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The Effect of Sulfite on the Yeast *Saccharomyces cerevisiae*

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Abstract. After a short period of tolerance, living cells of *Saccharomyces cerevisiae* were irreversibly damaged by low concentrations of sulfite. The length of the period of tolerance and the rate of the damaging effect depended on the concentration on sulfite, pH-value, temperature, the physiological state of the cells, and incubation time.

Inhibitors of protein synthesis and mitochondrial ATP synthesis did not alter the deleterious effect of sulfite on living cells. Furthermore, cell damage leading to inhibition of colony formation occured under aerobic as well as under anaerobic conditions.

Prior to cell inactivation sulfite induced the formation of respiratory deficient cells.

The active agent was shown to be SO_2.

Key words: Sulfur dioxide – Sulfite – Air polluting substances – *Saccharomyces cerevisiae* – Active agent of irreversible cell inactivation.

SO_2 is one of the most frequently occurring air pollutant substances. While uncontaminated air contains about 1.3×10^{-3} µg $SO_2 \times l^{-1}$, in polluted air $20 - 65 \times 10^{-3}$ µg $\times l^{-1}$, sometimes even 650×10^{-3} µg $\times l^{-1}$ were measured (Fishbein, 1976). Furthermore, SO_2 is widely used as antimicrobial agent and antioxidant in food preservation. It is rather important therefore, to know the possible dangerous effect of SO_2 on living cells.

The effect of sulfite on animals and man has been investigated (Hötzel et al., 1966; Pfleiderer et al., 1968; Hötzel et al., 1969; Cohen et al., 1973; Leivestadt and Muniz, 1976; O'Sullivan, 1976). In these organisms a sulfite oxidase rapidly converts sulfite to sulfate. Thus below certain concentrations – which are specific for each organism – no damaging effect of sulfite could be detected.

The effect of sulfite on plants (Ziegler, 1975) and bacteria (Babich and Stotzky, 1978a) has been reviewed recently. These organisms are more sensitive towards sulfite.

Some work has been done on the mechanism of sulfite action. Modification of nucleic acids (Hayatsu and Miller, 1972; Shapiro et al., 1974) and proteins (Cole, 1967; Yang, 1970) as well as reactions of sulfite with some cofactors (Adams, 1969; Leichter and Joslyn, 1969; Hevesi and Bruice, 1973; Tuazon and Johnson, 1977) have been reported.

Since these experiments were done with very high sulfite concentrations it is not certain whether these reactions can be primarily responsible for damage through air pollution.

In vitro investigations on enzyme inhibition by low concentrations of sulfite revealed competitive inhibition of reactions that are concerned with HCO_3^- fixation (Libera et al., 1975; Mukerji, 1977; Schimz and Holzer, 1977). Furthermore, competitive inhibition of α-glucan-phosphorylase from potatoe and rabbit muscle with regard to phosphate has been shown by Kamogawa and Fukui (1973).

The present work deals with the influence of sulfite concentrations comparable to those found in polluted air on intact eucaryotic cells. Experiments on the influence of sulfur dioxide on photosynthesis in chloroplasts led to the assumption that the physiological effect of $520 - 780$ ng $SO_2 \times l^{-1}$ air equals that of 1 mM sulfite in aqueous solution (Ziegler, 1975).

The experiments reported here try to explain the mechanism by which irreversible inhibition of colony formation in *Saccharomyces cerevisiae* cells by sulfite occurs.

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Materials and Methods

Materials

Cycloheximide, Carbonylcyanide-m-chlorophenylhydrazone and Chloramphenicol were purchased from Carl Roth, Karlsruhe (FRG), from Sigma Chemie GMBH., München (FRG) and from Boehringer Mannheim, Mannheim (FRG), respectively.

All other substances used were from the usual commercial sources of the purest grade available.

Organisms

Saccharomyces cerevisiae strain X2180 (2n) was received from the Yeast Genetic Stock Center, Berkeley/Calif. (USA) (R. Mortimer), and strain S288c (1n) α as well as strain 4450-1A (1n) a was from the stock of G. R. Fink, Cornell University, Ithaka/N.Y. (USA), kindly provided by Dr. D. Wolf, Biochemisches Institut der Universität, Freiburg (FRG).

Media

1. YEPD Medium for Cultivation

Ammmonium sulfate 6 g/l, potassium dihydrogenphosphate 6.8 g/l, disodium hydrogenphosphate · 2 H_2O 8.9 g/l, Bacto Peptone (Difco) 5 g/l, Yeast Extract (Difco) 25 g/l and D-glucose 20 g/l were dissolved in destilled water (final pH 6.8) and sterilized for 30 min at 120°C. After cooling, separately sterilized calcium chloride · 2 H_2O, 0.214 g/l, and magnesium sulfate · 7 H_2O, 0.122 g/l, were added. YEPD medium plates were prepared by adding 20 g/l Difco Agar.

2. Medium for Incubation of Cells with Sulfite

McIlvain buffer (disodium hydrogenphosphate/citric acid) of the desired pH value, prepared according to Bates and Paabo (1970), was mixed with water or YEPD medium (without Ca^{2+} and Mg^{2+}) respectively as will be described below.

Methods

Cultivation Conditions and Preparation of Cell Suspensions for Incubation Experiments

Cells (1/2 size of match head taken from solid YEPD medium, at most 10 days old) were cultivated at 30°C on a reciprocal shaker in 1 l Erlenmeyer flasks containing 0.2 l YEPD medium. Cells were grown for 5 h (early-log-phase), 13 h (late-log-phase), 24 h (stationary-phase) and 48 h (late-stationary-phase) respectively. Cells were harvested at times indicated by centrifugation (15 min, 5°C, 27,000 × g), washed with sterile 0.2 M sodium potassium phosphate buffer pH 6.8 and water respectively and resuspended in sterile water. Concentration of cells was about 2 to 6×10^8 cells/ml suspension.

Incubation Procedure

At time $t_{-15\ (min)}$ 1 ml of cell suspension was inoculated in 100 ml Erlenmeyer flask containing 17.5 ml incubation medium and 1.5 ml of YEPD-medium or destilled water (c.f. legends to figures). This incubation mixture was incubated at 30°C on a reciprocal shaker. At time t_0 the following steps were carried out:

a) Na_2SO_3 salt was added up to the desired final concentration (c.f. legends to figures);

b) 0.5 ml of this incubation mixture was diluted with 4.5 ml sodium potassium phosphate buffer (0.2 M, pH 6.8), further dilution steps were performed immediately before plating some minutes later;

c) pH value was corrected if necessary.

Incubation under anaerobic conditions was performed with incubation mixtures which had been made free of oxygen by degassing and subsequent washing with pure nitrogen repeated three times each for 5 min. Anaerobic conditions were verified by using a discolored methyleneblue solution.

Incubations in presence of inhibitors of protein biosynthesis were performed by using 100 µg per ml incubation mixture of cycloheximide, and chloramphenicol, respectively. Mitochondrial ATP synthesis was inhibited by addition of 10 µg per ml incubation mixture of carbonylcyanide-m-chlorophenylhydrazone. Each substance was applied as dry powder.

Determination of Colony Formation

After incubation of cells with Na_2SO_3 as described above aliquots of 0.5 ml were taken and appropriately deluted with 0.2 M sodium potassium phosphate buffer pH 6.8. Aliquots of 0.1 ml were taken and plated on YEPD agar-medium. Colonies appearing after 2–3 days of incubation at 30°C were counted (diameter of colonies about 1.5–2.0 mm). The very small colonies (diameter about 0.2–0.4 mm) appearing 2–3 days later were assumed to be respiratory deficient mutants and counted separately. These mutants should be unable to form colonies on YEP-glycerol agar medium (containing glycerol, 20 g/l, instead of glucose). Replica plating therefore was used as an assay for these mutants. In fact 92% ($n = 36$) of the very small colonies did not grow on glycerol and could therefore be assumed to be deficient in respiration.

From these counts concentrations of colony-forming cells per ml of incubation mixture were calculated.

Determination of Sulfite Concentration

To get some information on the stability of sulfite under the incubation conditions sulfite concentrations were determined in parallel using the pararosaniline reagent according to Tate et al. (1970).

Results and Discussion

Sulfur Dioxide in Aqueous Solutions

Sulfur dioxide is readily soluble in water. The aqueous solution contains H_3O^+, SO_2 (sulfur dioxide), HSO_3^- (bisulfite), SO_3^{2-} (sulfite), and $S_2O_5^{2-}$ (pyrosulfite) (Schmidt and Siebert, 1973).

Sulfurous acid (H_2SO_3) is not present in detectable amounts (Jones and McLaren, 1958); furthermore, pyrosulfite is formed in equilibrium with bisulfite exclusively when concentrations about 10 mM (and higher) are used (Golding, 1960).

Therefore during this work the term "sulfite" is defined by the following reactions:

1. $SO_2 + H_2O \rightleftharpoons HSO_3^- + H_3O^+$
2. $HSO_3^- + H_2O \rightleftharpoons SO_3^{2-} + H_3O^+$

with pK values of $pK_1 = 1.81$ and $pK_2 = 6.91$, respectively (Weast, 1974).

Using mass-action relations one can calculate the ratios of "sulfite" species present in an aqueous solution at a given pH value and a given concentration of e.g. disodium sulfite. From pH 3.6–5.6 the concentration of SO_2 decreases and the concentration of SO_3^{2-} increases both about 100fold, whereas the concentration of HSO_3^- remains approximately constant at a given concentration of Na_2SO_3 salt (c.f. Table 1).

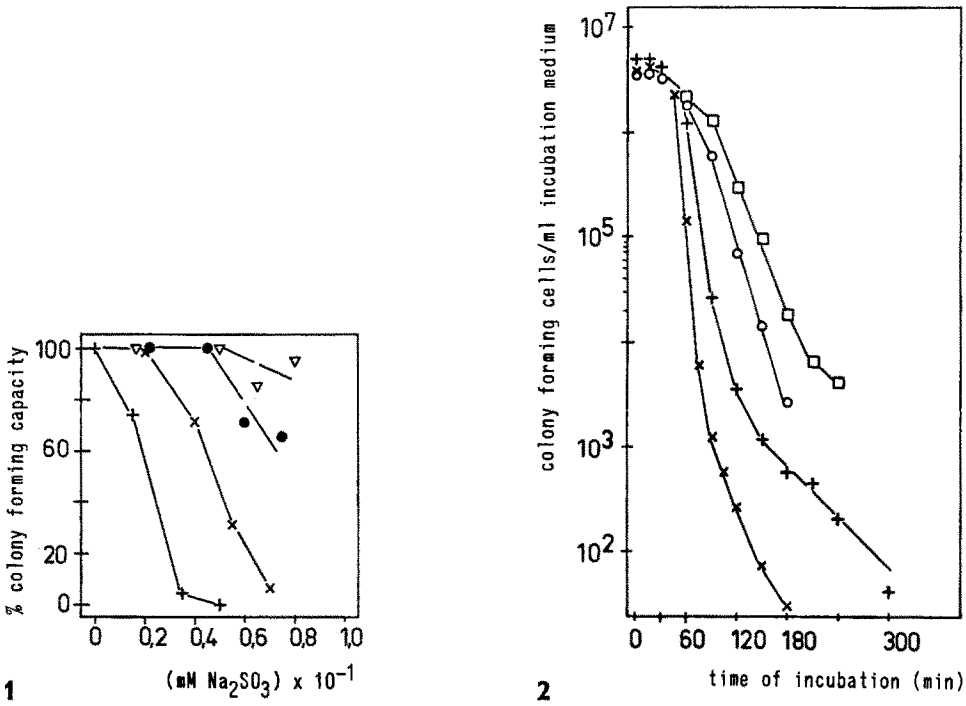


Fig. 1. Influence of Na_2SO_3 concentration on colony formation at various pH values: pH 3.33 (+), pH 3.68 (×), pH 4.05 (●), pH 4.44 (▽) (±0.02). Conditions of incubation: 30°C , aerobic, late-log-phase cells, YEPD-medium, reciprocal shaker, 6 h

Fig. 2. Influence of different "sulfite" concentrations on the time dependent decrease of colony forming capacity of yeast cells: mM Na_2SO_3 10 (×), 0.5 (+), 0.1 (○), 0.05 (□). Conditions of incubation: 30°C, pH 3.7, aerobic, early-log-phase cells, reciprocal shaker, YEPD-medium

The concentration of "sulfite" can be determined unequivocally even in the presence of sulfate and sulfide (not shown in a figure) according to Tate et al. (1970). "Sulfite" concentration decreases slightly in the presence of oxygen. pH value and the composition of the aqueous solution did not influence the stability of "sulfite", provided that the solutions were buffered. Within 6 h at 30°C on a reciprocal shaker an about 15% decrease of "sulfite" concentration was measured (data not shown).

Influence of pH Value on Cell Inactivation by "Sulfite"

The influence of the pH value and "sulfite" concentration on colony formation is shown in Fig. 1. Control experiments were performed with Na_2SO_4 instead of Na_2SO_3.

Decreasing pH values raised sensitivity of cells towards a given concentration of "sulfite". Significant reduction of colony formation could be found below pH 6.0 in the presence of 10 mM "sulfite" and below pH 4.5 with 1 mM "sulfite". The same results (not documented) were obtained under anaerobic conditions. Even in the presence of 10 mM "sulfite", no inhibition of colony formation occured at pH values above 6.0. Therefore, dilution of the incubation mixture with 0.2 M sodium potassium phosphate buffer pH 6.8 was sufficient to stop the action of "sulfite" immediately. It can be seen from these experiments, that concentrations of "sulfite" in the range of those found in polluted atmosphere led to an irreversible inhibition of colony formation of the yeast *Saccharomyces cerevisiae* X2180.

The time dependence of this inhibitory effect in the presence of different "sulfite" concentrations is shown in Fig. 2. Increasing incubation times and increasing concentrations of "sulfite" led to increasing inhibition rates of colony formation at a given pH value.

Influence of the Physiological State of the Cells on the Inactivation by "Sulfite"

The sensitivity of cells towards "sulfite" was shown to depend on their physiological state (Fig. 3). A phase of insensitivity against "sulfite" was visible, which was found to be prolonged when late-stationary-phase cells underwent "sulfite" treatment. Macris (1972) who has done his experiments with a different yeast strain – *Saccharomyces cerevisiae var. ellipsoideus* – did not find this phase of insensitivity. This may be due to differences between the strains compared. However, it has been shown (Schimz and Holzer, 1979) that the length of this phase of insensitivity is of importance for

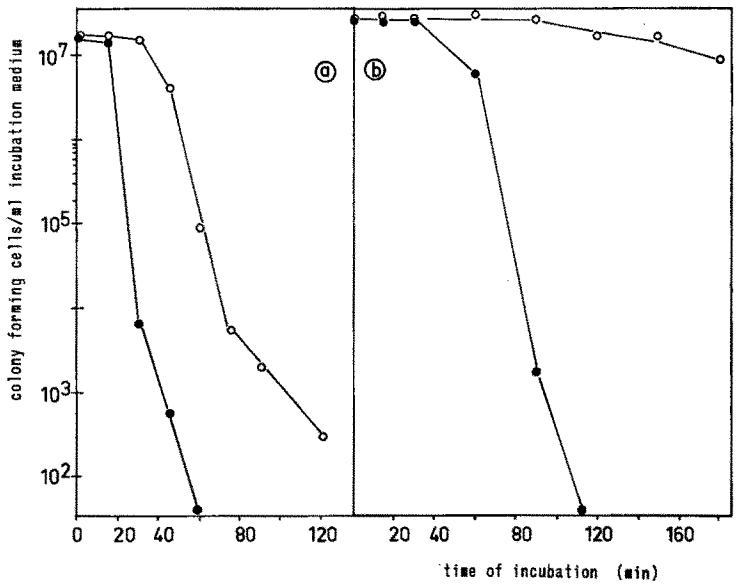


Fig. 3
Influence of "sulfite" concentration on colony formation of yeast cells from different growth phases. **a** Early-log-phase cells 10 mM Na_2SO_3 (●); early-log-phase cells 1 mM Na_2SO_3 (○). **b** Late-stationary-phase cells 10 mM Na_2SO_3 (●); late-stationary-phase cells 1 mM Na_2SO_3 (○). Conditions of incubation: 30°C, pH 3.35, aerobic, reciprocal shaker, YEPD-medium

the ability of the strain to overcome the deleterious effect of "sulfite" (see below for further discussion).

The rate of inhibition of colony formation was greatly reduced at low "sulfite" concentrations (1 mM) when late-stationary-phase cells were treated. At high concentrations of "sulfite" (10 mM) the inibiton rate was roughly equal in log-phase and stationary-phase cells.

Influence of Temperature on Cell Inactivation by "Sulfite"

The effect of temperature on the irreversible inhibition of colony formation is shown in Fig 4. Rising the temperature from 18° C to 28° C caused a 4.5–5-fold shortened period of insensitivity of cells against "sulfite" treatment which decreased from 180–200 min to 30–40 min (Fig. 4). This points to catalytic processes rather than to non-catalytic processes. Probably transport or enzyme activation or inactivation phenomena are involved in irreversible inhibition of colony formation.

Previous in vitro experiments showing that "sulfite" accelerates hydrolysis of ATP in the particulate fraction of a yeast cell extract support this suggestion (Schimz and Holzer, 1978). Further indirect evidence for the involvement of a surface located system in inhibition of colony formation by "sulfite" came from experiments with diploid and haploid strains, which are known to have different surface areas and different amounts of proteins located at the surface (Weiss et al., 1975). Small differences could be found in the periods of insensitivity of these strains (data not shown in a figure), the diploid organism, which has a bigger surface area being slightly more sensitive towards "sulfite".

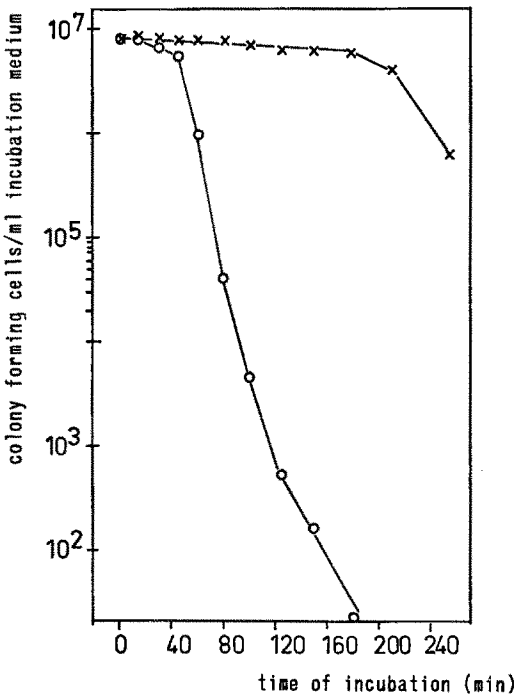


Fig. 4. Influence of temperature on the inhibition effect of "sulfite" at pH 3.6: 28°C (○), 18°C (×). Further conditions of incubation: 5 mM Na_2SO_3, aerobic, early-log-phase cells, reciprocal shaker, YEPD-medium

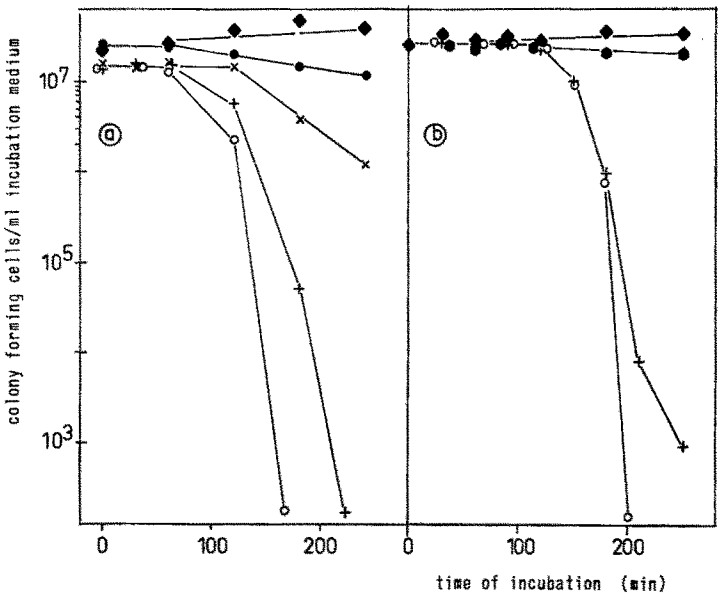
Influence of Intact Protein Biosynthesis and Oxidative Phosphorylation on Cell Inactivation by "Sulfite"

The effect of inhibitors of protein biosynthesis as well as of mitochondrial phosphorylation on the "sulfite" effect has been studied (Fig. 5). In the presence of growth promoting nutrients (e.g. YEPD) the rate of

Fig. 5
Influence of "sulfite" on colony formation under various conditions.
a late-log-phase cells starved for 2 h in McIlvain buffer; **b** late-stationary-phase cells

	Na_2SO_3 mM	CHx[a] CCP[a]	CHx[a] CAC[a]	YEPD-medium
● a	–	–	–	–
◆ a, b	–	–	–	+
● b	–	–	+	+
× a	1	–	–	–
+ a	1	–	–	+
+ b	5	–	–	+
○ a	1	+	–	+
○ b	5	–	+	+

Incubation conditions: pH 3.75, 30°C, aerobic, reciprocal shaker.
[a] Abbreviations (and concentrations used)
CHx Cycloheximide 100 µg/ml;
CCP Carbonylcyanide-m-chlorophenylhydrazone 40 µg/ml;
CAC Chloramphenicol 100 µg/ml



inhibition of colony formation after "sulfite" treatment was much higher than in the absence (Fig. 5a). Inhibitors of protein synthesis and oxidative phosphorylation did not alter the "sulfite"-induced inhibition rate of colony formation significantly.

During the first 50 min of incubation "sulfite" induced the production of respiratory deficient mutants; i.e. mutants defective in oxidative phosphorylation. Once having been induced these mutants were impaired in their growth potential by "sulfite" in the same way as the respiratory competent wild type strain (Fig. 6).

These finding further supports the suggestion that oxidative phosphorylation is not necessary for the inhibitory effect of "sulfite" on colony formation.

The Active Species of "Sulfite"

As concentrations of the different species of "sulfite" can be calculated using mass action relations at given concentrations of Na_2SO_3 and at a given pH value correlation of pH change with colony forming capacity under "sulfite" treatment should uncover the active agent.

Table 1 shows that increasing concentrations of SO_2 led to decreased capacity of colony formation, while the concentration of SO_3^{2-} falls and the concentration of HSO_3^- remains constant. No change in the concentration of colony forming cells could be seen in the presence of increasing HSO_3^- and SO_3^{2-} concentrations, when the concentration of SO_2 was kept constant. These results show that SO_2 is the most likely

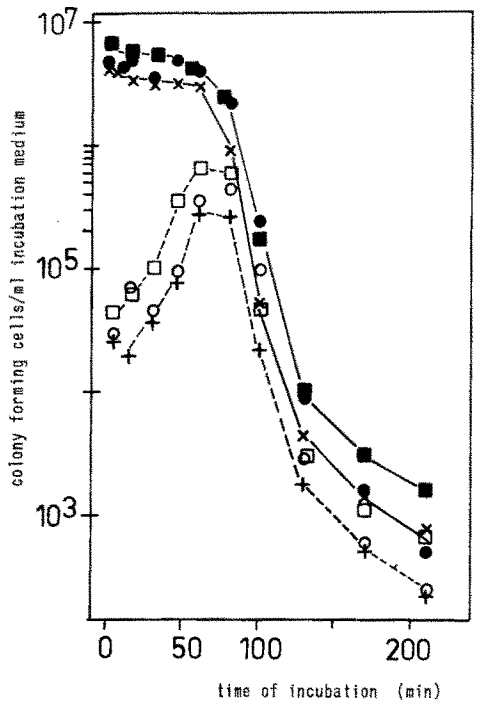


Fig. 6. Effect of "sulfite" on the increase of respiratory deficient mutants in wild type populations of yeast.

Strains used (Ploidy)	4450 1A (1 n)	S288c (1 n)	X2180 (2 n)
Total number of colony forming cells per ml	●	■	×
Number of respiratory deficient mutants per ml	○	□	+

Conditions of incubation: pH 3.7, 30°C, aerobic, 5 mM Na_2SO_3, early-log-phase cells, reciprocal shaker, YEPD-medium

Table 1. Influence of pH value and concentrations of SO_2, HSO_3^-, SO_3^{2-} and Na_2SO_3 on colony forming capacity of the yeast *Saccharomyces cerevisiae*. Conditions of incubation: 30°C, aerobic, late-log-phase cells, reciprocal shaker, YEPD-medium

	Concentration of "sulfite" species (mM)				Colony forming cells/ml after incubation time (min)				
pH	SO_2	HSO_3^-	SO_3^{2-}	Na_2SO_3	0	60	90	120	180
Constant HSO_3^- concentration									
4.0	0.025	3.91	0.004	3.95	1.0×10^7	0.3×10^7	1.0×10^5	1.0×10^1	–
4.6	0.006	3.91	0.019	3.94	0.9×10^7	0.5×10^7	2.1×10^5	1.0×10^1	–
5.2	0.0016	3.92	0.076	4.0	1.1×10^7	1.0×10^7	0.7×10^7	2.7×10^6	–
5.8	0	3.92	0.48	4.4	0.9×10^7	1.0×10^7	0.8×10^7	8.0×10^6	–
Constant SO_3^{2-} concentration									
4.0	0.102	15.752	0.018	15.873	1.4×10^7	1.4×10^7	–	–	3.0×10^1
4.4	0.015	5.786	0.018	5.819	1.3×10^7	1.2×10^7	–	–	1.1×10^6
5.0	0.00087	1.426	0.018	1.444	1.4×10^7	1.4×10^7	–	–	1.8×10^7
5.6	0	0.358	0.018	0.375	1.4×10^7	1.6×10^7	–	–	1.9×10^7
Constant SO_2 concentration									
4.0	0.0102	1.575	0.00175	1.587	1.5×10^7	2.0×10^7	–	–	1.4×10^6
4.4	0.0102	4.041	0.0122	4.064	1.7×10^7	2.4×10^7	–	–	1.6×10^6
5.0	0.0102	16.713	0.205	16.93	1.1×10^7	1.8×10^7	–	–	1.1×10^6

candidate as the active agent responsible for the deleterious effect on colony formation. Similar results have been obtained by Macris (1972) using another yeast, and by Carr et al. (1976) who used lactic acid bacteria from fermented apple juice.

The deleterious effect of SO_2 is in fact the result of a series of reactions:

1. in a fast reaction SO_2 activates an ATP hydrolyzing enzyme system – presumably located in the cell membrane – and causes a rapid decrease of ATP concentration within the cells (Schimz and Holzer, 1979);

2. secondly, SO_2 penetrates the cell membrane, most probably still in the uncharged form;

3. once having entered into the cell, SO_2 may possibly be transformed into a charged species again. In this way the cells may act as a "sulfite"-trap, and a charged species of "sulfite" may be responsible for intracellular actions either with metabolites or with DNA by acting as mutagen (Dorange and Dupuy, 1972) which causes primarily C-G to T-A transitions (Mukai et al., 1970; Hayatsu and Miura, 1970).

If the influence of SO_2 and the other species of "sulfite" on cell metabolism is not stopped within a certain period of time (i.e. during the phase of insensitivity) ATP depletion (Chapman et al., 1971) together with further events leads to cell death both in wild type and mutant strains.

Babich and Stotzky (1978b) did not find "sulfite" induced cell death in *Escherichia coli*. This may be due to the fact that their turbidity measurements were not able to discriminate between viable cells and irreversibly damaged but not yet lyzed cells.

Since there are many targets for "sulfite" attack in living cells, its action can certainly not be described by a simple model mechanism. Besides cell death and mutagenesis some adaptation phenomena have been reported even in higher organisms like plants (Ziegler, 1975; Horsman et al., 1978).

Sulfite producing and tolerating wine yeasts (Dott and Trüper, 1978) should be specially well suited for further investigations of the molecular events of "sulfite" action.

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