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Growth of Wine Yeast and Formation of Addition Compound of Sulfur Dioxide with Carbonyl Compounds under Weakly Acidic pH

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In order to examine the dependency of the concentration of pyruvate released from wine yeast on the bisulfite concentration in a growth medium, two kinds of weakly acidic media were prepared. One of them, the bufferized medium, was convenient, since it allowed yeast growth in the presence of bisulfite ranging from 0 to 25 mM (1600 ppm as SO_2). Analytical data revealed that the pyruvate concentration in the medium did not depend on the bisulfite concentration. Acetaldehyde concentration, on the other hand, increased with the increase of the concentration of bisulfite.

Numerous investigations have been made on the formation of addition compounds between bisulfite and carbonyl-containing compounds during wine fermentation[1~5]. But few attempts have been made to determine the formation of these compounds by wine yeasts under weakly acidic pH, since most grape must have a pH range between 3 and 4[6].

This report mainly describes the growth of wine yeast in weakly acidic media and the concomitant formation of the addition products of bisulfite and carbonyl-containing compounds (bound SO_2). To solve the problem, we used two kinds of media: the one was a usual medium for yeast growth but was neutralized before sterilization; the other was used to give strong buffer capacity between pH 6.0 and pH 6.6 throughout the culture. Special attention was paid to the concentration of the pyruvate released from yeast cells, since the dependency of the pyruvate concentration on the bisulfite concentration has not been examined in detail.

Materials and Methods

Organism *Saccharomyces cerevisiae* IAM 4274 (OC-2), a gift from Prof. Goto, Institute of Enology and Viticulture, Yamanashi University, was used. The maintenance of the strain and the preparation of seed culture were made as described previously[7].

Media The mineral composition of the two defined media was that of Wicker-

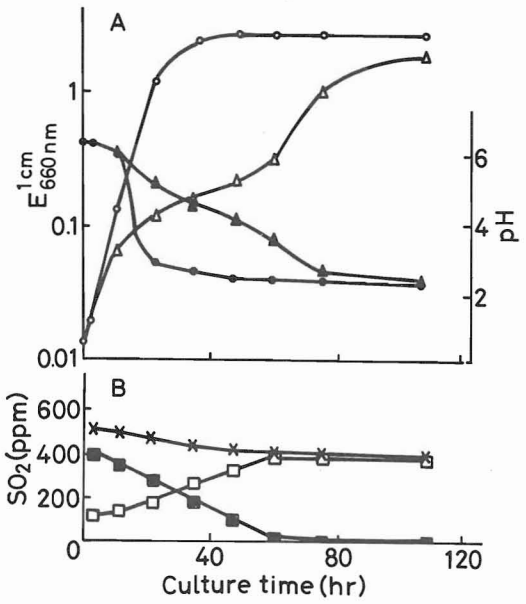

Fig. 1. Growth of *Saccharomyces cerevisiae* OC-2 in a weakly acidic medium in the presence or absence of bisulfite. A chemically defined medium was adjusted to pH 7.0 before sterilization. Cells were grown statically at 30℃. Symbols: growth (○) and pH (●) in the absence of bisulfite: growth (△) and pH (▲) in the presence of 10 mM sodium bisulfite: free SO_2 (■), bound SO_2 (□), total SO_2 (×).

ham[8], and biotin, calcium pantothenate, inositol, and thiamine hydrochloride were added as growth requirements. One of them, the neutralized defined medium, was prepared as follows: 3% glucose was added to the mineral and vitamin solution mentioned above, and neutralized with 2 N potassium hydroxide with the use of a glass electrode pH-meter (Hitachi-Horiba Model M-3) before sterilization. The preparation of the other medium, bufferized medium, was described in a previous paper[7]. In the earlier experiments, a 100 ml portion of the defined medium was poured into a 200-ml Erlenmeyer flask, and later a 400ml portion was poured into a 500-ml Erlenmeyer flask. The flasks were plugged with cotton and autoclaved for 10min at 115℃. All reagents used were of the highest grade available.

Culture The test flasks were inoculated and cultured statically at 30°C[7]. Freshly prepared sodium bisulfite solution was added at 2 hr of the culture time. Samples were taken aseptically at intervals, and the growth of the yeast was followed by E^{1cm}_{660nm}. The culture pH was determined by the pH-metter discribed above.

Determination of bisulfite The method of Rankine[9] was used to determine both "free" and "bound" SO_2. Total SO_2 was obtained by summing free SO_2 and bound SO_2.

Enzymatic estimation of pyruvate, α-ketoglutarate and acetaldehyde Pyruvate was enzymatically determined by the method described by Vassault[10]. α-Ketoglutarate was determined by using glutamate dehydrogenase[11]. Acetaldehyde was determined as follows; 2.00 ml of 0.1M potassium phosphate buffer (pH 7.8), 0.05 ml of 1.35mM NADH solution, and 0.05ml of sample solution were mixed in a quartz cuvette and equilibrated to 20℃ in a double beam spectrophotometer (Hitachi 323). After the addition of 0.05ml of alcohol dehydrogenase (from yeast, product of Oriental Yeast Co. Ltd. Tokyo) solution (150 units per ml) the reaction mixture was allowed to stand for 20 min. The difference of absorbance at the wave length of 340 nm before and after enzyme addition was recorded, and the amount of acetaldehyde was calculated from the difference.

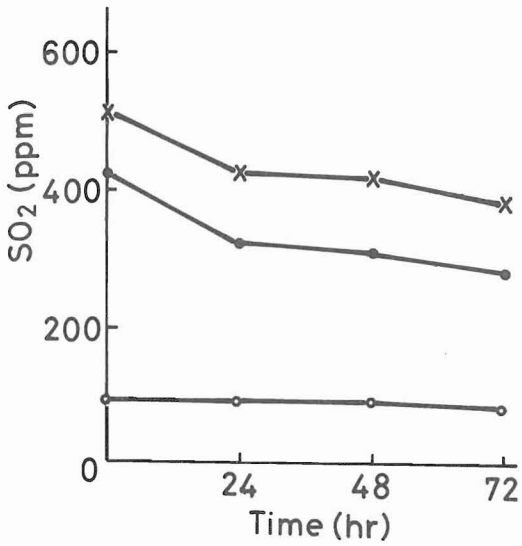

Fig. 2. Changes of free SO_2 bound SO_2 and total SO_2, in sulfite in potassium phosphate buffer (pH 6.0) solution. Freshly prepared sodium bisulfite solution was added to the potassium phosphate buffer, and aseptically incubated at 30℃. Symbols: (●), free SO_2; (○), bound SO_2; (×), total SO_2.

Results and Discussion

Growth of *S. cerevisiae* OC-2 in the neutralized defined medium with or without bisulfite Figure 1 A shows the variation profiles of growth and medium pH in the presence (10mM or 640 ppm as SO_2) and absence of sodium bisulfite. Figure 1 B shows the amounts of free SO_2 and bound SO_2 in the medium throughout the culture. In the presence of 10mM bisulfite, the yeast grew almost normally for about 10 hr, then gradually reduced its growth rate, and reached a stationaly phase of growth[12]. The pH of the medium showed a two-step decrease which reflected the growth profile. Cellular growth was greatly retarted when more bisulfite was added to the medium. The medium, therefore, was considered to be unsatisfactory.

Free SO_2 decreased almost linearly for about 60 hr and bound SO_2 conversely increased in the duration. The extent of bound SO_2 at the initiation of cellular growth was about 100 ppm (20% of total SO_2). King *et al*[13] reported that the percentage binding of SO_2 (300 ppm initial concentration) by 3% glucose in the pH range 2.5-4.5 was 20%. The coincidence of the two values suggested that the binding extent of SO_2 with glucose depended not on the concentration of SO_2, but mainly on the concentration of glucose over a wide (2.5-6.0) pH range. The increase in bound SO_2 with the progress of culture time (Fig. 1 B), thus, was considered to be not the addition compound of the remaining glucose, but those of other carbonyl compounds which were released by yeast cells. To verify this, 3% glucose was aseptically incubated at 30℃ with 10mM sodium bisulfite in potassium phosphate buffer (pH 6.0). No increase in bound SO_2 was observed for 72 hr (Fig. 2).

Alcohol dehydrogenase method for the determination of acetaldehyde The usual enzymatic method for acetaldehyde determination uses aldehyde dehydrogenase as a substrate specific enzyme[14]. But alcohol dehydrogenase is more available. The problems in the use of alcohol dehydrogenase are: firstly most samples contain alcohol which is not only the metabolic product of statically growing yeast, but also the reaction product of the enzyme reaction. Secondarily bisulfite in the samples is an enzyme inhibitor. The equilibrium constant (K) of the reaction[15]

$$\text{Ethanol} + \text{NAD}^+ \rightleftharpoons \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

is 8.0×10^{-12}. The value means that the reaction is very favorable for the for-

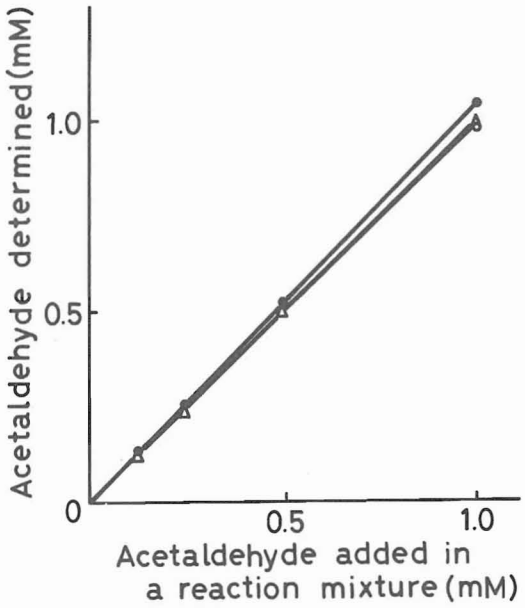

Fig. 3. Calibration of acetaldehyde determination by the use of alcohol dehydrogenase. Ethanol (2%) or sodium bisulfite (10 mM)was added to acetaldehyde before determination. Symbols: (●), standard system; (△), with 2% ethanol; (○), with 10 mM sodium bisulfite.

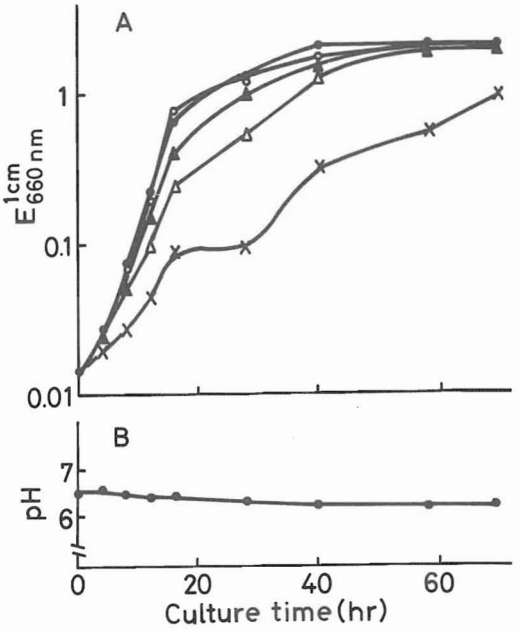

Fig. 4. Growth of S. cerevisiae OC-2 in the bufferized medium in the presence or absenc of bisulfite. Growth conditions were same as described in Fig. 1. Concentrations of sodium bisulfite were 0mM (●), 5mM (○), 10mM (▲), 15mM (△), and 25mM (×).

mation of ethanol around neutral condition. Alcohol contained in the samples, therefore, does not disturb the determination of acetaldehyde substantially. When the sample contains 10mM bisulfite, the bisulfite is diluted to 0.25mM with buffer solution in the quartz cuvette. As bisulfite is not a very potent inhibitor, the presence of bisulfite does not affect the proceeding of the reaction seriously. When a sufficient amount of alcohol dehydrogenase is used in the reaction mixture, the reaction reaches the equiliblium point within a short reaction period. Figure 3 shows that acetaldehyde can substantially be determined in the presence of 2% ethanol or 10mM bisulfite.

Growth of *S. cerevisiae* OC-2 in the bufferized medium (glucose 5%)

Unlike the results in Fig. 1 A, 10mM bisulfite did not cause a two-step growth (Fig. 4) whereas 25mM (1600 ppm as SO_2) bisulfite caused a two-step growth. The difference seems to be the result of a much slower decrease of pH in the bufferrized medium (Fig. 4 B) than that in the neutralized defined medium (glucose 3%). An additional reason was the difference in glucose concentration.

As representative of the experimental data obtained, the case of 25mM bisulfite was selected (Fig. 5) to show the amounts of free SO_2 and bound SO_2. At 48 hr of

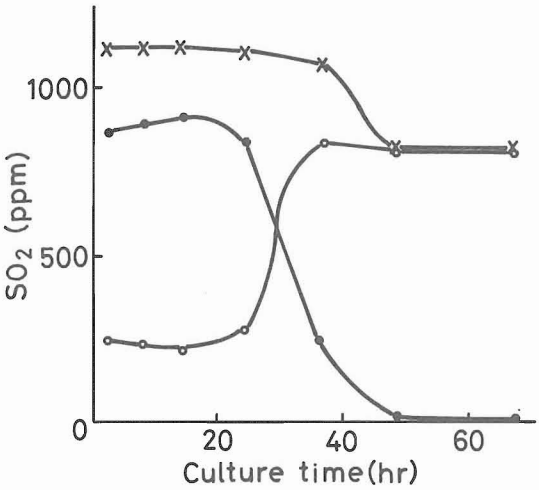

Fig. 5. Variation profiles of free SO_2, bound SO_2 and total SO_2 in the bufferized medim. 25mM sodium bisulfite was added at 2hr of culture time. Symbols: (●), free SO_2; (○), bound SO_2; (×), total SO_2.

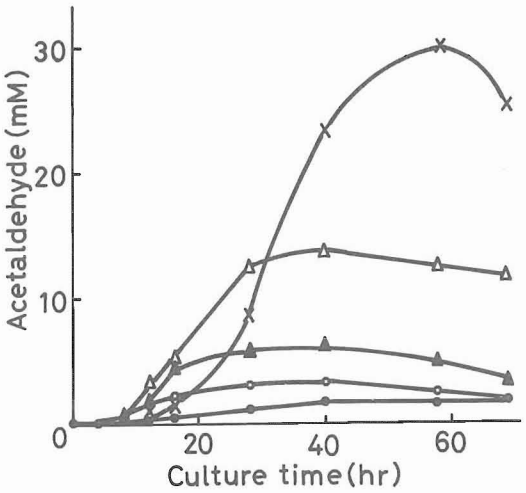

Fig. 6. Acetaldehyde concentration in the bufferized medium with the change of bisulfite concentration. Concentrations of sodium bisulfite were 0 mM (●), 5mM (○), 10mM (▲), 15mM (△), 25mM (×).

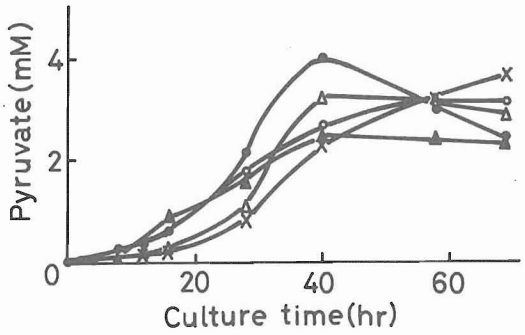

Fig. 7. Pyruvate concentration in the bufferized mdium with the change of bisulfite concentration. Concentrations of sodium bisulfite were 0mM (●), 5mM (○), 10mM (▲), 15mM (△), 25mM (×).

culture time, free SO_2 disappeared and the bound SO_2 reached the maximum amount. Figure 6 and 7 are the variation profiles of acetaldehyde and pyruvate concentrations in the bufferized medium. Increasing the amounts of bisulfite caused a corresponding increase in the quantity of acetaldehyde. The pyruvate quantity was not influenced by the amounts of bisulfite. Although trace amounts of α-ketoglutalate were detected in every sample, it was not enough to compare amounts.

Growing wine yeast releases organic acids, malic and citric, and to a lesser extent, lactic, succinic and pyruvic into the medium[6]. Although it is produced in small amounts, pyruvic acid has been considered to be important in wine making because of its capacity to bind bisulfite. In spite of efforts of several investigators[4,5], it has been unclear whether or not the amount of pyruvate formed depends on the amount of bisulfite added. When sulfite is dissolved in water, it forms various molecular species (H_2SO_3, HSO_3^-, SO_3^{2-}) and their percentage distributions are dependent on the pH value[1]. Undissoci-

ate sulfite (H_2SO_3) has a lethal effect on microorganisms and bisulfite form (HSO_3^-) is inhibitory to fermentation because of its ability to combine with acetaldehyde, the normal hydrogen acceptor in glycolysis. The pH value of grape must ordinarily lies between 3 and 4[1]. In this acidic range sulfite has a greater lethal effect. A little amount (100-200 ppm as SO_2) of bisulfite, therefore, is enough to suppress the growth of most undesirable microorganisms but does permit the growth of desirable wine yeast. The fact seems to be one of the reasons that the relationship between the amount of bisulfite added and that of pyruvate released have been obscure[4,5]. In order to clarify the relationship, we used the bufferized medium which can allow addition of a greater amount (25mM or 1600 ppm as SO_2) of bisulfite without lethal effects on yeast. The results in Fig. 7 proved that there is no relationship between the amounts of bisulfite and pyruvate.

With acetaldehyde, the addition reaction reaches equilibrium rapidly, and nearly complete conversion is obtained even in the absence of excess bisulfite[16]. This accepted fact and the results of Fig. 6 suggest that acetaldehyde is released from yeast cells as long as free SO_2 exists in the medium to form the addition compound. The process is identical to that of the commonly known glycerol fermentation or Neuberg's second form of fermentation[17].

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