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Mold bran and submerged culture fungal preparations for saccharifying corn fermentation mashes

Edwin Lewis Pool
Iowa State College

NOTE TO USERS

MOLD BRAN AND SUBMERGED CULTURE FUNGAL PREPARATIONS FOR SACCHARIFYING CORN FERMENTATION MASHES

by

Edwin Lewis Pool

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Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

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TABLE OF CONTENTS

INDEX OF TABLES

INDEX OF TABLES (Continued)

INDEX OF FIGURES

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I. INTRODUCTION

For many centuries ethanol has found use in the preparation of medicines, perfumes, cosmetics, and in alcoholic beverages. Within the last 100 years it has begun to find considerable use outside the beverage field and certainly within the last 40 years it has become one of the most vital raw materials in the world's chemical industry.

At the present time millions of gallons of ethanol are used yearly in the production of acetaldehyde, acetic acid, ethyl acetate, ethyl chloride, ethyl ether, and other similar compounds. As a solvent its use is surpassed by only one other liquid, water. It is an important starting material in the manufacture of many drugs, plastics, lacquers, polishes, plasticizers, and rubber accelerators. During wartime, its uses become even more varied and it has been used in the production of explosives (especially smokeless powder), chemical warfare gases, anti-freeze, and butadiene for synthetic rubber.

A Chemical and Engineering News Staff Report entitled "Industrial Alcohol" (1952) presents an interesting discussion including production statistics for recent years. It is of some interest to examine these alcohol production figures.

The peace-time production of ethanol in 1940 was approximately 250 million proof gallons, increasing during the war years to a high production of 1,200 million proof gallons in 1945. Following the war the production and use of ethanol dropped rapidly but new uses for ethanol and the recent industrial mobilization programs have once again started the demand on the upswing. The estimated production in the year ending in June, 1951, was about 456 million proof gallons, with the demand for the entire year of 1951 estimated at 700 million proof gallons.

Prior to 1929, and even through the next decade, alcohol from the fermentation of starches and sugars constituted nearly the entire commercial production. In 1935, for example, approximately 86 per cent of the commercial alcohol production was by fermentation of molasses, 3 per cent from grain fermentations and only about 10 per cent was synthesized from ethylene. During World War II the submarine menace greatly decreased the supply of blackstrap molasses, obtained mainly from Cuba. The synthetic alcohol plants, although expanded greatly, were unable to meet the increased alcohol demand. Consequently the Government called on all beverage manufacturers capable of producing 190-proof alcohol to switch to the production of industrial-grade material. Due to the fact that most of the beverage plants were equipped to handle grains only, there was a tremendous increase in the use of

grain for alcohol production during the war. Initially corn was used as the substrate, but later demands necessitated the use of wheat, barley, rye, oats, and sorghum grains. About 43 per cent of the alcohol production at that time was from the fermentation of grains.

The synthetic alcohol industry has continued to expand since its inception in 1929 and in 1951 was responsible for 48.5 per cent of the commercial alcohol production. Alcohol production by molasses fermentation, which must compete with the use of molasses in feeds, has leveled off at about 100 million proof gallons yearly, about 24 per cent of the 1951 production. High-cost grain has nearly disappeared as a source of industrial alcohol, dropping to about one per cent in 1950. However in 1951 the increased alcohol demands resulted in a temporary increase in alcohol production from grains up to about 26 per cent of the total production.

The future outlook indicates that the synthetic alcohol industry will continue its expansion and will dominate the alcohol production picture. There is little variation in raw material cost so the price of synthetic alcohol should level off at 25 to 30 cents a wine gallon (190-proof). Since blackstrap molasses is an agricultural product it is subject to some variation in price. The price fluctuation is somewhat dampened by the continuing demand of the fermentation industries; also the cost is at least partially keyed to the

selling price of the alcohol produced from it but it is still an expensive material. The supply of molasses is becoming limited due to competition by foreign markets and also to its increasing use as a feed supplement. In addition to the supply problem the higher cost of molasses alcohol, around 50 cents a wine gallon, indicates that this source of industrial alcohol will steadily decrease in importance.

Grains are subject to wild fluctuations in price. At the present time the cost of alcohol from grain is 70 cents or more per wine gallon. Although recovery of carbon-dioxide, corn oil, feeds, and other by-products can lower the production cost, alcohol from grain can not now compete with synthetic alcohol. However, the beverage industry with its facilities for grain-fermentations does represent an enormous industrial alcohol production potential. In times of sudden increased alcohol demand, like during World War II and the present emergency, grain-fermentation does become exceedingly important.

The importance, or at least potential importance, of the production of industrial alcohol by the fermentation of grains necessitates a continued interest and investigation in this field. Of extreme importance is the problem of reducing the cost of this type of production. This can be accomplished by either increasing the efficiency of the process or by decreasing the cost of materials used.

All starchy substrates, such as grains, must be converted to fermentable sugars, a process called saccharification, before alcoholic fermentation by yeast is possible. Barley malt, although it has been used for centuries for saccharification, is relatively expensive and does not give complete conversion into sugars. Therefore, any replacement for malt offers the hope of increasing the efficiency and/or decreasing the cost of raw materials.

One such replacement is 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. 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 especially at the Northern Regional Research Laboratory. It has been found that such submerged culture preparations can be used as efficient and inexpensive replacements for malt.

A number of problems still exist in regard to the use of

mold preparations for the saccharification of starchy materials. Of considerable interest is the testing of various organisms for use either in submerged culture or in the preparation of mold bran. Since Aspergillus oryzae 38-b had been found earlier to be the organism of choice for the preparation of mold bran at Iowa State College the investigation of its use in submerged culture seemed promising. Another equally promising possibility was the preparation of mold brans from two strains of Aspergillus niger which had been found to produce submerged culture preparations of high saccharifying efficiency.

In using these fungal preparations it was necessary to study the effect of varying the composition of the medium, the method of growing the cultures, time and temperature of saccharification, and other factors.

There remained the problem of how to evaluate the mold preparations. One obvious method involves the determination of enzyme activity in the preparations. Early workers soon realized that the method used for the evaluation of malts, Lintner (1886) and Lintner and Wirth (1908), was of no value when applied to fungal preparations. A later criterion used for the evaluation of such preparations was the alpha-amylase activity. Maltase and limit-dextrinase determinations were next tried and the former appeared to have greater reliability. The problem still remained, however, whether a simple

determination of enzyme activity was adequate or whether some type of an experimental saccharification and fermentation was necessary to evaluate the efficiency of fungal preparations as saccharifying agents.

II. REVIEW OF THE LITERATURE

A. Development and Use of Mold Amylase Preparations

In the production of ethanol by the fermentation of starchy materials such as grains, it is necessary first to convert the starch to a sugar (glucose or maltose) upon which the yeast can act. This conversion, generally referred to as saccharification, is necessary since the ordinary yeasts have no enzyme systems capable of hydrolyzing starches.

There are two main methods by which the saccharification of starches can be accomplished: acid hydrolysis and enzymatic hydrolysis. Acid hydrolysis of starch is carried out by heating with moderate concentrations of hydrochloric or sulfuric acid, usually at an elevated temperature and pressure. Acid saccharification is inconvenient and requires special acid-resistant equipment. The conversion to fermentable sugars is incomplete and possibly toxic factors are produced resulting in low alcohol yields. For these reasons this process has found little use in the industrial alcohol field.

Diastases or amylases comprise the group of enzymes which hydrolyze starch to dextrins and the sugars, maltose and glucose. They occur in a variety of biological forms, including the seeds of plants such as barley, wheat, and soybeans,

animal glands such as the pancreas, and microorganisms such as the bacteria and molds. Of these materials barley is utilized most commonly as a source of enzymes for the industrial saccharification of starch. Before barley can be used for saccharification its enzymatic activity must be enhanced by the malting operation. Carefully selected barley is steeped (i.e., soaked in water); allowed to germinate; and finally, when the desired stage of growth has been attained, dried under carefully controlled conditions to form barley malt. There are many critical factors in all stages of the malting process; the temperature, moisture, oxygen supply, and time all have their effect on the production of amylases and on the color, flavor, and aroma which are imparted to the final product. The latter is important because malt is used for the most part by brewers and distillers.

Despite its wide-spread use malt does have a number of very definite disadvantages. First, it is relatively expensive and large amounts are necessary for saccharification. The cost of the malt needed for the preparation of one wine gallon of alcohol from corn is 8 to 10 cents, or at least 10 per cent of the total production cost. Facilities for malting are limited so that in emergencies, as during World War II, when increased industrial alcohol demands must be satisfied by the beverage industry, sufficient high quality malt is not

available. A third disadvantage of malt is that it is a possible source of bacterial contamination. Even the best malts may have as many as 2 million organisms per gram. Such contamination may result in poor fermentations and the need for frequent clean-up and sterilization periods in the alcohol plant.

Although certain bacteria do produce amylases they have found little or no use in the saccharification of starchy material for alcoholic fermentation. On the other hand, molds and mold preparations have found rather extensive use, particularly in the Orient, as saccharifying agents.

It is of some interest to speculate briefly on the possible reasons for the geographical restriction on the type of saccharifying agent used in the fermentation industries. In Europe and the American continent the use of malt is nearly exclusive, while in the Orient one finds saccharification only by molds. Centuries ago, in Europe, the addition of yeast, either by chance or by design, to cooked grain a portion of which had sprouted undoubtedly resulted in the first alcoholic fermentation of grain. If not the first, it was certainly a much more successful fermentation, and the addition of sprouted grains must have been one of the earliest developments in the brewing art.

In the Orient, also many centuries ago, a chance exposure

of cooked rice to the air was undoubtedly followed by a prolific growth of a mixed culture of molds, bacteria, and yeasts. Saccharification and the spontaneous fermentation to alcohol then occurred. Similar methods are still in use in modern times in China and Japan for the preparation of alcoholic beverages from rice according to Owen (1933). In Japan a mixture of microorganisms from which many single strains of high saccharifying power have been isolated is grown on a steamed rice substrate and the resulting product is known as "kôji". Kôji is then used in the preparation of a rice beer "saké". In China a similar process is followed involving the exposure of rice flour dough and steamed rice to the air. A mixed culture growth occurs, and the material is dried to give "Chinese yeast" which may then be used for the saccharification of rice mashes and fermentation to form alcohol.

It was not until near the end of the 19th Century that much scientific interest was given to the investigation of fungal enzyme preparations. Korshelt (1878) during a study of the preparation of Japanese saké 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 (1887) investigated Aspergillus oryzae and several species of Mucor and found that Aspergillus oryzae

had the highest saccharifying ability. Calmette, in 1892, made the first scientific investigation of Chinese yeast. He found it to consist of a mixture of microorganisms in which one mold was predominate. This mold, to which he gave the name Amylomyces rouxii, was capable of converting starches into sugars which it was able to ferment slowly to alcohol.

Sanguinete (1897) continued the investigations, comparing the molds studied by Gayon and Calmette. He concluded that although Aspergillus oryzae was superior to Amylomyces rouxii in saccharifying power the latter organism was more suitable for industrial use since it fermented the starchy materials without the addition of yeast.

These investigations led Collette and Boidin (1897) to develop a process for the manufacture of alcohol involving saccharification and fermentation by molds. Their patent indicated that the mold used was Amylomyces rouxii, Aspergillus oryzae, or some other species of mold, and that yeast was added to hasten the fermentation. They claimed yields of alcohol which were much higher than those obtained by the process usually practised (i.e., a process involving malt-saccharification). The details of their process, which became known as the Amylo process, were described by Galle (1923) and by Owen (1933).

In the Amylo process the starchy material was first liquefield by a small amount of malt, acid, or mold. It was then

heated under pressure and cooled under aseptic conditions. The mold was then inoculated into the starch solution and allowed to grow for 24 hours in submerged culture with continued aeration by sterile air. At the end of this period yeast was added and the fermentation was continued for 48 hours. A great disadvantage of this process was the need for special aseptic techniques to prevent contamination.

Consequently, the original organism, Amylomyces rouxii (later classified as Mucor rouxii), was replaced by Mucor B., Mucor G, Rhizopus delemar, and finally Mucor Boulard No. 5. All of these molds were isolated from Chinese yeast or Japanese kôji. The last organism, Mucor Boulard No. 5, was found to have a greater tolerance for alcohol, produced less acid, shortened the time to 48 hours, held its own against contaminants, and allowed the addition of yeast and mold at the same time. It was used in the Boulard (or Boulard-Amylo) process, which differed from the original Amylo process in that it required less drastic conditions of sterility, and the yeast and mold were added simultaneously. This process, or other modifications of the Amylo process, has found use in southern Europe, South America, and Asia. Sakaguchi and Okasaki (1952) report that the amylo process is widely used in Japan at present. The organism is Rhizopus javanicus Takeda isolated by G. Takeda (1935b).

Jokichi Takamine (1914) attempted as early as 1891 to introduce the use of amylolytic mold preparations to the distilling industries of this country. He reviewed in this paper his attempts to find a substitute for malt in the saccharification of starch. His research yielded a mold preparation of Aspergillus oryzae on a bran substrate, which he designated as "taka-kôji" to differentiate it from the Japanese kôji which was the same mold grown on rice. Enzyme concentrates from extracts of taka-kôji were labeled "taka-diastase." His experiments resulted in his patenting about 15 processes prior to 1914 for developing or growing enzymes capable of converting starch into sugar. He considered three genera of molds, Aspergillus, Mucor, and Penicillium, but most of his work was with Aspergillus oryzae. Takamine developed methods for growing taka-kôji 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.

In discussing present fermentation methods in Japan, Sakaguchi and Okasaki (1952) note that a Kôji process based on Takamine's investigations was introduced into Japan in 1893 and is at present one of the 5 major methods of industrial saccharification.

Jean (1898) reviewed Takamine's process for saccharification

and fermentation by means of Aspergillus oryzae and compared it with the earlier Amylo process; he found that the Aspergillus oryzae had a greater saccharifying power than Mucor rouxii and could be used with stronger mashes.

Saito (1904) 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 also fermented it to alcohol. Saito (1907) also studied Aspergillus batatae, Aspergillus pseudoflavus, and Rhizopus japonicus, with no outstanding saccharifying ability being shown by any of these species.

In 1909, Woolner and Lassloffy described the use of a diastatic slop, or fungi-diastase, as a converting agent in the production of alcohol from grain mashes. Aspergillus oryzae was grown submerged under aerobic conditions in a medium of thin stillage. After a grain mash was partially converted with a small amount of malt, the mold culture and yeast were added.

Ortved (1912) tried out Takamine's preparation of taka-kôji on a plant scale at the Hiram Walker and Sons plant in Canada. He found that complete conversion was obtained by the use of 4 grams of taka-kôji in a mash containing 96 grams of corn and rye. He also reported that the alcohol yields were greater than those from malt-saccharified grain and that a

shorter fermentation time was required. However, disagreeable odors were noted during the saccharification and fermentation, and the fear that these odors would be imparted to the beverage product caused the investigation to be abandoned.

Kita (1913) studied the action of kôji 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 kôji resembled the beta-amylase of malt which converted starch into maltose. As will be noted later, recent investigations have supported the validity of Kita's observation.

A somewhat different type of enzyme activity was investigated by Acton (1911) who obtained a patent on a diastatic substance which transformed starch into dextrins but not into sugar. The organism, which was grown on bran, was a species of Amylomyces obtained from East Indian opium. In 1914, Okazaki studied members of the Aspergillus genus and found that Aspergillus okazakii and Aspergillus albus produced considerable amounts of amylase.

Scales (1914) prepared an enzyme concentrate from Aspergillus terricola, a typical soil fungus. He found that among the many enzymes which this organism produced were diastase and maltase. The enzyme preparation was able to produce over 82 per cent of fermentable sugars from a starch solution after

incubation for 3 days.

The efficiency of various methods for the saccharification of boiled sweet cassava roots was studied by Collens (1914). He found that 81.5 wine gallons of alcohol could be obtained per ton of dry material when saccharification was accomplished by taka-diastase. This was about 8 per cent higher than the yield obtained when malt was used for the saccharification.

The course of the formation of diastase by Aspergillus niger was studied by Went (1919). The organism was grown as a surface culture on a synthetic medium. Went found that the diastase was concentrated mainly in the mold mycelium. Euler and Asarnoj (1920), also working with Aspergillus niger, found that the addition of starch and peptone to the medium resulted in increased amylase and saccharase yields. This was the first indication that the amylolytic enzymes of molds were adaptive, and it has been corroborated by numerous later investigations.

Kashiwagi (1921) 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. Rice, wheat bran, or corn from which the starch had been removed could also be used. After growth, the diastase was extracted by water and then precipitated to form a solid enzyme product.

An investigation of the amylase produced by Rhizopus tritici was carried out by Harter (1921). The mold was grown on Czapek's nutrient solution or on sweet potato bouillon at 25° to 35° C. Amylase activity was determined by the production of reducing sugars through the action of the powdered mycelium, its extracts, or the mold filtrate on Irish potato or sweet potato starch. Harter noted that glucose inhibited the starch hydrolysis and also that amylase production was enhanced by the addition of starch to the mold medium. Harter and Weimer (1925) continued this study and also investigated the amylase produced by Rhizopus nigricans. They believed that amylase production was closely related to sporulation of the molds.

A review of a number of applications of mold enzymes in industry was made by Oshima and Church (1923). They also carried out an intensive study on molds isolated from kôji in regard to amylase and protease production. Aspergillus oryzae and certain other molds intermediate between Aspergillus flavus and Aspergillus oryzae were the best producers of amylase. They found great variation in amylase production between different strains of the same mold species.

Mold growth and enzyme production were compared on the following media: casein, ground dried codfish, dried yeast, soybean preparations, cottonseed cake, peanut meal, cocoanut meal, corn meal, wheat middlings, and wheat bran which turned out to be the best substrate. Later, in 1928, Oshima reported

the results of continued study on the protease and amylase of Aspergillus oryzae. He found that the production of amylase on an artificial medium was stimulated by the addition of casein or peptone and starch or glucose. The pH range for optimum amylase activity was found to be 4.8 to 5.2. The amylase was found to be heat labile and was inactivated by a temperature of 65° C. for one hour.

Nishimura (1925) in a study of takadiastase found it to contain the following enzymes: 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 later eluted with a phosphate solution at pH 8.0.

The formation of diastase by Aspergillus niger was investigated by Funke (1926). He found that the growth of the mold 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. Funke (1928) also carried out a similar investigation of diastase formation by Aspergillus oryzae. On a medium buffered by the addition of 0.5 per cent dibasic potassium phosphate, Aspergillus oryzae formed large amounts of diastase

and secreted it into the medium. The following substances were examined as possible substrates: glucose, galactose, mannose, fructose, sucrose, maltose, lactose, starch, inulin, lichenin, glycerol, and calcium lactate. The amount of diastase produced apparently was not related to the substrate or its concentration (0.5 to 5.0 per cent). There was less growth and also lower enzyme production when the medium was not buffered.

Yamagishi (1928) also 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. Diastase production was directly related to the starch concentration in the medium and was inhibited by the addition of glucose.

Ålvik (1931) studied the stability and action of mold diastases at various pH values. Aspergillus oryzae, Aspergillus niger, and 3 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 the optimum activity around 5.0.

In 1931, Harada made an extensive study of the preparation of amylolytic enzymes by the growth of Aspergillus oryzae on steam-cooked wheat bran. He grew the mold on bran containing 50.0 per cent moisture and obtained maximum growth in 48 hours. Enzyme activities were determined on water extracts of the mold bran. The optimum pH for amylolytic activity was 5.2 at temperatures up to 50° C. At higher temperatures the optimum pH increased becoming 6.6 at 65° C.

Muta, Nomoto, and Tanaka (1931), Muta and Tanaka (1933), and Muta and Tanaka (1936) compared the saccharifying power of Rhizopus pêka I, Rhizopus pêka II, 4 strains of Aspergillus awamori, 6 strains of Aspergillus oryzae, 8 strains of unidentified molds, and one strain of Rhizopus delemar. The Rhizopus delemar, Rhizopus pêka I, and 2 other unidentified species were found best for alcoholic fermentation by the Amylo process on a small industrial scale. Using these molds on a semi-industrial scale fermentation efficiencies of 87 to 91 per cent were obtained. The optimum concentration of the wort was 12 to 13 per cent for kaoliang, 12 to 15 per cent for dry sweet potato, and 16 to 17 per cent for cassava.

The diastatic power of 10 species of Aspergillus was determined by Wei and Chin (1934). Aspergillus oryzae (AOLD) was found to have the greatest diastatic power. Sakaguchi

and Okasaki (1952) reported that an Amylo-Kôji 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 amylase systems. Alcohol yields are about 85 per cent of theory.

Takeda (1935a, b) compared the amylolytic powers of 27 strains of Rhizopus which had been isolated from ragi-kôji and soybean-kôji produced in Java and Sumatra. Two species, Rhizopus semarangensis and Rhizopus javanicus had strong amylolytic properties. The latter organism was considered particularly promising because of its faster action on various types of starch. Satisfactory results in the Amylo process for alcoholic fermentation using Rhizopus javanicus were obtained on both laboratory and industrial scales, and it is still used industrially in Japan.

Shih (1937) compared the diastatic power of 45 strains of Aspergillus grown on wheat bran. Extracts of the mold brans were allowed to act upon starch and then the reducing sugars were determined. Aspergillus oryzae and Aspergillus flavus were found to have the highest diastatic power.

An Amylo process for the alcoholic fermentation of potatoes was investigated by Doi and Takahasi (1938). Rhizopus javanicus was used for the conversion of the potato mashes. Plevako and Cheban (1939) found that a new species of fungus,

Mucor D, in the presence of added organic nitrogen compounds saccharified, in the course of 6 hours, the starch of corn, rye, millet, and potatoes. Mucor D slowly forms ethanol by the decomposition of hexoses but the yields are less than 2 per cent of the theoretical. Consequently, yeast must be added for efficient production of alcohol.

Summarizing briefly at this stage, one finds that two methods for the production of amylolytic enzymes have been investigated: first, production by the growth of certain Aspergillus, Mucor, or Rhizopus strains on wheat bran; and second, by the growth of these organisms on a liquid medium. Both methods were shown to result in enzyme preparations of high amylolytic activity which could efficiently replace malt in saccharifying starchy materials for alcoholic fermentations. Actually, only one of the two methods has found extensive industrial application. This method was the Amylo process, or a modification, in which saccharification was brought about by actually growing the mold in the starch mash which was to be fermented. The use of this process, although extensive, was largely confined to the Orient.

Underkofler, Fulmer, and Schoene (1939) revived the taka-kôji process of growing molds on wheat bran for use in replacing malt for the saccharification of starchy substrates. A rotating drum technique, similar to that of Takamine (1914),

was used in the preparation of the mold bran. Seventeen strains of molds and 7 strains of bacteria were grown on wheat bran and tested by actual fermentation tests; the bacteria proved to be of little value, but all the molds gave good yields of amylase. The following molds were tested: Aspergillus oryzae, Aspergillus flavus, Mucor rouxii, Rhizopus delemar, Rhizopus oryzae, Rhizopus pêka I, Rhizopus tritici, Mucor circinelloides, and Mucor javanicus. Aspergillus oryzae and the Rhizopus species seemed equally as efficient in preliminary tests. Because of its superior cultural characteristics Aspergillus oryzae was picked for further study. Alcohol yields obtained following saccharification by Aspergillus oryzae mold bran were generally 90 per cent of the theoretical yield, about 10 to 12 per cent higher than the malt controls.

Schoene, Fulmer, and Underkofler (1940) compared malt, mold bran and soybean meal as saccharifying agents and found the mold bran to be superior and the soybean meal to be the least efficient. Schoene (1939) observed the action of malt, soybean, fungal, and bacterial amylases on 3 types of mashes: normal, thinned slightly by premalting or acid-cooking; thick, cooked with dilute acid and diluted to the proper concentration; and acid-saccharified mashes. In all cases, mold bran was first and malt was second in saccharifying efficiency. The addition of either mold bran or malt to acid-saccharified mashes increased the alcohol yield.

Goering (1941) also found that the addition of mold bran increased the yields of alcohol from acid-cooked corn. He suggested that some toxic factor was formed by the action of acid on corn bran, and that mold bran counteracted this factor.

Underkofler, Goering, and Buckaloo (1941) prepared mold brans from 9 strains of molds, using the rotating drum technique. They found that best amylase production occurred with 2 strains of Aspergillus oryzae, and with Rhizopus oryzae and Rhizopus tritici. They attempted to grow Aspergillus oryzae on a number of different substrates such as rice hulls, peanut hulls, sawdust, corn cobs, cotton-seed hulls, oat hulls, corn bran and wheat bran. Only the latter two supported adequate growth. These authors found no correlation between the Lintner values and the saccharification efficiency (from test fermentations) of the mold brans.

Hao, Fulmer, and Underkofler (1943) employed a new technique for the preparation of mold brans, which involved incubation in a pan with air under slight pressure passing through the bran mass. Mold brans were prepared from 27 strains of molds from 4 genera: Aspergillus, Mucor, Rhizopus, and Penicillium. Rhizopus cultures gave good amylase production but certain strains of Aspergillus oryzae were considered most suitable because of their better growth characteristics.

Alcohol yields of 95 per cent of theory were obtained. A method of preparing mold bran in closed containers was patented by Underkofler (1942a). Wheat bran containing 40 to 70 per cent 0.3 normal hydrochloric acid was used as the substrate. Severson, Underkofler, and Schoene (1942) patented a saccharification process in which soybean meal was used to supplement the action of taka-kôji.

Underkofler (1942b) and Underkofler and Fulmer (1943) reviewed the status of microbial amylases for the saccharification of starch in the alcoholic fermentation with particular attention to the use of mold bran. They noted that it had been demonstrated on a laboratory scale that mold bran was an inexpensive and efficient substitute for malt.

Beginning in the spring of 1945 tests were made on the use of mold bran in grain fermentations on a full plant scale at the alcohol plant of the Farm Crops Processing Corporation at Omaha, Nebraska. Reports on these tests were given by Boyer and Underkofler (1945) and Underkofler, Severson, and Goering (1946). These tests proved that mold bran was entirely satisfactory for use as a saccharifying agent in large scale production of industrial alcohol. Mold bran was found to be superior to malt for conversion in yeast culture mashes, resulting in excellent yeast growth. Conversion by 4 per cent mold bran resulted in higher yields than were

obtained when the mashes were converted by 9.0 to 9.3 per cent malt. The alcohol yields were slightly over 5.3 proof gallons per standard bushel for mold bran converted mashes and slightly over 5.1 for the malt converted mashes. A mixture of mold bran and malt was also found to give higher yields than malt alone.

Underkofler (1946) briefly reviewed and Underkofler, Severson, Goering, and Christensen (1947) gave a detailed report on the production and use of mold bran from the laboratory scale up to the full scale production of over 10 tons per day at the Mold Bran Company plant at Eagle Grove, Iowa. They discussed the economics of mold bran use. There was no difference in the quality of the alcohol produced by mold bran or malt saccharification and better yields were obtained with mold bran. The value of mold bran in replacing malt, which sold prewar for 3 cents a pound, was about 9 cents a pound, and mold bran could be produced commercially well below that figure. The cost of malt has nearly doubled at the present time but it is doubtful if the cost of mold bran production has increased as much percentagewise. Therefore, the use of mold bran is now even more favorable economically.

Banzon, Fulmer, and Underkofler (1949) found that Aspergillus oryzae mold bran was superior to malt for the saccharification of cassava mashes. They also considered the use of a bacterial enzyme preparation, but found it less efficient.

A patent for saccharification with mold bran has been obtained recently by Christensen and Underkofler (1952). The mold bran was prepared by mixing with an equal weight of 0.1 normal hydrochloric acid, adding a nutrient salt solution, and inoculating with Aspergillus oryzae spores.

Asai and Matsumoto (1942) examined 39 strains of Rhizopus for amylase activity and found Rhizopus javanicus and Rhizopus semarangensis to produce the greatest amounts of amylases. They felt that the existence of two pH and temperature optimums indicated the presence of two amylases, perhaps alpha- and beta-amylase.

Ram and Sreenivasan (1943) patented a process for the preparation of an amylase concentrate. The concentrate was prepared by alcohol precipitation from a water extract of Aspergillus oryzae on wheat bran. The saccharifying enzyme produced by black kôji (probably some strain of Aspergillus niger on rice) was studied by Ohara (1943). Ohara found that the enzyme could be extracted from the kôji more easily with 0.01 to 0.005 normal hydrochloric acid than with water. The addition of hydrochloric acid also increased the alcohol yields in the manufacture of Korean spirits with black kôji.

Roberts, Laufer, Stewart, and Saletan (1944) compared the action of Aspergillus oryzae mold bran and barley malt on corn, winter wheat, spring wheat, and 2 wheat flour mashes. Pressure cooking (125° C.) and atmospheric cooking (66° C.)

were used with saccharification by 8 per cent malt or 2.5 to 4 per cent mold bran. Malt apparently gave better conversion in pressure cooked mashes, but for the mashes cooked at the lower temperature 2.5 to 3.0 per cent mold bran was most efficient. The alcohol yields from mold bran saccharified mashes were 90 to 98 per cent of the theoretical yield. These authors also found that saccharification by mold bran at 30° C. was equal to or better than the conversion at 52.5° C.

A number of microbial amylases were tested for their efficiency as conversion agents by Hao and Jump (1945). The conversion temperature was 130° F. and the efficiency was measured by the alcohol yields in laboratory fermentations. They found the mold amylases to be efficient saccharifying agents. Aspergillus oryzae mold bran was studied by Pan and Liu (1944). They found that mashes of 12 to 14 per cent starch were completely saccharified in 4 to 6 hours. The optimum pH for saccharification was determined to be 5.0.

Bindal and Sreenivasaya (1945a, b) investigated the formation of amylase by fungi. They varied the soluble starch/nitrogen ratio in the medium for Aspergillus oryzae and found that the economic optimum ratio for enzyme production was 7/1. They also found that malt residues with the addition of 10 per cent peanut cake formed a good substrate for the production of amylases. They list a number of advantages for this process, including more efficient conversion.

The influence of the carbohydrate source in the medium for Aspergillus oryzae on amylase formation was investigated by Rao and Sreenivasaya (1946). They found that starch, glucose, and maltose stimulated amylase production. Other carbohydrates which were tested were found to have no effect. Rao and Sreenivasaya (1947) reported work on the influence of the form of nitrogen on the formation of amylase. Four groups of nitrogen compounds were observed: inorganic nitrates, ammonium salts of inorganic acids, ammonium salts of organic acids, and amino acids. They found good amylase production when ammonium salts of organic acids especially ammonium acetate were used as the nitrogen source. Bindal and Sreenivasaya (1945c) 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 a 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.

Since 1946 investigation of the use of fungal amylolytic preparations has followed two courses: first, the work on mold brans has continued, mainly in other countries; and second, the use of submerged fungal cultures has been given a great deal of attention. Recent developments in the two methods will be considered separately.

Montes (1946) has 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 (1947) investigated the use of mold bran prepared by growing Aspergillus oryzae on wheat bran. They recommended the use of 4 per cent mold bran for industrial saccharifications.

Guagnini and Jacovkis (1947a, b) 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 mold amylases were used to clarify grape and

other fruit juices in addition to saccharification of corn mashes. The alcohol yield was 93 per cent of the theoretical yield when 12 per cent fresh mold bran was the saccharifying agent.

The replacement of malt by Aspergillus oryzae mold bran was also considered by Soriano and Trucco (1948). They were able to obtain alcohol yields of 78 to 87 per cent of theory.

Reindel, Frey and Lottner (1950) compared the alcohol yields obtained following acid, mold amylase, and green malt conversion of potato flour, corn meal, and milo meal. In all but one case, the mold amylase gave best yields on corn and potato flour. They noted that saccharification by mold is fundamentally different from that by green malt, probably due to the presence of different enzyme systems. Lampe (1950) investigated the action of a number of saccharifying agents on various raw materials. The saccharifying agents were green malt, barley malt, Aspergillus niger mold, and combinations of the latter two. Rye, corn, milo maize, dried potatoes, and low-grade flour were used as substrates. The highest alcohol yields were obtained from mold saccharification.

Yamazaki and Ueda (1951) studied the amylase activity of malt, yellow kôji, and black kôji on cooked and uncooked starch from a number of natural sources. Yellow and black kôji probably consisted of Aspergillus oryzae and Aspergillus niger, respectively, on a steamed rice substrate. On cooked

starch the amylase activity decreased in the order malt, yellow kôji, and black kôji. This order was reversed for amylase activity on uncooked starches. The authors also listed a number of starchy materials in the order of their saccharification velocities. Uncooked corn and wheat starches were saccharified most rapidly and potato starch, the slowest. This order was also reversed for cooked starches.

Kôji was prepared by Sugiyama, Tanabe, and Fukuzako (1946) and Tanabe and Fukuzako (1948) by growing Aspergillus oryzae on dried slices of sweet potatoes. The sweet potato substrate was found to be deficient in nitrogen and phosphorus compounds. It was necessary to supplement the basic substrate by the addition of wheat or rice bran, peptone, and minerals. The addition of brans considerably increased the liquefying power of the preparation.

Somewhat of a departure from the Amylo-type process was made by Erb and Hildebrandt (1946) in their use of submerged cultures of Rhizopus delemar or Rhizopus boulard for the 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. They described the culturing techniques used in the preparation of large volumes of the saccharifying material. 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 wheat flour was pre-malted (about 2 per cent malt) before addition of the mold culture and yeast. 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 100.0 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. Essentially the same process was patented by Hildebrandt and Erb 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.

Following the discovery at the Northern Regional Research Laboratory that a strain of Aspergillus niger, numbered NRRL 337, was a superior producer of alpha-amylase Erb, Wisthoff, and Jacobs (1948) turned their attention to further work with that organism. They measured amylase production by the ferricyanide titration of reducing sugars formed on the addition of the mold filtrate to a solution of soluble starch and attempted, unsuccessfully, to correlate the enzyme activity to the alcohol yield in test fermentations. The mold was grown on an acid-cooked 10 per cent corn mash containing 0.25 per cent urea,

0.001 to 0.01 per cent sodium chloride, and sufficient calcium carbonate to adjust the final pH to 5.3. A saccharification volume of 20 per cent resulted in alcohol yields of 6.03 proof gallons per (dry) bushel. The best results were obtained when the mash was pre-malted. Alcohol yields on a pilot plant scale were 5.61 proof gallons per dry bushel, about 3 to 5 per cent greater than the corresponding yields from malt-saccharified mashes. These authors attempted to inhibit bacterial growth by the addition of ammonium fluoride or Dowicide G. Somewhat favorable results were obtained with the use of the latter substance.

The major portion of recent investigations on the use of submerged fungal cultures as saccharifying agents has been carried out at the Northern Regional Research Laboratory, Peoria, Illinois. Van Lanen and LeMense (1946) reported on studies which were made in the attempt to develop submerged culture methods for preparing fungal amylases, suitable for adaptation to large-scale production, for use in grain alcohol distillery operations. Over 350 organisms were cultivated under aeration in a medium composed of thin stillage, one per cent corn meal, and 0.5 per cent calcium carbonate. Enzyme production was determined both by the dextrinization of starch, using the Sandstedt, Kneen, and Blish (1939) method for alpha-amylase determination, and by the ability of the culture liquors to replace barley malt as the saccharifying

agent in the alcoholic fermentation of grains. Of the cultures tested only 7 produced practical concentrations of alpha-amylase under the conditions used. Aspergillus niger was superior both in the production of alpha-amylase and in replacing malt. In the alcoholic fermentation of corn, use of culture liquor at the rate of 10 to 15 per cent of the mash volume resulted in alcohol yields of 5.2 to 5.4 proof gallons per standard bushel as compared to 5.0 to 5.2 proof gallons per standard bushel obtained with commercial barley malt.

LeMense, Corman, VanLanen, and Langlykke (1947), in further work on this problem, attempted to develop an optimum medium for the production of amylases by submerged growth of a number of different molds. Enzyme production was determined by alpha-amylase activity, maltase activity (by hydrolysis of maltose monohydrate to glucose), and by actual fermentations of corn mashes. The best medium consisted of thin stillage, 2 per cent corn, and 0.5 per cent calcium carbonate. Maximum enzyme production was obtained when the initial pH of the mold medium was about 5.0. An increase in aeration of the submerged culture also resulted in increased enzyme production. A volume of submerged culture equal to 10 to 20 per cent of the final mash volume was used for the conversion and alcohol yields up to 5.4 proof gallons per standard bushel (5.15 per malt controls) were obtained. A positive correlation was found between maltase activity and alcohol yields provided a

certain minimum amount of alpha-amylase was present. Le Mense and Van Lanen (1948) patented essentially the same process for the preparation of fungal enzymes by Aspergillus niger NRRL 337.

The observation that the alcohol yields from the fermentation of corn mashes saccharified with mold culture filtrates correlated with the potency of a glucogenic enzyme system, as measured by maltose hydrolysis, led to a detailed study by Gorman and Langlykke (1948) of starch saccharification by fungal diastatic preparations. The molds were grown under aeration in a medium composed of distillers' thin stillage (about 5 per cent solids), 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 and found to produce variable amounts of alpha-amylase and glucogenic enzyme. The alcohol yields from the test fermentations correlated with the glucogenic activity for those molds which had more than a trace of alpha-amylase. The following molds gave alcohol yields greater than obtained in the malt controls: Aspergillus niger 330, Aspergillus phoenicus 363, Aspergillus wentii 378, Aspergillus oryzae 464, Aspergillus niger 605, Aspergillus niger 326, and

Aspergillus niger 337. The first and last of the molds listed were especially high in glucogenic activity and alpha-amylase activity, respectively.

The application of submerged mold amylase as a saccharifying agent on a pilot plant scale was described by Le Mense, Sohns, Corman, Blom, Van Lanen, and Langlykke (1949). They found that the volume of mold culture used for saccharification was more important than the amount of dextrinizing enzyme (alpha-amylase) and also that there was no correlation of alcohol yields and maltase (glucogenic) activity. Best results were obtained with the use of 10 to 20 volume per cent mold culture for saccharification following pre-liquefaction of the mash by small amounts of malt or mold culture. The saving calculated for the replacement of malt by submerged mold culture was approximately 2.4 to 3.6 cents per gallon of 190 proof alcohol.

Plant scale fermentations using submerged culture fungal amylase for saccharification of the mashes were carried out during 1949 at the Grain Processing Corporation, Muscatine, Iowa, under the direction of personnel from the Northern Regional Research Laboratory. The results were reported by the U. S. Production and Marketing Administration and the Bureau of Agricultural and Industrial Chemistry (1950) in the U. S. Department of Agriculture Technical Bulletin No. 1024.

The procedures used were those previously developed on the laboratory and pilot-plant scale at the Northern Regional Research Laboratory with only minor modifications. The addition of a small amount of ammonium bifluoride (0.018 per cent) to the mold medium decreased the amount of bacterial contamination. Some difficulty was encountered due to the slow liquefaction of mashes by the fungal amylases. This was overcome by the addition of small amounts of malt and/or increasing the conversion temperature to 67° C. and increasing the holding time. A fermentation time of 48 to 60 hours was allowed. The alcohol yields obtained from mashes saccharified by fungal amylase were 5.98 proof gallons per dry bushel compared to 5.87 for malt-saccharified mashes. In the fermentation of heat-damaged corn the yields were 6.04 for mold-saccharified mashes and 5.86 for malt-saccharified mashes. It was found that stillage from mold-converted mashes was as effective as that from malt-converted mashes for the preparation of fungal amylase. The quality of the alcohol and feeds (distillers' dried grains and solubles used for animal feeding) produced by the fungal amylase process was found to be equal to that from the usual fermentations using malt. Cost calculations comparing the production of alcohol with fungal amylase and with malt indicate that the replacement of malt by fungal amylase would result in savings of approximately 3.7 cents per gallon of 190 proof alcohol.

Tsuchiya, Corman, and Koepsell (1950) investigated certain factors affecting the production of alpha-amylase and maltase by Aspergilli. They found that the presence of calcium carbonate in the mold medium in amounts exceeding 0.05 molar (or approximately 0.05 per cent) inhibited maltase and alpha-amylase production. Alpha-amylase was found to be unstable below a pH of about 4.7. Consequently some method was needed to keep the pH of the mold medium above 4.7 without the addition of calcium carbonate. This was accomplished by varying the concentration of corn and distillers' dried solubles in the medium. The initial pH was adjusted to 5.2 using sodium hydroxide and the final pH was found to vary directly with the dried solubles concentration and inversely with the concentration of corn. The alpha-amylase production was generally 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 corn and 5 per cent distillers' dried solubles. The composition of the medium apparently had little effect on enzyme production by Aspergillus oryzae 458. Aspergillus niger 330 produced slightly more maltase than Aspergillus niger 337 but was the poorest alpha-amylase producer of the three organisms studied. Aspergillus oryzae 458 produced nearly as much alpha-amylase as Aspergillus niger 337 but was last in the production of

maltase.

Gorman and Tsuchiya (1951) confirmed earlier observations that the alcohol yield was proportional to the maltase activity if an adequate amount of alpha-amylase was present. In the preceding investigations at the Northern Regional Research Laboratory it had been found that Aspergillus niger 337 and Aspergillus niger 330 produced nearly equal amounts of maltase but the latter organism produced only very small amounts of alpha-amylase. Therefore, the effect of supplementing the action of these molds in submerged cultures by the addition of malt, a good alpha-amylase source, was investigated. The alcohol yields from mashes saccharified by amylase from Aspergillus niger 337 were not improved by the addition of malt. A significant improvement was noted when malt was added to Aspergillus niger 330 amylase. Apparently this improvement was due to the increase of the alpha-amylase activity to or above a necessary minimum level. These authors also reported that satisfactory results were obtained in the conversion of corn, moldy corn, wheat, milo sorghum, and dried potatoes by submerged cultures of Aspergillus niger 337.

During the period of these investigations at the Northern Regional Research Laboratory, similar work was being carried out at Joseph E. Seagram and Sons, Inc. Balanchura, Stewart, Scalf, and Smith (1946) screened 23 strains of the genera Aspergillus, Mucor, Penicillium, and Rhizopus which were known

to produce amylases. The best preparations as determined by the alpha-amylase activity of the submerged cultures were further evaluated by yeast fermentation of grain mashes using these preparations as converting agents. The best of the cultures was a strain of Aspergillus flavus grown for 48 hours on a medium of 3 per cent distillers' dried solubles with an initial pH of 6.0.

Adams, Balankura, Andreasen, and Stark (1947) found that growth and amylase production by Aspergillus niger in plant stillage were poor and were not improved by treatment with charcoal or aluminum powder. Further investigations were carried out using a culture of Aspergillus niger 337, from the Northern Regional Research Laboratory. The medium consisted of 5 per cent distillers' dried solubles, 1 per cent corn, and sufficient calcium carbonate to adjust the initial pH to approximately 5.0. Slightly better conversion was obtained at 145° F. than at 130° F. Alcohol yields of 6.16 to 6.27 proof gallons per dry bushel were obtained from mashes saccharified by 20 volume per cent of submerged culture. The yield for the malt controls was 5.95 proof gallons per dry bushel. They found no correlation between alcohol yields and alpha-amylase activity or total saccharifying power (determined by the measurement of reducing sugars formed from soluble starch).

Stark, Kolachov, Smith, Blankmeyer, and Willkie (1949) reviewed the use of amylase from submerged culture growth of

Aspergillus niger. Alcohol yields were 0.2 to 0.3 proof gallons per bushel greater than the yields from malt-converted mashes. Other advantages were the more rapid fermentation and the reduction of bacterial contamination. They estimated a savings of 2 cents a proof gallon would be obtained in replacing malt by fungal amylase. Kolachov, Smith, and Willkie (1950) issued a later progress report concerning the production and use of fungal amylase.

Pan, Andreasen, and Kolachov (1950b) investigated the fermentation rate in corn mashes converted by Aspergillus niger amylase. 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.

Various preparations were tested by Teixeira, Andreasen, and Kolachov (1950) for use in the conversion of cassava mashes for alcoholic fermentation. In the order of increasing efficiency the preparations (and their efficiencies) were: acid hydrolysis (43 to 74), barley malt (74), mold bran (80 to 85), and Aspergillus niger 337 in submerged culture (90 per cent of theoretical yield).

Blaisten (1948) reported that the incubation of Aspergillus oryzae or Aspergillus niger in semi-submerged culture on wheat bran gave a very active amylase preparation.

Goodman (1950) compared sucrose and soluble starch as carbon sources for amylase production by Aspergillus flavus Link, Aspergillus terreus Thom, 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 depended directly upon the starch concentration. Work of a similar nature was reported by Shu and Blackwood (1951). Aspergillus niger 558 (Prairie Regional Laboratory, Saskatoon, Saskatchewan) was grown in submerged culture on a basal medium of salts to which various carbon and nitrogen sources were added. There was no correlation between the amount of growth and the amount of enzyme production. The highest enzyme production was obtained when starch was used as the carbon source. Maltose or higher glucose polymers also favored enzyme production. Alpha-amylase was very dependent upon the carbon source, while limit-dextrinase was least dependent. The enzyme yield was also dependent upon the nitrogen-availability, and highest yields were obtained when protein hydrolyzates were used as the nitrogen source. By varying the composition of the medium and/or the time of growth the authors were able to control the relative enzyme activities in the medium.

Fukimbara, Yoshida, and Shibuya (1951a, b) screened 54 strains of Aspergilli and found 5 strains of Aspergillus niger and 3 of Aspergillus oryzae which gave high amylase production

in submerged culture. The mold culture liquor was used for the saccharification of sweet potato mashes for alcoholic fermentation. The alcohol yield was higher for black Aspergilli (Aspergillus niger) than for yellow (Aspergillus oryzae). The fermentation efficiency was found to parallel the maltase activity of the mold cultures.

An attempt has been made in this section to review the literature on the development and use of mold amylase preparations. Much additional work has been carried out on the problem of the separation and purification of the several enzymes found in fungal amylases. This work has been noted only incidentally, either when the enzyme concentrates were used for the purpose of saccharification or when some other phase of the work was of interest. In general, it may be stated that typical enzyme techniques have been applied to the problem. In the separation and purification of a particular enzyme the preparations were often subjected to certain temperatures, pH's, and salt concentrations which were intended to inactivate the other enzymes present. Preparations containing high enzyme activity were obtained by precipitation by the addition of salts, such as ammonium sulfate, or organic solvents, such as acetone or alcohols. Considerable progress has been made; numerous investigators have reported the preparation of enzyme concentrates relatively free from other enzyme activity. Roy (1950) and Underkofler and Roy (1951) have reported the

crystallization of alpha-amylase and limit-dextrinase from Aspergillus oryzae preparations. Roy and Underkofler (1951) have also prepared highly active maltase concentrates from the mold filtrate of a culture of Aspergillus niger 330. The preparation of crystalline alpha-amylase from Aspergillus oryzae has been recently repeated successfully by Fischer and de Montmollin (1951).

B. Methods for the Evaluation of Amylases

Since fungal amylase is in reality a mixture of a number of different enzymes it is necessary to consider briefly those enzymes and their action before discussing methods for the evaluation of amylases. In connection with amylases and their actions it is also necessary to examine their principal substrate, i.e., starch.

Despite an enormous volume of work on the problem it is not yet possible to state exactly the structure of starch. Most investigators are agreed that starch is heterogeneous, having two main components: amylose, and amylopectin. Amylose is a linear polymer of glucose, consisting of 300 or more glucose units joined in the same manner as in maltose, i.e., through 1,4'-alpha-D-glucosidic linkages. Amylopectin is a highly branched glucose polymer of a much higher molecular weight, perhaps over 200,000, or over 1300 glucose units. The

majority of linkages in amylopectin are the same as those in amylose, but it is believed that 1,6'-alpha-D-glucosidic linkages are found at the points of branching. The two components vary considerably in their ratio depending upon the source of the starch. The molecular weights and structure, such as the degree of branching of the amylopectin fraction, are similarly dependent upon the source.

There are a number of reviews on the subject of amylases. Two recent ones are by Bernfeld (1951) and Myrbäck and Neumüller (1950). Unfortunately only brief mention of fungal amylases is made by these authors, with their main attention being directed to animal and plant amylases, particularly those of malt. Miller (1951) has recently reviewed the literature on fungal amylases.

Early observers noted that a certain sequence of action took place when starch was treated with amylases. There was first a very rapid decrease in the viscosity of the starch solution. This was followed by a gradual change in the color of the starch-iodine complex from blue-purple through brown-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 the amylase action. Observations of the optimum temperatures and acidities for these different actions and the change in reaction rates accompanying purification or partial inactivation suggested

the presence of two or more enzymes in amylases. Two of the enzymes receiving early attention were alpha-amylase and beta-amylase.

Alpha-amylase is an enzyme of extremely wide distribution, being found in representatives of nearly all forms of life. In animals there have been found salivary, pancreatic, and blood alpha-amylases. Cereals are good sources of alpha-amylase, the activity increasing during germination. Bacteria and molds have also been found to be producers of this enzyme. 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 and any striking differences between alpha-amylases from different sources are probably due to the presence of additional enzymes in the various preparations.

Alpha-amylase is responsible for the rapid decrease in viscosity of starch solutions and is often referred to as the "liquefying" enzyme. It is also 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 1,4'-alpha-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 and greater solubility. This is the liquefaction stage. 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 1,6'-alpha-glucosidic linkages and are quite resistant toward further hydrolysis. The simplest limit dextrin would be isomaltose, and the average limit dextrin following extended alpha-amylase hydrolysis contains about 4 to 6 glucose units.

Methods of measuring alpha-amylase activity are suggested by the consideration of its action. The methods involve measurement of the change of viscosity or of the change in the color of the iodine complex. Methods involving measurement of the production of reducing materials have found limited use.

The second enzyme, beta-amylase, is found mainly in plants, especially the cereals. Malt, soybeans, and sweet potatoes are particularly good sources of this enzyme. 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 and that mold saccharification is accomplished mainly by the action of alpha-amylase, maltase, and perhaps other enzymes. However, beta-amylase is the principal saccharifying enzyme in malt.

Beta-amylase, as does alpha-amylase, acts by the hydrolytic cleavage of 1,4'-alpha-glucosidic linkages in starch. Its action differs, however, in that the attack is seemingly 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 1,6'-alpha-glucosidic bonds are resistant to both amylases and in addition inhibit the hydrolysis by beta-amylase of normal 1,4'-bonds in their vicinity. 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 high molecular weight dextrin of low reducing power. Alpha-amylase can hydrolyze 1,4'-bonds 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. It is quite possible that alpha-amylase, and perhaps even beta-amylase

can further hydrolyze limit-dextrins, but such action is extremely slow.

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. Consequently the methods used in the determination of alpha-amylase are of no value in regard to beta-amylase. Since the principal action of beta-amylase is the production of fermentable 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 (1886) method and consist of the measurement of the reducing substances formed by the action of the enzyme preparation on a soluble starch medium under certain specified conditions.

The enzyme which is considered by some observers to be responsible for the major portion of the saccharifying action of fungal amylase preparations is maltase. True maltase is classified as an alpha-D-glucosidase since it cleaves the alpha-1,4'-D-glucosidic linkage which joins the two glucose units in maltose. Actually, the maltases which have been found in many microorganisms seem to be somewhat less specific. For example, Pringsheim, Borchardt, and Loew (1931) found that yeast maltase hydrolyzed simple glucosides such as alpha-methyl-glucoside to a greater extent than maltose. The same

workers and also Hestrin (1940) found that impure maltase preparations from species of Aspergilli hydrolyzed glucosides only if the aglucones were of the complexity of the order of glucose, other carbohydrates, or their derivatives. Maltase activity is very readily measured by observing the production of glucose by the action of the enzyme preparation on a maltose substrate.

Still another carbohydrase has been shown to be present in fungal amylase preparations and to a lesser extent in malt. The enzyme is limit-dextrinase and its action is the hydrolytic cleavage of the alpha-1,6'-glucosidic linkages in amylopectin and dextrins which resist cleavage by the other carbohydrases. Back, Stark, and Scalf (1948) have developed a method for the measurement of limit-dextrinase activity. This method, which will be described in detail in a later section, involves treating limit dextrin (prepared by the action of barley malt diastase on cornstarch) with the enzyme preparation and then measuring the amount of glucose which was produced. These authors suggested that the higher limit-dextrinase activity of fungal amylase preparations compared to malt may be responsible for the higher alcohol yields obtained from fungal-saccharified mashes. Tsuchiya, Montgomery, and Corman (1949) reported that isomaltose was hydrolyzed to the extent of 90 per cent in 5 hours by the filtrate from an Aspergillus niger 330 culture. This suggests that the tedious method of

Back, et al (1948) could be replaced by a method using isomaltose in much the same manner as in the use of maltose monohydrate in the determination of maltase activity.

Although Kita (1913) suggested the presence of an enzyme in kôji diastase which converted starch directly to glucose, most workers continued to believe that maltose was the primary product in the action of fungal amylases on starch and that glucose, if present, was formed by the further hydrolysis of maltose by maltase. Corman and Langlykke (1948), realizing that their mold culture filtrates produced glucose from both maltose and the higher polymers of glucose, 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 preparation. Kerr, Cleveland, and Katzbeck (1951) investigated a similar enzyme which they named "amyloglucosidase." This enzyme produced glucose from amylose and amylopectin apparently by attacking the non-reducing terminals of the molecules. They did not report whether amyloglucosidase had any action on maltose. A semi-purified enzyme preparation from Rhizopus delemar was investigated by Phillips and Caldwell (1951a, b). This enzyme, first designated as "delta-amylase" and later as "gluc amylase", 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. Although the enzyme concentrate was subjected to numerous purification steps and also to partial inactivation the ratio of maltase activity to gluc amylase activity remained constant. However, the rate of hydrolysis of maltose was considerably less than that for the hydrolysis of starch.

Kitahara and Kurushima (1949) investigated an enzyme from a strain of Aspergillus awamori which they considered to be a beta-amylase. They found that it produced glucose and maltose in the ratio of 4/1 from starch and had practically no action on maltose. They suggested that the enzyme be named "gamma-amylase." In further work, Kitahara and Kurushima (1951) concentrated an enzyme obtained in a saline extract of Aspergillus awamori. The purified enzyme product, free of maltase and alpha-amylase activity, produced glucose and maltose from starch in the ratio of 2/1. They obtained a similar enzyme from Aspergillus cinnamomeus and Aspergillus batatae.

Pigman (1944) 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 molds only 70 to 80 per cent. He suggested that fungal enzyme 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 (1945).

The role of yeast in the secondary stage of fermentation was studied by Pan, Andreasen, and Kolachov (1951). During the secondary fermentation stage dextrins are hydrolyzed by fungal enzymes into fermentable sugars 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 that the role of the yeast in aiding secondary conversion was the removal of maltose. In other reports on this problem, Pan, Andreasen, and Kolachov (1950a) and Pan, Nicholson, and Kolachov (1951) found that a filtrate from a submerged culture of Aspergillus niger 337 converted maltose into an un-fermentable dextrin. This dextrin was found to be a tri-saccharide consisting only of 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 (1951) suggested, and Wolfrom, Thompson, and Galkowski (1951) confirmed that the tri-saccharide,

which was called "panose", was 4-alpha-isomaltopyranosyl-D-glucose.

Pazur and French (1951) 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 the 6-position of a co-substrate saccharide. From pure maltose the enzyme synthesized isomaltose, 6-(alpha-D-glucosyl) maltose, and 6-(alpha-D-glucosyl) isomaltose, and a tetrasaccharide of unknown constitution. This synthetic action very closely resembles the reverse of limit-dextrinase action. It seems entirely possible that only one enzyme is involved, in an equilibrium reaction, and that the direction of action is easily controlled by varying the concentrations of reactant and products.

In summary, it appears that fungal amylase preparations contain 3 or more enzymes. Alpha-amylase acts by a random hydrolytic cleavage of 1,4'-alpha-glucosidic bonds. There is first a rapid decrease in viscosity. This is followed by the loss of color of the iodine-complex as smaller dextrins are formed; and in the final stage limit dextrins, glucose, maltotriose, and maltose are slowly formed. The major saccharifying action is due to one or more enzymes capable of hydrolyzing 1,4'-alpha-D-glucosidic bonds. A small amount of

maltase may be present, but the major saccharifying enzyme has little or no action on maltose. This saccharifying enzyme produces glucose from starch and also in combination with alpha-amylase forms some limit dextrins. These dextrins are then hydrolyzed by limit-dextrinase to form reducing sugars. If maltose is not removed from the solution (by the action of yeast, dialysis, or by supplementing the maltase activity) limit-dextrinase may synthesize non-fermentable sugars.

Much of the work in regard to the evaluation of amylolytic preparations has been done on malt. Reese (1947) has reviewed the various methods used. They may be classified under the following headings: viscosity methods; iodometric methods; polarimetric methods; methods involving the determination of reducing power; direct determinations. The first 2 methods are concerned mainly with the action of alpha-amylase; the 4th method, with beta-amylase; the 3rd and the 5th consider the combinations of the enzyme-actions.

It is of little value to discuss here the methods used for the evaluation of malts, especially since the saccharifying actions of malt and molds are due to different enzymes. It is sufficient to state that the most popular methods for evaluating malts are based on the determination of beta-amylase activity, and involve the measurement of maltose produced from starch by the malt. Reese (1947) noted that in recent years it has

become the general opinion that the measurement of some isolated enzyme system, such as beta-amylase, is not an entirely satisfactory method of evaluating malts. The need for a method measuring the integrated enzymatic action was definitely indicated, and experimental laboratory fermentations appeared the only satisfactory manner of meeting that need. Thorne, Emerson, Olson, and Peterson (1945) evaluated malts used for the production of alcohol from wheat by extensive analytical and fermentation tests and came 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.

Very nearly the same situation has existed in regard to the evaluation of fungal enzyme preparations. In the early stages of the investigation of mold brans at Iowa State College Underkofler, Goering, and Buckaloo (1941) attempted to correlate the Lintner value of the mold preparations with the saccharifying efficiency as measured by the alcohol yield in a laboratory fermentation. Since the Lintner value corresponds to beta-amylase activity, which is nil in fungal enzyme preparations, it is not surprising that they obtained no positive correlation. It was widely observed that, in general, amylolytic preparations high in alpha-amylase activity were quite

apt to be satisfactory for the saccharification of grain mashes. Therefore, alpha-amylase activity became the criterion for the screening of organisms used in the production of mold brans and, later on, for use in submerged culture preparations. Practically all investigators in this field after a preliminary selection on the basis of alpha-amylase activity studied the promising organisms by the use of laboratory fermentations.

Adams, Balankura, Andreasen, and Stark (1947) reported that there was no correlation between the alpha-amylase activity of submerged cultures of Aspergillus niger 337 and the alcohol yields obtained from mahses converted by the fungal preparation. LeMense, Sohns, Corman, Blom, Van Lanen, and Langlykke (1949) agreed with this observation in stating that the volume of submerged fungal culture used for saccharification was apparently more important than the amount of dextrinizing enzyme (i.e., alpha-amylase).

LeMense, Corman, Van Lanen, and Langlykke (1947) 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. In the following year Corman and Langlykke (1948) indicated their preference for the term "glucogenic activity" instead of maltase activity. Glucogenic activity was measured by the same procedure as for maltase (i.e., by the hydrolysis of maltose monohydrate) and also correlated with the alcohol

yields. They further stated that the Lintner determination was too rapid for the slower acting mold amylase, or glucogenic enzyme. Gorman and Tsuchiya (1951) observed that the alpha-amylase activity should be greater than one unit per milliliter if the alcohol yield is to depend on the maltase activity. In their investigation of the use of mold culture liquors of certain Aspergilli for conversion of sweet potato mashes Fukimbara, Yoshida, and Shibuya (1951b) observed that the fermentation efficiency paralleled the maltase activity.

Another anlyytical method for the evaluation of submerged fungal cultures was investigated by Erb, Wisthoff, and Jacobs (1948). 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 (1950) for the plant scale experiments at the Grain Processing Corporation at Muscatine, Iowa. A positive correlation was found to exist between the saccharogenic power and the alcohol yields. It must be pointed out that their data also indicate just as good a correlation between maltase or alpha-amylase activity and the alcohol yield. Using essentially the same method Adams, Balankura, Andreasen, and Stark (1947) compared the total saccharifying power with the alcohol yields but found no dependence.

In summary, only two analytical methods for the evaluation of fungal enzyme preparations have been shown to have any promise. In one, maltase or glucogenic activity is determined by measuring the production of glucose by enzymatic hydrolysis of maltose monohydrate. In the other method, the saccharogenic power is measured by observing the production of reducing sugars by enzymatic action on soluble starch. Both methods leave much to be desired, especially in regard to the level of amylolytic preparation to be used, which must be determined by actual fermentation tests. The method of Erb, et al (1948) presumably measures the integrated action of alpha-amylase, maltase, and other saccharifying enzymes. Since the enzyme preparation is allowed to act upon the starch for a comparatively short time, the method may possibly penalize those preparations having low alpha-amylase activities. The early stage of saccharification for such preparations is slow and the amount of sugars produced would not accurately indicate the degree of saccharification which could be attained over a longer period of time.

Drews, Lampe, and Specht (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 starch-containing material with the sample to be tested.

Reese (1947) and Reese, Fulmer, and Underkofler (1948) described a short fermentation test for evaluating 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. For a number of different mold brans (all prepared from Aspergillus oryzae 38) the lines thus obtained were parallel but had different intercepts. The lower the intercept value, the higher was the saccharifying efficiency of that particular mold bran. It was also noted that 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 mold brans.

This procedure can be used to determine the levels of different mold brans to be used to obtain maximum yields but it does not indicate definitely which mold bran will give the highest alcohol yields. Generally, but not without exception, the mold bran with the lowest intercept will give the highest

alcohol yield. It may be seen then, that the enzymatic method of evaluation as discussed previously will indicate to some extent the alcohol yields which can be obtained and the short fermentation method indicates the level of saccharifying agent which should be used.

III. MATERIALS

A. Materials for Fermentations and Media

1. Corn meal

The corn which was used for the major portion of this investigation 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 corn and screwing the cover on tightly. Moisture determinations were run on the corn in each bottle as used.

2. Corn mill fractions

The following mill fractions from yellow corn were also obtained from the Quaker Oats Company in June of 1949. The weights received are equivalent to their corresponding percentages in whole corn. Moisture determinations were run on each fraction prior to its use.

Granulated meal	49.0 lbs.)	Total grits	70.0 lbs.
Golden maize	21.0 lbs.)		
Hominy feed			15.0 lbs.
Corn germ (dried)			15.0 lbs.
			100.0 lbs.

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, placed in screw-topped bottles, and moisture determinations were made on each portion before use.

4. 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.

5. Corn starch

The starch used in the short fermentation tests was Argo Corn Starch, a commercial pure food grade starch produced by the Corn Products Refining Co., Argo, Illinois. The contents of a number of small packages of this product were thoroughly

mixed and stored in a small drum with a tight-fitting cover. A moisture determination was carried out before use of the product.

6. Barley malt

Barley malt obtained from the Kurth Malting Company, Milwaukee, Wisconsin, was used as a control during a portion of the fermentations carried out during the course of this investigation. A small amount of the malt was freshly ground and its moisture determined prior to its use.

7. Malt extract

The malt extract used to prepare media for yeast cultures was obtained in a 140 pound keg from Anheuser Busch and Company, Saint Louis, Missouri in 1942. It was designated by the manufacturer as "light Budweiser non-diastatic malt syrup." Midway in this investigation it was found that the beer wort medium prepared from this material was resulting in very sluggish yeast cultures. This was attributed to the production of toxic materials in the malt extract by a very copious mold growth on its surface. A new supply of non-diastatic malt extract was obtained in a 60 pound drum from the Pabst Brewing C., Peoria Heights, Illinois, in October of 1951. This material was designated as "Bakers Syrup."

8. 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.

B. Chemicals

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

C. Special Materials

1. Soluble starch

Merck soluble starch, for use in the alpha-amylase determinations, was obtained in 1950 from Merck and Company, Rahway, New Jersey.

2. Maltose

The maltose used in the maltase determination was of C. P. grade, specific rotation of +130.4°, and was purchased from Pfanstiehl Chemical Co., Waukegan, Illinois in 1950.

3. Beta-amylase

This enzyme preparation, especially prepared for alpha-amylase determination, was obtained from Wallerstein Laboratories, New York, New York in 1950.

4. Limit dextrin

This dextrin was purchased from the Wahl-Henius Institute, Chicago, Illinois in 1950. It is prepared according to the method of Kneen, Beckord, Spoerl, and Foster (1948) by the action of bacterial alpha-amylase and beta-amylase on potato starch.

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 boiling tap water. The volume of the medium obtained is 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 150 ml. amounts were placed in 300 ml. Erlenmeyer flasks to serve as the inoculant for the experimental fermentations. For some of the early fermentations which required larger volumes of yeast culture for inoculation, 250 ml. of beer wort medium was placed in 500 ml. Erlenmeyer flasks. 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.

The stock culture was originally obtained from the Northern Regional Research Laboratory, Peoria, Illinois, as

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 at frequent intervals in order to maintain a vigorous yeast culture. The inoculated medium was incubated at 30° C. and 20 to 24 hour 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-b from the Biophysical Chemistry culture collection at Iowa State College.

Stock cultures of these molds were maintained on dextrose-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 gm. wheat bran and 10 gm. of 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, proteolysis occurs and the culture is spoiled.

Several procedures were used in the preparation of mold spore inocula. For the inoculation of small volume submerged cultures a spore suspension was prepared by the addition of sterile one per cent saline solution to a slant culture of the mold. The spores were scraped from the surface growth and suspended by the use of a sterile wire. Bran cultures were inoculated by removing a 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. Delayed drying

occasionally occurred when this method was used so an alternate procedure was developed. A small amount of inert material, such as filteraid (Dicalite Filteraid, Dicalite Co., Chicago, Illinois), was strongly heated in a test tube to sterilize it. After cooling, a small amount of this material was added to a slant culture of the mold. With the aid of a sterile wire mold spores were distributed throughout the inert material. This spore suspension was transferred by means of flamed spatulas into flasks containing the sterilized bran culture medium. The flasks were shaken and incubated as previously described. This method had the advantage of not introducing any moisture-retaining material which would delay drying of the bran cultures. After an initial bran culture of a mold had been prepared it was most convenient to use a small portion of it to inoculate succeeding bran cultures.

Two methods were used to make inoculations from the bran cultures. First, about 100 ml. of sterile one per cent saline 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 pipette. A simpler method consisted of merely shaking the bran culture and then pouring the spores and a small amount of the solid material into the flasks to be inoculated.

The exact details of the submerged fungal cultures will be discussed in more detail in the experimental section of this thesis. Briefly, the required amounts of corn, distillers' dried solubles, and calcium carbonate were placed in Erlenmeyer flasks and the proper volume of tap water was added. The flasks were stoppered with cotton plugs and sterilized at 15 pounds of steam pressure for 15 minutes. After cooling, the medium in each flask was adjusted to the desired pH by means of concentrated sodium hydroxide or sulfuric acid solutions. The pH was checked by means of a Macbeth continuous indicating line operated pH meter, manufactured by the Macbeth Corporation, New York, New York. Finally the medium was inoculated by one of the methods described previously and the flasks were placed in a reciprocal shaker in the 30° C. incubator.

Mold brans were prepared employing the method described by Hao, Fulmer, and Underkofler (1943). Briefly, the method consisted of mixing about 750 gm. of wheat bran with an equal weight of 0.3 N HCl 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 material was removed, cooled, and inoculated with 5 to 10 gms. 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.

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 500-ml. Erlenmeyer flasks equipped with water traps to decrease the loss of alcohol. Fifty grams of corn was placed in each flask, 125 ml. of 0.06 normal sulfuric acid added, and the flasks were cooked for one hour at 20 pounds steam pressure. The desired amount of saccharifying agent was mixed with tap water to make 125 ml. of slurry at 20° to 25° C. After completion of the cooking period, the autoclave was blown down immediately 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 gm. of calcium carbonate to bring the pH to approximately 5.0. The slurry of the saccharifying agent was next added with vigorous stirring, 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 8 to 10 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. 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 initial fermentations were carried out in a larger volume than those described in the standard method. The early experiments used 100 gms. of corn or corn fraction in a one-liter flask, 250 ml. of 0.06 normal sulfuric acid, 250 ml. of slurry of the saccharifying agent, and finally 20 ml. of yeast culture as the inoculum. Although there is a greater tendency toward erratic results in the smaller scale fermentations, they were adopted because of greater convenience and saving of materials.

Modifications of the standard method due to variation of certain factors were studied in this investigation and will be noted in connection with the particular fermentation in the experimental section. These modifications included such factors as the strength of the acid used in preparing the mash, amounts of saccharifying agent, and the saccharification time. In connection with the latter, when malt was used for saccharification, the mash was held at 55° C. for about 5 minutes before cooling to 30° C.

3. The short starch fermentation test

The procedure for a short fermentation test for saccharifying efficiency was described by Reese, Fulmer and Underkofler (1948). This method may be outlined briefly: to 100.0 gms. of pure food grade starch and 5.0 gms. of Difco yeast extract in a wide-mouth one-liter Erlenmeyer flask is added 250.0 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 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. cold water, and the entire contents are mixed with a high speed stirrer. 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, corn fractions, distillers' dried solubles, mold brans, and starch used during this investigation. Aluminum pans containing 3 to 5 gms. of the material were placed in an oven at 108° C. for 16 to 18 hours. The loss of weight during that treatment 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. In the small

volume fermentations (in 500 ml. flasks) the entire contents of each flask were added to 650-ml. Kjeldahl flasks along with 100 ml. of wash water. In the larger fermentation volumes, 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 was transferred to the Kjeldahl flasks. Calcium carbonate (0.5 to 1.0 gm.) was added to neutralize the acids present and about 99 ml. of distillate was collected from each flask. The volumetric flasks containing the distillates were brought to 25° C. by immersion 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) gives the total yield of alcohol in each flask.

The alcohol yields for the 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; i.e., the dry weight of the corn, distillers' dried solubles, malt, bran, etc. 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 proof gallons per standard bushel. Division by 1.9 will give yields in terms of wine gallons per standard bushel. (A wine gallon is a gallon of 190 proof alcohol.)

3. Determination of enzyme activities

a. Preparation of amylolytic materials for analysis. The mold bran samples were extracted with 0.5 per cent sodium chloride solution by adding 125 ml. of the saline solution to 12.5 gms. of the mold bran in a laboratory blender. After 5 minutes of vigorous stirring the slurries were allowed to stand at 30° C. for one hour and then filtered by means of suction. The extracts were kept in the refrigerator until time for analysis. It was necessary to further dilute the mold bran extracts for the alpha-amylase determination.

The submerged fungal cultures were prepared and grown in the usual manner. At the end of the incubation period the mold cultures were poured into a laboratory blender, stirred vigorously for 5 minutes, and then suction filtered to give an extract for analysis.

b. Determination of alpha-amylase.

(1) Reagents.

(a) Buffer solution No. 1. Anhydrous sodium acetate (164 gms.) was dissolved in water, 120 ml. of glacial acetic acid added, and the solution diluted to one liter with distilled water.

(b) Color standard. The hexahydrate of cobaltous chloride (25.0 gms.) and 3.84 gms. of potassium dichromate were dissolved in 100 ml. of 0.01 normal hydrochloric acid, according to the directions of Olson, Evans, and Dickson (1944).

(c) Beta-amylase. This enzyme preparation, especially prepared for alpha-amylase determination was obtained from the Wallerstein Laboratories, New York, New York.

(d) Stock iodine solution. Eleven gms. of resublimed iodine and 22 gms. of potassium iodide were made up to 500 cc. with distilled water.

(e) Dilute iodine solution. Twenty gms. of potassium iodide and 2 ml. of the stock iodine solution were diluted with distilled water to 500 ml. in a volumetric flask.

(2) Procedure. A standardized Wohlgemuth procedure for alpha-amylase determination as developed by Sandstedt, Kneen, and Blish (1939) with a modification by Olson et al. (1944) was used in this investigation. This method measures dextrinizing activity as compared to other methods which evaluate the liquefying or saccharifying powers.

The enzyme substrate, referred to as alpha-amylodextrin

solution, was prepared in the following manner. A slurry of 5.0 gms. of Merck soluble starch in about 20 ml. of water was prepared and added to approximately 120 ml. of boiling distilled water. The starch solution was transferred quantitatively to a 250-ml. volumetric flask, cooled to room temperature, and to it was then added 12.5 ml. of buffer solution No. 1. To the buffered starch solution was next added 125 mg. of beta-amylase which had been dissolved in a small amount of water. After dilution to volume a few milliliters of toluene was added to inhibit microbial contamination. The preparation was shaken and allowed to stand for 20 hours before using.

To start the analysis 20 ml. of the alpha-amylodextrin solution was pipetted into a 50-ml. Erlenmeyer flask, followed by 5 ml. of distilled water. The flask was placed in a water bath at 30° C. and allowed to reach equilibrium. A volume of 5 ml. of the enzyme solution to be analyzed was added to the substrate and the solutions were thoroughly mixed by swirling the flask. In certain instances it was necessary to use more than 5 ml. of distilled water and less than 5 ml. of the enzyme solution. The total volume must be kept equal to 30 ml.

After 10 minutes one ml. of the reaction mixture was pipetted into a 12-ml., plain, Pyrex centrifuge tube containing 5 ml. of the dilute iodine solution and the tube inverted twice to mix the solutions.

The tube was then placed in the color comparator between two similar tubes each containing 6 ml. of the color standard. If the color of the sample was too dark, one ml. aliquots of the reaction mixture were taken periodically until the color matched that of the standards. If the color was too light after 10 minutes, the entire procedure was repeated using a smaller volume of the enzyme solution. The number of alpha-amylase units per milliliter of enzyme solution was calculated from this equation:

$$\frac{0.4 \times 60}{V \times T} = \text{units per ml.}$$

where "V" equals the volume of enzyme solution in ml. and "T" is the total time in minutes from the addition of the enzyme solution to the alpha-amylodextrin solution to that time at which the colors were matched. Knowing the dilution, extraction ratio, weight of dry material per ml., etc., one can readily convert this figure to units of alpha-amylase per gram of dry material. One unit of alpha-amylase is the amount of enzyme required to dextrinize one gm. of beta-amylase treated starch in 1 hour at 30° C. This differs from the unit of Sandstedt et al (1939) which was based on the enzyme activity at 20° C.

c. Determination of maltase

(1) Reagents

(a) Stock acetate buffer solution No. 2. Anhydrous sodium acetate (183 gms.) was dissolved in about 500 ml. of distilled water, 217 ml. of glacial acetic acid was added, and the mixture diluted to one liter with distilled water. The final pH should be 4.4.

(b) Maltose substrate. Maltose monohydrate (2.35 gms.) was transferred to a 100-ml. volumetric flask, 50 to 60 ml. of distilled water was added, 5 ml. of stock acetate buffer (No. 2) was added, and finally dilution was made to 100 ml.

(c) Sulfuric acid and sodium hydroxide, each 1.0 normal; phenolphthalein indicator.

(d) Reagents for reducing-sugar analysis (see "determination of reducing sugars").

(2) Procedure. The method used here for the determination of maltase activity was the one described by Tsuchiya et al (1950). The unit of maltase is that amount of enzyme hydrolyzing one mg. of maltose monohydrate in one hour at 30° C.

Ten ml. of the buffered maltose substrate was pipetted into a test tube and placed in the water bath at 30° C. After about 5 minutes, 5 ml. of the enzyme solution, attempered to 30° C. was added to the tube and the contents were thoroughly mixed. After 15 minutes, a 3-ml. aliquot was withdrawn from

the reaction tube and transferred to a 100-ml. volumetric flask containing 5 ml. of one normal sulfuric acid. After allowing 10 minutes for the acid to inactivate the enzyme the solution was neutralized with one normal sodium hydroxode to the phenolphthalein end-point. Dilution was made to the mark with distilled water and after shaking, two 5-ml. aliquots were withdrawn for reducing value (R.V.) determinations, using the method for reducing-sugars described later. When the remainder of the enzyme-substrate solution had reacted for 120 minutes a similar procedure was followed for a second 3-ml. aliquot.

The calculation of maltase activity is based on the empirical rule that the conversion of maltose monohydrate into glucose is accompanied by a 78 per cent increase in reducing power. The following equation is used:

$$\frac{(b - a)}{0.78} \times (1.78 \times G) \times 20 \times \frac{60}{105} = \text{units of maltase per ml.}$$

where "a" equals the reducing value (R.V.) of the 15-min. reaction mixture; "b" equals the R.V. of the 120-min. reaction mixture; and "G" equals the glucose equivalent per ml. of the sodium thiosulfate used in the reducing-sugar determination.

d. Determination of limit-dextrinase

(1) Reagents

(a) Limit dextrin solution. This reagent had to be prepared fresh for each set of analyses. Limit dextrin

(2.25 gms.) was dissolved in water in a 250-ml. volumetric flask, 50 ml. of buffer solution (No. 3) was added, and the solution was diluted to the mark.

(b) Sulfuric acid and sodium hydroxide, each 1.5 normal; methyl red indicator.

(c) Buffer solution No. 3. Dibasic sodium phosphate (35.32 gms.) and 9.73 gms. of citric acid were dissolved in distilled water and diluted to one liter. If the resulting pH was not 4.8, one or the other of the components was added to adjust it to that value.

(d) Washed yeast. Fleischmann's yeast cake was washed with water and centrifuged until the supernatant became clear. This was accomplished by placing 1.5 cakes in each of 4 glass centrifuge tubes (250-ml.) and filling with distilled water. After thorough mixing, the tubes were centrifuged at 1600 r.p.m. for about 10 minutes. The supernatant liquid was poured off and the yeast mixed with a new volume of water. After 5 washings the supernatant liquid was clear. The washed yeast was spread on absorbent paper to remove the excess moisture. About 45 to 50 gms. of washed yeast was the yield from 6 cakes.

(e) Hydrochloric acid, 1.4 normal.

(f) Reagents for reducing-sugar determination.

(2) Procedure. The method used for limit-dextrinase activity was that of Back, Stark, and Scalf (1948). A

limit-dextrinase unit was defined as the amount of enzyme in one gm. of enzyme preparation necessary to produce one mg. of fermentable sugar from limit dextrin in one hour at 30° C. In connection with the submerged culture preparations this definition was modified to read ". . . one ml. of enzyme preparation . . . " instead of " . . . one gm. . . . ."

The analysis for limit dextrinase activity was started by pipetting 20 ml. of the buffered limit dextrin solution into a 50-ml. volumetric flask. The flask was placed in a 30° C. water bath and after equilibrium was attained, 5 ml. of the enzyme solution, attempered to 30° C., was added and the solutions were mixed. After 60 minutes 5 ml. of 1.5 normal sodium hydroxide was added to stop the enzyme action.

Thirty minutes later the solution was adjusted to pH 4.8 by the addition of 1.5 N sulfuric acid with methyl red as the indicator. The solution was diluted to 50 ml. and shaken. A 20-ml. aliquot was transferred to a plastic centrifuge tube containing 3 gms. of moist washed yeast. After mixing by means of a glass rod the tube was placed in a water bath at 30° C. for 2.5 hours with frequent stirring.

After fermentation the yeast was centrifuged out and a 10-ml. aliquot was removed from the clear supernatant liquid and transferred to a test tube containing 10 ml. of 1.4 normal hydrochloric acid. These solutions were mixed and the tube was placed in a boiling water bath for 2.5 hours. At the end

of this hydrolysis period the contents were quantitatively transferred to a 100-ml. volumetric flask, neutralized to a phenolphthalein end-point with 1.5 normal sodium hydroxide, and made to volume. Duplicate 5-ml. aliquots were taken for reducing sugar determinations.

A blank was run in a somewhat similar manner but in a different order. Five ml. of the enzyme solution was pipetted into a 50-ml. volumetric flask containing 5 ml. of 1.5 normal sodium hydroxide. After 30 minutes for inactivation of the enzyme, 20 ml. of the limit dextrin solution was added. The pH was next adjusted to 4.8 using 1.5 N sulfuric acid. After being made to volume and mixed, this solution was treated in a manner similar to the previous solution; i.e., yeast fermentation, acid hydrolysis, and finally the determination of reducing sugars. In addition, 10 ml. of this blank was removed before the yeast treatment and placed in a test tube containing 10 ml. of 1.4 normal hydrochloric acid. This second blank made it possible to correct for the slight dilution due to the added yeast in the sample and treated blank.

Calculations were made from the following equations. Both per cent conversion and the units per ml. were determined.

$$\frac{(B_2 - G_2)}{B_2} \times 100 = \text{per cent conversion}$$

$$\frac{(B_2 - G_2)100}{E \times \frac{(B_2)}{(B_1 L}} = \frac{(\%\ \text{conversion})B_1}{E} = \text{units of L.D./ml.}$$

where "B_1" is the mg. of glucose in 5 ml. of the blank before yeast treatment, "B_2" is the mg. of glucose in 5 ml. of the yeast-treated blank, "G_2" is the mg. of glucose in 5 ml. of the enzyme-treated, yeast-treated sample, and "E" is the volume of enzyme solution used (5 ml.). Knowing the weight of dry material represented by 5 ml. of the enzyme solutions it was a simple matter to calculate the units of limit dextrinase per gram of dry material.

e. Determination of reducing sugars. 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 (1943) describing a modification of the Shaffer-Somogyi method.

(1) Reagents

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

(b) Sulfuric acid, 7.5 normal.

(c) Standard 0.0500 normal sodium thiosulfate solution.

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

solution.

(e) Sugar reagent "G". One liter of this solution was composed of the following chemicals: 37.5 gms. of copper sulfate pentahydrate, 125.0 gms. of Rochelle salts, 53.0 gms. of anhydrous sodium carbonate, 1.0 gm. of potassium iodide, 50.0 gms. of anhydrous sodium sulfate, and 3.5665 gms. of potassium iodate. Saturated sodium hydroxide was used, if necessary, to adjust the final pH to 9.48.

(2) 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 stopper containing a short length of 1-mm. capillary tubing in the hole.

The test tube was next half-submerged 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 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.

V. EXPERIMENTAL

A. Investigation of a Toxic Factor

When starchy substrates for alcoholic fermentation are cooked with water, gelatinization of the starch results in highly viscous mashes which are extremely difficult to handle. This is particularly true in the case of corn. In addition to being viscous, water-cooked corn mashes also contain lumps of material which remain despite vigorous stirring and which are resistant to saccharification. Industrial plants attempt to eliminate this difficulty by partially liquefying the starch prior to or during the cooking operation. This is often accomplished by the addition of a small amount of malt (usually about one per cent) and is known as "premalting." Other methods involve the addition of small amounts of mineral acid or fungal amylase preparations. For laboratory scale fermentations the use of acid is the most convenient procedure.

It has been demonstrated a number of times that acid-saccharification of starch in grain mashes results in a lower alcohol yield than is obtained by the conventional malt-saccharified mashes. This may be due to any of a number of reasons; for example, the starch may be incompletely converted to fermentable sugars. Kerr and Schink (1941) have pointed

out that limit dextrins are resistant to moderate acid saccharification. Also, colored products are formed by the action of acids on starch or sugars. These products may resist fermentation or may even be toxic to yeast. Goering (1941) suggested that toxic material may be formed by the action of acid on the bran or germ portion of corn during cooking. It was therefore of some interest to observe the effect of acid concentrations which might be used in the liquefaction of corn mashes for laboratory fermentations.

The alcohol yields from mashes composed of the different fractions of corn cooked with varied acid concentrations were determined. These yields were compared with those obtained from whole corn mashes or mashes containing the appropriate proportions of the different fractions cooked under the same conditions. It seemed possible that any inhibitory material produced by the action of acid on a particular fraction should also have an inhibitory effect on the fermentation of either whole corn or composite mashes. This would result in yields lower than theoretical yields which could be calculated on the basis of experimentally determined alcohol yields from each fraction and the per cent of each fraction in whole corn or in the composite corn sample.

In the first series of fermentations the three mill fractions (i.e., grits, hominy feed, and corn germ) and a mixture of the fractions (referred to as the "composite") were

cooked with varied concentrations of sulfuric acid and fermented without the addition of an accessory converting agent. The composite was prepared in such a manner as to approximate whole corn by mixing the proper percentages of each fraction, that is, 70 per cent grits, 15 per cent hominy feed, and 15 per cent dried corn germ. The results are summarized in Table I.

Table I

Effect of Acid Concentration on Alcohol Yields From Corn Mill Fractions

Normality of Acid	Alcohol Yields, Proof Gallons per Standard Bushel				
	Grits Fraction	Hominy Feed	Germ Fraction	Composite Yield	Calculated Yield
0.02	0.23	2.50	0.30	0.57	0.58
0.04	0.26	2.43	0.30	--	0.59
0.06	0.42	2.48	0.27	1.66	0.71
0.08	0.78	2.51	0.29	--	0.86
0.10	1.48	2.45	0.49	2.33	1.37

The yields as reported here are in proof gallons of alcohol per standard bushel (i.e., 56 pounds and 12 per cent moisture). The calculated yield is a theoretical yield for the composite based on the yields from the different fractions composing it. This is best illustrated by a sample calculation. For example, the yield for a composite cooked with 0.02

normal sulfuric acid should be equal to:

$$(0.7)(0.23) + (0.15)(2.50) + (0.15)(0.30) = 0.58$$

It may be seen that the yields actually obtained from the composite cooked at various acid normalities were equal to or greater than the calculated yields. Extremely varied results were obtained in fermentations of the composites. The thickness and degree of lumping of the mashes seemed to vary irregularly with the acid concentration, giving rise to variable alcohol yields. The few successful fermentations were those having the least amount of lumping in the cooked mash.

The grits and germ fractions gave quite low yields of alcohol which increased as the concentration of acid increased, undoubtedly due to higher acid-conversion of starch during cooking. The higher alcohol yields associated with the hominy feed (or bran) fraction, remaining constant at different acid concentrations, suggest the presence of fermentable sugars and a relative lack of starch in this fraction.

When cooked with concentrations of sulfuric acid below 0.06 normal all of the materials gave mashes too thick for proper fermentation. In general, thinning of the mashes occurred with increased acid concentration. The dark color of the mashes cooked with 0.10 normal sulfuric acid indicated some caramelization had occurred. To avoid the possible loss of fermentable material in that manner the minimum acid concentration (0.06 normal) giving a thin mash was chosen for all

succeeding fermentations.

In the next phase of the work various concentrations of Aspergillus oryzae mold bran were added to the mashes which had been cooked with 0.06 normal sulfuric acid. The fermentations were run on the three mill fractions and the composite. The data are found in Table No. II.

Table II

Alcohol Yields From Corn Mill Fractions at Different Mold Bran Levels

Per Cent Mold Bran	Alcohol Yields, Proof Gallons per Standard Bushel				
	Grits Fraction	Hominy Feed	Germ Fraction	Composite Yield	Calculated Yield
0.0	0.42	2.48	0.27	1.66	0.71
2.1	5.90	3.57	2.56	4.81	5.05
2.6	5.95	3.43	2.59	4.89	5.06
3.2	5.95	3.56	2.60	4.99	5.06
3.7	5.87	3.63	2.62	5.06	5.04
4.2	5.75	3.55	2.59	5.00	4.95

All series showed increasing yields up to a certain mold bran concentration after which the yields decreased. Extremely high yields were obtained in the fermentation of the grits fraction indicating that the starch is concentrated in this fraction. The yields of alcohol from corn germ were significantly increased by the addition of mold bran. This indicates

the presence of some saccharifiable material in this fraction along with a considerable amount of non-fermentable material. There was only a small increase in alcohol yields from hominy feed following the addition of mold bran. This suggests that this fraction consists of approximately 50 per cent non-saccharifiable material, 35 per cent fermentable sugars, and 15 per cent starch.

The actual yields from the fermentation of the composite saccharified with different levels of mold bran were equal to or slightly less than the calculated yields, the differences probably being no more than the experimental error. The lack of any general inhibition would indicate that no toxic factor was produced by cooking with 0.06 normal sulfuric acid.

A similar series of fermentations was carried out using malt instead of mold bran for saccharification. The results are presented in Table III.

The alcohol yields in Table III, as in the previous tables, are in proof gallons per standard bushel and are calculated on the basis of the total fermentable material. That is, the weight of the saccharifying agent, either mold bran or malt, is included in the grain bill. Somewhat lower yields from corn grits were obtained using malt than had been previously obtained from the use of mold bran and slightly higher yields were found for the germ fraction. The results of this series of fermentations lead one to the same conclusions regarding

Table III

Alcohol Yields From Corn Mill Fractions at Different Malt Levels

Per Cent Malt	Alcohol Yields, Proof Gallons per Standard Bushel				
	Grits Fraction	Hominy Feed	Germ Fraction	Composite Yield	Calculated Yield
0.0	0.42	2.48	0.27	1.66	0.71
6.0	4.97	3.14	3.22	5.27	4.43
8.0	5.44	3.18	3.27	5.26	4.79
10.0	5.52	3.50	3.25	5.25	4.87
12.0	5.69	3.57	3.27	5.19	5.00
14.0	5.76	3.68	3.22	5.15	5.06

the fermentable materials in the different fractions as were stated following the mold bran series. The yields actually obtained from fermentations of the composites were considerably higher than the calculated yields. This difference may be largely due to experimental errors which differ from time to time. In any case, just as in the series saccharified with mold bran, there was no indication of the production of any toxic factor.

In an attempt to reduce the experimental errors two more series were run with each converting agent in which fermentations of the three mill fractions, the composite, and a whole corn mash were carried out simultaneously. All mashes were cooked with 0.06 normal sulfuric acid and saccharification

Table IV

Alcohol Yields From Corn Mill Fractions
Saccharified by Mold Bran or Malt

Substrate	Alcohol Yields, Proof Gallons per Standard Bushel When Saccharified by	
	Mold Bran	Malt
Grits fraction	5.85	5.70
Hominy feed	3.39	3.71
Corn Germ	2.50	2.95
Composite	5.08	5.22
Calc. yield	4.97	4.96
Whole corn	4.91	5.04

was effected with 3.64 per cent mold bran or 12.5 per cent malt. The average yields are given in Table IV.

The yields from the whole corn mashes are somewhat lower than those of the composites. The composites perhaps have slightly more of the high alcohol-producing grits fraction than is actually present in whole corn. The actual alcohol yields from the composites were slightly higher than the calculated yields. These results again indicate the absence of any toxic factor due to the action of acid on some material in corn during the cooking process.

A final series of fermentations was run in which whole corn and composite mashes were cooked with either water or with 0.06 normal sulfuric acid and then saccharified with 3.6

per cent mold bran or 12.0 per cent malt. The alcohol yields from mashes cooked with acid prior to saccharification were invariably higher than yields from the water-cooked mashes. This was particularly true of mashes converted with mold bran. When malt was used for conversion, the mashes were held at the converting temperature (approximately 55° C.) for 5 minutes before cooling to 30° C. This tended to decrease the importance of thinning by acid-cooking.

Two conclusions may be drawn at this point: first, the use of 0.06 normal sulfuric acid solutions for cooking corn mashes which are to be converted by mold bran increases alcohol yields; second, there is no significant production of a toxic factor by the action of such an acid concentration on corn during cooking.

B. Saccharification by Submerged Cultures

Before undertaking the study of Aspergillus oryzae 38 in submerged culture, some preliminary investigations were carried out using Aspergillus niger 337. This mold was grown according to the method of Adams, Balankura, Andreasen, and Stark (1947) on a medium consisting of 5 per cent distillers' dried solubles and one per cent corn, adjusted to a pH of 5.0 with calcium carbonate. The culture was transferred serially through 3 stages. In the first stage, 2 ml. of a spore suspension prepared by adding sterile saline solution to a slant

culture of the mold was inoculated into 50 ml. of mold medium in a 150-ml. Erlenmeyer flask. The flask was then placed in a shaker and incubated at 30° C. After 24 hours 5 ml. of the mold culture was transferred to 50 ml. of medium in a 150-ml. flask. At the end of a second 24-hour growth period, a 15 ml. inoculation was made into 150 ml. of medium in a 500-ml. flask. After 48 hours of growth the mold culture was ready for use.

In the first fermentation series using a submerged culture of Aspergillus niger 337 for saccharification, the effect of saccharification time on alcohol yield was studied. The flasks containing the acid-cooked mash and the mold culture were held for various lengths of time (0 to 30 minutes) at 55° C. before being cooled to 30° C. A volume of mold culture equal to 20 per cent of the final total mash volume was used for saccharification. Controls using about 3.7 per cent mold bran for saccharification were quick-cooled immediately after mixing while others using 12.5 per cent malt were held at 55° C. for 5 minutes before cooling. The results are summarized in Table V.

The value of 5.20 proof gallons per standard bushel for malt is an average of 5 fermentations at different times during the course of this investigation. The individual values varied only from 5.17 to 5.23. This value represents the upper limit in commercial production of alcohol using

Table V

Alcohol Yields From Corn Mashes Saccharified for Varied Times by Submerged Cultures of Aspergillus niger 337

Saccharification Time, Minutes at 55° C.	Alcohol Yield, Proof Gallons per Standard Bushel
30	5.30
20	5.31
10	5.29
0 (quick cool)	5.25
Mold Bran Control	5.25
Malt Control	5.20

malt saccharification and as such should be used as a basis for comparison of other yields given in this thesis.

Since the alcohol yield varied so little with saccharification time, the quick cool method was used during the succeeding fermentations. The volume of submerged culture of Aspergillus niger 337 necessary for optimum saccharification, as measured by alcohol production, was next investigated. The total yield of alcohol was nearly constant for saccharification by 10 volume per cent or greater. Since the yields are reported on the basis of the total amount of dry material there was a slight decrease for the higher volume per cents. Consequently, 25 ml. of mold culture (equal to 10 volume per cent) was chosen as the optimum and was used for the remainder of fermentations in which saccharification was accomplished by

Table VI

Alcohol Yields From Different Saccharification Levels of Submerged Cultures of Aspergillus niger 337

Volume per cent sub. cult.	Alcohol Yield, Proof Gallons per Standard Bushel
5	4.75
10	5.29
15	5.24
20	5.26
25	5.15

Aspergillus niger 337.

Submerged fungal cultures require aeration, either by bubbling air through the medium or by simply shaking the flasks. It is of some interest to observe the effect of different rates of aeration upon the production of amylases. Obviously, by decreasing the volume of medium in the mold culture flasks one can increase the degree of aeration. Therefore, the mold medium volume (in the final, 48-hour culture phase) was varied from 75 to 450 ml. in 500 ml. Erlenmeyer flasks and the effect on the amylase production was determined by measuring the alcohol yields following saccharification with the fungal cultures. A volume ratio exceeding 150 ml. in a 500 ml. flask was found to be unsatisfactory. This ratio was chosen in preference to the 75 ml./500 ml. ratio because of greater convenience.

Table VII

Effect of Aeration on Amylase Production by Submerged Cultures of Aspergillus niger 337

Volume of Mold Medium, ml/500-ml Flask	Alcohol Yield, Proof Gallons per Standard Bushel
75 ml./500 ml. flask	5.24
150 ml./ "	5.24
250 ml./ "	3.48
350 ml./ "	3.31
450 ml./ "	3.37

During later work with submerged cultures of Aspergillus oryzae 38-b there was some indication that the composition of the carrying medium had some effect on the production of amylase by the mold in submerged culture. Therefore the effect of carrying Aspergillus niger 337 on dextrose, glycerol, and dextrose-glycerol slants was investigated. The prior history was found to have no effect on amylase production by Aspergillus niger 337 in submerged culture.

After finding that calcium carbonate decreased the production of maltase and alpha-amylase by Aspergillus niger 337 and Aspergillus niger 330, Tsuchiya, Corman, and Koepsell (1950) developed a calcium carbonate-free medium. They reported that a medium consisting of 5 per cent corn and 5 per cent distillers' dried solubles gave the optimum production of maltase and alpha-amylase by both strains of Aspergillus niger.

For comparison with previous results a fermentation series was run using different volumes of submerged culture of Aspergillus niger 337 on the improved medium of Tsuchiya, et al (1950) for saccharification. The complete data are given in Table VIII.

Table VIII

Alcohol Yields From Different Saccharification Levels of Calcium Carbonate-Free Submerged Cultures of Aspergillus niger 337

Volume Per cent Mold Cult.	Dry Mat. in Sacch. Vol., Gms.	Total Dry Matter, Gms.	Alcohol Prod., Gms.	Alcohol Yield, Proof Gallons per Std. Bu.
5	1.07	44.68	13.20	4.40
10	2.15	45.76	15.75	5.12
15	3.22	46.83	16.58	5.28
20	4.30	47.91	16.65	5.17
25	5.37	48.98	16.99	5.19

The final mash volume was 250 ml. and the volume of mold culture used for saccharification varied from 12.5 ml. to 62.5 ml. The alcohol yield is in proof gallons per standard bushel based on the total grain bill. The total alcohol produced was somewhat higher than had been obtained in previous fermentations. The higher concentration of corn and dried solubles in the mold medium increased the total grain bill and thus tended to decrease the calculated alcohol yield. The results of this fermentation may also be found in Figures 2

and 4 in the Appendix.

A similar fermentation series was carried out using Aspergillus niger 330 in submerged culture for saccharification. This mold was grown, as in the preceding experiment, on a medium consisting of 5 per cent corn and 5 per cent distillers' dried solubles with the initial pH adjusted to 5.2 with sodium hydroxide and/or sulfuric acid. The mold culture medium was inoculated directly from a bran culture of the mold and was ready for use after 48 hours in a shaker at 30° C. The results are given in Table IX and in Figures 3 and 4.

Excellent results were obtained in saccharification with submerged cultures of Aspergillus niger 330. Ten volume per cent of mold culture was adequate for saccharification and

Table IX

Alcohol Yields From Different Saccharification Levels of Calcium Carbonate-Free Submerged Cultures of Aspergillus niger 330

Volume Per cent Mold Cult.	Dry Mat. in Sacch. Vol., Gms.	Total Dry Matter, Gms.	Alcohol Prod., Gms.	Alcohol Yield, Proof Gallons per Std. Bu.
5	1.07	44.38	15.36	5.16
10	2.14	45.45	16.61	5.44
15	3.21	46.52	16.86	5.40
20	4.28	47.59	16.96	5.31
25	5.35	48.66	17.02	5.21

high alcohol yields were obtained. Although the total alcohol production increased with increased volume of mold culture for conversion, the dry material in the mold culture increased the grain bill to the extent that the alcohol yield calculated on the total grains basis actually decreased.

In the first series of fermentations using submerged cultures of Aspergillus oryzae 38-b for conversion, the saccharification time was varied. The mold was grown as during the initial investigations with Aspergillus niger 337; i.e., it was carried through two 24-hour stages and a third 48-hour stage in a medium consisting of 5 per cent distillers' dried solubles and one per cent corn, adjusted to pH 5.0 with calcium carbonate. The data for the effect on alcohol yield of varying the saccharification time are found in Table X. The volume of mold culture used for saccharification was 20 volume per cent.

Table X

Alcohol Yields From Corn Mashes Saccharified for Varied Times by Submerged Cultures of Aspergillus oryzae 38-b

Saccharification Time, Minutes at 55° C.	Alcohol Yield, Proof Gallons per Standard Bushel
30	4.40
20	4.44
10	4.38
0 (Quick cool)	4.47
Malt Control	5.25

The quick-cool procedure was found to be adequate and was used in the remainder of the experimental fermentations. The effect of different volumes of mold culture on conversion was next studied and the results are given in Table XI. The data

Table XI

Alcohol Yields From Different Saccharification Levels of Submerged Cultures of *Aspergillus oryzae* 38-b

Volume per cent Sub. Cult.	Alcohol Yield, Proof Gallons per Standard Bushel
5	4.18
10	4.51
15	4.59
20	4.80
25	4.78

indicated that 20 volume per cent of mold culture for saccharification gave the maximum yields, and this volume was used throughout the remaining fermentations. The aeration effect was next studied by varying the volume ratio of the mold medium from 75 ml. to 300 ml. in 500 ml. Erlenmeyer flasks. As is shown in Table XII, the 150/500 volume ratio proved most satisfactory.

An accidental observation suggested that the composition of the carrying medium might have some effect upon the later amylase production by the mold submerged culture. This was

Table XII

Effect of Aeration on Amylase Production by Submerged Cultures of Aspergillus oryzae 38-b

Volume of Mold Medium, ml/500-ml Flask	Alcohol Yield, Proof Gallons per Standard Bushel
75	4.66
150	4.68
200	4.48
250	4.10
300	3.78

investigated in more detail and it was found that dextrose-agar was much superior to glycerol- or dextrose-glycerol-agar for carrying Aspergillus oryzae 38-b. As mentioned previously no such effect was observed for Aspergillus niger 337.

Thus far, Aspergillus oryzae 38-b had failed to show much value in the production of amylases in submerged culture. Since this particular strain produced an extremely efficient mold bran, it was hoped that if a proper medium could be developed the submerged culture would prove to be equally as effective. In an attempt to improve the medium, bran was added to the corn and dried solubles. The growth of the mold seemed to be improved but there was no increase in the alcohol yields. Another attempt involved the variation of the amounts of corn and distillers' dried solubles in the medium. With an increase in the corn/dried solubles ratio there was obtained a

significant increase in alcohol yield, the yield exceeding 5.0 proof gallons per standard bushel for the first time. It was then decided that a systematic investigation of the variation of the corn and distillers' dried solubles levels in the mold medium would be of value. Tsuchiya, Corman, and Koepsell (1950) published about this time the observation that calcium carbonate inhibited the production of alpha-amylase and maltase by molds. As a result, the previously outlined project was modified by the elimination of calcium carbonate from the mold medium.

Submerged cultures of Aspergillus oryzae 38-b were grown in 75 ml. of medium in 250 ml. Erlenmeyer flasks. The two 24-hour preliminary phases were eliminated and large spore inoculations were made directly into the final medium which was shaken for 48 hours at 30° C. and was then ready for use. The levels of corn and dried solubles were varied independently from 1 to 6 per cent and both the initial and final pH values of the mold medium were observed. Amylase production was measured by determining the production of alcohol in corn mashes saccharified by the various fungal cultures at a level of 20 volume per cent.

Using the method of least-squares, the following equations were found, expressing the initial pH (pH_i) and the final pH (pH_f) as functions of the weights of corn (C) and dried solubles (DDS) in the mold medium:

(1) $pH_i = 4.297 - 0.103\ DDS + 0.057\ C$

(2) $pH_f = 4.953 + 0.051\ DDS - 0.188\ C$

Thus it is seen that the initial pH increases slightly with an increase in the amount of corn and definitely decreases with an increase in dried solubles. The opposite effects are noted for the final pH. This is in accord with the usual rule that increasing the carbohydrate source (in this case, corn) decreases the final pH of the medium, while an increase in protein source (i.e., dried solubles) increases the final pH.

The alcohol yields from mashes saccharified by the various fungal cultures are presented in Table XIII. The

Table XIII

Alcohol Yields* From Corn Mashes Saccharified by Submerged Cultures of Aspergillus oryzae 38-b on Varied Media

Dry Weight Corn, Gms.	Dry Weight DDS, Gms.					
	0.65	1.29	1.94	2.58	3.23	3.87
0.62	4.71	4.87	4.90	4.92	4.88	4.85
1.25	4.69	4.93	4.95	4.94	4.87	4.89
1.87	4.69	4.80	4.91	4.96	4.84	4.81
2.50	4.74	5.06	5.06	4.95	5.04	5.03
3.07	4.64	4.89	4.88	4.95	4.91	4.94
3.67	4.76	4.95	4.98	4.96	4.94	4.97

*Yields are in proof gallons per standard bushel.

weights of corn and dried solubles are given in grams, while the alcohol yields are in proof gallons per standard bushel. The results were somewhat variable and no attempt was made to obtain an equation relating corn and dried soluble levels to alcohol production. It was sufficient to note that intermediate levels of both corn and dried solubles seemed to result in the maximum alcohol yields. A medium composed of 1.29 gms. dried solubles and 2.50 gms. of corn was chosen for further work. Such a medium had been found to have an initial pH of 4.33 and a final pH of 4.59.

Tsuchiya, Corman, and Koepsell (1950) had reported that alpha-amylase was sensitive to acidities below a pH of 4.75. It was thought that if the initial pH of the medium described above was adjusted to a higher level amylase production might be improved. Therefore, using the optimum medium, experiments were conducted in which the initial pH was adjusted to different values (4.3 to 5.5) using concentrated sodium hydroxide and sulfuric acid as required. At the same time it was decided to also grow the mold in a larger volume so all factors were doubled. When the final pH values of the cultures were measured it was found that the larger volume cultures were invariably more acid than the identical smaller volume cultures. Also the final pH values of all cultures depended on the pH to which they had been initially adjusted. As a result, a number of mold cultures were available which were

identical except that the final pH values varied from about 4.3 to 5.4. When these were used to saccharify corn mashes it was found that the alcohol yield was a linear function of the final pH of the mold culture over the pH range of 4.3 to 5 5.2. Therefore, it was expected that a change in the medium producing a higher final pH would result in greater alcohol production. This, as found earlier, could be accomplished by decreasing the amount of corn and increasing the dried solubles in the medium.

The medium as finally chosen consisted of 3.93 gms. corn and 4.23 gms. distillers' dried solubles in 150 ml. of solution in a 500 ml. Erlenmeyer flask. The initial pH was adjusted to 5.2 using sodium hydroxide or sulfuric acid. The saccharification efficiency at different levels was finally determined for submerged cultures of Aspergillus oryzae 38-b on the optimum medium. The complete results are given in Table XIV and also in Figures 1 and 4.

Intensive investigation of the problem resulted in the development of a medium for submerged culture growth of Aspergillus oryzae 38-b which gave considerably greater saccharification as indicated by a 10 per cent increase in alcohol yields. However, it appears that both Aspergillus niger 337 and Aspergillus niger 330 are superior to Aspergillus oryzae 38-b when used in submerged culture for the saccharification of corn mashes.

Table XIV

Alcohol Yields From Different Saccharification Levels of Submerged Cultures of Aspergillus oryzae 38-b on Optimum Medium

Volume Per cent Mold Cult.	Dry Mat. in Sacch. Vol., Gms.	Total Dry Matter, Gms.	Alcohol Prod. Gms.	Alcohol Yield, Proof Gallons per Std. Bu.
5	0.68	44.04	13.17	4.46
10	1.36	44.72	14.52	4.83
15	2.03	45.39	15.22	4.99
20	2.71	46.07	15.49	5.01
25	3.39	46.75	16.10	5.13

C. Saccharification by Mold Brans

Following the disappointing results found for the use of Aspergillus oryzae 38-b in submerged culture as a saccharifying agent, attention was next turned to the preparation and use of mold brans. Since both Aspergillus niger 337 and Aspergillus niger 330 had been shown to produce submerged cultures of high saccharifying efficiency, it was of considerable interest to investigate their use in mold bran preparations. Mold brans were prepared from Aspergillus oryzae 38-b and the two strains of Aspergillus niger according to the method of Hao, Fulmer, and Underkofler (1943). The saccharifying efficiencies of the mold bran preparations were studied by measuring the alcohol production from corn mashes saccharified

Table XV

Alcohol Yields From Mold Brans at Different Levels

Per cent Mold Bran	Dry Wt. Mold Bran, Gms.	Total Dry Matter, Gms.	Alcohol Prod., Gms.	Alcohol Yield, Proof Gallons per Std. Bu.
A. niger 337				
2.59	1.14	44.10	15.43	5.21
3.57	1.59	44.55	15.85	5.30
4.53	2.04	45.00	16.06	5.31
5.50	2.50	45.46	16.24	5.32
6.42	2.95	45.91	16.34	5.29
A. niger 330				
2.62	1.16	44.32	15.50	5.21
3.62	1.62	44.78	16.20	5.38
4.62	2.09	45.25	16.20	5.33
5.58	2.55	45.71	16.31	5.32
6.54	3.02	46.18	16.27	5.24
A. oryzae 38-b				
2.59	1.14	44.10	15.01	5.08
3.49	1.59	44.55	15.62	5.22
4.55	2.05	45.01	15.99	5.28
5.52	2.51	45.46	16.13	5.29
6.45	2.96	45.92	16.20	5.25
Malt Control				
10.90	5.25	48.28	16.86	5.20

by different levels of mold brans. The levels tested were from 2.6 to 6.5 per cent of the total dry material. Malt, at a level of 10.9 per cent, was used as a control for all series of fermentations. The complete results are listed in Table XV.

These data may also be found plotted in Figures 1, 2, 3, and 5 in the Appendix. All of the mold brans were shown to be 3 to 4 times as effective as malt on a weight basis and also

gave higher optimum yields. It has been reported a number of times, viz., Boyer and Underkofler (1945) or Corman and Tsuchiya (1951), that malt can supplement the action of mold bran in conversion, resulting in increased alcohol yields. The question of whether a similar supplementary effect would be found for mixtures of mold brans then arose. Six fermentation series were carried out using mixtures of mold brans at different levels for conversion of corn mashes. Within the limit of experimental error, the alcohol yields were the same as would be expected on the basis of a linear combination of the conversion activities of the individual mold brans.

D. Short Starch Fermentation Test

In analyzing the data for the fermentation method of evaluating the various fungal preparations, the following mathematical treatment was used. The dry weight of the saccharifying agent (either as mold bran or submerged culture) was divided by the weight of alcohol produced from the fermentation of a corn mash saccharified by that level of saccharifying agent. This was plotted against the dry weight of saccharifying agent. It was found that the data gave straight lines of essentially the same slope for all fungal converting agents tested, as shown in Figures 4 and 5.

It was on this basis that Reese (1947) developed the short starch fermentation test for the evaluation of mold

brans prepared from Aspergillus oryzae 38-b. It appeared that this method might prove effective as a method of evaluating not only other mold brans but also submerged culture preparations as well.

Consequently, mold brans and submerged culture preparations of the three molds under investigation were tested using this procedure. The results are indicated in Tables XVI and XVII.

Table XVI

Alcohol From Starch at Different Mold Bran Levels

(1) Dry Weight Mold Bran, Gms.	(2) Alcohol Produced, Gms.	(1)/(2)
A. niger 337		
0.91	20.18	0.0451
1.82	26.26	0.0693
2.28	27.93	0.0818
2.73	29.08	0.0939
3.63	30.50	0.1190
A. oryzae 38-b		
0.91	25.58	0.0356
1.82	31.28	0.0582
2.28	31.68	0.0720
2.73	31.59	0.0865
3.64	33.47	0.1087
A. niger 330		
0.93	7.25	0.1282
1.86	14.58	0.1276
2.79	20.23	0.1379
3.71	25.60	0.1450

Table XVII

Alcohol From Starch at Different Submerged Culture Levels

Vol. Per cent Mold Culture	(1) Dry Wt. in Sacch. Vol., Gms.	(2) Alcohol Produced, Gms.	(1)/(2)
A. niger 337			
5	2.15	27.25	0.0790
10	4.30	31.00	0.1388
15	6.45	32.15	0.2005
20	8.60	33.55	0.2565
A. oryzae 38-b			
4	1.09	25.23	0.0432
8	2.18	29.31	0.0744
10	2.72	30.93	0.0880
12	3.26	31.67	0.1030
16	4.35	33.20	0.1310
A. niger 330			
5	2.15	5.32	0.404
10	4.30	10.03	0.428
15	6.45	14.74	0.436
20	8.60	18.88	0.455

The data presented in Tables XVI and XVII are plotted in Figure 6 in the Appendix. It should be pointed out that the upper line for *Aspergillus niger* 330 in submerged culture was off the range of the graph and has been transposed downward. This was done to illustrate that the data for the submerged culture preparation resulted in a line parallel to that from the mold bran preparation. The fact that the data for *Aspergillus niger* 330 preparations gave lines of a different slope than those for the other molds suggests that starch saccharification by *Aspergillus niger* 330 may be due to the action of

different enzymes or at least to a different ratio of the same enzymes. However, actual fermentations using fungal preparations of this strain had indicated that high efficiency could be expected. This leads to the conclusion that the enzymes of Aspergillus niger 330 preparations act very slowly upon starch in the initial stages of saccharification.

According to the method of Reese (1947) the ratio of the graphical intercepts for two mold brans was 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 intercepts obtained in this investigation to see whether this relationship applies to mold brans prepared from different molds or to submerged culture preparations. This comparison is made in Table XVIII. The preparations are listed in the

Table XVIII

Comparison of Maximum Alcohol Yields, Optimal Levels, and Intercept Values for Various Mold Preparations

Preparation	Max. Alcohol Yield, Proof Gal. per Bu.	Level, Per cent	Intercept Value
330 sub.	5.44	4.72	0.289
330 mb.	5.38	3.62	0.110
337 mb.	5.30	3.57	0.0202
337 sub.	5.28	6.89	0.0202
38-b mb.	5.28	4.55	0.0108
38-b sub.	5.13	7.26	0.0150

order of decreasing maximum alcohol yield and are identified by the strain number with "sub." or "mb." for submerged culture or mold bran. The weight of dry material in the saccharifying preparation at the optimum level for each preparation is listed under "Level" as the per cent of the total dry material in the fermentation mash.

Examination of the values in Table XVIII leads to the conclusion that there is no over-all relation between the intercept values from the short starch fermentation test and the optimal levels for actual fermentations. Neither does this relation apply to submerged cultures or mold brans as classes. There is only a slight indication that this relation applies to different types of preparations from the same mold. It is possible that this method may be used for the evaluation of preparations of the same type from one mold in the same manner as Reese (1947) has used it for the evaluation of a number of Aspergillus oryzae 38-b mold brans. However, for the evaluation of fungal preparations of different types and from different molds the laboratory fermentation method appears preferable to the short starch fermentation test.

E. Enzyme Activity Determinations

In continuing the investigation of fungal amylolytic preparations attention was next directed toward the determination of enzyme activities. The first problem was the choice

of a procedure for the extraction of the mold brans to prepare an enzyme solution for analysis. Tokuoka (1937) reported that the best procedure for the extraction of amylase, maltase, protease, and sucrase from saké-kôji was to extract with one per cent sodium chloride solution for 3 to 5 hours at room temperature. A report by Caldwell and Doebbeling (1937) suggests that a 0.05 molar (approximately 0.3 per cent) sodium chloride solution would be desirable for the extraction since both amyloclastic and saccharogenic activities were found to be highest at that salt concentration. Gates and Kneen (1948, 1949) in the preparation of extracts of mold brans for amylase and maltase determinations used 10 ml. of distilled water for each gram of mold bran.

The following procedure was chosen for use in this investigation. A slurry was prepared by the addition of 125 ml. of 0.5 per cent sodium chloride solution to 12.5 grams of mold bran. This slurry was vigorously stirred in a laboratory blender for approximately 5 minutes and then allowed to stand for one hour at 30° C. The material was filtered and the enzyme-containing solution was stored in the refrigerator until used. It was necessary to dilute this solution prior to the determination of alpha-amylase activity.

Submerged fungal cultures of the various molds were prepared as previously described and shaken for 48 hours at 30° C. The entire mold culture was next transferred to a

laboratory blender, stirred vigorously for approximately 5 minutes, and finally filtered. These enzyme solutions were also stored in the refrigerator until used.

The activities of alpha-amylase, maltase, and limit-dextrinase were determined in the enzyme solutions using methods previously described. The enzyme activities are summarized in Table XIX. The preparations are grouped as mold

Table XIX

Enzyme Activities of Fungal Preparations

Preparation	Alpha-Amylase Units per Gm.	Maltase Units per Gm.	Limit-Dextrinase Units per Gm.
Mold Brans			
330	25	129	106.5
337	113	88.5	109.7
38-b	230	81.5	89.1
Sub. Cult.			
330	20.9 (1.8)*	70.4 (6.05)	60.5 (5.2)
337	192 (16.5)	59.1 (5.08)	123. (10.6)
38-b	259 (14.1)	41.1 (2.24)	58.6 (3.2)

*Values in parentheses are in units per ml.

brans and submerged cultures (sub. cult.). The organisms are identified by their strain number and are listed in the order of decreasing maltase activity. Enzyme activities are indicated in enzyme units per gram of dry weight of the preparation.

Each organism was found to be particularly efficient in the production of a certain enzyme. Aspergillus niger 330

produced the highest maltase activity and the lowest alpha-amylase. Aspergillus oryzae 38-b produced the highest amounts of alpha-amylase and the lowest amounts of the other enzymes. Aspergillus niger 337 produced high concentrations of all enzymes.

Table XX

Summary of Data for Mold Preparations

Mold Preparation	Alcohol Yield, Proof Gallons per Bu.	Optimal Level, Per cent	Intercept Value	Alpha-Amylase, Units per Gm.	Maltase, Units per Gm.	Limit-Dextrinase, Units per Gm.
Sub. Cult.						
330	5.44	4.72	0.289	20.9 (1.8)*	70.4 (6.06)	60.5 (5.2)
337	5.28	6.89	0.0202	192 (16.5)	59.1 (5.08)	123 (10.6)
38-b	5.13	7.26	0.0150	259 (14.1)	41.1 (2.24)	58.6 (3.2)
Mold Brans						
330	5.38	3.62	0.110	25	129	106.5
337	5.30	3.67	0.0202	113	88.5	109.7
38-b	5.28	4.55	0.0108	230	81.5	89.1

*Values in parentheses are in units per ml.

VI. DISCUSSION

Aspergillus niger 337 was grown in submerged culture on a medium consisting of 5 per cent corn and 5 per cent distillers' dried solubles. The pH was adjusted to 5.2 using sodium hydroxide and/or sulfuric acid. The optimum level of this preparation for the saccharification of corn mashes for alcoholic fermentation was found to be 15 volume per cent or 6.89 per cent on a dry materials basis. The maximum yield of alcohol obtained was 5.28 proof gallons per standard bushel. This is slightly higher than the normal yield of 5.20 from malt-saccharified mashes. The yields reported in the literature range from 5.09 by Corman and Langlykke (1948) to 5.46 by Adams, Balankura, Andreasen, and Stark (1947). The U. S. Production and Marketing Administration and the Bureau of Agricultural and Industrial Chemistry (1950) report that yields of 5.28 proof gallons per standard bushel were obtained on a plant scale basis using submerged cultures of Aspergillus niger 337 for saccharification. However an additional amount of malt (one to 2 per cent of the total grain bill) was added to aid in thinning the mashes.

Submerged cultures of Aspergillus niger 330 were grown on the same medium and were found to be more efficient as

saccharifying agents. The optimum level for this fungal preparation was only 10 volume per cent (4.72 per cent on a dry basis) and the maximum alcohol yield was 5.44 proof gallons per standard bushel. This was the highest yield obtained in the entire investigation, and it is considerably greater than the malt controls. Corman and Langlykke (1948) reported a maximum yield of 5.24 for submerged cultures of this strain. Later, Corman and Tsuchiya (1951) by adding small amounts of malt increased the yield to 5.4. They noted that their submerged culture had less than the minimum amount of alpha-amylase necessary for efficient saccharification, having only 0.7 units per ml. at 20° C. As indicated in the previous section the submerged culture of Aspergillus niger 330 prepared in the present investigation had 1.8 alpha-amylase units at 30° C. (approximately equivalent to 0.9 units at 20° C.) Possibly this extra alpha-amylase activity was just sufficient to bring about high conversion of starch without the addition of a supplemental converting agent such as malt.

Aspergillus oryzae 38-b was initially grown in submerged culture on a medium containing 5 per cent distillers' dried solubles, one per cent corn, and sufficient calcium carbonate to adjust the pH to 5.0. Extremely low yields, 4.6 to 4.8 proof gallons per standard bushel, led to the attempt to develop an improved medium. By changing the concentrations

to approximately 3 per cent each of corn and distillers' dried solubles the alcohol yields were improved to 5.13 proof gallons per standard bushel. Despite the fact that malt yields are slightly higher, the use of submerged cultures of Aspergillus oryzae 38-b for saccharification is more economical.

During the attempt to develop an improved medium for submerged culture growth of Aspergillus oryzae 38-b, a number of cultures differing only in their final pH became available. The alcohol yields obtained from mashes saccharified by these preparations indicated that the production of saccharifying enzymes in submerged fungal cultures depended on the acidity of the medium during growth. Tsuchiya, Corman, and Koepsell (1950) also made this same observation. The fact that increasing the corn from one per cent to 3 per cent brought about an increased alcohol yield seems quite reasonable since numerous observations have indicated that the amylases are adaptive enzymes. This is counter-balanced by the fact that increased corn concentrations also result in a more acidic medium. The acidity can be decreased by the addition of dried solubles but this increases the total grain bill. The optimum medium must have a proper balance of factors which result in high enzyme production at a pH ensuring stability and must make only a small addition to the total grain bill.

Since the optimum medium for submerged culture growth of Aspergillus oryzae 38-b only partially met these conditions, attention was turned to the use of mold brans. Mold brans of Aspergillus niger 337, Aspergillus niger 330, and Aspergillus oryzae 38-b were prepared and maximum alcohol yields of 5.30, 5.38, and 5.24 proof gallons per standard bushel were obtained, respectively. There have been no reports in the literature regarding mold brans prepared from these particular strains of Aspergillus niger. Optimum yields for Aspergillus oryzae mold brans vary from 4.98 obtained by Hao (1942) to 5.34 as reported by Underkofler, Severson, and Goering (1946).

A summary of the data for the various fungal preparations investigated is presented in Table XX. The preparations are grouped as mold brans and submerged cultures (sub. cult.). The organisms are identified by their strain number and are listed in the order of decreasing alcohol yield. The levels of saccharifying agents for maximum alcohol yield are indicated as per cent of the total dry weight. The intercept values were obtained by graphical analysis of the data from the short starch fermentation tests. The enzyme activities are expressed in terms of the appropriate units per gram of dry weight of saccharifying agent.

The amylase activities for submerged cultures as listed in Table XX agree quite well with the many determinations reported in the literature. Equally satisfactory results are

presumed for the activities of the mold bran preparations based on the very meager reports found in the literature. It appears that Aspergillus niger 330 produces the highest maltase yields both in submerged culture and as mold bran. It has also been found to produce only very small amounts of alpha-amylase. The reverse situation is true for Aspergillus oryzae 38-b which produces extremely high alpha-amylase activities in submerged culture and as mold bran while producing the lowest maltase activity. Both types of preparations of Aspergillus niger 337 produced intermediate amounts of alpha-amylase and maltase and the highest limit-dextrinase activities. In comparing the two methods of enzyme production, neither seemed clearly superior with the exception that mold bran preparations had considerably higher maltase activity for all molds. If the mold preparations are classified into types based on the approximate ratios of enzyme activities, both preparations of Aspergillus niger 337 and Aspergillus oryzae 38-b would be placed in a high alpha-amylase class. The two preparations of Aspergillus niger 330 would be in a low alpha-amylase class. If there is any significance to such a classification then the action of the Aspergillus niger 330 preparations on starch should differ from the action of the other preparations.

In this regard, it is of interest to examine the results of the short starch fermentations as shown in Figure 6. The

slope and intercepts of the straight lines for the two Aspergillus niger 330 preparations are quite different from those for the other fungal preparations. The slope of the lines for Aspergillus oryzae 38-b and Aspergillus niger 337 preparations was 0.0275 compared to the value, 0.0274, obtained by Reese (1947). The intercepts were in the same range as the better mold bran preparations investigated by Reese (1947) and according to his method of evaluation the optimal levels should be from about 2.2 to 4.0 per cent. Actually, the optimal levels were somewhat higher, 3.6 to about 7.3 per cent. A further examination of the data of Table XX leads one to the conclusion that there is no correlation between the intercept value and the optimal level. This applies to all comparisons: i.e., over-all; within a group, such as all submerged cultures; or between the two different types of preparations from the same mold. This does not deny that this method of evaluation can be applied to different preparations of the same type from the same organism. Actually, the fact that straight lines are obtained leads one to feel that the method may be applied to other mold preparations with the same degree of success as Reese attained in the evaluation of Aspergillus oryzae 38-b mold brans.

There remains the problem of why the slope of the lines for Aspergillus niger 330 was different and why the intercepts were so high. A high intercept value should indicate that the

amylolytic agent was inefficient. Actual fermentation tests proved that Aspergillus niger 330 preparations were very efficient.

As previously pointed out, when the mold preparations are grouped according to the enzyme activity ratios, Aspergillus niger 330 preparations were found to be different from the other preparations. The combined action of a group of enzymes upon a substrate must certainly vary as the ratio of the individual enzymes within the group varies. Therefore, since the short starch fermentation test really depends upon the combined enzyme action on starch, the different slope must be due to the different enzyme ratio.

The indication that the saccharifying efficiency at 24 hours is poor (high intercept value) and at 72 hours is high (high alcohol yields) is easily explained by assuming slow initial enzymatic action. This is undoubtedly due to the low alpha-amylase activity of the Aspergillus niger 330 preparations. This leads one to the comparison of alpha-amylase activities with the intercept values. It appears that a relationship between the two values does exist and that the intercept value is a parabolic function of the alpha-amylase activity. One might speculate then that the equation of the straight lines obtained in the short starch fermentation test might be written:

$$\frac{X}{Alc.} = \frac{K}{A} + K' \times \frac{1}{S}$$

where X is the weight of saccharifying agent, Alc. is the weight of alcohol produced, K and K' are constants, A is the alpha-amylase activity, and S is the activity of the saccharifying enzyme such as maltase.

The data in Table XX indicate that there is no correlation between alpha-amylase activity and either the alcohol yield or the optimal level. The product of the optimal level and the alpha-amylase activity is proportional to the total amount of alpha-amylase added to the mash. There is no correlation between the total amylase and alcohol yields. There is a similar lack of correlation between limit-dextrinase activity or total limit-dextrinase and alcohol yields.

When the classes of preparations are considered separately (i.e., either all submerged cultures, or all mold brans) a positive correlation is found between maltase activity or total maltase and the alcohol yields.

VII. SUMMARY AND CONCLUSIONS

1. The effect of different concentrations of acid for the cooking of whole corn mashes and mashes composed of the various mill fractions of corn has been studied. The minimum concentration of acid necessary for proper thinning of the mashes was found to be 0.06 normal sulfuric acid. No evidence was found indicating the production of a toxic factor by the action of such a concentration of acid on whole corn or any mill fraction during cooking.

2. Mold brans and submerged culture preparations were made from Aspergillus niger 330, Aspergillus niger 337, and Aspergillus oryzae 38-b. The preparations from Aspergillus niger 330 were found to be extremely efficient saccharifying agents and resulted in alcohol yields of approximately 5.40 proof gallons per standard bushel. Aspergillus oryzae 38-b preparations were the least efficient, giving yields from 5.13 to 5.28 proof gallons per standard bushel. With the exception of Aspergillus oryzae 38-b all of the fungal preparations resulted in alcohol yields considerably higher than the malt controls and it appears certain that all could surpass malt on an economic basis.

3. An optimum medium for the production of Aspergillus oryzae 38-b in submerged culture was developed and the alcohol yields were increased from the initial value of about 4.4 to the final value of 5.13 proof gallons per standard bushel.

4. The short starch fermentation test of Reese, Fulmer, and Underkofler (1948) was considered for the evaluation of the various fungal preparations. It was found 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 type preparations from the same organism. Further work should be carried out confining the attention to a particular type of preparation from one mold as Reese (1947) did in evaluating numerous mold brans all prepared from Aspergillus oryzae 38-b.

5. It further appears that the short starch fermentation test is too rapid for organisms such as Aspergillus niger 330, which produce only small amounts of alpha-amylase. It seems quite possible that the intercept value may be related inversely to the alpha-amylase activity and that the slope may depend on the activity of the saccharifying enzymes (maltase, glucogenic activity, etc.) or the ratio of such activity to alpha-amylase.

6. Determinations of alpha-amylase, maltase, and limit-dextrinase activities in the various fungal preparations were made and it was found that the enzyme production in mold brans

paralleled the production by the same mold in submerged culture. It is possible to divide the fungal preparations into two classes on the basis of enzyme activity ratios. In a class having a low ratio of alpha-amylase activity to other enzyme activity may be placed both the mold bran and submerged culture of Aspergillus niger 330. The remaining preparations belong in a class having high ratios of alpha-amylase activity. The same classes could also be obtained on the basis of intercept value and slope obtained in the short starch fermentation test.

7. No correlation was found between alpha-amylase activity or limit-dextrinase activity and the optimal level of saccharifying agent or the final alcohol yield. Considering only one type of preparation at a time (i.e., all the mold brans or all the submerged cultures) a correlation was found between maltase and the alcohol yield. However, no over-all correlation for all preparations was found.

8. It appears that the short starch fermentation test may find some application for the choice of the optimal level of certain saccharifying agents. The determination of maltase or saccharogenic activity of the preparation would allow the prediction of the expected alcohol yield. Such a method of evaluation would probably be of value in a control laboratory where it would be used on only one type of saccharifying agent. For the comparison and evaluation of a number of different

preparations the most reliable results will come from laboratory fermentations using different levels of the preparations for saccharification.

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IX. APPENDIX

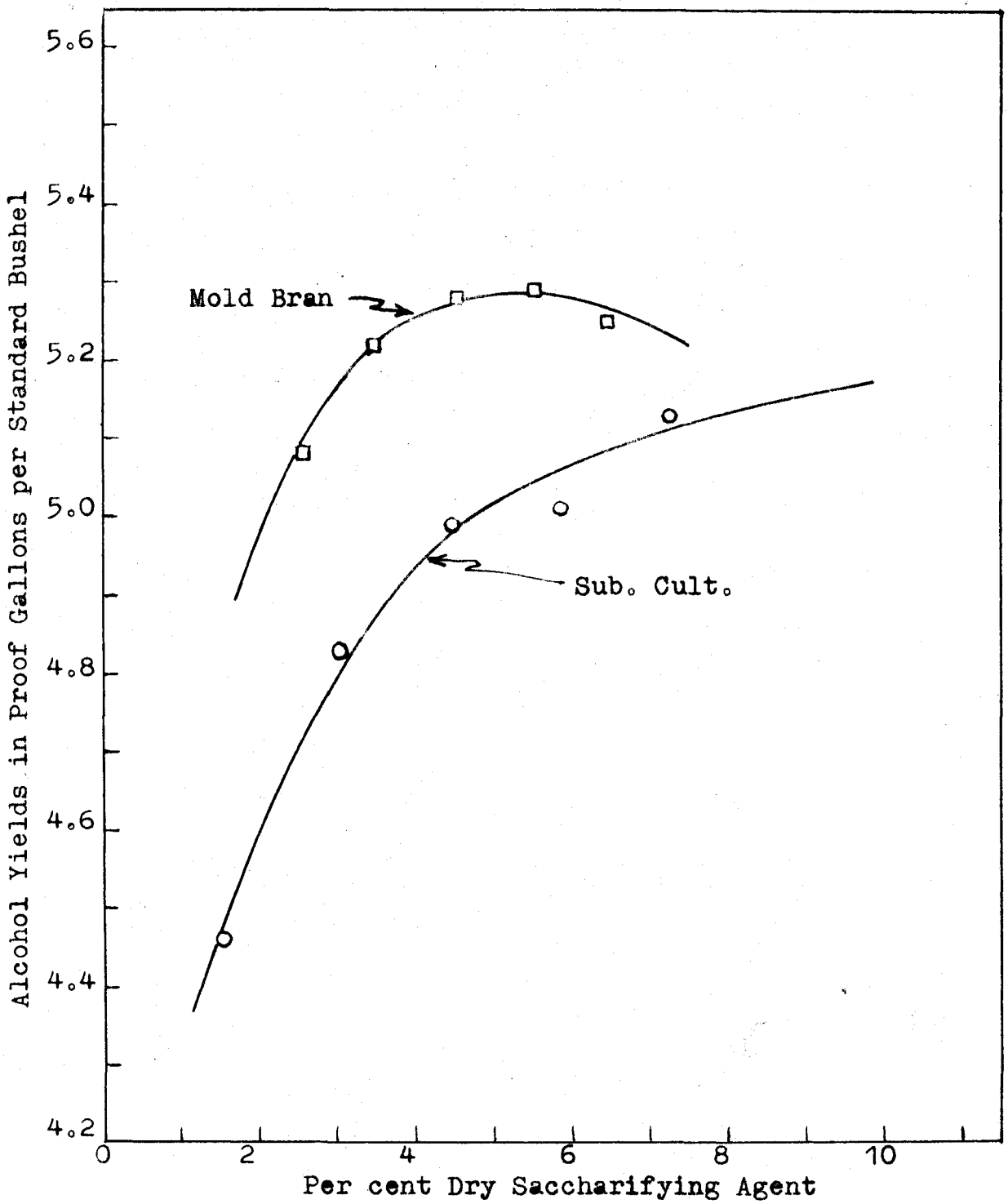

Figure 1. Alcohol Yields from Mashes Saccharified by Aspergillus oryzae 38-b Preparations

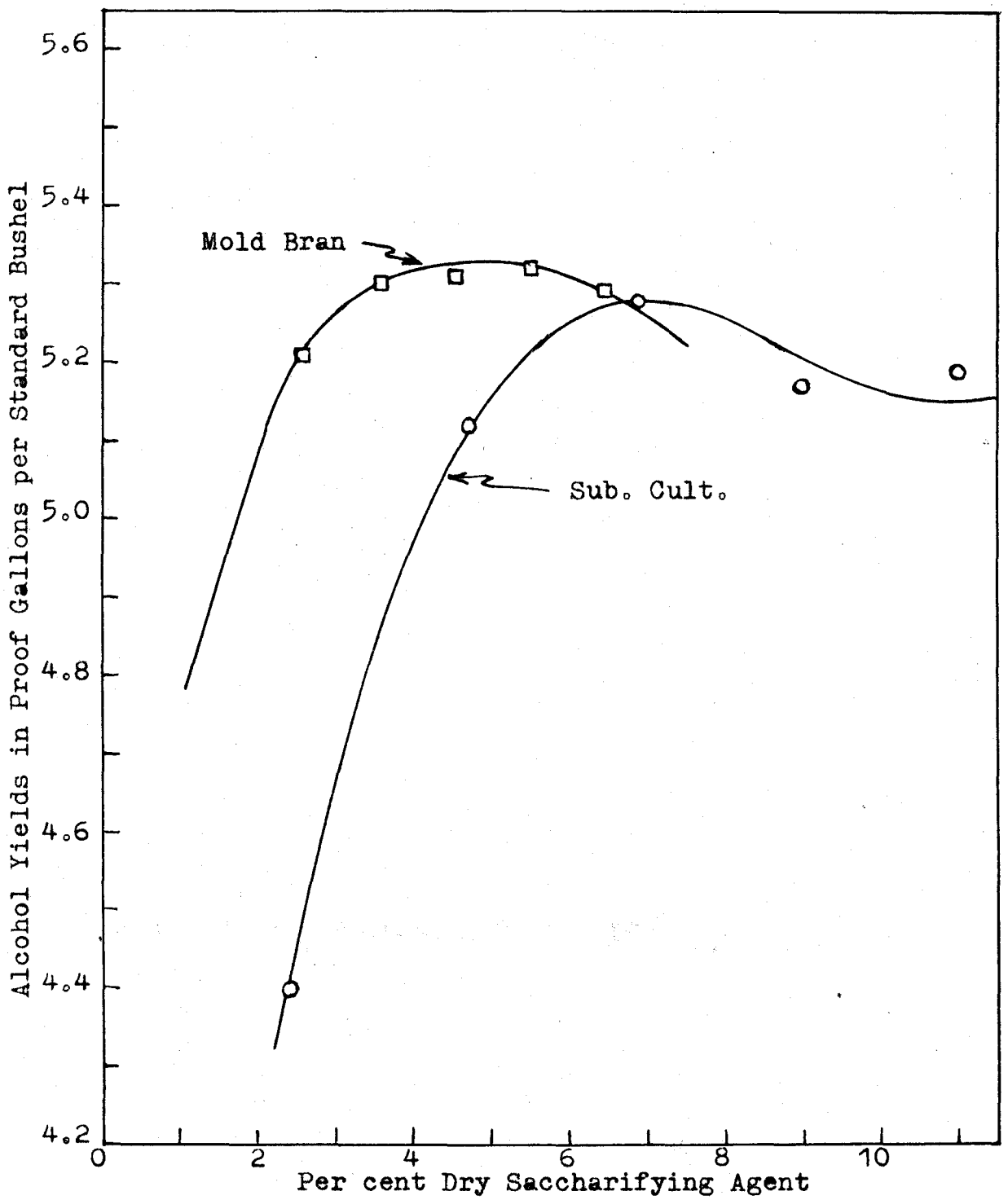

Figure2. Alcohol Yields from Mashes Saccharified by Aspergillus niger 337 Preparations

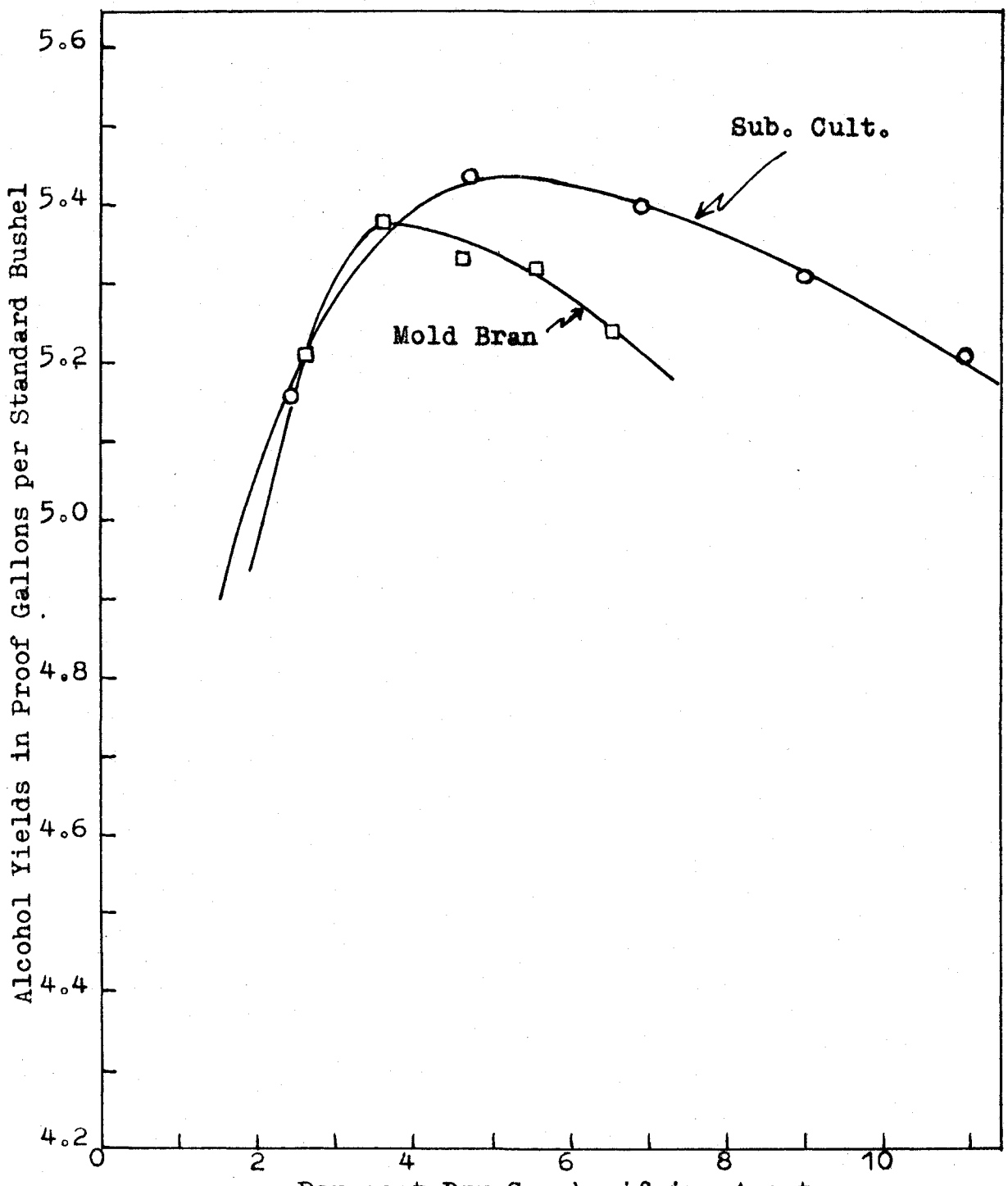

Figure 3. Alcohol Yields from Mashes Saccharified by Aspergillus niger 330 Preparations

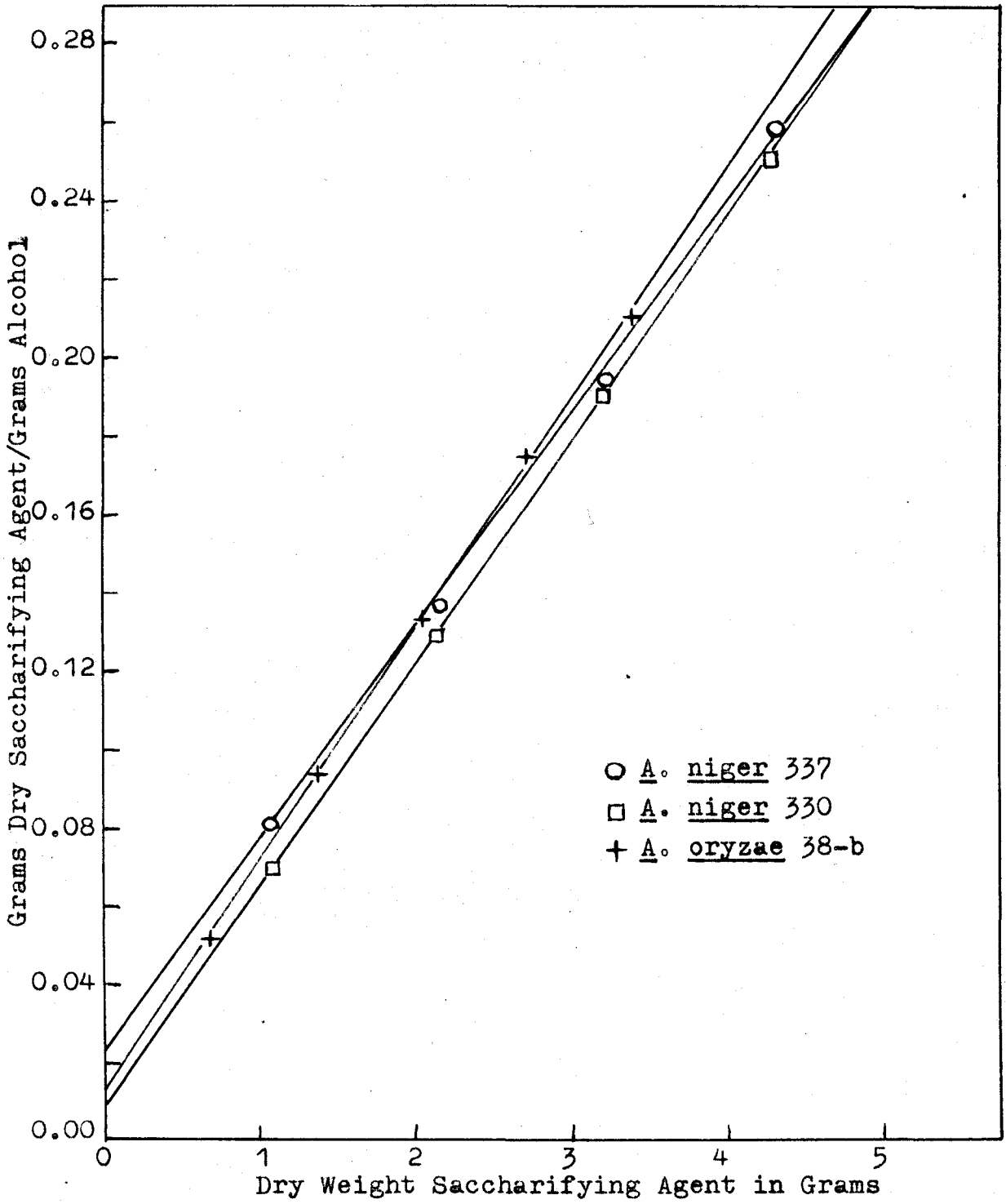

Figure 4. Curves for Corn Mashes Saccharified by Submerged Cultures

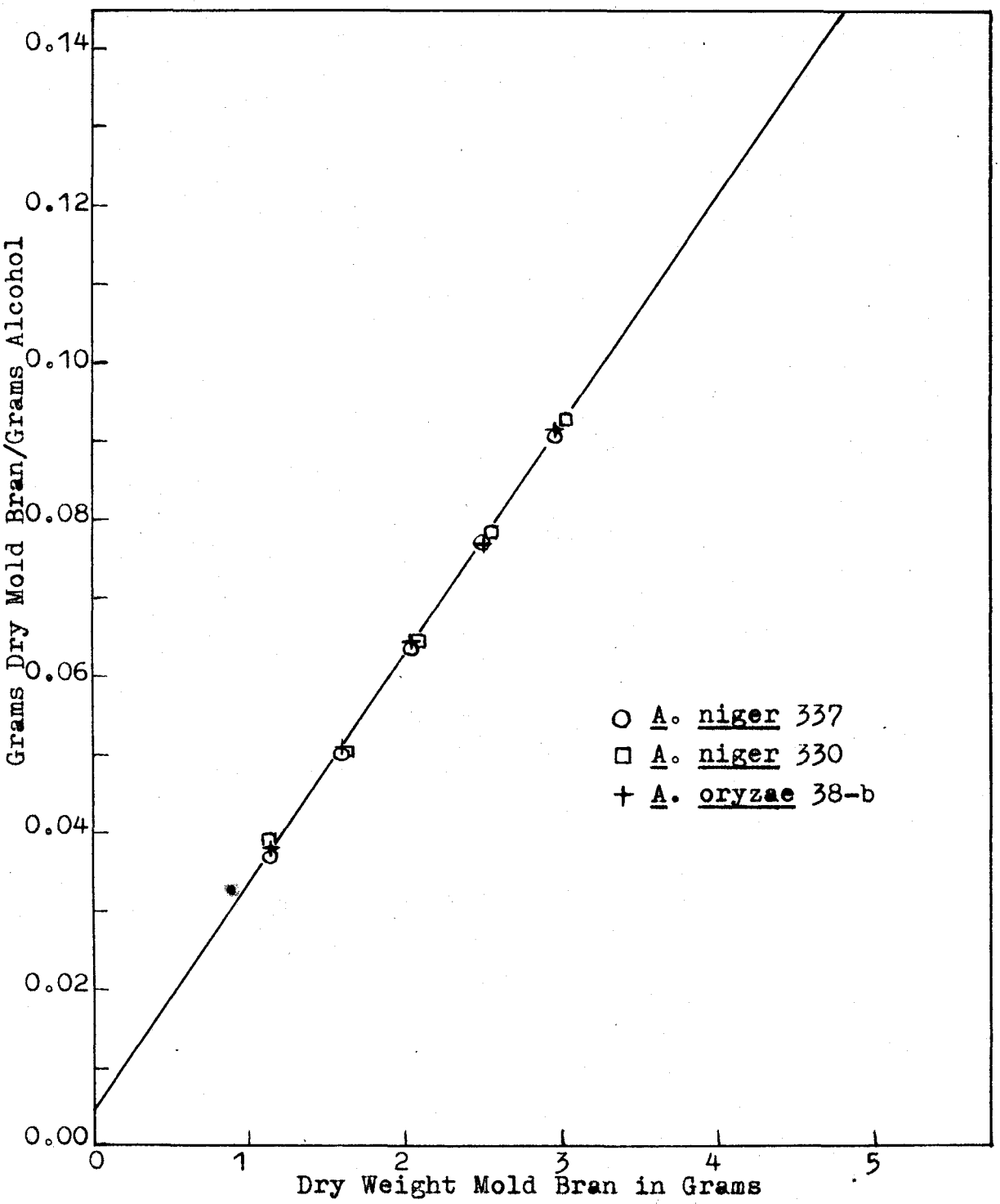

Figure 5. Curves for Corn Mashes Saccharified by Mold Brans

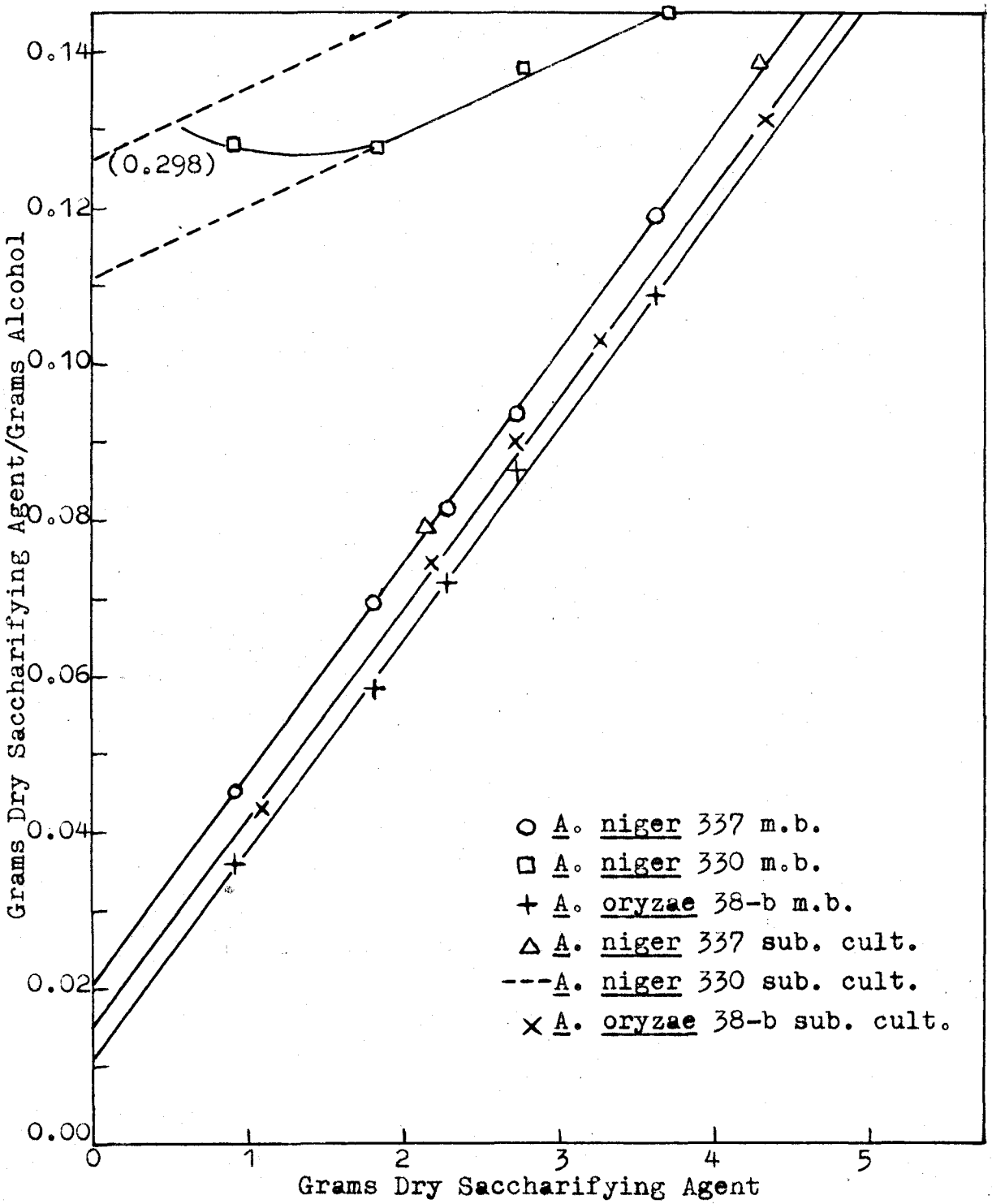

Figure 6. Curves for Short Starch Fermentation Test