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UMI

THE BUTYL-ACETONIC FERMENTATION OF THE SUGARS WITH SPECIAL REFERENCE TO XYLOSE

by

LELAND A. UNDERKOFLER

A Thesis Submitted to the Graduate Faculty

for the Degree

DOCTOR OF PHILOSOPHY

Major Subject Biophysical Chemistry

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1934

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I. INTRODUCTION

The butyl-acetonic fermentation, as a process of industrial importance, had its beginnings in the years just prior to the start of the World War. During the war the process was further developed for the production of acetone, and in the years following the war the demand for butyl alcohol resulted in the growth of the butyl alcohol fermentation industry until it now ranks with the largest chemical industries in the United States. This industry, just before the depression, utilized about 30,000,000 bushels of corn annually, the principal use of the butyl alcohol being in the preparation of nitrocellulose lacquers. The finish on the average automobile represents the fermentation products from one and three-quarters bushels of corn.

A mash of corn meal is the only substrate which has been used commercially with complete success for this fermentation. The starch of the corn is transformed into the solvents butyl alcohol, acetone, and ethyl alcohol in the approximate ratio of 6:3:1, together with carbon dioxide and hydrogen. The latter two products are used in the synthesis of methanol. Low grade corn, unfit for feeding purposes, as well as better grades of corn are suitable for use in the process.

The commercial development of this fermentation industry marks a big step in the utilization of agricultural products in the manufacture of industrial chemicals, and it is, of course, very gratifying to the chemist to observe how chemical industry is making more and more use of farm products. However, the chemistry department at Iowa State College, along with various other research agencies, has also turned considerable attention to the practical commercial utilization of agricultural wastes, such as corn stalks, corn cobs, oat hulls, peanut shells, cottonseed hull bran, and the like.

These wastes contain considerable amounts of cellulose, which, by acid hydrolysis, can be partially converted into dextrose, but the cost of the high concentration of sulfuric acid required is great, the yields of dextrose obtained are only a fraction of the theoretical, and the process, so far at least, has not proven commercially feasible. But it has also been discovered that from many of these important agricultural wastes considerable quantities of the pentose sugar xylose can be obtained rather easily and cheaply by hydrolysis. Emley (1930) gives the following figures for the xylose content of several of these wastes:

Material	Percent Xylose by Weight of Bone-dry Material
Bagasse	25 to 30
Artichoke tops	15
Cornstalks	29 to 31
Corncobs	31 to 37
Peanut shells	23
Oat hulls	31
Cotton burrs	19
Cottonseed hull bran	40

A semi-commercial plant at Anniston, Alabama, has made crystalline xylose, produced from cottonseed hull bran, available in quantity since 1929, and considerable attention has been devoted by various agencies to discovering possible uses for this sugar. Among the many research projects at Iowa State College endeavoring to make use of farm wastes are several which are attempting to find uses for xylose. One possible use for this sugar is the conversion of it into useful chemicals by fermentation processes.

In commercial fermentation processes utilizing xylose, the crystallized sugar will probably not be used, but instead the liquor obtained by the hydrolysis of the materials containing pentosans.[2] The hydrolytic products from pentosan-containing materials will probably prove to be the cheapest and most easily available fermentable materials unless the process for hydrolyzing cellulose to produce dextrose becomes more practical.

However, the availability of crystallized xylose is essential in order that fundamental information about the behavior of this substance may be obtained. Owing largely, no doubt, to the fact that the sugar has been economically available in considerable quantities for such a short time, the use of xylose in fermentations has not yet been investigated very thoroughly.

No extensive investigations to determine the possibility of using xylose in the butyl-acetonic fermentation have been reported. Fundamental investigations of the butyl-acetonic fermentation have been confined, with a very few exceptions, to studies of the fermentation of starch in corn meal. The information to be found in the literature concerning the fermentation of carbohydrates other than starch is extremely limited.

The primary purpose of the present investigation was to study the fermentation of xylose by the butyl organism. This problem was chosen, first, because of the great industrial importance of the butyl-acetonic fermentation, second, because of the meager information available concerning the butyl-acetonic fermentation of sugars, and third, because fermentation methods afford one of the most promising ways for utilizing farm wastes. However, in order to investigate adequately the fermentation of xylose, a knowledge of how the organism attacks other carbohydrates was necessary, and

therefore a secondary purpose of this investigation was to study the fermentation of sugars in general by the butyl organism.

In order to achieve these purposes, the course of the butyl-acetonic fermentation of several carbohydrates was followed carefully, particular attention being given to xylose; an attempt was made to develop the best possible medium of known composition for the utilization of xylose; and other factors which might influence the fermentation of xylose were studied.

II. HISTORICAL

A. The Fermentation of Xylose.

One of the characteristics used for the differentiation of micro-organisms is the ability to ferment various carbohydrates. Xylose is one of the sugars long used by the bacteriologists for this purpose. For many years this was almost the sole use for this sugar.

It has long been known that xylose could readily be obtained by the hydrolysis of xylan. This substance, next to cellulose and lignin, is probably the most widely distributed organic compound found in nature. It has been found in various grains, straws, gums, woods, parts of the corn plant, and other plant substances. The earliest methods for preparing xylose, for instance as given by Stone and Lotz (1891), isolated the xylan prior to hydrolysis, but Bertrand (1891) showed that isolation of the xylan before hydrolysis was unnecessary.

The ordinary laboratory methods for the preparation of xylose--Hudson and Harding (1917,1918)--rely upon the use of alcohol or glacial acetic acid to make the sugar crystallize. The demand of the bacteriologists was so small that no attempts

were made to put production on a commercial basis, and the resulting price of about one hundred dollars a pound quite effectually prevented much experimentation with this sugar.

In 1928, however, when the Bureau of Standards was authorized by Congress to investigate and to develop uses for the waste products from the land, among the projects begun was the recovery of xylose. A report of the preliminary investigations carried on in Washington was given by Hall, Slater, and Acree (1930). In 1929 the Bureau of Standards put into operation at Anniston, Alabama, a semi-commercial plant for the production of xylose from cottonseed hull bran. The plant and its operation was described by Schreiber, Geib, Wingfield, and Acree (1930). Considerable quantities of xylose have been made available by this plant.

Due mainly to the short time it has been economically available in quantities, comparatively few studies have been made of the fermentation of xylose in which the dissimilation products have been adequately identified. Buchanan and Fulmer (1930), and Fulmer and Werkman (1930) have reviewed the fermentation chemistry of xylose. Since the appearance of these summaries, papers by Werkman, Hixon, Fulmer, and Rayburn (1929), Guittonneau, De Laval, and Bejambes (1930), Breden and Fulmer (1931), and Weinstein and Rettger (1931, 1932, 1933) which report the dissimilation products from xylose when fermented by

various organisms, have appeared, and are listed here for the sake of completeness.

B. The Butyl-Acetonic Fermentation of Sugars.

According to Donker (1926) the first record of the discovery of butyl alcohol as a fermentation product is that of Pasteur in his "Etudes sur la biere". He observed its formation in small amounts during the butyric fermentation of sugars, mannite, lactates, and tartrates brought about by his Vibrio butyricus, later renamed Bacillus butyricus by Cohn.

Following the work of Pasteur, a large number of early investigators reported the production of butyl alcohol in fermentations carried out by them. The first attempts at industrial utilization of a butyl fermentation were made in England under the patents of Fernbach and Strange (1911-1912). During the war, the demands for acetone in large quantities for the production of munitions led to the establishment by the allied governments of large plants in England, Canada, India, and the United States for the butyl-acetonic fermentation of corn mash. With the later development, after the war, of the nitrocellulose lacquer industry which required butyl alcohol in the preparation of butyl acetate for the lacquer solvent, the butyl alcohol fermentation industry under private management has made rapid progress until it is now a major

chemical industry. The patents of Weizmann (1915,1919,1922) cover the war-time and post-war operations.

The commercial importance of the butyl-acetonic fermentation has led to a very considerable amount of research work, most of which has been confined to the fermentation of starch, particularly of starch in corn meal.

The first systematic study of the action of the butyl organism on various different carbohydrates was reported by Robinson (1922). This author investigated the butyl fermentation of the monosaccharides xylose, arabinose, glucose, fructose, mannose, galactose, rhamnose; of the disaccharides sucrose, maltose, lactose, melibiose, trehalose; of the trisaccharides raffinose, melezitose; of the polysaccharides starch, dextrin, inulin; and of the alcohols glycerol and mannitol. A number of sugar mixtures were also fermented. Robinson's nutrient solutions contained 0.1 percent monopotassium phosphate, 0.02 percent magnesium sulfate, 0.001 percent ferrous sulfate, 0.001 percent sodium chloride, and 0.5 percent Bacto-peptone. At first 5 percent carbohydrate was used, but as 3 percent concentrations gave more complete fermentations, this concentration was employed for most of the work. In no case was a complete analysis of the products made, but the course of the fermentations was followed by determining the titratable acidity at periodic intervals, and

the progress of carbohydrate consumption was determined by periodic sugar determinations.

Robinson found that, based on their reaction toward fermentation by the butyl organism, the sugars fell into two groups. Sugars of the first group were fermented normally. Characteristics of the normal fermentations were: rapid rise in acidity to a maximum, followed by a decline, and later by a second slight increase towards the end of the fermentation. Sugars of the second group behaved very differently. The acidity curves reached very high maximums, and then fell only a very little if at all, and the utilization of the carbohydrates was very incomplete. In the first group, according to Robinson's results, belonged the monosaccharides glucose, fructose, mannose, the disaccharides sucrose, maltose, lactose, and the polysaccharides starch and malt dextrin. Glucose, of the monosaccharides, seemed to ferment most readily. Of the disaccharides, maltose was most readily fermented, sucrose in a slightly longer time, and lactose required fully 24 hours longer than maltose. In the second group, giving abnormal fermentations, belonged the monosaccharides xylose, arabinose, galactose, the trisaccharides raffinose, melezitose, and the polysaccharides inulin and acid dextrin. The poly-alcohol, mannitol, gave an abnormal fermentation. Rhamnose, melibiose, trehalose and glycerol were not attacked by the butyl organism.

The fermentations of various sugar mixtures also were studied. A mixture of 3 percent sucrose and 2 percent glucose in the nutrient solution gave a rapid fermentation and an acidity maximum was reached in about 30 hours. This was followed by the characteristic fall, and later by a marked rise, showing evidence of a secondary fermentation. Sugar determinations showed that the organism had a marked preference for glucose, all glucose being consumed in 72 hours, while no sucrose had been utilized. After this the sucrose was utilized slowly. Normal fermentation of glucose or of sucrose lasted 3 to 4 days, but this double fermentation lasted for fully 9 days.

When a medium containing 2.4 percent lactose and 0.5 percent glucose was fermented the result resembled the glucose-sucrose fermentation in that the disaccharide was not fermented until after the last of the glucose had disappeared. A medium containing 2.4 percent maltose and 0.5 percent glucose was fermented normally, and no preference was shown for either sugar. Both were utilized at about the same rate. A half-and-half mixture of glucose and galactose showed a double fermentation. First the acidity curve behaved as in the normal glucose fermentation, but after the first break the acidity rose again until a higher acidity was reached than before, and this was maintained as in a pure galactose fermentation.

When toluene was added to vigorously fermenting cultures of the butyl organism in different media in order to stop cellular activities, Robinson's results showed that amylase, inulinase, and maltase were secreted by the organism into the media, but that neither sucrase nor lactase was secreted. Sucrose and lactose were therefore presumed to be hydrolyzed within the cell. Raffinose was believed to be hydrolyzed by sucrase within the cell into melibiose and fructose, the former of which was not fermented but was excreted and accumulated in the medium.

In the following year, from the same laboratory, appeared an extension of Robinson's investigations. This report by Speakman (1923) not only gave total acidity curves, but also the individual curves for butyric, acetic, and non-volatile acids. Quantitative determinations of neutral products, or solvents, were not made. Speakman again called attention to the two types of fermentation, (1) the normal fermentation characterized by practically complete utilization of carbohydrate, good solvent yields, and rapid rise in acidity curve followed by a fall, and (2) the abnormal fermentation, characterized by low consumption of carbohydrate, poor yields of solvents, and an acidity curve that does not fall markedly after the maximum is reached.

In addition to maize mash, Speakman submitted glucose,

xylose, arabinose, galactose, and mannitol to the butyl fermentation. Dulcitol was found not fermentable. All gave normal products, but the proportions of the neutral solvents were not determined. Speakman's nutrient solution differed only slightly from that used by Robinson. To distilled water was added 0.05 percent dipotassium phosphate, 0.05 percent monopotassium phosphate, 0.02 percent magnesium sulfate, 0.001 percent sodium chloride, 0.001 percent ferrous sulfate, 0.001 percent manganous sulfate, 0.5 percent peptone, and 3 percent carbohydrate.

Fermentations of glucose were much slower than of maize, and accumulation of non-volatile acid was most rapid in the first phase of the fermentation, contrary to the case in maize mash, in which the non-volatile acid accumulated only at the end of the fermentation. The maximum concentration of non-volatile acid corresponded to the peak of maximum acidity, and after this there was no marked change. After gas evolution had ceased, a slow rise in total acidity occurred, due entirely to the production of non-volatile acid.

Speakman believed he had demonstrated, on the basis of qualitative tests, that the non-volatile acid was mainly lactic acid, and that it was formed from sugar and not from protein. However, Schmidt, Peterson and Fred (1924) demonstrated conclusively that the non-volatile acid from maize

was chiefly l-leucic acid (alpha-hydroxy-isocaproic acid) although others might be found in traces also, and that the probable source was the protein, since the butyl organism is markedly proteolytic. These authors believed this might account for the fact that non-volatile acid was produced mainly at the end of the maize fermentation, but earlier in all the fermentations given by Speakman using his semi-synthetic medium containing peptone. In the latter case the protein was already partially broken down into simpler substances.

From his results with the fermentations of the other sugars, Speakman concluded that there were several important distinguishing features in the fermentations which appeared to be similar from the standpoint of final products. His pentose fermentations differed from the normal glucose fermentation in that production of acetic acid was higher than that of butyric acid, whereas the reverse was true for all of the six-carbon compounds investigated. In the case of the two pentose fermentations, the fermentation of arabinose was more rapid and more complete.

The fermentation of galactose was active for 40 hours, and then became slow and sluggish. In the first stage, production of acetic and butyric acid was rapid, but in the second stage the production of volatile acids rapidly diminished while non-volatile acid increased in amount. Speakman

regarded the last period as a true lactic fermentation. With mannitol, the results obtained were similar to those with galactose except that the total acidity was not so great, due mainly to the production of less acetic acid.

At the end of his paper Speakman advanced the theory that the butyl organism seemed to attack most rapidly those sugars in which hydroxyl groups attached to neighboring carbon atoms are also adjacent, as a possible explanation of the influence of structure of fermentable sugars on the fermentation products.

An important contribution from the standpoint of the present investigation was that of Peterson, Fred and Schmidt (1924), who investigated the fermentation of the pentoses xylose and arabinose by the butyl organism.

For Speakman both of these sugars gave abnormal fermentations on the basis of acid production; no data on solvent yields were given. Peterson, Fred and Schmidt obtained a good production of solvents from xylose, although not equal to that obtained from glucose. Sugar media of 2 percent concentration, using either yeast water or Robinson's peptone-salt nutrient, were used for the study. On the average, the xylose changed into solvents was 21.7 percent, while glucose averaged 27.0 percent changed into solvents. Glucose was found to ferment more rapidly than xylose. The fermentation of glucose in 2 percent concentration in Robinson's peptone-salt medium was

exceedingly rapid, being completed in about 30 hours. At the end of 66 hours 90 percent of the xylose was fermented in Robinson's medium, this rate being about the same as normal maize fermentations. Only a slight break in the acidity occurred in the case of xylose, but the authors state that a definite break in acidity in xylose fermentations was not uncommon. Breaks ranging from 0.5 to 1.3 cubic centimeters of 0.1 normal acid per 10 cubic centimeters of culture were observed in a dozen fermentations. The break in acidity for arabinose fermentations was also less marked than for glucose. The final acidity was uniformly high in the pentose fermentations, and was found to be due to larger amounts of volatile acids than were found in the glucose fermentation. These higher residual acidities accounted for the lower yield of solvents from the pentoses. The ratio of two parts butyl alcohol to one part acetone was about the same for glucose and xylose, but the proportion of acetone was considerably larger for arabinose.

Carbon balances for xylose, arabinose, and glucose in 2 percent concentration in yeast water media were obtained. Several assumptions were made in calculating the values. The difference between total solvents and acetone was called butyl alcohol, although it was recognized that 10 to 20 percent of this consisted of ethyl alcohol and minor products;

in calculating the volatile acids an average molecular weight of 70 was taken; the non-volatile acid was calculated as lactic acid. In all the cases a slightly larger weight of products was obtained than sugar destroyed, this discrepancy being laid by the authors to yeast water. In a fermentation containing 19.1 grams of xylose, 21.19 grams of products (butyl alcohol, acetone, volatile acids, non-volatile acids, and carbon dioxide) were obtained. From 19.2 grams of arabinose 22.15 grams of products were formed, and from 19.1 grams of glucose 21.40 grams of products were obtained.

Johnson, Peterson and Fred (1931) studied the fermentation of glucose, mannitol, calcium gluconate, and arabinose by the butyl organism with special reference to the relation between degree of oxidation of the compound fermented and the distribution of the various oxidized and reduced products of fermentation. The fermentations of these sugars were carried out in Speakman's medium using 2.5 percent substrate in each case. These workers summarized their findings as follows:

> "When mannitol, a reduced compound, is fermented, large amounts of hydrogen and of butyl alcohol are produced. The production of acetone is small, and almost as much butyric as acetic acid is formed. When glucose is fermented, less hydrogen and butyl alcohol and more acetone are produced than are formed from mannitol. The ratio of acetic to butyric acid is higher in the case of glucose. Calcium gluconate, which is more oxidized than glucose, is fermented largely into acids, because of the neutralizing effect of the calcium ion, and the oxidized nature of

> the substrate. Here much more acetone than butyl alcohol is formed, and there is a high acetic to butyric acid ratio. Hydrogen production, however, being a corollary of acid production, is high. When arabinose, which has the same degree of oxidation as glucose, is fermented, an 'oxidized' type of fermentation is produced. Acetone production is high and butyl alcohol production is low. The acid produced is largely acetic. The production of hydrogen is small.
>
> "The appearance of an oxidized type of fermentation from arabinose seems to be related to the ready splitting off of only one molecule of carbon dioxide for each molecule of pentose fermented. The resulting scarcity of hydrogen available for reduction results in a large production of a substance whose formation does not involve reduction reactions, namely acetone. The total amount of carbon dioxide evolved, is, however, more than can be accounted for by a hypothesis predicting the preliminary splitting of the pentose molecule into a two-carbon and a three-carbon compound, and with the subsequent fermentation of these fractions in the conventional manner."

In the experiments of Johnson, Peterson, and Fred, about 95 percent of the sugars were utilized when glucose and arabinose were fermented. No data were given as to the completeness of utilization of mannitol and calcium gluconate. The fermentation of mannitol is known to be abnormal (Robinson, Speakman), and one may well wonder if the utilization of this substrate, as well as of the calcium gluconate, were not very incomplete. In the fermentation of the latter compound it seems hardly advisable to compare it with that of sugars which do not have the calcium ion involved. It has been definitely shown by Speakman (1920) and by Stiles, Peterson and Fred (1929) that the presence of calcium carbonate in a

maize fermentation produces a marked change in the ratio of products formed, and a similar behavior might be expected in the fermentation of calcium gluconate which would yield calcium carbonate during the fermentation.

It is also rather difficult to reconcile the differences in the fermentations of arabinose and xylose reported in the two papers by Peterson, Fred and Schmidt (1924) and Johnson, Peterson and Fred (1931) just discussed. Being stereoisomers, both should have the same degree of oxidation, yet the ratio of the yields of solvents from xylose was very nearly the same as from glucose, as expected, while this was not true in the case of arabinose. This would seem to indicate a weakness in the theory of these authors.

The most recent contribution to our knowledge of the dissimilation of sugars by the butyl organism is that of Weinstein and Rettger (1933). These authors, in attempting to corroborate the observations of Robinson that the sugars could be put into two classes according to the kind of acidity curve they gave on butyl fermentation found that in no case when they fermented any of the sugars glucose, levulose, galactose, lactose, maltose, sucrose, xylose, arabinose, starch, or dextrin by any of six strains of the organism in Robinson's medium did the acidity materially decrease after the maximum had been reached. Normal yields of acetone were obtained, but

only very small amounts, if any, of butyl alcohol. Each of the six strains fermented corn mash in the normal way, producing the typical acidity curve with its definite break, and giving high yields of solvents in the ratio of approximately two parts of butyl alcohol and one part of acetone.

Investigation of several factors by these authors led them to the conclusion that an alcohol-soluble protein or prolamine, such as zein from corn, or a closely allied or associated substance appeared to be necessary for the production of normal amounts of acetone and butyl alcohol from carbohydrates in semi-synthetic medium. Each of the carbohydrates xylose, arabinose, and glucose yielded butyl alcohol and acetone in a ratio of approximately two to one when zein was added to the semi-synthetic medium. This is in exact agreement with the results of Peterson, Fred and Schmidt without the addition of zein for xylose and glucose. Peterson, Fred and Schmidt reported deficient amounts of butyl alcohol from arabinose in agreement with the findings of Weinstein and Rettger when zein was not added. These latter authors found that in the absence of alcohol-soluble protein normal amounts of acetone were produced, but little or no butyl alcohol was formed from any of the three sugars. They believed that Peterson, Fred and Schmidt must have added enough zein in the inoculum which they used to allow fermentation to proceed

normally with glucose and xylose. The difficulty noted in the discussion above concerning the difference in behavior of xylose and arabinose in the butyl fermentation seems to be cleared up by this investigation.

Other factors, such as hydrogen-ion concentration, method of securing anaerobiosis, addition of oils, addition of colloidal material, concentration of peptone, and the type of medium used, had no apparent influence on the production of butyl alcohol from carbohydrates in a semi-synthetic medium to which a prolamine in some form had not been added.

Acid hydrolysis of various raw materials, cottonseed hulls, peanut husks, Douglas fir sawdust, white pine sawdust, maple sawdust, birch sawdust, oak sawdust, and corn cobs gave good yields of reducing sugars, corn cobs giving the highest proportion and peanut husks the least. Fermentation of the acid hydrolysates in 3 percent sugar concentration in the absence of prolamine yielded appreciable amounts of acetone only, but the addition of 5 percent yellow corn mash to the neutralized hydrolysates was found to provide enough prolamine to allow the fermentation to proceed normally, and conversion of all carbohydrate in corn and hydrolysate took place. The unhydrolyzed raw materials could not be fermented by the butyl organism.

These authors showed that the prolamine probably did not

act as a catalyst in the reaction, or at least solely as a catalyst, and that the butyl alcohol was not derived from the prolamine. They state:

> "Alcohol-soluble proteins in all probability influence favorably the production of butyl alcohol from carbohydrate material in a semi-synthetic medium through a combination of their physical action and their direct influence on the metabolism of Clostridium acetobutylicum."

In addition to these five papers devoted to the investigation of the butyl-acetonic fermentation of sugars which have just been discussed, McCoy, Fred, Peterson and Hastings (1926) and Weyer and Rettger (1927) in their work on the cultural characteristics of various strains of the butyl organism, subjected a considerable number of carbohydrates to the action of the organism and found that many of them were fermented. The products were not determined.

No attempts to work out the most suitable synthetic medium for the growth of the butyl organism, to study factors which might improve the butyl-acetonic fermentation of the sugars, or to investigate in detail the fermentation of xylose by the butyl organism have been reported in the published literature.

III. METHODS

A. Bacteriological.

1. The culture.

Isolation and preliminary treatment. A single culture of the butyl organism was used throughout this investigation. This culture was isolated in 1931 from a sample of wheat by L. M. Christensen at Iowa State College. The method of isolation was as follows: Samples of the wheat were introduced into tubes of sterile corn mash, the tubes heated in a boiling water bath for two minutes to kill non-resistant organisms, cooled in running water, and incubated at 37° Centigrade. After two days the tubes showing best gas production and most pronounced starch liquefaction were selected and incubated for five days so that spores would be formed. Transfers were made from these tubes into fermentation tubes containing fresh corn mash, the tubes heated in the boiling water bath for two minutes, cooled promptly, and incubated at 37° Centigrade. After vigorous growth had begun, inoculation was made into malt agar, and anaerobic plates were made. After incubating the anaerobic plates for two days at 37° Centigrade, typical polyhedral colonies were picked from the plates and inoculated

into fresh corn mash tubes. The tubes were incubated until fermentation had ceased and then five days longer to ensure spore formation.

The culture obtained in this way did not at first produce strong fermentations and was improved by repeating the following cycle many times: From the spore culture inoculation was made into fermentation tubes containing fresh sterile corn mash, the tubes "heat-shocked" for two minutes in the boiling water bath, cooled in running water and incubated at 37° Centigrade. After 24 hours the active culture was transferred into fresh tubes of corn mash. Similar transfers were made each 24 hours and incubated at 37° until four transfers had been made. Fermentation in the last of these transfers was allowed to become complete, and the tubes then allowed to stand for five days for sporulation. From the "spore-stock" a fresh culture was started by inoculating into new tubes of corn mash, "heat-shocking", cooling, and incubating. Transfers were then made every 24 hours and the cycle repeated.

After repeating this cycle a large number of times a culture was finally secured which fermented corn mash vigorously and gave excellent yields of solvents. Since spores will not keep indefinitely when stored in the fermented liquid in which they are formed, although it is true that they will remain virile for a long time in this condition, a "soil-culture"

was made by pouring a liquid spore-culture upon a sterile mixture of soil with 10 percent calcium carbonate contained between two sterile porous plates, sealing the two plates containing the resulting mud with adhesive tape, and drying in the incubator. The resulting dry soil-culture was transferred, taking care to avoid contamination, to a dry sterile flask, the mouth of which was immediately closed with a sterile cotton plug. It was from this soil-culture, or from another soil-culture derived from it, by special treatment to improve its power to ferment sugars, that individual active cultures were started for the investigations reported in this thesis.

Active cultures for the fermentations reported under the experimental results were started from the soil-culture by transferring a small amount (0.2-0.4 gram) of the soil, by means of a sterile pipette of wide glass tubing, into a tube of sterile corn mash, heat-shocking for two minutes in the boiling water bath, cooling promptly, and incubating at 37° Centigrade. Transfers were made each 24 hours, the third or fourth transfer being made into a flask of sterile corn mash of sufficient volume to provide for inoculation of the flasks for the experimental fermentation. The experimental fermentations were in every case either the fourth or fifth transfer from the original soil-culture. Incubation of all cultures and fermentations was at 37° Centigrade.

Characterization of the culture. An abbreviated descriptive chart, following the outline of the Society of American Bacteriologists, is given below. The results of this characterization show this culture to be identical with that described by Weyer and Rettger (1927).

DESCRIPTIVE CHART.

Source. Wheat.
Date of Isolation. 1931.

Morphology.

Vegetative cells. Medium: 6 percent corn mash. Temperature: 37°. Age: 20 hours. Form: short rods. Arrangement: single. Ends: rounded. Stained with carbol fuchsin.

Sporangia. Present. Medium: 6 percent corn mash. Age: 48 hours. Temperature: 37°. Form: spindle.

Endospores. Present. Location: sub-polar. Form: elliptical. Wall: thick, non-adherent.

Motility. Very motile. Medium: 6 percent corn mash. Temperature 37°. Age: 20 hours.

Irregular forms. Present in old sugar media; cuneate.

Staining reactions. Stains deeply with common stains. Gram stain: positive. Lugol's iodine: granulose.

Cultural Characteristics.

Aerobic agar stroke. No growth.

Agar shake. Growth slow. Gas splits the medium.

Gelatin stab. Very slow, limited growth. Line of puncture: plumose in depth of medium. Liquefaction: slight.

Nutrient broth. No growth.

Sugars, etc., in peptone water containing pulped filter paper. Acid and solvents produced from starch, glucose, lactose, sucrose, levulose, maltose, xylose, mannitol, dextrin. No growth in glycerol medium.

Peptone starch paste. Growth: rapid. Diastatic action: rapid, complete.

Nitrogen source. Secured from protein and peptone, not from ammonium salts.

Best medium for growth. Six percent corn mash.

Quick differential test. Diastatic activity, odor.

Acids produced. Acetic, butyric, non-volatile.

Alcohols produced. Ethyl, butyl.

Acetylmethyl carbinol. Present.

Aldehyde. Slight.

Acetone. Strong.

Resistance to drying. Very resistant. Cultures best stored in the dry state.

2. The media.

The stock medium used for carrying the cultures and for controls was 6 percent corn mash. This medium was prepared by weighing out ground yellow corn meal, adding the required amount of cold tap water, cooking, either by bringing to a boil with constant stirring or by steaming for 30 minutes with occasional agitation, and sterilizing in the autoclave at 20 pounds steam pressure for two hours. For starting cultures and for early transfers, the corn mash was tubed in long 10 by 5/8 inch fermentation tubes after cooking, each tube containing approximately

20 cubic centimeters of the medium. The flasks of corn mash from which inoculations of the experimental fermentations were made were prepared by weighing the dry corn meal directly into the flasks, adding water, steaming, and sterilizing. The corn meal used for these flasks was analyzed for starch and moisture, and a sufficient weight used in each case so that a concentration of exactly 6 percent dry corn meal was obtained.

The composition and method of preparation of the media used for the individual experimental fermentations are given along with the experimental findings below. Sterilization of all sugar media was at 10 pounds steam pressure for 30 minutes unless otherwise stated. The carbohydrate content of all media was made equivalent to that in a 7 percent corn mash.

B. Chemical.

1. Determination of total acidity.

Total acidity in the fermentations was determined by titrating 10 cubic centimeters of culture with N/10 sodium hydroxide using phenolphthalein as indicator. Total acidities are expressed in recording results as cubic centimeters of 0.1 normal sodium hydroxide required for 10 cubic centimeters of fermented liquid.

2. Determination of solvents.

To a measured volume of the fermented liquid (usually 100 cubic centimeters) was added an approximately equal volume of water, and then powdered calcium carbonate in excess to neutralize the acidity. The resulting mixture was distilled rapidly, a measured volume of the distillate (about one-fourth the total volume) being collected in volumetric flasks which were cooled by running water. All of the solvents, acetone, butanol, and ethanol were distilled off and collected by this treatment.

Analyses for total mixed solvents were made by determining the specific gravity of the distillate at 25°/25°, using a Chainomatic Westphal balance. From the specific gravity the weight of total mixed solvents in 100 cubic centimeters of distillate was ascertained by means of the empirical relation

$$\text{Total mixed solvents} = \left(1 - \frac{\text{Specific gravity of distillate}}{0.00152}\right).$$

Since this equation is accurate only when the distillate contains solvents in the ratio six parts butanol, three parts acetone, and one part ethanol, the results obtained for the actual fermentations were probably usually slightly in error, but the values obtained were of sufficient accuracy for many

of the preliminary investigations. For greater accuracy the individual solvents were determined in most of the experiments.

Acetone was determined in the distillate by Goodwin's (1920) modification of the Messinger (1888) iodoform titration method.

For the determination of butanol and ethanol in the distillate several methods involving determination of index of refraction or dichromate oxidations have been worked out in these laboratories. These methods will be published elsewhere. Only the method which seems to give the best results is given here.

Ten cubic centimeters of the solvent distillate are diluted to 100 cc. and 10 cc. of this dilution mixed with 20 cc. of 1:1 sulfuric acid and exactly 25 cc. of 0.2 N potassium dichromate in a 10 by 1 inch Pyrex test tube, the tube stoppered with a one-hole rubber stopper fitted with a short piece of capillary glass tubing to allow an opening for expansion, and heated in a boiling water bath for 10 minutes. It is then cooled rapidly under the tap, the liquid transferred quantitatively to a 500 cc. Erlenmeyer flask, diluted to approximately 200 cc., and 10 cc. of 20 percent potassium iodide solution added. The mixture is then allowed to stand for about 3 minutes, and finally the iodine liberated by the excess potassium dichromate titrated with N/10 sodium thiosulfate, using starch

at the end as indicator. A blank is run for each series of determinations exactly as outlined, using 10 cc. of distilled water instead of a 10 cc. sample of solvent dilution. The difference between the blank and the titration gives the number of cubic centimeters of thiosulfate equivalent to the potassium dichromate reduced by the solvents in the sample of dilution.

Twenty cubic centimeters of the original distillate are mixed with 40 cc. of carbon tetrachloride in a large Pyrex test tube which is stoppered and shaken vigorously, then allowed to stand at 25° Centigrade in a water thermostat for two hours. Ten cc. of the aqueous layer are diluted to 50 cc., and 10 cc. of this dilution submitted to a similar dichromate oxidation and titration as that outlined above.

From the two titration values so obtained, along with the acetone values, the weight of butanol and the weight of ethanol in 100 cc. of distillate can be calculated by means of suitable equations.

3. Determination of volatile acids.

When volatile acids were to be determined, the solvents were distilled off for analysis as described above except that the acids were neutralized to phenolphthalein with sodium hydroxide instead of with excess calcium carbonate. The remaining liquid was acidified with normal sulfuric acid and

distilled at constant volume until 500 cubic centimeters of distillate had been collected. The volatile acids in the distillate were determined by the method of Virtanen and Pulkki (1929).

4. Determination of gases.

When analyses for the gases were to be made, the fermentation gases were collected over saturated salt brine in large bottles, and the volume and pressure recorded. Samples of the gas were taken from the bottles and analyzed for carbon dioxide, oxygen (air), and hydrogen, using William's modification of the Orsat apparatus.

5. Determination of sugars.

Reducing sugars were determined by the Marsh and Joslyn (1932) modification of the Shaffer-Hartmann (1921) method. The factors given by Stiles, Peterson and Fred (1926) were used in the calculation of results for sugars not included in the tables of Munson and Walker (1906). Sucrose and starch were hydrolyzed according to the official methods of the Association of Official Agricultural Chemists (1925) before titration by the Shaffer-Hartmann method.

6. Method of expressing the results of analyses.

Throughout this work the yields of the various products were always calculated, and are uniformly expressed in the tables included under Experimental Results below, as percent of total glucose equivalent, that is, on the basis of the quantity of carbohydrate required to furnish the same amount of carbon as the glucose. To illustrate, if the original medium contained a total of 20 grams of anhydrous glucose and, on analysis, the yield of total solvents was found to be 5 grams, the yield was 25 percent. If another carbohydrate, such as sucrose, was used, the quantity of glucose which would furnish the same amount of carbon as this sugar was calculated as the glucose equivalent. For sucrose, $C_{12}H_{22}O_{11}$, with a molecular weight of 342, the equivalent of glucose, $C_6H_{12}O_6$, with a molecular weight of 180, is 2(180)/342 = 1.082 grams of glucose per gram of sucrose. Hence, if the medium contained 20 grams of sucrose, this was equivalent to 20 x 1.082 = 21.64 grams of glucose, and if 4 grams of mixed solvents were obtained from this fermentation, the yield would be (4)(100)/21.64 or 18.48 percent. The glucose equivalents for starch and xylose were obtained in a similar manner. For starch the equivalent is $C_6H_{12}O_6/C_6H_{10}O_5$ or 180/162 = 1.111 grams of glucose per gram of starch. For xylose the equivalent is $5C_6H_{12}O_6/6C_5H_{10}O_5$ = 1

gram of glucose per gram of xylose. However, the crude xylose used was found by analysis (and also by fermentations in which xylose was substituted for starch in series as described under Experimental Results) to have a glucose equivalent of 0.984 gram of glucose per gram of xylose.

IV. EXPERIMENTAL RESULTS

A. The Action of the Butyl Organism on Various Carbohydrates.

1. Replacement of corn meal by pure carbohydrates.

A mash of corn meal seems to be the ideal medium for the growth of the butyl organism. In order to determine how the organism would attack various pure carbohydrates, namely starch, glucose, sucrose, and xylose, mashes were prepared in which these carbohydrates were used to replace various amounts of corn meal in a series, the amount of carbohydrate added being equivalent in each case to the amount of carbohydrate in the corn meal replaced. Fermentations were carried out in duplicate in 500 cubic centimeter Erlenmeyer flasks containing 300 cubic centimeters of medium. A 3 percent inoculation from an active 24-hour culture in corn mash was used in each case, that is, 3 cubic centimeters of culture were added for every 100 cubic centimeters of medium. Fermentation was complete in four to five days. The data for the analyses for solvents in these fermentations are given in Table 1. The values given are averages for the determinations in the duplicate fermentations. The data are plotted in Figures 1 to 5.

TABLE 1. Yields of Solvents from Fermentations of Carbohydrates added to Corn Mash.

Percent of Total Substrate		Yield, Percent of Glucose Equivalent			
Corn Meal	Carbohydr.	Butanol	Acetone	Ethanol	Total Solv.
	Starch				
100%	0%	15.35%	6.23%	2.88%	24.46%
80	20	15.56	5.97	3.19	24.72
60	40	14.75	6.00	3.56	24.21
40	60	14.93	6.11	4.45	25.49
30	70	14.00	5.71	4.74	24.45
20	80	14.49	6.15	4.24	24.88
10	90	14.57	5.48	3.81	23.86
0	100	7.18	2.38	2.42	11.98
	Glucose				
100	0	17.56	8.45	1.46	27.47
90	10	17.40	7.97	2.12	27.49
80	20	17.40	8.45	1.63	27.49
70	30	17.40	7.97	2.44	27.81
60	40	17.56	7.32	2.50	27.38
50	50	17.44	7.98	2.60	28.01
40	60	17.56	7.48	2.76	27.80
20	80	16.42	6.34	1.46	24.22
0	100	2.44	1.30	----	3.74
	Sucrose				
100	0	16.43	9.59	1.46	27.48
90	10	16.43	9.26	1.46	27.15
80	20	16.90	8.46	2.28	27.64
70	30	17.08	8.95	2.28	28.31
60	40	17.40	8.46	2.60	28.46
50	50	16.90	7.97	2.60	27.47
40	60	17.84	7.15	2.99	27.98
20	80	18.98	7.48	2.99	29.45
0	100	3.09	0.81	0.33	4.23
	Pure Xylose				
100	0	16.86	8.98	1.73	27.57
80	20	16.25	8.80	2.54	27.59
60	40	16.13	8.45	2.94	27.52
40	60	15.94	7.54	1.29	24.77
30	70	13.41	6.58	1.95	21.94
20	80	9.00	4.09	2.09	15.18
10	90	2.03	0.59	----	2.62
0	100	-----	----	----	-----

TABLE 1. (Concluded)

Percent of Total Substrate		Yield, Percent of Glucose Equivalent			
Corn Meal	Carbohydr.	Butanol	Acetone	Ethanol	Total Solv.
	Crude Xylose				
100	0	17.47	9.25	1.88	28.60
80	20	17.44	9.08	1.63	28.15
60	40	16.64	8.06	2.51	27.21
50	50	15.94	7.52	2.63	26.09
40	60	14.72	6.49	3.00	24.21
30	70	5.53	1.00	----	6.53
20	80	-----	----	----	-----
0	100	-----	----	----	-----

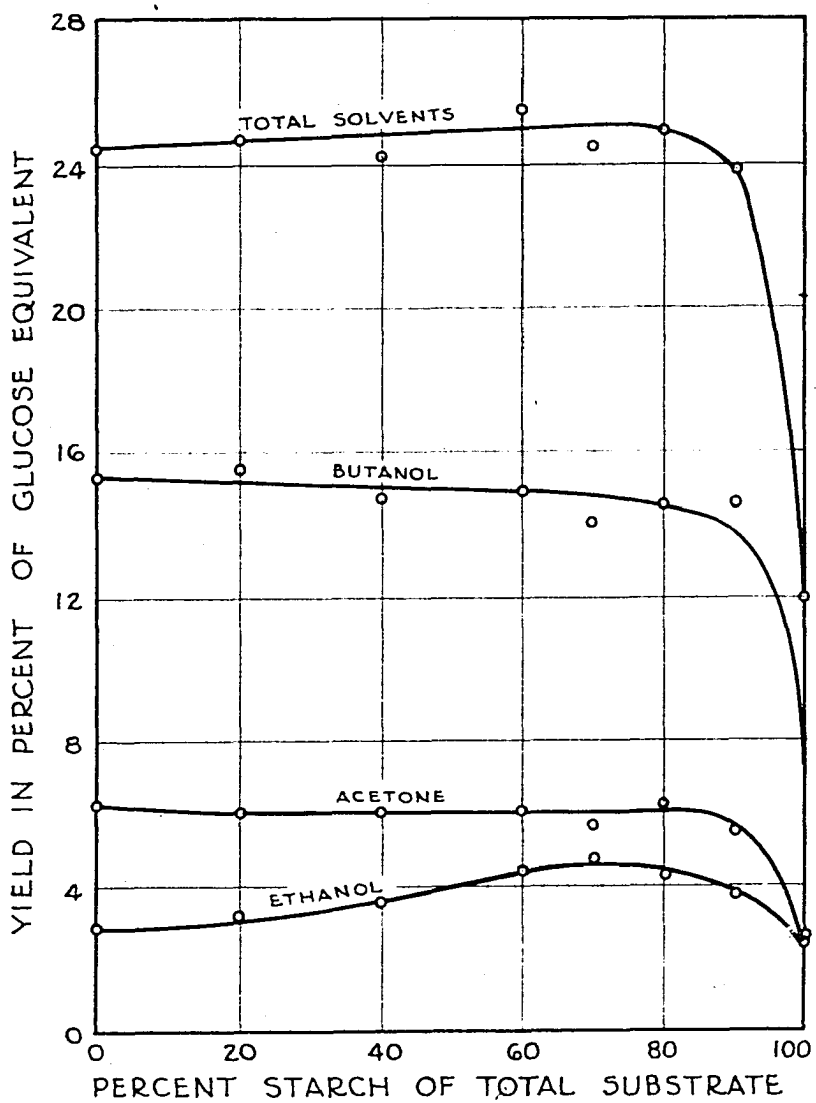

FIGURE 1. Solvents Produced in the Fermentation of the Starch-Corn Meal Mixture.

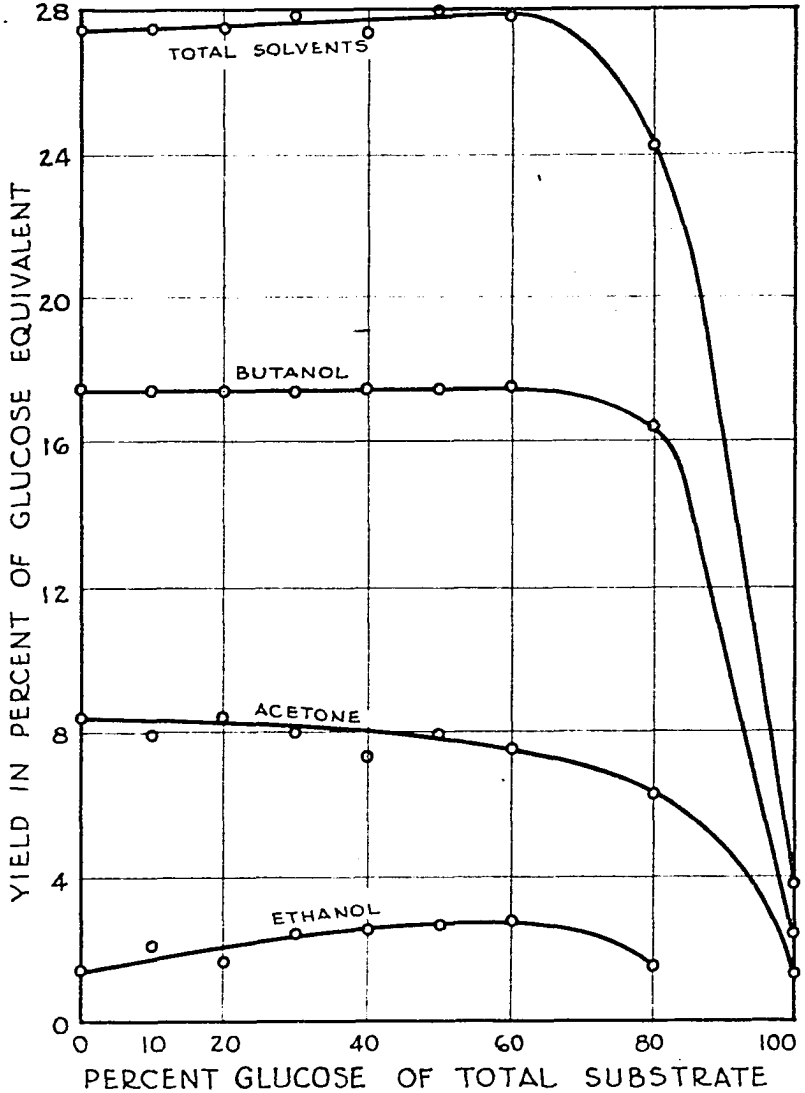

FIGURE 2. Solvents Produced in the Fermentation of the Glucose-Corn Meal Mixture.

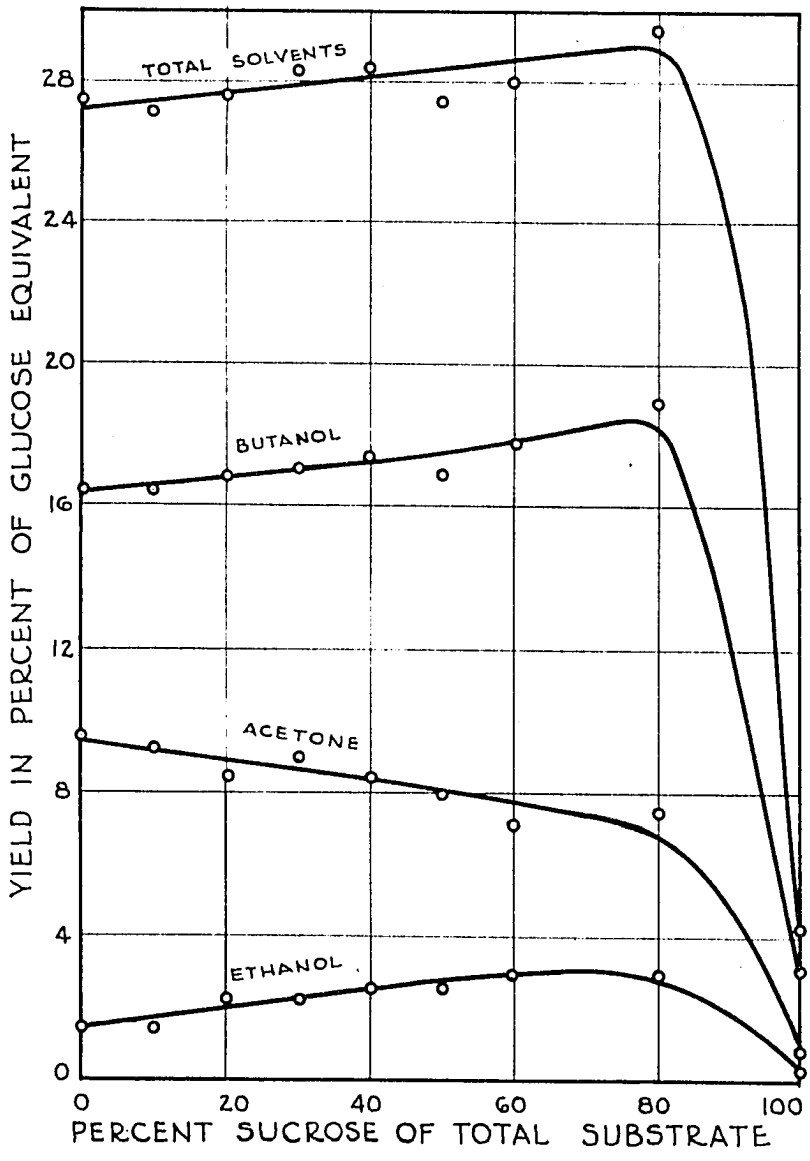

FIGURE 3. Solvents Produced in the Fermentation of the Sucrose-Corn Meal Mixture.

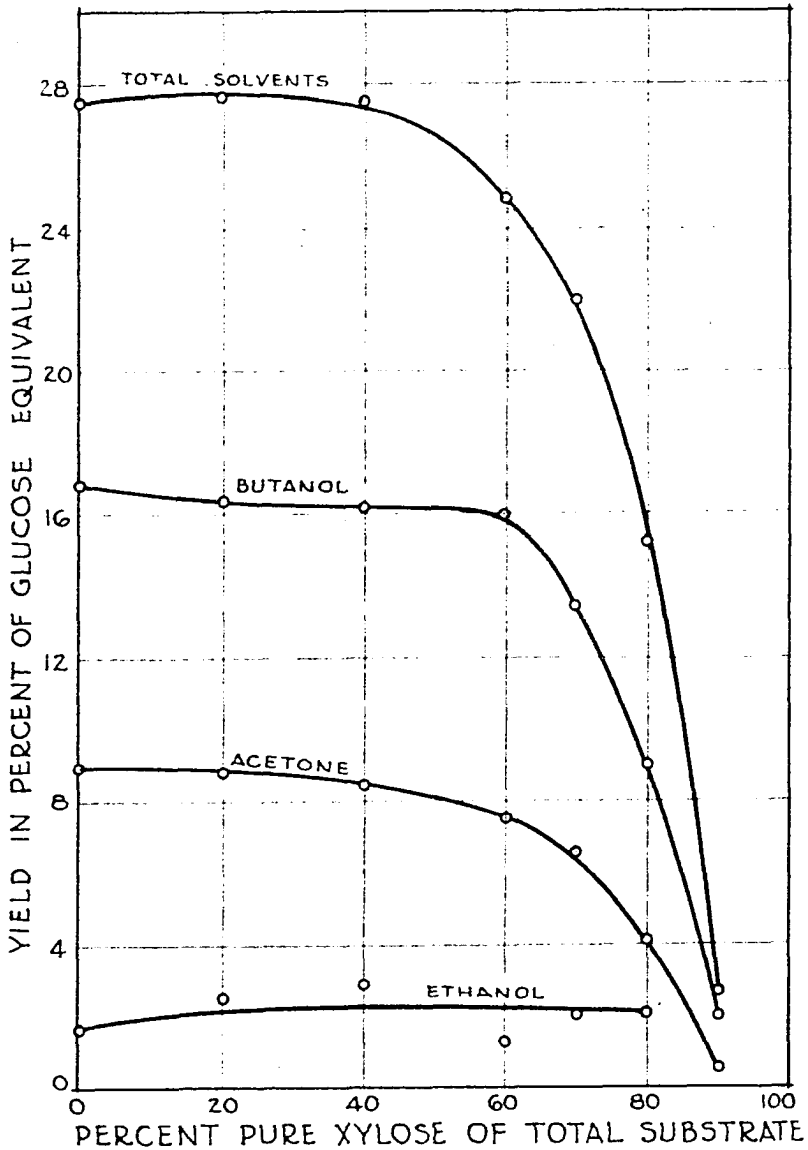

FIGURE 4. Solvents Produced in the Fermentation of the Pure Xylose-Corn Meal Mixture.

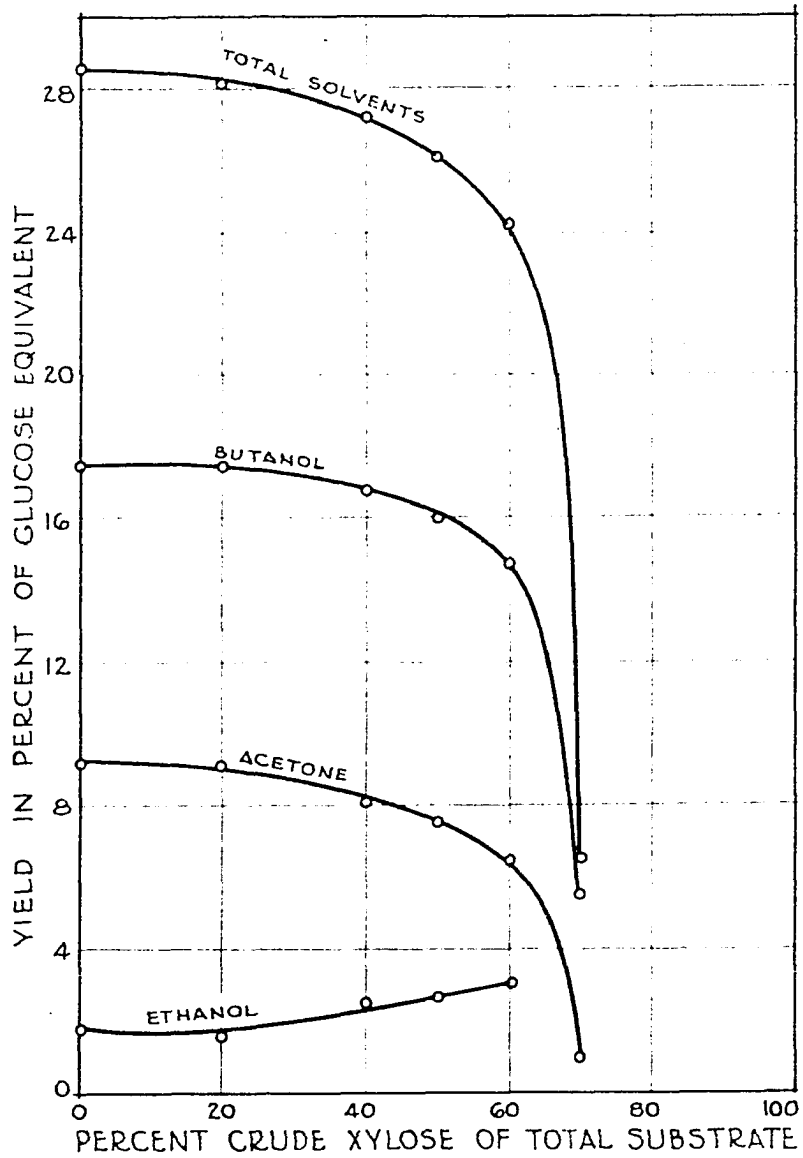

FIGURE 5. Solvents Produced in the Fermentation of the Crude Xylose-Corn Meal Mixture.

Since the carbohydrate in corn meal is starch, it was expected that replacement of corn meal by an equivalent quantity of starch would result in no change in yield until the amount of non-carbohydrate nutrients in the corn meal remaining was no longer sufficient to support the activities of the organism, resulting in a rapid drop in yield. Figure 1 shows that there was no marked drop in yield until more than 90 percent of the corn meal was replaced by starch.

When the corn meal was replaced by an equivalent amount of glucose, the results were the same as for starch, except that the drop in yield became marked when only 80 percent of the corn meal had been replaced as shown in Figure 2.

From Figure 3 it may be seen that not only did the yield of total solvents not drop when sucrose was used to replace the corn meal, but that it actually increased until 90 percent of the corn meal had been replaced. The yield of acetone fell off slightly, but the yields of butanol and ethanol, and the total yield of all solvents increased. A probable explanation of this is that the glucose equivalent of the corn mash was lower than shown by the carbohydrate analysis, or else the addition of sugar improved the fermentation of the corn mash.

In Figures 4 and 5 are given the results for fermentations of corn meal and xylose mixtures. Evidently xylose is not so readily available as the other carbohydrates investigated.

A great lowering of yield was observed when slightly more than 50 percent of corn meal had been replaced by recrystallized xylose, and the yield continued to drop off rapidly as more xylose was used until at 90 percent there was practically no yield. Crude xylose did not ferment as well as the purified carbohydrate. From the appearance of the curve it appears that some toxic agent must be associated with the crude xylose. No fermentation occurred when more than 70 percent of the corn meal was replaced by crude xylose.

2. Development of a medium of known composition.

Although it was apparent from the first that the butyl organism attacked xylose with some difficulty (see Figures 4 and 5), two practical considerations led to extended investigations with the objective of developing a medium suitable for the butyl fermentation using xylose, especially in the crude form, as the substrate. In the first place, xylose is a substance which could be readily made available on a large scale, since it is a product easily produced from several raw materials at present classed as agricultural wastes, such as corn cobs, oat hulls, and cottonseed hull bran. A possible utilization of these materials on a large scale would be very desirable. In the second place, it was thought that since xylose was attacked with difficulty, if a suitable medium

using this substrate could be worked out, a similar result could be arrived at by a similar method for the other sugars, if, indeed, the same medium, varying the carbohydrate, would not serve.

The first step in the development of a medium of known composition for the utilization of xylose was to discover a suitable source of nitrogen. The butyl organsim is markedly proteolytic. The proteolytic action of the organism has been extensively studied by Peterson, Fred and Domogalla (1924), Fulton, Peterson and Fred (1926), and Wilson, Peterson and Fred (1930). These authors, as well as Weyer and Rettger (1927), and Speakman (1926), state that inorganic nitrogen alone does not meet the requirements of the butyl organism, but that it requires, instead, nitrogen from complex protein substances. Wilson, Peterson and Fred (1930), Weyer and Rettger (1927), and Weinstein and Rettger (1933) report extensive investigations of the nitrogen requirements of the butyl organism. In view of the conclusions of these workers, experiments were carried out using ammonium chloride, peptone, corn-gluten meal, casein, steep water, and tankage in varied series as the source of nitrogen, with xylose as the substrate. It has been shown by Fulmer, Nelson and Sherwood (1921) that the best medium from known constituents could be developed only when constituents were varied in series to determine the

optimum, and this procedure was followed for all the substances used in these investigations.

The basal medium to which the nitrogenous nutrients were added contained 6.25 grams of recrystallized xylose, 0.2 gram of dipotassium phosphate, and 0.5 gram of pulped filter paper per 100 cubic centimeters of water. The filter paper was added to help maintain anaerobic conditions. Several authors have reported this procedure, notably Robinson (1922), Speakman (1923), Johnson, Peterson and Fred (1931), Weyer and Rettger (1927), and Weinstein and Rettger (1933). It was discovered later in the work here reported that if the medium were inoculated immediately after sterilization and subsequent cooling to the proper temperature, the addition of filter paper was unnecessary. Yields were identical both with the addition of filter paper and without such addition.

The fermentations were carried out in duplicate in 150 cubic centimeter Erlenmeyer flasks containing 100 cubic centimeters of medium. A 5 percent inoculation from an active 24-hour culture in corn mash was used in each case. Fermentation was allowed to continue until gas ceased to be evolved. Five days were sufficient for the fermentations using peptone, steep water, and ammonium chloride, but longer lengths of time, up to 10 days for some flasks, were required in the case of the other nutrients. Results of analyses for yields of solvents

are given in Table 2. The yields are expressed in percent of glucose equivalent. The values given are the averages for the duplicate fermentations. The data are plotted in Figure 6.

The ammonium salt gave very poor yields, as did the steep water in larger concentrations. The yields from the ammonium salt were somewhat better than might be expected, however, probably due to the heavy inoculation used, either by carrying over nutrients with the inoculation which allowed scanty growth and fermentation, or by attack of the substrate by the active organisms introduced in the inoculum without growth or multiplication occurring.

Peptone and gluten meal gave the best yields. The gluten meal gave a somewhat higher final yield, but required nine days for completion of the fermentation instead of five days which were required for the peptone. However, for all subsequent studies, corn-gluten meal was used as the basic nutrient to furnish nitrogen. This choice was made because from the practical standpoint of cost in large scale production the cost of peptone or similar substances would be entirely prohibitive.

TABLE 2. Yields of Total Solvents Produced in Fermentations of Xylose with Various Nitrogenous Nutrients.

Amount of Nutrient*	Yield, Percent Glucose Equiv.	Amount of Nutrient*	Yield, Percent Glucose Equiv.
Peptone		Corn-gluten	
0.2 g.	13.98%	0.3 g.	9.18%
0.4 g.	16.25	0.6 g.	17.89
0.6 g.	17.64	0.9 g.	19.83
0.8 g.	16.99	1.2 g.	19.83
1.0 g.	18.12	1.5 g.	19.60
1.2 g.	17.89	1.8 g.	20.18
1.4 g.	18.54	2.1 g.	20.40
1.6 g.	18.85	2.4 g.	19.43
1.8 g.	18.93	2.7 g.	11.78
2.0 g.	18.12	3.0 g.	11.87
Tankage		Casein	
0.3 g.	9.67	0.3 g.	12.75
0.6 g.	12.91	0.6 g.	16.50
0.9 g.	15.18	0.9 g.	17.15
1.2 g.	16.15	1.2 g.	17.80
1.5 g.	17.30	1.5 g.	16.99
1.8 g.	16.66	1.8 g.	17.40
2.1 g.	15.80	2.1 g.	17.89
2.4 g.	17.56	2.4 g.	17.23
2.7 g.	18.19	2.7 g.	19.28
3.0 g.	17.72	3.0 g.	18.70
Steep Water		Ammonium Chloride	
1.2 cc.	15.60	0.03 g.	8.37
2.4 cc.	16.80	0.06 g.	10.15
3.6 cc.	10.24	0.09 g.	10.48
4.8 cc.	8.78	0.12 g.	7.80
6.0 cc.	8.70	0.15 g.	8.61
7.2 cc.	8.62	0.18 g.	7.40
8.4 cc.	9.18	0.21 g.	8.70
9.6 cc.	9.26	0.24 g.	8.37
10.8 cc.	9.26	0.27 g.	7.56
12.0 cc.	9.34	0.30 g.	7.07

*Amount of nutrient in grams or cubic centimeters per 100 cubic centimeters of medium.

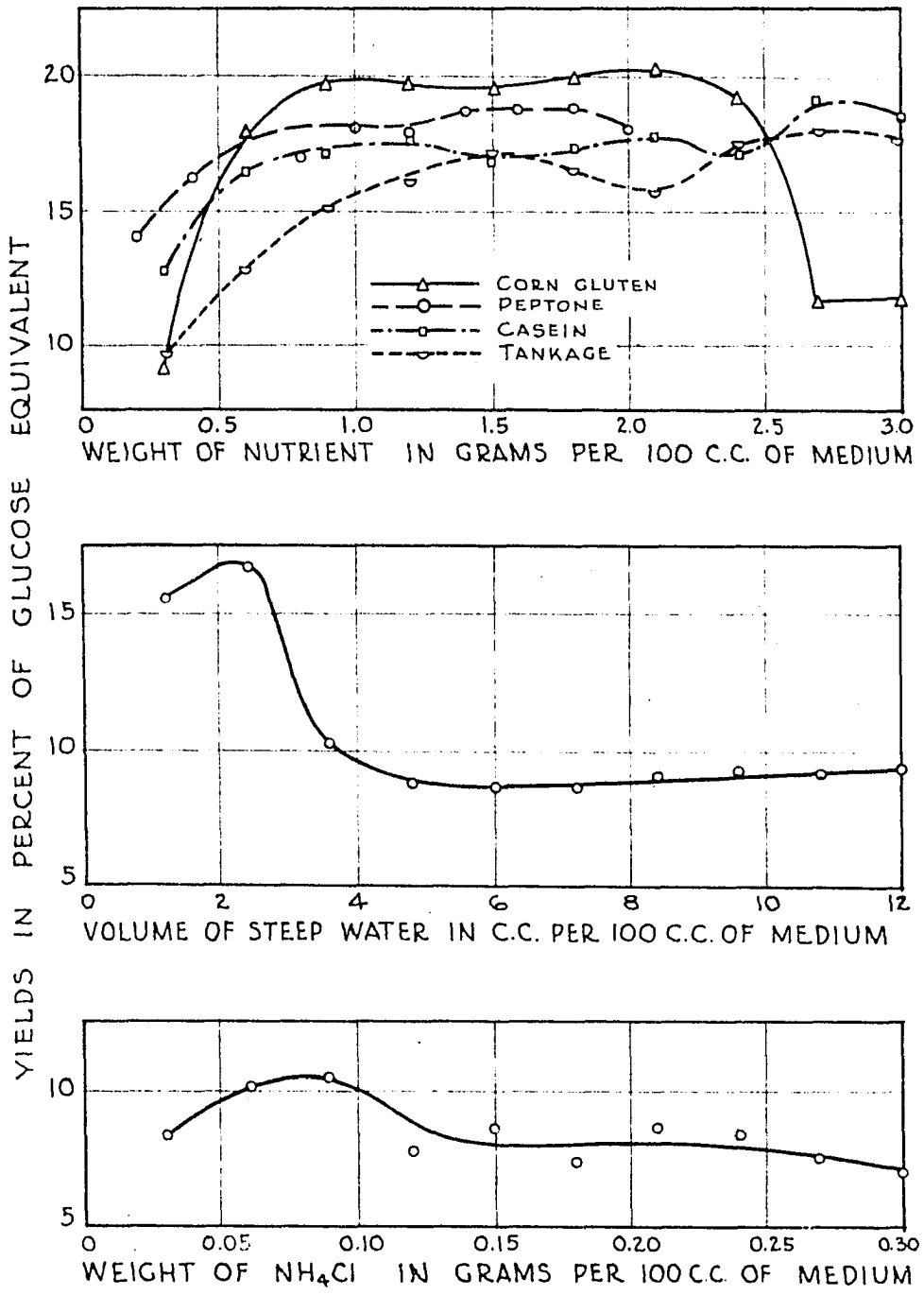

FIGURE 6. Solvents Produced in Fermentations of Xylose Media Containing Various Nitrogenous Nutrients.

In order to determine the optimum amount of corn-gluten to be used in the medium, two series of fermentations were run. In one series the same basal medium containing xylose was used as before, except that the filter paper was omitted, and varying amounts of gluten meal were added. In the second series glucose was substituted for xylose. The fermentations were carried out in duplicate in 500 cubic centimeter Erlenmeyer flasks containing 300 cubic centimeters of medium, since growth was found to be better and more uniform in larger flasks. A 3 percent inoculation from an active 24-hour culture in corn mash was used. The data obtained in this experiment are tabulated in Table 3 and are plotted in Figure 7. The yields given in the table are the averages for the analyses of the duplicate fermentations. Yields are expressed as percent of glucose equivalent.

The results show quite a distinct optimum in the xylose medium when the medium contains 2.0 grams of gluten meal per 100 cubic centimeters. This agrees with the preliminary results obtained in the previous experiment. The results show an optimum in the glucose medium when the medium contains 0.5 gram of gluten meal per 100 cubic entimeters, although the yield was not markedly less when as much as 1.0 gram of gluten meal per 100 cubic centimeters was used. The difference in the optimum amounts of corn gluten meal required in

the two media may be due to the need for more buffering for the xylose. The adverse effect of larger amounts of the corn-gluten meal is probably due to an unfavorable pH resulting from the buffering action of the large amount of protein.

TABLE 3. Yields of Total Solvents from Fermentations Containing Varying Amounts of Corn-Gluten Meal.

Weight of Gluten (g. per 100 cc.)	Xylose Fermentation		Glucose Fermentation	
	Time of Ferm. (Days)	Yield, Percent Glucose Equiv.	Time of Ferm. (Days)	Yield, Percent Glucose Equiv.
0.00	10	0.97%	3	2.45%
0.25	10	0.97	6	26.80
0.50	10	6.06	3	30.80
0.75	8	21.68	3	30.48
1.00	8	24.55	3	29.07
1.25	5	26.20	3	28.43
1.50	5	27.40	3	28.30
1.75	5	28.70	4	28.18
2.00	5	29.00	4	27.54
2.25	-	-----	4	26.39
2.50	6	27.22	-	-----
2.75	8	26.04	4	6.72
3.00	-	-----	4	6.43

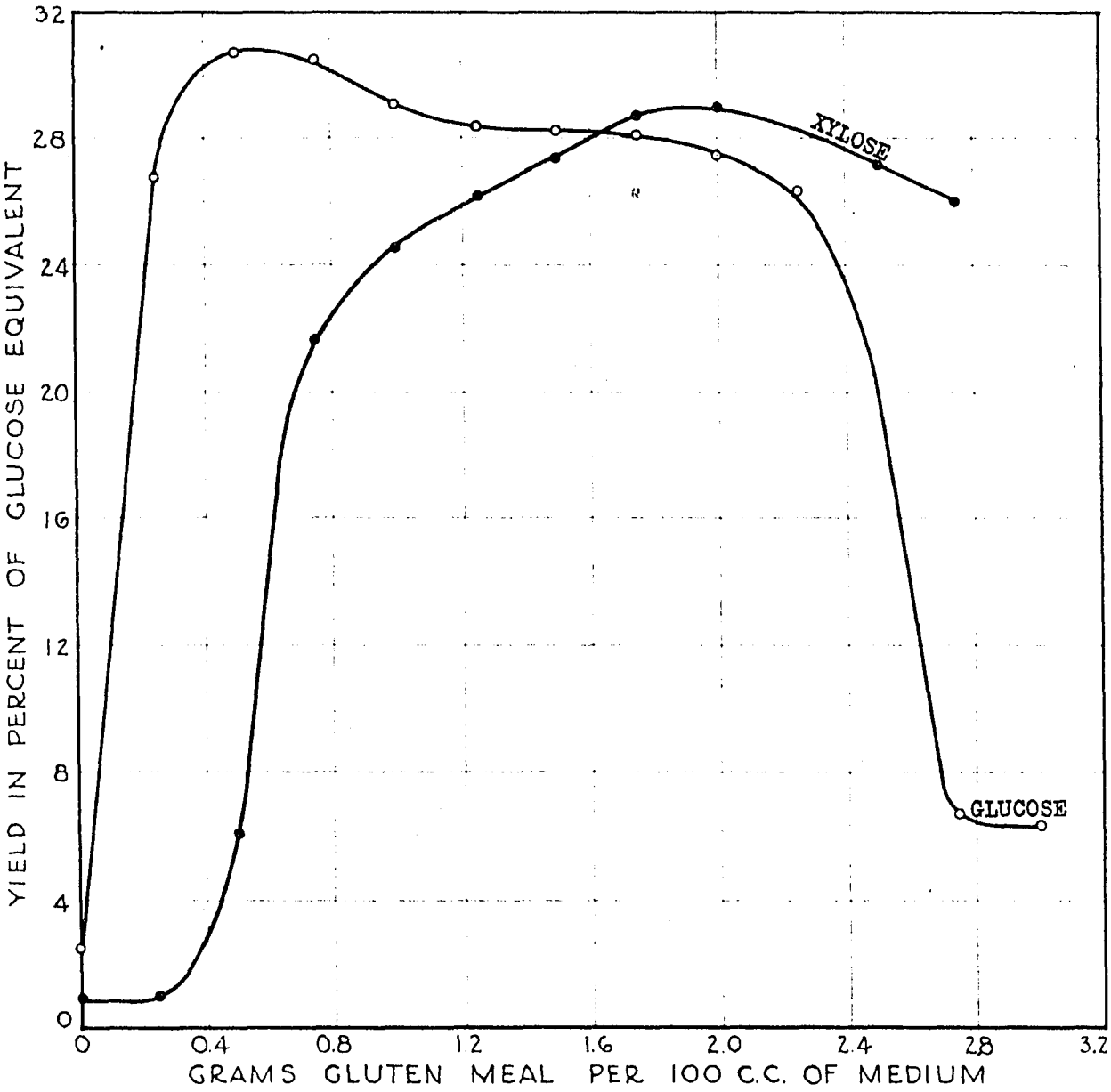

FIGURE 7. Yields of Total Solvents Produced in Fermentations of Media Containing Varying Amounts of Corn-Gluten Meal.

In order to test the effect of various salts on the fermentation of xylose in a medium containing gluten, experiments were undertaken in which the salts used by Speakman (1923) were separately tested in series. The composition of the medium which Speakman used for his investigations on the butyl fermentation of several carbohydrates was as follows: 0.05 gram of dipotassium phosphate, 0.05 gram of monopotassium phosphate, 0.02 gram of magnesium sulfate, 0.001 gram of sodium chloride, 0.001 gram of ferrous sulfate, 0.001 gram of manganous sulfate, 0.5 gram of peptone, 3.0 grams of carbohydrate, and distilled water to make a volume of 100 cubic centimeters.

The salts tested in the present experiments were dipotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, and manganous sulfate. The basal medium to which these salts were added separately contained 6.25 grams of crude xylose, 1.0 gram of corn-gluten meal, 0.2 gram of dipotassium phosphate (except in the experiments testing the effect of this salt), 0.5 gram of pulped filter paper, and 100 cubic centimeters of water. Fermentations were carried out in duplicate in 500 cubic centimeter Erlenmeyer flasks containing 300 cubic centimeters of medium. A 3 percent inoculation from an active 24-hour culture in corn mash was used in every case. Data are given in Table 4.

TABLE 4. Yields of Total Solvents from Xylose Fermentations Containing Varying Amounts of Different Salts.

Salt Added (g. per 100 cc.)	Yield, Percent Glucose Equivalent	Salt Added (g. per 100 cc.)	Yield, Percent Glucose Equivalent
K_2HPO_4		$MgSO_4$	
0.000	26.76%	0.000	26.08%
0.300	26.10	0.005	25.77
0.600	26.23	0.010	25.29
0.900	26.15	0.015	24.91
1.200	26.07	0.020	25.20
1.500	26.10	0.025	25.81
1.800	26.00	0.030	25.60
2.100	25.46	0.040	25.60
2.400	23.22	0.060	25.61
3.000	7.65	0.080	25.23
$FeSO_4$		$MnSO_4$	
0.0000	24.84	0.0000	25.71
0.0002	24.40	0.0002	25.35
0.0004	24.01	0.0004	25.49
0.0006	24.09	0.0006	25.71
0.0008	24.27	0.0008	25.73
0.0010	25.23	0.0010	26.16
0.0012	25.11	0.0012	26.46
0.0014	25.08	0.0014	26.38
0.0016	25.79	0.0016	26.05
0.0020	25.53	0.0020	25.37
0.0100	24.70	0.0100	23.95
0.1000	24.61	0.1000	25.10
NaCl			
0.0000	26.20		
0.0002	26.52		
0.0004	23.58		
0.0006	26.32		
0.0008	23.97		
0.0010	25.83		
0.0012	26.79		
0.0014	26.20		
0.0016	26.20		
0.0020	26.91		
0.0120	24.92		

The addition of dipotassium phosphate had no significant effect on the yield of solvents from xylose in a medium containing gluten until relatively large amounts had been added. Large amounts had an adverse effect on the fermentation. The variation of the concentrations of ferrous sulfate, sodium chloride, magnesium sulfate, and manganous sulfate within the limits tested had no significant effect on the yield of solvents. It is very likely that commercial gluten meal contains all the salts necessary for the well-being of the organism. The gluten meal used throughout this investigation was a commercial product, the manufacturer's analysis being as follows: crude protein not less than 40 percent, crude fat not less than 1 percent, crude fiber not less than 4 percent, and nitrogen free extract (carbohydrates) not less than 40 percent. No analysis for inorganic salts was given, and none was made in the course of this experimental work. The carbohydrates present in the gluten meal were evidently not fermentable, since, when tested, the meal alone did not ferment, nor did an extract from the meal.

3. Course of the butyl-acetonic fermentation of various carbohydrates.

Using the semi-synthetic medium developed for xylose, the course of the fermentations of the individual carbohydrates

starch, glucose, maltose, levulose, sucrose, and xylose as regards titratable acidity, pH, solvent formation, and sugar utilization was followed. The media contained, besides the carbohydrates, 0.25 gram of dipotassium phosphate and 1.0 gram of corn-gluten meal per 100 cubic centimeters of water. A fermentation was carried out in 7 percent corn mash in parallel with the other fermentations, as a check. The fermentations were all carried out in duplicate in 4 liter Erlenmeyer flasks containing approximately 3600 cubic centimeters of medium. A 2 percent inoculation from an actively growing 24-hour culture in corn mash was used in each case. The sugar concentrations varied slightly with the different sugars, but in each case was somewhat greater than 5 percent, and approximately equivalent to the 7 percent corn mash used in the industrial butyl-acetonic fermentation. Other investigators--Robinson (1922), Speakman (1923), Peterson, Fred and Schmidt (1924), Johnson, Peterson and Fred (1931), and Weinstein and Rettger (1933)--in general used much lower concentrations of carbohydrates. Their concentrations of from one to three percent sugar gave more complete fermentations than higher concentrations. However, it was the desire in these experiments to find a medium in which the organism would utilize sugars in sufficiently high concentration to be of practical

utility. Also experiments have shown that low carbohydrate concentrations give somewhat erratic yields. Samples were taken, using sterile pipettes, at the beginning and at intervals throughout the fermentation, and were analyzed for titratable acidity, pH, solvents, and carbohydrate remaining. Fermentation was judged complete when all gassing had ceased. The results of the analyses are given in Table 5.

TABLE 5. Course of the Butyl-Acetonic Fermentation of Various Substrates.

Time (hours)	Acidity (cc. 0.1 N NaOH/10cc.)	pH	Sugar (Glucose Equiv., g./100cc.)	Yield, Percent of Glucose Equivalent			
				Butanol	Acetone	Ethanol	Total
Corn Mash							
0	2.0	6.42	5.60	-----	----	----	-----
12	6.2	4.19	3.73	-----	----	----	-----
14	7.0	4.10	3.43	-----	----	----	-----
16	6.4	4.20	3.04	-----	----	----	-----
18	4.1	4.83	----	10.48	3.70	0.87	15.05
21	3.1	4.77	1.58	12.86	7.02	1.87	21.75
24	2.8	4.46	0.99	14.72	6.55	1.99	23.26
31	3.2	4.71	0.64	19.34	9.01	0.10	28.44
34	3.1	4.63	0.33	18.83	7.93	1.29	28.05
40	3.4	4.77	0.33	18.50	8.45	1.52	28.47
46	3.4	4.73	0.33	18.49	8.74	2.40	29.63
55	3.4	4.61	0.25	18.41	8.96	2.20	29.57
65	3.6	4.60	----	18.52	8.62	2.13	29.27
81	3.6	4.60	----	18.54	8.45	2.12	29.11
131	3.6	4.82	0.17	18.20	8.62	2.39	29.21
Starch							
0	2.4	6.54	5.15	-----	----	----	-----
12	3.4	4.55	3.47	-----	----	----	-----
14	3.8	4.53	3.41	-----	----	----	-----
16	4.4	4.44	3.34	-----	----	----	-----
18	4.7	4.48	3.21	4.80	1.61	0.99	7.40
21	5.0	4.44	2.94	-----	----	----	-----
24	4.3	4.85	2.14	8.12	3.43	1.31	12.86
31	3.2	4.78	1.33	14.50	7.02	3.75	25.27
34	3.3	4.71	0.96	16.15	6.97	3.82	26.94
40	3.4	4.78	0.48	18.62	7.51	3.66	29.79
46	3.2	4.78	0.38	19.38	8.01	4.31	31.70
55	3.4	4.70	0.25	19.00	7.92	4.73	31.65
65	3.4	4.66	0.34	20.28	7.98	3.49	31.75
81	3.4	4.66	0.31	19.40	7.91	4.05	31.36
131	3.8	4.77	0.23	19.68	7.86	4.28	31.82

TABLE 5. (Continued).

Time	Acidity	pH	Sugar	Butanol	Acetone	Ethanol	Total
Glucose							
0	1.4	6.39	6.13	-----	----	----	-----
12	3.6	4.44	5.27	-----	----	----	-----
14	4.1	4.44	4.70	-----	----	----	-----
16	4.6	4.34	4.97	-----	----	----	-----
18	4.8	4.41	5.07	-----	----	----	-----
21	4.9	4.33	4.46	-----	----	----	-----
24	4.7	4.38	2.91	3.42	1.08	0.33	4.83
31	4.0	4.51	2.61	7.92	3.16	1.27	12.35
34	3.6	4.61	1.72	9.11	3.64	1.85	14.60
40	3.4	4.67	2.02	14.10	4.66	2.66	21.42
46	3.4	4.51	1.38	14.40	5.14	3.32	22.86
55	3.4	4.60	1.59	17.10	6.87	3.17	27.14
65	3.8	4.51	1.40	16.69	6.99	3.98	27.66
81	4.2	4.56	1.05	19.01	7.42	2.98	29.41
131	4.1	4.56	0.95	19.60	7.73	2.70	30.03
Maltose							
0	1.2	6.56	5.57	-----	----	----	-----
12	3.6	4.61	4.95	-----	----	----	-----
14	4.5	4.46	4.91	-----	----	----	-----
16	5.0	4.36	4.55	-----	----	----	-----
18	5.6	4.33	4.74	3.11	0.87	----	3.98
21	5.6	4.33	4.66	-----	----	----	-----
24	5.2	4.41	3.16	5.67	1.74	----	7.41
31	3.4	4.82	3.00	10.76	5.41	2.18	18.35
34	3.2	4.75	1.64	14.79	6.93	2.52	24.24
40	3.5	4.66	1.55	17.05	6.89	3.92	27.86
46	3.4	4.60	1.46	17.56	7.94	3.90	29.40
55	3.4	4.65	1.37	17.34	8.51	5.58	31.43
65	3.4	4.60	1.55	18.27	7.68	5.01	30.96
81	3.6	4.63	1.39	19.38	6.98	3.70	30.06
131	3.5	4.65	1.43	18.15	7.15	4.65	29.95

TABLE 5. (Continued).

Time	Acidity	pH	Sugar	Butanol	Acetone	Ethanol	Total
Levulose							
0	1.4	6.31	5.44	-----	----	----	-----
12	3.6	4.61	4.64	-----	----	----	-----
14	4.0	4.49	4.60	-----	----	----	-----
16	4.4	4.46	4.54	-----	----	----	-----
18	4.7	4.38	4.49	-----	----	----	-----
21	5.0	4.39	3.94	-----	----	----	-----
24	4.6	4.41	3.80	4.15	1.60	0.86	6.61
31	4.0	4.49	2.10	9.28	3.74	1.97	14.99
34	4.0	4.56	1.00	13.30	4.80	1.16	19.26
40	3.6	4.66	0.39	17.07	7.78	3.66	28.51
46	3.4	4.68	0.29	17.61	8.43	6.00	32.04
55	3.2	4.80	0.54	19.00	7.90	5.49	32.39
65	3.5	4.65	----	19.65	7.39	4.99	32.03
81	3.8	4.61	0.56	19.85	7.78	4.83	32.46
131	4.0	4.68	0.56	19.55	8.71	6.00	34.26
Sucrose							
0	1.5	6.53	5.53	-----	----	----	-----
12	3.6	4.61	4.77	-----	----	----	-----
14	4.8	4.39	4.92	-----	----	----	-----
16	5.2	4.33	4.85	-----	----	----	-----
18	6.3	4.26	4.77	3.20	1.18	----	4.38
21	6.7	4.22	4.15	-----	----	----	-----
24	7.1	4.16	4.04	3.57	1.27	----	4.84
31	7.9	4.16	4.00	4.10	1.31	----	5.41
34	7.6	4.22	4.04	3.82	1.48	----	5.30
40	7.5	4.20	3.93	2.37	1.49	----	4.64
46	7.8	4.20	3.93	5.74	1.75	----	7.49
55	7.6	4.19	3.80	5.30	1.84	----	7.14
65	7.4	4.27	3.93	5.82	1.75	----	7.57
81	7.4	4.22	3.83	6.50	2.10	----	8.60
130	7.3	4.22	3.52	5.85	1.75	0.31	7.91
154	6.9	4.39	3.62	6.68	2.27	0.05	9.00
202	5.1	4.34	3.65	7.04	2.80	1.12	10.96

TABLE 5. (Concluded).

Time	Acidity	pH	Sugar	Butanol	Acetone	Ethanol	Total
Sucrose							
0	1.0	6.49	5.47	----	----	----	----
34	7.1	4.22	3.80	7.25	1.81	----	9.06
41	7.5	----	----	----	----	----	----
46	7.1	4.22	3.52	6.95	2.48	0.04	9.47
55	6.6	4.22	3.45	6.63	2.92	0.28	9.83
65	6.1	4.27	3.32	7.38	3.18	0.80	11.36
81	4.6	4.22	3.32	10.51	4.51	0.51	15.53
94	4.7	4.22	2.41	12.01	5.84	0.55	18.40
107	4.2	4.46	2.11	12.83	6.24	1.62	20.69
118	4.4	4.44	1.94	13.76	7.52	2.65	23.93
130	4.5	4.43	1.50	16.38	7.86	1.10	25.34
154	4.8	4.65	1.08	18.90	9.55	1.07	29.52
202	5.1	4.34	1.19	15.37	9.46	4.94	29.77
Xylose							
0	1.4	5.30	5.32	------	-----	-----	------
18	1.8	5.10	5.23	------	-----	-----	------
24	1.9	4.75	5.20	------	-----	-----	------
28	2.5	4.70	5.15	------	-----	-----	------
34	3.3	4.25	5.02	------	-----	-----	------
40	3.9	4.40	4.81	0.424	0.364	0.686	1.474
44	4.0	4.30	----	0.574	0.455	0.856	1.885
48	4.0	4.50	----	1.213	0.773	0.885	2.871
52	4.2	4.40	4.64	1.900	1.001	0.790	3.691
56	4.0	4.45	4.53	2.080	1.456	1.731	5.267
60	3.8	4.45	----	2.182	1.774	2.635	6.591
64	3.5	4.45	4.06	4.730	2.501	1.467	8.698
68	3.5	4.45	3.72	3.980	3.098	3.141	10.219
72	3.5	4.45	3.51	6.550	3.548	3.151	13.249
81	3.5	4.45	2.87	9.015	4.915	2.475	16.405
89	3.5	4.40	2.30	12.624	6.280	2.540	21.444
96	3.6	4.40	1.90	11.600	6.870	5.140	23.610
100	3.7	4.40	1.64	14.210	8.096	3.878	26.184
120	3.8	4.50	1.20	16.940	8.186	3.595	28.721
126	3.6	4.40	1.10	17.400	8.240	3.454	29.094
136	3.8	4.40	1.03	17.400	8.950	3.650	30.000
168	4.0	4.75	1.03	17.880	8.800	3.050	29.730

From Table 5 it may readily be seen that all the sugars investigated were attacked by the butyl organism in concentrations of sugar equivalent to the 7 percent corn mash used in the industrial process for making butyl alcohol and acetone by fermentation. However, there was great variation in the rates of fermentation, marked differences in the courses of the various fermentations, and considerable variation in the completeness of utilization of the carbohydrates. Table 6 will make these relations more evident.

TABLE 6. Proportion of Carbohydrate Utilized, and Yields of Solvents from the Fermentation of Various Substrates.

Substrate	Carbohydrate Utilized, Percent	Yield of Total Solvents	
		Percent Glucose Equivalent of Total Carbohydrate in Medium	Percent Glucose Equivalent of Carbohydrate Actually Utilized
Corn mash	91.5%	29.63%	31.2%
Starch	94.6	31.75	33.5
Glucose	84.5	30.03	35.8
Maltose	73.9	30.06	40.0
Levulose	91.5	32.03	35.0
Sucrose	77.4	29.77	38.5
Xylose	80.5	30.00	37.2

The corn mash fermentation showed the typical normal fermentation so often described in the literature. Speakman (192Φ) and Peterson and Fred (1932), among others, quite extensively

investigated the chemistry of the fermentation of this substrate. In the normal corn mash fermentation the titratable acidity curve rises very rapidly to a relatively high maximum (7.0 cubic centimeters of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium after 14 hours fermentation in the present experiment) and then drops again rapidly as the solvents increase in amount. A second slight rise in the acidity occurs in the last stage of the fermentation. The production of solvents is slight at the beginning, but becomes rapid after the acidity "break" and reaches a maximum after about 46 hours. A slight diminishing in the solvents from this maximum is probably due to evaporation in the incubator after the fermentation has ceased, although it is also possible that the alcohols are oxidized to acids to some extent. The sugar concentration rapidly drops throughout the fermentation, reaching the final value at about the time the yield of solvents reaches a maximum. Of the total carbohydrate, 91.5 percent was utilized in this experiment. The maximum yield of total solvents from the corn mash was 29.63 percent of the total glucose equivalent, or 31.2 percent of the glucose equivalent of the carbohydrate actually fermented.

In general, the fermentations of the carbohydrates in the semi-synthetic media were marked by greater required times, by less pronounced acidity breaks, by less complete utilizations

of carbohydrates, and by slightly better yields, based on amount of carbohydrate fermented, as compared with the corn mash fermentation.

The course of the fermentation of starch was about the same as that of corn mash, except that the acidity rise was slower, the peak coming after 21 hours of fermentation, and was not as great, only 5.0 cubic centimeters of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium being required at the peak. Of the total starch, 94.6 percent was used, and the yield of total solvents based on total glucose equivalent was 31.75 percent, while it was 33.5 percent of the glucose equivalent of the carbohydrate actually fermented.

The fermentation of glucose was much slower than the fermentation of starch. The acidity peak of 4.9 cubic centimeters of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium came at the twenty-first hour. These values are about the same as for the starch fermentation. However, there was a much slower decline after the "break", and a much greater "tail-end" acidity rise in the case of the glucose fermentation. The production of solvents after the acidity "break" was slower, and the maximum value of total solvents, 30.03 percent of total glucose, or 35.8 percent of sugar actually utilized, was found only after 131 hours. The sugar was not so completely utilized as was the starch; 84.5 percent of the

glucose was utilized.

The fermentations of maltose and of levulose were the most rapid of the sugars. The acidity curve for maltose was more like that of starch, except the higher peak value of 5.6 cubic centimeters of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium came a little earlier, between the eighteenth and twenty-first hours of fermentation. The utilization of the maltose was poor. Only 73.9 percent of the sugar was used. The final yield of 30.06 percent of the total glucose equivalent represents a 40.0 percent yield of total solvents on the basis of the glucose equivalent of sugar actually fermented. The acidity curve for levulose corresponds closely with that for glucose. The maximum in each case came at the twenty-first hour of fermentation, the decline was slow, and the "tail-end" rise was rather great. The "break" came after a maximum of 5.0 cubic centimeters of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium, 0.1 cubic centimeter higher than for glucose. However, the production of solvents was much more rapid in the case of levulose and the sugar utilization was much better; 91.3 percent of the levulose was fermented. The yield of 32.03 percent total solvents, based on the total glucose equivalent, represents a yield of 35.0 percent of the glucose equivalent of the sugar consumed in the fermentation.

The fermentation of sucrose by the butyl organism often produces anomalous results. In the experiment here reported the duplicate flasks of sucrose medium behaved quite differently. In both cases the acidity rose to a very high maximum. In the first flask the fermentation apparently stopped after the high maximum was reached, as evidenced by the cessation of gassing. The acidity remained at a high value, the sugar was very incompletely utilized, and the solvent yield was very poor. Only 34.9 percent of the sucrose was utilized. The yield of total solvents was 10.96 percent as based on total glucose equivalent but was 31.4 percent of the glucose equivalent of the sugar actually fermented. In the second flask, the maximum acidity was reached at the 40th hour of fermentation. The maximum value of 7.5 cubic centimeters of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium was very high. The decline in acidity after the "break" was very slow, and the final acidity was high. The production of solvents was very slow and continued to increase regularly to the 154th hour of fermentation. Only 77.4 percent of the sucrose was fermented. The yield of total solvents was 38.5 percent of the glucose equivalent of the sugar actually utilized, or 29.77 percent of the total glucose equivalent.

This curious behavior with sucrose was also noted several times in other experiments. In all cases, the butyl-acetonic

fermentation of sucrose was very slow and was attended with high acidities. In approximately half the cases the fermentation, which commenced rapidly enough, ceased after about two days, while in the remaining cases, the fermentation began rapidly but became slower and continued for a week or longer. The same results might not be observed in a different medium or with a different culture.

The fermentation of xylose was characterized by a very slow acidity rise, and slow initial utilization of sugar. The maximum acidity of 4.2 cubic centimeters of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium was very low, and was reached in this experiment only after the fermentation had been in progress for 52 hours. After the peak was reached sugar utilization became more rapid, and solvents began to form rapidly. The acidity decline was slight, being only 0.7 cubic centimeter of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium, and the "tail-end" rise brought the final acidity almost to the value at the peak. In certain other experiments, the final acidity was found to be greater than the peak acidity at the "break" for some xylose fermentations. In no case, however, when acidities were determined was a curve obtained such as that given by Speakman (1923) in which there was no break whatever for xylose fermentations. In the present experiment 80.5 percent of the xylose was used in the

fermentation, producing a yield of total solvents of 37.2 percent of the glucose equivalent of the fermented sugar, or a yield of 30.0 percent on the basis of total glucose equivalent.

B. Investigations of Various Factors which Influence the Butyl-Acetonic Fermentation.

1. Surface-volume ratio.

It has often been observed in practice that larger yields may be obtained in a large-scale fermentation than on a laboratory-scale. In the present investigation, during the development of the semi-synthetic medium for xylose, it was noted that yields seemed better when the fermentations were run in 500 cubic centimeter flasks than when run in 150 cubic centimeter flasks, the volumes of medium used being in each case 300 cubic centimeters and 100 cubic centimeters respectively. In order to investigate more thoroughly this tendency, the following experiment was carried out.

Fermentations of corn mash, glucose, and xylose were carried out in duplicate in Erlenmeyer flasks of 150, 500, 1000, 2000, and 4000 cubic centimeter capacities, the flasks being filled as full as practicable in every case. The flasks contained, in the cases of the glucose and xylose fermentations,

besides the carbohydrate, 1.0 gram of corn-gluten meal, 0.2 gram of dipotassium phosphate, and 0.5 gram of pulped filter paper per 100 cubic centimeters of water. A 3 percent inoculation from an active 24-hour culture in corn mash was used in all cases. After the fermentations were completed, the contents of the flasks were analyzed for total solvents, and yields correlated with the surface-volume ratio, that is, with the ratio of the surface of the medium which was exposed to air, in square centimeters, to the volume of the medium contained, in cubic centimeters. The results are given in Table 7, and are plotted in Figure 8. In every case the larger the flask, that is, the smaller the surface-volume ratio, the larger was the yield of solvents. This was probably due to the greater ease in maintaining anaerobic conditions when the ratio of surface to volume is small.

TABLE 7. Variation of Yields of Total Solvents with Change in the Surface-Volume Ratio of the Fermentations.

Flask Size (cc.)	Vol. of Medium (cc.)	Surface (Sq.cm.)	Surface-Volume Ratio*	Yield, Percent of Glucose Equiv.		
				Corn	Glucose	Xylose
150	100	22.5	0.225	20.59	20.40	19.58
500	300	50	0.167	24.66	24.42	22.50
1000	750	62	0.083	27.10	28.79	24.52
2000	1500	80	0.053	28.66	30.02	25.90
4000	3000	110	0.037	30.24	31.40	27.00

*Ratio of surface (sq.cm.) to volume (cc.).

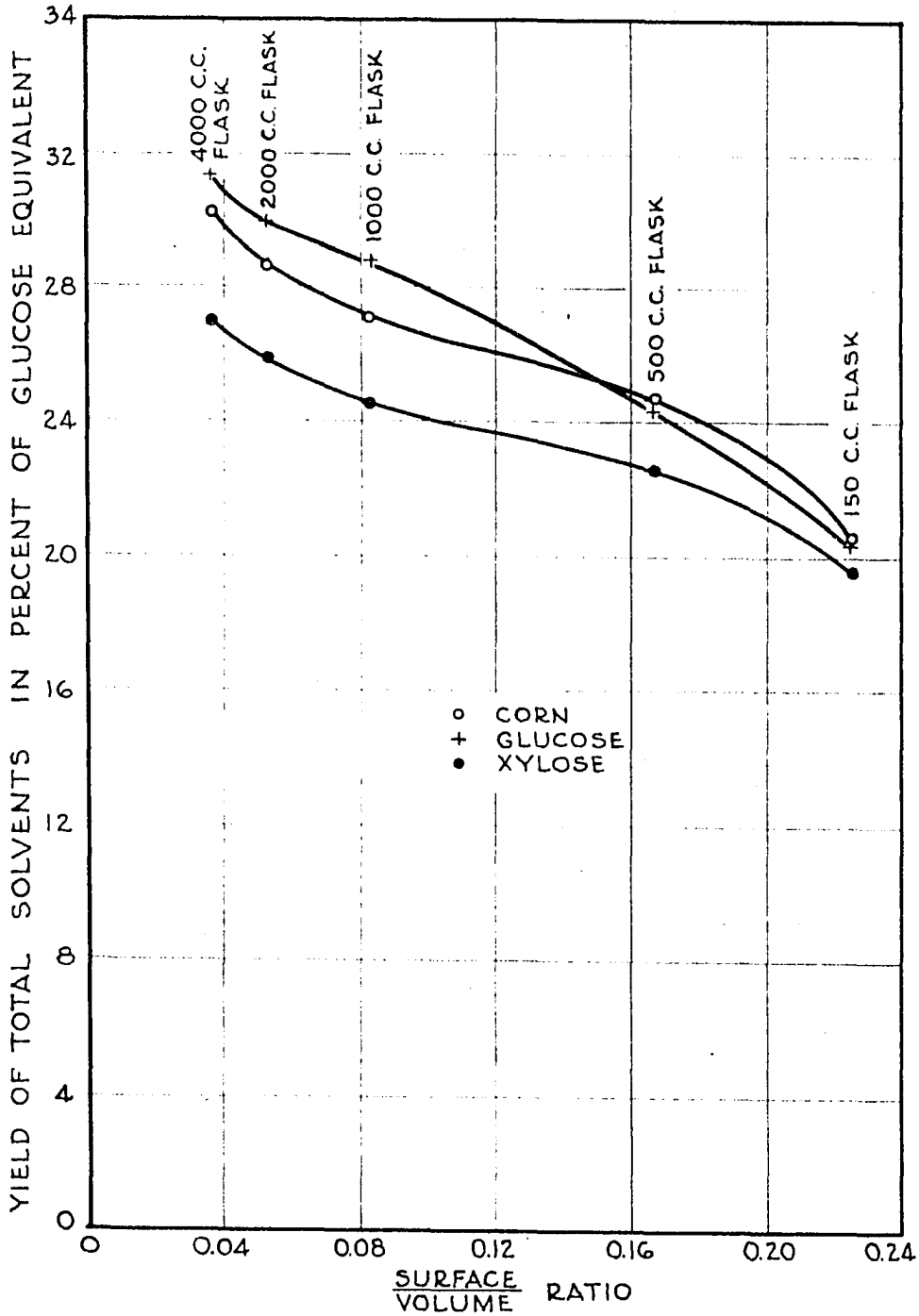

FIGURE 8. Variation of Yields of Total Solvents with Change in Surface-Volume Ratio.

2. Prolonged incubation.

In the industrial fermentation of corn mash to produce butanol and acetone it has been found that the fermentation beer must be distilled immediately after the fermentation is completed in order not to lose part of the solvents. After fermentation has stopped, solvents disappear and acidity is found to increase, probably due to oxidation of the alcohols. Gill (1919) is an early author who makes mention of this phenomenon. In the experiment with the fermentations of the various carbohydrates reported above, this same occurrence was observed. (See Table 5). Therefore, an experiment was carried out in order to determine the magnitude of this effect in the semi-synthetic xylose medium.

A number of 500 cubic centimeter Erlenmeyer flasks containing 300 cubic centimeters of the synthetic xylose medium (6.25 grams of crude xylose, 0.2 gram of dipotassium phosphate, 1.0 gram of corn-gluten meal, 0.5 gram of pulped filter paper per 100 cubic centimeters of water) were inoculated with a 3 percent inoculation from an active 24-hour culture of the butyl organism in corn mash. The flasks were set in an incubator at 37° Centigrade and after each 24 hours for thirteen days two flasks were selected at random and analyzed for titratable acidity, sugar remaining, and yield of total solvents.

The data (representing the averages for the two determinations) are presented in Table 8, and are plotted in Figure 9. The sugar was utilized rapidly for four days and then showed no further significant decrease, while the solvent yield increased rapidly for the like time, and then began to fall off gradually while the acidity increased.

TABLE 8. Effect of Prolonged Incubation.

Time of Incubation (days)	Acidity (cc. 0.1 N NaOH/10cc.)	Sugar Remaining (g./100cc.)	Yield of Total Solvents, Percent of Glucose Equivalent
0	1.50	5.73	----
1	4.37	4.75	6.41
2	2.57	3.17	16.41
3	2.35	1.26	25.00
4	2.35	0.96	27.41
5	2.45	0.93	27.08
6	2.87	0.91	25.90
7	3.10	0.88	25.30
8	2.97	0.78	24.76
9	3.35	0.81	24.08
10	3.35	0.88	23.38
11	3.40	0.81	23.38
12	3.35	0.88	22.29
13	3.40	----	21.48

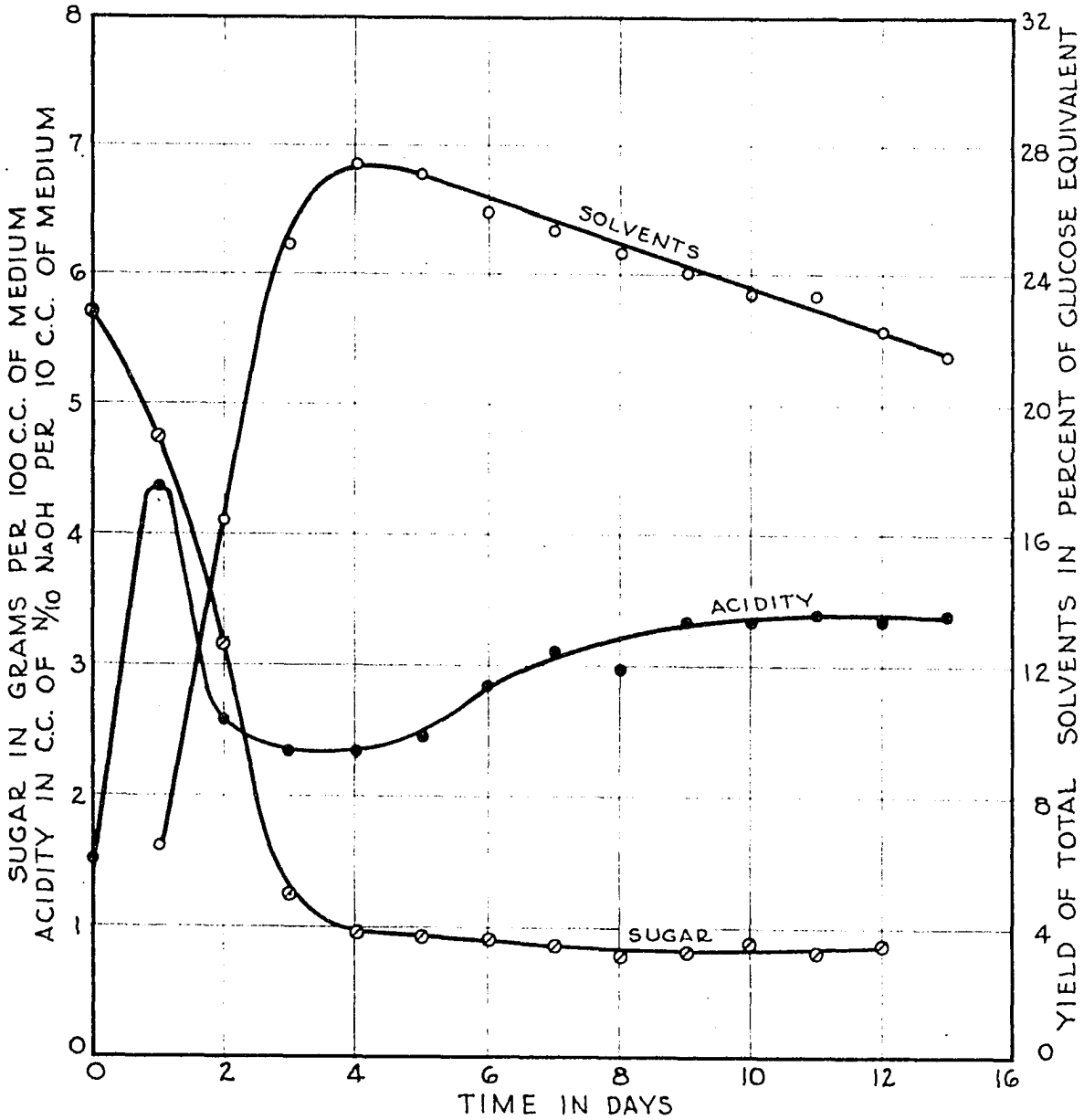

FIGURE 9. Behavior of the Xylose Fermentation on Prolonged Incubation.

3. Inoculation from different transfers.

The customary technique for handling a culture of the butyl organism is as follows: A fresh tube of medium (usually corn mash) is inoculated from a spore culture of the organism. This spore culture may be a tube of medium which has undergone the butyl fermentation and then has been allowed to stand for a period of a week or longer. More commonly, the spore culture obtained in this way is mixed with sterile soil and dried under sterile conditions. This "soil culture" will produce actively fermenting cultures of the butyl organism almost indefinitely, whereas the liquid spore culture, even when kept in a refrigerator, gradually deteriorates, and after long standing produces very sluggish fermentations.

After inoculation from the spore culture, the fresh tube of medium is "heat shocked" or pasteurized for two minutes in a vessel of boiling water, cooled rapidly, and incubated at 37° Centigrade. After 24 to 36 hours the culture so prepared should be gassing rapidly. From this original culture a second tube of medium is inoculated, using a sterile pipette for the transfer. After 24 hours, transfer is again made into another tube, or in industrial practice into a larger vessel. Transfers are made similarly into new medium every 24 hours,

and usually the fourth, fifth, or sixth culture from the original spore stock is used to produce the desired fermentation. Transfers farther removed from the original stock are found to become less vigorous, fermentation is slower and less complete, and the medium becomes viscous and slimy instead of quite liquid.

In order to observe the effect on yield from xylose medium when inoculation was from different transfers, two 500 cubic centimeter Erlenmeyer flasks containing 300 cubic centimeters of medium, made up with 6.25 grams of crude xylose, 1.0 gram of corn-gluten meal, 0.25 gram of dipotassium phosphate per 100 cubic centimeters of water, were inoculated on each of eight successive days from an active culture in corn mash carried along by repeated daily transfers as outlined above. Fermentations were allowed to continue until gas ceased to be evolved, the contents of the flasks were distilled, and the distillates analyzed for solvents. Results are given in Table 9, and are plotted in Figure 10, averages of the duplicate fermentations being recorded.

From the data it may be seen that the best yields of solvents were obtained when the fermentation was from the fourth to the seventh transfer removed from the original spore stock. Yields from the second and third transfers were quite good, although less than from later transfers, and the

fermentations were slower. The eighth and ninth transfers gave very slow fermentations with very much reduced yields of solvents. The medium in these cases became very slimy and viscous.

TABLE 9. Yields of Solvents from Xylose Medium Inoculated from Different Transfers.

Number of Transfers from Original Spore Stock	Time Required For Completion of Fermentation (hours)	Yield, Percent of Glucose Equivalent			
		Butanol	Acetone	Ethanol	Total
2	288	17.34	5.59	1.89	24.82
3	192	16.69	5.90	2.55	25.14
4	142	17.10	6.58	2.58	26.26
5	161	17.62	6.01	2.62	26.25
6	162	17.76	6.26	2.24	26.26
7	139	17.70	6.43	2.63	26.76
8	227	13.61	4.02	1.24	18.87
9	279	12.13	3.79	1.15	17.07

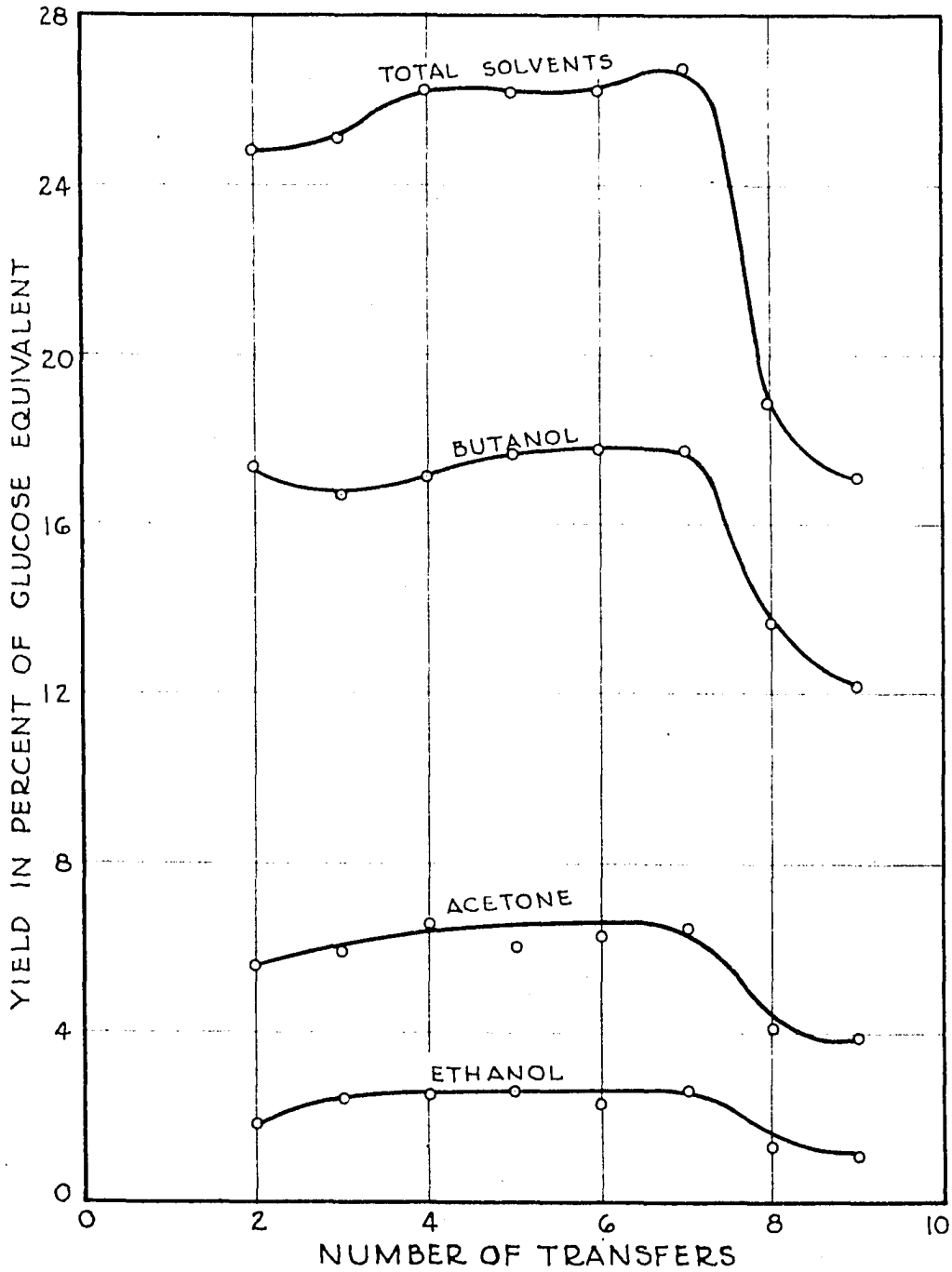

FIGURE 10. Solvents Produced from Xylose Medium Inoculated from Different Transfers.

C. Separation and Identification of the Products of the Butyl Fermentation of Xylose in the Semi-Synthetic Medium.

In all the analytical work during this investigation it was assumed that the only neutral products produced in any considerable amounts were butanol, acetone, and ethanol. This is known to be true for the butyl fermentation of corn mash, although Wilson, Peterson and Fred (1927) showed that acetyl-methyl carbinol was also formed in small quantities. The analytical method used in determining solvents was worked out for mixtures containing only the above mentioned three solvents. In order to determine whether any other neutral products besides the three mentioned were produced in the fermentation of the semi-synthetic xylose medium, and also in order to check the analytical results, a large fermentation was carried out, the solvents distilled off, dried and fractionated, and derivatives made for the identification of the fractions. The experiment was carried out as follows:

Nineteen liters of the xylose medium, containing 6.25 grams of crude xylose, 1.0 gram of corn-gluten meal, and 0.25 gram of dipotassium phosphate per 100 cubic centimeters of water, were prepared in a 22-liter flask and sterilized in the autoclave at 15 pounds steam pressure for 30 minutes after

the mixture had become thoroughly heated. After cooling to incubator temperature the medium was inoculated with a 2 percent inoculation from an active 24-hour culture in corn mash. Immediately after inoculation a sample was taken for sugar analysis and was found to contain 5.32 grams of glucose equivalent per 100 cubic centimeters of medium. Fermentation had ceased after 5 days, but it was necessary for the flask to stand at room temperature for two days longer before the contents were distilled. Samples of the fermented liquid were analyzed for sugar remaining and for solvents according to the usual methods. Unfermented sugar amounted to 1.04 grams per 100 cubic centimeters of medium, expressed as glucose equivalent. After neutralizing to phenolphthalein with normal sodium hydroxide solution, 17,000 cubic centimeters of the fermented liquid were distilled. Distillation was stopped after collecting 3,500 cubic centimeters of distillate in flasks cooled in ice water. To the distillate was added an excess of solid sodium chloride to salt out the solvents, and the mixture was redistilled. After 600 cubic centimeters of distillate had been collected the solvents were found to have distilled over completely. This distillate was shaken with excess of solid anhydrous potassium carbonate and allowed to stand in a refrigerator over night. Two layers were formed in the mixture and these were separated by means of a

separatory funnel. The aqueous layer was redistilled and 20 cubic centimeters of distillate collected. This was again treated with solid anhydrous potassium carbonate and set in the refrigerator for 18 hours. By this procedure there was obtained 1-2 cubic centimeters of solvents which were added to the oily layer previously collected, making the total volume of crude solvents 327 cubic centimeters. This solvent mixture was submitted to slow fractional distillation, using a long and efficient fractionating column. Five fractions were collected in graduates cooled in ice water. The total volume of all the fractions was 321 cubic centimeters.

The respective fractions were carefully refractionated, and in these refractionations a total of 78 cubic centimeters of distillate was collected which boiled at 56-7° Centigrade. In the range 57° to 77° a total volume of 16 cubic centimeters was received. From 77° to 80° the total volume of distillate was 25 cubic centimeters. From 80° to 116° there was a gradual increase in the temperature of distillation, but no representative fraction could be collected. A total of 40 cubic centimeters distilled over in this range. A constant boiling fraction of total volume of 161 cubic centimeters was obtained at 116-7°.

It was believed probable that the fraction boiling at 56-7° was acetone, the fraction boiling from 77-80° mainly

ethanol, and the fraction boiling at 116-7° butanol. The fraction collected within the range 57° to 77° was again carefully refractionated and the products added to the acetone and ethanol fractions. The final volume of the fraction boiling at 56-7° was 90 cubic centimeters, and that boiling at 77-80° was 29 cubic centimeters.

It was thought at first that the considerable quantity of distillate coming over in the range 80 to 116° might consist of a mixture of the propyl and butyl alcohols other than normal butanol. However, no success attended efforts to refractionate this portion, the temperature rising continuously until all was distilled. During the beginning of this distillation the distillate was very cloudy and tended to separate into two layers. Therefore the entire portion was again dried over night with anhydrous potassium carbonate and again distilled. The temperature rose almost at once to 116°, and 37 cubic centimeters of distillate was collected at 116-7°, making a total volume of 198 cubic centimeters for the combined fractions boiling at 116-7°.

Derivatives were made from the three fractions into which the solvents were thus ultimately separated. Solid derivatives were obtained from the two higher boiling fractions by treating with 3,5-dinitrobenzoyl chloride and recrystallizing the products. The melting points of these derivatives were 92° and

64°. These values agree exactly with the values for ethyl 3,5-dinitrobenzoate and n-butyl 3,5-dinitrobenzoate as given by Kamm (1923). Mixed melting points with known derivatives prepared in the same way from pure ethanol and butanol gave unchanged values.

A solid derivative from the acetone fraction was obtained by treating the lowest boiling fraction with benzaldehyde and dilute sodium hydroxide solution. The melting point was 111°. This agrees with the value given by Kamm (1923) for dibenzylidine acetone. A mixed melting point of the unknown with the known derivative from pure acetone gave an unchanged value.

From these results it was concluded that the three substances butanol, acetone, and ethanol constituted the only neutral volatile products produced in any considerable amounts by the fermentation of xylose by the butyl organism.

In Table 10 are given the yields of solvents produced in this fermentation, calculated on the basis of the solvents recovered by distillation and also from the usual methods of analysis.

In the butyl fermentation of corn mash the distribution of solvents produced is approximately 60 percent butanol, 30 percent acetone, and 10 percent ethanol. The distribution of solvents from the xylose fermentation seems to be the same as in the corn mash fermentation, both on the basis of solvents

obtained by distillation and by analysis, as shown in Table 10. The proportion of acetone was somewhat low, but that was probably due to the fact that the fermented medium was allowed to stand for nearly two days after fermentation was completed before it was distilled. The loss of the more volatile acetone was probably greater than for the other products, expecially butanol. The yields obtained by distillation were somewhat lower than the values obtained by the usual analytical methods, although correspondence was ~~ccise~~ close. The difference probably is due to the unavoidable losses during the distillations.

TABLE 10. Neutral Products Produced in the Butyl-Acetonic Fermentation of Xylose.

	Butanol	Acetone	Ethanol	Total
From Distillation of 17,000 cc. of Fermented Medium				
Volume (cc.)	198.0	90.0	29.0	317.0
Total Weight (grams)	160.4	71.3	22.9	254.6
Weight (g./100 cc. of medium)	0.9436	0.4195	0.1347	1.4978
Yield (percent of glucose equivalent fermented)	22.05	9.96	3.15	35.16
Percent distribution of solvents	62.97	28.02	9.01	100.00
By Usual Analytical Method				
Weight (g./100 cc. of medium)	0.9500	0.4325	0.1575	1.5400
Yield (percent of glucose equivalent fermented)	22.20	10.10	3.68	35.98
Percent distribution of solvents	61.69	28.08	10.23	100.00

D. The Course of the Fermentation of Xylose in the Semi-Synthetic Medium, Including a Continuous Carbon Balance for this Fermentation.

The main products of the butyl-acetonic fermentation are: butanol, acetone, ethanol, carbon dioxide, and hydrogen. In addition, rather considerable quantities of organic acids are found at the end of fermentation, these being mainly acetic acid, butyric acid, and non-volatile acids.

A 4-liter Erlenmeyer flask was filled with 3800 cubic centimeters of the xylose medium containing 6.25 grams of crude xylose, 1.0 gram of corn-gluten meal, and 0.25 gram of dipotassium phosphate per 100 cubic centimeters of water. After sterilization at 10 pounds steam pressure for 30 minutes and cooling to incubator temperature (37° Centigrade) a 2 percent inoculation from an active 20-hour culture in corn mash was added, and the flask equipped with a two-hole rubber stopper bearing a short glass tube (which had a plug of cotton in the bore to prevent contamination) to serve as a gas outlet tube for carrying away the fermentation gases, and a second long glass tube extending down into the medium and bent in an inverted U-shape for taking samples of the fermenting liquid. The end of this latter glass tube was fitted with a short piece of rubber tubing closed by a screw clamp and bearing

a shorter length of Pyrex glass tubing plugged at the end with cotton. The stopper and all the attendant equipment were put together and sterilized in the autoclave just before being placed in the flask. The rubber stopper was wired down to the flask so that the gas pressure which built up in the flask would not force it out.

A sample of the medium was taken immediately after inoculation and was analyzed for sugar content. The flask was set in the incubator and the gas outlet tube connected with large 5-gallon bottles of salt brine for collecting the gases. At intervals samples of the fermenting medium were taken by opening the screw clamp and allowing the gas pressure to force out some of the liquid. After closing the screw clamp the end of the Pyrex tube was flamed and plugged with a bit of cotton saturated with mercuric chloride solution. The samples were analyzed for solvents, pH, total acidity, volatile acids, non-volatile acids, and sugar remaining. The volume of gas collected in the bottles over the brine was also measured at intervals by means of gas measuring flasks and a gas burette, and samples were analyzed for carbon dioxide, hydrogen, and oxygen (air). The results of the analyses are given in Table 11, and are plotted in Figures 11 and 12. Figure 11 presents the products industrially important, butanol, acetone, ethanol, carbon dioxide, and hydrogen, and Figure 12 the acids produced and sugar utilized in this fermentation.

TABLE 11. Course of the Fer

Time (hours)	Total Acidity (cc. 0.1 N NaOH/10cc. of medium)	pH	Sugar (Xylose, g./100cc. of medium)	Sugar Fermented (Xylose, g./100cc. of medium)	Yield per 100 c				
					Butanol (grams)	Acetone (grams)	Ethanol (grams)	Carbon Dioxide (cc.)	Hydr (c
0	1.43	5.1	6.08	0.00	-----	-----	-----	----	-
30	2.04	4.6	6.04	0.04	0.013	0.007	0.017	19.2	2
44	3.06	4.4	5.74	0.34	0.043	0.011	0.011	68.3	7
54	4.19	4.4	----	----	-----	-----	-----	-----	--
60	4.70	4.0	5.03	1.05	0.180	0.069	0.029	254.5	20
68	4.08	4.2	4.33	1.75	0.339	0.164	0.068	461.0	32
80	3.57	4.5	2.80	3.28	0.642	0.274	0.133	888.7	57
84	3.47	4.5	2.42	3.66	0.736	0.330	0.133	986.3	63
92	3.67	4.3	1.90	4.18	0.821	0.371	0.167	1119.0	71
100	4.08	4.3	1.63	4.45	0.878	0.382	0.192	1175.1	74
108	4.18	4.3	1.53	4.55	0.885	0.386	0.206	1194.6	75
122	4.29	4.4	1.49	4.59	0.879	0.429	0.220	1200.0	76
144	4.49	4.3	1.53	4.55	0.970	0.462	0.180	1200.0	76

Course of the Fermentation of Xylose, and a Carbon Balance for this Fermentation.

Yield per 100 cc. of Medium						Mols of Products per Mol of Xylose						
anol ams)	Carbon Dioxide (cc.)	Hydrogen (cc.)	Butyric Acid (cc. N)	Acetic Acid (cc. N)	Non-Volatile Acid (cc. N)	Butanol	Acetone	Ethanol	Butyric Acid	Acetic Acid	Non-Vol. Acid	C D
---	----	----	----	----	----	-----	-----	-----	-----	-----	-----	
017	19.2	21.8	0.14	1.90	----	-----	-----	-----	-----	-----	-----	
011	68.3	75.8	0.70	2.41	----	0.256	0.084	0.105	0.309	1.064	-----	
---	-----	-----	----	----	----	-----	-----	-----	-----	-----	-----	
029	254.5	204.6	1.07	2.78	0.87	0.348	0.170	0.090	0.153	0.398	0.124	
068	461.0	322.4	0.75	2.17	1.25	0.394	0.242	0.127	0.064	0.186	0.107	
133	888.7	574.0	0.31	1.66	1.60	0.397	0.216	0.132	0.014	0.076	0.073	
133	986.3	631.6	----	----	----	-----	-----	-----	-----	-----	-----	
167	1119.0	711.4	0.30	1.81	1.60	0.399	0.230	0.131	0.011	0.065	0.058	
192	1175.1	746.6	----	----	----	-----	-----	-----	-----	-----	-----	
206	1194.6	758.5	----	----	----	-----	-----	-----	-----	-----	-----	
220	1200.0	761.9	0.27	2.06	1.90	0.392	0.244	0.158	0.009	0.068	0.063	
180	1200.0	761.9	0.28	2.08	2.01	0.432	0.263	0.129	0.009	0.069	0.066	

Carbon Balance for this Fermentation.

	Mols of Products per Mol of Xylose								Carbon Balance	
olatile cid c. N)	Butanol	Acetone	Ethanol	Butyric Acid	Acetic Acid	Non-Vol. Acid	Carbon Dioxide	Hydrogen	-C	C
---	-----	-----	-----	-----	-----	-----	-----	-----	---	-----
---	-----	-----	-----	-----	-----	-----	-----	-----	---	-----
---	0.256	0.084	0.105	0.309	1.064	-----	1.356	1.492	5.0	6.206
---	-----	-----	-----	-----	-----	-----	-----	-----	---	-----
.87	0.348	0.170	0.090	0.153	0.398	0.124	1.631	1.307	5.0	5.741
.25	0.394	0.242	0.127	0.064	0.186	0.107	1.777	1.233	5.0	5.496
.60	0.397	0.216	0.132	0.014	0.076	0.073	1.806	1.171	5.0	4.879
---	-----	-----	-----	-----	-----	-----	-----	-----	---	-----
.60	0.399	0.230	0.131	0.011	0.065	0.058	1.804	1.141	5.0	4.816
---	-----	-----	-----	-----	-----	-----	-----	-----	---	-----
---	-----	-----	-----	-----	-----	-----	-----	-----	---	-----
.90	0.392	0.244	0.158	0.009	0.068	0.063	1.778	1.121	5.0	4.881
.01	0.432	0.263	0.129	0.009	0.069	0.066	1.778	1.121	5.0	5.057

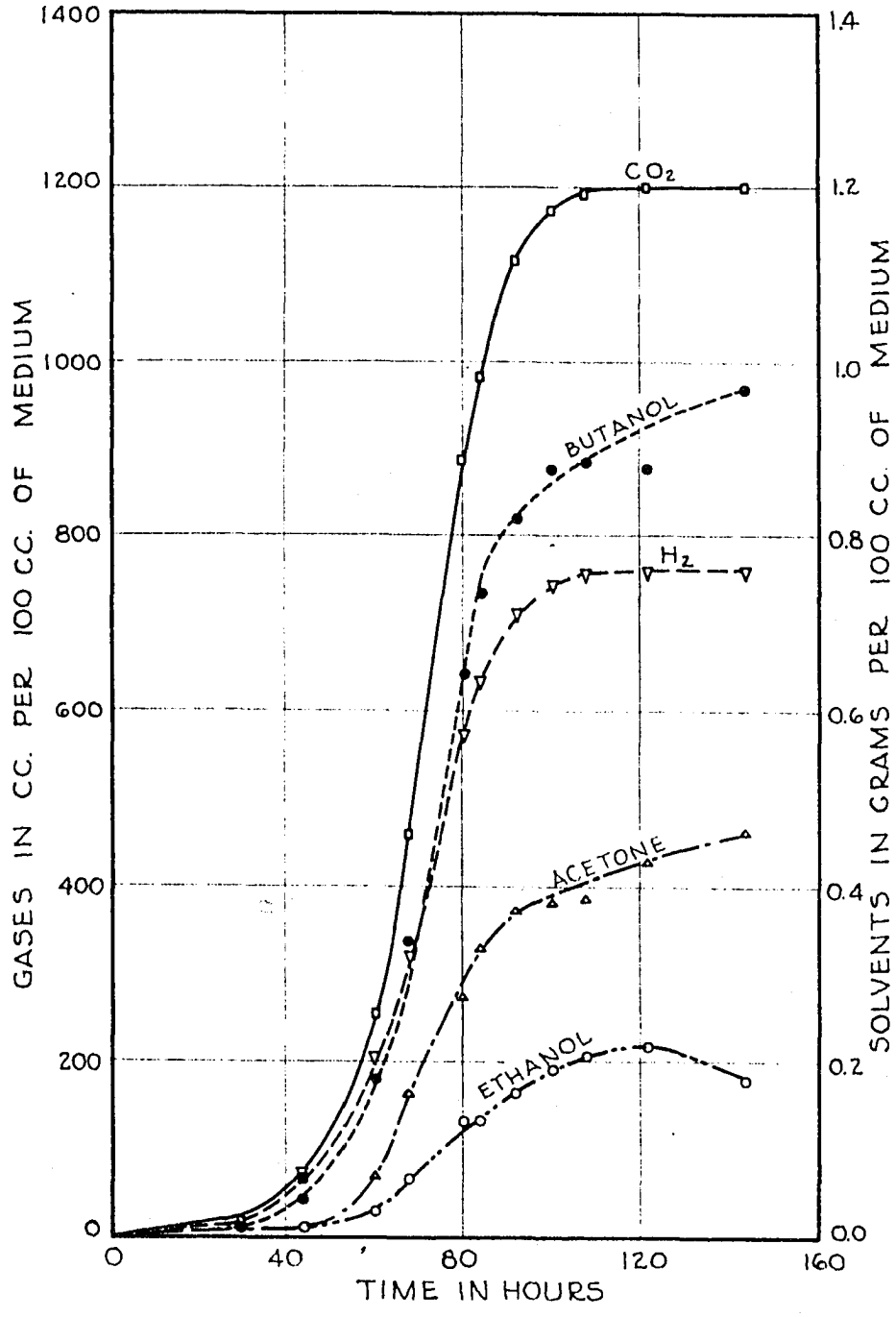

FIGURE 11. Products of Industrial Importance Produced in the Xylose Fermentation.

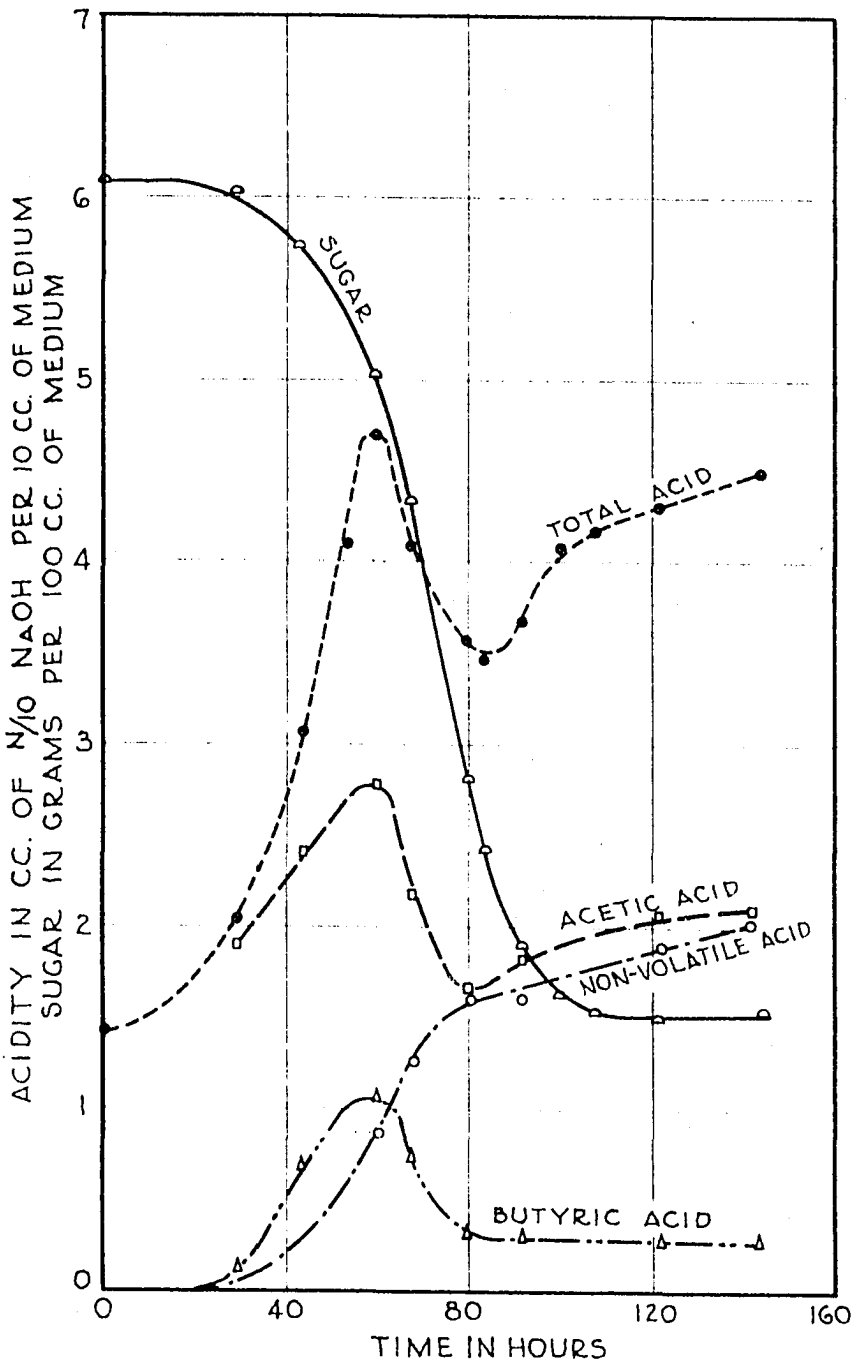

FIGURE 12. Acids Produced and Sugar Utilized in the Xylose Fermentation.

From Figures 11 and 12 it is evident that the course of this particular fermentation was essentially the same as that of the xylose fermentation previously followed, the results of which are given in Table 5, and which have previously been discussed. The acidity rise to the maximum was somewhat slower than for the previous fermentation, but the peak value of 4.70 cubic centimeters of 0.1 normal sodium hydroxide per 10 cubic centimeters of medium was 0.5 cubic centimeter higher, and the decline was somewhat greater, 1.23 cubic centimeters of 0.1 normal instead of 0.7 cubic centimeter. This fermentation did not last as long as the previous fermentation, but only 74.8 percent of the sugar was fermented instead of 80.5 percent as in the former fermentation, and the yield of total solvents was 36.0 percent of the glucose equivalent of the fermented sugar instead of 37.2 percent as in the former case. In the present fermentation gases and individual acids were determined, while this was not done in the previous experiment.

From the analytical data, the number of mols of each product per mol of xylose fermented was calculated and these values are also given in Table 11, as well as carbon balances derived from these results. In these calculations it was assumed that the non-volatile acids had an average of five carbon atoms per molecule. According to the work of Schmidt, Peterson and Fred (1924), and of Stiles, Peterson and Fred

(1929), the butyl-acetonic fermentation produces a mixture of non-volatile acids, including both alpha-hydroxy and beta-hydroxy acids. According to the former authorities l-leucic acid, which is an alpha-hydroxy isocaproic acid, a six-carbon compound, made up the main portion of the alpha-hydroxy portion, although other acids were present also. The latter investigators assumed in their calculations that the alpha-hydroxy fraction was entirely l-leucic acid, and that the beta-hydroxy fraction was beta-hydroxy butyric acid, a four-carbon compound, although they recognized that each of these fractions were mixtures of several acids. In view of these investigations, the assumption that the non-volatile acids average five carbon atoms per molecule seems justified, and the values obtained for the carbon balances in the present case bear out this conclusion. Also, part, if not all, of the non-volatile acids no doubt comes from the protein, which would account for the fact that in the carbon balance for the completed fermentation the products were in some slight excess over sugar fermented.

According to Wilson, Peterson and Fred (1927) small amounts of acetylmethyl carbinol are formed by the butyl organism in corn mash fermentations. Qualitative tests on the liquid from the xylose fermentation also showed the presence of acetylmethyl carbinol. However, since the amount of this

substance formed is very small, no quantitative estimations were made, and in the calculation of the carbon balances for this fermentation the acetylmethyl carbinol and other minor products were ignored.

The carbon recovered in the products during the early stages of the fermentation was considerably greater in amount than in the sugar consumed. This discrepancy was probably due to the fact that in the sugar analyses certain reducing intermediate compounds produced in the fermentation also reduced copper solution and caused the values obtained for sugar remaining to be too high; therefore the actual amounts of sugar consumed were greater than calculated from the analytical results. At the end of the fermentation the carbon balance approached closely the theoretical value.

SUMMARY AND CONCLUSIONS

1. The action of the butyl organism on various carbohydrates has been investigated by replacing corn meal, in series, by equivalent amounts of the carbohydrates starch, glucose, sucrose, and xylose. All of these carbohydrates were found to be fermented, starch the most readily, and xylose the least readily. When there was sufficient corn meal to supply the necessary nutrients to support the activities of the organism, normal yields of solvents were obtained from starch, glucose, and sucrose. Xylose, particularly in the crude form, was not so readily available as the other carbohydrates studied.

2. The best medium composed of several constituents was developed by determining the optimum concentration of each. Of the nitrogenous nutrients studied, peptone, tankage, steep-water, casein, ammonium chloride, and corn-gluten meal, the latter was chosen as most suitable. The optimum amounts of corn-gluten meal were 2.0 grams and 0.5 gram per 100 cubic centimeters of medium when xylose and glucose, respectively, were used as substrates. The salts dipotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, and manganous sulfate in the concentrations studied had no appreciable effect on the yields of solvents from the fermentation of

xylose in the presence of corn-gluten meal. The latter nutrient evidently contained all the constituents necessary to the development of the organism. For experiments during this investigation 0.2-0.25 gram of dipotassium phosphate per 100 cubic centimeters of medium was added for the buffering action of this substance.

3. The course of the fermentations of the individual carbohydrates starch, glucose, maltose, levulose, sucrose, and xylose as regards titratable acidity, pH, solvent formation, and sugar utilization has been followed in the semi-synthetic media containing corn-gluten meal. It was found that all these carbohydrates are available for the butyl organism, but that there was considerable variation in the rates of fermentation, and a marked difference in the course of the various fermentations. In general the fermentations of the carbohydrates in the semi-synthetic media were marked by greater required times, by less pronounced acidity breaks, by somewhat poorer utilizations of carbohydrates, and by slightly better yields, based on amounts of carbohydrates fermented, as compared with the corn mash fermentation. Sucrose and xylose gave the most abnormal fermentations, the very high acidity developed characterizing the former, and the slow rate of fermentation, particularly at the beginning, was especially characteristic of the latter.

4. Several factors which influence the butyl-acetonic fermentation have been investigated as they apply to the fermentation of xylose in the semi-synthetic medium. The yield of solvents was found to increase with decrease in surface-volume ratio of the medium, that is, when larger flasks were used and filled as completely as practicable, yields were better, due to the lessened difficulty of maintaining anaerobiosis in the medium. The yield of solvents decreased with prolonged incubation, while acidity increased, perhaps due to oxidation of the alcohols to acids as well as to evaporation. Yields of solvents were greatest when the fermentation was from the fourth to the seventh transfer removed from the original spore stock.

5. Employing a relatively large-scale fermentation of xylose, the neutral volatile products were determined by the usual analytical methods in a portion of the fermented liquid, and were also separated by fractionation of the main portion of the liquid. The fractions were identified by boiling points and by preparation of the customary derivatives, and it was concluded that the three substances butanol, acetone, and ethanol constituted the only neutral volatile products produced in any considerable amounts by the fermentation of xylose. The ratio of these products was approximately the same as has been reported from corn mash, and the amounts

obtained from the fractionation checked quite closely with those found by the analytical methods, the slightly lower values being due, probably, to unavoidable losses during the distillation.

6. The course of the fermentation of xylose in the semi-synthetic medium was followed as regards pH, total acidity, production of volatile acids, non-volatile acids, solvents, carbon dioxide, hydrogen, and utilization of sugar. From the data a continuous carbon balance was calculated. Although the carbon in the products obtained during the early stages of the fermentation was considerably greater in amount than in the sugar consumed, at the end of the fermentation the carbon balance approached closely the theoretical value. Production of acetylmethyl carbinol and of other minor products was ignored in calculating the carbon balances. The slight excess of products at the completion of the fermentation probably resulted from the production of non-volatile acids from the corn-gluten meal by proteolysis.

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