Fermentation Processes Leading to Glycerol

II. Studies on the Effect of Sulfites on Viability, Growth, and Fermentation of *Saccharomyces cerevisiae*

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The high concentrations of free sulfite and bisulfite necessary to direct ethanolic fermentation of hexoses towards formation of glycerol and acetaldehyde in the sulfite process cause considerable reduction in the rates of growth and rate and completeness of fermentation by yeast. In an investigation of variables influencing the sulfite fermentation of blackstrap molasses, Freeman and Donald (1957) showed that reducing sugar attenuation at a given time decreased with increase of sulfite dosage and free sulfite concentration and it was observed that little yeast cell division took place in the presence of sulfite at pH 6.7 to 7.0. In the present paper, the effects of sulfite-bisulfite on rate of fermentation of glucose by yeast and on growth rate and viability of *Saccharomyces cerevisiae* have been determined over a wide range of pH in simpler systems. The effect of some products of the sulfite fermentation, namely acetaldehyde and acetaldehyde-bisulfite complex, on rate of fermentation of glucose by yeast has also been studied.

The inhibition of the activities of normal yeast cells by sulfite-bisulfite raises the question of the possible isolation of sulfite resistant strains. The use of sulfite resistant yeast cultures in wine manufacture (Joslyn and Cruess (1934) as cited by Prescott and Dunn (1949)) is well known, where small quantities of sulfur dioxide or potassium metabisulfite are added to destroy or inhibit undesirable microorganisms including "wild" yeasts without serious injury to the pure culture of specific wine yeast subsequently added. The concentrations (around 0.022 per cent as Na_2SO_3) and pH values (about pH 3) are, however, of an entirely different order from those involved in the sulfite fermentation.

The effects of sulfites on yeast growth and fermentation have been investigated by Tomoda (1928), who reported that the presence of sodium sulfite or of $SO_3^{=}$ ion nearly arrested the propagation of yeast and that the fermentative activity was greatly diminished. It was stated that acetaldehyde-sodium bisulfite compound had no injurious effect upon the yeast cell or fermentation but in alkaline media the complex was slightly dissociated into its components, which produced injurious effects. In a further paper (Tomoda, 1929) the rate of fermentation in the presence of sulfite was reported. It was concluded that fermentation rate in a synthetic medium was seriously affected by the presence of sulfite, whereas in a molasses medium the corresponding effect was small. The maximum sodium bisulfite concentration which permitted fermentation was stated to be 0.2 per cent.

Experimental Methods

The analytical methods used were as described by Freeman and Donald (1957).

Isolation of Yeast Strains and Growth in Presence of Sulfite-Bisulfite

A series of yeast cultures was isolated from commercial pressed yeast and samples of brewers' and distillers' yeasts by suspension of the samples in sterile saline and plating on to beer wort agar medium in Petri dishes which were incubated at 35 C for 24 hr. Typical colonies of *S. cerevisiae* were picked on to beer wort agar slopes and the cultures stored at 5 C. The cultures were "acclimatized" to tolerate the presence of sulfite-bisulfite concentrations of 5.0 to 6.6 g/100 ml (as Na_2SO_3) at pH 6.7.

Effect of Sulfite-Bisulfite on Yeast Growth

(*a*) *In aerated cultures.* Diluted molasses medium (195 ml) containing reducing sugars (8 g/100 ml) and having an initial sulfite concentration of 2.4 g/100 ml (as Na_2SO_3) and pH 6.7 was inoculated with a beer wort culture (5 ml) of *S. cerevisiae* strain B.71 (isolated from an industrial alcohol distillery yeast). One series of cultures was aerated with air at 25 vol/vol/hr and another series at 80 vol/vol/hr. A similar series of cultures was prepared having an initial sulfite concentration of 3.6 g/100 ml. The cultures were incubated at 35 C in conical flasks, and yeast cell counts were determined at intervals by the hemocytometer method. The multiplication factor, K, was calculated from the formula

$$q = a \times K^t$$

where a is the initial number of cells per unit volume and q is the number after time, t, expressed in hours.

The values of K (table 1) were significantly smaller than those obtained in corresponding experiments with sulfite free medium where K = 1.4 to 1.5.

In comparative experiments with uninoculated controls, extensive oxidation of sulfite occurred under the experimental conditions, although the total of sulfite and sulfate remained constant.

(*b*) *In unaerated cultures.* A similar study of the rate of yeast growth was carried out in unaerated cultures. A series of diluted molasses media (195 ml) containing reducing sugars (5 g/100 ml) and having initial sulfite concentrations of 0, 0.5, 0.8, 1.6, 2.6, and 3.1 g/100 ml (as Na_2SO_3) and pH 6.7 were inoculated with a culture of yeast strain B.71 (5 ml), and incubated at 35 C for 56 hr without agitation. To minimize surface oxidation the experiments were carried out in cylinders (31 × 3.5 cm internal diameter).

The multiplication factor K (table 2) shows a sharp fall in the rate of yeast growth as the initial sulfite concentration was raised from zero to 1.6 g/100 ml. There was no growth with an initial sulfite concentration of 3.1 g/100 ml, and virtually none in the range 2 to 3.1 g/100 ml. Visible fermentation took place in all cases after an induction period (8 to 48 hr) which increased steadily with the initial sulfite concentration. The fermentation resulted in fixation of sulfite, but the reduction in free sulfite concentration did not lead to an increase in growth rate although the maximum yeast population had not been reached. This observation suggested that total sulfite concentration, free plus fixed sulfite, was the significant factor influencing rate of yeast growth under these conditions.

Effect of Sulfite-Bisulfite on Yeast Viability

The toxicity of sulfite-bisulfite to yeast at a series of pH values in the range 5 to 10 was determined as follows: A series of glucose-phosphate-sulfite media was prepared, containing glucose (5 g/100 ml), dipotassium hydrogen phosphate (0.3 g/100 ml) and sodium sulfite (3.5 g/100 ml) with adjustment of the media to pH 5, 6, 7, 8, 9, and 10 by addition of sodium hydroxide or hydrochloric acid. The media (500 ml) were each inoculated with 10^8 cells of *S. cerevisiae* strain B.71 and a similar series was inoculated with strain B.71 after acclimatization to the presence of 6.6 g/100 ml sulfite equivalent at pH 6.7. The media were incubated at 30 C for 24 hr and constant pH values were maintained by periodic addition of acid or alkali. Viable cell counts were determined at intervals by the agar plate method. The results are plotted in figure 1. Minimum toxicity occurred in the range pH 8 to 8.5 and the toxicity increased sharply at pH values below or above this. Even at pH 8.3 the cell counts fell to ¼ of the original after 7 hr and to $\frac{1}{40}$ after 24 hr. There was no significant difference between the acclimatized and unacclimatized strains. Similar results were obtained when the glucose-phosphate-sulfite media were replaced by

TABLE 1. *Rate of yeast growth in aerated sulfite-containing media*

Diluted molasses media (195 ml; 8 g/100 ml reducing sugars) containing the initial sulfite concentrations stated below (as Na_2SO_3) at pH 6.7 were inoculated with *Saccharomyces cerevisiae* strain B.71 (5 ml). The cultures were incubated at 35 C.

Aeration Rate	Time (t)	Free Sulfite Concentration	Cell Count	Multiplication Factor (K)	Mean Multiplication Factor (t = 0–24)
vol/vol/hr	*hr*	*g/100 ml*	× 10^6/*ml*		
25	0	2.4	3		1.11
	4	2.3	4	1.07	
	8	2.1	10	1.16	
	24	0.8	25	1.09	
	32	0.8	58	1.09	
	48	0.7	24	1.04	
80	0	2.4	3		1.14
	48	0.7	20	1.04	
25	0	3.6	2		1.08
	48	1.5	20	1.05	
80	0	3.6	2		1.10
	48	1.8	50	1.07	

TABLE 2. *Rate of yeast growth in unaerated sulfite containing media*

Diluted molasses media (195 ml; 5 g/100 ml reducing sugars) containing the initial sulfite concentrations stated below (as Na_2SO_3) at pH 6.7 were inoculated with *Saccharomyces cerevisiae* strain B.71 (5 ml). The cultures were incubated at 35 C.

Initial Free Sulfite Concentration	Time (t)	Cell Count	Multiplication Factor (K)	Mean Multiplication Factor (t = 0–24)	Final Free Sulfite Concentration
g/100 ml	*hr*	× 10^6/*ml*			*g/100 ml*
0	56	128	1.05	1.13	—
0.5	56	60	1.03	1.06	0.18
0.8	56	60	1.03	1.04	0.31
1.6	56	40	1.02	1.01	0.49
2.6	56	14	1.01	1.003	0.68
3.1	56	10	1.0	1.00	0.90

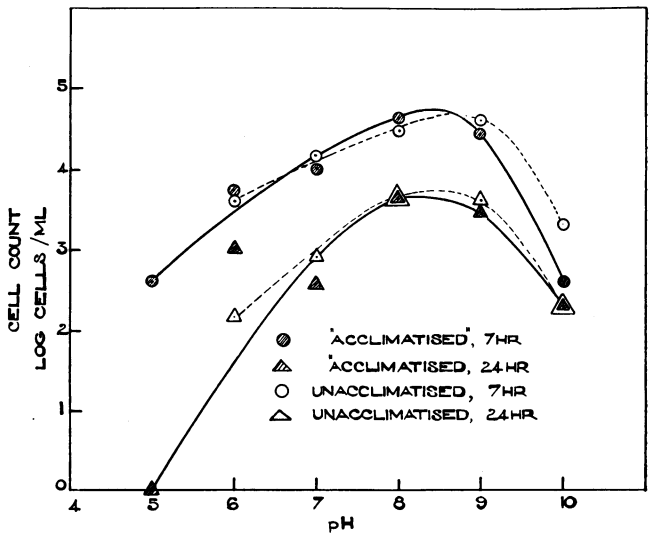

FIG. 1. Effect of pH on viability of *Saccharomyces cerevisiae* in glucose-phosphate-sulfite medium.

similar media containing diluted molasses instead of glucose, and when a culture isolated from commercial bakers' yeast was introduced into each series of media.

Effect of Sulfite-Bisulfite on Initial Rate of Fermentation

A series of media was prepared which contained glucose (5 g/100 ml), dipotassium hydrogen phosphate (0.3 g/100 ml) and sodium sulfite (at concentrations in the range of 0 to 2.9 g/100 ml). The pH was adjusted to pH 6.8 to 6.9. The media (500 ml) were inoculated with a commercial bakers' pressed yeast (10 g) and incubated at 35 C without aeration. Samples were withdrawn at intervals for determination of reducing sugars. The fall in rate of fermentation with increasing sulfite concentration is shown in table 3.

Effect of pH on Rate of Fermentation in the Presence of Sulfite-Bisulfite

A series of media was prepared containing glucose (5 g/100 ml), dipotassium hydrogen phosphate (0.3 g/100 ml) and sodium sulfite (3 g/100 ml). The pH of these media was adjusted to a series of values in the range pH 7.0 to 9.4 by addition of sodium hydroxide. The media (500 ml) were inoculated with commercial pressed yeast (10 g) and incubated at 35 C. The pH was restored to the initial value by addition of sodium hydroxide at 30 minute intervals. The rates of fermentation during the period 0 to 2 hr from inoculation were determined from the fall in sugar concentration. The results (table 4) showed that most rapid fermentation occurred in the range pH 7.8 to 8.1. Similar results were obtained using media based on molasses (table 5). Outside the range pH 6 to 9 the rate of fermentation fell extremely rapidly.

At high initial sugar concentrations (21 g/100 ml; table 5) the effect of pH on rate of fermentation appeared to be less sharply defined, and the optimum pH somewhat lower. This was substantiated by a series of fermentations carried out on a 7 L scale (initial sugar concentration of medium 21 g/100 ml) under the conditions described by Freeman and Donald (1957), in which pH values of 7, 8, and 9 were maintained by periodic addition of 30 per cent aqueous sodium hydroxide. At pH 9 fermentation was partially inhibited after 24 hr and ceased at about 48 hr. Fermentation at pH 7 was complete in 120 hr, but at pH 8 it was slower throughout and was incomplete at 144 hr. It was evident that under these conditions the fermentations were influenced by factors which are unimportant in the experiments described in tables 4 and 5.

Effect of Acetaldehyde and Acetaldehyde-Sodium Bisulfite Compound on Rate of Fermentation

(a) *Acetaldehyde.* The effect of acetaldehyde on the rate of fermentation of glucose by yeast was determined by Slator's (1906) method. The media contained glucose (5 g/100 ml), dipotassium hydrogen phosphate (0.3 g/100 ml) and acetaldehyde (0 to 1.0 g/100 ml). The acetaldehyde solution used in the preparation of the media was standardized by Ripper's (1900) method. The rate of fermentation was halved in presence of an acetaldehyde concentration of 0.1 to 0.25 g/100 ml (table 6). Similar results were obtained when laboratory strain B.71 was used in place of the commercial pressed yeast employed in the above experiments.

There was a drift of pH towards more acidic reactions during the fermentations, but the constancy of the rates observed at 10 min intervals showed that this drift had no important effect on the rates of fermentation.

TABLE 3. *Effect of sulfite-bisulfite on rate of fermentation of glucose by Saccharomyces cerevisiae at pH 6.8 to 6.9*

The media contained glucose (5 g/100 ml), K_2HPO_4 (0.3 g/100 ml) and sulfite-bisulfite (0 to 2.9 g/100 ml as Na_2SO_3). The medium (500 ml) plus commercial pressed yeast (10 g) was incubated at 35 C.

	Initial Sulfite Concentration (g/100 ml)					
	0	0.4	0.9	1.6	2.0	2.9
Rate of Fermentation (mg glucose/100 ml/min)						
a) 0–2 hr	13.3	9.2	7.5	6.7	6.7	5.8
b) 0–7 hr	10.5	9.8	5.0	3.5	3.3	2.8

TABLE 4. *Effect of pH on rate of fermentation of glucose in presence of sulfite-bisulfite*

The media contained glucose (5 g/100 ml), K_2HPO_4 (0.3 g/100 ml) and sodium sulfite (3 g/100 ml). The pH was adjusted by addition of sodium hydroxide to the values shown below. Medium (500 ml) plus commercial pressed yeast (10 g) was incubated at 35 C, and the pH of the fermenting liquors was maintained at the desired values by addition of sodium hydroxide at 30 min intervals. The rate of fermentation was determined from the fall in glucose concentration during the initial 2 hr period.

	pH					
	7.0	7.4	7.8	8.1	8.6	9.4
Rate of fermentation (mg glucose/100 ml/min)	7.3	10.9	11.3	11.8	9.3	8.8

TABLE 5. *Effect of pH on rate of fermentation of molasses media in presence of sulfite-bisulfite*

The media were based on molasses. Other conditions were as stated in table 4.

Initial Reducing Sugar Concentration	Rate of Fermentation (mg invert sugar/100 ml/min) at pH			
	7.2	7.6	8.0	9.2
g/100 ml				
5.0		11.2	11.8	3.3
8.8	6.2		12.6	
21.0	12.8	13.3	8.7	

(*b*) *Acetaldehyde-sodium bisulfiite compound.* The compound was prepared by mixing equivalent quantities of sodium bisulfite and acetaldehyde in aqueous solution. Free bisulfite in the solution was determined by addition of iodine to a portion and back-titration with sodium thiosulfate, and further acetaldehyde was then added until the amount of free bisulfite was negligibly small. To determine the concentration of acetaldehyde-sodium bisulfite compound the complex was decomposed by saturation of the solution with sodium bicarbonate and the liberated bisulfite was titrated with iodine (Underkofler *et al.*, 1951). The compound was not isolated in the dry state in which it was found to undergo rapid oxidation.

The rate of fermentation of glucose by yeast in the presence of concentrations of acetaldehyde-sodium bisulfite compound in the range 0 to 18.8 g/100 ml was determined as in the previous experiment. As before there was a downward drift of pH during the course of experiments. The results (table 6) showed the complex to possess a significantly lesser inhibitory effect than either of its components.

Discussion

The importance of the influence of sulfite-bisulfite on yeast growth, viability, and rate of fermentation in relation to the sulfite fermentation has been demonstrated by the work described above. The quantitative aspects of the presence of sulfite-bisulfite on yeast growth in aerated media are difficult to interpret because, under these conditions, oxidation of part of the sulfite to sulfate takes place. There is also loss of free bisulfite by combination with acetaldehyde. In unaerated cultures, however, the effect of sulfite-bisulfite is clearly defined and growth ceases when the sulfite equivalent concentration is raised above 3.0 g/100 ml at pH 6.7. pH has an important influence on viability and fermentation rate in the presence of sulfite-bisulfite since this factor determines the relative proportions of sodium sulfite and the highly toxic bisulfite component in the mixture. Acetaldehyde and acetaldehyde-sodium bisulfite compound, products of the sulfite fermentation, were shown partially to inhibit yeast fermentation of glucose, although Tomoda (1928) states that the bisulfite compound had no injurious effect on fermentation except in alkaline media.

Table 6. *Rate of fermentation of glucose in the presence of acetaldehyde and of acetaldehyde-sodium bisulfite compound*

The media contained glucose (5 g/100 ml), K_2HPO_4 (0.3 g/100 ml) and the reagent as shown below. Medium (50 ml) and commercial pressed yeast (1.0 g) were incubated at 35 C. The rate of fermentation was determined by Slator's (1906) method during the initial 20 to 60 minute period.

Reagent	Concentration	pH of Medium		Rate of Fermentation
		Initial	Final	
	g/100 ml			*mg glucose/100 ml/min*
Acetaldehyde	0	4.8	Approx 4.0	12.7
	0.1			8.8
	0.25			3.3
	0.5			1.7
	1.0			1.0
Acetaldehyde	0	7.0	5.3	9.0
	0.1		4.9	6.8
	0.25		5.6	2.8
	0.5		5.9	1.4
	1.0		6.2	1.0
Acetaldehyde-sodium bisulfite compound	0	4.8	Approx 4.0	12.4
	5			8.9
	10			5.4
	18.8			0.8

The inhibition of the activities of the yeast cell by sulfite-bisulfite stimulates interest in the question of isolation of sulfite-resistant strains. Earlier workers have suggested that use of yeast cultures which had previously been grown in the presence of sulfite-bisulfite led to improvement in the sulfite fermentation. They referred to such cultures as "sulfite-acclimatized." One of the aims of the present work was to make a critical assessment of these claims. It has been assumed that if any advantageous changes had taken place as a result of sulfite-acclimatization, which would be of value in carrying out the fermentation on a technical scale, they would have been evident in their effect on one or more of the following criteria: (a) yield of glycerol in terms of hexose fermented, (b) rate of fermentation of glucose in presence of sulfite, and (c) rate of growth in presence of sulfite. Experiments on criterion (a) are reported by Freeman and Donald (1957). It does not follow that strains acclimatized to give increased fermentation rates (b) would necessarily also have increased growth rates in the presence of sulfite-bisulfite (c). Production and maintenance of sulfite-resistant strains would necessitate the presence of sulfite-bisulfite at every stage since if a genetically mixed culture were grown in absence of sulfite-bisulfite the nonacclimatized would be expected to outgrow the sulfite-resistant cells. This raises an important experimental difficulty as none of the strains so far examined grew in unaerated media containing sulfite equivalent concentrations exceeding 3 g/100 ml. Growth took place in aerated cultures but this observation is ascribed to reduction of the free sulfite equivalent concentration by oxidation to sulfate.

A further possible method of acclimatization of yeasts to ferment in the presence of sulfite-bisulfite was investigated by our colleague, Mr. A. J. Baillie, in experiments undertaken in collaboration with Professor G. Pontecorvo, F.R.S.; part of the work was carried out in the Department of Genetics of Glasgow University and the authors wish to acknowledge the close cooperation of Professor Pontecorvo. The method em-

ployed was a modification of that of Spiegelman *et al.* (1944), originally used for detection of galactose-fermenting cells in yeast populations. Yeast colonies, embedded between two layers of agar, were grown in Petri dishes. The media contained relatively high concentrations of sulfite-bisulfite at pH 6.7 in order to determine the presence of cells which fermented under these conditions and provide a means of isolation. A colony which produced vigorous fermentation was readily distinguished by the typical starshaped crack produced in the agar in the immediate neighborhood. The tests were carried out with a "Yeastrel"-peptone-agar medium containing sulfite equivalent concentrations of 0.5 to 10 g/100 ml. Independently of the sulfite concentration, in this range, 90 to 99 per cent of the colonies fermented under these conditions and gave starshaped colonies. It was concluded that cells adapted to ferment in the presence of high sulfite equivalent concentrations were not detectable by this means.

We have failed to find any evidence of production of yeast strains acclimatized to ferment hexose in the presence of sulfite-bisulfite by any of the methods investigated. It is concluded that even if it were possible by techniques not yet known to develop such strains, their importance in technical scale fermentations would be very limited as yeast growth is powerfully inhibited in the presence of these reagents in propagation media.

Acknowledgment

We wish to express our thanks to Mr. A. J. Baillie for assistance in the experimental work.

Summary

In unaerated media, growth of *Saccharomyces cerevisiae* was powerfully inhibited by sulfite-bisulfite. At pH 6.7, no growth took place in the presence of sulfite equivalent concentrations exceeding 3.1 g/100 ml and virtually none in the range 2 to 3.1 g/100 ml.

The viability of yeast in the presence of 3.5 g/100 ml sulfite equivalent is dependent upon pH. Maximum viability was observed in the range pH 8 to 8.5 and the toxicity of these reagents increased sharply at pH values below and above this.

Rates of fermentation of glucose in the presence of sulfite-bisulfite were determined by rate of fall of substrate concentration. The initial rate of fermentation of glucose by yeast was halved by addition of a sulfite equivalent of 1.0 to 1.5 g/100 ml. In the presence of a sulfite equivalent concentration of 3.0 g/100 ml, the optimal rate of fermentation of glucose, both in synthetic and molasses media, was at pH 7.5 to 8.0. The rate of fermentation of glucose was inhibited by products of the sulfite fermentation. Acetaldehyde was much more toxic than acetaldehyde-sodium bisulfite compound. The former reduced the initial fermentation rate of glucose by one-half at a concentration of 0.1 to 0.25 g/100 ml and the latter at a concentration of approximately 10 g/100 ml.

None of the methods investigated led to the isolation of yeast strains adapted to increased growth or more rapid fermentation in the presence of sulfite-bisulfite. It is concluded that the use of "sulfite-acclimatized" yeast cultures leads to no advantages in the sulfite fermentation.

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