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The Production of 2,3-Butanediol by Fermentation of Sugar Beet Molasses[1]

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The production of 2,3-butanediol from natural materials by fermentation offers a practical and economical method for obtaining appreciable quantities of this important chemical. A variety of substrates have been used satisfactorily such as wood hydrolyzates (Perlman,1944), wheat (Katznelson, 1944 ; Ledingham *et al.*, 1945; Ward *et al.*, 1945) and corn (Ward *et al.*, 1945). The use of several grades of beet molasses prepared from home-grown beets has been reported by British (Freeman and Morrison, 1947) and Canadian workers (Anastassiadis and Wheat, 1953). Excellent yields were obtained which compared favorably with those resulting from the use of pure sucrose.

Although the characteristics of three distinct fermentations yielding this chemical have been determined (Freeman, 1947), of concern to us was the use of *Aerobacter aerogenes* since the fermentable carbohydrate in beet molasses is primarily sucrose. In this paper are presented the results of a study to determine the suitability of sugar beet molasses as a fermentable medium for the production of 2,3-butanediol.

Experimental Methods

Sugar beet molasses produced locally were found to contain 60 to 65 per cent sucrose; this material was diluted to various sucrose concentrations (4 to 22 per cent) by dissolving the desired amount of molasses in distilled water and heating to 50 C. Ten g of ground barley malt per liter of medium were added and the mixture stirred for 30 minutes at 50 C. The media were then transferred to 64-ounce bottles or 2-liter Erlenmeyer flasks fitted with openings for the aseptic introduction of liquids or solids and for the removal of samples of the fermenting media. All media were autoclaved for 20 min at 15 psi pressure.

Reducing sugars were determined by the method of Stiles, Peterson and Fred (1926) following acid inversion as recommended by the A.O.A.C. (1940). 2,3-Butanediol was determined by the periodic acid oxidation procedure of Brockman and Werkman (1933) following ether extraction of the alkaline media. By this method, quantitative recovery of pure 2,3-butanediol added to fermented media was realized. A very dilute aerosol OT solution (1 to 2 ml) was added during the ether extraction to prevent foaming and this in no way influenced the analyses. Acetoin was determined by the method of Langlykke and Peterson (1937). Recovery of pure acetoin added to fermented media was complete by this method. Routine analyses in the early stages of this study revealed that the amount of acetoin formed under varying conditions was very small (65 mg per cent). No further data were sought concerning the concentration of this end point. All pH measurements were made electrometrically.

All experiments reported here were conducted at 37 C. It was found during a preliminary experiment that, under the conditions in this laboratory, fermentations carried out at 37 C were more rapid and complete (although often at lower efficiency) than at 32 C.

A survey was made of the ability of nine strains of *A. aerogenes* to produce 2,3-butanediol. A 10-ml volume of a diluted beet molasses medium (10 per cent sucrose and 1 per cent malt) was inoculated with the test organism and incubated for 24 hr. The contents of this tube and 10 g of sterile calcium carbonate were added aseptically to a large flask containing 990 ml of the same medium. This fermentation, essentially anaerobic, was allowed to continue for 144 hr. At this time, the amount of sucrose fermented and butanediol produced were determined. The efficiency was calculated on the basis that 2 moles of diol may be produced from each mole of sucrose fermented (0.526 g diol per g sucrose fermented). Three strains fermented 78 to 90 per cent of the available sucrose and produced 44 to 60 per cent of the theoretical amount of diol under these conditions. Strain A-5 was used in these further studies.

Beresford and Christensen (1941) have indicated that the addition of small amounts of mold bran or malt to yeast fermentations involving beet molasses greatly improved the yield of ethanol. They suggested that these supplements supplied growth factors and nitrogenous components essential for the yeast and that enzymatic action may have increased the amount of fermentable carbohydrate. Our work demonstrated that the production of this diol was markedly improved by the addition of small amounts (1 per cent) of malt (McCall, 1943).

In the following experiments, other than the aeration

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studies, flasks containing 1.3 to 1.5 L of medium were employed and were inoculated with a 48-hr mass culture of *A. aerogenes*. The inoculum was prepared by washing the cells from the surface of a large flat bottle containing a 220-cm^2 area of nutrient agar (about 1 cm in depth).

In the remainder of this paper are presented the results of a survey to determine the effect of the initial sucrose concentration, the use of calcium carbonate for pH control and aeration on the fermentation of sugar beet molasses.

Effect of Sucrose Concentration

In determining the optimum sucrose concentration, a number of factors were of importance. The osmotic pressure of the medium could not be such as to interfere with the growth of the bacteria. When the sucrose concentration is high, the fermentation may be retarded because the bacteria are unable to tolerate their own end products. This effect is illustrated in figure 1.

In a typical experiment, sucrose concentrations of 4.2 to 16.4 per cent were used for fermentations over periods up to 4 days. Ten g of sterile calcium carbonate per liter of media were added after autoclaving. Media containing 4 to 6 per cent sucrose were almost completely fermented in 24 hr (figure 1). The actual yield of diol was above 90 per cent of theory on the basis of the sucrose added. With sucrose concentrations above

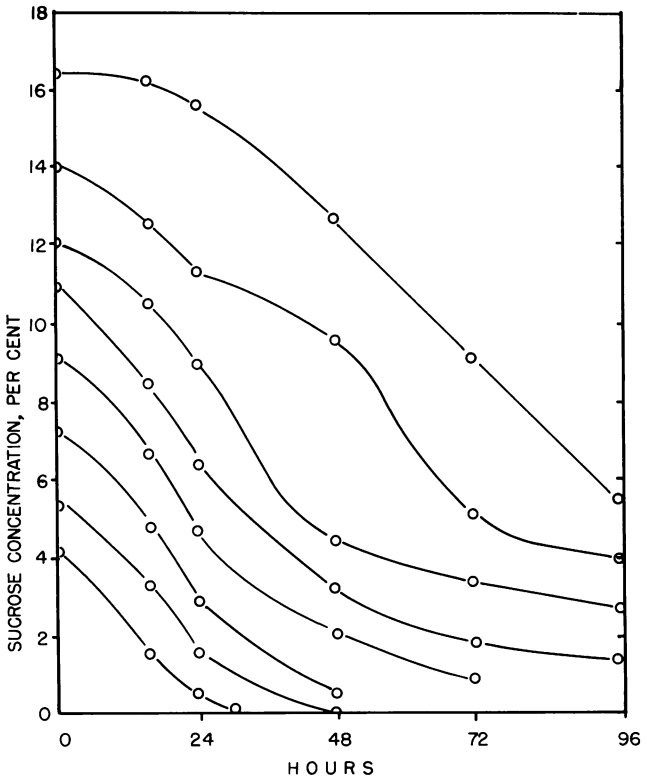

FIG. 1. Utilization of sucrose in solutions of varying initial concentrations.

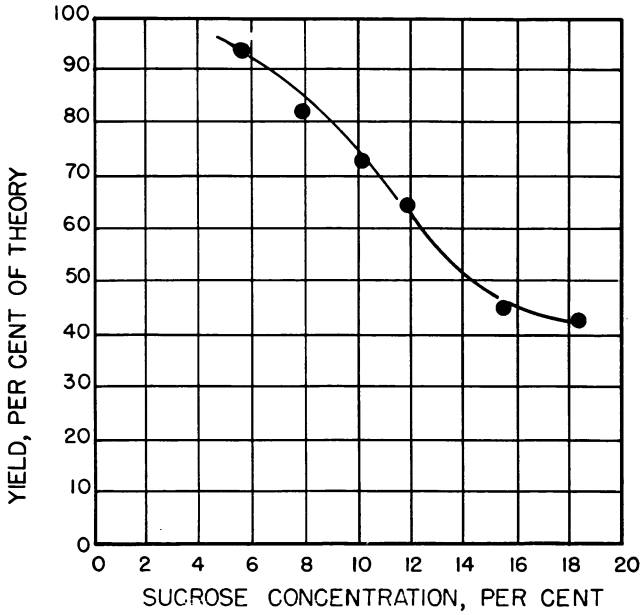

FIG. 2. 2,3-Butanediol production with different sucrose concentrations.

6 per cent, the fermentations were incomplete in many cases even after incubation periods of 98 hr and, although the actual yield per liter was greater, the efficiency was lower than that found for low sucrose concentrations (figure 2). With high sucrose concentrations, extending the fermentation period resulted in a more complete but always a less efficient fermentation.

Although it was possible that the incomplete fermentation of media containing high sucrose concentrations was due to the accumulation of the end products and their adverse effects on the bacteria, no studies were conducted in which diol was removed since the difficulties involved in its removal without producing deleterious effects on the unfermented residues are obvious.

Use of Calcium Carbonate

Although the pH of an unbuffered beet molasses medium (10 per cent sucrose) was 6.9 to 7.1 following sterilization, the production of organic acids and CO_2 during the fermentation caused the pH to drop rapidly to 5.7 within 5 hr after inoculation regardless of the strain of *A. aerogenes* used or the initial pH of the media (range 5.8 to 7.4; see figure 3). After 24 hr, the pH had risen to 6.0 and sucrose decreased rapidly during the next 24 hr until the pH reached 5.5 or below. At this point the fermentation practically ceased although the sucrose content was about 5 g per cent.

A simple method of preventing extensive lowering of the pH was to add an excess (1 per cent) of sterile calcium carbonate following sterilization of the medium. In these cases the pH dropped to 6.1 to 6.3 seven hr after inoculation (figures 3 and 4), and remained at 6.0 to 6.2 until the major portion (70–80 per cent) of

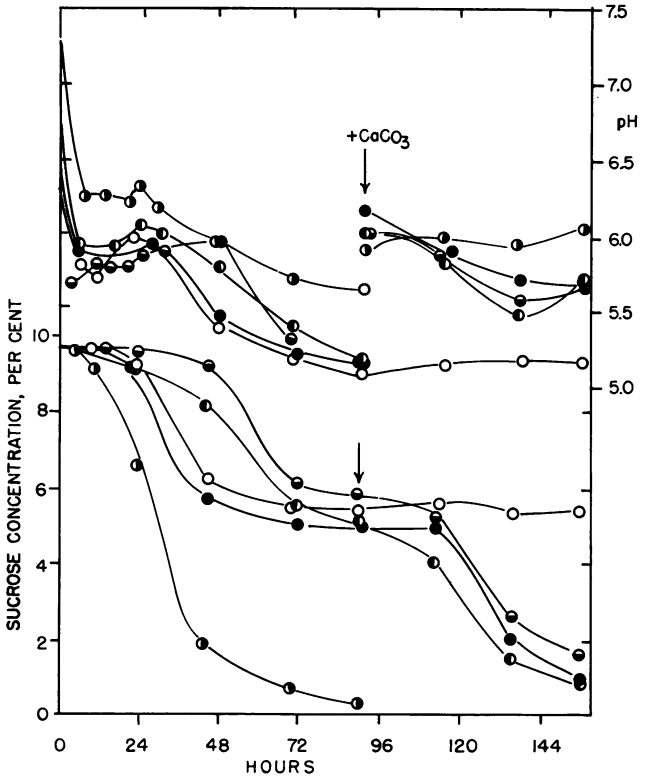

FIG. 3. Effect of $CaCO_3$ and initial pH on sucrose utilization ◑ contained $CaCO_3$ from start, initial pH 7.38; all other (except ○) received $CaCO_3$ after 91 hours (see arrow in figure). The initial pHs of each were: ○, 6.30; ●, 6.72; ◐, 7.04;, ◒, 5.83.

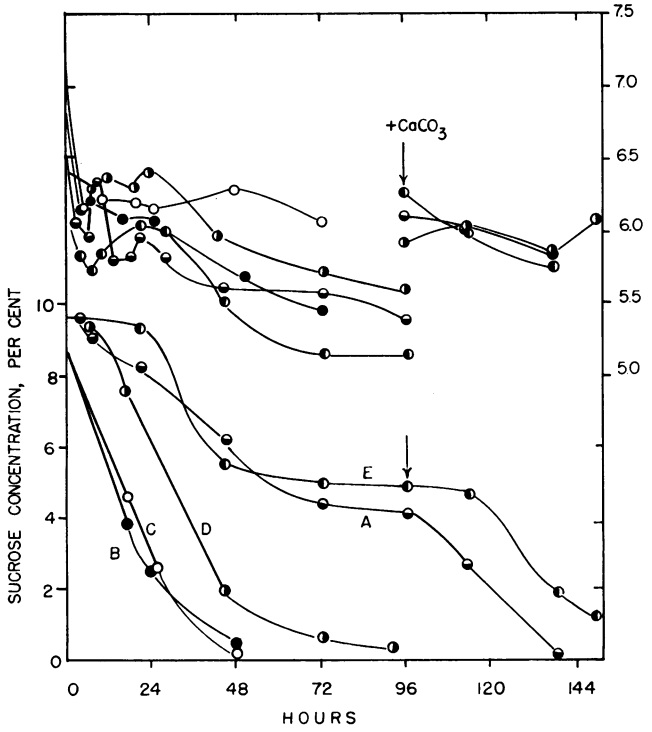

FIG. 4. Effect of aeration and $CaCO_3$ on sucrose utilization ◒ curve A, aerated, no $CaCO_3$ present until time indicated by arrow on curve; ● curve B, aerated for 24 hours only, with $CaCO_3$; ○ curve C, aerated for 72 hours, with $CaCO_3$; ◑ curve D, containing $CaCO_3$ but not aerated; ◐ curve E, aerated, no $CaCO_3$ present until time indicated by arrow on curve.

the sucrose was fermented. The pH then slowly dropped below 6.0. Vigorous fermentation occurred while the pH was above 6.0 to 6.2 and was detected by considerable gassing and "head" production during the first 18 hr and by the rapid decrease in the sucrose concentration.

When calcium carbonate was added at some time subsequent to inoculation (generally when the pH of the unbuffered medium dropped to 5.2), the yield of 2,3-butanediol was greater than when it was added with the inoculum (table 1). However, the time required for the complete utilization of the sucrose was considerably increased. A striking effect of the lack of carbonate during the early stages of the fermentation was that the fermentation was significantly delayed for the first 15 to 18 hr even though the pH of the medium was maintained at 5.8 or above during this time (figure 3). This same lag period also followed the first addition of carbonate (at 91 hr, figure 3). The fact that a less efficient fermentation resulted when the carbonate was added with the inoculum may be due in part to rendering unavailable for butanediol formation certain intermediates as they are formed during the early stages of the fermentation. This observation is in contrast to that of Ledingham *et al.* (1945) who reported that the best yields were obtained with *Aerobacillus polymyxa* fermentations when calcium carbonate was added at the start of the fermentation.

Effect of Aeration

Although it is evident that theoretical considerations of the fermentation mechanism (Kluyver, 1931; Stahly and Werkman, 1942) postulate an anaerobic process, aeration may be of value in removing carbon dioxide produced in the process and thus have a stimulatory effect on the fermentation. Aeration has been employed by others to increase the rates of fermentation of naturally occurring materials by *A. aerogenes* (N.R.R.L. Report, 1942; Kluyver and Scheffer, 1933). The retention of this gas in unaerated media may account in part for the higher acidity generally encountered. These experiments were undertaken to determine the effect of aeration on the fermentation in the presence and absence of added calcium carbonate.

Fermentations were conducted in pyrex test tubes (65 x 500 mm) containing 800 ml of beet molasses medium (10 per cent sucrose) and fitted with openings for the aseptic addition of solutions and withdrawal of samples. An automatic air pump (Wood *et al.*, 1940) was used to pass 65 ml of sterile air per min through a sintered glass disc at the bottom of the tube. This

TABLE 1. *2,3-Butanediol production by* Aerobacter aerogenes (A-5)

Initial Sucrose Concentration	Sucrose Utilized	Fermentation Time	2,3-Butanediol Yield		
				Per cent of theory	
				Sugar utilized*	Sugar added†
1 per cent $CaCO_3$ added with inoculum					
gm per cent	*per cent*	*hr.*	*gm per cent*		
4.22	90.0	24	1.54	77.3	69.5
5.66	71.0	24	2.01	95.4	67.4
7.35	92.8	48	2.01	56.2	52.1
9.23	90.8	72	2.22	50.5	45.7
10.60	82.5	72	2.16	46.9	38.8
12.10	72.8	72	2.71	58.5	42.7
14.15	63.6	72	3.18	67.0	42.7
16.40	45.2	72	3.64	62.2	42.2
16.40	68.0	96	—	—	—
1 per cent $CaCO_3$ added 91 hours after inoculum					
5.76	100.0	191	2.87	94.7	94.7
7.90	95.3	191	3.44	85.8	82.8
10.10	92.3	191	3.89	79.4	73.3
11.80	92.0	236	4.12	72.2	66.4
14.20	65.3	236	3.18	65.2	42.6
15.40	61.6	236	3.67	73.5	45.3
18.50	58.2	236	4.34	76.5	44.5

* See text; based on actual sucrose fermented.
† See text; based on initial sucrose concentration.

quantity of air was about one-half that used by Kluyver and Scheffer. The only organism used in these studies was *A. aerogenes* A-5. The preparation of the inoculum and the inoculation procedure were as described previously in the text.

The importance of calcium carbonate and aeration are apparent in the several experiments reported in figure 4 and table 2. These data support the hypothesis that either the addition of calcium carbonate or aeration alone produced the same initial stimulatory effect on the rate of sucrose utilization. The most rapid fermentation resulted with constant aeration in the presence of calcium carbonate. It is of interest to note that under anaerobic conditions, as reported above, fermentation of molasses containing 9 per cent sucrose and an excess of calcium carbonate was complete in 91 hr (curve D, figure 4) but that with continuous aeration the fermentation of the same medium was complete in 48 hr (curve C, figure 4). However, as in the previous experiments, higher efficiency of 2,3-butanediol production resulted when the addition of calcium carbonate was delayed until the fermentation was well under way.

Early in the course of these investigations it was noted that one sample of beet molasses (# 1376) was fermented much slower than others even in the presence of malt. Sucrose utilization did not proceed above 50 per cent of that when other samples were used. Several fermentations were carried out in which small amounts

TABLE 2. *Effect of aeration and $CaCO_3$ upon the production of 2,3-butanediol by* Aerobacter aerogenes (A-5)

Exp. No.	Curves in Figure 4	Conditions of Fermentation	Initial Sucrose Concentration	Fermentation Time*	2,3-Butanediol Production	
					Final concentration	Yield, per cent of theory
			gm per cent	*hr.*	*gm per cent*	
A-1	D	Nonaerated. 1% $CaCO_3$ added at time of inoculation	9.70	96	2.99	58.8
A-2	E	Nonaerated. 1% $CaCO_3$ added 91 hr. after inoculation	9.70	161	2.90	62.2
A-3	A	Aerated continuously. 1% $CaCO_3$ added 91 hr. after inoculation	9.70	138	3.50	68.6
A-4	B	Aerated 24 hr. 1% $CaCO_3$ added at time of inoculation	8.90	72	2.04	43.6
A-5	C	Aerated continuously. 1% $CaCO_3$ added at time of inoculation	8.90	72	2.46	52.5

* The time for 100 per cent utilization of sucrose.

TABLE 3. *Properties of recovered 2,3-butanediol*

Boiling point	178.3 C
Melting point	−17 to −12 C
Density d_{20}^{20}	1.108
Refractive index	1.4310 (29 C)
$[\alpha]_D^{27c}$	+1.50

of $MnSO_4$, $FeSO_4$ and $MgSO_4$ were added but neither this combination of salts nor KH_2PO_4 were effective in increasing the rate of fermentation. This sample of beet molasses contained 73.0 mg per cent residual SO_2 while the others which were readily fermented contained 65 mg per cent or less. No attempt was made to lower the SO_2 content and then test for fermentability.

Characteristics of Product

Using a rectifying column, a small amount of 2,3-butanediol was recovered from fermented media and purified by fractional distillation for characterization. The pure product had the properties recorded in table 3 which indicate that it was a mixture of the d- and meso forms with a preponderance of the latter (Ward, *et al.*, 1944).

Summary

The preparation and fermentation of media from molasses prepared from regionally grown sugar beets by several strains of *Aerobacter aerogenes* are described.

With low sucrose concentrations (4 to 6 per cent) and

in the presence of malt and excess calcium carbonate, fermentation proceeded rapidly to completion in 24 hr. At higher sucrose concentrations (to 17 per cent) fermentations were incomplete and less efficient.

One per cent calcium carbonate resulted in efficient control of the pH.

Continuous aeration stimulated the rapid utilization of sucrose and materially aided in pH control.

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