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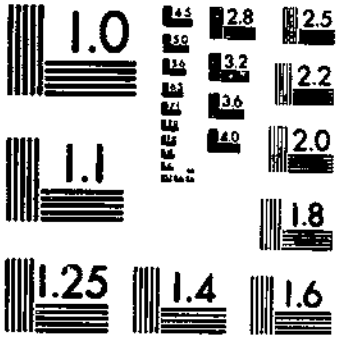
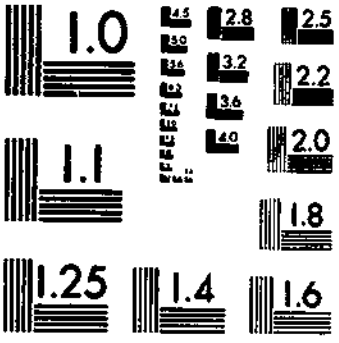


MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A



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NATIONAL BUREAU OF STANDARDS-1963-A

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UNITED STATES
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WASHINGTON, D. C.

METHODS AND COSTS OF PRODUCING ALCOHOL FROM GRAIN BY THE FUNGAL AMYLASE PROCESS ON A COMMERCIAL SCALE[1]

By Production and Marketing Administration
and
Bureau of Agricultural and Industrial Chemistry

CONTENTS

[1] Submitted for publication May 31, 1950.

INTRODUCTION[2]

For centuries the world's supply of ethyl alcohol was produced from starch or natural sugars by fermentation with micro-organisms. Recently, the production of synthetic alcohol from petroleum by-products has reached significant proportions in the United States. Nevertheless, the fermentation process remains a very important source of the product. At present the bulk of the industrial alcohol produced by fermentation is from molasses, whereas corn, rye, grain sorghum, wheat, and barley malt are used in the production of distilled spirits for beverage purposes.

During World War II, however, several hundred million bushels of grain were fermented to industrial alcohol that was used in our synthetic-rubber program. The shortage and comparatively high cost of barley malt, which was needed for the conversion of grain to alcohol, stimulated research for an enzymatic material to replace malt and lower the production cost of alcohol. In 1946 Van Lanen and Le Mense (*11*)[3] and in 1947 Le Mense, Corman, Van Lanen, and Langlykke (*4*) who carried out the pioneer work at the Northern Regional Research Laboratory, of the Bureau of Agricultural and Industrial Chemistry, at Peoria, Ill., announced the preparation of a fungal amylase agent that could be employed for the conversion of mash in the production of alcohol from grain. It was made by fermentation of a stillage-corn medium with the mold *Aspergillus niger*, NRRL 337.

A subsequent publication (*5*) gives details of pilot-plant studies on the production and utilization of fungal amylase, and includes a summary of the estimated cost of production of the enzymatic material. It was reported that on the basis of pilot-plant results a potential savings of 2.4 to 3.6 cents per gallon of 190 proof material is possible in the production cost of alcohol when fungal amylase is employed instead of malt.

During years of abundant production, the utilization of grains, both sound and damaged, can become a troublesome problem. Should a program ever be initiated to utilize such grains through conversion to industrial alcohol and animal feed, the most economical method of processing would be important. Production costs in an alcohol plant, it was believed, might be decreased significantly through the use of

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[2] The investigation described in this bulletin was conducted by the Grain Processing Corporation, Muscatine, Iowa, under a contract between the Corporation and the Production and Marketing Administration, U. S. Department of Agriculture. However, because the Northern Regional Research Laboratory of the Bureau of Agricultural and Industrial Chemistry developed the fungal amylase process, personnel of that laboratory served as consultants to the contractor during the course of the work. The important role played by the Northern Laboratory in the investigation is gratefully acknowledged. Particular credit is due Russell H. Blom, of the Northern Laboratory, who assembled the data presented in this report and prepared the bulletin for publication. The contributions made by the Nebraska Agricultural Experiment Station, University of Nebraska, Lincoln, Nebr., and the Bureau of Animal Industry, USDA, Beltsville, Md., are also acknowledged. This investigation is being continued under a second contract between the Grain Processing Corporation and the Agricultural Research Administration, U. S. Department of Agriculture.

The study on which this report is based was made under authority of the Research and Marketing Act of 1946.

[3] Italic numbers in parentheses refer to Literature Cited, p. 29.

fungal amylase. However, plant-scale tests of the process were necessary to provide more exact information on (1) the investment cost of a fungal amylase plant to operate in conjunction with a distillery of a given capacity; (2) the feasibility of producing pure-culture mold fermentations on a large scale in a distillery; (3) the process operations required in the alcohol plant when fungal amylase is substituted for barley malt; (4) the yields and quality of alcohol and byproducts obtained when the fungal amylase process is employed; and (5) the comparative production costs of alcohol made from grain converted with fungal amylase and with barley malt. Consequently, a contract was negotiated between the Production and Marketing Administration, United States Department of Agriculture, and the Grain Processing Corporation, Muscatine, Iowa, wherein the Corporation agreed to install a fungal amylase unit in the Government-owned alcohol plant at Muscatine, Iowa, of which it is the lessee, to operate the plant, and to utilize the enzymatic liquor in plant-scale fermentations of grain to ethyl alcohol. Because the mold process was developed at the Northern Regional Research Laboratory, personnel of that laboratory served as consultants to the contractor during the course of the investigation.

It is the purpose of this report to describe in detail the work conducted at the plant of the Grain Processing Corporation on the fungal amylase process; to present the data and information obtained; and to submit cost estimates and conclusions based on results of the experimental work.

DEVELOPMENT OF FUNGAL AMYLASES FOR THE SACCHARIFICATION OF GRAIN MASHES

In the production of ethyl alcohol by the fermentation of starchy materials such as grains, it is necessary first to convert the starch to a sugar (glucose or maltose) upon which the yeast can act. In this country enzymatic conversion or saccharification is employed entirely.

Diastases or amylases comprise the group of enzymes which hydrolyze starch to dextrins and the sugars maltose and glucose. They occur in a variety of biological forms, including the seeds of plants such as barley, wheat, and soybeans, animal glands such as the pancreas, and micro-organisms such as the bacteria and molds. Of these materials barley is utilized most commonly as a source of enzymes for the industrial saccharification of starch. The enzymatic activity of the grain is enhanced by the malting operation, and the dry desprouted product, known as barley malt, is used by distillers and brewers.

In the United States the producers of alcohol from grain have been dependent upon barley malt for the conversion of starch to fermentable sugars. However, since the latter part of the 19th century several attempts have been made to utilize fungal preparations for the saccharification of grain mashes. In 1914 Takamine conducted experiments in a distillery with a converting agent prepared by the traditional methods used by the Chinese and Japanese in the production of Koji. Indeed, the name Taka-Koji was given by Takamine to his product, which was a culture of *Aspergillus oryzae* on wheat bran, to distinguish it from the Japanese material that is a culture on steamed rice. The product is now called moldy bran or mold bran.

The production and utilization of mold bran has been investigated

extensively in the United States. In the process a mold, usually *Aspergillus oryzae*, is cultured on moistened wheat bran. After incubation under controlled conditions of temperature and aeration, the product is dried. Molds that may be employed have been tested by Underkofler, *et al.* (*9*), by determining the yields of alcohol from grain mashes saccharified with mold brans prepared with the various organisms. The plant-scale usage of mold bran in an alcohol plant is described by Underkofler, *et al.* (*10*). Considerable quantities of mold bran were produced, and the material was utilized in the production of industrial alcohol in 1945 and 1946 in at least one alcohol plant. However, at present little, if any, mold bran is being used for this purpose.

Mold bran is made by surface-culture fermentation; several processes for the production of enzymes by submerged culture methods have been suggested. The amylo process (*3*) was developed in 1895 by Calmette, and has been modified somewhat since then. A mold, generally a species of *Mucor* or *Rhizopus*, is grown submerged in the grain mash itself, and the starch, saccharified by the action of mold enzymes, is fermented to alcohol by yeasts. The process must be carried out under the most rigorously pure culture conditions. It is being used by alcohol plants in Asia, southern Europe, and South America.

In 1909, Woolner and Lassloffy (*12*) described the use of a diastatic slop, or fungi-diastase, as a converting agent in the production of alcohol from grain mashes. *Aspergillus oryzae* was grown submerged under aerobic conditions in a medium of thin stillage. After a grain mash was partially converted with a small amount of malt, the mold culture and yeast were added.

Erb and Hildebrandt (*1*) in 1946 described a somewhat similar process in which both fungal amylase produced in submerged culture and malt were used for saccharification purposes. They employed a strain of *Rhizopus delemar* and incorporated granular wheat flour, stillage, and malt in the mold propagation medium.

The process developed at the Northern Regional Research Laboratory for the preparation of fungal amylase and its utilization in the production of alcohol is similar in some respects to the Woolner-Lassloffy and the Erb and Hildebrandt processes, but differs from them in that no malt is used for conversion. Briefly, the Northern Laboratory process (*4, 5, 8, 11*) is carried out as follows:

A sterilized medium composed of thin stillage, grain, and calcium carbonate is inoculated with a liquid culture of *Aspergillus niger*, NRRL 337. (Recent investigations by Tsuchiya, Corman, and Koepsell (*8*) have shown that improved enzyme preparations are obtained when calcium carbonate is eliminated from the stillage medium and the concentration of grain is increased.) During incubation at 86° F., the submerged culture is aerated and agitated. The mold is propagated for 50 to 60 hours, when the production of fungal amylases is practically completed. The whole culture is added to the grain mash, previously cooked, and cooled to conversion temperature, at the rate of approximately 3.0 gallons per 56-pound bushel of corn. After primary conversion, the grain mash is cooled and inoculated with yeast. The alcoholic fermentation is finished in about 72 hours. Yield of alcohol from grain converted with the mold amylase is reported to be no less

than that obtained when barley malt is employed as the saccharifying agent.

FUNGAL AMYLASE LIQUOR, ALCOHOL, AND BYPRODUCT FEEDS

In the experimental work at the Muscatine plant of the Grain Processing Corporation, fungal amylase liquor was produced by the Northern Regional Research Laboratory process and utilized in the saccharification of grain to produce alcohol and byproduct feeds. During the preparation of enzyme liquor, analytical tests were performed to ascertain the presence or absence of contaminating organisms in certain stages of the process, and the enzyme potency of the fungal amylase was determined, in order to evaluate the material for subsequent use as a converting agent. As it was conceivable that the quality as well as the yield of products might be affected by using fungal amylase in place of barley malt, analytical and taste determinations were made on the alcohol and feeding tests on the byproduct feeds produced with the mold agent and with malt.

Fungal amylase liquor is known to display two types of activity: (1) A dextrinizing or liquefying action measured as alpha-amylase activity; and (2) a saccharifying action measured by enzymatic action on maltose. The latter type of action is apparently dependent on the presence of either an alpha-glucosidase or a transglycosidase, or both; and for convenience and simplicity it will be referred to in this report as maltase. Research has been done on the possible correlations between the alpha-amylase and maltase activities of fungal amylase preparations and the yields of alcohol obtained from corn mashes converted with these materials. No relationship has been found between alpha-amylase content and yield of alcohol, except that a preparation must contain at least a certain minimum activity of this type to insure good alcohol yields. However, it was determined that alcohol yields correlate well with the maltase activity of mold preparations, provided that the low, but significant, activity of alpha-amylase is present also. It is evident that both types of enzymatic activity are important and must be considered in the evaluation of fungal amylase.

A method for the determination of the combined effect of the enzyme systems in fungal amylase was utilized for plant control, because the procedure is simple and tests can be conducted rapidly. The method is based on the quantity of reducing sugars formed by the action of a measured amount of fungal amylase liquor on a given quantity of starch under controlled conditions of time and temperature. The extent of conversion of starch to sugars is thus ascertained. As the available data indicate a relationship between the percentage of conversion and the alcohol yield, the method affords a means of evaluating mold preparations comparable to the Lintner method of analysis for the evaluation of barley malt. The mold liquors produced during the large-scale work were analyzed, both for their alpha-amylase and maltase contents and for the combined effect of the enzymes, by methods which are given in Appendix A. Methods for the detection of contaminants in a medium or in filtered air also are described in Appendix A.

Samples of distilled alcohol (190 proof) produced from mold-

converted mashes were analyzed chemically for esters, aldehydes, and fusel oil, and the odor and taste of the products were evaluated organoleptically. Standard methods of analysis were employed for these determinations.

Distillers' dried grains and distillers' dried solubles were recovered in each of the test runs. Samples of these byproduct feeds were analyzed (routinely, and by standard procedures) for moisture, protein, fat, and fiber. Comparatively large quantities of certain batches of feeds were submitted to the University of Nebraska and to the Bureau of Animal Industry at Beltsville, Md., for evaluation by animal-feeding tests. A few of the samples of grains and solubles were analyzed for their content of the vitamins, riboflavin, thiamine, niacin, and pantothenic acid. Comparative data were obtained in all of the tests.

PREPARATION OF MOLD INOCULA

The methods and techniques employed for developing laboratory cultures of *Aspergillus niger*, NRRL 337, were those recommended and established by the Northern Regional Research Laboratory. The general procedure used to increase the quantity of culture and details of the various laboratory processes follow:

Parent or stock culture (slant)→Slant→Flask→Flask→
Carboy (2.5 gallons)→Seed tank (250 gallons)→
Amylase plant fermentor (about 25,000 gallons)

Sporulated cultures of *Aspergillus niger*, NRRL 337, obtained from the Northern Regional Research Laboratory,[4] are stored in a refrigerator. To initiate the production of an inoculum, a few spores are transferred from the parent culture to a test-tube slant. The culture medium and method of preparation are as follows:

Distillers' thin stillage fortified with solubles or sirup to a solids content of 5 percent.
2 percent corn starch.
2 percent agar.
Sodium hydroxide to adjust pH to 6.0.
Plug tubes with cotton and sterilize 30 minutes at 250° F. (15 pounds to the square-inch gage).

This transfer and all others must be conducted aseptically because no degree of contamination can be tolerated in laboratory or seed cultures. After the slant has been incubated for 24 hours at 87° F., a part of the vegetative growth produced is transferred to a 1-liter flask containing 200 milliliters of medium of the following composition:

Distillers' thin stillage fortified with solubles or sirup to a solids content of 5 percent.
2 percent cornstarch solubilized with 0.1 percent barley malt.
0.5 percent calcium carbonate.
Sodium hydroxide, if necessary, to adjust pH to 5.0 to 6.0.
Plug flasks with cotton and sterilize 30 minutes at 250° F. (15 p.s.i.g.).

In order to supply oxygen to the organism, the flask is shaken in a room or cabinet in which the temperature is controlled thermostatically at 87° F. The speed of the shaker is approximately 45 cycles per

[4] Cultures of this organism are available to industry in general.

minute. The flask is incubated and agitated for 24 hours, after which a small part of its contents (5 milliliters) is transferred to a second 1-liter flask which contains 200 milliliters of medium of the same composition. This flask is shaken at 87° F. for 24 hours, at which time vigorous and healthy growth is obtained.

The entire contents of the second flask are employed to inoculate 10 liters of sterile medium which is contained in a 4.5-gallon carboy. The medium is of the same composition as that used in the flasks; however, because of the large quantity of liquor, the bottle and contents are sterilized for 60 minutes at 267° F. (25 p.s.i.g.). The inoculated medium is aerated vigorously by the passage of air into the carboy through a glass or copper tube. Air for this purpose is sterilized by filtration through tubes packed with cotton or glass wool. In addition to the air inlet, the carboy is equipped with an air vent and a tube through which the contents of the bottle are transferred to the dona tank. After aeration and incubation at 85° to 90° F. for 24 hours, the carboy culture may be used to inoculate the dona, or seed, tank in the fungal amylase plant. Details of the methods and equipment employed to transfer culture liquors from flask to carboy and carboy to seed tank, and for the aeration of the carboy culture, are given in Appendix B.

DESIGN, INSTALLATION, AND OPERATION OF FUNGAL AMYLASE UNIT AT PLANT OF GRAIN PROCESSING CORPORATION, MUSCATINE, IOWA

In the pilot-plant work on the fungal amylase process conducted at the Northern Regional Research Laboratory, mold fermentations were carried out in copper 800-gallon tanks, and alcoholic fermentations were made in steel fermentors, each of which had a capacity of 4,000 gallons. During the experiments, approximately 300 gallons of fungal amylase was prepared per batch and a portion of this was employed to convert about 1,400 gallons of grain mash (40 bushels of corn). It was ascertained that 7 to 10 percent of the mold agent, based on the final volume of the grain mash, was sufficient for conversion, and that when batch cooking and conversion were used the fungal amylase could simply be substituted for a slurry of malt with no other change in mashing operations.

The effect of rate of aeration during fermentation on the enzyme potency of the fungal amylase liquor had been investigated. Although one-eighth volume of air per volume of medium per minute appeared to be sufficient for the preparation of a satisfactory product, it was recommended that an aeration rate of one-quarter be employed for the plant operation, as the minimum rate appeared to be a function of the shape of the vessel and the degree and type of agitation provided. A satisfactory medium had been developed and tested during the pilot-plant and laboratory investigations. This medium, to be used in the plant, consisted of thin stillage with a solids content of about 5 percent, supplemented with 1 percent of ground corn and 0.25 to 0.50 percent of calcium carbonate.

It had been determined that the yield of alcohol from corn converted with fungal amylase was no less than, and in some instances significantly higher than, the yield with malt as the saccharifying agent.

Much information had been obtained on the pure culture techniques needed in the large-scale production of fungal amylase. All available results and engineering data of the Northern Regional Research Laboratory were placed at the disposal of the operators of the Grain Processing Corporation to be used as bases for the design and operation of a large installation.

It had been decided that sufficient fungal amylase was to be produced per run to convert enough grain to set two large fermentors in the distillery. As the capacity of each alcohol fermentor is 100,000 gallons, and approximately 10 percent fungal amylase was to be employed for conversion, the required capacity of the fungal amylase plant was 20,000 gallons per run.

The design of the enzyme plant was influenced to a major extent by the type of tank or fermentor that could be obtained readily. The closed tank which was purchased is constructed of lightweight steel plates, and is cylindrical, with flat top and conical bottom. The tank has a total capacity of 30,000 gallons, and cannot be pressure-sterilized because of its construction. Although a simple installation was desirable for the experiments, it obviously was impossible to sterilize the medium batchwise in the fermentor; hence, a continuous cooker was provided and the tank was cleaned by mechanical and chemical means before each run.

Figure 1 is a diagrammatic flowsheet of the process as installed and used. Each item of equipment shown on the flowsheet is numbered and corresponding numbers are used in the following description.

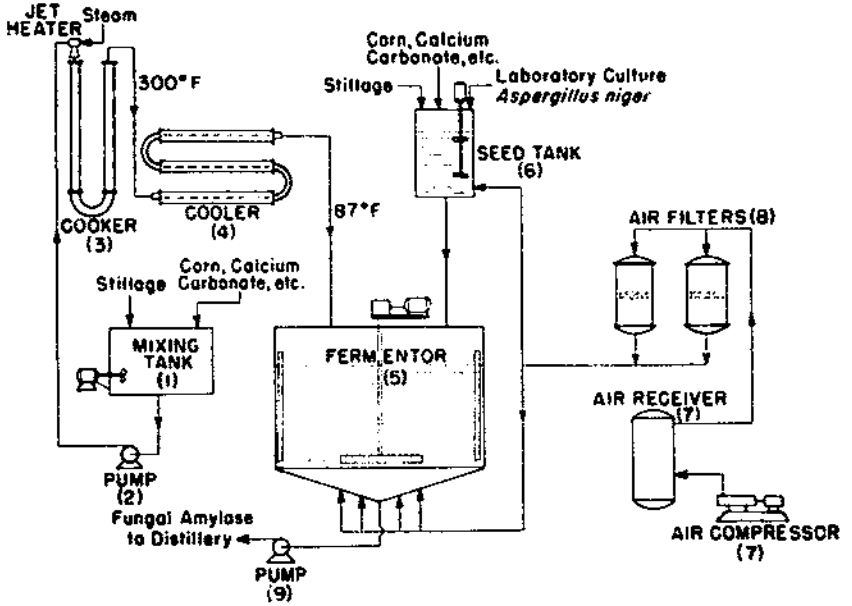

FIGURE 1.—Flowsheet for the production of fungal amylase.

(1) MIX TANK.—This unit is an open cylindrical tank equipped with a side-entering agitator. Its total capacity is 500 gallons. It is used for

blending supplementary materials with the stillage prior to sterilization of the medium.

(2) COOKER FEED PUMP.—The pump has a rated capacity of 50 g.p.m. against a head of 75 pounds to the square-inch gage and is driven with a 15-horsepower motor. It is employed to pump stillage with added ingredients through the cooker and cooling system and into the fermentor.

(3) COOKER.—This unit consists of a $1\frac{1}{2}$-inch jet heater and 40 feet of 6-inch standard iron pipe. The medium is heated instantaneously in the jet heater and is kept at the elevated temperature during its passage through the section of pipe.

(4) COOLER.—Hot, sterile medium is cooled continuously to fermentation temperature in this equipment. It consists of two double-pipe or concentric-pipe heat exchangers connected in series. The total surface for cooling in both units is 235 square feet.

(5) FERMENTOR.—This cylindrical closed tank is constructed of welded steel plate, with a flat top and conical bottom. It is 20 feet in diameter and 12 feet high on the straight side, and has a total capacity of approximately 30,000 gallons. The fermentor is equipped with a top-entering mixer, which is driven at the rate of 32 revolutions per minute by means of a 75-horsepower motor with chain and gear reduction. The mixer consists of one turbine-type unit, 84 inches in diameter; the tank is equipped with four vertical baffles to reduce swirling of the medium during agitation. Air is introduced into the fermentor by four 2-inch pipe lines, the discharge ends of which are just below the turbine agitator. All piped openings into the tank are protected against the accidental passage of contaminants by double valves, and by steaming the section of pipe between the valves. Air is exhausted from the tank through a 6-inch pipe welded in the top of the tank. This line is piped in the shape of an inverted "U" to prevent contaminating moisture or dust from entering the vessel.

(6) SEED TANK.—This is a closed cylindrical steel tank, $3\frac{1}{2}$ feet in diameter and 5 feet high, with flat heads; its total capacity is approximately 360 gallons. It is equipped with a top-entering mixer with 2-propeller agitators. Air is sparged into the fermentor through a $\frac{3}{4}$-inch tee near the bottom of the vessel under the lower propeller. All piped openings into the tank are steam-sealed. The inoculum inlet is described in Appendix B. Air is exhausted from the unit through a section of pipe, which is trapped by an inverted "U" bend to prevent the entrance of contaminated moisture or dust into the tank. A flowmeter is connected to the discharge end of the vent line and used to measure the volume of air that is passed through the medium.

(7) AIR COMPRESSOR.—This is a single-stage, reciprocating compressor with a rated capacity of 1,000 cubic feet per minute at 20 pounds to the square-inch gage. It is driven by a 75-hp. motor. An air receiver, 425 cubic feet in capacity, is provided. It was determined that the actual capacity of the compressor was about 650 c.f.m. at 20 p.s.i.g. and 900 c.f.m. at 15 p.s.i.g.

(8) AIR FILTERS.—Two filters are provided. Each consists of a tank 3 feet in diameter by 4 feet high (shell of Bowser filter), with a removable top head and equipped with a single horizontal support for the filter medium. This is nonabsorbent cotton, of which 16 1-inch pads compressed to a thickness of approximately $4\frac{1}{2}$ inches are used.

The tanks are constructed to withstand internal pressure; hence, they can be sterilized with steam at 15 pounds to the square-inch gage.

(9) FUNGAL AMYLASE PUMP.—This is a centrifugal pump with a capacity of 25 gallons per minute against a head of 50 feet. It is driven by means of a 5-horsepower motor, and is used for transferring fungal amylase from the fermentor to the distillery.

The final assembly and installation of the equipment were made with the knowledge that fungal amylase must be produced by pure culture fermentation. Items of equipment which must be sterile or free of contaminants during all or some portion of the fermentation cycle are the cooker, cooler, fermentor, air filters, seed tank, and pipe lines that connect these units. Therefore, provisions were made to sterilize the equipment and lines with steam at a pressure of 15 pounds to the square-inch gage when possible. Obviously, in an installation of this type, the equipment and pipe lines must be arranged so that they can be cleaned and kept clean if steam sterilization is to be completely effective. For example, if medium can enter the air line and particles of corn are deposited in the pipe between the air filter and the fermentor, these pockets of organic material may become centers of infection and cause contamination of the medium in later runs.

Although it appears improbable, it is possible that infectious material might leak past the gate of a closed valve in a pipe line connected to the fermentor or seed tank. For this reason sample outlets, mash inlet lines, drain outlets, etc. are double-valved and a steam seal is provided between the valves. In general, this method should be used as a safety measure in all piped connections between the fermentor or seed tank and the appurtenances which are or may be non-sterile.

The fungal amylase plant was erected and operated as an outdoor unit. Subsequently, however, it was enclosed in a frame building so that operations could be continued during winter.

PLANT OPERATION.—Thin stillage was pumped from a storage tank in the feed recovery house of the distillery to the mix tank in the fungal amylase plant. The solids content of the stillage was in most cases about 4 percent and it was increased to 6 to 8 percent by fortification of the stillage with distillers' sirup (evaporated stillage) that contained approximately 25 percent solids. Ground corn, calcium carbonate, and a solution of sodium hydroxide were added proportionally to the fortified stillage in the mix tank. These ingredients were added in quantities sufficient to give the medium a composition of approximately 1 percent corn and 0.25 to 0.50 percent calcium carbonate. Its initial pH was 5.0 to 5.5. A small amount of ammonium bifluoride also was added to the stillage at this time, for the purpose of inhibiting bacterial growth in the medium if it became contaminated during fermentation.

In a typical plant run (Experiment 12B), about 25,000 gallons of medium was prepared. To produce the liquor, fortified thin stillage was supplemented with 2,000 pounds of ground corn, 1,000 pounds of calcium carbonate, 325 pounds of sodium hydroxide, and 37 pounds of ammonium bifluoride. The pH of the medium after sterilization was 5.0. Based on the volume at this time, it contained 0.96 percent of corn, 0.48 percent of calcium carbonate, and 0.018 percent of ammonium bifluoride.

Stillage, mixed with corn and the other ingredients, was withdrawn

continuously from the mix tank and pumped through the cooker and cooler into the fermentor. In preparation for this operation, the cooker and cooler had been sterilized with steam under pressure, and the fermentor had been cleaned chemically with detergents and antiseptics and then steamed at atmospheric pressure. The temperature of the medium was increased instantaneously to 290° F. by mixing it with steam at 55 pounds per square-inch gage in the jet heater, and the medium was retained at this temperature for 6 minutes during its passage through the cooker. The rate of pumping was 30 to 35 gallons per minute. The hot medium passed from the cooker through an expansion valve to the cooler, where its temperature was reduced to 87° F., and thence to the fermentor. A total time of about 12 hours was required to sterilize 25,000 gallons of medium.

From 24 to 30 hours before the large fermentor was charged with sterile medium, the seed tank had been inoculated. The seed medium was prepared and sterilized batchwise. Approximately 250 gallons of fortified thin stillage was pumped into the seed tank, the agitator started, and ground corn, calcium carbonate, sodium hydroxide, and ammonium bifluoride were added. The volume of medium at this time was about 260 gallons. In experiment 12B, the quantities of ingredients used were as follows: 20 pounds of corn, 7.5 pounds of calcium carbonate, 2.0 pounds of sodium hydroxide, and 140 grams of ammonium bifluoride. The composition of the medium before sterilization was 0.92 percent corn, 0.35 percent calcium carbonate, and 0.014 percent ammonium bifluoride, and the pH after sterilization was 5.6.

The medium was sterilized in the seed tank by double cooking, with an intervening rest period. After the medium was cooked with open steam for 60 minutes at 240° F., it was cooled to 90° to 100° F. and kept at this temperature for several hours. The medium was recooked for 60 minutes at 270° F., then cooled to 87° F. and inoculated with 10 liters of a laboratory culture of *Aspergillus niger*, NRRL 337. Hot medium was cooled by means of a spray of cold water on the outside of the tank. Cold water or warm condensate was sprayed on the tank to control the temperature of the medium at 84° to 88° F. during fermentation.

Sterile air was supplied the seed fermentor while the cooked medium was being cooled. The contents of the tank were under atmospheric pressure only when the inoculum was transferred into it. During fermentation, the rate of aeration with sterile air was about one volume per volume of medium per minute.

After the seed culture had been incubated for 24 to 30 hours, it was transferred to the large fermentor. This was done by closing the vent valve of the seed tank, while aeration was continued, to increase the pressure in the vessel to 12 to 15 p.s.i.g. which was sufficient to force the culture into the large fermentor through a connecting pipe line. The transfer line had been cleaned and was steamed until used. The large fermentor was inoculated while it was being charged, and usually when it contained about 8,000 gallons, which was enough medium to cover the turbine agitator.

Aeration in the large fermentor was begun immediately after it had been steamed and just before sterile medium was pumped into the tank. In this way, the pressure inside the vessel always was slightly greater than atmospheric, a condition which is recommended for pure

culture fermentation. Aeration of the medium in the large fermentor was conducted at the rate of 650 to 950 c.f.m., which corresponded to a volumetric rate of 0.20 to 0.28 volumes of air per volume of medium per minute, when 25,000 gallons of liquor was fermented. The temperature of fermenting medium in the large tank was adjusted by means of a spray of cold water or warm condensate on the outside of the vessel. This temperature ranged usually between 85° to 89° F.

After fermentation for 48 to 60 hours, the fungal amylase liquor was ready for use. At this time, it was pumped to the distillery as needed during mashing operations there.

OPERATIONS IN THE ALCOHOL PLANT— USE OF FUNGAL AMYLASE

In order that the reader may fully comprehend the use of fungal amylase in the distillery, the system of mashing employed at the Grain Processing Corporation is explained here in detail. Figure 2 is a diagrammatic flowsheet of the process used at Muscatine to prepare grain for fermentation with yeast. Corn is ground in hammer mills, and the ground grain is mixed with water and stillage in the premix

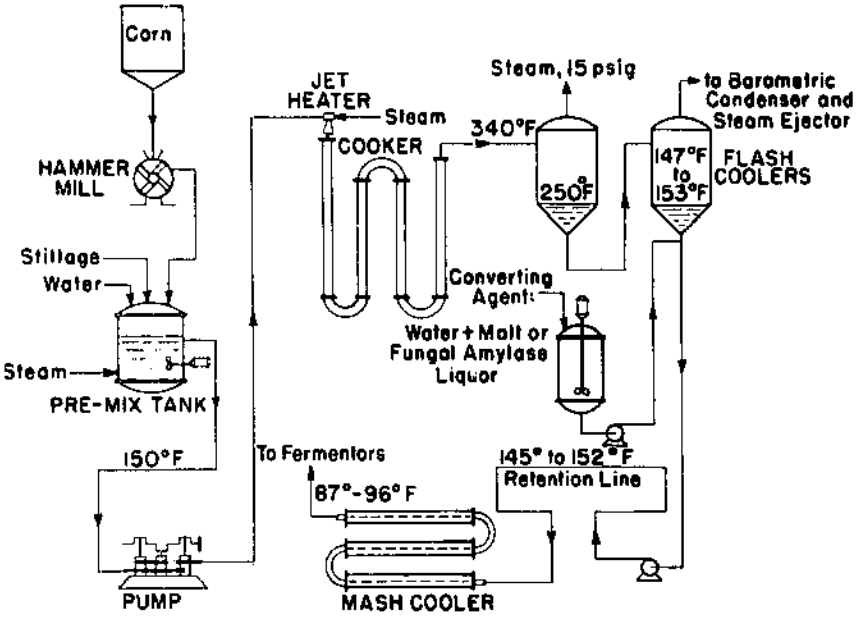

FIGURE 2. - Flowsheet showing how grain is mashed in the alcohol plant of the Grain Processing Corporation.

tank or precooker. The slurry is pumped from this vessel to a continuous cooker. A jet heater at the entrance to the pipe cooker instantaneously heats the mash to 340° F., and it remains at this temperature for about 5 minutes during its passage through the retention leg of the unit. The hot mash is cooled to 250° F. in a pressure flash cooler; then to 147° to 154° F. in a vacuum flash cooler. The converting

agent is introduced into the mash at this point, and the mixture is pumped through a length of pipe in which the mash is retained for 5 to 6 minutes at the conversion temperature that is usually 145° F. when malt is used. This is referred to as the period of primary conversion. Mash passes from the conversion line to the mash coolers, where its temperature is reduced to about 90° F., and thence to a fermentor.

When malt is used as the converting agent, it is ground, mixed with water in a malt slurry tank, and the mixture is pumped into the mash stream. To change from malt to fungal amylase was a simple operation; the mold liquor was pumped from the fungal amylase plant to the malt slurry tank, from which it was introduced in the regular way into the mash stream.

Several purposes are accomplished when grain is mashed. In grinding grain, the starchy portions of the kernel are exposed, but only a small part of the starch is available to enzyme action. When an aqueous slurry of ground grain is cooked, the starch granules swell and rupture; the starch becomes gelatinized and thus open to attack by enzymes. When the cooked mash is cooled, it becomes very viscous, and if the concentration of grain in the mash is normal (20 to 22 percent) it is impossible practically to transport the material mechanically; that is, by pumps and through pipe lines. Also, at this time, only a small amount of fermentable sugar is present in the mash, and some sugar is required for immediate use by the yeast in the subsequent alcoholic fermentation. To liquefy or thin the mash, and to degrade a portion of the starch to dextrins and fermentable sugars, amylolytic enzymes are added to the cooked and partially cooled material. When mashing is continuous, the conversion temperature employed must be high enough to saccharify the starch and to thin the mash to the desired extent in a few minutes. The temperature must not be so high as to cause destruction of the enzymes.

This initial treatment of starch at an elevated temperature with enzymes is called primary conversion. The mash has been only partially converted. Saccharification of the residual starch and of the dextrins takes place slowly at the temperature of and during the alcoholic fermentation. This is the period of secondary conversion.

In the pilot-plant work at the Northern Regional Research Laboratory, corn mashes were cooked batchwise and primary conversion with fungal amylase was batchwise in all instances. No difficulties were encountered; both liquefaction of the mash and saccharification of the starch were satisfactory. However, because the conversion step was a batch operation, time was not a factor. Experimental results indicated that fungal amylase did not thin a grain mash as rapidly as malt under conditions usually employed when malt was used as the converting agent. It was beneficial, in the tests at Muscatine, to increase the conversion temperature with fungal amylase to 152° F., in order to enhance the liquefaction power of the material. This development will be discussed in more detail later in this bulletin.

The alcoholic fermentation of mash converted with fungal amylase was conducted in the regular manner. Each fermentor in the plant of the Grain Processing Corporation has a capacity of 100,000 gallons, and in these experiments one tank was charged with 2,700 to 3,500 bushels of corn, the amount charged depending on the concentration of grain in the mash. One batch of fungal amylase liquor was used to

convert mash for two plant fermentors. Temperature of the fermenting mash was adjusted when necessary by circulation of the liquor through a heat exchanger at the base of a fermentor. The yeast inoculum was prepared from malt-corn mash which was lactic-soured. Most alcoholic fermentations of mash converted with fungal amylase were in process over a week end; hence, a beer was 4 or 5 days old at the time it was distilled.

At the time these contractual experiments were conducted at Muscatine, the plant was engaged in the production of neutral spirits for use in beverages. The distillation unit, a four-column still, gave a product of satisfactory quality as judged by organoleptic tests. The operation of the columns was not altered for the distillation of fungal amylase beer. Because neither the beer well nor the distillation unit was empty when it was time to distill mold beer, it was impossible to make a sharp separation between malt spirits and mold spirits. Samples taken during the latter part of a distillation were considered to be representative of mold alcohol.

Byproduct feeds were recovered at the plant by the regular methods. Whole stillage was screened, and the oversize particles of grains pressed and dried to make distillers' light grains. The thin stillage that passed through the screen was concentrated by evaporation to a sirup containing about 25 percent solids. Sirup was pumped to a double drum dryer where distillers' dried solubles were produced. During the experiments, some distillers' dark grains were made. In this case, the dried light grains were mixed with sirup and the mixture dried in a rotary unit. Again, there was some difficulty in sharply separating mold and malt byproduct feeds because of the constant flow of material through the plant.

PRODUCTION AND UTILIZATION OF FUNGAL AMYLASE

A total of 31 experiments on the production of fungal amylase was conducted during the course of the work at Muscatine. Of these runs, the first 15 were preliminary and served mainly to familiarize the operators with the process and with the techniques involved. The fungal liquors produced in the preliminary runs were unsatisfactory in most instances; only one batch was used in the distillery to convert mash. It would serve no useful purpose to discuss these runs further in this bulletin.

Information on the last 16 experiments is given in table 1. Approximately 25,000 gallons of fungal amylase liquor was prepared in each of these runs, and with the exception of material made in experiments 18B and 23B, each batch of mold liquor was used in the distillery for the conversion of 5,000 to 7,000 bushels of grain.

Fortified thin stillage was employed as the principal substrate of the medium in all mold fermentations. Because in regular operations grain was converted with malt, stillage most easily available was from malt beer. If a plant were to use fungal amylase regularly the stillage would be from mold beer. It seemed conceivable that malt stillage might contain nutrients for mold growth and that these factors might not be present in mold stillage. In order to determine the effect of recycling mold stillage on the quality of the fungal amylase liquor, experiments 13B, 14B, 15B, 16B, 17B, and 18B were conducted in

TABLE 1.—*Data on production of fungal amylase liquor on a semiplant scale*

Run number	Composition of medium [1]				Time of fermentation	Analysis of fungal amylase liquor				
	Type of stillage	Corn	Calcium carbonate	Acidity		Saccharification value, conversion	Alpha-amylase	Maltase	Acidity	
									Titratable [2]	Final
		Percent	*Percent*	*pH*	*Hours*	*Percent*	*Units (30° C.) /ml.*	*Units/ml.*		*pH*
9B	Corn-malt	0.96	0.48	5.5	52	24.0	12.0	4.0	5.2	4.6
10B	do	.96	.48	5.3	54	24.1	11.0	5.2	4.0	5.0
11B	do	.91	.48	5.2	60	24.5	15.0	N.D.	4.1	4.9
12B	do	.96	.48	5.6	58	29.4	15.0	?	4.0	4.9
13B	Corn-mold from 12B	.85	.44	5.3	60	19.8	6.7	2.0	6.6	4.5
14B	do	.98	.49	5.1	57	15.2	5.3	?	3.3	4.8
15B	Corn-mold from 13B	1.50	.60	5.0	48	28.4	10.0	2.3	2.8	5.0
16B	Corn-mold from 14B	.96	.48	5.2	60	29.4	15.0	4.6	5.1	4.6
17B	Corn-mold from 16B	.96	.48	5.0	54	32.1	19.2	4.0	2.6	5.0
18B	do	.96	.48	.....	68	22.0	8.6	2.1	3.2	5.2
21B	Part corn-malt, part corn-mold	1.20	.60	5.1	72	21.4	8.9	Less than 2	3.5	4.9
22B	Corn-malt	.96	.48	5.5	56	34.8	11.0	4.7	2.7	4.9
23B	do [3]	.....	.....	.....	.....	.....	.....	.....	.....	.....
25B	do	1.33	.44	5.6	52	39.3	13.8	N.D.	1.5	5.2
26B	do	1.15	.46	5.2	54	38.5	19.2	3.3	1.5	5.1
27B	do	1.11	.44	5.9	84	19.8	10.0	Less than 2	.25	6.7

[1] All media contained about 0.02 percent ammonium bifluoride to inhibit growth of bacteria if a liquor became contaminated during run.
[2] Milliliters of 0.1 N NaOH to titrate 10 ml. of filtered mold liquor to phenolphthalein end point.
[3] Discarded after 24 hours because it was badly contaminated. Trouble traced to laboratory culture.

TABLE 2.—*Data on the utilization of fungal amylase liquor for the conversion of corn mashes in the alcohol plant of the Grain Processing Corporation*

Converting agent		Grain mashed		Mashing operations					Fermentation						
Mold liquor produced in run number—	Saccharification value, conversion	Total corn mashed for two fermentors, as received	Grade of corn used [1]	Fermentor number	Quantity of mold liquor used per bu. corn as received	Malt added to help thin mash (percent of grain bill)	Conversion temperature	Type stillage for backset	Age of beer when fermentor emptied	Final specific gravity	Acidity		Alcohol in finished beer	Final concentration grain in beer per bu. (dry)	Yield of alcohol per 56 lb. dry grain
											Titratable [2]	Final			
	Percent	*Bushels*			*Gallons*	*Percent*	*°F.*		*Hours*	*°Balling*		*pH*	*Percent*	*Gallons* [3]	*Proof gallons*
9B.....	24.0	5,502	No. 3	9	4.0	0.0	145	Corn-malt	62	0.9	3.2	4.5	5.76	46.4	5.35
				2	3.4	1.0	145	...do.....	59	.8	4.3	4.9	8.10	37.1	6.01
10B....	24.1	6,003	..do...	1	2.8	1.2	151	...do.....	105	1.3	6.7	4.6	8.11	36.8	5.97
				3	3.8	1.0	151	...do.....	104	1.4	5.8	4.6	8.02	37.1	5.95
11B....	24.5	6,130	..do...	3	3.2	.9	152	...do.....	117	.8	4.7	4.7	7.81	38.1	5.95
				5	3.9	.1	152	...do.....	118	.8	5.6	4.6	7.58	40.6	6.16
12B....	29.4	5,176	..do...	2	4.3	.0	152	...do.....	107	1.3	6.6	4.6	7.55	41.3	6.24
				4	4.5	.0	152	...do.....	105	1.1	4.5	4.7	7.94	39.4	6.26
13B....	19.8	6,413	..do...	2	3.2	1.0	153	Corn-mold	109	2.0	9.4	4.0	6.48	38.5	4.99
				4	3.2	2.0	153	...do.....	110	.9	6.1	4.8	7.89	37.0	5.84
14B....	15.2	6,404	..do...	5	3.3	1.5	152	...do.....	54	2.5	8.4	4.9	7.14	36.4	5.20
				7	3.4	1.4	152	...do.....	49	3.1	7.9	5.0	6.32	36.7	4.64
15B....	28.4	6,473	..do...	2	3.1	1.3	151	...do.....	110	1.5	8.0	4.2	7.24	38.4	5.56
				4	3.1	1.1	151	...do.....	110	.7	5.3	4.7	8.31	35.3	5.87
16B....	29.4	6,030	..do...	3	3.3	2.0	153	...do.....	105	.7	5.4	4.4	7.45	39.6	5.90
				5	3.3	1.8	153	...do.....	93	.6	4.2	4.8	8.38	37.1	6.22

17B....	32.1	6,073	Sample, damage 50%	8	3.5	1.2	153	do.....	119	.9	5.0	4.9	8.78	35.3	6.20
				3	3.5	1.2	153	...do.....	114	1.0	7.8	4.1	7.87	36.6	5.76
18B....	22.0	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(4)
21B....	21.4	5,437	No. 3	4	2.2	2.0	150	Corn-malt	148	.3	5.7	4.1	7.44	36.9	5.49
				6	3.8	2.4	150	...do.....	142	.6	6.5	4.5	7.61	37.0	5.63
22B....	34.8	5,596	Sample, damage 50%	8	3.6	1.2	152	...do.....	110	.2	6.5	4.5	8.12	37.8	6.14
				3	3.9	1.3	152	...do.....	96	.2	6.5	4.5	6.89	44.0	6.06
23B....	.....	See Table 1.													
25B..	39.3	7,007	No. 3	2	3.3	1.4	151	None	106	.0	4.1	4.6	7.61	39.4	6.00
				4	3.3	1.3	151	...do.....	105	.1	4.6	4.0	7.81	37.4	5.84
26B...	38.5	6,961	...do...	6	3.2	1.1	153	...do.....	104	.1	2.1	4.8	7.76	39.0	6.05
				8	3.9	.3	153	...do.....	104	.2	4.0	4.6	7.91	38.3	6.07
27B...	19.8	6,999	...do...	5	3.4	4.6	145	...do.....	59	.2	3.2	4.6	7.94	37.8	6.00
				7	3.9	4.6	145	...do.....	52	1.7	4.5	4.6	7.93	36.6	5.79

[1] Grain was graded No. 3 because of moisture content.
[2] Milliliters of 0.1 N NaOH to titrate 10 ml. of filtered beer to phenolphthalein end point.
[3] This number was obtained by dividing total volume of finished beer in fermentor, in gallons, by total bushels (56 pounds) of dry grain with which fermentor was charged.
[4] Mold liquor contaminated badly; not used in alcoholic fermentation.

which mold stillage from a previous distillery operation was used for preparation of the medium. It was found that the use of recycled stillage from mold beers had no effect on the enzymatic potency of the resultant mold liquor or on the conversion power of the material when it was used in the distillery. These results are in accord with information obtained in pilot-plant work at the Northern Regional Research Laboratory (5).

The principal difficulty in the preparation of the fungal amylase liquor was contamination of the medium during fermentation. In only three experiments was the finished liquor free of contaminants. A thorough search was made for the source of the infection; filtered air was tested for sterility, the cooked medium was sampled aseptically and cultured to determine the presence or absence of contaminating organisms, and the seed culture was examined. As a result of these tests it was decided that the large fungal amylase fermentor was the cause of the trouble. Because of its construction this tank could not be sterilized with steam under pressure, and the interior surface of the vessel was rough and difficult to clean.

The enzyme potency of the fungal amylase liquors varied widely. Saccharification powers as low as 15.2 percent in experiments 14B and as high as 39.3 in run 25B were obtained. It is believed that contamination was the chief cause of the variation. In pilot-plant work at the Northern Laboratory consistent results were obtained, but contamination was infrequent.

Data on the utilization of fungal amylase liquor in the distillery are given in table 2. After a few runs in the distillery it became evident that the power of the mold liquor to liquefy mash was less than that of malt. This is of special significance to a plant in which continuous cooking and conversion are employed, as only about 6 minutes are provided for liquefaction and primary conversion of the mash. If the mash is too viscous at the time it enters the cooler the pressure drop across the cooler becomes excessive and the mash pump delivers less material. Thus, the over-all rate of mashing must be decreased in order to maintain steady-state conditions in the continuous system.

When malt-converted mash was pumped at the rate of 400 bushels of grain per hour the pressure at the discharge side of the mash pump was about 30 p.s.i.g. At the same rate, but with mash converted with fungal amylase, the pump pressure in most cases was 40 to 60 p.s.i.g. In one experiment, this pressure reached 100 p.s.i.g., which was sufficient to lower the capacity of the pump and mash accumulated in the vacuum flash cooler.

In order to improve liquefaction when fungal amylase was used for conversion, a small amount of malt was mixed with the mold liquor in most of the experiments. From 1 to 2 percent malt was employed on the basis of the total grain bill.

It was observed that when mash was converted with fungal amylase at a temperature of 152° F., rather than 145° to 146° F., liquefaction was improved. The higher temperature had no detrimental effect on the alcohol yields. It is believed that fungal amylase alone will liquefy mash satisfactorily if a conversion temperature of 152° to 155° F. and a retention time of 12 to 15 minutes are used. Indeed, recent plant-scale experiments have shown this to be true. Another factor which affects rate of liquefaction is the quality of the fungal amylase liquor.

Experiment 26B was a very successful run. Corn mash for the second fermentor of this experiment was converted with fungal amylase mixed with only a very small quantity of malt (0.3 percent of the total grain bill); yet the mashing operation was normal in all respects. It is significant that the mold liquor was of good quality. It is reasonable to assume that the use of fungal amylase of consistently good quality will help to eliminate the liquefaction problem in these runs.

On the average, about 3.5 gallons of mold liquor was used for the conversion of 1 bushel of corn, as received. The controlling factor in these experiments was liquefaction during mashing operations. No attempt was made to determine the effect on fermentation of smaller amounts of liquor. However, in pilot-plant work and in recent plant-scale experiments satisfactory results were obtained with 2.7 gallons and less of fungal amylase liquor per bushel of grain when the liquor was of good quality and under favorable conditions for primary conversion.

Secondary conversion and fermentation of mold-converted mashes appeared to proceed more slowly than with malt-converted material. This indicates that a longer fermentation time might be required when fungal amylase is used unless more favorable primary conversion were obtained.

Samples of mold-converted mash taken at various times during the alcoholic fermentation were examined for bacterial contamination. The extent of contamination was less than usually encountered in malt-converted mashes. This was to be expected, as many bacteria are added to the mash with the malt.

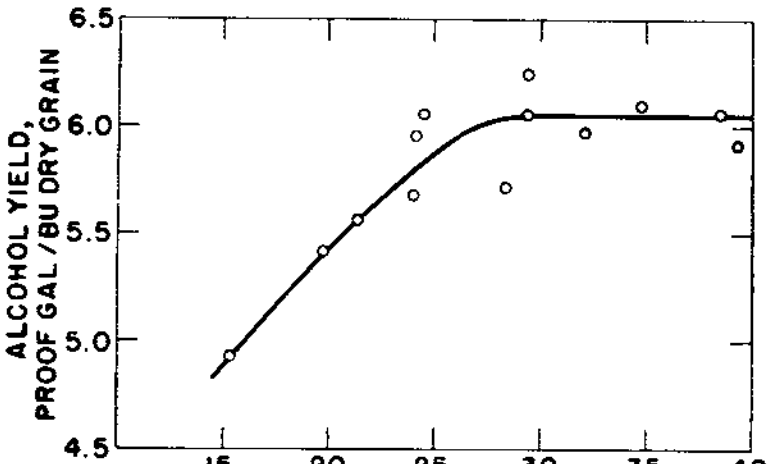

FIGURE 3.—Relationship between saccharogenic power of fungal amylase and yield of alcohol.

The results of the plant experiments indicate a relationship between saccharogenic power of the fungal amylase liquor and the yield of alcohol obtained from fermented mashes converted with this agent.

The correlation is not precise for two reasons: The quantity of mold liquor used for the conversion of 1 bushel of corn was not the same in all experiments; and a small amount of malt was employed with the fungal amylase in most of the runs. Figure 3 shows the superficial relationship between saccharogenic power of fungal amylase and the yield of alcohol. For the preparation of the graph the yield used was the average of those obtained in the two alcoholic fermentations conducted with a given batch of fungal amylase liquor. The data from plant run 27B were not used because of the inordinately large quantity of malt that was employed with the fungal amylase for conversion. It is indicated from the graph that to be a satisfactory converting agent the fungal amylase liquor should have a saccharification value of 25 percent or more. Of the 16 runs reported in table 1, 10 batches of mold liquor had a saccharification value of 24 percent or more. The other 6 preparations were poor because of contamination of the culture during the mold fermentation.

In the 10 plant runs in which fungal amylase of satisfactory quality was used, the average alcohol yield was 5.98 proof gallons per bushel of dry grain. During this same period of operation, the average yield of alcohol from malt-converted mashes was 5.87 proof gallons per bushel of dry grain. In experiments 9B, 12B, and 26B, 4 fermentors were set with mash converted with fungal amylase which had been supplemented with only a trace of malt, or in which no malt was used. It is significant that the average alcohol yield from these fermentors was 5.98 proof gallons per bushel of dry grain. This indicates that the 1 to 2 percent malt used in other runs to help thin the mash did not affect the alcohol yields.

Heat-damaged corn in experiments

In plant runs 17B and 22B, corn that was approximately 50 percent heat-damaged was mashed to determine whether fungal amylase could be used with this type of material. The results were satisfactory. An average alcohol yield of 6.04 proof gallons per bushel of dry grain was obtained from the four fermentors of these runs. The average yield from this type of corn converted with malt was 5.86 in fermentations conducted immediately before and after each of the experimental runs.

No difficulties or differences in operation were encountered in the distillation of beer from mold-converted mash. Feed recovery operations also were normal. Feed yields, although impossible to measure accurately, appeared to be the same as yields from malt stillage.

The use of fungal amylase for the conversion of yeast mashes was not investigated. All yeast cultures used in the plant experiments were prepared with malt-converted mash.

Quality of alcohol and feeds produced with fungal amylase

Samples of the alcohol and byproduct feeds recovered during the experimental runs were analyzed chemically for components which are used commonly as a means of evaluation. In addition, the alcohol was compared, organoleptically, with standard samples of high-grade spirits made with malt. The results of these tests are given in table 3. Included in this table is a typical analysis of products made with malt as the converting agent. There is no significant difference between these figures and the average values for the products made with fungal

TABLE 3.—*Properties of products made with fungal amylase as converting agent*

Run number	Alcohol			Organoleptic evaluation [1]		Feeds							
	Chemical analysis					Dried grains				Solubles			
	Esters	Aldehydes	Fusel oil	Odor	Taste	Moisture	Protein	Fat	Fiber	Moisture	Protein	Fat	Fiber
	G./100 l.	*G./100 l.*	*G./100 l.*			*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
9B	1.208	0.386	1.9	Good	Good	7.4	23.1	10.1	9.2	5.6	32.6	13.4	4.2
10B	.880	.385	2.3	do	do	8.0	24.1	10.2	9.2	4.3	26.7	12.1	3.8
11B	1.408	.375	2.1	Rejected	do	6.8	22.5	9.3	9.0	5.6	34.2	14.3	5.1
12B	.440	.380	2.3	Good	do	9.0	27.1	10.6	8.9	4.8	30.4	14.1	5.0
13B	1.320	.386	1.9	do	do	6.7	24.3	10.0	10.5	5.4	28.4	12.9	4.6
14B	2.476	.374	2.0	do	do	8.2	26.4	9.6	10.3	5.2	29.6	12.6	4.7
15B	1.584	.440	2.1	Rejected	Rejected	7.9	22.4	10.0	9.4	5.1	33.8	12.8	4.5
16B	.886	.397	2.2	Good	Good	8.6	24.1	10.2	8.8	4.6	27.6	14.2	4.8
17B	.528	.385	2.3	do	do	6.7	24.5	10.8	10.3	5.6	33.7	14.6	4.5
21B	2.204	.378	2.1	do	do	9.4	22.8	9.8	9.4	4.3	34.6	14.2	3.9
22B	1.606	.386	2.4	do	do	9.6	24.6	10.1	9.8	5.4	28.6	13.7	3.6
25B	2.486	.385	2.2	do	do	8.7	26.5	10.1	10.2	5.2	30.3	12.4	4.1
26B	.896	.392	2.1	do	do	9.1	23.4	10.4	9.4	4.9	31.0	14.5	3.8
27B	1.488	.384	2.3	do	do	6.4	25.1	9.6	9.2	5.1	28.7	13.7	4.2
Average	1.386	.388	2.14			8.0	24.4	10.1	9.5	5.1	30.7	13.5	4.3
Typical values [2]	1.220	0.392	2.20			9.4	24.9	9.5	9.9	6.0	32.0	12.5	4.5

[1] Evaluations relative to high-grade spirits made with malt. Frequency of rejection of mold alcohol no greater than that of spirits made with malt.
[2] Typical values for products made with malt as converting agent.

amylase as the saccharifying agent. The organoleptic evaluation of the alcohol produced from mold-converted mash showed it to be satisfactory.

Quantities of distillers' dried grains and distillers' dried solubles produced by the fungal amylase process and by malt conversion were supplied to the Experiment Station of the University of Nebraska and to the Bureau of Animal Industry, U. S. Department of Agriculture, for comparison by animal feeding tests. Preliminary reports have been made by each of these agencies on the progress of their experiments.[5, 6, 7] At the Nebraska Experiment Station steer calves which were fed grain and prairie hay along with the fungal amylase grains made an average daily gain of 1.8 pounds for 223 days, and required an average of 286 pounds of prairie hay, 599 pounds of ground shelled corn, and 146 pounds of distillers' product per 100 pounds of live-weight gain. The lot of calves fed the dried grains from malt-converted mash made an average daily gain of 1.68 pounds, and required an average of 302 pounds of prairie hay, 602 pounds of ground shelled corn, and 155 pounds of dried grains per 100 pounds of live-weight gain. A similar lot of cattle was fed prairie hay and ground shelled corn without any protein supplement. These animals made an average daily gain of 1.35 pounds and required 441 pounds of hay and 838 pounds of ground shelled corn for 100 pounds of gain.

In another series of tests by the Nebraska Experiment Station steer calves were wintered on a full feed of August-cut prairie hay, 2 pounds per head daily of ground shelled corn, and 1.72 pounds per head daily of the distillers' products. The lot fed fungal amylase grains made an average daily gain of 1.21 pounds and consumed an average of 880 pounds of hay, 165 pounds of corn, and 144 pounds of distillers' grains per 100 pounds of gain. The lot of calves fed grains from malt-converted mash made an average daily gain of 1.19 pounds and required an average of 876 pounds of hay, 168 pounds of corn, and 146 pounds of distillers' product per 100 pounds of live-weight gain.

The results of the Nebraska tests indicate that there is no significant difference in feed value between distillers' grains made with fungal amylase or with malt as the converting agent.

Investigations of the following type have been conducted or are in progress by the Bureau of Animal Industry on the byproduct feeds made with malt and with fungal amylase: A complete analytical study, including the amino acid and vitamin content of the byproducts produced by the two processes; toxicity and palatability studies with sheep and cattle; poultry- and swine-feeding tests where the solubles are used as vitamin carriers; and digestibility studies and practical sheep-feeding tests in which the dark grains are used as protein supplements.

The Bureau of Animal Industry found no significant difference in either chemical composition or amino acid content between compar-

[5] Baker, M. L., Col. of Agr. and Agr. Expt. Sta., Univ. of Nebr. (Private communication.

[6] U. S. Dept. Agr., Bur. of Anim. Indus. Prog. Rept. (Unpublished.)

[7] Dowe, T. W., and Arthaud, V. H., Col. of Agr. and Agr. Expt. Sta., Univ. of Nebr. Prog. Repts., No. 192 and No. 193. (Unpublished.)

able materials produced with malt and fungal amylase. The results to date of the palatability and toxicity tests with sheep indicate that the fungal amylase dark grains had no toxic or detrimental effect on the animals, although they were fed at 4 to 7 times the level ordinarily used in feeding work and for long periods of time. There are some indications that sheep have a slight preference for the conventional malt dark grains over the fungal amylase byproducts; however, the dark grains produced by the fungal amylase process were palatable, as they were consumed in large quantities throughout the test periods.

Practical sheep-feeding tests have shown that distillers' dark grains produced by either the malt or fungal amylase process can satisfactorily replace linseed oil meal as a protein supplement in a fattening ration for lambs.

No significant differences have been observed between solubles produced by either process when used in swine rations.

Preliminary chick feeding tests have been conducted by the Bureau of Animal Industry, in which the conventional malt solubles and also the fungal amylase solubles have been used as riboflavin carriers. Additional work is being done in order to obtain conclusive results.

ESTIMATED COSTS FOR INSTALLATION AND OPERATION OF FULL-SCALE FUNGAL AMYLASE PLANT

To calculate, for purposes of comparison, the cost of producing alcohol from grain mash saccharified with fungal amylase, an estimate has been made of the investment and production costs for a fungal amylase plant to operate in conjunction with the alcohol plant of the Grain Processing Corporation. It is emphasized that the estimates given are specific in that they apply to a possible installation at the Muscatine plant. Certain items of equipment and of operational expense pertain uniquely to this alcohol plant; hence, general application of the data should be made cautiously.

The design of the hypothetical plant and the cost estimates were based on the following considerations and assumptions:

1. The distillery is to mash 11,850 bushels (as received) of No. 2 corn per day for 27 days per month to produce 842,000 wine gallons of 190-proof or 1,600,000 proof gallons of alcohol per month. The yield of alcohol is assumed to be 2.63 wine gallons of 190-proof or 5.00 proof gallons of alcohol per bushel of grain (as received).

2. The estimates are based on the use of 3.1 gallons of fungal amylase liquor per bushel of corn. It is recognized that as little as 2.7 gallons of mold liquor per bushel of grain might be adequate; however, so that the costs might be conservatively high, the larger figure was used.

3. In view of considerations 1 and 2, the required average daily capacity of the enzyme plant is 36,800 gallons.

4. The maximum rate at which sterile air is supplied to the fermentors during production of the amylolytic liquor is 0.25 volume of air per volume of medium per minute. This rate of aeration is

probably higher than will be needed in actual practice with fermentors of favorable design.

5. The fermentors in which the fungal amylase liquor is produced are to operate on a cycle based on a 60-hour fermentation period.

PLANT OPERATIONS ON WHICH COSTS ARE BASED

Corn meal and calcium carbonate are fed quantitatively and continuously to a mixing tank along with fortified thin stillage from the feed recovery department of the distillery. The medium is sterilized by pumping it through a steam-jet heater and a holding pipe which constitute the cooker. The jet heater instantaneously raises the temperature of the medium to 300° F. (53 pounds per square-inch gage) and the liquor is retained at this temperature for 6 to 12 minutes. The sterile liquor is cooled to 86° by passage through a concentric-pipe heat exchanger. A portion of the medium is delivered to a seed-culture tank and the remainder to a fermentor which has been cleaned and sterilized.

Approximately 2.5 gallons of a culture of *Aspergillus niger*, NRRL 337, is prepared in the laboratory and used to inoculate 250 gallons of sterile medium in a seed tank. Aseptic precautions are observed throughout the plant and especially in the preparation of the inoculum. The seed culture is grown for 24 hours and then transferred to a fermentor that contains about 24,750 gallons of sterile medium. After a fermentation period of 60 hours at 86° or 87° F., the contents of the fermentor are pumped to the distillery as required.

Sterile air is supplied to both the fermentors and seed tanks at the rate of 0.25 volume per volume of medium per minute. The air is compressed and then sterilized by passing it through a column packed with activated carbon or through cotton filters.

During the period of operation (27 days per month) fermentors are set at the rate of approximately one every 16 hours. For one fermentor 2,080 pounds of corn, 520 to 1,040 pounds of calcium carbonate, and about 24,500 gallons of fortified thin stillage are required.

INVESTMENT AND OPERATING EXPENSES

The investment cost for the fungal amylase plant and for the necessary distillery modifications is estimated to be $305,000. An itemized account of this cost is given in table 4. No steam plant is included in the estimate, because the fungal amylase would be produced in conjunction with the alcohol plant of the Grain Processing Corporation, which has a steam plant of sufficient capacity to satisfy the requirements of the enzyme plant.

The estimated production costs for the mold amylase plant are presented in table 5. All charges are included except administrative expense. The cost to produce sufficient fungal amylase to convert 1 bushel of grain in the distillery is estimated to be 4.62 cents, which is equivalent to 1.76 cents per wine gallon of 190-proof alcohol, or 0.92 cents per proof gallon.

TABLE 4.—*Estimated cost of fungal amylase plant, equipment, and installation for operation in conjunction with alcohol plant of Grain Processing Corporation, 1949*

[Capacity of plant: 36,800 gallons of fungal liquor per day, operating 27 days per month]

Fungal amylase plant cost	Estimated total cost
Buildings and improvements:	*Dollars*
One building, tile, 80 x 60 x 30	75,000
One well; drilling, pump, motor, pumphouse, etc., 1,000 g.p.m.	14,000
Three transformers, 500 kv. = a., 13,200-440 volt	9,000
Total	98,000
Equipment:	
Six tanks, fermentor, working capacity—25,000 gal., with agitator, drive, and motor at $16,000	96,000
Six air compressors, 1,000 c.f.m. at 20 p.s.i.g. at $3,750	22,500
Four tanks, seed fermentor, working capacity 250 gal., with sparger, agitator, and motor at $1,500	6,000
One continuous cooker, complete with jet heater and controls	2,000
One heat exchanger, concentric pipe cooler, 2,000 sq. ft.	8,000
One bin, corn storage, with feeder	1,200
Two bins, chemical storage, with feeder	2,400
One hammer mill, with motor, complete	2,500
One tank, mixing, 2,000 gallons	750
Four air filters	2,000
One air receiver	1,000
Instruments and controls	5,000
Laboratory equipment	5,000
Total	154,350
Alterations in distillery:	
Stillage line from drier house	500
Pipe to increase capacity of conversion line	1,000
One controller (to control ratio of flow of fungal liquor to mash)	1,000
Total	2,500
Installation costs:	
Electric wiring and supplies	5,000
Pipe and pumps	15,000
Construction: Labor, exclusive of building	30,000
Total	50,000
Total	304,850
Total cost of plant, ready for operation	305,000

TABLE 5.—*Estimated monthly operating costs for the production of fungal amylase liquor in a full-scale plant, 1949*

[Capacity of plant: 36,800 gallons of fungal liquor per day, operating 27 days per month]

Fungal amylase liquor production cost	Estimated monthly cost	Cost per 3.1 gal. of liquor [1]
Raw materials:	*Dollars*	*Cents*
Corn, 82,900 lb. at $1.20 per bushel	1,776	0.555
Calcium carbonate, 41,400 lb. at $0.01 per lb.	414	.129
Total	2,190	0.684
Supplies	500	.56
Utilities:		
Electricity, 500,000 kw.-hr. at $0.005	2,500	0.781
Steam, 6,000,000 lb., coal cost only	2,150	.672
Total	4,650	1.453
Labor and supervision:		
One operator per shift, 720 hours at $1.40 per hour	1,008	0.315
Two laborers per shift, 1,440 hours at $1.25 per hour	1,800	.563
One chemist-bacteriologist, $400 per month	400	.125
One supervisor, $500 per month	500	.156
Total	3,708	1.159
Maintenance	500	0.156
Fixed charges:		
Depreciation—		
Equipment, 10 percent per year on $230,000	1,917	0.599
Building, 5 percent per year on $75,000	313	.098
Taxes and insurance, 4 percent per year on $305,000	1,017	.318
Total	3,247	1.015
Summary:		
Raw materials	2,190	0.684
Supplies	500	.156
Utilities	4,650	1.453
Labor and supervision	3,708	1.159
Maintenance	500	.156
Fixed charges	3,247	1.015
Total production cost	14,795	[2] 4.623

[1] 3.1 gallons is the quantity of liquor to be used for the conversion of 1 bushel of grain.

[2] Since the yield of alcohol per bushel is assumed to be 2.63 wine gallons of 190-proof or 5.00 proof gallons of alcohol, the cost for the fungal converting agent is 1.76 cents per wine gallon 190-proof or 0.92 cents per proof gallon of alcohol.

COMPARATIVE COSTS OF PRODUCING ALCOHOL

Table 6 shows the comparative costs of alcohol produced with malt and with fungal amylase. The assumptions used in making the calculations are stated. Operating costs to produce alcohol were based on data obtained from the Grain Processing Corporation and represent procedures and wage scales which prevailed during the experimental runs at Muscatine, Iowa.

TABLE 6.—*Comparative costs of alcohol produced with fungal amylase and with malt* [1]

Item	Distillery operation with	
	Malt	Fungal amylase
Bases:		
Alcohol yield per bushel No. 2 corn, as received.....wine gallons..	2.63	2.63
Dried byproduct feed recovered per bushel No. 2 corn, as received.....pounds..	16.0	16.0
Quantity of conversion agent used.....	0 percent	3.1 gal./bu.
Cost of corn, No. 2, per 56 lb. bushel, as received.....dollars..	1.20	1.20
Cost of malt, per 34 lb. bushel, as received...dollars..	1.70	.....
Cost of fungal amylase liquor, per 3.1 gal.....cents..	.....	4.62
COST PER WINE GALLON 190-PROOF ALCOHOL:		
Operating costs of alcohol plant:	*Dollars*	*Dollars*
Labor.....	0.0523	0.0523
Steam.....	.0349	.0349
Supplies.....	.0143	.0143
Electricity.....	.0077	.0077
General and administrative expense.....	.0316	.0316
Total operating costs.....	0.1408	0.1408
Raw material costs:		
Corn, 19.377 lb. and 21.293 lb.....	0.4152	0.4563
Malt, 1.916 lb.....	.0958	.....
Fungal amylase, 1.179 gal.....	.....	.0476
Total cost of raw materials.....	0.5110	0.4739
Total production cost [2].....	0.6518	0.6147
Savings by use of fungal amylase per wine gallon of 190-proof alcohol.....	.....	0.0371

[1] Figures based on 1949 costs and on information supplied by the Grain Processing Corporation, Muscatine, Iowa.

[2] These totals do not include credit for byproduct feed, rent, or profit, all of which would be the same for distillery operation with either malt or fungal amylase.

It should be noted that operating costs in the distillery are the same for both malt and fungal amylase. This is to be expected because the operations and yields of products are the same for both converting

agents. In order to use fungal amylase successfully, provision was made in the design of the fungal amylase plant to increase the retention time of the mash during conversion. This alteration is minor, and would not add to the cost of operation.

The savings in production cost of alcohol when fungal amylase is employed instead of malt amounts to 3.71 cents per wine gallon of 190-proof alcohol. If a distillery operated at a capacity of 11,850 bushels of grain per day, it is indicated that a total saving of $1,150 per day is possible.

The extent of the savings in production cost when fungal amylase is used instead of malt is the difference in price between barley malt and corn. This relationship is shown in figure 4. If 56 pounds of malt costs only 51.5 cents more than 56 pounds of corn there is no monetary advantage in using fungal amylase. However, if corn can be obtained for $1.00 per bushel and barley malt costs 6.0 cents per pound the potential savings with the fungal amylase process is 6.2 cents per wine gallon of 190-proof alcohol.

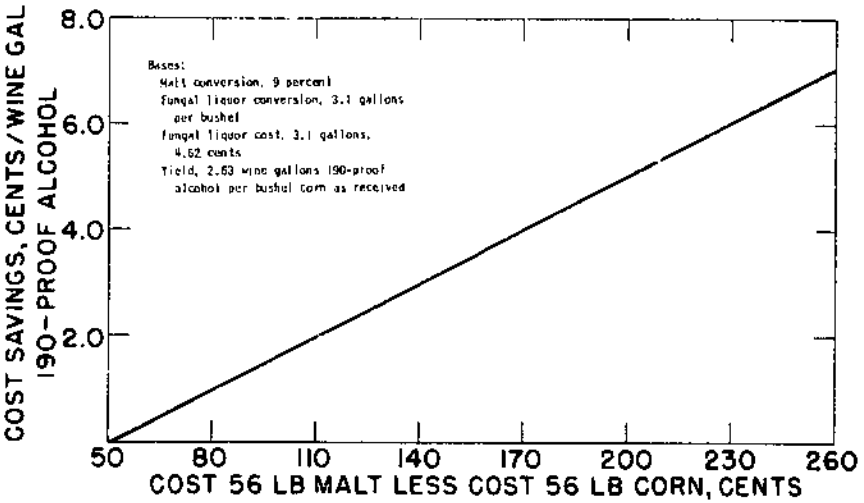

FIGURE 4.—Relationship between difference in costs of malt and corn, and the possible savings in production cost of alcohol by the fungal amylase process.

SUMMARY AND CONCLUSIONS

1. The preparation and utilization of fungal amylase by a distillery, and in conjunction with its operations, appear to be feasible and practicable. Only minor alterations in the distillery may be required in order to adapt the plant to the use of the mold liquor.

2. Satisfactory fungal amylase may be produced on a large scale, and in commercial equipment, in a medium consisting of distillers' thin stillage supplemented with 1 percent of ground corn and 0.5 percent of ground limestone. However, pure culture conditions must be maintained and the medium must be properly aerated and agitated during fermentation.

3. Fungal amylase liquor can be used to replace malt completely in mashing operations if the liquor is of good enzyme potency, and if proper conversion time and temperature are employed.

4. The yield of alcohol from sound corn saccharified with fungal amylase is at least equal to the yield obtained when malt is the converting agent.

5. When fungal amylase is employed in the mashing of heat-damaged corn, the yield of alcohol obtained is no less than that obtained when malt is used with the same type of corn.

6. There is no significant difference in enzyme potencies of fungal liquors prepared with stillage from mold-converted mash and from malt-converted mash. The alcohol yield is not affected when the fungal amylase is produced on stillage from mold-converted mash.

7. Mashes converted with fungal amylase contain fewer contaminants than do malt-converted mashes. Fermentation of mold-converted mash appears to proceed at a slower rate than with malt-converted material; however, conclusive data on this point were not obtained.

8. Fungal amylase liquefies mash at a slower rate than does malt under the conditions most favorable for the latter material. Higher conversion temperatures enhance the thinning power of the mold agent. It is believed that fungal amylase will liquefy mashes satisfactorily if a longer retention time during conversion and the higher conversion temperature are employed. This is of special significance to distilleries using continuous mashing systems.

9. The use of fungal amylase appears to have no detrimental effect on the operation of the distilling system or on the quality of the alcohol.

10. Feeds recovered from fermentations of mold-converted mash are, by appearance and chemical analysis, practically identical to feeds recovered from malt fermentations. The yields of feed appear to be the same. Limited information on the evaluation of fungal amylase feeds by animal-feeding tests indicates that these materials are of the same quality as malt feeds.

11. On the basis of cost calculations, there is a definite and significant economic advantage in using fungal amylase in place of malt for the conversion of grain mash.

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APPENDIX A

DETECTION OF BACTERIAL CONTAMINANTS IN FUNGAL AMYLASE MEDIUM AND IN FILTERED AIR

A sample of mold liquor is withdrawn aseptically from the fermentor or seed tank into a sterile flask. One ml. of this is transferred with a sterile pipette to a large test tube that contains 10 ml. of nutrient broth. The test medium is prepared in accordance with the directions given below. The broth culture is incubated for 24 hours at 88° F. A loopful of this culture is then transferred to a second tube of broth which is prepared just like the first one. The second tube is incubated for 24 hours at 88° F. and then examined for turbidity. If the broth is turbid, bacterial contaminants are present. The result should be confirmed by examination of the broth under the microscope.

DIRECTIONS FOR PREPARING TEST MEDIUM [1]

Nutrient broth		*Salt solution*	
Glucose	1 gm.	$MgSO_4 \cdot 7H_2O$	4 gm.
Yeast extract	1 gm.	$FeSO_4 \cdot 7H_2O$	0.2 gm.
Peptone	1 gm.	$MnSO_4 \cdot 4H_2O$	0.8 gm.
K_2HPO_4	0.5 gm.	NaCl	0.2 gm.
Sodium citrate	1 gm.	Conc. HCl	0.4 ml.
Sodium acetate	0.1 gm.	Water to make	100.0 ml.
Salt solution	2.0 ml.		
Water to make	100.0 ml.		

[1] Dispense 10-ml. quantities into test tubes; plug with cotton; sterilize 15 minutes at 250° F.; and cool.

Sterility of air used in the fermentor and seed tank is tested by the passage of a sample stream of it through 200 ml. of the described nutrient broth for 30 minutes. The broth is contained in a 1,000-ml. Erlenmeyer flask equipped with an air delivery tube, and with an opening plugged with cotton through which the air is vented. After aeration of the broth, it is incubated for 24 hours at 88° F. Sterility is shown by the absence of turbidity in the medium. The result should be confirmed by examination of the broth under the microscope.

Determination of Alpha-Amylase Activity in Fungal Amylase Preparations

The methods for the determination of alpha-amylase activity in fungal amylase preparations are based on the procedure of Sandstedt, Kneen, and Blish (*6*) and that given in the Journal of the Association of Official Agricultural Chemists, fifth edition (1947), page 96. The method depends on the length of time required for an enzyme preparation to hydrolyze beta-amylase treated starch to dextrins which give a characteristic color when the enzyme-substrate mixture is added to a solution of iodine and potassium iodide.

I. Reagents

(*a*) Stock iodine solution: 5.5 grams of iodine crystals and 11 grams of potassium iodide are dissolved in water and the solution is made up to 250 ml. The stock solution is stored in the dark. A fresh solution is made each month.

(*b*) Dilute iodine solution: 2 ml. of the stock iodine solution (I*a*) is added to 20 grams of KI dissolved in water and made up to 500 ml.

(*c*) Buffer solution: 120 ml. of glacial acetic acid and 164 grams of anhydrous sodium acetate are dissolved and made up to 1 liter.

(*d*) Beta-amylase: A special beta-amylase in dry form has been developed by Wallerstein Laboratories, New York, N. Y., for use in the preparation of the alpha-amylodextrin substrate in the determination of alpha-amylase in starch hydrolyzing materials by dextrinization procedures. The beta-amylase is standardized at 2,000° Lintner and to the specifications set by the Malt Evaluation Committee of the American Association of Cereal Chemists. The specifications require that "(1) at the addition level recommended there shall be a variation not greater than 5 percent in the dextrinization times of a given malt extract when one- and three-day old substrates are compared, and (2) a substrate prepared by adding twice the recommended level of beta-amylase shall not deviate by more than 5 percent from one prepared with the recommended level after 24 hours standing."

(*e*) Buffered beta-amylase limit dextrin (alpha-amylodextrin) solution: A suspension of 10 grams (dry weight) of soluble starch (accord-

ing to Lintner, "Special for Diastatic Power Determination") is poured slowly into boiling water. The solution is boiled for 1 to 2 minutes with stirring and then cooled. 25 ml. of buffer solution and 250 mg. of beta-amylase (*1d*) dissolved in a small amount of water are added to the starch solution. The volume is made up to 500 ml. and saturated with toluol. The solution is stored at, or close to, 30° C. for not less than 24 nor more than 72 hours before use.

(*f*) Standard solution: 100 ml. of 0.01 *N* HCl are added to 25 grams of $CoCl_2 \cdot 6H_2O$ and 3.84 grams of $K_2Cr_2O_7$. 10-ml. portions are placed in test tubes which are then sealed. These standard solutions may be kept indefinitely.

II. Enzyme Reaction

(1) The fungal amylase preparation (culture filtrate), the substrate (*1e*) and iodine (*1b*) solutions are all attempered to 30° C. Five ml. of fungal amylase preparation (or appropriate dilutions thereof) is added to 10 ml. of the substrate solution (*1e*). The instant that the fungal amylase preparation is added to the substrate solution, the time is noted. At appropriate time intervals, 1-ml. samples of the reaction mixture are transferred to test tubes containing 5 ml. of the iodine solution (*1b*). The optical density of the mixture is compared with that of the standard (*1f*) in a rapidly acting photometer (type similar to Lumetron model No. 400 with a 650μ filter). The instrument is set so that the standard (*1f*) gives 50 percent transmission. When the reaction has proceeded to the point where the enzyme-substrate solution gives 50 percent transmission when added to the iodine solution (*1b*) the end point has been reached and the time is noted. The elapsed time is referred to as the dextrinization time.

Samples should be taken and read every 15 seconds as the end point is approached. Although a photometer is desirable and convenient to use in this determination, visual reading may be made when such an instrument is not available.

III. Calculation

The alpha-amylase activity may be calculated from the following equation, where D.T. is the dextrinization time.

$$\frac{0.2}{1} \times \frac{60}{\text{D.T.}} \times \frac{\text{diln.}}{\text{ml. enz. sol.}} = \text{grams beta-amylase treated starch dextrinized per ml. of original enzyme solution per hour.}$$

Example No. 1:

5 ml. of 1:100 diln. of fungal amylase has a D.T. of 10 minutes.

$$\frac{0.2}{1} \times \frac{60}{10} \times \frac{100}{5} = 24 \text{ grams beta-amylase treated starch dextrinized per ml. of original enzyme solution per hour.}$$

Example No. 2:

1 ml. of 1:20 diln. of fungal amylase plus 4 ml. of water has a D.T. of 10 minutes.

$$\frac{0.2}{1} \times \frac{60}{10} \times \frac{20}{1} = 24 \text{ grams beta-amylase treated starch dextrinized per ml. of original enzyme solution per hour.}$$

For convenience, table 7 has been compiled from the foregoing equation.

TABLE 7.—*Grams of beta-amylase treated starch dextrinized per ml. of original enzyme solution per hour*

Minutes	0	¼	½	¾	Minutes	0	¼	½	¾
10	0.240	0.234	0.229	0.223	21	0.114	0.113	0.112	0.110
11	.218	.213	.209	.204	22	.109	.108	.107	.105
12	.200	.196	.192	.188	23	.104	.103	.102	.101
13	.185	.181	.178	.175	24	.100	.099	.098	.097
14	.171	.168	.166	.163	25	.096	.095	.094	.093
15	.160	.157	.155	.152	26	.092	.091	.091	.090
16	.150	.148	.145	.143	27	.089	.088	.087	.086
17	.141	.139	.137	.135	28	.086	.085	.084	.083
18	.133	.132	.130	.128	29	.083	.082	.081	.081
19	.126	.125	.123	.122	30	.080			
20	.120	.119	.117	.116					

The use of the table is demonstrated in the following examples:

5 ml. of 1:100 dilu. of fungal amylase has a D.T. of 10 minutes.
10 min. ÷0.24; dilu. is 1:100.
0.24 × 100 = 24 grams of beta-amylase treated starch dextrinized per ml. of original enzyme solution per hour.
1 ml. of 1:20 dilu. of fungal amylase plus 4 ml. of water has a D.T. of 10 minutes.
10 min. ÷0.24; dilu. is 1:20 and 1:5.
0.24 × 20 × 5 = 24 grams of beta-amylase treated starch dextrinized per ml. of original enzyme solution per hour.

IV. PRECAUTIONS

(1) The type of starch used is very important. Soluble starch prepared according to Lintner, "Special for Diastatic Power Determination," must be used. Emphasis is placed on the term "Special for Diastatic Power Determination." The starch must be of grade equivalent to Merck's soluble starch, according to Lintner, "Special for Diastatic Power Determination."

(2) In the preparation of buffered beta-amylase limit dextrin (Ic), the starch must be in complete solution after boiling for 1 to 2 minutes. Formation of starch film must be avoided by pouring while hot into the volumetric flask and cooling quickly prior to the addition of the buffer (Ic) and water to make up to volume.

DETERMINATION OF MALTASE ACTIVITY IN FUNGAL AMYLASE PREPARATIONS

The method, developed at the Northern Laboratory for the determination of maltase activity in fungal amylase preparations, is based on the observation that an increase of 78 percent in reducing power is obtained when maltose monohydrate is hydrolyzed to glucose under the conditions given below. There is a stoichiometric relationship between the amount of enzyme and the hydrolysis rate, when the rate is calculated from the difference in maltose hydrolyzed at 15 and 120 minutes.

I. REAGENTS

(*a*) pH 4.4 acetate buffer (6.0*M*), 217 ml. of glacial acetic acid, and 183 grams of anhydrous sodium acetate are dissolved and diluted to 1 liter with water.

(*b*) Acetate buffer (0.3*M*), maltose substrate (0.06*M*) solution. 2.35 grams of maltose monohydrate (92 percent pure as calculated on reducing value, for example, of Eastman Kodak Co. product) and 5 ml. of acetate buffer are diluted to 100 ml. with water.

(*c*) 1*N* sulfuric acid, 1*N* sodium hydroxide, phenolphthalein indicator.

(*d*) Reagents for sugar estimation by method of Somogyi (7).

II. Enzyme Reaction

Five ml. of fungal amylase preparation (culture filtrate) and 10 ml. of buffered substrate solution, both attempered to 30° C., are placed in a test tube and the tube is incubated in a water bath at 30° C. After 15 minutes, a 3-ml. aliquot of the reaction mixture is transferred to a 100-ml. volumetric flask containing 3 ml. of 1*N* H_2SO_4 to inactivate the enzyme. After 120 minutes, a second 3-ml. aliquot of the reaction mixture is treated in similar manner.

III. Analysis

After 10 minutes, the acidified reaction mixtures are adjusted to the phenolphthalein end point with 1*N* sodium hydroxide solution and made up to 100 ml. with water. Five ml. aliquots are taken for analyses for reducing value (R.V.) by the method of Somogyi (7), using the 20-minute heating period.

In this procedure, the R.V.'s of the reaction mixtures, obtained after 15 and 120 minutes' hydrolysis, are measures of glucose produced, residual maltose, and reducing sugars in the enzyme preparation.

IV. Calculation

a = R.V. of reaction mixture incubated for 15 minutes.

b = R.V. of reaction mixture incubated for 120 minutes.

$$\frac{(b-a)}{0.78} \times (\text{glucose equivalent of } Na_2S_2O_3 \times 1.78) \times 20 \times \frac{60}{105} = \text{mg. maltose hydrolyzed ml. of enzyme preparation in 1 hr.}$$

V. Precautions

Hydrolysis rate values should be between 2 and 10 mg. maltose hydrolyzed ml. enzyme preparation in 1 hr. to be acceptable. Values in higher range are preferred.

A pH of 4.4 must prevail in reaction mixture. Fungal amylase preparations highly buffered at pH values other than 4.4 must be adjusted to approximately this point before testing.

Determination of Saccharogenic Activity of Fungal Amylase

The method of analysis was adapted by Erb, Wisthoff, and Jacobs (2) from a combination of two analytical procedures — methods 20.61, p. 257, and 20.28, pp. 244-245 — given in the Journal of the Association of Agricultural Chemists, sixth edition (1945).

Reagents

(*a*) Starch solution (3 percent). Make a paste of 3 grams of Lintner soluble starch with cold water. Pour slowly into about 70 ml. of boiling water. Cool and add water to 100 ml.

(*b*) Buffer solution. Make 6 ml. of glacial acetic acid and 8.2 grams of anhydrous sodium acetate to 1 liter with water. The pH of this solution should be 4.6 to 4.8.

(*c*) Sulfuric acid solution. (3.58 *N*). 50 ml. conc. H_2SO_4 diluted to 500 ml. with water.

(*d*) Sodium tungstate solution. 12 grams diluted to 100 ml. with water.

(*e*) Ferricyanide solution. (0.1 *N*). 33.0 grams of pure dry $K_3Fe(CN)_6$ and 44.0 grams of anhydrous Na_2CO_3 per liter.

(*f*) Acetic acid-salt solution. Make up 200 ml. of glacial acetic acid, 70 grams of KCl, and 40 grams of $ZnSO_4 \cdot 7H_2O$ to 1 liter with water.

(*g*) Starch and KI solution. Add 2 grams of soluble starch to small quantity of cold water and pour slowly into boiling water with constant stirring. Cool thoroughly (or resulting mixture will be dark colored), add 50 grams of KI and make up to 100 ml. with water. Add 1 drop of NaOH solution (saturated).

(*h*) Thiosulfate solution. 24.82 grams of $Na_2S_2O_3 \cdot 5H_2O$ and 3.8 grams of $Na_2B_4O_7 \cdot 10H_2O$ per liter.

PROCEDURE

The following are placed in a 100-ml. Erlenmeyer flask:

20 ml. starch solution (3-percent Lintner soluble starch solution).
25 ml. buffer solution.
1 ml. fungal amylase (filtrate from mold culture).

Hold for 1 hour at 30° C. in a water bath. Add 2 ml. of 3.58 *N* H_2SO_4 and 2 ml. of sodium tungstate solution. Filter at once; discard first 8 to 10 drops. Pipette a 5-ml. aliquot into a 1- by 8-inch Pyrex test tube and add 10 ml. ferricyanide solution. Hold exactly 20 minutes in a boiling water bath. Cool to room temperature and empty the tube into a 100-ml. Erlenmeyer flask. Rinse with 25 ml. acetic salt solution, add 1 ml. starch + KI solution, and titrate with 0.1 *N* thiosulfate solution. Record ml. of thiosulfate used. Run a blank containing only starch and buffer in each test.

CALCULATIONS

Use table on page 245, A.O.A.C., sixth edition (p. 216, A.O.A.C., 5th edition), to find the amount of maltose corresponding to the 0.1 *N* ferricyanide used. Divide by 20 to give the mg. of maltose per 60 mg. of starch. This figure divided by 60 gives the percentage of conversion in 1 hour at 30° C.

Example:

Blank	9.68 ml.
Test	1.52 ml.
	8.16 ml. = 475 mg. maltose

$$\frac{475}{20} \times \frac{1}{60} = 39.58 \text{ percent conversion}$$

If the results show over 40 percent conversion, a smaller aliquot than 5 ml. may be taken for the ferricyanide test and the results calculated accordingly.

APPENDIX B

APPARATUS AND TECHNIQUES EMPLOYED IN THE PRODUCTION AND TRANSFER OF INOCULUM

The first steps in the preparation of inoculum involve culturing of the mold on slants. A few spores are transferred from the parent or stock slant cultures to a second slant which is incubated to propagate the fungus, and a small portion of the vegetative growth is then transferred to 200 ml. of broth in a 1-liter Erlenmeyer flask. No special techniques or equipment are required for these operations; standard bacteriological procedures are quite satisfactory.

After the flask-culture is incubated on a shaker for 24 hours part of the culture-liquor is used to inoculate a second flask identical in size

to the first flask, and which contains the same quantity of medium. A pipette is used to make this transfer and regular aseptic precautions are observed. The second flask, like the first, is simply plugged with cotton, as shown in figure 5-*A*.

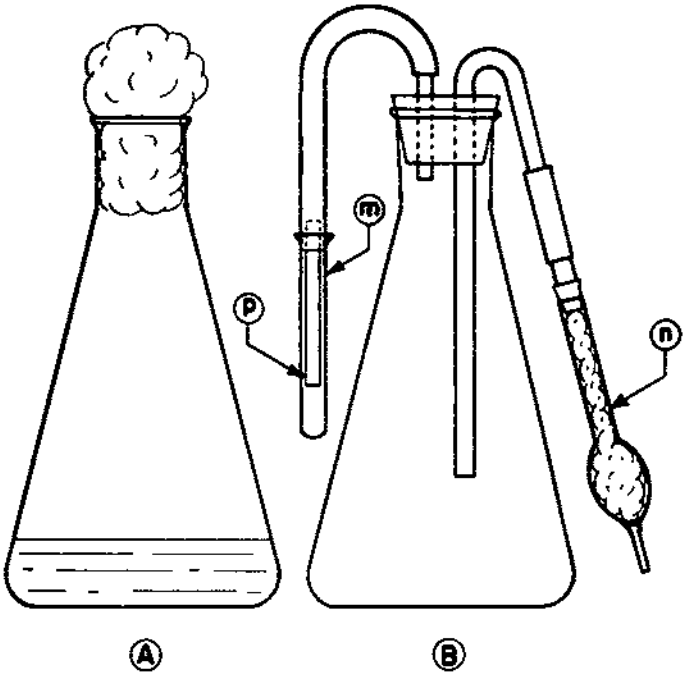

FIGURE 5.—*A*, Shaker-flask and, *B*, apparatus for transferring culture.

After incubation, the total contents of the second flask are used to inoculate a carboy of sterile medium. The carboy with special fittings is shown in figure 6-*A*. It is equipped with a tight-fitting stopper which has openings for three tubes. Tube (*a*) is a vent and serves also as the inoculum inlet; tube (*b*) is the line through which the carboy culture is discharged; and air is passed into the culture through tube (*c*). The length of tubing (*e*) is packed with cotton or glass-wool. Air that is passed into the culture during fermentation is sterilized by means of this filter.

To prepare the carboy culture the bottle is charged with medium and closed with the stopper that is complete with appurtenances. The stopper is fastened in place securely by means of a wire clamp. Vent-tube (*a*) is plugged with cotton and the special fitting (*d*) is plugged and wrapped with cotton. Pinch-clamps (*f*) and (*g*) are closed. The entire unit is then sterilized in an autoclave for 60 minutes at 267° F. (25 p.s.i.g.).

Inoculum is transferred from the second shaker flask into the carboy. For this purpose the apparatus shown in figure 5-*B* is used. The glass tube (*p*) is protected by means of the test tube (*m*) which fits snugly onto the rubber tubing. The filter tube (*n*) is packed with cotton. The

assembly, including the stopper, is placed in an empty 1-liter flask and the stopper and top of the flask are wrapped in cotton. The whole unit is then sterilized in an autoclave. To transfer inoculum from a shaker flask to a carboy the assembly is shifted from the empty flask to the

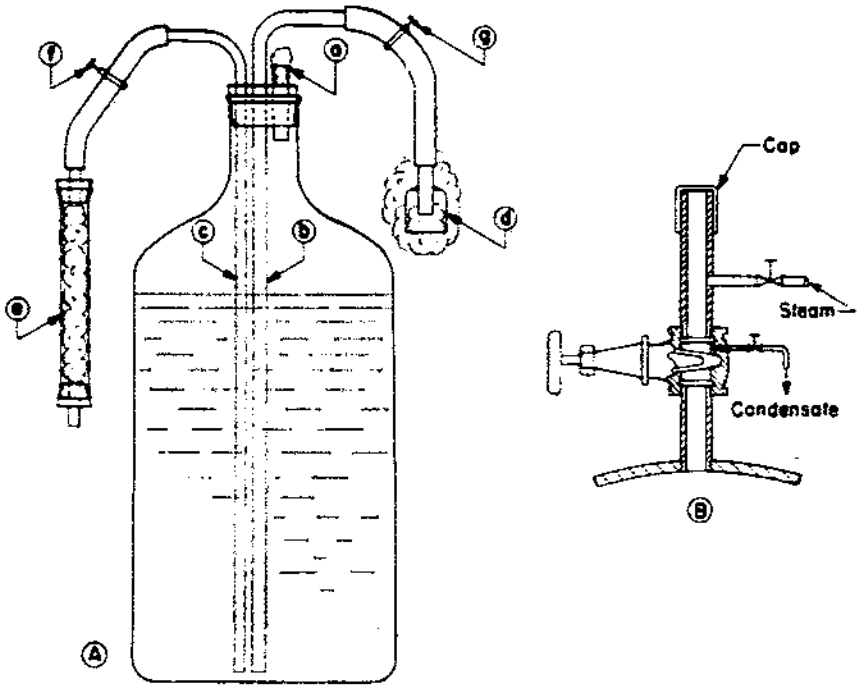

FIGURE 6. - Details of: *A*, Carboy in which culture is grown; and *B*, inoculum valve on seed tank.

shaker flask; the test-tube is removed from the glass delivery tube; the cotton plug is removed from the vent opening in the carboy; and the delivery tube of the flask assembly is inserted into the carboy vent. These operations are conducted aseptically. The flask is then inverted and the culture flows into the carboy. If clumps of mycelium plug the delivery line, air is pumped into the flask by means of an aspirator bulb attached to the end of the filter, in order to force the culture through the tube. After the transfer is made, the carboy vent is again plugged, an air line is attached to the end of the air filter (*e*), pinch-clamp (*f*) is opened, and the flow of air into the carboy is begun.

Inoculum is transferred from the carboy into a seed tank through fitting (*d*), (fig. 6-*A*) into the inoculum valve on the seed tank. A sketch of the valve assembly is given in figure 6-*B*. Except when the tank is being seeded the inoculum valve is closed, the drip valve is open, and sufficient steam is passed into the pipe nipple above the valve so that vapors escape from around the cap and through the drip line. At the time of inoculation, the cap is removed, the cotton covering and plug removed from fitting (*d*) of the carboy, and the adaptor inserted into the opening of the inoculum valve assembly. The

inoculum valve is opened, the steam and drip valves are closed, and pinch clamp (*g*) is opened. Air is passed into the carboy through the filter (*e*), the vent (*a*) is closed by covering it with a finger, and the pressure which develops forces the culture liquor into the seed tank.

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