{-1}: 10.0 yeast extract, 10.0 peptone and 20.0 glucose. The inoculum cultivation was realized by inoculation of *Z. mobilis* 10 % (w v^{-1}) in Erlenmeyer flasks containing the same medium and then, was incubated in orbital shaker (100 rpm) during 24 hours at 30°C. Fermentation broth was composed by yeast extract 5.0 g L^{-1}; KH_2PO_4 1.0 g L^{-1}; $(NH_4)_2SO_4$ 1.0 g L^{-1}; $MgSO_4$. 7 H_2O 1.0 g L^{-1} and initial pH of 4.95.

Immobilization on loofa sponge

Immobilization was performed by adsorption, with modifications in each experiment. In first experiment, immobilization was carried out in Erlenmeyer flasks containing 12.0 g L^{-1} of loofa sponge, previously cut in 2 cm × 2 cm pieces and autoclaved at 121°C for 15 minutes. After, loofa sponge pieces were inoculated with *Z. mobilis* suspension (10 %, w v^{-1}) and growth media. Erlenmeyer flasks were incubated during 24 hours at 30°C and 150 rpm^{-1}. The loofa sponge pieces were then washed in distilled water to remove non-immobilized cells and used for fermentation.

In the second experiment, immobilization was performed directly in the reactor and changes in the support fermentation design were required. Immobilization support was attached in stainless steel wire to prevent that loofa sponge pieces interrupt the agitation medium in the reactor. The same concentration of loofa sponge cited before was used and the immobilization medium was similar to the fermentation medium but using glucose (100 g L^{-1}). Then, the reactor was inoculated with *Z. mobilis* (10 %, w v^{-1}) and incubated for 24 hours at 30°C and 200 rpm for immobilization. After, immobilization medium was removed and the support containing immobilized cells was washed in distilled water. Then, fresh fermentation medium and sucrose (250 g L^{-1}) were added.

Experiment 1

Response surface methodology was used to evaluate the best conditions for ethanol and levan production. Fermentations were realized in Erlenmeyer flasks contained fermentation medium and sucrose (250 g L^{-1}) using 50 mL of final volume. All experiments were carried out without pH control. Evaluated variables are described in Table 1.

Table 1. Variables evaluated in statistical design 2^2 for levan-ethanol production.

Variables	Level		
	-1	0	+1
X_1: sucrose (g L^{-1})	150	250	350
X_2: temperature (°C)	30	35	40
X_3: agitation (rpm)	0	100	200

Experiment 2

The second experiment was based on the high production obtained in the first experiment. The bioreactor used was an in-house-design with jar. reactor with capacity of 500 mL and 6 cm in

diameter and 16 cm in height. The following parameters were employed: pH control absence; 200 rpm for medium homogenization; oxygenation absence and temperature at 30°C. Repeated recycle fermentations were carried out for ethanol and levan production during 10 days and using final volume of 200 mL in the reactor.

Fermentation medium at the end of each fermentation recycle was removed from the reactor and a fresh medium at a similar volume was transferred for the next cultivation recycle. Recycles were repeated till ethanol and levan production reduces to levels considerably low. After each recycle, was determined the ethanol and levan production and residual sugars in fermentation medium.

Analytical methods

Free biomass was removed by centrifugation (12000 rpm by 10 min.$^{-1}$ at 4°C) and the supernatant was used for determination of levan, ethanol and reducing sugar.

Ethanol concentration was measured by microdiffusion method, according described by Noriega-Medrano et al. (2016) with minor modifications. Reducing sugars were measured by dinitrosalicylic acid method (Miller, 1959) with previously sucrose hydrolysis using hydrochloric acid (HCl) concentrated for 9 minutes in boiling water and neutralized with sodium hydroxide (NaOH) 40%. All determinations were performed in triplicate.

Cellular growth in immobilization support was determined by optical density at 600 nm, using a standard curve of the absorbance against dry cell mass. Initially, the immobilized cell on loofa sponge pieces was removed from the support by fast agitation (240 rpm during 2 hours) in flasks with distilled water and then, the optical density was determined.

Levan was precipitated from fermentation medium (without cells) using three volumes of cold ethanol, overnight, followed by centrifugation at 12000 rpm for 20 minutes. It was further washed with distilled water and re-centrifuged under the same conditions mentioned before. Levan concentration was determined as fructose units by the dinitrosalicylic acid method (Miller, 1959), after hydrolysis with 0.13 M hydrochloric acid (HCl) solution, during 60 minutes in boiling water, following Borsari, Celligoi, Buzato, & Silva (2006), with adaptations.

Results and discussion

Effect of sucrose, temperature and agitation media on ethanol and levan production

According to ANOVA, agitation medium significantly affected ($p < 0.05$) ethanol and levan production using *Z. mobilis* CDBB 603 immobilized on loofa sponge. However, the agitation showed a negative effect since the best production was obtained in agitation absence.

On the other hand, the Response Surface Methodology (RSM) revealed that increase in levan production 13.56 g L^{-1} (0.56 g L h^{-1}) was accompanied by an increase in sucrose concentration (250 and 350 g L^{-1}) and a lower fermentation temperature (30 at 35°C). Maximum ethanol production (23.94 g L^{-1}; 0.98 g L h^{-1}) was also obtained using the same temperature range above cited, but, initial sucrose concentration did not affect ethanol production, as Figure 1.

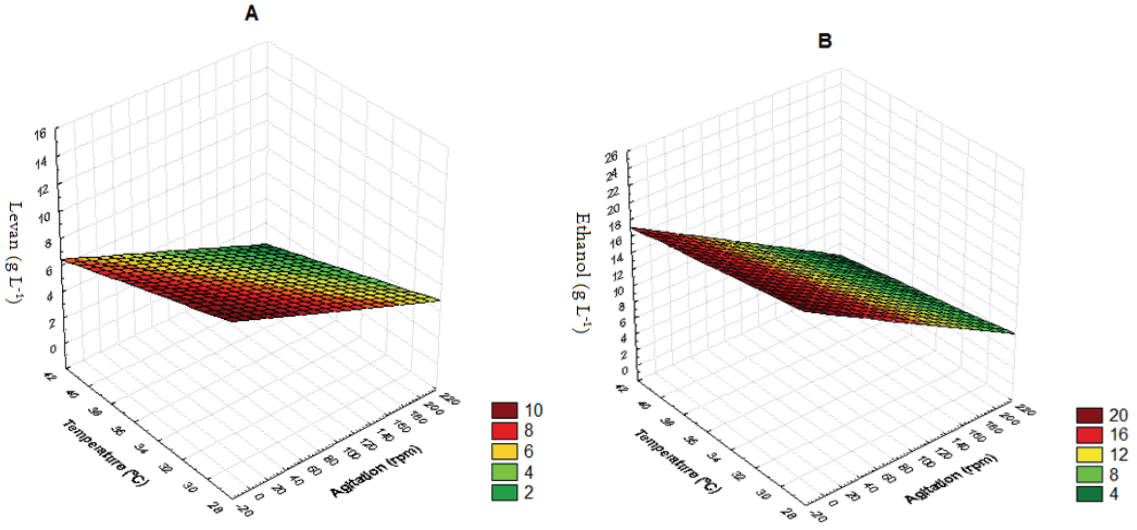

Figure 1. Response surfaces using loofa sponge as immobilization support for (A) Levan production (g L^{-1}) and (B) Ethanol production (g L^{-1}).

Microorganism growth on immobilization support and also free in culture medium, was not proportional to increase in levan and ethanol production and was not affected by the fermentation conditions tested. Immobilized biomass on supports did not had any statistically significant interference, even though, the MSR graphics showed more growth at 40°C, low sucrose concentrations and agitation absence (Figure 2).

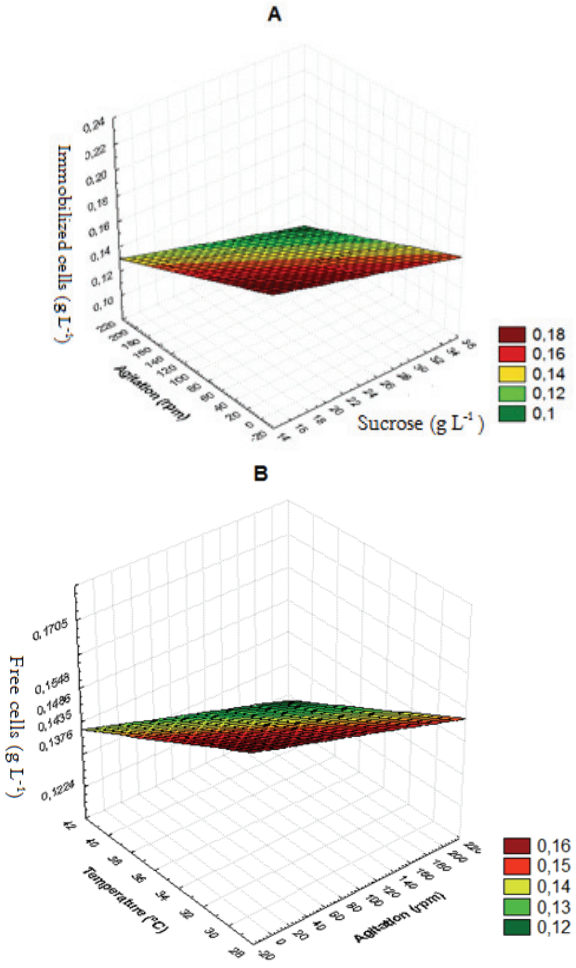

Figure 2. Response surfaces using loofa sponge as immobilization support for (A) Immobilized biomass (g L^{-1}) and (B) Free biomass (g L^{-1}).

On the other hand, the higher free biomass growth on cultivation medium (0.17 g L^{-1}) was significantly affected by low temperature and agitation absence ($p < 0.05$), due to the fact that *Z. mobilis* has cell growth independent of product synthesis. Consequently, this bacterium is promising for ethanol and levan production (Kalnenieks, 2006). The best temperature (between 30 and 35°C) observed by levan and ethanol production (Figure 1) was similar to that reported by several authors in the literature (Han & Clarke, 1990; Bekers, Laukevics, Upide, Kaminska, & Linde, 1999; Bandaru et al., 2006; Behera et al., 2010; Sreekumar, Chand, & Basappa, 1999).

The negative effect caused by high agitation in levan and ethanol production was also observed by other authors who attributed the anaerobic characteristics as the main inhibitory factor (Barros & Celligoi, 2006). The above behavior was also corroborated by Pinilla, Torres and Ortiz (2011) and Kannan, Sangiliyandi and Gunasekaran (1998).

Several authors have also reported the levan production with high sucrose concentration. They observed that in these conditions the microorganism change their metabolic pathway after sucrose hydrolysis into fructose and glucose units, and starts fructose transfructosylation in units linked by β (2, 6), forming levan (Viikari & Gisler, 1986; Shih, Chen, & Wu, 2010; Borsari et al., 2006; Vigants, Hicke, & Marx, 2001).

Bekers et al. (2001) obtained maximum ethanol production (14.4 g L^{-1}) using *Zymomonas mobilis* immobilized on stainless steel beads. On the other hand, sucrose concentration affected negatively the ethanol production because this carbon source forms byproducts such as levan and sorbitol that reduce ethanol yield (Bekers et al., 2000; Viikari, 1988).

Cazzeta et al. (2007) also reported a high ethanol production (54.83 g L^{-1}) using low molasses concentration and noted that ethanol production decreased with concentrations over 250 g L^{-1}.

Experiment 2: Scale up and repeated recycle fermentation

Based on the best parameters for ethanol and levan production obtained in Experiment 1, fermentation was scaled-up in the reactor (200 mL) and the immobilization support was reuse by semi-continuous fermentation during 10 days was evaluated. Best conditions comprised sucrose 250 g L^{-1}; fermentation medium without pH control, at 30°C and 200 rpm for medium homogenization, without oxygenation.

Immobilized cells on loofa sponge showed high ethanol and levan production during ten fermentation recycles of support reuse. According to Figure 2, there was an increase in ethanol concentration until the 8th recycle, with highest production (34.64 g L^{-1}); yield 0.32 g g^{-1}; theoretical yield of 0.63 g g^{-1} and productivity 1.44 g $L\ h^{-1}$ (Table 2).

Table 2. Ethanol production by *Z. mobilis* CDBB-603 immobilized on loofa sponge during ten fermentation recycles.

Recycle	Ethanol production ($g\ L^{-1}$)	Ethanol yield ($g\ g^{-1}$)	Theoretical ethanol yield ($g\ g^{-1}$)	Ethanol productivity ($g\ L\ h^{-1}$)
1	29.71	0.26	0.51	1.23
2	18.52	0.19	0.38	0.77
3	20.94	0.33	0.65	0.87
4	23.15	0.33	0.65	0.96
5	27.87	0.39	0.77	1.16
6	23.19	0.34	0.66	0.96
7	28.77	0.27	0.54	1.19
8	34.64	0.32	0.63	1.44
9	32.20	0.35	0.69	1.34
10	33.70	0.29	0.58	1.40

Results were higher than those by Chandel, Narasub, Chamdrasekhar, Manikyama and Venkateswar (2009) using *Saccharomyces cerevisiae* V3 immobilized on wild sugarcane, featuring 22.85 $g\ L^{-1}$ and 0.44 $g\ g^{-1}$ for ethanol production and yield, respectively, also after eight recycles.

On the other hand, Behera et al. (2010) used *Saccharomyces cerevisiae* immobilized in sodium alginate for ethanol production using Mahula flowers and had a higher yield than that obtained in current study, with a production of 154.5 $g\ kg^{-1}$ in the first recycle, and gradual decrease in the ethanol synthesis as from the second recycle.

Levan production during ten recycles remained almost constant, with reduction only in the second and fourth recycles (Figure 1). However, the fifth recycle showed the highest production rates (26.4 $g\ L^{-1}$, 0.37 $g\ g^{-1}$ and 1.1 $g\ L\ h^{-1}$ for production, yield and productivity, respectively), as Table 3 demonstrates. Better results were obtained by Shih et al. (2010) using *Bacillus subtilis* immobilized on alginate beads. These authors reported high production in the third recycle (70.6 $g\ L^{-1}$) using initial pH control and medium supplementation with organic nitrogen during the five recycles and observed that the beads remained stable during all fermentation recycles.

Table 3. Levan production by *Z. mobilis* CDBB-603 immobilized in loofa sponge during ten fermentation recycles.

Recycle	Levan production ($g\ L^{-1}$)	Levan yield ($g\ g^{-1}$)	Levan productivity ($g\ L\ h^{-1}$)
1	16.04	0.14	0.66
2	24.27	0.25	1.01
3	26.07	0.41	1.08
4	18.28	0.26	0.76
5	26.40	0.37	1.10
6	24.96	0.36	1.04
7	22.02	0.21	0.91
8	25.01	0.23	1.04
9	24.27	0.26	1.01
10	24.36	0.21	1.01

Low yield in ethanol and levan production might be due to reduced substrate conversion during 24 hours of fermentation. In this case, maximum substrate consumption of 45.43% and 43.03% was observed in the first and eight recycles (Table 4). The latter was the best for ethanol production and productivity. The substrate conversion low rate was possibly affected by initial pH (4.95) which was not controlled during fermentation recycles.

Initial pH 4.95 caused a fast reduction of the culture medium pH to rates below 3.5 between the first and the eighth recycle, as Table 4 shows. Below this rate, pH is an inhibitory factor for growth *of Z. mobilis* and for ethanol and levan production (Han & Clarke, 1990).

Similarly to current study, Shih et al. (2010) observed that levan production is inhibited without pH control after the third fermentation recycle. The behavior may have occurred because small amounts of fermented medium may have remained occluded on the immobilization support, particularly when the fermenter required adjustment in the immobilization support design. It may cause fresh medium initial pH decrease and affect the substrate consumption and ethanol and levan production.

Table 4. Profile of final pH, free biomass and substrate consumption during ethanol and levan production by *Z. mobilis* CDBB-603 immobilized on loofa sponge for ten fermentation recycles.

Recycle	pH final	Free cells ($g\ L^{-1}$)	Substrate consumption (%)
1	3.43	1.95	45.43
2	3.59	0.62	37.69
3	3.39	1.16	25.22
4	3.40	1.49	27.69
5	3.45	1.40	28.27
6	3.33	1.55	27.22
7	3.33	1.33	41.57
8	3.46	2.42	43.03
9	3.46	1.81	36.11
10	3.51	1.94	45.22

As shown in Table 4, *Z. mobilis* in very low initial pH may be inhibited by the high acidity generated. This behavior would explain the low conversion rates observed between the 3rd and 7th recycle (Tables 1 and 2). A higher rate observed in recycles 8, 9 and 10, possibly was due to the death of the immobilized microorganism following reduction in medium acidity during the last recycles.

Immobilized cell concentration after the 10 fermentation recycles was 0.27 $g\ L^{-1}$ and free biomass of each recycle showed results between 0.62 and 2.42 $g\ L^{-1}$. These results reveal that there was a low bacteria fixation on the support, which may have been caused by two factors. First, the above may be due to low affinity between *Z. mobilis* and immobilization support, since was not used any pretreatment to enhance adhesion; second, due the

microorganism do not be flocculant and therefore the ability of adhesion to the support is restricted.

On the other hand, the high growth rate of free biomass on the medium was observed in all fermentation recycles, indicating that immobilization by adsorption on solid surfaces occurred naturally with the detachment of immobilized cells to growth medium which help in production and lead towards a mixed culture.

Despite their low rise, immobilized cells remained viable until the last recycle, as a starter culture for the growth and detachment of free cells leading to ethanol and levan production. The above indicates that the immobilization of the loofa sponge is possible although adhesion to the support must be improved. It would be solved by pre-treatment and by using a gelling substance, such as sodium alginate, to increase microbial adhesion.

Conclusion

Z. mobilis immobilized on loofa sponge to ethanol and levan production, promoted a mixed culture between free and immobilized cells, due to low affinity of the microorganism and immobilization support. However, high ethanol and levan production was obtained with recycle fermentation in Erlenmeyer flasks and with the reuse of immobilized support by semi-continuous process in the fermenter. *Z. mobilis* remained viable until the last recycle day. Therefore, this bacteria may be used immobilized, requiring only adjustments, to the support to increase adhesion and can be used as a mixed culture of immobilized and free cells in continuous and semi-continuous fermentation.

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