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Fermentative utilization of cassava

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NOTE TO USERS

FERMENTATIVE UTILIZATION OF CASSAVA

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I. INTRODUCTION

The cassava is a plant possessing quite unusual characteristics. It has no known pests nor enemies. It grows in most soils, resists extreme droughts, and propagates easily. It is the cheapest source of starch known. When American technologists first came over to the Philippines the accounts of their work were filled with enthusiasm over this plant. To quote Bacon (1908): "it will furnish the cheapest source of starch in the world and also compete on even grounds with molasses for the manufacture of alcohol." But up to the present the industries have not made any use of cassava except for the manufacture of starch which is found in the market throughout the world because it can undersell any other starch.

A very difficult local situation has arisen in the Philippines. The Islands are found to be almost devoid of petroleum resources. However, the demand for liquid fuel has steadily increased with the years. In 1932 some five million dollars' worth of liquid fuels was imported. This was not the entire fuel bill because ethyl alcohol, either straight or blended with 50 per cent gasoline, was and is being used quite extensively to power tractors, locomotives, trucks, and busses. The alcohol is manufactured from cane molasses. But the entire molasses output of the country if fermented would hardly meet the domestic fuel requirement.

The cassava as a potential source of power alcohol has been much discussed and fermentations on laboratory scale have been tried. But up to the present there is no known large scale production of alcohol from this source. There are several reasons for this failure of the commercial development of the above outlet. Cassava starch is still in great demand. The manufacture of starch is a comparatively simple matter compared to the processes required in the conversion of starch to alcohol. Starch requires a special treatment, that of saccharification, before it can be acted upon by yeast. The current methods of saccharification which employ acids or barley malt are too costly and are inapplicable to local Philippine conditions. Barley is not raised in the country and will have to be imported. The acid process requires special equipment. Even in the United States "The use of acids to convert starches for a fermentation process has never been practicable".[1]

The fermentation industry is one of the largest consumers of carbohydrate material aside from the food industries. The diversion of cassava from the starch market would seem to lie in its fermentative utilization. At present the two fermentations that are being carried out on a large industrial scale

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1. U. S. 76th. Congress, 1st. Session. Regional research laboratories; report of a survey. U. S. Congress. Senate document 65. p. 31. 1939.

are the alcoholic and the butyl-acetonic fermentations. The products of these fermentations, ethyl alcohol, acetone, and butyl alcohol, find extensive uses in the arts and industries. The Philippines is an extremely agricultural country. The commercial production of chemicals by fermentation is a step towards industrialization.

In view of the circumstances presented, the fermentative utilization of cassava was made the object of this investigation.

II. HISTORICAL

The cassava plant is botanically known as Manihot utilissima Pohl. It belongs to the family Euphorbiaceae. It is also called tapioca or manioc although the word tapioca is often used to designate certain forms of cassava products. The plant itself is a perennial shrub which attains a height of six to twelve feet at the age of one year. At the base of its stem it produces a cluster of long fleshy roots. According to Mendiola (1931) the number of roots varies from 3 to 5 and the average length of the roots is from 27.7 centimeters to 43.3 centimeters, and the diameter, from 4.6 centimeters to 7.4 centimeters. A single root may weigh 4 kilograms and all the roots in a plant may weigh 12 kilograms. Copeland (1908) gave an excellent description of the cassava together with its history, varieties, toxicity, culture, and uses. In the Philippines the yield of cassava was reported by Mendiola (1931) to be 16,100 kilograms to 38,800 kilograms per hectare (7.18 tons to 17.3 tons per acre); in Java yields as high as 50,000 kilograms to 55,000 kilograms per hectare (22.3 tons to 24.7 tons per acre) have been obtained.

Several chemical analyses of the cassava root and flour have been published. Archbold (1903) gave an analysis of Jamaican cassava. Analyses by Reibling of Philippine cassava have been reported by Bacon (1908). Galang (1931) compiled

the results of analyses of 33 varieties by the Philippine Bureau of Science. Adriano (1933) made a comparative analysis of flours obtained from cassava, wheat, rice, and corn. Adriano, Ramos, and Ynalvez (1932) found the fresh cassava root to be composed of 63.80 per cent moisture, 27.65 per cent starch, 0.96 per cent protein, 1.44 per cent ash, and 0.02 per cent hydrocyanic acid.

Hydrocyanic acid is a characteristic constituent of the cassava. Collens (1914) found the peel of freshly dug root to contain 0.055 per cent hydrocyanic acid; the pulp, 0.053 per cent. After 3 days the peel analyzed 0.245 per cent hydrocyanic acid and the pulp 0.1114 per cent hydrocyanic acid. The increase in the percentage of hydrocyanic acid was ascribed to the loss of water by the roots. According to Furnock (1937) the toxic principle in cassava is a cyanogenetic glucoside. This author found that the processes used for the preparation of cassava foods do not eliminate the hydrocyanic acid. Collens (1914) however found that the hydrocyanic acid was entirely removed by boiling, roasting, or drying.

The fatty acids associated with cassava starch were found by Lehrman (1932) to be palmitic, oleic, linoleic, and linolenic; this last fatty acid was stated to be peculiar to cassava starch. According to this investigator the fatty acids were combined with the amylose component of the starch. Data by Walton (1929) gave the amylose component of cassava as 16 per cent to 17.5 per cent, the total fatty acids as

0.10 per cent, and phosphorous pentoxide as 0.137 per cent. According to this author, "The unusually low content of phosphorous and of fatty acids in tapioca starch may possibly be connected with its reported superiority in certain industrial processes. This starch has been considered by some operators as being the most suitable source material for the manufacture of alkali-starch adhesives, nitrostarches and certain types of dextrin."

Studies in other properties of cassava starch were reported by Archbold (1903), by Harvey (1924), by Ophof (1936), and by Etorma (1936).

The commercial utilization of cassava was given attention a great many years ago by Ewell and Wiley (1893). Moore (1907) and Lange (1909) also pointed out its industrial possibilities for the manufacture of glucose and industrial alcohol. Moore (1911) obtained a patent covering the manufacture of alcohol from a starch milk prepared from cassava. French breweries were employing cassava some 35 years ago according to Petit (1917). This practice was discontinued because of a heavy import duty placed upon cassava which was believed to be cyanogenetic. Monier-Williams (1922), in his monograph on power alcohol, considered cassava as one of the more important raw materials from the point of view of yield and availability. Calculated on the per acre basis the data of Monier-Williams placed cassava as the highest yielder in gallons alcohol, when compared with cereals, tubers, roots, and trees.

Collens (1914), at the Government Laboratory in Trinidad, conducted a few experiments on the alcoholic fermentation of cassava flour; malt and taka diastase were employed for saccharification. The experiments were very brief.

The only detailed and lengthy investigation on the alcoholic fermentation of cassava as reported in the literature is that of Roxas and Manio (1921). The work of these investigators was concentrated on acid hydrolysis and subsequent yeast fermentation. They investigated the effect of acid concentration, time of heating, mash concentration, time of fermentation, and the use of stimulants. Yields over 90 per cent were claimed.

The alcoholic fermentation of fresh cassava roots was undertaken by De Leon and Valentin (1938). Acid hydrolysis was employed but the yields were low, the highest being only 43.24 per cent of theoretical.

Tubangui and coworkers (1939) investigated the butyl-acetonic fermentation of cassava flour. They found that cassava can replace 90 per cent of corn. The ratio of the solvents butanol, acetone, and ethanol was found to be normal, that is, in the ratio 60:30:10. They experienced difficulty in fermenting mashes of concentrations above 5.23 per cent dextrose equivalent.

Summarizing, the literature on the fermentative utilization of cassava is quite brief. A detailed study had been made of alcoholic fermentation using acid saccharification.

Cassava was used once with satisfactory results in the brewing industry. There has been no work on the use of molds for saccharification.

For the prosecution of the investigation as reported in this thesis it was necessary to review not only the literature on cassava but also the literature on the tools of the investigation: the amylases, mold-bran, starch, and related subjects.

The literature on starch is very voluminous. Walton (1927) made a comprehensive survey of the field and collected in book form the literature from 1811 to 1925.

Pringsheim (1932) credited Irvine with the discovery of malt amylase in 1785; a better characterization of this amylase was made by Kirchhoff in 1815. It was found then that the product obtained from the action of malt amylase on starch was a sugar which was not identical with glucose, and Dubrunfaut (1847) proved the sugar to be maltose.

The action of amylase on starch was recognized to consist of two stages. First there was liquefaction observed followed by the formation of reducing substances. Märcker (1878) explained this action of amylase by postulating the presence of two components, a liquefying fraction and a maltose forming fraction. Ohlsson (1922) and Kuhn (1925) contributed much of the work that led to the present acceptance of Marker's theory. According to Hopkins and Krause (1937) the liquefying component of malt amylase splits the starch particle into

smaller particles with very little formation of sugars. The cleavage products are dextrins. The maltose-forming fraction attacks the colloidal starch particle and splits off maltose without causing a marked decrease in viscosity. The liquefying component which is also known as α-amylase is more resistant to heat than the maltose forming component (β-amylase).

The saccharification of starch by amylase has been known not to yield quantitative amounts of maltose; it was found to stop at a limiting value of 75 to 80 per cent of the sugar. Pringsheim (1932) considers the process as belonging to an equilibrium reaction, the sugar already formed interfering with further saccharification.

The use of malt for saccharification is little known in the Orient. Instead, various fungus preparations are used, in the production of alcoholic beverages. The most widely known of these preparations are taka koji and Chinese yeast. This latter is a dried rice cake rich in a microflora which is strongly amylolytic. Since it is found in many parts of the Orient, its method of preparation varies with the locality. In the northern Philippines the procedure is as follows: A dough is made of steamed rice and mixed thoroughly with pieces of an old cake. The mixture is laid over young banana leaves and set aside overnight. The dough partially liquefies and becomes sweetish in taste; later it turns strongly alcoholic. The liquid portion, which is separated, constitutes

an alcoholic beverage. The solid residue is pressed into round flat cakes and when dried it becomes the so-called Chinese yeast cake. Lafar (1911) gave descriptions of the methods used by the people in Java.

Calmette in 1892 made a study of the microflora of Chinese yeast cake. He found a Mucor which he named Amylomyces rouxii always present in his samples and to this fungus he ascribed the amylolytic activity of the cake. A process of alcoholic fermentation employing this organism was developed by Calmette and Boidin. It is known as the Amylo process. Owen (1933) gave an account of the details of the process, which consisted of three steps: (1) gelatinization of the starch under pressure; (2) saccharification of the starch by diastatic action of the mold; and (3) fermentation of the sugar by yeast. The advantages derived from the process are economy due to saving in malt, decrease in losses due to infection introduced with the malt, and a higher purity of alcohol found in the mash. Also the adaptability of the process to temperatures as high as 38° C. makes it of value in tropical countries.

The trend of development in the Amylo process is in the isolation and employment of more vigorous species of organisms. In the Boulard process use is made of Mucor Boulard Number 5. Boidin (1933) reported the employment of Mucor delemar as giving the best results in industrial application. Yeasts, like Saccharomyces anamensis, isolated from sugar cane in Cochin China, and yeasts known as Boulard Numbers 21-30 have been used.

Some of these yeasts are active at high temperatures and live in symbrosis with the mold.

According to Neubauer (1933) the Amylo process suffers several disadvantages. The power consumption greatly exceeds that of the malt process. An expert personnel and a complete bacteriological laboratory are required.

In the perfection of the Amylo process Boidin (1933) found that it is not sufficient to bring about favorable conditions for the development of the molds. The accomplishment of diastatic action was also important. The physico-chemical state of the wort, the satisfactory liquefaction, and the reaction, were deemed essential. The fermentation by the Amylo process of corn, sorghum grain, and other starchy materials were reported by Galle (1923).

The saccharification of starchy mashes for alcoholic fermentation by Aspergillus oryzae has lately been revived. This mold has been used in Japan for a number of years in the preparation of rice beer, soy sauce, and other fermented products. It remained for Takamine to bring this mold to the attention of Western countries. In a review of his own work, Takamine (1914) traced the history of his efforts from 1891 on, to produce an amylolytic material which would take the place of malt. Wheat bran was found satisfactory as the substrate for growing Aspergillus oryzae. As soon as vigorous growth ceased, the bran was dried and the product thus formed was called taka koji.

When taka koji was employed instead of malt, good yields of alcohol were obtained. The new product cost much less than malt and required only one-third to one-fourth the time needed for preparing malt.

Takamine (1894, 1896, 1910, 1911, 1913, 1918, 1923) took numerous patents in the United States for his diastatic product and its use in the fermentation industry. Oshima and Church (1923) studied in detail the enzymes in taka koji. Strains of the Aspergillus flavus-oryzae group, which were isolated from koji used in soy sauce manufacture, were found to vary in their ability to produce amylases. Even in soybean flour which contains no starch, strong amylolytic activity was produced by some strains. The intracellular enzymes passed out into the culture medium soon after sporulation. Of the media studied, which included corn meal, cocoanut meal, peanut meal, ground dried codfish, casein, and crushed soybeans, the best found was wheat bran.

Oshima (1928) extended this study. He found the activity of the enzyme to be greatest at pH 4.8 to 5.2 and to be most stable to heat at pH 6.4. At 85° C. and one hour heating it became completely inactivated.

The preparation and properties of Aspergillus oryzae enzymes were further studied by Harada (1931). A culture of the mold was ground in cooked wheat bran containing 50 per cent water. It took two days to obtain maximum growth. The

enzyme was prepared by alcoholic precipitation. As important factors for enzyme production, the following were listed: quality of bran, water content of bran, hydrogen ion concentration, time of incubation, temperature, humidity of chamber, and sterilization. Details, however, were not given. An interesting point in Harada's results was the finding 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.

Studies on Aspergillus oryzae until very recently were concentrated on the enzymes. The original proposition of Takamine to use the mold-bran itself in the alcoholic fermentation process was largely forgotten. Underkofler, Fulmer, and Schoene (1939), revived the idea and undertook a detailed study of the problem. Eight species of bacteria and 21 species or strains of molds were tested for amylase production. Molds of the Rhizopus species as well as strains of the Aspergillus flavus-oryzae group were found to possess high amylolytic activity. The Aspergillus cultures were more rapid and uniform in their growth and attention was concentrated on them. Mold-bran was prepared in aerated five gallon rotating pyrex bottles. An important contribution was the employment of acid, up to 0.3 normal hydrochloric acid, instead of water. This prevented growth of contaminating organisms. A detailed

procedure of the process was developed. The prepared mold-bran was used in place of malt for the saccharification of corn mash. The amount necessary was found to be 8 to 10 per cent of the weight of corn. Saccharification was satisfactory within the range 45° to 60° C. Ninety per cent conversion to alcohol was obtained in fermentations of 85 gallons of mash. The investigation pointed out advantages in the use of mold-bran instead of malt: cheapness of the material, high alcohol yields, and shorter time of preparation.

Schoene (1939) and Schoene, Fulmer, and Underkofler (1940) compared malt, mold-bran, and soybean meal as saccharifying agents. Of these three, mold-bran was the most effective and soybean least. Two types of mashes, normal and thick, were also studied in conjunction with combinations of the three amylolytic materials. No advantage was found using combinations over mold-bran alone. With acid-saccharified mashes, the addition of mold-bran gave considerable increase in alcohol yield. This result was ascribed to enzymatic action, the conversion of the non-fermentable polysaccharides remaining in the acid-saccharified mashes to fermentable sugar. The greater effectiveness of mold-bran over malt was associated with the greater variety of enzymes present in the former. The addition of soybean meal to mold-bran was found to increase alcohol yields markedly in acid-saccharified mashes. It was suggested, however, that the employment of mold-bran alone without

preliminary acid hydrolysis might be less difficult and more economical.

Buckaloo (1940) investigated various mold amylases in the saccharification of corn mash for ethanol fermentation. Four strains of Aspergillus oryzae, two strains of Rhizopus species, a Mucor, and two unidentified molds were grown on moistened wheat bran in a rotating drum apparatus. It was found that two strains of Rhizopus species were as high amylase producers as the reference mold Aspergillus oryzae.

III. MATERIALS

The more important materials used in this investigation were the following:

Cassava flour

Chips of peeled and dried cassava roots were obtained from the College of Agriculture, University of the Philippines, Philippines. The chips were ground in a burr mill to a flour. One batch analyzed 88.1 per cent glucose or 79.2 per cent starch; another batch analyzed 91.8 per cent glucose or 82.5 per cent starch.

Ground cassava

In this investigation the term ground cassava is used to designate the material obtained by grinding the sliced and dried unpeeled root. Because the peel or skin of the root was not removed, this material would cost very much less than cassava flour. It should be of greater industrial importance than the flour. A 100 pound lot was obtained from the College of Agriculture, University of the Philippines, Philippines. One batch of ground cassava analyzed 79.1 per cent glucose, another batch analyzed 85.2 per cent glucose, and a third batch analyzed 82.8 per cent glucose. These percentages correspond,

respectively, to 71.2, 76.6, and 74.5 per cent starch.

Mold-bran

The mold-bran used in this investigation was prepared according to the method of Underkofler, Fulmer, and Schoene (1939). It was air dried and passed through a Wiley mill.

Barley malt

The barley malt, obtained from the Fleischmann Malting Company, was ground to a coarse powder in a burr mill.

Bacterial enzyme "Rapidase"

The sample of the bacterial enzyme "Rapidase" was obtained from Dr. Underkofler of the Chemistry Department of this College. "Rapidase" is prepared by the Wallerstein Company, New York City.

Malt extract

The malt extract used in this investigation to prepare beer wort for yeast cultures was Blue Ribbon Malt Extract commercially available from the Premier-Pabst Corporation, Peoria Heights, Illinois.

Miscellaneous materials

Shrimp powder: Dried shrimp was obtained from the open

market in Chicago. It was ground by passing once through a burr mill.

Compressed yeast was of the Fleischmann brand.

Soybean, corn, and corn gluten were of the commercial grades. The soybean was ground in a burr mill.

Water: Distilled water was used in all experiments.

IV. METHODS

A. Microbiological Procedures

Yeast culture

One hundred grams of malt extract was dissolved in distilled water to make 1 liter of solution. Such beer wort medium was placed in Erlenmeyer flasks: 30 ml. in 50 ml. flasks for carrying the cultures, and 300 ml. in 500 ml. flasks 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 a 50 ml. beer wort flask and incubated at 30° C. for 24 hours. By means of a sterile pipette 3 to 5 ml. of this yeast culture was transferred to another flask and incubated at the same temperature and time. Transfers were made in a similar manner, daily, throughout the course of this investigation, to maintain a vigorous culture of yeast.

The inoculum for experimental mashes was prepared by inoculating 500 ml. of beer wort with 15 to 20 ml. of a vigorous yeast culture.

Culturing of the butyl organism

About 0.2 to 0.4 gram of a spore culture in sand of Clostridium acetobutylicum designated in the collection of this laboratory as POS, was transferred by means of a sterile pipette of wide glass tubing into the bottom of a tube of sterile corn mash (6 per cent). The culture was given a heat shock by placing the tube in a beaker of vigorously boiling water, allowing it to remain in the bath for exactly two minutes, and cooling promptly in cold running water. The tube was then incubated at 37.5° C. After about 36 hours, when vigorous fermentation had begun, about two ml. of this culture was transferred by means of a sterile pipette into the bottom of a new tube of sterile corn mash and incubated as before. The third transfer was made into a flask of sterile corn mash containing sufficient volume to serve as inoculum for the experimental flasks. In some experiments, the third transfer was made into a flask containing a mash of the same composition as the experimental mashes; for example, a six per cent cassava mash containing a quantity of shrimp powder equal to three per cent of the weight of cassava. This was used for inoculating mashes containing shrimp powder. In all experiments the butyl cultures were started from one source which was a sample of a spore culture designated as POS. All inoculations were made from the third transfer.

Preparation of mold-bran

The method of preparing mold-bran was that developed by Underkofler, Fulmer, and Schoene (1939). The method essentially was as follows: a five gallon pyrex bottle was charged with 800 grams of wheat bran and thoroughly mixed by means of a wooden stick with 1000 ml. of 0.05 normal sulfuric acid. The bottle was plugged with cotton and sterilized under steam pressure for an hour. Upon cooling to room temperature, it was inoculated with 60 grams of a well sporulated culture of Aspergillus oryzae, designated in this laboratory as Number 2, prepared in the following manner: Sixty grams of wheat bran was thoroughly mixed with 60 ml. of water contained in a flask of liter capacity. The mixture was heated for an hour at 20 pounds steam pressure. Upon cooling, it was heavily inoculated with the stock culture of Aspergillus oryzae. After four to five days incubation at 30° C. a well sporulated culture was obtained, which was used for the inoculation of the five gallon drum, containing a sterilized mixture of 1000 grams of wheat bran and 1000 ml. of water.

Mixing of the contents was accomplished by rotating the drum for a few minutes. It was then allowed to remain at rest for 12 hours with continuous aeration. After this period, growth was well started and the drum was then slowly rotated, about one revolution per minute. The white mycelial development stopped and yellow spores began to appear, after forty to fifty hours.

The mold-bran thus formed was spread out on sheets of paper to dry in the air. The air dried preparation was ground in a burr mill and in this form was used for the experimental mashes.

B. Analytical Procedures

Determination of starch and of sugars

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 method of Shaffer and Hartmann (1921). The reagents were standardized by means of a sample of pure glucose.

All experiments in this thesis were carried out in duplicate or in triplicate.

Determination of ethanol

The entire volume of fermented mash or a measured aliquot of it was distilled and the first 100 ml. was collected. The volume distilled was kept below 300 ml. for preliminary runs had shown that with such volumes the ethanol was recovered quantitatively in the first 100 ml. About 0.5 gram calcium carbonate was added to the mash to neutralize acids present. The specific gravity ($25^{\circ}/25^{\circ}$) of the distillate was determined by means of a Chainomatic Westphal balance and the ethanol concentration in grams per 100 ml. of solution was read from a table. Temperature was controlled in the following manner: the distillate was cooled in running water to about 24° C.; on pouring into the specific gravity cylinder the temperature

was raised to exactly 25° C. by warming the cylinder with the hands and stirring by gentle rotation. The specific gravity float acted as a stirrer.

Determination of corrections for inoculum, mold-bran, and malt

The inoculum for the alcoholic fermentation was made of a solution of malt extract. When inoculated with yeast an amount of alcohol was formed and this was the correction for inoculum. However, it was found that in the presence of mold-bran or malt, a larger quantity of ethanol was obtained. A differential method was used to evaluate the corrections for the beer wort and the mold-bran. For beer wort the calculation was as follows:

4 g. mold-bran + 200 ml. beer wort = 6.14 g. ethanol
4 g. mold-bran + 100 ml. beer wort = 3.13 g. ethanol
100 ml. beer wort = 3.01 g. ethanol

The average of two determinations was 100 ml. beer wort = 3.03 g. ethanol. The direct fermentation of beer wort gave 100 ml. = 2.01 g. ethanol.

For mold-bran, the calculation was as follows:

8 g. mold-bran + 200 ml. beer wort = 6.29 g. ethanol
4 g. mold-bran + 200 ml. beer wort = 6.14 g. ethanol
4 g. mold-bran = 0.15 g. ethanol

The average of two determinations was 1.0 g. mold-bran = 0.0325 g. ethanol. From the direct fermentation of

mold-bran, 1 g. mold-bran = 0.0320 g. ethanol. Similarly, for barley malt 1 g. malt = 0.334 g. ethanol.

Calculation of ethanol yield

In an experiment the original mash contained the following: 40 grams cassava with a glucose equivalent of 85.2 per cent; 20 ml. beer wort as inoculum; and 4.2 grams mold-bran. The entire fermented mash (300 ml.) was distilled and the first 100 ml. distillate collected. The specific gravity ($25^{o}/25^{o}$) of the distillate was 0.9753 corresponding to 15.22 grams ethanol per 100 ml. distillate. The ethanol corrections are as follows:

for the 20 ml. inoculum:	= 0.606 grams ethanol
for the mold-bran: 4.2 x 0.0325	= 0.136 grams ethanol
Total	= 0.74 grams ethanol

Therefore, the quantity of ethanol from the cassava is 15.22 grams minus 0.74 grams or 14.48 grams. From the equation $C_6H_{12}O_6 \longrightarrow 2CO_2 + 2C_2H_5OH$, 180 grams of glucose would yield 92 grams of ethanol. Forty grams of cassava should then yield

$$(40 \times 0.852) \times 92/180 = 17.40 \text{ grams ethanol.}$$

The ethanol yield is therefore 14.48/17.40 = 83.1 per cent.

Determination of total solvents in the butyl-acetonic fermentation

The fermented butyl mash (the entire volume or an aliquot if it is larger than 300 ml.) was distilled. The first 100 ml. of distillate was collected and its specific gravity ($25^{\circ}/25^{\circ}$) determined. The total solvents were calculated from the Christensen-Fulmer equation (1935):

Total solvents, grams/100 ml. = 698 (1.0000 - sp. gr. $25^{\circ}/25^{\circ}$).

Correction for inoculum in the butyl-acetonic fermentation was determined by actual fermentation. For example, 370 ml. of a corn inoculum yielded 5.06 grams of total solvents; a ten ml. inoculum therefore was equivalent to 0.137 gram solvents.

Correction for additions of shrimp powder, soybean, peptone, compressed yeast, urea, and corn gluten were evaluated by determining the increase in total solvents produced by adding a known amount of the material to the corn mash. For example, 21 grams corn plus 1 gram shrimp powder yielded 4.06 grams solvents; 21 grams corn alone yielded 3.80 grams solvents. Therefore one gram of shrimp powder was equivalent to 0.26 grams total solvents. The corrections per gram of material found were 0.26 gram for shrimp, 0.48 gram for peptone, 0.002 gram for compressed yeast, and zero for urea, corn gluten, and soybean.

Calculation of yield in the butyl-acetonic fermentation

Yield in the butyl-acetonic fermentation was calculated as grams total solvents per 100 grams glucose equivalent. For example, from 21 grams of cassava having a glucose equivalent of 17.4 grams, 4.79 grams total solvents were obtained after deductions had been made for inoculum. The yield was therefore, (4.19/17.4) x 100 = 27.6 grams solvents per hundred grams glucose in the sample.

V. EXPERIMENTAL RESULTS

A. Alcoholic Fermentation

1. Studies on some factors affecting acid hydrolysis and the fermentation of acid hydrolysates.

The conversion of starch to glucose by acid hydrolysis has been used for years on an industrial scale. There have been numerous reports in the literature on the employment of acid saccharification in alcoholic and other fermentations. These studies have been only on laboratory scale and as far as known the alcoholic fermentation industry does not find the use of acids feasible. Nevertheless, data on the behavior of ground cassava root towards acid hydrolysis were deemed desirable.

a. Effect of acid concentration and the ratio sample: acid on the liquefaction of mash and on the per cent conversion to sugars. Ten grams of ground cassava root were thoroughly mixed with sulfuric acid solutions. The concentrations of acid were: 0.1, 0.2, 0.4, and 0.8 normal; the ratio grams sample: ml. acid solution was varied according to the proportions of 1:1, 1:1.5, 1:2, 1:2.5, and 1:3; for example, 10 grams of sample were mixed with 30 ml. of acid solution to make a 1:3

ratio. All the mixtures were heated under a steam pressure of 20 pounds for two hours. The degree of liquefaction of the samples was noted. If the sample was definitely liquid and flowed at room temperature it was designated + in Table I; if it caked solid and appeared dry, then it was designated — .

Sufficient water was added to the flasks to dissolve or suspend the hydrolysate and the solution diluted to exactly 100 ml. Sugars were determined from this solution by the Shaffer-Hartmann method. The data are collected in Table I.

TABLE I

Effect of Acid Concentration and the Ratio Sample: Acid on Liquefaction of Mash and on the Per Cent Conversion to Sugars

Acid Conc.	Sample: Acid (Dilution)	Milli-equivalent Acid	Liquefaction	% Conversion to Sugars
0.1	1:1	1	−	not determined
	1:1.5	1.5	−	"
	1:2	2	−	"
	1:2.5	2.5	−	"
	1:3	3	+	"
0.2	1:1	2	−	"
	1:1.5	3	−	"
	1:2	4	±	"
	1:2.5	5	+	19.8
	1:3	6	+	33.8
0.4	1:1	4	−	not determined
	1:1.5	6	±	33.4
	1:2	8	+	75.4
	1:2.5	10	+	93.0
	1:3	12	+	94.5
0.8	1:1	8	±	68.2
	1:1.5	12	+	87.0
	1:2	16	+	90.0
	1:2.5	20	+	94.5
	1:3	24	+	96.0

This experiment was carried out to determine the limiting value of the ratio sample: acid for satisfactory conversion to sugars. The results obtained indicated that sulfuric acid concentrations of 0.1 and 0.2 normal were not satisfactory. With 0.4 normal acid, 93 per cent of theoretical glucose was obtained when the sample: acid ratio was 1:2.5. When the acid concentration was raised to 0.8 normal, 87 per cent of theoretical glucose was obtained at a sample: acid ratio of 1:1.5 and conversions of 90 per cent or above were obtained at higher dilutions.

At equal quantities of sulfuric acid, the higher dilution (lower sample: acid ratio) favored higher saccharification. The conversion from 0.4 normal acid and 1:2 dilution was 75.4 per cent which is higher than the value obtained with 0.8 normal acid and a dilution of 1:1 although in both cases the total quantity of acid was 8 milliequivalents. Again with 0.4 normal acid and 1:3 dilution the conversion was higher than that with 0.8 normal acid and 1:1.5 dilution, the quantity of acid in both cases being constant at 12 milliequivalents.

It is to be noted also that 10 milliequivalents from a 0.4 normal acid gave a 93 per cent conversion while 12 milliequivalents from 0.8 normal acid gave only 87 per cent conversion; even 16 milliequivalents of the latter acid concentration did not give quite as high a result as the first acid concentration. That the higher dilution favored hydrolysis was also

borne out by later experiments.

b. Effect of acid concentration and of the ratio sample: acid on per cent conversion to sugars and to ethanol. Using the preceding data as a guide in selecting suitable sample: acid ratios, and employing the same procedure, the experiment was repeated. The weight of sample was increased to 30 grams, the cooking pressure to 25 pounds, and the cooking time to two and one-half hours. The hydrolysates were diluted to 200 ml. and 2 ml. of the solution was pipetted off for sugar determinations. To the main volume of hydrolysate (198 ml.) a sufficient quantity of concentrated ammonium hydroxide was added to bring the pH to 5. Inoculation was made with 10 ml. yeast culture. After incubation at 30° C. for seventy-two hours the alcohol was determined.

As in the preceding experiment the amount of sugars actually obtained by the hydrolysis was compared with the theoretical amount derivable from the starch. The comparisons are expressed in Table II as "% conversion of starch to glucose". The actual weight of ethanol obtained by fermentation was also compared with that which would have been obtained from the glucose present had the conversion been quantitative; the values so calculated are recorded in Table II under the heading "% conversion of glucose to Et OH". Similarly the per cent conversion of theoretical glucose to ethanol was also calculated.

TABLE II

Effect of Acid Concentration and of the Ratio Sample: Acid on Per Cent Conversion to Glucose and to Ethanol

Sample Number	Acid Conc.	Sample: Acid (Dilution)	Milliequivalents Acid	% Conversion Starch to Glucose	% Conversion Glucose to Et OH	% Conversion Theoretical Glucose to Et OH
1	0.1	1:4	12	17.3	41.4	7.2
2	0.1	1:6	18	37.4	72.3	27.0
3	0.2	1:2.5	15	32.2	52.4	16.8
4	0.2	1:3	18	40.1	72.8	29.6
5	0.4	1:1	12	19.7	71.3	14.1
6	0.4	1:1.5	18	61.2	81.4	49.6
7	0.4	1:2	24	86.4	76.1	65.8
8	0.4	1:2.5	30	98.8	69.5	68.9
9	0.4	1:3	36	95.6	73.6	70.8
10	0.8	1:1	24	83.2	75.5	63.0
11	0.8	1:1.5	36	90.8	71.6	65.1

Table II shows that under the conditions of the experiment satisfactory conversions of starch to sugars were obtained with 0.4 normal sulfuric acid at sample:acid ratios of 1:2, 1:2.5, and 1:3 and with 0.8 normal acid, at proportions as high as 1:1 and 1:1.5. The advantage of using as high a ratio of sample to acid as possible, is economy of autoclave space especially when the autoclave has to be of especial material resistant to acids.

As in the previous experiment, it is shown by the data in Table II that at equal quantities of acid the lower ratio of sample:acid favored higher saccharification as demonstrated by sample numbers 7 and 10, and 10 and 11. However, this was not always the case, for sample numbers 1 and 5, and sample numbers 2, 4, and 6 exhibit the reverse tendency. Both concentration of acid and dilution of sample seem to be important in saccharification by sulfuric acid. High acid concentrations promote hydrolysis of the starch to sugars but have a tendency to destroy the sugars formed. High dilutions of the sample prevent destruction of the sugars but slow down hydrolysis. In the present experiment an acid concentration of 0.4 normal and dilution of 1:2.5 or 1:3 showed the highest degree of conversion of starch to sugars.

The conversion of the sugars (formed by the acid hydrolysis) to ethanol by fermentation did not run parallel to the quantity of sugars present. Thus sample number 6 with only

61.2 per cent hydrolysis gave the highest ethanol yield of 81.4 per cent. This result is in accord with the findings of Severson (1937) who worked with the acid hydrolysis of corn.

The per cent conversion of theoretical glucose to ethanol seemed to increase as the ratio sample:acid decreased. This was invariably the case irrespective of the acid concentration. Thus sample numbers 5, 6, 7, 8, and 9 show a progressive increase of per cent conversion of theoretical glucose to ethanol.

c. Effect on ethanol yield of adding varying amounts of mold-bran to acid hydrolysate. Thirty grams of cassava flour were thoroughly mixed with 250 ml. of 0.1 normal sulfuric acid, gelatinized by heating over a burner, and autoclaved for two hours under a steam pressure of 20 pounds. Upon cooling, the pH was adjusted to 5 by means of concentrated ammonium hydroxide. The mash was saccharified with various amounts of mold-bran for one hour at 60° C. and then was inoculated with yeast. Alcohol was determined after incubation at 30° C. for fifty-two hours.

The results as shown in Table III indicate that the addition of mold-bran increased the alcohol yield. This is in accord with the findings of Schoene (1939) and of Schoene, Fulmer, and Underkofler (1940) who worked with corn. Under the conditions of the present experiment, the quantity of

mold-bran required to increase alcohol yield is about 10 per cent. In later experiments satisfactory yields were obtained with mold-bran alone without recourse to the use of acid. Acid hydrolysis, under the conditions adhered to in the present experiment, offered no advantage over straight mold saccharification.

TABLE III

Effect on Alcohol Yield of Adding Varying Amounts of Mold-bran to Acid Hydrolysate

Mold-bran, Per Cent by Weight Sample	Per Cent Et OH
0	74.4
1.7	76.2
5	76.4
10	79.2
20	81.6

d. Effect on ethanol yield of adding mold-bran, barley malt, inactivated mold-bran, inactivated barley malt, wheat bran, and yeast extract to acid hydrolysates. The method and quantities of materials used in this experiment were the same as in the preceding one. During saccharification, however, different materials were added to the samples. The mold-bran and barley malt were inactivated by mixing them with water and boiling for fifteen minutes.

The results as given in Table IV indicate that the function of mold-bran is that of a saccharifying agent. It seemed to serve as a source of nutrients only to a slight extent because the addition of inactivated mold-bran raised the alcohol yield somewhat; so did the wheat bran. Inactivation of mold-bran and malt lowered yields.

TABLE IV

Effect on Ethanol Yield of Adding Mold-bran, Barley Malt, Inactivated Mold-bran, Inactivated Malt, Wheat Bran, and Yeast Extract to the Acid Hydrolysate

Material Added	Material Added, % by weight of sample	Per Cent Et OH
none (control)	-	74.4
mold-bran	20	81.4
inactivated mold-bran	20	77.2
barley malt	20	68.8
inactivated barley malt	20	64.7
wheat bran	7.5	77.4
yeast extract (and 10% mold-bran)	2.5	80.0
yeast extract (and 10% mold-bran)	3.3	80.8

e. Effect on ethanol yield of varying the mash concentration of acid hydrolysates. Weights of cassava flour (15, 30, 45, and 60 grams) corresponding to 6, 12, 18, and 24 per cent

mashes were mixed in 500 ml. flasks with 250 ml. of 0.1 normal acid. The mixtures were gelatinized by heating and then heated under a steam pressure of 20 pounds for two and one-half hours. Upon cooling the hydrolysates were adjusted to pH 5 by means of concentrated ammonium hydroxide and inoculated with yeast. Incubation was made at 30° C. for seventy-two hours.

The results as given in Table V show that the highest alcohol yield was obtained with the lowest mash concentration studied. This is in accord with the results of the experiment recorded in Table II: the higher the dilution the greater is the conversion of theoretical sugars to alcohol. The higher dilutions studied in the present experiment may account for the higher conversions. With a 6 per cent mash, which would give a maximum of only 2.6 per cent beer, the actual conversion was 81.3 per cent. Proper dilution of acid hydrolysate seems to furnish as high alcohol yield as that obtained with mold-bran. The dilution necessary is so low however as to be of a distinct disadvantage in large scale fermentations.

TABLE V

Effect on Ethanol Yield of Varying the Mash Concentration of Acid Hydrolysates

Mash Concentration	Ratio Sample: Acid	Per Cent Et OH
6%	1:16.6	81.3
12	1:8.3	72.0
18	1:5.6	58.4
24	1:4.2	48.3

f. Effect of ethanol yield of employing various yeasts in the fermentation of acid hydrolysates containing mold-bran. Thirty grams of cassava flour were heated with 250 ml. of 0.1 normal acid for one hour at 20 pounds steam pressure. Upon cooling the samples were adjusted to pH 5 by means of ammonium hydroxide and treated with 6 grams of mold-bran for one hour at 60° C. The mixture was then cooled to 30° C. and inoculated with yeast. Yeasts designated as numbers 2, 16, 21, 35, and 43 were employed.

As shown in Table VI, all the yeasts studied, except number 2, gave alcohol conversions very close to or above 80 per cent. The highest yield was obtained from number 43 which is the yeast used throughout this study.

TABLE VI

Effect of Employing Various Yeasts on Acid Hydrolysate Containing Mold-bran

Yeast Number	Per Cent Et OH
2	70.8
16	79.8
21	79.0
35	80.6
43	81.8

g. Effect on ethanol yield of varying the acid concentration and the cooking pressure. Fifteen grams of cassava flour were gelatinized by heating with 250 ml. of acid solutions of various concentrations. The cooking pressures used were 0 (atmospheric pressure), 15, and 25 pounds. The cooking time was also varied: 15, 30, and 60 minutes (at 15 and 25 pounds steam pressure); and 60, 120, and 180 minutes (at atmospheric pressure). Cooking was considered started ten minutes after the autoclave had reached the desired pressure. Steam was turned off after the desired heating time had elapsed and the autoclave was cooled as rapidly as possible by a current of air. Twenty-five minutes were required to bring the pressure down to atmospheric without causing the samples to boil over. The pH of each mixture was then adjusted to 5 by means of ammonium hydroxide. Saccharification was carried out at 60° C. for an hour with mold-bran equal to 10 per cent by weight of the sample. The mash was cooled to 30° C. and inoculated with 20 ml. of yeast culture. Alcohol was determined after incubation for sixty hours. The results are tabulated in Tables VII, VIII, and IX.

TABLE VII

Effect on Ethanol Yield of Acid Concentration and Time of Cooking (at 25 pounds)

Acid Concentration (Normality)	Time of Cooking, Minutes (25 pounds)		
	15	30	60
0.0	75.6	78.6	78.6
0.02	71.6	75.5	77.4
0.05	79.7	80.5	84.5
0.10	84.0	84.5	86.4

TABLE VIII

Effect on Ethanol Yield of Acid Concentration and Time of Cooking (at 15 pounds)

Acid Concentration (Normality)	Time of Cooking, Minutes (15 pounds)		
	15	30	60
0.0	80.4	86.4	79.6
0.02	81.0	84.5	74.3
0.05	83.5	83.2	77.0
0.10	83.2	80.0	76.0

TABLE IX

Effect on Ethanol Yield of Acid Concentration and Time of Cooking at Atmospheric Pressure

Acid Concentration (Normality)	Time of Cooking, Minutes (Atmospheric Pressure)		
	60	120	180
0.0	80.9	84.1	83.6
0.1	85.4	83.6	84.1
0.2	78.4	79.6	84.5
0.5	81.0	80.1	83.6

At a cooking pressure of twenty-five pounds (Table VII), alcohol yields improved with increasing acid concentrations and increasing cooking times. This is readily explainable for both of these factors promote hydrolysis of the starch to fermentable sugars. When the cooking pressure was fifteen pounds the results were not so uniform. Alcohol yields increased with acid concentration only when the cooking time was fifteen minutes; with thirty minutes cooking the trend was the opposite. With sixty minutes cooking, the yields were comparatively low.

When the cooking was done at atmospheric pressure the results did not indicate any marked trend, although alcohol yields attained a quite uniform value when the cooking time was prolonged to one hundred and eighty minutes. Cooking at

atmospheric pressure seemed to give as good results as pressure heating. In general it may be concluded that the conversion of cassava flour to alcohol under the conditions studied, is a process in which the cooking pressure, acid concentration, and cooking time are not the only controlling factors.

h. Effect on ethanol yield of acid concentration and cooking pressure, the cooking time being constant at one hour. The results recorded in Tables VII, VIII, and IX are not directly comparable because the experiments were made on different days. To study the relation between cooking pressure and acid concentration, runs were made employing the same materials and methods of the preceding experiment. Mashes of different acid concentrations were cooked at the same pressure. For example, mashes having acid concentrations of 0.0, 0.05, and 0.1 normal were steamed at the same uniform pressure of 15 pounds.

TABLE X

Effect on Ethanol Yield of Acid Concentration and Cooking Pressure; Time of Cooking One Hour

Acid Concentration (Normality)	Cooking Pressure, Pounds		
	0	15	25
0.00	81.4	73.4	73.4
0.05	75.2	77.7	78.4
0.10	75.5	80.0	84.5

According to the results given in Table X, at cooking pressured of 15 and 25 pounds, the trend was for improved ethanol yield as acid concentration was increased. When the cooking pressure was atmospheric, the results were rather irregular. This may be due to a factor which was being eliminated by higher cooking pressures and stronger acid solutions.

On the whole these experiments show that ethanol yields of over 80 per cent can be obtained from the alcoholic fermentation of cassava without recourse to pressure cooking or acid hydrolysis.

1. Comparison of alcohol yield from cassava flour and from ground cassava root, acid hydrolysed at varying pressures. Fifteen gram samples of cassava flour and of ground cassava root were acid hydrolyzed as in preceding experiments with 0.1 normal acid for one hour. Cooking pressures of 0 (atmospheric), 15, and 25 pounds were studied. Upon cooling the acid hydrolysates were adjusted to pH 5 with concentrated

ammonium hydroxide. Mold-bran equal to 10 per cent by weight of sample was added and the mash was inoculated with yeast. Alcohol was determined after incubation at 30° C. for fifty hours. The results are summarized in Table XI.

TABLE XI

Comparison of Alcohol Yield from Cassava Flour and from Ground Cassava Root, Acid Hydrolyzed at Varying Cooking Pressures.

Sample	Pressure, Pounds		
	0	15	25
Cassava Flour	75.5	80.0	84.5
Ground Cassava Root	78.5	83.0	86.0

The above data show that the entire cassava root, peel and all ground together, is a better raw material for alcoholic fermentation than is cassava flour. The alcohol yields were invariably higher with the ground root than with the flour. This may be partly due to the higher percentage of protein in the root. Archbold (1903), in his analysis of Jamaican cassava, found the whole root to contain 2.45 per cent protein and the flour only 1.31 per cent. Monier-Williams (1922) also states that fermentation of concentrated worts are assisted by the presence of a certain amount of solid matter. The bark particles might be performing this role.

Besides offering improved yields of alcohol the ground root is much cheaper than the flour. A large part of the cost of preparing cassava flour comes from the peeling of the roots. As far as known ground cassava root has not been used in the industries nor studied in the laboratory.

2. Studies on methods of thinning cassava pastes.

Mashing may be defined as the process which renders a starchy material ready for alcoholic fermentation. The process may be considered to involve three steps: gelatinization of the starch, liquefaction of the starch, and saccharification. The final objective is a soluble transformation product which can be readily acted upon by yeast. Low mash concentrations of cassava do not present much of a problem; the higher concentrations offer difficulties mainly due to the thick, mucilaginous pastes obtained. Concentrated mashes are desirable because of resulting economy of space of cooker, fermenter, and still.

a. Gelatinizing temperature. In a 25 ml. Erlenmeyer flask, 2 grams of ground cassava root were mixed with 20 ml. of distilled water and heated slowly in a water bath. The temperature of the bath was gradually raised, the contents of the flask being stirred in the meantime. The temperature at which the mixture stiffened to a paste was taken as the gelatinizing temperature. This was found to be about 70° C.

For the purposes of mashing the above value was considered sufficiently accurate because all that was desired was the approximate temperature to which a cassava-water mixture must be heated to achieve gelatinization of the starch. The low value of this temperature is of significance. The starch-liquefying components of some amylases are still active at 70° C. although for only a short time. Amylases are known which are capable of attacking starch even at 85° C. For ease of mashing it would be desirable to have a gelatinizing temperature low enough not to inactivate amylase or to locate an amylase, sufficiently resistant to heat, to attack starch at or above its gelatinizing temperature. Two general procedures for the liquefaction of starch may be followed: First, the amylase may be heated together with the starch (and water) up to the gelatinizing temperature; the amylase would attack the starch granules during the process of swelling and the starch-water mixture would be expected to hardly pass through the gelatinized state at all. Second, the amylase may be added to the starch at a temperature above its gelatinizing temperature. These procedures are designed to avoid carrying out of the liquefying operation at temperatures lower than the gelatinizing temperature because then pastes encountered are thick and difficult to work with.

It was noted, however, that cassava pastes were fairly workable at 60° to 65° C. when they were used at once and not

allowed to cool down to room temperature.

b. pH of cassava mash. Cassava mashes were found to have a pH of about 7. As expected it varied from sample to sample, but only slightly. Thus 30 grams of cassava flour gelatinized with 250 ml. of distilled water gave a pH of 6.8. Ground cassava root under similar conditions gave a pH of 7.0. To determine the amount of acid required to obtain a desired pH, the following experiment was carried out: To 36-gram portions of ground cassava root, 250 ml. of sulfuric acid solution, containing a definite volume of 2 normal sulfuric acid, were added and the mixture was heated. The gelatinized mixture thus formed was autoclaved for forty minutes at 20 pounds steam pressure. The pH was determined upon cooling to room temperature (26° to 27° C).

TABLE XII

The pH of Gelatinized Cassava Containing Varying Amounts of Sulfuric Acid

ml. 2 Normal H_2SO_4 per 250 ml.	pH
0	7.07
1	6.55
2	6.10
3	5.28
4	4.68

c. Saccharification at 55° to 60° C. Cassava pastes were found to be difficult to mash at 55° to 60° C., the usual saccharifying temperatures, due to the stiffness of the pastes. To obtain a final alcohol concentration of 5 per cent, the original cassava mash must have at least 12 grams of material per 100 ml. mash. An even higher mash concentration would be advantageous and desirable. To overcome or avoid the formation of thick mashes, the operation known as thinning was given careful study.

d. Thinning with acid. When 40 grams of cassava was cooked with 200 ml. of 0.1 normal sulfuric acid for one hour at 20 pounds pressure, the result was a thin mash. Using 0.05 normal acid instead of 0.1 normal acid, the resulting product was not so fluid, but was sufficiently thinned to be workable.

Attempts were also made to employ sulfuric acid concentrations as high as 0.8 normal. At these high acid concentrations, the ratios of sample to acid studied were increased up to 1:1. Using 10 grams of sample and autoclaving for an hour at 20 pounds steam pressure, the ratio of sample to acid that gave a product that was more or less fluid and not caked hard were the following: with 0.8 normal acid, 1:1; with 0.4 normal acid, 1:1.5; with 0.2 normal acid, 1:2; and with 0.1 normal acid, 1:3. The product from this acid treatment was diluted with water and formed a rather thin mash. One objection

to the employment of acids is the necessity of neutralizing the excess acid.

e. Thinning with dry mold-bran. With 40 grams of cassava was mixed thoroughly 0.8 grams of dry mold-bran (2 per cent of weight of sample). Two hundred ml. of water at 85° C. were then added with continued stirring of the paste formed. The temperature of the mixture was a little above 70° C., which is the gelatinizing temperature. The mash was at first thick but liquefied sufficiently upon standing for fifteen to thirty minutes.

Mashes as concentrated as 24 grams per 100 ml. of mash were prepared with this method of thinning and found subsequently to give good yields of alcohol. However, 16 per cent mash is about the satisfactory upper limit with regards to ease of working. For the thicker mashes a larger amount of thinner could be used with advantage. In experiments on mold-bran, it was found that the amount of thinner could be increased to 3 per cent of the weight of the sample without detriment to the alcohol yield, although the amount of mold-bran for saccharification was reduced to 7 per cent. While this method was found to be very satisfactory with reference to the preparation of mashes, it suffers the disadvantage of losing a good portion of the active amylase of the mold-bran through the cooking subsequent to thinning. In this method the mash is first thinned and the cooking is performed later.

f. Thinning with mold-bran suspension. Takamine, Inc. recommend the use of 0.5 per cent by weight of sample of their taka-koji (a material similar to mold-bran) for thinning, the koji being made into a suspension in water. In line with this procedure, experiments were tried with mold-bran suspensions. To 100 ml. of distilled water warmed to 40° C., 1.8 grams of mold-bran were added and shaken vigorously. The bran particles settled to the bottom of the container; the supernatant liquid was light greenish yellow and somewhat turbid. Ten ml. of this suspension was used to thin cassava pastes in the following manner: The heated and gelatinized cassava paste was cooled to 74° C. and at this temperature, a volume of the prepared thinner containing a weight of mold-bran equal to 0.5 per cent of the weight of the sample was added and the mixture stirred. Liquefaction was found to take place speedily. While this method was found to be effective, it worked best at mash concentrations in the neighborhood of 12 per cent.

There were times when liquefaction by the above method did not produce consistent results, especially when the pH of the mash was lower than 5 and the temperature of thinning was greater than 70° C. In this case, the mash did not liquefy well and remained as a thick semiliquid. Such a mash, for some reason, refused to liquefy well even when additional mold-bran was added. The resulting alcohol yields were also

abnormally low. A properly thinned mash always separated into two layers, a water-thin supernatant liquid and a sediment layer. Such mashes consistently gave very satisfactory yields upon subsequent alcoholic fermentation. To obtain such a mash the procedure employed was as follows: The cooked cassava paste, fresh from the autoclave, was allowed to cool to 65° C. At this temperature the thinner, in the form of a suspension in water as described earlier, was added and the mixture was stirred. Liquefaction took place in a few minutes. After letting the mash stand for fifteen to thirty minutes a separation of the layers started to take place. This method was found to give consistently good results.

g. Temperature for thinning. A 12 per cent cassava paste prepared with sufficient acid to bring the pH to 5 and cooked for thirty minutes at 15 pounds pressure was found to be of sufficient consistency at 65° C. to allow mixing in of the thinner. More concentrated pastes were somewhat more difficult to handle. The pastes were more workable the higher the temperature but enzymic activity decreased quite rapidly at these high temperatures. The following experiment was undertaken to determine the upper limit of temperature for thinning. Thirty-six grams of cassava were gelatinized with 250 ml. of water containing 3 ml. of 2 normal sulfuric acid (to adjust pH to 5) and the sample was autoclaved for thirty minutes at 15

pounds steam pressure. The flask containing the sample was allowed to cool. As soon as the temperature reached 85° C. 10 ml. of mold-bran suspension containing 0.18 grams mold-bran was added and the mixture stirred with a glass rod. After fifteen minutes the sample was rapidly cooled to 30° C. by means of running tap water (14° C.). Employing the same procedure the temperature of the paste, at the instant of adding the mold-bran suspension, was varied from 80° C. to 65° C. The paste was considered well thinned if a separation into two layers was obtained after two hours standing. The results of these experiments are shown in Table XIII.

TABLE XIII

Effect of Temperature on the Thinning of Cassava Paste.

Initial Temperature	Temperature after Adding Thinner	Minutes Standing	Degree of Thinning
85°	83°	15	unsatisfactory
80°	78.5°	15	"
75°	74°	15	satisfactory
75°	74°	30	"
70°	69°	15	"
70°	69.5°	30	"
65°	64.5°	15	"
65°	64°	30	"

It is seen from Table XIII that satisfactory thinning was

obtained even at 75° C. This was the upper limit of temperature for the materials employed and the conditions of the experiment. The thinning or liquefaction of the pastes seemed to proceed more rapidly at the lower temperatures. This is probably due to the inactivation of a large part of the enzyme at the higher temperatures. For this reason the lowest feasible temperature is advisable in the thinning operation.

h. Thinning with bacterial enzymes. The mold-bran employed in the experiments described previously contained enzymes which were active at not higher than 75° C. An enzyme from Bacillus mesentericus has been known to liquefy starch even at as high a temperature as 95° C. Such an enzyme, known by the trade name of "Rapidase 10X", was obtained from the Wallerstein Company and its suitability for mashing cassava was determined in the following manner: Five-tenths of a gram of the enzyme in the form of a powder, was dissolved in 20 ml. of distilled water. A weighed amount of ground cassava root was mixed with enough distilled water at 70° C. to make 80 ml. of mixture. One ml. of the "Rapidase" solution was added by means of a pipette. The mixture was heated on a water bath to 80° C. The consistency of the mixture after ten minutes heating was noted. The results are shown in Table XIV.

TABLE XIV

Liquefying Action of the Bacterial Enzyme "Rapidase" on Cassava Pastes

Per Cent Mash	Grams Sample Per 80 ml. Mixture	Grams "Rapidase" Per 100 gram Sample	Liquefaction *
15	12	0.208	very satisfactory
17.5	14	0.179	" " "
20	16	0.156	" " "
25	20	0.125	slow, but satisfactory
30	30	0.104	quite thick; thinned slowly

The above results demonstrate the suitability of the bacterial enzyme "Rapidase" for thinning cassava pastes. The advantage of this enzyme preparation over mold-bran lies in its ability to act on the cassava starch at temperatures much higher than the gelatinizing temperature. Cassava mashes as concentrated as 30 per cent were obtainable when the amount of enzyme used was only 0.104 per cent of the weight of sample. This bacterial enzyme however has no saccharifying power. The starch is transformed only into dextrins. Mold-bran is therefore still needed for the formation of fermentable sugars. However, this bacterial enzyme is a very satisfactory solution to the mashing problem presented by the thick, mucilaginous character of concentrated cassava pastes.

3. Saccharification by mold - Bran

a. Mash concentration. Five hundred ml. Erlenmeyer flasks were marked to contain 250 ml. Various amounts of ground cassava root (20, 30, 40, 50, and 60 grams) corresponding to 8, 12, 16, 20, and 24 per cent mash were weighed in. A quantity of mold-bran corresponding to 1 per cent of the sample was added to the flask and mixed with the sample to act as thinner. Two hundred ml. of distilled water, heated to 85° C. were added with continued stirring by means of a glass rod. The pH was adjusted to a value of 5 by the addition of 2 normal sulfuric acid. The mixture was cooked for one hour at 20 pounds steam pressure. After cooking, saccharification was allowed to proceed at 60° C. for an hour, with 9 per cent by weight of mold-bran. Distilled water was added to make 250 ml. The mashes were inoculated with 10 ml. of yeast culture, incubated at 30° C. for 72 hours, and distilled. The results are shown in Table XV.

TABLE XV

Ethanol Yield From Cassava Mashes of Various Concentrations

Per Cent Mash	Grams Sample/250 ml.	Per Cent Et OH
8	20	83.8
12	30	83.8
16	40	87.2
20	50	82.7
24	60	81.8

Glucose in Sample: 79.1 Per Cent

The above results indicate that highest ethanol yields were obtained from 16 per cent cassava mash. The slightly lower yields at higher mash concentrations were perhaps due to the high concentration of ethanol obtained. Thus, the 24 per cent mash after fermentation, contained 20.36 grams of alcohol per 250 ml. of mash. This is equivalent to a concentration of 8.14 per cent ethanol which is about the limit for the growth of yeast unacclimatized to high alcohol concentrations. The higher mash concentrations were difficult to prepare due to the stiffness of the paste or gel formed. Without very vigorous stirring lumps were likely to form and remain unacted upon by the amylase. A mash concentration of 16 per cent seemed to be about the upper limit for this method of mashing in regard to ease of working.

b. Effect of mold-bran concentration on alcohol yields. Forty grams of ground cassava root were mixed with 0.8 gram mold-bran as thinner and mashed with 200 ml. of distilled water at 85° C. The pH was adjusted to 5 by means of 2 normal sulfuric acid. The mixture was autoclaved at 20 pounds for one hour and cooled. Saccharification was carried out at 60° C. for an hour using various amounts of mold-bran. The mashes were inoculated with 10 ml. of yeast culture, diluted to 250 ml., incubated at 30° C. for 72 hours, and distilled. The data obtained are given in Table XVI.

TABLE XVI

Effect of Mold-Bran Concentration on Ethanol Yields

Per Cent Mold-Bran Total	Per Cent Mold-Bran for Saccharification	Per Cent Et OH
2.5	0.5	64.0
5.0	3.0	71.4
7.5	5.5	78.5
10.0	8.0	82.0
12.5	10.5	88.8
15.0	13.0	84.5
Glucose in sample: 79.1 per cent		

The above results show that, for the sample of mold-bran employed, the highest yields of ethanol were obtained with a total of 12.5 per cent mold bran. Of this amount, only 10.5 per cent actually acted as saccharifying agent because the 2.5 per cent employed as a thinner was rendered inactive by subsequent cooking.

c. Effect of varying the amount of thinner. The amount of mold-bran employed as thinner plays an important part in the mashing process for the fluidity of the resulting mash depends on the amount of thinner used. The thinner, however, is later rendered inactive due to the subsequent cooking process. In the following experiment the total weight of mold-bran was kept constant at 10 per cent by weight of sample, but the weight of mold-bran employed as thinner was varied.

Forty grams of ground cassava root were mixed with a given weight of mold-bran as thinner and mashed with 200 ml. of water at 85° C. After the pH was adjusted to 5 the mixture was autoclaved at 15 pounds steam pressure for an hour and cooled. The mash was saccharified with sufficient mold-bran to make the total 10 per cent, inoculated with 10 ml. of yeast culture, and distilled after incubation for 72 hours at 30° C. The results are given in Table XVII.

TABLE XVII

Effect of Varying the Amount of Thinner on Ethanol Yields from Cassava Mashes Saccharified with Mold-Bran

Thinner		Ratio	Yield
Per Cent of Substrate	Per Cent of Total Mold-Bran	Thinner/Saccharifier	Per Cent Et OH
1	10	1:9	73.5
2	20	1:4	80.3
3	30	3:7	80.3
4	40	2:3	76.4
5	50	1:1	73.9

These data indicate that with this method mashing, and with the materials employed, 2 to 3 per cent mold-bran as thinner give the highest yields of alcohol if the total weight of mold-bran amounts to 10 per cent of the sample. With 3 per cent thinner, the mash is so fluid as to be easily workable. There is therefore a distinct advantage in increasing the thinner from 2 to 3 per cent, for there is a gain

in the workability of the mash without any accompanying decrease in yield. Since the thinner is not fully utilized for saccharification, being rendered inactive by subsequent autoclaving, these results indicate that, for the materials here employed, 7 per cent mold-bran is sufficient to carry on saccharification. The data also show the importance of thinning the mash, for in the runs employing 1 per cent thinner, fully 9 per cent was available for saccharification, but the alcohol yield was lower than in a well thinned mash in which only 7 per cent mold-bran was present for saccharification. It is, therefore, to be anticipated that if the mash could be thinned by some procedure to the same extent as that produced by 3 per cent mold-bran, the mold-bran required for subsequent saccharification could be reduced to 7 or 8 per cent. It might be pointed out, however, that it is not so much the amount of thinner used, as the degree of thinning obtained, for as was demonstrated by experiments to be described later, even 0.5 per cent mold-bran, properly employed, produced very satisfactory mashes.

d. Length of time of fermentation. Forty grams of ground cassava root, mixed with 0.8 gram mold-bran as thinner, were mashed with 200 ml. of water at 85° C. The pH was adjusted to 5 with 2 normal sulfuric acid. The mash thus obtained was heated at 15 pounds steam pressure for an hour and then cooled. Saccharification was carried out with 3.2 grams mold-bran at 60° C. for one hour. Inoculation was made

with 10 ml. yeast culture and incubation was at 30° C. as usual. A pair was distilled every 24 hours. Table XVIII presents results of these experiments.

TABLE XVIII

Effect on Ethanol Yield of Time of Fermentation of Cassava Mash Saccharified with Mold-Bran

Time of Fermentation, Hours	Per Cent Et OH
0	0
24	44.3
48	69.7
72	78.8
96	80.3
120	80.1

Glucose in sample: 82.8 Per Cent

The data show that beers should be distilled after 3 to 4 days incubation. This particular run was observed to be rather slow, but there was practically no change in the yield even after 120 hours or 5 days. Fermented mashes have a tendency to become sour due to contamination with acetic organisms. Since these fermentations employing mold-bran are not carried under strictly aseptic conditions, there is always the danger of acetification of the alcohol. The beer in this experiment attained a concentration of 5.15 per cent ethanol,

which is low enough to be conducive to attack by acetic organisms.

e. Effect of inoculum ratio. Forty grams of ground cassava root were mashed with 200 ml. water at 85° C. A weight of mold-bran equal to two per cent of the sample was used as thinner. The mixture was autoclaved at 15 pounds for 40 minutes. Upon cooling it was saccharified with 8 per cent mold-bran at 60° C. for one hour, inoculated with yeast culture in varying amounts, and diluted to 250 ml. Incubation was at 30° C. for 72 hours. The results are given in Table XIX.

TABLE XIX

Effect of Per Cent Inoculum on Ethanol Yield

Inoculum Per cent of Total Volume	Per Cent Et OH
2	77.2
4	78.2
6	80.1
8	77.6
10	77.6
12	77.2

The above data show that the volume of inoculum, in the range studied, does not appreciably affect the yields. The

highest yield was obtained, however, at 6 per cent. The amount of inoculum is a measure of the number of yeast cells introduced.

f. Effect of fineness of sample and of stirring during saccharification. A sample of cassava root was passed through a burr mill four times to increase the fineness of subdivision. Another sample was passed just once as usual. Mashes were prepared from these samples using 36 grams in 250 ml. water at 80° C. Thinning was carried out at 72° C. by the addition of 10 ml. of mold-bran suspension prepared by stirring 1.8 grams mold-bran in 100 ml. water at 40° C. The following variables were studied: fineness of sample, stirring during thinning, and cooking pressure. Saccharification was carried out in all samples by means of 10 per cent by weight of mold-bran at 60° C. for 1 hour. All were inoculated with 20 ml. of yeast culture incubated at 30° C. for 72 hours. The results are shown in Table XX.

TABLE XX

Effect on Ethanol Yield of Fine-Grinding, Stirring and Pressure of Cooking of Cassava Mash

Fineness	Stirring Time, Minutes	Cooking Pressure, Pounds	Per Cent Et OH
Fine	0	20	80.9
Coarse	0	0	78.6
"	30	0	80.9
"	0	20	77.1
"	15	20	78.7
"	30	20	77.6

Glucose in sample: 85.2 Per Cent

This investigation was carried out because the slop or residue after fermentation contained particles of the original material unacted upon by the mold amylase. A microscopic examination revealed pieces of the original root still retained their shape although the starch granules were swollen. It was thought that fine-grinding and stirring should eliminate these particles, for fine-grinding should give small particles that would be readily acted upon by mold amylase, and stirring should break any lumping that may occur. The pressure of cooking may also be a factor in the disintegration of the particles.

Higher yields were obtained when the sample was ground fine. High cooking pressure was of doubtful value; the lower

pressure even showed an advantage. This result is perhaps explained on the basis that the cassava starch granule is small, gelatinizes at a low temperature and thus "cooks" easily without the need of pressures above atmospheric. The advantage of stirring was not demonstrated in a clear cut manner. There was no improvement in alcohol yields with stirring when the cooking pressure was 20 pounds. But there was a slight gain with stirring when cooking was done at atmospheric pressure.

g. Length of time in the saccharifying bath. Thirty-six grams of ground cassava root was gelatinized with 250 ml. of water heated to 80° C. and autoclaved at 20 pounds for an hour. After cooling to 74° C. it was thinned with 10 ml. of a 1.8 per cent suspension of mold-bran and allowed to attain the temperature for saccharification by setting the flasks in the saccharifying bath which was held at 60° C. When this temperature was reached, 10 per cent by weight of mold-bran was added and the mixture was well stirred from time to time. The samples were allowed to remain in the bath for varying lengths of time. They were then removed and quickly cooled under the tap to room tamperature. The cooling required no more than two minutes for the tap water was at 13° C. The samples were inoculated with 20 ml. yeast culture, incubated at 30° C. for 72 hours and distilled. The data obtained are given in Table XXI.

TABLE XXI

Effect on Ethanol Yield of Length of time of Saccharification at 60° C. of Cassava Mash

Saccharification Time, Minutes	Per Cent Et OH
15	76.0
30	73.3
45	73.1
60	72.6

The results seem to indicate that a saccharification time as short as 15 minutes is conducive to higher yields. The lower conversions at longer periods may be due to the growth of thermophilic bacteria or inactivation of the enzyme.

h. Temperature of saccharifying bath. An experiment was carried out using the same method and amount of materials as in the preceding experiment, but the saccharification temperature was varied. The time of saccharification was kept constant at one hour. Results are given in Table XXII.

TABLE XXII

Effect of Temperature of Saccharifying Bath on Ethanol Yield from Cassava Mashes

Temperature °C.	Per Cent Et OH
30	75.5
40	68.2
50	73.5
60	72.6

While the yields in this experiment were rather low, the data show a tendency for higher yields at 30° C. Further trials at various times were run at the two temperatures, 30° and 60° C. The results, as shown in Table XXIII indicate that the saccharification process may as well take place at 30° C. as at 60° C.

TABLE XXIII

Comparison of Ethanol Yields from Cassava Mashes Saccharified at 30° C. and at 60° C.

Trial Number	30° C.	60° C.
1	76.4	79.0
2	76.6	72.4
3	80.2	78.4
4	80.2	78.9
5	84.2	83.5

The improvement in yield in the later trials, especially the last, is due to the thinning technique. The above results demonstrate that, under the conditions used in this experiment, the saccharification procedure wherein the mash is kept at 60°C. is a superfluous process and may be eliminated. That a temperature of 30° C. works just as well as 60° C. may be explained in the following manner: It is well known that saccharification of starch by amylase does not go to completion but reaches a state of equilibrium between the reactants and the products of reaction. Yeast enzymes act on the products, setting up another reaction. The process of conversion of starch to alcohol in the presence of amylase and yeast consists therefore, of two consecutive reactions which may be formulated thus:

Reaction 1 $\quad \text{Starch} + H_2O \xrightleftharpoons{\text{Amylase}} \text{Maltose}$

Reaction 2 $\quad \text{Maltose} + H_2O \xrightarrow{\text{Yeast}} C_2H_5OH + CO_2$

The first reaction proceeds forward and approaches completion because the product of reaction, maltose, is removed through the second reaction. If the second reaction is slow enough, that is, slower than the first reaction at 30° C. then there will be no difference in the alcoholic fermentation whether saccharification (Reaction 1) takes place at 60° C. or 30° C.

According to the above experiment, the role of the thinner is to convert the starch into soluble forms, commonly

designated as dextrins. The literature records this conversion to be optimum at about 60° C. and this is found to be the case. But the results obtained in this experiment tend to show that the conversion of the dextrins to maltose is better achieved at 30° C. rather than 60° C. The higher temperature for such a prolonged time as 1 hour may inactivate the mold enzymes. Since there is reason to expect that inactivation at the 60° C. bath is conditioned by the length of time the sample is kept at this temperature, this factor was further studied.

1. Interrelation of mold-bran concentration, length of time in the 60° C. bath, and yield of ethanol. Thirty-six grans of ground cassava root were gelatinized with 250 ml. of water, at 80° C., containing 3 ml. of 2 normal sulfuric acid (to adjust the pH to 5). Twenty-four such samples were prepared in 500 ml. flasks and heated at 15 pounds steam pressure for an hour. The samples were allowed to cool to 65° C. Ten ml. of a 1.8 per cent mold-bran suspension were admixed as thinner, and the mixture was allowed to stand for 15 minutes with frequent shaking. Three concentrations of mold-bran were studied for saccharification: 5, 7.5, and 10 per cent. The lengths of time in the saccharifying bath were 0, 15, 60, and 120 minutes. In the case of the "0" minutes saccharification the flasks were first cooled to 30° C. and then mold-bran was added. All the flasks were inoculated with yeast and incubated as in previous experiments. The results are given in Table XXIV.

TABLE XXIV

Effect on Ethanol Yield of Varying the Mold-Bran Concentration and the Time of Saccharification at 60° C. of Cassava Mashes.

Mold-Bran Conc.	Length of Time in the 60° C. Bath, Minutes			
	0	15	60	120
5.0	78.3	79.4	76.4	75.6
7.5	81.4	84.0	80.3	80.8
10.0	84.2	84.5	83.5	80.4

The results obtained in this experiment indicate a slight advantage of carrying out saccharification for just 15 minutes at 60° C. With these conditions of time and temperature it is found that 7.5 per cent mold-bran was sufficient for obtaining good yields of ethanol. The data given in Table XXIV show that alcohol yields are affected adversely when saccharification was allowed to proceed at 60° C. for one or two hours. In fact better results were obtained when the saccharifying or "malting" bath was entirely eliminated and the mold-bran introduced into the cassava mash at 30° C.

From the above results it was recognized that proper conditions for saccharification have a decided effect on yield. For this reason a study was carefully made of the process. Various temperatures of the saccharifying bath and different lengths of time in the bath were investigated. The method of preparing and thinning the samples was as in the preceeding

experiment. The proportion of mold-bran employed was 7.5 per cent of the weight of the sample. The runs at 30° C. were made in the following manner: To the prepared thinned sample, the required amount of mold-bran was added and the flask was allowed to remain at 30° C. for 60 minutes in one case and 120 minutes in the other before inoculation with yeast was carried out. All runs were in duplicate as usual. The results are presented in Table XXV.

TABLE XXV

Effect on Ethanol Yield, of Temperature and Length of Time in the Saccharifying Bath

Temperature of Saccharifying Bath	Time in the Saccharifying Bath, Minutes	Per Cent Et OH
30°	60	84.7
30°	120	84.6
40°	15	84.4
40°	30	84.0
40°	60	83.5
40°	120	81.8
55°	15	83.2
55°	30	83.2
55°	60	81.3
55°	120	80.6
60°	15	83.4
60°	30	80.0
60°	60	78.3
60°	120	73.2

The results obtained from this experiment as presented in Table XXV, demonstrate once more that the saccharifying or "malting" bath is not only unnecessary but has a tendency to lower yields. For this reason it may be eliminated, thus simplifying the alcoholic fermentation of starchy mashes by the elimination of one operation. The "malting" operation has always been considered very essential in the brewing and alcoholic fermentation processes employing malt for conversion of starch to sugars. According to Jacobs and Newton (1938), one objection to the use of malt as saccharifying agent in the commercial production of ethanol is the time and careful control required to hold the cooked starchy mash at a certain temperature to insure complete diastatic action. From the results obtained in the present experiment, it seems that this objection could be removed by the replacement of malt by mold-bran. With the employment of mold-bran, the saccharifying or "malting" bath may be eliminated. The mold-bran is simply introduced into the mash at 30° C. The temperature is not critical, for as demonstrated by the data in Table XXV, there is no practical difference in final ethanol yields whether the mold-bran is added at 30° C. or 40° C.

j. Saccharification at temperatures above 60° C. Employing a procedure essentially identical with that used in the preceeding experiment higher saccharifying temperatures were investigated: 65°, 70°, 75°, and 80°. The thinning was

started at 65° C. and saccharification was carried out at the desired temperature. For comparison, runs were also made at 60°. Saccharification at 60°, 65°, and 70° were carried for an hour. Saccharification at 65° was carried out for 1 minute and 3 minutes. Saccharification at 80° was only for 1 minute. The results are given in Table XXVI.

TABLE XXVI

Effect on Ethanol Yield of Employing Temperatures Above 60° C. for Saccharification of Cassava Mash

Temperature	Minutes	Per Cent Et OH
60	60	75.2
65	60	64.7
70	60	57.5
75	1	56.5
75	3	57.4
80	1	57.2

The above data demonstrate that temperatures above 60° C. give low yields of alcohol. These low results may be ascribed to destruction or inactivation of the mold enzymes.

k. Effect of employing different yeasts. The materials and methods in this experiment were the same as in previous runs. The improved method of thinning at 65° with 10 ml. of 1.8 per cent mold-bran suspension for 1 hour and saccharifying with 7.5 per cent mold-bran at 30° was employed.

The results are shown in Table XXVII.

TABLE XXVII

Effect on Ethanol Yield, of Employing Various Yeasts in the Fermentation of Cassava Mash

Yeast Number	Per Cent Et OH
2	78.0
16	80.2
21	82.2
35	80.9
42	84.0
43	83.5

The data obtained indicate yeasts Numbers 21, 42 and 43 were found best suited to the cassava substrate used in this investigation. Numbers 42 and 43 were slightly better than Number 21.

4. Saccharification with barley malt.

a. Effect on ethanol yields of varying the malt concentration. Thirty grams of cassava flour was gelatinized with 250 ml. of water containing 2.5 ml. of 2 normal sulfuric acid and heated for 40 minutes at 15 pounds steam pressure. After cooling, saccharification was carried out at 60° C. for an hour. Various proportions of barley malt were used. The mashes were inoculated with 20 ml. of yeast culture and alcohol was determined after incubation at 30° C. for 72 hours. Two runs were made on different days, each run being in duplicate as usual. The results are shown in Table XXVIII.

TABLE XXVIII

Ethanol Yield from Cassava Mash Saccharified with Various Concentrations of Barley Malt

Malt Concentration	Per Cent Et OH	
	1	2
1.7	45.5	49.3
5	59.6	55.0
10	68.4	62.3
20	69.5	66.7
Corn with 20 per cent malt	59.9	59.6

The above data show that ethanol yields increased slowly with increasing proportion of malt in the mash. The yields

were rather low even when the amount of malt used was 20 per cent of the cassava sample.

b. Effect on ethanol yield of varying the cassava mash concentration. Into flasks, marked to contain 250 ml. of mash, 20 grams of ground cassava and 0.2 grams of malt (1 per cent of sample) were introduced and mashed with sufficient water at 85° C. to make up a volume of 250 ml. In the same manner, mashes containing 30 grams, 40 grams and 50 grams cassava per 250 ml. of mash were prepared. All the samples were allowed to stand for 2 hours to complete the preliminary liquefaction. The mashes were then autoclaved at 15 pounds for 40 minutes. After cooling, saccharification was carried out at 60° C. for an hour employing a quantity of malt equal to 9 per cent of the sample. The inoculation with yeast and incubation at 30° C. were carried out as usual. The results are given in Table XXIX.

TABLE XXIX

Ethanol Yield from Cassava Mash of Various Concentrations Saccharified with Barley Malt.

Mash Concentration Per Cent	Ground Cassava per 250 ml. Mash	Per Cent Et OH
8	20	65.6
12	30	70.5
16	40	74.0
20	50	73.0

The data show that cassava mashes prepared with this sample of barley malt gave almost identical ethanol yields when the mash concentration was 12, 16, or 20 per cent. The yields were low indicating that malt does not act well on cassava mash.

c. Effect of varying the proportion of thinner to saccharifier on alcohol yields. The procedure employed in this experiment is identical with the preceeding. The amount of malt as thinner, however, was varied so that the thinner constituted 0, 1, 2, 3, 4, and 5 per cent of the sample. The corresponding amounts of malt employed for saccharification were 10, 9, 8, 7, 6, and 5 per cent of the sample. In other words, the total amount of malt was held constant at 10 per cent of sample. The results are given in Table XXX.

TABLE XXX

Ethanol Yield from Cassava Mash When the Proportion Thinner:Saccharifier was Varied; Barley Malt Used.

Thinner		Ratio	
Per Cent of Sample	Per Cent of Total Malt	Thinner:Saccharifier	Per Cent Et OH
0	0	0:10	67.2
1	10	1:9	67.9
2	20	1:4	64.6
3	30	3:7	67.2
4	40	2:3	66.9
5	50	1:1	67.5

The data show that, varying the proportion of thinner to saccharifier has no apparent effect upon alcohol yield. With mold-bran there was a definite gain in yield when the ratio of thinner to saccharifying agent was 1:4 or 3:7. All the mashes in the present experiment were quite thin and fluid. The apparent inability of barley malt to give higher ethanol yield may be due to the nature of the amylase or amylases present in malt. The difference in behavior between mold-bran and malt in this type of experiment lends support to this view.

d. Comparison of mold-bran and barley malt as saccharifying agents in the alcoholic fermentation of cassava mash. To 40 grams of ground cassava, 0.4 gram of mold-bran or barley malt was added as thinner and the mixture was mashed with 200 ml. of water at 85° C. The samples were then heated for an hour at 20 pounds steam pressure. Saccharification was carried out at the 60° bath for an hour; mold-bran or barley malt was employed depending upon which material was used as thinner. In one experiment the samples were thinned with malt and saccharified with mold-bran. As usual all the samples were inoculated with yeast and incubated for 72 hours at 30° C. The ethanol yields are presented in Table XXXI.

TABLE XXXI

Comparison of Mold-Bran and Barley Malt as Saccharifying Agents in the Alcoholic Fermentation of Cassava Mash.

Saccharifying Agent	Thinner	Per Cent Et OH
Mold-Bran	1 Per Cent Mold-Bran	86.5
Barley Malt	1 Per Cent Barley Malt	73.8
Barley Malt	1 Per Cent Mold-Bran	79.9

In this experiment the effectiveness of malt as a liquefying and as a saccharifying agent was demonstrated separately. The ethanol yield was low in the mash thinned and saccharified with malt. The yield increased when the thinning was carried out by means of mold-bran instead of malt. It may be concluded that malt amylase is rather deficient in the component which liquefies starch. It was observed however that the mashes which were thinned with malt were as fluid as those thinned with mold-bran. A possible explanation to these rather contradictory results may be offered: The amylase in malt differs in nature from that in mold-bran and the difference lies mainly in the starch-liquefying component. It is therefore probable that the primary conversion products from starch are not identical. The thinning action is then not only physical but also chemical. The products formed when mold-bran is employed to liquefy cassava starch,

are of such a nature as to be readily acted upon further by the amylase in both malt or mold-bran. When malt is used as the thinner the primary transformation products are probably more resistant to further action of the enzyme in malt.

Whatever the cause, the superiority of mold-bran over malt as saccharifying agent in the alcoholic fermentation of cassava mash has been demonstrated by this experiment.

B. Butyl-Acetonic Fermentation

1. Replacement of corn by cassava.

Three hundred ml. mashes were prepared in which the corn was progressively replaced by ground cassava. The weights of corn and of cassava are given in Table XXXII. The procedure was as follows: The weighed quantities of corn and cassava were placed in 500 ml. erlenmeyer flasks and water at 70° C. added and thoroughly mixed. The mixture was made to gelatinize by heating. The samples were then autoclaved at 20 pounds for 40 minutes, allowed to cool and inoculated with 10 ml. of a culture of the butyl organism in corn mash. The flasks were incubated at 37.5° C. for 72 hours and then total solvents determined by distillation. The results are given in Table XXXII.

TABLE XXXII

Yield of Total Solvents in the Butyl-Acetonic Fermentation of Corn Mash in Which 0 to 100 per cent of the Corn was Progressively Replaced by Ground Cassava

Per Cent Corn	Grams Corn	Grams Cassava	Grams Total Solvents	Grams Solvents per 100 grams of Glucose in Sample
100	18.0	0.0	4.16	30.22
80	14.4	3.6	3.95	28.30
60	10.8	7.2	4.50	31.70
40	7.2	10.8	4.61	31.93
20	3.6	14.4	4.44	30.21
0	0.0	18.0	0.74	5.00

Glucose in Corn: 76.5 Per cent; in Cassava: 82.8 Per cent

Table XXXII shows that butyl-acetonic fermentation did not take place to any large extent in a mash containing only cassava. However, a cassava mash containing 20 per cent corn ferments satisfactorily with yields of total solvents comparable to those obtained from a mash containing corn only.

Cassava mashes containing 0 to 30 per cent corn were further studied. Employing the same procedure as in the preceeding experiment and using a sample weight of 48 grams to make 800 ml. mash, the experiment was repeated. The replacement of corn in the mash was made in steps of 5 per cent. The results are given in Table XXXIII.

TABLE XXXIII

Yield of Total Solvents from the Butyl-Acetonic Fermentation of Corn Mash in Which Corn was Replaced by Cassava

Per Cent Corn	Grams Corn	Grams Cassava	Grams Total Solvents	Grams Solvents per 100 Grams of Glucose in Sample
30	14.4	33.6	11.16	29.0
25	12.2	35.8	11.03	28.6
20	9.6	38.4	10.64	27.4
15	7.2	40.8	8.95	22.9
10	4.8	43.2	6.66	17.0
5	2.4	45.6	3.98	10.1
0	0.0	48.0	0.76	1.9
100	21.0	0.0	4.92	31.7

Glucose in Corn: 74.0 Per cent; in Cassava: 82.8 Per cent

Table XXXIII shows that in order to obtain good yields of total solvents in the butyl-acetonic fermentation of cassava, at least 20 per cent of the mash should be corn. A deficiency of cassava in protein or nutrients may explain its low fermentability.

2. Effect of the addition of shrimp powder, corn gluten meal, soybean flour, compressed yeast, peptone, and urea to cassava mashes on the yield of total solvents. To 21 grams of ground cassava, 350 ml. of water were added and thoroughly mixed. The pH was adjusted to 6.5 by the addition of 0.5 ml. of 2 normal sulfuric acid. The shrimp powder, corn gluten meal, or soybean flour was then added. The soybean was best introduced by shaking the weighed quantity with a small amount of water in a test tube and pouring the resulting suspension into the mixture of cassava and water. Small amounts of soybean left in the test tube were rinsed out into the flask by means of a wash bottle. In the case of peptone, urea, and compressed yeast solutions or suspensions of known concentration were made and the desired quantity was pipetted off into the cassava flask. For example, 5 grams of peptone was dissolved in water to make 100 ml. of solution and 2 ml. were used for introducing 0.1 gram of peptone into a cassava mash. The mixtures were heated under steam pressure for 40 minutes at 15 pounds. Upon cooling the mashes were inoculated with 10 ml. of a culture of the butyl organism. The total solvents were determined after incubation for 72 hours at 37.5° C. In these experiments the sample of cassava employed had a glucose equivalent of 82.8 per cent. The correction for inoculum as determined by actual fermentation was 0.139 gram total

solvents per 10 ml. of inoculum. The other corrections, in grams solvents per gram material, were: for shrimp, 0.26; for peptone, 0.48; for soybean, compressed yeast, corn gluten meal, and urea, the correction was found to be negligible.

The results of this experiment are presented in Tables XXXIV, XXXV, XXXVI, XXXVII, XXXVIII, and XXXIX.

TABLE XXXIV

Yield of Total Solvents in the Butyl-Acetonic Fermentation of Cassava Mash Containing Various Amounts of Shrimp Powder

Per Cent Shrimp	Grams Shrimp	Grams Total Solvents	Grams Solvents per 100 grams Glucose
0.0	0.0	0.76	1.9
0.5	0.1	1.67	9.6
1.0	0.2	2.38	13.7
2.0	0.4	3.85	22.1
3.0	0.6	4.80	27.6
4.0	0.8	5.26	30.2
5.0	1.0	4.79	27.6
Corn	-	4.92	31.7

TABLE XXXV

Yield of Total Solvents in the Butyl-Acetonic Fermentation of Cassava Mash Containing Various Amounts of Corn Gluten Meal

Per Cent Corn Gluten Meal	Grams Corn Gluten Meal	Grams Total Solvents	Grams Solvents per 100 grams Glucose
0.0	0.0	0.76	1.9
0.5	0.1	0.69	3.9
1.0	0.2	0.69	3.9
2.0	0.4	2.28	13.1
3.0	0.6	3.32	19.1
4.0	0.8	4.01	23.1
5.0	1.0	5.07	29.2
Corn	-	4.92	31.7

TABLE XXXVI

Yield of Total Solvents in the Butyl-Acetonic Fermentation of Cassava Mash Containing Various Amounts of Soybean Flour

Per Cent Soybean	Grams Soybean	Grams Total Solvents	Grams Solvents per 100 grams Glucose
0.0	0.0	0.26	1.5
0.5	0.1	0.43	2.5
1.0	0.2	1.13	6.5
2.0	0.4	1.98	11.4
3.0	0.6	3.17	18.2
4.0	0.8	3.68	21.2
5.0	1.0	4.45	25.6
Corn	-	4.36	28.1

TABLE XXXVII

Yield of Total Solvents in the Butyl-Acetonic Fermentation of Cassava Mash Containing Various Amounts of Compressed Yeast

Per Cent Yeast	Ml. of 10 Per Cent Suspension	Grams Total Solvents	Grams Total Solvents per 100 grams Glucose
0.0	0.0	0.14	1.5
0.5	0.6	0.25	2.5
1.0	1.2	0.49	4.9
2.0	2.4	0.70	7.0
3.0	3.6	0.70	7.0
4.0	4.8	1.26	12.7
5.0	6.0	1.61	16.2
Corn	-	2.36	26.6

(Weight of Sample: 12 grams)

TABLE XXXVIII

Yield of Total Solvents in the Butyl-Acetonic Fermentation of Cassava Mash Containing Various Amounts of Peptone

Per Cent Peptone	Ml. of 5 Per Cent Peptone Solution	Grams Total Solvents	Grams Total Solvents per 100 grams Glucose
0.0	0	0.67	3.9
0.25	1	0.91	5.2
0.5	2	1.75	10.0
1.0	4	2.55	14.7
1.5	6	3.17	18.2
2.0	8	3.36	19.3
2.5	10	4.09	23.5
Corn	-	3.66	23.6

TABLE XXXIX

Yield of Total Solvents in the Butyl-Acetonic Fermentation of Cassava Mash Containing Various Amounts of Urea

Per Cent Urea	Ml. of 10 Per Cent Urea Solution	Grams Total Solvents	Grams Total Solvents per 100 grams Glucose
0.0	0	0.68	3.9
0.5	1	0.85	4.9
1.0	2	1.06	6.1
2.0	4	1.12	6.4
3.0	6	1.16	6.6
4.0	8	1.36	7.8
5.0	10	1.69	9.7
Corn	-	3.66	23.6

Tables XXXIV, XXXV, XXXVI, XXXVII, XXXVIII, and XXXIX present the results of attempts to locate substances which may serve as nutrients when added to cassava mash. As repeatedly shown in these experiments cassava by itself is a very poor substrate for the butyl-acetonic fermentation; the yields of total solvents were always very low. The addition of small quantities of shrimp powder, corn gluten meal, and soybean flour was found to increase the amount of total solvents to values comparable to those from corn. Corn was used as the reference material because it is known to be one of the best substrates for the butyl-acetonic fermentation. Shrimp powder as far as known has not been used previously in an investigation of this kind. Its effectiveness in promoting fermentation may be ascribed to its highly proteinous character. It is abundant in some localities like the Philippines and this fact would favor its employment as a nutrient in the conversion of cassava into butanol, acetone and ethanol.

Compressed yeast and urea in the quantities studied were not found suitable as nutrients. Peptone in small amounts served as an excellent nutrient. On a large scale fermentation however cost is a drawback to its employment.

SUMMARY AND CONCLUSIONS

1. A study of the fermentative utilization of cassava was undertaken. Both the alcoholic and the butyl-acetonic fermentation processes were applied to cassava. Acid, mold-bran, and malt were investigated as saccharifying agents.

2. In studies on the acid hydrolysis of very concentrated mashes, in which the proportion of sample to acid was carried as high as 1:1, it was found that conversion of starch to sugars was maximum when the ratio of sample to acid was 1:2.3 to 1:3 and when the concentration of the sulfuric acid was 0.4 normal. Yeast fermentation of the acid hydrolysates gave poor yields of ethanol; the addition of mold-bran improved the yields somewhat.

3. When mold-bran (in conjunction with acid) was employed for saccharification of mashes, cooking pressure and acid concentration were found to have little effect on the final alcohol yield.

4. Several methods of thinning cassava mash were studied because the usual method of introducing the mold-bran or malt into the mash at 55°-60° C. was found to be difficult. The difficulty lay in the mucilaginous character of cassava paste. Satisfactory thinning was accomplished by stirring the paste with a suspension of mold-bran in water. The process was

carried out between 60° and 75° C. (best at 65° C.) at which temperatures the cassava paste was sufficiently fluid for easy mixing. A bacterial enzyme known commercially as "Rapidase" was found to be a satisfactory thinning agent. Mashes which had been thinned by means of mold-bran gave consistently high ethanol yields upon saccharification and fermentation. In the thinning process the chemical change brought about by mold-bran is believed to differ from that brought about by malt because, although both of these amylolytic materials were able to liquefy cassava paste to the same extent, the ethanol yields varied according to which material was employed as saccharifying agent.

5. Yields well above 80 per cent of the theoretical were obtained in the alcoholic fermentation of cassava under laboratory conditions. The procedure which gave consistently good yields is as follows: A cassava paste is prepared containing 12 grams of cassava per 100 ml. of water. The paste is liquefied at 65° C. by means of a suspension of mold-bran in water; the amount of mold-bran is 0.5 per cent of the weight of the sample. A quantity of mold-bran equal to 7.5 per cent of the weight of the cassava, is introduced into the thinned mash at 30° to 40° C. The mash is inoculated with yeast and distilled after 72 hours.

6. The standard saccharification operation for other starchy mashes, known as "malting", was found to be unnecessary for cassava. A study was made of the effect on ethanol yields

of the length of time, the temperature, and the concentration of mold-bran for saccharification. The higher the temperature and the longer the time for saccharification the lower were the ethanol yields obtained. The best results were attained when the mold-bran was introduced into the mash at 30° C. No temperature control was necessary because yields were almost identical whether the operation was carried out at 30° C. or at 40° C.

7. The saccharification of cassava mash with barley malt was found to be unsatisfactory. The final ethanol yields were low. Mold-bran was found to be much more effective than barley malt.

8. The butyl-acetonic fermentation of cassava by itself was found to give very low yields of total solvents. The replacement by corn of 20 per cent of the weight of the cassava resulted in yields comparable to those from corn alone. The effect of the addition of various amounts of shrimp powder, corn gluten meal, soybean flour, compressed yeast, peptone, and urea on the yield of total solvents was investigated. Of these, shrimp powder was found particularly promising because of its ready availability in the Philippines and because it gave satisfactory yields of solvents when it was employed in small quantities.

9. For the alcoholic and the butyl-acetonic fermentations, it was found that the cassava root need not be peeled as is done customarily. The peeling process entails expense

and causes the loss of fermentable material. The unpeeled root when ground, was found to be as suitable for fermentations as the flour prepared from peeled roots.

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