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Production of the 2, 3-butanediols by the fermentation of starch

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PRODUCTION OF THE 2,3-BUTANEDIOLS BY THE FERMENTATION OF STARCH

by

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I. INTRODUCTION

2,3-Butanediol, commonly known as 2,3-butylene glycol, has also been called β-butylene glycol, symmetrical dimethyl ethylene glycol, symmetrical butylene glycol, 2,3-dihydroxybutane, and pseudo-butylene glycol. The general formula of the compound is $CH_3 \cdot CHOH \cdot CHOH \cdot CH_3$. 2,3-Butanediol has received special attention in recent years because of its possible conversion to 1,3-butadiene for use in the manufacture of synthetic rubber. With the loss of our sources of natural rubber to Japan, intensive research was carried out by many organizations for the purpose of investigating all possible materials, including 2,3-butanediol, which could be converted into 1,3-butadiene, the basic material of the more important synthetic elastomers.

Three raw materials, petroleum, ethanol, and 2,3-butanediol, attained major importance as possible sources of the butadiene. The relative advantages of each material with respect to the production of butadiene were the subject of numerous congressional debates and committee investigations. The Baruch committee compiled a critical survey of our rubber supplies and of the proposed methods for the production of synthetic rubber, and presented evidence showing the necessity for a method of production of 1,3-butadiene which would

meet the demand for a supply of a large amount of the butadiene in as short a time as possible. Inasmuch as the commercial production of the butadiene from ethanol had already been established as an industrial process in Europe, and the semi-commercial production of the butadiene from cracked gasoline had been shown to be feasible, it was logical that these materials became the major sources of the 1,3-butadiene for the production of our synthetic rubber. The rate at which new plants were built and existing plants were converted to the production of these raw materials is a recorded tribute to the ingenuity and perseverance of the fermentation and petroleum chemists and engineers. Rubber Director Dewey (1944) reported that in the second quarter of 1944, about sixty per cent of the butadiene produced in this country was prepared from ethanol, and the remainder from cracked petroleum. From the first quarter of 1943 to the second quarter of 1944, the production of butadiene from ethanol was increased from one and a half short tons to ninety-nine short tons and the production from petroleum was increased from five and a half to sixty-four short tons. Thus, in a little over a year, butadiene production increased approximately twenty-three-fold.

When the synthetic rubber program was organized, the conversion of 2,3-butanediol to 1,3-butadiene was still in the laboratory or pilot-plant stages. It was, therefore, a

wise choice not to replace the more established processes with the butanediol process when the need for synthetic rubber was so critically immediate. However, the possibility remained of converting the ethanol distilleries to the production of 2,3-butanediol by fermentation with very minor changes should the butanediol process prove to be more feasible or more economical. Furthermore, the physical and chemical properties of 2,3-butanediol and its derivatives are such that it might well find application in the preparation of a variety of products such as antifreezes, solvents, plastics, resins, humectants, pharmaceuticals, and coatings.

The production of chemicals by fermentation has long been advocated because of the huge potential supply of agricultural raw materials. Of equal importance is the possibility that volume production of chemicals by fermentation would provide a stabilizing factor in agricultural economy by utilization of surplus crops. 2,3-Butanediol can be produced by fermentation from a variety of agricultural materials; any material containing sugars, starch, pentosans, or cellulose can serve as the ultimate raw material for the production of the diol. In the event that the process is adapted to industrial production, the raw materials used will necessarily depend upon their availability and the economic feasibility of production from selected materials.

The method of attack relating to the production of chemicals by fermentation which has been used in these laborato-

ries for many years involves the following essentials: first, selection of an organism which is known to produce substantial quantities of the chemical desired; second, selection of a raw material which is readily available in substantial quantities; third, selection of a medium and conditions suitable for growth of the organism; and fourth, variation, one at a time, of the variety and of the concentration of the constituents of the fermentation medium and of the physical conditions of cultivation until the optimum conditions for production of the desired chemical have been established. The advantages of this method have been discussed by Fulmer (1943).

The use of starch or, in general, any pure carbohydrate as the raw material for the production of chemicals by fermentation lends itself well to the above procedure. The amount and type of nitrogen supply, growth factors, and inorganic materials present can be varied at will within wide limits. An additional factor to be considered is the possibility that constituents present in the original raw material before the isolation of the pure carbohydrate may be detrimental to maximum production of the chemical desired. On an industrial scale, separation of the starch from the other constituents of the corn kernel prior to fermentation also permits recovery of valuable by-products; that is, corn oil, gluten, germ meal, and bran. There is a ready market

for these products, and, in many cases, a saleable by-product can determine the economic feasibility of an industrial process.

The experimental investigations in this work have been divided into five major parts. The first part, the production of 2,3-butanediol from dextrose by fermentation with Aerobacter aerogenes, was conducted in an effort to determine some of the factors involved in the fermentation of comparatively large volumes of media, with particular reference to decreasing the time required for fermentation. Concurrent with this phase, but included under a separate heading, was the recovery of the 2,3-butanediol from the fermentation liquors.

The second part, the production of 2,3-butanediol from corn by fermentation with Aerobacter aerogenes, was undertaken at the request of the Doane Agricultural Service of St. Louis, Missouri. The work was done in cooperation with the Columbia Brewing Company of St. Louis for the purpose of determining if brewery equipment and procedures could be adapted to the production of 2,3-butanediol by fermentation.

The third part, the production of 2,3-butanediol from corn by fermentation with Aerobacillus polymyxa, was undertaken for the purpose of preparing the levo-2,3-butanediol, and also to determine some of the factors and relations

involved in the fermentation of starchy substrates by Aerobacillus polymyxa in order that the information thus obtained might be applied to the investigations concerning the fermentation of corn starch.

The fourth part, the preparation of 2,3-butanediol from corn starch by fermentation with Aerobacillus polymyxa, was carried out in order to investigate the optimal conditions for the formation of 2,3-butanediol from corn starch, and to determine if conditions could be discovered under which the fermentation of starch could be conducted more efficiently than the fermentation of the whole grain.

The fifth part was conducted concurrently with the production of 2,3-butanediol from dextrose and from corn, and involved a study of the recovery procedures best suited to the isolation of the diol from fermented mashes. These investigations were conducted in order to recover the 2,3-butanediols in order that other members of the research group in Biophysical Chemistry might study the physical and chemical properties of the purified materials.

There is also included, in the Appendix, the design of semi-pilot plant fermenter units and a semi-pilot plant extraction unit which were constructed for the use of members of the research group in Biophysical Chemistry for large-scale study of the production and recovery of fermentation products.

II. REVIEW OF LITERATURE

A. Preparation of the 2,3-Butanediols

1. Chemical methods

Probably the earliest reported preparation of 2,3-butanediol is that of Wurtz (1859), who prepared the compound from 2-butene by conversion of the butene to the dibromide, the dibromide to the diacetate with silver acetate, and the diacetate to 2,3-butanediol by means of aqueous potassium hydroxide. Adaptations of this method have been used by a number of workers including Maruyama and Higasi (1928), Moureu and Dode (1937), and Taufen, Murray, and Cleveland (1943).

Eltekoff (1882) prepared 2,3-epoxybutane from 2-butene and found that the epoxybutane could be completely converted to 2,3-butanediol by reaction with boiling water. Similar methods were employed by Wilson and Lucas (1936), Batalin and Ugryumov (1936), and Winstein and Wood (1940).

There has been considerable controversy concerning the formation of 2,3-butanediol by the action of magnesium amalgam upon acetaldehyde. Meunier (1902) reported that a small amount of the 2,3-butanediol was formed by the above reaction. However, Tischtschenko and Woronkow (1906), in

an attempt to duplicate the results of Meunier, were unable to detect 2,3-butanediol among the reaction products but did report the formation of an acetate of 1,3-butanediol. Kling and Roy (1907) reported a fifteen per cent yield of 1,3-butanediol by the above reaction and assumed that this was the compound which Meunier had considered to be the 2,3-diol. The controversy was finally clarified by Ciusa and Milani (1913, 1915) who found the main product of the reaction to be 1,3-butanediol, while 2,3-butanediol was also formed in small amounts. The 2,3-butanediol was identified by Ciusa and Milani by oxidation to diacetyl and by the phenylurethan derivative.

The preparation of 2,3-butanediol by hydrogenation of diacetyl in the presence of nickel was reported by Sabatier and Mailhe (1907). Acetylmethylcarbinol was also formed.

Ciamician and Silber (1911) found a small amount of 2,3-butanediol among the products resulting from the exposure of a mixture of acetone and ethanol to light for nine months.

2,3-Butanediol may also be prepared by direct hydrolysis of the dihalogen derivatives of 2-butene. This method was used by Böeseken and Cohen (1928). Schierholtz and Staples (1935) reported a yield of fifty per cent of theory by the hydrolysis of 2,3-dibromobutane in the presence of

lead oxide. Dobryanskiĭ, Gutner, and Schigel'skaya (1937) obtained a forty per cent yield of 2,3-butanediol by hydrolysis of 2,3-dichlorobutane in the presence of sodium bicarbonate.

Smoler (1930) studied the reduction of acetaldehyde by the dropping mercury electrode and stated that the probable reduction product was 2,3-butanediol. Subsequently Semerano and Polacsek (1938) positively identified 2,3-butanediol as the product formed.

Fichter and Metz (1936) reported the formation of a small amount of 2,3-butanediol as a secondary product resulting from the electrolysis of the sodium salt of 2-methylbutanoic acid in the presence of sodium nitrate. Fichter and Sutter (1938) found that eleven grams of 2,3-butanediol dinitrate was formed from a hundred grams of 2-methylbutanoic acid under similar conditions. Rudin (1942) reported that some 2,3-butanediol was formed upon the electrolysis of a mixture of the sodium salt of pentanoic acid and sodium nitrate. Miolati and Semerano (1937) found 2,3-butanediol among the products resulting from agitation of the sodium salt of 2-hydroxybutanoic acid with carbon.

2. Microbiological methods

a. Mechanism of formation

In the study of the mechanism of the formation of

fermentation products, it is advantageous to know the various minor products which are formed by the organism under consideration in order that these products may be examined for their possibilities as precursors leading to the formation of the main product. It may be generally assumed that closely related species follow the same mechanism if the major products formed are the same.

The products of fermentation of xylose and sucrose by Aerobacter faeni were found by Breden and Fulmer (1931) to be 2,3-butanediol, acetylmethylcarbinol, ethanol, acetic acid, formic acid, succinic acid, l-lactic acid, butyric acid, carbon dioxide, and hydrogen. Patrick (1931) found acetone, acetylmethylcarbinol, ethanol, acetic acid, formic acid, succinic acid, lactic acid, carbon dioxide, and hydrogen to be formed from levulose by Aerobacillus polymyxa. It is, of course, well known that Aerobacillus polymyxa also produces 2,3-butanediol by fermentation of carbohydrates.

The available literature concerning the mechanism of the formation of 2,3-butanediol deals mainly with the immediate precursor for 2,3-butanediol and the relation between the diol and acetylmethylcarbinol. Investigators have not agreed as to the mechanism of the biological origin of these products. Harden and Norris (1912) suggested that 2,3-butanediol was formed from dextrose by Aerobacter aerogenes by reduction and condensation of acetaldehyde but presented

no evidence to substantiate this theory. Neuberg, Nord, and Wolff (1920) presented data showing that acetaldehyde was an intermediate product of the action of Aerobacter aerogenes on levulose. Nagai (1923) found that acetaldehyde accumulated in considerable quantities when calcium sulfite was added to an Aerobacter aerogenes fermentation.

On the other hand, evidence given by Reynolds, Jacobson, and Werkman (1937) indicated that in the case of Aerobacter indologenes, acetic acid was reduced and condensed to 2,3-butanediol, although these investigators suggested that the conversion may go through acetaldehyde and acetylmethylcarbinol as intermediate stages. Barritt (1937) found that many bacteria were able to oxidize 2,3-butanediol to acetylmethylcarbinol and suggested that in the bacterial fermentation of dextrose, formation of 2,3-butanediol may precede the formation of acetylmethylcarbinol.

Silverman (1941) and Silverman and Werkman (1941) reported that a cell-free enzyme preparation from Aerobacter aerogenes converted pyruvic acid into acetylmethylcarbinol and carbon dioxide without detectable intermediate formation of acetaldehyde. These results indicated that the addition of acetaldehyde and acetic acid increased yields of acetylmethylcarbinol by inhibiting the enzyme system by which pyruvic acid is converted to acetic acid and formic acid. The above workers emphasized the possibility that the

mechanism of dissimilation with proliferating cells and with enzyme preparations may differ. Gross, Wood, and Werkman (1942) added acetaldehyde containing heavy carbon to enzyme preparations from Aerobacter aerogenes; all the heavy carbon added was found in the recovered acetaldehyde, showing that acetaldehyde as such was not an intermediate in the formation of the 2,3-butanediol, although the possibility of nascent acetaldehyde acting as an intermediate was not precluded.

The investigations of Slade and Werkman (1943) constitute the most direct evidence for the participation of acetaldehyde as an intermediate in the formation of 2,3-butanediol. By adding acetic acid containing heavy carbon to Aerobacter indologenes fermentations, it was found that the acetic acid was reduced and condensed to 2,3-butanediol. Therefore, acetaldehyde, or a closely related derivative, was involved in the synthesis of the diol. The carbon-carbon linkage created in the synthesis involved the carbon atom originally present in the carboxyl group of the acetic acid.

Stahly and Werkman (1942) studied in detail the mechanism involved in the formation of acetylmethylcarbinol and 2,3-butanediol by Aerobacillus polymyxa. These studies were made using as cultural media both dextrose-peptone and dextrose-peptone plus several probable intermediary compounds. The above investigators found that whether the

fermentations were conducted aerobically or anaerobically, the addition of acetaldehyde resulted in increased yields of ethanol, acetylmethylcarbinol, and 2,3-butanediol. Furthermore, acetaldehyde was fixed by addition of calcium sulfite to a normal fermentation. The addition of acetate likewise resulted in increased yields of acetylmethylcarbinol and 2,3-butanediol. Thus, it would appear that the mechanism of the formation of butanediol by Aerobacillus polymyxa is similar to that for species of the Aerobacter genus.

With respect to the relation between the microbiological formation of 2,3-butanediol and acetylmethylcarbinol, Stahly and Werkman (1942) found that a marked drop in oxidation potential and rapid fermentation of the dextrose followed inoculation with Aerobacillus polymyxa, of a vigorously oxygenated dextrose medium. The potential rose only after most of the dextrose was fermented. Large quantities of 2,3-butanediol accumulated while the oxidation potential was low, and part of the butanediol was oxidized to acetylmethylcarbinol after the oxidation potential attained a fairly high level. Furthermore, added acetylmethylcarbinol was reduced to 2,3-butanediol, but only when the oxidation potential was low. From these results it was concluded that acetylmethylcarbinol and 2,3-butanediol comprise a reversible oxidation-reduction system with a low oxidation-reduction potential favoring the accumulation of 2,3-butanediol and a high potential favoring the formation of acetylmethylcarbinol.

It would thus appear that with both Aerobacter aerogenes and Aerobacillus polymyxa, acetylmethylcarbinol and 2,3-butanediol are formed by reduction and condensation of preformed acetic acid, probably through the intermediate formation of acetaldehyde or a closely related intermediate. Likewise, with both organisms, acetylmethylcarbinol and 2,3-butanediol constitute a reversible oxidation-reduction system with the ratio of the two products depending to a large extent upon the oxidation-reduction potential existing toward the completion of the fermentation.

b. Yields of 2,3-butanediol. 2,3-Butanediol and the associated product, acetylmethylcarbinol, are formed by a large number of microorganisms and occur in a wide variety of natural products. Only those investigations will be reviewed in which quantitative data were obtained under standardized conditions.

Henninger (1882) fractionated fifty liters of red Bordeaux wine and succeeded in isolating about six grams of a diol which he assumed to be 2-methyl-1,2-propanediol. Since Henninger did not examine the constitution of the diol but drew his conclusions from the physical properties of the product, the butanediol which he isolated may conceivably have been 2,3-butanediol.

Pere (1896) examined the products of the action of

Bacillus subtilis, Bacillus mesentericus vulgatus, and Tyrothrix tenuis on dextrose and sucrose and found in the distillates from the fermentation liquors a levorotatory substance which reduced Fehling's solution. Although Pere attributed the reduction to glyceraldehyde, there is little doubt that the compound was actually acetylmethylcarbinol. Grimbert (1901) found that up to 0.09 gram per 100 ml. of acetylmethylcarbinol was produced from various sugars by Bacillus tartricus. Desmots (1904) identified acetylmethylcarbinol among the volatile products formed by the action of a number of bacteria of the Bacillus group upon a variety of substrates.

The first significant contribution to the production of 2,3-butanediol by fermentation was made by Harden and Walpole (1906) who isolated about eight grams of 2,3-butanediol per liter from a solution of five per cent dextrose subjected to fermentation by Aerobacter aerogenes. Making a correction for losses which occurred during distillation, the authors estimated that the total yield of the 2,3-butanediol was 27 per cent of the weight of dextrose added. The fermentation was conducted at 37°C. under anaerobic condition in a medium containing peptone and chalk. Walpole (1911) conducted additional fermentations under similar conditions and recovered 21 grams of 2,3-butanediol from fermentation of 100 grams of dextrose. Similar results were obtained by Harden

and Norris (1912) and by Thompson (1912).

Lemoigne (1912, 1913a, 1913b, 1919a, 1919b, 1923a, 1923b) studied the formation of 2,3-butanediol and acetylmethylcarbinol by several bacteria of the Bacillus genus. The yields obtained were very small.

By adding diacetyl to yeast fermentations, Neuberg and Nord (1919) found that 35 per cent of the diacetyl added was converted to 2,3-butanediol. Neuberg, Nord, and Wolff (1920) obtained 1.5 grams of 2,3-butanediol and 0.7 gram of acetylmethylcarbinol by fermentation of 100 grams of dextrose with Aerobacter aerogenes. Neuberg and Kobel (1925) added ten grams of acetylmethylcarbinol to a fermenting yeast and sugar mixture and after a few days were able to isolate 5.8 grams of 2,3-butanediol.

In studying the classification of Aerobacillus polymyxa, Donker (1924) found that this organism formed 25 per cent of 2,3-butanediol and 19 per cent of ethanol in fermentation of dextrose. Donker (1926) reported that in the fermentation of a two per cent dextrose medium by Aerobacillus polymyxa, twenty per cent by weight of the dextrose utilized could be accounted for as 2,3-butanediol and twenty per cent as ethanol. With Bacillus macerans only a trace of 2,3-butanediol was formed.

A number of patents, evidently evolving from the initial efforts of Donker (1924, 1926), Harden and Walpole (1906),

Walpole (1911), Harden and Norris (1912), and of Thompson (1912), have been issued to Verhave and his associates. Several of these patents - Verhave (1928, 1929a, 1929b, 1933), Kluyver and Scheffer (1933), and N. V. Nederlandsche Gist-en Spiritusfabriek (1934) - were issued in separate countries and appear to be nearly identical. The patent of Kluyver and Scheffer (1933) comprises fermenting a mash containing carbohydrates, nitrogen compounds, phosphates, and a carbonate with an organism capable of forming 2,3-butanediol. The improved process represented in the patent involves development of the process to a technical scale by substitution of starchy materials or molasses for dextrose, substitution of ammonium sulfate or malt for peptone, use of carbohydrate concentrations up to 15 to 20 per cent, and decreasing the time of fermentation to less than two days by the use of aeration. Thus, in an example representing the above process, potatoes saccharified by malt were fermented by Aerobacter aerogenes, in the presence of superphosphate and limestone, in 33 to 39 hours. Calculations made from the data given indicate a yield of approximately 34 per cent of 2,3-butanediol and 18 per cent ethanol by weight of the starch present. Scheffer's process (1936) recommends replacement of air by hydrogen recovered from butanol fermentations.

Pederson and Breed (1928) found that Serratia marcescans and Serratia indica formed 2,3-butanediol from dextrose.

Yields were high as 16 per cent by weight of dextrose fermented.

Work at Iowa State College on the preparation of 2,3-butanediol by fermentation was first reported by Breden (1930) and Breden and Fulmer (1931). These authors investigated the aerobic and anaerobic fermentation of xylose and sucrose by Aerobacter faeni at 37°C. The yields of products from a synthetic medium containing two per cent carbohydrate may be summarized as follows, in terms of grams of products per 100 grams of sugar added:

	Xylose		Sucrose	
	aerobic	anaerobic	aerobic	anaerobic
Carbon dioxide	60.5	38.9	82.2	52.8
2,3-Butanediol	10.6	13.7	15.1	16.6
Acetylmethyl-carbinol	2.6	0.3	6.5	0.6
Ethanol	6.3	12.9	11.3	19.3
Lactic acid	1.2	12.7	2.3	14.3

Essentially all of the sugar was fermented at the end of ten days. Several factors are particularly worthy of note: the increase in conversion to carbon dioxide under aerobic conditions, the increase in diol yield and corresponding decrease in acetylmethylcarbinol under anaerobic conditions, and the increase in the yields of ethanol and lactic acid under anaerobic conditions. It is also noteworthy that the same products are formed from xylose as from sucrose. Any theory of the mechanism of the fermentation must, therefore, take into consideration the dissimilation of the five-carbon

as well as the six-carbon sugars.

The work of Breden and Fulmer was extended by Fulmer, Christensen, and Kendall (1933) and Kendall (1934a, 1934b). These investigators studied the utilization of sucrose by several bacteria of the Aerobacter genus and determined optimal conditions for fermentation of sucrose under essentially anaerobic conditions at 37°C. Results obtained by fermentation of solutions of various sucrose concentrations are shown below:

Sucrose added, grams per 100 ml.	Sucrose fermented, per cent	Fermentation period, days	2,3-Butanediol formed, grams per 100 grams of sucrose added
2	99	7	42.5
4	99	12	43.5
6	99	14	45.3
8	99	18	46.4
10	88	23	41.1
12	85	23	44.5

The medium used by the above authors was developed according to the methods later described by Fulmer (1943). In addition to sucrose, the medium contained:

	grams per 100 ml.
NH_4Cl	0.250
$K_2HPO_4 \cdot 3\ H_2O$	0.175
$CaCl_2$	0.015
$MgSO_4$	0.175

The optimum pH was found to be 6.0. The yields of 2,3-butanediol obtained were higher than any previously reported and

amounted to about 90 per cent of theory.

Birkinshaw, Charles, and Clutterbuck (1931) found that Bacillus asiaticus-mobilis gave approximately 27 per cent of 2,3-butanediol by weight of dextrose added in fermentation of a modified Czapek-Dox medium. Brockmann (1933) studied the role of oxidation-reduction in the dissimilation of dextrose by species of Aerobacter and concluded that while the addition of hydrogen acceptors tended to increase the oxidation potential, the variation had little or no effect on yields of 2,3-butanediol and acetylmethylcarbinol. Chappell (1935) obtained yields of twenty to thirty per cent of 2,3-butanediol in the fermentation of sucrose by Aerobacter faeni. Canepa and de la Serna (1935) found that a two per cent solution of dextrose was completely fermented by Aerobacter aerogenes, giving 26 per cent of 2,3-butanediol.

Working with cell suspensions of Aerobacter indologenes, Michelson and Werkman (1938) found that in a dextrose medium above pH 6.3, ten per cent by weight of dextrose fermented was converted to 2,3-butanediol, while at or below pH 6.3, 39 per cent of the dextrose fermented was converted to the diol.

Sakaguti, Ohara, and Kobayasi (1939) isolated strains of Aerobacter aerogenes and Aerobacter cloacae from soil which were capable of producing thirty to forty per cent

of 2,3-butanediol from an eight per cent solution of dextrose at pH 6 to 7 and a temperature of 37-38°C. Sakaguchi, Ohara, and Kikuti (1940) obtained similar yields from various sugars.

With the advent of World War II and the increasing interest in 2,3-butanediol as a precursor for butadiene, studies on the production of 2,3-butanediol by fermentation were initiated in many laboratories, including those of Commercial Solvents Corporation, Heyden Chemical Corporation, Joseph E. Seagram and Sons, the National Research Council of Canada, the Northern Regional Research Laboratory, Schenley Distillers Corporation, and others. Much of the work done was put under governmental secrecy orders and has not been reported, although the secrecy orders have since been lifted.

Elder (1942) mentioned the fermentation of saccharified grain mashes by Aerobacter aerogenes. According to an anonymous article in Chemical Industries (1943), 2,3-butanediol was at that time being produced on a large pilot-plant scale by the Schenley Distillers Corporation. Adams (1943) stated that members of the National Research Council in Canada were studying the production of 2,3-butanediol by fermentation of wheat with Aerobacillus polymyxa.

Hendlin (1943) studied the fermentation of dextrose by Aerobacter aerogenes and obtained up to 40 per cent of 2,3-butanediol by weight of dextrose added. The medium used contained 5 per cent of dextrose in addition to inorganic

salts, and was aerated for 84 hours and then allowed to stand without aeration for an additional 12 hours. During this period of standing after aeration, the 2,3-butanediol content of the fermentation liquor increased while the acetylmethylcarbinol content decreased; the sum of the two products remained constant. Hendlin also found that corn mashes could be fermented to 2,3-butanediol by the use of Aspergillus oryzae in conjunction with Aerobacter aerogenes. From a medium containing 15 per cent corn meal, one per cent calcium carbonate, and 0.4 per cent dipotassium phosphate, Hendlin obtained up to 38 per cent of 2,3-butanediol by weight of starch added.

A patent issued to Christensen (1944) involved the fermentation of sugar or molasses solutions by Aerobacter aerogenes; the sugar concentration was held above 6 per cent by periodic addition of sugar.

By using the starch-hydrolyzing yeast Endomycopsis fibuliger in conjunction with Aerobacter aerogenes, Wickerham et al (1944) obtained 27 per cent of 2,3-butanediol and 13 per cent of ethanol by weight of starch present. The substrate used was a 15 per cent wheat mash.

Although much of the work which is reviewed in the following paragraphs was reported too late to be of assistance in the selection of experiments in conducting the present investigations, it is included here in order that

the survey of other work done may be brought up-to-date.

Studies on the fermentation of wheat mashes with Aerobacillus polymyxa were reported by Ledingham and Adams (1944). The yields obtained upon fermentation of 15 per cent wheat mashes were about 35 per cent of 2,3-butanediol and 19 per cent of ethanol by weight of starch present. It was found that the most concentrated mash which could be fermented efficiently contained about 15 per cent wheat. The maximum yields were obtained after 4½ days incubation at 32.5°C.

Katznelson (1944a) studied additional factors affecting the fermentation of wheat by Aerobacillus polymyxa and found that neither the age of the inoculum nor the nature of the inoculating medium had much effect on the yields of 2,3-butanediol. The surface-volume ratio was found to have a considerable effect on the yield of products; a high surface-volume ratio favored the production of 2,3-butanediol. Addition of yeast extract to the medium likewise effected an increase in the yield of 2,3-butanediol. Katznelson (1944b) also studied the effect of bacteriophage on Aerobacillus polymyxa and recommended aseptic technique and the use of non-lysogenic strains as precautions. Katznelson and Lockhead (1944) found that Aerobacillus polymyxa required biotin for growth.

The production of 2,3-butanediol from wheat was also studied by Ledingham, Adams, and Stanier (1945), who found that the addition of yeast extract, malt extract, dried

yeast, or corn steep liquor was essential for the preparation of an active inoculum. Pure wheat starch with inorganic supplements was only partially utilized; even combinations of starch wash water, bran, gluten, and yeast extract added to the wheat starch failed to produce as good results as were obtained by fermentation of the whole grain. Aerobic conditions were found to inhibit the fermentation, particularly in regard to the rate of fermentation. Passing nitrogen or hydrogen through the medium was found to decrease the time of fermentation but was also found to increase the yield of ethanol and decrease the yield of 2,3-butanediol. In fermentations conducted on 15 per cent whole wheat mashes, yields of 2,3-butanediol plus ethanol were as high as 50 per cent by weight of available starch.

Stanier, Adams, and Ledingham (1945) found that the filterability of the fermented mash varied considerably with the strain of Aerobacillus polymyxa used. By selection of proper strains, it was possible to obtain a readily filterable mash and still obtain high yields of the diol.

Rose and King (1945) compared yields of 2,3-butanediol and ethanol obtained when 15 per cent wheat mashes were fermented with Aerobacillus polymyxa in small flasks and in five-gallon fermenters. Yields of the 2,3-butanediol were consistently higher when the fermentations were conducted in small flasks, while the ethanol yields were con-

sistently higher when the fermentations were conducted in the five-gallon fermenters.

Studies on the production of 2,3-butanediol from wood hydrolyzates were conducted by Perlman (1944). Yields obtained were about 35 per cent by weight of the sugar present in the hydrolyzate. The fermentation was carried out using aerated cultures of Aerobacter aerogenes.

According to Stanier and Adams (1944), Aeromonas hydrophila produces considerable quantities of 2,3-butanediol in the fermentation of sugars; 37 grams of the diol were obtained from 100 grams of fermented dextrose. Neish, Blackwood, and Ledingham (1945a, 1945b) found that a strain of Bacillus subtilis formed 57 millimoles of 2,3-butanediol for each 100 millimoles of dextrose fermented.

Adams and Stanier (1945) found that the ratio of 2,3-butanediol to ethanol resulting from fermentation of various sugars with Aerobacillus polymyxa varied with the sugar used as the source of carbon. Liebmann (1945) gives the following equation for the dissimilation of dextrose by Aerobacillus polymyxa:

$$5\ C_6H_{12}O_6 \longrightarrow 3\ C_4H_{10}O_2 + 4\ C_2H_5OH + 10\ CO_2 + 3\ H_2.$$

This equation probably represents the results usually obtained rather than the theoretical conversion.

Production of 2,3-butanediol from acid-hydrolyzed starch was studied by Ward, Pettijohn, and Coghill (1945).

Yields obtained in fermentations with Aerobacter aerogenes were from 40 to 41 per cent by weight of starch added.

3. Methods of recovery

The problems involved in the recovery of 2,3-butanediol from fermentation liquors are similar to those involved in the recovery of glycerol. Like glycerol, 2,3-butanediol is miscible with water in all proportions and has a boiling point considerably above that of water. 2,3-Butanediol is also very hygroscopic and therefore is difficult to obtain free from water. Two general methods seem to be applicable to the recovery of the 2,3-butanediol, distillation and solvent extraction. Of these two methods, solvent extraction following evaporation seems to have been more generally used. Walpole (1911), Thomson (1912), Harden and Norris (1912), and Neuberg and Nord (1919) recovered the 2,3-butanediol by evaporation of the fermentation liquor to a syrupy liquid, followed by extraction of the residual diol with ethanol and fractionation of the resulting solution. Losses of 2,3-butanediol by this method were rather high.

Ether extraction has been employed by Chappell (1935) and Akabori (1938). This method has also been used in connection with the work of Kolfenbach, Kooi, Fulmer, and Underkofler (1944). Further details of this method will be discussed later.

According to Elder (1942) and an anonymous article in

Chemical Industries (1943), butanol extraction has been used in the pilot-plant recovery of the 2,3-butanediol from fermentation liquors. The previously mentioned patents of Verhave and his associates involve recovery by vacuum distillation.

Recently, Rose and King (1945) studied the recovery of 2,3-butanediol from wheat mashes fermented by Aerobacillus polymyxa. Considerable difficulty was experienced in coagulating the wheat proteins, and as much as 20 per cent of the diol remained in the filter cake. Recovery of the diol was conducted by passing the vaporized liquor through a packed fractionating column and refractionating the distillate. The overall recovery was 50 to 55 per cent of the 2,3-butanediol present in the fermented mash.

Blom et al (1945) described a recovery process which involved steam-stripping a concentrated liquor at an elevated pressure. The feed liquor was obtained by fermenting saccharified wheat or corn mash with Aerobacter aerogenes and concentrating the filtered mash. The concentration was carried out by multiple-effect evaporation, the vapors from each effect being rectified to retain all of the 2,3-butanediol in the evaporator syrup. The proposed recovery process, which is based on pilot-plant investigations, is described by Blom et al as follows:

> The evaporator sirup containing all of the glycol is fed to the top of a packed stripping

> column, which is operated at a pressure of 55 pounds per square inch gage. Steam is passed up the column, in the ratio of 5 pounds of steam per pound of sirup, and absorbs or strips the glycol from the sirup during the countercurrent passage. After every 11 hours of operation the sirup fed to the stripping column is diverted to a storage tank . . . and water is pumped through the column for 1 hour to remove the deposited sludge. Steam flow is not interrupted during the washing cycle; hence, the continuity of the operation as a whole is not disturbed. The stripped sirup, which is discharged from the base of the stripping column, is similar to distillers' evaporator sirup and is drum-dried for the production of poultry and cattle feed. The vapor from the top of the column contains butylene glycol, water, and an insignificant amount of impurities. It is led to the base of a bubble-cap scrubbing column, which is also operated under pressure, and is washed free of glycol by a stream of water introduced at the top of the column. A reflux ratio (overflow to vapor) of approximately 0.286 is used in this operation. The vapor from the top of the scrubbing column has a pressure of approximately 50 pounds per square inch gage and is divided into two streams; one stream is used for process work in other parts of the plant, and the other stream is compressed to a pressure of 55 pounds per square inch gage by a thermocompressor with steam at a pressure of 250 pounds per square inch gage. The compressed vapor is re-used for stripping. Steam withdrawn from the system for process use is approximately equal to that required for compression; hence, the steam required for stripping is practically only that which is condensed because of heat losses in the system. The glycol-water product from the scrubbing column contains approximately 8% butylene glycol and is easily rectified to make a product containing 99% butylene glycol.

Pilot plant data showed that the condensate from the stripping column contained as much as 90 per cent of the 2,3-butanediol present in the concentrated liquor.

B. Properties of the 2,3-Butanediols

1. Chemical properties

Although a large number of compounds closely related in structure to 2,3-butanediol have been described in the literature, those considered here will include only such as have been prepared from 2,3-butanediol directly.

The esterification of 2,3-butanediol is important in that butadiene prepared from the diol is usually made by catalytic decomposition of the diacetate. It is well known that the diacetate of 2,3-butanediol can be prepared by esterification with acetic acid or with acetic anhydride. Schniepp, Dunning, and Lathrop (1945) described a continuous process for the acetylation of 2,3-butanediol. The operation was carried out by feeding the diol, along with a catalytic amount of sulfuric acid, into the top of a reaction column while introducing a continuous stream of glacial acetic acid into the base of the column. The diacetate of 2,3-butanediol was produced in a 97 per cent of theory yield. Shlechter, Othmer, and Marshak (1945) studied the kinetics of the esterification of the diol with acetic acid and described a continuous esterification process utilizing an entraining agent to remove the water formed. Yields of the diacetate were about 95 per cent of theory.

According to an advertisement of the Lucidol Corporation

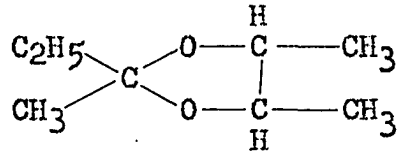
(1933), 2,3-butanediol forms nitrates with nitric acid and resins with phthalic anhydride. Matignon, Moreu, and Dodé (1932-1933) prepared the dinitrate of 2,3-butanediol by esterification with nitric acid in the presence of sulfuric acid.

Denivelle (1939) prepared the neutral sulfite of 2,3-butanediol by treating the diol with thionyl chloride in the presence of pyridine. Rippere and La Mer (1943) found that 2,3-butanediol reacted with boric acid to yield a slightly volatile ester. Kolfenbach, Fulmer, and Underkofler (1945) described the properties of the inner carbonate of 2,3-butanediol. The carbonate was prepared by the interaction of the diol with phosgene.

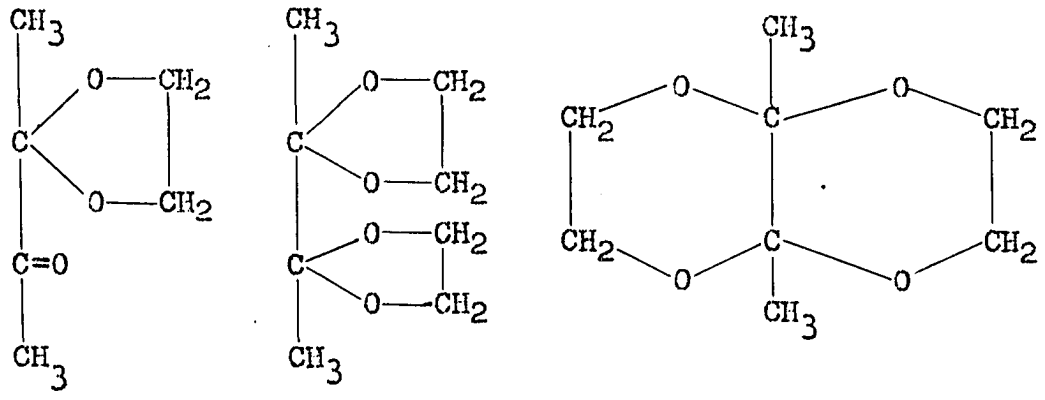
Oxidation of 2,3-butanediol may lead to a variety of substances. Walpole (1911) examined the oxidation products obtained by the action of dilute nitric acid on the diol and reported finding acetylmethylcarbinol, diacetyl, lactic acid, and carbon dioxide. The oxidation of 2,3-butanediol to diacetyl by reaction with bromine in water was studied in detail by Matignon, Moreu, and Dodé (1934). This reaction has often been used in the analytical determination of the diol; the diol is oxidized to diacetyl and the diacetyl converted to nickel dimethylglyoxime. McAllister and de Simo (1936) were issued a patent involving the catalytic oxidation of 2,3-butanediol to diacetyl. The reaction was carried out in a stream of oxygen over a copper catalyst. According

to Suknevich and Chiligaryan (1936), 2,3-butanediol is converted to chloroform by reaction with calcium hypochlorite. Evdokimov (1938) found that the diol gave carbon dioxide, water, and formic acid upon warming with anhydrous oxalic acid.

The acetals of 2,3-butanediol are readily formed in the presence of aldehydes or ketones with hydrochloric acid as catalyst. Backer (1936) attempted to prepare butadiene by heating 2,3-butanediol with aluminum oxide and obtained instead an appreciable amount of methyl ethyl ketone. An acetal was also formed in the reaction, resulting from interaction of the diol and the methyl ethyl ketone. Backer showed the structure of the acetal to be:



Böeseken and Tellegen (1938) studied various acetals of 2,3-butanediol. Reaction of the diol with diacetyl in the presence of such dehydrating agents as phosphorus pentoxide led to the formation of three different acetals whose structural formulas may be represented as follows:



The direct dehydration of 2,3-butanediol to butadiene is a reaction which involves merely the removal of two molecules of water from the diol molecule. This reaction has been attempted by many investigators including Backer (1936), Akabori (1938), Denivelle (1939), and Kolfenbach (1944). Invariably the main product obtained in the reaction was methyl ethyl ketone, although Kolfenbach did obtain a 7.5 per cent yield of the butadiene.

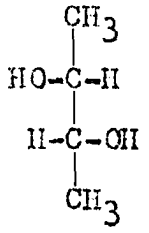
Catalytic decomposition of the esters of 2,3-butanediol to butadiene, however, has been proved to be a feasible process. Tishchenko and Kosternaya (1937) and Kosternaya (1938) obtained butadiene by the decomposition of the xanthate of 2,3-butanediol. Denivelle (1939) obtained 8 to 10 per cent of butadiene by the decomposition of the neutral sulfite of the diol. On a practical scale, however, the diacetate appears to be the best starting material for the preparation of butadiene. A patent issued to Hill and Isaacs (1938) involves the preparation of butadiene by passing the hot

vapor of the diacetate of 2,3-butanediol through a tube packed with quartz. In a report on the progress of butadiene production Elder (1942) indicated that an 88 per cent conversion to butadiene could be obtained by the catalytic decomposition of the diacetate. An outstanding feature of the diacetate process is the fact that the butadiene produced is of sufficiently high quality for polymerization so that further purification is unnecessary. Recently, Morell, Geller, and Lathrop (1945), Schniepp et al. (1945), and Shlechter, Othmer, and Brand (1945) have reported the details of the process.

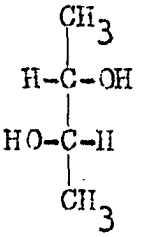
Neish (1944) found that substantial amounts of acetylmethylcarbinol could be produced by passing 2,3-butanediol over a hot copper catalyst. Diacetyl was also formed. Neish, Haskell, and Macdonald (1945) studied the formation of methyl ethyl ketone by dehydration of 2,3-butanediol with sulfuric acid.

2. Physical properties

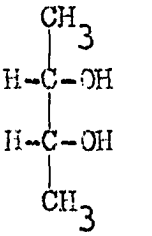
Inasmuch as 2,3-butanediol contains two asymmetric carbon atoms in a symmetrical molecule, three stereoisomeric forms are possible, the configuration of which, according to conventional practice, may be represented as follows:



d-2,3-butanediol



l-2,3-butanediol



meso-2,3-butanediol

It would be expected that the d- and l-2,3-butanediols would have identical physical properties, differing only in the sign of rotation, whereas the meso form would be expected to have physical properties differing from those of the d and l forms. Early data concerning the physical properties of the 2,3-butanediols are not reliable, since most of these data are given without reference to the stereoisomeric form or forms present.

Wilson and Lucas (1936) prepared the dl mixture and the meso-2,3-butanediol starting with the cis- and trans-butene, respectively. The diols were purified by recrystallization from isopropyl ether. The properties of the two forms prepared by these investigators were given as follows:

Configuration	dl	meso
Boiling point at 742 mm., °C.	176.7	181.7
Boiling point at 16 mm., °C.	86	89
Melting point, °C.	7.6	34.4

Wilson and Lucas also found that the dl mixture and the meso isomer formed a eutectic melting at -1.5°C. and containing about 44 per cent of the meso form. Ward et al (1944)

determined the physical properties of the 2,3-butanediols isolated *en masse* from fermentations with *Aerobacter aerogenes* and *Aerobacillus polymyxa*. The properties reported by these authors were:

Fermentation by	*A. polymyxa*	*A. aerogenes*
Specific rotation, 25°C.	-13.0	1.0
Refractive index, 25°C.	1.4307	1.4384
Melting point, °C.	19	25
Viscosity, 25°C., centipoises	41.0	118.0
Hydrate formation	----	pentahydrate (melting point 16.8°C.)

The physical constants of the diols as given by Morell and Auernheimer (1944) agree substantially with those given by Ward *et al*.

Neish (1945) has presented evidence showing that the 2,3-butanediol produced by *Aerobacillus polymyxa* is the pure *levo* isomer. Neish reported the density of the product to be 0.9880 at 26°C. (water at 15°C. = 1) and the refractive index measured in daylight to be 1.4318.

The 2,3-butanediol formed by *Aerobacter aerogenes* has been shown by Lees, Fulmer, and Underkofler (1944) to consist of about 90 per cent of the *meso* form. Fulmer, Underkofler, and Bantz (1943) have shown that the remaining 10 per cent consists almost entirely of the *d* form.

Wurtz (1859) reported 2,3-butanediol to be soluble in all proportions in water, ethanol, and ether. The Lucidol Corporation (1933) reported that 2,3-butanediol was a nearly colorless solid or liquid, hygroscopic, a good

solvent for dyes, and possessed remarkable ability to penetrate woods and textiles.

Chappell (1935) isolated 2,3-butanediol from fermentations by Aerobacter aerogenes and obtained two fractions upon fractionally distilling the product. The highest boiling fraction, which was undoubtedly the meso form, had only a slight positive rotation. Pressure-temperature relationships for this fraction, as given by Chappell, were as follows:

°C.	100	110	120	130	140
Pmm.	24.0	44.5	76.0	120.0	177.0

These data may be expressed in the form of two equations, as follows:

Temperature range	Equation
100-120°C.	$\log P_{mm} = -\frac{3731}{T} + 11.38$
120-140°C.	$\log P_{mm} = -\frac{2924}{T} + 9.326$

Schierholtz and Staples (1935) determined pressure-temperature relationships of 2,3-butanediol prepared by hydrolysis of 2,3-dibromobutane. The relationships, as expressed by the authors, were:

Temperature range	Equation
80-130°C.	$\log P_{mm} = -\frac{3023.9}{T} + 9.5521$
130-182.5°C.	$\log P_{mm} = -\frac{2907.1}{T} + 9.2616$

Inasmuch as Schierholtz and Staples fractionated the diol until the refractive indices for successive fractions agreed

to not more than 0.00030, the boiling point datum (182.5°C.$^{760\ mm.}$) indicates that the 2,3-butanediol was rather pure meso. The density was reported as $d_4^{20} = 1.0033$, and the refractive index as $N_D^{25} = 1.43637$.

Blom et al (1945) determined liquid-vapor equilibria of 2,3-butanediol-water mixtures at various pressures. The 2,3-butanediol used was the meso mixture obtained from Aerobacter aerogenes fermentations. Additional data for the diol-water and other systems involved in butadiene manufacture from 2,3-butanediol are given by Othmer, Shlechter, and Koszalka (1945).

The specific heat of 2,3-butanediol was determined by Khokhlovkin and Kalacheva (1936). The experimental values given were:

At 30°C., 0.6009 calories per gram
At 140°C., 0.8311 calories per gram

The data were also expressed as:

$c = 0.5381 - 0.0010464t$, where t is the temperature in °C., and c is the specific heat in calories per gram.

Moureu and Dode (1937) determined the heat of combustion of 2,3-butanediol under carefully controlled conditions and found Q_v to be 588.3 kilocalories per mole and Q_p to be 589.1 kilocalories per mole.

Marvel and Denoon (1938) determined the ultraviolet absorption spectra for various compounds and found that 2,3-butanediol did not absorb in the ultraviolet region. Kraus (1941) found 2,3-butanediol to be a fairly good solvent for

nitrocellulose. Of a list of twenty-five solvents, 2,3-butanediol was fourteenth in time required for solution of equal quantities of nitrocellulose. Willard (1941) determined the extent of sound absorption and the velocity of sound in numerous liquids including 2,3-butanediol. Barnes, Liddell, and Williams (1943) determined the infrared spectrum of a number of compounds relating to synthetic rubber and found that 2,3-butanediol showed little absorption in the unsaturated region from 1900 to 1600 $cm.^{-1}$. This is important in spectroscopic analysis for unsaturated compounds in materials containing 2,3-butanediol.

Lees, Fulmer, and Underkofler (1944) investigated the hygroscopicity of 2,3-butanediol (prepared by Aerobacter aerogenes fermentation) and found that the anhydrous diol would absorb as much as 10 per cent of its weight of water in 16 hours. These investigators also determined specific viscosities of mixtures of meso-2,3-butanediol and l-2,3-butanediol and found the specific viscosity to be an exponential function of the percentage of meso-2,3-butanediol present. The results were expressed by the equation,

$$\% \text{ meso-glycol} = 140.0 \log (s - 2.87) - 55.2,$$

where s is the specific viscosity. The specific conductivities and pH values of mixtures of meso-2,3-butanediol and l-2,3-butanediol in boric acid solution were also determined. The equations derived expressing the relations for a mixture

of the two diols in a 0.5 molar boric acid solution were,

$$\%\ \underline{\text{meso}}\text{-glycol} = 153.6 - (22.3 \times L \times 10^5),$$

where L is the specific conductivity, and

$$\%\ \underline{\text{meso}}\text{-glycol} = 123.6 - (4.81 \times a_{H^+} \times 10^5)$$

Liquid-liquid extraction data for systems composed of meso-2,3-butanediol, water, and various solvents were compiled by Othmer et al (1945). The solvents studied included butanol, butyl acetate, 2,3-butanediol acetate, and methylvinylcarbinol acetate.

3. Biological properties

Many microorganisms are capable of carrying out their normal metabolism with 2,3-butanediol as the sole source of carbon. According to Sakaguchi and Kambayasi (1939), 2,3-butanediol is assimilated by bacteria, yeast, and molds almost as well as is glycerol.

One of the most interesting microbiological reactions of 2,3-butanediol is its oxidation to acetylmethylcarbinol. Kling (1905a, 1905b) obtained a 50 per cent yield of acetylmethylcarbinol by the action of Mycoderma aceti, using as the substrate the diol produced by fermentations by Aerobacter aerogenes. Visser't Hooft (1926) reported a 77 per cent yield of acetylmethylcarbinol using Acetobacter suboxydans. Both workers assumed that they were dealing with substantially equimolar quantities of the d and l forms of 2,3-butanediol and since in each case the residual diol was

dextrorotatory, Kling concluded that the l form only was attacked, while Vissert Hooft concluded that both the d and l forms were attacked. It is now well established that the 2,3-butanediol produced by Aerobacter aerogenes consists of a mixture of the stereoisomers containing about 90 per cent of the meso-2,3-butanediol. Fulmer, Underkofler, and Bantz (1943) obtained yields of better than 90 per cent of acetylmethylcarbinol by the action of Acetobacter suboxydans on the diol produced by the Aerobacter aerogenes. The residual diol recovered showed a rotation of $[\alpha]_D^{25} = 10.15$, showing that the stereoisomeric mixture of the 2,3-butanediols produced by Aerobacter aerogenes consists of the meso-diol and the d-diol with little or none of the l-diol. In addition, Underkofler, Fulmer, Bantz, and Kooi (1944) have shown that Acetobacter suboxydans will preferentially oxidize the meso- and levo-diols, while the dextro-diol is not attacked. This represents another example of the biochemical specificity of living organisms toward one of a pair of enantiomorphs.

Stanier and Fratkin (1944) found that Aerobacter aerogenes was unable to oxidize the l-isomer but did oxidize both the meso- and d-isomers. Aeromas hydrophila oxidized the meso-2,3-butanediol but not the l-isomer and probably not the d-isomer. Aerobacillus polymyxa oxidized both the l-and meso-2,3-butanediols, but the l-isomer was oxidized four

times as rapidly as the meso-isomer.

There are few actual data concerning the physiological action of 2,3-butanediol upon mammals. Neuberg and Gottschalk (1925) found that 2,3-butanediol injected into rabbits was excreted as a glycuronic acid compound. No toxic effects were observed. Hooper (1936) included 2,3-butanediol among examples of water-miscible solvents of vitamin D suitable for theurapeutic use. Zwikker (1942) stated that 2,3-butanediol could be substituted for glycerol in suppositories and all pharmaceutical preparations for external use, but should not be added to preparations for internal use, since it is somewhat toxic and in sufficient concentration causes kidney damage. Doisy and Westerfield (1943) found that the acetylation of o-aminobenzoic acid by rabbits was significantly increased by the administration of 2,3-butanediol. It should be interesting to determine the relative toxicity of the stereoisomeric forms of 2,3-butanediol.

III. PRODUCTION OF 2,3-BUTANEDIOL FROM DEXTROSE BY FERMENTATION WITH AEROBACTER AEROGENES

A. Introduction

This phase of the work was begun in January, 1942, and was undertaken for the primary purpose of preparing relatively large quantities of 2,3-butanediol. The recovered and purified diol was used by other members of the research group in Biophysical Chemistry in studying the physical and chemical properties of the compound.

B. Materials

The dextrose used was anhydrous "cerelose" obtained from the American Maize-Products Company, Roby, Indiana. Inorganic constituents used in the media were reagent grade chemicals. In the preparation of the media, distilled water was used unless otherwise specified.

C. Cultures

Two strains of Aerobacter aerogenes were used in this series of experiments. The numbers assigned in the Biophysical Chemistry culture collection and the sources of the

organisms are shown below:

Name	Culture Number	Source
Aerobacter aerogenes	B16	American Type Culture Collection No. 211
Aerobacter aerogenes	B24	Northern Regional Research Laboratory, Peoria, Illinois (NRRL B199)

D. Methods of Analysis

1. Determination of 2,3-butanediol

In the early part of the work, no convenient method was available for the rapid determination of 2,3-butanediol in a large number of samples. Later, an unpublished method made available by the Northern Regional Research Laboratory was used. This method involves the oxidation of the 2,3-butanediol to acetaldehyde, absorbing the acetaldehyde in excess sodium bisulfite, oxidizing the excess bisulfite with a strong iodine solution, liberating the bound bisulfite with sodium bicarbonate, and titrating the liberated bisulfite with a standard iodine solution, using starch as the indicator. Acetylmethylcarbinol, dextrose and maltose interfere with this method. Where the butanediol:acetylmethylcarbinol ratio is 5:1 or greater, acetylmethylcarbinol reacts quantitatively to yield one mole of acetaldehyde per mole of acetylmethyl-

carbinol; thus, the appropriate correction may be applied after the determination of the acetylmethylcarbinol. Maltose and dextrose also react to yield small amounts of bisulfite-binding materials. By conducting analyses on pure solutions of dextrose and of maltose, it was found that consistent results were obtainable over a fairly wide range of concentrations. Curves for dextrose and maltose corrections were thus plotted which permitted corrections to be applied for these two substances. Ethanol and lactic acid do not interfere to an appreciable extent. This method for the determination of 2,3-butanediol is similar to the one described by Johnson (1944).

2. Determination of residual dextrose

The modified Shaffer-Somogyi method of Underkofler, Gumon, Rayman, and Fulmer (1943), which has been used in these laboratories for a number of years and found applicable to the determination of a variety of easily oxidizable fermentation products, was used for the determination of dextrose. The reagents used were standardized periodically against pure anhydrous dextrose.

3. Determination of ethanol

During the early portion of the work, ethanol was determined by distilling a portion of the fermentation medium after the addition of calcium carbonate and determining the

specific gravity of the distillate. The per cent ethanol was ready from a standard table for specific gravity of pure ethanol-water solutions. It was found later that ammonia also distilled from the fermentation liquor, thus making this method unreliable for the determination of ethanol. Hence ethanol analyses conducted in this manner are not reported. It was necessary, therefore, to develop a suitable method for the determination of ethanol which would be applicable to rapid determination of the ethanol content of a large number of samples. The method used was adapted from the dichromate oxidation method of Kozelka and Hine (1941). Certain changes were made, since it was not necessary to observe all the precautions involved when determining the amount of ethanol in blood for medicolegal purposes.

Procedure used

A sample of fermentation liquor containing from 40 to 2000 mg. of ethanol was diluted to 300 ml. and placed in a 500-ml. Kjeldahl distillation flask. A small amount of calcium carbonate was then added to prevent volatilization of acids, and the flask was fitted with a spray trap and attached to a Kjeldahl distillation rack. The liquid was then distilled, and the first 100 ml. of distillate was collected in a volumetric flask. An aliquot of the distillate containing from 2 to 20 mg. of ethanol was placed in a 200x75 mm. pyrex test tube, and 5.00 ml. of 0.4000 N potassium dichromate was added. Sufficient water was added to bring the total volume to 10 ml., and 2.0 ml. of concentrated sulfuric acid was allowed to run down the side of the tube to avoid mixing and liberation of excess heat before stoppering. The tube

> was then stoppered with a rubber stopper fitted with a short section of fine bore capillary tubing, and the tube shaken quickly, care being taken to prevent the solution from touching the rubber stopper. After standing for 20 minutes, the tube was placed in an ice bath to prevent further oxidation. When the tube was cool, 10 ml. of water was added, the liquid transferred to a 125-ml. Erlenmeyer flask, using 10 to 15 ml. of water in the transfer. Crystalline potassium iodide (0.4 g.) was added, and the liberated iodine titrated with 0.05 normal sodium thiosulfate, using soluble starch as indicator.

The differences between this oxidation procedure and that of Kozelka and Hine lie in the use of a more concentrated dichromate solution to extend the allowable variation in amount of ethanol in the sample, the use of a capillary stopper instead of an airtight seal, and allowing the heat of solution of the sulfuric acid to furnish the heat necessary for the reaction rather than heating the mixture in a water bath.

Tests were run to determine the effect of various factors on the determination. It was found that quantitative results were obtained with pure alcohol solutions when the period of standing during the oxidation procedure was from 10 to 30 minutes. Stoppering with a capillary stopper was equally as effective as an airtight stopper.

Table 1 shows the results obtained when pure ethanol-water solutions were subjected to the oxidation procedure. It is evident that virtually quantitative recovery was obtained.

It would be expected that acetylmethylcarbinol would interfere with the method, since it is somewhat volatile with steam. Acetylmethylcarbinol was prepared according to the procedure of Fulmer, Underkofler, and Bantz (1943) and was purified by fractionation. The material thus obtained had a boiling range of 142-143°C. Solutions of the acetylmethylcarbinol were subjected to the dichromate oxidation

Table 1

Analysis of Ethanol-water Solutions by Dichromate Oxidation

Ethanol added, mg.	Ethanol by analysis, mg.	Recovery, per cent
2.04	2.06	101.0
2.04	2.07	101.5
4.08	4.05	99.3
4.08	4.05	99.3
8.16	8.14	99.8
8.16	8.18	100.2
12.2	12.2	100.0
12.2	12.2	100.0
16.3	16.3	100.0
16.3	16.3	100.0
20.4	20.3	99.5
20.4	20.3	99.5

Table 2

Analysis of Acetylmethylcarbinol Solutions by Dichromate Oxidation

Acetylmethylcarbinol added, mg.	Acetylmethylcarbinol by analysis*, mg.	Recovery, per cent
1.01	1.38	137
1.01	1.49	148
2.02	2.44	121
2.02	2.34	116
3.03	3.30	109
3.03	3.30	109
4.04	4.15	103
4.04	4.15	103
5.05	5.10	101
5.05	5.10	101
6.06	6.06	100
6.06	6.06	100
7.07	7.03	99.5
7.07	7.03	99.5
8.08	8.15	101
8.08	8.05	99.6
9.09	9.05	99.7
9.09	9.05	99.7

*Calculated on the basis of oxidation of the acetylmethylcarbinol to diacetyl.

procedure. It is evident from the results given in table 2 that in samples containing over 5 mg. of acetylmethylcarbinol, the carbinol is quantitatively oxidized to diacetyl; whereas for lower quantities, the oxidation proceeds farther.

Since the effective concentration of dichromate toward acetylmethylcarbinol would be reduced if a small amount of acetylmethylcarbinol were present with a relatively large amount of ethanol, a series of solutions containing various amounts of ethanol and acetylmethylcarbinol were prepared and subjected to the oxidation procedure. The results of the analysis of these solutions, shown in table 3, indicate that in the presence of ethanol, acetylmethylcarbinol is essentially quantitatively converted to diacetyl. The correction for acetylmethylcarbinol present in distillates from fermentation may thus be applied on a quantitative basis.

In carrying out the determinations, blank analyses were conducted with each series of analyses. The amount of sodium thiosulfate required for each sample was subtracted from that required for the blank, and the titer value thus obtained was converted to its equivalent of 0.4000 normal potassium dichromate. Using a dichromate solution containing 19.612 grams of potassium dichromate per liter of solution,

Table 3

Analysis of Ethanol-Acetylmethylcarbinol Mixtures

by Dichromate Oxidation

Ethanol added, mg.	Acetylmethylcarbinol added, mg.	Ethanol recovered, mg.	Ethanol recovered, per cent
4.08	1.01	3.98	97.5
4.08	1.01	4.08	100.0
4.08	2.02	4.04	99.1
4.08	2.02	4.04	99.1
4.08	5.05	4.05	99.1
4.08	5.05	4.01	98.1
20.4	1.01	20.2	99.0
20.4	1.01	20.2	99.0
20.4	2.02	20.2	99.0
20.4	2.02	20.2	99.0
20.4	5.05	20.0	98.0
20.4	5.05	20.0	98.0

1.00 ml. of the dichromate solution is equivalent to 4.61 mg. of ethanol or 8.80 mg. of acetylmethylcarbinol. One milligram of acetylmethylcarbinol is therefore equivalent to 0.113 ml. of 0.4000 normal potassium dichromate, or 0.529 mg. of ethanol.

4. Estimation of acetylmethylcarbinol

During the course of the study on the interference of acetylmethylcarbinol with the determination of ethanol, it was found that the amounts of acetylmethylcarbinol appearing in the same fractions of the distillate for solutions of identical concentrations were surprisingly constant. In order to determine if this phenomenon could be applied to the determination of acetylmethylcarbinol in fermentation liquors, a solution of acetylmethylcarbinol was prepared, and appropriate quantities of this solution diluted to 300 ml. The solutions were then distilled under the same conditions as employed for the determination of ethanol, and successive 100-ml. fractions of distillate were collected from each solution. Aliquot portions of the distillates were analyzed for acetylmethylcarbinol content by the method of Underkofler et al (1943). The reagents were standardized against purified acetylmethylcarbinol. Results of these analyses, shown in table 4, indicate that an average of 44.5 per cent of the acetylmethylcarbinol present in the solution was contained in the first 100 ml. of distillate. The fraction present in the second 100 ml. of distillate was also constant. It should be noted that for the solution containing 0.167 grams of acetylmethylcarbinol per 100 ml., a slight decrease occurred in the percentage of acetylmethylcarbinol recovered.

Table 4

Effect of Acetylmethylcarbinol Concentration on the Amount of Acetylmethylcarbinol Distilled

Acetylmethylcarbinol added, mg.	Acetylmethylcarbinol in distillates, mg.		Recovery of acetylmethylcarbinol, per cent	
	1st 100 ml.	2nd 100 ml.	1st 100 ml.	2nd 100 ml.
50	22.4	18.9	44.8	37.8
50	23.4	18.9	46.8	37.8
100	44.8	36.4	44.8	36.4
100	44.8	36.4	44.8	36.4
200	87.6	71.5	43.8	35.8
200	89.4	71.9	44.7	36.0
300	134	109	44.7	36.3
300	131	107	43.7	35.7
400	180	144	45.0	36.0
400	177	147	44.7	36.7
500	214	178	42.5	35.6
500	216	178	43.2	35.6

However, Blom and Efron (1945) have shown that the equilibrium relation between the liquid and vapor composition for acetylmethylcarbinol-water solutions is linear for concentrations of acetylmethylcarbinol up to one per cent. Therefore, the above method should be applicable to solutions containing up to one per cent of acetylmethylcarbinol.

In the analysis of fermentation liquors, the acetylmethylcarbinol was determined in the distillate from the ethanol determination, thus affording at the same time an accurate estimate of the amount of acetylmethylcarbinol present in the samples taken for the determination of ethanol.

Langlykke and Peterson (1937) have previously applied a method similar to the method discussed above to the determination of acetylmethylcarbinol in fermentation liquors and have shown that the presence of other materials in solution has little effect on the fraction of acetylmethylcarbinol appearing in the distillate.

5. Determination of pH

The pH values of fermentation liquors were determined with a Cameron glass electrode apparatus. The meter was adjusted against a potassium acid phthalate buffer, pH 4.10. Determinations of pH were carried out at room temperature.

E. Experimental Results

1. Study of the factors affecting the rate of utilization of dextrose by Aerobacter aerogenes

The time required for completion of fermentation becomes increasingly important as the scale of production increases. On an industrial scale the man-hours of work required and the equipment necessary for the production of a given amount of substance depend to a great extent upon the rate of fermentation. Since it was desired to produce the 2,3-butanediol on a large laboratory scale and at the same time to develop some information concerning the feasibility of industrial production, it was considered advisable to make a preliminary study of the factors affecting the rate of fermentation of dextrose by Aerobacter aerogenes.

a. Effect of addition of calcium carbonate. Kendall (1934a) obtained up to 48 per cent by weight (on the basis of the sugar added) conversion of sucrose to 2,3-butanediol. Kendall conducted the fermentations in a synthetic medium at 37°C. and adjusted the pH daily to 6.10. The fermentation required about 14 days for completion. Kluyver and Scheffer (1933) claimed 30 to 35 per cent by weight conversion to 2,3-butanediol of the sugars present in molasses. In this case the medium was aerated continuously, and calcium carbonate was added to maintain the desired pH. The fermenta-

tion period was from 33 to 39 hours. In view of these results, it was desired to determine the effect of the addition of calcium carbonate on the rate of dextrose fermentation by Aerobacter aerogenes. The conditions of the experiment are given below:

Organism: Aerobacter aerogenes, I.S.C. B16

Medium:

NH_4CL	0.25 grams
$MgSO_4$ (anh.)	0.20 grams
K_2HPO_4	0.15 grams
$CaCl_2$	0.015 gram
Dextrose	10.0 grams
$CaCO_3$	as shown in table 5

Distilled water to 100 ml.

Inoculum:

Five ml. of a 48-hour culture of Aerobacter aerogenes per 100 ml. of medium, grown at 37°C. on the above medium containing 20 grams of calcium carbonate per liter.

Scale of fermentation:

100 ml. of medium in 300-ml. Erlenmeyer flasks.

Sterilization:

Constitutents for each fermentation sterilized together for 30 minutes at 15 pounds steam pressure.

Incubation:

At 37°C., with shaking several times daily.

The results, given in table 5, indicate clearly that the addition of calcium carbonate definitely increased the rate of utilization of dextrose. It should be noted that

no attempt was made to adjust the pH of the control fermentations; it should therefore not be assumed that the addition of calcium carbonate is a more effective means of pH control than the periodic addition of alkali. However, the addition of calcium carbonate prior to inoculation is a more convenient means of pH control and lessens the danger of contamination which might be introduced by periodic sampling and addition of alkali.

Table 5

The Effect of Addition of Calcium Carbonate on the Rate of Utilization of Dextrose by Aerobacter aerogenes B16

Age of fermentation, hours	Utilization of dextrose, per cent	
	$CaCO_3$ added, none	$CaCO_3$ added, 2.0 grams per 100 ml.
0	--	--
52	5	69
125	11	79
200	20	84

b. Effect of aeration and nitrogen agitation. Since Kluyver and Scheffer (1933) claimed complete fermentation of molasses by Aerobacter aerogenes in 33 to 39 hours and ascribed the decrease in time required for completion of the fermentation to the effects of aeration, an effort was made to duplicate these results using conditions similar to those given

in the preceding experiment. The media used were of the same composition as the medium used in the preceding experiment containing 2.0 grams of calcium carbonate per 100 ml. The organism and the ratio of inoculum to medium volume were the same. However, tap water was used instead of distilled water. The fermentations were conducted on a larger scale than in the previous experiment in order that the 2,3-butanediol formed could be recovered. Three 4-gallon fermentations were included in the series: number 1 was aerated with finely dispersed air; number 2 was agitated with dispersed nitrogen, and number 3 was neither aerated nor agitated for the first 44 hours. At the end of 44 hours, the conversion of dextrose was so slight in fermentation number 3 that aeration was started in this fermentation also. The results of the experiment, as expressed in table 6 and figure 1, show clearly that aeration is very effective in increasing the rate of utilization of dextrose. The fact that the nitrogen agitation also increased the rate of fermentation indicates that at least a portion of the effects of aeration may be due to the agitation and/or sweeping out of the carbon dioxide formed.

Although the fermentations were not analyzed for 2,3-butanediol content, the recovery yield (obtained by concentrating the fermented liquors under vacuum, extracting the butanediol with diethyl ether, and fractionally distilling

the resulting extract) from fermentation number 1 was 27 per cent by weight of dextrose added. Yields from fermentations 2 and 3 were somewhat less.

Table 6

Effect of Aeration and Nitrogen Agitation upon the Rate of Dextrose Utilization by *Aerobacter aerogenes* B16

Age of fermentation, hours	Utilization of dextrose, per cent		
	1 Aeration*	2 Nitrogen agitation*	3 Aeration* after 44 hours
18	33.5	8.8	2.7
24	51.5	19.6	3.3
37	82.8	33.5	4.5
44	95.0**	39.0	5.0***
72	95.0	85.1	71.2

* 50 ml. per liter per minute.
** Aeration stopped at 44 hours.
*** Aeration started at 44 hours.

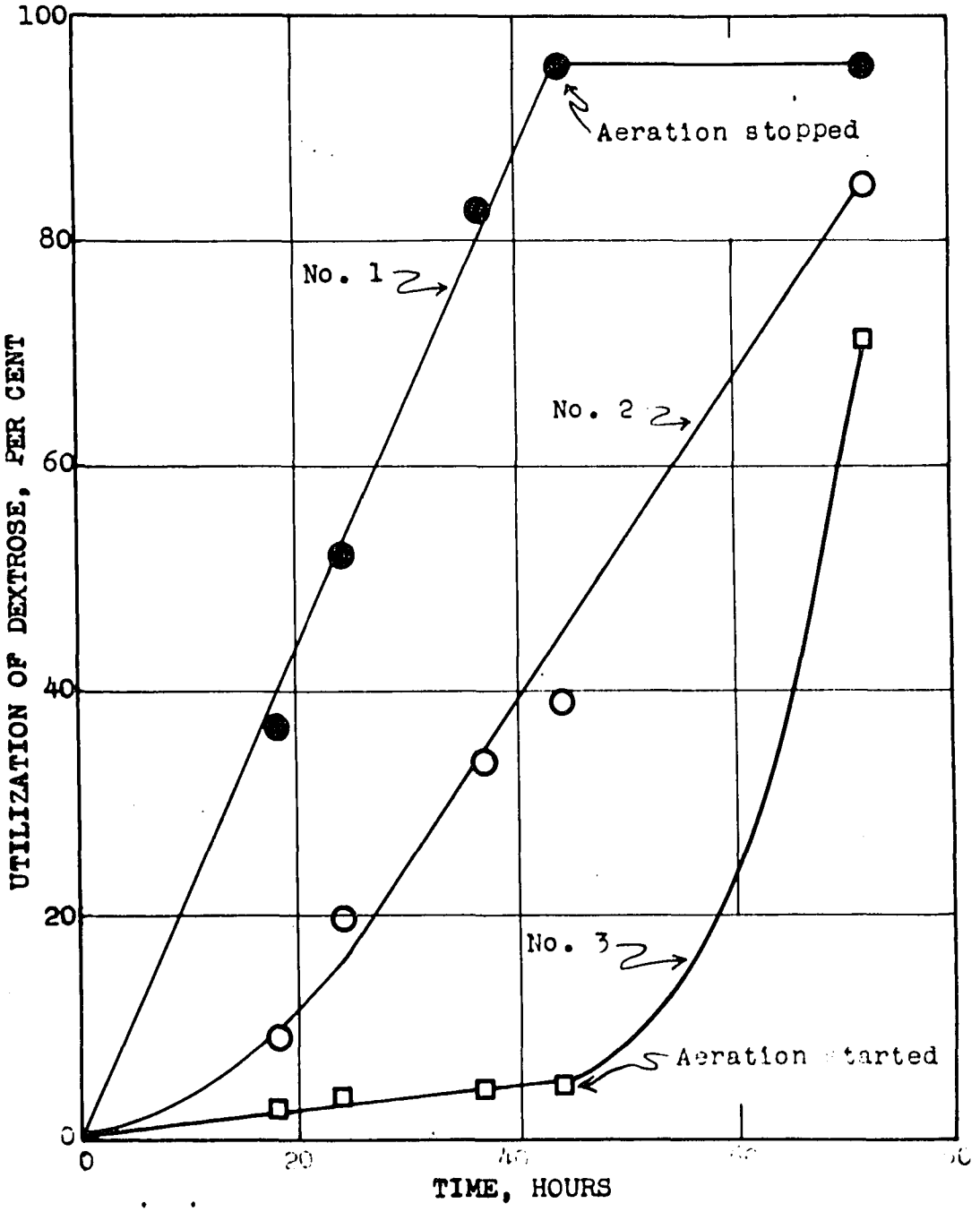


Fig. 1. Effect of aeration and nitrogen agitation on the rate of dextrose utilization by Aerobacter aerogenes.

2. Laboratory preparation of 2,3-butanediol from dextrose by fermentation with Aerobacter aerogenes

a. Sterilization apparatus. Sterilization of large volumes of dextrose media in an autoclave usually causes excessive caramelization of the carbohydrate due to the long period of heating necessary to bring the solution to the sterilization temperature and also due to the long period required to cool the solution below its boiling point. Inasmuch as it was desired to conduct the fermentations in 5-gallon bottles, the need for apparatus to provide rapid sterilization and cooling of large volumnes of fermentation media was evident. The apparatus shown in figure 2 was constructed from discarded equipment but was found adequate for the intended purpose. Several improvements could obviously be made if the equipment were to be fabricated rather than adapted.

Sterilization of media was carried out by dissolving the dextrose and soluble salts in 9 liters of water, placing the solution in the tank, and sparging with live steam until the solution boiled and the air was driven from the tank. The tank outlet valve was then closed and the steam pressure maintained at 15 pounds for 30 minutes. The steam was then shut off, and the pressure was allowed to fall to atmospheric. The pipe and tube below the drain valve were sterilized

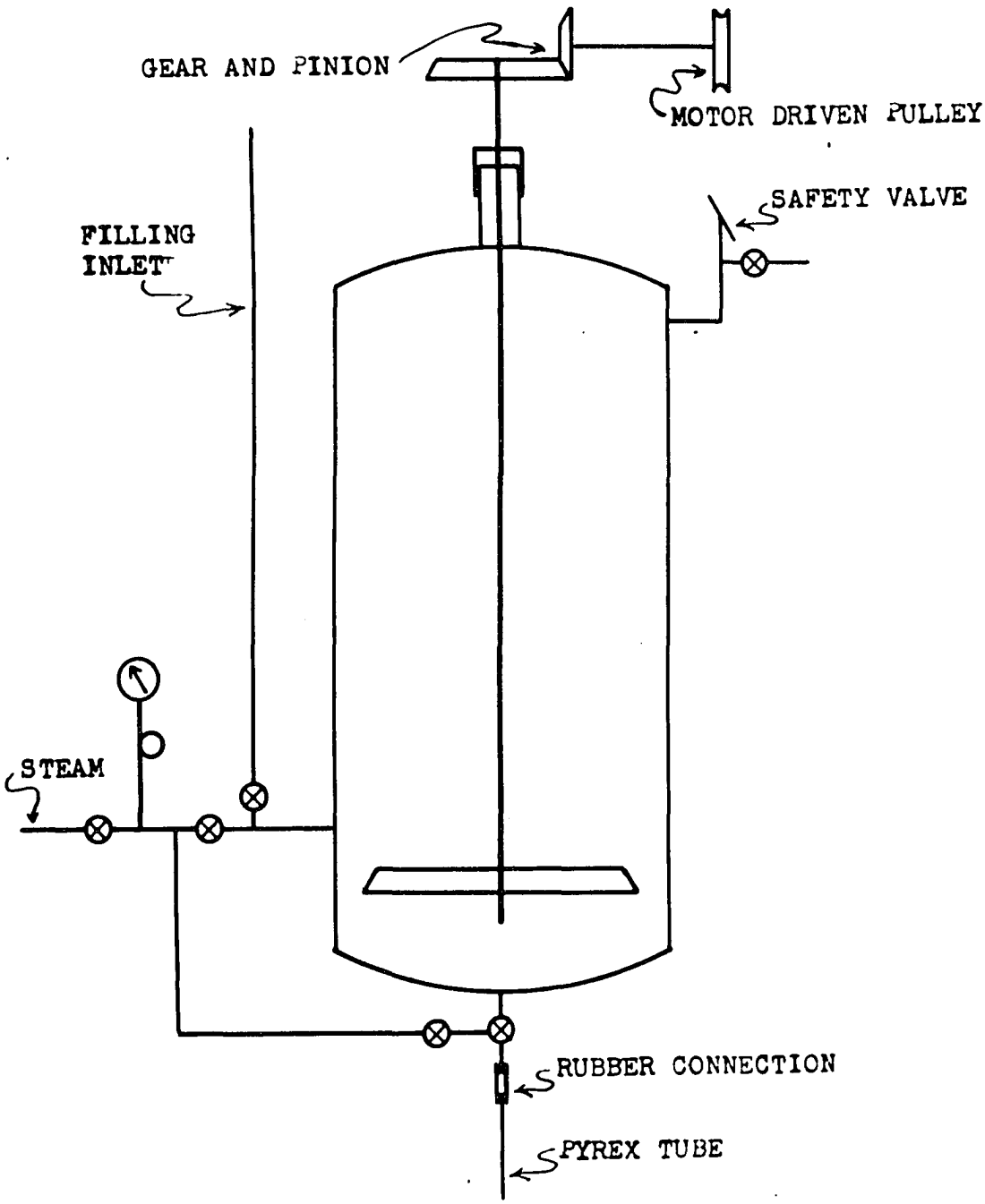


Fig. 2. Sterilization apparatus.

by flowing steam during this cooling period, and the solution was drained into previously sterilized 5-gallon bottles. The amount of condensate obtained brought the liquid volume to between 15 and 16 liters. The liquid was stirred continuously while in the tank.

b. Fermentation apparatus. The fermentations were carried out in 5-gallon bottles, each containing 15 to 16 liters of medium. All bottles were equipped with Aloxite air dispersers, thermometers, and gas outlet tubes, as shown in figure 3. The temperature of the room in which the fermentations were carried out was controlled to within 2°C. of the temperature specified. The air used in aerating the fermentations was taken from the compressed air line and was sterilized by passing through a sterilized cotton filter.

c. Results of fermentations. During the course of the work, a number of 15-liter fermentations were carried out. The medium used during the first portion of the work was identical with that employed in the preceding 4-gallon fermentations. The calcium carbonate was sterilized separately and added after sterilization of the medium. All fermentations were conducted at a room temperature of 37°C., and were aerated at the rate of 50 to 100 ml. per liter per minute. The averages of the results of five fermentations thus conducted are shown in table 7.

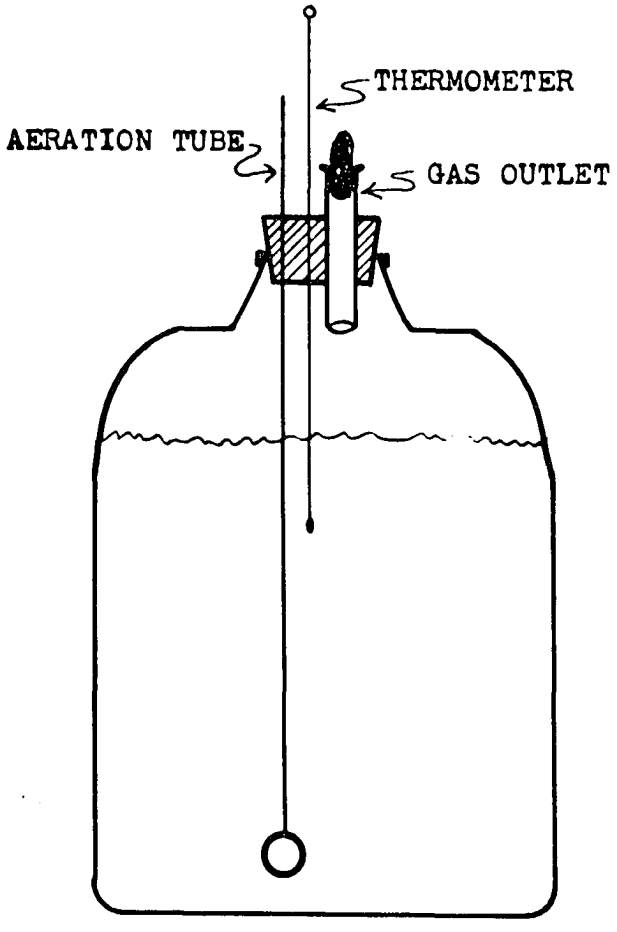


Fig. 3. Fermentation apparatus.

Table 7

Production of 2,3-Butanediol by Fermentation of Dextrose by *Aerobacter aerogenes* B16

	Average	Range
Initial dextrose, grams per 100 ml.	10.0	--
Utilization of dextrose, per cent	93	89 to 97
Yield of 2,3-butanediol, grams per 100 ml.	3.37	3.06 to 3.53
per cent by weight*	31.8	29.6 to 34.6
Time for completion, hours	52	34 to 109

* Based on total dextrose added, corrected for dextrose added in inoculum and for volume change.

The results obtained indicate that the fermentation can readily be conducted on a large laboratory scale. The yield of 2,3-butanediol was less than that reported by Kendall (1934a), but the decrease in fermentation time partially offsets this difference for purposes of production.

During the latter stages of the investigation, a medium recommended in a report issued by the Northern Regional Research Laboratory was used in the preparation of the 2,3-butanediol. The conditions of the experiment were as given below:

Organism: Aerobacter aerogenes B16

Medium:

Dextrose	150 grams
$MgSO_4 \cdot 7H_2O$	0.25 gram
KH_2PO_4	1.80 grams
Urea	2.0 grams
$CaCO_3$	5.0 grams
Tap water to 1000 ml.	

Inoculum:

Dextrose	75 grams
Concentrated corn steep liquor	5.0 ml.
$CaCO_3$	5.0 grams
Distilled water to 1000 ml.	

The organism was transferred from a stock slant to 10 ml. of the above medium contained in a 50-ml. Erlenmeyer flask and incubated at 30°C. for 48 hours. This culture was transferred to 100 ml. of the same medium contained in a 300-ml. Erlenmeyer flask, which in turn was used to inoculate 1000 ml. of the same medium contained in a 2-liter Erlenmeyer flask. After 48 hours of incubation at 30°C., 1000 ml. of this culture was used to inoculate each of the 16-liter fermentations.

Scale of fermentations: 15 liters of medium contained in 5-gallon pyrex bottles.

Sterilization: The urea was sterilized separately as a 20 per cent solution. The fermentation bottles, containing the required amount of calcium carbonate, were sterilized in an autoclave. The media were sterilized in the sterilization tank previously described. After the sterile solution had been placed in the bottles, the urea was added and the pH of the media adjusted to 6.1 to 6.2.

Incubation: At a room temperature of 30 to 32°C.

Aeration: Air was passed through the Aloxite dispersers at the rate of 50 to 100 ml. per liter per minute. Aeration was continued until the utilization of dextrose ceased, after which the fermentations were

allowed to stand for 6 to 12 hours without aeration before final analysis.

The average of results of three such fermentations are shown in table 8.

Table 8

Production of 2,3-Butanediol by Fermentation of Dextrose by Aerobacter aerogenes B16

	Average	Range
Initial dextrose, grams per 100 ml.	15.0	--
Utilization of dextrose, per cent	77	76 to 78
Yields of 2,3-butanediol, grams per 100 ml.	4.06	4.04 to 4.08
per cent by weight*	25.7	25.4 to 25.9
Time for completion, hours	84	66 to 150

*Based on dextrose added.

A number of fermentations were also conducted using a strain of Aerobacter aerogenes obtained from the culture collection of the Northern Regional Research Laboratory. The organism is designated in the culture collection of the Biophysical Chemistry department of Iowa State College as B24 and was obtained as NRRL B 199. The medium and condi-

tions under which the fermentations were conducted were identical with those of the preceding experiment. The results of two of these fermentations conducted at an initial dextrose concentration of 10 per cent are shown in table 9, and the results of four fermentations conducted at an initial dextrose concentration of 16 per cent are shown in table 10.

Table 9

Production of 2,3-Butanediol by Fermentation of Ten Per Cent Dextrose Medium by Aerobacter aerogenes B54

	Average	Range
Initial dextrose, grams per 100 ml.	10.0	---
Utilization of dextrose, per cent	85	84 to 85
Yield of 2,3-butanediol, grams per 100 ml.	3.16	3.10 to 3.22
per cent by weight*	29.0	28.0 to 30.0
Time for completion, hours	45	40 to 50

*Based on dextrose added.

Table 10

Production of 2,3-Butanediol by Fermentation of Sixteen Per Cent Dextrose Medium by Aerobacter aerogenes B24

	Average	Range
Initial dextrose, grams per 100 ml.	16.0	---
Utilization of dextrose, per cent	92	85 to 93
Yield of 2,3-butanediol, grams per 100 ml.	6.30	6.08 to 6.60
per cent by weight*	35.4	34.2 to 36.5
Time for completion, hours	93	93

*Based on dextrose added.

F. Conclusions

Aeration and the addition of calcium carbonate to the fermentation medium substantially decrease the time required for completion of the fermentation of dextrose by Aerobacter aerogenes. Under the conditions employed, over 30 per cent by weight of the dextrose added has been converted to 2,3-butanediol in as little as 34 hours. These results, obtained in fermentations conducted on a large laboratory scale, indicate that the fermentative production of 2,3-butanediol from dextrose could be feasibly conducted on an industrial scale.

IV. PRODUCTION OF 2,3-BUTANEDIOL FROM CORN BY FERMENTATION WITH AEROBACTER AEROGENES

A. Introduction

This phase of the work was undertaken in cooperation with the Doane Agricultural Service of St. Louis, Missouri. The semi-pilot plant and plant fermentations were carried out at the plant of the Columbia Brewing Company, St. Louis, Missouri.

The purpose of this investigation was to determine whether brewery equipment and techniques could be adapted to the production of 2,3-butanediol by Aerobacter aerogenes. In the manufacture of beer, saccharification of starch-containing materials is followed by filtration of the saccharified mash. The filtrate, called beer wort, is then subjected to fermentation by yeast. The problem thus resolved itself into studies on the production of 2,3-butanediol by the fermentation of wort prepared by the saccharification and filtration of corn mash.

The saccharification process, as carried out in the

preparation of beer, involves two steps: pre-thinning and the main saccharification. The starch-containing material (corn in the present experiments) and the malt for pre-thinning are ground and mixed with water at 70°C. After two hours at this temperature, the temperature is raised to 105°C. (4 pounds per square inch), and the mixture is boiled for one and one-half hours. The saccharification is carried out by suspending ground malt in cold water (28°C.) and adding the hot corn mash from the cooker. The mash is then agitated at 65°C. until the iodine test for starch shows only a faint pink color. The mash is then pumped to a Lauter tub, which is a copper tub with a false bottom composed of a slit screen. When the agitation is stopped, the malt and corn hulls settle and form a filter bed. When the tub is tapped, the filtrate is returned to the top of the bed until a sparkling clear filtrate is obtained. The filtrate is then run into a copper boiling kettle and concentrated to the desired sugar content.

The first Aerobacter aerogenes fermentation conducted was carried out in the plant. There was no opportunity to precede the plant fermentation with laboratory investigations on fermentation of filtered wort, but since the first important factor to be determined was whether the brewery equipment could be adapted to the handling of the corn mash and filtration of the wort, the plant experiment gave the necessary information. The mash filtered readily through

the Lauter tub, and a sparkling clear, yellowish filtrate was obtained.

The fermentation, however, was unsuccessful. The pH of the fermentation liquor dropped rapidly from 6.0 to 4.1, and utilization of maltose ceased after 50 per cent of that present in the wort had been utilized. The yield of 2,3-butanediol was about 10 per cent by weight of the total maltose present.

The reasons for the unsuccessful fermentation could have been any number of variables which could not be controlled in the first trial. Most of these difficulties were mechanical such as inadequate control of air flow, limitations in apparatus for sterilization of the calcium carbonate and urea which were added to the medium, and inadequate size of inoculum tanks. While the necessary changes in equipment were being made, it was decided to conduct laboratory investigations on the preparation of 2,3-butanediol from filtered corn wort.

B. Experimental Results

1. Laboratory investigations

During the course of the laboratory experiments, a number of variables which could conceivably have an effect on the yield of 2,3-butanediol were studied. By far the great-

est number of these were found to have little or no effect on the results of the fermentations. Thus it was found that the addition of magnesium sulfate and potassium phosphate was not essential. The fermentations of worts containing 7 to 12 per cent maltose could be fermented efficiently but worts containing greater than 15 per cent maltose fermented slowly and resulted in decreased yields. Aeration was found essential for rapid fermentation; consistently good results were obtained using air flow rates of from 20 to 100 ml. per liter per minute. The addition of an excess of lard oil, added to prevent foaming, was not detrimental. The presence of ferric ions in concentrations of 10 to 25 parts per million had no effect on the results. The pH of the medium could be adjusted with either calcium hydroxide or ammonium hydroxide before or after sterilization with equally good results. A drop of the pH of the mash from 6.1 to 3.9 during saccharification was not detrimental to the fermentation. The presence of the cupric ion in a concentration of 5 parts per million likewise had no effect on the results.

The yields obtained in the initial laboratory experiments varied from 10 to 20 per cent of 2,3-butanediol by weight of maltose present in the filtered wort. By lowering the temperature at which the mash was held during saccharification to 58°C. and allowing the mash to stand for 12 hours during

saccharification, yields of 20 per cent were obtained rather consistently. Addition of manganous sulfate to the medium at times appeared to cause a further increase in the yield, but the results were not consistent.

Significant improvements in the yield of 2,3-butanediol were obtained, however, by combining the above improvements with the addition of malt or malt extract to the medium after active fermentation had commenced. The experiment conducted in determining the effect of the addition of malt extract is shown below.

To prepare the wort for the fermentation media, 2000 grams of corn and 120 grams of malt were added to 9 liters of tap water, and the mixture was heated in a water bath at 68°C. for two hours. The mash was then heated to boiling by direct steaming, cooked in the autoclave for two hours at 5 to 6 pounds steam pressure, and allowed to cool overnight. After cooling, 1357 grams of malt were added, and the mash was placed in a water bath held at 56 to 58°C. for 6 hours and allowed to cool overnight. The mash was then filtered through a fruit press, and the cake was washed with two liters of water. The combined filtrates were then refiltered through muslin and then through heavy filter paper. A clear filtrate was thus produced. The filtrate so obtained was diluted to the desired maltose concentration.

To the filtered wort, the following constituents were added:

Urea	2.0 grams per liter
$MnSO_4$	0.05 grams per liter
$CaCO_3$	5.0 grams per liter

The urea was sterilized separately as a 20 per cent solution and the calcium carbonate as a 20 per cent slurry. The manganous sulfate was dissolved in the wort and 1000 ml. of the resultant solution placed in each 2-liter Erlenmeyer flask. The flasks were fitted with Aloxite air dispersers, and the media were then sterilized for 45 minutes at 5 pounds per square inch. After cooling, the urea solutions and the calcium carbonate slurries were added, and the media were adjusted to pH of 6.1 to 6.2 with ammonium hydroxide.

The media were then inoculated with 50 to 60 ml. of a 48-hour culture of Aerobacter aerogenes B24, grown at 30°C. on an unaerated medium of the following composition:

Dextrose	75 grams
Concd. corn steep liquor	5 ml.
$CaCO_3$	5 grams
Distilled water	1000 ml.

Air, filtered through sterile cotton, was passed through the air dispersers at a rate of 40 to 50 ml. per liter per minute. Incubation was at 30°C.

After 24 hours of fermentation, 25 ml. of malt extract, prepared by stirring 50 grams of distillers malt with 500 ml. of tap water for 30 minutes and filtering the resulting sus-

pension, was added to each fermentation. When maltose utilization ceased, the air was shut off and the fermentation allowed to stand without aeration for an additional 12-hour period, at which time final analysis was conducted.

The results of the fermentations are shown in table 11. These results clearly indicate that not only was utilization more complete when malt extract was added, but conversion of the utilized maltose to 2,3-butanediol was more efficient when the extract was added.

The more complete utilization of maltose when malt extract was added may have been due in part to the conversion of partially saccharified dextrins to maltose by the active malt diastase. The probability of this occurrence is substantiated by the fact that some fermentations analyzed a few hours after the addition of malt extract showed an actual increase in maltose concentration greater than that due to the maltose present in the malt extract added. It should be noted that if the above supposition is correct, an error is introduced in the expression of the per cent of theory yield of 2,3-butanediol, since the calculations have been made on the basis of initial and final maltose concentrations with a correction for the maltose equivalent of the malt extract added. The weight per cent yield is also subject to this error, but the weight per cent of 2,3-butanediol based on the total starch present in the corn and malt would be about 90

Table 11

Effect of the Addition of Malt Extract on the Fermentation of Filtered Corn Wort with Aerobacter aerogenes B24

Malt extract added, ml. per liter	None	25
Initial maltose concentration, grams per 100 ml.	11.9	11.7
Utilization of maltose, per cent	55	85
Yield of 2,3-butanediol, grams per 100 ml.	1.82	3.88
per cent by weight*	16	33
per cent by theory**	56	78
Time for completion, hours	100	90

* Based on maltose added, as maltose hydrate
** Based on maltose utilized

per cent of the weight per cent yield as expressed above, since about 90 per cent of the starch present in the corn and malt were consistently recovered as maltose in the filtrate and washings after saccharification. It will be noted that the amount of malt added for saccharification was much greater than that ordinarily used in the saccharification of corn mash for alcohol production. This amount of malt is commonly added in saccharification of starch for the preparation of beer, and

since the filter bed in the Lauter tub is formed mainly by malt husks, it was decided to use this amount for experimental investigations.

The results of fermentations conducted at three different maltose concentrations are shown in table 12. The fermentations were conducted in the same manner as the fermentations in the preceding experiment to which the malt extract was added. Yields obtained by this procedure were at times as high as 38 per cent by weight of maltose present, but these results were not obtained consistently. The results given in table 12 represent more fairly those which may be consistently obtained.

Table 12

Fermentation of Filtered Corn Wort by Aerobacter aerogenes B24 at Various Maltose Concentrations

Initial maltose concentration, grams maltose hydrate per 100 ml.	11.7	9.4	7.6
Utilization of maltose, per cent	85	82	85
Yield of 2,3-butanediol, grams per 100 ml.	3.88	3.16	2.34
per cent by weight*	33	34	32
per cent of theory**	78	82	72
Time for completion, hours	90	47	47

* Based on total maltose present, as maltose hydrate
** Based on maltose utilized

2. Semi-pilot plant fermentation

Subsequent to the completion of a number of successful laboratory fermentations, the fermentation of filtered corn wort was conducted on a semi-pilot plant scale under conditions similar to those used in the laboratory experiments.

The development of the inoculum in order to build up the volume of the culture was carried out on media of the same composition as the inoculum for the preceding experiment, transferring every 24 hours to 10 ml., 100 ml., and 1000 ml., respectively. The final inoculum contained the following constituents:

Beer wort	607 ml.
$MnSO_4 \cdot 4H_2O$	0.34 gram
$CaCO_3$	5.0 grams
Urea	2.0 grams
Tap water to	1000 ml.

Sufficient wort and water were mixed in a 5-gallon pyrex bottle to give a total volume of 15 liters. The manganous sulfate was then added, the bottle equipped with an aerator stone as shown in figure 3, and the solution sterilized for 30 minutes at 15 pounds per square inch. The calcium carbonate was sterilized dry and the urea in a 20 per cent solution. After cooling, the urea and calcium carbonate were added. The resultant pH of the solution was 5.9, and the maltose concentration was 8.2 grams per 100 ml. The medium was inoculated with 1000 ml. of a 28-hour culture of Aero-

bacter aerogenes B24 and incubated at 30 to 31°C. A slow stream of sterile air was passed through the aerator stone.

In order to prepare the corn wort for the fermentation medium, 15 gallons of tap water were heated to 60°C. in the mash tub shown diagrammatically in figure 4. Seventy pounds of ground corn and 10 pounds of ground brewers malt were then added, and the mash stirred manually with a wooden paddle. The mash was then heated to 100°C. and boiled for two and a half hours. After cooling to 69°C., sufficient water was added to bring the temperature to 64°C., and 40 pounds of ground brewers malt were added. The mash was stirred manually for 30 minutes and then allowed to stand for 12 hours, during which time the temperature decreased to 56°C. The liquor was then separated from the insoluble solids by pressing the mash through a fruit press. No attempt was made to make a quantitative recovery of the maltose, since only sufficient wort for a 45-gallon fermentation was needed.

The final fermentation medium was composed of the following constituents:

Maltose (anh.) (in filtered wort)	91.5 pounds
$MnSO_4 \cdot 4H_2O$	0.28 pound
$CaCO_3$	4.2 pounds
Urea	1.7 pounds
Tap water to	100 gallons

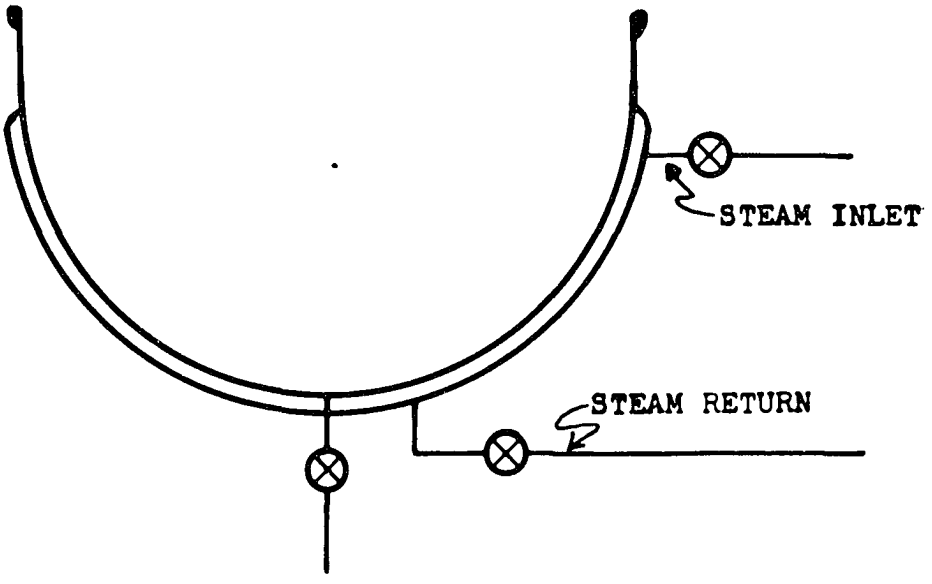


Fig. 4. Laboratory mash tub.

The filtered wort was placed in the 60-gallon yeast culture tank shown diagrammatically in figure 5, and sufficient tap water was added to bring the maltose concentration to 91 pounds per 100 gallons. The total volume at this point was 43 gallons. To this solution, 0.15 pound of manganous sulfate (tetrahydrate) was added, and the liquid was heated by direct steam to 212°F. and then sterilized for 60 minutes at 10 pounds. Amounts of 0.86 pound of urea and 2.15 pounds of calcium carbonate were sterilized separately, the urea in a 20 per cent solution and the calcium carbonate in a 30 per cent slurry; both were added to the fermentation mash after it had cooled to 88°F. The resultant pH was 6.0.

The medium was then inoculated with a 3-gallon culture of Aerobacter aerogenes cultured on the inoculum indicated above. The age of the inoculating culture was 25 hours. Analysis of the wort after inoculation showed 42.6 pounds of anhydrous maltose per 100 gallons. Air was passed through a steam jacket and a cotton filter and introduced into the fermentation tank through the Lamsen stone in the bottom of the tank. The aeration rate was controlled at 0.40 cubic foot per gallon per hour for the first 12 hours of fermentation and at 0.16 cubic foot per gallon per hour for the next 24 hours. Two pounds of ground distillers malt was added to the fermentation 16 hours after inoculation. The temperature of the medium was maintained at 89 to 92°F. by running cold

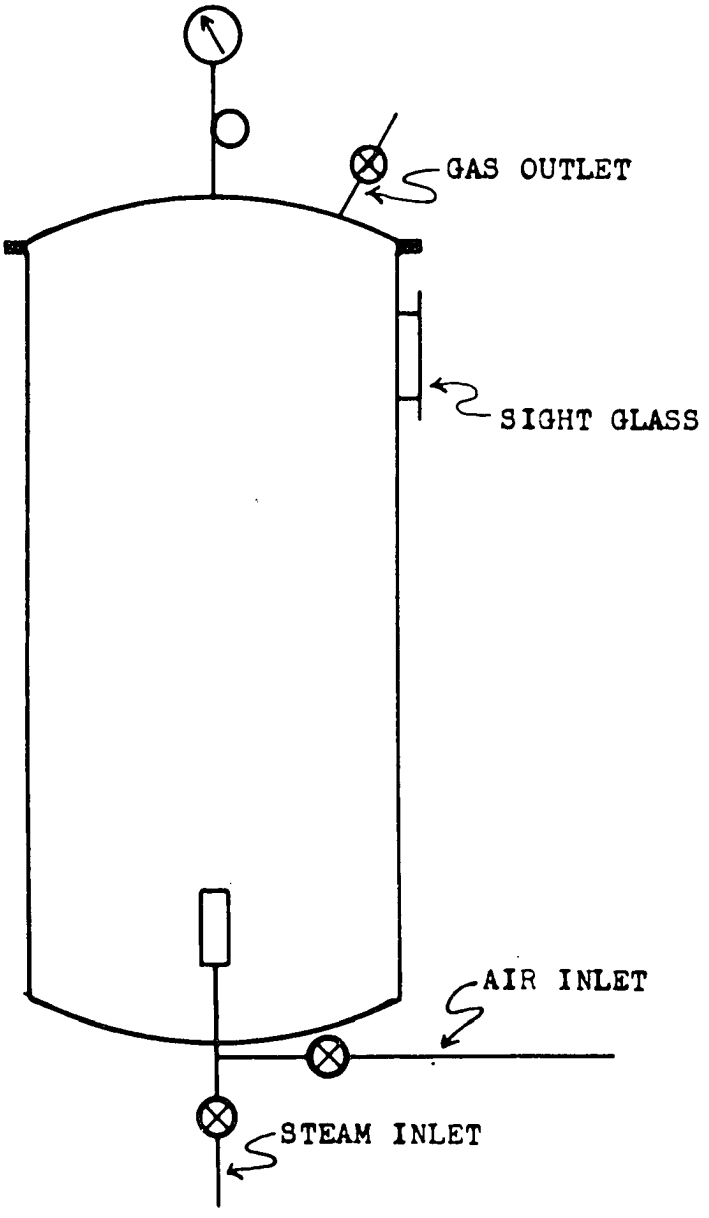


Fig. 5. Semi-pilot plant fermentation tank.

water over the tank when the temperature rose. At 36 hours the air was shut off; final analysis was made 42 hours after inoculation.

The course of the fermentation with respect to pH, maltose utilization, and production of 2,3-butanediol is shown in table 13. Acetylmethylcarbinol was determined at 42 hours and was found to be 0.17 pound per 100 gallons. Maltose concentrations have not been corrected for interference due to the acetylmethylcarbinol present. The course of the fermentation with respect to the maltose utilization and production of 2,3-butanediol is shown graphically in figure 6. A summary of the results of the fermentation is shown in table 14. It will be noted that the yield compares favorably with those obtained in laboratory fermentations.

It was originally planned to conduct additional plant fermentations, but interest in the production of butadiene from the diol abated with the development of the ethanol and petroleum processes. In addition, a critical shortage of corn developed, and as a result this phase of the research was discontinued.

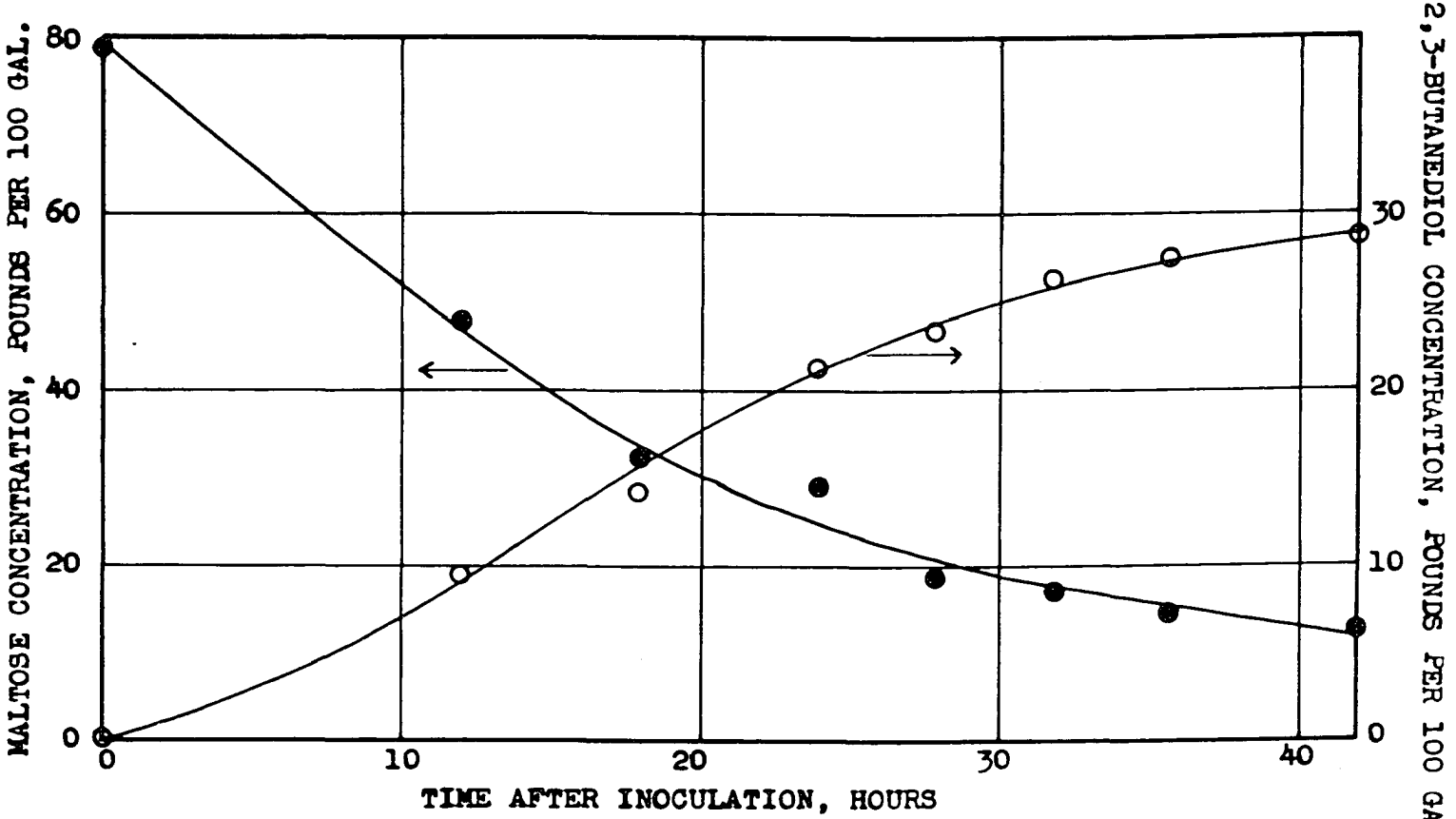


Fig. 6. Utilization of maltose and production of 2,3-butanediol during fermentation of filtered corn wort with Aerobacter aerogenes B24.

Table 13

Semi-pilot Plant Fermentation of Filtered Corn Wort by *Aerobacter aerogenes* B24

Time after inoculation, hours	pH	Maltose concentration, pounds per 100 gallons	2,3-Butanediol concentration, pounds per 100 gallons
0	6.0	78.4	0
12	5.9	46	9.5
16	5.2	(malt added)	
18	5.2	32	14.3
24	5.2	28	21.0
28	5.2	18	22.2
32	5.1	16	25.5
36	5.1	14	26.5
42	4.9	11.1	27.2

Table 14

Semi-pilot Plant Fermentation of Filtered Corn Wort by Aerobacter aerogenes B24

Initial maltose concentration, pounds maltose (anh.) per 100 gallons	78.4
Utilization of maltose, per cent	86
Yield of 2,3-butanediol pounds per 100 gallons	27.2
per cent by weight*	35.0
per cent of theory**	77.0
Time for completion, hours	42

* Based on initial maltose (anhydrous)
** Based on utilized maltose

C. Conclusions

Addition of malt extract to the fermenting medium substantially improves the yield of 2,3-butanediol obtained in fermentation of corn wort by Aerobacter aerogenes. Thirty-five per cent (by weight) of the maltose present in the wort has been converted to 2,3-butanediol in a fermentation period of 42 hours. These results, obtained on a semi-pilot plant scale, show a definite possibility of production of 2,3-butanediol by adaptation of brewery equipment and techniques to the fermentation process.

V. PRODUCTION OF 2,3-BUTANEDIOL FROM CORN BY FERMENTATION WITH AEROBACILLUS POLYMYXA

A. Introduction

The use of Aerobacillus polymyxa in the preparation of 2,3-butanediol from starchy materials has at least one distinct advantage over the use of Aerobacter aerogenes, namely, the ability of the organism to utilize starch directly without preliminary saccharification of the starch. This factor is important industrially, because the saccharification step would add considerably to the cost of production of the 2,3-butanediol. In addition, the 2,3-butanediol produced by Aerobacillus polymyxa is the levo-diol. Since the levo-diol does not form a hydrate such as is formed by the meso-isomer, the former is suitable for use as an anti-freeze agent.

The production of 2,3-butanediol by Aerobacillus polymyxa was first mentioned by Donker (1926). The group of patents issued to Verhave and his associates (1928a, 1929b, 1929c, 1933) and to Kluyver and Scheffer (1933) also mention the use of Aerobacillus polymyxa but not in connection with its starch-hydrolyzing ability. In a search for starch-hydrolyzing organisms capable of producing 2,3-butanediol, members of the research staff of the Northern Regional Research Laboratory

found that Aerobacillus polymyxa (NRRL-B510) possessed the ability to attack unsaccharified starch in grain mashes very rapidly, converting the starch to 2,3-butanediol, acetylmethylcarbinol, and ethanol. With respect to the nutrient requirements of this organism, the above workers found that the fermentation of corn or wheat mashes containing calcium carbonate could be conducted successfully. The addition of a supplementary nitrogen source was found to be unnecessary, as was also the addition of inorganic salts other than calcium carbonate.

Regarding operating conditions, aeration was found to be neither necessary nor desirable. Agitation was likewise unnecessary. The optimum temperature of fermentation was found to be 30°C. The fact that aeration is not essential gives rise to the possibility of recovery of the gaseous products, carbon dioxide and hydrogen, on an industrial scale.

Fermentations were conducted at the Northern Regional Research Laboratory on a pilot-plant scale. The yields obtained were as follows:

Substrate	Corn	Wheat
Yields, per cent by weight*		
2,3-Butanediol	29.0	27.0
Acetylmethylcarbinol	0.5	0.9
Ethanol	22.2	24.7

*Based on total starch present

The total yield, calculated as per cent of theory from starch utilized, was 10[illegible] per cent, suggesting that other components of the mash, such as pentosans and hemicelluloses, might be utilized by the organism. The time required for the fermentation of an 8 per cent mash was two to three days. The 2,3-butanediol recovered from the fermentation mashes was found to be the levo-form having a specific rotation at 25°C. of -13.0°, a viscosity about one-half that of the meso-form, and a density of 1.4305^{23} as compared with 1.4384^{23} for the diol producted by Aerobacter aerogenes.

The following investigations were carried out for the purpose of preparing and recovering levo-2,3-butanediol from corn mash fermented with Aerobacillus polymyxa and to determine some of the factors involved in the production of 2,3-butanediol by fermentation of corn mash with Aerobacillus polymyxa.

B. Materials

The corn used was Iowa No. 2 yellow corn and was ground in a Wiley mill with the medium screen in place. Analysis showed the following partial composition:

Starch	68.5 per cent
Moisture	11.2 per cent
Nitrogen	1.28 per cent
Ash	1.15 per cent

The malt used was distillers' malt and was ground in a Wiley mill just before use. Analysis showed the following partial composition:

Starch	57.3 per cent
Nitrogen	1.28 per cent

The calcium carbonate used was powdered U.S.P. grade.

The bacterial bran used was obtained from Dr. L. M. Christensen of the University of Nebraska.

C. Cultures

The organism used was Aerobacillus polymyxa I.S.C. B25. It was obtained from the culture collection of the Northern Regional Research Laboratory and was designated as NRRL B510. The stock cultures were stored on corn mash containing 5 per cent corn and 0.5 per cent calcium carbonate.

D. Methods of Analysis

1. Determination of starch

Starch in the raw materials was determined by direct acid hydrolysis according to the method of the Association of Official Agricultural Chemists (1940), followed by the determination of dextrose in the hydrolyzate by the method of Underkofler et al (1943).

2. Determination of nitrogen

Nitrogen in the raw materials was determined by the Kjeldahl method as described by Koltoff and Sandell (1938).

3. Determination of moisture

Moisture in the raw materials was determined by drying a sample of the material to constant weight at 105°C.

4. Determination of ash

Ash was determined in the raw materials by igniting a sample of the material to constant weight over a Meker burner.

5. Determination of residual carbohydrate

The residual carbohydrate in the fermentation mashes was determined by the same method as that used for the determination of starch in the raw materials. It was necessary to apply a correction for the acetylmethylcarbinol present in the hydrolyzates, since analysis of samples before and after hydrolysis showed that the acetylmethylcarbinol present was not affected by the acid treatment.

6. Determination of maltose, 2,3-butanediol, ethanol, and acetylmethylcarbinol

These substances were determined by the methods previously discussed. All 2,3-butanediol analyses have been corrected for maltose and acetylmethylcarbinol interference; all ethanol analyses have been corrected for acetylmethylcarbinol interference.

7. Preparation of samples for analysis

Inasmuch as the samples contained varying amounts of insoluble solids, the direct determination of constituents present in the filtrate obtained from such a mash, recalculated to the volume of the mash, would be subject to error due to the volume occupied by the solids present. In order to minimize the effect of the presence of solids, the fermentation mash was well agitated, and a sample of 200 ± 1 ml. withdrawn and this sample diluted to 500 ml. in a volumetric flask. When the original fermentation mashes consisted of 200 ml. of medium or less, the total mash was diluted to 500 ml. In this manner the error due to the volume occupied by the solids was reduced to not more than two-fifths of its former value.

In order to remove samples for the various analyses, the diluted mashes were well shaken, and samples of the agitated mash were removed for residual carbohydrate analysis and for the ethanol and acetylmethylcarbinol distillation procedures. The remainder of the diluted mash was allowed to

settle, and samples for the determination of 2,3-butanediol and of free maltose were taken from the supernatant liquid.

E. Experimental Results

1. Laboratory production of 2,3-butanediol

The initial phases of this portion of the investigation were concerned with the preparation and recovery of levo-2,3-butanediol for purposes of the study of the physical and chemical properties of the purified material by other members of the research group in Biophysical Chemistry. The fermentation procedures followed were those recommended by the Northern Regional Research Laboratory. A summary of the conditions used and the results obtained is shown below:

Medium:

Corn	100 grams
$CaCO_3$	5.0 grams
Tap water and steam condensate to	1000 ml.

Sufficient corn and calcium carbonate to make 15 liters of medium were placed in 5-gallon bottles. Eleven liters of tap water were then added and the starch gelatinized by introducing live steam. The mash was then diluted to 15 liters, the bottle plugged with cotton, and sterilized for 60 minutes at 15 pounds. After cooling to 30°C., the mash was inoculated with 1000 ml. of a 24-hour culture of Aerobacillus polymyxa, I.S.C. B25, grown on a medium containing 5 per cent corn and 0.5 per cent calcium carbonate. Analyses were conducted periodically for 2,3-butanediol; when no further increase occurred, the fermentation was discontinued. Average results of a number of fermentations conducted in this manner are shown in table 15.

Table 15

Production of 2,3-Butanediol by Fermentation of Corn Mash with Aerobacillus polymyxa B25

Yield of 2,3-butanediol, per cent by weight*	29.4
Yield of ethanol, per cent by weight*	17.8
Time of fermentation, days	4½

*Based on the starch added.

2. Fermentation of corn mash by Aerobacillus polymyxa

In order to plan the procedure to be followed in the investigations of the factors involved in the fermentation of corn starch by Aerobacillus polymyxa, the following experiments were conducted on corn mash to determine some of the relationships existing between the products of fermentation.

a. Effect of variation in the concentration of corn. This experiment was conducted in an effort to determine the highest concentration of mash which could be fermented efficiently.

The mashes were prepared as follows. The corn was placed in a 300-ml. Erlenmeyer flask. Calcium carbonate equal to 5 per cent of the weight of the corn (but not less than 0.5 grams per 100 ml.) was added. One hundred ml. of

distilled water was then added, and the mash was heated to boiling to gelatinize the starch. The flasks were then plugged with cotton and sterilized for 30 minutes at 15 pounds.

The inoculum for the experiment was prepared as follows: The organism was transferred from a stock culture to 10 ml. of a medium containing 5 per cent corn and 0.5 per cent calcium carbonate in a six by one-half inch test tube and incubated 48 hours at 30°C. One ml. of this culture was then transferred to 10 ml. of fresh medium, and transfers were made every 24 hours thereafter to fresh 10-ml. portions of the same medium. The final inoculum was made up of 200 ml. of the same medium contained in a 500-ml. flask, inoculated with 10 ml. of the culture and incubated for 24 hours at 30°C. The inoculum was the fourth transfer from the stock culture. Each 100 ml. of mash was inoculated with 8 ml. of the above inoculum. The fermentations were then incubated at 30°C. for 19 days. The long fermentation period was used in order that the more concentrated mashes might be fermented as completely as possible. From the results of the experiment, as expressed in table 16, several interesting observations can be made. Figures 7 and 8 show some of these data in graphical form. In figure 7 is shown the relationship between the yields of 2,3-butanediol and acetylmethylcarbinol, expressed as per cent by weight of the starch added. It is evident that an increase in the yield

Table 16

The Effect of Variation in Mash Concentration on the Fe

Corn added, grams per 100 ml.	2.5	5.0	7.5	10.0	12.5	15.0
Yield of products, grams per 100 ml.						
2,3-Butanediol	0.25	0.53	0.88	1.35	1.88	1.91
Acetylmethylcarbinol	0.30	0.48	0.60	0.61	1.04	1.14
Total	0.55	1.01	1.48	1.97	2.22	2.45
Ethanol	0.10	0.22	0.49	0.58	0.58	0.70
Yield of products, per cent by weight*						
2,3-Butanediol	12.6	14.3	16.2	19.0	13.3	12.1
Acetylmethylcarbinol	15.0	13.3	11.0	8.5	11.7	11.6
Total	27.6	27.6	27.2	27.5	25.0	24.0
Ethanol	5.1	6.0	9.0	8.1	6.5	6.6
Yield of products, per cent of theory**						
2,3-Butanediol	25.0	28.8	31.6	37.6	27.5	25.6
Acetylmethylcarbinol	30.8	27.3	22.8	17.2	24.8	22.4
Total	55.8	56.1	54.4	54.8	52.3	48.0
Ethanol	9.8	11.7	17.7	15.7	13.2	13.4
Total yield of products, per cent of theory**	65.6	67.8	72.1	70.5	65.5	61.4
Utilization of starch, per cent	90.5	89.8	89.7	91.1	87.3	87.3

* Based on starch added
** Based on starch utilized

Table 16

tion on the Fermentation of Corn by *Aerobacillus polymyxa* B25

.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5	35.0
.88	1.31	2.09	2.56	3.38	3.55	4.30	4.85	4.91	5.39
.04	1.14	0.71	0.70	0.45	0.69	0.50	0.35	0.23	0.24
.22	2.45	2.80	3.26	3.83	4.24	4.80	5.10	5.14	5.63
.58	0.70	0.88	1.02	1.20	1.27	1.40	1.66	1.70	1.71
.3	12.1	17.1	18.3	21.6	20.6	22.6	23.2	21.9	22.2
.7	11.6	5.8	5.0	2.9	4.0	2.6	1.7	1.0	1.0
.0	24.0	22.9	23.3	24.5	24.6	25.2	24.9	22.9	23.2
.5	6.6	7.1	7.3	7.6	7.3	7.3	8.0	7.7	7.0
.5	25.6	40.5	44.0	51.9	49.5	53.5	56.3	54.6	59.1
.8	22.4	14.1	12.3	6.9	9.8	6.3	4.2	2.6	2.6
.3	48.0	54.6	56.3	58.8	59.3	59.8	60.5	57.2	61.7
.2	13.4	16.6	17.2	17.7	17.2	16.9	19.0	18.8	18.0
.5	61.4	71.2	73.5	76.5	76.5	76.7	79.5	76.0	79.7
.3	87.3	76.0	75.0	76.4	74.8	75.8	74.4	72.2	68.8

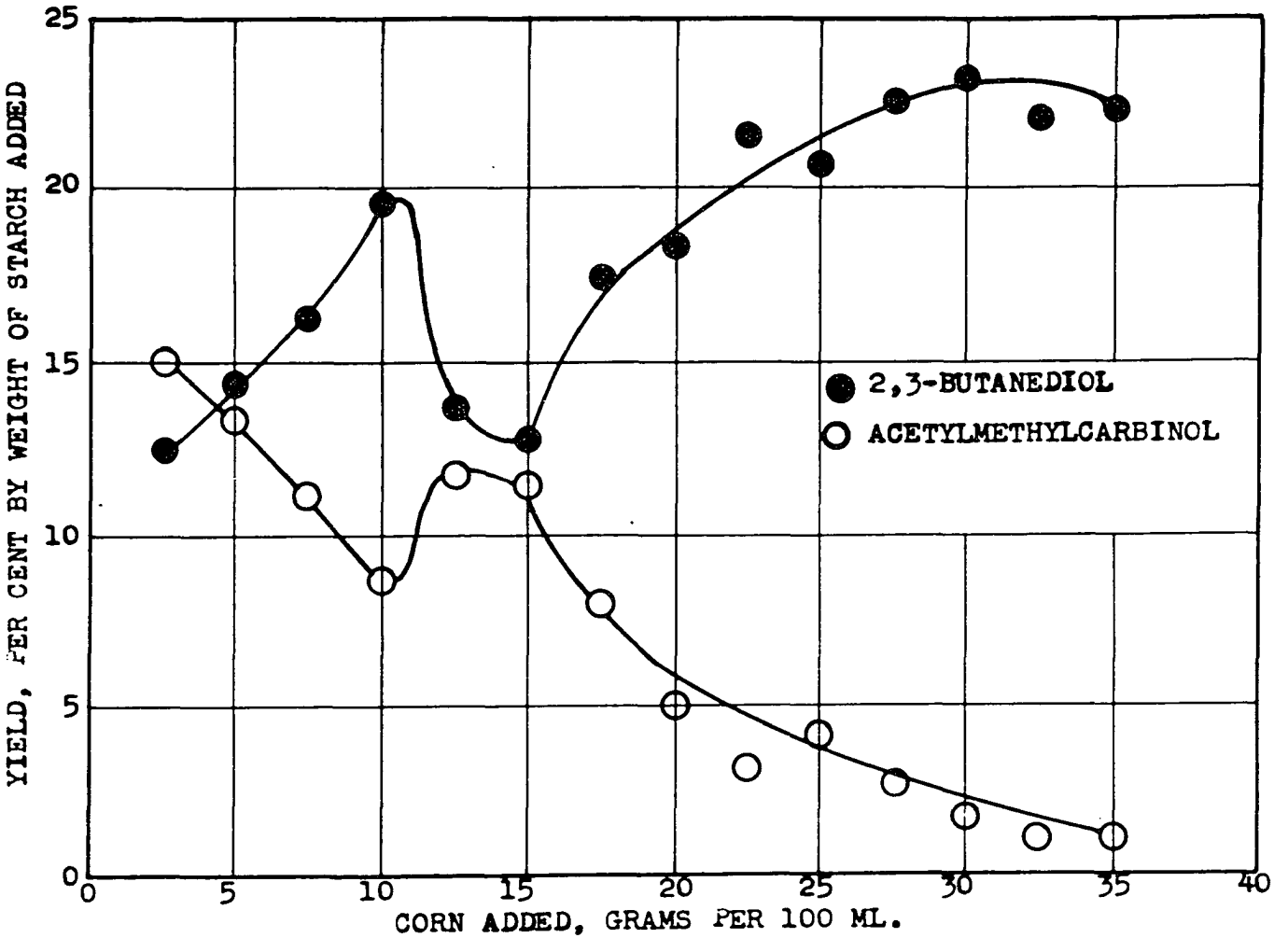


Fig. 7. Effect of the concentration of corn on the yields of 2,3-butanediol and acetylmethylcarbinol obtained by fermentation of corn with Aerobacillus polymyxa B25.

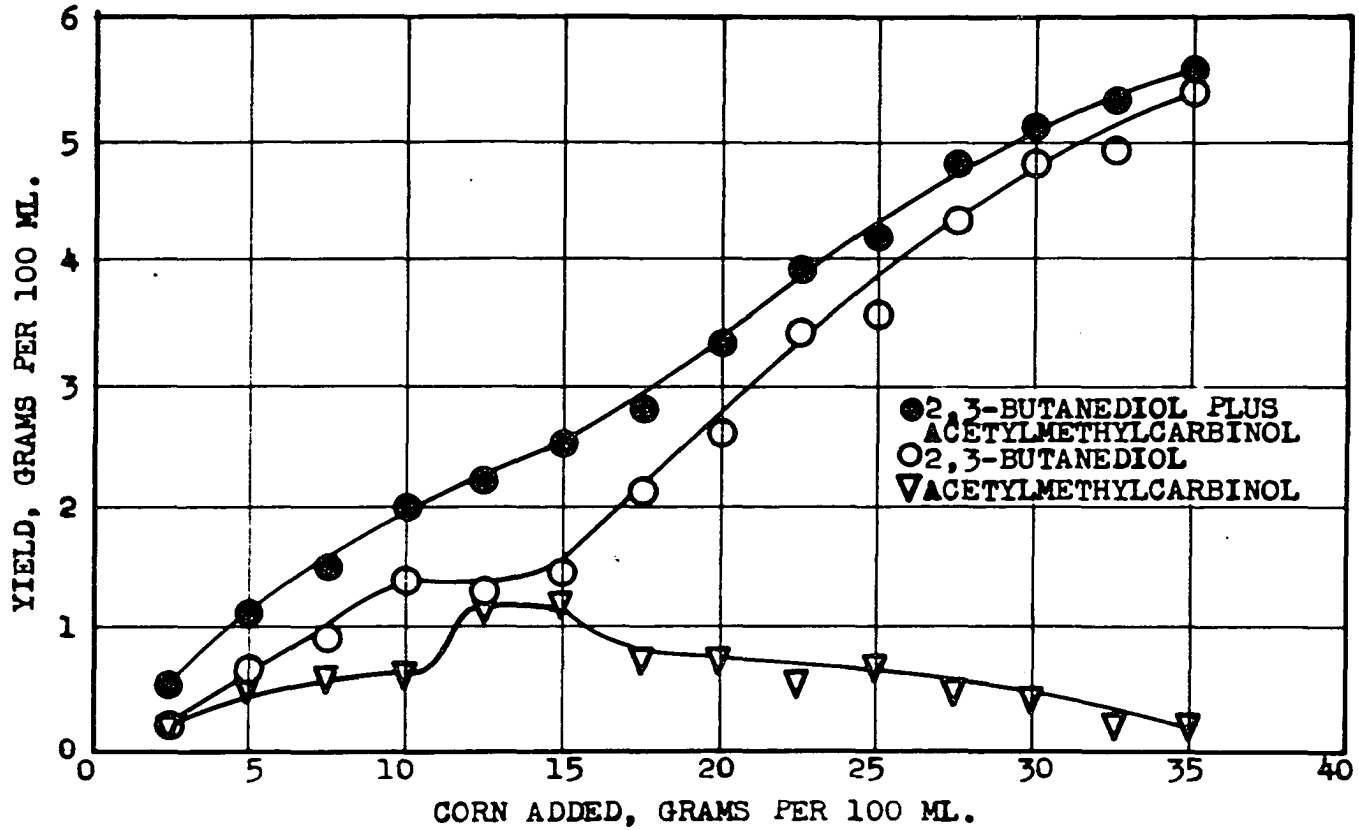


Fig. 8. Effect of the concentration of corn on the yields of 2,3-butanediol and acetylmethylcarbinol obtained by fermentation of corn with Aerobacillus polymyxa B25.

of 2,3-butanediol was accompanied by a decrease in the yield of acetylmethylcarbinol, and vice versa. These results may be explained on the basis of the investigations of Stahly and Werkman (1942), who presented evidence which showed the existence of a reversible oxidation-reduction system between the above products. This relation is further shown by figure 8; although the yields of 2,3-butanediol and acetylmethylcarbinol, expressed as grams per 100 ml. of mash, are not smooth functions of the concentration of corn, the yield of the sum of these two products is almost a straight line function of the concentration of corn.

The best weight per cent yields of 2,3-butanediol plus acetylmethylcarbinol were obtained from mashes containing up to 10 per cent of corn. The yields of ethanol were considerably lower than ordinarily obtained by fermentation of corn with Aerobacillus polymyxa. Since the mashes were incubated for 19 days, the low yields of ethanol were probably due to loss of the alcohol by volatilization.

b. Effect of the length of the period of fermentation. The following experiment was conducted in an effort to determine the length of fermentation period required for the maximum conversion of the starch in corn mashes to 2,3-butanediol and to study further the relations existing between the products formed.

The mashes were prepared in the same manner as those in the preceding experiment, using an amount of calcium carbonate equivalent to 5 per cent of the weight of corn in the mash. The fermentations were conducted at corn concentrations of 10, 20, 30, and 40 grams per 100 ml. of water. Since analyses were to be conducted at periodic intervals, a number of flasks of each concentration were prepared. This procedure was used rather than preparing a large volume of fermentation mash of each concentration, since mashes prepared by the latter procedure would be subject to variance in surface-volume ratio and depth of fermentation liquor due to the withdrawal of samples.

The inoculum for the series was prepared in the same manner as for the preceding experiment, except that the final inoculum was the fifth transfer from the refrigerated stock culture. At various intervals, duplicate flasks of each concentration of mash were analyzed for maltose, 2,3-butanediol, acetylmethylcarbinol, ethanol, and residual carbohydrate.

The results of the analyses at various intervals are shown in tables 17, 18, 19, and 20 for mash concentrations of 10, 20, 30, and 40 grams per 100 ml., respectively. The yields of 2,3-butanediol, acetylmethylcarbinol, and ethanol are shown graphically in figures 9, 10, 11, and 12. Examination of these figures shows that the concentration of

Table 17

The Effect of the Length of the Period of Fermentation on the Fermentation of Ten Per Cent Corn Mash by *Aerobacillus polymyxa* B25

Time of fermentation, days	1.3	2.1	3.0	5.0	8.0	15.2
Yield of products, grams per 100 ml.						
2,3-Butanediol	1.15	1.54	1.68	1.59	1.71	1.60
Acetylmethyl-carbinol	0.11	0.09	0.16	0.30	0.28	0.43
Total	1.26	1.63	1.84	1.89	1.99	2.03
Ethanol	0.78	0.90	0.93	0.85	0.78	0.63
Yield of products, per cent by weight*						
2,3-Butanediol	16.1	21.6	23.6	22.3	24.0	22.4
Acetylmethyl-carbinol	1.5	1.2	2.2	4.2	3.9	6.0
Total	17.6	22.8	25.8	26.7	27.9	28.4
Ethanol	11.1	12.8	13.2	12.1	11.1	9.0
Yield of products, per cent of theory**						
2,3-Butanediol	51.0	59.3	58.3	51.4	52.2	45.9
Acetylmethyl-carbinol	4.9	3.5	5.6	9.9	8.7	12.6
Total	55.9	62.8	63.9	61.3	60.9	58.5
Ethanol	34.4	34.5	31.9	27.2	23.6	17.9
Total yield of products, per cent of theory**	89.3	97.3	95.8	88.5	84.5	76.4
Utilization of starch, per cent	57.0	66.5	73.0	78.2	82.7	88.2

*Based on total starch added.
**Based on starch utilized.

Table 18

The Effect of the Length of the Period of Fermentation on the Fermentation of Twenty Per Cent Corn Mash by *Aerobacillus polymyxa* B25

Time of fermentation, days	2.1	5.0	8.0	11.5	15.2
Yield of products, grams per 100 ml.					
2,3-Butanediol	2.62	3.28	3.41	3.26	3.08
Acetylmethyl-carbinol	0.15	0.22	0.30	0.67	0.96
Total	2.77	3.50	3.71	3.93	4.04
Ethanol	1.48	1.51	1.57	1.34	1.22
Yield of products, per cent by weight*					
2,3-Butanediol	18.8	23.5	24.4	23.3	22.0
Acetylmethyl-carbinol	1.0	1.6	2.2	4.9	7.0
Total	19.8	25.1	26.6	28.2	29.0
Ethanol	10.6	10.8	11.2	9.6	8.7
Yield of products, per cent of theory**					
2,3-Butanediol	61.1	60.5	57.1	53.2	50.1
Acetylmethyl-carbinol	3.2	4.1	5.1	11.1	15.9
Total	64.3	64.6	62.2	64.3	66.0
Ethanol	33.2	27.3	25.7	21.4	19.4
Total yield of products, per cent of theory**	97.7	91.9	87.9	85.7	85.4
Utilization of starch, per cent	56.3	70.0	77.0	79.0	79.4

*Based on starch added.
**Based on starch utilized.

Table 19

The Effect of the Length of the Period of Fermentation on the Fermentation of Thirty Per Cent Corn Mash by *Aerobacillus polymyxa* B25

Time of fermentation, days	2.1	5.0	8.0	11.5	15.2
Yield of products, grams per 100 ml.					
2,3-Butanediol	3.39	4.74	4.96	5.10	4.95
Acetylmethyl-carbinol	0.20	0.24	0.32	0.32	0.35
Total	3.59	4.98	5.28	5.42	5.30
Ethanol	1.90	2.04	2.04	1.88	1.62
Yield of products, per cent by weight*					
2,3-Butanediol	16.3	22.8	23.8	24.4	23.8
Acetylmethyl-carbinol	1.0	1.1	1.5	1.5	1.7
Total	17.3	23.9	25.3	25.9	25.5
Ethanol	9.1	9.8	9.8	9.0	7.8
Yield of products, per cent of theory**					
2,3-Butanediol	52.5	62.3	62.4	62.5	62.0
Acetylmethyl-carbinol	3.2	3.2	4.1	4.0	4.5
Total	55.7	65.5	66.5	66.5	66.5
Ethanol	28.8	26.3	25.1	22.6	19.9
Total yield of products, per cent of theory**	84.5	91.8	91.6	89.1	86.4
Utilization of starch, per cent	55.8	65.8	68.9	70.6	69.0

*Based on starch added.
**Based on starch utilized.

Table 20

The Effect of the Length of the Period of Fermentation on the Fermentation of Forty Per Cent Corn Mash by *Aerobacillus polymyxa* B25

Time of fermentation, days	5.0	8.0	11.5	15.2
Yield of products, grams per 100 ml.				
2,3-Butanediol	5.50	6.07	6.27	6.29
Acetylmethylcarbinol	0.20	0.28	0.28	0.28
Total	5.70	6.35	6.55	6.57
Ethanol	2.38	2.29	2.19	1.75
Yield of products, per cent by weight*				
2,3-Butanediol	19.9	22.0	22.6	22.7
Acetylmethylcarbinol	0.7	1.0	1.0	1.0
Total	20.6	23.0	23.6	23.7
Ethanol	8.6	8.3	7.7	6.3
Yield of products, per cent of theory**				
2,3-Butanediol	61.7	64.4	66.2	66.1
Acetylmethylcarbinol	2.2	3.1	3.1	3.1
Total	63.9	67.5	69.3	69.2
Ethanol	26.2	23.8	21.9	18.0
Total yield of products, per cent of theory**	90.1	91.3	91.2	87.2
Utilization of starch, per cent	58.0	61.3	61.6	61.9

*Based on starch added.
**Based on starch utilized.

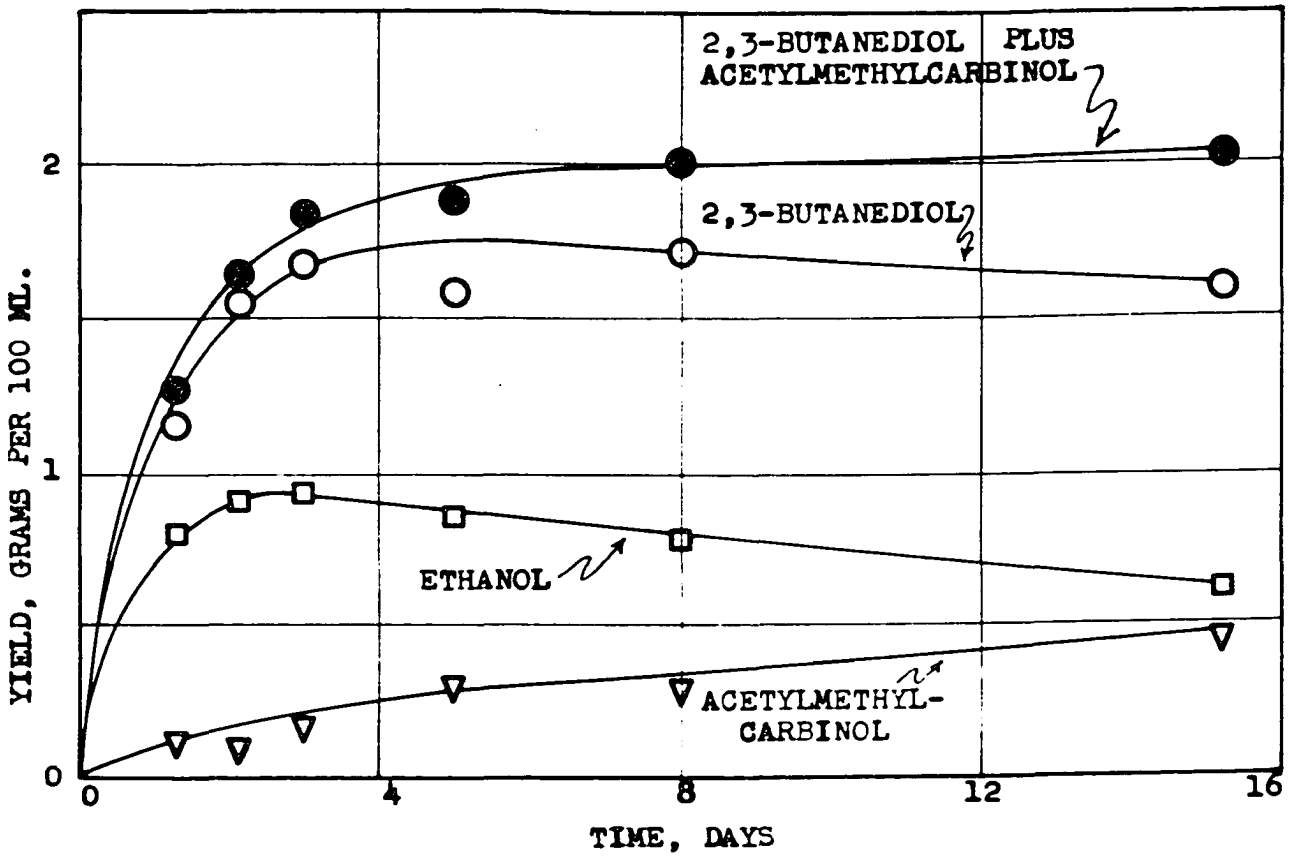


Fig. 9. Effect of the length of the period of fermentation on the fermentation of 10% corn mash by Aerobacillus polymyxa B25.

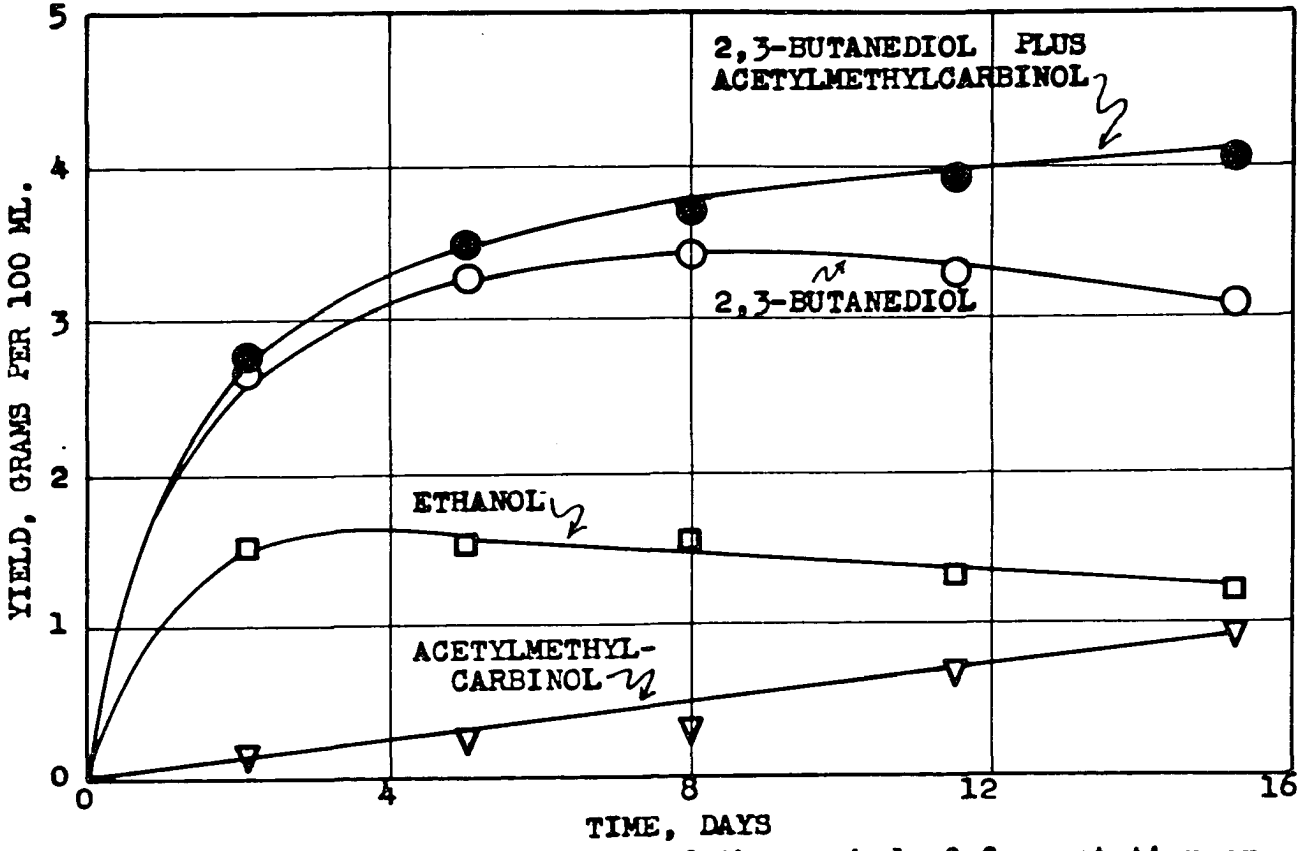


Fig. 10. Effect of the length of the period of fermentation on the fermentation of 20% corn mash by Aerobacillus polymyxa B25.

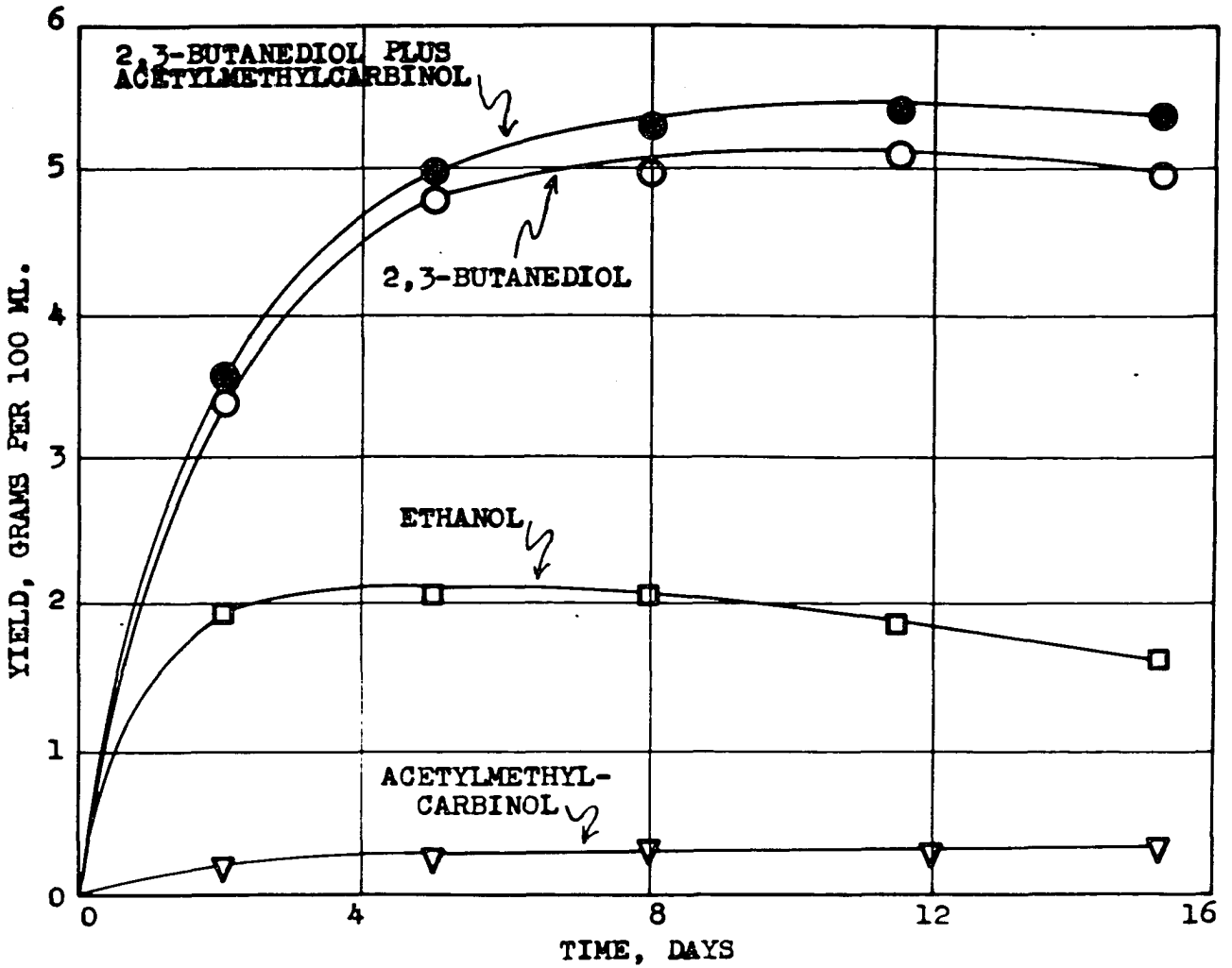


Fig. 11. Effect of the length of the period of fermentation on the fermentation of 30% corn mash by Aerobacillus polymyxa B25.

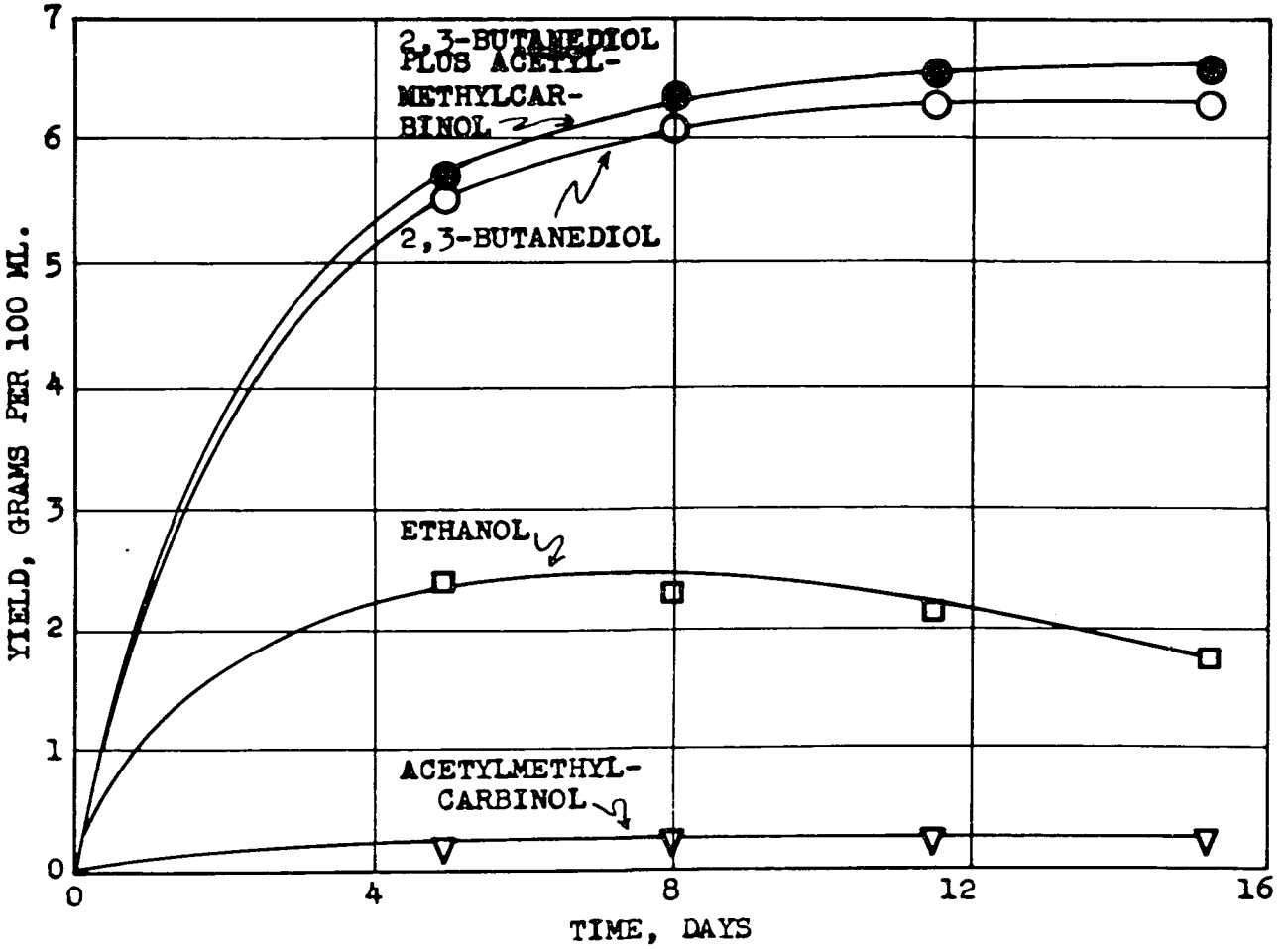


Fig. 12. Effect of the length of the period of fermentation on the fermentation of 40% corn mash by Aerobacillus polymyxa B25.

ethanol in the mash quickly reached a maximum and then gradually decreased. As has been mentioned previously, this decrease in the concentration of ethanol was probably due largely to evaporation. The formation of 2,3-butanediol was rapid during the first few days of the fermentation at all of the corn concentrations used. In the fermentation of the 10 per cent and 20 per cent corn mashes, the 2,3-butanediol concentration reached a maximum and then decreased, the decrease being accompanied by an increase in the concentration of acetylmethylcarbinol. This relation is further shown by the sum of the concentrations of 2,3-butanediol and acetylmethylcarbinol, which generally came to a maximum and then remained constant or increased gradually after the initial period of rapid fermentation. An exception to this relationship is the point at 15.2 days for the 30 per cent corn mash, but this point is of doubtful accuracy, since the results of the duplicate fermentations were not consistent. As compared with the analysis at 11.5 days, one of the duplicates showed an increase in the concentration of 2,3-butanediol and an increase in the utilization of starch, while the other showed a decrease in the concentration of 2,3-butanediol and a decrease in the utilization of the starch.

The yields of acetylmethylcarbinol, expressed in grams per 100 ml. of water added, were considerably higher at the

end of the fermentation period for mashes of 10 per cent and 20 per cent than for mashes of 30 per cent and 40 per cent corn. This would indicate that a higher oxidation-reduction potential exists toward the end of the fermentation period in mashes of lower corn concentration.

The yields of 2,3-butanediol plus acetylmethylcarbinol, expressed as per cent by weight of starch present in the mash, are shown in figure 18. In fermentation of 10 and 20 per cent mashes, the final yields were essentially equal. The time required to obtain the maximum yield, however, was considerably longer in the case of the 20 per cent mash. The yield obtained from fermentation of the 30 per cent mash was only slightly less than that obtained at lower concentrations, but a considerable decrease in yield was obtained in the fermentation of the 40 per cent mash.

c. Effect of pre-thinning the mash. Considering the fermentation from a practical standpoint, it would appear that the production of 2,3-butanediol by fermentation of mashes containing 30 grams of corn per 100 ml. of water would be the most efficient. As compared with the fermentation of a mash containing 10 per cent of corn, production of the 2,3-butanediol by fermentation of a 30 per cent mash would involve one-third the fermenter volume and one-third the evaporator capacity. However, corn mashes of 20 per cent and above are so thick as to preclude handling in industrial equipment.

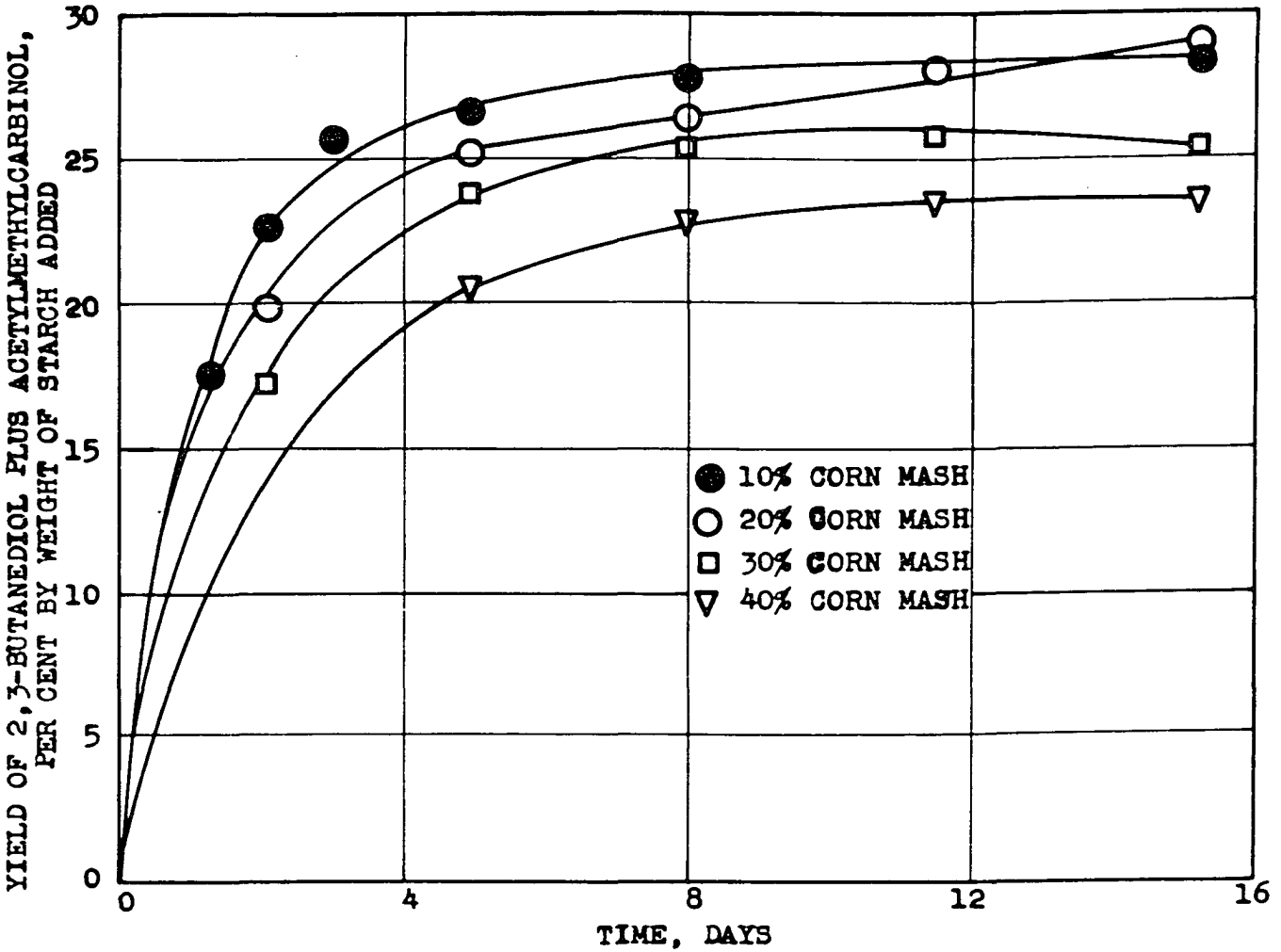


Fig. 13. Effect of the length of the period of fermentation on the fermentation of corn mash by Aerobacillus polymyxa B25.

Even assuming that the entire operations of gelatinization and sterilization of the mash could be carried out in the fermenters, the 30 and 40 per cent mashes in the preceding experiment were so thick after fermentation that special equipment would be required to transfer and filter the fermented mash. Since 2,3-butanediol, in order to compete with similar products, such as glycerol and ethylene glycol, would have to be produced on a low-cost basis, the use of specialized equipment would have to be kept at a minimum.

It is evident, therefore, that the practical fermentation of concentrated mashes will depend to a great extent on whether the mash can be thinned before transfer of the mash to the fermenter.

The method generally used for thinning of corn mashes is to add a small amount of malt to the mash before cooking and to heat the agitated mixture to approximately 70°C. However, according to the report issued by the Northern Regional Research Laboratory, the presence of reducing sugars in the fermentation mash inhibits the diastatic activity of Aerobacillus polymyxa. Hence, if a pre-thinned mash is to be fermented with this organism, the thinning must be done in such a manner that little of the starch is converted to maltose or dextrose.

The following experiment was conducted in an effort to determine if the mash could be pre-thinned with malt or bac-

terial-bran without seriously decreasing the yield of 2,3-butanediol.

Thinning of the corn mash with malt was conducted at corn concentrations of 20 and 40 grams per 100 ml. of water and was carried out by placing the corn in 500-ml. Erlenmeyer flasks, adding 100 ml. of distilled water, and placing the flasks in a water bath held at 77°C. After the temperature of the mash had reached 75°C., varying amounts of the malt were added to separate flasks and the temperature maintained at 75 ± 1°C. for 30 minutes. The flasks were shaken frequently during this period. The mashes were then heated to boiling over a Bunsen flame, the calcium carbonate added (equivalent to 5 per cent of the weight of the corn), the flasks plugged with cotton and sterilized for 30 minutes at 15 pounds.

Thinning of the corn mash with bacterial-bran was conducted at a corn concentration of 20 grams per 100 ml. of water. The thinning was carried out by placing the corn in 500-ml. Erlenmeyer flasks, adding 100 ml. of distilled water which had been heated to 65°C., and adding varying amounts of the bacterial-bran. The flasks were then placed in a water bath held at 65°C., and the temperature of the water bath was gradually increased so that the temperature of the mash increased to 85°C. in 30 minutes. One gram of calcium carbonate was then added to each flask, and the mashes were then

heated to boiling over a Bunsen burner. The mashes were sterilized for 30 minutes at 15 pounds.

After cooling to room temperature, each flask was inoculated with 8 ml. of a culture of Aerobacillus polymyxa B25, which was grown 24 hours on a medium containing 5 per cent corn and 0.5 per cent calcium carbonate. The inoculum was the 24th transfer from the refrigerated stock culture. The mashes were incubated at 30°C. and were shaken several times daily.

The mashes containing 20 grams of corn per 100 ml. were analyzed 10 days after inoculation; those containing 40 grams of corn per 100 ml. were analyzed 21 days after inoculation. The results of the fermentations conducted on the media containing 20 grams of corn per 100 ml. and pre-thinned with varying amounts of malt are shown in table 21. The results given are averages of duplicate fermentations. Since it was expected that a decrease in yield would be obtained when the mashes were pre-thinned with malt, it is somewhat surprising to observe that a substantial increase in the yield of 2,3-butanediol occurred when the mash was so pre-thinned. The increase amounted to as much as 4.7 per cent by weight of total starch present when two grams of malt were added per 20 grams of corn. Even when 0.3 gram of malt was added per 20 grams of corn, an increase of 3.6 per cent of butanediol by weight of starch present was obtained.

Table 21

The Effect of Pre-malt Treatment on the Fermentation of Twenty Per Cent Corn Mash by Aerobacillus polymyxa B25

Malt added, grams per 100 ml.	0.0	0.3	1.0	2.0	3.0
Yield of products, grams per 100 ml.					
2,3-Butanediol	3.53	4.08	4.25	4.48	4.55
Acetylmethyl-carbinol	0.50	0.51	0.53	0.57	0.57
Total	4.03	4.59	4.78	5.05	5.12
Ethanol	1.02	0.86	0.85	0.90	0.98
Yield of products, per cent by weight*					
2,3-Butanediol	25.2	28.8	29.2	29.7	29.0
Acetylmethyl-carbinol	3.6	3.6	3.6	3.8	3.6
Total	28.8	32.4	32.8	33.5	32.6
Ethanol	7.1	6.1	5.9	6.0	6.2
Yield of products, per cent of theory**					
2,3-Butanediol	58.0	67.5	69.6	71.3	70.3
Acetylmethyl-carbinol	8.5	8.6	8.9	9.3	9.0
Total	66.5	76.1	78.5	80.6	79.3
Ethanol	16.0	13.9	13.6	14.0	14.8
Total yield of products, per cent of theory**	82.5	89.0	92.1	94.6	94.1
Utilization of starch, per cent	78.1	77.0	75.7	75.0	74.4

*Based on starch added.
**Based on starch utilized.

There was little difference in the weight per cent yields of either ethanol or acetylmethylcarbinol in the presence or absence of malt, nor was there a significant difference in the amount of starch utilized. The drop in per cent utilization of starch with the addition of more malt may have been due to the presence of more starch (added as malt) in the initial mash.

The increased production of 2,3-butanediol obtained upon addition of malt would appear to be due to a more efficient utilization of the starch. This is shown by the increase in the per cent of theory yield of 2,3-butanediol.

Table 22 shows the results obtained when the mashes containing 20 grams of corn per 100 ml. of water were thinned with bacterial-bran. The results parallel those obtained with mashes pre-thinned with malt. Use of increasing quantities of bacterial-bran, however, seemed to stimulate the production of acetylmethylcarbinol, while the yields of acetylmethylcarbinol obtained with the addition of increasing quantities of malt were substantially constant. The increased yields of acetylmethylcarbinol are, therefore, apparently not related to the viscosity of the mash, since in this case an increase in the yield of acetylmethylcarbinol would also be expected upon the addition of increasing quantities of malt. In addition, the mashes which were prepared with

Table 22

The Effect of Pre-Thinning the Mash with Bacterial-bran on the Fermentation of Twenty Per Cent Corn Mash by Aerobacillus polymyxa B25

Bacterial bran added, grams per 100 ml.	0	0.1	0.2	0.3	0.6
Yield of products, grams per 100 ml.					
2,3-Butanediol	3.53	3.93	3.84	3.58	3.43
Acetylmethyl-carbinol	0.50	0.78	0.86	1.01	1.32
Total	4.03	4.71	4.70	4.59	4.75
Ethanol	1.02	1.38	1.28	1.34	1.18
Yield of products, per cent by weight*					
2,3-Butanediol	25.2	28.0	27.4	25.6	24.5
Acetylmethyl-carbinol	3.6	5.7	6.1	7.2	9.5
Total	28.8	33.7	33.5	32.8	34.0
Ethanol	7.1	9.6	8.6	9.4	8.2
Yield of products, per cent of theory**					
2,3-Butanediol	58.0	64.0	64.8	59.0	57.2
Acetylmethyl-carbinol	13.0	13.0	14.8	17.0	22.6
Total	66.5	77.0	79.6	76.0	79.8
Ethanol	16.0	21.5	20.6	21.1	18.8
Total yield of products, per cent of theory**	82.5	98.5	100.2	97.1	98.6
Utilization of starch, per cent	78.1	79.2	76.3	78.2	77.2

*Based on starch added.
**Based on starch utilized.

bacterial-bran were more viscous than those prepared with malt. This would indicate that the increase in the yield of acetylmethylcarbinol obtained upon the addition of bacterial-bran was due to an increase in the oxidation-reduction potential of the medium, resulting from the addition of the bacterial-bran.

The results of fermentations conducted on mashes containing 40 grams of corn per 100 ml. of water and pre-thinned with various amounts of malt are shown in table 23. The weight per cent yields of 2,3-butanediol obtained were not as high as those obtained by fermentation of the 20 per cent mashes, but in this case also, an improvement in the yield was obtained by pre-thinning the mash. The yields of 2,3-butanediol plus acetylmethylcarbinol, however, were as high as 81.3 per cent of theory from the starch fermented.

F. Conclusions

Nearly 30 per cent of the starch present in a 10 per cent corn mash can be converted to 2,3-butanediol in four and one-half days by fermentation with Aerobacillus polymyxa B25.

A long period of fermentation leads to an increase in the yield of acetylmethylcarbinol and a decrease in the yield of 2,3-butanediol, particularly at low mash concentrations.

Table 23

The Effect of Pre-malt Treatment on the Fermentation of Forty Per Cent Corn Mash by *Aerobacillus polymyxa* B25

Malt added, grams per 100 ml.	0	0.5	1.0	2.0	3.0	5.0
Yield of products, grams per 100 ml.						
2,3-Butanediol	7.00	7.65	7.72	8.17	7.82	7.55
Acetylmethyl-carbinol	0.25	0.21	0.27	0.25	0.28	0.34
Total	7.25	7.86	7.99	8.42	8.10	7.89
Yield of products, per cent by weight*						
2,3-Butanediol	24.4	27.4	27.4	28.2	26.6	24.7
Acetylmethyl-carbinol	1.0	0.8	1.0	0.9	0.9	1.1
Total	25.4	28.2	28.4	29.1	27.5	25.8
Yield of products, per cent of theory**						
2,3-Butanediol	63.8	73.5	78.0	79.0	78.6	77.5
Acetylmethyl-carbinol	2.6	1.5	2.8	2.5	2.9	3.6
Total	66.4	75.0	80.8	81.5	81.5	81.1
Utilization of starch, per cent	69.0	67.0	63.2	64.4	60.0	57.3

*Based on starch added.
**Based on starch utilized.

However, the sum of the yields of 2,3-butanediol and acetylmethylcarbinol at all corn concentrations either shows a gradual increase or reaches a maximum and then remains constant, indicating conversion of the 2,3-butanediol to acetylmethylcarbinol after active fermentation has subsided.

Fermentation of corn mashes containing as much as 40 grams of corn per 100 ml. can be efficiently conducted, and good yields of 2,3-butanediol can be obtained if the mashes are pre-thinned with malt. However, in the production of 2,3-butanediol by fermentation of concentrated mashes, considerable difficulty would undoubtedly be encountered in the recovery of the diol from the fermented mash, due to the large amounts of residual carbohydrate and other solids present. It is doubtful if the mash could be filtered readily because of the grain residues present; the presence of large quantities of soluble carbohydrate would certainly interfere with recovery of the diol by distillation.

VI. PRODUCTION OF 2,3-BUTANEDIOL FROM CORN STARCH BY FERMENTATION WITH *AEROBACILLUS POLYMYXA*

A. Introduction

As has been previously mentioned, production of 2,3-butanediol by fermentation of starch rather than by fermentation of the whole grain would possess certain advantages. If fermentation processes were carried out in conjunction with a corn milling plant, it would be possible to eliminate certain procedures connected with the preparation of the substrate materials and thereby decrease the cost of production of fermentation products. It must be admitted, of course, that the market price of starch is higher than the price of corn on the basis of carbohydrate content. This difference, however, would be at least partially offset by eliminating the drying step in the production of commercial starch. There is no reason to believe that a starch slurry could not serve as a substitute for the dried starch with equally good results.

The production of 2,3-butanediol from starch rather than from the whole grain would possess an additional practical advantage, *viz*, the absence of grain residues from the fermentation mash. If the yields of 2,3-butanediol from the

two media were the same, based on the starch contents of each material, the recovery of the diol from the starch mash should be more efficient, since the ratio of residual solids to 2,3-butanediol would be much smaller in the fermented starch mash than in the fermented corn mash.

The first step in determining whether 2,3-butanediol can be produced more economically from starch than from corn is to ascertain whether the fermentation process can be conducted as efficiently on starch mashes as on corn mashes. Unless the yield of the diol from fermentation of starch mashes is comparable to that which can be obtained by fermentation of corn, the advantages given above for using starch as the substrate material will be nullified. The following portion of the research, therefore, was conducted in order to determine the conditions favorable to the formation of 2,3-butanediol by the action of Aerobacillus polymyxa on corn starch.

B. Materials

The starch, corn gluten, and spray-dried corn steep liquor were furnished by the American Maize-Products Company of Roby, Indiana. Analysis of the starch showed the following partial composition:

Starch	82.0 per cent
Nitrogen	0.04 per cent
Ash	0.06 per cent

The corn gluten was ground to a fine powder in a ball mill. Analysis of the ground material showed:

Moisture	6.4 per cent
Nitrogen	8.70 per cent
Ash	1.29 per cent

Analysis of the dried corn steep liquor showed 6.96 per cent nitrogen and 15.1 per cent ash.

The dried yeast and the malt sprouts were furnished by the Pabst Brewing Company of Milwaukee, Wisconsin. The corn and malt were the same as those employed in the preceding section. The alfalfa meal was prepared by grinding cured alfalfa hay, and the peptone and yeast extract were the Difco dehydrated products.

C. Cultures

The organism used in this portion of the work was Aerobacillus polymyxa I.S.C. B32, obtained from the culture collection of the Northern Regional Research Laboratory as NRRL B510-R18. The stock cultures were stored on corn mash containing 5 per cent corn and 0.5 per cent calcium carbonate. The cultures were kept active by daily transfers to fresh medium of the same composition.

D. Methods of Analysis

The analytical methods used were identical with those employed in the preceding section. Unless otherwise indicated, the results of the analyses have been corrected for the presence of interfering substances as shown below.

E. Methods of Calculation

Since the methods for calculation of the yields of various products are complicated by the necessity of applying corrections for the various interfering substances, the details involved in applying these corrections are shown below.

The results of standardization of the sugar reagents against pure samples of dextrose, maltose hydrate, and acetylmethylcarbinol are shown graphically in figure 14. It will be noted that when the amount of acetylmethylcarbinol present in the sample is less than 6 mg., the amount of acetylmethylcarbinol present is a linear function of the amount of sodium thiosulfate required. It will also be noted that the amount of maltose hydrate (or dextrose) present in the sample is a linear function of the amount of sodium thiosulfate required. Since the amount of acetylmethylcarbinol present in the samples taken for the analysis of dextrose or maltose was always less than 6 mg., it was possible to use a numerical

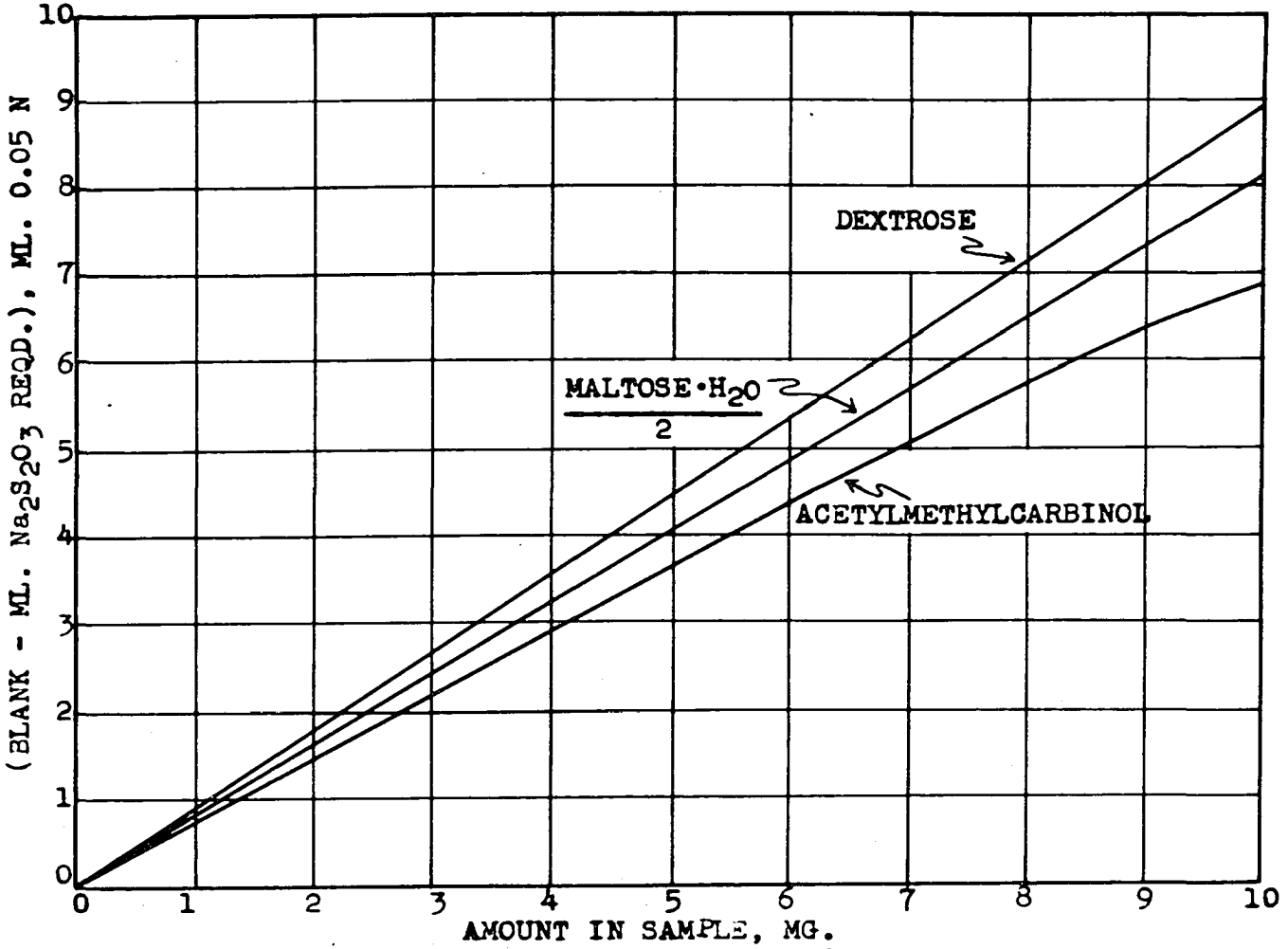


Fig. 14. Results of calibration of sugar reagents.

factor to correct the apparent maltose and dextrose concentrations for the interference due to the presence of acetylmethylcarbinol. Thus, from the relations shown in figure 14, one mg. of acetylmethylcarbinol is equivalent to 1.71 mg. of maltose hydrate or 0.80 mg. of dextrose.

The graph of figure 15 was constructed on the basis of the results obtained when solutions of pure maltose hydrate were subjected to the procedure employed for butanediol analysis. Since the ratio of the amount of maltose to 2,3-butanediol-equivalent varied with the amount of maltose present in the sample, the correction for maltose was based on the actual amount of maltose present in the samples taken for 2,3-butanediol analysis, and the extent of the correction was read from figure 15.

It has been previously mentioned that in the ethanol determination, one mg. of acetylmethylcarbinol is equivalent to 0.529 mg. of ethanol, and also that the amount of acetylmethylcarbinol appearing in the distillate resulting from the distillation of 100 ml. from a total volume of 300 ml. of solution is equal to 44.5 per cent of the total acetylmethylcarbinol present in the 300-ml. sample.

Since acetylmethylcarbinol reacts quantitatively with the reagents as used in the determination of 2,3-butanediol, yielding one mole of acetaldehyde per mole of acetylmethylcarbinol, one mg. of acetylmethylcarbinol

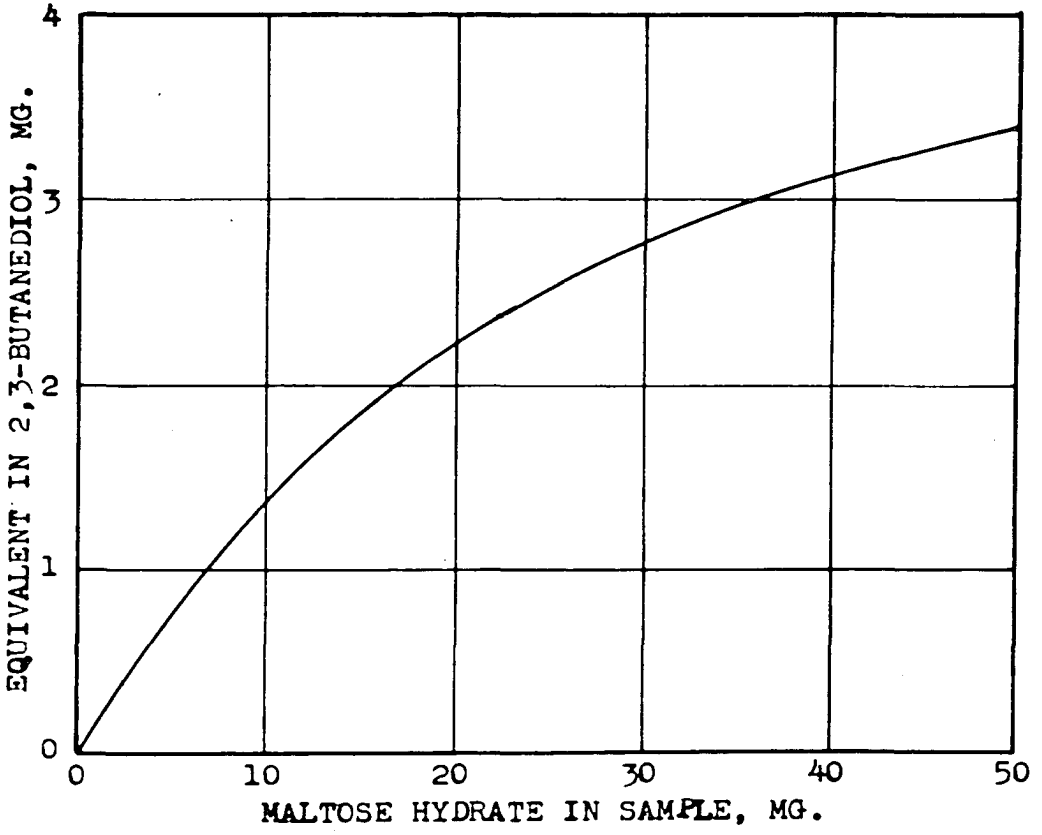


Fig. 15. Extent of maltose interference with the determination of 2,3-butanediol.

is equivalent to 0.51 mg. of 2,3-butanediol in analysis for the diol.

Following is given a typical example of the calculations involved in the determination of the yields of products and the concentrations of residual carbohydrate and free maltose. A fermentation was conducted in a medium containing 15.00 grams of starch[1] per 200 ml. of water. The inoculum was 8 ml. of a culture grown on 5 per cent corn medium. After 4 1/2 days of fermentation, the mash was transferred to a 500-ml. volumetric flask and diluted to 500 ml. This mixture was well shaken, and two 200-ml. samples were poured off before the solids could settle. One of the 200-ml. samples was hydrolyzed with hydrochloric acid according to the method previously mentioned for the determination of residual carbohydrate. After hydrolysis, the sample was neutralized to methyl orange with concentrated sodium hydroxide, transferred to a 500-ml. volumetric flask, diluted to volume, and filtered. Analysis of a 2-ml. sample of the filtrate showed an apparent dextrose concentration of 6.24 mg. per 2 ml.

[1]It should be noted that where the amount of starch added to the medium is stated, the term "starch" refers to the commercial starch described under Materials. The amount of pure starch added is 82.0 per cent of the amount of commercial starch added. However, yields expressed as per cent by weight based on starch added are based on the amount of pure starch added, including the starch contained in the inoculum.

The other 200-ml. sample of the diluted mash was transferred to a Kjeldahl flask, and 100 ml. of distilled water was added. Calcium carbonate and a small amount of castor oil were then added and the mixture distilled until 100 ml. of distillate was collected. Analysis of a 5-ml. sample of the distillate for acetylmethylcarbinol showed the presence of 1.64 mg. of acetylmethylcarbinol per 5 ml. Analysis of a 2-ml. sample of the distillate for ethanol by the dichromate oxidation method showed an apparent ethanol concentration of 14.2 mg. of ethanol per 2 ml.

The solids in the remainder of the original diluted mash were allowed to settle, and a small amount of the supernatant liquor was filtered. Analysis of a 3-ml. sample of this filtrate showed an apparent 2,3-butanediol concentration of 21.2 mg. per 3 ml.; analysis of a 2-ml. sample showed an apparent maltose hydrate concentration of 7.61 mg. of maltose hydrate per 2 ml.

Since the analysis for acetylmethylcarbinol is the only determination which is not subject to interference due to the presence of the other constituents, the calculation of the amount of this constituent present in the fermentation liquor must be made first. The calculations are made as follows:

Acetylmethylcarbinol in 5 ml. of distillate	1.64 mg.
Acetylmethylcarbinol in 100 ml. of distillate $\frac{1.64 \times 100}{5}$	32.8 mg.
Total acetylmethylcarbinol in sample distilled $\frac{32.8 \times 100}{44.5 \times 1000}$	0.074 gram
Total acetylmethylcarbinol in fermentation liquor $\frac{0.074 \times 500}{200}$	0.19 gram

The apparent amount of ethanol is calculated and corrected for interference due to the presence of acetylmethylcarbinol as follows:

Apparent ethanol in 2 ml. of distillate	14.2 mg.
Apparent ethanol in 100 ml. of distillate $\frac{14.2 \times 100}{1000 \times 2}$	0.710 gram
Total apparent ethanol in sample distilled	0.710 gram
Total apparent ethanol in fermentation liquor $\frac{0.710 \times 500}{200}$	1.77 grams
Acetylmethylcarbinol correction 0.19 x 0.529	0.10 gram
Total ethanol in fermentation liquor 1.77 - 0.10	1.67 grams

The apparent amount of residual carbohydate (as dextrose) is calculated and corrected for interference due to the presence of acetylmethylcarbinol as follows:

Apparent dextrose in 2 ml. of hydrolyzate	6.24 mg.
Total apparent dextrose in hydrolyzate $\frac{6.24 \times 500}{1000 \times 2}$	1.56 grams
Total apparent dextrose in fermentation liquor $\frac{1.56 \times 500}{200}$	3.90 grams
Acetylmethylcarbinol correction 0.19 x 0.80	0.15 gram
Total dextrose in fermentation liquor 3.90 - 0.15	3.75 grams

The apparent amount of free maltose in the fermentation liquor is calculated and corrected for interference due to the presence of acetylmethylcarbinol as follows (all maltose concentrations are expressed as maltose hydrate):

Apparent maltose in 2 ml. of filtrate	7.61 mg.
Total apparent maltose in fermentation liquor $\frac{7.61 \times 500}{2 \times 1000}$	1.90 grams
Acetylmethylcarbinol correction 0.19 x 1.71	0.32 gram
Total maltose in fermentation liquor 1.90 - 0.32	1.58 grams

The apparent amount of 2,3-butanediol in the fermentation liquor is corrected for interference due to the presence of maltose and acetylmethylcarbinol as follows:

Apparent 2,3-butanediol in 3 ml. of filtrate	21.2 mg.
Maltose present in 3 ml. of filtrate $\frac{1.58 \times 3 \times 1000}{500}$	9.5 mg.
Maltose correction (from fig. 15)	1.3 mg.
Apparent 2,3-butanediol in 3 ml. of filtrate (corrected for maltose present) 21.2 - 1.3	19.9 mg.
Total apparent 2,3-butanediol in fermentation liquor (corrected for maltose present) $\frac{19.9 \times 500}{3 \times 1000}$	3.32 grams
Acetylmethylcarbinol correction 0.19 x 0.51	0.10 gram
Total 2,3-butanediol in fermentation liquor 3.32 - 0.10	3.22 grams

In order that the extent of the corrections may be shown, a summary of the corrected amounts of the above constituents and values which have been calculated without applying corrections are given below:

Constituent	Amount present in fermentation liquor, grams	
	Uncorrected	Corrected
2,3-Butanediol	3.54	3.22
Acetylmethylcarbinol	0.19	0.19
Ethanol	1.77	1.67
Carbohydrate (as dextrose)	3.90	3.75
Free maltose (as maltose hydrate)	1.90	1.58

Since the starch added contained 82.0 per cent pure starch, and the corn added in the inoculum contained 68.5 per cent starch, the total amount of pure starch present was 13.57 grams, equivalent to 13.97 grams of dextrose. The theoretical yields of 2,3-butanediol, acetylmethylcarbinol, and ethanol, based on total starch available, are therefore 6.97, 6.83, and 7.14 grams, respectively. The calculations involved in the determination of the per cent of theoretical yields of these products on the basis of utilized starch are made as follows:

Utilization of starch

$$\frac{(13.97 - 3.75) \times 100}{13.97}$$ 72.8 %

Yield of 2,3-butanediol

$$\frac{3.22 \times 100}{6.97 \times 0.728}$$ 63.5 % of theory

Yield of acetylmethylcarbinol

$$\frac{0.19 \times 100}{6.83 \times 0.728}$$ 3.8 % of theory

Yield of ethanol

$$\frac{1.67 \times 100}{7.14 \times 0.728}$$ 32.1 % of theory

Yield of total products

$63.5 + 3.8 + 32.1$ 99.4 % of theory

Calculations of the yields of the above three products, in per cent by weight of pure starch added, are made as follows:

Yield of 2,3-butanediol $\frac{3.22 \times 100}{12.57}$ 25.6 % by weight

Yield of acetylmethylcarbinol $\frac{0.19 \times 100}{12.57}$ 1.5 % by weight

Yield of ethanol $\frac{1.67 \times 100}{12.57}$ 13.3 % by weight

F. Experimental Results

1. Effect of varying concentrations of inorganic constituents

In order to determine the optimum conditions for the formation of a fermentation product from a given substrate material, it is advisable to begin with an arbitrarily chosen basal medium and then to study the effect of variation in the concentration of a single constituent. The optimum quantity of this constituent is then added to the basal medium and the effect of varying concentrations of other constituents determined in a similar manner.

In applying the above procedure to the determination of optimum concentrations of inorganic constituents for the formation of 2,3-butanediol from corn starch by fermentation with Aerobacillus polymyxa, the basal medium chosen consisted of:

Starch	7.5	grams
$CaCO_3$	0.5	gram
Distilled water	100	ml.

In preparing the fermentation mashes, double the amounts of the above materials, together with varying amounts of other added materials as will be indicated, were placed in each 500-ml. Erlenmeyer flask. The starch was then gelatinized by heating the mashes over a Bunsen burner, following which the flasks were plugged with cotton and sterilized for 30 minutes at 15 pounds steam pressure. Included in some of the experiments as a control were fermentations conducted on corn media containing 20 grams of corn and 1.0 gram of calcium carbonate per 200 ml. of water.

The media were inoculated with 8 ml. each of a 24-hour culture of Aerobacillus polymyxa B32, grown on a medium containing 10 grams of corn and 1.0 gram of calcium carbonate per 200 ml. of water. After 4½ days incubation at 30°C., the fermentations were analyzed by the methods previously mentioned. Results given are averages of duplicate fermentations.

a. Effect of variation in the concentration of ammonium chloride. Since a source of nitrogen is necessary to support growth of any organism, ammonium chloride was the first constituent added to the basal medium. The inoculum for the experiment was the 25th transfer from the refrigerated stock culture. The results obtained upon addition of varying

concentrations of the ammonium chloride are shown in table 24. It will be noted that when no ammonium chloride was added to the medium, little utilization of the starch occurred. The highest utilization of starch and the highest weight per cent yield of 2,3-butanediol plus acetylmethylcarbinol occurred at an ammonium chloride concentration of 0.2 gram per 100 ml.

Comparison of the data obtained from the starch fermentations containing 0.2 gram of ammonium chloride per 100 ml. with those obtained from the corn fermentations shows that the weight per cent yield of 2,3-butanediol plus acetylmethylcarbinol obtained by fermentation of starch was only about one-third of the yield of these products obtained by fermentation of the whole corn. However, the per cent of theory yields of the three products, 2,3-butanediol, acetylmethylcarbinol, and ethanol, are comparable to those obtained in the fermentation of corn, showing that the lower weight per cent yields obtained in the starch fermentations were due to a lesser extent of utilization of the starch in the starch mash rather than to less efficient conversion of the starch to 2,3-butanediol, acetylmethylcarbinol, and ethanol.

b. Effect of variation in the concentration of potassium dihydrogen phosphate. The preceding experiment showed the optimum concentration of ammonium chloride to be about 0.2 gram per 100 ml. In the present experiment, this amount of ammonium chloride was added to the basal medium previously

Table 24

Effect of Variation in the Concentration of Ammonium Chloride on the Fermentation of Corn Starch by Aerobacillus polymyxa B32

NH_4Cl added, grams per 100 ml.	0	0.1	0.2	0.3	0.4	10% Corn Mash
Yield of products, grams per 100 ml.						
2,3-Butanediol	0.16	0.69	0.79	0.78	0.72	1.82
Acetylmethyl-carbinol	0.04	0.06	0.07	0.06	0.05	0.14
Total	0.20	0.75	0.86	0.84	0.77	1.96
Ethanol	0.14	0.47	0.47	0.45	0.43	0.95
Yield of products, per cent by weight*						
2,3-Butanediol	2.5	11.0	12.6	12.4	11.5	26.1
Acetylmethyl-carbinol	0.6	1.0	1.1	1.0	0.8	2.0
Total	3.1	12.0	13.7	13.4	12.3	28.1
Ethanol	2.2	7.2	7.5	7.2	6.8	13.7
Yield of products, per cent of theory**						
2,3-Butanediol	29.6	48.9	55.6	55.5	55.6	57.9
Acetylmethyl-carbinol	7.5	4.3	5.0	4.4	3.9	4.6
Total	37.1	53.2	60.6	59.9	59.5	62.5
Ethanol	25.2	32.4	32.2	31.2	32.4	30.0
Total yield of products, per cent of theory	62.3	85.6	92.8	91.1	91.9	92.5
Utilization of starch, per cent	15.5	40.5	40.7	40.3	37.1	81.1

* Based on starch added
**Based on starch utilized

indicated, and the concentration of potassium dihydrogen phosphate was varied as shown in table 25. The inoculum was the 25th transfer from the refrigerated stock culture.

The addition of the potassium dihydrogen phosphate to the medium resulted in increased utilization of starch and a corresponding increase in the weight per cent yield of the products. The optimum concentration of potassium dihydrogen phosphate was 0.10 to 0.15 gram per 100 ml.

The maximum yield obtained in the present experiment, 20.5 per cent of 2,3-butanediol plus acetylmethylcarbinol by weight of starch added, was still considerably less than the yield obtained from corn in the preceding experiment, showing that the addition of other nutrients of the proper type might cause a further increase in yield.

c. Effect of variation in the concentration of magnesium sulfate. Under the conditions of the preceding experiments, the optimum concentrations of ammonium chloride and potassium dihydrogen phosphate were found to be about 0.2 and 0.15 gram per 100 ml., respectively. These amounts of the above materials were therefore added to the basal medium, and the amount of magnesium sulfate added was varied as shown in table 26. The final inoculum was the 33d transfer from the refrigerated stock culture. The results of the experiment show that the addition of magnesium sulfate (heptahydrate) in quantities up

Table 25

Effect of Variation in the Concentration of Potassium Dihydrogen Phospha

polymyxa B3

KH_2PO_4 added, grams per 100 ml.	0	0.05	0.10	0.15
Yield of products, grams per 100 ml.				
2,3-Butanediol	0.99	1.23	1.25	1.26
Acetylmethyl-carbinol	0.07	0.07	0.07	0.07
Total	1.06	1.30	1.32	1.33
Ethanol	0.61	0.74	0.77	0.77
Yield of products, per cent by weight*				
2,3-Butanediol	15.2	18.9	19.2	19.4
Acetylmethyl-carbinol	1.1	1.1	1.1	1.1
Total	16.3	20.0	20.3	20.5
Ethanol	9.4	11.4	11.9	11.9
Yield of products, per cent of theory**				
2,3-Butanediol	55.3	54.3	54.6	54.5
Acetylmethyl-carbinol	4.0	3.1	3.1	3.1
Total	59.3	57.4	57.7	57.6
Ethanol	33.2	30.9	32.8	32.4
Total yield of products, per cent of theory**	92.5	88.3	90.5	90.0
Utilization of starch, per cent	51.3	65.0	65.6	66.4

* Based on starch added
** Based on starch utilized

Table 25

.hydrogen Phosphate on the Fermentation of Corn Starch by Aerobacillus polymyxa B32

0.15	0.20	0.25	0.30	0.35
1.26	1.22	1.24	1.20	1.22
0.07	0.06	0.05	0.05	0.05
1.33	1.28	1.29	1.25	1.27
0.77	0.74	0.74	0.71	0.76
19.4	18.8	19.1	18.5	18.8
1.1	0.9	0.8	0.8	0.8
20.5	19.7	19.9	19.3	19.6
11.9	11.4	11.4	10.9	11.7
54.5	54.0	53.2	52.2	53.3
3.1	2.7	2.2	2.2	2.2
57.6	56.7	55.4	55.4	55.5
32.4	31.9	31.0	30.1	32.4
90.0	88.6	86.4	84.5	87.9
66.4	64.8	66.8	65.9	65.6

Table 26

Effect of Variation in the Concentration of Magnesium Sulfate on the Ferment

$MgSO_4 \cdot 7H_2O$ added, grams per 100 ml.	0	0.03	0.05	0.10	0.15
Yield of products, grams per 100 ml.					
2,3-Butanediol	1.22	1.23	1.22	1.23	1.22
Acetylmethyl-carbinol	0.07	0.07	0.07	0.07	0.07
Total	1.29	1.30	1.29	1.30	1.29
Yield of products, per cent by weight*					
2,3-Butanediol	19.4	19.6	19.4	19.6	19.4
Acetylmethyl-carbinol	1.1	1.1	1.1	1.1	1.1
Total	20.5	20.7	20.5	20.7	20.5

*Based on starch added

Table 26

Sulfate on the Fermentation of Corn Starch by Aerobacillus polymyxa B32

0.10	0.15	0.20	0.25	0.30	0.35	10% corn mash
1.23	1.22	1.23	1.21	1.20	1.20	1.86
0.07	0.07	0.07	0.07	0.07	0.07	0.14
1.30	1.29	1.30	1.28	1.27	1.27	2.00
19.6	19.4	19.6	19.2	19.1	19.1	26.6
1.1	1.1	1.1	1.1	1.1	1.1	2.0
20.7	20.5	20.7	20.3	20.2	20.2	28.6

to and including 0.35 gram per 100 ml. was neither beneficial nor detrimental to formation of the 2,3-butanediol. The mashes were not analyzed for ethanol or residual carbohydrate.

2. Effect of varying concentrations of organic nutrients

Following the preceding experiment, a number of fermentations were conducted employing a basal medium containing 7.5 grams of starch, 0.5 gram of calcium carbonate, 0.2 gram of ammonium chloride, and 0.15 gram of potassium dihydrogen phosphate per 100 ml. of water. A variety of materials were added to the basal medium, a single concentration of a single constituent to separate flasks. The materials added included a number of inorganic materials and a variety of nitrogenous organic materials such as corn gluten, malt, alfalfa meal, etc. The addition of complex nitrogenous materials to the basal medium resulted in increases in the yields of 2,3-butanediol, whereas no significant increases in yields were obtained upon addition of inorganic materials.

In view of the results obtained, it was obvious that the development of a medium containing an organic nitrogen source held more promise than further development of a medium containing inorganic nutrients only.

a. Effect of the kind of organic nitrogen source. The first step in the development of a medium containing a complex organic nitrogen source was the comparison of alfalfa

meal, corn steep liquor solids, corn gluten, and peptone. The basal medium for the experiment was composed of the following constituents:

Starch	5.0 grams
$CaCO_3$	0.5 gram
Distilled water	100 ml.

To separate flasks were added varying quantities of alfalfa meal, steep liquor solids, corn gluten, and peptone. The potassium dihydrogen phosphate and ammonium chloride previously employed were not added to the media, since it is not necessarily true that the addition of these materials is essential when a complex organic source of nitrogen is added. The starch concentration of 5.0 grams per 100 ml. was used in order that differences due to the extent of utilization of the starch might not be exaggerated.

The media were prepared and inoculated in the manner previously indicated and were analyzed after $4\frac{1}{2}$ days incubation. The results given are average value for duplicate fermentations.

Addition of increasing quantities of alfalfa meal, as shown in table 27, resulted in increased utilization of the starch and in a corresponding increase in the weight per cent yield of products.

Addition of increasing quantities of steep liquor solids, as shown in table 28, also caused an increase in the amount

Table 27

Effect of Variation in the Concentration of Alfalfa Meal on the Fermentation of Corn Starch by *Aerobacillus polymyxa* B32

Alfalfa meal added, grams per 100 ml.	0	0.1	0.3	0.5	1.0	10% Corn mash
Yield of products, grams per 100 ml.						
2,3-Butanediol	0.13	0.21	0.43	0.57	0.87	1.81
Acetylmethyl-carbinol	0.04	0.05	0.06	0.08	0.09	0.20
Total	0.17	0.26	0.49	0.65	0.96	2.01
Ethanol	0.10	0.17	0.28	0.38	0.55	0.98
Yield of products, per cent by weight*						
2,3-Butanediol	3.1	5.0	10.2	13.5	20.6	26.0
Acetylmethyl-carbinol	1.0	1.2	1.4	1.9	2.1	2.9
Total	4.1	6.2	11.6	15.4	22.7	28.9
Ethanol	2.4	4.0	6.6	9.0	13.0	14.0
Yield of products, per cent of theory**						
2,3-Butanediol	36.5	44.5	55.9	54.6	53.7	60.7
Acetylmethyl-carbinol	12.0	11.0	7.8	7.9	5.6	6.9
Total	48.5	55.5	63.7	62.5	59.3	67.6
Ethanol	27.6	35.0	35.4	35.6	33.2	32.0
Total yield of products, per cent of theory**	76.1	90.5	99.1	98.1	92.5	99.6
Utilization of starch, per cent	15.3	20.2	32.9	44.5	69.1	77.2

* Based on starch added
** Based on starch utilized

Table 28

Effect of Variation in the Concentration of Steep Liquor Solids on the Fermentation of Corn Starch by Aerobacillus polymyxa B32

Steep liquor solids added, grams per 100 ml.	0	0.1	0.3	0.5	1.0	10% corn mash
Yield of products, grams per 100 ml.						
2,3-Butanediol	0.13	0.65	0.97	1.07	1.18	1.81
Acetylmethyl-carbinol	0.04	0.11	0.11	0.12	0.16	0.20
Total	0.17	0.76	1.08	1.19	1.34	2.01
Ethanol	0.10	0.41	0.63	0.63	0.63	0.98
Yield of products, per cent by weight*						
2,3-Butanediol	3.1	15.3	22.9	25.3	27.9	26.0
Acetylmethyl-carbinol	1.0	2.6	2.6	2.8	3.8	2.9
Total	4.1	17.9	25.5	28.1	31.7	28.9
Ethanol	2.4	9.7	14.9	14.9	14.9	14.0
Yield of products, per cent of theory**						
2,3-Butanediol	36.5	56.0	53.4	55.7	60.0	60.7
Acetylmethyl-carbinol	12.0	9.7	5.9	6.3	8.4	6.9
Total	48.5	65.7	59.3	62.0	68.4	67.6
Ethanol	27.6	34.8	34.0	32.2	31.4	32.0
Total yield of products, per cent of theory**	76.1	100.5	93.3	94.2	99.8	99.6
Utilization of starch, per cent	15.3	49.2	77.3	81.8	83.7	77.2

* Based on starch added
** Based on starch utilized

of starch utilized and an increase in the weight per cent yield of products. It is interesting to note that the weight per cent yield of 2,3-butanediol plus acetylmethylcarbinol obtained in the presence of 1.0 gram of steep liquor solids per 100 ml. is greater than that obtained from the fermentation of the 10 per cent corn mash. These results are not strictly comparable, however, since the corn mash contained 6.38 grams of available starch per 100 ml., while the starch mashes contained only 4.23 grams of available starch per 100 ml.

The results obtained by the addition of corn gluten, as shown in table 29, were comparable to those obtained with steep liquor solids. Addition of peptone, the results of which are shown in table 30, also caused an increase in the utilization of starch and an increase in the yield of products with increasing concentration of peptone.

A more direct comparison of the weight per cent yields of 2,3-butanediol plus acetylmethylcarbinol obtained upon the addition of the various nitrogen sources is shown in figure 16. Alfalfa meal was decidedly inferior to the other three nutrients. Peptone was superior to either corn gluten or steep liquor solids when the three nutrients were present in concentrations of 0.1 gram per 100 ml. but somewhat inferior at higher concentrations. Corn gluten and steep liquor solids gave approximately the same yields at all concentra-

Table 29

Effect of Variation in the Concentration of Corn Gluten on the Fermentation of Corn Starch by *Aerobacillus polymyxa* B32

Corn gluten added, grams per 100 ml.	0.	0.1	0.3	0.5	1.0	10% corn mash
Yield of products, grams per 100 ml.						
2,3-Butanediol	0.13	0.61	1.07	1.13	1.18	1.81
Acetylmethyl-carbinol	0.04	0.09	0.09	0.09	0.10	0.20
Total	0.17	0.70	1.16	1.22	1.28	2.01
Ethanol	0.10	0.38	0.61	0.63	0.64	0.98
Yield of products, per cent by weight*						
2,3-Butanediol	3.1	14.4	25.3	26.8	27.9	26.0
Acetylmethyl-carbinol	1.0	2.1	2.1	2.1	2.4	2.9
Total	4.1	16.5	27.4	28.9	30.3	28.9
Ethanol	2.4	9.0	14.4	14.9	15.1	14.0
Yield of products, per cent of theory**						
2,3-Butanediol	36.5	52.2	56.1	57.9	60.5	60.7
Acetylmethyl-carbinol	12.0	7.7	4.8	4.6	5.3	6.9
Total	48.5	59.9	60.9	62.5	65.8	67.6
Ethanol	27.6	31.8	31.2	31.3	32.0	32.0
Total yield of products, per cent of theory**	76.1	91.7	92.1	93.4	97.8	99.6
Utilization of starch, per cent	15.3	49.8	81.3	84.0	83.2	77.2

* Based on starch added
**Based on starch utilized

Table 30

Effect of Variation in the Concentration of Peptone on the Fermentation of Corn Starch by Aerobacillus polymyxa B32

Peptone added, grams per 100 ml.	0	0.1	0.3	0.5	1.0	10% corn mash
Yield of products, grams per 100 ml.						
2,3-Butanediol	0.13	0.71	0.87	0.99	1.09	1.81
Acetylmethylcarbinol	0.04	0.10	0.11	0.11	0.11	0.20
Total	0.17	0.81	0.98	1.10	1.20	2.01
Ethanol	0.10	0.47	0.58	0.63	0.63	0.98
Yield of products, per cent by weight*						
2,3-Butanediol	3.1	16.8	20.6	23.4	25.8	26.0
Acetylmethylcarbinol	1.0	2.4	2.6	2.6	2.6	2.9
Total	4.1	19.2	23.2	26.0	28.4	28.9
Ethanol	2.4	11.1	13.7	14.9	14.9	14.0
Yield of products, per cent of theory**						
2,3-Butanediol	36.5	53.2	49.0	53.6	55.3	60.7
Acetylmethylcarbinol	12.0	7.8	6.2	6.1	5.7	6.9
Total	48.5	61.0	55.2	59.7	61.0	67.6
Ethanol	27.6	34.4	31.8	33.4	31.2	32.0
Total yield of products, per cent of theory**	76.1	75.4	87.0	93.1	92.2	99.6
Utilization of starch, per cent	15.3	56.8	75.8	78.6	84.1	77.2

* Based on starch added
** Based on starch utilized

tions. The results of the experiment, as expressed in figure 16, emphasize the principle stated by Fulmer (1943) that a comparison of the effect of the addition of different materials upon microbiological processes should be made at various concentrations.

Inasmuch as an increase in yield was obtained upon addition of increasing quantities of corn gluten and steep liquor solids up to and including 1.0 gram of either constituent per 100 ml., it would appear that the optimal concentration of each of these constituents is in excess of 1.0 gram per 100 ml. However, part of the increase in yield was probably due to small amounts of carbohydrate present in the corn gluten and steep liquor solids.

It should be emphasized that the experiment was conducted at a starch concentration of 5.0 grams per 100 ml., and that the most effective concentration of nutrients may vary with the concentration of starch used. However, the relative efficiency of the various nutrients with respect to increasing the yield of 2,3-butanediol, on a semi-quantitative basis at least, should hold for various concentrations of starch.

b. Effect of variation in the concentration of corn gluten. The preceding experiment showed that corn gluten and corn steep liquor solids are effective nutrients for the production of 2,3-butanediol from corn starch by fermentation with Aerobacillus polymyxa. Peptone was also found to be fairly

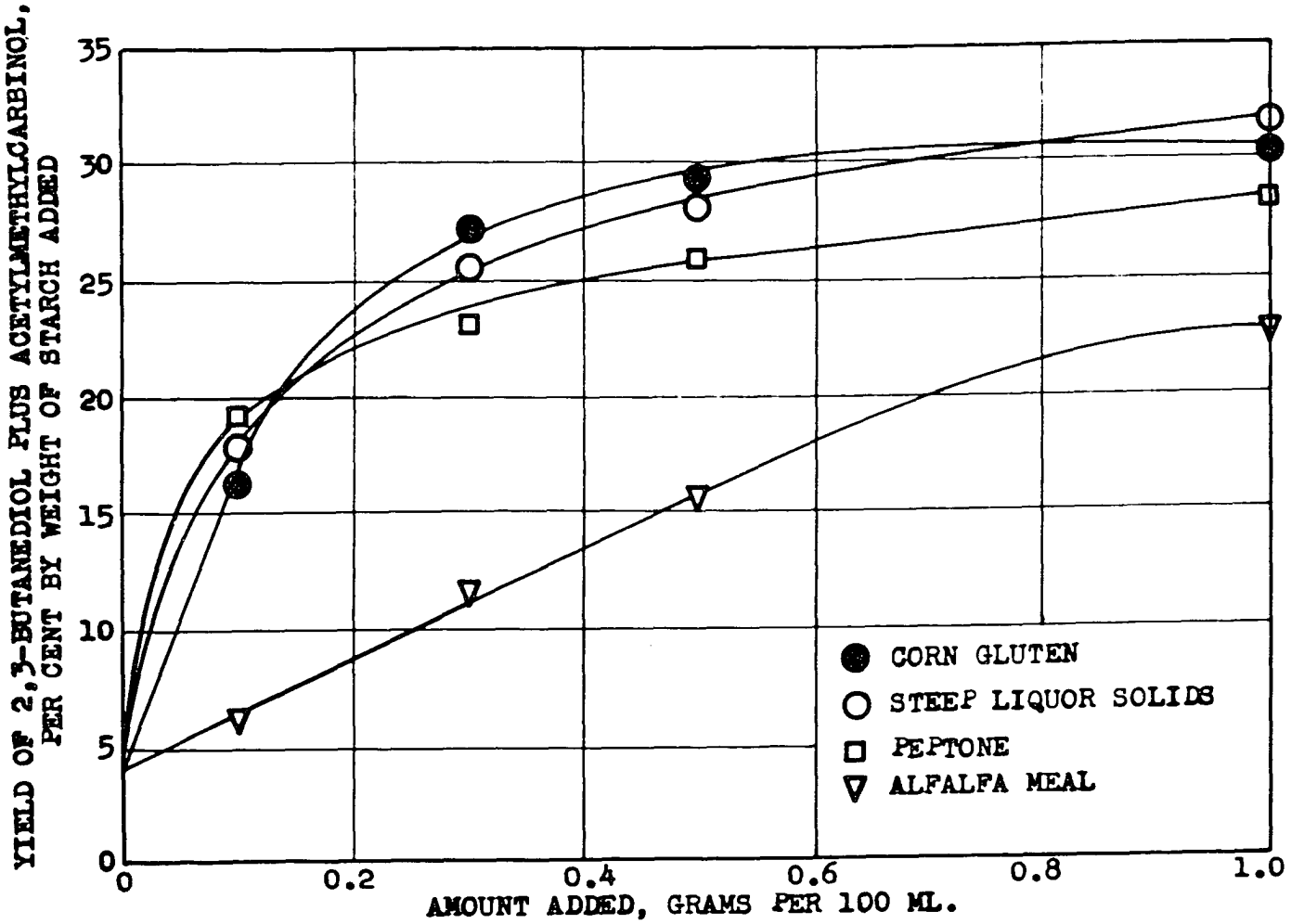


Fig. 16. Effect of addition of organic nitrogen sources on the fermentation of corn starch by Aerobacillus polymyxa B32.

effective but the cost of this material would be prohibitive for use as a nutrient on an industrial scale. Corn gluten and corn steep liquor, on the other hand, are by-products of the corn milling industry and would be readily available at a comparatively low cost. In order to investigate further the effect of adding increasing quantities of corn gluten on the yield of products, fermentations were conducted in media containing 7.5 grams of starch per 100 ml.

The media were prepared and inoculated in the same manner as the previous experiments, each 500-ml. Erlenmeyer flask containing 15.0 grams of starch and corresponding amounts of the other constituents. The composition of the basal medium used in this experiment was as follows:

Starch	7.5	grams
$CaCO_3$	0.5	gram
Distilled water	100	ml.

To separate flasks of the basal medium were added varying quantities of corn gluten. The culture used for the inoculum was the 32nd transfer from the refrigerated stock culture. The fermentations were analyzed after 4½ days incubation.

The results of the experiment are shown in table 31. The yields of 2,3-butanediol plus acetylmethylcarbinol obtained from mashes containing 0.5 and 0.7 grams of corn gluten per 100 ml. were comparable to the yield obtained by fermentation of corn, even though a higher percentage of starch

Table 31

Effect of Variation in the Concentration of Corn Gluten on the Fermenta

Corn gluten added, grams per 100 ml.	0	0.1	0.2	0.3	0.4
Yield of products, grams per 100 ml.					
2,3-Butanediol	0.23	0.84	1.00	1.32	1.47
Acetylmethyl-carbinol	0.04	0.07	0.09	0.08	0.07
Total	0.27	0.91	1.09	1.40	1.54
Ethanol	0.16	0.49	0.57	0.65	0.73
Yield of products, per cent by weight*					
2,3-Butanediol	3.7	13.4	15.9	21.0	23.4
Acetylmethyl-carbinol	0.6	1.1	1.4	1.3	1.1
Total	4.3	14.5	17.3	22.3	24.5
Ethanol	2.5	7.8	9.1	10.3	11.6
Yield of products, per cent of theory**					
2,3-Butanediol	40.2	58.6	55.7	63.0	64.5
Acetylmethyl-carbinol	7.2	5.1	5.1	3.8	3.2
Total	47.4	63.7	60.8	66.8	67.7
Ethanol	27.4	33.4	31.2	30.4	31.2
Total yield of products, per cent of theory**	74.8	97.1	92.0	97.2	98.9
Utilization of starch, per cent	16.4	41.1	51.4	60.0	65.3

* Based on starch added
** Based on starch utilized

Table 31

on the Fermentation of Corn Starch by Aerobacillus polymyxa B32

0.4	0.5	0.7	10% corn mash
1.47	1.53	1.60	1.71
0.07	0.07	0.08	0.14
1.54	1.60	1.68	1.85
0.73	0.75	0.80	0.94
23.4	24.5	25.4	24.5
1.1	1.1	1.3	2.0
24.5	24.6	26.7	26.5
11.6	11.9	12.7	13.5
64.5	64.7	65.7	55.2
3.2	3.1	3.4	4.6
67.7	67.8	69.1	59.6
31.2	31.0	32.0	30.1
98.9	98.8	101.1	89.9
65.3	67.9	69.9	79.8

was utilized in the corn mash. However, the starch fermentations contained only 6.29 grams of available starch per 100 ml. while the corn fermentations contained 6.98 grams of available starch per 100 ml. This difference in the carbohydrate concentration prevents an accurate comparison of the results obtained by fermentation of the two substrates.

The relation between the yield of 2,3-butanediol plus acetylmethylcarbinol and the amount of corn gluten added is shown in figure 17. A fairly rapid increase in the yield of 2,3-butanediol plus acetylmethylcarbinol occurred upon addition of increasing amounts of corn gluten up to 0.4 gram of corn gluten per 100 ml., whereas the addition of amounts in excess of 0.4 gram per 100 ml. resulted in only a slight increase in the yield of the two products over that obtained upon the addition of 0.4 gram.

Figure 17 also shows the relation between the per cent of starch utilized and the amount of corn gluten added. It is interesting to note how closely the utilization of starch parallels the yield of 2,3-butanediol plus acetylmethylcarbinol. This is reflected in the per cent of theory yields of 2,3-butanediol plus acetylmethylcarbinol, which were substantially constant when corn gluten was added to the medium.

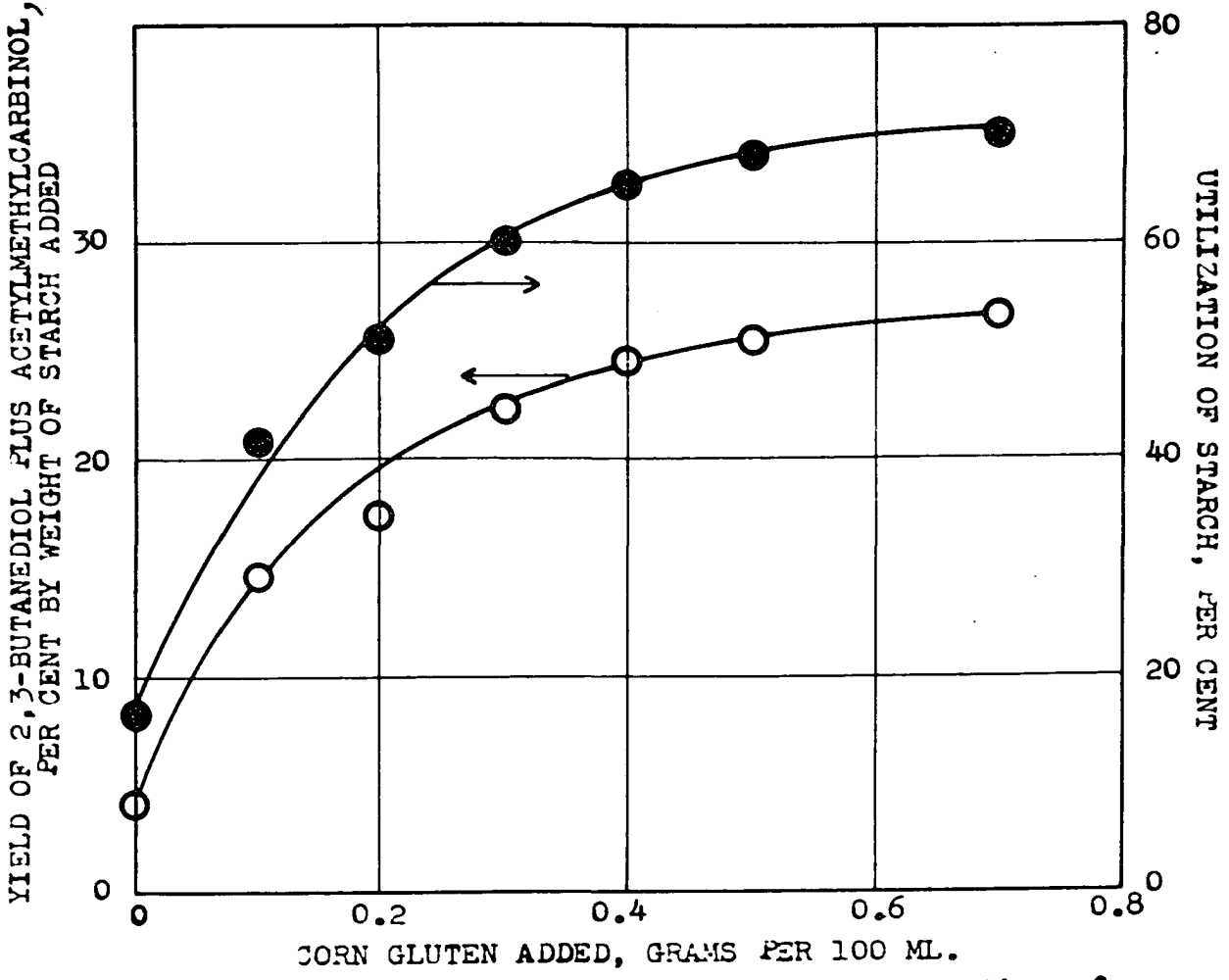


Fig. 17. Effect of variation in the concentration of corn gluten on the fermentation of corn starch by Aerobacillus polymyxa B32.

3. Effect of variation of other constituents in the presence of corn gluten

Corn gluten was chosen as the basal nutrient for further investigations. If the fermentation were to be conducted on an industrial scale and in conjunction with a corn processing plant, and if the fermentation of corn starch could be conducted successfully using corn gluten as the main nutrient, it might not be necessary to separate the starch and gluten in the milling process, thus saving an additional step in the processing of the raw materials.

The basal medium for the following series of experiments was composed of the following:

Starch	7.5	grams
$CaCO_3$	0.5	grams
Corn gluten	0.5	grams
Distilled water	100	ml.

Each 500-ml. Erlenmeyer flask contained double the amount of the constituents shown above. The media were prepared in the same manner as in the previous experiments and the fermentations were analyzed after 4½ days incubation at 30°C.

a. Preliminary survey. This experiment was conducted as a preliminary survey of the effect of the addition of various substances on the production of 2,3-butanediol from corn starch media containing corn gluten as nutrient. The purpose of the survey was to determine if any of the materials added would effect an increase in the amount of 2,3-butanediol

formed. To separate flasks of the basal medium were added the various substances shown in table 32. The inoculum was the 25th transfer from the refrigerated stock culture.

The results of the experiment, as given in table 32, show that the results were not greatly affected by addition of ammonium chloride, potassium dihydrogen phosphate, magnesium sulfate, or ferrous ammonium sulfate at either of the concentrations of these materials used. Addition of potassium ferrocyanide, potassium ferricyanide, or copper sulfate had little effect at the lower of the two concentrations used for these materials but caused a decrease in yield at the higher concentrations. The addition of cobalt sulfate resulted in decreased yields at both of the concentrations employed. Increases in the yields of 2,3-butanediol plus acetylmethylcarbinol were obtained upon addition of manganous sulfate, potassium permanganate, ferric chloride, and yeast extract, indicating that a more detailed study of the effect of the addition of these substances might be worthwhile.

b. Effect of variation in the concentration of manganous sulfate. The preceding experiment showed that an increase in the yield of 2,3-butanediol was obtained when manganous sulfate was added to the mash. It was therefore decided to investigate the effect of the addition of manganous sulfate over a wider range of concentrations in order to determine the optimum concentration of this constituent. Various

Table 32

Effect of Addition of Various Substances on the Fermentation of Corn Starch by *Aerobacillus polymyxa* B32

Constituent added	Amount added, grams per 100 ml.	Yield of 2,3-butanediol plus acetylmethylcarbinol	
		grams per 100 ml.	per cent by weight*
None	0.0	1.45	23.0
NH_4Cl	0.05	1.46	23.2
	0.10	1.46	23.2
KH_2PO_4	0.05	1.43	22.8
	0.10	1.44	22.9
$MgSO_4 \cdot 7H_2O$	0.05	1.47	23.4
	0.10	1.45	23.0
$MNSO_4$	0.0025	1.57	25.0
	0.025	1.46	23.2
$KMnO_4$	0.0025	1.68	26.7
	0.025	1.60	25.4
$FeSO_4(NH_4)_2SO_4 \cdot 7H_2O$	0.005	1.49	23.7
	0.025	1.46	23.2
$FeCl_3 \cdot 6H_2O$	0.005	1.54	24.5
	0.025	1.55	24.6
$K_4Fe(CN)_6$	0.0025	1.40	22.2
	0.025	1.06	16.9
$K_3Fe(CN)_6$	0.0025	1.46	23.2
	0.025	1.05	16.7
$CoSO_4 \cdot 7H_2O$	0.0025	1.27	20.2
	0.025	0.53	8.4
$CuSO_4 \cdot 7H_2O$	0.0025	1.41	22.4
	0.005	1.20	19.1
	0.05	1.70	27.0
Yeast Extract	0.25	1.76	28.0

*Based on starch added

amounts of manganous sulfate were added to separate flasks of the basal medium which were then sterilized, inoculated, incubated, and analyzed in the manner previously indicated. The inoculum for the experiment was the 30th transfer from the refrigerated stock culture.

The results of the experiment, shown in table 33, show that the maximum yield of 2,3-butanediol plus acetylmethylcarbinol was obtained in the presence of 0.0006 gram of manganous sulfate per 100 ml. The optimal range of manganous sulfate appears to lie between 0.0003 and 0.003 gram per 100 ml. Concentrations higher than 0.03 gram per 100 ml. resulted in decreased yields.

c. Effect of variation in the concentration of potassium permanganate. Since potassium permanganate had also been found to stimulate the production of 2,3-butanediol, it was considered desirable to investigate the effect of the addition of this substance over a wider range of concentrations than was used in the preliminary experiment.

Ordinarily, after the optimum concentration of manganous sulfate had been determined, this amount of the material would be added to the basal medium when determining the effect of the addition of subsequent constituents. However, the manganous sulfate was not added to the mash in the present experiment, since the effects of the two constituents,

Table 33

Effect of Variation in the Concentration of Manganous Sulfate o

$MnSO_4$ added, grams per 100 ml.	0	0.0003	0.0006	0.0010	0.0030
Yield of products, grams per 100 ml.					
2,3-Butanediol	1.59	1.65	1.66	1.64	1.64
Acetylmethyl-carbinol	0.05	0.06	0.07	0.07	0.08
Total	1.64	1.71	1.73	1.71	1.72
Ethanol	0.81	0.81	0.75	0.76	0.76
Yield of products, per cent by weight*					
2,3-Butanediol	25.3	26.2	26.4	26.1	26.1
Acetylmethyl-carbinol	0.8	1.0	1.1	1.1	1.3
Total	26.1	27.2	27.5	27.2	27.4
Ethanol	12.9	12.9	11.9	12.1	12.1
Yield of products, per cent of theory**					
2,3-Butanediol	63.5	65.9	65.1	65.5	64.9
Acetylmethyl-carbinol	2.1	2.5	2.9	2.9	3.2
Total	65.5	68.4	68.0	68.4	68.1
Ethanol	32.1	32.0	28.2	30.0	29.8
Total yield of products, per cent of theory**	97.7	100.4	96.2	98.4	97.9
Utilization of starch, per cent	71.6	71.9	73.0	71.9	72.5

* Based on starch added
** Based on starch utilized

Table 33

as Sulfate on the Fermentation of Corn Starch by Aerobacillus polymyxa B32

0.0030	0.0060	0.0100	0.030	0.060	0.100
1.64	1.59	1.62	1.54	1.48	1.24
0.08	0.07	0.08	0.07	0.09	0.07
1.72	1.66	1.70	1.61	1.57	1.31
0.76	0.71	0.70	0.69	0.60	0.56
26.1	25.3	25.8	24.5	23.6	19.7
1.3	1.1	1.3	1.1	1.4	1.1
27.4	26.4	27.1	25.6	25.0	20.8
12.1	11.3	11.1	11.0	9.6	8.6
64.9	66.1	66.1	66.1	68.7	66.1
3.2	3.1	3.3	3.2	4.2	3.9
68.1	69.2	69.4	69.3	72.9	70.0
29.8	29.4	28.4	29.4	27.5	29.5
97.9	98.6	97.8	98.7	100.4	99.5
72.5	68.9	70.2	66.6	61.8	53.9

which are both manganese compounds, might not be additive. It was indeed found to be true subsequently that the addition of potassium permanganate to a mash containing the optimum amount of manganous sulfate did not effect a further increase in the yield of products.

The results obtained upon addition of varying quantities of potassium permanganate to the basal medium are shown in table 34. The inoculum was the 27th transfer from the refrigerated stock culture. The optimum concentration of potassium permanganate was approximately 0.003 grams per 100 ml., with the optimal range lying between 0.001 and 0.006 grams per 100 ml.

It will be noted that the utilization of the starch in the present experiment was as high as 80 per cent. This extent of utilization and the yield of 2,3-butanediol plus acetylmethylcarbinol of 27.9 per cent by weight of starch added are the highest which have been obtained by fermentation of a 7.5 per cent mash in the present investigations. This yield compares favorably with the results obtained by fermentation of corn mashes containing equivalent quantities of starch.

4. Effect of cultural conditions

a. Effect of variation in the number of transfers of the culture. It will be noted that in the preceding experiments the number of times the organism was transferred on

Table 34

Effect of Variation in the Concentration of Potassium Permanganate

$KMnO_4$ added, grams per 100 ml.	0	0.0003	0.0006	0.0010
Yield of products, grams per 100 ml.				
2,3-Butanediol	1.63	1.63	1.61	1.69
Acetylmethyl-carbinol	0.05	0.06	0.06	0.07
Total	1.68	1.69	1.67	1.76
Ethanol	0.81	0.80	0.79	0.86
Yield of products, per cent by weight*				
2,3-Butanediol	25.7	25.7	25.6	26.8
Acetylmethyl-carbinol	0.8	1.0	1.0	1.1
Total	26.5	26.7	26.6	27.9
Ethanol	12.9	12.7	12.6	13.7
Yield of products, per cent of theory**				
2,3-Butanediol	63.0	63.0	63.5	63.3
Acetylmethyl-carbinol	2.0	2.4	2.5	2.7
Total	65.0	65.4	66.0	66.0
Ethanol	31.0	30.6	30.8	31.9
Total yield of products, per cent of theory**	96.0	96.0	96.8	97.9
Utilization of starch, per cent	74.2	74.1	72.8	76.5

* Based on starch added
** Based on starch utilized

Table 34

Permanganate on the Fermentation of Corn Starch by *Aerobacillus polymyxa* B32

010	0.0030	0.0060	0.0100	0.0300	0.060	0.100
69	1.68	1.69	1.64	1.57	1.48	1.39
07	0.07	0.07	0.08	0.09	0.09	0.09
76	1.75	1.76	1.72	1.66	1.57	1.48
86	0.88	0.93	0.92	0.87	0.80	0.85
8	26.7	26.8	26.1	25.0	23.6	22.1
1	1.1	1.1	1.3	1.4	1.4	1.4
9	27.8	27.9	27.4	26.4	25.0	23.5
7	14.0	14.8	14.6	13.8	12.7	13.5
3	62.0	59.9	58.4	58.0	58.0	56.5
7	2.7	2.6	2.9	3.3	3.6	3.7
0	64.7	62.5	61.3	61.3	61.6	60.2
9	32.2	32.7	32.4	31.8	31.2	35.7
9	96.9	95.2	93.7	93.1	92.8	95.9
5	77.8	80.8	80.5	77.6	73.1	70.6

5 per cent corn medium was recorded. In order to determine whether variation of the activity of the culture occurred during these transfers, with resulting decrease or increase in ability to form 2,3-butanediol, fermentations were conducted which were inoculated with cultures which had been transferred varying numbers of times. The cultures were transferred in the same manner as in the preceding experiments; i.e., the stock culture was transferred to a medium containing 5 per cent corn and 0.5 per cent calcium carbonate. This first transfer was incubated at 30°C. for 48 hours, and the culture was thereafter transferred every 24 hours to fresh medium of the same composition.

The media used in the experiment contained:

Starch	7.5	grams
$CaCO_3$	0.5	gram
Corn gluten	0.5	gram
Distilled water	100	ml.

The media were prepared in the same manner as indicated in the preceding experiments and were analyzed for 2,3-butanediol only. The yields of 2,3-butanediol expressed in table 35 represent the apparent yields of 2,3-butanediol obtained, since they have not been corrected for interference due to the presence of maltose and acetylmethylcarbinol. The results indicate that there was no loss of activity of the culture upon extensive subculturing, and possibly a slight increase in activity. Inasmuch as the yields have not been

Table 35

Effect of Variation in the Number of Transfers on the Yield of 2,3-Butanediol from Fermentation of Corn Starch by Aerobacillus polymyxa B52

Number of transfers preceding inoculation	Apparent yield of 2,3-butanediol* grams per 100 ml.	Apparent yield of 2,3-butanediol* per cent by weight of starch added
3	1.68	26.8
4	1.68	26.8
5	1.70	27.0
10	1.70	27.0
19	1.77	28.2
49	1.77	28.2

*Not corrected for interference due to the presence of acetylmethylcarbinol of maltose.

corrected for interference due to the presence of acetylmethylcarbinol and maltose, the conclusion that an increase in the yield of 2,3-butanediol does occur when the inoculum has been extensively is not entirely justified, but the possibility of an increase in activity upon extensive subculturing is worthy of further investigation.

b. Effect of variation in the surface-volume ratio of the fermentation mash. The amount of exposed surface and the depth of the fermentation mash often have a decided effect upon the results obtained in fermentation processes. This is frequently one of the main difficulties encountered when attempts are made to extrapolate results obtained in the

laboratory to the fermentation of large volumes of mashes. The present experiment was conducted in an effort to determine the effect of variation in the surface-volume ratio on the fermentation of corn starch by Aerobacillus polymyxa.

The fermentation media were composed of:

Starch	7.5 grams
Corn gluten	0.5 gram
Calcium carbonate	0.5 gram
Distilled water	100 ml.

The fermentations were conducted in 3000-ml. Fernbach flasks, containing varying volumes of mash. The media were prepared by placing 150, 112.5, 75, 56.2, and 37.5 grams of starch (in duplicate) in separate flasks and adding corresponding quantities of the other constituents. After sterilization and cooling, each flask was inoculated with a culture of Aerobacillus polymyxa B32; the amount of inoculum used was 4 per cent by volume of the water added to the medium.

After incubation at 30°C. for 4½ days, the surface, the volume, and the weight of the mashes were measured, and 300 grams of each mash diluted to 500 ml. for analysis. The results of the experiment, shown in table 36, have been recalculated from the results of the analyses so that the concentration of the constituents of the mash might be expressed as grams per 100 ml. of water added. The results show that the yield of products and the extent of utilization of the starch are markedly affected by the surface-volume

Table 36

Effect of Variation in the Surface-volume Ratio of the Fermentation Mash on the Fermentation of Corn Starch by *Aerobacillus polymyxa* B32

Surface-volume ratio, sq. cm. per ml.	0.075	0.15	0.27	0.39	0.60
Yield of products, grams per 100 ml.					
2,3-Butanediol	1.22	1.30	1.37	1.48	1.56
Acetylmethylcarbinol	0.005	0.015	0.025	0.027	0.027
Total	1.22	1.31	1.39	1.51	1.59
Ethanol	0.75	0.80	0.83	0.77	0.75
Yield of products, per cent by weight*					
2,3-Butanediol	19.4	20.7	21.8	23.6	24.8
Acetylmethylcarbinol	0.1	0.2	0.4	0.4	0.4
Total	19.5	20.9	22.2	24.0	25.2
Ethanol	11.9	12.7	13.2	12.2	11.9
Yield of products, per cent of theory**					
2,3-Butanediol	59.6	59.0	55.8	59.5	63.1
Acetylmethylcarbinol	0.2	0.7	1.0	1.1	1.1
Total	59.8	59.7	56.8	60.6	63.1
Ethanol	35.8	35.4	33.0	30.2	29.0
Total yield of products, per cent of theory**	95.6	95.1	89.8	90.8	92.1
Utilization of starch, per cent	58.6	63.1	70.4	71.2	72.2

* Based on starch added
** Based on starch utilized

ratio. The weight per cent yield of 2,3-butanediol and the extent of utilization of the starch increased with an increase in the surface-volume ratio. This relation is shown graphically by figure 18; the increase in yield of 2,3-butanediol plus acetylmethylcarbinol closely parallels the increase in the extent of utilization of the starch, showing that the efficiency of conversion of utilized starch to 2,3-butanediol plus acetylmethylcarbinol is not greatly affected by the surface-volume ratio.

The weight per cent yield of ethanol reached a maximum at a surface-volume ratio of 0.27 and then decreased, while the per cent of theory yield decreased with increasing surface-volume ratio. Since the per cent of theory yield of 2,3-butanediol was essentially constant, it is probable that the decrease in the per cent of theory yield of ethanol was due to more evaporation when the depth of the mash was less.

The effect of the surface-volume ratio may be due to an increased air supply and a more rapid release of carbon dioxide at higher surface-volume ratios. The fact that the final pH of the medium was higher at higher surface-volume ratios (ranging from 5.9 at a surface-volume ratio of 0.075 to 6.2 at a surface-volume ratio of 0.60) lends credence to the theory that more rapid release of carbon dioxide may be one of the factors involved.

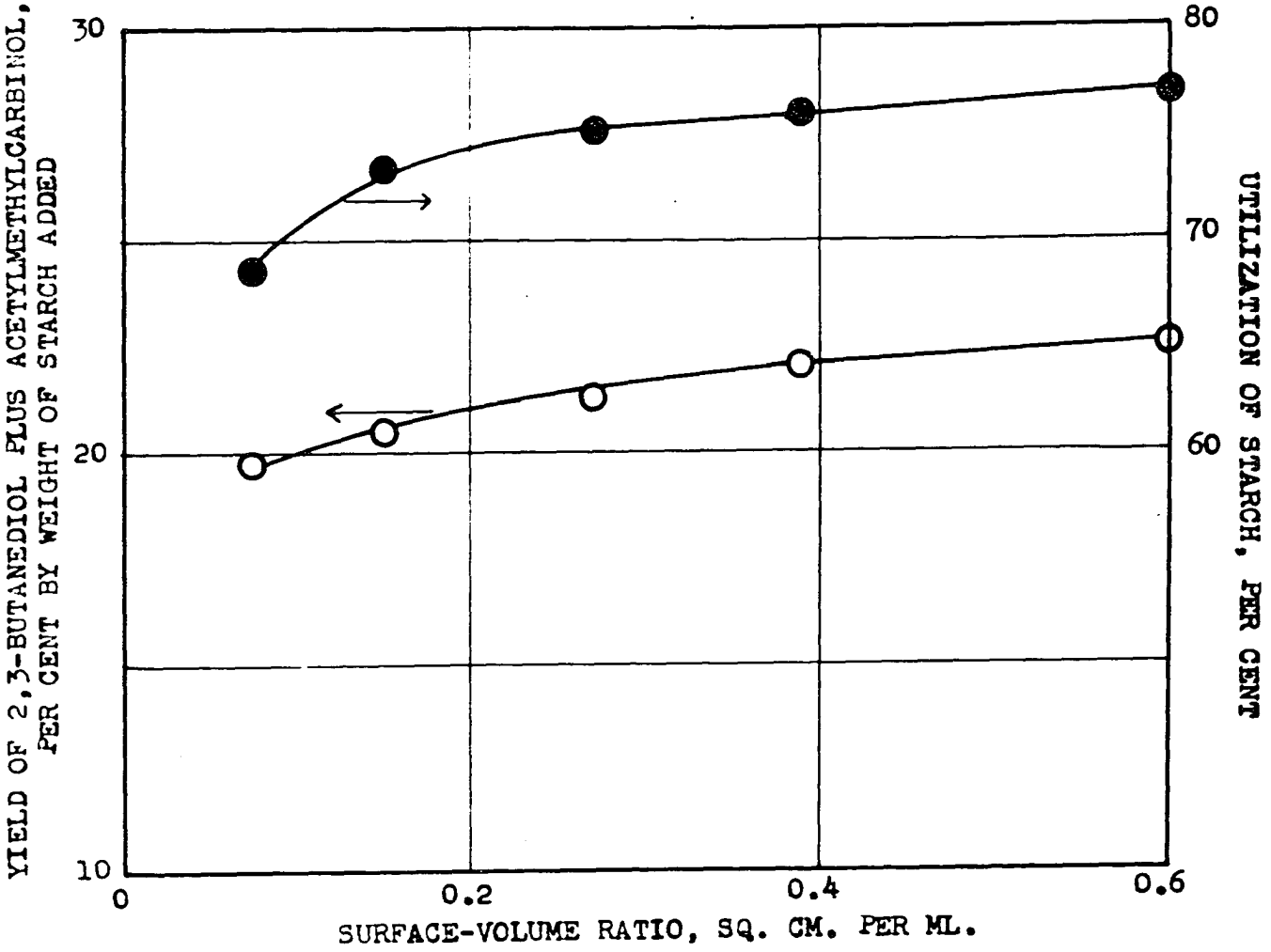


Fig. 18. Effect of variation in the surface-volume ratio upon the fermentation of corn starch by Aerobacillus polymyxa B32.

The results of the experiment show that the yields obtained when the fermentation is conducted in small flasks will probably be dissimilar to those which would be obtained on a larger scale; therefore, although the factors involved in the fermentation of corn starch may be studied on a laboratory scale, conclusions thus derived cannot be extrapolated to show the feasibility of industrial production.

c. Effect of the nature of the inoculum medium. A previous experiment has shown that when yeast extract was added to a medium containing corn gluten as the main source of nitrogen, an increase in the yield of 2,3-butanediol and in the extent of utilization of starch was obtained. Thus it was apparent that the nutritional requirements of Aerobacillus polymyxa were not completely satisfied by the corn gluten. Since yeast extract contains a number of growth-promoting substances, it is possible that the increase in utilization of the starch which resulted when yeast extract was added may have been due to an increase in the number of cells of the organism developed in the fermentation medium. In this event, the possibility of the development of a more active inoculum by the addition of yeast extract to the inoculum would be worth investigating.

The cost of yeast extract would prohibit its use on an industrial scale. It was therefore decided to substitute

malt sprouts and brewers' yeast, both low-cost materials, for the yeast extract and to determine the effect of the addition of these substances to the inoculum medium and to the fermentation medium.

The mashes and the inocula were prepared as previously indicated, all of the constituents being added prior to sterilization. Carbohydrate contents of the corn and starch employed have been given previously. Each inoculum contained an amount of corn or starch equivalent to a pure starch content of 3.42 grams per 100 ml. of water added. Each fermentation medium contained an amount of corn or starch equivalent to a pure starch content of 6.15 grams per 100 ml. of water added. The constituents of the various inoculating media were as follows:

Inoculum C

Corn	5.0 grams
Calcium carbonate	0.5 gram
Distilled water	100 ml.

Inoculum C,Y

Corn	5.0 grams
Calcium carbonate	0.5 gram
Dried brewers' yeast	0.5 gram
Distilled water	100 ml.

Inoculum C,M S

Corn	5.0 grams
Calcium carbonate	0.5 gram
Malt sprouts	0.5 gram
Distilled water	100 ml.

Inoculum C,Y,M S

Corn	5.0 grams
Calcium carbonate	0.5 gram
Dried brewers' yeast	0.5 gram
Malt sprouts	0.5 gram
Distilled water	100 ml.

Inoculum S,Y,M S

Corn starch	4.17 grams
Calcium carbonate	0.5 gram
Dried brewers' yeast	0.5 gram
Malt sprouts	0.5 gram
Distilled water	100 ml.

The fermentation mashes contained the following constituents:

Medium S

Corn starch	7.5 grams
Calcium carbonate	0.5 gram
Corn gluten	0.5 gram
Distilled water	100 ml.

Medium S,Y

Corn starch	7.5 grams
Calcium carbonate	0.5 gram
Corn gluten	0.5 gram
Dried brewers' yeast	0.5 gram
Distilled water	100 ml.

Medium S,M S

Corn starch	7.5 grams
Calcium carbonate	0.5 gram
Corn gluten	0.5 gram
Malt sprouts	0.5 gram
Distilled water	100 ml.

Medium S,Y,M S

Corn starch	7.5 grams
Calcium carbonate	0.5 gram
Corn gluten	0.5 gram
Dried brewers' yeast	0.5 gram
Malt sprouts	0.5 gram
Distilled water	100 ml.

Medium C

Corn	8.97 grams
Calcium carbonate	0.5 gram
Distilled water	100 ml.

Medium C,Y,M S

Corn	8.97 grams
Calcium carbonate	0.5 gram
Dried brewers' yeast	0.5 gram
Malt sprouts	0.5 gram
Distilled water	100 ml.

Each 200 ml. of inoculum was seeded with 10 ml. of a 24-hour culture of Aerobacillus polymyxa B32 grown on medium of the same composition as Inoculum C. Each 200 ml. of fermentation medium was seeded with 8 ml. of the inoculum indicated in table 37.

The fermentations were analyzed after 3⅛ days incubation. Results of the analyses are shown in table 37. It is evident that the addition of either malt sprouts or brewers' yeast, or a combination of the two, to the medium or to the inoculum stimulated the production of 2,3-butanediol by increasing the extent of utilization of the starch. The yield of 2,3-butanediol obtained when yeast and malt sprouts were added to the inoculum only was as good as the yield obtained when

Table 37

Effect of Addition of Malt Sprouts and Brewers' Yeast to the Inoculum

Aerobacillus polymyxa

Inoculum	C	C,Y	C,M S	C,Y,M S	C
Medium	S	S	S	S	S,Y
Yield of products, grams per 100 ml.					
2,3-Butanediol	1.30	1.45	1.53	1.62	1.63
Acetylmethyl-carbinol	0.08	0.09	0.09	0.10	0.11
Total	1.38	1.54	1.62	1.72	1.74
Ethanol	0.68	0.73	0.82	0.84	0.75
Yield of products, per cent by weight*					
2,3-Butanediol	20.7	23.0	24.4	25.8	26.0
Acetylmethyl-carbinol	1.3	1.4	1.4	1.6	1.8
Total	22.0	24.4	25.8	27.4	27.8
Ethanol	10.8	11.6	13.0	13.4	11.9
Yield of products, per cent of theory**					
2,3-Butanediol	63.9	64.0	63.5	63.9	67.2
Acetylmethyl-carbinol	4.1	4.0	3.8	4.0	4.6
Total	68.0	68.0	67.3	67.9	71.8
Ethanol	32.6	31.4	33.4	32.4	30.2
Total yield of products, per cent of theory**	100.6	99.4	100.7	100.3	102.0
Utilization of starch, per cent	58.4	64.8	69.0	72.8	69.4

* Based on starch added
** Based on starch utilized

Table 37

the Inoculum and to the Medium on the Fermentation of Corn Starch by

lus polymyxa B32

C	C	C,Y,M S	S,Y,M S	C	C,Y,M S	C
S,Y	S,M S	S,Y,M S	S	C	C	C,Y,M S
1.63	1.51	1.62	1.45	1.49	1.58	1.73
0.11	0.15	0.12	0.06	0.12	0.13	0.12
1.74	1.66	1.74	1.51	1.61	1.71	1.85
0.75	0.76	0.72	0.74	0.84	0.80	0.85
26.0	24.0	25.8	23.0	23.7	25.2	27.5
1.8	2.4	1.9	1.0	1.9	2.1	1.9
27.8	26.4	27.7	24.0	25.6	27.3	29.4
11.9	12.1	11.5	11.8	13.4	12.7	13.5
67.2	57.0	63.0	60.8	58.0	58.6	65.4
4.6	5.8	5.2	2.6	4.8	4.9	4.6
71.8	62.8	68.2	63.4	62.8	63.5	70.0
30.2	28.0	29.8	30.2	31.8	28.9	31.4
102.0	90.8	98.0	93.6	94.6	92.4	101.4
69.4	76.0	67.8	68.4	73.6	77.2	75.8

yeast was added to the medium but not to the inoculum, and was equally as good as that obtained when yeast and malt sprouts were added to both the inoculum and to the medium. Replacement of the corn in the inoculum by an equivalent amount of starch resulted in a decreased yield of 2,3-butanediol. In the fermentation of corn mashes, the addition of yeast and malt sprouts to the inoculum or to the medium also caused an increase in the yield of 2,3-butanediol obtained.

The results of the experiment show that the nature of the inoculum medium has a considerable effect on the results of the fermentations. Inasmuch as the addition of malt sprouts and yeast to the inoculum medium effected an increase in the yield of 2,3-butanediol without adding appreciably to the residual solids content of the fermented mash, this factor might well be taken into consideration as a starting point in future investigations.

It should be noted that the mashes were analyzed at 3½ days, rather than at 4½ days as in the preceding experiments. The differences in the extent of starch utilization and yields of 2,3-butanediol obtained upon the addition of yeast and malt sprouts to the inoculum and to the medium may be due mainly to differences in the rates of fermentation. Further experiments in which the mashes were analyzed at various

intervals would show whether the differences obtained would be less as the length of the fermentation period was increased. Even if the increase in the yield of 2,3-butanediol obtained upon addition of growth-promoting substances to the inoculum were due to a difference in the rate of fermentation, the increase in the rate of fermentation would be important industrially, since less equipment would be necessary if a shorter period of fermentation could be used.

G. Discussion of Results

The fundamental problem dealt with in this section was the preparation of 2,3-butanediol from corn starch by fermentation with Aerobacillus polymyxa. The advantages in using the starch rather than the whole grain as the substrate are: (1) the recovery of by-products from the raw material in separating the starch; (2) the absence of grain residues from the fermented mash. Whether or not these advantages would offset the difference in cost of the corn and starch as a substrate material would be to a great extent dependent upon the possibility of obtaining as high a yield of the 2,3-butanediol from starch mashes as from corn mashes of equivalent starch content.

A medium containing ammonium chloride as the source of nitrogen, supplemented by other inorganic materials, holds little promise as an effective medium for the production of 2,3-butanediol from corn starch. A medium containing complex organic materials as the source of nitrogen, on the other hand, shows great promise as a nutrient medium for producing the diol. Since the addition of either corn steep liquor solids or corn gluten to the medium is effective in bringing about high yields of the diol, the use of either of these two materials is to be particularly recommended. Both are by-products of the commercial starch separation process, and both are low-cost materials. The use of corn gluten as the source of nitrogen may have an additional advantage; since it is the last constituent of the corn to be removed from the starch in the starch separation process, it might not be necessary to effect a complete separation of the starch and gluten, thus saving an additional step in the processing of the starch.

The yield of 2,3-butanediol obtained by fermentation of a medium containing 7.5 grams of starch, 0.5 gram of calcium carbonate, 0.5 gram of corn gluten, and 0.003 gram of potassium permanganate per 100 ml. of water was equally as high as that obtained by fermentation of a 10 per cent corn mash containing 0.5 per cent calcium carbonate. These results show that the fermentation of corn mashes or starch mashes

can be conducted with equal efficiency.

The results obtained at varying surface-volume ratios indicate that it would not be practical to extrapolate the laboratory results to a prediction of the results which could be obtained in large-scale production.

Preliminary experiments were conducted at various starch concentrations. The results showed that mashes containing up to 10 grams of starch per 100 ml. could be fermented in 6 days with little variation in the yield of the 2,3-butanediol, while a decrease in the yields of all products occurred when the starch concentration was 12.5 per cent or greater.

The main difficulty foreseen for adaptation of the fermentation of corn starch by Aerobacillus polymyxa to commercial production of 2,3-butanediol is the incomplete utilization of the starch. In the present experiments, the maximum final concentration of 2,3-butanediol obtained was 1.69 grams per 100 ml. of water, while the residual carbohydrate, calculated as starch, was 1.3 grams per 100 ml. This amount of residual carbohydrate would undoubtedly interfere with the recovery of the 2,3-butanediol, particularly if the diol were to be recovered by distillation. Further investigations, therefore, might well be conducted in an effort to discover means of increasing the utilization of the starch. A few

preliminary experiments were conducted with this factor in mind, including inoculation of the fermented mash with yeast, reinoculation of the fermenting mash with Aerobacillus polymyxa, and inoculation of the fermented mash with Aerobacter aerogenes. However, none of these exploratory experiments met with success, although further investigations might be warranted.

H. Conclusions

Under the proper conditions, nearly 27 per cent (by weight) of the starch present in a 7.5 per cent starch mash can be converted to 2,3-butanediol in 4½ days by fermentation with Aerobacillus polymyxa B32.

The addition of ammonium chloride to a mash containing starch and calcium carbonate results in an increase in the yield of 2,3-butanediol. The addition of potassium dihydrogen phosphate causes a further increase. The addition of magnesium sulfate has little effect on the results.

The addition of organic sources of nitrogen results in higher yields of the 2,3-butanediol than the addition of ammonium chloride. Corn gluten and corn steep liquor solids are particularly effective.

The addition of a small amount of manganous sulfate or potassium permanganate to a starch medium containing corn gluten as the source of nitrogen effects a further increase

in the yield of the 2,3-butanediol.

The ability of Aerobacillus polymyxa B32 to form 2,3-butanediol does not vary appreciably upon extensive subculturing on 5 per cent corn mash.

The formation of 2,3-butanediol by Aerobacillus polymyxa B32 is considerably affected by a change in the surface-volume ratio of the medium. At higher surface-volume ratios, more of the starch present is utilized and more 2,3-butanediol is formed.

The addition of malt sprouts or brewers' yeast to the inoculum or to the medium causes an increase in the utilization of the starch and an increase in the yield of 2,3-butanediol.

VII. RECOVERY OF 2,3-BUTANEDIOL FROM FERMENTATION MASHES

A. Introduction

The recovery of 2,3-butanediol from fermented mashes is complicated by the high boiling point of the diol and by its affinity for water. In addition, fermented mashes usually contain appreciable amounts of solid material in solution and in suspension. The solids content of the fermented media, particularly if the fermentation is conducted on whole grain mashes, is often higher than the diol content.

The main purpose of this portion of the investigation was to recover the 2,3-butanediol from the fermented mashes. The 2,3-butanediol recovered was used in the previously mentioned investigations of Fulmer, Underkofler and Bantz (1943), Kolfenbach (1944), Lees, Fulmer, and Underkofler (1944), and Underkofler, Fulmer, Bantz, and Kooi (1944).

B. Experimental Results

The recovery experiments were carried out on fermented media resulting from the fermentation of dextrose media by Aerobacter aerogenes and from the fermentation of corn mashes by Aerobacillus polymyxa. The initial treatment of the fermented media was the same in all cases. The medium was

adjusted to pH 8 to 10 by the addition of concentrated sodium hydroxide and filtered through a diatomaceous earth filter cake. The filtrate was then readjusted to pH 7 and concentrated under vacuum to about one-fourth the original volume. This concentrated solution will hereafter be referred to as the concentrated liquor.

1. Recovery by distillation

A number of distillation procedures were tried for the recovery of the 2,3-butanediol from the concentrated liquor. By employing ordinary vacuum distillation, followed by fractional distillation of the distillate, approximately 60 per cent of the diol present in the fermentation liquor was recovered.

One variation of the vacuum distillation technique attempted involved introducing the concentrated solution dropwise into a distillation flask connected through a condenser and a receiver to the vacuum source. By keeping the temperature of the distillation flask at about 200°C., the diol-water mixture could be distilled continuously until a large amount of solids was deposited in the distillation flask. Upon fractional distillation of the resulting distillate, about 60 per cent of the diol present in the fermentation liquor was recovered. An additional 12 per cent was recovered

by extracting the distillation residue with 95 per cent ethanol and fractionating the resulting solution.

2. Recovery by solvent extraction

Solvent extraction was found to be a more practical recovery method on a laboratory scale than was vacuum distillation. Diethyl ether was used as the extraction solvent. A number of commercially available laboratory continuous liquid-liquid extractors were tried but most of these were poorly designed and the extractions became time-consuming operations. As a result, a laboratory continuous countercurrent liquid-liquid extractor was designed by Kolfenbach, Kooi, Fulmer, and Underkofler (1944). Using this extractor, over 90 per cent of the 2,3-butanediol present in a concentrated liquor containing 15 per cent of 2,3-butanediol was extracted in about 75 hours of continuous operation. The diol in the extract was recovered by evaporating the ether over a water bath and fractionally distilling the residual liquid. Overall recovery of the 2,3-butanediol by this procedure was 85 to 90 per cent of that present in the fermentation liquor.

VIII. SUMMARY

1. A study of the production of 2,3-butanediol from dextrose by fermentation with Aerobacter aerogenes was undertaken. By adding calcium carbonate to the medium and aerating, the fermentation time was decreased to as little as 34 hours. In fermentations conducted on a large laboratory scale, yields of 2,3-butanediol as high as 35 per cent by weight of the dextrose added have been obtained.

2. A study of the production of 2,3-butanediol from corn by fermentation with Aerobacter aerogenes was undertaken. The use of brewery equipment and techniques for conducting the fermentation was investigated. The addition of manganous sulfate to the fermentation medium, the lowering of the saccharification temperature, and the addition of malt or malt extract to the fermenting medium effected an increase in the yield of 2,3-butanediol obtained. In fermentations conducted on a laboratory scale and on a semi-pilot plant scale, 35 per cent of the weight of maltose present in a filtered wort has been converted to 2,3-butanediol.

3. The production of 2,3-butanediol from corn by fermentation with Aerobacillus polymyxa has been investigated. Long periods of fermentation led to an increase in the yield

of acetylmethylcarbinol and a decrease in the yield of 2,3-butanediol, particularly at low mash concentrations. The weight per cent yields of 2,3-butanediol obtained from pre-thinned mashes containing up to 40 grams of corn per 100 ml. were nearly as high as the yields obtained from more dilute mashes. However, considerable residual carbohydrate remained when the mash concentration was high. Nearly 30 per cent of the weight of starch present in a 10 per cent corn mash was converted to 2,3-butanediol in 4½ days.

4. A study of the production of 2,3-butanediol from corn starch by fermentation with Aerobacillus polymyxa was undertaken. The effect of the addition of a number of inorganic and organic materials to the medium was investigated. The addition of complex sources of nitrogen, such as corn gluten or corn steep liquor solids, was essential to producing high yields of 2,3-butanediol. By the addition of a small amount of potassium permanganate to a medium containing corn starch, calcium carbonate, and corn gluten, the yield of 2,3-butanediol obtained was as high as that obtained in the fermentation of a corn mash equivalent in starch content to the starch mash. Yields of 2,3-butanediol obtained were nearly 27 per cent by weight of starch added.

Extensive subculturing of the organism was found to have little effect upon the yields of 2,3-butanediol obtained.

Higher yields of 2,3-butanediol were obtained from mashes with high surface-volume ratios than from mashes with low surface-volume ratios.

The addition of malt sprouts and brewers' yeast to the inoculum medium resulted in higher yields of 2,3-butanediol than those obtained when these substances were not added to the inoculum medium.

5. The recovery of 2,3-butanediol from fermented media by solvent extraction was found to be more efficient than recovery by distillation. It was found possible to recover 90 per cent of the 2,3-butanediol present in the fermentation liquor.

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X. APPENDIX

DESIGN AND CONSTRUCTION OF SEMI-PILOT PLANT FERMENTER AND EXTRACTOR UNITS

The semi-pilot plant fermenters and the extractor shown in the diagrams below were designed and constructed in connection with the foregoing investigations. It was originally planned to follow each phase of the investigation with semi-pilot plant fermentations in order to determine if the results obtained in the laboratory could be duplicated on a larger scale. The difficulty in obtaining materials prevented the realization of this objective during the course of the present investigation.

The floor plan and the location of the equipment are shown in figure 19. The still, condenser, and vacuum pump were commercial semi-pilot plant units. The floor was constructed of concrete, and all of the equipment was anchored on raised concrete blocks. The floor was so constructed that all points sloped toward the drain.

Figure 20 shows the line diagram of the 50-gallon fermenter unit. All outlets to the fermentation tank were equipped with tapped valves. Provision was thereby made for sterilization by passing steam through the portions of the pipes which could not be sterilized by steam under pressure.

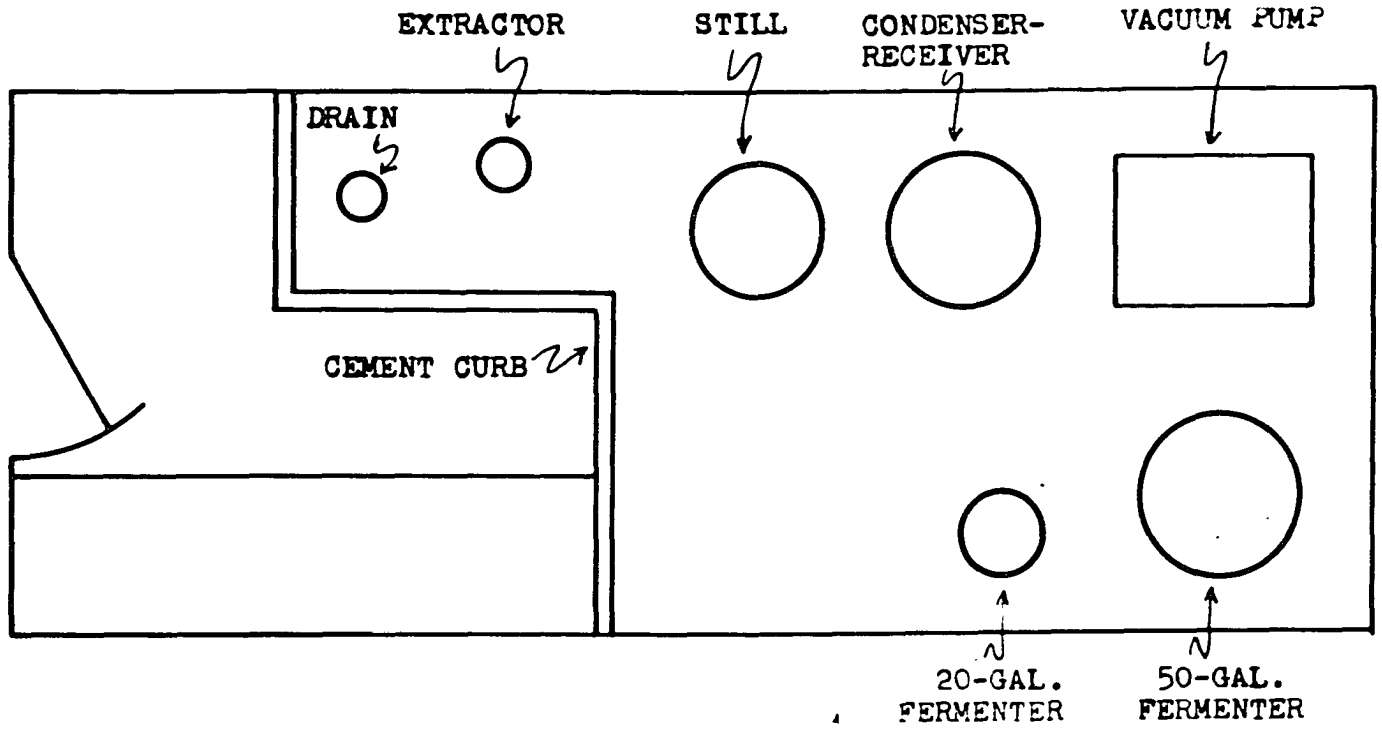

Fig. 19. Floor plan.

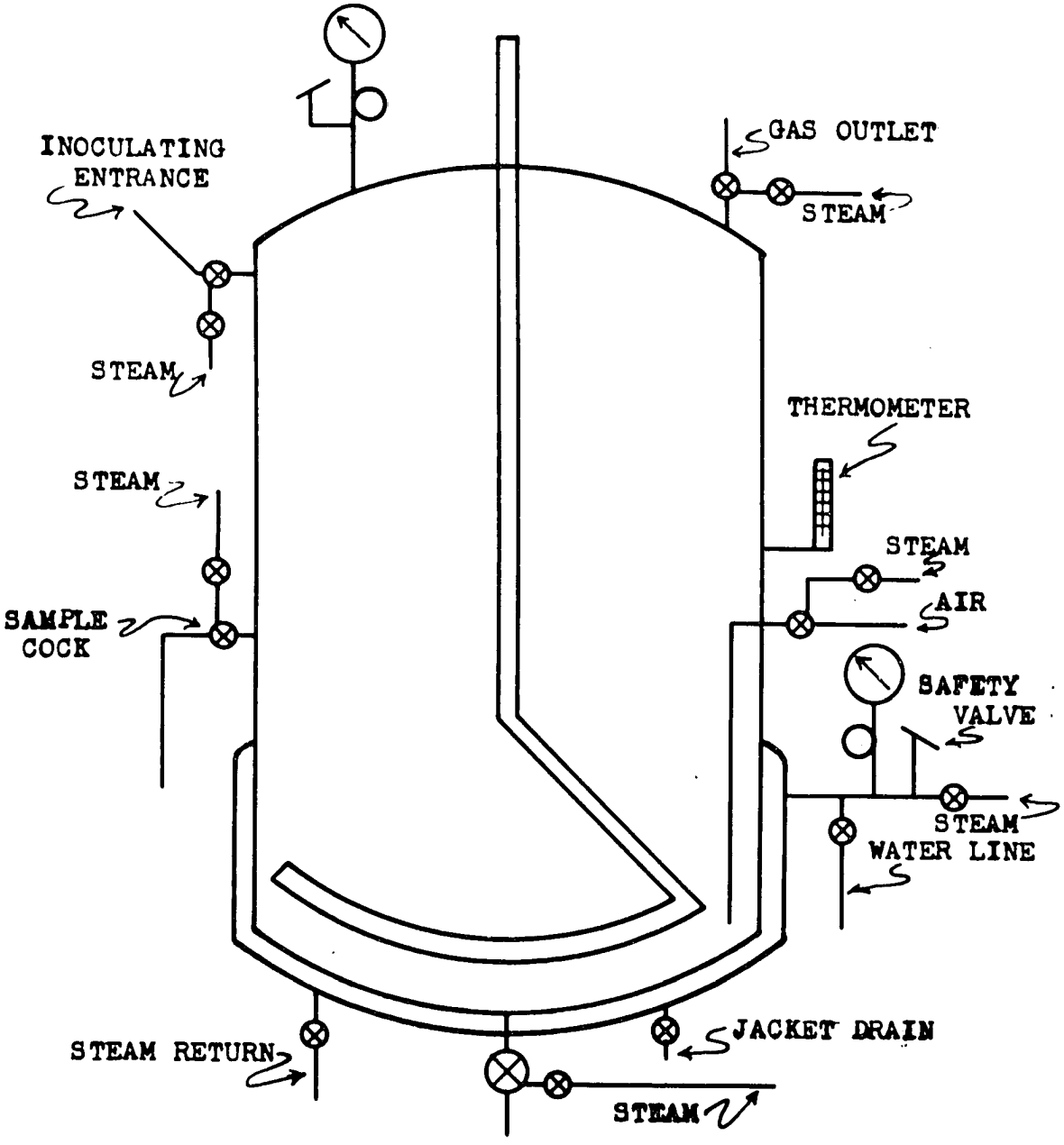

Fig. 20. Fifty-gallon fermenter. Piping diagram.

Provisions were also made for running cooling water through the water jacket.

The line diagram of the 20-gallon fermenter is shown in figure 21. The piping detail is similar to that for the larger fermenter, except that the steam is introduced into a coil rather than into a jacket. Direct steaming of the medium can be carried out by capping the fermenter drain and introducing live steam through the steam line connected to the drain valve.

The line diagram for the proposed construction of a semi-pilot plant extractor unit is shown in figure 22. The design has been patterned after the laboratory continuous counter-current liquid-liquid extractor of Kolfenbach, Kooi, Fulmer, and Underkofler.[1] The column is a 6-foot section of 5-inch inside-diameter cast iron pipe. Three 12 by 1 inch sight glasses are spaced at intervals on the front of the column to permit observation of the solvent and feed levels and of the degree of dispersion. The piping details allow for the use of either lighter-than-water solvents or heavier-than-water solvents. This extractor has been designed for use with a packed column and discontinuous feed.

[1]Kolfenbach, J. J., E. F. Kooi, E. I. Fulmer, and L. A. Underkofler. Ind. Eng. Chem., Anal. Ed. 16, 473-474 (1944).

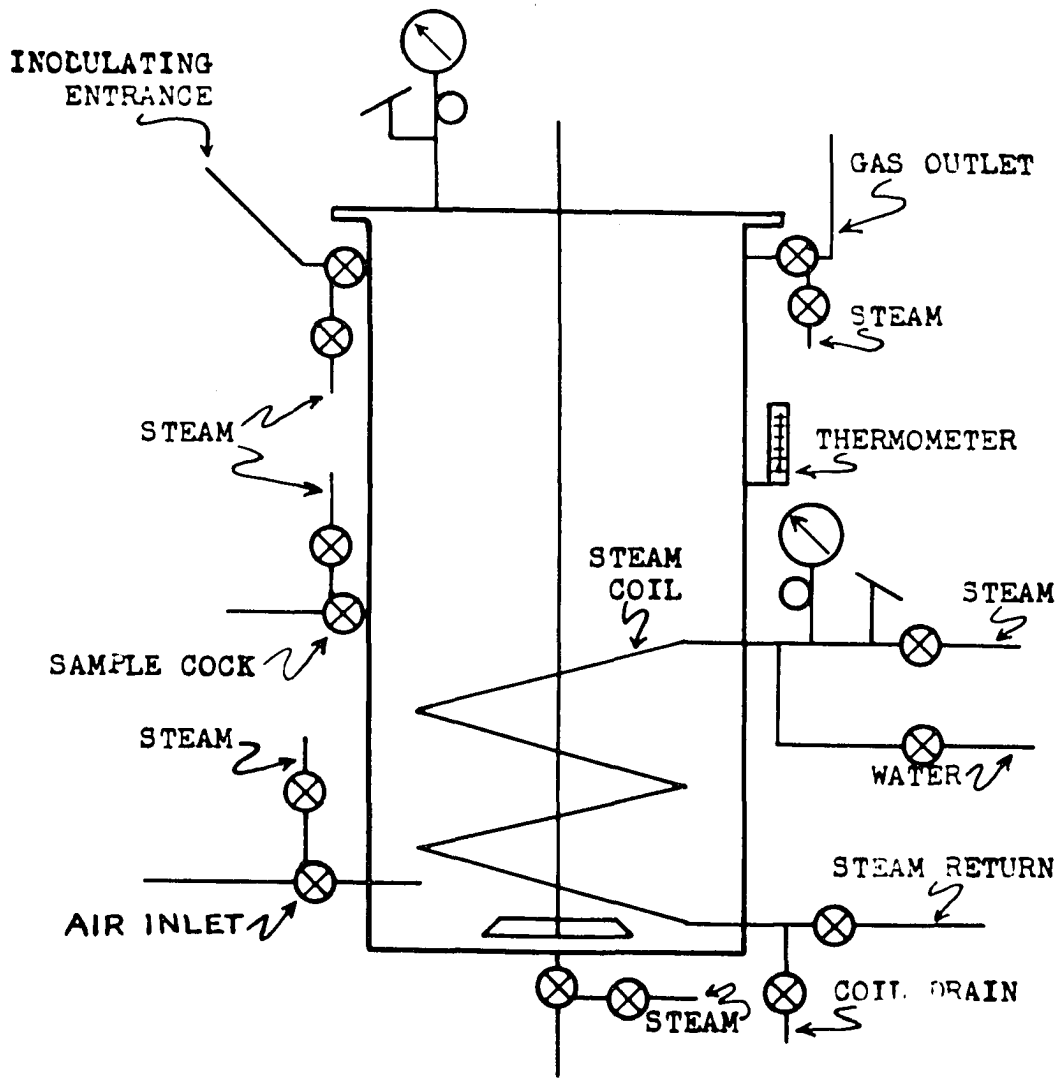

Fig. 21. Twenty-gallon fermenter. Piping diagram.

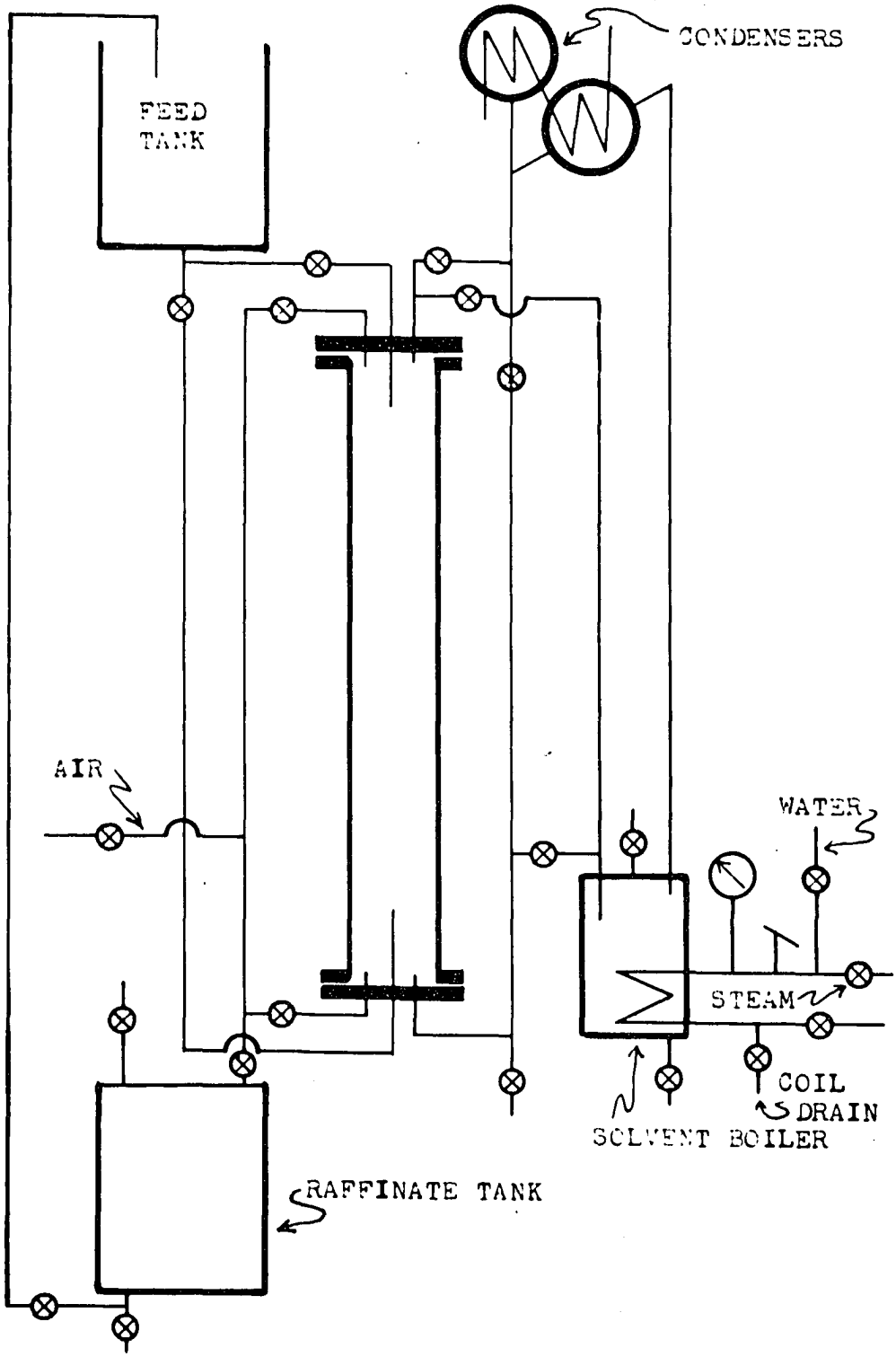

Fig. 22. Semi-pilot plant extractor. Piping diagram.

The solvent is continuously vaporized in the boiler shown at the right in figure 22. The feed is placed in the tank shown at the top of the figure and after passing through the column is collected in the raffinate tank shown at the bottom. When the feed tank has been emptied, the raffinate is forced up to the feed tank by air pressure and recirculated.