*Journal of Food Protection, Vol. 65, No. 6, 2002, Pages 1033–1037*
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# Survey of Hydrogen Sulphide Production by Wine Yeasts

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MS 01-392: Received 23 October 2001/Accepted 27 January 2002

## ABSTRACT

Twenty-one strains of commercial wine yeasts and 17 non-*Saccharomyces* species of different provenance were surveyed for their ability to produce hydrogen sulphide in synthetic grape juice medium indicator agar with different nitrogen sources, as well as in natural grape juice. Bacto Biggy agar, a commercially available bismuth-containing agar, was used to compare our results with others previously reported in the literature. Under identical physiological conditions, the strains used in this study displayed similar growth patterns but varied in colony color intensity in all media, suggesting significant differences in sulphite reductase activity. Sulphite reductase activity was absent for only one strain of *Saccharomyces cerevisiae*. All other strains produced an off-odor to different extents, depending significantly ($P < 0.05$) on medium composition. Within the same species of some non-*Saccharomyces* yeasts, strain variation existed as it did for *Saccharomyces*. In natural musts, strains fell into three major groups: (i) nonproducers, (ii) must-composition-dependent producers, and (iii) invariable producers. In synthetic media, the formation of sulphide by strains of *S. cerevisiae* results from the reduction of sulphate. Therefore, this rapid screening methodology promises to be a very useful tool for winemakers for determining the risk of hydrogen sulphide formation by a given yeast strain in a specific grape juice.

The formation of hydrogen sulfide ($H_2S$) by yeasts during the fermentation of grape juice is a longstanding and serious problem in the winemaking process *(3)*. Hydrogen sulphide is a malodorous compound (smelling like rotten eggs) with a low sensory threshold (50 to 80 μg/liter) and can be produced by yeasts in excessive amounts during the fermentation of grape musts *(13)*. $H_2S$ is an intermediate in the biosynthesis of sulphur-containing compounds, including the amino acids methionine and cysteine and the methyl-group donor *S*-adenosylmethionine *(7, 13)*. In most wine-producing countries, inoculation with suitable strains has so far been the most successful way of limiting excessive $H_2S$ production. Apart from yeast strain *(14)*, nitrogen and sulphur composition of grape musts most strongly affects $H_2S$ production during must fermentation *(5, 6, 8–10, 12, 15, 16, 18)*. Winemakers usually add an assimilable nitrogen, such as diammonium phosphate, to must to reduce the risk of $H_2S$ formation during grape juice fermentation. A rapid method for screening the ability of a given yeast to produce hydrogen sulphide in a particular grape juice would be preferable to unnecessary nitrogen addition, which may not always be successful *(15)*. Zambonelli *(21)* employed a solid medium containing bismuth sulphite to show a wide variation in yeast strains in terms of their ability to reduce sulphite to sulphide. Since then, other researchers have found a strong correlation between colony color intensity in bismuth-containing indicator media and direct measurements of sulphite reductase activity *(9, 16)*. The purpose of this study was to evaluate the ability of several commercially available strains of *Saccharomyces cerevisiae* to produce hydrogen sulphide in a chemically defined medium and in natural grape juice. This ability was also screened in non-*Saccharomyces* species of the genera *Dekkera, Pichia, Kloeckera/Hanseniaspora,* and *Metschnikowia.*

## MATERIALS AND METHODS

**Microorganisms.** A total of 38 strains of different origins were used in this study (Table 1). Yeast cultures were maintained at 4°C on slants of YPD (glucose at 20 g/liter, peptone at 10 g/liter, yeast extract at 5 g/liter, and agar at 20 g/liter).

**Culture media.** The chemically defined grape juice media used in this study were based on grape juice medium (GJM), developed at the Australian Wine Research Institute (AWRI) *(7)*. A solid medium consisting of GJM supplemented with agar (20 g/liter) and bismuth citrate (11 g/liter) (as an indicator) was prepared. To evaluate the effect of nitrogen source on $H_2S$ production, the amino acids were replaced with ammonium sulphate (GJM 2), diammonium phosphate (GJM 3), and cysteine (GJM 4), all with the same final concentration of nitrogen as in the original formulation (805 mg of N per liter). Also, sulphide production was evaluated in yeast malt extract medium (yeast extract at 3 g/liter, malt extract at 3 g/liter, peptone at 5 g/liter, and glucose at 10 g/liter) and in two white and two red grape juices in which assimilable nitrogen content ranged from 245 to 289 mg/liter; all of these juices were supplemented with bismuth citrate (11 g/liter) and agar (20 g/liter). The pH was adjusted to 3.7 in all media with 1 M KOH or 1 M HCl as necessary. The media were heated to dissolve the agar and dispensed in petri dishes. Sulphur dioxide was not added to any media. Bacto Biggy agar, a commercially available bismuth-containing agar (Difco Laboratories, Sparks, Md.), was prepared according to the manufacturer's instructions. All assays were carried out in duplicate.

To evaluate the effects of nitrogen and sulphate on sulphide

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TABLE 1. *Yeast strains used in this study*

| Species | Strain(s) |
|---|---|
| *Saccharomyces cerevisiae* | AWRI 792,[a] AWRI 1091,[a] UCD 522,[b] IGC 4072[c]; commercial strains[d]—S19, S20, S21, S22, S23, S24, S25, S26, S27, S28, S29, S30, S31, S32, S33, S34, S35 |
| *Pichia membranaefaciens* | IGC 3796[c] |
| *P. anomala* | IGC 4321,[c] IGC 4380,[c] UTAD 16[e] |
| *Hanseniaspora uvarum* | UTAD 9,[e] UTAD 11,[e] UTAD 12,[e] UTAD 14,[e] UTAD 15[e] |
| *Metschnikowia pulcherrima* | UTAD 10,[e] UTAD 13[e] |
| *Dekkera anomala* | 5133,[c] IGC 5153,[c] 5153T[c] |
| *D. bruxellensis* | 4179,[c] IGC 5162[c] |
| *D. naardenensis* | 5163[c] |

[a] AWRI strains were kindly provided by Dr. Paul Henske, of the Australian Wine Research Institute.
[b] From the Department of Enology and Viticulture of University of California, Davis.
[c] Portuguese Collection of Yeast Cultures.
[d] Commercial strains are from Laffort, Lallemand, and Gist-Brochades.
[e] Strains isolated from natural grape juice from Douro Region, identified by physiological and morphological tests performed according to the keys proposed by Barnett et al. (*2*) and Yarrow (*20*) and supported by the Barnett et al. (*1*) computer program.

production, GJM's nitrogen source was replaced with ammonium sulphate and diammonium phosphate at different concentrations (267, 402, and 805 mg of N per liter), while potassium sulphate (0.92, 1.38, and 2.78 g of $SO_4$ per liter) was added to ensure the same final concentration of sulphate. Synthetic grape juice medium with diammonium phosphate as the nitrogen source, with and without sulphate (the $MgSO_4$ used in the original recipe was replaced with $MgCl_2$), provided controls. After the addition of bismuth citrate and agar, the pH was adjusted to 3.7 with 1 M KOH or 1 M HCl as necessary.

**Growth conditions.** Yeast strains were pregrown in YPD agar plates for 24 to 48 h at 25°C. The streak plate technique was then used to inoculate the strains on agar surface media. This procedure was carried out in duplicate for all media and for all strains, which were inoculated and immediately incubated for 48 h at 30°C.

**Evaluation of hydrogen sulphide.** $H_2S$ formation on the indicator media was determined by the color of the streaks, which turn brown to black or remain white, depending on the extent of production. Different rankings (0 to 4) were given according to the coloration of the colonies: 0, white; 1, light brown; 2, brown; 3, dark brown; 4, black.

**Estimation of assimilable nitrogen content of grape musts.** The assimilable nitrogen content of grape juice was evaluated by the formol titration procedure *(11).* After adjusting the pH of grape juice (50 ml) to 8.5 with 1 M NaOH, 20 ml of formaldehyde (37%, vol/vol) was added. After a few minutes, the mixture was retitrated to pH 8.5 with 0.1 M NaOH. The amount of assimilable nitrogen, expressed as milligrams of N per liter, was determined by multiplying by 28 the volume of 0.1 M NaOH used.

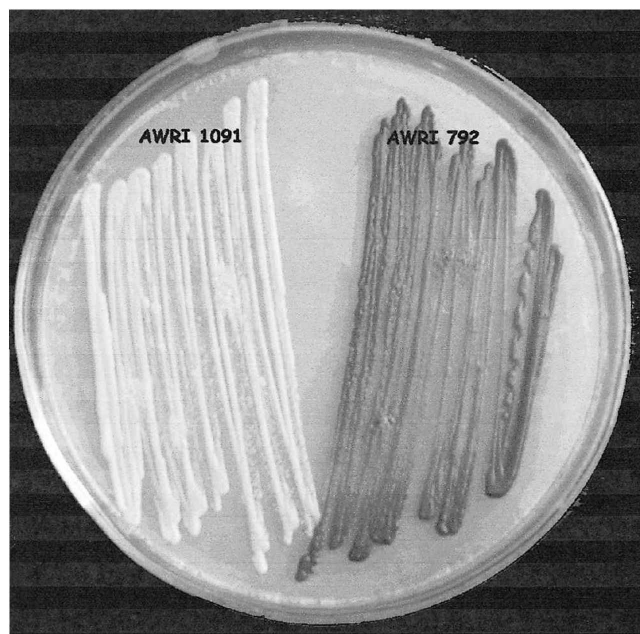


FIGURE 1. *A negative (*S. cerevisiae *AWRI 1091) and a positive (*S. cerevisiae *AWRI 792) sulphide-producer strain in natural grape juice bismuth indicator agar.*

**Statistical analysis.** The analysis of variance of the results was carried out with the SUPERANOVA program (version 1.11 for Macintosh, Abacus Concepts Inc.). As data consist of small whole numbers, $\sqrt{y + 1/2}$ was calculated for each observation preceding the analysis of variance *(17).* For paired comparison, the Duncan New Multiple Range feature of the SUPERANOVA program was used.

## RESULTS AND DISCUSSION

As has been reported, relative levels of sulphite reductase activity in yeast strains can be estimated by determining colony color in Biggy agar, and there seems to be a strong correlation between colony color and direct measurements of sulphite reductase activity *(5, 8, 9, 15).* The strains under study all produced similar growth patterns but displayed variable colony color intensities in Biggy agar as well as in other media modified from GJM and in natural grape juice (Fig. 1), suggesting significant differences ($P < 0.05$) in sulphite reductase activity. Five distinct colors were observed in all media. The 38 strains used in this work can be classified as high, medium, and low sulphide producers. The higher and the lower group include, respectively, *S. cerevisiae* Montrachet 522 and *S. cerevisiae* Prise de Mousse EC1118, as previously reported *(5, 15, 18),* which is consistent with our findings and validates our data. As shown in Table 2 for *S. cerevisiae* and in Table 3 for non-*Saccharomyces* species, yeast strains differ significantly ($P < 0.05$) with regard to the levels of hydrogen sulphide they produce under identical physiological conditions. *S. cerevisiae* AWRI 1091 was the only strain that showed no sulphite reductase activity in any of the chemically defined media or even in the natural grape juices used in this study. Only a very light brown was observed in the medium in which cysteine was the nitrogen source (GJM 4), which agrees with the previously reported finding that cysteine can

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TABLE 2. *Mean values for hydrogen sulphide produced by* Saccharomyces cerevisiae *in different media*

| Strain[a] | Value[b] in medium | | | | | |
|---|---|---|---|---|---|---|
| | GJM | GJM 2 | GJM 3 | GJM 4 | Biggy | YME[c] |
| AWRI 109 A | 0.0 | 0.0 | 0.0 | 1.5 | 0.5 | 0.0 |
| S26 B | 1.0 | 1.9 | 1.7 | 3.0 | 2.0 | 0.0 |
| S24 B | 1.0 | 2.1 | 1.6 | 3.0 | 2.0 | 0.0 |
| S21 BC | 1.0 | 2.4 | 1.7 | 3.0 | 2.5 | 0.0 |
| S33 BCD | 1.5 | 2.2 | 1.9 | 3.0 | 2.4 | 0.0 |
| S34 BCD | 1.5 | 2.2 | 1.7 | 3.0 | 2.5 | 0.0 |
| S27 BCDE | 1.2 | 2.6 | 2.6 | 3.0 | 2.4 | 0.0 |
| S20 BCDEF | 2.0 | 2.9 | 2.0 | 3.0 | 2.2 | 0.0 |
| AWRI 792 CDEFG | 2.5 | 2.9 | 1.5 | 2.5 | 3.0 | 0.2 |
| S35 CDEFGH | 2.0 | 2.1 | 1.9 | 3.0 | 2.6 | 1.1 |
| S28 CDEFGH | 2.0 | 2.6 | 2.6 | 3.0 | 2.5 | 0.2 |
| S25 CDEFGH | 2.0 | 3.0 | 2.6 | 3.0 | 2.5 | 0.0 |
| S32 DEFGH | 2.5 | 2.9 | 2.9 | 3.0 | 2.5 | 0.0 |
| S31 DEFGH | 2.0 | 3.0 | 2.6 | 3.0 | 2.5 | 0.4 |
| S30 EFGH | 2.5 | 3.0 | 2.5 | 3.0 | 2.5 | 0.2 |
| IGC 4072 EFGHI | 2.7 | 3.0 | 3.0 | 3.0 | 2.4 | 0.0 |
| S23 FGHI | 2.5 | 3.2 | 3.0 | 3.2 | 2.6 | 0.0 |
| S19 FGHI | 3.0 | 3.0 | 3.0 | 3.0 | 2.5 | 0.2 |
| UCD 522 GHI | 3.0 | 2.9 | 2.9 | 3.0 | 2.6 | 0.5 |
| S29 HJ | 2.7 | 2.9 | 2.6 | 3.0 | 3.0 | 1.1 |
| S22 I | 3.0 | 3.2 | 3.0 | 3.2 | 2.9 | 1.4 |

[a] Strains with different letters are significantly different ($P < 0.05$).
[b] $H_2S$ production is expressed in arbitrary units according to the coloration of colonies, as described in ''Materials and Methods.'' Each value is a mean of two replicates.
[c] YME, yeast malt extract.

TABLE 3. *Mean values for hydrogen sulphide produced by non-*Saccharomyces *species in different media*

| Strain[a] | Values[b] in medium | | | | | |
|---|---|---|---|---|---|---|
| | GJM | GJM 2 | GJM 3 | GJM 4 | Biggy | YME[c] |
| *Dekkera bruxellensis* IGC 5162 A | 1.5 | 1.4 | 1.2 | 4.0 | 1.7 | 0.0 |
| *Hanseniaspora uvarum* UTAD 11 A | 1.0 | 0.6 | 1.5 | 3.7 | 2.4 | 1.0 |
| *H. uvarum* UTAD 12 A | 1.0 | 1.2 | 1.3 | 3.7 | 2.0 | 1.0 |
| *H. uvarum* UTAD 9 A | 1.0 | 1.1 | 1.3 | 3.7 | 2.2 | 1.0 |
| *H. uvarum* UTAD 14 A | 0.5 | 1.2 | 1.7 | 3.7 | 2.2 | 1.0 |
| *Dekkera naardenensis* 5163 A | 2.7 | 1.7 | 2.0 | 1.9 | 1.9 | 0.5 |
| *D. bruxellensis* 4179 AB | 1.2 | 1.4 | 1.4 | 3.5 | 2.5 | 1.0 |
| *H. uvarum* UTAD 15 AB | 0.5 | 1.6 | 2.0 | 3.7 | 2.2 | 1.0 |
| *Metschnikowia pulcherrima* UTAD 13 AB | 1.2 | 2.7 | 2.0 | 2.0 | 2.3 | 1.0 |
| *Pichia anomala* UTAD 16 AB | 2.0 | 1.5 | 1.5 | 4.0 | 2.0 | 1.0 |
| *M. pulcherrima* UTAD 10 ABC | 1.5 | 2.3 | 2.1 | 2.0 | 2.6 | 1.0 |
| *P. anomala* IGC 4121 ABCD | 2.0 | 2.1 | 2.1 | 4.0 | 2.1 | 1.0 |
| *P. anomala* IGC 4380 BCD | 3.5 | 1.2 | 1.7 | 4.0 | 2.6 | 1.5 |
| *Pichia membranaefaciens* IGC 3796 CD | 2.7 | 1.2 | 2.0 | 4.0 | 4.0 | 1.0 |
| *Dekkera anomala* 5133 DE | 3.0 | 2.1 | 2.1 | 3.5 | 3.9 | 1.0 |
| *D. anomala* 5153T E | 3.5 | 3.0 | 3.0 | 3.5 | 3.5 | 1.7 |
| *D. anomala* IGC 5153 E | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 1.7 |

[a] Strains with different letters are significantly different ($P < 0.05$).
[b] $H_2S$ production is expressed in arbitrary units according to the coloration of colonies, as described in ''Materials and Methods.'' Each value is a mean of two replicates.
[c] YME, yeast malt extract.

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TABLE 4. *Differences in $H_2S$ production by yeast in different media*[a]

| Medium | *S. cerevisiae* ($n$ = 42) | Non-*Saccharomyces* sp. ($n$ = 34) |
|---|---|---|
| YME[b] | 0.3 A[c] | 1.0 A |
| GJM | 2.0 B | 1.8 B |
| GJM 2 | 2.6 D | 1.7 B |
| GJM 3 | 2.3 C | 1.9 B |
| GJM 4 | 2.9 E | 3.5 D |
| Biggy | 2.4 CD | 2.5 C |

[a] $H_2S$ production is expressed in arbitrary units according to the coloration of colonies, as described in "Materials and Methods."
[b] YME, yeast malt extract.
[c] Means with different letters in the same column are significantly different ($P < 0.05$).

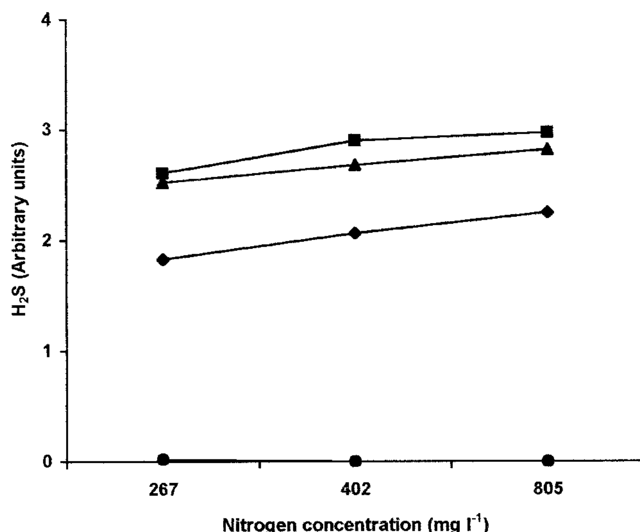


FIGURE 2. *Effect of nitrogen and sulphate in sulphide production (n = 42) by* Saccharomyces cerevisiae *in synthetic grape juice media. $H_2S$ production is expressed in arbitrary units according to colony coloration, as described in "Materials and Methods."* ◆, *nitrogen supplied as diammonium phosphate, with the sulphate content of the original formulation (480 mg/liter);* ●, *nitrogen supplied as diammonium phosphate, with no sulphate (the $MgSO_4$ used in the original recipe was replaced with $MgCl_2$);* ■, *nitrogen supplied as ammonium sulphate;* ▲, *nitrogen supplied as diammonium phosphate, with additional sulphate ($K_2SO_4$ was added to ensure the same final concentration achieved with ammonium sulphate).*

be degraded by yeast to hydrogen sulphide, pyruvate, and ammonium by cysteine desulphydrase *(3)* or to hydrogen sulphide and serine by the reverse reaction of cysteine synthetase *(7)*. All of the other strains produced variable amounts of sulphide, suggesting variable levels of sulphite reductase activity, depending significantly ($P < 0.05$) on media composition. The use of a chemically defined medium permits standardization of conditions and eliminates variations due to must composition.

**Sulphide production in synthetic grape juice with different nitrogen sources.** As shown in Table 4, for *S. cerevisiae,* the lowest sulphide production was observed in media with the original formulation (GJM), in which a mixture of amino acids and diammonium phosphate was used as the nitrogen source. A significant increase in sulphide production was detected when ammonium sulphate (GJM 2) was the nitrogen source. Giudici and Kunkee *(5)* previously provided preliminary evidence that sulphate could be a mechanism of $H_2S$ production by yeast during grape juice fermentation when a nutrient deficiency exists. It seems that even with excess nitrogen, sulphate can play a role in sulphide production. The highest production was observed for GJM 4, in which cysteine was the nitrogen source. Exceptions were strains UCD 522 and S19, whose sulphite reductase activity was not affected by the nitrogen source, as shown in Table 2.

Non-*Saccharomyces* yeasts are an ecologically and biochemically diverse group capable of altering the fermentation dynamics, composition, and flavor of wine *(4)*. Nothing was found in the literature with regard to their ability to produce sulphide. $H_2S$ produced by non-*Saccharomyces* species was variable among strains and species (Table 3). The strongest producers were the three strains of *Dekkera anomala,* while the weaker producers were *Dekkera bruxellensis* and *Kloeckera/Hanseniaspora.* However, contrary to what was observed for *Saccharomyces,* no significant differences were detected between nitrogen sources (amino acids, diammonium phosphate, and ammonium sulphate) (Table 4). The highest sulphide production was observed for media with cysteine as the only nitrogen source (as found in *Saccharomyces*), in spite of the delay in yeast growth caused by this *S*–amino acid, as previously reported *(7)*. For all strains, the lowest sulphite reductase activity was found in the complex yeast malt extract medium. Strains within some species showed variation in sulphide, suggesting that its production is not a species-specific trait.

**Sulphide production in natural grape juice.** The strains behaved similarly with respect to $H_2S$ production in synthetic and in natural grape juice. The exception was *D. anomala* 5133, which was a nonproducer in natural grape juice and a strong producer in synthetic grape juice media (Table 3). In natural grape musts, strains fell into three major groups: (i) nonproducers (*S. cerevisiae* AWRI 1091, *D. anomala* 5133, and *D. bruxellensis* IGC 5162), (ii) must-composition-dependent producers (*S. cerevisiae* S20, S21, S24, S26, S33, and S35, *Pichia membranaefaciens* IGC 3796, and *Pichia anomala* UTAD 16), and (iii) invariable producers (results not shown). Inoculation with selected strains therefore emerged as the most preferred way of eliminating excessive $H_2S$ production. This rapid screening method promises to be a very useful tool for the winemaker in determining the risk of hydrogen sulphide formation by a given strain for a specific grape juice.

**Effect of nitrogen and sulphate concentration in synthetic grape juice medium.** Evidence has been presented that $H_2S$ production during grape juice fermentation is inversely correlated with the nitrogen content of musts *(19)* and that following nitrogen starvation of yeast culture, sulphate and sulphite are potent substrates for $H_2S$ liberation *(6)*. The results of the present study show that sulphate

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could be the main substrate for hydrogen sulphide production, as an increase in sulphate leads, to a certain degree, to an increase in sulphide, and none was detected in the absence of sulphate, as illustrated in Figure 2. On the other hand, with nitrogen concentrations ranging from 267 to 805 mg of N per liter, the formation of sulphide by the strains of *S. cerevisiae* was almost independent of nitrogen concentration. These results do not agree with previous observations for natural grape juice *(19),* probably because the role of assimilable nitrogen in the regulation of sulphide production is not as important in solid media as it is in liquid cultures, as reported by Jiranek et al. *(9).* Again, AWRI 1091 with either nitrogen source, even at the lowest nitrogen concentration and the highest sulphate level, did not produce $H_2S$. Accordingly, sulphate seems to be the main substrate for sulphide formation in the strains of *S. cerevisiae* used in this study. However, for most of the strains studied, it seems that there are factors other than nitrogen content and sulphate that regulate hydrogen sulphite production. The mechanism involved in sulphide formation by yeast will be further investigated.

## ACKNOWLEDGMENTS

The authors thank Ecofiltra, Proenol, and A. Freitas Vilar for providing the commercial wine yeast strains. The authors also thank Dr. Paul Henschke for numerous suggestions and much assistance during this study.

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