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FUNGAL AMYLASES AS SACCHARIFYING AGENTS IN THE ETHANOL FERMENTATION OF STARCHY MATERIALS

by

Lu Cheng Hao

A Thesis Submitted to the Graduate Faculty

for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Biophysical Chemistry

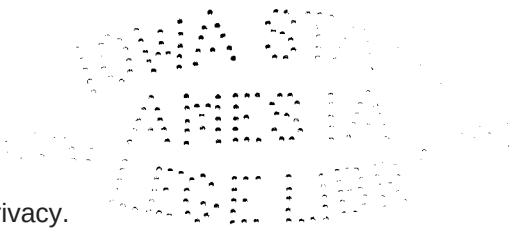

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TABLE OF CONTENTS

I. INTRODUCTION

Ethanol fermentation, or the alcoholic fermentation, is one of the oldest chemical processes related to human history, and is probably the oldest fermentation carried out by mankind. The early interest in alcoholic fermentation was limited to the use of the product as a beverage. Increased knowledge of the chemical, physical, and physiological properties of ethanol led to its present wide application in industry and medicine. Now it is one of the six most important substances in the world.

In the past decade, interest in the production of ethanol has been growing rapidly because of the increased attention being given to the use of ethanol as motor fuel. Most of the European and some of the Asiatic countries have used alcohol-gasoline blends in various quantities as fuel for motor-vehicles. Ethanol has become an indispensable liquid fuel in countries where the supply of gasoline is limited. Since the present war broke out increase in the production of ethanol has become extremely important because tremendous quantities of ethanol are needed for many defense industries particularly in the production of smokeless powder. This has led the government of the United States to take the action of ordering the distilleries in this country either to reduce or to stop the production of whiskey, and

to use their facilities to produce industrial alcohol. Government officials have stated that this country must increase the annual production of industrial alcohol to twice or thrice the quantity produced in 1941, which was about 125,000,000 gallons, in order to meet the expanding requirements of munitions manufacture.

The first item which must be considered in the problem of increasing ethanol production is, of course, the availability of raw material. About 85 per cent of all the ethanol manufactured in this country is produced by fermentation. The balance is produced by synthesis from ethylene derived from the cracking of petroleum. Since the amount of ethylene available is limited, any large expansion in ethanol production must be in the fermentation industry.

Nearly 90 per cent of the industrial alcohol made by fermentation has been produced from blackstrap molasses which is normally the cheapest raw material. Due to the tremendous increase in the demand for alcohol the molasses supply is inadequate. Moreover, the molasses shortage is rendered even more acute since most of the supply must be shipped to this country by tankers. Hence it has become necessary to use other raw materials on a large scale. Raw sugar and grains are the only immediately available raw materials for increased alcohol production. It is because of the allocation of a million tons of raw sugar for alcohol production that sugar rationing has become necessary.

Next to raw sugar, the most feasible raw materials for ethanol fermentation are corn and wheat. Corn or wheat, like all other starchy materials, can not be fermented directly by yeasts, because yeasts do not secrete amylase, which is the enzyme that hydrolyzes starch into fermentable sugars. Therefore, starch has first to be converted into sugars before it can be subjected to yeast fermentation. This process is called saccharification. Two types of catalytic agents can be used for saccharification, chemical and biological. The chemical agents commonly used are dilute mineral acids. The biological agent of saccharification is an enzymic complex called amylase. Amylase is present in small amounts in most cereals and in larger quantities in soybeans and sprouting grains. Most fungi produce amylase in varying amounts.

Barley malt has been the only saccharifying agent used in the alcoholic fermentation industries in the United States. It not only has a high content of amylase, but also gives a characteristic flavor to the fermented liquor. However, the production of malt is laborious and time consuming, and the cost is high. It has been estimated by Shepherd, McPherson, Brown and Hixon (1940) that the cost of malt alone adds over four cents a gallon to the price of alcohol produced from grain.

The use of mold amylases in alcoholic fermentation of grains has been practiced in the Orient for many centuries.

Chinese have always used "yeast cake" in making alcoholic beverages from grains. The so called "yeast cake" actually contains a group of molds and yeasts. After "yeast cake" is added to grain mash, saccharification and fermentation go on simultaneously.

"Koji" is the most popular mold enzyme preparation used in the Japanese fermentation industries. Koji is prepared by growing a strain of mold of the Aspergillus-flavus-oryzae group on any starchy material. Steamed rice is most commonly employed. The use of koji in the United States was introduced by Takamine, who manufactured several commercial amylase preparations which have been marketed under a variety of trade names.

Takamine also suggested the use of mold amylase to replace malt in alcoholic fermentation. Large-scale tests at the Canadian plant of Hiram Walker and Sons, Inc., proved entirely successful, yields of alcohol being better than with malt. However, a slight off-flavor or odor was produced in the alcohol, and since the flavor is of paramount importance in beverage alcohol, Takamine's preparation has not found favor in the alcohol industry. However, this objection would not apply to alcohol employed for industrial purposes. It would seem that the use of mold amylase should hold much promise for the production of industrial alcohol.

The Biophysical Division of the Department of Chemistry of Iowa State College has always been interested in the

fermentative utilization of agricultural products. "Saccharification of starchy mashes for the alcoholic fermentation industry" has been a continuous research project in its laboratories. Since previous studies of the use of mold amylases in the saccharification of grain mashes have only dealt with a few strains of molds, and since a simultaneous comparison of the activities of different mold amylase preparations has been lacking, a more comprehensive study was deemed to be of both theoretical and practical interest. It was the major purpose of this investigation to make a comparative study of the relative activities of the amylase preparations produced from a number of selected representative strains of molds. In the course of making a comparative study of these amylase preparations, it was necessary to develop a standard procedure both for the growth of the molds and for saccharification of the grain mashes. Therefore, as minor objects, this investigation also included the following studies: (1) optimum conditions for the growth of molds; (2) optimum conditions for the saccharification of grain mashes with mold-bran.

II. HISTORICAL

The use of mold amylases as saccharifying agents in the alcoholic fermentation of starchy materials is a relatively new science, but is an old art. It is probably one of the earliest chemical processes employed by mankind. Manufacturing alcoholic beverages from grain has been practiced for many centuries in the Orient.

According to the review of Fang and Sun (1940), as early as 1700 B.C., during the Hsia and the Shang dynasties of China, wine was a popular beverage and had also been used as a solvent for medicine. About a thousand years later, one emperor lost his empire on account of intoxication. The succeeding emperor issued the first Law of Prohibition. The use of various kinds of grains and the use of both grain sprouts and molds for making wine were recorded in the Chinese classics.

Through all of the succeeding centuries the use of malt and mold in fermentation has always been an art. The scientific study of malt and mold enzymes has had a history of only about one and a half centuries.

According to Pringsheim (1932), amylase was discovered in malt by Irvine in 1785, but no literature reference is given. The first recorded observation of the enzymic hydrolysis of starch was dated in 1814 when Nasse found that starch

extracted from living plants is capable of effecting its own conversion to sugar. In 1815, Kirchoff observed that a gluten-meal obtained from wheat was able to liquefy starch paste, fermentable sugar being formed. He made the concrete statement that a starch-sugars transformation is a necessary step in the alcoholic fermentation of amylaceous materials.

In 1830, Dubrunfaut made an extract of malt which converted starch into sugar, and in 1833 Payen and Persoz precipitated from malt extract, by means of alcohol, a substance capable of digesting starch. This substance which they called "diastase" could be dried and preserved. "Diastase" is a term which still persists in the literature. However, "amylase" appears preferable, since it avoids confusion that sometimes results from the fact that "diastase" is the French term for enzyme. Inasmuch as amylase is concerned with the hydrolysis of starch and is the active principle in malt, amylase has probably been studied more extensively than any other enzyme. The observations of Payen and Peroz, therefore, also marked the real beginning of enzyme study, and opened a new field in chemistry.

Continued studies of the action of malt extract on starch made by Dubrunfaut (1847), O'Sullivan (1872) and Schulze (1874) led to the discovery of the conversion of starch to maltose and laid the foundations for our knowledge of the enzymic degradation of starch.

Since that time, a very extensive literature concerning

the action of enzymes on starch has developed based upon research on the structure of starch and on enzyme action. Walton (1928) has compiled the titles, and in many cases an abstract, of twelve hundred articles dealing with the study of amylases and their action on starch. This compilation covers the period from 1811 to 1925. Research work in this field has undoubtedly further intensified since that time. The author of this thesis has no intention of making a comprehensive review of the literature devoted to the study of amylase or even of that related to the enzymic degradation of starch, but an attempt will be made to survey the knowledge relating to the application of amylase preparations in alcoholic fermentation.

The nature of the enzymic hydrolysis of starch was first postulated by Maercker (1878), who suggested the existence of two diastase ferments. This was not confirmed until Ohlsson (1922) succeeded in separating two individual enzymes from malt extract based on their different stability toward heat at different pH. These two fractions are a "dextrinogenase" (α-amylase) which hydrolyzes starch into dextrins, and a "saccharogenase" (β-amylase) which converts starch and dextrins to maltose. Blom, Bak and Braae (1937) also believed that α-amylase is responsible for the liquefying action, whereas the action of β-amylase is purely saccharogenic.

During the last decade, the study of amylases has

extended to their purification and fractionation, their activators, inhibitors and complements. Since this investigation is focused primarily on the relative activities of mold preparations as saccharifying agents in ethanol fermentation of starchy materials, the review will not be extended to the chemistry dealing with the saccharification of pure starch by relatively pure types of amylase, and those factors which influence enzymic reactions. However, that factor which Pringsheim (1923) called "complement" is of interest in alcoholic fermentation.

When maltose-free amylases are employed, the end product of the saccharification of starch is maltose. It has long been known, however, that this process does not yield the theoretical amount of maltose. The explanation has been that this is an equilibrium reaction, and the sugar already formed interfered with the further saccharification. Pringsheim and Schmalz (1923) have shown that at the point of limiting degradation there remains a "limiting dextrin" which has been proved to be identical with the starch-trihexosan. This amylolytic remainder is, however, saccharified after the amylases have been activated by their complement. Such an activator, of a protein origin, was found in fresh yeast. It was found to accelerate only that portion of the saccharification of starch lying beyond the limits of degradation. This investigation was confirmed by Kuhn (1925), Sjöberg (1925) Lüers and Wieninger (1925), and Hoop and von Laer (1925).

The latter authors reported that starch may be hydrolyzed to maltose by amylase to the extent of 96 per cent, providing sufficient coenzyme is present.

The amylases and their activators occur together in nature. It was shown by Pringsheim and Beiser (1924) that the complement could be secured from barley-malt extract by dialysis. The varying proportion of the activators present with natural amylases, explains why the limiting degradation does not always stop at exactly the same stage.

Regarding the methods of estimating the activity of an enzymic preparation, there does not appear to be any entirely satisfactory and rapid method for determining the activity of enzymes although there are several of great indicative value. Such measurements may be broadly classed under four main headings:

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

1. Viscosity methods. Chrzaszcz (1931) and with Janicki (1932) have examined the methods in use for determining the liquefying power of amylases, and give 15 methods, together with 18 proposed modifications of these methods.

2. Methods involving determination of reducing power.

The method adopted by the Institute of Brewing is that of Lane and Eynon (1923), using Fehling's solution. The amount of reducing sugar formed may be determined volumetrically or by gravimetric methods, preferably by the method of Sherman and his co-workers (1910, 1916). Lane and Eynon's (1923) method is one of the standard methods used by workers in England. Norris and Carter (1935) titrated the sugar solution against alkaline ferricyanide, and obtained results which are comparable with those obtained with Lane and Eynon's method. However, so far as fermentation is concerned, Harada's method seems to be more applicable.

Harada's (1931) method is a modification of that of Lintner (1886), being based on the principle of the production of a definite quantity of reducing sugar by the action of amylase upon a definite amount of Lintner's starch solution under specified conditions. The sugar is determined by the reduction of Fehling's solution. An objection is that many amylase preparations also contain maltase, the enzyme which converts maltose to dextrose. The final product then may be a mixture of the two sugars of different reducing powers. Thus, the reducing value obtained might be misleading.

3. Iodometric Methods. The degradation of starch may be followed by observation of the iodine reaction, a method first suggested by Wohlgemuth (1908) modified and advanced by Blom, Bak, and Braae (1937) and Hanes and Cattle (1938).

The latter workers studied the absorption spectra of starch-iodide in various stages of hydrolysis by means of a spectrophotometer.

Chzaszcz (1913) has repeatedly stressed that the activities of diastases should be considered separately according to whether they are liquefying, dextrinizing, or saccharifying in action.

4. Direct determinations. The choice of method naturally depends upon the circumstances and aim of the work. From the point of view of factory control it is preferable to have some direct method of getting the result wanted. Clibbens and Geake (1931) developed a method for determining the activity of a desizing enzyme on the cloth upon which it is subsequently to be used.

In all the work related to the saccharification of starchy mashes for the alcoholic fermentation done at Iowa State College, the activities of the mold amylase preparations have always been measured by running fermentation experiments. This direct method undoubtedly furnishes the most accurate and conclusive results.

The above review may provide a fundamental background for the understanding of the action of enzymic preparations applied to alcoholic fermentation. The remaining part of this historical treatment will be devoted to tracing the development of the use of mold amylase preparations in alcoholic fermentation. No attempt will be made here to

discuss either the history of the use of malt in fermentation or the history of yeast fermentation. For yeast fermentation, excellent reviews can be found in publications of Guilliermond and Tanner (1920), Harden (1932) and Prescott and Dunn (1940). For the use of malt in fermentation industry, books by Hopkins and Krause (1937) and Ross-Mackenzie (1934) provide theoretical and practical discussions.

Molds have been widely employed for the production of various dietary articles in almost all the Oriental countries. Nevertheless their utilization in Occidental countries is lacking. Scientifically, Aspergillus oryzae attracted the attention of Occidental investigators as far back as 1875. Kozai (1900) reviewed the literature regarding the early investigation of Aspergillus oryzae and its industrial applications, and gave credit to Hoffmann and Korshelt as the first writers upon this subject. Korshelt (1878) reported that in the preparation of "sake", a Japanese alcoholic beverage, an amylolytic enzyme developed during the culture of the fungus which he named Eurotium. In his "Chemistry for Sake Brewing", Atkinson (1881) discussed the function of the enzyme just named. The fungus was then known as Eurotium oryzae, having been first identified by Ahlburg in 1876 but Cohn's later investigation led to renaming it Aspergillus oryzae. A Japanese, Jokichi Takamine, was chiefly responsible for the introduction of the application of amylolytic enzymes of Aspergillus oryzae to the Occidental countries.

He advocated the use of the enzymes of Aspergillus oryzae in the distilling industry. He obtained a number of patents in England, the United States and several other countries for manufacturing enzymic preparations under the trade names: "moyashi" (1891, 1894), "koji" (1891, 1894), "taka-koji" (1894), "moto" (1894) and "taka-koji diastase" (1894).

"Moyashi" is prepared by mixing mold spores with steamed cereals. After the grain is well covered by green mold, the product is dried at a low temperature, and the grain sifted from the green powder, which consists of the mold spores. The green spores may be preserved by mixing with some hygroscopic inert substance, such as roasted starch or anhydrous calcium sulfate.

"Koji" is prepared from any starchy material by first steaming it, in order to thoroughly gelatinize the starch, allowing this to cool, and seeding with 1/50,000 part of pure "moyashi". The mass is placed in a chamber ranging from 20° to 40° C., and after 24 hours is spread out in a thin layer, and is allowed to remain thus until the koji ferment develops. This is indicated by the formation of a white mold over the mass. It is then to be used as a diastatic and alcoholic ferment. A comprehensive study of the nature of koji diastase has been reported by Ito (1932).

Instead of growing the fungus on rice or other grains, Takamine also employed bran of grains. He found that wheat bran is the best for this purpose, as it is of a loose and

coarse nature, thus affording a large surface for the growth of the fungus and a ready access of air, besides being rich in albuminoids and nitrogeneous matter, so forming a nutritious material. The fungus is sown and developed on the moist bran medium. To the whole mass, upon and through which the fungus has developed, which possesses both diastatic and fermenting properties, Takamine applied the name "taka-koji" to distinguish it from "koji" which is usually a culture on steamed rice.

"Moto" is a liquor containing ferment cells, prepared from a mixture of rice-koji, steamed rice and water. "Taka-koji diastase" is the water extract of "taka-koji".

Takamine (1918) tried the isolation of the diastase from the water extract of "taka-koji". From precipitation by alcohol, he obtained a yellowish-white amorphous powder, perfectly odorless, possessing a pleasant nutty taste. It is marketed under the name "taka-diastase".

Takamine (1896, 1906, 1910, 1911) also patented his improvements and modifications in the manufacture and application of these diastatic preparations in converting starch into sugar.

More recently Harada (1931) made a relatively comprehensive study of the cultivation of Aspergillus oryzae on cooked wheat bran for the preparation of diastatic enzymes, and of some of the properties of the latter. He pointed out that the essential factors in the production of the enzymes

by growth of Aspergillus oryzae may be specified as follows: (1) quality of bran; (2) water content of bran; (3) hydrogen ion concentration; (4) time of incubation; (5) temperature; (6) humidity of chamber; and (7) sterilization. He also demonstrated the presence of certain other enzymes associated with the diastatic enzyme preparation.

In studying technical improvements of the process for producing "taka-koji", Takamine (1913) used a pneumatic malting drum within which the mass is tumbled by rotation while being subjected to a current of moistened air. The mycelial growth was better than any method used before. The filements were shorter and thicker. Takamine (1914) stated that the "taka-koji" made in this way forms an efficient substitute for malt in alcoholic fermentation. It possesses a great advantage over malt diastase as a medicinal agent by retaining its activity almost unchanged for several years.

The process was further improved (1913, 1915, 1918, 1921, 1923) by using fungus acclimatized to an antiseptic such as formaldehyde, thus eliminating contamination; by using salt water instead of pure water in mixing the bran mash; and by keeping the bran medium neutral or acidic.

Oshima and Church (1923) studied in detail the enzymes produced by strains of molds of the Aspergillus-flavus-oryzae group, which were isolated from tane-koji used in shoyu manufacture and related industries. These organisms produced individually amylases and proteases of widely different

strengths. Culture experiments on various food substances showed that strongest amylase and protease were produced with bran. The ratio of amylase and protease did not seem to change much with the culture medium. Enzymes were present in the mycelium of the fungus in greatest amount at the period of sporulation of the fungus. Practically all the intracellular enzymes passed out into the culture medium soon after sporulation. Oshima (1928) made an extensive study of the quantitative estimation and the properties of the protease and amylase of Aspergillus oryzae. He concluded that the extra-cellular and intra-cellular enzymes of Aspergillus oryzae are fundamentally the same. After spore formation, almost all intra-cellular enzyme was excreted into the medium. The optimum pH for amylo-saccharification was found to be pH 4.8 to 5.2 and that for amylo-liquefaction about the same. The amylases were found most stable to heat at pH 6.4. They became completely inactivated on heating for one hour at 85°C.

Concerning the application of taka-koji in distilleries, Ortved (1912) carried out large-scale tests at the plant of Hiram Walker and Sons, in Canada, and reached the conclusion:

> "Therefore, I should say as a final conclusion that in distilleries which make commercial or potable neutral spirit, the Taka-Koji process could be introduced to advantage. Besides from a probable higher yield in spirit, the saving in malt cost would be worth while in years with normal malt prices and very considerable in years when the malt prices become abnormal."

Paralleling the study of the molds used in Japanese

fermentation industries, the use of "Chinese Yeast" has also attracted the attention of Western scientists. In China, the saccharifying material is prepared by starting with dough made from rice flour. This is made into cakes and mixed with aromatic herbs, the whole covered over with rice husks and allowed to undergo spontaneous fermentation. A white mold grows on the cakes, which are then dried in the sun. The product is known as Chinese yeast cake. In 1892, Calmette made the first scientific study of the microflora of this product. As a result of his extensive investigation, he found that a certain species of Mucor always predominated in this conglomeration of microorganisms. He termed this species of mold Amylomyces Rouxii. It possessed the property of saccharifying starch and of slowly converting sugar into alcohol.

However, a few years earlier, Gayon and Dubourg (1887) had investigated the molds Eurotium oryzae, Mucor circinelloides, Mucor racemosus, and Mucor alternans. Their observations showed that Eurotium oryzae had the highest saccharifying power; Mucor alternans had considerable saccharifying activity but somewhat less than Eurotium oryzae. The other molds had some saccharifying ability but were quite inferior to Eurotium oryzae and Mucor alternans.

At Seclin distillery near Lille, Collette and Boidin (1897) found that Amylomyces Rouxii, when grown in one hectolitre of the residuary liquors of yeast factories,

produced 1 litre of alcohol. Other similar residues could be utilized for the production of alcohol in this way, and the above mentioned mold could be replaced by the Chlamydo-mucor of Java or the Aspergillus oryzae of Japan.

For fermenting starchy material by molds, the same authors (1897) described the process as follows: the starchy raw material was well boiled, and the starch liquefied by a little malt, acid, or mold; it was then well boiled and cooled and a pure culture of Amylomyces Rouxii, Aspergillus oryzae, or other mold was added. Saccharification and fermentation of the starch occurred, the latter being hastened by the addition of a little yeast. It was stated that process gave much better yields of alcohol than the one usually practiced. Boidin and Rolants (1897) also reported that in the saccharification of rice by Amylomyces Rouxii, the activity of the diastase was greatest in a faintly acid medium.

Sanguinetti (1897) made comparative experiments with Aspergillus oryzae, Gayon's Mucor alternans, and Amylomyces Rouxii, he found that the saccharifying power was greatest in Aspergillus oryzae, the Amylomyces Rouxii taking second place. However, Amylomyces Rouxii had greater fermentative power, rendering it alone, out of the three, suitable for industrial employment, whether for the direct fermentation of starchy material or for utilizing distillery residues.

Jean (1898) compared the relative merits of Takamine's

process for saccharification and fermentation by means of Aspergillus oryzae and Collette and Boidin's process for the use of Amylomyces Rouxii of Calmette; he remarked that the former organism had a much greater saccharifying power, and could be used with stronger mashes than the latter.

Boidin (1899) remarked, however, that the fungi were deficient in starch liquefying power; the grains in the distillery were, therefore, treated with 1 to 2 per cent of malt before sterilization in order to liquefy the gelatinized starch.

The use of molds in fermentation has attracted so much attention that by the end of the nineteenth century, molds were utilized extensively in southern Europe where fermentations of amylaceous materials were carried out. At first, Amylomyces Rouxii was used exclusively, but subsequently Mucor β, Mucor γ, and Rhizopus delemar were employed, all of which were isolated either from Chinese yeast cake or from Japanese koji. A higher saccharifying efficiency was claimed for the Mucor β, by Colette and Boidin (1900).

From the study of the growth and morphology of the so called Amylomyces Rouxii, Wehmer (1900) decided that it was a true Mucor. He also isolated Mucor circinelloides, Mucor japonicus, and Rhizopus from the Chinese yeast cake. Wehmer's opinion was shared by Turquet (1902), who studied the ways of reproduction of Amylomyces Rouxii, and concluded that it belonged to the genus Mucor and should henceforth

be termed Mucor rouxii, its nearest relations being Mucor racemosus and Mucor circinelloides.

From the wheat-flour cake used in the preparation of the Chinese beverage "Shao-hing-Chew", Saito (1904) found beside the ordinary mold fungi, two new kinds of Rhizopus, named Rhizopus chinensis and Rhizopus tritici. They saccharified starch with the production of alcohol and ester, and also liquefied gelatin.

Will (1913) isolated a new yeast "Saccharomyces anamensis" from a mixture of wild yeast found on the sugar cane in Cochin, China. He applied it to the fermentation of saccharified mashes, and this organism was found to be particularly useful because it fermented actively at the same temperature at which the mold developed in the Amylo process (35°-38°C.), and could therefore be employed advantageously in mashes of the Amylo process.

Grove (1914) made a comprehensive review of the Amylo process of fermentation. Some of its important points can be summarized as follows: (1) The amylo-process, which is used in many of the largest distilleries of France, Italy, and Spain, is characterized by the use of mold fungi instead of malt for the saccharification of starch and by the rigorous application of pure culture methods on a large scale. The organisms employed, originally isolated from "Chinese yeast" belong to the groups Mucor and Rhizopus. The most satisfactory one, Rhizopus delemar, converts starch into dextrose

and also slowly ferments the latter, but in practice a special yeast is added to effect fermentation. (2) The principal raw materials of the amylo-process are rice, maize, manioc, dari, millet, and potatoes. (3) The starch has to be liquefied as completely as possible. It is done by hydrochloric acid or malt, according to its origin. In the case of maize, it is coarsely ground and agitated for one hour with two parts of water containing hydrochloric acid (usually 6-8 litres of concentrated acid per 1,000 kilos of maize), and then introduced into the first of the two cookers, in which the pressure is raised to 60 lbs. per sq.in. in 15-20 minutes and maintained for 15-30 minutes. Whilst a small quantity of steam is allowed to blow off in order to keep the mash in agitation. (4) The yield of alcohol obtained by amylo-process is considerably higher than by other methods, for no dextrins are left unfermented and the fermentation is pure.

Boulard (1918) used Mucor Boulard No. 5 for saccharification in the alcoholic fermentation of grain. The yeast was introduced a few hours after the mold was added in contrast to the relatively long interval of 18 to 24 hours generally employed at that time. Boulard claimed that both saccharification and fermentation were completed within 48 hours.

In 1918, Baud reported that the use of Mucor for the saccharification of carbohydrates had extended considerably,

having been adopted by more than 24 plants; the quantity of alcohol produced by this means was 10,000 hectoliters (2,640,000 gallons) in 1914 and 182,962 hectoliters (48,312,000 gallons) in 1917. Mucor may also be used in the preliminary saccharification in the manufacture of vinegar.

Delemar (1919) reviewed the development of diastase-secreting fungi, particularly Amylomyces Rouxii, in the preparation of alcohol from cereals. He stated that in the period 1910-1919, progress had been in the direction of accelerating the speed of the saccharifying process by using the much more rapidly germinating conidia of the Mucor instead of the spores hitherto employed, so that the yeast could be added at the same time as the Mucor culture. The yield obtained by this process amounted to 97.5 per cent of the theory, 100 kilos of maize giving 36-42 liters and 100 kilos of rice 42-47 liters of alcohol.

Galle (1925) reviewed in detail the production of alcohol in a maize distillery where 200 hectoliters (52,800 gallons) of alcohol was produced daily by Amylo process from maize and other grains, and malt diastase was completely replaced by the Amylomyces and acid. The yields obtained were 39-40 liters of alcohol per 100 kg. of maize as against 34 liters by the malting process. In addition to maize, the following substitutes were successfully treated during the war by the Amylo process: damaged maize, seeds of Sorghum vulgare, Setaria Beauvois, and Vicia sativa.

Almost theoretical yields of alcohol were obtained.

Since the method of calculating the yield of alcohol from fermentation was not given in the reports of the above authors, it is difficult to compare their yields with those of the present investigation. At any rate, their yields were very good as compared with the yields from malt used at the same time.

Concerning the advantages of the Amylo process over the malt process, Horn (1917) gave the Amylo process credit in increasing the yield of alcohol; eliminating the cost of malt; and saving steam, water, and labor on account of the fact that higher concentrations of mashes could be employed. In addition, Foth (1929) reported that the residue from the Amylo process contained more fat than the malt process yielded.

Owen (1933) summarized the most important advantages in the processes using species of Mucors for the saccharification of starch which are as follows: (1) economy due to saving in malt; (2) decreases in losses due to infection introduced with the malt; (3) increased yields of alcohol; and (4) higher purity of alcohol formed in the mash.

Prompted by Owen's article, Neubauer (1933) stated that the Amylo process suffers several disadvantages. The higher yield of alcohol was offset by the higher consumption of power and special installations. Besides, an expert personnel and a complete bacteriological laboratory are

required.

Following the lead of Owen, Boidin (1933) published the improvements in the development of the Amylo process. He reported that Mucor Delemar was of high saccharifying power and gave the best results in industrial application. The duration of the fermentation was comparatively long when the Amylo process was first used. He reported that the duration had been cut down to 2.5 to 3 days by the use of a very active yeast which ferments at high temperature and which, living in symbiosis with the molds, enters into action at the very moment when saccharification has reached the desired point.

In addition to Aspergillus oryzae and the Mucor and Rhizopus species listed above, a number of other fungi have been studied from the point of view of their amylolytic enzymes. Among them may be mentioned: Aspergillus batatae, Aspergillus pseudoflavus and Rhizopus japonicus studied by Saito (1907); Aspergillus albus, Aspergillus candidus, and Aspergillus Okazakii by Okazaki (1914); Aspergillus terricola by Scales (1914); and Aspergillus niger by Funke (1922). All of these fungi produced more or less amylase, with a few of them producing enough to warrant commercial exploitation.

Muta and co-workers (1931, 1933, 1936) compared the saccharifying power of Rhizopus pêka I, Rhizopus pêka II, four strains of Aspergillus Awamori, six strains of

Aspergillus oryzae and eight strains of unidentified molds with one strain of Rhizopus delemar. They found that Rhizopus delemar, Rhizopus peka I, and two unidentified species were found to be best for alcoholic fermentation by the Amylo process. These organisms gave 87 to 91 per cent yields on a semi-industrial scale. 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.

Hemmi and Tsukitart (1933) studied the alcoholic fermentation by Rhizopus delemar, Rhizopus tritici, Rhizopus tonkinensis, Rhizopus oryzae, Rhizopus japonicus, and Rhizopus acidus. When they were cultivated in koji water, the first three varieties produced more alcohol. The addition of secondary potassium phosphate increases the production of alcohol.

Wei and Chin (1934) examined the diastatic activity by both Bertrand's and Lindner's methods of ten species of Aspergillus including six strains of Aspergillus oryzae, one strain each of Aspergillus niger, Aspergillus Luchuensis, and Aspergillus glaucus, and an unidentified Aspergillus species. They found that Aspergillus oryzae (AOLD) had the greatest diastatic power.

Takeda (1935) made a comparison of the amylolytic power of 27 stocks of Rhizopus isolated from rogi and soybean-koji produced in Java and Sumatra. Strains of

Rhizopus semarangensis and Rhizopus javanicus were said to have strong amylolytic power, and the latter was especially valuable for the Amylo process for alcoholic fermentation. Satisfactory results were obtained on both the laboratory and the industrial scales.

The above review has dealt with two processes in the use of fungal amylases as saccharifying agents in the ethanol fermentation of starchy materials, namely, Takamine's "taka-koji" process and the Amylo process. The difference between these two processes is that in the "taka-koji" process the mold is grown on wheat bran, and the product is in turn used for the saccharification of grain mash, while in the Amylo process the mold is grown directly on the grain mash.

Although both processes were found to be superior to malt processes, the "taka-koji" process possesses advantages over the Amylo process in that the former takes a shorter time in its operation and requires no special installations, which are needed in the Amylo process. But, strangely enough, the Amylo process has been widely applied industrially for more than thirty years while the "taka-koji" process of using mold-bran in the alcoholic fermentation has been totally forgotten.

Recently, Underkofler, Fulmer and Schoene (1939) revived the idea of using mold amylase preparations to replace malt and undertook a detailed study of the use of

mold-bran in the saccharification of starchy grain mashes for the alcoholic fermentation. By using a rotating drum technic, they developed a procedure for the production of active amylolytic preparations from the growth of molds on wheat bran. Two strains of the mold Aspergillus oryzae were found very satisfactory for producing amylase for use in saccharifying corn mashes. With mold-bran produced from either of the two strains of mold alcohol yields were, on the average, about 12 per cent higher than with malt. Alcohol yields of at least 90 per cent of the theory were obtained by the use of mold-bran for saccharification in fermentations of 85 gallons of corn mash. The amount of mold-bran required for maximum alcohol production was 8 to 10 per cent of the weight of corn.

Schoene (1939) made a preliminary investigation of amylase production by 17 species or strains of molds and by 7 species or strains of bacteria grown on wheat bran in flasks. He concluded that none of the bacteria produced useful quantities of amylase, but all of the molds, which included 2 strains of the Aspergillus flavus-oryzae group, 2 strains of Mucor rouxii, 2 strains of Rhizopus delemar, 4 strains of Rhizopus oryzae, one strain of each of Rhizopus pêka I, Rhizopus tritici, Mucor circinelloides, Mucor javanicus and Diplodia zeae, and 2 strains of unidentified yellow mold, yielded considerable amounts of amylase. From fermentation results, the mold-bran prepared by using

Aspergillus oryzae was proved to be definitely superior to that from any of the other molds tested.

Schoene, Fulmer, and Underkofler (1940) compared malt, mold-bran, and soybean meal as saccharifying agents. Of these three, mold-bran was the most effective and soybean least. Two types of mashes, normal and thick, were also studied in conjunction with combinations of the three amylolytic materials. No advantage was found using combinations over mold-bran alone. With acid-saccharified mashes, the addition of mold-bran gave considerable increase in alcohol yield. This result was ascribed to enzymic action, causing the conversion of the non-fermentable polysaccharides remaining in the acid-saccharified mashes to fermentable sugar. The greater effectiveness of mold-bran over malt was associated with the greater variety of enzymes present in the former.

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

Buckaloo (1940), and Underkofler, Goering and Buckaloo (1941) continued the investigation of various mold amylases in the saccharification of corn mash for ethanol fermentation. Four strains of Aspergillus oryzae, one strain each of Rhizopus oryzae, Rhizopus tritici and Mucor javanicus, and two strains of unidentified molds were grown on moistened wheat bran in the rotating drum apparatus. The two strains of Aspergillus oryzae, Rhizopus oryzae, and Rhizopus tritici were found to produce amylase preparations which gave high saccharification values in the alcoholic fermentation of corn mash. The alcohol yields obtained were approximately 10 per cent greater than those secured from malt. Attempts were made to grow Aspergillus oryzae on various fibrous materials including wheat bran, corn bran, oat hulls, cotton-seed hulls, corn cobs, sawdust, peanut hulls, and rice hulls. Wheat bran and dry-milled corn bran were the only substrates of those tested which adequately supported the growth of this strain of mold.

Goering (1941) investigated the use of mineral acids and of mold-bran as saccharifying agents for the production of fermentable sugars from starch. He found that when mold-bran was added to corn mashes partially saccharified by dilute hydrochloric acid very high ethanol yields were obtained. The addition of 4 per cent mold-bran to these mashes produced an ethanol yield of 91.5 per cent of theoretical. Mold bran produced higher yields of ethanol from corn starch than

it did from corn meal. At the optimum concentration of 15 per cent mold-bran, an ethanol yield of 92.2 per cent of theoretical was obtained from 16 per cent starch mashes. The use of 6 per cent mold-bran produced an ethanol yield of 86.7 per cent of theoretical. He accounted for the fact that lower ethanol yields were obtained from corn meal than from corn starch, when both were subjected to acid hydrolysis under the same conditions, as due to the presence of corn bran. Apparently the hydrolysis of the corn bran produced something toxic to yeast.

III. MATERIALS

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

Corn meal

The corn meal used in this investigation was obtained in several lots at different times from the storage bins in the rat laboratories of the Physiological Division of the Chemistry Department. The average moisture content of the corn meal was about 11 to 14 per cent, and average starch content 56 to 60 per cent.

Wheat bran

The wheat bran used was purchased from a local grain elevator. The analytical data given to it by the grain company are as follows: Protein not less than 15 per cent, fat not less than 3 per cent, crude fiber not more than 11 per cent, nitrogen free extract 39 per cent, and total carbohydrate 50 per cent.

Barley malt

The barley malt, obtained from Hiram Walker Company, Peoria, Illinois, was ground to a coarse powder in a Wiley mill.

Malt extract

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

Wheat and oats

The wheat and oats used were obtained from the rat laboratories of the Physiological Division of the Chemistry Department. The wheat had a moisture content of 12 per cent and a starch content of 53.2 per cent. The moisture content for oats was 10 per cent, and the starch content 52.4 per cent.

Barley and sorghums

The Spartan barley, Leoti red sorghum, and pink Kafir sorghum used were obtained from Dr. Leo M. Christensen at the University of Nebraska. The moisture and starch contents were as follows:

	Starch	Moisture
Barley	50.2	7.2
Leoti red sorghum	60.1	7.6
Kafir pink sorghum	56.6	7.0

Rice

The rice used was bought from a local grocery. It had a moisture content of 9.3 per cent, and a starch content of 64.0 per cent.

IV METHODS

A. Microbiological Procedures

Yeast culture

A weighed amount of malt extract was dissolved in ten times its weight of boiling water, and the precipitate was allowed to settle. After cooling, the supernatant liquid was placed in Erlenmyer flasks and designated as beer wort medium. For carrying the cultures, 60 ml. of the wort was used in each 125-ml. flask, and for cultures employed for inoculating experimental mashes 300 ml. in each 500-ml. flask. The flasks were plugged with cotton and sterilized for 30 minutes under a steam pressure of 15 pounds.

A stock culture of Saccharomyces cerevisiae, designated in this laboratory as yeast number 43, was carried in beer wort in 125-ml. flasks incubated at 30°C. Transfers were made daily in order to maintain a vigorous yeast culture; by means of a sterile pipette 1 to 3 ml. of the yeast culture were transferred to another flask each time.

The inoculum for experimental mashes was prepared by inoculating 300 ml. of beer wort with 5 to 8 ml. of an active yeast culture and incubating at 30° C. for 20 to 24 hours.

Preparation of mold-bran

Amylase production by 27 strains of molds has been

studied in this investigation. These molds, together with their laboratory numbers and their sources, are listed in Table 1 under Experimental Results. The stock cultures of the molds were kept on wort-agar slants, and the molds were grown in flasks on wheat bran mash to provide inoculum for larger batches.

In the course of making a comparative study of the relative activities of the amylases produced by different molds, it was necessary to develop a standard procedure for both the growth of the molds and for saccharification. From the results of preliminary studies, the following method was found to be optimum for the growth of molds on wheat bran, and was adopted as the standard method for the preparation of mold-bran throughout this investigation.

For cultivating molds in flasks, well-sporulated stock cultures of molds were transferred to wheat bran mashes. The latter were prepared by mixing 25 g. of wheat bran and 25 ml. 0.3 N hydrochloric acid in 500-ml. Erlenmyer flasks and sterilizing for 30 min. at 15 lbs. steam pressure. The flasks were placed in the incubator lying on their sides and incubated at 30° C., and were shaken twice or thrice every day to diminish lumping and matting. After abundant spores had formed on the bran mashes, the flasks were removed from the incubator and the spore cultures used as inoculum for larger batches of bran mash. It has been found that these well sporulated mold cultures on wheat bran can be kept at room

temperature for months without deterioration in their usefulness as inoculum.

In the preparation of large batches of mold-bran, special 3-qt. aluminum pots equipped for aeration were used throughout this investigation instead of the rotating drum previously used in this laboratory. This apparatus is a slight modification of that developed by Leo M. Christensen (1940) and is shown in Figure 1.

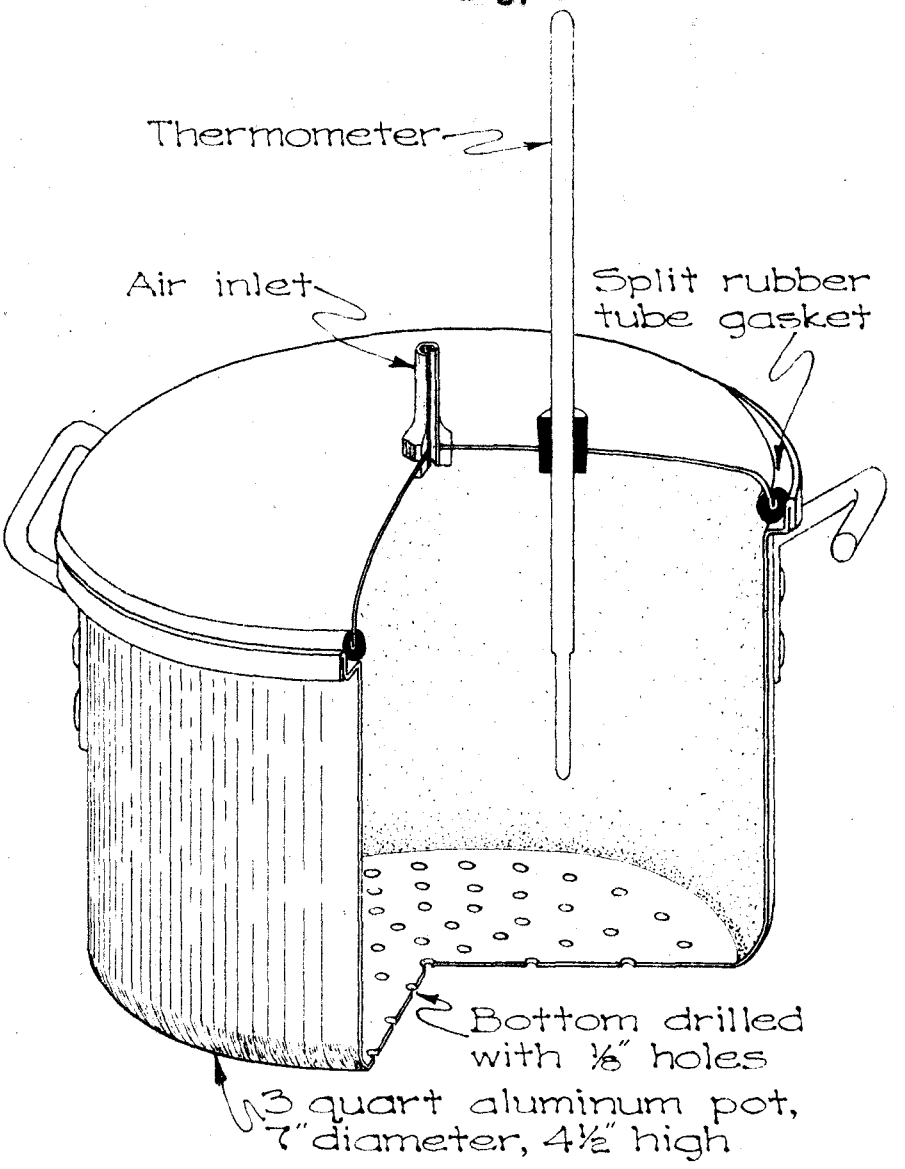

Fig. 1. Special Aluminum Pot Aerator Used in Growing Mold-bran

The pot has several advantages over the drum. It takes less space, and requires no special mechanical devices. There is no disturbance during the growth of the molds and more uniform aeration is obtained. The growth of the molds is more rapid, and, above all, the pot preparations give more consistent and higher alcohol yields.

The method of culturing molds in these pots is as follows: The bran mash used as a medium is prepared by moistening 750 g. of wheat bran with an equal weight of 0.3 N hydrochloric acid. The wet bran is packed into the pot and sterilized in the autoclave at 15 lbs. for 30 minutes. The cooled bran mash is mixed with 10 g. of well-sporulated mold culture grown in flasks on wheat bran mash, and the inoculated material is packed firmly into the pot. The culture is incubated at 30° C. until the temperature rises to 37-40° C., thus indicating rapid growth of the mold. The growing mold mass is then aerated by passing air through the pot at a pressure of one-half inch of water. After aeration for 16 to 24 hours the bran is well covered with mold mycelium and the material, which is designated as mold-bran, is removed, spread out on paper and dried at room temperature. The dried material is ground in a Wiley mill, and is used as such to saccharify grain mashes for fermentation tests.

B. Saccharification and Fermentation

According to the results obtained from preliminary tests, the following procedures were found to represent the optimum conditions for saccharification and fermentation and were adopted as the standard methods used for comparative studies of the activities of the different mold-bran preparations throughout this investigation.

Twenty per cent grain mash was used exclusively as the substrate for fermentation. It was prepared by mixing 60 g. of grain of known starch content with 300 ml. of 0.04 N hydrochloric acid in a 500-ml. Erlenmeyer flask, gelatinizing by heating on a hot plate with occasional shaking, and sterilized at 20 lbs. for 30 minutes. After cooling, the grain mash was neutralized with concentrated sodium hydroxide solution and adjusted to a pH of 4.5 to 5.0. The desired amount of mold-bran and the grain mash were then mixed in a Whiz-mixer for one minute. After the mixed mash had been incubated at 30° C. for one hour, it was inoculated with 20 ml. of a 20 to 24 hour old yeast culture in 10 per cent malt extract solution. The mash was then incubated at 30° C. The fermentation was generally completed in 72 hours.

C. Analytical Procedures

Determination of starch and of sugar

Starch was determined by the "Diastase Method with Subsequent Acid Hydrolysis" in accordance with the Official and Tentative Methods of Analysis of the Association of Official Agriculture Chemists (1940). The reducing substances formed in the hydrolysis were estimated according to the modified Shaffer and Somogyi (1933) method developed by Guymon (1939). The reagents were standardized by means of a sample of pure glucose. All determinations were carried out in duplicate or triplicate.

Determination of ethanol

The total volume of the fermented mash was distilled in a 500-ml. Kjeldahl flask after 0.5 g. of sodium carbonate had been added to neutralize the acids present. The first 100 ml. of the distillate were collected in a 100 ml. volumetric flask.

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

Determination of corrections for inoculum, mold-bran and malt

The yeast inoculum for the alcoholic fermentation was grown in a solution of malt extract. Since this inoculum contributes a certain amount of alcohol, this quantity must be deducted from the total amount of alcohol obtained from the fermented grain mash. This was the correction for inoculum. Banzon (1940) found that in the presence of mold-bran or malt, a larger quantity of ethanol was obtained from malt extract medium than in their absence. Hence, a differential method was used to evaluate the corrections for the beer wort and the mold-bran. For beer wort the calculation was as follows:

4 g. mold-bran + 200 ml. beer wort = 6.78 g. ethanol

4 g. mold-bran + 100 ml. beer wort = 3.36 g. ethanol

100 ml. beer wort = 3.42 g. ethanol

Different mold-bran preparations gave slightly different results in the determination of correction for beer wort. The amount of ethanol produced from 100 ml. of beer wort ranged from 3.25 g. to 3.5 g. Accordingly, the amount of ethanol produced from 20 ml. of beer wort, which was the amount used as inoculum, varied from 0.65 to 0.7 g. An average value of 0.68 g. was used throughout this investigation. A difference of 0.03 or even 0.05 g. of ethanol is negligible in comparison with the amount of ethanol produced from 60 g. of grain which is usually 18 to 20 g.

For the correction for mold-bran, the calculation was as follows:

8 g. mold-bran + 200 ml. beer wort	= 6.75 g. ethanol
4 g. mold-bran + 200 ml. beer wort	= 6.57 g. ethanol
4 g. mold-bran	0.18 g. ethanol

Again, different mold-bran preparations gave slightly different results in the determination of the correction for mold-bran. The differences were negligible, and 0.045 g. ethanol was taken as the average value for 1.0 g. of mold-bran used. The value for malt determined by Banzon (1940) using similar method was 0.334 g. ethanol per 1.0 g. malt.

Calculation of ethanol yield

In a typical experiment the fermentation mash contained the following: 60 g. of corn with a starch content of 58.1 per cent; 3.6 g. of mold-bran; and 20 ml. beer wort as inoculum. The entire fermented mash (365 ml.) was distilled and the first 100 ml. of distillate collected. The specific gravity (25°/25°) of the distillate was 0.9695 corresponding to 19.19 g. ethanol, which was the total ethanol obtained from the corn, mold-bran and beer wort. The ethanol corrections are as follows:

for the 20 ml. inoculum	= 0.68 g. ethanol
for the mold-bran: 3.6 x 0.045	= 0.16 g. ethanol
Total	= 0.84 g. ethanol

Therefore, the quantity of ethanol from the corn alone is 19.19 minus 0.84 or 18.35 g. From the equation

$$C_6H_{10}O_5 + H_2O \longrightarrow 2\ C_2H_5OH + 2\ CO_2,$$

162 g. of starch yield 92 g. of ethanol. In 60 g. corn, there are 60 x 0.581 = 34.86 g. starch, which should theoretically give:

$$34.86 \times 92/162 = 19.85 \text{ g. ethanol.}$$

The ethanol yield of the above fermentation is therefore 100 x 18.35/19.85 = 92.5 per cent.

V. EXPERIMENTAL RESULTS

A. Optimum Conditions for the Growth of Molds on Wheat Bran

1. Ratio of bran and acid

There are two reasons for using acid in preparing the bran mash for the growth of the molds. First, the acid may release some of the nutrients in the bran which are essential for the optimum growth of molds. Second, the acid will bring the wetted bran to a desirable pH, namely between 4.0 and 5.0 at which the molds grow normally while the contamination by other microorganisms may be largely prevented. Among the different acids, hydrochloric acid and sulfuric acid are equally good although hydrochloric acid was used exclusively throughout this investigation.

The alcohol yields obtained from corn mash saccharified with mold amylase preparations made by growing mold on bran with different amounts and strengths of hydrochloric acid, are given in Table 1.

TABLE 1

ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH MOLD AMYLASE PREPARATIONS PRODUCED FROM BRAN MASHES CONTAINING DIFFERENT AMOUNTS AND STRENGTHS OF ACID

Mold	Mold Culture No.	Bran in g.	Normality of HCl	Vol. of HCl in ml.	pH	Alcohol Yield, % of Theory
Aspergillus oryzae	2	750	0.2 N	750	5	92.0
Aspergillus oryzae	2	750	0.2 N	900	5	93.5
Aspergillus oryzae	2	750	0.3 N	750	4	93.8
Malt Control						82.0

These data indicate that equal weights of bran and 0.3 N hydrochloric acid give satisfactory results.

2. Period of incubation

After the bran is inoculated with sporulated mold culture, it should be incubated at 30° before aeration is begun. The incubation period depends on the strain of the mold and the age of the sporulated culture used as inoculum. The rise of temperature of the bran can be considered as an index of the propagation of the mold. The incubation period may be considered to be sufficient when the temperature rises to 37° to 40° C., which usually takes from 8 to 24 hours.

3. Period of aeration

Mold amylase preparations are found to be most active at the stage just prior to the sporulation of the mold. This stage is reached after passing air through the pot for 16 to 24 hours depending on the strain of the mold. Strains of Aspergillus oryzae grow faster than most strains of other molds. The pressure of the air is kept at one-half inch of water. The temperature of the culture generally rises to 45° C. or slightly higher; sometimes, it goes to as high as 50° C. The temperature remains at a high level for several hours, but this seems to cause no deterioration of the activity of the amylases produced. After aeration, the mold is well covered with mold mycelium and this material is designated as mold-bran.

4. Period of drying

After aeration, the bran is removed from the pot, spread out on paper and dried at room temperature. The period of drying depends on the moisture content of bran when it is taken out of the pot, and on the room temperature. Drying is considered to be completed when the moisture content of the bran is reduced to less than 10 per cent. This usually takes about 3 or 4 days. The period of drying can be shortened by leaving the bran at room temperature for 24 hours (this is important, because the growth of the mold will be more complete), then completing the drying at 33 to 37° C.

(about eight hours). The dried material is ground in a Wiley mill, and is used as such for saccharification.

5. Addition of certain mineral salts

It is well known that the presence of mineral salts stimulates the growth of molds. In certain parts of this investigation, the salts recommended by Steinberg (1920) were added to the acid used in preparing the bran mash for the growth of the molds. To each liter of dilute acid was added 0.000625 g. each of ferrous sulfate and zinc sulfate.

Among the eight strains of molds tested for the effect of the addition of mineral salts to bran mash, stimulation of growth was found in the cases of molds of the genus Aspergillus, while not in the cases of molds of the genus Rhizopus. The data on alcohol yields from corn mash saccharified by means of these preparations will be tabulated in section C, "Comparison of the activities of amylase preparations produced by different molds."

B. Optimum Conditions for Saccharification

1. Concentration of mold-bran

From preliminary studies using portions of dried ground mold-bran of 2,4,6,8, and 10 per cent of the weight of corn in saccharifying corn mash, 6 and 8 per cent were found to be optimum, and these concentrations were used in most of the

experiments in this investigation. The optimum concentration of mold-bran may vary slightly from one preparation to another with the same mold, or with the same preparation in different experiments. However, the yields of ethanol obtained by using 6 and 8 per cent of mold-bran differed generally less than 1 per cent.

2. Time and temperature

In most of the previous work relating to the saccharification of starchy mashes for the alcoholic fermentation done at Iowa State College, saccharification at 50 to 55° C. for one hour was generally practiced. However, Banzon (1940) found that in the case of mold-bran, saccharification at 30° C. gave the highest alcohol yields from cassava. For sake of confirmation, time and temperature for the saccharification of corn mash with mold-bran were re-examined in the present investigation. Representative results are given in Table 2.

TABLE 2

EFFECT OF SACCHARIFICATION TEMPERATURE AND TIME ON ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED BY MEANS OF SEVERAL MOLD PREPARATIONS

Saccharification Temperature, °C.	Saccharification Time, Hours	Mold-bran, % of Corn	Alcohol Yield, % of Theory
Aspergillus Oryzae No. 2			
30	1	4	88.6
30	1	6	91.5
30	1	8	91.3
55	1	4	87.0
55	1	6	88.4
55	1	8	89.5
55	2	4	86.1
55	2	6	88.0
55	2	8	89.5
55-30	2	4	86.0
55-30	2	6	88.0
55-30	2	8	90.0
Aspergillus oryzae No. 38			
30	1	6	94.5
30	1	8	94.8
55	3	6	93.5
55	3	8	93.7
Rhizopus oryzae No. 15			
30	1	6	92.0
30	1	8	93.5
55	3	6	93.0
55	3	8	94.0
Rhizopus delemar No. 34			
30	1	6	92.5
55	3	6	91.0

The data show that alcohol yields are just as good when the conversion is performed at 30°C. for one hour as at 55°C. for 1, 2, or 3 hours as may be commonly used with malt. With some molds the yields are consistently higher when saccharification takes place at the lower temperature. The fact that saccharification with mold-bran can be accomplished satisfactorily at fermentation temperature, in contrast with the higher temperatures commonly used with malt, is an important advantage in the use of mold-bran in industrial processing. In commercial practice it may be of advantage to add the mold-bran at 55° C. to lower the mash viscosity, and then to pump immediately through the mash coolers into the fermenters.

3. Partial acid hydrolysis

It should be pointed out that in preparing the fermentation mashes the viscosity was lowered by the partial hydrolysis of the mash by 0.04 N hydrochloric acid. The use of dilute acid in cooking the mash not only simplified the procedure of saccharification but also eased the handling of the mash. The 20 per cent grain mashes were generally very thick when cooked with plain water, while they were much thinner when dilute acid was employed.

The optimum concentration of the acid was determined by running a series of fermentations using 60 g. of corn autoclaved at 20 lbs. for 30 minutes with 300 ml. of hydrochloric

acid of different concentrations. After cooking, the mashes were cooled and the pH adjusted to about 4.5 with concentrated sodium hydroxide in accordance with the standard procedure described above in "Methods and Procedures." Saccharification was done by the addition of 8 per cent of an amylase preparation produced by Rhizopus delemar No. 12 The corresponding alcohol yields are listed in Table 3.

TABLE 3

EFFECT OF CONCENTRATION OF HCl IN COOKING CORN MASH ON THE ALCOHOL YIELDS

Normality of HCl	Mold-bran, % of Corn	Alcohol Yield, % of Theory
0.005	8	86.5
0.01	8	86.7
0.02	8	87.5
0.04	8	93.5
0.08	8	90.0
0.16	8	88.8
0.32	8	85.8

These results indicate that 0.04 normal is the optimum concentration of the hydrochloric acid to be used. There was a distinct difference in the consistency of the mash cooked with hydrochloric acid of 0.02 normal and that of 0.04 normal. The former was quite thick a mash while

the latter was very thin. The mashes cooked with acid of higher concentrations appeared brown in color and the color was deeper as the acid contrations went higher. The color was due to caramelization of sugars derived from the starch and this degradation accounts for the fact that mashes cooked with acid of higher concentrations did not give higher or equally good alcohol yields as mash cooked with 0.04 N. HCl. In addition, the toxic effect produced in cooking corn meal with acid, as described by Goering (1941), may become more pronounced at higher concentrations.

4. Physical condition and age of mold-bran.

It was of interest to know whether mold-bran of different physical conditions would give different results in saccharification. Four different conditions of mold-bran were studied: (1) Dry lumps--lumps contained less than 10 per cent moisture; (2) wet lumps--lumps contained more than 15 per cent moisture; (3) dry powder; and (4) slurry--a water suspension of dry powers.

The data in Table 4 show the results of fermentation tests employing a mold-bran from Aspergillus oryzae No. 38 in the different physical conditions.

TABLE 4

EFFECT OF PHYSICAL CONDITION OF THE MOLD-BRAN ON ALCOHOL YIELDS

Condition of Mold-bran	Mold-bran, % of Corn	Alcohol Yield, % of Theory
Wet lumps	6	91.2
Dry lumps	6	94.0
Dry powder	6	94.0
Wet powder	6	94.3

Evidently the physical condition of the mold-bran preparation is of no practical significance except that wet lumps are somewhat inferior in saccharifying activity.

It was also of interest to determine the effect of storage on the amylolytic activity of the amylase preparations. Table 5 shows the typical results of fermentation tests run with amylase preparations which had been stored in the dry powdered form from 1 to 24 months.

TABLE 5

EFFECT OF STORAGE ON THE AMYLOLYTIC ACTIVITY OF MOLD-BRAN PREPARATIONS

Mold	Mold Culture	Pot Run No.	Time of Storage, Months	Mold-bran % of Corn	Alcohol Yield, Mold-bran Stored	Alcohol Yield, Mold-bran Fresh
A. oryzae	2	17	1	6	92.5	92.8
A. oryzae	2	15	3	6	94.2	94.0
A. oryzae	2	13	3.5	8	93.3	93.2
A. oryzae	2	8	6	8	90.6	90.2
A. oryzae	2	8	10	8	91.0	90.2
A. oryzae	2	3	7.5	8	92.2	90.5
A. oryzae	2	1	9	8	90.6	91.0
A. oryzae	2	1	13	8	91.0	91.0
A. oryzae	2	Drum	24	6	91.5	92.0
A. oryzae	38	00	4.5	6	93.5	93.8
R. oryzae	33	33	3	8	93.0	93.5

No significant deterioration in the activity of any of the preparations was observed. It should be pointed out that extensive data on the keeping qualities of the preparations from molds other than from Aspergillus oryzae No. 2 are not available since the other strains have been under investigation only the past few months.

C. Comparison of the Activities of Amylase Preparations Produced by Different Molds

Twenty-seven representative strains of molds were chosen for test. These were from four genera, namely Aspergillus, Mucor, Penicillium and Rhizopus. All the strains of molds are listed in Table 6. With the exception of two unidentified black molds, probably Rhizopus species, which were isolated in the laboratories of this Chemistry Department, all cultures were obtained from carefully kept culture collections and are designated by the names under which they were received.

TABLE 6

MOLDS TESTED FOR AMYLASE PRODUCTION

Lab No.	Name	Source
1	Aspergillus niger	Botany Dept., I.S.C.
2	Aspergillus oryzae	A.T.C.C.[1] No. 4814
3	Aspergillus niger	N.R.R.L.[2] No. 3
4	Mucor rouxii	A.T.C.C., No. 4855
7	Penicillium chrysogenum	Thom,[3] No. 5034·11
8	Penicillium purpurogenum	Thom, No. 413·2670
11	Rhizopus nigricans	A.T.C.C., No. 1210
12	Rhizopus delemar	A.T.C.C., No. 4859
13	Rhizopus delemar	A.T.C.C., No. 4858
14	Rhizopus oryzae	Lockwood,[4] No. 649
15	Rhizopus oryzae	Lockwood, No. 660
16	Rhizopus oryzae	Lockwood, No. 664
17	Rhizopus oryzae	Lockwood, No. 704
18	Rhizopus pêka I	Lockwood, No. 839
19	Rhizopus tritici	Lockwood, No. 654
20	Mucor circinelloides	Lockwood, No. 840
21	Mucor javanicus	Lockwood, No. 718
32	Rhizopus oryzae	N.R.R.L., No. 395
33	Rhizopus oryzae	N.R.R.L., No. 1034
34	Rhizopus delemar	N.R.R.L., No. 1472
35	Rhizopus shanghaiensis	N.R.R.L., No. 1518
38	Aspergillus oryzae	Röhm and Haas[5] No. 38
40	Aspergillus oryzae	Röhm and Haas No. 40
42	Aspergillus oryzae	Röhm and Haas No. 42
67	Aspergillus niger	N.R.R.L., No. 67
K_1	Unidentified black mold	Isolated from Lab., I.S.C.
K_2	Unidentified black mold	Isolated from Lab., I.S.C.

1. American Type Culture Collection, Georgetown University Medical School, Washington, D. C.
2. Northern Regional Research Laboratory, Peoria, Illinois.
3. Thom, Charles, U. S. Department of Agriculture, Bureau of Chemistry, Washington, D. C.
4. Lockwood, L. B., U. S. Department of Agriculture, Bureau of Chemistry and Soils, Washington, D. C.
5. Röhm and Haas Company, Bristol, Pennsylvania.

The activities of the amylase preparations produced from these molds are expressed in terms of percentages of the theoretical yield of conversion of starch into alcohol. Corn was selected as the standard starchy material for this comparative study, because corn mash is more easily handled and gives more uniform results than the mashes of other starchy materials. All the data presented in this thesis are averages of the results obtained from duplicate fermentation flasks, and all of these data have been confirmed by repeated experiments.

Since laboratory facilities did not allow parallel fermentations employing all the amylase preparations in a single series, it was necessary to choose a reference amylase for comparison. One amylase preparation of Aspergillus oryzae No. 2 was chosen as the reference substance, because it not only gave excellent ethanol yields but also gave very consistent results.

1. Amylase preparations produced by molds of the genus Aspergillus

Three strains of Aspergillus niger designated as cultures No. 1, No. 3, and No. 67; and four strains of Aspergillus oryzae designated as cultures No. 2, No. 38, No. 40, and No. 42, were tested. The alcohol yields from corn mash saccharified with the amylase preparations produced from these strains of Aspergillus are summarized in Table 7.

TABLE 7

ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH AMYLASE PREPARATIONS FROM STRAINS OF ASPERGILLUS

Mold	Mold Culture No.	Mold-bran, % of Corn	Alcohol Yield, % of Theory
A. niger	1	4	86.4
		6	91.4
		8	91.3
A. niger	3	6	90.0
		8	91.6
A. niger	63	6	84.1
		8	89.5
A. oryzae	2	4	88.6
		6	91.5
		8	93.2
A. oryzae	2	4	90.5
		6	92.8
		8	92.5
A. oryzae	38	4	93.2
		6	93.8
		8	93.5
A. oryzae	40	4	92.5
		6	93.5
		8	93.0
A. oryzae	42	4	89.4
		6	91.1
		8	91.6
Barley Malt Control		10	82.5

All of the strains tested were found to be very active in saccharifying fermentation mashes under the conditions employed. Cultures of Aspergillus oryzae, Nos. 2, 38, and 40 were especially good.

The amylase preparations produced by these three strains of Aspergillus oryzae also gave more consistent conversion of starch into ethanol than those produced by other species or strains of molds. The average yield of ethanol produced by these amylase preparations was, in general, about 93.0 to 93.5 per cent of the theoretical. Occasionally, yields as high as 94.5 to 95 per cent were also obtained.

2. Amylase preparations produced by molds of the genus Mucor

Among the Mucors, one strain each of Mucor rouxii, Mucor circinelloides and Mucor javanicus designated as cultures No. 4, No. 20, and No. 21, respectively, were tested. The results of the alcohol yields obtained from corn mash saccharified with amylase preparations from these strains of Mucor are summarized in Table 8.

TABLE 8

ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH AMYLASE PREPARATIONS FROM STRAINS OF MUCOR

Mold	Mold Culture No.	Mold-bran, % of Corn	Alcohol Yield, % of Theory
M. rouxii	4	6	87.5
		8	92.7
M. circinelloides	20	6	91.0
		8	92.8
M. javanicus	21	6	72.8
			82.8

The results in Table 8 show that the amylase preparations produced by Mucor rouxii and Mucor circinelloides possessed considerable activity although they did not give as high conversions of starch into alcohol as the best strains of Aspergillus oryzae.

3. Amylase preparations produced by molds of the genus Penicillium

Two species, Penicillium chrysogenum designated as culture No. 7 and Penicillium purpurogenum designated as culture No. 8 were tested. The alcohol yields from corn mash saccharified with amylase preparations from these species

of Penicillium are summarized in Table 9.

TABLE 9

ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH AMYLASE PREPARATIONS FROM STRAINS OF PENICILLIUM

Mold	Mold Culture No.	Mold-bran, % of Corn	Alcohol Yield, % of Theory
P. chrysogenum	7	6	64.7
		8	73.4
P. purpurogenum	8	6	86.1
		8	90.5

These results show that amylase preparations produced by these species of Penicillium were less active in converting starch into alcohol in comparison with the preparations from other genera of molds tested. Penicillium chrysogenum was particularly poor.

4. Amylase preparations produced by molds of the genus Rhizopus

Altogether thirteen strains of Rhizopus were tested. These consist of three strains of Rhizopus delemar designated as cultures No. 12, No. 13, and No. 34; six strains of Rhizopus oryzae designated as cultures No. 14, No. 15, No. 16, No. 17, No. 32, and No. 33; and one strain each of Rhizopus nigricans No. 11, Rhizopus pêka I No. 18, Rhizopus shanghaiensis

No. 35, and Rhizopus tritici No. 19. All three strains of Rhizopus delemar and most strains of Rhizopus oryzae gave very active amylase preparations. The alcohol yields from corn mash saccharified with amylase preparations from these molds are summarized in Table 10.

TABLE 10

ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH AMYLASE PREPARATIONS FROM STRAINS OF RHIZOPUS

Mold	Mold Culture No.	Mold-bran % of Corn	Alcohol Yield, % of Theory
R. delemar	12	6	92.5
		8	93.9
R. delemar	13	6	92.5
		8	93.7
R. delemar	34	4	93.8
		6	93.8
		8	93.4
R. oryzae	14	6	90.5
		8	90.5
R. oryzae	15	6	92.0
		8	93.5
R. oryzae	16	6	85.2
		8	90.5
R. oryzae	17	6	71.6
		8	84.0
R. oryzae	32	4	85.0
		6	90.6
		8	93.0
R. oryzae	33	4	92.8
		6	93.5
		8	94.0
R. nigricans	11	6	81.0
		8	89.5
R. shanghaiensis	35	6	86.9
		8	91.0
R. tritici	19	6	90.8
		8	89.6

The above data show that most of the amylase preparations obtained from Rhizopus were very active. Preparations from Rhizopus delemar No. 12, No. 13, and No. 34; and those from Rhizopus oryzae No. 15, No. 32, and No. 33, were particularly good. Their average maximum conversion of starch into alcohol was about 93.5 to 94.0 per cent of the theoretical yield. Individual fermentation flasks often gave alcohol yields as high as 94.5 to 95.0 per cent of the theoretical. Occasionally, the yields reached or exceeded 95.5 per cent.

The alcohol yields of the amylase preparations obtained from the best strains of Rhizopus were as good as those obtained from the best strains of Aspergillus although generally preparations from strains of Aspergillus seemed to give more consistent results.

When amylase preparations from Rhizopus were used for saccharification, a longer time was usually required to complete the fermentations than in the cases of preparations from strains of Aspergillus. In the latter cases three days were sufficient, while in the former cases an additional half a day to a day was required.

It is difficult to say which ones of the better strains of Rhizopus are superior. From repeated experiments, it seems that Rhizopus delemar No. 34 and Rhizopus oryzae No. 33 give more consistent results. They also stand out in the fact that they give high yields of alcohol even with four per cent of moldy bran.

5. Amylase preparations produced by unidentified molds

Two strains of unidentified black molds isolated from wheat bran in this laboratory and designated as K_1 and K_2 were also tested. The alcohol yields from corn mash saccharified with amylase preparations from these unidentified molds are given in Table 11.

TABLE 11

ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH AMYLASE PREPARATIONS FROM STRAINS OF UNIDENTIFIED MOLDS

Mold	Mold Culture No.	Mold-bran % of Corn	Alcohol Yield, % of Theory
Unidentified	K_1	8	88.5
Unidentified	K_2	6	90.3
		8	90.3

These results indicate that the amylase preparations obtained from these unidentified black molds had fairly good saccharifying power although the ethanol yields were lower than those obtained with amylase preparations from the better strains of Aspergillus, Rhizopus or Mucor.

6. Amylase preparations produced by molds with the addition of mineral salts to the bran mash.

It has been mentioned in the discussion of "Optimum

conditions for the growth of molds on wheat bran" that iron and zinc salts stimulate the growth of molds. For the study of the effect of these salts on the activities of the amylase preparations produced by molds, 0.000625 g. of each of ferrous sulfate and zinc sulfate was added to each liter of 0.3 N hydrochloric acid used in preparing the bran mash.

Eight of the best strains of the molds previously tested were selected and grown on bran to which the mineral salts had been added. These molds were: Aspergillus oryzae No. 2, No. 38, and No. 42; Rhizopus delemar No. 12, No. 13, and No. 34, and Rhizopus oryzae No. 32 and No. 33. The alcohol yields from corn mash saccharified with amylase preparations from these molds grown on bran with and without addition of mineral salts are compared in Table 12.

TABLE 12.

COMPARISON OF ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH AMYLASE PREPARATIONS FROM MOLDS GROWN ON BRAN WITH AND WITHOUT ADDITION OF MINERAL SALTS

Mold	Mold Culture No.	Mold-bran, % of Corn	Alcohol Yield, % of Theory	
			Bran with Salts	Bran without Salts
A. oryzae	2	4	91.0	90.5
		6	94.2	92.8
		8	95.5	93.2
A. oryzae	38	4	94.0	93.2
		6	94.5	93.8
		8	95.0	93.5
A. oryzae	40	4	92.5	92.5
		6	94.4	93.5
		8	92.0	93.0
R. delemar	12	4	91.o	----
		6	93.0	92.5
		8	92.0	93.9
R. delemar	13	4	88.4	----
		6	90.2	92.5
		8	90.6	93.7
R. delemar	34	4	92.5	93.8
		6	91.9	93.8
		8	90.3	93.4
R. oryzae	32	4	90.4	85.0
		6	90.2	90.6
		8	90.0	93.0
R. oryzae	33	4	89.3	92.8
		6	89.1	93.5
		8	88.9	94.0

These data indicate that the addition of the salts to the bran mash increased the amylolytic activity of mold-bran preparations from strains of Aspergillus oryzae, whereas the addition of the salts appeared actually to retard the effectiveness of the strains of Rhizopus. However, this does not prove that mineral salts are not essential for the maximum growth of the Rhizopus cultures; perhaps a different formula might give different results. With the cultures of Aspergilli the preparations made with bran mash containing the salts consistently gave alcohol yields of about 95 per cent of theory as contrasted with about 93.5 per cent yields from preparations on bran mash not containing the salts.

From the results obtained it appears that certain strains of Aspergillus oryzae are the most satisfactory molds to employ for the saccharification of fermentation mashes. Although some of the Rhizopus species give almost equally good results, the cultures of Aspergillus are much easier to handle. The Aspergilli produce more abundant sporulation, which facilitates growth of the inoculum and makes possible heavier inoculation of the bran mash, thus minimizing danger of contamination. Also the mycelium produced by an Aspergillus is more dense, and the mold-bran is easier to handle. Hence, the three best strains of Aspergillus oryzae were selected, and two mold-bran preparations from each compared, along with malt, in the same fermentation series. The results are given in Table 13.

TABLE 13

ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH DIFFERENT AMYLASE PREPARATIONS FROM STRAINS OF ASPERGILLUS ORYZAE

Mold Culture No	Pot Run No.	Mold-bran % of Corn	Alcohol Yield, % of Theory
2	15	6	95.7
		8	96.0
2	38	6	94.3
		8	95.0
38	00	6	95.5
		8	96.0
38	44	6	95.0
		8	96.0
40	18	6	94.5
		8	95.5
40	41	6	95.3
		8	95.5
Barley Malt Control		10	87.6

The mold amylase preparations showed remarkable uniformity in results, and gave about 8 per cent better alcohol yields than did the malt. It is unlikely that better yields than those shown may be expected under laboratory conditions. Without question some of the carbohydrate must be utilized in building up the protoplasm of the yeast, some alcohol will be lost by evaporation, and small amount of glycerol are always produced in normal yeast fermentations.

D. Some Modifications in Saccharification Procedures

1. Thinning of corn mash

In the discussion of optimum conditions for saccharification, it has been mentioned that the use of dilute acid greatly lowered the viscocity of the cooked corn mash. Other means have also been tried to reduce the consistency of the corn mash.

Processes used to reduce the consistency of the mash, called "thinning" were carried out by the following means:

a. Thinning by premolding. Premolding is a term applied to the addition of a small amount of mold-bran to the starchy material before gelatinization. In the case of corn 1.2 g. of a mold-bran preparation were added to 60 g. of corn meal. To this were added 300 ml. of distilled water which had previously been warmed to 75° C. This mash was heated on a hot plate until the mash was gelatinized. The gelatinizing

temperature of the corn mash was about 78° C.

Amylases are known to be able to attack starch at temperature as high as 85° C. It is conceivable that the activity of the amylases will be gradually destroyed at temperatures between 70° and 80° C. Nevertheless, from preliminary studies, 1.2 g. of mold-bran were found to be sufficient to liquefy the corn mash to a workable state. Malt can also be used in place of the mold-bran and the process is called premalting.

Both mold-bran and malt were found to give good results in thinning. The effect of premolding and premalting on the final alcohol yield has been studied. Corn mashes premolded and premalted with 2 per cent mold-bran and 2 per-cent malt respectively were sterilized, saccharified with 6 per cent of an amylase preparation produced by Aspergillus oryzae No. 38, and fermented by yeast. The alcohol yields obtained with these thinning processes together with that obtained with the usual process of partial acid hydrolysis are compared in Table. 14.

TABLE 14

EFFECT OF PREMOLDING AND PREMALTING
ON
THE YIELDS OF ALCOHOL OBTAINED FROM
CORN MASH SACCHARIFIED BY MOLD-BRAN

Thinning Agents	Mold-bran, % of Corn	Alcohol Yields, % of Theory
2 % malt	6	92.5
2 % mold-bran	6	93.5
0.04 N HCl (Control)	6	94.5

These results indicate that both premolding and premalting are adaptable processes. However partial hydrolysis with dilute acid is the most favorable process.

b. Thinning by adding mold-bran to cooked mash. Thinning can also be done by the addition of either dry mold-bran powder or the water extract of the same to the cooked mash which has been cooled to about 75° C. By this method 1.2 g. of mold-bran were mixed with the cooked mash containing 60 g. of starchy material as soon as the temperature had dropped to 70° C. Then, when the mash had cooled to 55° C., it was mixed with 6 per cent of mold-bran in a Whiz-mixer for 2 to 3 minutes. The mash was then transferred to an Erlenmyer flask and kept in a 55° C. water bath. It was believed that

the mashes cooked with plain water might require a higher temperature and a longer time in saccharification than those cooked with dilute acid. The saccharification temperature employed in this experiment was 55° C.; periods of saccharification time tested were 0, 1, and 3 hours. The effect of thinning with mold-bran and with water extract of the same on the alcohol yield from corn mash saccharified with mold-bran was studied. Two per cent of a mold-bran preparation produced from Aspergillus oryzae No. 38 was used for thinning, and 6 per cent of it was used for saccharification. The pH of the mash was adjusted to 5 before saccharification.

Alcohol yields obtained from corn mashes treated by these different thinning and saccharification methods are summarized in Table 15.

TABLE 15

EFFECT OF METHOD OF THINNING COOKED MASH AND OF SACCHARIFICATION TIME ON ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED BY MOLD-BRAN

Thinning Agents	Saccharification Temp., °C.	Time, hours	Alcohol Yield, % of Theory
2% malt	55	3	90.2
2% mold-bran	55	3	89.5
2% mold-bran	55	1	90.2
2% mold-bran extract[1]	55	3	91.2
2% mold-bran	55	0[2]	92.5
0.04 N HCl (Control)	30	1	94.5

1. Mold-bran extract was made up by stirring 12 g. of mold-bran with 300 ml. of distilled water using an electric mixer for about 20 minutes. The extract was filtered and one tenth of its total volume, which was equivalent to 1.2 g. of mold-bran, was added to the mash containing 60 g. of corn.

2. "Zero hour" means that after the mash and mold-bran extract had been mixed in the Whiz-mixer at 55° C., the mash was set aside at room temperature. As soon as the mash had cooled down to 30° C., it was inocculated with yeast culture.

The data in Table 15 indicate that the mold-bran extract was a better thinning agent than either dry mold-bran powder or malt. There was very little difference between the latter two agents. Concerning the saccharification time, keeping

the mash for one or more hours at 55° C. did not seem to aid the saccharification. On the other hand, letting the mash cool gradually from 55° to 30° right after the mold-bran had been added and mixed, gave a higher alcohol yield. Nevertheless, all these processes of thinning cooked mash gave considerably lower yields of alcohol than the method of partial acid hydrolysis.

c. Thinning by quick cooling. Christensen (1940) suggested the method of quick cooling to reduce the viscosity of the mash of starchy material. This was done by cooking the mash containing 60 g. of corn with 150 ml. of distilled water. After sterilization, the hot mash was thoroughly mixed with another 150 ml. of cold distilled water. This generally brought the temperature of the mash down to about 55° C. The pH of the mash was adjusted to 5, and 6 per cent of mold-bran was added. The mash was then thoroughly mixed for about two minutes in a Whiz-mixer.

The effect of quick cooling together with the effect of saccharification time on the alcohol yield from corn mash saccharified with mold-bran were studied. Mash containing 60 g. of corn, and a mold-bran preparation produced from Aspergillus oryzae No. 38 were used in these experiments. The results are given in Table 16.

TABLE 16

EFFECT OF QUICK COOLING AND SACCHARIFICATION TIME ON ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED BY MOLD-BRAN

Treatment	Saccharification Temp., °C.	Saccharification Time, hours	Alcohol Yield % of Theory
Quick cooling	55	0	89.5
Quick cooling	55	3	88.7
0.04 N HCl	30	1	94.5

These results show that quick cooling was less efficient in thinning than were the other processes.

Since all of these experiments on thinning of corn mash discussed above were performed at the same time, and the same mold-bran preparation was used in all cases, the best alcohol yields obtained from these experiments furnish an accurate comparison of the efficiency of the different thinning processes. This is shown in Table 17.

TABLE 17

COMPARISON OF THE EFFICIENCY OF THE DIFFERENT THINNING PROCESSES

Processes	Saccharification Temp., °C.	Saccharification Time, hours	Alcohol Yield, % of Theory
Premolding	30	1	93.5
Premalting	30	1	92.5
Thinning cooked mash with mold-bran extract	55	0	92.5
Thinning cooked mash with dry mold-bran	55	1	90.2
Quick cooling	55	0	89.5
0.04 N HCl (Control)	30	1	94.5

This comparison shows that next to partial acid hydrolysis, premolding is the best among all of the thinning processes. Thinning cooked mash with mold-bran extract is the second best. It should be noted here that these experiments on the effect of thinning on the alcohol yields from corn mash saccharified by mold-bran can be considered only as a preliminary study. Modifications on the mechanical methods such as stirring and time of mixing of the mash may improve

and vary the final yield of alcohol. However, this preliminary study shows that if it is desirable to eliminate the use of dilute acid, alternative processes can be employed without sacrificing much of the alcohol yield and without complicating the procedure to any considerable extent.

2. Secondary addition of mold-bran

From the investigation of the activities of mold amylases produced from twenty-seven representative strains of molds discussed above, it can be concluded that the highest ethanol yields consistently obtained from corn mash saccharified with amylase preparations from molds grown on bran without addition of mineral salts were about 93.5 per cent, and 95.0 per cent in cases where mineral salts were added to the bran. These yields are 5 to 6.5 per cent lower than the theoretical.

Several factors may contribute to the failure to obtain theoretical yields. Carbohydrates are utilized by growth of yeast, some evaporation of alcohol occurs during the incubation period, by-products such as glycerol are formed, and the saccharification of the starch may be incomplete. Of these factors, the most important in the present study is the possibility of incomplete saccharification. Some modifications in procedure were made to attempt to diminish the last named factor.

Incomplete saccharification may be due to either or both of the following causes: First, the activity of the

amylase preparations may be insufficient to break down the starch completely to fermentable sugars; second, the mold enzymes may have the ability to resynthesis simple fermentable sugars to unfermentable polysaccharides.

In the previous attempt to improve the activity of the amylase preparations, addition of mineral salts to the bran mash was found to increase the amylolytic activity of amylase preparations from strains of Aspergillus oryzae as already discussed. An attempt was also made to reduce the possible loss due to resynthesis of simple fermentable sugars to unfermentable polysaccharides by some of the mold enzymes. It was thought that if amylase preparation were added at intervals, in amounts that would maintain the concentration of the enzymes at a level where the resynthesis would be held at a minimum, the alcohol yield might be improved. This was done by adding part of the total amount of the mold-bran before beginning the fermentation and part of it during the fermentation. This is what is called "secondary addition" of mold-bran.

Preliminary tests showed that 2 per cent of mold-bran was an adequate amount for the secondary addition. The desirable time for the secondary addition was between the 20th and 24th hour of fermentation. One amylase preparation from Aspergillus oryzae No. 38 and one from Rhizopus oryzae No. 33 were selected for this study. The alcohol yields from corn

mash saccharified with these amylase preparations with and without the modification of secondary addition are compared in Table 18.

TABLE 18

COMPARISON OF ALCOHOL YIELDS FROM CORN MASH SACCHARIFIED WITH AMYLASE PREPARATIONS WITH AND WITHOUT SECONDARY ADDITION

Mold	Mold Culture No.	Mold-bran % of Corn 1st Add.	Mold-bran % of Corn 2nd Add.	Alcohol Yield, % of Theory
A. oryzae	38	4	0	92.0
		2	2	92.5
A. oryzae	38	6	0	93.5
		4	2	94.3
A. oryzae	38	8	0	94.8
		6	2	95.0
R. oryzae	33	4	0	92.8
		2	2	93.9
R. oryzae	33	6	0	93.5
		4	2	94.2
R. oryzae	3	8	0	93.1
		6	2	93.5

The above data show that, in general, the secondary addition of mold-bran gave an increase of 0.7 to 1.1 per cent of alcohol yield. These data also indicate that in cases of alcohol yields between 94.5 to 95.0 per cent obtained

without secondary addition of mold-bran, the effect of secondary addition became much less significant. It is conceivable that secondary addition would do little or no good with amylase preparations which, without secondary addition, give alcohol yields already close to the highest yields obtainable.

Tertiary addition of mold-bran, i.e., dividing the amount of mold-bran to be added into three portions, and adding one portion before the fermentation starts, the other two portions at 20 to 24 hour intervals, and secondary addition of 10 ml. of active yeast culture have also been tried. Neither these modifications nor any combination of these modifications further improved the alcohol yields.

3. Use of mold-bran mixtures

The effect of mixtures of mold-bran preparations on the yield of alcohol has also been studied. A series of different proportions of mixtures of amylase preparations from mold belonging to different genera, have been tested. None of these mixtures with or without the modification of secondary addition gave a higher alcohol yield than that obtained by using the corresponding amount of the better strain alone.

E. Fermentation of Various Starchy Materials

The objective of this thesis, as indicated by its

title, was to make a comparative study of the relative activities of the amylase preparations from different molds as saccharifying agents in the ethanol fermentation of starchy materials. In making this comparative study, it was necessary to choose a standard substrate. Corn was selected as the representative starchy material, because corn mash is easy to handle and gives more uniform fermentation results. In addition, corn is the primary starchy material used in ethanol fermentation at the present time. The results obtained from the study of the relative activities of the amylase preparations from different molds in saccharifying corn mash should give valuable indications of the behaviors of the corresponding amylase preparations toward other starchy materials, although the results may not be entirely parallel. In addition to the relatively extensive investigation of the activities of mold amylases as saccharifying agents in the ethanol fermentation of corn, preliminary studies of the use of mold amylases to saccharify mashes of wheat, oats, barley, two varieties of sorghum and rice have also been undertaken.

1. Amylase preparation produced by Aspergillus oryzae No. 38

One series of fermentations was run by using an amylase preparation produced by Aspergillus oryzae No. 38 to saccharify 20 per cent mashes of corn, wheat, Spartan barley, oats, Leoti red sorghum, Kafir pink sorghum and rice under identical conditions employing the standard procedure. The

relative alcohol yields obtained from the fermentation of these mashes with 6 and 8 per cent mold-bran for saccharification are summarized in Table 19.

TABLE 19

COMPARISON OF ALCOHOL YIELDS FROM VARIOUS STARCHY MATERIALS SACCHARIFIED WITH AMYLASE PREPARATIONS PRODUCED BY ASPERGILLUS ORYZAE NO. 38

Materials	Mold-bran % of Corn	Alcohol Yield, % of Theory
Corn (Control)	6	91.2
	8	92.9
Wheat	6	80.0
	8	83.6
Spartan Barley	6	79.5
	8	81.0
Oats	6	89.0
	8	89.5
Leoti red sorghum	6	87.5
	8	89.0
Kafir pink sorghum	6	95.5
	8	96.7
Rice	6	95.7
	8	97.0

These results show that wheat and barley gave low alcohol yields. It was noticed that the wheat and barley mashes were still quite thick after they had been autoclaved

with 0.04 N hydrochloric acid. The fermentations of these mashes were also slow and took about five days to complete. Oats and Leoti red sorghum gave fairly good alcohol yields although they were about 3 per cent lower than those from corn. The mashes of oats were quite thick after autoclaving with dilute acid, while the mashes of both varieties of sorghum and rice were as thin as those of corn.

Saccharification of rice mashes appeared to be particularly smooth and the fermentations were completed in a little over two days. Rice is considerably more expensive than other grains; its use in industries is probably out of question. The high alcohol yields obtained from Kafir pink sorghum look particularly promising. Its feasibility in the manufacture of industrial alcohol deserves consideration and study.

2. Amylase preparation produced by Aspergillus oryzae No. 2

An amylase preparation produced from Aspergillus oryzae No. 2 was used to saccharify 20 per cent mashes of oats, rice, Leoti red sorghum and Kafir pink sorghum. Corn was used as a control. The alcohol yields obtained from these mashes with 6 per cent mold-bran for saccharification are summarized in Table 20.

TABLE 20

COMPARISON OF ALCOHOL YIELDS FROM VARIOUS STARCHY MATERIALS SACCHARIFIED WITH AMYLASE PREPARATION PRODUCED BY ASPERGILLUS ORYZAE NO. 2

Materials	Mold-bran, % of Corn	Alcohol Yield, % of Theory
Corn (Control)	6	91.5
Oats	6	89.0
Rice	6	93.4
Leoti red sorghum	6	86.7
Kafir pink sorghum	6	96.2

Again, rice and Kafir pink sorghum gave alcohol yields considerably higher than from corn, while oats and Leoti red sorghum gave yields lower than from corn.

3. Amylase preparation produced by Mucor rouxii No. 4

Twenty per cent mashes of rice, Leoti red sorghum and Kafir pink sorghum were further studied. Six per cent of an amylase preparation produced by Mucor rouxii No. 4 was used for saccharification of these mashes. Corn mash was used as control. The results are summarized in Table 21.

TABLE 21

COMPARISON OF ALCOHOL YIELDS FROM VARIOUS STARCHY MATERIALS SACCHARIFIED WITH AMYLASE PREPARATION PRODUCED BY MUCOR ROUXII NO. 4

Materials	Mold-bran, % of Corn	Alcohol Yield, % of Theory
Corn (Control)	6	87.4
Rice	6	95.0
Leoti red sorghum	6	91.6
Kafir pink sorghum	6	97.5

It is interesting to note that in using the amylase preparation produced from Mucor rouxii No. 4, Leoti red sorghum gave a higher alcohol yield than corn did, and Kafir pink sorghum gave a particularly high yield of 97.5 per cent. The yields obtained from mashes of rice and the two varieties of sorghum saccharified by the amylase preparation produced from this Mucor were higher than the corresponding yields obtained from mashes saccharified by the amylase preparations from the two strains of Aspergillus oryzae tested.

It should be remarked, however, that these data can

be considered only as results of preliminary study. More extensive experiments, such as the fermentation of these grain mashes using a series of concentrations of mold-bran are required to confirm these results and their consistency.

3. Amylase preparation produced by Rhizopus delemar No. 13 and Rhizopus oryzae No. 32

Amylase preparations produced from Rhizopus delemar No. 13 and Rhizopus oryzae No. 32 were then used to saccharify the mashes of rice and the two varieties of sorghum. Six per cent mold-bran was used for 20 per cent mashes. Corn was employed as the control. The alcohol yields obtained from these mashes are compared in Table 22.

TABLE 22

COMPARISON OF ALCOHOL YIELDS FROM VARIOUS STARCHY MATERIALS SACCHARIFIED WITH AMYLASE PREPARATIONS PRODUCED BY *RHIZOPUS DELEMAR* NO. 13 AND *RHIZOPUS ORYZAE* NO.32

Material	Mold-bran, % of Corn	Alcohol Yield % of Theory
R. delemar No. 13		
Corn (Control)	6	92.2
Rice	6	93.4
Leoti red sorghum	6	86.7
Kafir pink sorghum	6	93.4
R. oryzae No. 32		
Corn (Control)	6	87.4
Rice	6	88.5
Leoti red sorghum	6	89.5
Kafir pink sorghum	6	92.0

These results show generally that mashes of rice and the two varieties of sorghum saccharified by the amylase preparation produced from Rhizopus delemar No. 13 gave good alcohol yields, while the activity of the amylase preparation produced from Rhizopus oryzae No. 32 was considerably lower in saccharifying these corresponding mashes.

From these preliminary studies of the fermentation of various starchy materials, it can be concluded that all the various starchy materials tested produced considerable amounts of alcohol. Alcohol yields obtained from fermentation mashes of Kafir pink sorghum and rice saccharified with the different amylase preparations were consistently higher than those obtained from corresponding fermentations of corn mashes. Kafir pink sorghum deserves special attention, because it not only gave particularly high alcohol yields but is also a cheap raw material for industrial application. Leoti red sorghum and oats gave alcohol yields of about 90 per cent or slightly higher, while wheat and barley generally gave alcohol yields below 85 per cent. The cooked mashes of wheat and barley appeared quite thick. It is probable that some modifications in liquefication of the mashes such as premolding or using acid of higher concentration in cooking the mashes, or modifications in the saccharification procedure or proportion of mold-bran employed would increase the alcohol yields. The use of higher concentrations of mold-bran in saccharification would likely improve the alcohol

yields since in these experiments only 6 per cent of mold-bran was used.

It should be noted here that only one sample of each of the starchy materials other than corn has been employed in these experiments. It is difficult to say whether these single samples are adequately representative. More extensive studies are needed to confirm these results and the consistency of these results before proper general conclusions can be drawn on the fermentation of these various starchy materials. At any rate the results obtained from these preliminary experiments provide indications of the relative activities of the amylase preparations produced from representative species of molds toward the various starchy materials and the feasibility of the various starchy materials as substrates for ethanol fermentation.

VI. SUMMARY AND CONCLUSIONS

1. The amylolytic activities of the amylases produced from 27 strains of molds in saccharifying corn mash for ethanol fermentation have been compared. Ninety per cent or higher yields of alcohol were obtained from 20 per cent corn mash saccharified by amylase preparations from 23 of the 27 strains of molds tested.

2. A new and efficient laboratory method for culturing molds on wheat bran mash in special 3-quart aluminum pots equipped for aeration has been developed. Growth of the molds in pots is more rapid and uniform, and the amylolytic activities of the products are greater than when grown in the rotating drums previously employed.

3. The method for culturing the molds is as follows: The bran mash used as medium is prepared by moistening 750 g. of wheat bran with an equal weight of 0.3 N hydrochloric acid. The wet bran is packed into the pot and sterilized in the autoclave at 15 lbs. for 30 minutes. The cooled bran mash is mixed with 10 g. of well-sporulated mold culture grown in flasks on wheat bran mash, and the inoculated material is packed firmly into the pot. The culture is incubated at 30° C., until the temperature rises to 37° to 40° C., thus indicating rapid growth of the mold. The growing mold mass is then aerated by passing air through the pot at a

pressure of one-half inch of water. After aeration for 16 to 24 hours the bran is well covered with mold mycelium and the material, which is designated as mold-bran, is removed, spread out on paper and dried at room temperature. The dried material is ground in a Wiley mill, and is used as such to saccharify corn mashes for fermentation tests.

4. The procedure for saccharification is as follows: The corn mash is prepared by mixing 60 g. of grain of known starch content with 300 ml. of 0.04 N hydrochloric acid in a 500-ml. Erlenmeyer flask, gelatinized by heating over a hot plate with frequent shaking and sterilized at 20 lbs. for 30 minutes. After cooling, the grain mash is neutralized with concentrated sodium hydroxide solution and adjusted to a pH of 4.5 to 5.0. The desired amount of mold-bran and the grain mash are then mixed in a Whiz-mixer for one minute. After the mash is incubated at 30° C. for one hour, it is inoculated with 20 ml. of a 20 to 24 hour old yeast culture, Saccharomyces cerevisiae, in 10 per cent malt extract solution. The mash is then incubated at 30° C. The fermentation is generally completed in 72 hours.

5. Saccharification performed at 30° C. for one hour has been found to be just as good as at 55° C. for one or more hours. With some molds the yields were consistently higher when saccharification was performed at the lower temperature. The fact that saccharification with mold-bran can

be accomplished satisfactorily at fermentation temperature, in contrast with the higher temperatures commonly used with malt, should be an important advantage in the use of mold-bran in industrial processing.

6. Physical condition of the mold-bran preparation is of no practical significance. However, slurry seems to be most satisfactory in practice. Mold-bran has excellent keeping quality. In the case of amylase preparations produced by Aspergillus oryzae No. 2, no significant deterioration was observed after a period of two years.

7. Among the 8 strains of Aspergillus tested, the use of preparations from Aspergillus oryzae No. 2, No. 38 and No. 40 gave highest conversion of starch into ethanol. The average maximum yield of ethanol produced by using amylase preparations from these molds was, in general, about 93.0 to 93.5 per cent.

8. Amylase preparations produced by Mucor rouxii and Mucor circinelloides were quite active while that produced by Mucor javanicus was relatively inferior.

9. The amylase preparations produced by species of Pencillium were less active in converting starch into alcohol in comparison with the preparations from other genera of molds. The amylase preparations produced by Penicillium chrysogenum were particularly poor.

10. Most of the amylase preparations obtained from the 12 strains of Rhizopus were very active. Amylase preparations

from Rhizopus delemar No. 12, No. 13 and No. 34, and those from Rhizopus oryzae No. 15, No. 32, and No. 33 were particularly good. The alcohol yields from the amylase preparations obtained from the best strains of Rhizopus were as good as those obtained from the best strains of Aspergillus. However, preparations from the best strains of Aspergillus oryzae seemed to give more consistent results than preparations from any other molds.

11. The amylase preparations from the two unidentified molds were quite effective although not as active as those produced from the better strains of Aspergillus, Rhizopus, and Mucor.

12. Different processes have been tried to reduce the viscosity of the fermentation mash. Partial acid hydrolysis, i.e. use of 0.04 N hydrochloric acid to cook the mash was the best process. Premolding, premalting and thinning cooked mash with mold-bran extract all gave satisfactory results. Thinning cooked mash with dry mold-bran was not as satisfactory as the use of mold-bran extract. Quick cooling has not yet been proved to be very effective.

13. Secondary addition of mold-bran gave, in general, an increase of 0.7 to 1.1 per cent alcohol yield. In cases where alcohol yields between 94.5 to 95.0 per cent were obtained without secondary addition of mold-bran, the effect of secondary addition became much less significant. It is conceivable that secondary addition would do little or no

good with those amylase preparations which, without secondary addition, give alcohol yields close to the highest yields obtainable.

14. Tertiary addition of mold-bran, secondary addition of yeast, and the use of mold-bran mixtures have also been tried in attempting to further improve the conversion of starch to alcohol. None of these modifications was found to be effective.

15. The addition of traces of ferrous and zinc sulfates to the bran mash increased the amylolytic activity of mold-bran preparations from strains of Aspergillus oryzae, while the same salts appeared to retard the effectiveness of the strains of Rhizopus. However, this does not prove that mineral salts are not essential for the maximum growth of the Rhizopus cultures; perhaps a different formula would give different results.

16. From the activities of mold amylases produced from the twenty-seven representative strains of molds investigated, it can be concluded that the consistent highest ethanol yield obtained from corn mash saccharified with amylase preparations from molds grown on bran without addition of mineral salts was about 93.5 per cent, and 95.0 per cent in cases when mineral salts were added to the bran. These yields are 5 to 6.5 per cent lower than the theoretical. It is unlikely that better yields than those shown may be expected under

laboratory conditions. Without question some of the carbohydrate must be utilized in building up the protoplasm of the yeast, some alcohol will be lost by evaporation, and small amounts of glycerol are always produced in normal yeast fermentations.

17. Besides corn, the other starchy materials investigated were wheat, oats, Spartan barley, oats, Leoti red sorghum, Kafir pink sorghum and rice. Amylase preparations from 5 strains of molds were used to saccharify the mashes of these materials, using 6 per cent mold-bran in each case. These molds were Aspergillus oryzae No. 38 and No. 2, Mucor rouxii No. 4, Rhizopus delemar No. 13 and Rhizopus oryzae No. 32.

18. From these preliminary studies of the fermentation of various starchy materials, it can be concluded that all the various starchy materials tested produced considerable amounts of alcohol. Alcohol yields obtained from fermentation mashes of Kafir pink sorghum and rice saccharified with the different amylase preparations were consistently higher than those obtained from corresponding fermentations of corn mashes. Kafir pink sorghum deserves special attention, because it not only gives particularly high alcohol yields but is also a cheap raw material for industrial application. Leoti red sorghum and oats gave alcohol yields of about 90 per cent or slightly higher, while wheat and barley generally gave alcohol yields below 85 per cent. These results do not

necessarily imply that wheat and barley are poor starchy materials for ethanol fermentation. The cooked mashes of wheat and barley appeared quite thick. It is very probable that some modifications in liquefication of the mash, such as premalting or using acid of higher concentration in cooking the mashes, would increase the alcohol yields. Very likely the use of higher concentrations of mold-bran in saccharification would also improve the alcohol yields since only 6 per cent mold-bran has so far been tried.

19. As a final conclusion, it can be said that fungal amylases are very satisfactory saccharifying agents in the ethanol fermentation of starchy materials. Besides producing higher alcohol yields than malt, mold-bran is easily prepared and is made from abundant and cheap raw material. It would seem that the use of mold-bran to replace malt in the production of industrial alcohol should hold much promise, particularly in meeting the urgent need of saccharifying agents in the expanded grain alcohol industry of to-day.

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