1934

The biochemistry of the production of 2,3-butylene glycol by fermentation

Anson R. Kendall
Iowa State College

NOTE TO USERS

THE BIOCHEMISTRY OF THE PRODUCTION OF 2,3-BUTYLENE GLYCOL BY FERMENTATION

By

Anson R. Kendall

A Thesis Submitted to the Graduate Faculty

for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject - Bio-Physical Chemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College

1934

ACKNOWLEDGMENT

The author wishes to acknowledge his indebtedness to Dr. E. I. Fulmer, for suggesting the problem and for his advice and assistance throughout the course of this investigation. Thanks are also due to Dr. L. M. Christensen, for his many helpful suggestions, and to Mr. Kenneth Dykstra, for his help in certain phases of the analytical work.

TABLE OF CONTENTS

I. INTRODUCTION

One of the fields of research now occupying the attention of many agencies is the utilization of agricultural products, including the so-called wastes, as raw materials for the manufacture of industrial chemicals. From a chemical viewpoint, the principal surplus consists of the carbohydrate materials. The carbohydrates lend themselves admirably to elaboration into useful chemicals by means of fermentation processes. This process of the production of chemicals by fermentation has been described thus by Fulmer (1930): "From a chemical viewpoint, zymology (fermentation) deals with catalysis (or rather autocatalysis) in heterogeneous system. Industrial catalyses and zymotechnical syntheses differ in that in the former case the catalyst, usually a simple type of chemical, is manufactured outside the reacting mixture and then added to the reactant or reactants under controlled conditions. In the latter processes the catalysts, the enzymes, are manufactured during the course of the reaction. This involves a knowledge of the nutrition and the characteristics of the organism and the conditions under which it will produce in the highest degree the particular catalysts required.••••••••• The problem resolves itself into the bringing together of the right organism or organisms and the right medium under optimum conditions".

In the paper under consideration, a table is presented,

based upon compilations of Buchanan and Fulmer (1930) and Fulmer and Werkman (1930) showing the fermentative inter-relationships of the microbiological dissimilation products of the carbohydrates. Forty-eight chemicals are listed. An examination of this list shows that only a very few have been commercially exploited or systematically studied with a view to their large scale production. One of the most striking of recent developments is the large scale production of butyl alcohol and acetone by fermentation.

Of the chemicals listed, attention has been directed in these laboratories to the development of methods for obtaining maximum yields of 2,3-butylene glycol. Preliminary studies were made by Breden (1930) and Breden and Fulmer (1930) on the action of Aerobacter faeni upon xylose and sucrose. Since there are large quantities of pentosans in the agricultural wastes, xylose was included in order to obtain information on the utilization of the pentose sugars. The authors found that the products formed from the two sugars are practically identical.

The purpose of this thesis was to extend these preliminary findings with special reference to the production of maximum yields of 2,3-butylene glycol.

II. HISTORICAL SURVEY

There are many papers in the literature involving the determination of 2,3-butylene glycol and acetylmethyl carbinol; most of these determinations were qualitative and incidental. Hence, there will be briefly reviewed only those communications in which quantitative data were obtained under standardized conditions.

One of the earliest references is that of Péré (1896), who identified acetylmethyl carbinol as produced from mannitol by B. subtilis and B. mesentericus vulgatus, and from dextrose and glycerol by Tyrothrix tenuis. Grimbert (1901) identified this chemical as produced from various sugars by B. tartricus. Desmots (1904) proved this material to be produced from various substrates by several bacteria including B. mesentericus vulgatus, B. fuscus, B. flavus, B. ruber, B. subtilis and Tyrothrix tenuis.

Harden and Walpole (1906) were the first to prove the production of acetylmethyl carbinol and 2,3-butylene glycol by bacterial action on sugars. They found that about 27.2 per cent of the dextrose fermented by B. lactis aerogenes, under anaerobic conditions, was converted into 2,3-butylene glycol. Walpole (1911), using B. lactis aerogenes in a nutrient medium containing 5 per cent sugar (dextrose or levulose), under anaerobic conditions, obtained yields of two optically active forms of the

glycol, the diphenylurethan derivatives melting at 199.5° and 157°C., respectively, with the former composing about 90 per cent of the mixture. Eight grams of the crude glycol were obtained, presumably from a liter fermentation.

Thompson (1911) obtained 9.5 grams of 2,3-butylene glycol from the anaerobic fermentation of glucose by the organism B. cloacae; the boiling point of the fraction was 178°-184°C. The medium contained 5 per cent glucose, 1 per cent peptone and 1 per cent calcium carbonate and was allowed to ferment at 37°C. for 6 weeks. Harden and Norris (1912) found that B. coli communis converted 33 per cent of the dextrose into the glycol, calculated on the basis of sugar carbon.

Lemoigne (1913) found that the relative amounts of 2,3-butylene glycol and acetylmethyl carbinol varied with the time of fermentation. The ratio of carbinol to glycol was 860 to 1718 at the end of 3 days, and at the end of the seventh day was 5772 to 3371. Data obtained by Harden and Norris (1913) showed that Aerobacter aerogenes converted 9.9 per cent of the glycerol used into 2,3-butylene glycol, the fermentation taking place under anaerobic conditions. Lemoigne (1923) reported the action of three strains of the Bacillus proteus group upon dextrose. The amounts of the carbinol and glycol in milligrams per liter produced after various time intervals were

	3 days	6 days	18 days
Acetylmethyl carbinol	16	60	110
2,3-butylene glycol	60	140	50

Breden and Fulmer (1931) studied the fermentation of sucrose and xylose by Aerobacter faeni. The yields of glycol and carbinol may be summarized as follows, in terms of grams of each chemical produced per 100 grams of sugar fermented:

	Xylose		Sucrose	
	Aerobic	Anaerobic	Aerobic	Anaerobic
2,3-butylene glycol	10.5	13.7	15.0	16.7
Acetylmethyl carbinol	2.6	0.2	6.4	0.5

Verhave (1933) found the organisms Aerobacillus polymyxa and Aerobacter aerogenes especially active in the production of 2,3-butylene glycol from carbohydrates.

Brockman (1933), in studying the oxidation-reduction potentials of biological systems, obtained yields of 2,3-butylene glycol as high as 64.9 per cent of the dextrose used. This was calculated as molar conversion and is equivalent to 32.45 grams of glycol per 100 grams of dextrose. The organisms Aerobacter salicinovorum and Aerobacter decolorans each gave yields of the glycol as high as 64.6 per cent, and Aerobacter indologenes gave yields as high as 64.9 calculated on the molar basis.

The production of 2,3-butylene glycol and acetylmethyl carbinol by the action of yeast upon various substrates has been

studied especially by Neuberg and Reinfurth (1923), Neuberg and Rosenthal (1924), Kluyver and Donker (1924), Neuberg and Gorr (1924), Neuberg and Simon (1925), Kluyver, Donker and visser't Hooft (1925), Elion (1926), and others.

III. DESCRIPTION OF METHODS

1. The cultures

A. Aerobacter faeni

This organism was isolated and identified by Burkey (1928) in his studies on bacteria attacking constituents of the corn-stalk.

Burkey describes the organism as follows:

"Non-motile rods, 1.0μ broad and 1.0μ to 3.0μ long, conforming to the generic diagnosis. Acid and gas produced from the mono- and di- saccharides, including melezitose, from pentose sugars, raffinose, rhamnose, trehalose, salicin, aesculin, all the alcohols except erythritol, from glycogen, soluble starch, and pectin. No fermentation from amygdalin, inulin, or the pentosans. Acid and gas in litmus milk. Indol produced. Gelatin not liquefied. Isolated from hay infusion".

The generic diagnosis of Aerobacter is given by Weldin (1927) as follows:

"Motile or non-motile, non-sporeforming rods, fermenting both glucose and lactose with both acid and gas. Produce acetyl-methyl-carbinol (Voges-Proskauer reaction positive); reverse the reaction of 0.5 per cent glucose-phosphate-peptone solution relatively rapidly; generally able to utilize uric acid as an available source of nitrogen. Pathogenicity usually slight or absent".

B. Aerobacter motorium

This organism was also isolated and characterized by Burkey (1928) as follows:

"Motile rods, 0.6μ to 0.8μ by 0.8μ to 2.0μ in size, conforming to the generic diagnosis. Acid and gas produced from the common hexose sugars, the di-saccharides, raffinose, rhamnose, trehalose, and the pentose sugars. The alcohols are fermented

with the exception of glycerol and erythritol. Pectin is fermented. Acid and gas is produced from many glucosides, but there is no fermentation of the poly-saccharides. Amygdalin is not fermented. Litmus milk is fermented with the production of acid, gas, a coagulation and reduction of the litmus. Indol is produced. Gelatin is not liquefied. Isolated from rotted potato".

C. Aerobacter pectinovorum

This organism was isolated and described by Burkey (1928) as follows:

"Non-motile rods, 0.8 μ broad and 1.0 to 3.0 μ long, conforming to the generic diaghosis. Acid and gas from the mono- and di-saccharides, pentose sugars, raffinose, rhamnose, trehalose, salicin, aesculin, glycerol, dulcitol, and other alcohols, but not erythritol, glycogen, most poly-saccharides and pectin. No fermentation from melezitose, amygdalin, or pentosan. Acid and gas produced in litmus milk. Indol is produced. Gelatin is not liquefied. Isolated from creek water".

Cultures of the three organisms just described were kindly furnished by Dr. C. H. Werkman of the Department of Bacteriology.

D. Aerobacter cloacae

Cultures were obtained from the American Type Culture Collection.

The characterization given by Weldin (1927) for this organism is as follows:

"Motile rods, 0.5 to 1.0 μ broad by 0.8 to 2.0 μ long, conforming to the generic diagnosis. Sucrose is fermented with acid and gas production; glycerol, starch, dulcitol and inositol are rarely attacked and adonitol is not fermented. Gelatin is usually liquefied. Indol is usually produced. Litmus milk is acidified and coagulated. Originally isolated from sewage. Found in the alimentary tract".

Burkey (1928) suggested the following modification to the above diagnosis:

"Motile rods, 0.5 to 1.0 μ broad by 0.8 to 2.0 μ long, conforming to the generic diagnosis. Acid and gas produced from sucrose, maltose, raffinose, galactose, arabinose, and mannitol. No fermentation of glycerol, dulcitol, inositol, adonitol, salicin, and inulin. Gelatin liquefied. Indol is produced. Litmus milk is acidified and coagulated. Originally isolated from sewage. Found in the alimentary tract".

E. Aerobacter aerogenes

This organism was furnished by the American Type Culture Collection. It was described by Weldin (1927) as follows:

"A non-motile rod 0.5 to 0.8 broad by 1.0 to 2.0 long, conforming to the generic diagnosis. Acid and gas are formed from sucrose, glycerol, inositol, adonitol and usually from starch; dulcitol is not attacked. Gelatin is rarely liquefied. Indol is rarely formed. Litmus milk is made acid and coagulated. The organism is found in the alimentary tract of man and animals and widely distributed in nature".

Burkey (1928) suggested the above be modified as follows:

"A non-motile rod, 0.5 to 0.8 μ by 1.0 to 2.0 μ in size, conforming to the generic diagnosis. Acid and gas are produced from sucrose, maltose, glycerol, inositol, adonitol, mannitol, salicin, and aesculin; dulcitol, inulin, glycogen, and melezitose are not fermented. Gelatin is not liquefied. Indol is not formed. Litmus milk is made acid and coagulated. The organism is found in the alimentary tract of man and animals and is widely distributed in nature".

2. The Preparation of Media

Since each series of experiments usually involved but one variant, the medium was prepared in one large container and aliquot portions taken for each of the various media. It was

then a simple matter to add to each medium the desired amount of the variable constituent, and to dilute each to a definite volume. This procedure had the advantage of yielding media exactly identical except in regard to the variant.

Erlenmeyer flasks of 500 cc. capacity, closed with cotton plugs, were used as containers. After the media were adjusted to the desired pH, they were sterilized at 15 lbs. pressure for 30 minutes.

3. Methods of Analysis

2,3-Butylene glycol. The first method used for the determination of 2,3-butylene glycol was the ether extraction method described by Breden (1930). This procedure, however, was too laborious to yield itself well to the analysis of a large number of samples. Moreover, a method of eliminating troublesome emulsions that sometimes formed was imperative. After considerable experimentation, these objections were overcome by means of the following method:

To each flask, after fermentation was completed, as evidenced by the cessation of the formation of acid, was added 1 1/2 cc. of 12 N sodium hydroxide. The alkali caused a precipitation of suspended material, including bacteria, leaving a clear solution for analysis. A 20 cc. portion of the clear supernatant liquid was placed, together with 21 grams of powdered

potassium carbonate, in a glass extraction tube which was so constructed that it, together with a small funnel, could be suspended from an A. S. T. M. extraction apparatus, and hence was adapted for the continuous extraction of liquids with an immiscible solvent. Stirring the mixture until all the salt was dissolved gave approximately 26 cc. of a saturated potassium carbonate solution.

The temperature of the water bath was so regulated that about 2 drops of ether condensed each second (45-50°C.), and the extraction was continued for 5 days at this rate. This prolonged extraction was found advisable for complete removal of the glycol. The ether was then evaporated at 45-50°C. and the flask allowed to stand un-stoppered until attaining constant weight (about 15 hours). The amount of impurities in the glycol separated by this method is quite small, as was found when this fraction from a large amount of fermentation mixture was examined.

By the use of some carefully fractionated and remarkably pure glycol (B.P. 182.5°C. corrected), data were obtained on the readings of a dipping refractometer in various concentrations of the glycol in water (Table I).

Table I.

Dipping Refractometer Readings for 2,3-Butylene Glycol at Various Concentrations at 25°C.

Grams of Glycol plus 100 cc. water	Refractometer Reading
0	13.26
1.0	16.19
2.0	19.12
3.0	22.05
4.0	24.98
5.0	29.91

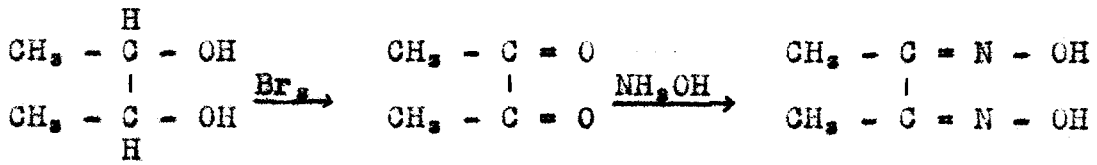
It is evident that the refractometer reading is a linear function of the concentration of the glycol. In order to check the gravimetric method, 20 cc. of water were added to the weighed residues from the extractions, and the solutions were analyzed by the refractometric method. In general, the refractometric methods give somewhat lower results than those obtained by weighing. In the data presented in this thesis, the yields of the glycol represent the average of the values obtained by the two procedures.

The purified material extracted by the above method was characterized by the specific test devised by Lemoigne (1920) and modified by Kluyver, Donker and visser't Hooft (1925). The test was carried out as follows: About 2 drops of the glycol

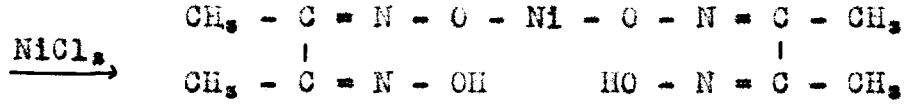
were mixed with 15 cc. of water, 2 cc. of bromine, 5 cc. of a 45 per cent ferric chloride solution, 1 cc. of 0.5 N acetic acid and 3 g. of solid sodium acetate. This solution was refluxed in a soil flask for 20 minutes on a water bath. The soil flask consists of an Erlenmeyer flask with a ground glass stopper in which is sealed a straight tube about 100 cm. in length. The water jacket of a condenser can be attached to it to make a water cooled reflux condenser. After cooling to room temperature the solution was decanted from any liquid bromine remaining. The bromine in the solution was exactly neutralized with a saturated solution of sodium thiosulfate, using starch potassium iodide test papers to determine the neutralization point. The solution was then slowly distilled, 10 cc. were collected and neutralized to litmus with sodium hydroxide. This was mixed with 1 cc. of a 20 per cent water solution of hydroxylamine hydrochloride, 2 cc. of a 20 per cent solution of sodium acetate and about 5 drops of a 10 per cent nickel chloride solution. The mixture was then boiled for a few minutes. A precipitate of fine red needles was formed, showing the presence of 2,3-butylene glycol in the original solution.

The test is due to the oxidation, by bromine in the presence of ferric chloride, of the 2,3-butylene glycol to the easily volatilized diacetyl which is distilled off. The diacetyl reacts with the hydroxylamine to form dimethylglyoxime which in

turn reacts with the nickel chloride to form the characteristic red insoluble nickel dimethyl-glyoxime. These transformations can be represented as follows:



2,3-Butylene Glycol — Diacetyl — Dimethylglyoxime



Nickel Dimethylglyoxime

Analysis of Unfermented Sucrose

Samples of the medium containing 12 per cent of sucrose were hydrolyzed for various periods of time with varying concentrations of hydrochloric acid. The reducing sugars were determined by the Shaffer and Hartmann (1920) method. The most satisfactory procedure was found to be as follows: A 5 cc. sample of the fermented medium, clarified as noted above, was diluted with 35 cc. of water and 5 cc. of concentrated hydrochloric acid. The solution was heated at 75°C. for 9 minutes, cooled quickly to 20°C., and immediately neutralized by the addition of 5 cc. of 12 N sodium hydroxide. The reducing sugar was then determined.

Determination of Acid Produced.

Each fermenting medium was adjusted daily to a definite pH by the addition of 1 N sodium carbonate solution under sterile conditions. The total acid produced is expressed in terms of the total amount of the sodium carbonate solution added during the course of fermentation.

IV. THE EFFECT OF CHEMICAL ENVIRONMENT UPON THE YIELD OF 2,3-BUTYLENE GLYCOL

1. General Discussion

In developing synthetic media for the growth of yeast, Fulmer, Nelson, and Sherwood (1921) and Sherwood and Fulmer (1926) systematically varied the concentrations of the salts used in order to determine optimum conditions for growth at the given temperature. A similar procedure was adopted here in developing the medium optimum for the maximum production of 2,3-butylene glycol by the organisms tested of the genus Aerobacter at 37.5°C.

Such a study presented several difficulties. For example, the analytical procedure outlined above for the determination of the glycol after fermentation, had it been available from the first, would have eliminated the necessity for repeating a considerable number of experiments. Again, the development of a systematized bacteriological technique that will enable a worker to check consistently his results requires considerable time and practice. Moreover, there is always the uncertainty of whether the optimum concentration of a given salt will be the same with varying concentration of the other salts in the medium.

2. Effect of pH

The medium for this investigation was made up as follows:

$CaCl_2$ — 0.2 g.

$MgSO_4$ — 0.1 g.

NH_4Cl — 0.25 g.

K_2HPO_4 — 0.1 g.

Na_2CO_3 — varying amounts

Sucrose — 5.0 g.

Water — up to 100 cc.

After sterilizing and cooling, each medium was inoculated with 2 cc. of a 24 hour culture of Aerobacter pectinovorum. Each medium was maintained at a predetermined pH by the daily addition of sufficient 1 M sodium carbonate solution. These additions were made under sterile conditions. The fermentations were allowed to proceed as long as acid was being produced. The results are summarized in Table II and shown graphically in Figure I.

From this experiment it is evident that there is a definite maximum conversion of sucrose to glycol at a pH of about 6.2 at which value the yield is 49 per cent, by weight, of the sugar fermented.

3. Effect of Sucrose Concentration

The media used in this fermentation were made up as follows:

Table II

Effect of Varying pH Upon the Production of 2,3-Butylene Glycol by Action of *Aerobacter pectinovorum*

Flask Number	pH Maintained	Total cc. of 2 N acid per 100 cc.	Total Days	Average cc. of 2 N acid per Day	Grams of Sucrose Used per 100 cc.	Grams of Glycol/100 cc.	Grams of Glycol per 100 grams of Sucrose
1	4.5	0.10	2	0.050	0.20	0.05	25.0
2	5.0	0.62	15	0.041	2.00	0.51	25.5
3	5.4	1.52	35	0.043	5.84	1.27	21.8
4	5.7	2.92	35	0.083	7.24	2.49	34.4
5	5.9	3.55	35	0.101	7.58	2.98	39.3
6	6.0	4.00	35	0.114	7.78	3.45	44.3
7	6.1	4.53	35	0.129	7.82	3.84	49.1
8	6.3	5.37	34	0.158	7.94	3.89	49.0
9	6.6	6.40	32	0.200	7.94	3.52	44.3
10	7.0	8.57	29	0.295	7.95	2.71	34.1
11	7.5	11.97	29	0.412	7.95	1.49	18.7
12	8.0	15.95	22	0.725	7.95	0.09	1.1

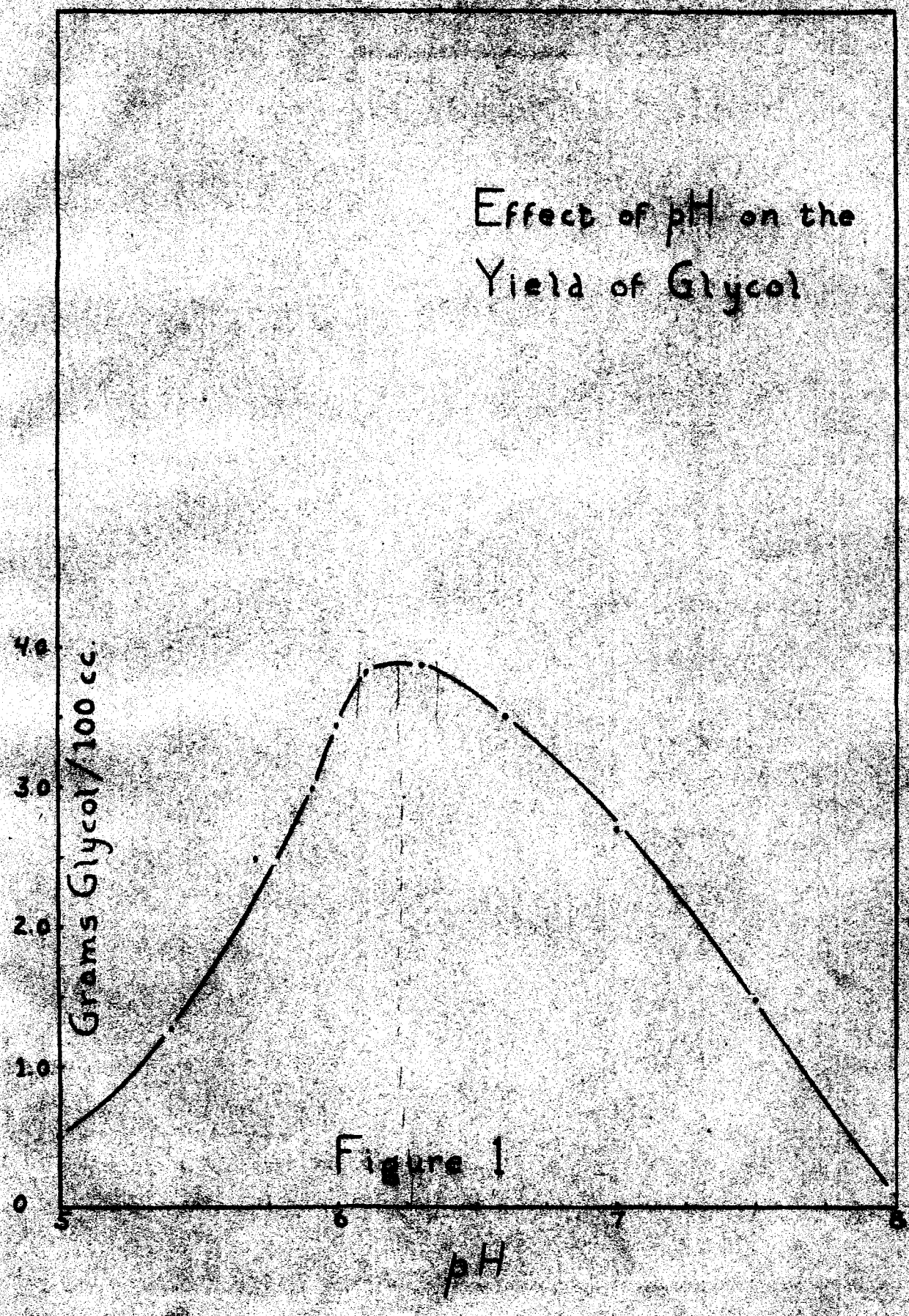

Effect of pH on the
Yield of Glycol
Grams Glycol/100 cc.
4.0
3.0
2.0
1.0
0
5
6
7
8
pH

Figure 1

$MgSO_4$	—	0.2 g.
NH_4Cl	—	0.25 g.
$CaCl_2$	—	0.15 g.
K_2HPO_4	—	0.15 g.
Sucrose	—	varying amounts
Na_2CO_3	—	to a pH of 6.0
H_2O	—	up to 100 cc.

To each of the 100 cc. portions of the medium, with varying concentrations of sucrose, was added 1 cc. of inoculum of a 2-day old culture of Aerobacter pectinovorum grown on a similar medium containing 6 per cent sucrose. The pH of each flask was adjusted to 6.0 by the addition of 1 M sodium carbonate solution. The cultures were incubated at 37.5°C. and the medium analyzed for 2,3-butylene glycol and sucrose when no further acidity developed. The results of these experiments are given in Table III and previously reported by Fulmer, Christensen and Kendall (1933). The graphical representation of these data are shown in Figure 2.

The data show the following:

1. Up to and including 8 per cent sucrose, all of the sugar is fermented; at higher concentrations (8 to 12 per cent) the percentage of sucrose fermented falls from 100 to 85 per cent.

Table III

Effect of Varying Concentrations of Sucrose upon the Production of 2,3-Butylene Glycol by Action of *Aerobacter pectinovorum*

Sucrose per 100 cc: Grams	Sucrose Fermented: Grams	: %	Fermentation Time: Days	Glycol per 100 cc.: Weighed: Grams	Refractive Index: Grams	Ave.: Grams	Glycol per 100 grams Sucrose: Grams	Total 2 N acid/100cc: cc.	Acid per 100g sucrose:	Sucrose per Day: Gram	Days to Ferm. 1 gm. Sugar	Acid per gram Glycol: cc.
1.00	0.98	98	4	--	--	--	--	0.85	87	0.245	4.04	--
2.00	1.97	99	7	0.90	0.80	0.85	43.1	1.60	81	0.282	3.54	1.88
3.00	2.99	100	10	--	--	--	--	2.00	67	0.299	3.34	1.54
4.00	3.97	99	12	1.82	1.65	1.74	43.8	2.30	58	0.331	3.02	1.32
5.00	4.97	99	13	--	--	--	--	2.65	53	0.382	2.62	1.19
6.00	5.97	99	14	2.96	2.47	2.72	45.6	2.80	47	0.426	2.35	1.03
7.00	6.96	99	15	--	--	--	--	3.10	45	0.453	2.21	0.97
8.00	7.91	99	18	3.92	3.49	3.71	46.9	3.70	46	0.439	2.28	0.98
9.00	8.41	93	23	--	--	--	--	3.90	46	0.366	2.75	0.98
10.00	[illegible]	88	23	4.42	3.80	4.11	46.7	4.25	48	0.382	2.61	1.03
11.00	9.42	86	23	--	--	--	--	4.30	46	0.410	2.43	1.00
12.00	10.24	85	23	4.92	4.20	4.56	44.5	4.10	41	0.445	2.25	0.92

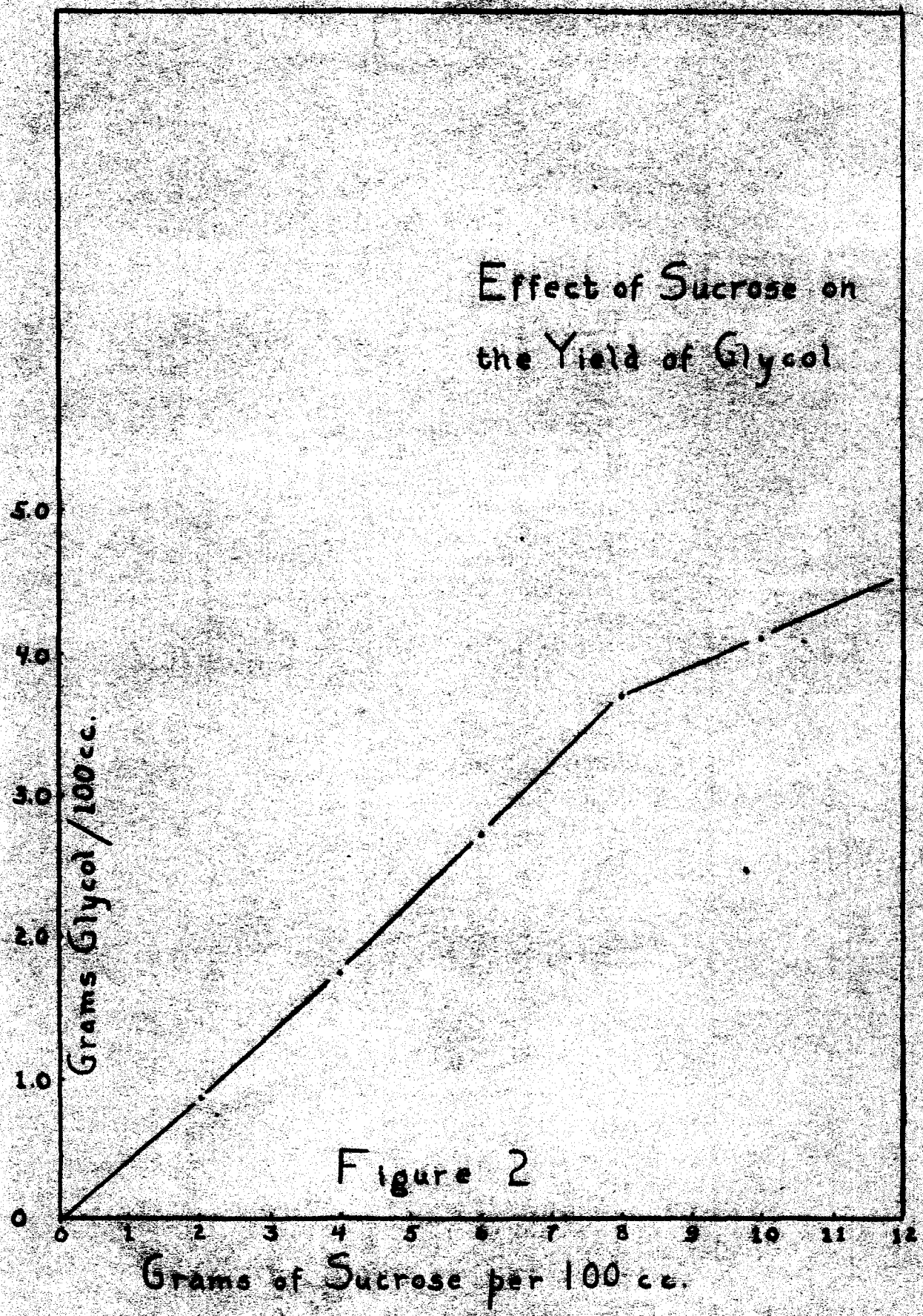

Effect of Sucrose on
the Yield of Glycol
5.0
4.0
3.0
2.0
1.0
0
Grams Glycol / 100 cc.
0 1 2 3 4 5 6 7 8 9 10 11 12
Grams of Sucrose per 100 cc.

Figure 2

2. The rate of fermentation of the sucrose, that is, the average amount of sugar fermented per day is at a definite maximum at about 8 per cent. The average rate of fermentation at 8 per cent is nearly double that at 1 per cent.

3. The 2,3-butylene glycol produced per 100 grams of sucrose fermented is at a maximum of 47 grams at 8 per cent sucrose.

4. The acid produced per 100 grams of sucrose fermented decreases at first rapidly and then slowly with increase in concentration of sucrose.

5. The ratio of acid to glycol is markedly affected by the concentration of the sucrose, dropping from a value of 1.88 for 1 per cent sucrose to a constant low level of 1.00 at 8 per cent sucrose.

4. Effect of $MgSO_4$

The medium consisted of:

$CaCl_2$	—	0.15 g.
NH_4Cl	—	0.25 g.
K_2HPO_4	—	1.0 g.
Sucrose	—	8.0 g.
$MgSO_4$	—	varying amounts
Na_2CO_3	—	to a pH of 6.0
Tap water	-	up to 100 cc.

After being sterilized at 15 lbs. pressure for 30 minutes and cooling, each medium was inoculated with 1 cc. of a 24-hour

culture of Aerobacter aerogenes grown on a similar medium. The latter organism was used here instead of Aerobacter pectinovorum, as in earlier experiments, inasmuch as preliminary work had indicated that there is practically no difference in the inorganic nutrient requirements of, and the amount of glycol produced by the various species of the genus Aerobacter, and it was thought advisable therefore to use the better known test organism. The results of this experiment are given in Table IV and Figure 3.

From these experiments it is evident that the maximum conversion of sucrose to glycol (38.8 per cent by weight) occurs in a medium containing 0.1 to 0.2 per cent of magnesium sulfate.

5. Effect of NH_4Cl

In this investigation the media were composed of the following constituents:

$CaCl_2$	—	0.15 g.
$MgSO_4$	—	0.2 g.
K_2HPO_4	—	1.0 g.
Sucrose	—	8.0 g.
NH_4Cl	—	varying amounts
Na_2CO_3	—	to a pH of 6.0
Tap water	—	up to 100 cc.

Table IV

Effect of Varying Concentrations of $MgSO_4$ upon the Production of 2,3-Butylene Glycol by Action of *Aerobacter aerogenes*.

Flask Number	Grams $MgSO_4$ per 100 cc.	Total cc. of 2 N acid per 100cc	Total Days	Average cc. of 2 N acid per Day	Grams of Sucrose Used per 100 cc.	Glycol per 100 cc.(Grams) Weighed	Refractive Index Method	Average	Grams of Glycol per 100 grams of Sucrose
1	0	6.80	16	0.425	7.37	2.435	2.37	2.40	32.5
2	0.05	7.50	16	0.470	7.60	2.770	2.71	2.74	36.0
3	0.10	7.80	16	0.485	7.74	3.000	2.94	2.97	38.3
4	0.15	8.05	16	0.505	7.82	3.050	3.01	3.03	38.7
5	0.20	8.25	16	0.515	7.86	3.070	3.04	3.05	38.8
6	0.25	8.45	16	0.530	7.88	2.950	2.88	2.91	36.9
7	0.30	8.65	16	0.540	7.89	2.850	2.82	2.83	35.9
8	0.40	8.60	16	0.535	7.89	2.849	2.78	2.81	35.6
9	0.70	8.65	15	0.575	7.88	2.847	2.78	2.81	35.7
10	1.00	8.65	12	0.720	7.88	2.850	2.75	2.80	35.5

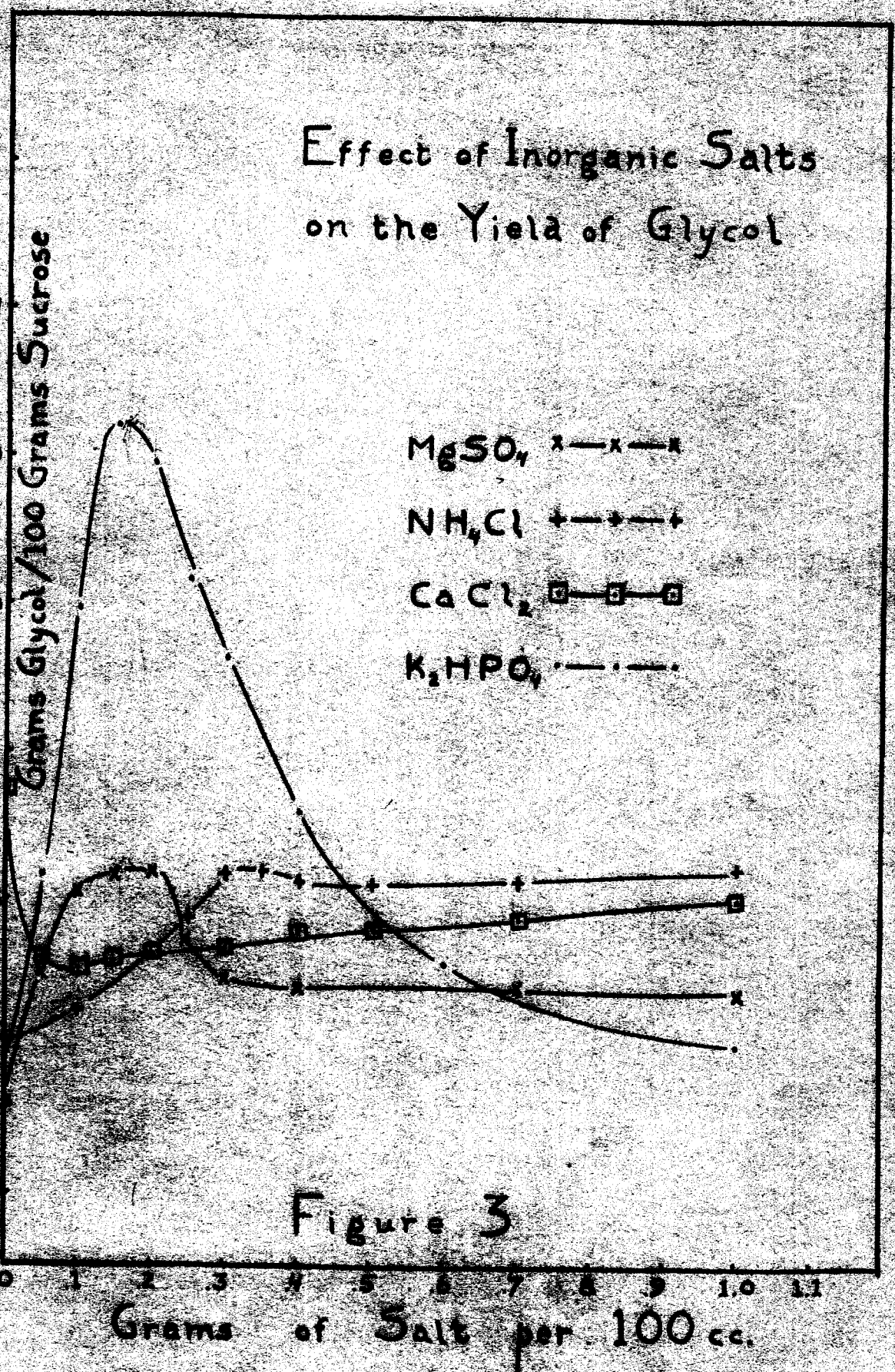

Effect of Inorganic Salts
on the Yield of Glycol
Grams Glycol/100 Grams Sucrose
54
50
46
42
38
34
30
$MgSO_4$
NH_4Cl
$CaCl_2$
K_2HPO_4
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.1
Grams of Salt per 100 cc.

Figure 3

In these, and all of the following experiments, except where noted, the media were sterilized under the same conditions (15 lbs. pressure for 30 minutes) and were all inoculated and fermented under like conditions; that is, inoculated with 1 cc. of 24 hour cultures of Aerobacter aerogenes grown on a similar medium and incubated for a maximum period of 16 days, at a temperature of 37.5°C. The pH was adjusted to 6.0 each day by the addition of 1 M sodium carbonate solution. Tap water was substituted for distilled water in these experiments since at this time there were indications that the distilled water available contained traces of copper or other substances inimical to cell growth.

The results of this study, given in Table V, and Figure 3, indicate that there is a very marked increase, in the percentage conversion of sucrose to glycol, with increase in the ammonium chloride concentration, reaching a maximum at 0.3 per cent of the salt. In the range of 0.3 to 1.0 per cent ammonium chloride there is almost a constant yield of glycol amounting to about 38.5 grams per 100 grams of sucrose utilized. It should be noted that flask number 1 contained a small amount of ammonium chloride, introduced in the inoculum.

6. Effect of K_2HPO_4

Each flask was composed of the following ingredients:

Table V

Effect of Varying Concentrations of NH_4Cl upon the Production of 2,3-Butylene Glycol by Action of Aerobacter aerogenes.

Flask Number	Grams NH_4Cl per 100 cc.	Total cc. of 2 N acid per 100cc	Total Days	Average cc. of 2 N acid per Day	Grams of Sucrose Used per 100 cc.	Grams of Glycol per 100cc: Weighed	Refractive Index Method	Average	Grams of Glycol per 100 grams of Sucrose
1	0	5.70	16	0.355	7.08	2.430	2.41	2.42	34.2
2	0.10	7.35	16	0.460	7.46	2.630	2.60	2.61	35.0
3	0.20	7.90	16	0.495	7.71	2.835	2.82	2.83	36.7
4	0.25	7.95	16	0.495	7.80	2.960	2.91	2.93	37.5
5	0.30	8.00	16	0.500	7.84	3.060	3.01	3.03	38.7
6	0.35	8.00	16	0.500	7.86	3.070	3.04	3.05	38.8
7	0.40	8.05	16	0.505	7.87	3.050	3.01	3.03	38.5
8	0.50	8.00	16	0.500	7.88	3.050	3.01	3.03	38.4
9	0.70	8.05	16	0.505	7.88	3.070	3.01	3.04	38.5
10	1.00	8.00	16	0.500	7.88	3.110	3.04	3.07	38.9

$CaCl_2$	—	0.15 g.
$MgSO_4$	—	0.2 g.
NH_4Cl	—	0.25 g.
Sucrose	—	8.0 g.
+ K_2HPO_4	—	varying amounts
Na_2CO_3	—	to a pH of 6.0
Tap water	—	up to 100 cc.

The flasks were sterilized, inoculated and incubated as noted above for the experiments on the effect of ammonium chloride concentration.

From the results given in Table VI, and diagramed in Figure 3, it is evident that there is a definite optimum concentration of secondary potassium phosphate for maximum conversion of sucrose into glycol. At a concentration of 0.15 per cent potassium phosphate, the yield of glycol is slightly better than 50 grams per 100 grams of sugar utilized. This yield is considerably higher than any obtained in the experiments on the effect of varying the concentration of magnesium sulfate, ammonium chloride and calcium chloride (discussed below). This can be easily explained, however, inasmuch as in these other experiments, none of the media contained this optimal concentration of K_2HPO_4.

+ The secondary potassium phosphate used, though labeled anhydrous, has since been found to contain three molecules of water. Therefore, in this thesis, K_2HPO_4 should be understood to be $K_2HPO_4.3H_2O$.

Table VI

Effect of Varying Concentrations of K_2HPO_4 upon the Production of 2,3-Butylene Glycol by Action of Aerobacter aerogenes.

Flask Number	Grams K_2HPO_4 per 100 cc.	Total cc. of 2 N acid per 100cc	Total Days	Average cc. of 2 N acid per Day	Grams of Sucrose Used per 100 cc.	Grams of Glycol/100cc Weighed	Grams of Glycol/100cc Refractive Index Method	Grams of Glycol/100cc Average	Grams Glycol per 100 g. of Sucrose
1	0	2.00	16	0.125	6.06	2.060	2.02	2.04	33.7
2	0.05	2.75	16	0.170	6.13	2.450	2.30	2.37	38.7
3	0.10	3.30	16	0.205	6.21	2.950	2.74	2.85	45.9
4	0.15	3.75	16	0.235	6.32	3.255	3.18	3.22	50.9
5	0.20	4.10	16	0.255	6.44	3.265	3.15	3.21	49.9
6	0.25	4.40	16	0.275	6.61	3.150	3.04	3.09	46.7
7	0.30	4.70	16	0.295	6.76	3.045	2.97	3.01	44.6
8	0.40	5.20	16	0.325	7.06	2.890	2.82	2.85	40.4
9	0.60	6.20	16	0.390	7.52	2.780	2.68	2.73	36.3
10	1.00	7.65	16	0.475	7.85	2.715	2.64	2.68	34.1

7. Effect of $CaCl_2$

The medium consisted of:

$MgSO_4$	—	0.2 g.
NH_4Cl	—	0.25 g.
K_2HPO_4	—	1.0 g.
Sucrose	—	8.0 g.
$CaCl_2$	—	varying amounts
Na_2CO_3	—	to a pH of 6.0
Tap water	—	up to 100 cc.

The results are given in Table VII and shown in Figure 3. It appears that the addition of calcium chloride tends to somewhat decrease the yield of glycol. However, when it was attempted to ferment a medium containing no calcium chloride, either in the medium as made up or in the inoculum added, very poor growth of the organism resulted. This fact indicates, therefore, that a trace of calcium chloride aids glycol yield by supplying elements necessary for luxurient cell growth, whereas appreciable amounts, although not inimical to cell growth, result in a chemism unfavorable to high glycol yields. It should be noted that in flask number 1 a trace of calcium chloride (0.0015 per cent) was introduced when adding the inoculum, besides any contained in the tap water used. It seems then, that a trace of calcium chloride (about 0.01 per cent) is necessary for high yields.

Table VII

Effect of Varying Concentrations of $CaCl_2$ upon the Production of 2,3-Butylene Glycol by Action of Aerobacter aerogenes.

Flask Number	Grams $CaCl_2$ per 100 cc.	Total cc. of 2 N acid per 100cc	Total Days	Average cc. of 2 N acid per Day	Grams of Sucrose Used per 100 cc.	Grams of Glycol/100 cc. Weighed	Grams of Glycol/100 cc. Refractive Index Method	Grams of Glycol/100 cc. Average	Grams of Glycol per 100 grams of Sucrose
1	0	5.30	12	0.440	7.80	3.265	3.11	3.20	41.0
2	0.05	7.15	16	0.445	7.80	2.880	2.81	2.84	36.4
3	0.10	7.35	16	0.460	7.81	2.865	2.77	2.82	36.1
4	0.15	7.30	16	0.455	7.81	2.875	2.81	2.84	36.3
5	0.20	7.30	16	0.455	7.81	2.885	2.84	2.86	36.6
6	0.30	7.45	16	0.465	7.81	2.900	2.84	2.87	36.7
7	0.40	7.70	16	0.480	7.82	2.925	2.88	2.90	37.1
8	0.50	8.00	16	0.500	7.82	2.950	2.88	2.91	37.2
9	0.70	8.50	16	0.530	7.83	2.975	2.91	2.94	37.5
10	1.00	9.35	12	0.780	7.86	3.035	2.94	2.99	38.0

V. COMPARISON OF THE YIELDS OF GLYCOL BY VARIOUS SPECIES OF THE GENUS AEROBACTER.

The above experiments indicated that the medium giving maximum yields of 2,3-butylene glycol should have the following concentrations of salts:

$MgSO_4$	—	0.175 g.
NH_4Cl	—	0.350 g.
K_2HPO_4	—	0.175 g.
$CaCl_2$	—	0.015 g.
Na_2CO_3	—	to a pH of 6.0
Tap water	—	up to 100 cc.

Accordingly, the above medium was prepared and used to test the action of two strains of Aerobacter cloacae, two of Aerobacter aerogenes and one of Aerobacter pectinovorum .

The sucrose concentration was reduced to 3.45 grams per 100 cc. This low concentration of sugar made possible a comparison of the above bacterial types with a short time fermentation. The results are given in Table VIII.

From these data it is evident that:

1. There is no significant difference in the ability of the various species of the genus Aerobacter to produce 2,3-butylene glycol from sucrose. This conclusion has further support in the studies made by Brockman (1933), and previously

Table VIII

Action of Various Species of the Genus Aerobacter on Identical Media

Flask Number	Organism Used	Total cc. of 2 N Acid per 100cc.	Total Days	Average cc. of 2 N Acid per Day	Grams of Sucrose used per 100 cc.	Grams of Glycol/100cc. Weighed	Grams of Glycol/100cc. Refractive Index Method	Grams of Glycol/100cc. Average	Grams of Glycol per 100 grams of Sucrose
1	A. cloacae	2.35	10	.235	2.845	1.220	1.165	1.192	41.9
2	A. cloacae	1.90	7	.271	3.324	1.370	1.365	1.367	41.1
3	A. pectinovorum	2.75	6	.458	3.436	1.392	1.370	1.381	40.2
4	A. aerogenes	2.45	6	.408	3.395	1.275	1.260	1.267	37.3
5	A. aerogenes	2.50	6	.417	3.395	1.370	1.315	1.342	39.5

noted, where A. indologenes, A. salicinovorum and A. decolorans were found to produce almost identical yields of the glycol under standardized conditions.

2. The medium used in this experiment was optimum for high yields of glycol, in regard to salt concentrations, as evidenced by the rapidity of the fermentation (6 days) as compared to a previous experiment time (11 days, c.f., Table III), with practically the same yields in both cases; 41.9 per cent conversion (Table VIII) compared to 43.4 per cent conversion (Table III).

VI. SUMMARY

It has been shown that for the maximum conversion of sucrose into 2,3-butylene glycol, in an inorganic medium:

1. There is a definite optimum pH of about 6.2.

2. The most efficient conversion of the sugar occurs at a concentration of 8 per cent.

3. There is a definite optimum concentration of magnesium sulfate at 0.175 per cent.

4. Ammonium chloride is very essential and at least 0.3 per cent must be present. A higher concentration has very little effect.

5. There is a definite optimum concentration of secondary potassium phosphate at 0.175 per cent.

6. A trace of calcium chloride is essential, but any appreciable concentration is somewhat harmful, a 0.1 per cent concentration being slightly more harmful than a 1.0 per cent concentration.

7. Various species of the genus Aerobacter produce like yields of glycol under like conditions.

8. Under optimum conditions the yield of glycol amounts to about 50 per cent of the sucrose fermented.

VII. LITERATURE CITED

Breden, C. R. The chemical action of Aerobacter faeni on xylose and on sucrose. Published doctoral thesis. Iowa State College (1930).

Breden, C. R., and Fulmer, E. I. The chemical action of Aerobacter faeni on xylose and sucrose. Iowa State Coll. J. Sci. 5, 133-153 (1931).

Brockmann, M. C. Oxidation-reduction in the dissimilation of glucose by species of the genus Aerobacter. Unpublished thesis. Iowa State College (1933).

Buchanan, R. E. and Fulmer, E. I. The physiology and biochemistry of bacteria. Vol. III, Williams and Wilkins, Baltimore (1930).

Burkey, L. A. The fermentation of cornstalks and their constituents. I. Studies on the pectin-fermenting bacteria Iowa State Coll. J. Sci. 3, 57-100 (1928).

Desmots, H. Production de l'acetylmethylcarbinol par les bactèries du groupe du B. mesentericus. Compt. rend. soc. biol., 56, 344-46 (1904).

Elion, L. Zur Kenntnis der Acetoinbildung bei der Vergärung von Zucker durch Hefe. Biochem. Z. 169, 471-7 (1926).

Fulmer, E. I. The chemical approach to problems of fermentation. Ind. Eng. Chem. 22, 1148-1150 (1930).

Fulmer, E. I., Christensen, L. M., and Kendall, A. R. Production of 2,3-butylene glycol by fermentation. Ind. Eng. Chem. 25, 798-800 (1933).

Fulmer, E. I., Nelson, V. E., and Sherwood, F. F. The nutritional requirements of yeast. II. The effect of the composition of the medium on the growth of yeast. J. Am. Chem. Soc. 43, 191-9 (1921).

Fulmer, E. I., and Werkman, C. H. An index to the chemical action of microorganisms on the non-nitrogenous organic compounds. C. C. Thomas, Springfield (1930).

Grimbert, L. Production d'acetylmethylcarbinol par le Bacillus tartricus. Compt. rend. 132, 706-9 (1901).

Harden, A., and Norris, D. The bacterial production of acetylmethylcarbinol and 2,3-butylene glycol from various sources. Proc. Roy. Soc. (London) B 84, 492-99 (1912).

Harden, A., and Norris, D. The bacterial production of acetylmethylcarbinol and 2,3-butylene glycol from various sources. Proc. Roy. Soc. (London) B 85, 73-8 (1912).

Harden, A., and Walpole, G. S. Chemical action of Bacillus lactis aerogenes (Escherich) on glucose and mannitol: Production of 2,3-butylene glycol and acetylmethylcarbinol. Proc. Roy. Soc. (London), B 77, 399-405 (1906).

luyver, A. J., and Donker, H. J. L. De vorming van acetylmethylcarbinol en 2,3-butyleenglycol bij de fermentatieve ontleding van suikers door alcoholgisten en ware melkzuurbacteriën. Verslag. Akad. Wetensch. Amsterdam. 33, 915-19 (1924).

Kluyver, A. J., Donker, H. J. L., and Hooft, F. v't., Über die Bildung von Acetylmethylcarbinol und 2,3-butyleneglykol im Stoffwechsel der Hefe. Biochem. Z. 161, 361-78 (1925).

Lemoigne, M. Fermentation butylèneglycolique du glucose par les staphylocoques et les tetragènes. Compt. rend. 157, 653-5 (1913).

Lemoigne, M. Assimilation du saccharose par les bactèries du groupe du B. subtilis fermentation butylèneglycololique. Ann. inst. Pasteur. 27, 856-85 (1913).

Lemoigne, M. Reaction specifique du 2,3-butyleneglycol et de l'acetylmethylcarbinol, produits de la fermentation butyleneglycolique. Compt. rend. 170, 131-2 (1920).

Neuberg, C., and Gorr, G. Neue Untersuchungen über die Vergärung der Oxalessigsäure. Biochem. Z., 154, 495-502 (1924).

Neuberg, C., and Reinfurth, E. Eine neue Form der Umwandlung des Acetaldehyds durch gärende Hefe. Biochem. Z. 143, 553-65 (1923).

Neuberg, C., and Rosenthal, O. Zusammenhang von carboligatischer Synthese mit carboxylatischem Abbau. (Verwendung von Acetaldehyd als Abfangmittel). Ber. 57, 1436-41 (1924).

Neuberg, C., and Simon, E. Zur Kenntnis der biochemischen Acyloinsynthese. VIII. Mitteilung über Carboligase. Biochem. Z. 156, 374-8 (1925).

Péré, A. Mécanisme de la combustion des corps ternaires par une groupe de microbes aerobies. Ann. Inst. Pasteur 10, 417-48 (1896).

Shaffer, P. A., and Hartmann, A. F. The iodometric determination of copper and its use in sugar analysis. II. Methods for the determination of reducing sugars in blood, urine, milk and other solutions. J. Biol. Chem. 45, 365-90 (1921).

Sherwood, F. F., and Fulmer, E. I. The effect of temperature upon the growth of yeast in varibus media. J. Phys. Chem. 30, 738-56 (1926).

Thompson, J. The chemical action of Bacillus cloacae (Jordan) on glucose and mannitol. Proc. Roy. Soc. (London) B 84, 500-04 (1912).

Verhave, T. H. Process for the preparation of 2,3-butylene glycol from carbohydrates. U. S. Patent 1,899,156. Feb. 28, 1933.

Walpole, G. S. The action of Bacillus lactis aerogenes on glucose and mannitol. II. The investigation of 2,3-butanediol and the acetylmethylcarbinol formed; the effect of free oxygen on their production; the action of B. lactis aerogenes on fructose. Proc. Roy. Soc. (London) B 83, 272-86 (1911).

Weldin, J. C. The colon-typhoid group of bacteria and related forms. Relationships and classifications. Iowa State Coll. J. Sci. 1, 121-97 (1927).