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The production of alcohols by thermophilic fermentations

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THE PRODUCTION OF ALCOHOLS BY THERMOPHILIC FERMENTATIONS

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INTRODUCTION

Increasing attention is being paid by research workers and various agencies to the development of industries based upon the production of industrial chemicals from agricultural materials as raw products. The conversion of the carbohydrates into useful chemicals by fermentation processes offers particularly great promise of large scale application. Up to the time of the World War the only chemicals produced on a large scale by fermentation processes were ethanol and acetic acid. Under pressure of shortage for the production of munitions, methods were developed for the production of glycerol and of acetone by fermentation. Normal butyl alcohol is likewise formed in the latter process and for a period of years found no large outlet. Now the butyl-acetonic fermentation is one of the large chemical industries. It is interesting to note that the finish on the average automobile represents the fermentation products of about one and three-quarters bushels of corn.

Considerable attention has been paid to the commercial production of combustible gases and of acetic acid by the thermophilic fermentation of cellulosic materials. Many patents have been taken out for these processes. A consideration of the literature shows the production of ethanol by the acid forming thermophilic cultures. However, practically no systematic work

has been done on the subject, the production of ethanol being incidental. It is obvious that if a method could be developed for giving considerable yields of ethanol by the thermophilic fermentation of cellulose, such a process would be of great value in the utilization of agricultural products in the production of industrial chemicals. Cellulosic materials are very abundant in nature and large quantities are wasted annually in the form of oat hulls, corn cobs, corn stalks, beet pulp, and similar materials. In the event of a shortage of petroleum it is not at all impossible that alcohol from this source will be of great importance as a motor fuel.

The purpose of this thesis is to make a systematic study of the production of ethanol by the thermophilic fermentation of cellulose and to determine the optimum conditions of medium and temperature for a variety of cultures.

HISTORICAL SURVEY

The history of the fermentation of cellulose dates back to 1950 when Mitscherlich (1850) observed that the cell membranes of potatoes were destroyed when allowed to rot in wet places.

Later, van Tieghem (1877, 1879) reported on the destruction of cellulose by Bacillus amylobacter at temperatures of 25°C. to 35°C. The organisms were able to withstand a temperature of 100°C. for short periods of time. Considerable amounts of gas were given off during the fermentation. He believed Bacillus amylobacter to be the principal agent in the destruction of cellulose in nature.

Omelianshy (1902) published a series of articles on the fermentation of cellulose at ordinary temperatures. He found two types of fermentations. In one carbon dioxide and hydrogen were the gaseous products and in the other carbon dioxide and methane were formed. The fermentations were very slow and often lasted for months.

Boruff and Buswell (1929) and Buswell and Boruff (1930) developed a method of obtaining large quantities of methane from corn stalks by fermentation. The ratio of carbon dioxide to methane varied from 1-1 to 1-10. They calculated that a ton of corn stalks would produce from 10,000 to 20,000 cubic feet of gas and a circle of 8 mile radius planted 30 per cent to corn

would yield sufficient corn stalks to supply gas to a city of 80,000 population. The literature on the fermentation of cellulose at ordinary temperatures has been reviewed by McBeth and Scales (1913) and by Simola (1932).

The first mention of thermophilic cellulose decomposition by bacteria was by MacFayden and Blaxall (1899). Soil was added to a nutrient solution containing paper and incubated at 60°C. The paper was destroyed in 10 to 14 days. Bacilli as well as many spores were detected in the culture. They stated that acetic acid, butyric acid, and furfural were formed, and that the action seemed to be symbiotic.

Woodman (1917) and Woodman and Stewart (1928, 1932) studied the relation of thermophilic cellulose fermentation to digestion in the ruminant organism. They were able to demonstrate the presence of considerable amounts of glucose during the fermentation of cellulose in the presence of toluene, but no glucose was detected during the course of an ordinary fermentation. They are of the opinion that the thermophilic cellulose fermenters play an important part in the utilization of cellulose in the ruminant organism.

Khouvine (1923) studied the cellulose decomposing organisms found in the intestinal flora of man. Thermophilic bacteria were found that would decompose cellulose. Several different media were used, but one containing organic nitrogen, sodium chloride, and di-potassium phosphate was considered the best.

Khouvine claims to have isolated a pure culture, which she named Bacillus cellulosae dissolvens, by washing a fragment of fermenting cellulose with sterile physiological salt solution. It was found that organisms were present which were attached to the cellulose by their non-sporulating end. This culture grew more slowly than did crude cultures and had an optimum temperature range of 35° and 51°C., but would withstand 62°C. Many common carbohydrates were not attacked by this culture. From one gram of cellulose there were obtained 0.001 g. of hydrogen, 0.023 g. of carbon dioxide, 0.02 g. of ethanol, and 0.008 g. of volatile acids. The amount of cellulose decomposed was 0.175 g., so the yield of ethanol was 11.4 per cent of the cellulose decomposed. There is some doubt as to whether this culture may be considered a pure strain of a thermophilic cellulose fermenter.

Langwell and his co-workers in England have done a great deal of work on the thermophilic fermentation of cellulose and cellulosic materials, particularly with reference to commercial applications. Lymn and Langwell (1923) presented a paper as a part of a "Discussion of the Action of Bacteria on Cellulosic Materials." They claim that the organisms grow from 37° to 68°C., and that they have isolated a pure cellulose fermenter by plating and picking from a colony. The optimum temperature range is reported to be from 60° to 68°C. The cultures fermented resistant celluloses such as filter paper, sulfite half stuff, and linen fiber, modified resistant cellulose such as parchmet-

ised filter paper and mercerized filter paper, hemicelluloses, starches, and sugars. Alcohol yields of 27 per cent were obtained from sulfite pulp, 8.3 per cent from filter paper, and 15.5 per cent from xylose from rice straw. Butyric acid, lactic acid, carbon dioxide, and methane were also produced. In the view of later work it is doubtful whether these authors were working with pure cultures.

Langwell and Hind (1923) claim that for the thermophilic cellulose fermentation potash and phosphate should be present in the medium and that ammonium salts suffice as a source of nitrogen. They note the extreme susceptibility of the organisms to copper and that the organisms cannot ferment cellulose in the presence of lignin. They claim that 40 gallons of a mixture of alcohol and acetic acid can be obtained from a ton of fermentable material such as spent hops.

Langwell (1932) states that a mobile medium is necessary for the rapid decomposition of cellulose. Thus, when beet pulp is converted to a thick mucilaginous mass by stirring, fermentation ceases. He claims also that the presence of calcium carbonate prevents the fermentation from going to completion by converting the phosphate into the insoluble tri-calcium phosphate, and that sodium carbonate or bicarbonate is much better for the neutralization of the acids formed. The presence of metallic iron was also found to be injurious. Iron steam coils were rapidly corroded and fermentation would not progress during

stirring in their presence. Later, some fermentation vats were constructed entirely of aluminum. The results of some fermentations in 40 gallon aluminum fermenters are given. Yields as high as 7.75 per cent alcohol and 40 per cent acetic acid are reported using a 6.2 per cent corn cob medium. Butyric acid, carbon dioxide, hydrogen, and methane are also listed as products. As nutrients, di-sodium phosphate, potassium sulphate, and ammonium sulphate were used. Langwell notes that small laboratory fermentations generally gave relatively high yields of alcohol, while in large fermenters only small yields were obtained. The effect of aeration was tested by blowing 20 to 40 liters of air per minute through the 40 gallon fermenters for 6 hours a day. On corn cob media the aeration increased the alcohol yield from 3.2 to 7.1 per cent and decreased the acetic acid yield from 31.6 to 25.2 per cent. Later, 1000 gallon fermenters were built but the recovery of alcohol was abandoned to avoid excise regulations. Beet pulp was then employed and yields of approximately 50 per cent of acetic and butyric acids were obtained. Data from these tests justified the erection of a 60,000 gallon plant. Langwell suggests the use of ammonia for neutralization with subsequent recovery of the ammonia by treatment of the fermented mash with lime. The mechanical details of such an arrangement had not been worked out. Langwell used a temperature of 60°C. for his fermentations and adjusted the pH to a value of 7.0.

By far the largest number of publications in recent years dealing with the thermophilic fermentation of cellulose have come from the University of Wisconsin. Among the investigators at this institution, whose work is discussed below, are Viljoen, Fred, Peterson, Martin, Tetrault, Scott, Sarles, Snieszko, and Kimball. Although they worked on other phases of the subject, their main object was to develop a method for isolating a thermophilic cellulose fermenter in pure culture.

Viljoen, Fred, and Peterson (1926) used a medium consisting of 2.0g. of microcosmic salt, 1.0 g. of mono-potassium phosphate, 0.3 g. of calcium chloride, 5.0 g. of peptone, and 15 g. of cellulose in 1000 cc. of tap water with an excess of calcium carbonate. At a temperature of 65°C. they obtained 70 to 95 per cent decomposition of cellulose. Of the cellulose decomposed, 50 to 55 per cent was obtained as acetic acid, and 5 to 25 per cent as ethanol. Small amounts of butyric acid, carbon dioxide, hydrogen, and pigment were also obtained. They claimed that they had isolated a pure culture by making deep agar shakes at a dilution of 1-10,000 and removing the agar around a single gas bubble. They claim that organic nitrogen is necessary for the functioning of the organisms. They called the supposedly pure culture Clostridium thermocellum.

Peterson, Fred, and Martin (1926) investigated the effect of molecular complexity on the end-products formed by the above culture. They found that cellulose, starch, and lactose yielded

very little lactic acid while mono-saccharides, with the exception of galactose, gave considerable quantities of this acid. These facts tend to disprove the formation of glucose as an intermediate product in the formation of alcohols and acids.

Tetrault (1930) continued the work on the culture. It was apparent by this time that the culture was not pure. Although several methods of purification were tried, Tetrault did not consider that he obtained a pure culture. Anaerobic plating, dilution, pasteurization, and single-cell isolations were tried. When the plating was attempted, cleared areas and colonies resulted, and apparently the cellulose fermenters were present in the cleared areas while the colonies were formed by contaminants. Transfers from the cleared areas were not different from the original cultures. Growth was observed at higher dilutions than digestion of cellulose so this method had to be abandoned. Although 114 single cells were picked, only 4 developed and none fermented cellulose. Tetrault found that his cultures would grow in agar concentrations up to 1.5 per cent and that higher concentrations were inhibitory. He developed a method of purification by repeated anaerobic plating whereby at least some of the contaminants were eliminated. He obtained considerable glucose from all his purified cultures; yields of glucose as high as 0.328 g. per 100 cc. were obtained with a 3 per cent cellulose medium.

Scott, Fred, and Peterson (1930) reported on the various

products obtained from crude, enrichment, and purified cultures at 55°C. Purification of the culture decreased the yield of ethanol from 9.8 to 0.5 per cent. Volatile acid yields were greatest in the enrichment or semi-purified cultures. Either ammonium sulphate or peptone was used as a source of nitrogen.

Sarles, Fred, and Peterson (1932) reported on the continuous fermentation by the "addition withdrawal" process but less vigorous fermentation resulted with each successive step.

Śnieszko and Kimball (1933) studied the bacteria found in association with the thermophilic cellulose fermenting organisms. They prepared enrichment cultures in liquid media by heating and plating according to the method of Tetrault. At dilutions of 1-100,000,000 only 1 to 5 per cent of the cultures fermened cellulose, but more than 50 per cent contained bacteria of some kind, showing that the contaminants were present in much greater numbers than were the cellulose fermenters. For the purposes of isolation, cultures were grown in 0.3 per cent agar or liquid media, ground with sterilized sand to secure uniform suspensions, diluted in physiological salt solution, and used to seed agar plates. Two organisms were isolated. Both were facultative anaerobes, obligate thermophiles, Gram positive, and withstood heating in boiling water for 20 hours. One was considerably larger than the other.

Śnieszko (1933) attempted the isolation of a thermophilic cellulose fermenting organism. All ordinary methods of isolation

had failed. A purified culture was transferred to a nutrient broth medium and incubated at 60°C. At intervals of 4 to 6 hours the culture tubes were placed in boiling water for 10 to 15 minutes, dilutions were made, and tubes containing cellulose medium with 1 to 2 cc. of a rich suspension of a pure culture of young yeast cells were inoculated. It was thought that the cellulose fermenter did not grow in nutrient broth and therefore would remain in the spore stage while the contaminants would germinate and be reduced in number by the repeated pasteurizations. Eventually a culture was obtained which fermented cellulose and not glucose. The usual tests for pure cultures could not be applied to this culture as it did not grow in ordinary culture media, but from the difference in properties between this culture and crude cultures and between this culture and the contaminants isolated, it seemed probable that it was pure. Of course there is a possibility of more than one cellulose fermenting organisms being left. The resulting culture does not grow at 45°C. or below. The organisms are obligate anaerobes, slender rods, Gram positive, spore forming and catalase negative. If one or both contaminants are present the catalase test is strongly positive. No growth was obtained in any medium except cellulose medium. In a medium containing 2.86 g. of cellulose per 100 cc., 2.01 to 2.30 g. of cellulose were decomposed, and 1.11 to 1.30 g. of acetic acid and 0.25 to 0.31 g. of ethanol were produced. The maximum yield of ethanol was thus 13.1 per

cent of the cellulose decomposed. The fermentations with this pure culture were somewhat slower than those in which enrichment cultures were employed. This culture did not show nearly as wide variations as did the crude and enrichment cultures. Mixtures of this culture with the contaminants isolated by Śniesźko and Kimball were tried but only preliminary experiments were performed. It may be that excellent fermentations may be induced by the proper mixing of cultures.

Carter (1929) and Werkman and Carter (1930) conducted experiments to determine the optimum pH of the fermentation of corn cobs at 61°C. They obtained an optimum at a pH value of 9.0. Their main object was to obtain high volatile acid yields, and they succeeded in obtaining yields up to 21.8 per cent of the corn cobs added.

Stritar (1931) conducted experiments on the fermentation of beet pulp with crude cultures at 56° to 58°C. and obtained an optimum at a pH value of 9.0; 56.86 per cent of the beet pulp was fermented, of which 41.64 per cent was converted to volatile acids.

Tomoda (1932, 1933) reported on some data at 65°C. Using a 2 per cent filter paper medium the following yields were obtained on the basis of cellulose fermented: ethanol, 15-17 per cent; acetic acid, 21-26 per cent; butyric acid, 6-8 per cent; lactic acid, 0.5-1.5 per cent; carbon dioxide, 18-19 per cent; hydrogen, 0.2-0.5 per cent; and no methane. It was also found

that the unfermented cellulose had the same physical and chemical properties as did the original cellulose.

The literature on the fermentation is so copious that in the preceding pages no attempt was made to give a complete review of the subject. For an almost complete bibliography on the topic the reader is referred to the articles by Simola (1932). It is evident that the previous researches on the thermophilic fermentation of cellulose have been confined almost entirely to the isolation of pure cultures and to the production of gases and acetic acid. In a few instances the production of ethanol has been noted, but no systematic quantitative work has been reported on the development of optimum yields of this chemical.

EXPERIMENTAL METHODS

Preparation of Cultures

The cultures employed were obtained from various sources including the feces of cows, horses, goats, sheep, rabbits, and guinea pigs; compost; leaf mold; rotting logs; and corn stalks in plowed ground. All these sources gave cultures that were able to ferment cellulose at 55° to 60°C. Usually the cultures were grown in media consisting of 3 g. of cellulose, 0.25 g. of ammonium chloride, and 0.25 g. of di-potassium phosphate per 100 cc. Sometimes the cultures were grown in the medium described by Viljoen, Fred, and Peterson (1926) which consisted of 2 g. of cellulose, 0.2 g. of microcosmic salt, 0.14 g. of monopotassium phosphate, 0.03 g. of magnesium sulphate, 0.01 g. of calcium chloride, and 0.5 g. of peptone per 100 cc. of tap water. The cultures were then selected on the basis of rate of growth and ability to produce ethanol and acids. Culture number 20 was obtained from horse manure, number 22 from a compost pile, and number 24 from a pile of rotting horse manure and straw. Cultures 20 and 24 were very much alike and produced considerable amounts of alcohols. Culture 22 produced more acids and less alcohols than did numbers 20 and 24.

The usual procedure was to transfer the cultures about every

sixth day. When a culture was not to be used for a while, the flask was capped with tinfoil and placed in a refrigerator or at room temperature. In quantitative experiments, inoculations were made from a culture two to five days old and which exhibited good "heading." This procedure insured a rapid initiation of the fermentation.

The cultures thus obtained are obviously mixtures, and various attempts have been made in other laboratories to effect a separation of the various organisms present in cultures of this type. These attempts have not proven of value in the preparation of more active cultures than the original mixture. In the researches herein reported no attempts looking toward such a separation were made; instead it was deemed desirable to find means for maintaining the mixed cultures in a satisfactory degree of vigor and sufficiently uniform so that results could be duplicated.

Preparation of Media

The cellulose media were usually made up in 3 liter quantities. The required salts were weighed into a flask and a little water added to dissolve them. The required amount of filter paper was placed in a two liter beaker and one liter of water was added. The paper was worked into a pulp by rubbing and squeezing by hand. The pulp was then placed in a large flask with the salt solution and more water added until the total amount of water was three liters. The mixture was shaken and

measured into smaller flasks. These flasks were then sterilized with steam for three hours under twenty pounds pressure.

When calcium carbonate was used for neutralizing the fermentations, it was sterilized separately for three hours at 163°C. in test tubes capped with lead foil. Scrap filter paper purchased from the Wilkins Anderson Company of Chicago was used as the supply of cellulose. It should be noted that the concentrations of the materials used in the preparation of the media are expressed in grams per hundred cc. of water added and not in grams per hundred cc. of medium.

The Fermentation

Inoculations were made from warm to cold flasks. If transfers were being made simply to carry a culture, about one-fifteenth of the volume of the flask being inoculated was transferred by pouring from one flask to the other. If a series was to be inoculated uniformly, a graduated cylinder was used to measure equal amounts into each flask. It had been found that the fermentation would start more quickly if transfers were made from flasks two to five days old, when a considerable amount of the paper remained. Pipettes could not be used as it was impossible to get representative portions of the thick medium into the pipettes.

If calcium carbonate was employed for neutralization, it was added immediately after the inoculation. If the fermenta-

tion was not to be analyzed, it was plugged with cotton. If the flasks were to be analyzed only once and calcium carbonate was the neutralizing agent, the cotton plugs were removed and the flasks were capped with tinfoil held in place with rubber bands. This procedure decreased the evaporation of the media and alcohol, and also gave a little more room for the fermentation to "head." If the flasks were to be opened several times during the fermentation, as when the pH was controlled by sodium hydroxide or sodium carbonate, cork stoppers were used. The use of cork stoppers and tinfoil allowed the fermentation gases to escape and at the same time did not permit any great circulation of air in and out of the flasks.

All flasks were shaken once just after inoculation. They were then allowed to stand until active fermentation was indicated by the evolution of gas. From that time they were shaken from three to five times daily. The purpose of the shaking was to insure the proper neutralization of the acids formed by the reaction with the calcium carbonate and to keep the cellulose down into the liquid. During active fermentation, sufficient gas was evolved in four to six hours to raise the cellulose entirely out of the liquid to form the characteristic "head."

Methods of Analysis

Alcohols.

A 300 cc. sample of the culture was placed in a 500 cc.

Kjeldahl flask and 50 cc. were distilled over. The distillate was made just acid to congo red with sulphuric acid and again distilled to eliminate any ammonia that might be present. Unless otherwise indicated, the amounts of ethanol and butanol in the distillate were measured by a method developed by others in these laboratories, the details of which will be published later. This method is based upon wet oxidation with potassium dichromate. Occasionally the alcohols were estimated from refractive index measurements made upon the distillate. The wet oxidation method gave excellent results for ethanol, but the percentage error is likely to be considerable with butanol because of the extremely small amounts present.

In order to verify the quantitative results, the neutral distillates from a number of fermentations were collected. The concentration of the alcohols was increased by further distillation and by saturating with potassium carbonate. The alcohols were then fractionated; from 75 cc. about 3 cc. of distillate were obtained with a boiling range of 114° to 118°C. Practically all the remainder distilled over between 77° and 85°C. The 3, 5 dinitrobenzoate derivatives were prepared; that from the larger portion had a melting point of 91° to 92°C.; and that from the smaller portion 62° to 64°C. These data indicate the presence of ethanol and n-butanol in the ratio of about twenty-five to one, which was approximately that found by the oxidation method.

Volatile acids.

The volatile acids were separated from the rest of the fermentation products by distilling an acidified sample. The fermentation liquid was shaken vigorously and 50 cc. were measured out into a 300 cc. Erlenmeyer flask; 50 cc. of 1N. sulphuric acid were added and the flask was allowed to stand open to the air for about 20 minutes to allow any carbon dioxide to escape. The sample was then washed into a 500 cc. Kjeldahl flask. The volatile acids were distilled on a Kjeldahl distilling rack. Five hundred cc. of distillate were collected; the volume of the liquid in the Kjeldahl flask was maintained at about 100 cc. by additions of distilled water.

The distillate was mixed thoroughly and 100 cc. were titrated with N/10 sodium hydroxide using phenolphthalein as the indicator. Usually this was as far as the analysis was carried and the volatile acid was calculated as acetic acid.

Two methods were tried for the determination of the relative amounts of acetic and butyric acids. The distillation method of Virtanen and Pulkki (1928) did not prove entirely satisfactory. The procedure of Fyleman (1924) gave much more consistent results, hence this method, with a slight variation, was used in all the work on determining acetic and butyric acid ratios. The method employed was as follows:

A portion of the distillate equivalent to about 2.5 cc. of N/10 sodium hydroxide was accurately measured into a 500 cc.

Erlenmeyer flask. One drop of phenolphthalein was added and the solution was made alkaline with N/10 sodium hydroxide. The flask was then placed on a steam plate and allowed to evaporate to dryness over night to remove alcohols. On the following day the flask was cooled, and 20 cc. of distilled water and 25 cc. of N/4 potassium dichromate were added. The flask was placed under a reflux condenser and 30 cc. of concentrated sulphuric acid (36 N.) were added through the condenser. The liquid was refluxed for exactly one hour, cooled, and diluted with distilled water to about 250 cc.; 25 cc. of a 10 per cent solution of potassium iodide were added and the solution was titrated immediately with standard N/4 sodium thiosulphate with starch as the indicator. A blank with distilled water was run on the reagents and potassium dichromate solution.

By this procedure the butyric acid is apparently oxidized quantitatively to acetic acid but a correction must be applied for the amounts of acetic acid present and formed, as it is also somewhat oxidized. The equation used in calculating the amount of butyric acid is as follows:

$$0.00183(b-0.2n) = B$$

where b indicates the difference between the blank and the amount of N/4 sodium thiosulphate required to titrate the excess dichromate in cc., n the cc. of N/10 sodium hydroxide equivalent to the acids in the sample, and B the grams of butyric acid in the sample. Acetic acid was calculated by difference from the

original titration with sodium hydroxide.

Non-volatile acids.

The samples used for the determination of volatile acids were employed in the determination of non-volatile acids. After the distillation, the sample was removed from the Kjeldahl flask, evaporated to a volume of about 25 cc., and extracted with ether for 48 hours. The ether was then allowed to evaporate, the residue was dissolved in water, and titrated with N/10 sodium hydroxide using phenolphthalein as the indicator. The non-volatile acids were reported as lactic acid. The amounts found were too small to be of any importance in these studies and consequently this determination was generally omitted.

Residual cellulose.

The amount of residual cellulose at the conclusion of an an experiment was occasionally determined in order to be able to calculate the amount of cellulose decomposed by the organisms. The determinations were made on the same sample used for the determinations of alcohols and which represented 300 cc. of fermentation liquor. After the removal of the neutral distillates, the sample in the Kjeldahl flask was washed into an Erlenmeyer flask, and two portions of concentrated hydrochloric acid of 10 cc. each were added to dissolve any calcium carbonate. The contents of the flask were boiled about two minutes, allowed to

settle, filtered hot on a Buchner funnel, and washed with water. The material on the filter was covered with a hot 3 per cent sodium hydroxide solution, which was allowed to remain on the filter for a minute or so and then drawn off. The treatment with 3 per cent sodium hydroxide was repeated, and the residue was washed with water. If possible, the filter paper was peeled off, but if not possible it was allowed to remain and a correction applied later. The residue was dried in the incubator at 55°C. for a day or more and weighed.

The weight of residue divided by 3 gave the number of grams of cellulose left per 100 cc. of the fermentation liquor. This value subtracted from the grams of cellulose originally present per 100 cc. (usually 3 g.) gave the number of grams of cellulose decomposed per 100 cc. of medium.

The chief source of error in this method lies in the sampling of a fermentation when most of the cellulose is unfermented. The medium is then very thick and the cellulose tends to clump. In such a case, when the amount of cellulose decomposed is calculated by subtraction, relatively larger errors are likely to occur. When only very small amounts of cellulose remain unfermented, the correction applied for the extra paper added during filtration is likely to be in error by about 20 milligrams but this is not a serious error when the final calculations are made. When only small amounts remain, the residue is usually yellow in color and contains a condiderable proportion of gums and bacteria.

In spite of these limitations, this method gives a good indication of the amount of decomposition of cellulose.

Reducing sugars.

Reducing sugars were determined by the method outlined by Shaffer and Hartmann (1920). In this procedure Fehling's solution is used and the reduction is carried out in the standard manner. The reduced copper is determined by an iodometric titration.

Gaseous products.

Carbon dioxide, hydrogen, and methane were determined with a Williams gas analysis apparatus.

The gases evolved during fermentation were collected over saturated sodium chloride solution. The volumes of gas collected were measured, and a 100 cc. sample was drawn into the apparatus for analysis. The gas was first bubbled through a solution of potassium hydroxide, and the decrease in volume of the sample was recorded as the percentage of carbon dioxide. Oxygen was then added, the volume again read, and the mixture in the tube ignited by a spark from a spark coil. The gas was allowed to cool, and the volume was read. The gas was then passed through the potassium hydroxide to remove carbon dioxide, and the volume again determined. In some instances, when the percentage of hydrogen was high, it was necessary to let some of the sample

escape before adding oxygen in order to keep the gases within the apparatus during the explosion.

The amount of carbon dioxide present was calculated as indicated above. The percentage of methane was equal to the decrease in volume during the last absorption with potassium hydroxide. The percentage of hydrogen was calculated by taking the decrease in volume during the explosion less twice the percentage of methane and multiplying the result by 2/3. If part of the sample was allowed to escape before adding oxygen, the proper factor was applied in calculating the amounts of hydrogen and methane.

THE EFFECT OF VARIOUS FACTORS UPON THE PRODUCTION OF ALCOHOLS AND ACIDS

The Effect of Temperature

The purpose of these experiments was to determine the effect of temperature upon the production of ethanol. The medium contained 3 g. of cellulose, 0.25 g. of di-potassium phosphate and the appropriate concentration of ammonium chloride. The concentration of ammonium chloride was, at each temperature, calculated from the relation established by Sherwood and Fulmer (1926) for the optimum concentration of the salt for the growth of yeast. The optimum concentration was expressed by these authors as $0.0960 + 0.00306\ t°C$. At each temperature 8 liters of medium were used in a 12 liter flask fitted with a capillary vent. Each flask was inoculated with 500 cc. of culture number 24, which had been incubated at 60°C. Immediately after inoculation, 200 g. of sterile calcium carbonate were added. During the fermentation the flasks were shaken four times daily. A portion of 350 cc. was used for the analysis of the following products: ethanol, butanol, acetic acid, butyric acid, and cellulose.

The first experiment was run at 37.5°, 45°, 55°, and 60°C. The data showed a distinct optimum production of ethyl alcohol at 55°C. There was practically no action at 37.5°C. even after

50 days. This flask was then placed at 60°C. and a normal fermentation followed, showing that the effect was solely one of temperature.

In order to verify the above results through a narrower range, experiments were conducted at 65°, 60°, 55°, and 50°C. The data are given in Tables 1, 2 and 3. The data for ethanol, acetic acid, and cellulose decomposed are plotted in Figures 1, 2 and 3 respectively. In Table 4 are given the data showing the production of ethanol and acetic acid per gram of cellulose fermented.

There was a decided maximum production of ethanol and butanol at 55°C. The yield of the latter was about one-tenth that of the former. The maximum yield took place in about 8 days. At 50°C. the reaction was slower and the maximum was reached in about 11 days. The maximum yield of acetic and butyric acids was obtained at 60°C. The time required for the maximum yield of butyric acid was about 8 days and for acetic acid about 11 days. The maximum yield of acetic acid was about 20 times that of butyric acid. Under these conditions the yield of acetic acid was about 33 per cent of the cellulose fermented as compared to 26 per cent at 55°C. While the total yield of acetic acid was at a maximum at 60°C. the yield per gram of cellulose was greatest at 65°C. and amounted to about 47 per cent of the cellulose fermented. The maximum decomposition of cellulose took place at 55°C. and 60°C. the values being practically identical after the eighth day, at

Table 1. The effect of temperature upon the production of ethanol and butanol.

Age in hours	Grams C_2H_5OH per 100 cc.				Grams C_4H_9OH per 100 cc.			
	65°C.	60°C.	55°C.	50°C.	65°C.	60°C.	55°C.	50°C.
0	0.0037	0.0037	0.0037	0.0037	0.0008	0.0008	0.0006	0.0008
12	0.0037	0.0038	0.0037	0.0037	0.0008	0.0009	0.0001	0.0001
24	0.0093	0.0049	0.0045	0.0039	0.0008	0.0003	0.0003	0.0001
36	0.0381	0.0254	0.0111	0.0073	0.0017	0.0005	0.0003	0.0001
48	0.0540	0.0537	0.0253	0.0173	0.0018	0.0003	0.0003	0.0001
60	0.0650	0.0880	0.0530	0.0388	0.0035	0.0006	0.0013	0.0001
72	0.0697	0.0216	0.108	0.0418	0.0047	0.0087	0.0028	0.0001
84	0.0792	0.276			0.0053	0.011		
96	0.0800	0.317	0.287	0.0787	0.0056	0.012	0.0037	0.0034
120			0.325	0.187			0.015	0.0083
144	0.1015	0.368	0.455	0.261	0.0088	0.029	0.018	0.0087
192	0.0900	0.363	0.503	0.403*	0.0078	0.020	0.058	0.0113*
264	0.0667	0.370	0.505	0.435	0.0100	0.016	0.056	0.0050
360	0.0967	0.368	0.465	0.433	0.0102	0.025	0.042	0.0088

*Age 220 instead of 192 hours.

The Effect of Temperature on the Yields of Ethanol.

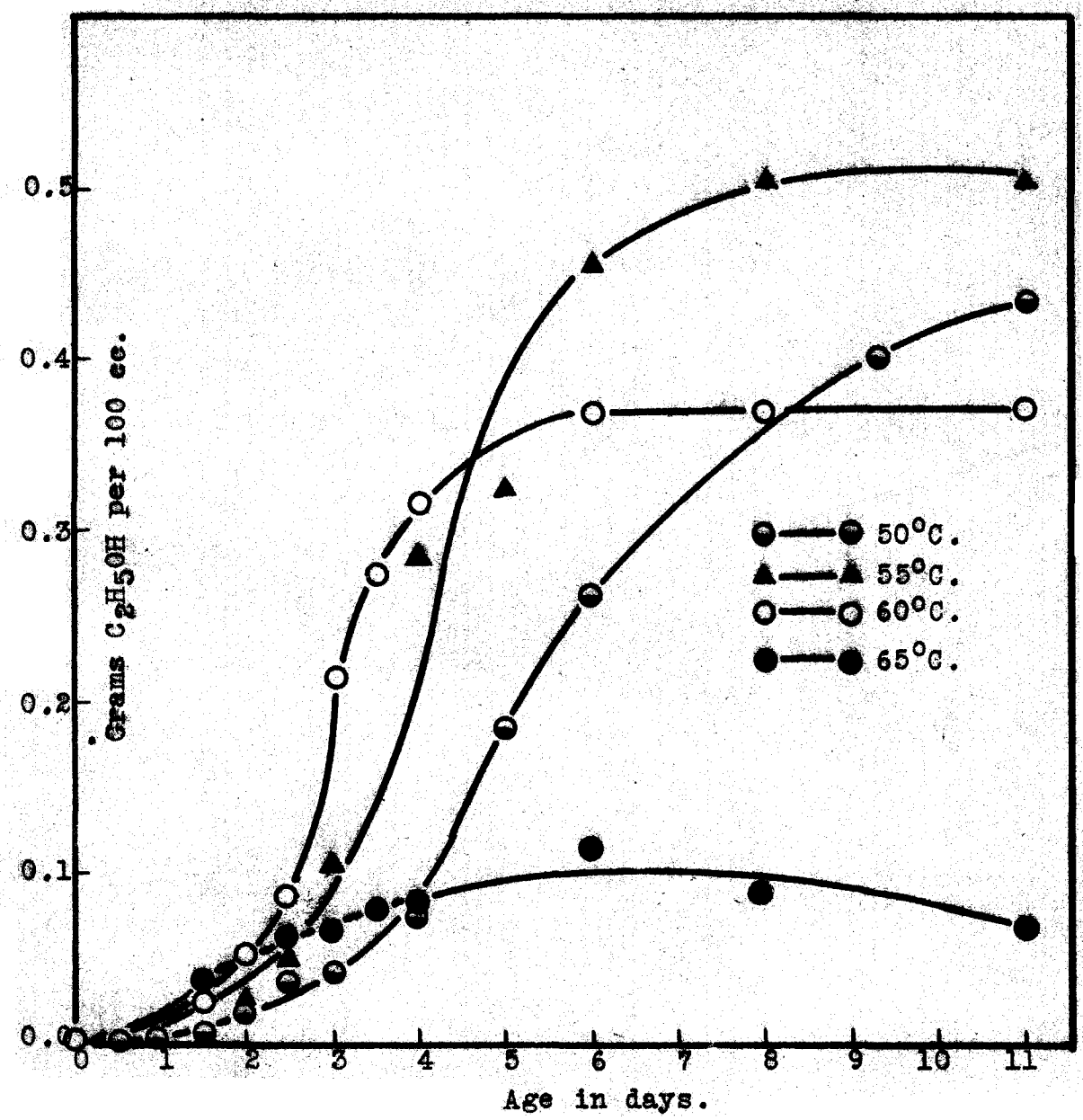


Figure 1.

Table 2. The effect of temperature upon the production of acetic and butyric acids.

Age in hours	Grams CH_3COOH per 100 cc.				Grams C_3H_7COOH per 100 cc.			
	65°C.	60°C.	55°C.	50°C.	65°C.	60°C.	55°C.	50°C.
0	0.011	0.011	0.011	0.011	0.0015	0.0015	0.0015	0.0015
12	0.017	0.017	0.023	0.017	0.0018	0.0013	0.0015	0.0014
24		0.023	0.023	0.023	0.0030	0.0013	0.0013	0.0014
36	0.131	0.107	0.035	0.035	0.0019	0.0012	0.0013	0.0014
48	0.189	0.172	0.101	0.071	0.0055	0.0022	0.0016	0.0012
60	0.278	0.227	0.148	0.101	0.0142	0.0021	0.0024	0.0018
72	0.461	0.346	0.208	0.140	0.0202	0.0023	0.0031	0.0023
84	0.602	0.355			0.0316	0.0072		
96	0.612	0.507			0.0359	0.0126		
144	0.810	0.813	0.635	0.385	0.0350	0.0312	0.0196	0.0156
192	0.815	0.906	0.730	0.485	0.0372	0.0442	0.0332	0.0273
264	0.840	0.980	0.770	0.635	0.0349	0.0446	0.0326	0.0316
360	0.842	0.980	0.781	0.750	0.0323	0.0448	0.0335	0.0424

The Effect of Temperature on the Yields of Acetic Acid.

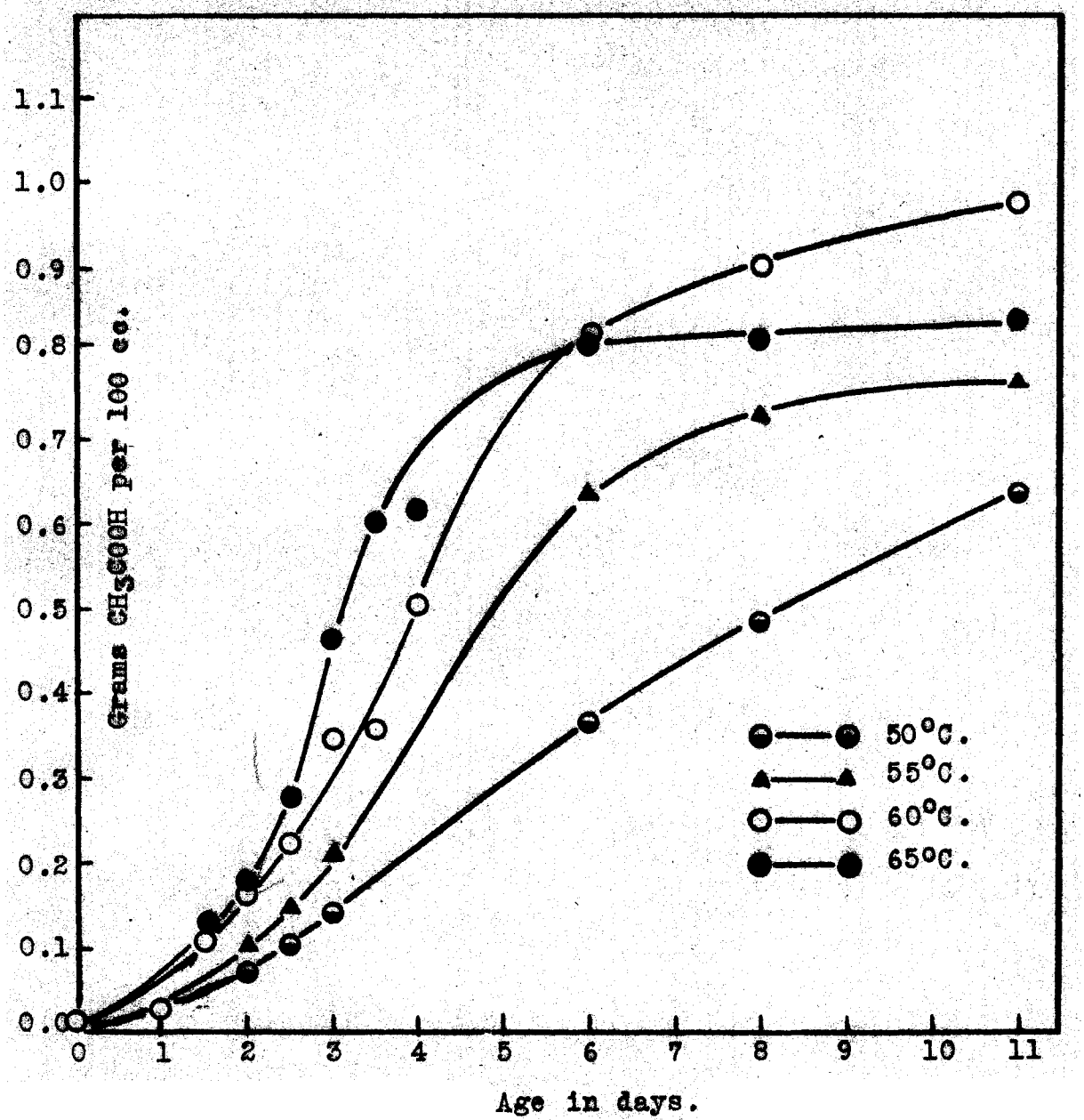


Figure 2.

Table 3. The effect of temperature upon the digestion of cellulose.

Age in hours	Cellulose dissolved, g./100 cc.			
	65°C.	60°C.	55°C.	50°C.
0	0.10	0.10	0.10	0.10
12	0.11	0.06	0.36	0.15
24	0.21	0.17	0.21	0.23
36	0.55	0.38	0.35	0.32
48	0.80	0.73	0.61	0.43
60	1.06	1.17	0.73	0.38
72	1.44	1.57	0.77	0.74
84	1.58	1.79		
96	1.66	2.16	1.50	0.74
120			2.11	1.13
144	1.74	2.64	2.45	1.35
192	1.80	2.75	2.81	2.36*
264	1.77	2.71	2.92	2.43
360	1.89	2.96	2.97	2.76

*Age 220 instead of 192 hours.

The Effect of Temperature on

the Decomposition of Cellulose.

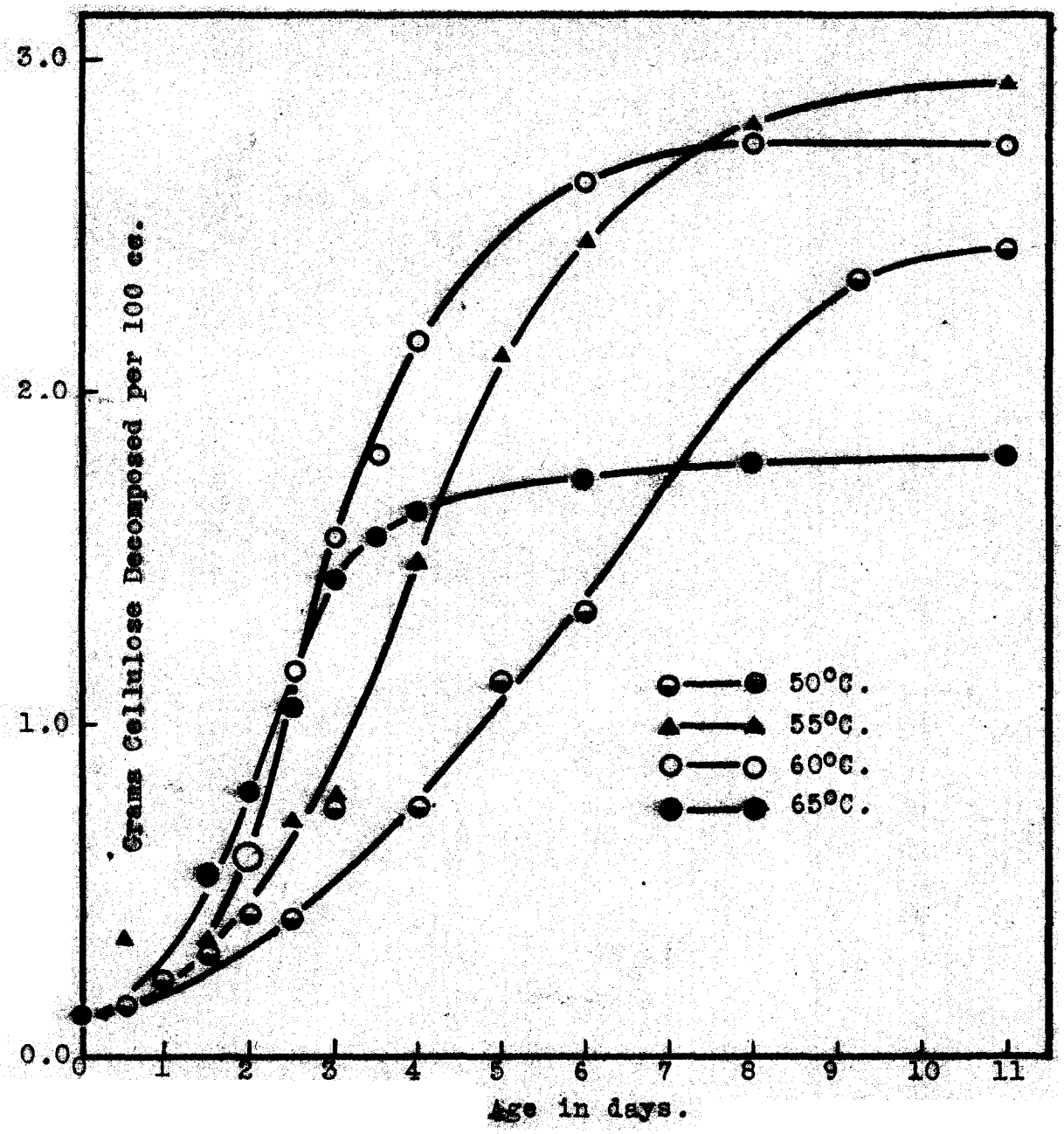


Figure 3.

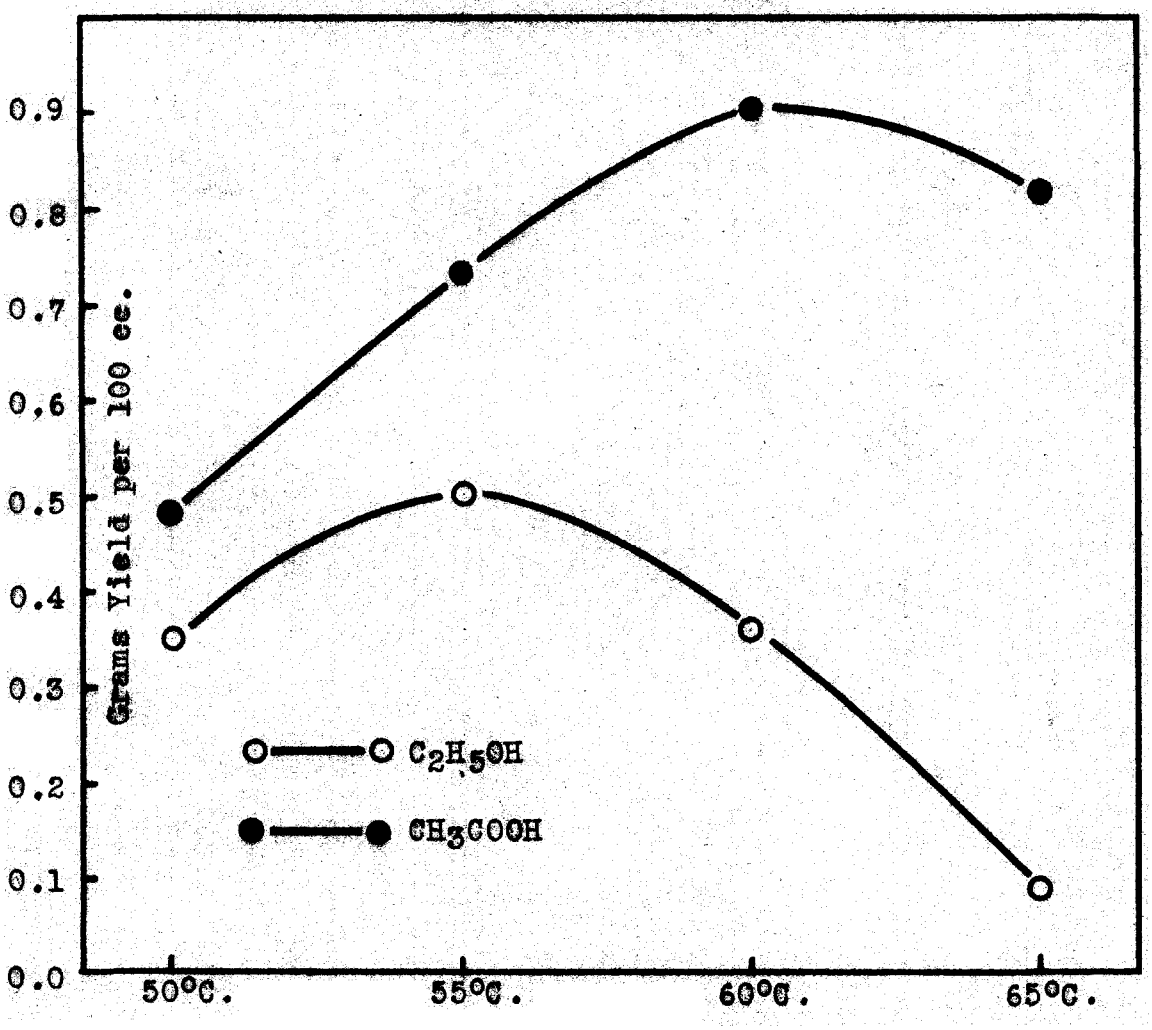
Table 4. The effect of temperature upon the amounts of ethanol and acetic acid produced per gram of cellulose decomposed.

Age in hours	Grams yield per gram cellulose decomposed							
	C_2H_5OH				CH_3COOH			
	65°C.	60°C.	55°C.	50°C.	65°C.	60°C.	55°C.	50°C.
12	0.033	0.063	0.010	0.025	0.15	2.83	0.06	0.11
24	0.044	0.029	0.021	0.017	1.09	0.14	0.11	0.10
26	0.069	0.067	0.032	0.023	0.24	0.28	0.10	0.11
48	0.068	0.073	0.041	0.040	0.24	0.24	0.17	0.16
60	0.061	0.075	0.073	0.102	0.26	0.19	0.20	0.10
72	0.048	0.138	0.139	0.056	0.32	0.22	0.27	0.19
84	0.050	0.154			0.38	0.20		
96	0.048	0.147	0.191	0.106	0.37	0.23		
120			0.154	0.165				
144	0.058	0.140	0.185	0.193	0.47	0.31	0.26	0.29
192	0.050	0.132	0.180	0.171*	0.45	0.33	0.26	0.21
264	0.038	0.137	0.173	0.179	0.47	0.36	0.26	0.26
360	0.051	0.124	0.156	0.157	0.45	0.33	0.26	0.27

*Age 220 instead of 192 hours.

The Effect of Temperature.

Yields on the eighth day.



Temperature.

Figure 4.

which time about 93 per cent of the cellulose was decomposed. The decomposition was practically complete on the fifteenth day.

In Figure 4 the yields of acetic acid and ethanol on the eighth day are plotted against temperature. This figure shows particularly well the effect of temperature on the yields of these products.

The Effect of Inorganic Salts

The effect of ammonium chloride.

In the previous section it was shown that the maximum production of ethanol was obtained at 55°C., the yield being about 20 per cent of the cellulose fermented. For each temperature the concentration of ammonium chloride was that calculated as optimum for the growth of yeast. The following experiments were designed to test quantitatively the effect of varying concentrations of the ammonium salt upon the production of ethanol. In each case 750 cc. of medium were prepared in a one liter Erlenmeyer flask. The medium contained 3 g. cellulose, 0.25 g. dipotassium phosphate, about 3.3 g. calcium carbonate per 100 cc. and varying concentrations of ammonium chloride. The culture employed, number 24, was the same as that used in the temperature experiments. Each flask was inoculated with 50 cc. of an active fermentation of this culture.

The data obtained are given in Table 5. At 60°C. there was very little difference in the yield of alcohol or acid within

Table 5. The effect of ammonium chloride.

	60°C. 12 days			55°C. 8 days				
NH_4Cl g./100 cc.	C_2H_5OH* g./100 cc.	Volatile acids g./100 cc.	Ratio C_2H_5OH to volatile acids	C_2H_5OH g./100 cc.	Volatile acids g./100 cc.	Cellulose decomposed g./100 cc.	Ratio C_2H_5OH to volatile acids	C_2H_5OH g./g. cellulose decomposed
0.016				0.052	0.12	0.41	0.433	0.127
0.025				0.075	0.20	0.72	0.375	0.104
0.050				0.089	0.25	0.87	0.356	0.102
0.075				0.187	0.37	1.37	0.505	0.137
0.100				0.200	0.41	1.58	0.488	0.126
0.150				0.376	0.46	2.12	0.816	0.177
0.200	0.39	1.01	0.387	0.520	0.54	2.47	0.963	0.210
0.225	0.36	0.96	0.375					
0.250	0.39	0.95	0.400	0.508	0.55	2.61	0.924	0.196
0.275	0.38	0.98	0.388					
0.300	0.36	0.96	0.375	0.533	0.60	2.69	0.888	0.198
0.325	0.47	1.02	0.471					
0.350	0.40	1.02	0.392					
0.375	0.40	0.95	0.421					
0.400	0.43	0.96	0.437	0.518	0.58	2.66	0.893	0.196
0.450	0.42	0.97	0.433					
0.500	0.45	0.98	0.459	0.443	0.62	2.57	0.714	0.172
0.550	0.39	0.92	0.424					
0.600				0.438	0.49	2.42	0.894	0.191

*These results are calculated from refractive index measurements.

the range of 0.200 to 0.550 g. of ammonium chloride per 100 cc. and the ratio of alcohol to acid was nearly constant at about 0.4. The range of concentrations of the ammonium salt employed at 55°C. was wider than at 60°C. The yields of alcohols and acids were practically constant for concentrations of the salt from 0.200 to 0.400 g. per 100 cc. at 55°C. Through this range the ratio of ethanol to acid was considerably more than twice that at 60°C.; the yield of alcohol amounted to about 20 per cent of the cellulose fermented. If the concentration of the salt was less or greater than the above concentrations the yields were lowered.

According to the formula established by Sherwood and Fulmer (1926), the optimum concentration of ammonium chloride at 55°C. is 0.264 g. per 100 cc., and 0.290 g. per 100 cc. at 60°C. These values fall well within the range found to be optimum for the thermophilic cellulose fermenters. A concentration of 0.25 g. of ammonium chloride per 100 cc. was used in all later experiments.

The effect of potassium phosphate.

Twelve one-liter flasks, with 750 cc. of medium in each, were prepared for this experiment. Each contained 3 g. of cellulose, 0.25 g. of ammonium chloride per 100 cc., and varying amounts of phosphate ($K_2HPO_4,3H_2O$); 25 g. of sterile calcium carbonate were used per flask for neutralization. Each flask

was given an inoculation of 50 cc. of culture number 24. The flasks were incubated at 55°C., and determinations of ethanol, butanol, volatile acids, and cellulose were made on the eighth day. The results of this experiment are given in Table 6.

Maximum yields of ethyl alcohol were obtained between the limits of 0.20 and 0.40 g. of phosphate per 100 cc. Maximum yields of volatile acids were also obtained within this range. The yields of butyl alcohol were too small to be determined with any degree of accuracy or to be of any great importance. The range of maximum cellulose decomposition was about the same as that of maximum yields of ethyl alcohol and of volatile acids. A concentration of 0.25 g. of di-potassium phosphate per 100 cc. was used in all later experiments.

The effect of calcium sulphate.

It had been repeatedly observed that the cultures did not grow as well in media made with distilled water as they did when tap water was used. It was known that the tap water contained about 0.0125 g. of calcium sulphate per 100 cc. and it was thought that the poor fermentations in distilled water media might be due to the lack of this salt. Experiments were therefore undertaken to determine the effect of calcium sulphate upon the fermentation.

One liter Erlenmeyer flasks with 750 cc. of medium in each were prepared for this experiment. Series I was prepared with ordinary distilled water from the regular supply. Each flask

Table 6. Effect of phosphate.

Grams $K_2HPO_4,3H_2O$ per 100 cc.	Grams C_2H_5OH per 100 cc.	Grams C_4H_9OH per 100 cc.	Grams acids per 100 cc.	Grams cellulose decomposed per 100 cc.
0.016	0.046	0.003	0.148	0.52
0.025	0.117	0.005	0.282	0.72
0.050	0.175	0.006	0.246	0.60
0.075	0.228	0.019	0.294	1.25
0.100	0.293	0.029	0.348	2.05
0.150	0.344	0.004	0.450	1.58
0.200	0.442	0.016	0.515	2.31
0.250	0.487	0.000	0.535	2.63
0.300	0.355	0.000	0.535	1.94
0.400	0.474	0.013	0.550	2.24
0.500	0.238	0.015	0.300	1.25
0.600	0.060	0.001	0.136	0.28

contained 3 g. of cellulose, 0.0272 g. $MgCl_2,6H_2O$, 0.001 g. $FeCl_3$, $6H_2O$, and 0.25 g. of di-potassium phosphate per 100 cc. This amount of magnesium salt was equivalent to that in the tap water. Varying proportions of a saturated solution of calcium sulphate and distilled water were used in preparing the media. The amounts of the saturated calcium sulphate solution employed varied from none to 750 cc. per flask. To each flask there were added 25 g. of sterile calcium carbonate as a neutralizing agent. Series II was exactly like Series I except that all the water used was redistilled in glass. The flasks in both series were inoculated with 50 cc. of culture number 22 and incubated at 55°C. This culture was not a very good alcohol producer, but an active culture of number 24 was not available at the time. Determinations of ethanol, butanol, volatile acids, and residual cellulose were made on the eighth day after inoculation. The results of these determinations are given in Table 7.

Although the alcohol yields were low, the results indicate quite definitely that there is an optimum concentration of calcium sulphate in the neighborhood of 50 cc. of saturated calcium sulphate per 800 cc. of medium, which is equivalent to 0.0129 g. of anhydrous calcium sulphate per 100 cc. Larger yields of ethanol, butanol, and volatile acids were obtained at this concentration than at any other concentration used. More cellulose decomposition also occurred under these conditions. A small amount of calcium sulphate appears to be beneficial and a larger amount

Table 7. The effect of calcium sulphate.

cc. Saturated $CaSO_4$	cc. Distilled water	Series I. Distilled water				Series II. Redistilled water			
		Grams C_2H_5OH per 100 cc.	Grams C_4H_9OH per 100 cc.	Grams volatile acids per 100 cc.	Grams cellulose decomposed per 100 cc.	Grams C_2H_5OH per 100 cc.	Grams C_4H_9OH per 100 cc.	Grams volatile acids per 100 cc.	Grams cellulose decomposed per 100 cc.
00	750	0.145	0.006	0.58	1.62	0.178	0.011	0.98	2.28
50	700	0.194	0.017	0.80	2.09	0.231	0.015	1.21	2.50
75	675	0.092	0.008	0.56	1.22	0.163	0.016	0.95	2.12
100	650	0.075	0.008	0.45	-	0.088	0.007	0.60	1.38
150	600	0.073	0.008	0.34	0.99	0.036	0.004	0.38	1.17
200	550	0.047	0.005	0.29	0.92	0.063	0.005	0.41	0.93
300	450	0.062	0.010	0.34	0.62	0.052	0.010	0.36	1.53
400	350	0.072	0.010	0.26	0.83	0.035	0.005	0.40	0.74
500	250	0.058	0.005	0.30	0.72	0.039	0.004	0.26	0.94
600	150	0.014	0.001	0.10	0.17	0.030	0.000	0.13	0.53
700	50	0.022	0.002	0.27	0.90	0.039	0.005	0.31	0.96
750	00	0.006	0.000	0.09	0.00	0.040	0.007	0.22	0.94

very harmful. The amount of sulphate present in the tap water was almost identical to the optimum value obtained above.

A very distinct odor of hydrogen sulphide was noticeable when 200 cc. or more of saturated calcium sulphate were used per flask. The calcium carbonate employed in this experiment was of the precipitated technical grade, and apparently contained little sulphate. Good fermentations were nearly always obtained when this grade was used with tap water. Sometimes when the "reagent" grade was used, poor fermentations resulted, and the odor of hydrogen sulphide was usually noticeable in such cases. The lables on the "reagent" grade usually list a maximum SO_3 content of 0.02 per cent. If the maximum amount were present, the concentration of sulphate in the medium from this source would be equivalent to 0.00113 g. of calcium sulphate per 100 cc. for the usual amount of calcium carbonate employed. This quantity added to the amount present in tap water makes 0.0136 g. of calcium sulphate per 100 cc. This may be the explanation for the fact that some lots of calcium carbonate gave poor fermentations. At least this experiment indicates that the amount of sulphate in the calcium carbonate may be a factor of considerable importance.

Higher yields were obtained by using water distilled in glass than by using ordinary distilled water as it came from the tap. Although experiments were not undertaken to test this point, it is probable that the decreased activity of the culture is due to small traces of copper and tin from the pipe line.

The Effect of Variations of pH

Two series of experiments were run in the pH studies. In Series I the pH was adjusted by additions of 5 normal sodium hydroxide or 5 normal hydrochloric acid. Such adjustments were made twice daily for the first four days and daily thereafter. Culture number 24 was used and the flasks were incubated at 60°C. After 12 days the alcohols and volatile acids were determined. In Series II the pH was controlled by the use of 5 normal sodium carbonate and 5 normal hydrochloric acid. The pH values were adjusted twice daily throughout the experiment. Culture 20 was employed and the flasks were incubated at 55°C. On the eighth day the media were analyzed for ethanol, butanol, volatile acids, and residual cellulose. The media in both series contained 3 g. cellulose, 0.25 g. ammonium chloride, and 0.25 g. di-potassium phosphate per 100 cc. of tap water.

The results are given in Table 8. For Series I the yield of ethanol increased up to a pH of 6.75; from 6.75 to 8.00 there was very little difference in yield; and at a pH value above 8.00 the yield very materially decreased. The yield of acid paralleled that of ethanol except that there was indicated a slight maximum at pH values from 7.75 to 8.00. In Series II the yield of ethanol increased up to a pH value of 7.50, remained practically constant to pH value 8.00 and then decreased. The acid yield paralleled that of ethanol except that there was a high yield at pH 8.50.

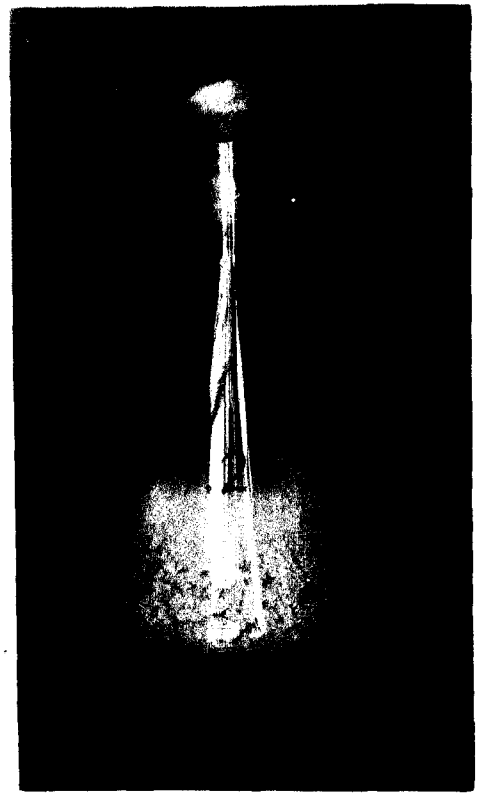
Table 8. The effect of variations of pH.

pH	Yields in grams per 100 cc.						
	Series I. 60°C.		Series II. 55°C.				
	C_2H_5OH	Volatile acids	C_2H_5OH	C_4H_9OH	Volatile acids	Cellulose decomposition	C_2H_5OH g. per g. cellulose decomposed
5.00	0.033	0.06	0.005	0.001	0.02	0.16	0.03
5.50	0.051	0.06	0.007	0.001	0.04	0.16	0.03
6.00	0.057	0.19	0.036	0.004	0.06	0.33	0.11
6.50	0.291	0.38	0.236	0.017	0.07	0.38	0.62
6.75	0.351	0.62	0.121	0.000	0.15	0.63	0.19
7.00	0.333	0.62	0.256	0.008	0.26	1.39	0.18
7.25	0.432	0.64	0.435	0.035	0.54	2.44	0.18
7.50	0.333	0.62	0.613	0.022	0.73	2.80	0.22
7.75	0.366	0.71	0.623	0.000	0.71	2.75	0.23
8.00	0.391	0.72	0.673	0.003	0.68	2.76	0.24
8.50	0.084	0.14	0.572	0.005	0.94	2.30	0.25
9.00	0.042	0.04	0.006	0.001	0.03	0.56	0.00

These results indicate that with the technique employed the pH for highest yields of ethanol should be adjusted within the range of 7.5 to 8.0. The data again show the higher yield of ethanol at 55°C. as compared to that at 60°C.

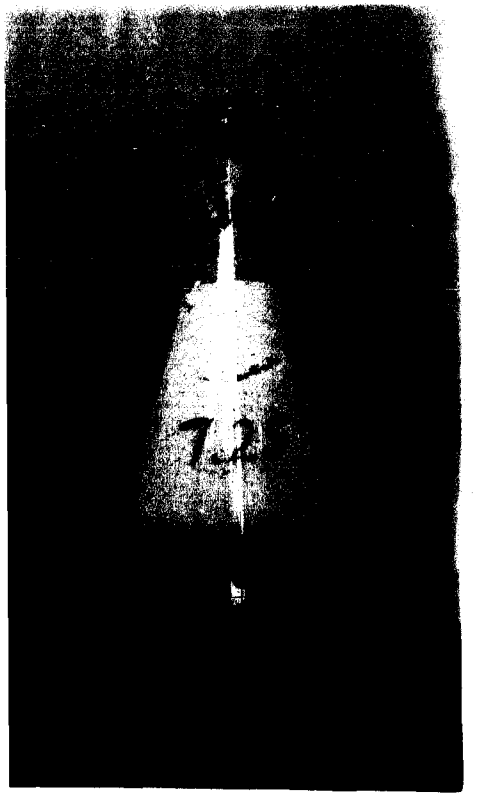
There are several factors which make a more exact recommendation impossible without obtaining further data. A high pH appears to delay the start of the fermentation, but when once under way, the action may be particularly vigorous. In Series II the flask at pH 7.75 started first and was followed within 12 hours by those at 7.5, 7.25, and 7.0. The flask at 8.0 did not start until 24 hours later than the one at pH 7.75. The flask at pH 8.5 had been actively fermenting only 4½ days at the conclusion of the experiment, and once under way it was the most rapid of all. Another disturbing factor is the fluctuation in pH between adjustments. During the active fermentation the usual decreases in pH at a pH of 7.0, 7.25, 7.50, 7.75, 8.00, and 8.50 were about 0.3, 0.4, 0.7, 0.8, 0.9, and 1.3 units respectively during 12 hour periods. This effect was even more pronounced in Series I, using sodium hydroxide as the neutralizing agent. In the ideal experiment the pH would be kept constantly at the specified value, but this is rather difficult to do in the laboratory when large quantities of acid are produced.

In Figure 5 are shown photographs of the flask in Series II at the pH value of 7.25. These show the flask 0, 3 and 8 days after inoculation. The second shows the typical "head" formation.



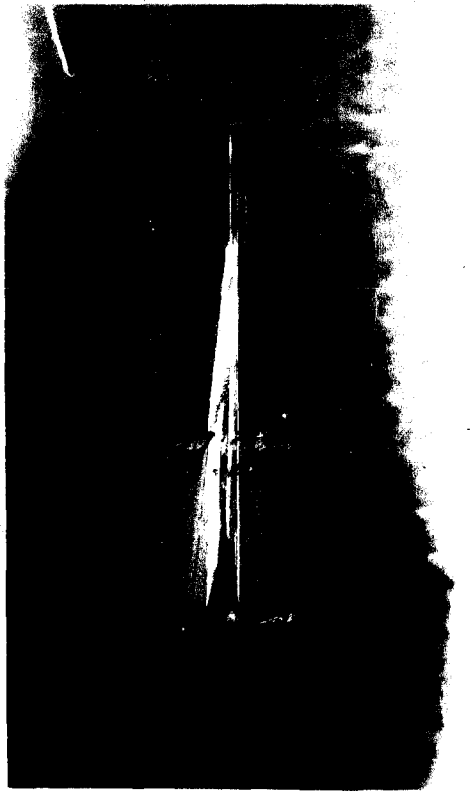
Photograph 1.

Immediately After Inoculation.



Photograph 2.

Three Days After Inoculation.



Photograph 3.

Eight Days After Inoculation.

Figure 5.

The Effect of Variations of Concentrations of Cellulose

In these experiments 12 one-liter flasks were used, each containing 750 cc. of medium of the composition employed in the previous studies. The concentration of cellulose was varied from 1.5 to 6.0 g. per 100 cc. Each flask was inoculated with 50 cc. of culture number 24, followed by the addition of 25 g. of sterile calcium carbonate. On the eighth day after inoculation analyses were made for ethanol, butanol, and residual cellulose. The data are given in Table 9.

While the results are somewhat irregular, due to difficulties in the analysis for cellulose, and other difficulties inherent in this type of experiment, the following general trends may be observed. With an increase in cellulose concentration there was an increase in the yields of ethanol and volatile acids; the yield of these chemicals per gram of cellulose fermented was practically constant with indications of a maximum between 2 and 5 g. of cellulose per 100 cc. The flasks containing more than 5 g. of cellulose per 100 cc. were too thick to allow efficient stirring. A concentration of 3 g. per 100 cc. was considered the most satisfactory for quantitative studies since it gave sufficient products to be conveniently analyzed and gave a medium sufficiently fluid to be easily handled.

Table 9. The effect of variations of concentrations of cellulose.

Grams cellulose per 100 cc.	Grams C_2H_5OH per 100 cc.	Grams C_2H_5OH per 100 cc.	Grams volatile acids per 100 cc.	Grams cellulose decomposed per 100 cc.	Grams C_2H_5OH per gram cellulose decomposed	Grams volatile acids per gram cellulose decomposed	Grams C_2H_5OH per gram cellulose added	Grams volatile acids per gram cellulose added	Per cent cellulose decomposed
0.5	0.050	0.002	0.11	0.36	0.14	0.31	0.10	0.22	72
1.0	0.079	0.002	0.17	0.57	0.14	0.30	0.08	0.17	57
1.5	0.174	0.003	0.22	1.04	0.17	0.21	0.12	0.15	70
2.0	0.371	0.013	0.31	1.87	0.20	0.17	0.18	0.15	94
2.5	0.322	0.012	0.28	1.53	0.21	0.18	0.13	0.11	61
3.0	0.392	0.012	0.53	2.23	0.18	0.24	0.13	0.18	74
3.5	0.624	0.006	0.68	3.00	0.21	0.23	0.18	0.19	86
4.0	0.458	0.069	0.62	2.44	0.19	0.25	0.12	0.16	61
4.5	0.726	0.009	0.77	3.45	0.21	0.22	0.16	0.17	77
5.0	0.722	0.009	0.95	3.84	0.19	0.25	0.14	0.19	76
5.5	0.755	0.007	0.91	4.71	0.16	0.19	0.14	0.17	86
6.0	0.763	0.002	0.85	4.80	0.16	0.18	0.13	0.14	80

The Effect of Aeration

During the course of an ordinary fermentation the medium naturally becomes anaerobic due to the formation of large quantities of carbon dioxide and hydrogen. Langwell (1932) has stated that aerobic conditions favor alcohol formation in a corn cob medium at 60°C.

In Experiment I, two 4 liter flasks containing 3 liters of medium each were used. The medium contained 3 g. of cellulose, 0.25 g. of ammonium chloride, and 0.25 g. of di-potassium phosphate per 100 cc. Each flask was inoculated with 200 cc. of culture number 24, and to each flask 100 g. of sterile calcium carbonate were added. One flask was capped with tinfoil. A Chamberland-Pasteur filter candle, 155 mm. by 15 mm. of porosity L2, was suspended vertically in the medium in the other flask. Compressed air at 17 inches of mercury was conducted to the candle. The air and fermentation gases escaping from this flask were passed through two bubbling towers filled with water at room temperature to trap any alcohol present. Both flasks were incubated at 55°C. Determinations of ethanol, butanol, volatile acids, and residual cellulose were made on the eighth day.

During the early part of the above experiment the rising air bubbles simply cleared a path through the thick medium and it was believed that the aeration was not very effective. A second experiment was therefore conducted with the filter candle

at an angle of approximately 45° from the vertical. In this experiment culture number 22 was used and only 2,700 cc. of medium were employed per flask. Otherwise the experiment was exactly like the first.

The results of the two experiments are given in Table 10. Both series indicate that aeration decreases the yield of ethanol, but the effect is most pronounced in the second experiment where the aeration was more effective. In addition to the effect indicated by the figures, there must have been more loss of alcohol with the flasks not aerated than those that were and this would only increase the difference. As far as digestion of cellulose and the yields of butanol and volatile acids are concerned, these experiments are not conclusive. During the early stages of the fermentations aeration seemed to produce a more rapid digestion of cellulose but this is not evident in the final results.

The Effect of Peptone

This experiment was undertaken to determine the effect of supplying a source of organic nitrogen. Four 4 liter flasks were prepared with 3000 cc. of medium in each. The medium contained 3 g. of cellulose, 0.25 g. of ammonium chloride, and 0.25 g. of di-potassium phosphate per 100 cc. The amount of peptone was varied from none to 1.0 g. per 100 cc. Each flask was inoculated with 175 cc. of culture number 20 and incubated at 55°C. Culture number 20 was similar to number 24 and gave good alcohol yields.

Table 10. The effect of aeration. (All quantities are in grams for the entire fermentation).

	Experiment I		Experiment II	
	Aerated	Not aerated	Aerated	Not aerated
C_2H_5OH	9.01	9.42	4.68	9.38
C_4H_9OH	1.05	0.18	0.10	0.03
Volatile acids	16.8	20.3	27.6	23.5
Cellulose decomposed	71.9	74.4	78.9	75.5
Cellulose added	96.0	96.0	87.0	87.0

During the fermentation the pH was adjusted every 12 hours to a value of 7.5 with 5 normal sodium hydroxide. A quinhydrone electrode was used to determine the pH. Determinations for ethanol, butanol, volatile acids, and residual cellulose were made on the eighth day.

The results are given in Table 11. The addition of peptone increased the yields of ethanol, butanol, and volatile acids, and also increased the decomposition of cellulose. The addition of peptone favored somewhat the production of volatile acids as the increase in yield of volatile acids was relatively greater than the increase in yield of ethanol. The yield of ethanol was increased 28.8 per cent by the addition of 0.50 g. of peptone per 100 cc. The corresponding increase in volatile acids yield was 36.5 per cent.

The data indicate that the presence of organic nitrogen is beneficial to the production of alcohol. The ethanol produced per gram of cellulose fermented increased from a value of 23 per cent to a value of 36 per cent.

The Effect of Glucose

Two experiments were carried out with glucose. In one, glucose was added in varying amounts to the ordinary cellulose medium; in the other, the culture was carried in glucose media containing no cellulose and was then transferred to the ordinary cellulose medium.

Table 11. The effect of peptone.

Peptone, g./100 cc.	0.0	0.25	0.50	1.00
C_2H_5OH, g./100 cc.	0.59	0.70	0.76	0.78
C_4H_9OH, g./100 cc.	0.0083	0.0062	0.0115	0.0135
Volatile acids, g./100 cc.	0.52	0.65	0.71	0.80
Cellulose decomposed, g./100 cc.	2.13	2.34	2.70	2.73
C_2H_5OH, g./g. cellulose decomposed	0.23	0.30	0.32	0.36

For the first experiment twelve one liter flasks were prepared containing 750 cc. of medium each. The media contained 3 g. of cellulose, 0.25 g. of ammonium chloride, 0.25 g. of di-potassium phosphate and varying amounts of glucose per 100 cc. Three flasks contained no glucose and were used as controls. Twenty-five g. of sterile calcium carbonate were added to each flask for neutralization. The flasks were inoculated with 50 cc. of culture number 24 and incubated at 55°C. Determinations of ethanol, butanol, volatile acids, and residual cellulose were made on the eighth day. At the completion of this series another series was prepared and inoculated in the same manner as the above, except that the concentrations of glucose represented a narrow range. The results of this experiment are given in Table 12.

For the second experiment, 11 500 cc. flasks containing 300 cc. of medium each were used. In six the medium consisted of 5 g. of glucose, 0.25 g. of ammonium chloride, and 0.25 g. of di-potassium phosphate per 100 cc. In the other 5 the medium consisted of 3 g. of cellulose, 0.25 g. of ammonium chloride, and 0.25 g. of di-potassium phosphate per 100 cc. Ten g. of sterile calcium carbonate were used per flask for neutralization. The glucose flasks were sterilized only 30 minutes at 15 pounds. One glucose flask was inoculated with about 15 cc. of culture number 24. The culture was transferred successively through the remaining five glucose flasks at the intervals of 6, 3, 5, and 6 days. Each time the culture was transferred to a glucose flask

Table 12. The effect of glucose.

Glucose added	Grams per 100 cc. C_2H_5OH	C_4H_9OH	Volatile acids	Cellulose decomposed	Per cent cellulose decomposed
			Series I		
0.0	0.513	0.0020	0.66	2.81	94
0.0	0.512	0.0021	0.76	2.79	93
0.0	0.546	0.0000	0.70	2.76	92
0.2	0.562	0.0105	0.71	2.64	88
0.2	0.528	0.0130	0.74	2.83	94
0.4	0.442	0.0003	0.80	2.65	88
0.4	0.394	0.0042	0.80	2.43	81
0.6	0.284	0.0032	0.75	1.54	51
0.6	0.270	0.0041	0.69	1.35	45
0.8	0.109	0.0032	0.45	0.18	6
0.8	0.136	0.0034	0.48	0.40	13
1.0	0.101	0.0029	0.44	0.00	00
			Series II		
0.00	0.488	0.0162	0.70	2.82	94
0.025	0.542	0.0000	0.70	2.87	96
0.050	0.530	0.0000	0.77	2.83	94
0.075	0.533	0.0008	0.71	2.83	94
0.100	0.580	0.0010	0.74	2.88	96
0.125	0.543	0.0002	0.80	2.84	95
0.150	0.498	0.0077	0.80	2.79	93
0.175	0.568	0.0053	0.81	2.89	96
0.200	0.548	0.0000	0.78	2.82	94
0.225	0.516	0.0062	0.82	2.80	93
0.250	0.577	0.0000	0.82	2.75	92

a transfer was also made to a cellulose flask. A determination of ethanol was made on the first glucose flask when it was 8 days old and determinations of volatile acids and residual glucose were made on the second glucose flask when it was 8 days old. Both experiments were carried out at 55°C.

In the first experiment fermentation started much more rapidly in the flasks containing glucose, but later slowed down. This phenomenon was particularly noticeable in Series I. All the flasks in this series which contained glucose headed much more rapidly during the first two days than did those containing no glucose. On the third day the three flasks containing no glucose were fermenting the more rapidly. The flask containing 1.0 g. of glucose per 100 cc. headed rapidly during the first two days, then it ceased to head until the sixth day. Apparently the cultures were first attacking the glucose and were not using the cellulose until the supply of glucose was exhausted. The more glucose present the longer it took for the culture to begin the fermentation of the cellulose.

The results of the analyses of the first experiment are given in table 12. The data obtained with Series I indicate an optimum of 0.2 g. of glucose per 100 cc. for the production of ethanol and 0.40 g. per 100 cc. for volatile acid. In no case did the presence of glucose increase the decomposition of cellulose; in fact the decrease in the decomposition of cellulose was very marked at concentrations of glucose greater than 0.40 g. per 100 cc.

The data obtained with the first series indicated that there might be an optimum between 0.0 and 0.2 g. of glucose per 100 cc. and the second series was prepared with this in mind. There was a slight increase in the alcohol yields and a somewhat greater increase in the yield of volatile acids, but no appreciable difference in the decomposition of cellulose was noticeable within this range.

The addition of a small amount of glucose increases the yield of both ethanol and volatile acids, but the increase is probably due to the fermentation of the glucose rather than to an increase in the fermentation of cellulose. It had been hoped that the presence of a small amount of glucose would aid the fermentation by producing a large number of bacteria in a short time which would then attack the cellulose. Instead, glucose seems to delay the action on the cellulose.

From the above results it would appear that either the actual cellulose fermenter does not grow well on glucose or that its growth on glucose reduces its ability to ferment cellulose. Further proof of this was obtained in the second experiment with glucose. When the culture was transferred to the medium containing glucose and no cellulose, a rapid fermentation set in, a considerable amount of gas was given off, and the medium became dark in color. The successive transfers on the glucose medium continued to ferment rapidly. When the flask of ordinary cellulose medium was inoculated from the first glucose flask, the fermentation of cellulose was evident within two days. An inocu-

lation from the second glucose flask also fermented cellulose, but the action was not apparent until the fourth day. Transfers from the third, fourth, and fifth glucose flasks produced no fermentation of cellulose even though allowed to remain in the incubator for more than a month. If the true cellulose fermenter does not grow on glucose, the fact that transfers from the first two glucose flasks fermented cellulose can be explained as being due to the large inoculation ratios used.

The determination of ethanol on the first glucose flask, when it was 8 days old, indicated 0.08 g. of ethanol per 100 cc. The determinations on the second glucose flask indicated 0.74 g. of volatile acids and 3.04 g. of reducing sugars. Both the volatile acid and ethanol values were low compared to the usual values obtained from the fermentation of cellulose, but the effect was the most marked with ethanol. The same phenomenon was indicated in the first experiment in which the flasks containing glucose yielded relatively more volatile acids than ethanol. In all cases when transfers were made from the glucose medium to the cellulose medium considerable gas evolved during the first day or so. Evidently the cultures were using the glucose transferred in the inoculum.

These experiments indicate that the presence of glucose up to about 0.25 per cent is not harmful to the fermentation while larger amounts are deleterious. This fermentation cannot be regarded as a good means of converting glucose into ethanol as yeasts give greater yields in less time and at lower temperatures.

THE ANALYSIS OF THE GASES PRODUCED

The gaseous products of this fermentation consist mainly of carbon dioxide and hydrogen. Small amounts of methane may also be formed, but not in sufficient quantities to be of any great importance. The gaseous products of several fermentations were collected and analyzed. The results of two such experiments will be discussed in detail. These results are typical of all the results obtained.

In the Experiment I, 750 cc. of medium were prepared in a one liter Erlenmeyer flask and inoculated with 50 cc. of culture number 22; 25 g. of calcium carbonate were added for neutralization. This culture was known to give a rather high yield of volatile acids. The gases produced were collected over saturated salt water and analyzed from time to time in a Williams gas analysis outfit according to the procedure previously described. In the second experiment 2750 cc. of medium were prepared in a four liter flask and inoculated with 200 cc. of culture number 24. The pH was controlled by additions of 5 N. sodium hydroxide to a pH value of 7.5 twice daily. This fermentation is the same as that discussed in pages 68-72. In both experiments, the medium consisted of 3 g. of cellulose, 0.25 g. of ammonium chloride, and 0.25 g. of di-potassium phosphate per 100 cc. of tap water.

The results of the Experiment I are given in Table 13. The

Table 13. An analysis of the gases produced by the fermentation. An 800 cc. fermentation with $CaCO_3$ neutralization. Experiment I.

Age in hours:	cc. gas evolved	:Per cent: CO_2	:Per cent: H_2	cc. gas per hour
24	367	15.6	17.9	15
37	743	50.0	44.2	57
44	520	52.0	45.7	74
61	1080	59.0	38.6	64
70	483	65.8	32.1	54
87	862	88.0	11.5	51
93	230	90.4	8.3	38
112	841	91.9	7.5	44
138	720	89.4	6.6	28
162	291	87.6	10.3	12
229	120	89.9	6.0	1.7
276	50	90.0	6.0	1.3
Left in ferm. flask	300	90.0	6.0	
Total volume gas collected	6307			

Yields:

Volatile acids as acetic	1.49 g./100 cc.
Total carbon dioxide	0.987 g./100 cc.
Carbon dioxide from reaction of acids and calcium carbonate	0.546 g./100 cc.
Net carbon dioxide from fermentation	0.441 g./100 cc.
Hydrogen	0.00734 g./100 cc.

yield of volatile acids was quite high and amounted to almost 50 per cent of the cellulose added. At first the production of hydrogen exceeded that of carbon dioxide, but as the fermentation proceeded there was a gradual decrease in the amounts of hydrogen relative to those of carbon dioxide. The rate of gas production was at a maximum during the second day, then gradually decreased, and by the eighth day had practically ceased. The total volume of gases collected was about seven times that of the medium, but if an allowance is made for the amount of carbon dioxide produced by the action of the volatile acids on calcium carbonate, the ratio is only about five. The yield of carbon dioxide was about 15 per cent by weight of the cellulose added.

The results of the Experiment II are given in Table 14. The yield of ethyl alcohol was high in this experiment and even exceeded that of the volatile acids. The yield of ethyl alcohol was 26 per cent by weight and that of volatile acids was 24 per cent. In this experiment the time intervals between the several gas analyses were greater than those in the preceeding experiment, and the gas was removed in units of 1042 cc.; hence the rates of production cannot be calculated with any degree of accuracy. In general, the results are like those obtained in the first experiment. The main difference is the lower percentage of carbon dioxide, but this is due to the use of sodium hydroxide as the neutralizing agent in place of calcium carbonate. The ratios of carbon dioxide to hydrogen were practically the same in both experiments when allowance was made in the first experi-

Table 14. An analysis of the gases produced by the fermentation. Experiment II. A 2950 cc. fermentation with NaOH neutralization.

Age in hours	cc. gas evolved	Per cent CO_2	Per cent H_2	Per cent CH_4
25	2084	18.2	18.2	0.40
71	3126	52.3	31.1	0.38
97	4168	84.2	11.5	0.10
144	3126	70.8	24.3	0.40
216	2084	58.6	22.5	0.08
294	880	56.0	28.7	0.35
Left in ferm. flask	1000	56.0	28.7	0.35
Total volume gas collected	15,468			

Yields:

Ethanol	0.78 g./100 cc.
Butanol	0.021 g./100 cc.
Volatile acids as acetic	0.72 g./100 cc.
Carbon dioxide during fermentation	0.55 g./100 cc.
Hydrogen	0.0096 g./100 cc.
Methane	0.0000083 g./100 cc.

ment for carbon dioxide from calcium carbonate. The volume of gas collected in the second experiment was just slightly over five times the volume of the medium.

THE DETERMINATION OF THE PRODUCTS OF FERMENTATION UNDER OPTIMUM CONDITIONS AT 60°C.

In this experiment a four liter flask containing 2750 cc. of medium was prepared. The medium was composed of 3 g. of cellulose, 0.25 g. of ammonium chloride, and 0.25 g. of dipotassium phosphate per 100 cc. of tap water. The inoculum consisted of 200 cc. of culture number 24. The flask was incubated at 60°C. The pH was adjusted twice daily to a value of 7.5 by the addition of 5 N. sodium hydroxide. The gases evolved during the fermentation were collected over a saturated solution of sodium chloride and analyzed from time to time for carbon dioxide, hydrogen, and methane. Twelve days after inoculation the contents of the flask were analyzed for carbon dioxide, volatile acids, non-volatile acids, butanol, ethanol, acetone, reducing sugar, reducing sugar after hydrolysis, and crude fiber.

The amount of carbon dioxide remaining in the medium was determined by acidifying a 200 cc. sample, boiling the liquid, collecting the escaping gases over a saturated salt solution, and determining the amount of carbon dioxide with the Williams gas analysis apparatus. Acetone was determined by the usual iodometric method. The hydrolysis was effected by refluxing a 100 cc. sample for two and a half hours with 7 cc. of concentrated hydrochloric acid (specific gravity 1.187). The amount

of crude fiber was determined by the standard A.O.A.C. method in which a sample was refluxed with 1.25 per cent sulphuric acid for 30 minutes, filtered, refluxed with 1.25 per cent sodium hydroxide for 30 minutes, filtered, washed, dried, and weighed. During the process of this determination a considerable amount of material was noticed that was soluble in the acid and insoluble in the base. Since the material could not have been any of the other products determined, it was also filtered, dried, and weighed. The exact nature of this material is unknown, but it is always present in quite large quantities. It precipitates as a flocculent precipitate and when dried it is an amorphous powder. It is light grey in color. The other determinations were made in the usual manner.

The results of this experiment are given in Table 15. The yield of ethanol was exceptionally large, amounting to 0.78 g. per 100 cc. of fermented liquor. The yields of methane, hydrogen, non-volatile acids, butanol, acetone and reducing sugars were small. The increase in the amount of reducing sugar upon hydrolysis indicates the presence of some carbohydrates of higher molecular weight than glucose.

In Table 16 the percentage yields of the various products and the percentage of the original carbon recovered in the various products are given. More than 86 per cent of the carbon was recovered and the total weight of products constituted more than 95 per cent of the original weight of cellulose. These values do not take into account the moisture present in the cellu-

Table 15. The products of fermentation under optimum conditions at 60°C. (The figures given are for the entire fermentation).

Carbon dioxide evolved during fermentation	16.5 g.	
Carbon dioxide remaining in the liquor	8.84	
Assumed carbon dioxide evolution from inoculum before inoculation	1.14	
Total carbon dioxide	28.48 g.	
Less carbon dioxide from calcium carbonate	2.93	
Net carbon dioxide from fermentation		23.55 g.
Methane		0.0243
Hydrogen evolved during fermentation	0.288	
Assumed hydrogen from inoculum before inoculation	0.027	
Total hydrogen from fermentation		0.315
Volatile acids as acetic acid		21.6
Non-volatile acids as lactic acid		0.216
Butanol		0.633
Ethanol		23.27
Acetone*		0.12
Reducing sugar as glucose		0.80
Reducing sugar after hydrolysis	1.68	
Crude fiber		2.844
Material soluble in acid and insoluble in alkali		11.36
Total weight of products		84.7323 g.

*Not positively identified.

Table 16. The products of fermentation under optimum conditions at 60°C.

The results are expressed in the percentage of the carbon recovered in the various products and percentage by weight.

	Carbon per cent	Weight per cent
CO_2	16.35	26.6
CH_4	0.0465	0.0275
H_2	0.000	0.356
$CH_3CHOHCOOH$	0.220	0.244
CH_3COOH	22.0	24.40
C_2H_5OH	30.0	26.30
C_4H_9OH	1.05	0.715
$(CH_3)2CO$	0.19	0.135
Reducing sugar	0.58	0.71
Residual cellulose	3.22	3.22
Insol. KOH, sol. H_2SO_4	12.85 ?	12.85
Total	86.5065%	95.5575%

lose. The moisture in the paper was about 2.5 per cent. If the moisture is taken into account the recovery of carbon was 88.7 per cent and the recovery by weight 98.0 per cent. In addition to this, small amounts of liquor were removed from time to time for the determinations of pH. If these amounts were considered the values would be slightly higher, but the difference would not be great.

MISCELLANEOUS OBSERVATIONS AND EXPERIMENTS

The Production of Pigment

Various colors were noticed in the media during fermentation, including yellow, bright orange, grey, and intermediate shades. Sometimes very little pigment was noticeable. In one experiment black spots, about a half inch in diameter, appeared simultaneously in several cultures. Because they occurred simultaneously it was thought they were due to something present in the media, but as far as could be determined the media had been prepared as usual. One of the black spots was transferred to a fresh medium, and it grew and fermented cellulose, but the black color disappeared. No relationship was found between pigment production and the production of alcohols and acids. The usual color was a dull yellow.

The Effect of Heat Shocking

Because heat shocking is so desirable in certain fermentations, particularly in the butyl-acetonic fermentation, it was thought that it might be beneficial to this fermentation. Several portions of a culture were placed in test tubes and placed in boiling water for periods varying from 2½ to 30 minutes. These

portions were then used to inoculate flasks. The treatment was without influence; the yields of products were practically the same in each case.

The Effect of Change of Temperature

In the experiment on the effect of temperature it was found that cultures at 65°C. started more rapidly and produced greater quantities of products during the first few days than at lower temperatures. Consequently it was thought that the fermentation might be speeded up by placing it at 65°C. for 48 hours and then at 55°C. Contrary to expectations, at the end of 8 days a fermentation treated in this manner had produced only half as much ethanol and about the same amount of volatile acids as one maintained at 55°C. throughout the course of the fermentation.

The Effect of Magnesium Chloride

It had been repeatedly shown that distilled water was not as good as tap water for the preparation of media. It was known that the tap water contained about 34 parts per million of magnesium and it was thought that the deleterious effect of the distilled water might be due to a lack of magnesium salts. In one case a series of media were prepared with distilled water and the content of $MgCl_2,6H_2O$ was varied from zero to 0.55 g. per 100 cc. In another similar experiment the content of the magnesium salt was varied from zero to 0.10 g. per 100 cc. From

the data obtained it appears that the presence of magnesium salts in the concentrations used is neither harmful or beneficial. The yields were still very low in these media.

The Effect of Ferric Chloride

Because of the presence of some iron salts is necessary in many fermentation, it was thought that the presence of iron salts might aid this fermentation. Two series of flasks were prepared. In one series the content of $FeCl_3,6H_2O$ was varied from zero to 0.64 g. per 100 cc. and in the other from zero to 0.0050 g. per 100 cc. The presence of more than 0.10 g. per 100 cc. was harmful, but otherwise no definite trend was noticeable. In both these series the yields were still very low. Distilled water was used in the preparation of these media.

Losses by Evaporation

During the course of a fermentation there was a decrease in volume of between 2 and 3 per cent. This was not taken into account in the calculations in most experiments as it was almost exactly compensated by the moisture content of the cellulose, which was also between 2 and 3 per cent. In addition to the volume effect, some alcohol was certain to be lost during the fermentation. In order to estimate this loss, flasks were prepared with about 0.6 g. of ethanol per 100 cc. of water. The approximate volume of gas that would have been evolved during fermentation of this size was aspirated through the solutions

while the flasks were in the incubator at 55°C. Approximately 2.5 per cent of the ethanol was lost during the operation. Losses from this source are therefore not significant.

GENERAL DISCUSSION OF RESULTS

The foregoing experiments show that it is possible to ferment cellulose at thermophilic temperatures and to obtain considerable amounts of ethanol. A medium has been systematically developed consisting of 3 g. of cellulose, 0.25 g. of ammonium chloride, and 0.25 g. of di-potassium phosphate per 100 cc. of tap water. The best temperature appears to be 55°C., and when adjustments are made twice daily the medium should be adjusted to pH 7.5 to 8.0. Using this medium, yields of ethanol as high as 27 per cent of the cellulose decomposed have been obtained.

In a large proportion of the experiments calcium carbonate was used as the neutralizing agent in order to eliminate the laborious adjustment of flasks twice daily. Calcium carbonate gave a pH of 6.5 to 6.8 and the yields were correspondingly lower than for pH values of 7.5 to 8.0.

One of the characteristics of the fermentation is the wide variation in yields. This can be illustrated very well by reference to Table 6. It will be noted that the yield of ethanol and the decomposition of cellulose were much less with a concentration of 0.300 g. of phosphate per 100 cc. than with the two adjacent concentrations. From the general trend the results at this concentration should be practically the same as at the adjacent concentrations. Similar irregularities were often observed. While

general trends may be evident from a single series of flasks, a considerable number of series must be averaged before smooth curves can be plotted.

As yet the problem of obtaining large yields of ethanol from cellulose by thermophilic fermentation is far from solved. The fact that distilled water does not yield good results when used in preparing the medium has not been satisfactorily explained. Further experiments should be conducted with a wide variety of cultures before generalizations are made. The application of the fermentation to crude cellulosic materials or the determination of the treatment of crude cellulosic materials to make them more fermentable should receive thorough study. The investigation reported in this thesis was carried out with filter paper as the source of cellulose in order to eliminate as many unknown factors as possible. The data presented show that the yield of ethanol can be very considerably increased by the use of the proper concentration of nutrients, regulation of pH and the employment of the proper temperature.

SUMMARY

The data presented in this investigation may be summarised as follows:

1. Under the conditions used and with the culture employed the optimum temperature for the production of ethanol was 55°C.

2. A concentration of 0.25 g. per 100 cc. of ammonium chloride sufficed as a source of nitrogen.

3. A concentration of 0.25 g. per 100 cc. of di-potassium phosphate sufficed as a source of phosphate.

4. Calcium sulphate in concentrations greater than 0.019 g. per 100 cc. greatly decreased the yield of ethanol.

5. Under the conditions of the experiments, with adjustments twice daily, and with the cultures employed, a pH of 7.5 to 8.0 gave the highest yields of ethanol.

6. A concentration of cellulose of 3 g. per 100 cc. was found to be most satisfactory for the experimental work.

7. Under the conditions of the experiment and with the cultures employed, aeration decreased the yield of ethanol.

8. Additions of peptone increased the yield of ethanol. It also increased the decomposition of cellulose. The yield of acids increased slightly more than that of ethanol.

9. The addition of glucose did not increase the decomposition of cellulose. Concentrations up to about 0.25 g. of glucose

per 100 cc. increased the yield of ethanol, but larger amounts decreased the yield. Glucose favored the production of volatile acids. Repeated culturing in glucose media with no cellulose caused the culture to lose its cellulose decomposing power.

10. The principal gaseous products of the fermentation were found to be carbon dioxide and hydrogen. The percentage of hydrogen in the gases evolved was greater during the early part of the fermentation. The amount of carbon dioxide in the gases was equal to about 18 per cent of the cellulose by weight, depending upon the neutralizing agent used. The yield of hydrogen was about 0.25 per cent by weight of the cellulose added.

11. A fermentation at 60°C., under otherwise optimum conditions, yielded 26.6 per cent carbon dioxide, 24.4 per cent volatile acids, 26.3 per cent ethanol, 0.715 per cent butanol, 0.71 per cent reducing sugar, 0.356 per cent hydrogen, 0.244 per cent non-volatile acids, 0.027 per cent methane, 12.85 per cent of a material soluble in acids and insoluble in bases, and 3.22 per cent of residual cellulose based on the cellulose originally present. The total recovery was 95.422 per cent by weight.

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