2,3-BUTYLENE GLYCOL AS A FERMENTATION BY-PRODUCT FROM CITRUS WASTES

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The compound, 2,3-butylene glycol, can be produced by bacterial fermentation from almost any sugar source under the proper conditions, however, cost is an important factor. During World War II this fermentation was studied intensively and developed through the pilot plant stage using wheat as a substrate (9). The fermentation never became of industrial importance because of the high cost of wheat.

In past years studies on citrus wastes have indicated the suitability of these liquors for fermentation. Some of the products obtained in this manner include ethyl alcohol, feed yeast (8), and citrus vinegar (4). Similarly, the use of these wastes as sugar sources for the 2, 3-butylene glycol fermentation has much to recommend it. Disposal of liquid citrus cannery wastes is a difficult and expensive problem of the processing industry and, with expected increased production, will become even more so. At present the primary method of disposal is through manufacture of citrus molasses. It is felt that fermentation of these wastes to compounds of potentially greater marketability and profit to the processor, e.g., butylene glycol, may be a more satisfactory answer to the disposal problem. This study was designed to provide the basis for commercial production of 2,3-butylene glycol from citrus wastes.

MATERIALS AND METHODS

Cultures. — Two known butylene glycol-producing organisms and one soil isolate were used in this study. *Bacillus polymyxa* NRRL-B-510 and *Aerobacter aerogenes* B-199 were obtained from the Northern Regional Research Laboratory, Peoria. The soil isolate, a species of *Aerobacter*, designated A-101, was obtained in the vicinity of a citrus feed mill. A. *aerogenes* B-199 and culture A-101 were markedly similar in rapidity of fermentation and quantity of glycol produced and may be considered equivalent in all other aspects.

Preparation of starter cultures. — All stock cultures were maintained on nutrient agar slants. Prior to use, the cultures were activated by transfer to fresh nutrient agar slants at 18-24 hr. intervals with incubation at 30°C. Cultures were then acclimatized to the substrate by one or more transfers to citrus press liquor or diluted molasses agar slants. These media consisted of the following: plain citrus press liquor (8-12° Brix) or citrus molasses (diluted to 20° Brix) containing 0.3 per cent yeast extract and 2.0 per cent agar. The media were adjusted to pH 7.0-7.5 with 10N NaOH before sterilization which gave a final pH of 6.5-7.0 after sterilization. After obtaining vigorous growth on either of these media a final transfer was made and incubated for 18-20 hr. at 30°C. This growth was suspended in sterile water and transferred to 1-L Erlenmeyer flasks containing 300 ml of molasses or press liquor medium. Flasks were incubated on a reciprocating shaker at 30°C. for 12-18 hr. and transferred to the fermentation substrate at a rate of 5-7 per cent (v/v).

Fermenter. — Fermentations were run in a 15-L fermenter containing a total volume of 13.4-L (12.5-L substrate +0.9-L starter culture). The fermenter was constructed entirely of glass and stainless steel (Figure 1). The

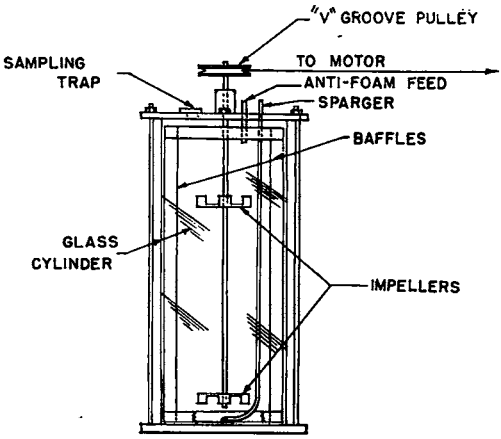

Fig. 1. Schematic diagram of fermenter.

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various environmental conditions affecting the fermentation, e.g., aeration, stirring, and temperature were controlled by appropriate devices. Optimum pH for fermentation was maintained by addition of 10N NaOH and foaming was prevented by addition of Dow-Corning Antifoam B at predetermined intervals and dosing rates.

Fermentation substrate. – The medium found most favorable to good glycol production by all species, with low residual sugar, consisted of 20° Brix citrus molasses or press liquor. These concentrations were obtained by dilution or concentration as required. The 12.5-L quantities of either of these substrates employed per fermentation run were sterilized at 121°C. for 30 minutes. After cooling, 0.4 per cent urea was added, the pH adjusted to 6.2, and the starter culture was added.

Miscellaneous determinations. – The effect of a number of variables upon the efficiency of the fermentations were determined includ-

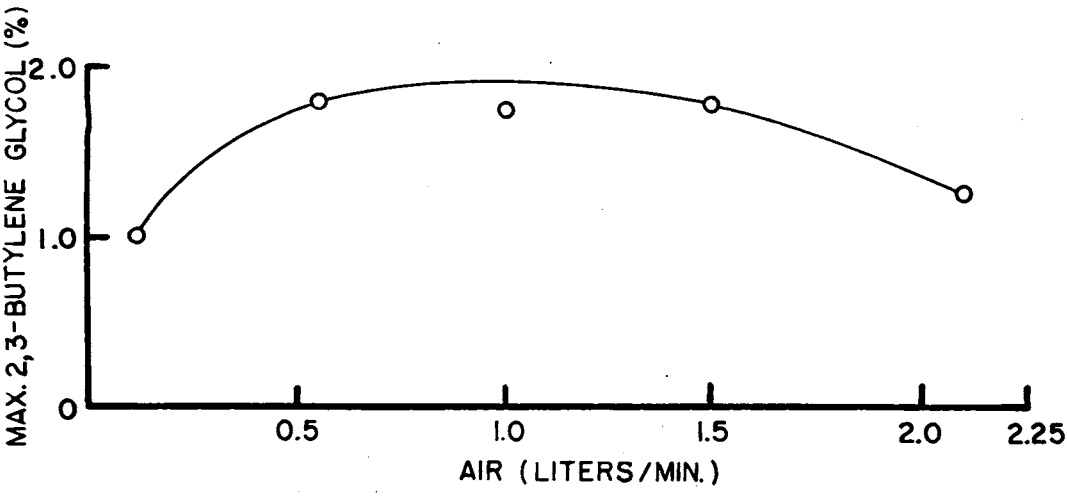

Fig. 2.....Effect of aeration upon production of 2,3-butylene glycol by Aerobacter spp.

Table 1. Effect of sugar concentration on glycol production

Type substrate	° Brix	Total sugar*	Maximum glycol produced	Fermentation time	Residual sugar
		per cent	per cent	hr	per cent
Press liquor	10	7-10	1.3-2.4	24	0.8-1.9
Press liquor (conc.)	20	21-22	4.9-5.3	56-64	1.4-2.2
Molasses	20	17-21	4.8-4.9	48	3.0-3.1
Molasses	25	23	5.7	40	3.8
Molasses	30	35	0	48	25.0

Culture: Aerobacter spp. A-101
Basal medium: KH_2PO_4, 0.2%; urea, 0.4%
Aeration rate: 1,000 ml/min
Stirring rate: 420 rpm

* Total carbohydrates: sugars and polysaccharides calculated as glucose.

ing volume of inoculum, aeration rate, stirring rate, temperature, nitrogen source and quantity, sugar concentration, pH, buffers, and mineral supplementation of the substrate.

Analytical methods. — The course of the fermentation was followed by periodic analysis of the substrate for total sugar by the method of Morris (6) and for 2,3-butylene glycol content by the method of Desnuelle and Naudet (2).

Recovery of glycol from fermentation beer.— Two characteristics of 2,3-butylene glycol make recovery of this compound from the fermentation beer rather difficult. Butylene glycol has a high boiling range (180-182°C.) which results in development of a number of problems when direct distillation is attempted. The extreme water-solubility of the glycol also interferes with solvent extraction as a method of recovery.

Two methods were employed for recovery of glycol. Distillation of glycol at atmospheric pressure was attempted but was found to be unsatisfactory due to charring of the residual sugar in the beer and subsequent binding of the glycol. Distillation under reduced pressure (25 mm) using glycerol as a supporting solvent was more satisfactory.

Table 2. Effect of sugar concentration on glycol production

Type substrate	° Brix	Total sugar*	Maximum glycol produced	Fermentation time	Residual sugar
		per cent	per cent	hr	per cent
Molasses	20	17.5	4.2	40	2.4
Molasses	20	17.0	2.3	48	4.5
Molasses	20	17.8	3.5	48	2.1
Molasses	20	22.0	4.4	46	3.2

Culture: Bacillus polymyxa B-510
Basal medium: urea, 0.4%
Aeration rate: 1,500 ml/min
Stirring rate: 420 rpm

* Total carbohydrates: sugars and polysaccharides calculated as glucose.

Table 3. Liquid-liquid extraction of 2,3-butylene glycol

Glycol content of beer (%)	Treatment	Solvent	% of original glycol extracted
4.4	None	Chloroform	20.8
4.4	Filtration, pH 3.0	Chloroform	27.7
4.4	None	Ethyl acetate	11.6
4.4	pH 3.0, 3% NaCl	Ethyl acetate	32.8
4.4	pH 3.0, 3% NaCl, filtered	Ethyl acetate	35.0
2.25	Two-fold conc., 3% NaCl, extract 2X	Ethyl acetate	63.4
2.6	15% NaCl, filtered	Ethyl acetate	50.0

Table 4. Physical characteristics of 2,3-butylene glycol

Source/ reference	Boiling range	Refractive index	Optical rotation
Aerobacter spp. A-101	180-182° C	$n_D^{23} = 1.4332$	$[\alpha]_D^{23°} = +0.88°$
Commercial (Tech.) redistilled	180-182° C	$n_D^{23} = 1.4359$	$[\alpha]_D^{23°} = 0.0°$
Morell and Auernheimer (5)	180-182° C	$n_D^{18} = 1.4381$	$[\alpha]_D^{23°} = +1.06°$
Clendenning (1)	- -	$n_D^{25} = 1.4366$	$[\alpha]_D^{20°} = +0.8°$
Freeman (3)	183° C	$n_D^{21.5} = 1.4371$	$[\alpha]_D^{23°} = +1.80°$
B. polymyxa B-510	180-182° C	$n_D^{23} = 1.4345$	$[\alpha]_D^{23°} = -1.01°$
B. polymyxa B-510	180-182° C	$n_D^{23} = 1.4314$	$[\alpha]_D^{23°} = -8.87°$
Neish (7)	180-182° C	$n_D^{26} = 1.4318$	$[\alpha]_D^{23°} = -13.34°$

Counter-current liquid-liquid extraction was also attempted using chloroform or ethyl acetate as extracting solvents in a York-Scheibel Multi-Stage Liquid-Liquid Extractor.

Results and Discussion

Starter cultures. — Prior adaptation of *B. polymyxa* and *Aerobacter* cultures to molasses or press liquor exhibited only slight effect on maximum glycol production but contributed to a shortened lag period and increased rate of fermentation. Only slight differences in rate and extent of growth of the cultures were found upon varying the sugar concentration of media between 8° and 20° Brix. Both yeast extract and urea were adequate nitrogen sources for growth of slant and starter cultures although yeast extract was preferred due to its heat stability. The volume of shake culture medium had no appreciable effect upon the rate or quantity of growth of starter cultures. Addition of buffers, e.g., $CaCO_3$, to molasses or press liquor agar slants and shake cultures was found to be unnecessary and had little effect upon quantity of growth of the cultures for the relatively short incubation periods employed. Initial adjustment of the media to pH 6.5-7.0 was adequate for growth of these cultures.

Sugar concentration. — The term "sugar" used in this discussion refers to total carbohydrates (sugars and polysaccharides). Although single-strength press liquor contained sufficient sugar for fermentation by *Aerobacter spp.* (Table 1), higher sugar concentrations were considered desirable due to an increased amount of glycol produced which gave greater efficiency in recovery. The sugar concentration giving the best glycol production (*Aerobacter*—4.8-5.3 per cent in 48-64 hr., *B. polymyxa* — 3.5-4.4 per cent in 40-48 hr.) for both cultures was approximately 17-22 per cent and was obtained by diluting molasses or concentrating press liquor to 20° Brix. It should be noted that use of °Brix as the sole means of measuring sugar content of press liquor or molasses can be misleading due to the high content of dissolved solids other than sugars, however it was a convenient and relatively reproducible means of diluting molasses. Residual sugar was somewhat greater and maximum glycol content lower for *B. polymyxa* (Table 2) than for *Aerobacter* cultures. A 25 per cent sugar content gave slightly better glycol production but with a higher sugar residue. A sugar concentration of 35 percent apparently was excessive for all cultures since glycol could not be detected after 48 hr.

Nitrogen supply. – A number of compounds were tested for suitability as sources of nitrogen for these cultures. Yeast extract, 0.3 per cent, permitted vigorous growth of all cultures and good glycol production by the *Aerobacter* cultures but gave a low maximum glycol with *B. polymyxa*. This supplement would, however, be impractical in large scale operations due to cost. Ammonium hydroxide, used with the dual purpose of maintaining optimum pH as well as supplying nitrogen, was uniformly unsuccessful. Inorganic nitrogen, e.g., $NaNO_3$, gave poor glycol production with high levels of residual sugar. Ammonium phosphate was also unfavorable for glycol production although residual sugar was low after 65 hr. Urea was found to be the most satisfactory nitrogen source for all cultures at a concentration of 0.4 per cent. It should be further noted that somewhat greater glycol levels were obtained using an ordinary urea-containing fertilizer than with reagent or technical grades of this compound.

Mineral supplements. – Addition of $MgSO_4$, $CaCl_2$, NaCl, $CuSO_4$, $ZnSO_4$, and $FeSO_4$ had no effect upon the fermentation in terms of sugar utilized or glycol produced. Apparently adequate concentrations of these minerals were naturally present in press liquor and molasses.

pH. – Calcium carbonate, as a buffer, resulted in reduced glycol levels and high sugar residues for all species. Satisfactory pH control, within the optimum range 6.0-6.2, was obtained by periodic adjustment with 10N NaOH. This treatment gave low total fermentation times and lower sugar residues. Maintenance of pH within the optimum was a problem only during the early, very active stages of the fermentation and generally occurred within 6-24 hr. after starting. As production of glycol approached a maximum and began to level off, the pH of the fermentation beer increased slowly and frequently attained pH 7.2 and above. Without periodic adjustment, the pH of the fermentation fell rapidly to a minimum of 5.2 to 5.5, resulting in inhibition of the organisms and, consequently, reduced rate of glycol formation.

Temperature. – Temperature had much the same effect upon *B. polymyxa* as upon the *Aerobacter* cultures. The optimum temperature for efficient fermentation was 28°-30°C. Variation of 5°C. above or below this optimum resulted in a marked decrease in total glycol production, as well as an increase in the fermentation time by 6-8 hr.

Stirring and aeration. – Rate of stirring of the fermentation was not critical for any of the cultures. Agitation sufficient to provide good mixing of the medium and to aid in air dispersion and retention was adequate. In general, a stirring rate of 420 rpm was satisfactory. As would be expected, the agitation supplied by aeration alone was vigorous but proved to be inadequate and was reflected in reduced glycol formation. High stirring or aeration rates were undesirable due to increased foaming which necessitated addition of large amounts of antifoam to prevent loss of medium. At the rate employed (420 rpm), foaming was no problem.

While aeration was required for maximum production of glycol by *Aerobacter spp.*, variation in rate over a broad range had little effect upon total yields (Figure 2). The most effective range approximated 500-1,500 ml/min, with a commonly employed rate of 1,000 ml/min. Aeration in the range of 2,000 ml/min resulted in a slight decrease in glycol but with a low sugar residue. *B. polymyxa* was less tolerant of variation in aeration rate with an optimum of approximately 1,500 ml/min. Reduced or increased rates resulted in decreased production of glycol. In the absence of aeration, stirring alone was inadequate for glycol formation by *Aeobacter* and *B. polymyxa* cultures.

Aeration also seemed to play some part in the rapid conversion of glycol to acetoin after the sugar concentration of the medium reached a given minimum level. The concentration at which this phenomenon was observed depended upon the initial sugar level of the medium, and consequently, upon the maximum level of glycol attained. In the presence of high concentrations of sugar with low levels of glycol, conversion to glycol proceeded normally. When the concentration of glycol exceeded that of the sugar, however, glycol concentration progressively decreased while apparent sugar content increased. In runs in which low initial levels of sugar were employed, glycol content of the beer rarely exceeded the residual sugar and competition was not evident.

Recovery of glycol. – Distillation of glycol from the fermentation mixture was useful for obtaining samples for analysis but would be

impractical for large-scale operations. Dehydration of the beer prior to distillation of the glycol (boiling range 180°-182°C) produces a thick gummy mass composed essentially of glycol and residual sugar which interfered with further distillation. Continued heating, sufficient to remove all glycol, resulted in formation of a vitreous char of sugar which was difficult to remove from the apparatus. Addition of glycerol to the beer, as a supporting solvent, aided in maintaining a liquid state but had no effect upon charring. The same difficulties were encountered with distillation under reduced pressure (25mm).

Liquid-liquid extraction of glycol from the fermentation beer seems to be the most promising method of recovery (Table 3) although the percentage of glycol extracted was very low. Adjustment of the pH of the beer and addition of NaCl have resulted in increased recovery although considerable improvement in percentage recovery will be required to give the method commercial application.

Physical characteristics of fermentation glycol. — As would be expected, the technique employed for distillation and fractionation of the glycol had a marked effect upon the physical properties of the compound. While the boiling range of the major fraction of the glycol was 180°-182° C., significant quantities distilled at 177°-180°C., presumably due to contaminants or mixtures of isomers. This may account for the variation in characteristics reported by other workers (Table 4). In the glycol obtained from *Aerobacter spp.*, there is no doubt that mixtures of dextro- and meso-isomers are always obtained since approximately only 1°C. difference in boiling range is found between the isomers.

The glycol obtained from *B. polymyxa* cultures appeared to be the pure levorotatory isomer (Table 4) which is in agreement with the results of Neish (6). Considerable variation in degree of optical rotation was observed for samples of this glycol from different fermentation runs. No explanation can be offered for this apparent discrepancy, however it seems unlikely that it was due to variation in technique since all samples were treated in the same manner. This problem is being subjected to further investigation.

SUMMARY

Citrus molasses and press liquor were found to be excellent substrates for 2,3-butylene glycol fermentation by two *Aerobacter* species and *Bacillus polymyxa*. Prior adaption of cultures to these substrates had little effect upon total glycol production although the initial lag period was decreased by this treatment. High aeration rates and stirring were required for maximum glycol production by *Aerobacter spp.* although rates were not critical. *Bacillus polymyxa* was less tolerant of variation in aeration rate with an optimum of approximately 1,500 ml/min. The most favorable sugar concentration in the fermentation medium was approximately 17-22 per cent. The optimum temperature for fermentation was 29°-30°C. The substrates used for the fermentation were deficient in nitrogen, and of the sources tested, yeast extract or urea were the most satisfactory supplements. Use of $CaCO_3$ as a buffer resulted in decreased glycol production by all species while addition of NaOH to control pH exhibited no adverse effects on the fermentation. Recovery of glycol by direct distillation of the fermentation beer was successful although unsatisfactory due to charring. Continuous liquid-liquid extraction using ethyl acetate appears to be the most promising method of recovery. *Aerobacter spp.* produced a mixture of dextro- and meso- rotatory 2,3-butylene glycol while *B. polymyxa* produced only the levo-isomer. The degree of optical rotation was variable between samples of like isomers.

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