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MINERAL ACIDS AND MOLD AMYLASE AS SACCHARIFYING AGENTS FOR PRODUCTION OF FERMENTABLE SUGARS FROM STARCH

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I. INTRODUCTION

Next to water, ethanol is the most useful chemical compound known to mankind. Since it enters either directly or indirectly into the preparation or processing of more materials than any other organic compound the chemical investigation of the methods of its production is of extreme importance. This is especially true in times of national emergency like the present when large quantities of ethanol are needed for the defense industries such as in the production of smokeless powder. Nearly 125,000,000 gallons of ethanol were manufactured during 1940, the largest production on record. The 1941 demand is expected to be in the neighborhood of 165,000,000 gallons which is practically the limit that industry can produce with the plants now available.

Approximately 85 per cent of all the ethanol made in the United States is obtained by fermentation. The balance is made by synthesis from ethylene derived from the cracking of petroleum. Since the amount of ethylene available is limited as to amount produced as well as by other uses, any large expansion in ethanol production must be in the fermentation industry. Nearly 90 per cent of the ethanol made by fermentation has been produced in recent years from by-product molasses, the remaining 10 per cent being produced primarily from grain. The recent increases in alcohol production have caused larger demands for molasses as evidenced by a 40 per cent increase in price, and statistics of production given by Shepherd, McPherson, Brown and Hixon (1940) led to the conclusion that probably not enough by-product molasses will be available to meet the needs of the alcohol industry. Thus it will become necessary either to use high-test molasses at a substantial increase in cost or to turn to some other raw material. For these reasons it is imperative that other sources of alcohol be investigated. The cheapest raw material for fermentation alcohol (after molasses) that is available in adequate quantities is corn.

Before corn or any other starchy material can be fermented by yeast the starch must first be converted into fermentable sugars. This process is called saccharification. Saccharification is not essential in the production of ethanol from molasses because it is composed largely of fermentable sugars. This is one of the chief reasons that molasses is used for ethanol production instead of corn. The conventional saccharification process requires the use of sprouted barley which is known as malt. It has been estimated by Shepherd, McPherson, Brown and Hixon (1940) that the cost of malt alone adds over four cents a gallon to the price of alcohol produced from grain. Therefore it is quite evident that before grains can compete with molasses it is necessary to find a less expensive method of saccharification.

In view of the above facts, the use of mineral acids and mold amylase as saccharifying agents for the production of fermentable sugars from starch was made the subject of this investigation.

II. HISTORICAL

The first recorded observation that starch could be converted into sugars by treatment with acids was made by Kirchhoff (1811) while searching for a substitute for gum. He found that dilute sulfuric acid converted potatoes or wheat into sugar. He also reported that 100 pounds of potatoes would yield fifty pounds of syrup having the same sweetness as grape sugar.

The literature for the next decade is crowded with the work of other investigators repeating and confirming Kirchhoff's data. Vogel (1812) carried out experiments proving that the sugars produced by acid saccharification could be fermented. He used fermentation tests to determine the amount of reducing sugars produced. For the next fifty years investigators became concerned with the products of acid-hydrolyzed starch and neglected further efforts toward increasing the efficiency of the process. Musculus (1862) discovered the fact that much better conversion was obtained if the starch was hydrolyzed under pressure.

Allihn (1880) completed a very exhaustive investigation on the effect of dilute sulfuric acid on starch. He found that the rapidity of saccharification is proportional to the concentration of the acid and, within certain limits, to the duration of its action and to the temperature. Allihn's results were based on the amount of reducing sugars present as determined by the use of Fehling's solution.

Many reports on the preparation of dextrose from starch followed this work. Rolfe and Defren (1899) described a process for the production of dextrose by treating starch obtained by the wet milling process with 1 per cent hydrochloric acid at a steam pressure of thirty pounds. Although there were many reports at this period on the preparation of dextrose by acid hydrolysis, many of them are incomplete in regard to time of processing. No reports were found until 1900 of any attempt to produce alcohol from acid-hydrolyzed starch other than to use this as a method for determining the amount of conversion effected.

Van Laer (1900) discussed a process which had been developed by Callebaut (1899) to save the cost of malt in beer making. Ground maize at a mash concentration of nearly 50 per cent was cooked under a steam pressure of 37 pounds per square inch with approximately 0.20 normal hydrochloric acid until the mash gave a violet color with iodine. At this point the major product was dextrins, and the saccharification was completed with malt after the acid had been neutralized with sodium carbonate. The malt was necessary in order to obtain a normal fermentation by yeast. Although the process yielded a beer with good keeping qualities, the absence of further literature indicates that the process was discarded. This is probably due to the fact that the flavor imparted by the malt is essential in the manufacture of beer. For that reason, even, if the acid process would allow a substantial saving in cost of the beer, it would not be economically sound.

Barbet (1912) described a process for saccharifying corn with 5 to 6 per cent hydrochloric acid. On fermenting this product with yeast it was reported to yield almost as much alcohol as is obtained from the conventional process. The details of this work are unavailable, and it is probable that the mechanical difficulties encountered would not allow economic competition with malt hydrolysis. Moreover, the cost of the acid and alkali required for its neutralization when used at these high concentrations would nearly equal the cost of the malt used in the conventional process.

Duryea (1911) made a report on the acid hydrolysis of starch dealing primarily with the products obtained. Since this work was carried out at 55° C. with 1 per cent hydrochloric acid, the yields of sugars were extremely low and of no practical importance to the fermentation industry.

Defren (1912) studied the fermentability of mixtures of glucose and dextrins resulting from the acid saccharification of starch. The presence of dextrins was found to retard the fermentation of glucose. From these results it is apparent that a high degree of conversion must be attained before good alcohol yields can be expected.

Wesener and Teller (1916) determined the fermentability of commercial glucose solutions manufactured by the acid hydrolysis of starch. These investigators found that the dextrins present, although originally unfermentable, were converted into fermentable sugars by the action of Taka-diastase or malt extract.

In 1921 acid hydrolysis was employed again for the conversion of starchy materials into fermentable sugars with the intention of using the process for the production of alcohol. Roxas and Manio (1921) made a study of the fermentation of cassava using acid hydrolysis. Upon the addition of 200 ml. of 0.8 normal sulfuric acid to 204 grams of starch and cooking at 120° C. for 2.5 hours a yield of 79.5 per cent glucose, based on cassava, was reported. They claimed an alcohol yield of 90 per cent of theoretical when certain inorganic salts were used as nutrients. These investigators assumed that complete conversion of starch to sugar was obtained under these conditions. Since no official sugar determinations on the cassava were reported, it is not possible to confirm this assumption.

Although the production of dextrose from starch is an established industry, very little information on the process is available in the literature. Even the patent literature (Dextrin Automat Gesellschaft (1923), Ghevenot (1925), and Dreyfus (1930)) is so brief that nothing of any value can be learned from it. However, Williams (1930) made a rather detailed report on the acid hydrolysis of starch studying the influence of time and pressure on the yield of dextrose obtained. The data show that the maximum yield was obtained by treating a starch suspension (Sp. G. 1.120) with a concentration of hydrochloric acid equal to 0.4 per cent of the weight of the dry starch for 50 minutes at a steam pressure of either 30 or 40 pounds per square inch. Although considerable data were tabulated the choice of a method for analysis was very unfortunate. The method used was as follows:

> "10 cc. cooled sample poured into 90 cc. of alcohol contained in a 100 cc. graduated glass cylinder. The maltose and glucose dissolve in the alcohol, but the dextrin, being insoluble, is precipitated and will sink rapidly to the bottom when the percentage may be quickly ascertained. With practice it is not necessary to wait for the dextrin to settle; the percentage can be judged roughly by the amount of discoloration on adding the liquor to the alcohol." p. 497.

The entire investigation is of little value because of the inexact procedure followed.

Severson (1937) studied the saccharification of cereals by hydrochloric acid and the alcoholic fermentation of the hydrolyzates. Each hydrolysis was carried out in a 300 ml. Erlenmeyer flask containing 15 grams of the grain and 100 ml. of the acid solution. The cereals investigated were wheat, corn, oats and barley. The factors studied were the acid concentration, time and temperature of hydrolysis. Severson found that, in general, better alcohol yields were obtained using lower acid concentrations and higher temperatures over longer periods of time. The best alcohol yields from corn were obtained by hydrolysis with 0.10 normal acid for three hours at a steam pressure of 25 pounds per square inch. Under these conditions only about 90-95 per cent conversion of starch to dextrose was obtained. The maximum alcohol yields were not obtained from mashes which showed the highest titer for reducing sugars. In all cases the yields of alcohol from the acid saccharified mashes were on the average nearly 12 per cent less than the yields from malt-saccharified mashes.

Yabuta and Aso (1939) investigated the saccharification of starch by 24 organic acids. One part of starch was heated with 5 parts of 1 per cent organic acid solution at 130° C. for three hours. They concluded that the amount of hydrolysis obtained in general paralleled the pH.

Schoene, Fulmer and Underkofler (1940) investigated the effect of addition of malt and mold-bran to acid-hydrolyzed corn mashes. It was found in general that the addition of malt did not increase the alcohol yield to any appreciable extent, and thus it may be concluded that acid hydrolysis is just as effective as malt hydrolysis. The addition of mold-bran did cause a marked increase in alcohol yield, and this would indicate that it was more effective. Unfortunately, however, they did not run a series testing the three methods simultaneously under similar laboratory conditions.

Banzon (1940) studied the acid hydrolysis of very concentrated cassava mashes, in which the proportion of the sample to acid was carried as high as 1:1. The maximum conversion of starch to sugars was obtained when the ratio of sample to acid was 1:2.3 to 1:3 and when the concentration of sulfuric acid was 0.4 normal. However, yeast fermentation of the acid hydrolyzates gave poor yields of ethanol. The best yield was only 70.8 per cent.

The above review definitely indicates that the literature on acid hydrolysis of starch for production of fermentable sugars is meager indeed. And from the results obtained, it seems logical to conclude this part of the historical section of this thesis with the following quotation from the report of the survey for the Regional Research Laboratories (1939):

> "The use of acids to convert starches for a fermentation process has never been practicable." p. 31.

Since this thesis deals with both mineral acids and mold amylase as agents for conversion of starch to fermentable sugars, it is also necessary to review briefly the history of the use of mold amylase. Although molds have been used for centuries in the Orient for saccharification of starchy grains prior to fermentation, they were introduced into other countries only very recently.

In the Orient no attempt is made to use pure cultures of molds. Instead a mixed culture of microorganisms which are active in the hydrolysis of starch is employed. The culture is grown on a cooked rice substrate, and the resulting product is known as "koji". A considerable number of molds with high saccharifying power have been isolated from this source. The predominating mold species in koji is Aspergillus oryzae although some Mucor and Rhizopus species are also present.

The production of amylase by molds has been studied by many investigators since the latter part of the nineteenth century, but a great deal of this work has been carried on in the Orient and most of the material has been published in unavailable journals. Too frequently mixed cultures were used, thus a large proportion of this material is of questionable value. There is some question about who actually was the first to do any scientific research in this field as several important observations were reported in the same year.

Gayon and Dubourg (1887) investigated the molds Aspergillus oryzae, Mucor circinelloides, Mucor spinosus, Chlamydomucor oryzae, and Mucor alternans. Their observations were that Aspergillus oryzae had the highest saccharifying power; Mucor alternans had considerable saccharifying activity but somewhat less than Aspergillus oryzae. The other three molds showed some saccharifying ability but were quite inferior to Aspergillus oryzae and Mucor alternans.

Sanquineti (1897) continued this investigation and made a quite thorough study of three molds. As a result the molds were listed in order of saccharifying power as follows: Aspergillus oryzae, Amylomyces rouxii, and Mucor alternans. He concluded that in all probability Amylomyces rouxii would be the only one to be used commercially because it fermented the starchy materials directly without the addition of yeast. Amylomyces rouxii is now called Mucor rouxii, although in some of the literature it is designated as Amylomyces Mucor rouxii.

Collette and Boidin (1897) took out a patent for the production of alcohol from starchy substrates by the use of a mold which they called Mucedineae. It is probable that this mold was really Mucor rouxii. This was the origin of the Amylo process which has been used in Europe.

Most of the literature dealing with the Amylo process is not accessible owing to patent restrictions, but Galle (1923) and Owen (1933) have published articles on the details of the methods used. As the process developed the original organism, Mucor rouxii, was replaced by other molds. Mucor B., Mucor G., and finally Rhizopus delemar were used in the order named. All of these organisms were isolated from either "Chinese Yeast" or "Japanese Koji". These organisms were more rapid, had a greater tolerance for alcohol, and produced less acid than the original mold. The organism used at the present time, in this process, is Mucor boulard Number 5. It is a very rapid growing mold and thus has the ability to hold its own against contaminants. Shorter time for fermentation is required (complete in 48 hours), and the yeast and mold may be added at the same time. Whether or not this process is economically sound is still debated.

Takamine (1898) isolated a pure culture of Aspergillus oryzae from koji and used it for the production of amylase. In a more recent report Takamine (1914) claims to have investigated on a practical scale in 1891 the application of Aspergillus oryzae for the saccharification of grain. This project was carried out at Peoria, Illinois, and was run on a 2,000 bushel scale for several months. The experiment was partially successful but did not attain general recognition of its merit "because it still lacked means to overcome various impediments due to trade conditions and difficulties in adapting the process in the new field of application".

Saito (1907) reported the isolation of a new species of mold from koji which he named Aspergillus batatae. Although it was reported to produce amylase, the information given is rather meager and would lead one to believe that its capacity to produce amylase is probably quite limited.

Okazaki (1914) studied three members of the genus Aspergillus, namely Aspergillus okazakii, Aspergillus albus, and Aspergillus candidus. The method used was to treat starch with these molds and then determine the amount of sugars produced. He claimed Aspergillus okazakii and Aspergillus candidus produced considerable amounts of amylase, but actual values were not given.

Scales (1914) prepared an enzyme powder by growing Aspergillus terricola for four days on an artificial medium. After the mycelium was well washed with water, acetone and ether, it was allowed to dry and then ground in a mill. This powder was added to a starch solution, and after an incubation period of three days 82 per cent of the starch was found to have been converted into sugar.

Takamine (1914) found <u>Aspergillus</u> <u>oryzae</u> grew very well on wheat bran. He named this product "Taka-Koji", and carried out experiments to determine its efficiency for saccharification as compared with malt. Due to a very favorable report from Takamine, Taka-Koji was tried on a plant scale by Hiram Walker and Sons in Canada under the direction of Ortved (1912). The alcohol yields were better than those obtained from malt, and although Taka-Koji was considerably less expensive than malt, the process was discarded. Why it was is unknown since Ortved (1912) made a very favorable report on its use. He claimed that although the first distillation produced a product which had a musty taste, this was entirely removed by redistillation and failed to develop again on aging. Takamine (1894, 1896, 1910, 1911, 1913, 1918, 1923) obtained numerous patents in the United States for his diastatic product and its use in the fermentation industry.

Collens (1915) investigated the possibility of producing industrial alcohol from cassava. Experiments were carried out using both malt and taka-diastase as saccharifying agents. Taka-diastase produced nearly 8 per cent more alcohol than did malt.

Oshima and Church (1923) made an intensive investigation of the molds isolated from koji in order to determine which ones produced the largest quantities of amylase. Aspergillus oryzae and a mold form intermediate between Aspergillus flavus and Aspergillus oryzae were found to be the most potent producers of amylase. This investigation was the first to show the great variation which may be found in different strains of the same mold. They also studied mold growth and enzyme production on the following media: wheat bran, wheat middlings, corn meal, cocoanut meal, peanut meal, cottonseed cake, oil-extracted soybean meal, soybean meal, crushed soybeans, dried yeast, ground dried codfish, and casein. They found wheat bran to be the best substrate. Oshima (1928) extended this study and found the activity of the enzyme to be greatest at pH 4.8 to 5.2. The enzyme was found to be heat laible becoming completely inactivated when heated at 85° C. for one hour.

Harada (1931) studied the preparation and properties of Aspergillus oryzae. The culture of mold was grown on cooked wheat bran containing 50 per cent water. Maximum growth was attained in two days. Harada found that the optimum pH for enzymatic activity increased with increasing temperature. At 30° C. the optimum pH was 5.2; at 65° C., it was 6.6. However, at temperatures below 50° C. the optimum pH remained practically constant at a value of 5.2.

Wei and Chin (1934) studied ten species of Aspergillus. They concluded that Aspergillus oryzae Aold had by far the greatest saccharifying power of all the species examined.

Takeda (1935) isolated twenty-seven stocks of *Rhizopus* from ragi-koji and soybean-koji produced in Java and Sumatra. Of these, only two had strong amylolytic powers. They were named *Rhizopus semarangensis* and *Rhizopus javanicus*. The latter was found to be especially valuable for the Amylo process. *Rhizopus javanicus* was tried on a commercial scale in Japan and pronounced entirely satisfactory. Takeda claims that the amylolytic action of *Rhizopus javanicus* was more rapid than that of *Rhizopus delemar Wehmer et Hanzawa*.

Recently Muta and Tanaka (1936) revived investigations on the possibilities of the Amylo process. They reported that *Rhizopus delemar* and *Rhizopus pêka I* were the best molds for use in this process.

Underkofler, Fulmer and Schoene (1939) studied *Aspergillus oryzae*, *Aspergillus flavus*, *Mucor rouxii*, *Rhizopus delemar*, *Rhizopus oryzae*, *Rhizopus pêka I*, *Rhizopus tritici*, *Mucor circinelloides*, and *Mucor javanicus* for amylase productions. From the preliminary results *Aspergillus oryzae*, and the three *Rhizopus* molds had about the same activity. *Aspergillus oryzae* was used in further investigations on the suitability of using mold preparations for saccharifying fermentation mashes. Unfortunately *Aspergillus oryzae* was not compared directly with *Rhizopus triciti* and *Rhizopus oryzae* by running a series of fermentations at the same time and under exactly the same conditions. However, they found that by the use of *Aspergillus* oryzae cultured on wheat bran a 90 per cent conversion of starch into alcohol could be obtained. This was a 10 per cent greater yield than could be obtained from malt. The mold-bran was prepared in aerated five-gallon rotating pyrex bottles. A detailed procedure was developed. The most important contribution was the employment of dilute acid instead of water; this prevented growth of contaminating organisms. The advantages of using mold-bran in place of malt were pointed out in the report of this investigation, namely, mold-bran is less expensive, more quickly prepared, and gives higher alcohol yields.

Schoene, Fulmer and Underkofler (1940) compared malt, mold-bran, and soybean meal as saccharifying agents. Mold-bran was found to be the most effective. The use of various combinations of these three saccharifying agents was proven to be little, if any, better than the use of mold-bran alone. However, the addition of mold-bran or combinations of mold-bran and soybean meal to acid-saccharified mashes gave a considerable increase in alcohol yield.

Banzon (1940) investigated the use of mold-bran as a saccharifying agent for the production of alcohol from cassava. By the use of a quantity of mold-bran equal to 7.5 per cent of the weight of the cassava, alcohol yields well above 80 per cent of the theoretical were obtained under laboratory conditions. The best results were attained when the mold-bran was introduced into the mash at 30° C. This discovery would eliminate the customary malting procedure carried on at elevated temperature and would thus result in a substantial reduction in the cost of the process.

Underkofler, Goering and Buckaloo (1941) continued the investigation of Underkofler, Fulmer, and Schoene (1939). Four strains of *Aspergillus oryzae*, two strains of *Rhizopus* species, a *Mucor* and two unidentified yellow molds, probably members of the *Aspergillus flavus-oryzae* group were investigated. Two strains of *Aspergillus oryzae*, *Rhizopus oryzae*, and *Rhizopus tritici* were especially good producers of amylase. The alcohol yields obtained were approximately 10 per cent greater than those obtained from malt. Attempts were made to grow *Aspergillus oryzae* on various fibrous materials including wheat bran, corn bran, oat hulls, cottonseed hulls, corn cobs, sawdust, peanut hulls, and rice hulls. Wheat bran and dry-milled corn bran were the only substrates of those tested which adequately supported the growth of this strain of mold. At the present time this investigation of various other molds for amylase production and different methods of growing them are being studied in this laboratory.

Since this thesis deals only with the production of fermentable sugars from starch, no attempt has been made to review the extensive chemical investigations on starch. However, the chemical structure of this substance is still not definitely settled. A very comprehensive survey on this field has been made by Walton (1928), who published in book form, a review of the literature from 1811 to 1925 on this subject.

III. MATERIALS

A. Important Materials Used In This Investigation

1. Corn starch

The corn starch used in this investigation was obtained in two lots from the American Maize-Products Company, Roby, Indiana. The first 25 pound lot was obtained June 21, 1940. It was very carefully mixed, sampled, and stored in well stoppered bottles until used. This sampled material yielded 92.5 grams of glucose per 100 grams of starch when hydrolyzed with acid according to the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists. The second 25 pound lot arrived on March 3, 1941. This material was Pearl starch and had to be ground through a burr mill before it could be mixed and sampled. The glucose equivalent of this material was 95.0 per cent.

2. Corn-gluten meal

Three different lots of corn-gluten meal were used in this investigation. The first lot was designated as gluten A. It is of unknown origin being obtained prior to February 1939. Its appearance and odor suggest that it probably contains the steep water concentrates. It was used because at that time it was the only gluten available in the laboratory. Gluten B was obtained from the American Maize-Products Company, Roby, Indiana on June 21, 1940. It was lighter in color than gluten A and did not have the same odor. Gluten C was obtained from the same source on March 3, 1941. It was slightly darker than gluten B.

3. Steep water

The steep water was obtained from the American Maize-Products Company, Roby, Indiana on April 21, 1941. It was designated as heavy steep water and contained between 40 and 50 per cent solids.

4. Cassava

The cassava used was obtained by grinding the sliced and dried unpeeled root. It was obtained from the College of Agriculture, University of the Philippines, Philippine Islands. The material used had a glucose equivalent of 85.0 per cent.

5. Mold-bran

The mold-bran used in this investigation was prepared by growing *Aspergillus oryzae Number 40* on wheat bran according to the method of Underkofler, Fulmer, and Schoene (1939). It was air dried and passed through a Wiley mill. This particular lot had been prepared and stored over one year before use. Experiments carried out when the material was first prepared and those conducted after storing for one year proved that very little amylolytic power had been lost.

6. Barley malt

The barley malt was obtained from the Fleischmann Malting Company. It was ground to a coarse powder in a burr mill and stored in a stoppered bottle until used.

7. Malt extract

The malt extract used to prepare beer wort for yeast cultures was Blue Ribbon Malt Extract. This product is made by the Premier-Pabst Corporation, Peoria Heights, Illinois.

8. Corn meal

The corn meal used in this investigation was obtained from the storage bins in the animal laboratories of Physiological Chemistry in this Department. The three lots obtained had a glucose equivalent of 74.0, 68.0, 70.7 per cent.

IV. METHODS

A. Microbiological Procedures

1. Yeast culture

One hundred grams of malt extract were dissolved in distilled water, and the volume made up to one liter. The solution was brought to a boil, and then the precipitate was allowed to settle. This supernatant liquid was then placed in Erlenmeyer flasks and designated as beer wort media: 50 ml. of the wort was used in each 125-ml. flask for carrying the cultures, and 300 ml. in each 500-ml. flask for cultures employed for inoculating experimental mashes. The flasks were plugged with cotton and sterilized for 30 minutes under a steam pressure of 15 pounds.

A stock culture of *Saccharomyces cerevisiae* designated in this laboratory as yeast number 43, was transferred to beer wort in a 125-ml. flask and incubated at 30° C. for 24 hours. In previous work in this laboratory smaller flasks were used for carrying the culture, but it was found that the yeast was apparently more vigorous when allowed to grow in larger quantities of medium. By means of a sterile pipette 1 to 3 ml. of this yeast culture were transferred to another flask, and the subculture incubated for the same length of time and at the same temperature. Transfers were made daily in this manner throughout the course of the investigation to maintain a vigorous yeast culture.

The inoculum for experimental mashes was prepared by inoculating 300 ml. of beer wort with 5 to 8 ml. of a vigorous yeast culture and incubating at 30° C. for 24 hours.

2. Preparation of mold-bran

The mold-bran used in this investigation was prepared by the method developed by Underkofler, Fulmer, and Schoene (1939). The method was essentially as follows: 800 grams of wheat bran were placed in a five gallon pyrex bottle and thoroughly mixed with 1,000 ml. of 0.05 normal sulfuric acid. The bottle was plugged with cotton and sterilized with steam at a pressure of 20 pounds per square inch for two hours. Upon cooling to room temperature, the bran mash was inoculated with 50 grams of a well sporulated culture of *Aspergillus oryzae* designated in this laboratory as Number 40.

The inoculum was previously prepared by mixing 25 grams of wheat bran and 25 grams of 0.5 normal sulfuric acid in 500-ml. Erlenmeyer flasks. These flasks were sterilized in an autoclave for 30 minutes at 20 pounds steam pressure. After cooling down to room temperature, the bran mixture in the flasks was inoculated from a stock culture of *Aspergillus oryzae*. The stock cultures are maintained on agar slants in a refrigerator. The bran cultures were now incubated at 30°

C. until well sporulated, usually five to six days. Such a culture was then used to inoculate the five gallon drum.

After inoculation the contents of the drum were mixed by allowing it to rotate for half an hour. When well mixed it was allowed to remain at rest for 12 hours with continuous aeration. By this time the growth was well started, and the drum was rotated slowly. After 36 to 48 hours the mold was removed from the drum and air dried. The time allowed in the drum varies with different preparations, but it was found that removal just prior to sporulation produced the best product. The air dried preparation was ground in a Wiley mill and stored in stoppered bottles until used.

B. Analytical Procedures

1. Determination of the sugar content of the starch

The glucose equivalent of the starch was determined by acid hydrolysis in accordance with the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists. The reducing substances formed in the hydrolysis were estimated according to the modified Shaffer and Somogyi method developed by Guymon (1939). The reagents were standardized by means of a sample of pure glucose. All experiments in this thesis were carried out in duplicate or triplicate. Any questionable analyses were repeated.

2. Determination of ethanol

The entire volume of fermented mash was distilled after approximately 0.5 gram of calcium carbonate had been added to neutralize the acids present. The first 100 ml. of the distillate were collected in 100-ml. volumetric flasks.

The distillates were placed in a thermostat at 25° C. and allowed to attain that temperature. The volume was then adjusted and the specific gravity determined by means of a Chainomatic Westphal balance. The ethanol concentration in grams per 100 ml. of solution was read from an appropriate table.

3. Determination of corrections for inoculum, mold-bran, malt, wheat bran, and steep water

The inoculum for the fermentation was made of a solution of malt extract. When yeast is grown in this medium, alcohol is produced; thus it is necessary to apply a correction for the inoculum. With acid-hydrolyzed mashes only a correction for the beer wort was necessary. This varied with the batch of beer wort prepared; the values obtained ranging from 2.05 to 2.30 grams ethanol per 100 ml. of beer wort. For this reason a sample of each beer wort preparation was inoculated with yeast and this correction value determined.

It was found that in the presence of mold-bran or malt a slight increase in the correction value was obtained. By keeping the amount of mold-bran constant and varying the volume of beer fermented, it was found that 100 ml. of beer wort yielded 3.00 grams of ethanol. The amount of alcohol produced from mold-bran is independent of the presence of beer wort. From direct fermentation 1 gram of mold-bran produced 0.032 gram of ethanol. Similarly 1 gram barley malt, 1 gram wheat bran, and 1 gram of heavy steep water produced respectively 0.334, 0.02, and 0.02 gram ethanol.

4. Calculation of ethanol yield

In a typical experiment the mash contained the following: 30 grams of corn starch with a glucose equivalent of 95.0 per cent glucose; 3.8 grams of mold-bran; and 20 ml. of beer wort. The entire fermented mash was distilled, and the first 100 ml. of distillate were collected. The specific gravity (25°/25°) of the distillate was 0.9780 which corresponded to 13.34 grams of ethanol per 100 ml. of distillate. The ethanol corrections were made as follows:

20 ml. of inoculum	= 0.60 gram ethanol
3.8 grams mold-bran (3.8 x 0.03)	= 0.11 gram ethanol
Total	= 0.71 gram ethanol

13.34 - 0.71 = 12.63 grams ethanol from corn starch. From the equation

$C_6H_{12}O_6 \longrightarrow 2CO_2 + 2C_2H_5OH$, 180 grams of glucose should yield 92 grams of ethanol. Thus thirty grams should yield (30)(0.950)(92/180) = 14.57. The ethanol yield is therefore 12.63/14.57 = 86.7 per cent theoretical.

In the case of acid hydrolyzed mashes the yield is calculated in the same manner except that there is no correction for mold-bran.

V. EXPERIMENTAL RESULTS

A. Acid Saccharification of Corn Meal

1. Effect of different acids at various concentrations on ethanol yields obtained from acid-saccharified corn mashes.

Since all previous attempts to use acids for saccharification of starchy substrates for fermentation had been reported in the literature as failures, very little information was available on the conditions necessary for acid hydrolysis. The first experiment was carried out in the following manner with the object of determining the effectiveness of different mineral acids at various concentrations.

Thirty grams of corn meal were weighed into 500-ml. Erlenmeyer flasks, and 200 ml. of acid solutions of various concentrations as given in Table I were added to each flask. The starch was gelatinized by heating the mixtures over a burner until thick, being very careful not to char any of the material. This preliminary gelatinization was found necessary, for if the samples were autoclaved without this treatment they became lumpy, especially at the lower acid concentrations; as a result poor alcohol yields would be obtained, because all the starch would not be hydrolyzed under these conditions. The mashes were then autoclaved the required time. The hydrolyzates were adjusted to pH 5 by the addition of sodium carbonate. The pH of each sample was determined by using a Cameron meter. The cell containing the glass electrode was designed so that the samples could be washed back into the reaction flasks after the measurements were made. This was a distinct advantage over the quinhydrone electrode system used previously, since it eliminated the necessity of running a control sample for each acid concentration. Moreover, the actual pH of every sample was known. The mashes were then inoculated with a 24 hour yeast culture in 10 per cent beer wort and fermented 72 hours. At the end of this time the mash was distilled, and the alcohol in the distillate determined as described in the section on methods. The data are collected in Table I.

TABLE I

Ethanol Yields From Corn Meal Saccharified With Phosphoric, Sulfuric and Hydrochloric Acids of Various Concentrations.

Time of hydrolysis (hrs.)	3¼	3¼	3½
Steam pressure (lbs./ sq. in.)	10	12	12
Acid used	H_3PO_4	H_2SO_4	HCl
Conc. of acid, normality	EtOH (yield) per cent	EtOH (yield) per cent	EtOH (yield) per cent
0.05	0.6	9.6	----
.10	4.6	63.9	80.2
.20	12.6	76.6	80.2
.30	28.5	77.0	76.6
.50	42.1	72.0	72.8
.60	54.0	71.0	----
.80	64.6	66.2	64.8
1.00	74.1	64.7	9.5
1.50	75.2	26.3	3.2
2.00	73.0	----	----
3.00	63.8	----	----

Control sample, 10 per cent malt, 75.3 per cent EtOH.

Theoretical yield, 10.85 grams ethanol.

These results were very encouraging since, under the most favorable conditions, the yields obtained were better than those from malt by the conventional process.

With phosphoric acid a gradual increase in the ethanol yield was observed with increasing concentrations of acid reaching 75.2 per cent of theoretical with 1.50 normal acid. Up to this point the color of the hydrolyzates ranged from a water white to a light yellow with very little evidence of any charring. At higher acid concentrations charring became apparent, the mashes were dark brown, and the alcohol yields decreased rapidly. This decrease was to be expected since, at this point, some of the sugar was destroyed by caramelization. From the data of Table I it is evident that phosphoric acid is not suitable for acid saccharification, even though yields of ethanol are equal to those obtained from malt, because of the high concentration of acid necessary for the conversion.

Sulfuric acid produced the maximum yield of ethanol, 77.0 per cent theoretical, at a concentration of 0.30 normal. The hydrolyzates at this acid concentration were light yellow. As the acid concentration increased above this value the mashes became darker, and the alcohol yields decreased accordingly as more of the sugar was destroyed by caramelization. Either 0.20 or .30 normal sulfuric acid, under the conditions of this experiment, produced nearly 2 per cent greater ethanol yields than did malt.

Hydrochloric acid produced a maximum ethanol yield of 80.2 per cent theoretical at a concentration of either 0.10 or .20 normal. This was nearly a 5 per cent greater yield than was obtained by the use of malt, and therefore acid hydrolysis was considered to be worthy of further investigation.

At higher acid concentrations charring began to occur, and the ethanol yields decreased in proportion to the amount of charring observed. Undoubtedly, the acid concentration necessary for optimum yields was a factor of temperature, time, the acid used, and probably of the particular sample of corn meal used.

2. Studies on the effect of time and pressure on ethanol yields from acid-hydrolyzed corn.

From the results presented in Table I it was quite evident that hydrochloric acid was effective in the hydrolysis of the starch present in corn meal. However, the acid concentrations necessary for good hydrolysis were rather high. It was thought that it might be possible to use more dilute acids if the temperature was increased by using a higher steam pressure. For this reason the following investigation was undertaken: Six hundred grams of finely ground corn were introduced into a 6-liter round bottom flask with 4,000 ml. of 0.05 normal hydrochloric acid. The flask was fitted with a glass tube leading nearly to the bottom. This tube was designed so that it could be attached to another tube, which projected through the side of the autoclave. The flask was placed in an autoclave which had a stirrer attached to the lid so that the contents of the flask could be constantly agitated. The tube projecting from the side of the autoclave was attached to a Leibig condensor by means of heavy rubber tubing. When the clamp on the tubing was released the pressure inside the autoclave would force the sample out. A diagram of this apparatus was given by Bryner, Christensen, and Fulmer (1936).

The starch was gelatinized by heating with a burner, placed in the apparatus described above and heated at the desired steam pressure. At one hour intervals about 700 ml. of mash were drawn off and cooled down to room temperature. Ten minutes were required to draw off these samples, so the process was started five minutes before the end of the hour and completed five minutes after. After cooling, 200 ml. aliquots were placed in 500-ml. Erlenmeyer flasks, the pH adjusted to 5 with sodium carbonate, and inoculated with 20 ml. of active yeast culture. The mashes were allowed to ferment 72 hours before distilling off the alcohol. After the run was complete, the mash remaining in the large flask was allowed to cool. The volume remaining was measured. Thus by knowing the total volume of the solution the amount of corn meal in each fermentation flask was known, and the ethanol yields could be calculated. Table II presents the results of these experiments.

TABLE II

Effect of Time and Pressure on Ethanol Yields From Corn Hydrolyzed with 0.05 N. Hydrochloric Acid

Time in hours	Per cent EtOH at the indicated pressures 10 lbs.	20 lbs.	30 lbs.
1	----	11.0	9.5
2	18.7	52.5	65.0
3	26.6	66.3	74.8
4	34.1	67.3	73.2
5	41.5	68.6	73.8
6	48.2	68.5	----

Malt control (10 per cent wt. of corn), 75.3 per cent of theoretical.

Theoretical yield 10.85 grams ethanol from 30 grams corn.

At a steam pressure of 10 pounds the ethanol yields increased quite regularly with the time of heating. However, even at the end of 6 hours only a 48.2 per cent yield of ethanol was obtained. Further study at this pressure was abandoned as it would not be economically sound to use longer periods of heating for the conversion.

At 20 pounds steam pressure the yield of ethanol increased with the time of heating, attaining a value of 68.5 per cent at 5 hours. Slight charring was apparent at this point, and a longer period of heating did not improve the alcohol yield. In all probability, the amount of increased conversion by longer heating was just compensated for by more rapid destruction of the sugars produced by caramelization. This would account for the fact that the same ethanol yield was observed at either 5 or 6 hours.

At 30 pounds steam pressure the ethanol yield increased with time attaining a value of 74.8 per cent at 3 hours. Increased periods of heating did not increase the ethanol yield, but actually caused a slight decrease in yield. A reason for this observation has been postulated above.

The above data illustrate that the treatment of corn meal with 0.05 normal hydrochloric acid at a steam pressure of 30 pounds per square inch for 3 hours produces approximately the same amount of fermentable sugars as the use of 10 per cent malt by the conventional process.

3. Effect of addition of mold-bran to acid-hydrolyzed corn meal.

The addition of mold-bran to acid-hydrolyzed mashes was reported by Schoene, Fulmer and Underkofler (1940) to result in very high alcohol yields. The acid concentration used by these investigators was 0.10 normal. At this concentration, as shown by the data of Table I of this thesis, the acid alone is quite effective. Therefore, it was thought desirable to investigate the result of addition of mold-bran to mashes hydrolyzed with more dilute acids. In this case, the amount of fermentable sugars present before the addition of mold-bran would be relatively small. The experiment was carried out in the following manner: Thirty grams of corn meal were added to each 500-ml. Erlenmeyer flask containing 200 ml. of 0.05 normal hydrochloric acid. The starch was gelatinized over a burner, and the flasks and contents autoclaved for 4 hours at 12 pounds steam pressure. After cooling, the pH was adjusted in the usual manner, the required amount of mold-bran added, and 20 ml. of active yeast culture added to each flask. After incubation for 72 hours the contents were distilled, and the distillate analyzed for alcohol. These results are tabulated in Table III.

TABLE III

Effect on Ethanol Yield of the Addition of Mold-Bran to Acid-Hydrolyzed Corn Mash.

Proportion of mold-bran added, per cent of corn	EtOH yield, per cent	Increase in yield, per cent
0	30.2	---
2	79.5	163
4	91.5	203
8	91.5	203
10	91.4	203

Theoretical yield 10.41 grams alcohol.

These data indicated that the ethanol yields of mashes partially saccharified by dilute acids are greatly increased on the addition of mold-bran. The amount of mold-bran required is quite small, 4 per cent giving an optimum yield of 91.5 per cent theoretical. The addition of larger quantities of mold-bran did not increase the alcohol yield.

4. Effect on ethanol yield of the addition of ammonium chloride and sodium chloride to corn saccharified by amylase.

Fulmer, Sherwood and Nelson (1924) reported that although small amounts of ammonium salts actually stimulated yeast growth, larger quantities were detrimental. It was thought that the ammonium salts produced by neutralization of the acid used for hydrolysis might be retarding yeast growth. The following experiment was run in order to test out this theory: Thirty grams of corn meal were weighed into each 500-ml. Erlenmeyer flask, and 200 ml. of distilled water added. The mashes were gelatinized, autoclaved at 20 pounds steam pressure for 1 hour, cooled and the pH determined. The pH was found to be in the desired range so no neutralization was necessary. Three grams of malt were added to each, and then the flasks were placed in a malting bath where they were held at a temperature of 55° C. for 1 hour, being shaken occasionally during the process. After cooling to 30° C. the required amount of salts were added to each along with 20 ml. of active yeast culture. The mashes were allowed to ferment 72 hours, and the alcohol determined in the usual manner. The results of this investigation are shown in Table IV.

TABLE IV

Effect on Ethanol Yield of the Addition of Ammonium Chloride and Sodium Chloride to Corn Saccharified by Malt.

NH_4Cl added to 200 ml. of mash, grams	NaCl added to 200 ml. of mash, grams	Yield EtOH, per cent
0	0	73.0
0	0.15	72.8
0	0.30	73.3
0.15	0	71.9
0.30	0	71.3

Theoretical yield 11.35 grams ethanol.

Table IV indicates that small amounts of sodium chloride do not influence the ethanol yield, and therefore are not toxic to yeast. The addition of small amounts of ammonium chloride apparently do affect yeast and result in slightly lower alcohol yields. The acid hydrolyzates might contain even higher concentrations of salts than those used above if high concentrations of acid are used, which are known to be necessary for saccharification, at low pressures. For this reason the following series were run exactly like the one in Table IV except that 1.5 grams of mold-bran were used for saccharification in place of malt. These data are presented in Table V.

TABLE V

Effect on Ethanol Yield of the Addition of Ammonium Chloride and Sodium Chloride to Corn Saccharified by Mold-Bran.

NH_4Cl added to 200 ml. of mash, grams	NaCl added to 200 ml. of mash, grams	EtOH yield, per cent
0	0	80.1
0	0.5	78.2
0	1.0	78.2
0	2.0	77.7
0	2.5	78.5
0	3.0	77.5
0	3.5	80.1
0.5	0	78.2
1.0	0	75.5
1.5	0	75.8
2.0	0	75.6
2.5	0	75.6
3.0	0	75.0
3.5	0	73.3

Theoretical yield 11.35 grams ethanol.

It is significant that the addition of sodium chloride did not appreciably alter the ethanol yield obtained even when present in concentrations as high as 16.5 grams per liter. The addition of ammonium chloride caused a decided lowering in the ethanol yield approaching 6 per cent when present in concentrations in excess of 5 grams per liter. This experiment indicates that lower yields of ethanol would be obtained if acid hydrolyzates were neutralized with ammonium hydroxide, and for this reason sodium carbonate was used for neutralization in all subsequent work.

B. Studies on the Saccharification of Corn Starch by the Use of Dilute Mineral Acids

The preliminary work on corn meal was so encouraging that it was decided to attempt the hydrolysis of pure starch by the use of dilute mineral acids. Starch was chosen for the substrate to continue this investigation for two reasons: (1) it is a more uniform product than corn meal, and (2) it would be possible to use corn starch industrially since it would cost very little more per pound of fermentable sugars produced than corn meal.

1. Preliminary studies on nutrient requirements of acid-hydrolyzed starch.

Since starch does not contain sufficient nutrients for yeast growth even after it is hydrolyzed, an attempt was made to discover just what is necessary for good alcohol yields. Various materials which were known to be necessary for yeast growth were added to the hydrolyzates. The hydrolyzates were prepared in the following manner: Thirty grams of corn starch were mixed with 200 ml. of 0.15 normal sulfuric acid in 500-ml. Erlenmeyer flasks, and autoclaved at a steam pressure of 12 pounds per square inch for 3 1/2 hours. The materials indicated in Table VI were then added, the pH adjusted to 5, and each flask inoculated with 20 ml. of active yeast culture. The data are tabulated in Table VI.

TABLE VI

Effect of Addition of Various Materials for Nutrients on Ethanol Yields Obtained from Acid-Saccharified Starch.

Mash No.	Grams of the following materials added to 200 ml. of mash					EtOH yield, per cent
	NH_4NO_3	KH_2PO_4	$MgSO_4$	$Ca(NO_3)_2$	corn-gluten meal	
1	0.24	----	----	----	----	33.0
2	----	0.30	----	----	----	45.7
3	----	----	0.08	----	----	31.3
4	----	----	----	0.01	----	3.1
5	.24	.30	.08	.01	----	18.4
6	.24	.30	.08	.01	2.00	56.3
7	----	----	----	----	5.00	87.3
Control sample (0.70 gram yeast extract)						84.0

Theoretical yield of ethanol 14.20 grams for 30 grams starch.

On the basis of the results of Table VI it was evident that corn-gluten meal alone not only supplied all the necessary nutrients, but actually gave better yields than any other combination tried. Calcium nitrate apparently had a detrimental influence on yeast growth. Since corn-gluten meal is a by-product of the starch industry and is available in any desired quantities at an extremely low price, no further investigation was taken in this direction at this point.

2. Effect on ethanol yield of the addition of various amounts of corn-gluten to acid-hydrolyzed starch mashes.

Although the preceding work proved corn-gluten meal to be a very effective nutrient, the problem still remained to ascertain the amount of corn-gluten meal necessary to obtain maximum yields from the starch hydrolyzates. Moreover, it was desirable to know if any appreciable difference would result if the gluten was added before or after autoclaving the mashes. Mashes were prepared by adding 15 grams of starch to 200 ml. of 0.15 normal hydrochloric acid in 500-ml. Erlenmeyer flasks. To one half of the mashes the required amount of gluten meal was added at this point. The mashes were then gelatinized, autoclaved for 4 hours at 12 pounds steam pressure and cooled. The required amount of gluten meal was then added to those not having had it added before autoclaving. The pH of each mash was adjusted to 5, and they were inoculated with 20 ml. of active yeast culture. The data from this experiment are tabulated in Table VII.

TABLE VII

Effect on Ethanol Yield of the Addition of Various Amounts of Corn Gluten to Acid-Hydrolyzed Starch Mashes.

Gluten added to 200 ml. of mash before autoclaving, grams	Gluten added to 200 ml. of mash after autoclaving, grams	EtOH yield, per cent
0	0	35.4
1	0	74.1
2	0	89.3
3	0	90.5
0	1	82.7
0	2	90.5
0	3	90.5

Theoretical yield 7.10 grams ethanol.

The results from Table VII indicate that 2 grams of gluten meal provided sufficient nutrients if it was added after autoclaving the starch, and between 2 and 3 grams of gluten meal were necessary if it was added before autoclaving. The final yield of ethanol was the same in either case, independent of whether or not the gluten meal was autoclaved, if sufficient gluten meal was added.

It was thought that the amount of gluten meal necessary might be dependent on the total concentration of fermentable sugars present. In order to test this fact the following experiment was undertaken: Thirty-two grams of starch were weighed into 500-ml. Erlenmeyer flasks containing 200 ml. of 0.10 normal sulfuric acid. To one series the gluten meal was added at this point before the starch was gelatinized. To the other the gluten meal was added after autoclaving. After being gelatinized, both series were autoclaved at the same time for 4 hours at 20 pounds steam pressure. This would eliminate any differences which might result if the steam pressure varied slightly on two different runs. After adjusting the pH to 5, the flasks were inoculated with 20 ml. of active yeast, allowed to ferment 90 hours, and then distilled. The ethanol was determined in the usual manner. These data are collected in Table VIII.

TABLE VIII

Effect on Ethanol Yield of the Addition of Various Amounts of Corn-Gluten to Concentrated Starch Mashes Hydrolyzed by Acid.

Gluten added to 200 ml. of mash before autoclaving, grams	Gluten added to 200 ml. of mash after autoclaving, grams	EtOH yield, per cent
1	0	61.3
2	0	79.4
3	0	79.4
4	0	80.7
5	0	80.3
6	0	81.5
0	1	71.3
0	2	81.0
0	3	81.0
0	4	81.0
0	5	81.5
0	6	81.0

Theoretical yield 15.54 grams ethanol.

The fact that the addition of gluten meal before or after autoclaving the mashes makes little difference in the final ethanol yield was definitely confirmed in Table VIII. Moreover, the concentration of the mash used had no effect on the amount of gluten meal necessary since 2 grams of gluten meal proved to be sufficient even when the mash concentration was doubled over that used in Table VII. The slight differences in yield of ethanol obtained from mashes in which the gluten meal was not autoclaved over those in which the gluten meal was autoclaved were not significant, and were offset by the more rapid rate of fermentation of the latter. The above facts excluded the possibility that the low yields from acid-hydrolyzed grains reported by Severson (1937) were due to toxic decomposition products from the gluten present in the grain.

3. Effect on ethanol yield of the addition of ammonium chloride to acid-hydrolyzed starch mashes.

Ammonium chloride caused a decided decrease in ethanol yield when added to corn mashes. Thus, it was desirable to study the effect of the addition of this salt to acid-hydrolyzed starch mashes. The following experiment was carried out to test this point: Fifteen grams of starch were weighed into 500-ml. Erlenmeyer flasks containing 200 ml. of 0.15 normal hydrochloric acid. The mashes were autoclaved for 4 hours at 12 pounds steam pressure. The pH was then adjusted with sodium carbonate after the addition of 2.5 grams corn-gluten meal and the required amount of ammonium chloride. The mashes were inoculated, and the alcohol determined in the usual manner after 72 hours incubation. These data are collected in Table IX.

TABLE IX

Effect on Ethanol Yield of the Addition of Ammonium Chloride to Acid-Hydrolyzed Starch Mashes.

NH_4Cl added to 200 ml. of mash, grams	EtOH yield, per cent
0	83.8
1.0	83.5
1.5	83.5
2.5	77.2
3.0	77.4
4.0	77.4

Theoretical yield 7.10 grams ethanol.

The results in Table IX indicated that even in the presence of sodium chloride, ammonium chloride had a slight depressing effect on ethanol yields obtained from acid-hydrolyzed starch. In concentrations up to 7.5 grams per liter no appreciable lowering in the ethanol yield was observed, but at higher concentrations nearly an 8 per cent lower yield was obtained. The fact that a much larger concentration of ammonium salts was necessary to reduce ethanol yields obtained from starch than was observed in the case of corn meal might be explained in the following manner. The amount of nitrogen in the starch mashes even after the addition of the gluten meal was probably a great deal less than the concentration found in corn meal, and therefore, more ammonium salts must be added before the critical nitrogen content of the medium is reached.

4. Effect on the ethanol yield of the neutralization of acid-hydrolyzed starch mashes with sodium carbonate and ammonium hydroxide.

Since the above experiment was not quite comparable to experimental conditions, it was desirable to continue the investigation of the effect of ammonium salts. Although sulfuric acid is not used industrially in the hydrolysis of starch for the production of dextrose, an attempt was to be made using it in this research problem, because it is considerably less expensive than hydrochloric acid of the same normality.

It was for the above reasons that the following experiment was undertaken studying the effect of ammonium sulfate on the ethanol yields: Thirty-two grams of starch were weighed into 500-ml. Erlenmeyer flasks containing 200 ml. of sulfuric acid of various concentrations. The mashes were autoclaved at 20 pounds steam pressure for 4 hours. Then after the addition of 2.5 grams of corn-gluten meal to each, the pH of one series was adjusted with concentrated ammonium hydroxide, and the other with sodium carbonate. After incubating for 96 hours the mashes were distilled, and the alcohol determined in the usual way. These data are tabulated in Table X.

TABLE X

Effect on Ethanol Yield of the Use of Sodium Carbonate and Ammonium Hydroxide for Neutralization of Acid-Hydrolyzed Starch Mashes.

Conc. of sulfuric acid, normality	Neutralized with Na_2CO_3, EtOH yield, per cent	Neutralized with NH_4OH, EtOH yield, per cent
0.02	44.3	36.2
.05	85.4	46.2
.10	85.2	55.3
.15	78.5	63.3

Theoretical yield 15.54 grams ethanol.

The data of Table X illustrate very effectively the detrimental influence of the use of ammonium hydroxide for adjusting the pH of acid hydrolyzed mashes. When ammonium hydroxide was used instead of sodium carbonate for adjusting the pH of the hydrolyzate obtained by using 0.05 normal sulfuric acid on starch mashes, the decrease in ethanol yield was nearly 46 per cent. These extremely low yields were questioned at first on the basis of results reported in Table IX. However, similar values were obtained on repeating this experiment. The yields obtained on different days by the use of ammonium hydroxide were of the same magnitude but could not be considered good checks even though good checks were obtained on the series using sodium carbonate run at the same time. It was found accidently that the pH of the mashes neutralized with ammonium hydroxide changed on standing. It might be possible that these mashes became too acid after a day or two for good yeast growth. Since this effect would be eliminated by the use of sodium carbonate, further investigation of this phenomenon was omitted because of lack of sufficient time.

5. Effect on the ethanol yield of the concentration of the acid used to hydrolyze starch mashes.

The acid concentration employed for hydrolysis was found to have a decided influence on the ethanol yields obtained from corn meal. It was suspected that the same factor would play an important part in the hydrolysis of starch. To test this point the following experiment was undertaken: Fifteen grams of starch were weighed into 500-ml. Erlenmeyer flasks containing 200 ml. of the hydrochloric acid of the concentrations given in Table XI. The mashes were autoclaved at a steam pressure of 12 pounds for 4 hours, 3 grams of corn-gluten meal added, and the pH adjusted with sodium carbonate. Then they were fermented, and the alcohol determined in the usual manner. These data are collected in Table XI.

TABLE XI

Effect on Ethanol Yield of the Concentration of the Acid Used to Hydrolyze Starch Mashes.

Conc. of HCl, normality	EtOH yield, per cent
0.05	83.5
0.10	83.5
0.15	82.2
0.20	80.9
0.30	76.6
0.40	73.2
0.50	73.2

Theoretical yield 7.10 grams ethanol.

From Table XI it is apparent that acid concentrations above 0.20 normal are not desirable as the yield decreased with increasing acid concentration. That this was due to the caramelization of the sugar was evident since the mashes became increasingly dark as the acid concentration increased. The maximum yield of 83.5 per cent at acid concentration of either 0.05 or 0.10 normal was very encouraging as it was equal to that obtained by Underkofler, Goering and Buckaloo (1941) with some of their best mold preparation.

6. <u>Effect of time of hydrolysis on the amount of reducing sugars produced, and ethanol yields obtained on subsequent fermentation of these hydrolyzates.</u>

Severson (1937) reported that the maximum alcohol yields were not obtained from acid hydrolyzates of cereals which showed the maximum amount of reducing sugars. It was thought desirable to test this fact with regard to acid hydrolyzates from starch. It was also necessary to find the optimum time of hydrolysis. To determine this information the following experiment was set up: Three hundred grams of starch were added to a 6-liter round bottom flask containing 4,000 ml. of 0.10 normal hydrochloric acid. This material was gelatinized and placed in the autoclave described previously, from which samples could be taken at the required time. The steam pressure was run up to 20 pounds, and 700 ml. samples were taken out at 1 hour intervals. After cooling to room temperature three 200 ml. aliquots were fermented in the usual manner after the addition of 2.5 grams of corn-gluten meal, and a sugar analysis was run on the remaining material according to the modified method of Shaffer and Somogyi described by Guymon (1939). The theoretical amount of ethanol was calculated on the basis of all the reducing sugar being dextrose. From this value and the actual amount of ethanol obtained by fermentation, the per cent conversion of reducing sugars present to alcohol, and the per cent conversion of starch to sugar could be calculated. These results are tabulated in Table XII.

TABLE XII

Effect of Time of Hydrolysis on the Amount of Reducing Sugars Produced, and Ethanol Yields Obtained On Subsequent Fermentation of These Hydrolyzates.

Time, hours	EtOH produced by ferment-ation, grams	Theoretical yield of EtOH, per cent	Theoretical EtOH calc. from sugar analysis, grams	Conversion of reducing sugar to EtOH, per cent	Conversion of starch to sugar, per cent
1	5.42	76.4	7.05	77.0	99.4
2	5.66	79.7	7.05	80.3	99.4
3	5.84	82.2	6.97	83.8	98.2
4	5.69	80.1	6.87	82.8	96.7
5	5.45	76.6	6.87	79.4	96.7

Theoretical yield of ethanol 14.10 grams.

Two interesting observations were indicated in this work. The first was that although the maximum concentration of reducing sugars, 99.4 per cent, was produced after autoclaving for only 1 hour, the maximum yield of ethanol was obtained from the hydrolyzate which had been autoclaved for 3 hours. This observation confirms the findings of Severson (1937). The second observation was that the maximum period of heating was around three hours. The difficulty of obtaining representative samples from the autoclave by this method was recognized, and the values of ethanol obtained at the different time intervals were not too significant. However, this is not true with regard to the comparison of the ethanol obtained by fermentation and that calculated from sugar analysis, since the solution used for sugar analysis was removed from the same material that was fermented.

7. Effect of time of fermentation on ethanol yield.

Before an extensive investigation could be undertaken studying the acid-hydrolysis of starch mashes, an experiment had to be made to determine how long the mash must be allowed to ferment before distilling in order to obtain the maximum yield. From usual observations it was thought that a 7.5 per cent starch mash would be completely fermented in 72 hours. A 16 per cent mash seemed to require around 90 hours. A series of mashes containing 16 per cent starch and one containing 7.5 per cent starch were made up in order to determine this value.

They were hydrolyzed at the same time by heating for 4 hours at a steam pressure of 20 pounds with 0.05 normal sulfuric acid. The mashes were prepared in the usual way using 2.5 grams of corn-gluten meal for nutrient. Every 24 hours after inoculation two duplicate samples of each series were distilled, and the ethanol determined. The results of these experiments are tabulated in Table XIII and XIV.

TABLE XIII

Effect of Time of Fermentation on Ethanol Yields Obtained From 7.5 Per Cent Acid-Hydrolyzed Starch Mashes.

Time of fermentation, hours	EtOH yield, per cent
24	60.5
48	88.4
72	89.3
96	86.3
120	85.5

Theoretical yield 7.27 grams ethanol.

TABLE XIV

Effect of Time of Fermentation on Ethanol Yields Obtained From 16 Per Cent Acid-Hydrolyzed Starch Mashes.

Time of fermentation, hours	EtOH yield, per cent
24	32.4
48	70.9
72	76.3
96	80.2
120	80.2

Theoretical yield 15.54 grams ethanol.

The data from Tables XIII and XIV indicated that 7.5 per cent mashes should be allowed 3 days for fermentation, and 16 per cent mashes should be fermented 4 days before distilling. These periods were used for all the following fermentations.

8. Effect of acid concentration and steam pressure on the ethanol yields obtained from acid-hydrolyzed starch.

As a result of the data tabulated in Table XII 4 hours was selected as the time interval to be used for all subsequent work. The object of this investigation was to lower the acid concentration as much as possible, and select the steam pressure which would give maximum yields of ethanol. Periods of heating longer than 4 hours were not desirable since this would result in tying up the cooking equipment too long to be used industrially.

In this series of experiments the required amount of starch was weighed into 500-ml. Erlenmeyer flasks containing 200 ml. of the acid indicated at the specified concentrations. The flasks were placed in the autoclave, and 10 minutes were allowed for the flasks to come up to the temperature of the autoclave before beginning the timing. After autoclaving 4 hours at the required steam pressure, the samples were cooled immediately by passing compressed air rapidly through the autoclave. As soon as the mashes had cooled to room temperature 2.5 grams of corn-gluten meal were added, and the mash prepared in the usual way. The time of fermentation used for the different mash concentrations was that found to be optimum preceding experiment. In fermentation work there are usually slight variations in results obtained one day as compared with duplicate experiments carried out at some other time. These variations are probably due to differences in yeast inoculum, and even though every precaution is taken to keep a vigorous and uniform yeast culture, such slight variations occur. Since this next experiment required a time of several weeks, the data from a series of fermentations conducted at the same time using the same inoculum will be given in each separate table. The data are collected in Tables XV, XVI, XVII, XVIII, and XIX.

TABLE XV

Effect of Various Concentrations of Hydrochloric and Sulfuric Acid on the Ethanol Yields Obtained From Starch Mashes Hydrolyzed for 4 Hours at a Steam Pressure of 5 Pounds.

Acid used	Conc. of acid, normality	Conc. of mash, per cent	EtOH yield, per cent
hydrochloric	0.05	7.5	79.7
"	.10	7.5	85.0
"	.15	7.5	84.4
"	.20	7.5	84.7
sulfuric	.05	7.5	52.2
"	.10	7.5	74.9
"	.15	7.5	82.8
"	.20	7.5	84.4

Theoretical yield 7.10 grams ethanol.

TABLE XVI

Effect of Various Concentrations of Hydrochloric and Sulfuric Acid on the Ethanol Yields Obtained From Dilute and Concentrated Starch Mashes Hydrolyzed for 4 Hours at a Steam Pressure of 10 Pounds.

Acid used	Conc. of acid, normality	Conc. of mash, per cent	EtOH yield, per cent
hydrochloric	0.02	7.5	73.5
"	.05	7.5	78.6
"	.10	7.5	83.3
"	.15	7.5	78.6
"	.02	16.0	67.0
"	.05	16.0	74.8
"	.10	16.0	80.4
"	.15	16.0	73.4
sulfuric	.02	7.5	46.9
"	.05	7.5	72.2
"	.10	7.5	82.0
"	.15	7.5	82.0
"	.02	16.0	39.6
"	.05	16.0	71.2
"	.10	16.0	73.0
"	.15	16.0	77.5

Theoretical yield 7.10 grams ethanol from 7.5 per cent mash, and 15.14 grams ethanol from 16 per cent mash.

TABLE XVII

Effect of Various Concentrations of Hydrochloric and Sulfuric Acid on the Ethanol Yields Obtained From Dilute and Concentrated Starch Mashes Hydrolyzed for 4 Hours at a Steam Pressure of 15 Pounds.

Acid used	Conc. of acid, normality	Conc. of mash, per cent	EtOH yield, per cent
hydrochloric	0.02	7.5	86.2
"	.05	7.5	88.2
"	.10	7.5	80.7
"	.15	7.5	75.8
"	.02	16.0	78.8
"	.05	16.0	79.7
"	.10	16.0	79.7
"	.15	16.0	78.5
sulfuric	.02	7.5	65.7
"	.05	7.5	84.0
"	.10	7.5	83.4
"	.15	7.5	80.0
"	.02	16.0	64.1
"	.05	16.0	76.1
"	.10	16.0	81.3
"	.15	16.0	79.3

Theoretical yield 7.10 grams ethanol from 7.5 per cent mash, and 15.14 grams from 16 per cent mash.

TABLE XVIII

Effect of Various Concentrations of Hydrochloric and Sulfuric Acid on the Ethanol Yields Obtained From Dilute and Concentrated Starch Mashes Hydrolyzed for 4 Hours at a Steam Pressure of 20 Pounds.

Acid used	Conc. of acid, normality	Conc. of mash, per cent	EtOH yield, per cent
hydrochloric	0.02	7.5	95.0
"	.05	7.5	96.5
"	.10	7.5	91.8
"	.15	7.5	90.4
"	.02	16.0	85.7
"	.05	16.0	86.2
"	.10	16.0	82.5
"	.15	16.0	75.7
sulfuric	.02	7.5	95.6
"	.05	7.5	97.5
"	.10	7.5	95.3
"	.15	7.5	93.1
"	.02	16.0	76.2
"	.05	16.0	86.2
"	.10	16.0	86.2
"	.15	16.0	84.2

Theoretical yield 7.10 grams ethanol from 7.5 per cent mash, and 15.14 grams from 16 per cent mash.

TABLE XIX

Effect of Various Concentrations of Hydrochloric and Sulfuric Acid on the Ethanol Yields Obtained From Dilute and Concentrated Starch Mashes Hydrolyzed for 4 Hours at a Steam Pressure of 25 Pounds.

Acid used	Conc. of acid, normality	Conc. of mash, per cent	EtOH yield per cent
hydrochloric	0.02	7.5	98.2
"	.05	7.5	96.5
"	.10	7.5	90.5
"	.15	7.5	85.2
"	.02	16.0	82.7
"	.05	16.0	79.7
"	.10	16.0	63.8
"	.15	16.0	59.6
sulfuric	.02	7.5	100.0
"	.05	7.5	97.4
"	.10	7.5	97.4
"	.15	7.5	93.2
"	.02	16.0	76.9
"	.05	16.0	86.3
"	.10	16.0	66.4
"	.15	16.0	49.9

Theoretical yield 7.10 grams ethanol from 7.5 per cent mash, and 15.14 grams from 16 per cent mash.

The above data illustrate the fact that the alcohol yields from concentrated mashes are quite inferior to those obtained from more dilute mashes. This may be due to the fact that when the sugar concentration reaches a certain point, the acid acts more readily on the sugar than it does on the starch. Hydrochloric acid is more effective than sulfuric acid at the same concentration for the hydrolysis of starch, but at the same time it causes charring at lower acid concentrations than does sulfuric acid. This accounts for the fact that at a certain steam pressure and acid concentration sulfuric acid was found more effective than hydrochloric acid. In general the ethanol yields were found to increase with increasing steam pressure if the acid concentrations were not over 0.05 normal. At higher acid concentrations a maximum was obtained, and then the alcohol yields began to fall off as the sugar was destroyed by caramelization.

The alcohol yield attained 100 per cent of theoretical when a 7.5 per cent mash was treated with 0.02 normal sulfuric acid at 25 pounds steam pressure for 4 hours. The best yield obtained from a 16 per cent mash was 86.3 per cent treated at a steam pressure of 25 pounds for 4 hours with 0.05 normal sulfuric acid.

Since in industry it is desirable to use more concentrated mashes, because it takes less equipment to yield the same amount of ethanol, the possibility of obtaining a higher ethanol yield from thick mashes was investigated. It was thought that the use of higher temperatures for shorter intervals with more dilute acid might yield the desired results. For that reason the following investigation was undertaken in the same manner as the preceding experiments on 16 per cent mashes except that the high pressure autoclave was used.

This autoclave did not have any provision for rapid cooling by the introduction of compressed air so it was necessary to set an arbitrary time value during which the autoclave was allowed to come down slowly to atmospheric pressure in order to prevent the samples from boiling over. The values used were 1/2 hour for 30 pounds, 3/4 hour for 40 pounds and 1 hour for 50 pounds steam pressure. The samples were introduced into the autoclave, the steam pressure raised as rapidly as possible, usually requiring about 5 minutes, up to the desired pressure. When this pressure was reached, they were allowed to remain the indicated time, and then the autoclave was turned off, and allowed to cool down in the manner specified above. The autoclave would only hold 8 samples at one time so each run contained all the mashes that were to be autoclaved at one pressure for one time interval. The data are tabulated in Tables XX and XXI.

TABLE XX

Effect of Steam Pressure and Time of Heating on Ethanol Yields Obtained From Starch Mashes Saccharified by Dilute Sulfuric Acid.

Conc. of acid, normality	Time of heating, hours	Steam pressure, lbs./sq. in.	EtOH yield, per cent
0.01	1	30	6.7
.01	2	30	49.0
.01	3	30	66.6
.01	1	40	57.6
.01	2	40	67.0
.01	3	40	74.5
.01	1	50	51.8
.01	2	50	69.0
.01	3	50	79.8
.02	1	30	24.4
.02	2	30	75.0
.02	3	30	76.0
.02	1	40	72.0
.02	2	40	74.4
.02	3	40	81.3
.02	1	50	81.3
.02	2	50	80.3
.02	3	50	75.0

Theoretical yield 15.54 grams ethanol.

TABLE XXI

Effect of Steam Pressure and Time of Heating on Ethanol Yields Obtained From Starch Mashes Saccharified by Dilute Hydrochloric Acid.

Conc. of acid, normality	Time of heating, hours	Steam pressure, lbs./sq. in.	EtOH yield, per cent
0.01	1	30	19.2
.01	2	30	74.2
.01	3	30	75.1
.01	1	40	70.6
.01	2	40	72.9
.01	3	40	79.8
.01	1	50	76.8
.01	2	50	80.9
.01	3	50	80.1
.02	1	30	48.6
.02	2	30	78.0
.02	3	30	81.6
.02	1	40	79.8
.02	2	40	79.8
.02	3	40	81.1
.02	1	50	83.2
.02	2	50	76.8
.02	3	50	57.1

Theoretical yield 15.54 grams ethanol.

Although the data from Table XX and XXI show that the yields obtained were less than those reported in previous tables, there was still one very interesting observation. An examination of the data will show that as the steam pressure is increased the time of heating must be decreased to obtain the maximum yield. The maximum yield was 83.2 per cent of theoretical when the mash was autoclaved with 0.02 normal hydrochloric acid at 50 pounds pressure for 1 hour. At higher pressures and shorter time intervals even better yields might be obtained. This was not tested experimentally, because it was impossible to raise the pressure in this autoclave above 50 pounds steam pressure. On the basis of these data a flash process might be developed for the saccharification of starch before fermentation. This would result in considerable saving in time and would be of great value should this method be used industrially.

9. Effect of varying the mash concentration on the ethanol yields obtained from acid-hydrolyzed starch mashes.

From the data presented above, it was apparent that the mash concentration was a very important factor in the acid-saccharification of starch mashes. The object of the following experiment was to examine this factor. For commercial reasons it would be desirable to increase the mash concentration as far above 7.5 per cent as possible without causing too great a decrease in the ethanol yield. The mashes were made up as usual except that varying amounts of starch were used. They were hydrolyzed by 0.02 normal sulfuric acid at a steam pressure of 20 pounds for 4 hours. The fermentation was carried out in the usual manner. These data are presented in Table XXII.

TABLE XXII

Effect of Varying the Mash Concentration on the Ethanol Yields Obtained From Acid-Hydrolyzed Starch Mashes.

Starch added, grams	Conc. of mash, per cent	Time of fermentation, hours	EtOH yield, per cent
10	5.0	72	85.4
15	7.5	72	91.0
20	10.0	72	84.9
25	12.5	96	82.5
30	15.0	96	76.8
35	17.5	96	75.9
40	20.0	96	72.1
45	22.5	96	68.5

Theoretical yield 4.85 grams ethanol for each 10 grams starch.

The above results indicate that under the conditions of this experiment a 7.5 per cent mash concentration is optimum for the acid-hydrolysis of starch. Undoubtedly the optimum mash concentration depends on several factors such as time of heating, concentration of acid, and temperature at which the hydrolysis takes place. However, the application of dilute acids is necessary if this process is to be used commercially. Since higher acid concentrations were shown experimentally to cause a decrease in the yields in most cases under similar conditions, their effect on the ethanol yield obtained from various mash concentrations was not investigated. The low yield observed in the case of 5 per cent mashes was thought to be due to loss of alcohol by evaporation before distilling. This particular series fermented very rapidly and probably could have been distilled 24 hours earlier than usual.

10. Effect on the ethanol yields obtained from acid-hydrolyzed starch in the presence of corn bran.

Throughout this investigation it has been quite evident that starch mashes hydrolyzed more completely with acid; at least, they produced more ethanol on the subsequent fermentation of the hydrolyzates than did corn mashes of equal starch content. From the observations reported previously in this thesis, it was apparent that the corn gluten was not causing the decreased yields. The next logical step was to investigate the effect of corn bran on the ethanol yields obtained from acid hydrolyzed starch, since it was thought that this might be the substance responsible for these low yields.

The following experiment was designed to test this theory: Thirty-two grams of starch were weighed into 500-ml. Erlenmeyer flasks containing 2.5 grams of gluten meal and 200 ml. of acid at the concentrations specified in Table XXIII. At the same time a series containing 36 grams of corn meal was made up in a similar manner except that no gluten meal was added. To one series of starch mashes prepared as described above, corn bran was added before autoclaving, and to the other the corn bran was added after autoclaving. All three series of mashes were hydrolyzed by autoclaving at a steam pressure of 20 pounds for 4 hours. They were then inoculated and fermented in the usual manner. These results are collected in Table XXIII.

TABLE XXIII

Effect of the Presence of Corn Bran on the Ethanol Yield Obtained From Acid-Saccharification of Corn and Starch Mashes Using Various Concentrations of Acid at 20 Pounds Steam Pressure for 4 Hours.

Materials added	Conc. of acid, normality	EtOH yield, per cent
36 grams corn meal	0.02	3.9
	.05	18.9
	.10	68.6
32 grams corn starch + 2 grams corn bran added after autoclaving	.02	83.5
	.05	83.5
	.10	88.3
32 grams starch + 2 grams corn bran added before autoclaving	.02	19.2
	.05	81.5
	.10	84.7

Theoretical yield of ethanol from starch 15.54 grams.

Theoretical yield of ethanol from corn meal 13.02 grams.

From the above data it is quite obvious that starch mashes, even with gluten present, hydrolyze more readily than do corn mashes under identical conditions. A noticeable decrease in ethanol yield was observed when the corn bran was added to starch mashes before autoclaving. The extremely low yield obtained with 0.02 normal sulfuric acid when corn bran was present during autoclaving was in all probability due to the fact that some of the acid was absorbed in the corn bran and thus effectively removed from the reaction mixture. At higher acid concentrations the decrease in ethanol yield observed when corn bran was present during autoclaving was believed to be due to the production of some material toxic to yeast. This theory was advanced because the yield observed with 0.10 normal acid, when the bran was added before autoclaving, was 3.6 per cent lower than that obtained when the bran was added afterwards, while with 0.05 normal the yield was only 2.0 per cent lower. If the decreased yields were due to absorption of acid, it would be expected to be greater with 0.05 normal acid than with 0.10 normal acid, since the corn bran could only absorb a definite amount of acid.

Because such a small amount of corn bran caused noticeable decreases in the ethanol yield when it was hydrolyzed with starch, a further investigation was thought desirable testing the influence of the concentration of corn bran on the ethanol yields obtained from acid-saccharified starch. Two series of starch mashes were made up and run in the same manner as in the previous experiment except that varying amounts of corn bran were added. These results are collected in Table XXIV.

TABLE XXIV

Effect on Ethanol Yield of Varying Concentrations of Corn Bran When Added Before and After Autoclaving Starch Mashes with 0.05 Normal Sulfuric Acid at 20 Pounds Pressure for 4 Hours.

Corn bran added before autoclaving, grams.	Corn bran added after autoclaving, grams	EtOH yield, per cent
0	2	84.7
0	4	84.7
0	6	84.9
0	8	85.2
2	0	82.2
4	0	78.2
6	0	65.3
8	0	57.0
36 g. corn meal hydrolyzed under identical conditions		19.0
36 g. corn meal hydrolyzed with 0.10 N. H_2SO_4		69.0

Theoretical yield of ethanol from starch 15.54 grams.

Theoretical yield of ethanol from corn meal 13.02 grams.

The results presented above very clearly indicated why the acid-hydrolysis of corn meal was not as successful as the acid-hydrolysis of starch. The exact percentage of corn bran in corn meal varies with the corn used, but the largest amount of corn bran used in this experiment was not equal to that present in corn meal containing an amount of starch equivalent to that used in this experiment. A thorough investigation of this problem will undoubtedly show that corn meal and starch containing the same amount of corn bran per gram of starch as is present in the corn meal will yield nearly identical amounts of ethanol per gram of starch present when hydrolyzed under identical conditions. Lack of sufficient time did not permit further investigation in this direction.

It is believed that these low yields may be due to the formation of toxic materials from the hydrolysis of the corn bran. This might explain why Melson (1940) was unable to ferment successfully the acid-hydrolyzates obtained from corn bran even though they contained considerable amounts of reducing sugars.

C. Studies on the Saccharification of Starch by the Use of Mold-Bran.

Since acid-hydrolysis gave much better yields of ethanol from starch than it did from corn meal, a comparison of the ethanol yields from mold-saccharification of the two substrates was thought desirable. As far as is known no previous attempt had been made to use mold-bran on starch for the express purpose of producing ethanol.

1. <u>Effect on ethanol yield of the addition of mold-bran to acid-hydrolyzed starch</u>.

Schoene, Fulmer, and Underkofler (1940) reported that maximum ethanol yields were obtained when acid-hydrolyzed corn was treated with 10 per cent mold-bran. Although good yields of ethanol have been obtained by the acid hydrolysis of starch as described previously in this thesis, it was desirable to select conditions under which the ethanol yields were still low and to test the effect of mold-bran on these hydrolyzates. It was for this purpose that the following experiment was undertaken: Fifteen grams of starch and 2 grams of corn-gluten meal were weighed into 500-ml. Erlenmeyer flasks containing 200 ml. of 0.05 normal hydrochloric acid. The mashes were then autoclaved at a steam pressure of 15 pounds for 4 hours, cooled, and the pH adjusted to 5 with sodium carbonate. At this point the indicated amounts of mold-bran or malt were added. The malt-saccharified mashes throughout this investigation were always heated at 55° C. for 1 hour, then cooled and inoculated. Since it was reported by Banzon (1940) that the ethanol yields from cassava were just as good when mold-saccharification was carried out at 30° C. as at elevated temperatures, in all the experiments carried out in this investigation requiring the use of mold for saccharification, the mold was introduced into the mash with the yeast and incubated at 30° C. The mashes were then inoculated with 20 ml. of active yeast culture. After fermenting for 72 hours the alcohol was determined in the usual manner. These data are collected in Table XXV.

TABLE XXV

Effect on Ethanol Yield of the Addition of Various Amounts of Mold-Bran to Acid-Hydrolyzed Starch.

Amylolytic material added	Amount of amylolytic agent per cent wt. of mash	EtOH yield per cent
none	0	89.4
mold-bran	2	94.0
"	4	96.0
"	6	96.0
"	10	97.3
"	20	95.7
malt	10	89.4

Theoretical yield 7.27 grams ethanol.

From the data presented in Table XXV, it is apparent that the addition of mold-bran to acid-hydrolyzed starch causes a considerable increase in the ethanol yield obtained by fermentation of these mashes. This observation was in complete agreement with the results reported on corn meal by Schoene, Fulmer, and Underkofler (1940). Four per cent mold-bran appeared to be sufficient as the ethanol yield was 96 per cent of theoretical. The slight increase observed with 10 per cent mold-bran over that obtained with 4 per cent was not significant, and it would not be economically sound to more than double the amount of saccharifying agent used to obtain this small increase in alcohol yield. It is also apparent, from the data, that the addition of malt to acid-hydrolyzed starch was a waste of material as the yield was not increased. This again agrees with the previous work of Schoene, Fulmer and Underkofler (1940) on the saccharification of corn meal. They reported no increased ethanol yield on the addition of malt to acid-hydrolyzed corn. However, a comparison of the data of Table XXV with the data of Table XIX indicates that if acid is to be used for the hydrolysis of starch, it would not be economically sound to use it in conjunction with mold-bran.

Previous work in this investigation indicated that concentrated starch mashes failed to give ethanol yields comparable with those obtained by the use of more dilute mashes. In industry it is desirable to use the highest possible mash concentrations in order to conserve equipment. For this reason it was deemed necessary to examine the possibility of acid and mold-bran saccharification of thick mashes. The following experiment was undertaken to test this point: The mashes consisted of 32 grams starch and 2.5 grams of gluten meal made up in the usual way. They were hydrolyzed with 200 ml. of 0.05 normal sulfuric acid at a steam pressure of 18 pounds for 4 hours. After adjusting the pH, the required amount of malt or mold-bran was added. After fermenting for 96 hours the mashes were distilled, and the ethanol determined in the usual manner. The data from this investigation are given in Table XXVI.

TABLE XXVI

Effect on Ethanol Yield of the Addition of Various Amounts of Mold-Bran To Concentrated Starch Mashes Partially Hydrolyzed by 0.05 Normal Sulfuric Acid.

Amylolytic material added	Amount of amylolytic agent per cent wt. of mash	EtOH yield, per cent
none	0	78.9
mold-bran	2	87.2
"	4	87.8
"	6	88.5
"	10	88.5
"	15	88.4
malt	10	78.2

Theoretical yield 15.54 grams ethanol.

The results in Table XXVI confirm the findings of the previous work using more dilute mashes. These results are: (1) the addition of malt to acid-hydrolyzed starch does not increase the ethanol yields; (2) the addition of mold-bran to acid-hydrolyzed starch results in considerable increase in the ethanol yields obtained; and (3) the addition of 4 per cent mold-bran is sufficient to obtain good yields from acid-hydrolyzed starch. The maximum yields obtained were 88.5 per cent of theoretical. These are a little better than the best obtained under acid-hydrolysis alone with 16 per cent starch mash. Referring to Table XVIII it was observed that 86.2 per cent ethanol yield was obtained by treating a 16 per cent mash 4 hours at 20 pounds steam pressure.

2. Effect on ethanol yield of varying the concentration of mold-bran used for saccharification.

For economic reasons if mold-bran is to be used commercially for saccharification, it is almost essential that it be used alone and not in connection with acid hydrolysis. The most logical attack on this problem was to first determine the amount of mold-bran necessary to obtain maximum yields from starch mashes. This problem was investigated in the following manner: Thirty-two grams of starch were weighed into 500-ml. Erlenmeyer flasks containing 200 ml. of distilled water. Then 0.18 gram mold-bran was added to each, and the mash was heated up to about 85° C. This process is called "premalting". It was used to liquefy the starch and thus prevent the lumping which would otherwise occur while autoclaving the mashes. The malt control was treated in a similar manner except that malt was substituted for mold-bran. The mashes were then autoclaved at 20 pounds steam pressure for 1 hour, cooled, the pH adjusted, and the indicated amounts of mold-bran and malt added. They were saccharified in the same manner as described in the preceding experiments, inoculated, and after fermenting for 96 hours the alcohol was determined in the usual manner. These data are collected in Table XXVII.

TABLE XXVII

Effect on Ethanol Yield of Varying the Concentration of Mold-Bran Used for Saccharification of Starch.

Mold-bran added, per cent wt. of starch	EtOH yield, per cent
2	74.6
4	82.5
6	86.7
8	87.2
10	89.8
15	92.2
20	91.9
Malt control (10 per cent wt. starch)	76.8

Theoretical yield 15.54 grams ethanol.

From the above data it is apparent that good ethanol yields can be obtained by using mold-bran for the saccharification of starch mashes. A maximum yield of 92.2 per cent theoretical was obtained when 15 per cent mold-bran was used. This concentration was too high to be used industrially. However, 10 per cent mold-bran produced a 90 per cent of theoretical yield. This same mold preparation was reported by Underkofler, Goering and Buckaloo (1941) to produce a maximum yield of 83.3 per cent of theoretical from corn meal. This indicated that the pure starch was more readily attacked by amylase than was the starch present in corn. Malt was apparently quite inferior to mold-bran for saccharification of starch as it produced only 76.8 per cent of theoretical yield of ethanol. By comparing the above data with that presented in Table XVIII, it is also apparent that acid hydrolysis is superior to malt saccharification.

3. Effect of mash concentration on the yields of ethanol obtained from starch mashes saccharified with mold amylase.

The mash concentration has been shown to have a profound influence on the yields of ethanol obtained by fermenting acid-hydrolyzed starch mashes. This effect should not show up if mold amylase were used for conversion unless the alcohol content in the concentrated mashes became great enough to be toxic to yeast. The following experiment was undertaken in order to test the above mentioned assumption: The mashes were prepared and analyzed in the same manner as those in the preceding section except that various concentrations of starch were used, and in every case the amount of mold-bran added was 10 per cent by weight of the starch used. A sample of corn meal was run at the same time to check the activity of the mold preparation. The data are collected in Table XXVIII.

TABLE XXVIII

Effect of Mash Concentration on the Yields of Ethanol Obtained from Starch Mashes Saccharified with 10 bran and Mold Amylase.

Starch added, grams	Mash conc., per cent	EtOH yield, per cent
20	10	87.2
30	15	86.8
35	17.5	88.5
40	20.0	84.8
45	22.5	84.8
36 (corn meal)	18.0	79.8

Theoretical yield 16.99 grams ethanol from 35 grams starch.

Theoretical yield 13.10 grams ethanol from 36 grams corn.

The results from Table XXVIII indicate that the mash concentration had little influence on the amount of ethanol produced when mold-bran was used. This was rather important since it would mean that industrially the capacity of the plant could be increased without changing the size of the equipment. Probably the most significant observation resulting from this experiment was the 79.8 per cent yield obtained from corn meal as compared with the 88.5 per cent yield for starch. This was another observation proving that starch was a better substrate for ethanol fermentation than corn meal. It was also evident that the concentration effect observed in the case of acid hydrolysis was not due to the toxicity to yeast of the alcohol in the mashes. However, at mash concentrations of 20 per cent or more this fact may be the reason for the lower yields obtained.

D. Studies on the Ethanol Yields Obtained by the Acid Saccharification of Cassava.

A detailed investigation of the possibility of using cassava for the production of ethanol was made by Banzon (1940). He investigated the use of both mold-bran and dilute mineral acids for the saccharification of cassava mashes. The best yields claimed for acid saccharification were slightly above 70 per cent of theoretical. A combination of acid hydrolysis with mold saccharification employing the use of 10 per cent mold-bran gave ethanol yields of 86 per cent of theoretical. Ethanol yields of this same magnitude were produced by using mold-bran alone under the most favorable conditions.

1. Effect on ethanol yields of varying the concentration of acid used for saccharification of cassava mashes.

In view of the work presented on corn starch, it was thought desirable to investigate the acid hydrolysis of cassava using the conditions found optimum for corn starch. The following experiment was set up to obtain this data:

Thirty-two grams of ground cassava root and 2.5 grams of corn-gluten meal were weighed into 500-ml. Erlenmeyer flasks containing 200 ml. of sulfuric acid of various concentrations specified in Table XXIX. The cassava starch was gelatinized, autoclaved at a steam pressure of 20 pounds for 4 hours, cooled and prepared for fermentation in the usual manner. After fermenting for 96 hours the mashes were distilled, and the alcohol determined in the usual manner. These data are collected in Table XXIX.

TABLE XXIX

Effect on Ethanol Yields of Varying the Concentration of Acid Used For Saccharification.

Conc. of acid, normality	Conc. of mash, per cent	EtOH yield, per cent
0.05	16	1.8
.10	16	19.8
.15	16	66.7
.05	7.5	9.2
.10	7.5	77.5
.15	7.5	79.8

Theoretical yield 13.92 grams ethanol.

The data in Table XXIX indicate that cassava starch is much more difficult to saccharify than is corn starch. At first glance one might be led to believe that something in the root when hydrolyzed produced materials toxic to yeast. However, on second thought this possibility was ruled out because the ethanol yields keep increasing as the acid concentrations became greater. The yield of 79.8 per cent is much higher than any obtained by Banzon (1940) with straight acid-saccharification. Since he used a heating period of only 2 1/2 hours this indicates that longer heating periods were more desirable. The same concentration effect was observed with the acid hydrolysis of cassava as already reported for corn starch.

2. <u>Effect of varying mash concentrations on the ethanol yields obtained from acid-hydrolyzed cassava</u>.

The mash concentration was shown to exert a great deal of influence on the yield of ethanol obtained from acid-hydrolyzed corn starch. It was thought desirable to test this point in regard to cassava starch, and for this reason the following experiment was undertaken: The amounts of ground cassava root indicated in Table XXX were added to 200 ml. of 0.20 normal sulfuric acid. The rest of the procedure was carried out in exactly the same manner as in the preceding experiment. The data are collected in Table XXX.

TABLE XXX

Effect of Varying Mash Concentration on the Ethanol Yields Obtained from Cassava Hydrolyzed with 0.20 Normal Sulfuric Acid for 4 Hours at 20 Pounds Steam Pressure.

Cassava added, grams per flask	Mash conc., per cent	EtOH yield, per cent
10	5.0	81.4
15	7.5	82.3
20	10.0	82.1
25	12.5	81.8
30	15.0	79.8
35	17.5	79.1
40	20.0	75.7
45	22.5	69.8

Theoretical yield 0.434 gram ethanol per gram cassava root.

The mash concentration did not influence the ethanol yield obtainable from acid-hydrolyzed cassava starch as much as it did in the case of corn starch. The decided decrease in yield observed with 22.5 per cent mash was probably due to the alcohol content of the mash reaching the point where it became toxic to the yeast. It was interesting to note from Table XXII that this point was reached with corn starch at a mash concentration of 20.0 per cent. The fact that these two mashes have nearly the same starch content was reasonably good proof of this theory. The yields of ethanol obtained were quite good and rather encouraging. The fact that the supply of cassava root was exhausted prevented further investigation of this problem.

E. Investigation of Commercial Nutrient Sources for Yeast Growth in Acid Hydrolyzates of starch.

1. Studies on various gluten meals as nutrients for yeast growth.

In all the experiments previously presented in this thesis the corn-gluten meal used for nutrient was that designated in the section on materials as gluten A. The supply of this material was becoming low so it was necessary to test a new supply of gluten. Preliminary investigation on the use of one sample of new gluten meal designated as gluten B yielded rather discouraging results. The following series was run in order to determine the relative effectiveness of the three samples of gluten meals available. The source of these materials has been given in the section on materials. Thirty-two grams of corn starch were hydrolyzed by heating with 200 ml. of 0.10 normal sulfuric acid at a steam pressure of 15 pounds for 4 hours. After cooling the various amounts of corn-gluten meal indicated in Table XXXI were added, and the pH adjusted to 5. The mashes were then inoculated with 20 ml. of active yeast, allowed to ferment 96 hours, and the ethanol then determined in the usual manner. These data are collected in Table XXXI.

TABLE XXXI

Ethanol Yields Obtained From Starch Hydrolyzates Containing Varying Amounts of Different Corn-Gluten Meals as Nutrients.

Gluten used, grams	Yield of alcohol in per cent from use of		
	Gluten A	Gluten B	Gluten C
1	61.3	49.0	46.3
2	79.4	49.3	49.3
3	79.4	46.3	49.7
4	80.7	52.6	67.2
5	80.3	65.3	70.6
6	81.5	72.0	74.6

Theoretical yield 15.54 grams ethanol.

The results of Table XXXI indicated that the old gluten meal used was far superior to either of the two new samples obtained. This fact was disconcerting since it was now necessary to reinvestigate the problem of obtaining a cheap source of nutrients. Because of the color and odor of gluten A, and also because of the fact that a few years ago the steep water concentrates were added in preparing corn-gluten meal, it was suspected that better yields might be due to the fact that it contained steep water. Since steep water was not immediately obtainable a further investigation of corn-gluten meal was undertaken.

2. <u>Effect on the ethanol yield of the addition to acid hydrolyzates of wheat bran and corn-gluten meal for nutrients.</u>

Wheat bran was known to be a very effective nutrient for the growth of molds. It was thought that combinations of wheat bran and corn-gluten meal might prove to be good nutrients. The following experiment was undertaken to test this theory: Thirty-two grams of starch were hydrolyzed with 200 ml. of 0.05 normal sulfuric acid by heating at a steam pressure of 20 pounds for 4 hours. After cooling the various amounts of corn-gluten meal and wheat bran which are indicated in Table XXXII were added, and the pH adjusted to 5. The mashes were then inoculated with 20 ml. of active yeast, allowed to ferment 96 hours, and the ethanol then determined in the usual manner. The results of this experiment are collected in Table XXXII.

TABLE XXXII

Effect on Ethanol Yield of the Addition to Acid Hydrolyzates of Combinations of Corn Gluten and Wheat Bran for Nutrients.

Amount of gluten A added, grams	Amount of gluten C added, grams	Amount of wheat bran added, grams	EtOH yield, per cent
2.5	0.0	0.0	79.6
0.0	2.5	0.0	63.5
0.0	2.0	0.5	79.8
0.0	1.5	1.0	82.3
0.0	1.0	1.5	81.9
2.0	0.0	0.5	85.0
0.0	0.0	2.5	81.6
0.0	0.0	20.0	84.5

Theoretical yield of ethanol 15.54 grams.

This experiment illustrated the remarkable stimulating effect of a small amount of wheat bran even on the good gluten used in previous work. It is significant that the yields obtained from a mixture of gluten and wheat bran were better than those obtained with either material when used alone.

3. Effect on ethanol yields of the addition of various nutrients to acid-hydrolyzed starch.

The stimulating influence of wheat bran might be due to the presence of inorganic salts or due to the presence of certain growth factors. For this reason it was desirable to repeat some of the previous work reported in this thesis, but this time eliminating the use of nitrates because they did not seem to be good nitrogen sources for yeast growth. The conditions used in this experiment were the same as those for the data in Table VI; namely, 30 grams of starch were treated for 3 1/2 hours at 12 pounds steam pressure with 0.15 normal sulfuric acid. The data are collected in Table XXXIII.

TABLE XXXIII

Effect on Ethanol Yields of the Addition of Various Nutrients to Acid-Hydrolyzed Starch.

Mash No.	Grams of the following added to 200 ml. of mash, $(NH_4)_2SO_4$	$CaCl_2$	$MgSO_4$	K_2HPO_4	corn gluten	wheat bran	EtOH yield, per cent
1	0.24	----	----	----	----	----	27.6
2	----	0.01	----	----	----	----	27.5
3	----	----	0.08	----	----	----	22.5
4	----	----	----	0.30	----	----	35.7
5	0.24	0.01	0.08	0.30	----	----	45.8
6	0.24	0.01	0.08	0.30	2.50	----	79.6
7	----	----	----	----	2.50	----	73.2
8	----	----	----	----	2.00	0.50	81.1

Theoretical yield 14.57 grams of ethanol.

The results from Table XXXIII confirm the data reported in the preceding experiment; namely, that corn-gluten plus a little wheat bran were most effective as nutrients. The stimulating influence of wheat bran was apparently due in some respect to inorganic salts present, because in mash Number 6 the total yield was nearly 9 per cent higher than in Number 7 where only corn-gluten was present. Mash Number 8 gave nearly 2 per cent better yields than did Number 6; thus indicating that something other than inorganic salts was responsible for the higher ethanol yields obtained by the use of wheat bran with corn-gluten as a nutrient.

4. Effect on ethanol yields of the addition of various amounts of steep water to acid-hydrolyzed starch.

Steep water has been reported by Wells, Lockwood, Stubbs, Roe, Porges, and Gastrock (1939) to be a useful nutrient for the growth of *Acetobacter suboxydans*. Thus it was suspected to be a good nutrient for yeast growth, and as this would be a very cheap source of nutrient, it was desirable to investigate the possibilities of using this material. The following experiment was carried out to examine the use of steep water for yeast nutrient: Thirty-two grams of starch were weighed into 500-ml. Erlenmeyer flasks containing a total volume of 200 ml. 0.05 normal sulfuric acid solution containing heavy steep water of the concentrations specified in Table XXXIV.

Hydrolysis was carried out at 20 pounds steam pressure for 4 hours. After cooling, the pH was adjusted to 5, and the flasks were inoculated with 20 ml. of an active yeast culture. They were allowed to ferment for 96 hours. The ethanol content was then determined in the usual manner. The data are collected in Table XXXIV.

TABLE XXXIV

Effect on Ethanol Yields of the Addition of Heavy Steep Water of Various Concentrations To Starch Mashes Before Autoclaving.

Conc. of steep water g./l.	EtOH yield, per cent
1.5	70.0
2.0	73.6
2.5	77.4
3.0	76.9
3.5	76.6
4.0	78.2
4.5	78.2

Theoretical yield 15.54 grams ethanol.

These data illustrate that concentrations of steep water greater than 2.5 grams per liter have relatively little effect on the ethanol yield obtained. The yields of ethanol were somewhat lower than those obtained by the use of gluten A as a nutrient. Higher yields might be obtained if the steep water concentrates were added after autoclaving, because some of the valuable substances might be destroyed as a result of autoclaving. This point was tested in the following experiment: It was carried out exactly like the preceding one except that the steep water was added after hydrolysis. The results are collected in Table XXXV.

TABLE XXXV

Effect on Ethanol Yield of the Addition of Steep Water Concentrates of Various Concentrations to Starch Mashes After Being Hydrolyzed.

Conc. of steep water g./l.	EtOH yield, per cent
1.5	78.0
2.5	80.6
3.5	81.4
4.5	83.2
5.0	83.5
6.0	83.5
7.0	83.6

Theoretical yield 15.54 grams ethanol.

The results of Table XXXV indicate rather clearly that steep water could be used as a nutrient for yeast growth if it is added after the hydrolysis of the starch mashes. A concentration of 4.5 grams per liter appeared sufficient to obtain maximum yields of ethanol. If the steep water was added before autoclaving, it apparently lost some of its activity. This is illustrated by a comparison of Tables XXXIV and XXXV.

Steep water alone was proven very effective in the above experiments, but combinations of steep water and other nutrients had not been tried. The following experiment was designed to test these effects: Unfortunately not enough starch remained to permit using the concentrations which were used above so 15 grams of starch were added to 200 ml. of 0.05 normal sulfuric acid. The mashes were intended to be hydrolyzed for 4 hours at 20 pounds steam pressure, but due to a change in line pressure this went up to 23 pounds. The fermentation and subsequent analysis of ethanol were carried out in the usual manner. The data are collected in Table XXXVI.

TABLE XXXVI

Effect on Ethanol Yield of the Addition of Combinations of Steep Water, Wheat Bran and Corn-Gluten to Acid-Hydrolyzed Starch Mashes.

Gluten A added to 200 ml. mash, grams	Wheat bran added, to 200 ml. mash, grams	Steep water concentrations, grams/ l.	EtOH yield, per cent
2.5	0.0	0.0	90.5
0.0	2.5	0.0	87.1
0.0	0.0	4.0	91.4
1.0	0.0	4.0	91.4
0.0	1.0	4.0	90.8
1.0	1.0	4.0	90.0
1.5	1.0	0.0	90.2

Theoretical yield of ethanol 7.27 grams.

The results from Table XXXVI indicated that steep water alone was more effective as a nutrient than either wheat bran, corn-gluten meal or a combination of both. However, since wheat bran did not exert a stimulating effect on corn-gluten meal in this experiment, more work is necessary on this problem. It appeared as if stimulation by wheat bran occurred only when mash concentrations were rather high. For this reason the above results might or might not be particularly significant.

F. Suggested New Method for Obtaining Ethanol from Corn.

From the findings of this thesis it seems that starch is a better substrate than corn meal for the production of ethanol. Therefore, it would probably be economically feasible to remove the starch from the corn, either by a wet-milling or dry-milling process before subjecting the starch to ethanol fermentation. Such a procedure would result in greater ease in processing, better alcohol yields, and more rapid fermentation, while valuable by-products such as corn oil could also be recovered.

VI. SUMMARY AND CONCLUSIONS

1. A study of the use of mineral acids and of mold amylase as saccharifying agents for the production of fermentable sugars from starch was undertaken. The use of phosphoric, hydrochloric and sulfuric acid was investigated.

2. Fermentation of corn mashes saccharified with 0.10 normal hydrochloric acid and a steam pressure of 30 pounds for 3 hours produced amounts of ethanol equal to those obtained in the conventional process using 10 per cent malt.

3. Either hydrochloric or sulfuric acid could be used for acid saccharification. The concentration of hydrochloric acid necessary for good conversion of starch to fermentable sugars is somewhat less than the amount of sulfuric acid necessary. However, hydrochloric acid at lower concentrations caused greater caramelization of the sugars produced than did sulfuric acid. For this reason, under certain conditions, sulfuric acid produced more fermentable sugars than did hydrochloric. Phosphoric acid was found effective only at extremely high concentrations.

4. In general higher steam pressures and shorter periods of heating produced the highest ethanol yields from corn meal which had been saccharified with dilute mineral acids.

5. When mold-bran was added to corn mashes partially

saccharified by hydrochloric acid very high ethanol yields were obtained. The addition of 4 per cent mold-bran to these mashes produced an ethanol yield of 91.5 per cent of theoretical.

6. A search was made for an economical source of the nutrients necessary for yeast growth on acid-saccharified starch mashes. Steep water, corn-gluten meal, and a mixture of corn-gluten meal with wheat bran were very effective. If the steep water was added before the hydrolysis of the starch, it became somewhat less effective. A concentration of 4.5 grams of heavy steep water per liter of mash when added after hydrolysis was found most effective. The effectiveness of corn-gluten meal was not appreciably altered whether added to the mashes before or after hydrolysis. The slight loss in activity when it was added before hydrolysis was more than compensated for by the increased rate of fermentation of the mashes. Variations were found when different gluten meal preparations were used. More uniform results could probably be expected if heavy steep water was used instead of corn-gluten meal for nutrient.

7. Corn starch was found to be more readily saccharified by dilute mineral acids than corn meal. The yield of ethanol reached 100 per cent of theoretical when 7.5 per cent starch mashes were hydrolyzed with 0.02 normal sulfuric acid at a steam pressure of 25 pounds for 4 hours.

8. Neutralization of acid-saccharified starch mashes with ammonium hydroxide instead of sodium carbonate resulted in a 40 per cent decrease in the ethanol yield. The addition of ammonium salts to either corn or starch mashes saccharified by mold-bran also resulted in lower ethanol yields.

9. The ethanol yield obtained from acid-saccharified corn starch was a function of mash concentration, acid concentration, kind of acid employed, steam pressure and time of hydrolysis.

10. The maximum ethanol yields were not obtained from acid-saccharified starch mashes which showed the greatest concentration of reducing sugars, but instead mashes in which 99.4 per cent of the starch had been converted into reducing sugars produced an ethanol yield of only 79.7 per cent of theoretical while those heated for a longer period showing 98.2 per cent conversion of starch to reducing sugars produced an ethanol yield of 82.2 per cent theoretical.

11. The lower ethanol yields obtained from corn meal than from corn starch when both were subjected to acid hydrolysis under the same conditions were found to be due to the presence of corn bran. Apparently the hydrolysis of the corn bran produces something toxic to yeast.

12. The addition of small amounts of mold-bran greatly increased the yield of ethanol obtained from starch mashes partially saccharified by dilute mineral acids. The addition

of 4 per cent mold-bran to 7.5 per cent starch mashes saccharified with 0.05 normal sulfuric acid at a steam pressure of 15 pounds for 4 hours increased the ethanol yield from 89.4 to 96.0 per cent of theoretical. When 16 per cent mashes were hydrolyzed under similar conditions the addition of 4 per cent mold-bran increased the ethanol yield from 78.9 to 87.8 per cent of theoretical. The addition of larger amounts of mold-bran to either 7.5 or 16 per cent mashes did not increase the ethanol yield obtained to any appreciable extent. The addition of malt to acid-saccharified mashes did not increase the ethanol yield obtained.

13. Mold-bran produced higher yields of ethanol from corn starch than it did from corn meal. At the optimum concentration of 15 per cent mold-bran an ethanol yield of 92.2 per cent theoretical was obtained from 16 per cent starch mashes. The use of 6 per cent mold-bran produced an ethanol yield of 86.7 of theoretical.

14. Cassava starch was much more difficult to hydrolyze than corn starch. When a 10 per cent ground cassava mash was hydrolyzed for 4 hours at 20 pounds steam pressure with 0.20 normal sulfuric acid the ethanol yield obtained was 82.1 per cent of theoretical. This was nearly equal to that obtained by Banzon (1940) with the use of mold-bran. The mash concentration did not influence the yields of ethanol obtained from cassava as much as it did in the case of corn starch.

15. Acid saccharification under optimum conditions, 4 per cent mold-bran in conjunction with acid, and 8 per cent mold-bran alone produced respectively ethanol yields of 86.3, 87.8, and 87.2 per cent of theoretical when used on 16 per cent starch mashes. Therefore any one of the three methods may be considered satisfactory. On 7.5 per cent starch mashes acid-saccharification is the most satisfactory of these three methods.

16. Corn starch was found to be a better substrate than corn meal for the production of ethanol. The yields of ethanol from corn meal and corn starch hydrolyzed with 0.10 normal sulfuric acid and fermented under the same conditions were respectively 68.6 and 88.3 per cent of theoretical. The yields of ethanol from corn meal and corn starch saccharified with 10 per cent mold-bran were respectively 79.8 and 88.5 per cent theoretical.

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