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Evaluation of saccharifying methods for alcoholic fermentation of starchy substrates

Alice Lee
Iowa State College

NOTE TO USERS

EVALUATION OF SACCHARIFYING METHODS FOR ALCOHOLIC FERMENTATION OF STARCHY SUBSTRATES

by

Alice Lee

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Head of Major Department

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TABLE OF CONTENTS

I. INTRODUCTION

Ethanol fermentation is probably the oldest chemical process carried out by mankind. As a solvent of widespread application, alcohol is second only to water. Each year, alcohol by the millions of gallons goes into the making of acetaldehyde, acetic acid, ethyl ether, ethyl chloride, ethyl acetate, plastics, lacquers, polishes, plasticizers and rubber accelerators. Particularly in wartime, this versatile reagent is used in the production of explosives, chemical warfare gases, antifreeze and butadiene for synthetic rubber. In Europe and other foreign countries where petroleum must be imported, alcohol has long served as essential fuel.

Prior to about 1929, the fermentation of sugars and starches was responsible for virtually the entire commercial output of ethyl alcohol in the United States. Gradually, however, the manufacture of synthetic alcohol from ethylene began to account for a steadily increasing share of the nation's alcohol production. During World War II, the demands for industrial ethyl alcohol were multiplied four or fivefold above the prewar normal demands and it is interesting to note the tremendous increase in the use of grain for industrial alcohol production during that period.

Following the war the production and use of ethanol dropped rapidly but new uses for ethanol have again amplified the demand resulting in an increase in the total production of 300 million proof gallons in 1947 to 485 million proof gallons in 1953, of which approximately 40 per cent came from fermentation.

Grains are subject to wide fluctuations in price. At the present time, because of cost alcohol from grain in this country cannot compete with synthetic alcohol. However, in times of suddenly increased alcohol demand, like during the war, grain-fermentation does become exceedingly important.

Utilization of surplus grains by fermentation to produce ethanol has been considered and discussed for many years. Recently (25, 36, 53, 71, 107, 108, 109, 110), considerable interest has developed in the possibility of employing alcohol-water injection as a means of raising the "effective" octane number of gasoline. Automobiles equipped with high-compression engines should operate very efficiently with a dual fuel system, using conventional motor fuels and alcohol injection to prevent detonation and promote full efficiency under peak load and open throttle. Should this system be generally adopted, with all automotive equipment in the United States so equipped, the consumption of alcohols would reach about a billion gallons per year for this service.

This would be a tremendous outlet for fermentation ethanol and synthetic alcohols, which might well take care of all foreseeable grain surpluses.

At present considerable interest has developed in the possibility of using surplus wheat for wheat gluten and alcohol production. The wheat surplus continues to increase each year in spite of acreage control by government. Coupled with this surplus is a great demand for increased supplies of wheat gluten from which to make monosodium glutamate. Increased needs for gluten and wheat surpluses, with possibility of disposal of stored wheat by the government at low prices, keep up interest in a possible wheat alcohol program.

In the production of ethyl alcohol by the fermentation of starchy materials such as grains, it is necessary first to convert the starch to sugars upon which the yeast can act. In the United States the producers of alcohol from grain have been dependent upon barley malt for the conversion of starch to fermentable sugars. However, several replacements for malt have been developed.

Mold bran, the result of a 10 year pioneering research program conducted in four laboratories, Iowa State College, University of Idaho, University of Nebraska and Farm Crops Processing Corporation, was used very successfully as a

malt supplement toward the end of World War II. Large plant scale usage has proven that it gives slightly better yields of alcohol, shortens the time for complete fermentation, produces more yeast cells, and can be produced cheaply from abundant raw materials.

Another possible replacement for malt is found in the use of submerged fungal preparations. A great deal of work on this problem has been done by investigators at U. S. Industrial Chemicals Co., Joseph E. Seagram and Sons, and the Northern Regional Research Laboratory. It has been found that such submerged culture preparations can be used as efficient and inexpensive replacements for malt.

Since the saccharifying agents are much more expensive than the starchy materials, it is of great economical value to find a means for evaluating mold preparations other than the usual laboratory fermentation method. Reese, Fulmer and Underkofler (115) have reported the details of a rapid fermentation method for evaluating the ability of fungal amylase preparations to saccharify grain mashes. They found that the graphical intercepts for their mold brans were proportional to the optimum level of saccharifying agent for maximum alcohol production. Pool and Underkofler (106) on further studies on the method of Reese, Fulmer and Underkofler reported that the method may be used for comparative

evaluation of preparations of the same type from one mold.

The purposes of this investigation were to further check the method of Reese, Fulmer and Underkofler and to determine the optimum conditions for alcoholic fermentation of wheat flour.

II. REVIEW OF THE LITERATURE

A. Development and Use of Fungal Amylolytic Preparations

Although molds have been used for centuries in the Oriental countries for the saccharification of starchy grains prior to fermentation, it was not until near the end of the 19th Century that much scientific interest was given to the investigations of fungal enzyme preparations. Kozai (64) in his review of the early literature regarding the early studies of Aspergillus oryzae and its important industrial applications gave credit to Hoffman and Korshelt as the first writers on this subject. Korshelt (63), during a study of the preparation of Japanese sake found that an amylolytic enzyme was produced by a mold which he called Eurotium oryzae. Later this mold was renamed Aspergillus oryzae by other investigators.

Gayon and Dubourg (44) investigated Aspergillus oryzae and several species of Mucor and found that Aspergillus oryzae had by far the highest saccharifying ability. Calmette (19), in 1892, made the first scientific investigation of the microflora associated with the Chinese yeast cake. He found that a certain species of Mucor was the predominant mold which he gave the name Amylomyces rouxii.

Sanguinete (124) comparing the molds studied by Gayon and Calmette, found that Aspergillus oryzae was superior to Amylomyces rouxii in saccharifying power. However, Amylomyces rouxii had greater fermentative power and he concluded that Amylomyces rouxii was the most suitable for industrial use since it fermented starchy materials directly without the aid of yeast.

A large scale fermentation was carried out in a distillery by Collette and Boidin (24) using Amylomyces rouxii. A patent on the process was issued to them and this was the beginning of the Amylo process which has been used rather extensively in Europe. The details of the Amylo process were described by Galle (43) and Owen (95). Amylomyces rouxii later became known as Mucor rouxii. Sakaguchi and Okasaki (122) reported that the Amylo process is still widely used in Japan at present. The organism is Rhizopus javanicus Takeda isolated by G. Takeda (139).

Jokichi Takamine (136) was responsible for the introduction of amylolytic mold enzymes to the distilling industries of this country. He designated his mold preparation of Aspergillus oryzae on a bran substrate as "taka-koji" to differentiate it from the Japanese Koji which was the same mold grown on rice. Enzyme concentrates from extracts of taka-koji were labeled "taka-diastase". Takamine

developed methods for growing taka-koji on trays and later in rotating drums. He was also able to acclimatize the mold to the presence of certain disinfectants and was able to reduce contamination during the preparation of the amylolytic material.

Saito (120) isolated from wheat-flour cake used in the preparation of a Chinese beverage, two new species of Rhizopus, Rhizopus chinensis, and Rhizopus tritici which not only saccharified starch but fermented it to alcohol. Saito (121) also studied Aspergillus batatae, Aspergillus pseudoflavus, and Rhizopus japonicus with no outstanding saccharifying ability by any of these species.

Kita (59) studied the action of koji on starch and reported the presence of some enzyme system which converted starch directly to glucose. Despite his observation, it was generally believed that the saccharifying enzyme of koji resembled the beta-amylase of malt which converted starch into maltose. As will be noted later, recent investigators have supported Kita's observation.

Scales (125) prepared an enzyme concentrate by growing Aspergillus terricola on an artificial medium for 4 days, washing the mycelium with water, acetone, and ether, drying, and then grinding in a mill. This enzyme preparation was

able to produce over 82 per cent of fermentable sugars from a starch solution after three days incubation.

Collens (23) was able to produce 8.0 per cent more alcohol from the use of taka-diastase as saccharifying agent than from malt in his experiments dealing with the possibility of producing industrial alcohol from cassava.

The first indication that the amylolytic enzymes of molds were adaptive was reported by Euler and Asarnoj (35) in their studies with Aspergillus niger.

Kashiwagi (56) obtained a patent for the production of diastase by Aspergillus oryzae. The mold was grown on a substrate consisting of a residue following water extraction of germinated barley or wheat. After growth, the diastase was extracted by water and then precipitated to form a solid enzyme product. Rice, wheat bran or corn from which the starch had been removed could also be used to grow the mold.

Oshima and Church (93) made an intensive investigation of the molds isolated from koji in order to determine which ones produced the largest quantities of amylase. Aspergillus oryzae and a mold form intermediate between Aspergillus flavus and Aspergillus oryzae were found to be the most potent producers of amylase. This investigation was the first to show the great variation which may be found in

different strains of the same mold. They also studied mold growth and enzyme production on the following media: wheat bran, wheat middlings, corn meal, cocoanut meal, soybean meal, crushed soybeans, dried yeast, ground dried codfish, and casein. They found wheat bran to be the best substrate. Oshima (92) extended this study and found the activity of the enzyme to be greatest at pH 4.8 to 5.2. The enzyme was found to be heat labile becoming completely inactivated when heated at 85° C. for one hour.

Nishimura (88) found the following enzymes in taka-diastase: amylase, invertase, maltase, proteases, catalase, lipase, pepsin, lactase, inulase, sulfatase, and amidase. He was able to separate these enzymes from extracts of the taka-diastase by adsorption on fuller's earth from which they were eluted with a pH 8.0 phosphate solution.

Yamagishi (163) studied diastase formation by Aspergillus oryzae. The mold was grown on an autoclaved starch culture medium, using 0.2 to 2.0 per cent starch. He reported that the diastase production was directly related to the starch concentration in the medium and was inhibited by the addition of glucose.

Funke (42) found that the growth of Aspergillus niger and subsequent formation of amylase were inhibited by a brown substance formed from reducing sugars on sterilization

in the presence of alkali (or glass). Amylase production was apparently stimulated by the presence of glucose or starch while fructose, mannose, lactose, and inulin were inhibitory.

Alvik (4) studied the stability and action of mold diastases at various pH values. Aspergillus oryzae, Aspergillus niger, and three species of Penicillium isolated from soil were grown on a synthetic medium containing salts, glycine, and soluble starch. Glycine was found to improve the production of diastase. The ratio of diastase production to the mycelial mass of the mold was found to increase as the final pH of the culture increased. The pH ranges for diastase activity and the range for optimum activity varied with the source of the enzyme. Generally, the diastases were active over a pH range of 2.0 to 8.0 with optimum activity at around 5.0.

Harada (52) studied the preparation and properties of Aspergillus oryzae. The culture of mold was grown on cooked wheat bran containing 50 per cent water. Maximum growth was attained in two days. Harada found that the optimum pH for enzymatic activity increased with increasing temperature. At 30° C. the optimum pH was 5.2; at 65° C., it was 6.6. However, at temperatures below 50° C., the optimum pH remained practically constant at a value of 5.2.

Wei and Chin (157) studied ten species of Aspergillus and concluded that Aspergillus oryzae (AOLD) had by far the greatest saccharifying power of all the species examined.

Sakaguchi and Okasaki (122) in 1952 reported that an Amylo-koji process was developed in 1934 by S. Miyazaki and is the major method of saccharification in Japan at the present date. This method involves the saccharification of starch by the joint action of Rhizopus and Aspergillus oryzae. Alcohol yields are about 85 per cent of theory.

Takeda (139, 138) isolated twenty-seven strains of Rhizopus from raji-koji and soybean koji produced in Java and Sumatra. Only Rhizopus semarangensis and Rhizopus javanicus had strong amylolytic powers. Rhizopus javanicus was tried on a commercial scale in Japan and pronounced entirely satisfactory, and it is still used industrially in Japan.

Underkofler, Fulmer, and Schoene (149) in 1939 revived the taka-koji process of growing molds on wheat bran for replacing malt. They studied Aspergillus oryzae, Aspergillus flavus, Mucor rouxii, Rhizopus delemar, Rhizopus oryzae, Rhizopus peka I. Rhizopus tritici, Mucor circinelloides, and Mucor javanicus for amylase productions. The mold bran was prepared in aerated five-gallon rotating pyrex bottles. On the average, the use of mold bran for the saccharification

of corn mash for the alcoholic fermentation gave yields of ethanol about 12 per cent higher than those obtained with good dried barley malt.

The above work was extended by the same group of workers in 1940 (126) with a view to developing the commercial possibilities. They compared malt, mold bran and soybean meal as saccharifying agents and found the mold bran to be superior and the soybean to be the least efficient. Combinations of two of the enzymic materials gave somewhat increased alcohol yields over those obtained with a single preparation in some cases; and in other instances there was no change. Lower alcohol yields were obtained from grain mashes saccharified with dilute acids rather than with amylase. The addition of active mold bran to such acid-hydrolyzed corn mash resulted in normal alcohol yield.

Banzon (8) found that malt saccharification gave very poor alcohol yields from cassava whereas mold bran saccharification gave rather satisfactory results. The best alcohol yields were obtained when the mold bran was introduced into the mash to effect saccharification at 30° C. This procedure eliminates the customary malting conducted at elevated temperatures.

This study of the use of mold bran and acid hydrolysis was carried further by Goering (45). He worked on corn,

corn starch, and cassava starch, first hydrolyzing with acids and then fermenting. Mold bran was used in addition to hydrolysis by acids in some experiments. He observed that hydrolysis of corn meal gave a toxic material due to acid action on corn bran. Mold bran increased the yield of alcohol from the acid hydrolyzed starch mashes but malt did not, thus adding evidence to support the superior converting action of mold amylase.

Underkofler, Goering, and Buckaloo (150) attempted to grow Aspergillus oryzae on a number of different fibrous materials as substrates such as rice hulls, wheat bran, corn bran, oat hulls, cottonseed hulls, corn cobs, sawdust and peanut hulls. Only wheat bran and dry-milled corn bran supported adequate growth. They found the Lintner value, which is useful in the case of malt as an index of saccharifying power, was meaningless with regard to mold-amylase preparations.

Hao, Fulmer and Underkofler (50) studied the use of mold bran produced from a large number of amylolytic fungi by an improved laboratory technic. Batches from 27 strains of molds of four different genera were used to saccharify mashes made from pressure-cooked ground yellow corn. Low temperature saccharification (30° C.) proved satisfactory. In this investigation no malt was used and rather high

percentages of mold bran were required to obtain maximum alcohol yields. Yields of 95 per cent of theory were obtained from 20 per cent corn mash saccharified with mold preparations from twenty-three of the strains tested. The molds were cultivated on wheat bran medium in aluminum pots perforated on the bottom and equipped with aeration. Growth of molds in the pots was found to be more rapid and uniform, and the amylolytic activities were greater than when grown in the rotating drum used previously. Preparations from strains of Aspergillus oryzae, Rhizopus delemar and Rhizopus oryzae gave the best results. However, Aspergillus oryzae was considered most suitable because it gave more consistent results and can be handled more easily.

Underkofler in 1942 (144) patented a method of preparing mold bran in closed containers using wheat bran containing 40 to 70 per cent 0.3 normal hydrochloric acid as substrate. Severson, Underkofler, and Schoene (129) in the same year patented a saccharification process in which soybean meal was used to supplement the action of taka-koji.

Underkofler (145) and Underkofler and Fulmer (148) noted in their reviews of the status of the application of microbial enzymes to alcoholic fermentation that mold bran had been proven on a laboratory scale as a satisfactory substitute for malt for industrial alcohol production.

Their conclusions were later substantiated by Pan and Liu (99), Roberts, Laufer, Stewart, and Saletan (118), and Hao and Jump (51).

Hao and Jump (51) undertook a study of the mold and bacterial amylase preparations available on the market or potentially available. Their object was to settle the relative merit of these products as compared to malt. They showed the effect of using the various converting agents simply as liquefying materials, then of finishing the conversion with a single agent, "Converzyme". Successful liquefaction was obtained in all cases. The various agents were then tested for converting power and were shown to be equivalent to malt. They also showed that combinations of mold and malt gave yields above those obtained when preparations were used singly.

In 1943 and 1944 Christensen (20) was granted a number of patents on process and apparatus details connected with mold bran production.

Beginning in the spring of 1945, full plant scale tests were made on the use of mold bran in grain fermentations at the alcohol plant of the Farm Crops Processing Corporation at Omaha, Nebraska. A brief report on the economic and operating aspects were reported by Boyer and Underkofler (16) in 1945. A more detailed report was given by

Underkofler, Severson and Goering (153) in 1946. These tests confirmed the previous laboratory reports and proved conclusively that mold bran was entirely satisfactory for use on plant scale in that it results in slightly higher yields of alcohol than malt. Furthermore, the amount of mold bran required was considerably less than the malt requirement.

Underkofler (147) briefly reviewed, and Underkofler, Severson, Goering and Christensen (154) gave a detailed report on the production and use of mold bran from laboratory scale up to the full scale production of over 10 tons per day at the Mold Bran Company plant at Eagle Grove, Iowa. The economics of using mold bran were discussed in these papers. It has been shown by many investigators that there is no difference in the quality of the alcohol obtained either by malt or by mold bran saccharification. On an overall basis, somewhat better yields are obtained with mold brans.

Recently, Christensen and Underkofler (22) obtained a patent for saccharification with mold bran. The mold bran was prepared by mixing an equal weight of 0.1 normal hydrochloric acid, adding a nutrient salt solution and inoculating with Aspergillus oryzae spores.

The influence of the carbohydrate source in the medium for Aspergillus oryzae on amylase formation was investigated by Rao and Sceenivasaya (111). They found that starch, glucose, and maltose stimulated amylase production. Other carbohydrates which were tested were found to have no effect. In 1947, the same workers (112) reported work on the influence of inorganic nitrates, ammonium salts of inorganic acids, ammonium salts of organic acids and amino acids on the formation of amylase. They found good amylase production when ammonium salts of organic acids especially ammonium acetate were used as the nitrogen source. Bindal and Sreenivasaya (11, 12, 13) paid particular attention to amino acids and proteins as nitrogen sources. Aspergillus oryzae was cultured on acid-washed asbestos plus a nitrogen- and vitamin-free basal medium to which various forms of nitrogen were added. After growth at 30° C. for 4 days the material was extracted and the amylase activity was determined. They found that inorganic ammonium and nitrate compounds supported the mold growth and induced formation of amylase. Asparagine was better than tyrosine or glycine as a nitrogen source. A peptic digest of fibrin with complexity of 13 induced the formation of more amylase than papain digests with a complexity of 7, or hydrogen sulfide-activated papain digests with a complexity of 3.6.

Montes (78) in 1946 described promising experiments using mold bran on a laboratory scale for saccharification. He found the alcohol yields from mold bran saccharified mashes to be greater than were obtained by other methods of saccharification. Blaisten and Saad (15) in 1947 investigated the use of mold bran. They recommended the use of 4 per cent mold bran for industrial saccharifications.

Guagnini and Jacovkis (47, 48) in their studies on the utilization of enzymes of Aspergillus oryzae obtained 64 strains of Aspergillus, mostly Aspergillus flavus-oryzae, from natural sources. Promising organisms were chosen for further investigation on the basis of their amyloclastic power on 5 per cent potato starch. The mold preparations were found to contain a number of enzymes including amylase, invertase, maltase, lactase, glucosidase, proteinase and peptidase. The best culture medium was found to be wheat bran treated with an equal weight of 0.3 normal hydrochloric acid. They also described methods of extracting and preserving the enzyme activity. The alcohol yield was 93 per cent of the theoretical yield when 12 per cent fresh mold bran was used to saccharify corn mash.

The replacement of malt by Aspergillus oryzae mold bran in the alcoholic fermentation of corn was also studied

by Soriano and Trucco in 1948 (132). They obtained alcohol yields of 78 to 87 per cent theory.

Reindel, Frey and Lottner (116) in 1950 compared alcohol yields obtained from acid, mold amylase, and green malt conversion of potato flour milo meal and corn meal. In all, but one case, the mold amylase gave the best yields on corn and potato flour. The action of green malt, barley malt and Aspergillus niger and combinations of the latter two on various raw materials was investigated by Lampe (65) in 1950. The highest alcohol yields were obtained from mold saccharification. Milo maize, low-grade flour, rye, corn and dried potatoes were used as substrates.

A typical commercial method for preparing fungal enzymes on wheat bran was briefly described by Shellenberger (130) in 1947. Jeffreys (55) in 1948 described a tray method for commercial mold bran preparation.

Rao and Sreenivasaya (113) in 1950 obtained a patent on a process for growing Aspergillus oryzae on fibrous material for 3 to 6 days, then drying the entire mass to procure material having a high amylase activity.

Roy (119) reported combined barley malt and mold enzyme increased alcohol yield from corn starch fermentation to the maximum. Addition of crystalline limit destrinase to barley also increased the yield to maximum, which proved that limit

dextrinase is essential for high alcohol yield over and above those amylases present in malt.

With the development of submerged-culture methods for the production of antibiotics by molds, similar cultural procedures were successfully developed for making enzymes. Erb and Hildebrandt (33) in 1946 described a method somewhat similar to the amylo process in which both the fungal amylase produced in submerged culture and malt were used for saccharification purposes. They used a strain of Rhizopus delemar for saccharification of starch in the alcoholic fermentation of granular wheat flour mashes. The molds were grown on a medium consisting of grain stillage, nutrient salts, a small amount of aluminum powder or charcoal, and granular wheat flour. The wheat flour was pre-malted (about 2 per cent malt) before addition of the mold culture and yeast. The volume of submerged mold culture used for saccharification amounted to 6 to 12 per cent of the total volume of the fermenter mash. The mash was aerated for 3 to 4 hours and then allowed to ferment for 40 to 55 hours. About 12.1 proof gallons of alcohol were obtained per 100 pounds of dry grain, a 91.2 per cent fermentation efficiency, instead of the usual 11.0 proof gallons obtained by malt alone. The addition of charcoal or aluminum powder to the stillage was necessary in order to decrease its toxicity

toward mold growth. Essentially the same process was patented by Hildebrandt and Erb (54) in 1951. Submerged cultures of Aspergillus oryzae, Rhizopus boulard, or Rhizopus delemar were grown on grain stillage from wheat, corn, or rye mashes with the addition of 0.005 per cent aluminum powder and were used as saccharifying agents in alcoholic fermentations.

Van Lanen and LeMense (156) in 1946 reported on studies which were made in the attempt to develop submerged culture methods for preparing fungal amylase, suitable for adaptation to large scale production, for use in grain alcohol distillery operations. Over 350 various cultures of fungal amylases grown in submerged culture on thin stillage supplemented with 1.0 per cent corn meal and 0.5 per cent calcium carbonate to adjust the pH were tested. Enzyme production was determined both by their dextrinizing action on starch under specific conditions and the alpha-amylase values obtained according to Sandstedt, Kneen and Blish (123) method. Only 7 samples gave commercial possibility with a strain of Aspergillus niger being superior both in the production of alpha-amylase and in replacing malt.

This work was further studied by LeMense, Corman, Van Lanen and Langlykke (66). They attempted to develop an optimum medium for the production amylases by submerged

growth of a number of different molds. The best medium found consisted of thin stillage, 2 per cent corn, and 0.5 per cent calcium carbonate. Enzyme production was determined by alpha-amylase activity, maltase activity, and by actual fermentation. Maximum enzyme production was obtained when the initial pH of the mold medium was about 5.0. A volume of submerged culture equal to 10 to 20 per cent of the final mash was used for the conversion and alcohol yields up to 5.4 proof gallons per standard bushel was obtained. This process was patented by LeMense and Van Lanen (68) in 1948 for the preparation of fungal enzymes by Aspergillus niger NRRL 337.

Adams, Balankura, Andreasen, and Stark (1) in 1947 used Aspergillus niger NRRL 337 in a submerged culture growth on a medium containing 5.0 g. distillers' dried solubles and 1.0 g. ground corn meal per 100.0 ml. They obtained an average of 6.26 proof gallons per standard bushel as compared with 5.95 proof gallons from the malt controls. They found no correlations between alcohol yields and alpha-amylase activity or total saccharifying power determined by measurement of reducing sugars formed from soluble starch.

Corman and Langlykke (26) in 1948 reported that the efficiency of alcohol fermentation of grain was more closely related to the maltase content of the fungal amylase

preparation employed as conversion agent than to the alpha-amylase content. The molds were grown under aeration in a medium composed of distillers' thin stillage, one per cent corn, and 0.5 per cent calcium carbonate, having an initial pH of approximately 5.0. Laboratory scale fermentations were carried out using a volume of mold culture equal to 10 per cent of the final mash volume for saccharification. Several species and strains of Aspergilli were tested. Aspergillus niger 330, Aspergillus phoenicus 363, Aspergillus niger 326, Aspergillus niger 605, Aspergillus niger 337, Aspergillus oryzae 464, and Aspergillus wentii 378 gave greater alcohol yields than were obtained in the malt controls.

Le Mense, Sohns, Corman, Blom, Van Lanen and Langlykke (67) in 1949 described the application of submerged mold amylase on a pilot plant scale. They found that best results were obtained with the use of 10 and 20 volume per cent of mold culture or saccharification following pre-liquefaction of the mash by small amounts of malt and mold culture. They also found that the volume of mold culture used for saccharification was more important than the amount of dextrinizing enzyme (alpha-amylase) and that there was no correlation of alcohol yields with maltase activity. Their results showed that saccharification was satisfactory and

alcohol yields were comparable to those obtained with malt when mold culture liquor equivalent to 6 to 10 per cent of the final mash volume was used. The calculated saving for the replacement of malt by submerged mold culture was approximately 2.4 to 3.6 cents per gallon of 190 proof alcohol.

In 1949, plant scale fermentations using submerged culture fungal amylase for saccharification of the mashes was carried out at the Grain Processing Corporation, Muscatine, Iowa, under the direction of the Northern Regional Research Laboratory personnel. The results were reported in 1950 (155). They concluded that satisfactory fungal amylase may be produced on a large scale and in commercial equipment, in a medium consisting of distillers' thin stillage supplemented with 1 per cent ground corn and 0.5 per cent ground limestone. The yield of alcohol from sound corn saccharified with fungal amylase was at least equal to the yield obtained when malt was the converting agent. When fungal amylase was employed in the mashing of heat damaged corn the yield of alcohol obtained was no less than that obtained when malt was used. They also concluded that on the basis of cost calculations, there was a definite and significant economic advantage in using fungal amylase in place of malt for the conversion of grain mash.

Stark, Kolachov, Smith, Blankmeyer, and Willkie (134) in 1949 reviewed the use of amylase from submerged culture growth of Aspergillus niger. They estimated a savings of 2 cents per proof gallon would be obtained in replacing malt by fungal amylase. Alcohol yields were found to be 0.2 to 0.3 proof gallon per bushel greater than the yields from malt-converted mashes. Other advantages were more rapid fermentation rate and less bacterial contamination.

A very active amylase preparation was reported by Blaisten (14) in 1948 by incubation of Aspergillus oryzae of Aspergillus niger in a semi-submerged culture on wheat bran.

Manufacture of ethyl alcohol from sweet potato starch and tapioca starch was studied extensively by Japanese workers (76, 77, 90, 57) in 1948 and 1949 with yields approaching those obtained with starch from other sources. The Amylo process in which the substrate was hydrolyzed by Rhizopus was used.

Tsuchiya, Corman and Koepsell (143) in 1950 studied factors affecting the production of alpha-amylase and maltase by certain Aspergilli. The alpha-amylase production was found to be very dependent on the dried solubles concentration. Highest production of both alpha-amylase and maltase by Aspergillus niger 337 and Aspergillus niger 330 was

obtained in a medium containing 5 per cent distillers' dried solubles and 5 per cent corn. The composition of the medium had little effect on enzyme production of Aspergillus oryzae 458. They found, contrary to previous results, that if the pH was not allowed to drop below 4.0, calcium carbonate was not needed in the medium. A pH lower than this figure markedly curtailed the production of alpha-amylase, whereas the effect on maltase production was not so great. By using proper amount of corn and distillers' thin stillage, enzyme yields were substantially increased.

Pan, Andreasen and Kolachov (98) in 1950 reported that the fermentation of corn mashes, hydrolyzed by barley malt or submerged cultures of fungal amylase, consisted of a rapid initial phase and a slow secondary phase. In the secondary phase, dextrin slowly underwent hydrolysis and fermentation. When fungal amylase were used in combination with yeast, a rapid fermentation of the dextrins resulted, and they considered that this rapid fermentation was more closely related to the maltase activity of the culture than to the alpha-amylase or limit dextrinase contents. They found that the replacement of 3 per cent distillers' dried solubles by 2 per cent wheat bran in the mold medium resulted in better conversion. Their best alcohol yields were about 5.3 to 5.4 proof gallons per standard bushel.

Teixeira, Andreasen and Kolachov (140) in 1950 studied the conversion of cassava starch for alcoholic fermentation by malt, mold bran and submerged fungal culture. They showed that it is possible to obtain plant efficiencies of 90 per cent from cassava mashes converted with submerged Aspergillus niger 337 culture.

Dekenbrock (28, 29) in 1950 compared the malt process with the fungal amylase process and found that the main advantages of the fungal amylase process as compared with the malt process were greater alcohol yields, decreased fermentation time, and decreased foaming.

Goodman (46) compared sucrose and soluble starch as carbon sources for amylase production by Aspergillus terreus Thom, Aspergillus flavus Link and Penicillium notatum Westling. The molds were grown in submerged culture in a basal medium to which variable amounts of sucrose and/or soluble starch were added. The production of alpha-amylase was found to be dependent directly on the starch concentration.

Fukimbara, Yoshida, and Shibuya (40, 41) screened 54 strains of Aspergilli and found 5 strains of Aspergillus niger and 3 of Aspergillus oryzae gave high amylase production in submerged culture. The mold culture was used for the saccharification of sweet potato mashes for alcoholic

fermentation. The alcohol yield was higher for Aspergillus niger than for Aspergillus oryzae.

Ono and Dazai (89) reported the application of the amylo process to the alcoholic fermentation of wheat. Steam boiling with 0.66 per cent hydrochloric acid under 2.5 kg. per cm. pressure for 45 to 60 minutes was used. The best yield obtained was about 80 per cent of theory which agrees well with the yield obtained when the koji method was applied to wheat fermentation.

Ono and Tanaka (91) in their studies on the necessary concentration of amylase in alcohol mash found that the moromi mash, for the mother yeast, should be at pH 5.6 for the koji process with Aspergillus oryzae, pH 4.7 for the amylo process and pH 4.2 for the black koji process with Aspergillus awamori. They found the amylase of Aspergillus oryzae was unstable in comparison with that of Aspergillus awamori, therefore the acidification of the moromi mash must be prevented in the koji process. According to the abstract of this paper, "the necessary concentration of amylase for the saccharification of mash was found to be 30 for koji process, 5 for the amylo process and 10 for the black koji process."

Tomikanehara (142) reported on the results from the industrial scale application of liquid koji in alcoholic

fermentation. Three strains of Aspergillus oryzae, 3 of Aspergillus awamori, and 5 strains of Aspergillus usamii were selected by the alpha-amylase test and maltose activity. He found the cost of saccharification to be less, the acidity of the moromi mash was lower and fewer lees were produced.

Murota, Saruno, and Ano (79, 80, 81, 82, 83, 84) studied the production of alcohol by use of submerged cultures of fungi. Several strains of Aspergilli were selected on the basis of their saccharogenic and dextrinogenic activities on 1 per cent starch solution. They found a small amount of nitrogen source in the culture medium gave weak amylase activity, for industrial use 2 to 3 per cent wheat bran was the best nitrogen source. The optimum pH was found to be 4 to 5 and optimum temperature 40 to 60° C. They obtained satisfactory saccharification and fermentation of black-rotted sweet potato. Brandy was produced from rice as raw material. Submerged mold culture was kept from contamination during the preparation and storage by the addition of ammonium bifluoride.

In 1954, the same authors (85) reported the pilot plant scale use of submerged mold culture for saccharification in alcoholic fermentation. Experiments were made in a pilot plant tank used for penicillin production. Yields equivalent

to 88 per cent of theory were obtained with Aspergillus awamori.

Otani and Takahashi (94) reported that for saccharification with liquid koji, higher pressure in cooking gave higher saccharification rates, particularly with corn and rice starches.

B. Methods for the Evaluation of Fungal Amylolytic Materials

Starches are composed of two main components called amylose and amylopectin. Amylose molecules are composed of a large number of repeating glucose units in helical spirals connected by alpha-1,4-glucosidic linkages, while amylopectin is considered to be an irregular network of glucose units connected largely by alpha-1,4-glucosidic linkages but with a large number of branched positions at irregular intervals. The branched positions are points at which a glucose unit in a chain is attached to another chain through an alpha-1,6-glucosidic link.

The enzymes associated with the breakdown and synthesis of starch have been thoroughly studied. There are a number of reviews on the subject of amylase (10, 17, 75, 87).

Early workers noted that when starch was treated with amylases, there was first a very rapid decrease in the viscosity of the starch solution. This is followed by a gradual change in the color of the starch-iodine complex from blue-purple through red to colorless. There was a simultaneous increase in the reducing power of the solution. Also it was found that fermentable sugars were produced from starch through amylase action.

The more important carbohydrases in the saccharification of starch for alcoholic fermentation are alpha-amylase, beta-amylase, maltase and limit dextrinase.

Alpha-amylase is an enzyme of extremely wide distribution. There are some differences in the alpha-amylases from various sources. These differences are often due to varying substrate affinities and result in different reaction rates and degree of hydrolysis of starch. However, the over-all action is the same.

Alpha-amylase is described as a "dextrinizing" enzyme due to the production of lower molecular weight reducing dextrins from starch. The action of alpha-amylase is apparently a random hydrolytic cleavage of alpha-1,4-glucosidic linkages found in starch. There are three stages in the action on starches. First, the breaking of a very small percentage of the total glucosidic bonds results in

the formation of dextrins of considerably smaller molecular weight. In the next stage continued hydrolysis occurs with the production of smaller dextrins and a loss of the ability to form a colored iodine complex. In the final stage, limit dextrins and reducing sugars are slowly formed. Amylose is converted nearly completely into glucose, maltotriose, and maltose, the latter being the principal product. Amylopectin is converted into these reducing sugars in addition to limit dextrins. Limit dextrins are low molecular weight carbohydrates containing alpha-1,6-glucosidic linkages. A number of crystalline alpha-amylases have been prepared recently (2, 3, 18, 37, 73, 74, 86, 128, 131, 137, 152).

Methods of measuring alpha-amylase activity involve measurement of the change of viscosity, change in the color of the iodine complex and the production of reducing materials.

Beta-amylase is found mainly in plants, especially malt, soybeans and sweet potatoes. There has been considerable debate concerning the presence of this enzyme in molds. At present it is generally believed that molds do not produce beta-amylase. However, beta-amylase is the principal saccharifying enzyme in malt.

Beta-amylase, as does alpha-amylase, acts by the hydrolytic cleavage of alpha-1,4-glucosidic linkages in

starch. Its action differs, however, in that the attack is not random. There is a step-wise removal of two glucose units (i.e., maltose) from the non-reducing ends of the starch molecules, proceeding until the entire molecule is degraded, as is the case for amylose, or until some obstruction such as branching is encountered. Apparently alpha-1,6-glucosidic bonds are resistant to both amylases. Amylose is converted quantitatively into maltose and a very small amount of maltotriose. Amylopectin is converted into maltose and beta amylase limit dextrin, also referred to as alpha-amylodextrin. The latter is a higher molecular weight dextrin with low reducing power. Alpha-amylase can hydrolyze alpha-1,4-glucosidic linkages located between the branching points, thus freeing new non-reducing end groups for further beta-amylase action. The combined action of alpha- and beta-amylases yields the previously mentioned limit-dextrin.

Beta-amylase has very little effect on the viscosity of starch solutions. There is also little or no change in the iodine-complex color since alpha-amylodextrin forms a deeply colored complex with iodine. Since the principal action of beta-amylase is the production of reducing sugars, particularly maltose, its activity can be measured by any method which involves the determination of reducing sugars. The most widely used methods are various modifications of

the Lintner (69, 70) method and consists of the measurement of the reducing substances formed by the action of the enzyme preparation on a soluble starch medium under certain specified conditions.

Maltase is classified as an alpha-D-glucosidase since it cleaves the alpha-1,4-D-glucosidic linkages which joins the two glucose units in maltose. However, the maltases which have been found in many microorganisms seem to be somewhat less specific. They are able to hydrolyze higher carbohydrates such as starch and dextrins into lower molecular weight dextrins and glucose (26, 39, 58, 60, 103, 104, 159). Maltase activity may be measured by the change in optical rotation resulting from the disappearance of maltose and the appearance of glucose, or by measuring the increase in the reducing power.

Corman and Langlykke (26) found that their mold filtrates produced glucose from both maltose and higher polymers of glucose. They preferred the term "glucogenic enzyme" over "maltase". They measured the glucogenic activity by the extent of hydrolysis of maltose monohydrate into glucose in the presence of the enzyme solution.

Kerr, Cleveland, and Katzbeck (58) investigated a similar enzyme which they named "amyloglucosidase". This enzyme was found to produce glucose from amylose and amylo

pectin apparently by attacking the non-reducing terminals of the molecules.

Phillips and Caldwell (103, 104) investigated a semi-purified enzyme preparation from Rhizopus delemar. They designated this enzyme as "gluc amylase" since it produced only glucose from amylose and hydrolyzed maltose quantitatively to glucose. Glucose was also produced from amylopectin and no large dextrins remained. Gluc amylase was unable to hydrolyze isomaltose but it can apparently by-pass 1,6 linkages present in dextrins.

Kitahara and Kurushima (60) found evidence of an amyloglucosidase in a filtrate of Aspergillus awamori which they referred to as gamma-amylase. This enzyme produced glucose and maltose in the ratio of 4/1 from soluble starch and thus should be classified as an amyloglucosidase. In their further work, the same workers (61) obtained a purified enzyme from Aspergillus awamori. The enzyme was free of maltase and alpha-amylase activity and produced glucose and maltose in the ratio of 2/1 from soluble starch. They obtained a similar enzyme from Aspergillus cinnamoneus and Aspergillus betatae.

Limit dextrinase is still another enzyme shown to be present in the fungal amylase preparations and to a lesser extent in malt. The action of limit dextrinase is the

hydrolytic cleavage of the alpha-1,6-glucosidic linkages in amylopectin and dextrins which resist cleavage by the other carbohydrases. Limit dextrinase activity may be measured by the method developed by Back, Stark and Scalf (7). This method involves treating malt dextrin (prepared by the action of barley malt diastase on corn starch) with the enzyme preparation and then measuring the amount of glucose produced.

Pigman (105) studied the extent of hydrolysis of starches by amylases in the presence and absence of yeasts. He found that malts and mold enzymes (Aspergillus oryzae and Aspergillus niger) generally gave 97 to 100 per cent conversion of corn starch in the presence of yeast. In the absence of yeast, malt gave about 90 per cent conversion and mold only 70 to 80 per cent. He suggested that fungal enzymes preparations were able to synthesize non-fermentable materials from maltose. Removal of maltose by the action of yeast prevented this synthesis. This observation was later confirmed by Schwimmer (127).

Pan, Andreasen, and Kolachov (96, 97, 98) studied the role of yeast in the secondary stage of fermentation. They found that dextrins are hydrolyzed by fungal enzymes into fermentable sugars during the secondary fermentation stage which are then utilized by the yeast. In the absence of

yeast, this conversion is inhibited by the accumulation of reducing sugar products. They found that maltose, but not glucose, was responsible for the inhibition and the role of the yeast in aiding secondary conversion was the removal of maltose.

Pan, Andreasen, and Kolachov (97) and Pan, Nicholson, and Kolachov (100) found that a filtrate from submerged culture of Aspergillus niger 337 converted maltose into an unfermentable dextrin. This dextrin was found to be a trisaccharide consisting of only glucose residues. The dextrin formation was reversible, as indicated by the fact that in the presence of yeast, the mold filtrate was able to convert the dextrin into fermentable sugars. French (38) suggested and Wolfrom, Thompson, and Galkowski (161) confirmed that the tri-saccharide which was called "panose", was 4-alpha-isomaltopyranosyl-D-glucose.

Pazur and French (102) described a carbohydrate-synthesizing enzyme present in the filtrate of the mold, Aspergillus oryzae. The mechanism postulated for the synthetic action was termed transglucosidation and involved a transfer of the terminal glucose residue of maltose to 6-position of a co-substrate saccharide. The synthetic action resembles closely the reverse of limit dextrinase action.

Weill, Burch and Van Dyk (158, 159) reported an amylase preparation from Aspergillus niger NRRL 599 which yielded large amounts of glucose as an end product in the hydrolysis of starch. Hydrolysis of both starch and maltose by this enzyme produced solutions which exhibited rising mutarotation.

Much of the work in regard to the evaluation of amylolytic preparations has been done on malt. The various methods used may be classified under the following headings:

1. Polarimetric methods.
2. Viscometric methods.
3. Iodometric methods.
4. Methods involving the determination of reducing power.
5. Direct determinations.

The first and the last methods are concerned with the combinations of enzyme actions, the 2nd and 3rd with the action of alpha-amylase and the 4th method with saccharifying action such as action of beta-amylase and amyloglucosidase.

The diastatic power test for malt, which is principally a measurement of beta-amylase, was at one time the most commonly used criterion for measuring the potential performance of barley malts used in the distilling industry.

Thorne, Emerson, Olson and Peterson (141) in 1945 evaluated malts used for the production of alcohol from wheat by extensive analytical and fermentation tests and come to the conclusion that only a fermentation test can give an accurate evaluation. For example, some malts that a distiller would have discarded on the basis of Lintner analyses proved in fermentation tests to be as good as other malts having much higher Lintner values.

Four research laboratories (160) collaborated in a program to study the relationship between alcohol yield and such factors as beta-amylase, alpha-amylase, wort nitrogen, Kjeldahl nitrogen, and proteolytic activity. Their results showed that the beta-amylase test was not a reliable index and that, of all the factors investigated, alpha-amylase is the most significant criterion for predicting alcohol yield.

Later in 1948, Kneen and Spoerl (62) published a method for determining limit dextrinase activity in barley malts. They analyzed samples of barley malt for alpha-amylase, beta-amylase, and limit dextrinase. Alcohol yield was checked on grain starch fermentation. They pointed out that their data were limited, but concluded that it is significant that, for the malts used, limit dextrinase is the only one of the three carbohydrase activities determined

that would be of value in predicting potential efficiency in alcohol production.

Whitehouse and Adams (160) in 1954 investigated the relation of alpha-amylase and limit dextrinase of barley malt to the production of ethyl alcohol from grains. They analyzed their data statistically, and concluded that measurement of alpha-amylase activity provides a reliable index of malt performance, and that limit dextrinase, because of its direct proportionality to alpha-amylase, may also be used as a reliable index of performance. They found alcohol yield is independent of limit dextrinase activity; the utility of the limit dextrinase determination is apparently dependent upon its relationship to alpha-amylase activity.

With regard to evaluation of fungal enzyme preparations, Underkofler, Goering and Buckaloo (150) found no correlation between Lintner value of the mold preparations with the saccharifying efficiency. Since the Lintner value corresponds to beta-amylase activity, it is not surprising that they obtained no positive correlation. It was widely observed that, in general, amylolytic preparations high in alpha-amylase activity became the criterion for the screening of organisms used in the production of mold bran and submerged culture preparations.

Adams, Balankura, Andreasen and Stark (1) reported that there was no correlation between the alpha-amylase activity of submerged cultures of Aspergillus niger 337 and the alcohol yields obtained from the mashes converted by the fungal preparation. LeMense, Sohns, Corman, Blom, Van Lanen, and Langlykke (67) agreed with this observation and found that the volume of submerged fungal culture used for saccharification was more important than the amount of dextrinizing enzyme.

LeMense, Corman, Van Lanen, and Langlykke (66) observed a positive correlation between maltase activity and the alcohol yields. It was important that the mold cultures, however, had a measurable amount of alpha-amylase.

Corman and Tsuchiya (27) in 1951 confirmed their previous findings that the alcohol yield from grain mashes converted with fungal amylase was positively correlated with the maltase content of the latter provided an adequate amount of alpha-amylase was present. Pool and Underkofler (106) in 1953 reported that they could find no direct correlation between alcohol yields from grain mashes saccharified with fungal amylase preparations and the absolute amounts of alpha-amylase, maltase, and limit dextrinase in either bran or submerged culture preparations of Aspergillus oryzae or two strains of Aspergillus niger.

Erb, Wisthoff, and Jacobs (34) in 1948 investigated another method for the evaluation of submerged fungal cultures. They determined the saccharogenic activity of fungal amylases by the ferricyanide titration of the reducing sugars formed by the action of enzyme preparations on soluble starch. The same method was reported by the U. S. Production and Marketing Administration and Bureau of Agricultural and Industrial Chemistry (155) in 1950 for the plant scale experiments at the Grain Processing Corporation at Muscatine, Iowa. They found a positive correlation between saccharogenic power and the alcohol yields. However, their data also indicate just as good a correlation between maltase or alpha-amylase activity and alcohol yield.

Drews, Lampe, and Specht (31) in 1950, after comparing methods for the evaluation of amylolytic activity, stated that the most reliable method was the determination of the alcohol yield in a fermentation test after saccharifying the starchy material with the sample to be tested.

Reese (114) and Reese, Fulmer, and Underkofler (115) described a short starch fermentation test for evaluation of fungal amylolytic materials. The alcohol yield is determined following a 24-hour fermentation of cornstarch saccharified by the amylolytic material to be evaluated. The ratio of mold bran/alcohol yield was plotted against

the weight of mold bran, and straight lines were obtained. Parallel lines with different intercepts were obtained with a number of Aspergillus oryzae 38 mold brans investigated. They found the lower the intercept value, the higher was the saccharifying efficiency of that particular mold bran. The ratio of the intercepts for two mold brans was equal to the ratio of the weights of the same mold brans necessary to obtain optimum alcohol yields. Thus, by running a standard mold bran at different levels to find the optimum and then comparing its intercept with the intercepts of other mold brans in the short fermentation test, the optimum levels for other mold brans can be calculated. Excellent results were obtained using this procedure for the evaluation of Aspergillus oryzae 38 mold brans. This procedure can be used to determine the levels of different mold brans to be used to obtain maximum yields but does not indicate definitely which mold bran will give the highest alcohol yields.

Pool and Underkofler (106) further investigated the short starch fermentation test and concluded that the method could not be used for the evaluation of different preparations, i.e., for the comparison of preparations from two different organisms or the comparison of different types of preparations from the same organism. They also noted

that this test was too rapid for organisms such as Aspergillus niger 330, which produce only small amounts of alpha-amylase.

III. MATERIALS

A. Materials for Fermentations and Media

1. Corn meal

The corn which was used for the early corn fermentations was obtained as No. 1 whole kernel yellow corn in a 100 pound lot from the Quaker Oats Company, Cedar Rapids, Iowa in June of 1949. It was ground, thoroughly mixed, and stored in screw-topped bottles until used. The corn was fumigated by pushing a test tube containing about 5 ml. of carbon disulfide upright into the ground corn and screwing the cover on tightly. As this supply ran out, a 100 pound lot of fine ground whole yellow corn was ordered from Gilchrist Feed Co., Ames, Iowa in August, 1953. The corn was again stored and fumigated in screw-topped bottles until used. Moisture and starch determinations were run on the corn in each lot.

2. Wheat bran

The history of the bran used for the preparation of bran cultures and mold brans in this investigation

is unknown except that it was obtained prior to 1946.

3. Distillers' dried solubles (DDS)

This material was obtained in a 100 pound lot from Joseph E. Seagram and Sons, Inc., Louisville, Kentucky. It was described as "A dried concentrate of the soluble portion of stillage obtained in the distilling of cereal grains, principally corn, also some barley malt." The following analysis accompanied the dried solubles:

Protein	not less than 30%
Crude fat	not less than 5%
Fiber	not more than 5%
Ash	not more than 6%
Moisture content	not more than 7%

This material had a tendency to take up water, forming hard cakes. Consequently it was divided into smaller portions and placed in screw-topped bottles until used.

4. Corn starch

The starch used in the short starch fermentation tests was obtained in a 100 pound lot from American Maize-Products Company in July, 1946. The glucose equivalent was 92.50 per cent on starch "as received" with moisture content of 11.40 per cent on the starch as it came from the sack.

5. Barley malt

Barley malt was obtained from Columbia Malting Co., Chicago, in February, 1953 as Distillers malt. The following analysis accompanied it:

6.8% Moisture

177-188° Lintner

6. Malt extract

The malt extract used to prepare media for yeast cultures was obtained in a 60 pound drum from the Pabst Brewing Co., Peoria Heights, Illinois, in October, 1951. This material was designated as "Bakers Syrup".

7. Yeast extract

The yeast extract employed in this investigation was the dehydrated powder form of Difco Bacto Yeast Extract manufactured by the Difco Laboratories, Detroit, Michigan.

8. Flour

The "Gold Medal" brand flour used in this investigation was obtained in four 50 pound lots from a grocery. The first two lots were received in June, 1954 and the last two lots in September, 1954. Starch and moisture analyses were carried out in triplicate for each sack.

9. Wheat germ

Two kinds of wheat germ were used in this investigation.

a. Hard spring wheat germ obtained from International Milling Co., Davenport, Iowa, in 5 pound lot in June, 1954. The germ was stored in screw-topped bottles and fumigated by pushing a test tube containing about 5 ml. of carbon disulfide upright into the germ and screwing the cover on tightly until used.

b. Kretschmer wheat germ obtained from a grocery in June, 1954. The following analysis was found on the label:

One oz. supplies the following percentage of minimum daily adult requirements:

Thiamine	49%	Iron	22%
Riboflavin	7.5%	Phosphorus	36.5%
Niacin	1 mg.	Protein	9.35 g.

Natural oil of wheat germ retained also contains the following, per ounce:

Choline	114 mg.
Inositol	201 mg.
Pyridoxine	.34 mg.
Pantothenic acid	.32 mg.
Folic acid	.13 mg.
Biotin	.003 mg.
Vitamin E	11.5 mg.

10. Ground whole wheat

The wheat used in this investigation was hard winter wheat received from Robert S. Ogden of the Chemurgy Department, University of Nebraska, Lincoln, Nebraska, in

summer, 1953. The whole wheat was stored in screw-topped bottles until January, 1954. It was then ground in the Wiley mill, thoroughly mixed and stored in screw-topped bottles until used. Moisture and starch determinations were run on the ground whole wheat.

B. Chemicals

All chemicals used during this investigation were of the C. P. grade and were obtained through ordinary commercial sources.

IV. METHODS

A. Microbiological Procedures

1. Yeast cultures

A 15 per cent "beer wort" medium was used for carrying the yeast culture and also for the preparation of cultures used for inoculating experimental mashes. This medium was prepared by dissolving a weighed amount of malt extract in approximately 5.6 times its weight of tap water. The malt extract was first dissolved in a little boiling water and then diluted to volume. The volume of the medium obtained was about 1.1 times the volume of the water used. From this medium, 20 ml. aliquots were distributed in 50-ml. Erlenmeyer flasks for use as carrying medium and 300 ml. amounts were placed in 500-ml. Erlenmeyer flasks to serve as medium for the innoculant for the experimental fermentations. After the distribution of the beer wort, the flasks were plugged with cotton and sterilized for 15 minutes at a steam pressure of 15 pounds per square inch.

The stock culture was originally obtained from the Northern Regional Research Laboratory, Peoria, Illinois, as yeast No. 567. It is listed as No. 51 in the Biophysical

Chemistry culture collection at Iowa State College. It is a strain of Saccharomyces cerevisiae which is extremely vigorous and produces high yields of alcohol. Transfers were made daily in order to maintain a vigorous yeast culture. The inoculated medium was incubated at 30° C. and 20 to 24 hour yeast cultures were used to inoculate the experimental fermentation mashes.

2. Mold cultures

The following molds were used in this investigation: Aspergillus niger No. 337 from the Northern Regional Research Laboratory, Peoria, Illinois; Aspergillus niger No. 330 from the same source; and Aspergillus oryzae No. 38 from the Biophysical Chemistry culture collection at Iowa State College.

Stock cultures of these molds were maintained on dextrose-yeast extract-agar slants. It was found more convenient to make most of the inoculations during the course of this work from intermediate bran cultures. Bran cultures were prepared by mixing 100 g. of wheat bran and 10 g. of ground corn. To this dry material there was added 60 ml. of 0.2 normal hydrochloric acid containing 0.62 p.p.m. of zinc sulfate heptahydrate, 0.63 p.p.m. of ferrous sulfate

heptahydrate, and 0.08 p.p.m. of cupric sulfate pentahydrate. Ten gram portions of the moistened material were placed in 150-ml. wide-mouth Erlenmeyer flasks and the flasks were plugged with cotton. The flasks were sterilized for 15 minutes at 15 pounds steam pressure, cooled, and inoculated with mold spores. The flasks were laid horizontally in the incubator at 30° C. After about a week, during which mycelial growth, sporulation, and finally drying of the material occurred, the bran cultures were ready for use. The moisture content is very critical and it is sometimes necessary to vary the amount of acid-salt solution depending on the weather conditions so that the material is neither too dry to support good growth nor so moist that drying is delayed. If drying does not occur soon after sporulation, autolysis occurs and the culture is spoiled.

Bran cultures were inoculated by removing a small portion of the mold growth from the surface of the agar slants by means of a sterile wire and placing it in the sterilized bran preparation. The flask was then shaken to distribute the mold spores throughout the moistened bran and then incubated at 30° C.

Mold brans were prepared employing the method described by Hao, Fulmer, and Underkofler (50). Briefly, the method consisted of mixing about 750 g. of wheat bran with equal

weight of 0.3N hydrochloric acid and packing it into an aluminum pot equipped with a tight fitting cover and a perforated bottom. The pot and contents were sterilized for 30 minutes at 15 pounds steam pressure. The pot was cooled, the material removed, and inoculated with 10 g. of a bran culture of the proper mold. The inoculated bran was repacked in the pot and incubated at 30° C. for about 8 hours. After this incubation period aeration was used to keep the temperature below 45° C. The direction of flow of the air was alternated periodically. After 12 to 24 hours the contents of the pots were spread on paper to dry. After drying, the material was ground and the mold bran was ready for use. Moisture determinations were run on each mold bran.

Submerged cultures were prepared by shake-flask culture of the molds upon media containing 5 per cent distillers' dried solubles and 5 per cent corn meal. The ingredients were mixed with tap water in the required proportions in flasks. A volume of 150 ml. of medium in each 500-ml. Erlenmeyer flask was employed. The flasks were stoppered with cotton plugs and sterilized at 15 pounds steam pressure for 15 minutes. After cooling, the medium in each flask was adjusted to pH 5.2 with concentrated sodium hydroxide solution, employing a glass electrode pH meter. The media were then inoculated with the molds spores and incubated at

30° C. in a reciprocal shaker. Inoculations were made from the bran cultures. About 100 ml. of sterile 0.05 per cent Tween 80 solution was added to the bran cultures and the flask was shaken to suspend the mold spores and a portion of the solid material. Inoculations were made from this suspension by means of a sterile pipet. The submerged culture preparations were incubated for 48 hours, and employed immediately after this incubation period.

B. Saccharification and Fermentation

1. Standard method

The major portion of the experimental fermentations in this investigation followed a procedure which has been in use in this laboratory for a number of years. The fermentations were carried out in 1-liter wide-mouth Erlenmeyer flasks equipped with water traps to decrease the loss of alcohol. One hundred grams of starchy material was placed in each flask, 250 ml. of 0.06 normal sulfuric acid added, and the flasks were held for 10 minutes in the water bath at 63° C. The water bath was then heated at such a rate that the temperature of the mashes was raised to 90° C. in about 30 minutes. The flasks were then transferred to the

hot autoclave and cooked immediately for 45 minutes at 20 pounds steam pressure. The desired amount of saccharifying agent was mixed with cold tap water to make 250 ml. of slurry at 15° to 20° C. After completion of the cooking period, the autoclave was blown down to atmospheric pressure, and the flasks were steamed continuously in the autoclave at atmospheric pressure until their removal, one at a time, for saccharification.

To the hot mash was added 0.4 g. of calcium carbonate to bring the pH to approximately 5.0. The cold slurry of the saccharifying agent was next added and the mash mixed rapidly with a high speed stirrer resulting in a mash temperature at this time of about 55° C. The mash was then quickly cooled to 30° C. by placing the flask in a cold water bath. When all the flasks in the series had been adjusted to the desired pH, saccharified, and cooled, the mashes were inoculated with 20 ml. of an active 20 to 24 hour culture of yeast. The flasks were swirled to distribute the yeast throughout the mash, the stoppers and traps were added, and the flasks were placed in the 30° C. incubator for 3 to 4 days until completion of fermentation. The flasks were swirled daily to resuspend the yeast which had settled to the bottom of the flasks.

2. Modifications of the standard method

The early experiments were carried out according to the standard method described. Due to greater convenience, the following modifications were adopted in the later experiments. One hundred grams of starchy material was placed in each 1-liter wide-mouth Erlenmeyer flask, 250 ml. of 0.06 normal sulfuric acid added, and the flasks were transferred to the hot autoclave and cooked at atmospheric pressure with occasional shaking until all the starch had gelatinized. The flasks were then cooked for 45 minutes at 20 pounds steam pressure, blown down to atmospheric pressure, and steamed continuously until removed one at a time for saccharification. To the hot mash was added 2.0 ml. of saturated sodium carbonate solution to adjust the pH to 5.0 and a slurry containing the desired amount of saccharifying agent in 250 ml. of cold water, and mixed with a high speed stirrer for 2 minutes. The mashes were finally cooled and inoculated with 20 ml. of an active 20 to 24 hour culture of yeast.

Modifications of this method due to variation of certain factors were studied in flour and starch slurry fermentations. These will be noted in connection with the particular fermentations in the experimental section. These

modifications included such factors as the strength of the acid used in preparing the mash, and the amounts of saccharifying agent used.

3. The starch slurry fermentation

Three hundred ml. of starch slurry was placed in each 1-liter flask and the slurry adjusted with 6 normal sulfuric acid to 0.06 normal by assuming 300 ml. of slurry contained 250 ml. of water. The mashes were gelatinized, cooked, saccharified and inoculated according to the modified standard method.

4. The short starch fermentation test

The procedure for a short fermentation test for saccharifying efficiency was described by Reese, Fulmer and Underkofler (115). This method is outlined briefly here. To 100 g. of pure food grade starch and 5 g. of Difco yeast extract in a wide-mouth one-liter Erlenmeyer flask is added 250 ml. of 0.05 normal hydrochloric acid previously heated to 70° C., and the contents are stirred with a glass rod to facilitate mixing. This is repeated for each flask in the series. The flasks are then heated in a water bath and

stirred continuously until the starch is gelatinized. The mashes are then cooked for one hour at 20 pounds steam pressure, blown down to atmospheric pressure, and steamed continuously until removed one at a time for saccharification. To the hot mash is added the requisite amount (about 1.9 ml.) of saturated sodium carbonate solution to adjust the pH to 5.0 to 5.3 and a slurry containing the amylolytic agent in 250 ml. of cold water, and the entire contents are mixed with a high speed stirrer for 2 minutes. The slurry should be cooled in an ice bath before using so the final temperature of the mash will be 55° C. The mashes are then cooled in a cold water bath to 30° C. and each flask is inoculated with 20.0 ml. of an active 24-hour yeast culture. The series is incubated at 30° C. for the 24 hour test period.

C. Analytical Procedures

1. Determination of moisture

Moisture determinations were carried out on the corn meal, flour, starch, distillers' dried solubles, ground whole wheat and starch used during this investigation. Weighing bottles each containing 3 to 5 g. of the material

were placed in an oven at 105° C. and heated until constant weight was obtained. The loss of weight was assumed to be due to the loss of water and the per cent moisture was calculated on that basis.

2. Determination of alcohol

At the end of the fermentation period the water in the traps was added to the fermentation flasks. The final volume of the beer in each flask was measured and a 250 ml. aliquot from each flask along with 100 ml. of wash water were transferred to a Kjeldahl flask. Calcium carbonate (0.5 to 1.0 g.) was added to neutralize the acids present and about 99 ml. of distillate was collected in a volumetric flask. The distillates were brought to 25° C. by immersion of the volumetric flasks in a constant temperature water bath, the volumes made to exactly 100.0 ml. with distilled water, and the specific gravities determined at 25°/25° with a Chainomatic Westphal balance. Ethanol contents of the distillates were then read from an appropriate table. Multiplication of the weight of alcohol thus determined by the ratio (beer volume/aliquot volume) gave the total yield of alcohol in each flask.

The alcohol yields for the early experimental fermentations were calculated in terms of gallons of 100 proof ethanol per standard bushel. This calculation is based on the total amount of dry material involved in the fermentation. The total alcohol yield multiplied by (100/total dry weight) gives the pounds of alcohol per 100 pounds of dry material. This figure divided by 3.31 gives proof gallons (i.e., gallons of 100 proof alcohol) per 100 pounds of dry material. A standard bushel of corn weighs 56 pounds and contains 12 per cent moisture, so multiplication of proof gallons per 100 pounds of dry material by (56)(0.88)/100 will give yields in terms of proof gallons per standard bushel. Division by 1.9 will give yields in wine gallons per standard bushel. (A wine gallon is a gallon of 190 proof alcohol.)

For comparison purpose, the yields of alcohol for flour, starch slurry and ground whole wheat fermentations were calculated in terms of grams of alcohol per 100 grams of starch. The total alcohol yield multiplied by (100/total starch content per flask) gives grams of alcohol per 100 grams of starch. This figure multiplied by (100/theoretical alcohol from 100 grams of starch) gives per cent theoretical yield. Theoretical alcohol from 100 grams of starch was calculated from the equation:

$$(C_6H_{10}O_5)_x + xH_2O \longrightarrow x\ C_6H_{12}O_6 \longrightarrow 2x\ C_2H_5OH + 2x\ CO_2 .$$

Hence, 100 multiplied by 92/162 gives the theoretical yield of alcohol from 100 grams of starch.

3. Determination of starch content

Starch content was determined by direct acid hydrolysis in accordance with the Official Method of Analysis of the Association of Official Agricultural Chemists (6).

A quantity of 2.5 to 3 g. of dry material was heated with 200 ml. of water and 20 ml. of hydrochloric acid (specific gravity 1.125) for 2.5 hours in a flask provided with a reflux condenser, cooled and nearly neutralized with sodium hydroxide. The solution was transferred quantitatively to a 500-ml. volumetric flask and diluted to volume, filtered and glucose determined in an aliquot of filtrate. Weight of glucose obtained multiplied by 0.90 gives the weight of starch. Glucose was determined according to the method of Underkofler, Guymon, Rayman, and Fulmer (151). The reagents were standardized with pure glucose.

4. Determination of glucose

For a more complete discussion of the method used in determining reducing sugars the reader may consult the article by Underkofler, Guymon, Rayman, and Fulmer (151) describing a modification of the Shaffer-Somogyi method.

a. Reagents

(1) Potassium iodide (125 g.) and potassium oxalate monohydrate (250 g.) were dissolved in one liter of water to furnish the source of iodine.

(2) Sulfuric acid, 7.5 normal.

(3) Standard 0.0500 normal sodium thiosulfate solution

(4) Starch indicator solution. This contained one per cent soluble starch in a saturated sodium chloride solution.

(5) Sugar reagent "G"

Components	Weight in grams per liter
$CuSO_4 \cdot 5H_2O$	37.5
$NaKC_4H_4O_6 \cdot 4H_2O$	125
Na_2CO_3 (anhydrous)	53
KI	1.0
Na_2SO_4 (anhydrous)	50

KIO_3	3.5665 (exactly)
NaOH	Approximately saturated solution added to adjust to pH 9.48

The sugar reagent "G" was prepared as follows: The sodium carbonate and Rochelle salt were dissolved in about 500 ml. of distilled water after which the copper sulfate, dissolved in about 500 ml. of distilled water, was added with continuous stirring so that no free carbon dioxide was evolved. The potassium iodide and sodium sulfate were then added and stirred until dissolved. The solution was then made up to a volume of about 960 ml. with distilled water. The strong sodium hydroxide solution was next added slowly, with stirring, until the pH reached the value of 9.48. The pH was determined at 25° C. with the glass electrode, the salt error being neglected. The resulting solution was then heated to boiling and was boiled gently for 10 minutes in a covered container. It was then cooled to 20° C. The potassium iodate, accurately weighed, was added, dissolved completely, and the volume made up to exactly one liter in a volumetric flask.

b. Procedure. Five milliliters of the solution to be analyzed for reducing sugars was pipetted into a test

tube (25 mm. x 145 mm.) and a similar volume of reagent "G" was added. The solutions were mixed by swirling the tube which was then stoppered with a one-hole, No. 4 rubber stopper containing a short length of 1 mm. capillary tubing in the hole.

The test tube was next immersed (at least two-thirds of its length) in a boiling water bath for 20 minutes. After cooling the tube and contents to room temperature in a cold water bath, 2 ml. of the iodide-oxalate solution was added and the solutions were thoroughly mixed. One ml. of 7.5 normal sulfuric acid was carefully added down the side of the inclined tube. After the effervescence had nearly subsided the tube was shaken until no undissolved material remained.

The excess iodine was titrated with standard sodium thiosulfate using the starch indicator. Titration was continued until the typical starch-iodine purple color changed to a light blue. A blank was run using 5 ml. of distilled water. The glucose equivalent (mg. glucose/ml. thiosulfate) was determined by following the above procedure using various dilutions of a standard glucose solution.

D. Methods for Preparing the Materials

1. Starch slurry

For the early experiments, the starch slurry was obtained by the following method:

One thousand grams of flour was mixed with 100 ml. of tap water at 52° C. It was allowed to stand for 30 minutes and then mixed carefully with 2000 ml. of tap water at 52° C. without unduly violent agitation. The gluten was then separated by filtering the slurry through muslin cloth and the starch slurry collected for fermentation. Another method was adopted during the later experiments due to greater convenience. Briefly, the flour was made up to a dough and allowed to develop for thirty minutes. The dough was then washed with several portions of tap water by hand, the washings were filtered through a muslin cloth and the starch slurry collected for fermentation.

2. Extracted wheat germ

Wheat germ extraction was accomplished in Soxhlet extraction with both hexane and alcohol as solvents for 24 hours and then air dried.

V. EXPERIMENTAL RESULTS

A. Short Starch Fermentation Test

Reese (114) in 1947 developed a short starch fermentation test for the evaluation of mold brans prepared from Aspergillus oryzae 38-b. He reported parallel straight lines were obtained when the ratios of the weight of mold bran used per weight of alcohol produced were plotted against weight of mold bran used. The intercept values from these straight lines were directly proportional to the optimum level of mold bran required for maximum alcohol production.

Further work on the application of this method for comparative evaluation of fungal preparations from different molds cultivated on bran and submerged was reported by Pool and Underkofler (106). They studied mold bran and submerged preparations of Aspergillus oryzae 38-b, Aspergillus niger 337 and Aspergillus niger 330, and found that the method could not be applied to Aspergillus niger 330. They further concluded that the method could not be used for the evaluation of preparations from two different organisms or for the comparison of different types of preparations from the same organism. They felt that the method ought to be tested

for possible application to mold preparations other than those of Aspergillus oryzae 38-b mold bran so successfully evaluated by Reese.

To check further the applicability of Reese's short starch fermentation test, 5 preparations of Aspergillus niger 337 mold bran were prepared according to the method of Hao, Fulmer and Underkofler (50). It was hoped that the 5 preparations of mold bran might have significant differences in their saccharifying efficiency for good checking of Reese's short starch fermentation test. The saccharifying efficiencies of these preparations were studied by measuring the alcohol production from corn mashes saccharified by different levels of the mold brans. The levels tested were from 3 to 7 per cent (wet basis) of the corn used. Alcohol yields were calculated in proof gallons per standard bushel (i.e., 56 pounds and 12 per cent moisture). The results are summarized in Table 1 and plotted in Figure 1.

Another series of fermentations was run with corn starch saccharified by different levels of the 5 mold brans following the procedure for Reese's short starch fermentation test method. The results are indicated in Table 2 and plotted in Figure 2.

According to the method of Reese (114) the ratio of the graphical intercepts for two mold brans was directly

Table 1. Alcohol yields from corn meal saccharified by different levels of Aspergillus niger 337 mold brans

Dry wt. mold bran, g.	Total dry matter, g.	Mold bran, per cent	Alcohol yields, proof gallons per standard bushel
Mold Bran I			
2.69	90.49	2.93	4.98
3.58	91.38	3.92	5.30
4.48	92.28	4.86	5.45
5.38	93.18	5.77	5.40
6.27	94.07	6.67	5.29
Mold Bran II			
2.69	90.49	2.93	5.03
3.59	91.39	3.93	5.33
4.49	92.29	4.87	5.46
5.38	93.18	5.77	5.52
6.28	94.08	6.68	5.50
Mold Bran III			
2.71	91.31	2.97	5.47
3.62	92.22	3.93	5.56
4.52	93.12	4.85	5.66
5.43	94.03	5.78	5.59
6.33	94.93	6.67	5.58
Mold Bran IV			
4.41	92.21	4.78	5.22
4.94	92.74	5.32	5.33
5.29	93.09	5.68	5.32
5.64	93.44	6.04	5.28
5.99	93.79	6.24	5.23
Mold Bran V			
4.55	92.35	4.93	5.05
4.91	92.71	5.30	5.16
5.26	93.06	5.65	5.25
5.61	93.41	6.01	5.24
5.96	93.76	6.36	5.22

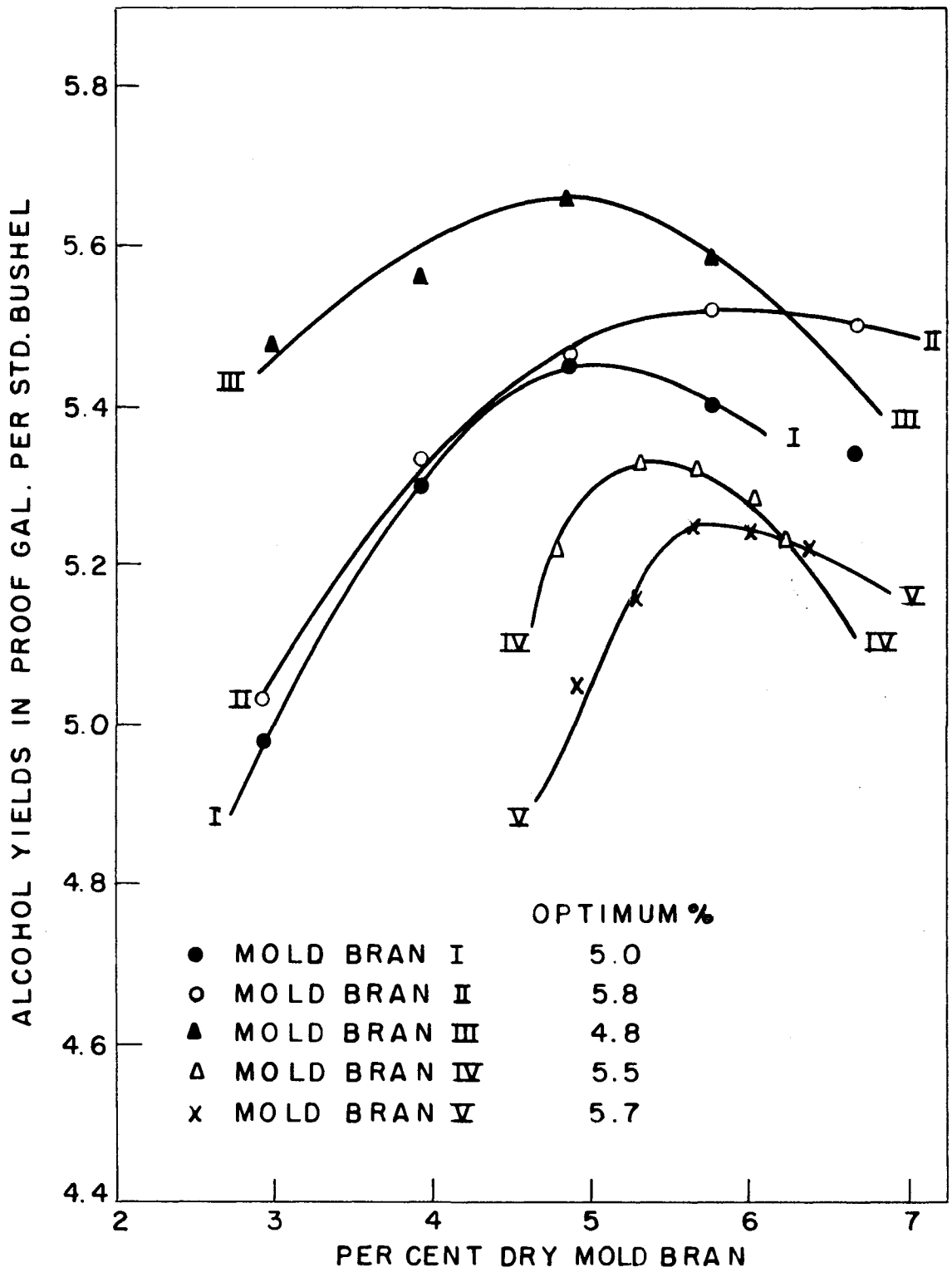

Figure 1. Alcohol yields from corn mashes saccharified by *Aspergillus niger* 337 mold brans

Table 2. Alcohol yields from corn starch saccharified by different levels of *Aspergillus niger* 337 mold brans

(1) Dry weight mold bran, g.	(2) Alcohol yield, g.	(1)/(2)
Mold Bran I		
1.79	23.87	.0750
2.69	25.02	.1075
3.58	26.55	.1349
4.48	27.52	.1628
Mold Bran II		
1.79	21.04	.0851
2.69	24.02	.1120
3.59	25.90	.1386
4.49	26.93	.1667
Mold Bran III		
.91	16.87	.0539
1.81	22.40	.0808
2.71	25.26	.1083
3.62	26.64	.1358
Mold Bran IV		
.88	17.07	.0516
1.76	22.47	.0785
2.64	25.09	.1052
3.53	26.45	.1335
Mold Bran V		
1.75	20.83	.0844
2.63	23.81	.1105
3.50	25.63	.1369
4.38	26.74	.1638

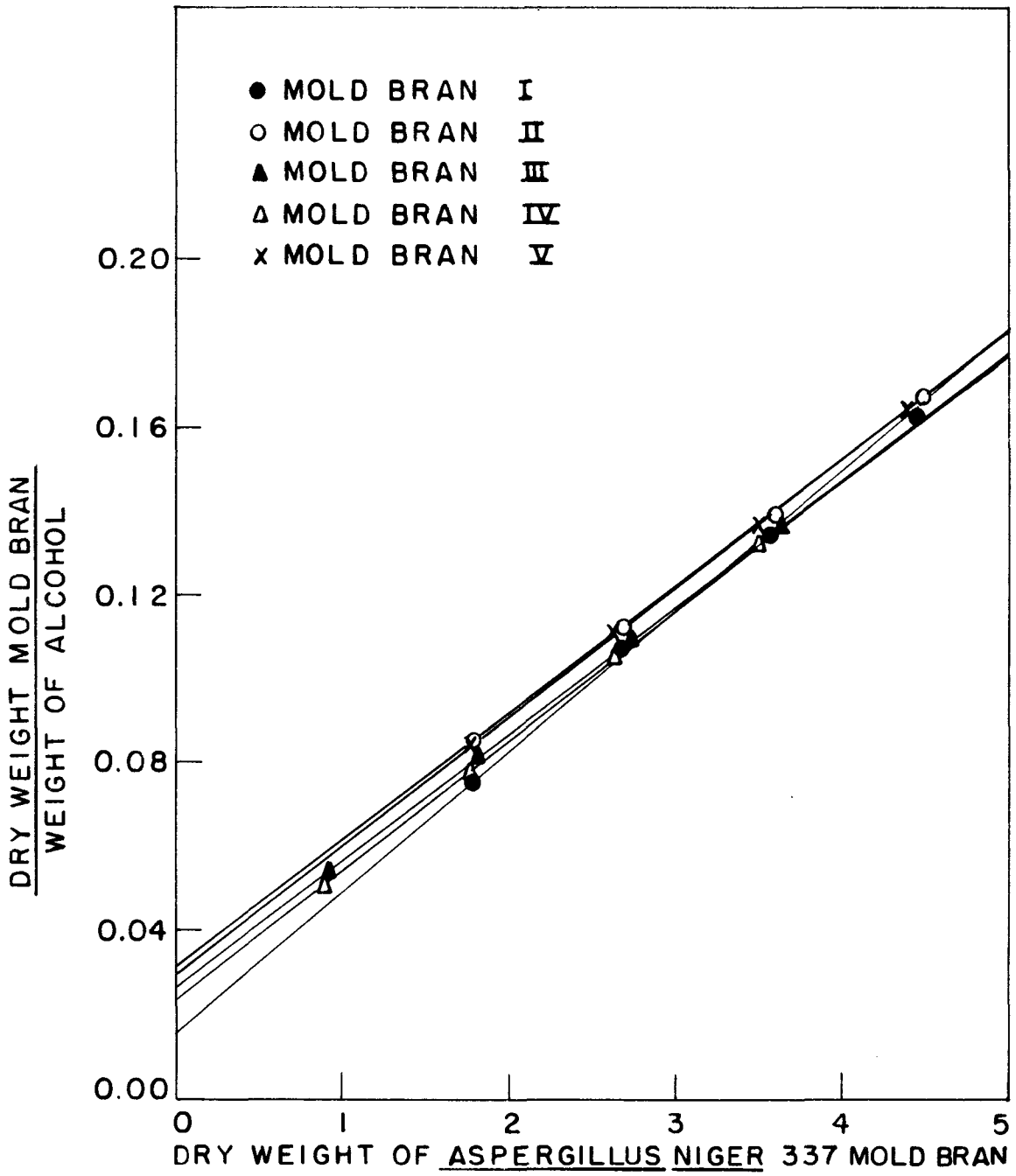

Figure 2. Curves for short starch fermentation test

related to the ratio of the levels of the two mold brans required for optimum alcohol production. A comparison of the optimum levels obtained from Figure 1 with the intercepts obtained from Figure 2 is made in Table 3. The optimum level for each preparation listed is the per cent of the total dry material in the fermentation mash. The slopes of the lines obtained in Figure 2 are also given in Table 3.

Unfortunately, the differences in the saccharification efficiencies of the 5 mold bran preparations are not very great. Examination of the values in Table 3 leads to the conclusion that there is no over-all relation between the

Table 3. Comparison of optimal levels, maximum alcohol yields, intercept values and slopes for Aspergillus niger 337 mold brans

Preparations	Max. alc. yield, proof gal. per bu.	Opt. level per cent	Intercept value	Slope
I	5.45	5.0	.0153	.0335
II	5.52	5.8	.0295	.0306
III	5.66	4.8	.0265	.0301
IV	5.33	5.5	.0236	.0312
V	5.25	5.7	.0316	.0301

intercept values from the short starch fermentation test and the optimal levels for actual fermentations. Although straight lines were obtained in Figure 2, they are not all parallel to each other. This may be due to the fact that the enzyme system in Aspergillus niger 337 is different from the enzyme system in Aspergillus oryzae 38-b. It must be pointed out that the data are very limited. Several attempts to prepare mold brans of higher potency than those already tested were unsuccessful. Since the data obtained were not very encouraging, no special effort was exerted to prepare more mold bran.

B. Alcoholic Fermentation of Wheat Flour

Utilization of surplus grains by fermentation to produce ethanol has been considered and discussed for many years. Research to obtain maximum yields of alcohol from different raw materials has been carried out in a number of laboratories. At present considerable interest has developed in the possibility of using surplus wheat for alcohol production. The surplus continues to increase each year in spite of acreage control by government. Coupled with this surplus is a great demand for increased supplies of wheat gluten from which to make monosodium glutamate. For recovery of wheat gluten the wheat must be milled to flour, the gluten

separated, and the starch slurry remaining is a suitable substrate for fermentation.

1. Optimum acid concentration for initial cook

In processing starchy substrates for ethanol fermentation gelatinization of the starch during cooking with water results in highly viscous mashes which are extremely difficult to handle. Industrial plants attempt to eliminate this difficulty by partially liquefying the starch prior to or during the cooking operation. This is usually accomplished by the addition of a small amount of malt (premalting), mineral acid, or fungal or bacterial amylase preparations. For laboratory scale fermentations the use of acid is the most convenient procedure. In a fermentation process it is necessary first to determine the optimum conditions for liquefying the starchy mashes so that they can be readily handled.

To determine the optimum acid concentration for initial cook, fermentation mashes prepared from wheat flour and starch slurry were cooked with various concentrations of sulfuric acid and fermented with the addition of sufficient amount of saccharifying agent (6 g. of Aspergillus niger 337

mold bran per flask). The results are shown in Table 4.

Since the starch contents of flour from different sacks are not the same, the alcohol yields are reported in grams of alcohol per 100 g. of starch for comparison purposes. In general, thinning of the mashes occurred with increased acid concentration. Alcohol yields from wheat flour cooked with 0.02 and 0.03 normal acid could not be determined

Table 4. Effect of sulfuric acid concentration on alcohol yields from wheat flour and starch slurry

Normality of acid	Alcohol yields, g. of alcohol per 100 g. starch	
	Starch slurry	Wheat flour
.02	47.21	overflowed
.03	47.48	overflowed
.04	48.08	47.90
.05	48.18	48.11
.06	48.01	48.12
.07	48.16	48.18
.08	46.24	46.52
.10	44.56	44.68

because the mashes overflowed during the initial cook due to excessive foaming. The dark color of the mashes cooked with 0.08 and 0.10 normal acid indicated that some caramelization had occurred. The minimum acid concentration giving a thin mash (0.05 normal) was chosen as the optimum acid concentration for initial cook for all succeeding fermentations.

2. Optimum level of saccharifying agent

a. Malt. Various concentrations of malt were added to the mashes which had been cooked with 0.05 normal sulfuric acid. The data are presented in Tables 5 and 6. Both series showed increasing yields up to a certain malt concentration. Due to differences in starch content of wheat flour and starch slurry, malt concentrations are reported as grams of malt per 100 grams of starch. Extremely low yields were obtained from starch slurry fermentations. The lower alcohol yield from wheat flour at malt concentration of 16.32 grams per 100 grams of starch was probably due to some experimental error.

b. Mold bran. The mold cultures employed in this investigation were Aspergillus oryzae 38b, an organism that has been employed in commercial production of mold bran

Table 5. Alcohol yields from wheat flour at different malt levels

Malt, g. per 100 g. starch	Alcohol yield, g. per 100 g. starch	Theoretical yield, per cent
8.16	46.31	81.6
10.88	47.85	84.3
13.61	47.58	83.8
16.32	47.11	83.1
19.05	47.74	84.1
21.77	47.80	84.2

Table 6. Alcohol yields from starch slurry at different malt levels

Malt, g. per 100 g. starch	Alcohol yield, g. per 100 g. starch	Theoretical yield, per cent
10.12	35.83	63.1
13.50	35.94	63.3
16.88	35.93	63.3
20.26	36.16	63.7
23.63	38.20	67.3
27.01	38.00	67.0

(153), Aspergillus niger 337, the organism employed in commercial scale tests of the submerged culture saccharification procedure (66, 155), and Aspergillus niger 330, reported to produce high maltase activity (27, 143). Mold brans were prepared according to the method of Hao, Fulmer, and Underkofler (50). The optimum levels of the mold brans for saccharification were determined by measuring the alcohol production from mashes saccharified by different levels of mold brans. The results are summarized in Tables 7 and 8.

All series showed increasing yields up to a certain mold bran concentration after which the yields decreased. All the mold brans proved to be better than malt. However, comparison of the above experiments were of little value because they were run during summer when the temperature inside the incubator could not be controlled most of the time (instead of 30° C. it went as high as 40° C.). Another series of fermentations was run using the optimum levels found in the above experiments to determine which saccharifying agent gave the best yields. The data are presented in Tables 9 and 10.

The results of this series of fermentations again indicate that malt was the poorest saccharifying agent tested, although somewhat higher yields were obtained from this series of fermentation than had been previously obtained.

Table 7. Alcohol yields from wheat flour at different mold bran levels

Mold bran, g. per 100 g. starch	Alcohol yield, g. per 100 g. starch	Theoretical yield, per cent
A. niger 330		
4.08	46.79	82.4
5.44	46.87	82.6
6.80	48.29	85.0
8.16	49.29	86.9
9.52	48.65	85.7
A. niger 337		
4.08	48.41	85.2
5.44	49.67	87.5
6.80	49.95	87.9
8.16	49.48	87.1
9.52	49.85	87.8
A. oryzae 38b		
4.08	44.16	77.8
5.44	47.17	83.1
6.80	48.56	85.5
8.16	49.21	86.7
9.52	49.02	86.3

Table 8. Alcohol yields from starch slurry at different mold bran levels

Mold bran, g. per 100 g. starch	Alcohol yield, g. per 100 g. starch	Theoretical yield, per cent
A. niger 330		
4.18	44.22	77.9
5.58	44.12	77.5
6.97	45.38	79.9
8.37	45.96	81.0
9.76	45.68	80.4
A. niger 337		
5.62	45.43	80.0
7.49	46.31	81.6
9.37	49.72	87.8
11.24	50.17	88.4
13.11	50.17	88.4
A. oryzae 38b		
4.51	40.54	71.4
6.02	42.43	74.7
7.52	43.27	76.2
9.03	43.79	77.4
10.53	43.48	76.6

Table 9. Alcohol yields from wheat flour at optimum levels of different mold brans and malt

Saccharifying agent, g. per 100 g. starch	Alcohol yield, per cent of theoretical
A. niger 330	
8.16	86.5
9.52	86.8
A. niger 337	
6.80	87.5
8.16	87.4
A. oryzae 38b	
8.16	85.6
9.52	86.0
Malt	
10.88	85.7
13.61	85.6

It is evident from the data that mold bran from *Aspergillus niger* 337 is the best saccharifying agent for both starch slurry and wheat flour fermentations. Extremely low yields were obtained from starch slurry using malt. Except when saccharified by mold bran from *Aspergillus niger* 337, the yields from starch slurry were always lower than the yields

Table 10. Alcohol yields from starch slurry at optimum levels of different mold brans and malt

Saccharifying agent, g. per 100 g. starch	Alcohol yield, per cent of theoretical
A. niger 330	
10.48	81.5
12.24	81.3
A. niger 337	
8.72	87.1
10.48	87.5
A. oryzae 38b	
10.48	77.0
12.24	76.9
Malt	
24.47	70.0
27.97	70.1

from wheat flour. These results suggest some growth factor must be present in the gluten. Even though mold bran from *Aspergillus niger* 337 gave the best yields, the yields were still unsatisfactory compared to those obtained from corn fermentations.

Submerged mold cultures were prepared according to the conditions used by Pool and Underkofler (106). The mold was grown on a medium consisting of 5 per cent distillers' dried solubles and 5 per cent corn meal. The ingredients were mixed with tap water. A volume of 150 ml. of medium in each 500 ml. Erlenmeyer flask was used. The medium in each flask was adjusted to pH 5.2 with concentrated sodium hydroxide solution after autoclaving, inoculated with mold spores and incubated in a reciprocal shaker for 48 hours. The mold cultures in different flasks were put together in a big flask and thoroughly mixed before being used. An accidental observation indicated that enzyme solutions mixed by a high speed mechanical stirrer gave better yields than when mixed by hand shaking. Therefore in all the experiments with submerged culture, the enzyme solution was thoroughly mixed with a high speed stirrer for 2 minutes before being used.

The volume of submerged culture of Aspergillus niger 337 necessary for optimum saccharification, as measured by the alcohol production, was then investigated. The volume per cent of enzyme solution used in starch slurry fermentation was computed by assuming 300 ml. of starch slurry contained 250 ml. of water. Therefore 25 ml. of mold

culture was considered as 10 volume per cent. Mold bran from Aspergillus niger 337 at a level of 6 g. per flask was used as control. The results are listed in Tables 11 and 12.

From the data, it appeared that submerged culture is a better saccharifying agent than mold bran since it gave better optimum yields for both starch slurry and wheat flour. Significant increase in optimum yield was observed with the use of submerged Aspergillus niger 337 culture especially in starch slurry fermentation.

It was observed that enzyme solution prepared from medium that was prepared several days prior to inoculation

Table 11. Alcohol yields from starch slurry at different levels of Aspergillus niger 337 submerged culture

Mold culture, volume per cent	Alcohol yield, g. per 100 g. starch	Theoretical yield, per cent
5	41.79	71.8
10	44.85	79.0
15	50.60	89.1
20	52.50	92.5
25	52.52	92.5
control	50.93	89.7

Table 12. Alcohol yields from wheat flour at different *Aspergillus niger* 337 submerged culture levels

Mold culture, volume per cent	Alcohol yield, g. per 100 g. starch	Theoretical yield, per cent
5	40.21	70.8
10	46.88	82.5
15	51.20	90.2
20	51.83	91.3
25	51.61	90.9
control	49.69	87.5

with the mold culture gave lower alcohol yield than enzyme solution prepared from freshly prepared medium. This is probably due to retrogradation of starch as observed by other workers. Such retrograded starch is not saccharified by amylases, and hence cannot be fermented.

One hundred ml. or 20 volume per cent of submerged mold culture was chosen as the optimum volume per cent for both starch slurry and wheat flour fermentation.

3. Effect of adding ammonium salt

In alcoholic fermentation of molasses, addition of small quantities of ammonium salts is usually desirable to increase the rate and efficiency of the fermentation. The amount of ammonium salt, such as ammonium sulfate, varies from 0.5 to 3 lb. per 1000 gal. of mash depending on the optimum amount as determined by laboratory fermentation tests. It was therefore of some interest to observe the effect of adding ammonium salts to both starch slurry and wheat flour mashes.

A first series of fermentations was run using the optimum amounts of saccharifying agents as determined in the previous experiments and varying the amount of ammonium sulfate added. The results are summarized in Tables 13 and 14.

Six grams of Aspergillus niger 330 mold bran was used per flask which is equivalent to 8.16 g. per 100 g. of starch in wheat flour mash or 10.47 g. per 100 g. of starch in starch slurry mash. Five grams Aspergillus niger 337 was used per flask which is equivalent to 7.14 g. per 100 g. of starch in wheat flour mash or 9.63 g. per 100 g. of starch in starch slurry mashes. Six grams Aspergillus oryzae 38b per flask was used which is equivalent to 8.16 g. per 100

Table 13. Effect of ammonium sulfate on alcohol yields from wheat flour at optimum level of different saccharifying agents

Ammonium sulfate, g. per flask	Theoretical yield, per cent
Aspergillus niger 330 mold bran	
0	86.5
.04	86.0
.08	86.3
.12	85.7
.16	84.9
Aspergillus niger 337 mold bran	
0	90.2
.04	90.3
.08	88.1
.12	86.2
.16	85.0
Aspergillus oryzae 38b mold bran	
0	88.0
.04	88.1
.08	87.8
.12	88.0
.16	88.0
Malt	
0	82.3
.04	83.1
.08	81.9
.12	80.1
.16	80.0
Submerged *Aspergillus niger* 337	
0	92.6
.04	91.9
.08	91.5
.12	90.2
.16	88.6

Table 14. Effect of ammonium sulfate on alcohol yields from starch slurry at optimum levels of different saccharifying agents

Ammonium sulfate, g. per flask	Theoretical yield, per cent
Aspergillus niger 330 mold bran	
0	84.9
.04	84.5
.08	84.4
.12	84.5
.16	84.1
Aspergillus niger 337 mold bran	
0	90.4
.04	89.7
.08	89.5
.12	88.6
.16	87.9
Aspergillus oryzae 38b mold bran	
0	82.1
.04	82.3
.08	82.2
.12	81.8
.16	82.2
Malt	
0	72.0
.04	72.6
.08	71.4
.12	70.6
.16	70.2
Submerged *Aspergillus niger* 337	
0	93.2
.04	93.8
.08	92.7
.12	91.7
.16	90.8

g. of starch in wheat flour mash or 10.52 g. per 100 g. of starch in starch slurry mash. Eight grams of malt or 10.88 g. of malt per 100 g. of starch was used in each flask of wheat flour mash. Fifteen grams of malt or 25.49 g. of malt per 100 g. of starch was used in each flask of starch slurry mash. Twenty volume per cent or 100 ml. of submerged culture per flask was used for both wheat flour and starch slurry.

No significant effect was observed when ammonium sulfate was added to mashes containing optimum levels of saccharifying agents except lowering of yields at higher concentrations of ammonium sulfate. Other ammonium salts such as nitrate and phosphate also were investigated with similar results.

Since Aspergillus niger 330, Aspergillus oryzae 38b and malt had failed to give high yields in the production of alcohol from starch slurry, attention was centered on Aspergillus niger 337. Since saccharifying agents are always more expensive than ammonium salt, it was of some interest to investigate if it is possible to obtain maximum yields with less than optimum amounts of saccharifying agent by the addition of ammonium salt. The results are summarized in Table 15. Five grams of mold bran per flask of wheat flour was run as control for the fermentation

Table 15. Effect of ammonium sulfate on alcohol yield from starch slurry saccharified with less than the optimum amount of saccharifying agent

Ammonium sulfate, g. per flask	Theoretical yield, per cent
2 g. *A. niger* 337 mold bran/flask or 3.61 g./100 g. starch	
0	83.1
.04	83.1
.08	81.6
.12	80.4
.16	76.8
control	90.2
3 g. *A. niger* 337 mold bran/flask or 5.33 g./100 g. starch	
0	85.6
.04	85.7
.08	82.1
.12	81.3
control	90.1
4 g. *A. niger* 337 mold bran/flask or 7.92 g./100 g. starch	
0	88.3
.01	88.0
.02	89.0
.03	89.5
.04	89.0
.05	87.6
.06	87.1
control	90.2
15 volume per cent submerged *A. niger* 337 culture	
0	89.9
.01	90.3
.02	91.1
.03	91.7
.04	90.7
.05	90.5
control	93.5

saccharified by mold bran and 20 volume per cent submerged culture was run as control for mashes saccharified with submerged culture.

Examination of the data obtained with ammonium sulfate addition leads to the conclusion that ammonium sulfate is not useful in the alcoholic fermentation of starch slurry. Although a slight increase in yields was observed with the addition of 0.03 to 0.04 g. of ammonium sulfate when the saccharifying agent was slightly less than the optimum amount, the yields were still lower than the controls containing the optimum amount of saccharifying agent. A lowering of yields at higher ammonium sulfate concentration was again observed.

4. Effect of adding wheat germ, both extracted and unextracted

Christensen (21) in 1953 patented a process for obtaining high yields of alcohol from corn. In his process the oil from the germ fraction of the degerminated corn was extracted. Fifty per cent of the extracted germ fraction was added to the degerminated starchy fraction, cooked at pH 1.6 to 2.5, pH adjusted to 5.5, saccharified, cooled and fermented as usual. He stated that the extraction of

the oil from the germ appears to remove one or more substances acting as amylase inhibitors. The next step in this investigation was to find out if a similar procedure could be used for wheat.

In the first series of fermentations with germ, the effect of extracted and unextracted germ was studied. A two gram portion of germ was added to each flask of wheat flour mash saccharified with optimum amount of Aspergillus niger 337 mold bran. Two grams of germ was chosen because wheat contains about 2 per cent germ. Alcohol yields are listed in Table 16.

Table 16. Effect of germ on alcohol yields from wheat flour saccharified with optimum amount of Aspergillus niger 337 mold bran

Germ added*	Theoretical yield, per cent
None (control)	88.6
Germ from Int. Milling Co.	90.2
Extracted germ from Int. Milling Co.	88.0
Kretschmer wheat germ	90.3
Extracted Kretschmer wheat germ	88.3

*Two grams germ added per flask.

Contrary to what was expected, extracted germ had no significant effect on the alcohol yield while unextracted germ gave an increase in yield. There was no difference between the two germ samples obtained from different sources although Kretschmer wheat germ must had been roasted.

Better yields were obtained when the germ was added to the mash before the initial acid hydrolysis than when it was added after the acid hydrolysis together with the saccharifying agent. Therefore in all the subsequent experiments with germ, the germ was added to the mash before the initial acid hydrolysis.

A series of fermentations was run to study the effect of germ on alcohol yields from mashes saccharified by optimum amount (20 volume per cent) of submerged Aspergillus niger 337 culture. Unextracted germ from International Milling Company was used in all the succeeding experiments with germ. The results are presented in Table 17.

Addition of germ to both starch slurry and wheat flour caused increasing yields up to 1 g. of germ added, after which the yields leveled off with addition of more germ. Excellent yields were obtained with the addition of germ. The next step was to study the effect of germ on the alcohol yields from mashes saccharified with less than the optimum amount of saccharifying agent. The results are listed in Table 18. Twenty volume per cent was run as control.

Table 17. Effect of germ on alcohol yields from mash saccharified by optimum level of submerged *Aspergillus niger* 337 culture

Germ added, g.	Theoretical yield, per cent
Starch slurry	
0	93.6
.5	93.8
1.0	95.1
1.5	94.9
2.0	95.3
2.5	94.9
Wheat flour	
0	92.2
.5	92.5
1.0	93.8
1.5	93.8
2.0	93.6

The alcohol yields from starch slurry saccharified by less than the optimum amount of submerged culture were significantly increased by the addition of germ. However, a yield higher than the control was obtained only in the case when starch slurry was saccharified with 15 volume per cent of submerged culture. With mash saccharified with 15 volume per cent of submerged culture, a 5 per cent increase in yield was observed. With mash saccharified by

Table 18. Effect of germ on alcohol yields from starch slurry saccharified by less than the optimum level of submerged Aspergillus niger 337 culture

Germ added, g.	Theoretical yield, per cent
15 volume per cent saccharifying agent	
0	88.9
.5	91.1
1.0	92.4
1.5	93.8
2.0	93.6
control	93.1
10 volume per cent saccharifying agent	
0	83.9
.5	86.2
1.0	86.9
1.5	88.5
2.0	90.2
2.5	90.7
control	93.9
13 volume per cent saccharifying agent	
0	84.8
1.0	86.6
1.5	90.7
2.0	87.7
control	93.1

less than 15 per cent submerged culture an increase of around six per cent in yield was obtained. The increase in yield indicates the presence of some growth factor rather than inhibitor. For better comparison purposes, another series of fermentations was run with optimum amount of germ and less than optimum amount of submerged Aspergillus niger 337 culture. Twenty volume per cent of submerged culture was run as control. The results are given in Table 19.

Table 19. Alcohol yields from starch slurry saccharified by less than the optimum volume of submerged Aspergillus niger 337 culture with the addition of optimum amount of germ

Mold culture, volume per cent	Germ added, g.	Theoretical yield, per cent
20 (control)	0	92.7
15	0	89.1
15	1.5	93.2
13	0	87.1
13	1.5	91.8
10	0	84.8
10	2.0	90.5

The results again showed that a yield higher than from the control was obtained when starch slurry mash with germ added was saccharified with 15 volume per cent of submerged culture. Following the excellent results obtained with the addition of germ, it was then decided to run a series of fermentations substituting ground whole wheat for flour instead of studying the effect of adding germ to wheat flour mash. Whole wheat was ground in the Wiley mill and thoroughly mixed before being used. Twenty volume per cent of submerged Aspergillus niger 337 culture was used as saccharifying agent. The results are listed in Table 20.

Table 20. Effect of substituting ground whole wheat for wheat flour on the alcohol yields from saccharification with 20 volume per cent of submerged Aspergillus niger 337

Wheat flour, g. per flask	Ground whole wheat, g. per flask	Theoretical yield, per cent
0	100	82.7
20	80	85.9
40	60	86.3
60	40	87.8
80	20	89.7
100	0	90.1

Instead of the expected increase in yield, a steady decrease in yield was obtained with increasing amount of substituted ground whole wheat. Furthermore, there was excessive foaming, especially at around 50 per cent substitution. The decrease in yields obtained may be due to inhibitor as found by other worker (21) in corn germ. Substitution of whole wheat is definitely not a desirable step in alcoholic fermentation.

5. Effect of adding vitamins

Attention was next turned to ascertain the growth factors present in the germ. Anderson (5) reported for wheat germ a range of 19 to 44 mcg. of thiamine, 5 to 12 mcg. of riboflavin and 34 to 69 mcg. of niacin per gram. Solutions of the above vitamins were prepared. An amount equivalent to the upper limit of vitamin in 2 g. of germ was added to each flask of slurry and saccharified with 15 volume per cent of submerged Aspergillus niger 337 culture. Twenty volume per cent was run as control. Alcohol yields are given in Table 21.

Of the three vitamins investigated, only thiamine showed a slight increase in yield. The effect of thiamine cannot alone account for the increase in yield due to the

Table 21. Effect of thiamine, niacin and riboflavin on alcohol yields from starch slurry saccharified by Aspergillus niger 337 submerged culture

Enzyme, volume per cent	Substance added per flask	Theoretical yield, per cent
20	0	93.7
15	0	90.2
15	2 g. germ	93.7
15	24 mcg. riboflavin	90.2
15	88 mcg. thiamine	91.3
15	138 mcg. niacin	90.5
15	24 mcg. riboflavin, 88 mcg. thiamine and 138 mcg. niacin	91.0

addition of germ, nor can the combination of thiamine, niacin and riboflavin.

In continuing the investigation on the growth factor(s) present in the germ, the effect of adding the other vitamins listed on the commercial Kretschmer wheat germ label was studied. No significant increase in alcohol yield was observed when choline, inositol, pyridoxine, pantothenic acid, niacin, thiamine, riboflavin or folic acid in the concentrations as listed on the Kretschmer wheat germ label was

added either alone or as a mixture to the fermentation mashes. The results are given in Table 22.

The favorable effect of germ addition on fermentation could very well be due to the combined effect of protein

Table 22. Effect of vitamins on alcohol yields from starch slurry saccharified by Aspergillus niger 337 submerged culture

Enzyme, volume per cent	Substance added per flask	Theoretical yield, per cent
20	0	92.3
15	0	89.8
15	2 g. germ	93.2
15	8.0 mg. choline	90.2
15	14 mg. inositol	89.9
15	24 mcg. pyridoxine	90.0
15	22 mcg. pantothenic acid	89.7
15	8.9 mcg. folic acid	90.0
15	M (Mixture of choline, inositol, pyridoxine, pantothenic acid and folic acid in concentrations similar to above)	90.5
15	M plus thiamine, niacin and riboflavin in concentrations similar to Table 21	91.2

and vitamins present in the germ. No evidence of inhibitor present in the wheat germ was found. The inhibitor present in the whole wheat must have been removed or destroyed during the milling or storage of the germ.

6. Evaluation of data by Reese's intercept method

Reese (114) developed his short starch fermentation test on the basis that when dry weight of saccharifying agent divided by the weight of alcohol produced from the fermentation of corn mash saccharified by that level of saccharifying agent was plotted against the dry weight of saccharifying agent, straight lines of essentially the same slope were obtained. He found the ratio of the intercepts for two mold brans were directly related to the ratio of the levels of the two mold brans required for optimum alcohol production. It is of interest then to compare the data obtained in the wheat fermentations with the intercepts. The comparison is made in Table 23. The alcohol yields for different saccharifying agents at different levels and the ratio of dry weight of saccharifying agent over the alcohol produced from which the values of Table 23 were obtained are summarized in Tables 24 and 25.

Table 23. Comparison of maximum alcohol yields, optimum levels, intercept values, and slopes from different starchy substrates saccharified by different mold preparations

Preparation	Optimum level, g./100 g. starch	Max. alc. yield, % of theoretical	Inter-cept	Slope
Slurry				
A. niger 330 mb.	8.37	81.0	.0066	.0294
A. niger 337 mb.	11.24	88.4	.0151	.0350
A. oryzae 38b mb.	9.03	77.4	.0036	.0340
A. niger 337 sub.	12.53	93.6	.0382	.0262
Wheat flour				
A. niger 330 mg.	8.16	86.9	.0104	.0257
A. oryzae 38b mb.	8.16	86.7	.0198	.0237
A. niger 337 mb.	6.80	87.9	.0043	.0265
A. niger 337 sub.	12.45	90.1	.0196	.0240
Ground whole wheat				
A. niger 337 sub.	10.80	83.2	.0152	.0283

The preparations listed in Table 23 are identified by the strain number with "sub" or "mb" for submerged culture or mold bran, respectively. Maximum alcohol yields are given in per cent of theoretical yield based on the starch analysis. Optimum levels of saccharifying agents are given in grams of saccharifying agent per 100 g. of starch.

Table 24. Alcohol yields from starch slurry and wheat flour at different mold bran levels

(1) Dry wt. mold bran, g.	Wheat flour		Slurry	
	(2) Alcohol produced, g.	(1)/(2)	(2) Alcohol produced, g.	(1)/(2)
A. niger 330				
2.64	34.39	.0768	31.70	.0833
3.52	34.45	.1022	31.56	.1116
4.40	35.49	.1240	32.53	.1353
5.28	36.23	.1457	32.95	.1602
6.16	35.76	.1723	32.75	.1881
A. niger 337				
2.59	35.62	.0729	24.25	.1068
3.46	36.51	.0948	24.72	.1399
4.32	36.71	.1178	26.54	.1628
5.19	36.37	.1427	26.89	.1931
6.05	36.64	.1652	26.89	.2304
A. oryzae 38b				
2.66	32.46	.0816	26.95	.0986
3.54	34.67	.1022	28.20	.1257
4.43	35.89	.1233	28.76	.1539
5.31	36.17	.1469	29.11	.1825
6.19	36.03	.1720	28.90	.2144

Table 25. Alcohol yields from different starchy substrates at different *Aspergillus niger* 337 submerged culture levels

Culture, vol. per cent	(1) Dry weight culture, g.	(2) Alcohol produced, g.	(1)/(2)
Ground whole wheat			
5	1.88	27.02	.0694
10	3.75	30.09	.1246
15	5.63	31.98	.1759
20	7.50	32.74	.2292
25	9.38	33.01	.2840
Wheat flour			
5	1.88	29.95	.0626
10	3.75	34.80	.1077
15	5.63	36.85	.1526
20	7.50	37.57	.1996
25	9.38	38.51	.2434
Slurry			
5	1.89	22.49	.0841
10	3.78	27.37	.1381
15	5.68	30.16	.1882
20	7.57	32.12	.2356
25	9.46	33.22	.2848

From examination of the values in Table 23 it appears that there is no over-all relation between the intercepts and the optimum levels of saccharifying agent for actual fermentations. Straight lines were obtained when dry weight of the saccharifying agent divided by the weight of alcohol produced was plotted against the dry weight of the saccharifying agent for all preparations. However, no correlation whatsoever could be found between intercept values and the optimum levels or optimum alcohol yields in any comparison.

VI. DISCUSSION AND CONCLUSIONS

The short starch fermentation test of Reese, Fulmer, and Underkofler (115) was considered for the evaluation of 5 mold brans prepared from Aspergillus niger 337. A summary of the data for the 5 mold brans was presented in Table 3, page 25. The levels of mold brans for maximum alcohol yields were indicated as per cent of the total dry weight. The intercept values were obtained by graphical analysis of the data from short starch fermentation tests. Although straight lines were obtained, they were not all parallel to each other. Examination of the data leads to the conclusion that there is no correlation between the intercept value and optimum level of mold brans from Aspergillus niger 337. However, it must be pointed out that graphical analysis of meager fermentation data covering only a limited range of saccharification efficiency cannot very well justify the conclusion that absolutely no statistical correlation exists between the intercept and optimum level. For more proper checking of the method, more mold brans of greater differences in saccharification efficiencies should be studied.

Next phase of this investigation was to determine whether starch slurry could be fermented efficiently after separation of gluten for the production of monosodium

glutamate. Comparative studies were made on wheat flour and starch slurry (wheat flour after removal of gluten).

In the studies of the optimum conditions for alcoholic fermentation of wheat flour and starch slurry, the optimum acid concentration for initial cooking was considered first. Sulfuric acid concentration of 0.05 normal was chosen as the optimum for initial cook. Next to be considered was the saccharifying agent. The different saccharifying agents investigated were mold brans prepared from Aspergillus niger 330, Aspergillus oryzae 38b, and Aspergillus niger 337, malt, and submerged culture from Aspergillus niger 337. Extremely low yields were obtained from starch slurry saccharified by malt. Maximum alcohol yields from optimum levels of mold brans and malt were given in Tables 9 and 10, pages 83 and 84. Aspergillus niger 337 was found to be the best.

When saccharified by Aspergillus niger 337 mold bran, both wheat flour and starch slurry gave about 88 per cent of theoretical yield. Optimum alcohol yields from wheat as reported by Stark, Kolachov and Willkie (135) vary from 82 to 90 per cent of theoretical yield depending on the kind of wheat. These yields are lower than those obtained from corn. Lower alcohol yield from wheat was also reported by Stark, Adams, Scalf, and Kolachov (133). In the present

investigation wheat flour always gave better yields than starch slurry when saccharified by malt, Aspergillus niger 330 or Aspergillus oryzae 38b. In all cases, higher optimum levels of saccharifying agent was required for starch slurry than for wheat flour for maximum alcohol production. This may be due to the presence of gluten or its degradation products which may serve as growth factors.

Saccharification by submerged culture of Aspergillus niger 337 gave higher yields than saccharification by mold bran. Approximately 92 per cent of theoretical yield was obtained from both starch slurry and wheat flour saccharified by submerged Aspergillus niger 337. This may be due to the fact that Aspergillus niger 337 produced higher alpha-amylase in submerged culture than surface culture as reported by Dingle and Solomons (30) and Pool and Underkofler (106). Furthermore submerged culture was grown in 5 per cent distillers' dried solubles which contains various vitamins and proteins as reported by Underkofler (146). These vitamins and proteins could very well serve as activators or growth factors. The favorable effects of various vitamins, especially thiamine, and protein degradation products on fermentation have been reported by several groups of workers (32, 72, 162, 164, 165). Addition of distillers' dried solubles resulting in more superior and consistent performance

in the production of amylase was reported by Adams, Balankura, Andreasen and Stark (1).

Addition of ammonium salts did not prove to be of any value in the alcoholic fermentation of wheat flour or starch slurry. A decrease in yield was observed at higher concentrations of ammonium salts.

Research by Christensen (21) has shown an inhibitor to be present in corn germ which can be eliminated by extraction of the germ prior to fermentation. The effect of adding both extracted and unextracted germ was therefore investigated for wheat flour and starch slurry fermentations. No evidence of the presence of inhibitor was found in either extracted or unextracted germ. Addition of unextracted germ resulted in increase in alcohol yield. Alcohol yield of 95 per cent of theoretical was obtained from starch slurry saccharified by 20 volume per cent of Aspergillus niger 337 submerged culture with the addition of 1.5 g. of germ per flask. In general, addition of germ to both starch slurry and wheat flour fermentations showed increasing yields up to a certain concentration of germ, after which the yields leveled off with the addition of more germ.

The effect of addition of germ to mashes saccharified by less than the optimum amount of submerged culture was next investigated. The results have been given in Table

19, page 98. The results indicate that it is possible to obtain maximum alcohol yields with 15 volume per cent (5 per cent less than the optimum) of submerged Aspergillus niger 337 culture with the addition of 1.5 g. of unextracted germ per flask. Whether this step will be economically feasible or not will depend on the relative cost of alcohol, germ and enzyme solution.

Since excellent results were obtained with the addition of germ, it was of some interest to study the effect of partly substituting ground whole wheat for wheat flour. The results were listed in Table 20, page 99. A steady decrease in yield was observed with increasing amount of ground whole wheat added. It seems the inhibitor observed by other worker (21) is still present in the whole wheat. It must have somehow been removed or destroyed during the milling or storage of the germ.

Attention was next turned to attempts to ascertain what ingredients in the germ were responsible for the stimulation of alcohol production. All the vitamins listed on the Kretschmer wheat germ label were investigated. Only addition of thiamine showed a slight increase in yield. The effect of thiamine alone cannot account for the increase in yield due to the addition of germ. Addition of choline, inositol, pyridoxine, pantothenic acid, niacin, thiamine, riboflavin,

and folic acid either alone or as a mixture did not result in appreciable increase in alcohol yield.

The increase in alcohol yield due to addition of germ may be due to the combined effect of the vitamins and proteins present in the germ. Whatever the growth factor(s) may be, it is stable under the experimental conditions, that is, cooking for 45 minutes at 20 lbs. steam pressure in 0.05 normal sulfuric acid solution.

It was considered of some interest to evaluate the mold brans and submerged cultures by Reese's intercept method from the wheat fermentation data. Reese developed his short starch fermentation test on the basis that when dry weight of saccharifying agent divided by the weight of alcohol produced was plotted against the dry weight of the saccharifying agent, straight lines of essentially the same slope were obtained. He found the ratio of the optimum levels of two mold brans to be directly related to the ratio of the intercepts for the two mold brans. The intercept values and optimum levels of the saccharifying agents employed in the present study have been given in Table 23, page 104. Optimum levels of saccharifying agents were expressed in grams (dry weight) of saccharifying agent per 100 g. of starch. Although graphical analysis gave straight lines for all mold preparations on all the three different substrates,

no correlation between the intercept values and the optimum levels was found. This applies to all comparisons: i.e., over all, between 2 different types of preparation from the same mold, or the same type of preparation from the same mold on different kind of substrates, or within a group such as all mold bran preparations from different molds. This confirms and extends the observation of no correlation previously reported by Pool and Underkofler (106).

VII. SUMMARY

1. The short starch fermentation test of Reese, Fulmer, and Underkofler (115) was considered for the evaluation of five Aspergillus niger 337 mold bran preparations. Optimum level of each mold bran for maximum alcohol yield was determined experimentally on corn fermentations. No correlation between the intercept value and optimum level of mold bran was found.
2. Comparative studies were made on wheat flour and starch slurry to determine whether the starch slurry remaining after separation of gluten for the production of monosodium glutamate could be fermented efficiently. The concentration of acid necessary for proper thinning of the mashes was found to be 0.05 normal sulfuric acid.
3. Saccharification of fermentation mashes by malt, mold bran and submerged culture was investigated. Extremely low yields were obtained from starch slurry saccharified by malt. All the three mold brans investigated proved to be better than malt.
4. Mold bran from Aspergillus niger 337 was shown to be better than mold brans from Aspergillus niger

330 and Aspergillus oryzae 38b. Approximately 88 per cent of theoretical yields were obtained from both wheat flour and starch slurry saccharified by Aspergillus niger 337 mold bran.

5. Wheat flour always gave better alcohol yields than starch slurry when saccharified by malt, or mold brans from Aspergillus niger 330 or Aspergillus oryzae. In all cases, higher optimum levels of saccharifying agent were required for starch slurry than for wheat flour for maximum alcohol yields.
6. Saccharification by submerged culture of Aspergillus niger 337 gave higher yields than saccharification by mold bran. Approximately 92 per cent of theoretical yields were obtained from both starch slurry and wheat flour saccharified by submerged Aspergillus niger 337 culture.
7. Addition of ammonium salts did not prove to be of any value in the alcohol fermentation of wheat flour or starch slurry saccharified by either malt or mold bran. Decreases in yield were observed at higher concentrations of ammonium salts.
8. Addition of unextracted wheat germ resulted in increases in alcohol yields from wheat flour or starch slurry saccharified by either malt, mold

bran or submerged culture. Highest alcohol yield (95 per cent of theoretical yield) was obtained from starch slurry saccharified by optimum volume (20 volume per cent) of submerged Aspergillus niger 337 culture with the addition of 1.5 g. of germ per 300 ml. of starch slurry. No evidence of the presence of an inhibitor was found in either extracted or unextracted germ.

9. Alcohol yield higher than control was obtained from starch slurry saccharified by less than optimum level (15 volume per cent) of submerged culture with the addition of unextracted germ.

10. The effect of partly substituting ground whole wheat for wheat flour was investigated. A steady decrease in alcohol yields was observed with increasing amounts of ground whole wheat added. The very low alcohol yield from ground whole wheat indicated the presence of inhibitor.

11. Addition of vitamins present in germ (thiamine, inositol, pyridoxine, pantothenic acid, niacin, riboflavin and folic acid) either alone or as a mixture did not increase the alcohol yields appreciably.

12. Evaluation of mold brans and submerged cultures by Reese's intercept method from the wheat fermentation data did not show any correlation between the intercept values and the optimum levels of saccharifying agents required for maximum alcohol yields. Graphical analysis gave straight lines for all mold preparations on all the three different substrates.

VIII. LITERATURE CITED

1. Adams, S. L., Balankura, B., Andreasen, A. A., and Stark, W. H. Ind. Eng. Chem. 39:1615. 1947.

2. Akabori, S., Hagihara, B. and Ikenaka, T. Proc. Japan Acad. 27:350. 1951

3. Akabori, S., Ikenaka, T. and Hagihara, B. J. Biodhem. (Japan). 41:577. 1954.

4. Alvik, G. Bergens Museums Arbok. Naturvidenskapelig Rekke. 5:52. 1931.

5. Anderson, G. K. J. Am. Med. Assoc. 125:848. 1949.

6. Association of Official Agricultural Chemists. Methods of Analysis. 7th ed. p. 348. 1950.

7. Back, T. M., Stack, W. H. and Scalf, R. S. Anal. Chem. 20:56. 1948.

8. Banzon, J. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1940

9. Banzon, J., Fulmer, E. I. and Underkofler, L. A. Iowa State College J. of Sci. 23:219. 1949.

10. Bernfeld, P. In Nord, F. Advances in Enzymol. 12:380. 1951. New York, Interscience Publishers, Inc.

11. Bindal, A. N. and Sreenivasaya, M. J. Sci. Ind. Res. (India). 3:287. 1945.

12. Bindal, A. N. and Sreenivasaya, M. J. Sci. Ind. Res. (India). 3:386. 1945.

13. Bindal, A. N. and Sreenivasaya, M. Current Sci. 18:21. 1945.

14. Blaisten, R. J. Rev. asoc. bioquim. Argentina. 15:47. 1948 (Original not available; abstracted in Chem. Abst. 43:1571. 1949).

15. Blaisten, R. J. and Saad, E. Rev. asoc. bioquim. Argentina. 14:183. 1947. (Original not available; abstracted in Chem. Abst. 42:5163. 1948).

16. Boyer, J. W. and Underkofler, L. A. Chem. and Met. Eng. 52, No. 12:110. 1945.

17. Bovard, F. C. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1952.

18. Caldwell, M., Adams, M., Tung-Kung, J. and Toralballa, G. J. Am. Chem. Soc. 74:4033. 1952.

19. Calmette. La levure chinoise. Ann. inst. Pasteur. 6:604. 1892.

20. Christensen, L. M. U. S. Patent 2,325,368 July 27, 1943; 2,352,168 June 27, 1944; 2,356,218 Aug. 22, 1944; 2,362,451 Nov. 14, 1944.

21. Christensen, L. M. U. S. Patent 2,662,842 Dec. 15, 1953.

22. Christensen, L. M. and Underkofler, L. A. U. S. Patent 2,584,258 Feb. 2, 1952.

23. Collens, A. E. Trinidad and Tobago, Bull. 14:56. 1914. (Original not available; abstracted in Chem. Abst. 9:2284. 1915).

24. Collette, A. and Boidin, A. British Patent 19,858 Aug. 28, 1897. (Abstracted in J. Soc. Chem. Ind. 17:483. 1898).

25. Colwell, A. T., Cummings, R. E. and Anderson, D. E. S. A. E. J. (Trans.). 53:358. 1945.

26. Corman, J. and Langlykke, A. F. Cereal Chem. 25:190. 1948.

27. Corman, J. and Tsuchiya, H. M. Cereal Chem. 28:280. 1951.

28. Deckenbrock, W. Chem. Z. 121, pt. 2:475. 1950.

29. Deckenbrock, W. Chem. Z. 121, pt. 2:715. 1950.

30. Dingle, J. and Solomons, G. L. J. Appl. Chem. (London). 2:395. 1952.

31. Drews, B., Lampe, B. and Specht, H. Branntweinwirtshaft. 4:293. 1950.

32. Dupuy, P. and Flanzy, M. Compt. rend. acad. agr. France. 40:273. 1954.

33. Erb, N. M. and Hildebrandt, F. M. Ind. Eng. Chem. 38:792. 1946.

34. Erb. N. M., Wisthoff, R. T. and Jacobs, W. L. J. Bact. 55:813. 1948.

35. Euler, H. von and Asarnoj, S. Fermentforschung. 3:318. 1920.

36. Fuel Alcohol Consumption Tops 200 Millions Gallons. Chem. Eng. News. p. 4694. 1953.

37. Fischner, E. H. and de Montmollin, R. Nature (London) 168:606. 1951.

38. French, D. Science 113:352. 1951.

39. French, D. and Knapp, D. W. J. Biol. Chem. 187:463. 1950.

40. Fukimbara, T., Yoshida, H. and Shibuya, M. J. Agr. Chem. (Japan). 24:133. 1951.

41. Fukimbara, T., Yoshida, H. and Shibuya, M. J. Agr. Chem. (Japan). 24:157. 1951

42. Funke, G. L. Rec. trav. botan. Neerlandais. 24:583. 1928.

43. Galle, E. Z. angew. Chem. 36:17. 1923.

44. Gayon, U. and Dubourg, E. Ann. Aci. agron 1 (pt. 1):419. 1887.

45. Goering, K. J. Iowa State College J. of Sci. 16:55. 1941.

46. Goodman, J. J. Science. 112:176. 1950.

47. Guagnini, O. and Jacovkis, D. Industria y quimica (Buenos Aires) 9:207. 1947. (Original not available; abstracted in Chem. Abst. 42:6881. 1948).

48. Guagnini, O. and Jacovkis, D. Industria y quimica (Buenos Aires) 9:357. 1947. (Original not available; abstracted in Chem. Abst. 42:7485. 1948).

49. Hao, L. C. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library.

50. Hao, L. C., Fulmer, E. I. and Underkofler, L. A. Ind. Eng. Chem. 35:814. 1943.

51. Hao, L. C. and Jump, J. A. Ind. Eng. Chem. 37:521. 1945.

52. Harada, T. Ind. Eng. Chem. 23:1424. 1931.

53. Heldt, P. M. Automotive Inds. 98, No. 2:32. 1948.

54. Hildebrandt, F. M. and Erb, N. M. U. S. Patent 2,556,084 June 5, 1951.

55. Jeffreys, G. A. Food Ind. 23, No. 3:80. 1951.

56. Kashiwagi, K. British Patent 179,012. Feb. 24, 1921. (Abstracted in Chem. Abst. 16:3364. 1922).

57. Kawamura, S. J. Ferm. Assoc. (Japan). 7:86. 1949.

58. Kerr, R. W., Cleveland, F. C. and Katzbeck, W. J. J. Am. Chem. Soc. 73:3916. 1951.

59. Kita, G. Ind. Eng. Chem. 5:220. 1913.

60. Kitahara, K. and Kurushima, M. J. Ferm. Tech. (Japan). 27:1. 1949.

61. Kitahara, K. and Kurushima, M. Mem. Res. Inst. Food Sci. (Kyoto Univ.) No. 1:28. 1951.

62. Kneen, E. and Spoerl, J. M. Am. Soc. Brewing Chemists Proc. 20:6. 1948.

63. Korshelt, O. Dingler's Polytech. J. 230:76. 1878.

64. Kozai, Y. Z. Untersuch. Nehr. u. Genussm. 4:211. 1901.

65. Lampe, B. Branntweinwirt 4:152. 1950. (Original not available; abstracted in Chem. Abst. 45:3116. 1951.).

66. LeMense, E. H., Corman, J., Van Lanen, J. M., and Langlykke, A. F. J. Bact. 54:149. 1947.

67. LeMense, E. H., Sohns, V. E., Corman, J., Blom, R. H., Van Lanen, J. M. and Langlykke, A. F. Ind. Eng. Chem. 41:100. 1949.

68. LeMense, E. H. and Van Lanen, J. M. U. S. Patent 2,451,567 Oct. 19, 1948.

69. Lintner, C. J. J. Prakt. Chem. ser. 2, 34:378. 1886.

70. Lintner, C. J. and Wirth, C. Z. ges. Brauw. 31:421. 1908.

71. Mack, R. C. Automotive Inds. 98, No. 3:36. 1948.

72. Maesen, Th. J. M. Biochim. et Biophys. Acta. 12:445. 1953.

73. Meyer, K. H., Fischer, E. H., Bernfeld, P. and Duckert, F. Arch. Biochem. 18:203. 1948.

74. Meyer, K. H., Fuld, M. and Bernfeld, P. Experientia. 3:411. 1947.

75. Miller, R. E. Unpublished M.S. Thesis. Ames, Iowa, Iowa State College Library.

76. Miyaji, N. J. Agr. Chem. Soc. (Japan). 22:79. 1948.

77. Miyaji, N. J. Agr. Chem. Soc. (Japan). 22:81. 1948.

78. Montes, A. E. Industria y quimica. (Buenos Aires). 8:37. 1946. (Original not available; abstracted in Chem. Abst. 40:5197. 1946.).

79. Murota, S., Saruna, R. and Ano, T. J. Ferm. Tech. (Japan). 31:179. 1953.

80. Murota, S., Saruna, R. and Ano, T. J. Ferm. Tech. (Japan). 31:222. 1953.

81. Murota, S., Saruna, R. and Ano, T. J. Ferm. Tech. (Japan). 31:282. 1953.

82. Murota, S., Saruna, R. and Ano, T. J. Ferm. Tech. (Japan). 31:305. 1953.

83. Murota, S., Saruna, R. and Ano, T. J. Ferm. Tech. (Japan). 31:357. 1953.

84. Murota, S., Saruna, R. and Ano, T. J. Ferm. Tech. (Japan). 31:392. 1953.

85. Murota, S., Saruna, R. and Ano, T. J. Ferm. Tech. (Japan). 32:8. 1954.

86. Muta, K. and Tanaka, S. J. Agr. Chem. Soc. (Japan). 12:129. 1936.

87. Myrbäck, K. and Neumüller, G. The enzymes. Chemistry and mechanism of action. 1st. ed. Vol. I, p. 653-724. New York, Academic Press, Inc. 1950.

88. Nishimura, S. Chem. Zelle U. Gewebe. 12:202. 1925. (Original not available; abstracted in Chem. Abst. 20:52. 1926.).

89. Ono, H. and Dazai, C. Hakko Kyokai-shi. 9:139. 1951.

90. Ono, H. and Misono, M. J. Ferm. Assoc. (Japan). 7:253. 1949.

91. Ono, H. and Tanaka, M. Hakko Kyokai-shi. 9:14. 1951.

92. Oshima, K. J. Coll. Agr. Hokkaido Imp. Univ. 19, pt. 3: 135. 1928. (Original not available; abstracted in Chem. Abst. 22:2383. 1928.).

93. Oshima, K. and Church, M. B. Ind. Eng. Chem. 15:67. 1923.

94. Otani, Y. and Takahashi, S. J. Ferm. Tech. (Japan). 32:392. 1954. (Original not available; abstracted in Chem. Abst. 49:2017. 1955.).

95. Owen, W. L. Ind. Eng. Chem. 25:87. 1933.

96. Pan, S. C., Andreasen, A. A., and Kolachov, P. Ind. Eng. Chem. 42:1783. 1950.

97. Pan, S. C., Andreasen, A. A. and Kolachov, P. Science 112:115. 1950.

98. Pan, S. C., Andreasen, A. A. and Kolachov, P. Arch. Biochem. 30:6. 1951.

99. Pan, S. C. and Liu, A. H. Biochem. Bull. (China). (Original not available; abstracted in Chem. Abst. 39:2619. 1945.).

100. Pan, S. C., Nicholason, L. W. and Kolachov, P. J. Am. Chem. Soc. 73:2547. 1951.

101. Pazur, J. H. Science, 117:355. 1953.

102. Pazur, J. H. and French, D. J. Am. Chem. Soc. 73:3536. 1951.

103. Phillips, L. L. and Caldwell, M. L. J. Am. Chem. Soc. 73:3559. 1951.

104. Phillips, L. L. and Caldwell, M. L. J. Am. Chem. Soc. 73:3563. 1951.

105. Pigman, W. W. J. Research Natl. Bur. Standards. 33:105. 1944.

106. Pool, E. L. and Underkofler, L. A. Agr. and Food Chem. 1:87. 1953.

107. Porter, J. C., Gilbert, M. M., Lykins, H. A. and Wiebe, R. Agr. Eng. 31:71. 1950.

108. Porter, J. C., McCloud, A. P. and Wiebe, R. Automotive Inds. 102, no. 1:195. 1952.

109. Porter, J. C., Roth, W. B. and Wiebe, R. Automotive Inds. 98, no. 8:34. 1948.

110. Porter, J. C. and Wiebe, R. Ind. Eng. Chem. 44:1098. 1952.

111. Rao, M. R. R. and Sreenivasaya, M. Current Science. 15:249. 1946.

112. Rao, M. R. R. and Sreenivasaya, M. J. Sci. Ind. Research. (India). 6B:96. 1947.

113. Rao, M. R. R. and Sreenivasaya, M. India Patent 39,991. June 7, 1950.

114. Reese, H. D. Unpublished Ph.D. Thesis, Ames, Iowa, Iowa State College Library. 1947.

115. Reese, H. D., Fulmer, E. I., and Underkofler, L. A. Anal. Chem. 20:348. 1948.

116. Reindel, F., Frey, A. and Lottner, E. Branntweinwert. 4:179, 1950.

117. Riberau-Gayon, J. and Peynaud, E. Compt. rend. acad. agr. (France). 39:444. 1952. (Original not available; abstracted in Chem. Abst. 48:12367. 1954.).

118. Roberts, M., Laufer, S., Stewart, E. D. and Saletan, L. T. Ind. Eng. Chem. An. Ed. 36:811. 1944.

119. Roy, D. K. Ann. Biochem. and Exptl. Med. (India). 12:115. 1952. (Original not available; abstracted in Chem. Abst. 48:3628. 1954.).

120. Saito, K. Centr. Bakt. Parasitenk. II, 13:153. 1904.

121. Saito, K. Centr. Bakt. II, 18:30. 1907.

122. Sakaguchi, K. and Okasaki, H. Unpublished report. Univ. of Tokyo, Bankyoku, Tokyo, Japan, 1952.

123. Sandstedt, R. M., Kneen, E. and Blish, M. J. Cereal Chem. 16:712. 1939.

124. Sanguinete, J. Ann. Inst. Pasteur. 11:269. 1897.

125. Scales, F. M. J. Biol. Chem. 19:459. 1914.

126. Schoene, L., Fulmer, E. I. and Underkofler, L. A. Ind. Eng. Chem. 32:544. 1940.

127. Schwimmer, S. J. Biol. Chem. 161:219. 1945.

128. Schwimmer, S. and Balls, E. K. J. Biol. Chem. 179:1063. 1949.

129. Severson, G. M., Underkofler, L. A. and Schoene, L. U. S. Patent 2,289,080 July 14, 1942.

130. Shellenberger, J. A. Chem. Eng. 54:130. 1947.

131. Shimada, S., Sugita, O. and Mizumoto, K., J. Ferm. Tech. (Japan). 31:498. 1953.

132. Soriano, S. and Trucco, R. E., Rev. facultad agron. y vet. Univ. Buenos Aires 12:26. 1948. (Original not available; abstracted in Chem. Abst. 43:5901. 1949.).

133. Stark, W. H., Adams, S. L., Scalf, R. E. and Kolachov, P. Ind. Eng. Chem. Anal. ed. 15:443. 1943.

134. Stark, W. H., Kolachov, P., Smith, L. A., Blankmeyer, H. C. and Willkie, H. F. Rept. Proc. 4th Intern. Congr. Microbiol. 1947:550. 1949.

135. Stark, W. H., Kolachov, P. and Willkie, H. F. Ind. Eng. Chem. 35:133. 1943.

136. Takamine, J. Ind. Eng. Chem. 6:824. 1914.

137. Takaoka, Ken-ichi, Fuma, H. and Nikuni, Ziro. Mem. Inst. Sci. and Ind. Res. (Japan). 10:199. 1952.

138. Takeda, Y. J. Agr. Soc. (Japan). 11:845. 1935. (Original not available; abstracted in Chem. Abst. 30:2316. 1936.).

139. Takeda, Y. J. Agr. Soc. (Japan). 11:943. 1935. (Original not available; abstracted in Chem. Abst. 30:2316. 1936).

140. Teixeira, C., Andreasen, A. A., and Kolachov, P. Ind. Eng. Chem. 42:1781. 1950.

141. Thorne, C. B., Emerson, R. L., Olson, W. J. and Peterson, W. H. Ind. Eng. Chem. 37:1142. 1945.

142. Tomikanehara, T. Hakko Kyokai-shi. 9:325. 1951.

143. Tsuchiya, H. M., Corman, J. and Koepsell, J. Cereal Chem. 27:322. 1950.

144. Underkofler, L. A. U. S. Patent 2,291,009 July 28, 1942.

145. Underkofler, L. A. Brewers Digest 17, No. 12:29. 1942.

146. Underkofler, L. A. Reprint from the Feed Bag. April, 1945.

147. Underkofler, L. A. The Frontier. 9, No. 1:6. 1946.

148. Underkofler, L. A. and Fulmer, E. I. Chronica. Botan. 7:420. 1943.

149. Underkofler, L. A., Fulmer, E. I. and Schoene, L. Ind. Eng. Chem. 31:734. 1939.

150. Underkofler, L. A., Goering, K. J. and Buckaloo, G. W. Proc. Iowa Acad. Sci. 48:225. 1941.

151. Underkofler, L. A., Guymon, J. F., Rayman, M. M. and Fulmer, E. I. Iowa State Coll. J. of Sci. 17:251. 1943.

152. Underkofler, L. A. and Roy, D. K. Cereal Chem. 28:18. 1951.

153. Underkofler, L. A., Severson, G. M. and Goering, K. J. Ind. Eng. Chem. 38:980. 1946.

154. Underkofler, L. A., Severson, G. M., Goering, K. J. and Christensen, L. M. Cereal Chem. 24:1. 1947.

155. U. S. Production and Marketing Administration and Bureau of Agricultural and Industrial Chemistry. U. S. Dept. of Agr. Technical Bull. No. 1024. Aug. 1950.

156. Van Lanen, J. M. and LeMense, E. H. J. Bact. 51:595. 1946.

157. Wei, N. S. and Chin, K. S. Science (China) 18:1193. 1934. (Original not available; abstracted in Chem. Abst. 29:2977. 1935.).

158. Weill, C. E., Burch, R. J. and Van Dyk, J. W. Cereal Chem. 31:150. 1954.

159. Weill, C. E., Burch, R. J. and Van Dyk, J. W. Abstr. 118th. mtg., Am. Chem. Soc. 1950:14B.

160. Whitehouse, K. and Adams, S. L. Agr. and Food Chem. 2:1040. 1954.

161. Wolfrom, M. L., Thompson, A. and Galkowski, T. T. J. Am. Chem. Soc. 73:4093. 1951.

162. Wolnak, B. and Miner, C. S. Agr. and Food Chem. 2:790. 1954.

163. Yamagishi, H. Science Repts. Tohoku Imp. Univ. 3:179. 1928. (Original not available; abstracted in Chem. Abst. 22:1613. 1928.).

164. Zabrodskii, A. G. and Kordyukova, N. S. Mikrobiologia. 23:57. 1954.

165. Zabrodskii, A. G. and Leshchinskaya, S. S. Mikrobiologia. 23:313. 1954.

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