NOTE TO USERS

EVALUATION OF AMYLOLYTIC AGENTS EMPLOYED IN THE ALCOHOLIC FERMENTATION

By

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I. INTRODUCTION

Ethanol has become the most useful industrial organic compound known to our present civilization. The only other chemical compound that surpasses it in number of uses is water. Human history gives it the distinction of being the product of one of the oldest chemical processes known, namely, fermentation. The chemical investigation of the methods of its production are of extreme importance since ethanol enters either directly or indirectly into the preparation or processing of more materials than any other organic compound.

During the past emergency the production of ethanol surpassed that of any other pure industrial liquid chemical. The peace time production of 247,000,000 proof gallons in 1940 was increased to more than 1,200,000,000 proof gallons in 1945. Prior to 1941, blackstrap molasses, an imported by-product from the sugar industries abroad, had been used almost entirely for the fermentative production of ethanol. Its unavailability after late 1941 made the use of corn of prime importance, and for some time corn was used exclusively.

Increased use of ethanol in the synthetic rubber program and in the manufacture of explosives made an increased production imperative. Such an increase was only possible

through a huge expansion program in the industrial yeast fermentation of starchy substrates to ethanol. This expansion necessitated the use of other starchy substrates such as wheat, barley, rye, oats, and sorghum grains to supplement the inadequate supply of corn.

Since all starchy substrates must be converted to fermentable sugars, a process called saccharification, before fermentation by yeast is possible, there was an increased demand on the barley malt supply which is generally used for the saccharification of starchy materials prior to fermentation. The desperate need for barley malt caused a deterioration in the quality of the malt amylases, since the time-consuming processes used to make good barley malt were speeded up and also less satisfactory barley had to be used for malting. Mold bran, the result of a 10 year pioneering research program conducted in four laboratories, Iowa State College, University of Idaho, University of Nebraska, and Farm Crops Processing Corporation, was used very successfully as a malt supplement. Large plant scale usage has proven that it gives slightly better yields of alcohol, shortens the time for complete fermentation, produces more yeast cells, and can be produced cheaply from abundant raw materials.

It has been known for many years that amylases from various sources and prepared differently show marked variation

in properties, such as extent of liquefaction, dextrinization, and saccharification of starch; temperature for optimum activity; thermal stability; and optimum pH range. These differences in properties have occasioned much interest in recent years and investigations have been made to determine more accurately the special properties of cereal, bacterial, and fungal amylases in an effort to be able to select or adapt the most favorable for specific industrial uses. Chemical tests have been devised for determining the amylase content of amylolytic agents. The methods commonly used to measure the activity of amylolytic preparations include polarimetric, iodometric, reducing power, and viscosity measurements.

These chemical tests in general measure the α-amylase, β-amylase, or the total α- and β-amylase activity of the agent employed, and, although they afford a very quick measure of the potency, there can be found no correlation between any of these test methods and actual performance in a complete fermentation. Such measurements of amylase activity are indeed helpful, but they give neither accurate information as to the suitability of the amylolytic agent for fermentation mashes nor any indication of the required levels for most efficient use. Obviously, factors other than those measured by chemical tests enter into the effectiveness of amylolytic agents for saccharifying fermentation mashes.

Since the saccharifying agents are much more expensive than the starchy materials processed, for most efficient and economical use it is necessary that a means be afforded for testing total amylolytic activity, and thus make it possible to adjust the proportions to be employed for maximum yield and minimum expense.

Since most of the chemical tests have been devised for measuring the amylolytic activity of malt, the correlation of these chemical tests for total amylase activity with the fermentation results using mold bran was even less favorable than with malt. The chemical test method commonly used for mold bran is the α-amylase evaluation of Sandstedt, Kneen, and Blish (1939). Mold brans with high α-amylase values are quite certain to be satisfactory for saccharifying fermentation mashes, but no prediction can be made from these α-amylase values of the optimum level of mold bran to be used for maximum ethanol production.

It has been stated by many competent observers that the most conclusive test for any saccharifying agent to be used with fermentation mashes is an actual fermentation on a series of mashes with several levels of the amylolytic agent. This requires much labor and three or four days for completion. There is a great need for a test that would have all the advantages of the tedious three to four day fermentation method, but would require a shorter length of time and would give an

exact fermentation evaluation. It was the major purpose of this investigation to develop such a method for the evaluation of amylolytic agents used for the saccharification of mashes in the alcoholic fermentation.

II. REVIEW OF LITERATURE

A. Methods for the Evaluation of Amylases

The art of using malt and molds as amylolytic agents in the fermentation of starchy materials is centuries old, but the scientific study of malt and mold enzymes has a history of only about one and a half centuries.

Irvine in 1785 is, according to Pringsheim (1932), given credit for discovering amylase in malt, but no further details are available since there is no literature reference cited. The first recorded observation involving an enzymatic action upon starches was that of Guyton (1798), who noted that the fermentation of starches gave some unidentified sugars. He was undoubtedly not aware of the amylolytic processes involved.

The first known amylolytic reaction on starch, converting it to sugars, was recorded by Nasse (1814) who noted that starch extracted from living plants was capable of effecting its own conversion into sugars. This transformation was not observed when the starch was taken from plants which had been killed by boiling in water. Kirchoff separated a glutinous constituent from wheat meal which was able to liquefy potato starch paste, fermentable sugars being formed. It is unique also that Kirchoff (1815) should have been the first to re-

port the conversion of starch to a different type of sugar by a glutinous component of malt; the true catalytic effect of the enzyme was noted and the new substance, maltose, clearly characterized. He concluded that the starch-sugar transformation was a necessary step in the alcoholic fermentation of amylaceous materials. These fundamental observations constitute the beginning from which all other investigations on the subject have developed.

In 1830, Dubrunfaut made an extract of malt which converted starch into fermentable sugars, and in 1833, Payen and Persoz precipitated from malt extract, by using alcohol, a substance capable of hydrolyzing starch. They called this substance, which was dried and preserved, "diastase", the French term for enzyme. However, to avoid confusion, "amylase" appears preferable inasmuch as "amylase" is concerned with the hydrolysis of the carbohydrate components in starch and is the most active principle in malt and mold brans. A new field of chemistry was opened by the observations of Payen and Persoz, and the methodical study of amylolytic enzymes was begun.

In 1847, Dubrunfaut definitely proved that maltose was one of the products of the action of malt extract on starch by a series of studies on the optical rotation of the resultant solutions. This was confirmed by Musculus (1860) and Payen (1865), who added that dextrins are among the hydrolysis

products, and definitely stated that in the alcohol production from grains practically all of the starch is converted to fermentable sugars and subsequently to alcohol. The work of O'Sullivan (1872, 1879) and Schulze (1874) culminated nearly a century of preliminary examination and firmly laid the foundations for our knowledge of the enzymic degradation of starch.

Since that time, a very extensive literature has developed concerning the actions of enzymes on starch based upon research on the structure of starch and on enzymic action. The survey which follows will be limited to the literature pertinent to the evaluation of the amylolytic agents employed in the alcoholic fermentation and there is no intention of making a comprehensive review of the literature devoted to the study of amylases, modes of action, and hydrolysis products. However, in this connection the comprehensive survey of Walton (1928) who compiled the titles, and in many cases an abstract, of 3,485 pertinent articles covering the period from 1811 to 1925, and books by Samec (1927), Euler (1928), and Pringsheim (1932) are worthy of mention. The many papers given over a period from 1939 to date by Karl Myrbäch go into much detail on some phases of starch degradation. A review by Preece (1941) listing many references is also worthy of note.

The exact nature of the enzymic hydrolysis of starch was first postulated by Maercker (1878), who, from his studies on

the hydrolytic products, suggested the existence of two "diastase ferments" in malt. This idea seemed almost forfotten for the next 40 years until experimental evidence of his own and other workers convinced Chrzaszcz (1913) that the amylases in malt had at least a threefold effect, namely, starch-liquefying, starch-dextrinizing, and starch-saccharifying. One enzyme did not seem to be adequate. In his study of hydrolytic products from the action of amylase on starch Friedrichs (1913) came to the conclusion that malt amylase was a β-enzyme and assumed that maltose residues were connected by β-linkages, since he obtained dextrins in the early stages of the hydrolysis and maltose and glucose later.

The two "diastase ferments" of Maercker were not confirmed until Ohlsson (1922) was able to show their existence using iodine and reduction reactions to follow the progress of starch hydrolysis by malt amylase. He separated both of them on the basis of their relative stability toward heat at different pH values. He called these two fractions a "dextrinogenase" (α-amylase) which hydrolyzes starch into dextrins, and a "saccharogenase" (β-amylase) which converts starch and dextrins to maltose. Sjöberg and Erikson (1924) confirmed the work of Ohlsson by noting the variation in proportion between dextrinizing power and saccharifying power in different enzyme preparations and the different inhibition of these two functions by maltose and glucose. Euler and Helle-

berg (1924) gave support to Ohlsson and to Sjöberg and Eriksson by discovering from polarimetric studies that the specific rotation of the hydrolyzate from the action of malt extract on starch was 121°, while that for α-maltose was 168°, for β-maltose was 118°, and for an equal mixture was 136°, thus showing the maltose liberated from starch was predominately the β-form.

Following up his earlier work Ohlsson (1926) prepared very pure "dextrinogenase" and "saccharogenase" and studied their optimal activity at various pH values, noting that for the former the optimal pH is 5.5-6, and for the latter 3.8-5.5. Kuhn (1924), Pringsheim and Leibowitz (1925), and Polak and Tychowski (1929) adopted the view that α-amylase and β-amylase split α-glucosides and β-glucosides respectively. Blom, Bak, and Braae (1937) believed that α-amylase was responsible for the liquefying action, whereas the action of β-amylase was purely saccharogenic. A decade of intensive investigation led Myrbäch and Lundén (1946) to believe that the liquefying, dextrinizing, and some saccharifying action was attributed to α-amylase, while the β-amylase was purely saccharogenic.

Kirchoff (1815) clearly demonstrated that the action of maltose-free amylase on starch did not give a complete conversion to maltose, but that a portion of the starch was resistant to amylolytic action, this portion later being given

the name dextrin. Musculus (1860) and later O'Sullivan (1872, 1879) demonstrated that there are several dextrins formed by the amylolytic action of malt extract. They, being unable to obtain the theoretical amount of maltose, explained that this is an equilibrium reaction, and the sugar already formed interfered with further saccharification. Sjöberg and Erikson (1924) were mentioned earlier as having discovered that maltose and glucose inhibited the two functions of dextrinizing and saccharifying. A year earlier Pringsheim and Schmalz (1923) stated that at the point of limiting degradation there remains a "limiting dextrin" which has since been proven to be identical with the starch-trihexosan. They obtained from a fresh yeast extract an activator or coenzyme of protein origin that together with malt amylases would hydrolyze starch to an almost theoretical yield of maltose. Their name of "complement" remains in the literature today. Confirmation by Kuhn (1925), Sjöberg (1925), Lüers and Wieninger (1925), and Hoop and van Laer (1925) of the investigations of Pringsheim and Schmalz gave another factor to be considered in the evaluation of the amylases. These studies also brought out the fact that over 96 per cent of the starch is converted to maltose, provided enough "complement" is present during the hydrolysis.

Pringsheim and Fuchs (1923) and Pringsheim and Beiser (1924) were able to show that the amylases and their activa-

tors occur together in nature since they obtained crude "complement" from barley malt extract by dialysis followed by purification by an ethanol precipitation procedure. The variance in proportion of activators to amylases in nature helps to explain why the limiting degradation does not stop at exactly the same stage for various malt samples using identical procedures. An additional note of interest was supplied by Myrbäch and Örtenblad (1939) and Snider (1941) who suggested that part of the β-amylase in barley malt was bound to an insoluble protein making it inactive until freed by the action of a proteolytic enzyme. Thus the elucidation of Pringsheim's "complement" may be accomplished soon.

A natural consequence of the work of Kirchoff (1815), who demonstrated that amylases will convert starch to fermentable sugars, was the development of methods for estimation of the activity of any and all enzymic preparations. Such measurements may be broadly classed under the following headings:

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

1. Polarimetric methods. The application of angular rotation of plane-polarized light to the estimation of the

amylolytic power of malt extract was suggested by Biot and Persoz (1833) who obtained, by acid hydrolysis, a gum they called "dextrine" because it gave a strong rotation to the right of the plane of polarized light. Dubrunfaut (1847) used this method to show that a sugar, later called maltose, produced by the action of malt extract on starch, gave an angle of optical rotation about three times as great as that of glucose. Following these early leads O'Sullivan (1872) in the first real concise investigations used this method to identify as well as determine the amounts of the hydrolytic products from the action of malt extract on starch paste.

Brown and Heron (1879), Wiley (1882), and Brown and Morris (1895) confirmed and supplemented the earlier reports with the use of this method. The latter authors were able to correctly determine the percentage of maltose in the end products of starch hydrolysis by malt amylases, their measure of amylolytic activity.

Twenty years later Wolff (1915) using a Zeiss interferometer obtained data on various mixtures of pure starch and glucose, plotted the scale divisions against the grams of glucose, and was thus able to determine the amount of glucose produced by the action of malt extract upon starch. Gore (1924) obtained an estimation of the amylolytic action of malt extract on starch by noting the decrease in the angle of rotation of their solutions over a 25 minute period of time,

and from a mathematical equation computed the amylolytic power in degrees Lintner. Euler and Helleberg (1924) were able to show from optical activity studies on pure α- and β-maltose that the maltose obtained by enzymic action on starch was mainly the β-form and thus the measurement was primarily one of the β-activity of malt.

Effront (1932) followed the lead of Gore (1924) and developed the now classical method of determining amylase activity by noting the decrease in the angle of rotation of a starch solution being acted upon by malt extract under specific conditions. Little else seems to have been added to this method in the intervening years.

2. Viscosity methods. Although it had been known for many decades that starch was liquefied by the action of malt amylases, no method of merit seems to have appeared until Northrop and Hussey (1923) developed a quantitative method for the determination of amylase activity using an Ostwald viscosimeter and the principle that the time to give a certain per cent change in viscosity is inversely proportional to the amount of the enzyme present. Their test was based on relative viscosity. Davison (1925) repeated the work of Northrop and Hussey and modified their method using Lintner starch and a boiled enzyme solution as a blank and comparison standard. His unit is the amount of enzyme which will reduce the viscosity of the starch 20 per cent in 60 minutes. Ches-

ley (1931) examined the validity of Davison's viscosimetric method for the determination of amylase comparing it with the iodine method of Wohlgemuth (1908). Chesley found no comparitive basis upon which the two methods could give the same values for an evaluation number.

Jozsa and Gore (1930) used a special 5 per cent starch paste of very stable and consistent viscosity for their method and expressed the results in terms of the enzyme and substrate. Fletcher and Westwood (1930) modified the method of Jozsa and Gore using a higher amylase concentration and obtained better results. Thompson, Johnson, and Hussey (1931) revised the original Northrop and Hussey method to give results especially for concentrated amylolytic solutions. At about the same time Chrzaszcz (1931) was developing his method using varying increments of malt extract on 10 ml. of 10 per cent starch paste, and the first tube in which the starch would just flow was used in his evaluation scheme. His results are expressed as the quantity of enzyme necessary to liquefy one gram of starch under specified conditions.

A very critical review by Chrzaszcz and Janicki (1932) of fifteen methods used to determine the liquefying power of amylases gave experimental evidence showing many of them to be very unreliable and proposed a total of eighteen modifications to improve them. The review covers the principles of liquefying, dextrinizing, and saccharifying as applied to

viscosity. An interesting modification of the viscosimetric principle is the one proposed by Krijgsman (1934) who used a Pulfrich photometer and noted the time required by the α-amylase of malt to decrease the opacity of a standard 3.0 per cent glycogen solution.

Blom and Agnete (1938), noting the difficulty of preparing a perfectly reproducible starch paste for any viscosity measurement, used a 45.0 per cent sucrose solution as the standard and then compared the reduction of the viscosity of a standard 3.0 per cent starch paste containing 0.1 ml. amylase and 1.0 ml. acetate buffer to keep the pH at or near 5.0. An equation was given to express their results.

The firm establishment of α-amylase as the liquefying portion of malt amylases gave impetus to future work and the results of a new modification by Blom (1939) were given in terms of the liquefying power of α-amylase. Blom used a saccharification method to determine the activity of the β-amylase component. In a review of many of the methods of determining the liquefying power of a malt Teplitskiĭ and Vodyagina (1939) came to the conclusion the viscosimetric methods are more objective and are much faster than most of the other methods employed.

3. Iodometric methods. According to Scholz (1814) the first use of iodine in connection with starch was made by Stromeyer in 1813 who discovered that iodine in solution

would stain starch a mixture of colors described as violet, blue, and bluish-black. Colin and Claubry (1814) confirmed Stromeyer's discovery and added that upon boiling the colors would disappear and then return upon cooling. All starches used gave this reaction.

Apparently for the next half century little was done with regard to using this iodine reaction for the determination of amylolytic action and it was not until 1881 that Roberts made use of it in his estimation of the amylolytic activity of pancreatic extracts, malt extracts, and human saliva. He expressed the activity as the number of ml. of standard 1.0 per cent starch paste which is converted, so as to give no color with a solution of iodine, by 1.0 ml. of enzymic solution during 5 minutes at 40°C. Jungk (1884) repeated and confirmed Roberts's work but expressed his results as the time required for 10.0 ml. of extract to convert 10.0 g. of starch paste at 40°C to complete disappearance of any color reaction with iodine. A good malt should do this in 10 minutes.

Nearly a quarter of a century elapsed before any modification of the existing iodine methods for the determination of amylolytic activity appeared. Wohlgemuth (1908) based his now well known method on the disappearance of the blue color as starch is hydrolyzed by various increments of the enzyme solution. To several test tubes containing 5 ml. of 1.0 per

cent starch increasing amounts of enzyme solution are added, 0.1 ml. increments for weak solutions and 0.01 ml. increments for strong solutions, and all tubes are incubated for 30 minutes at 35°C. One drop of 0.1 N iodine solution is then added to each tube and the tube just showing the nuance of blue color is picked as the correct one. For example, if 0.02 ml. of enzyme solution hydrolyzed 5.0 ml. of 1.0 per cent starch, then 1.0 ml. of enzyme would hydrolyze 250 ml. of 1.0 per cent starch to the dextrin stage, and Wohlgemuth's value is expressed as $D^{temp.\ 35^{\circ}}_{time\ 30'} = 250$.

Johnson (1908) used much the same method as Wohlgemuth but stipulated that the tube just showing no iodine color in 10 minutes was the correct one to use in computing the amylolytic power. Sherman, Kendall, and Clark (1910) on reviewing many methods for determination of the amylolytic power decided that the iodine method was most objective, but modified it to suit their conditions. The weight of the enzyme which gave no iodine color in 30 minutes under specified conditions was divided into 2.5 g. starch paste, and gave figures which were 2.7 times higher than the figures obtained by an analogous determination using Lintner's method and scale. A division of Sherman, Kendall, and Clark's scale by 2.7 will give the same numbers as those obtained by Lintner's method.

Ellrodt (1914) gave a method using iodine which is quite direct in its application to alcoholic fermentations. He

placed 3.0 ml. of 1.0 per cent starch paste solution into a stoppered flask, added 1.0 ml. of clear fermenting mash just undergoing secondary fermentation, suspended the flask into the mash, and tested the contents of the flask from time to time with a pale solution of iodine in potassium iodide. A yellow color with iodine denotes sufficient amylase; a reddish color indicates just enough amylase, but the quantity of amylase should be increased somewhat to be on the safe side; and a blue or violet color definitely demonstrates that more malt must be added to completely hydrolyze the mash for maximum alcohol production.

Baker and Hulton (1921) found difficulties using a copper reduction method for determining the reducing power of the hydrolytic products produced by malt extract and formulated a new iodometric titration, which, modified by Windisch and Kolbach (1921), is still used today. However, it has been modified by Windisch, Dietrich, and Kolbach (1922), Lampe (1922), Windisch, Dietrich, and Beyer (1923), Windisch and Kolbach (1925), and Lampe and Deplanche (1935). It is generally called the Windisch-Kolbach method for the estimation of the diastatic power of malt. Briefly, the method consists of adding 5.0 ml. of pale malt extract to 200.0 ml. of 2.0 per cent buffered and specially prepared starch solution, let stand 30 minutes at 20°C, add 10.0 ml. of 0.1 N NaOH, remove a 30.0 ml. aliquot to which is added 20.0 ml. of

standard 0.1 N iodine solution and 30.0 ml. 0.1 N NaOH, let stand 10 minutes at room temperature, add 4.0 ml. of N sulfuric acid, and titrate the excess iodine with 0.05 N sodium thiosulfate. A blank is run, and 1.0 ml. of 0.05 N iodine solution is equal to 855 mg. of maltose. The amylolytic power is expressed as the number of grams of maltose formed by the enzyme in 100.0 g. of malt.

The application of a colorimetric method involving iodine was used by Sym (1932) to follow the hydrolysis of a starch solution, but no amylolytic evaluation was given. Fischer (1933) used the same colorimetric method to determine the amount of undecomposed starch and dextrin after amylolytic action, but again no evaluation was made.

When it became clear that there were at least three distinct actions of malt amylases on starch, namely liquefying, dextrinizing, and saccharifying, new interest in modifying older methods of determining amylolytic action became evident when Andreev (1936), Tarasova (1937), Sokovych (1937), and Mihalkovics (1938) used methods reminiscent of the methods of Johnson (1908) and of Sherman, Kendall, and Clark (1910) for the determination of the saccharifying power of malt. Hanes and Cattle (1938), Sandstedt, Kneen, and Blish (1939), and Ehrnst, Yakish, and Olson (1939) used a modified Wohlgemuth (1908) method for the determination of α-amylase or the dextrifying and liquefying power of malt. Sandstedt,

Kneen, and Blish (1939) evaluated the activity in terms of units representing the number of grams of soluble starch which under the influence of excess β-amylase are dextrinized by 1.0 g. of malt in 60 minutes at 30°C under the specified conditions. This method showed many advantages and has wide current usage.

Altman and Nesterenko (1938) modified the test of Effront (1922), based upon the coagulating effect iodine has upon starch paste, to determine the saccharifying power of sweet malt. Dorfman (1938) also used a modified Effront method for a rapid determination of the saccharifying power of malt. Fertman and Kaler (1938) also modified Effront's method.

Hanes and Cattle (1938) studied the absorption spectra of the starch-iodine complex in various stages of hydrolysis by means of a spectrophotometer. Rundle and Baldwin (1943) did further work on starch involving the starch-iodine complex.

An ingenious method for determining amylolytic activity was the cup assay method of Lulla and Sreenivasaya (1946) which used a small cup of specified dimensions placed in the middle of a Petri dish containing 25.0 ml. of 0.4 per cent starch agar medium and filled with the enzyme preparation. After 12-24 hours incubation at 37°C the dish was flooded with 0.01 N iodine solution and the area not stained measured the diastatic activity.

In general the various iodometric methods seem to cover the three effects of malt amylases outlined by Chrzaszcz (1913), but no one method gives all three separately.

4. Methods involving the determination of reducing power. Trommer (1841) is generally given credit as being the first investigator to observe the reducing action of the starch hydrolysis products upon an alkaline copper solution. Eight years later Fehling (1849), using the accumulated data on copper reagents, formulated his well known alkaline copper solution from which most of the reagents used today have had their origin. The determination of the saccharogenic activity of malt was carried out by Kjeldahl (1880) who added a known amount of malt extract to an excess of starch, heated the mixture for 20 minutes at 57-9°C, determined the reducing sugars formed using Fehling's solution, and postulated that the resultant reduction was proportional to the amount of diastase present as long as not over 40.0 per cent of the starch was converted.

Lintner (1886) and Lintner and Wirth (1908) modified Kjeldahl's method and expressed the amylolytic power of malt amylase as the number of grams of maltose produced by the action of 100.0 g. of malt on soluble starch. In the Lintner method 2.0 to 8.0 ml. of clear malt extract, prepared by mixing 25.0 g. of finely ground malt with 500.0 ml. of water for 6 hours, is added to 100.0 ml. of 2.0 per cent soluble

starch made neutral to rosalic acid, incubated 30 minutes at 20°C, and 10.0 ml. of 0.1 N NaOH and water is added to make up to 150.0 ml. An aliquot of this is titrated using Fehling's copper reagent. Many modifications have been made and only rudiments of the original are still used in present day Lintner analyses.

One of the first modifications upon the Lintner method was that of Sykes and Mitchell (1896) who speeded up the method and estimated the copper reduction gravimetrically instead of by titration. Jones (1908) suggested improvements in the pH of the hydrolysis, preparation of the starch used, standardization of temperatures, and standard filtering techniques to avoid variation in the malt extract preparation. Oshima (1920), using essentially the original Lintner method in his modification, computed his results on the first tube containing 5.0 ml. of Fehling's solution that was completely reduced by a measured amount of malt extract-starch hydrolyzate. Other modifications have been added by Roeder (1921), Lampe (1929), Silbernagel (1930), and Harada (1931), the latter author using carefully controlled pH, temperature, and specially prepared Lintner starch solution.

Sherman, Kendall, and Clark (1910), and Sherman and Baker (1916) perfected a gravimetric copper method which has been used extensively for the determination of the reducing sugars formed by the hydrolysis of starch.

Lane and Eynon (1923) modified the original Fehling's titration by the use of methylene blue as an inside indicator, the method being adopted by the American Society of Brewing Chemists as their official volumetric method. Mortiz (1932) lauded the Lane and Eynon method and stated that it gave a very good estimate of the transforming power of any malt extract and was much more indicative than any determination for maltose alone. An inovation of the titration method was made by Ritter (1929) who dissolved the precipitated copper oxide with a solution of ferric sulfate, obtaining ferrous sulfate in the oxidation-reduction reaction, and titrated the resultant ferrous sulfate with potassium permanganate.

Blish and Sandstedt (1933) adapted the potassium ferricyanide titration method of Hagedorn and Jensen (1923) to the estimation of the amylolytic action of flour and related cereal product. The use, modifications, interpretations, and applications by Cole (1933), Ohlsson and Rosén (1934), Norris and Carter (1935), Teller (1937), Gore and Steele (1935), Anderson and Sallans (1937), Laufer, Schwarz, and Laufer (1938), Hildebrand (1938), Hildebrand and McClelland (1938), Rask (1939), Burkert and Dickson (1939), Dickson (1940), and Popov (1939) provided rapid and efficient methods for the determination of the saccharogenic property of malt.

An interesting development was that of Somogyi (1937), who modified the Shaffer-Hartman (1921) technique for deter-

mination of reducing sugars into a more useful micromethod. For the estimation of amylolytic activity, he assumed a linear relationship for the copper reducing power obtained with the hydrolyzates from the action of malt enzymes on starch. Underkofler, Guymon, Rayman, and Fulmer (1943) further modified the Somogyi procedure to include the determination of reducing sugars in fermentation media.

Hildebrand (1938) reported collaborative studies of the Blish and Sandstedt method and perfected a ceric sulfate titration for comparison, finding very little difference on most samples, but small deviations with samples of extremely high or low activity. These results were confirmed by Burkert and Dickson (1939).

An electrometric determination of the reducing power of the hydrolyzate from the action of malt extract on starch was proposed by Burkhart (1939), who, by using a modified quinhydrone pH meter as a potentiometer, was able to determine the reducing power of the various samples more than three times as rapidly as with the official method of the American Society of Brewing Chemists. The method is fast, simple, and gives concordant results.

The definite establishment of the α- and β-amylase components in malt increased interest in methods for the separate determination of these components. Following the lead of Sandstedt (1938), who studied the relation of α- and β-amylase

to the evaluation of malt, Sandstedt, Kneen, and Blish (1939) modified the Wohlgemuth procedure to give an exact determination of α-amylase. This α-amylase determination involves adding an excess of β-amylase until there is no further effect and determining the number of grams of soluble starch which is dextrinized by 1.0 g. of malt in 60 minutes at 30°C, since a linear relationship between α-amylase activity and dextrinizing time was indicated under the specified conditions. The β-amylase activity may be determined rapidly by the method of Kneen and Sandstedt (1941) which applies when the β-amylase present has at least twice the saccharogenic activity of the α-amylase, as is true for most malts. Both α-amylase and β-amylase may therefore be determined by the following procedure: the sum of the α- and β-amylase activities is determined by the potassium ferricyanide titration method and the α-amylase value, determined by the method of Sandstedt, Kneen, and Blish (1939), is subtracted to ascertain the β-amylase activity. Reports on collaborative studies of methods for the determination of amylase activity by Dickson (1942), Dickson, Wilcox, Singruen, and Schoenfelt (1944), and Dickson (1944) gave statistical evidence to confirm the work of Sandstedt and his collaborators, and have recommended the methods for adoption by the American Society of Brewing Chemists as tentative procedures. Slight modifications have been recommended by Olson, Evans, and Dickson (1944) and

Graesser and Dax (1946).

5. Direct methods. The choice of any method for an analysis depends upon the circumstances and the aim of the work. Most commercial control work requires a direct method accurately and precisely perfected to its specifications. The complex nature of any enzyme reaction makes a determination of any one of the factors of only limited importance for any practical use. Realizing, from a study of the methods for the determination of the amylolytic power of malt and the actions thereof, that malt exhibits more than one property, Chrzaszcz (1913) stated that a method for malt evaluation should at least take into account the powers of liquefying, dextrinizing, and saccharifying, along with any others not yet elucidated.

Matthews (1922) discovered that finely ground malt mixed with water had a much greater activity than the water extract of the same amount of malt when added to a soluble starch solution. His major conclusion was that the aqueous extract used in most determinations did not contain all the active principles which contribute to the so called amylolytic activity. Many observers such as Windisch (1922), Effront (1932), Fink (1934), Laufer (1937), Rossatkevitch (1937), Redfern and Johnston (1938), Sallans and Anderson (1939), Heintz (1939), Tanner and Englis (1940), and Raev (1940) have noted that variance in malts due to type of barley and loca-

tion where grown, in the water used in the analysis, fineness of grind of the malt sample, chemical and physical nature of starch substrate, amount of liquefaction obtained in comparison to the amount of saccharification, temperature, pH, and time all have a profound effect upon results obtained with the standard methods used in malt evaluation. Ketelbant (1939) simply concluded that there are too many variable factors to justify any real reliance on analytical results as a basis for malt evaluation.

Extensive investigations of the more common methods for malt analysis have led such workers as Davison and Maslow (1926), Hertel (1934), Gore (1936), Drews (1936), Davis and Tremain (1938), Menzel (1938), and Selec (1938) to believe that analytical results give much valuable information, but that there is very poor correlation between the various methods, and that concordant results cannot be obtained unless run by the same method, by the same operator, with the same samples, and same precise conditions. Since each method usually measures predominately only one of the many actions of an amylolytic material there can be little hope of thus obtaining an overall evaluation for practical usages. Petit (1935) stated that for brewery operations, since the analysis of the malt was carried out under conditions vastly differing from those of the brewing process, the interpretation of analytical results depended upon the experience the analyst

had with the method and with the brewery conditions and operations. Blish, Sandstedt, and Kneen (1938), Harreis (1941), Christensen (1943), Kneen, Beckord, and Sandstedt (1941), Preece (1942), and Kneen (1943, 1945) agreed with this same general viewpoint in connection with several practical uses of amylolytic materials.

Kneen, Beckord, and Sandstedt (1941) concluded that the general methods of analysis give no complete picture of the amylolytic potentialities of malts, but that in general high amylolytic activity is associated with high values for both α-amylase and β-amylase with no prediction of the liquefying and saccharifying powers in a fermentation. Nelson and Dickson (1942) confirmed these findings and added that high amylolytic values do not necessarily mean high ratios of α-amylase to β-amylase. Kneen (1943) stated that of the two most useful components so far studied, α-amylase and β-amylase, the former is probably the most important since it can liquefy, dextrinize, and saccharify, while β-amylase can only saccharify.

When Drews and Lampe (1940) substituted "maltspirits" and diastase-grits for malt in an actual fermentation they found from 3.0 to 10.0 per cent "maltspirits" was as good as 10.0 per cent malt and about 6.0 per cent diastase-grits was needed to equal 10.0 per cent malt, but analyses by the Lintner and Windisch-Kolbach methods gave the diastase-grits

twice the value of the malt and three times that of the "maltspirits". The analytical method used gave no indication of the fermentative possibilities. Kneen (1945) substituted sorghum amylase, analyzed as having high values for α-amylase and low values for β-amylase, for malt amylase in a fermentation, and found no correlation between the analyses and the potentialities for producing fermentable sugars over the entire period of saccharification. Redfern and Landis (1946) tried to compare three sources of α-amylase, namely, malt, bacteria, and molds, and found the relative strengths were independent of the degree of liquefaction, and that all three sources differed quantitatively in their action on starch.

Edlbacher (1946) in discussing the shortcomings of investigations on isolated enzymic reactions stated that all enzymes of an interrelated system act on the substrate simultaneously forming many complexes, and the study of isolated systems can have but limited value. The "totality" of the system should not be overlooked.

Evaluation methods using a direct approach, that is, methods employing specified conditions the same as those finally used in practical applications, are very limited. Clibbens and Geake (1931) developed a method for determining the activity of a desizing enzyme on the cloth upon which it is subsequently to be used. Kneen and Sandstedt (1942), when evaluating malts to be used as flour supplements, used actual

baking tests to determine the value of the malt added. Pigman (1944) used yeast along with malt amylase to get a complete hydrolysis of the starchy substrate. Thorne, Emerson, Olson, and Peterson (1945) evaluated malts used for the production of alcohol from wheat by extensive analytical and fermentation tests and came to the conclusion that only a fermentation test can give an accurate evaluation. For example, some malts that a distiller would have discarded on the basis of Lintner analyses proved in fermentation tests to be as good as other malts having much higher Lintner values.

In all of the work at Iowa State College related to the saccharification of starchy mashes for the alcoholic fermentation, the activities of the amylase sources used have been measured by actual fermentation experiments. This direct method undoubtedly furnishes the most accurate and conclusive evaluation of amylolytic materials for saccharifying starchy fermentation mashes.

B. Development and Use of Mold Amylase Preparations

Since this thesis is mainly concerned with the evaluation of mold amylases employed in the saccharification of starchy substrates for use in the alcoholic fermentation, it is necessary to briefly review the history of such use of mold amylases. Although molds have been used for centuries in the Oriental countries for the saccharification of starchy

grains prior to fermentation, it has only been very recently that they have been introduced and gained any importance in other countries.

Scientifically, Aspergillus oryzae first attracted attention outside the Oriental countries about 1875. Kozai (1901) in his review of the early literature regarding the early investigations of Aspergillus oryzae and its important industrial applications gave credit to Hoffman and Korshelt as the first writers on this subject. Korshelt (1878) first called the mold Eurotium oryzae since an amylolytic enzyme developed during the culturing of this fungus in the preparation of the Japanese alcoholic beverage "saké". Later this mold was renamed Aspergillus oryzae by other investigators.

It should also be noted that in the Orient no attempt was made to utilize pure cultures of molds. A mixture of micro-organisms from which many single strains of high saccharifying power have been isolated was grown on steamed rice substrate and the resulting product was known as "koji". Species of Mucor and Rhizopus as well as the predominating species of Aspergillus oryzae have been isolated from "koji". This mixture of molds made much of the early work of questionable character and hence of little value except of a historical nature.

Gayon and Dubourg (1887) investigated the molds Aspergillus oryzae, Mucor circinelloides, Mucor racemosus, Mucor

alternans, and Chlamydomucor oryzae. According to his results Aspergillus oryzae had by far the highest saccharifying ability, Mucor alternans slightly less, and the others were very inferior but still showed some saccharifying ability.

Calmette, in 1892, made the first scientific investigation of the microflora associated with the Chinese yeast cake. He found that a certain species of Mucor was the predominate mold in the conglomeration of microorganisms and gave it the name of Amylomyces rouxii, since it possessed the property of saccharifying starch and slowly converting the resultant sugar into alcohol.

Sanguinete (1897) continued the investigations and from the study of the molds Aspergillus oryzae, Gayon's Mucor alternans, and Amylomyces rouxii found that in saccharifying power Aspergillus oryzae was best with Amylomyces rouxii second. However, Amylomyces rouxii had greater fermentative power and Sanguinete concluded that in all probability Amylomyces rouxii was the most suitable for industrial employment since it fermented starchy materials directly without the aid of yeast. Amylomyces rouxii later became known as Mucor rouxii.

A large scale fermentation was carried out in a distillery by Collette and Boidin (1897) using the mold Mucor rouxii for the conversion of the residuary liquors of a yeast factory into ethanol. A patent on the process was issued to

these men and this was the beginning of the Amylo process which has been used rather extensively in Europe. Galle (1923) and Owen (1933) have published articles giving complete details of the Amylo process and reviewing the important developments. It was stated by these authors that the original organism, Mucor rouxii, was replaced by Mucor B., Mucor G., Rhizopus delemar and finally Mucor Boulard Number 5, since this last organism was found to have a greater tolerance for alcohol, produced less acid, shortened the time to 48 hours, held its own against contaminants, and allowed the addition of yeast and mold at the same time. Whether the process is economically sound is still being debated.

Jokichi Takamine was responsible for the introduction of amylolytic mold enzymes to the distilling industries of the Occidental countries. He obtained a number of patents in England, the United States, and several other countries for manufacturing enzymic preparations using Aspergillus oryzae and among the better known preparations are "koji" (1894), and"taka-koji diastase" (1894). A very comprehensive study of the nature of "koji" amylase has been made by Ito (1932).

Jean (1898) reviewed Takamine's process for saccharification and fermentation by means of Aspergillus oryzae and compared it with the utilization of Mucor rouxii by Collette and Boidin; he found that the Aspergillus oryzae appeared to have a greater saccharifying power and could be used with

stronger mashes. Boidin (1899) remarked that the molds were deficient in starch liquefying power and recommended, therefore, that starchy substrates used in Amylo distilleries should be treated with 1.0 to 2.0 per cent of malt before sterilization to liquefy the gelatinized starch. At the turn of the century molds were being utilized extensively in southern Europe for the fermentation of amylaceous materials. At first, Mucor rouxii was used exclusively, but later Mucor β, Mucor α, and Rhizopus delemar were employed.

Saito (1904) isolated from a wheat-flour cake used in the preparation of a Chinese beverage, two new species of Rhizopus, Rhizopus chinensis, and Rhizopus tritici which not only saccharified starch but fermented it to alcohol. Saito (1907) also studies Aspergillus batatae, Aspergillus pseudoflavus, and Rhizopus japonicus with no outstanding saccharifying ability shown by any of these species.

Okazaki (1914) studied three members of the genus Aspergillus, namely Aspergillus okazakii, Aspergillus albus, and Aspergillus candidus, and determined the amounts of sugar they produced from their reaction upon a starchy substrate. The first two were considered to have produced sizeable amounts of amylase, but actual values are not available.

Scales (1914) prepared an enzyme powder by growing Aspergillus terricola on an artificial medium for 4 days; washing the mycelium with water, acetone, and ether; drying well;

and then grinding in a mill. This powder was found to produce over 82.0 per cent of fermentable sugars from a starch solution after incubation for 3 days.

Takamine (1914) found that Aspergillus oryzae grew very well on wheat bran under specified conditions, gave the product the name of "Taka-Koji" and carried out experiments to determine its efficiency for saccharification as compared with malt. Such favorable results were obtained by Takamine that Ortved (1912) tried out the preparation on a plant scale at the Hiram Walker and Sons plant in Canada. Alcohol yields were better than any obtained with malt, "Taka-Koji" was much less expensive than malt, and Ortved gave a very favorable report, but fear that undesirable flavors or odors would be imported by the mold caused the project to be discontinued.

Collens (1915) was able to produce 8.0 per cent more alcohol from the use of taka-diastase as a saccharifying agent than from malt in his experiments dealing with the possibility of producing industrial alcohol from cassava.

An intensive study was made by Oshima and Church (1923) on the molds isolated from koji in order to ascertain those strains which would produce the largest quantities of amylase and thus have the most value as saccharifying agents. Aspergillus oryzae and mold forms intermediate between Aspergillus flavus and Aspergillus oryzae were the most potent producers of amylase. A noteworthy accomplishment of the investigation

was the discovery of the great variation which is found in different strains of the same mold. Mold growth and enzyme production was tried on the following media: casein, ground dried codfish, dried yeast, crushed soybeans, soybean meal, oil-extracted soybean meal, cottonseed cake, peanut meal, cocoanut meal, corn meal, wheat middlings, and wheat bran which turned out to be the best substrate. Oshima (1926) followed up this work and found the activity of the enzyme grown on wheat bran using Aspergillus oryzae was the greatest at pH 4.8 to 5.2. The enzyme was also heat labile and became completely inactive when heated for one hour at 85°C.

In 1931, Harada studied the cultivation of Aspergillus oryzae on cooked wheat bran for the preparation of amylolytic enzymes, and investigated some of the properties of the latter. Specific attention was given to the factors involved in the production of enzymes on wheat bran by the growth of Aspergillus oryzae such as quality of the bran, water content of the bran, hydrogen ion concentration, sterilization, time of incubation, temperature, and humidity of the chamber. Harada grew the mold on bran containing 50.0 per cent moisture obtaining maximum growth in 48 hours. The optimum pH for amylolytic activity was determined to be 5.2 from 30°C to 50°C, but at 65°C it was 6.6 thus showing that optimum pH increased with increasing temperature only above 50°C.

Muta, Nomoto, and Tanaka (1931), Muta and Tanaka (1933), and Muta and Tanaka (1936) compared the saccharifying power of Rhizopus pêka I, Rhizopus pêka II, 4 strains of Aspergillus awamori, 6 strains of Aspergillus oryzae, 8 strains of unidentified molds, and one strain of Rhizopus delemar. The Rhizopus delemar, Rhizopus pêka, and two of the unidentified species were the best for alcoholic fermentation by the Amylo process, and gave 87.0 to 91.0 per cent yields on a semi-industrial scale.

Analytical methods were used by Wei and Chin (1934) to determine the amylolytic activity of 10 species of Aspergillus including 6 strains of Aspergillus oryzae, one strain each of Aspergillus niger, Aspergillus luchuensis, and Aspergillus glaucus, and an unidentified species of Aspergillus. The greatest amylolytic power was exhibited by Aspergillus oryzae (AOLD).

In 1935, Takeda made comparisons of the amylolytic activities of 27 strains of Rhizopus isolated from ragi-koji and soybean-koji produced in Sumatra and Java. The only two showing strong amylolytic powers were given the names of Rhizopus semarangensis and Rhizopus javanicus. The latter was found to do particularly well in the Amylo process and was actually tried on a commercial scale with excellent results.

Two processes employing fungal amylases for saccharification of grain fermentation mashes have been evolved over

the years. In the "taka-koji" process the mold was grown on wheat bran, and the resultant product was used for saccharifying the grain mashes, while in the Amylo process the mold was grown directly in the grain mash.

Actual plant scale usage of the two processes have shown them to be superior to the use of malt, with the "taka-koji" best of all since it takes a shorter time in its operation, requires no special installations as are needed in the Amylo process, and gives higher yields. Strangely enough, though, the Amylo process has been used industrially for nearly 40 years, while the "taka-koji" process has until the last 4 or 5 years been almost completely ignored.

In 1939, Underkofler, Fulmer, and Schoene revived the "taka-koji" process of growing molds on wheat bran for use in replacing malt in the saccharification of starchy substrates for the alcoholic fermentation. A rotating drum technique was used to produce the active amylolytic preparations by growing molds on wheat bran. Seventeen strains of molds and 7 strains of bacteria were grown on wheat bran and tested by actual fermentation tests; the bacteria gave no useful quantities of amylase, but all the molds gave good yields of amylase. The molds employed were Aspergillus oryzae, Aspergillus flavus, Mucor rouxii, Rhizopus delemar, Rhizopus oryzae, Rhizopus pêka I, Rhizopus tritici, Mucor circinelloides, and Mucor javanicus. Preliminary results gave equal

ratings to the Aspergillus oryzae and the Rhizopus molds. Aspergillus oryzae was picked for further study because of its cultural characteristics, vigorous growth, and consistant high production of amylases, higher in most tests than any other in the group being tested. Over 90.0 per cent conversion of starch to alcohol became common for the Aspergillus oryzae strain, with malt yields about 10.0 per cent lower. It was clearly pointed out that mold bran was less expensive, more quickly prepared, and gave higher yields of alcohol than malt.

Subsequently Underkofler and his coworkers have published a number of papers covering various aspects of the use of mold amylase preparations in saccharifying fermentation mashes. Schoene, Fulmer, and Underkofler (1940) compared malt, mold bran, and soybean meal as saccharifying agents, and found the mold bran superior. Underkofler, Goering, and Buckaloo (1941) investigated amylases from 9 strains of molds grown on bran by the rotating drum technique. Best amylase production occurred with 2 strains of Aspergillus oryzae, and with Rhizopus oryzae and Rhizopus tritici. Attempts were made by these authors to grow Aspergillus oryzae on rice hulls, peanut hulls, sawdust, corn cobs, cotton-seed hulls, oat hulls, corn bran, and wheat bran. Of these only wheat bran and dry-milled corn bran supported adequate growth.

Hao, Fulmer, and Underkofler (1943) employed a new tech-

nique for growing molds on wheat bran, which involved incubation in a pan with air under slight pressure passing through the bran mass. These authors prepared mold brans with 27 different strains of molds selected from the 4 genera Aspergillus, Mucor, Rhizopus, and Penicillium. Although Rhizopus cultures gave good amylase production, the best strains of Aspergillus oryzae were judged to be most suitable because of their superior cultural characteristics, including denser mycelium, better sporulation, and vigorous growth.

Underkofler (1942) and Underkofler and Fulmer (1943) reviewed the status of microbial amylases for the saccharification of starch in the alcoholic fermentation and gave many valuable statistics concerning mold bran and its industrial applications. It had been demonstrated on the laboratory scale that mold bran could be made cheaply from abundant raw materials, did not lose its potency upon being stored in a dry condition, saccharified well at 30°C, could be prepared in about one fifth the time required for malt, and produced slightly more alcohol under optimal conditions for use of mold bran than did malt under optimal conditions for malt. These results were later substantiated by Roberts, Laufer, Stewart, and Saletan (1944), Barinova (1944), Pan and Liu (1944), Hao and Jump (1945), and Schwimmer (1945).

Beginning in the spring of 1945 tests were begun on the use of mold bran in grain fermentations on the full plant

scale at the alcohol plant operated by the Farm Crops Processing Corporation at Omaha, Nebraska. A brief report on these tests was given by Boyer and Underkofler (1945) and a detailed report by Underkofler, Severson, and Goering (1946). These tests proved conclusively that mold bran was entirely satisfactory for use as a saccharifying agent in large scale production of industrial alcohol, both when used alone and when used in combination with malt. The fermentation time was reduced from 48 to 36 hours when using mold bran in place of malt. The actual number of yeast cells produced per ml. of yeast mash was at least double the number produced when malt was used alone. When a mixture of 2 parts of mold bran to 4 parts of malt was used, improved yields of alcohol were noted, probably due to supplemental or synergistic action, and the requirements of the two cannot be predicted on the basis of a linear relation.

Underkofler (1946) briefly reviewed and Underkofler, Severson, Goering, and Christensen (1947) gave a detailed report on the production and use of mold bran from the laboratory, through the pilot plant and semi-commercial plant scales to the full scale production of over 10 tons per day at the new Mold Bran Company plant at Eagle Grove, Iowa. The economics of using mold bran were discussed in these papers. It has been shown by many investigators that there was no difference in the quality of the alcohol obtained by either malt or mold

bran saccharification and somewhat better yields on an overall basis are obtained with mold bran. The value of mold bran in replacing malt, which sold prewar for 3 cents a pound, is about 9 cents a pound, and mold bran could be produced commercially well below that figure, hence, the economics are definitely favorable.

A very recent trend in the use of molds in the fermentation industry has been outlined by Erb and Hildebrant (1946) who used a submerged culture of Rhizopus delemar or Rhizopus boulard, grown on a nutrient media consisting of grain stillage, nutrient salts, a small amount of aluminum powder, and granular wheat flour, for the saccharification of starch in the alcoholic fermentation of granular wheat flour mashes. Culturing techniques were described and the volume of mold medium used varied from 6.0 to 12.0 per cent of the total volume of the fermenter mash. The infusion method was used in the preparation of the fermentation mashes and four-fifths of the malt usually used in such a process was eliminated when using the mold medium. Approximately 12.1 proof gallons of alcohol per 100.0 pounds of dry grain was obtained, a 91.2 per cent fermentation efficiency, instead of the usual 11.0 proof gallons of alcohol per 100.0 pounds of dry grain obtained by malt alone. The use of plant stillage was recommended and with very little processing should be adequate as the medium for mold growth.

Van Lanen and LeMense (1946) tested 350 various cultures of fungal amylases grown in submerged culture on thin stillage supplemented with 1.0 per cent corn meal and 0.5 per cent calcium carbonate to adjust the pH. The amylolytic preparations were tested by their dextrinizing action on starch under specified conditions, and the α-amylase values obtained according to Sandstedt, Kneen, and Blish (1939). Only 7 samples gave commercial possibilities, with a strain of Aspergillus niger being superior. The liquid fungal amylase preparation was used at a rate of 10.0 to 15.0 per cent by volume of the corn mash to be saccharified, completely replacing the malt formerly used. The yield of ethanol was from 5.2 to 5.4 proof gallons compared with 5.0 to 5.2 proof gallons from malt controls. The fungal amylase was approximately 12 times as potent as the malt on a comparable basis. Adams, Balankura, Andreasen, and Stark (1947) used Aspergillus niger, obtained from the Northern Regional Research Laboratories as No. 337, in a submerged culture growth on a medium containing 5.0 g. distillers' dried solubles and 1.0 g. ground corn meal per 100.0 ml. They obtained an average of 6.26 proof gallons of ethanol from the fungal amylase preparations as compared with 5.95 proof gallons of ethanol from the malt controls. This was about a 5.0 per cent increase in ethanol production, corresponding to an overall fermentation efficiency of nearly 93.0 per cent.

III. MATERIALS

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

Corn meal

The corn meal used in the preliminary work in this investigation was hammer mill ground No. 1 yellow corn obtained in 100 pound lots from the Ames Grain and Coal Company. The first 100 pound lot was obtained November 1, 1943. It was carefully mixed and stored in well stoppered bottles until used. A test tube containing 2 to 3 ml. of carbon disulfide was pushed upright into the corn and the lid screwed down tight, the fumes acting as a fumigant. This sample had a glucose equivalent of 64.70 g. per 100.0 g. of corn "as received" and a moisture content of 12.84 per cent. The second lot of 100 pounds arrived in February, 1946. The glucose equivalent was 68.50 g. per 100.0 g. of corn "as received" and the moisture was 11.35 per cent. The starch analyses were carried out using the official diastase method with subsequent acid hydrolysis of the Association of Official Agricultural Chemists (1940).

Corn starch

The corn starch used in this investigation was obtained

in four 100 pound lots. The first lot was received from the American Maize-Products Company, Roby, Indiana at an unknown date, but probably in early 1944. It had a glucose equivalent of 92.00 g. per 100.0 g. of starch "as received" with a moisture of 11.82 per cent. The other three lots were obtained from the American Maize-Products Company on February 10, May 3, and July 11, 1946. The glucose equivalents respectively were 93.20, 92.60, and 92.50 per cent on starch "as received" with moistures of 11.71, 11.33, and 11.40 per cent on the starch as it came from the sack. The starch analyses were carried out by the official direct acid hydrolysis method of the Association of Official Agricultural Chemists (1940).

Malt extract

The malt extract used to prepare media for yeast cultures was obtained in a 140 pound keg from Anheuser Busch and Company, Saint Louis, Missouri in 1942. It was designated by the manufacturer as "light Budweiser non-diastatic malt syrup".

Yeast extract

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

Amylolytic preparations

The 19 amylolytic preparations used in this investigation were obtained from 6 sources over a period of 3 to 4 years with most of the samples being obtained in the last year and a half. Two of the oldest samples employing strains of Aspergillus oryzae were grown in the Biophysical Chemistry Laboratories at Iowa State College employing the method described by Hao, Fulmer, and Underkofler (1943). Twelve samples were secured from the commercial plant of the Mold Bran Company, Eagle Grove, Iowa at various intervals from September 1945 to December 1946. These samples were all prepared with one strain of Aspergillus oryzae. Two preparations using a strain of Aspergillus oryzae were obtained from the Farm Crops Processing Corporation, one, a laboratory preparation, in late 1944 and the other, a semi-commercial sample, in late 1945. One pilot plant preparation of Aspergillus oryzae mold bran was received from Dr. Leo M. Christensen at the University of Nebraska in November 1943. Two commercial preparations designated as "Protozyme" and "Polidase" were obtained respectively from Jacques Wolf Corporation, Brooklyn, New York, and Schwarz Laboratories, New York City, New York.

IV. METHODS

A. Microbiological Procedures

Yeast culture

A weighed amount of malt extract was dissolved in five times its weight of boiling tap water, and, after cooling somewhat, the liquid was placed in Erlenmeyer flasks and designated as yeast culture medium. For carrying the cultures and for cultures employed for inoculating experimental mashes, 300 ml. of the medium was used in each 500 ml. Erlenmayer flask. The flasks were plugged with cotton and sterilized for 30 minutes under a steam pressure of 15 pounds.

The stock culture was obtained from the Northern Regional Research Laboratories, Peoria, Illinois, as No. 567. It is listed as No. 51 in the Biophysical Chemistry collection at Iowa State College. It is a strain of *Saccharomyces cerevisiae* which is extremely vigorous and produces high yields of alcohol. Transfers were made daily in order to maintain a vigorous yeast culture; a sterile pipette was used to aseptically transfer 10 to 12 ml. of an active yeast culture to another flask each time. The inoculated medium was incubated at 30°C for 24 hours.

Preparation of amylolytic agents

The laboratory preparations used in this investigation were prepared employing the method described by Hao, Fulmer, and Underkofler (1943). The commercial preparations were prepared according to the methods discussed by Underkofler, Severson, Goering, and Christensen (1947).

B. Saccharification and Fermentation

According to the results obtained from several years' work, the following procedures were found to represent the optimum conditions for saccharification and fermentation and were adopted as the standard methods used throughout this investigation for the evaluation studies on the various amylolytic preparations.

The starchy substrate was prepared by mixing with a glass stirring rod a weighed amount of grain, or starch and yeast extract, with 250 ml. of hydrochloric acid (various normalities were employed depending upon the experiment) previously heated to 70°C, in a wide-mouthed 1-liter Erlenmeyer flask. This was repeated for each flask in the series. The flasks were then placed in a water bath which was heated by means of Fisher burners until the temperature of the mashes had risen from about 60°C to about 85°C; the temperature of the water bath rose from about 70°C to the boiling point of the water. The mashes were stirred occasionally during the heating process to facilitate even gelatinization of the starch. The mashes must be kept above 80°C to prevent irreversible retrogradation of the starch. The glass rods were removed and the openings of the flasks were then covered with Petri dishes and the flasks transferred to the heated

autoclave. The mashes were cooked for various lengths of time, depending on the series, at a steam pressure of 20 pounds. After completion of the cooking period, the autoclave was blown down immediately to atmospheric pressure, and the flasks were steamed continuously in the autoclave at atmospheric pressure until their removal, one at a time, for saccharification.

To the hot mash in the flask were quickly added the requisite amount of a concentrated solution of sodium carbonate to adjust the pH between 5.0 and 5.3 and a slurry of the desired quantity of amylolytic agent in 250 ml. of cold water, and the contents of the flask were mixed for one minute with a specially designed high speed mixer. The original temperature of the amylolytic slurry was adjusted so that the temperature of the mixed mash was about 55°C. The flask was then cooled in a running cold water bath to reduce the mash temperature quickly to 30°C; the time required was about 10 to 15 minutes. When all the mashes for a series had been saccharified and cooled, each was inoculated with 20.0 ml. of an active 24-hour culture of yeast. The fermentation mashes were mixed with an air-driven stirrer and the flasks were closed with rubber stoppers having water traps containing 30 to 32 ml. of water. The flask assembly is shown in Figure 1.

All of the fermentations were incubated at 30°C for various periods of time depending on the specific series. At

12-hour intervals the contents of each flask were gently swirled by shaking the flask in a rotating motion by hand, taking care that no air was whipped into the mash to disrupt anaerobic conditions. This shaking was done to obtain a homogenous distribution of the yeast throughout the mash.

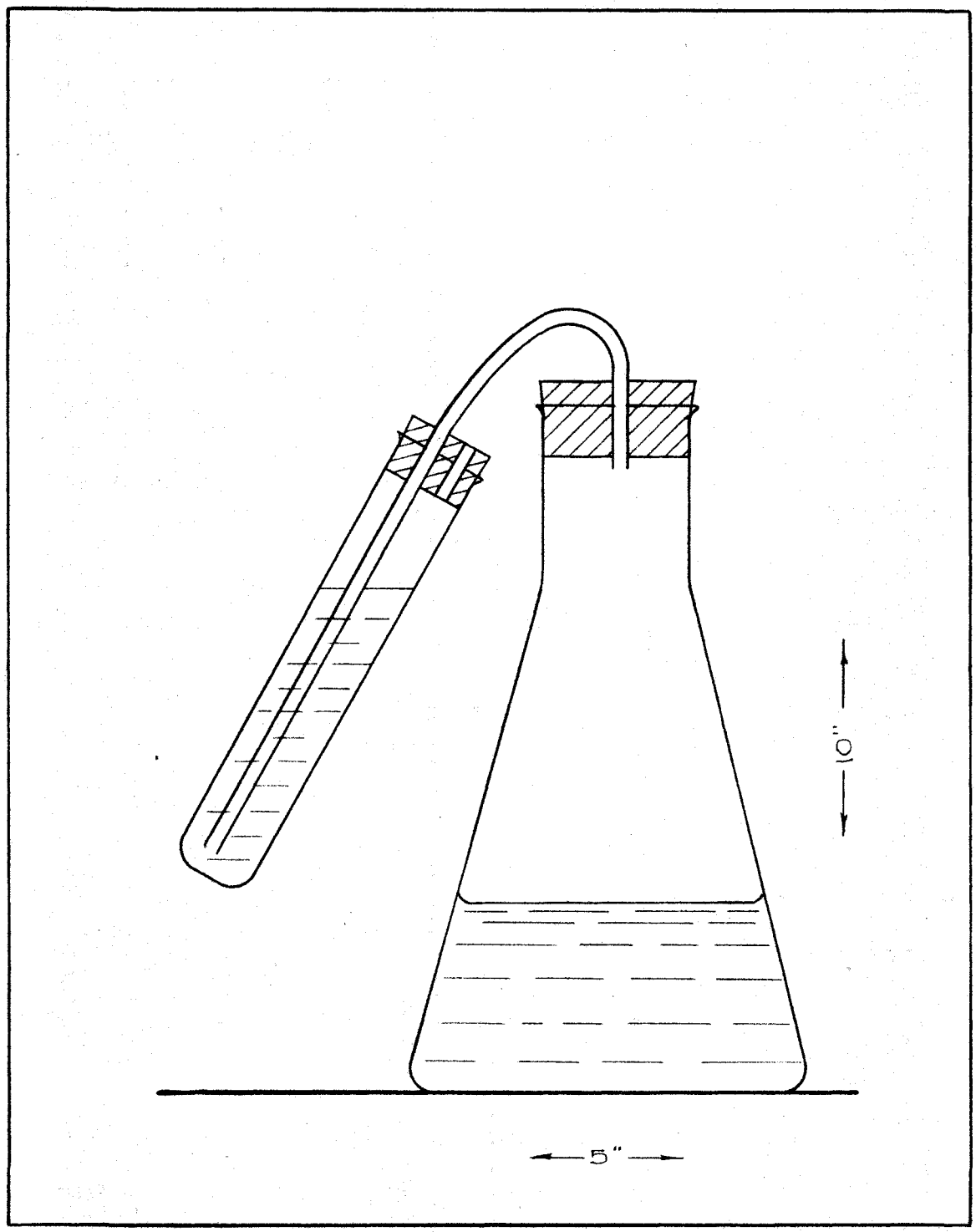

Figure 1. Wide-mouthed 1-liter Erlenmeyer flask assembly.

C. Analytical Procedures

Determination of the sugar equivalent of the starch

The glucose equivalent of the starch was determined by acid hydrolysis in accordance with the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists. The reducing substances formed in the hydrolysis were estimated according to the method of Underkofler, Guymon, Rayman, and Fulmer (1943). The reagents were standardized by means of a sample of pure glucose. All determinations were carried out in duplicate or triplicate. Any questionable analyses were repeated.

Determination of ethanol

At the end of the fermentation period the water in the trap was added to the fermentation flask and the final volume of each beer was measured. An aliquot of 250.0 ml. was distilled from a 650 ml. Kjeldahl flask after having added approximately 0.5 g. of calcium carbonate to neutralize the acids present and 200 ml. of wash water. The distillates were collected in 100 ml. volumetric flasks until about 99 ml. of distillate had been collected in each case. The volumetric flasks containing the distillates were brought to 25°C by immersion in a constant temperature water bath, the

volume made to exactly 100.0 ml. with distilled water, and the specific gravities determined at 25°/25° with the Chainomatic Westphal balance. Ethanol contents of the distillate were then read from an appropriate table.

Calculation of ethanol yield

In a typical experiment the fermentation mash contained the following: 100.0 g. starch, as received, with a glucose equivalent of 92.60 g., 3.0 g. of mold bran, 5.0 g. yeast extract, and 20.0 ml. of yeast inoculum. After fermentation a 250.0 ml. aliquot was distilled, and the first 99 ml. of the distillate collected in a 100 ml. volumetric flask. The flasks containing the distillate were brought to 25°C by immersion in a constant temperature water bath, the volume made up to exactly 100.0 ml., and the specific gravity at 25°/25° was found to be 0.9771 with the Chainomatic Westphal balance. The ethanol content of the distillate as read from the table was 13.96 g. per 100.0 ml. of distillate or per 250.0 ml. of fermented mash. The total volume of the mash was 579.0 ml.

The calculations for total ethanol are:

$$13.96 \times \frac{579.0}{250.0} = 32.36 \text{ g. of ethanol.}$$

D. Manner of Employing Materials

Materials used in the course of this investigation, such as corn meal, corn starch, yeast extract, and amylolytic agents, vary somewhat in composition as received, especially as regards moisture content. It would be possible to compensate for these variations by employing all materials on the dry weight basis. However, this would complicate procedures by making necessary a moisture determination on each material before it was used. Moreover, the industrialist is interested in the results obtainable with the materials as received by him. Hence, during the course of this investigation all materials used were measured out as needed in the form and condition received from the manufacturers without making any corrections for their composition or altering them in any way. This particular approach was made in an attempt to devise a method which would be most useful for practical application and would eliminate or compensate for as many variables as possible while still retaining accuracy and speed.

V. EXPERIMENTAL RESULTS

A. Experiments with Corn Mashes

1. Optimum conditions for liquefying corn mashes.

In processing starchy substrates for the ethanol fermentation gelatinization of the starch during cooking with water results in mashes so thick that they are exceedingly difficult to handle. Industrial plants attempt to obviate this difficulty by partially liquefying the starch prior to or during the cooking operation, usually by addition of a little malt (premalting), or, in a few plants, by addition of small amounts of mineral acid. For laboratory work the use of acid is much more convenient than premalting. In developing a fermentation test medium for use in evaluating amylolytic agents it was first necessary to determine optimum conditions for liquefying the starchy mashes so that they could be readily handled and fermented with low levels of the saccharifying agent. However, in such a test medium it is necessary that inappreciable amounts of fermentable sugars be formed during the liquefying operations so that subsequent fermentation results will be truly representative of the amylolytic activity of the agent under test.

Preliminary studies were first undertaken to obtain a

suitable test fermentation medium using ground whole yellow corn as a substrate. To be adequately cooked and properly thinned by using dilute mineral acid to achieve a uniform and readily fermentable mash, the proper proportions were found to be 100 g. of ground whole yellow corn with 500 ml. of added liquid. Mashes of this concentration when cooked with plain water were very thick and lumpy and did not give reliable data upon fermentation. All series of mashes throughout this investigation were prepared by the procedure given in the "Methods" section of this thesis.

The first series of mashes was prepared to determine the optimum concentration of acid to be employed. To each flask containing 100.0 g. of ground whole yellow corn was added 250 ml. of hydrochloric acid of different normalities. The resultant mashes were cooked for 30 minutes at 20 pounds steam pressure, and 4.0 g. per flask of mold bran sample MB#1 were employed for the saccharification. After cooling to 30°C the pH was first roughly checked with "Accutint" pH paper and later adjusted to a pH of 5.0 to 5.3 using a Cameron pH meter. To adjust the pH the required amounts of standard 1.0 normal hydrochloric acid were added to the flasks using the lower normalities of acid, while the flasks employing the higher normalities of acid required the addition of small amounts of a standard concentrated sodium carbonate solution.

The results of using various concentrations of hydro-

chloric acid in thinning corn mashes on the ethanol yields after 48 hours fermentation are given in Table 1. The 48-hour fermentation time was chosen for two reasons, namely, (1) a test method was needed to give an exact fermentation evaluation in a shorter period of time than the conventional three to four day fermentation, and (2) a shorter period would emphasize the difficiencies of a poor amylolytic agent.

Table 1

Effect of Concentration of Hydrochloric Acid Used in Thinning Corn Mashes on the Ethanol Yields

Normality of acid	Mold bran, g. per 100 g. corn	Ethanol, g. per flask
0.00	4.0	27.1
0.02	4.0	27.1
0.04	4.0	27.7
0.06	4.0	27.5
0.08	4.0	26.2
0.10	4.0	27.2

These results indicated that 0.04 normal hydrochloric acid was near the optimum concentration of acid to be used when the mash was cooked for 30 minutes at 20 pounds steam pressure. There was a distinct difference in the consistency

of the mashes cooked with water and with 0.02 normal and 0.04 normal hydrochloric acid. The mash containing only water was very thick and lumpy, the mash containing 0.02 normal acid was somewhat thinner but still too thick to handle well or ferment rapidly, while the mash cooked with 0.04 normal acid was quite thin and had a light tan color. The mashes cooked with acid of higher concentration appeared light brown in color and the color deepened as the acid concentration increased. This color was due to the carmelization of the starch hydrolytic products, and this degradation accounts for the fact that mashes in which higher acid concentrations were employed gave lower ethanol yields than the mash cooked with 0.04 normal acid. In addition, the toxic effect produced in cooking corn meal with acid, as described by Goering (1941), may become more pronounced at higher concentrations.

2. Effect on ethanol yields of time and variable mold bran levels.

It was of interest to determine the possible yields of ethanol at various levels of mold bran after fermentation for different periods of time, using the same procedure as with the previous series, but employing the 0.04 normal hydrochloric acid. The effects of fermentation time and varying levels of mold bran MB#1 on the ethanol yields are shown in Table 2.

Table 2

Effect on Ethanol Yields of Fermentation Time and Various Levels of MB#1

Mold bran, g. per 100 g. corn	Ethanol, g. per flask				
	24 hrs.	36 hrs.	48 hrs.	60 hrs.	72 hrs.
1.0	15.4	18.7	18.8	20.8	21.8
2.0	19.8	21.3	23.7	25.3	25.8
3.0	20.7	24.7	27.3	28.6	28.1
4.0	22.3	25.4	27.7	29.5	28.8
5.0	22.0	27.6	29.0	29.6	29.6
6.0	23.0	28.6	29.6	30.8	30.5

The data showed that the greatest spread of ethanol yields occurred with the 36 hour fermentation period. As the fermentation time increased from 36 to 72 hours the lower levels of mold bran gave yields of ethanol showing higher increments than those of the higher levels of mold bran; that is, the ethanol yields from lower levels of mold bran tend to approach the ethanol yields of the higher levels of mold bran if long enough fermentation times are allowed. In the same way ethanol yields obtained with a mold bran of poor amylase activity would tend to approach the ethanol yields produced

by a mold bran of higher amylolytic activity with long fermentation periods. That is, a mold bran of poor amylase activity would show less difference than a better mold bran if compared on a long fermentation period rather than on a shorter one. From a practical viewpoint it would be advantageous to adopt the shortest possible length of time to obtain significant data upon which an evaluation scheme could be based. For corn mash, using the specified conditions, 36-hour fermentation times gave the desired data.

3. Effect on ethanol yields of cooking time and concentration of hydrochloric acid.

The corn mashes of the series shown in Table 2 were observed to have a thick portion of improperly cooked mash in the center of each flask upon its removal from the autoclave prior to quick cooling and saccharification. This led to somewhat lumpy mashes which, unable to be completely saccharified, gave somewhat erratic ethanol yields. A series using a longer cooking time and various normalities of hydrochloric acid was judged necessary. The effects on the ethanol yields of 45 minutes cooking time at 20 pounds steam pressure with various normalities of hydrochloric acid, using mold bran MB#1 as the saccharifying agent, are shown in Table 3.

Table 3

Effects on the Ethanol Yields of 45 Minutes Cooking Time With Various Concentrations of Hydrochloric Acid Used in Thinning Corn Mashes

Normality of acid	Mold bran, g. per 100 g. corn	Ethanol, g. per flask
0.00	4.0	27.6
0.01	4.0	28.2
0.02	4.0	28.3
0.03	4.0	28.8
0.04	4.0	27.8
0.05	4.0	27.8

The mashes were adequately cooked in the 45 minutes cooking time at 20 pounds steam pressure and the data indicated that the 0.03 normal hydrochloric acid rather than 0.04 normal acid, was the proper concentration to give maximum ethanol yields under these conditions of cooking. The fact that a longer cooking period required less acid for maximum ethanol yields was noted many times throughout this investigation, and apparently the toxic effect produced in cooking corn meal with acid, as previously mentioned, increased with a longer cooking period using the same acid concentration.

4. Test medium using ground whole yellow corn.

Two 36-hour fermentation series employing wider ranges of mold bran levels using samples MB#1 and FCPC#1 were carried out to obtain a complete range of ethanol yields. The cooking time was 45 minutes at 20 pounds steam pressure with 0.03 normal hydrochloric acid. The data are presented in Table 4.

Table 4

Ethanol Yields from Mold Brans MB#1 and FCPC#1 Employing a 36-hour Fermentation Period

Mold bran MB#1, g. per 100 g. corn	Ethanol, g. per flask	Mold bran FCPC#1, g. per 100 g. corn	Ethanol, g. per flask
0.2	5.10	0.1	8.90
0.5	13.95	0.2	15.31
1.0	19.34	0.5	20.75
2.0	22.70	1.0	24.55
3.0	24.52	1.5	26.50
4.0	26.15	2.0	28.25
5.0	28.00	2.5	29.05
6.0	29.00	3.0	30.05
8.0	29.25	4.0	30.75

The results of Table 4 showed a wide range of ethanol yields for both mold bran samples, and no maximum yields were reached since the fermentations were not allowed to go to completion.

From meager data, obtained previous to this investigation, covering only limited levels of mold bran and with the conventional 72-hour fermentation period using corn mashes, mathematical and graphical analysis indicated a straight line function. This important discovery gave promise of furnishing a method for an accurate evaluation of the requirement of a given sample of mold bran for saccharifying fermentation mashes. This straight line function was obtained by plotting the weight of mold bran used divided by the weight of total ethanol produced, against the weight of mold bran used. This method was applied to the data from Table 4 which were used to compute the numerical values for $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ tabulated in Table 5.

Table 5

Calculation of the Numerical Values for

$$\frac{\text{Weight of Mold Bran}}{\text{Weight of Ethanol}}$$

Mold bran, g. per 100 g. corn	Ethanol, g. per flask	Weight of mold bran / Weight of ethanol
Mold bran, MB#1		
0.2	5.10	0.0392
0.5	13.95	0.0358
1.0	19.34	0.0518
2.0	22.70	0.0882
3.0	24.52	0.1222
4.0	26.15	0.1528
5.0	28.00	0.1786
6.0	29.00	0.2072
8.0	29.25	0.2732
Mold bran, FCPC#1		
0.1	8.90	0.0112
0.2	15.31	0.0131
0.5	20.75	0.0241
1.0	24.55	0.0407
1.5	26.50	0.0567
2.0	28.25	0.0708
2.5	29.05	0.0859
3.0	30.05	0.0977
4.0	30.75	0.1300

The data from Table 5, when plotted in the manner outlined above, gave the curves shown in Figure 2. It was very evident that, thus plotted, the straight lines obtained were almost parallel. The data at very low levels of mold bran did not fit the curves very well, which was not surprising,

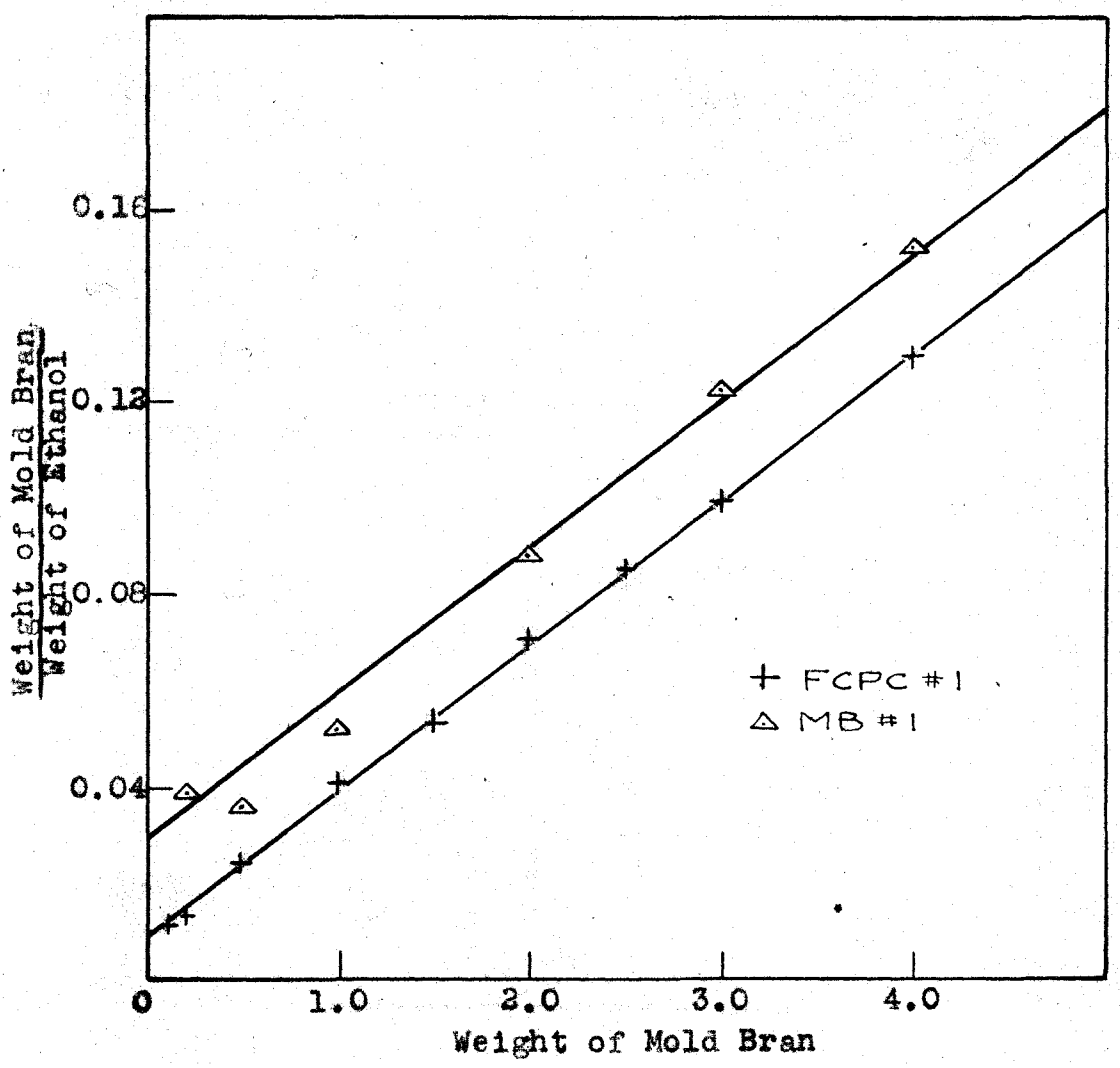

Figure 2. Curves for samples FCPC#1 and MB#1 from 36-hour corn fermentation.

since under the specific conditions employed saccharification was quite poor, and small experimental variations probably had more influence on the results than did the amylolytic activity of the mold bran.

The graphical analysis of the data of Table 5 with MB#1 and FCPC#1, the former a medium-quality mold bran and the latter a high-quality mold bran, indicated that the more efficient the mold bran was in saccharifying fermentation mashes the lower was the intercept value obtained by extrapolating to the Y-axis. It became the purpose of this investigation, therefore, to attempt to devise a method, based upon this mathematical and graphical principle, which would have all the advantages of the previous tedious fermentation method but would require the use of a minimum number of levels of the saccharifying agent, and would give exact fermentation evaluation in a much shorter period.

B. Establishment of a Standard Test Medium and Procedure

It was judged that corn, as a test medium, was unsatisfactory for future work because its lack of uniformity would render any consistent standardization almost impossible, and hence it would be useless for rapid and dependable evaluation measurements. The time required to obtain reproducible test data from corn mashes, namely 36 hours, was also considered undesirably long. In order to overcome these two main ob-

jections to the use of corn mashes, another substrate of more uniform composition was sought which could be prepared consistently and also could be handled with ease and fermented well. Corn starch of a pure food grade quality was selected because of its homogeneity and wide availability. It will henceforth be designated simple as "starch". Difco yeast extract, a uniform product of high quality which can be obtained readily, was used to supply the necessary nutrients for yeast growth and activity.

1. Ratio of acid, starch, and yeast extract.

Preliminary studies were undertaken to determine the optimum concentration of starch, yeast extract, and hydrochloric acid which would provide a mash not too thick to handle and ferment at the lower levels of mold bran, but still containing inappreciable amounts of fermentable sugars. Since the corn mashes employed in the preliminary work contained about 60 to 65 g. of starch per flask, it was decided that mashes containing 50.0 g., 75.0 g., or 100.0 g. of starch would be investigated as possible test media. The amount of yeast extract commonly employed in many fermentation media is 0.5 per cent of the total medium. Since it was necessary to keep the number of variables at a minimum, after preliminary experiments, some of which will be described later, had shown that 1.0 per cent of yeast extract gave as good or perhaps

slightly better results than the 0.5 per cent level, it was finally decided to employ 5.0 g. of yeast extract per flask containing 500 to 600 ml. total volume of mash to eliminate any effects of possible variation in the yeast extract and any possibility that insufficient nutrients were supplied for maximum yeast growth and activity. The limiting factor then would depend solely on how efficiently the amylolytic agent could saccharify the substrate.

In a preliminary experiment it was found that for mashes containing 50.0 g., 75.0 g., and 100.0 g. of starch with 250 ml. of liquid, the most satisfactory periods for cooking at 20 pounds steam pressure were 30, 45, and 60 minutes, respectively. Using these cooking periods three series of mashes were prepared, employing various concentrations of hydrochloric acid, one containing 50.0 g. of starch and 2.5 g. of yeast extract per flask, one employing 75.0 g. of starch and 5.0 g. of yeast extract per flask, and one containing 100.0 g. of starch and 5.0 g. of yeast extract per flask. The regular procedure for preparing mashes described under the section on "Methods" in this thesis was employed. Observations and analyses were made on the mashes after cooling to 30°C. In Table 6 are summarized the data on the visual appearance of the individual mashes and the reducing substances, determined by the method of Underkofler, Guymon, Rayman, and Fulmer (1943) and computed as glucose, produced during the

cooking periods.

Table 6

Effects of Concentrations of Hydrochloric Acid Used in Thinning Starch Mashes and the Reducing Substances Produced by the Resultant Hydrolysis

Normality of acid	Visual observation of mash	Reducing substances, g. per flask
50.0 g. starch per flask		
0.01	stiff gel	----
0.02	just thin	2.52
0.03	too thin; yellow color	5.88
0.04	too thin; brown color	----
0.05	too thin; dark brown	----
75.0 g. starch per flask		
0.03	stiff gel	----
0.04	too thick; thin gel	----
0.05	just thin	2.08
0.06	too thin; brown color	5.06
100.0 g. starch per flask		
0.04	stiff gel	----
0.05	just thin	2.63
0.06	too thin; yellow color	4.59
0.07	too thin; brown color	12.42

It was judged from the data of Table 6 that the hydrochloric acid concentration of 0.02, 0.05, and 0.05 normal adequately thinned the starch mashes containing 50.0 g., 75.0 g., and 100.0 g. of starch per flask, respectively, so that

these mashes could be handled readily with the lowest levels of amylolytic agents employed. These acid concentrations were adopted for future work and were considered optimum for thinning their respective starch mashes. It should be pointed out that the reducing substances were not evaluated in the mashes which were too thick, because of the difficulty of obtaining a measureable mash sample, and likewise the reducing substances were not determined in the mashes which were judged too thin and whose color indicated carmelization of some of the hydrolytic products derived from the starch. The actual amounts of reducing substances formed in the mashes judged to be just adequately thinned were less than 3.0 g. per flask.

In an attempt to ascertain what portion of the reducing substances, as indicated in Table 6 for the adequately thinned mashes, was contributed by the starch and yeast extract, it was necessary to determine the amount of reducing substances in not only the yeast extract before and after autoclaving, but also in the starch sample itself. Three related investigations were conducted in an attempt to elucidate this point. To 500.0 ml. of water was added 5.0 g. of yeast extract, the solution was stirred with a glass rod until homogenous, and the reducing substances were analyzed by the method of Underkofler, Guymon, Rayman, and Fulmer (1943). To 250.0 ml. of 0.05 normal hydrochloric acid was added 5.0 g.

of yeast extract and the general procedure for preparing mashes was followed. After cooking at 20 pounds steam pressure for 60 minutes, a 250.0 ml. portion of cold water was added and the pH adjusted to 5.3 with the requisite amount of a concentrated solution of sodium carbonate. Upon cooling to 30°C analysis was made to determine the reducing substances. To 500.0 ml. of water was added 100.0 g. of starch, the mixture was stirred to effect even distribution of the starch, and the starch was allowed to settle. The supernatant liquid was then used for the analysis of the reducing substances. The results of these studies are summarized in Table 7.

Table 7

Reducing Substances Obtained from Yeast Extract, Before and After Autoclaving, and from Starch

Material	Treatment	Reducing substances, g. per flask
Yeast extract, 5.0 g. per flask	water solution	0.20
Yeast extract, 5.0 g. per flask	0.05 normal acid, autoclaved	0.18
Corn starch, 100.0 g. per flask	water solution	0.01

The results of Table 7 indicated that the reducing substances obtained in cooking starch mashes, as shown in Table 6, are in a large measure due to the hydrolysis of the starch by the hydrochloric acid used to thin the fermentation mashes, since less than 7.0 per cent of the total reducing substances could have come from the yeast extract and the starch.

With the small amounts of reducing substances produced in the mashes judged just adequately thinned, for all practical fermentative purposes the amylolytic agent must saccharify the mashes rather extensively to obtain any significant ethanol yields. That is, the amount of less than 3.0 g. of reducing substances per flask was judged inappreciable under the conditions employed.

To determine whether 5.0 g. of yeast extract per flask supplied adequate nutrients for the yeast fermentation of starch, two 36-hour fermentation series on media containing 50.0 g. of starch per flask with 2.5 g. of yeast extract per flask in one series and 5.0 g. per flask in the other were conducted. The regular procedure for preparing mashes was used, and the mashes were cooked for 30 minutes at a steam pressure of 20 pounds, using 0.02 normal hydrochloric acid. The mold bran FCPC#1 was used in saccharifying the mashes of both series. The effects of varying amounts of yeast extract on the production of ethanol from starch are shown in Table 8.

Table 8

Effect of Two Levels of Yeast Extract on Ethanol Yields from Starch Employing Mold Bran Sample FCPC#1

Mold bran FCPC#1, g. per 50 g. starch	Ethanol, g. per flask	
	Yeast extract	
	2.5 g. per flask	5.0 g. per flask
0.2	12.33	13.40
0.5	16.75	16.89
1.0	18.17	18.50
1.5	19.28	19.33
2.0	20.16	20.33
4.0	21.07	20.60

The data of Table 8 indicated that within limits of experimental error there was little if any effect on ethanol yields upon the addition of more yeast extract. Thus, the 1.0 per cent concentration finally chosen more than adequately supplied the essential nutrients.

It was observed that the addition of more yeast extract to the mashes caused the pH to become slightly more basic, and the resultant mashes were somewhat more viscous, indicating possibly that a higher concentration of acid should be used when adding more yeast extract in a procedure standardized for a less amount.

2. Test medium employing 50.0 g. of starch.

The previous work using ground whole yellow corn indicated that the time needed to give a consistent wide spread in ethanol yields for various levels of mold bran was 36 hours. Since this was longer than desirable for a rapid method, the next few series were designed to determine if there would not be a wide range of ethanol yields at a shorter fermentation period using essentially the same levels of samples MB#1 and FCPC#1 as previously used. The optimum concentration of 0.02 normal hydrochloric acid, as determined from the data in Table 6, was used to thin the mashes consisting of 50.0 g. of corn starch and 2.5 g. of yeast extract per flask, the mashes being cooked for 30 minutes at 20 pounds steam pressure. The ethanol yields for various periods and levels of mold bran MB#1 and FCPC#1 are summarized in Table 9.

Table 9

Effect of Fermentation Time and Levels of Mold Bran MB#1 and FCPC#1 on Ethanol Yields from Starch

Mold bran, g. per 50 g. starch	Ethanol, g. per flask			
	12 hrs.	24 hrs.	36 hrs.	48 hrs.
Mold bran, MB#1				
0.5	7.73	8.75	10.60	11.50
1.0	8.17	13.79	14.70	17.19
2.0	8.98	16.83	17.26	19.77
3.0	10.05	18.13	18.33	20.80
4.0	10.30	19.41	19.42	21.76
5.0	10.48	19.98	21.03	22.85
Mold bran, FCPC#1				
0.2	6.08	12.32	12.33	13.59
0.5	8.02	15.53	16.25	15.88
1.0	10.50	17.50	18.17	18.39
1.5	10.90	19.11	19.28	19.62
2.0	11.79	19.60	20.16	20.90
4.0	12.96	21.53	21.07	21.43

The ethanol yields shown in Table 9 had leveled off by 24 hours and apparently had nearly reached their maximum with the higher levels of both mold bran MB#1 and FCPC#1. The fermentations were much faster than with ground whole yellow corn and the desired wide spread of ethanol yields was obtained in the 24-hour fermentation period. The data of Table 9 for mold brans MB#1 and FCPC#1 were used to compute the values for $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ which are tabulated in Table 10.

Table 10

Calculated Values for $\frac{\text{Weight of Mold Bran}}{\text{Weight of ethanol}}$

Mold bran, g. per 50 g. starch	$\frac{\text{Weight of mold bran}}{\text{Weight of ethanol}}$			
	12 hrs.	24 hrs.	36 hrs.	48 hrs.
Mold bran, MB#1				
0.5	0.0647	0.0572	0.0472	0.0435
1.0	0.1228	0.0726	0.0680	0.0582
2.0	0.2125	0.1187	0.1159	0.1013
3.0	0.2980	0.1654	0.1637	0.1440
4.0	0.3890	0.2060	0.2060	0.1839
6.0	0.5703	0.3010	0.2855	0.2624
Mold bran, FCPC#1				
0.2	0.0328	0.0162	0.0162	0.0147
0.5	0.0623	0.0321	0.0307	0.0316
1.0	0.0950	0.0571	0.0550	0.0543
1.5	0.1378	0.0786	0.0778	0.0764
2.0	0.1735	0.1020	0.0992	0.0957
4.0	0.3080	0.1854	0.1903	0.1870

The data in Table 10 were plotted in Figure 3, with $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ against weight of mold bran, and reliable straight lines were obtained with the data for fermentation periods of 24 hours or longer, while the 12-hour data gave a curve that was erratic and of only limited value. It was interesting to note that on extrapolation of the curves in Figure 3 to the Y-axis they tended to intersect at that point, and that the slopes of the curves became somewhat less as the

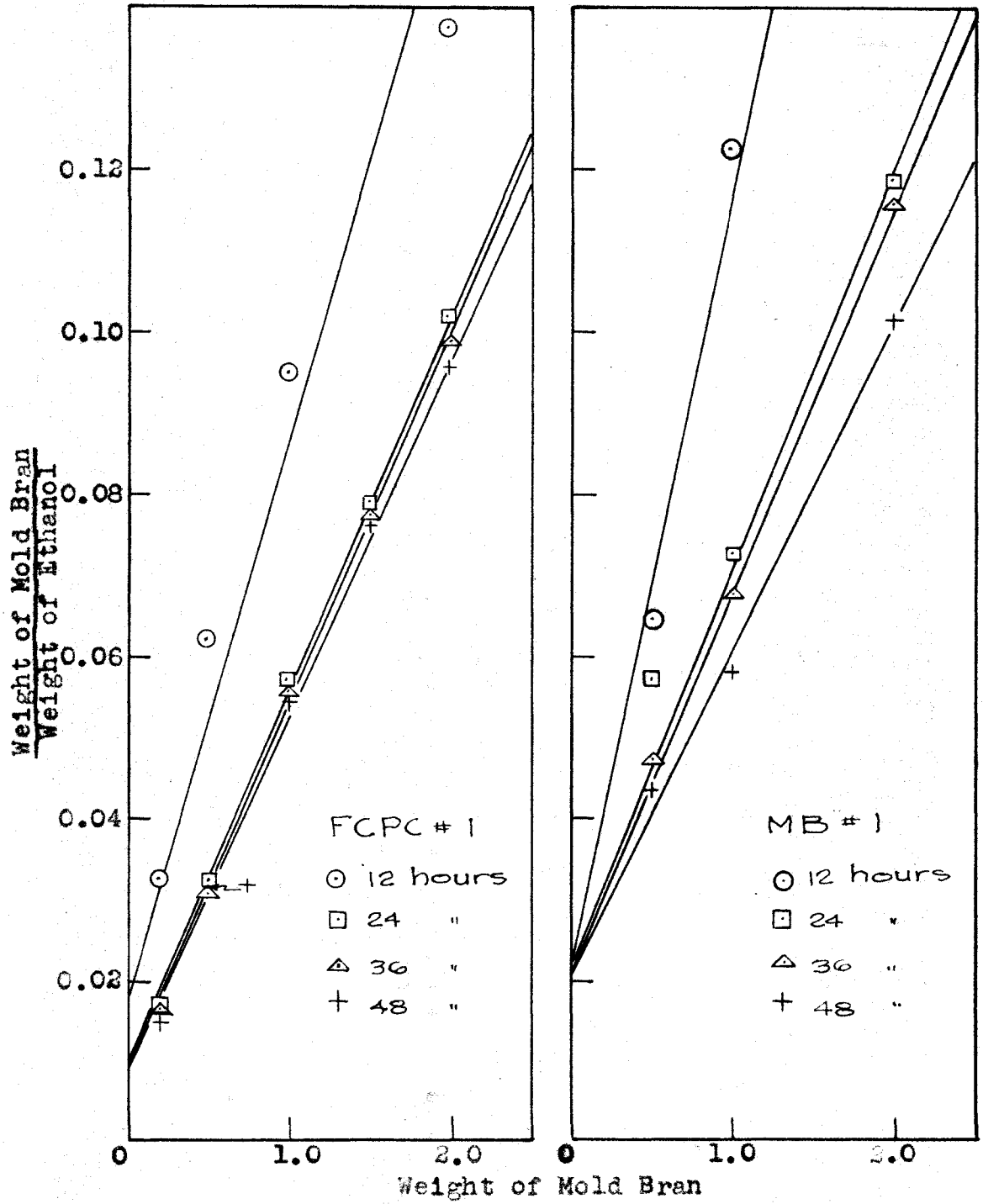

Figure 3. Curves for samples FCPC#1 and MB#1 from fermentations of 50.0 g. of starch for various time periods.

fermentation periods increased.

3. Test medium employing 75.0 and 100.0 g. of starch.

Since the ethanol yields at the 24-hour fermentation period were near the maximum which can be obtained from 50.0 g. of starch, the use of a higher concentration of substrate suggested itself, since this might possibly give a better interpretation of the amylolytic power of the agent employed. Consequently, series with 75.0 g. and 100.0 g. of starch per flask were investigated employing the optimum concentrations of acid and yeast extract as determined from the data in Table 6. The previously determined optimal cooking times were 45 and 60 minutes, respectively, at 20 pounds steam pressure. The tabulated data are given in Table 11. Only sample MB#1 was used because of the limited supply of mold bran FCPC#1.

Table 11

Effect of Fermentation Time and Levels of Mold Bran MB#1 on Ethanol Yields from Starch

Mold bran, g. per flask	Ethanol, g. per flask				
	12 hrs.	18 hrs.	24 hrs.	36 hrs.	48 hrs.
Starch, 75.0 g. per flask					
0.5	4.13	6.33	8.85	11.69	18.60
1.0	5.81	12.09	17.77	19.26	23.62
2.0	7.54	16.59	22.30	24.45	25.58
3.0	8.19	18.78	24.00	25.99	28.25
4.0	9.04	20.99	24.75	26.79	29.22
6.0	10.50	24.00	26.85	28.85	31.93
Starch, 100.0 g. per flask					
0.5	3.93	7.34	11.48	11.62	12.13
1.0	5.52	15.42	20.95	21.73	22.15
2.0	7.30	20.06	26.27	27.82	29.40
3.0	7.61	23.00	29.70	31.26	31.55
4.0	8.60	26.46	30.34	32.55	35.20
6.0	9.50	28.70	33.00	35.80	37.78

The results of Table 11 indicated that the higher starch concentration of 100.0 g. per flask gave a more satisfactory mash than either the concentration of 50.0 g. or 75.0 g. per flask. The 100.0 g. of starch per flask, when used as a substrate, not only appeared to give the desired excess of starch, but also supplied mashes from which the ethanol yields for the highest levels of mold bran had not begun to level off or reach a maximum in the 24-hour fermentation test period.

However, this was not true of the ethanol yields from the mashes containing 75.0 g. of starch per flask, since there was a leveling-off with the highest levels of mold bran after the 18-hour fermentation period. Hence, the amount of 100.0 g. of starch per flask was chosen for all future studies, and only the data for this starch concentration from Table 11 were used to compute the values for $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ which are tabulated in Table 12.

Table 12

Computed Values for $\frac{\text{Weight of Mold Bran}}{\text{Weight of Ethanol}}$

Mold bran MB#1, g. per 100 g. starch	$\frac{\text{Weight of mold bran}}{\text{Weight of ethanol}}$				
	12 hrs.	18 hrs.	24 hrs.	36 hrs.	48 hrs.
0.5	0.1275	0.0682	0.0436	0.0473	0.0413
1.0	0.1815	0.0649	0.0479	0.0461	0.0453
2.0	0.2740	0.0999	0.0761	0.0721	0.0681
3.0	0.3940	0.1300	0.1010	0.0959	0.0941
4.0	0.4650	0.1570	0.1317	0.1222	0.1135
6.0	0.6320	0.2090	0.1809	0.1675	0.1590

The data of Table 12 were plotted in Figure 4 as $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ against the weight of mold bran. Erratic results were obtained for the 12-hour and the 18-hour fermentation periods, and the data for the lower levels of mold bran did not fit the curves very well, which was not surprising since saccharification was very poor under the

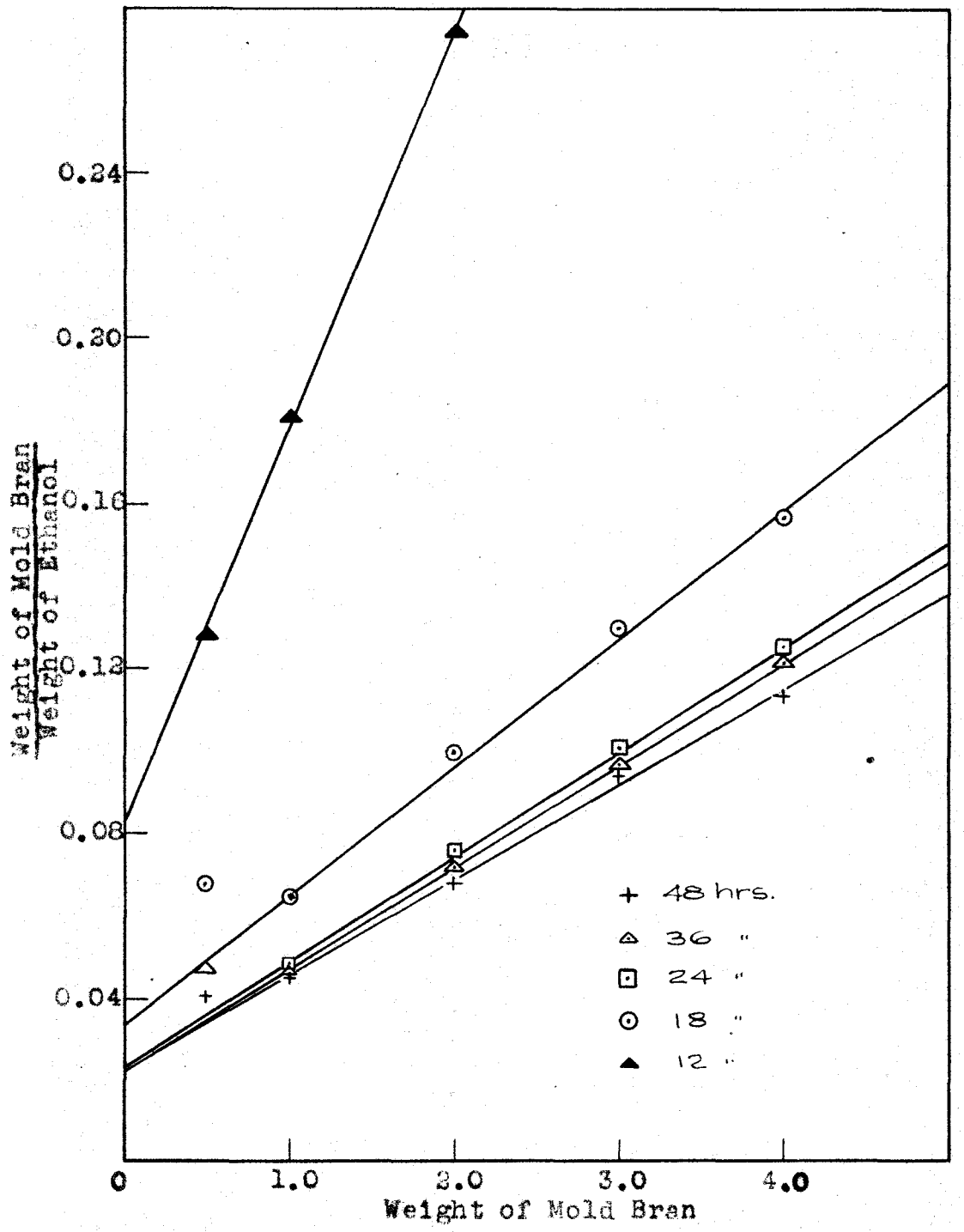

Figure 4. Curves for sample MB#1 from fermentations of 100.0 g. of starch for various time periods.

conditions employed. Significant thinning and saccharification must be produced by the enzymic action of the lower levels of the amylolytic agents before reliable fermentations can be obtained.

An attempt was made to show that there was an excess of starch in the mashes containing 100.0 g. of starch per flask at the end of the 24-hour fermentation period by determining the reducing substances and the ethanol yields at various time intervals. The mold bran FCPC#1 was employed because of its high amylolytic activity, assuming, of course, that this sample would saccharify as much or more starch than any other amylolytic agent tested. Two grams per flask of the sample FCPC#1 were employed since previous conventional 72-hour corn fermentations indicated that 2.0 g. per flask would be about optimum. The data are given in Table 13.

Table 13

Reducing Substances and Ethanol Yields at Various Fermentation Periods Employing Mold Bran FCPC#1

Time, hours	Reducing substances, g. per flask	Ethanol, g. per flask
12	17.06	8.43
24	7.69	23.80
36	3.32	29.80
48	3.08	33.40
60	2.33	34.78
72	2.52	36.75

The results from Table 13 indicated that even at the end of 72 hours the starch mash contained unused starch or its fractions since less than 80 per cent of the total starch had been converted to ethanol. The spent mash when tested with dilute iodine solution gave a bluish-purple color, indicating that some starch and dextrins still remained. The results also indicated that with this mold bran sample there were more than enough reducing substances produced by the amylolytic action in the 24-hour fermentation period, with the limiting factor on ethanol production apparently being the ability of the yeast to utilize the available sugars. Since this was

only a preliminary exploration, a more thorough investigation should be made before many concrete conclusions should be drawn.

Since the 24-hour fermentation test period gave enough time to smooth out unavoidable fluctuations in procedures and reagents and produce a vigorously fermenting mash, it was adopted as the most suitable time for a test period to give reliable and consistent data under the specific conditions employed. With this time period an exact fermentation evaluation of an amylolytic agent can be secured the next day after starting the test instead of three to four days later as when employing orthodox fermentation procedures.

4. The standard evaluation test medium and procedure.

The final test medium and method adopted, therefore, was to use 100.0 g. of starch and 5.0 g. of Difco yeast extract per flask, cook with 250.0 ml. of 0.05 normal hydrochloric acid for 60 minutes at 20 pounds steam pressure, and then follow the general procedure outlined in the "Methods" section of this thesis. The incubation time was 24 hours. This test method will henceforth be designated as the "Standard Evaluation Test".

This Standard Evaluation Test was devised to evaluate the total enzymic activity exhibited by an amylolytic agent in the saccharification of fermentation mashes for the pro-

duction of ethanol. As was pointed out in a previous section of this thesis, there are many known and unknown reactions which are not evaluated or determined by the several methods currently used for the determination of enzymic activity. Many of the present methods merely evaluate one or at best two isolated reactions, and no correlation can be found between the values obtained and the actual activity observed in the complex saccharification and subsequent fermentation reactions. The Standard Evaluation Test attempted to eliminate these major shortcomings common to conventional methods currently employed.

C. Standard Evaluation Test on Amylolytic Preparations

Having developed a satisfactory test medium and procedure, it was next necessary to determine whether the method could be used to evaluate amylolytic preparations having different enzymic activities for the saccharification of fermentation mashes. Samples of 19 different amylolytic preparations were obtained from the various sources indicated in the "Materials" section of this thesis. Each is listed in Table 14 along with the type or name designation and the number used in the laboratory work.

Table 14

Amylolytic Preparations Tested by the Standard Evaluation Test

Source	Designation	Lab No.
L. A. Underkofler	Lab preparation	FCPC#1
Farm Crops Processing Corp.	Semi-commercial	FCPC#2
L. M. Christensen	Pilot plant	UN
Iowa State College	Lab preparation	ISC#1
Iowa State College	Lab preparation	ISC#2
Jacques Wolf Corp.	Commercial "Protozyme"	JWC
Schwarz Laboratories, Inc.	Commercial "Polidase"	SLI
Mold Bran Co., Inc.	Commercial "Eaglezyme"	MB#1
Mold Bran Co., Inc.	Mycelium concentrate	MB#2
Mold Bran Co., Inc.	Plant experimental	MB#3
Mold Bran Co., Inc.	Plant Experimental	MB#4
Mold Bran Co., Inc.	Plant experimental	MB#5
Mold Bran Co., Inc.	Plant experimental	MB#6
Mold Bran Co., Inc.	Commercial "Eaglezyme", Feed Quality	MB#7
Mold Bran Co., Inc.	Plant experimental, contaminated	MB#8
Mold Bran Co., Inc.	Commercial "Eaglezyme", Blue Label	MB#9
Mold Bran Co., Inc.	Commercial "Eaglezyme", Blue Label	MB#10
Mold Bran Co., Inc.	Commercial "Eaglezyme", Red Label	MB#11
Mold Bran Co., Inc.	Commercial "Eaglezyme", Green Label	MB#12

All of the samples of amylolytic preparations in Table 14 except those from the Jacques Wolf Corporation and the Schwarz Laboratories were prepared from a culture of Aspergillus oryzae. This strain is designated as No. 38 in the

Iowa State College Biophysical Chemistry culture collection. No information was available regarding the cultures employed in the preparation of the samples from the Jacques Wolf Corporation and Schwarz Laboratories, but it is believed these also were manufactured with strains of Aspergillus oryzae.

1. Intercepts obtained by graphical methods.

Since laboratory facilities did not allow parallel fermentations employing all of the 19 amylolytic agents listed in Table 14 in a single series, it was necessary to choose a reference agent for comparison. The preparation FCPC#2 was chosen, because it not only gave excellent ethanol yields but also gave very consistent results the several times it was used as a comparison standard. The results obtained with the Standard Evaluation Tests on the 19 amylolytic agents tested have been arranged so that the graphical plotting of the data gave enough space between the curves for legibility and interpretation. Thus, the data from no more than four amylolytic agents are plotted on any one graph.

Since the preliminary studies seemed to indicate that four levels of amylolytic agent gave concordant results in the 24-hour test period, the levels of 1.0 g., 2.0 g., 3.0 g., and 4.0 g. of amylolytic agent were adopted as standard for each agent tested. Triplicate fermentations and determinations were carried out with each level, and any questionable series were repeated.

The ethanol yields as well as the computed values of $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ are given in Table 15 for amylolytic samples FCPC#2, UN, MB#1, and ISC#1 when employing the Standard Evaluation Test.

Table 15

Results of Standard Evaluation Tests with Samples FCPC#2, UN, MB#1, and ISC#1

Mold bran, lab No.	Mold bran, g. per 100 g. starch	Ethanol, g. per flask	Weight of mold bran / Weight of ethanol
FCPC#2	1.0	24.98	0.0402
	2.0	28.65	0.0698
	3.0	31.07	0.0965
	4.0	32.50	0.1230
UN	1.0	23.00	0.0435
	2.0	28.47	0.0703
	3.0	30.41	0.0988
	4.0	32.18	0.1244
MB#1	1.0	20.95	0.0479
	2.0	26.27	0.0761
	3.0	29.70	0.1010
	4.0	30.34	0.1317
ISC#1	1.0	18.15	0.0550
	2.0	24.55	0.0815
	3.0	27.28	0.1095
	4.0	29.89	0.1338

The corresponding ethanol yields for sample FCPC#2 were somewhat higher than for any of the other samples in Table 15,

indicating it to have a higher amylolytic activity. This difference was still more apparent when the results of Table 15 were plotted in Figure 5. Parallel lines were obtained within the limits of experimental error, and the low numerical value of the Y-axis intercept for sample FCPC#2 was indicative of its high amylolytic potency as compared with the other samples. The representative data for the reference mold bran, FCPC#2, given in Table 15 and plotted in Figure 5 are not repeated in subsequent tables and figures, but are referred to for comparison in discussing the results with the other amylolytic agents tested.

The samples FCPC#1, SLI, JWC, and ISC#2, when tested by the Standard Evaluation Test, gave the ethanol yields and the computed values of $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ which are summarized in Table 16.

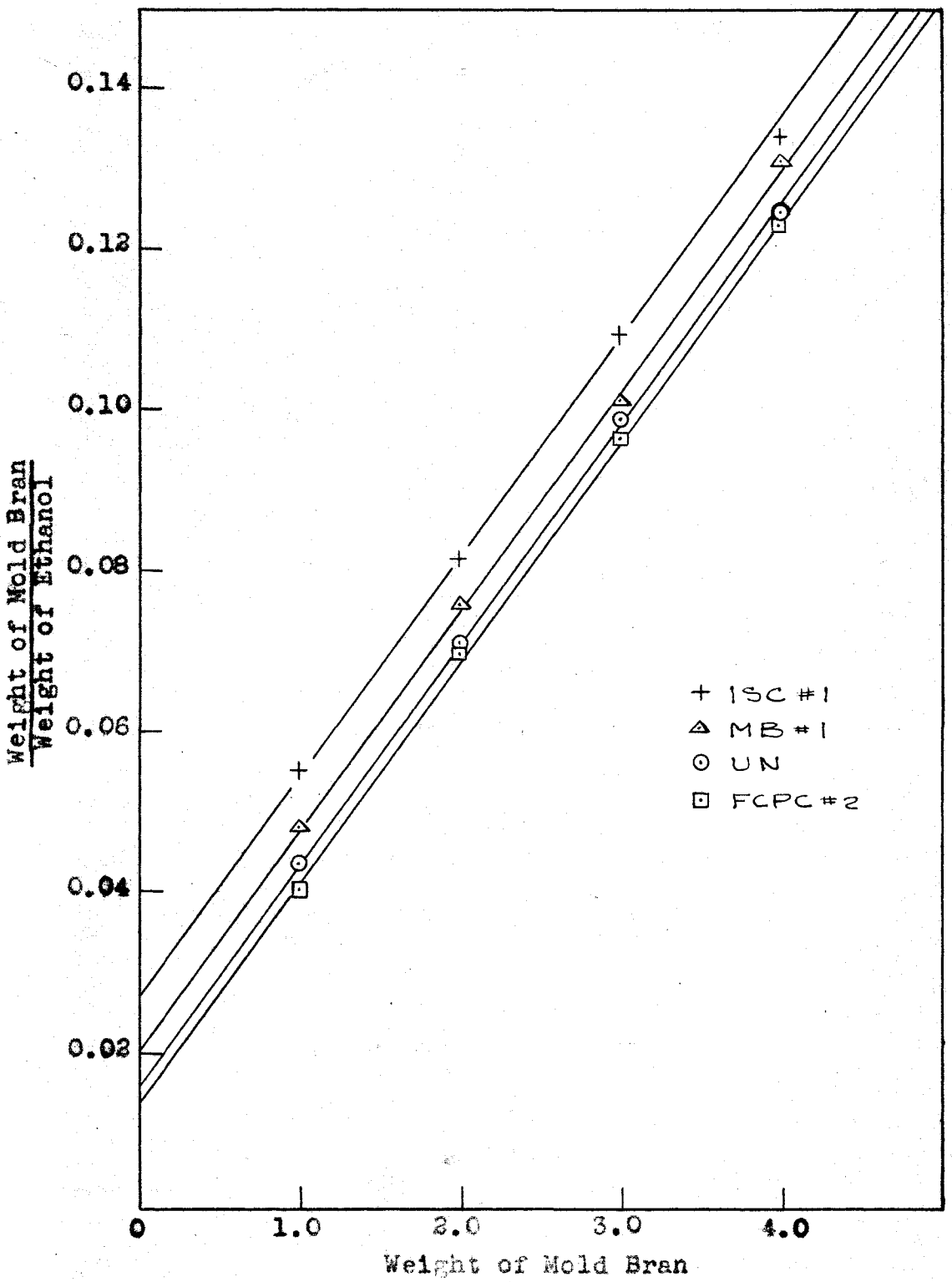

Figure 5. Curves for Standard Evaluation Tests with samples FCPC#2, UN, MB#1, and ISC#1.

Table 16

Results of Standard Evaluation Tests with Samples FCPC#1, SLI, JWC, and ISC#2

Mold bran, lab No.	Mold bran, g. per 100 g. starch	Ethanol, g. per flask	Weight of mold bran / Weight of ethanol
FCPC#1	1.0	26.30	0.0380
	2.0	30.57	0.0653
	3.0	32.36	0.0927
	4.0	33.33	0.1200
SLI	1.0	25.87	0.0387
	2.0	29.08	0.0688
	3.0	30.69	0.0977
	4.0	32.50	0.1230
JWC	1.0	25.48	0.0392
	2.0	28.45	0.0704
	3.0	30.83	0.0972
	4.0	31.72	0.1260
ISC#2	1.0	19.44	0.0513
	2.0	25.58	0.0780
	3.0	27.79	0.1096
	4.0	30.30	0.1320

The data of Table 16 showed that sample FCPC#1 gave slightly higher yields of ethanol than the reference FCPC#2, while the two commercial samples SLI and JWC gave somewhat lower yields, and the older ISC#2 sample the lowest. These small differences in enzymic potency as noted by ethanol yields in Table 16 were more noticeable when the data were plotted in Figure 6. Within the limits of experimental error

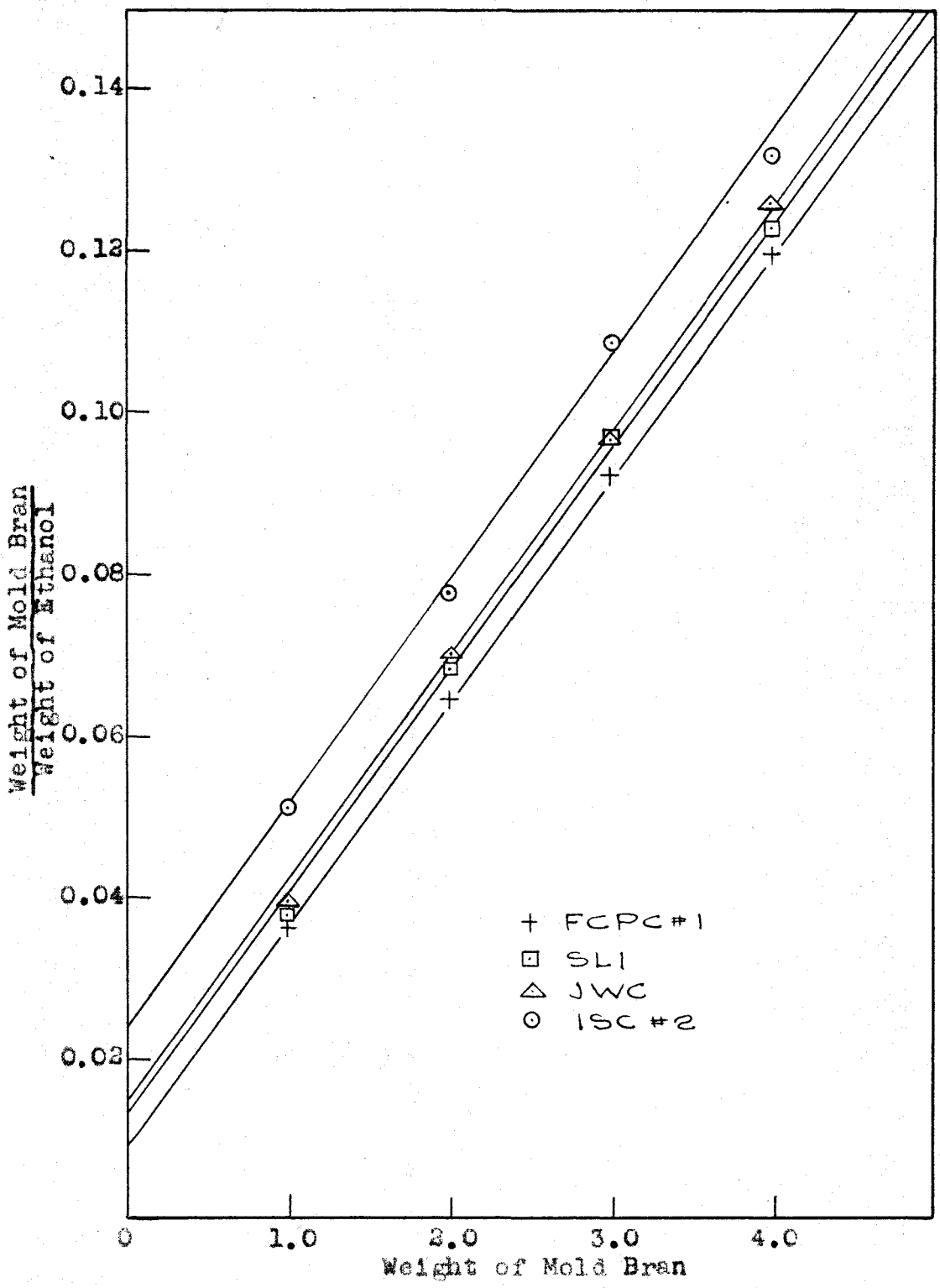

Figure 6. Curves for Standard Evaluation Tests with samples FCPC#1, SLI, JWC, and ISC#2.

parallel lines were again obtained, and the smaller the numerical value of the Y-axis intercept, the higher was the amylolytic activity.

The Standard Evaluation Tests on the four levels of MB#9, MB#2, MB#5, and MB#3 gave the data which are compiled in Table 17. Since MB#2 was a concentrate, smaller increments and levels of this sample were employed.

Table 17

Results of Standard Evaluation Tests with Samples MB#9, MB#2, MB#5, and MB#3

Mold bran, lab No.	Mold bran, g. per 100 g. starch	Ethanol, g. per flask	Weight of mold bran / Weight of Ethanol
MB#9	1.0	24.51	0.0408
	2.0	29.26	0.0673
	3.0	31.73	0.0944
	4.0	33.03	0.1210
MB#2	0.5	17.45	0.0286
	1.0	23.08	0.0433
	1.5	25.65	0.0583
	2.0	27.51	0.0726
MB#5	1.0	21.03	0.0476
	2.0	25.30	0.0789
	3.0	27.85	0.1076
	4.0	30.20	0.1325
MB#3	1.0	19.90	0.0502
	2.0	23.92	0.0836
	3.0	26.31	0.1139
	4.0	28.26	0.1412

It was noted from the ethanol yields in Table 17 that the MB#2, a concentrate of unknown potency, gave slightly lower yields than MB#9 and the reference FCPC#2; thus the standard levels of the Standard Evaluation Test could have been employed with MB#2. However, when the data were plotted in Figure 7, a fairly good straight line was obtained for MB#2 practically parallel to the other curves. The lower levels of MB#5 and MB#3 were erratic and the data did not fit the curves in Figure 7 very well, which is typical for samples having poor amylolytic activity, since saccharification is quite poor with resultant unreliable fermentations.

The Standard Evaluation Tests with MB#8, MB#7, MB#6, and MB#4 gave the data presented in Table 18.

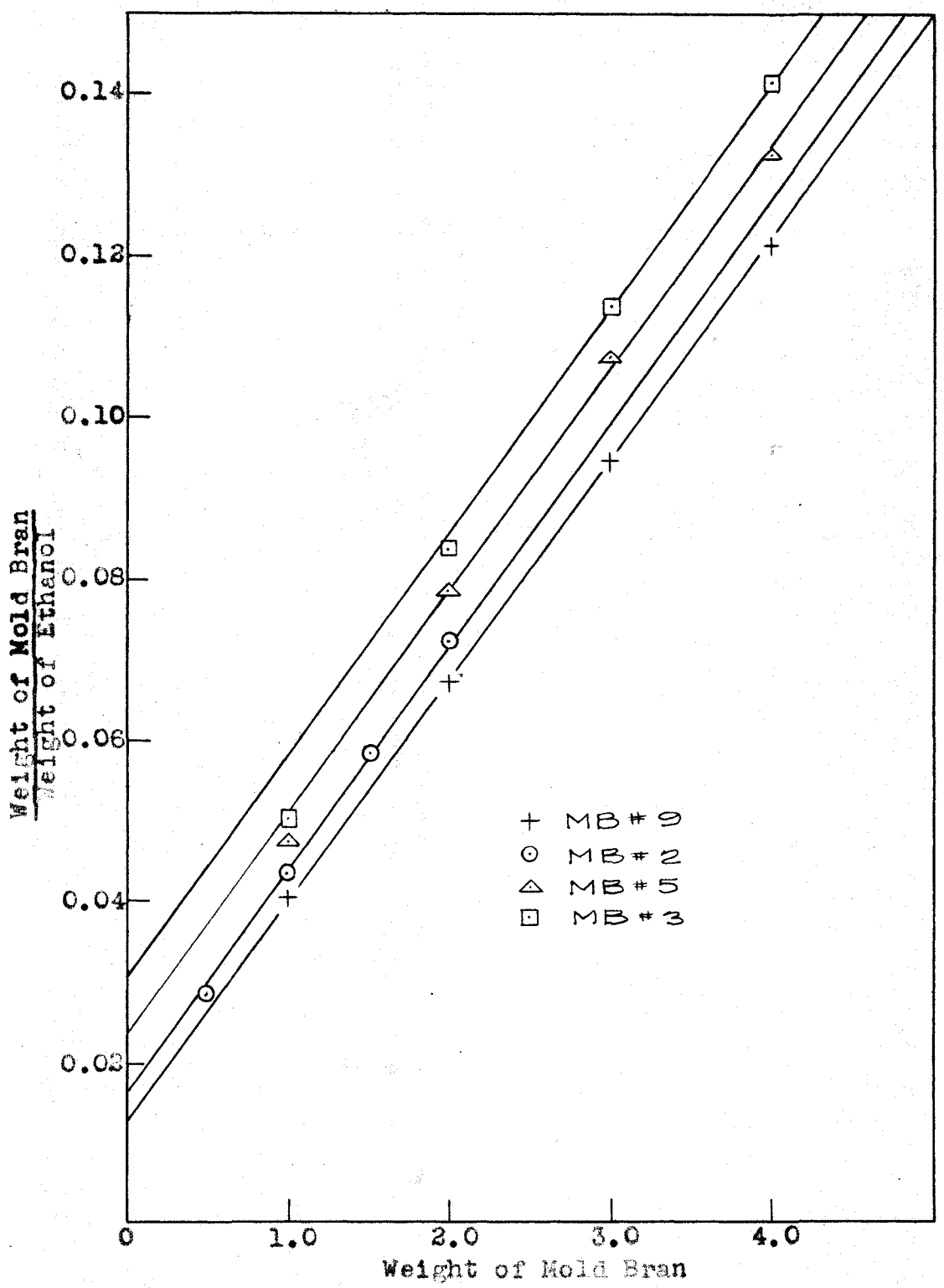

Figure 7. Curves for Standard Evaluation Tests with samples MB#9, MB#2, MB#5, and MB#3.

Table 18

Results of Standard Evaluation Tests with Samples MB#8, MB#7, MB#6, and MB#4

Mold bran, lab No.	Mold bran, g. per 100 g. starch	Ethanol, g. per flask	Weight of mold bran / Weight of ethanol
MB#8	1.0	6.21	0.1610
	2.0	13.85	0.1445
	3.0	19.79	0.1519
	4.0	23.21	0.1722
MB#7	1.0	7.53	0.1328
	2.0	15.83	0.1264
	3.0	22.27	0.1350
	4.0	24.90	0.1603
MB#6	1.0	21.17	0.0472
	2.0	24.21	0.0827
	3.0	27.83	0.1079
	4.0	29.82	0.1340
MB#4	1.0	22.90	0.0436
	2.0	27.00	0.0740
	3.0	28.89	0.1039
	4.0	31.38	0.1278

None of the ethanol yields from the corresponding levels of mold bran samples given in Table 18 were as high as for the reference FCPC#2, in fact, the MB#7 and MB#8 samples gave very low ethanol yields, indicating poor fermentations and very low amylolytic activities. The low activities of MB#7 and MB#8 were even more noticeable when the data were plotted in Figure 8, and straight lines were not obtained. Had high-

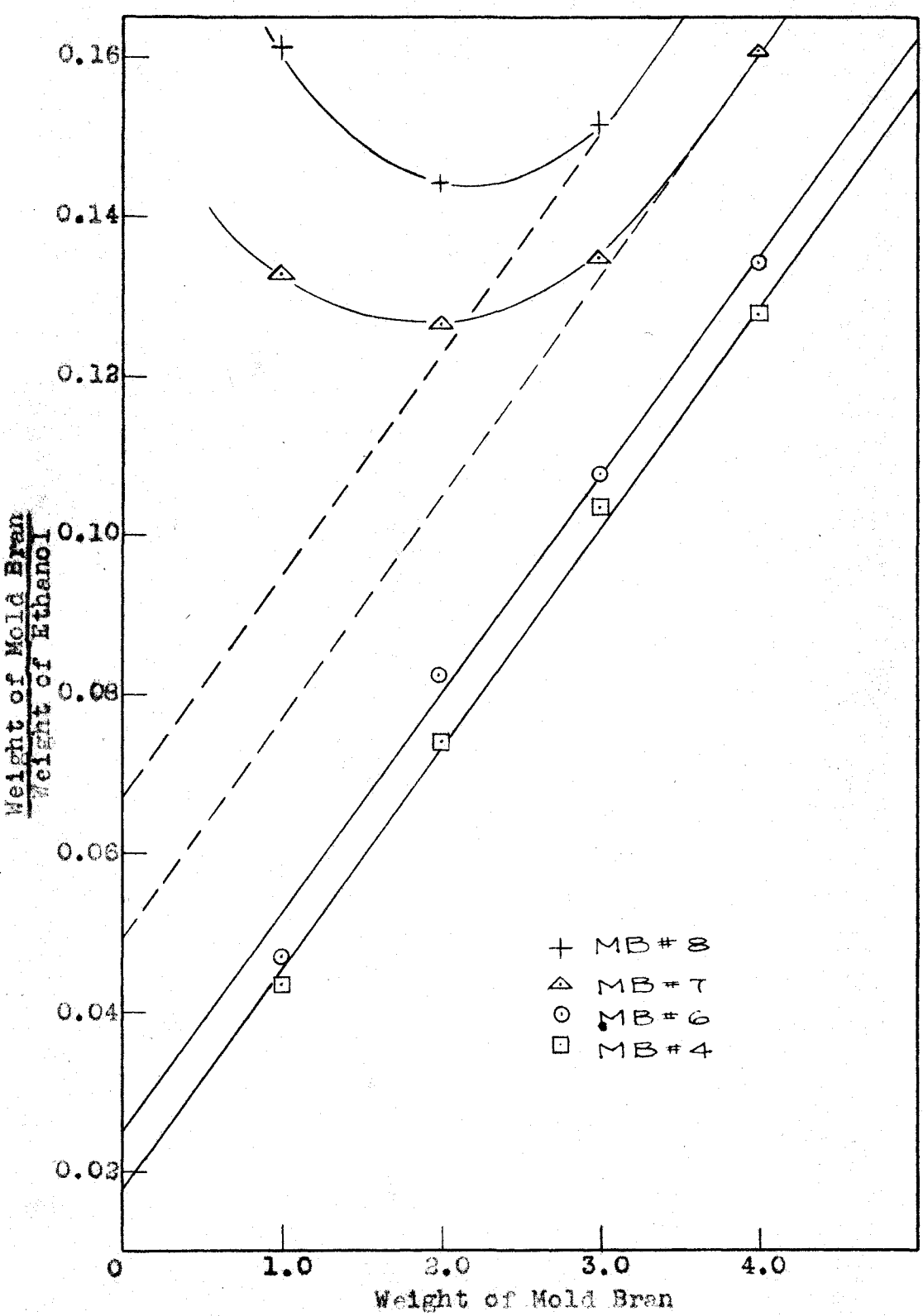

Figure 8. Curves for Standard Evaluation Tests with samples MB#8, MB#7, MB#6, and MB#4.

er levels of these mold brans been employed, it might be predicted that the dotted lines would have been obtained by extrapolation. However, samples of such low activity would be useless in commercial practice in saccharifying fermentation mashes for ethanol production.

The weights of samples employed, ethanol yields, and the computed values of $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ are tabulated in Table 19 for the Standard Evaluation Tests with MB#10, MB#11, and MB#12.

Table 19

Results of Standard Evaluation Tests with Samples MB#10, MB#11, and MB#12

Mold bran, lab No.	Mold bran, g. per 100 g. starch	Ethanol, g. per flask	Weight of mold bran / Weight of ethanol
MB#10	1.0	21.48	0.0466
	2.0	26.49	0.0757
	3.0	29.46	0.1019
	4.0	30.61	0.1309
MB#11	1.0	20.92	0.0477
	2.0	25.92	0.0772
	3.0	28.60	0.1048
	4.0	30.60	0.1308
MB#12	1.0	15.81	0.0632
	2.0	21.03	0.0950
	3.0	24.47	0.1226
	4.0	27.03	0.1478

The data in Table 19 showed low yields of ethanol for the MB#12 and higher ethanol yields for the MB#10 and MB#11, but none gave as good ethanol yields as the reference FCPC#2. The differences in amylolytic activity were clearly illustrated in Figure 9 when the data were plotted for the three samples, MB#10, MB#11, and MB#12.

The Y-axis intercepts for all samples except MB#7 and MB#8, for which straight lines were not obtained, were read from the graphs of Figures 5, 6, 7, 8, and 9. These intercepts in the order of their increasing numerical value, but decreasing amylolytic activity, are tabulated in Table 20.

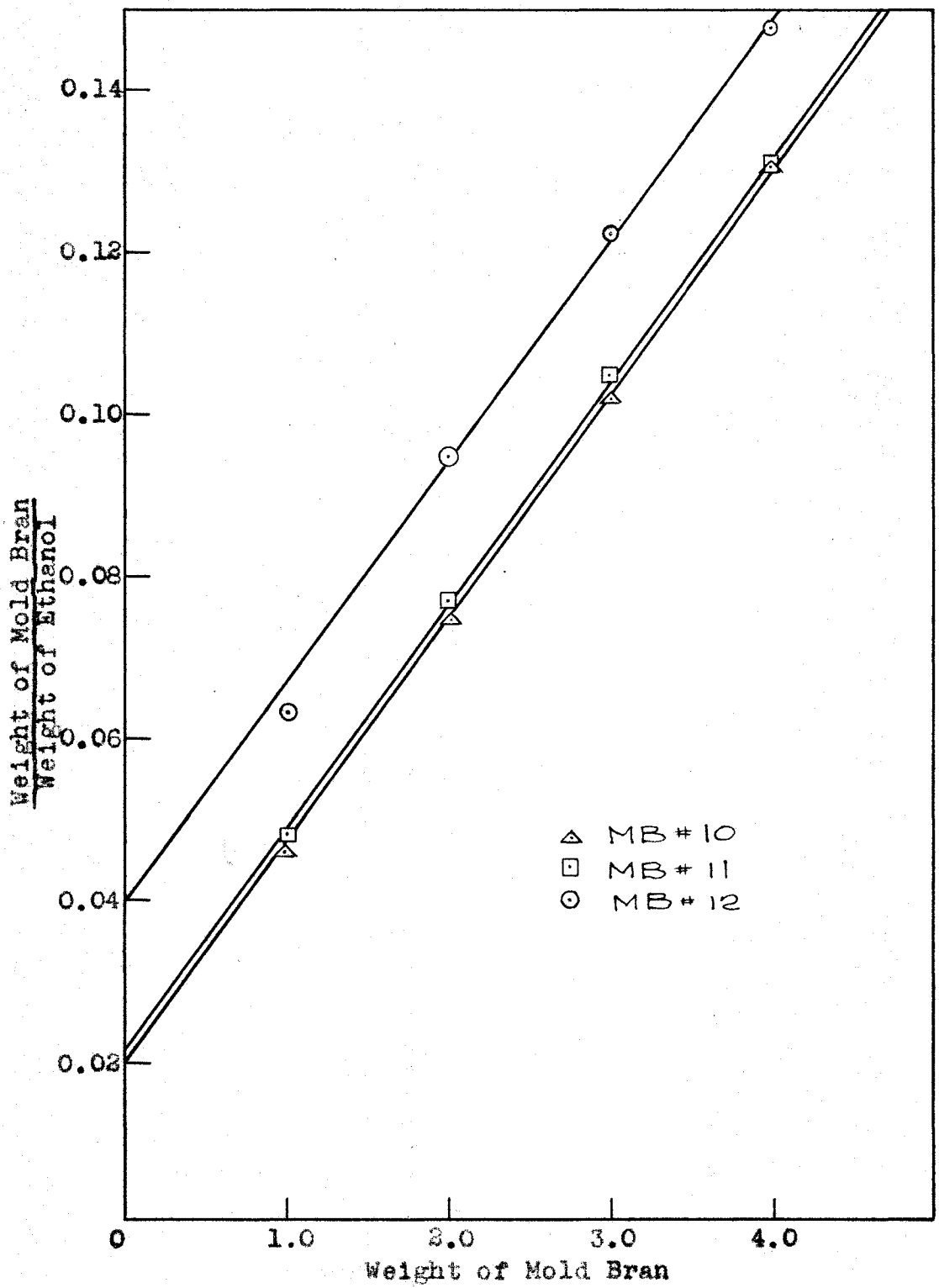

Figure 9. Curves for Standard Evaluation Tests with samples MB#10, MB#11, and MB#12.

Table 20

Numerical Intercept Values Obtained by Graphical Analysis for the Amylolytic Agents Tested by the Standard Evaluation Test

Mold bran, lab No.	Intercept value, graphical
FCPC#1	0.0100
MB#9	0.0131
SLI	0.0132
FCPC#2	0.0133
JWC	0.0144
UN	0.0156
MB#2	0.0163
MB#4	0.0177
MB#1	0.0200
MB#10	0.0210
MB#11	0.0213
MB#5	0.0236
ISC#2	0.0240
MB#6	0.0250
ISC#1	0.0264
MB#3	0.0303
MB#12	0.0378

It is apparent from a study of Figures 5, 6, 7, 8, and 9 that the curves tend to have approximately the same slopes, that is, the lines are essentially parallel within the limits of experimental error, and the higher the amylolytic activity of the mold bran, as measured by the Standard Evaluation Test, the lower is the intercept value obtained by extrapolat-

ing the curves to the Y-axis. These intercepts thus obtained give numbers, the relative value of which indicate the relative efficiencies of the amylolytic agents for saccharifying fermentation mashes, the lower the intercept the greater the efficiency. The equation for a straight line is $y = mx + b$, and, applied to the curves given in Figures 5, 6, 7, 8, and 9, y is the $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$, x is the weight of mold bran, m is the slope, and b is the intercept. The slopes for the curves are practically identical, having a value of about 0.0274, but the intercepts, b, are inversely proportional to the amylolytic activities of the different samples, and, therefore, these intercepts serve to evaluate the total enzymic activity of the amylolytic agents for saccharifying fermentation mashes.

2. Intercepts obtained by mathematical methods.

Since the slopes for all the Standard Evaluation Test curves were practically the same, namely, 0.0274, a simplification in the procedure suggested itself. The Standard Evaluation Test could be modified by using a single level of mold bran, say 3.0 g. per flask, instead of four levels, and then computing the intercept from the straight line equation, $y = mx + b$. The mold bran level of 3.0 g. per flask was chosen to avoid the erratic results obtained with many of the lower levels of some of the samples investigated. Three

samples of mold bran, MB#10, MB#11, and MB#12, were selected for testing this procedure, since they were the last samples obtained from the Mold Bran Co., and were judged to be representative. The fermentations were carried out in quadruplicate using 3.0 g. of each sample and the regular test procedure. The ethanol yields obtained from the 24-hour fermentations as well as the computed values of $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ are given in Table 21.

Table 21

Modified Standard Evaluation Test with Samples MB#10, MB#11, and MB#12

Mold bran, lab No.	Mold bran, g. per 100 g. starch	Ethanol, g. per flask	$\frac{\text{Weight of mold bran}}{\text{Weight of ethanol}}$
MB#10	3	29.15	0.1028
MB#11	3	28.71	0.1045
MB#12	3	25.09	0.1199

The data of Table 21 showed ethanol yields within limits of experimental error, comparable to those given in Table 19 for the same samples at the 3.0 g. per flask level. Using the straight line equation, $y = mx + b$, and slope 0.0274 obtained from the curve for the reference sample FCPC#2, the

intercepts were calculated from the data given in Table 21. These values are compared in Table 22 with the graphical intercepts which had previously been obtained (Table 20).

Table 22

Intercepts Determined Graphically from Four Mold Bran Levels and Calculated from a Single Level for the Samples MB#10, MB#11, and MB#12

Mold bran, lab No.	Intercept	
	Graphical	Calculated
MB#10	0.0210	0.0206
MB#11	0.0213	0.0223
MB#12	0.0378	0.0377

The results presented in Table 22 indicate that the greatest variance between graphical and computed intercept values for the three representative samples was for MB#11, and this difference is within the limits of experimental error. This mathematical method, although apparently not quite as accurate as the graphical method employing four levels of mold bran, seems promising for use in commercial control laboratory procedures for the rapid evaluation of the enzymic activity of amylolytic agents employed in the saccharification of fermentation mashes.

It should be pointed out that the Standard Evaluation Test has been devised to eliminate the troublesome variables that are commonly encountered in working with agricultural products. Such variables include the determination of moisture and of starch in both the starchy substrate and the amylolytic agent. It is well known that rather widely varying results are frequently obtained in these analyses on check samples sent to different laboratories even when the determinations are carried out by the accepted methods of the Association of Official Agricultural Chemists. Hence such analyses are of only limited practical value. Starch analyses were only helpful in this investigation to determine the approximate maximum ethanol yields which might theoretically be obtained from a substrate.

Adoption of pure food grade starch because of its consistent homogeneity and wide availability and use of a large excess in the Standard Evaluation Test made the starch and moisture analyses of inconsequential importance. However, to obtain reliable data on a comparative basis, the same substrate should be used for all the samples of amylolytic agents evaluated. That is, a standard curve with the reference amylolytic agent should be obtained with each new batch of starch employed.

Likewise the moisture and composition of the amylolytic agents need not be determined since the relative potency of

each agent is evaluated on the "as received" basis just as it is to be used in the actual conventional fermentations. In other words, the moisture content and composition are compensated for in the intercept obtained by the Standard Evaluation Test. For example, if two agents have the same amylolytic potency on a dry basis but one has a higher moisture content, the numerical intercept value will be larger for the one with the more moisture, other variables being constant.

D. Optimum Requirements of Amylolytic Agents for Maximum Ethanol Production

Preliminary information, obtained from the Standard Evaluation Tests in the previous section, indicated that, since the amylolytic activities were inversely proportional to the intercept values, the optimum requirements of two different amylolytic agents for maximum ethanol production should be proportional to the two intercept values. This correlation of the intercept values with optimum requirements of the amylolytic agents was necessary for a complete evaluation scheme that would give an exact fermentation evaluation. According to this postulate, if any good sample were selected as a standard and the conventional 72-hour corn fermentation carried out employing various levels of this standard sample to determine the minimum optimal level for maxi-

mum ethanol production, the minimum optimal level of a second agent could be computed by direct proportion from the intercept values obtained by the Standard Evaluation Test Method. The proportion used in this calculation would be:

$$\frac{\text{intercept (standard agent)}}{\text{intercept (unknown agent)}} = \frac{\text{optimum level (standard agent)}}{\text{optimum level (unknown agent)}}.$$

The conventional 72-hour corn fermentation procedure adopted for determining the minimum optimal level for maximum ethanol production for the standard sample, as well as for the other samples, followed the regular procedure used in the Standard Evaluation Test as closely as conveniently possible. This close adherence of analogous procedures for both the 24-hour Standard Evaluation Test and the 72-hour conventional corn fermentation was one of the principles upon which this investigation was based, and thus the correlation between the enzymic activity as shown by the Standard Evaluation Test and the actual performance in the conventional 72-hour corn fermentation was more easily made.

In general, any method which is used for a test has greater importance when the procedures of the method are very similar to the practical conditions under which the material tested is to be used, and the more closely the two coincide the more valuable becomes the test method. This principle has been stressed wherever possible in this investigation.

Utmost consideration should be given to the selection of the substrate to be used in the final 72-hour fermentation.

Corn was selected in this investigation because corn is the most widely used grain employed as a substrate for ethanol production by the yeast fermentation. If any other substrate, such as wheat, oats, rye, sorghum grains, barley, sweet potatoes, white potatoes, cassava starch, bananas, or any other suitable starchy material were to be used in the final fermentation, then it should be employed as the substrate in the so-called conventional 72-hour fermentation. Only in this way can a truly exact evaluation be made as to the total effectiveness of the amylolytic agent employed in the saccharification of fermentation mashes. Since this investigation used only the one substrate corn, future work might well include the use of various other starchy materials used to prepare fermentation mashes for the complete evaluation of an amylolytic agent.

The minor changes in the Standard Evaluation Test procedure necessary for the 72-hour corn fermentations were: 100.0 g. of ground whole yellow corn was substituted for the 100.0 g. of starch per flask; the Difco yeast extract was eliminated; 0.02 normal instead of 0.05 normal hydrochloric acid produced adequate thinning and gave a final pH of 5.0 to 5.3, thus eliminating the necessity of neutralizing with concentrated sodium carbonate solution; the fermentation time was increased from 24 to 72 hours; and six different levels of each amylolytic agent were employed over a range which in-

cluded the optimum of each agent as computed by proportion from the intercept data compiled in Table 20, using FCPC#2 as the standard sample. The other details of the procedure were followed precisely as given for the Standard Evaluation Test method.

1. Minimum optimal level of sample FCPC#2 for maximum ethanol yields from corn.

A series of preliminary 72-hour conventional corn fermentations using several levels of the reference sample FCPC#2 indicated that a maximum yield of ethanol was obtained with a weight of between 2.5 g. and 3.0 g. per 100.0 g. of corn. Series using 0.1 g. increments between those two limits were repeated until concordant results gave the average ethanol yields for each level of sample summarized in Table 23.

Table 23

Results of Conventional 72-hour Corn Fermentations with the Reference Sample FCPC#2

Mold bran FCPC#2, g. per 100 g. corn	Ethanol, g. per flask
1.5	28.53
2.0	30.50
2.5	32.11
2.6	32.27
2.7	32.25
2.8	32.25
2.9	32.21
3.0	32.23
3.5	32.09
4.0	32.17

The results from Table 23 indicated that the minimum optimal level of reference sample FCPC#2 was between 2.6 g. and 2.7 g. per flask. These data were plotted in Figure 10 to aid in determining the exact minimum level of sample FCPC#2 which gave the maximum yield of ethanol in the 72-hour corn fermentation. The arrow above the curve in Figure 10 indicates the minimum optimal level chosen for the reference sample FCPC#2, namely, 2.66 g. per flask.

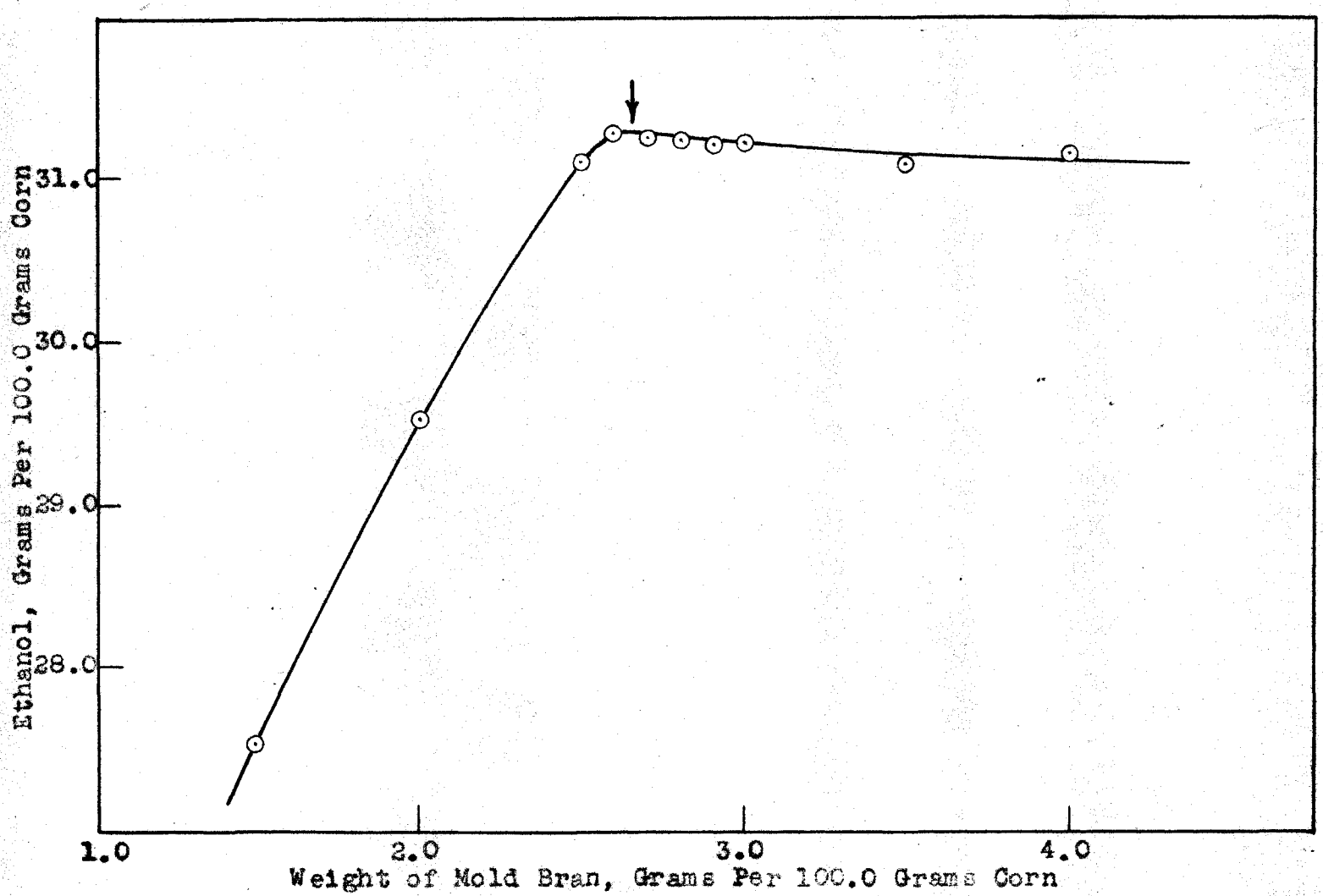

Figure 10. Minimum optimal level of sample FCPC#2 for maximum ethanol production from corn mashes.

2. Calculated minimum optimal levels of 19 amylolytic agents for saccharifying corn mashes.

The minimum optimal levels for the samples listed in Table 14 to give maximum ethanol production employing the conventional 72-hour corn fermentation were computed from the proportion

$$\frac{\text{intercept (standard agent)}}{\text{intercept (unknown agent)}} = \frac{\text{optimum level (standard agent)}}{\text{optimum level (unknown agent)}},$$

using the intercept data of Table 20, with the intercept of 0.0133 and the minimum optimal level of 2.66 g. per 100.0 g. of corn for the reference sample FCPC#2 as determined from Figures 5 and 10, respectively. These computed minimum optimal levels for maximum ethanol production from corn are tabulated for each sample in Table 24.

Table 24

Computed Minimum Optimal Levels of Amylolytic Agents for Maximum Ethanol Production from Corn

Mold bran, lab No.	Optimum level mold bran, g. per 100 g. corn
FCPC#1	2.00
MB#9	2.62
SLI	2.64
FCPC#2	(2.66)*
JWC	2.88
UN	3.12
MB#2	3.26
MB#4	3.54
MB#1	4.00
MB#10	4.20
MB#11	4.26
MB#5	4.72
ISC#2	4.80
MB#6	5.00
ISC#1	5.28
MB#3	6.06
MB#12	7.56

*Determined experimentally.

3. Minimum optimal levels of 12 amylolytic agents from conventional 72-hour corn fermentations.

For the conventional 72-hour corn fermentations, increments of 0.5 g. of sample were judged sufficiently accurate to secure a good curve when the ethanol yields were plotted against the weight of amylolytic agent, particularly

when the computed minimum optimal level was included nearly midway in the series of six levels employed.

The mold bran levels employed and the ethanol yields for the conventional 72-hour corn fermentations are given in Table 25 for the samples FCPC#2, JWC, and SLI.

Table 25

Results of Conventional 72-hour corn Fermentations with Samples FCPC#2, JWC, and SLI

Mold bran, lab No.	Mold bran, g. per 100 g. corn	Ethanol, g. per flask
FCPC#2	1.16	27.17
	1.66	29.22
	2.16	30.81
	2.66*	32.29
	3.16	32.16
	3.66	32.17
JWC	1.38	27.78
	1.88	29.37
	2.38	30.92
	2.88*	31.74
	3.38	31.84
	3.88	32.60
SLI	1.14	28.11
	1.64	29.36
	2.14	30.16
	2.64*	31.89
	3.14	31.90
	3.64	31.90

*Computed minimum optimal level.

The results of Table 25 indicated that the minimum op-

timal level for standard sample FCPC#2, as read from Figure 10, did give the maximum ethanol yield as a further increase in mold bran gave no further increase in ethanol. Sample JWC did not give the maximum ethanol production for the computed minimum optimal level of the sample, indicating either experimental error or a discrepancy in the Standard Evaluation Test data, although, a leveling-off of ethanol yields was indicated in the vicinity of the levels employed. Sample SLI gave maximum yields of ethanol for the computed level of mold bran thus adding substantial support to this evaluation method.

The data from Table 25 for samples FCPC#2, JWC, and SLI were used in Figure 11 with the ethanol yields plotted against the weight of mold bran. The arrow above each curve in Figure 11 indicates the computed minimum optimal level for that particular sample.

The mold bran levels employed with samples ISC#2, ISC#1, and UN gave the ethanol yields shown in Table 26 in the conventional 72-hour corn fermentations.

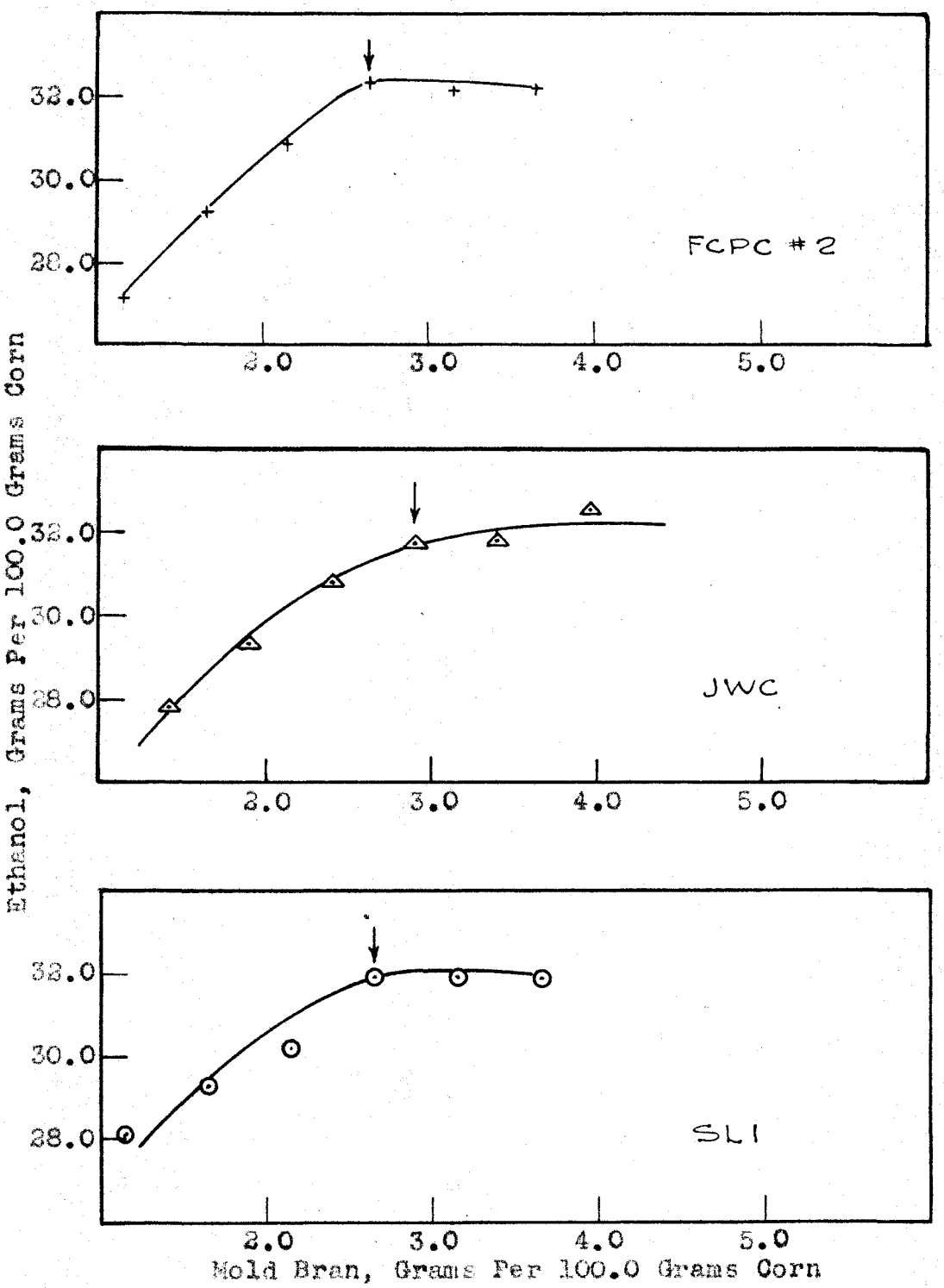

Figure 11. Minimum optimal levels of samples FCPC#2, JWC, and SLI for maximum ethanol production.

Table 26

Results of Conventional 72-hour Corn Fermentations with Samples ISC#2, ISC#1, and UN

Mold bran, lab No.	Mold bran, g. per 100 g. corn	Ethanol, g. per flask
ISC#2	3.30	28.75
	3.80	29.55
	4.30	30.79
	4.80*	31.70
	5.30	31.50
	5.80	31.62
ISC#1	3.78	32.01
	4.28	32.44
	4.78	32.88
	5.28*	33.84
	5.78	33.65
	6.28	33.74
UN	1.62	26.27
	2.12	28.20
	2.62	29.32
	3.12*	30.88
	3.62	31.19
	4.12	31.65

*Computed minimum optimal level.

The results of Table 26 indicated that the computed minimum optimal levels for the samples ISC#2 and ISC#1 gave the maximum ethanol yields, but the sample UN gave small increases in ethanol production for all increments of sample, although there was a general leveling-off of yields indicating a maximum was being attained. These conclusions were more noticeable when the ethanol yields were plotted against the weight

of mold bran in Figure 12. The arrow above each curve indicates the computed minimum optimal level for that particular sample.

Using the conventional 72-hour corn fermentations, samples FCPC#1, MB#1, and MB#9 gave ethanol yields for the various levels as tabulated in Table 27.

Table 27

Results of Conventional 72-hour Corn Fermentations with Samples FCPC#1, MB#1, and MB#9

Mold bran, lab No.	Mold bran, g. per 100 g. corn	Ethanol, g. per flask
FCPC#1	1.50	31.50
	2.00*	32.50
	2.50	33.14
	3.00	33.10
	3.50	33.26
	4.00	33.11
MB#1	2.50	26.90
	3.00	28.65
	3.50	30.15
	4.00*	30.98
	4.50	30.67
	5.00	30.65
MB#9	1.12	26.62
	1.62	27.98
	2.12	30.42
	2.62*	31.82
	3.12	31.15
	3.62	32.01

*Computed minimum optimal level.

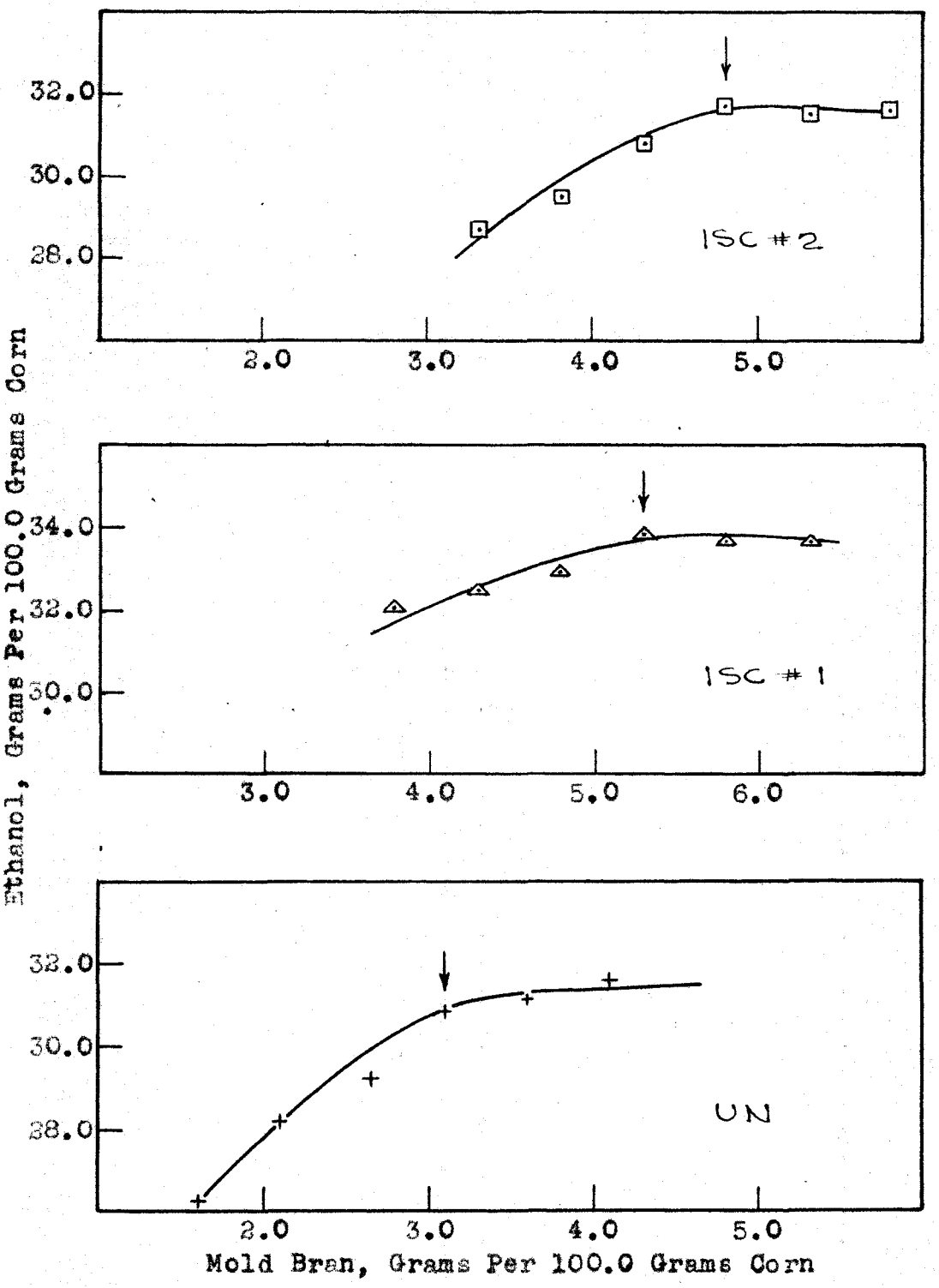

Figure 12. Minimum optimal levels of samples ISC#2, ISC#1, and UN for maximum ethanol production.

The results from Table 27 indicated there was a slight difference between the computed and the actual minimum optimal level for sample FCPC#1 to give maximum ethanol production. Either experimental error or unreliable data from the Standard Evaluation Test may be responsible, but possibly the few months time which elapsed between the Standard Evaluation Test and the 72-hour corn fermentations may have caused a slight deterioration in the sample, although usually a sample may be stored for two to three years with no appreciable loss in potency. Samples MB#1 and MB#9 gave, within limits of experimental error, maximum ethanol yields for the computed minimum optimal levels of sample. When ethanol yields were plotted against the weight of mold bran, the curves in Figure 13 were obtained. The arrow above each curve indicates the computed minimum optimal level for that particular sample.

The levels of samples MB#12, MB#11, and MB#10 and corresponding ethanol yields are given in Table 28 for the conventional 72-hour corn fermentations with these mold brans.

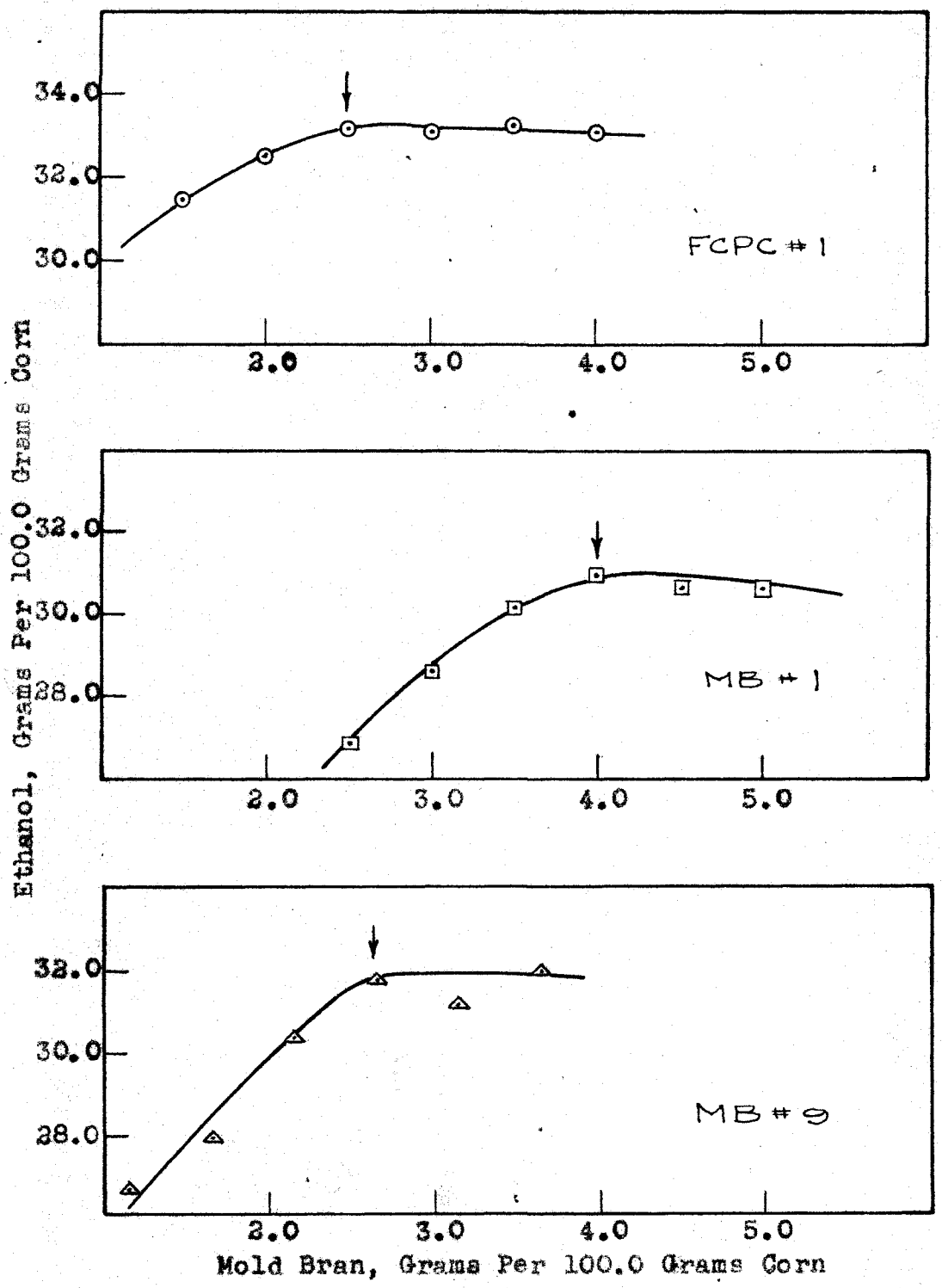

Figure 13. Minimum optimal levels of samples FCPC#1, MB#1, and MB#9 for maximum ethanol production.

Table 28

Results of Conventional 72-hour Corn Fermentations with Samples MB#12, MB#11, and MB#10

Mold bran, lab No.	Mold bran, g. per 100 g. corn	Ethanol, g. per flask
MB#12	6.06	27.04
	6.56	28.17
	7.06	28.75
	7.56*	29.20
	8.06	29.05
	8.56	29.07
MB#11	2.76	30.80
	3.26	31.90
	3.76	31.96
	4.26*	32.38
	4.76	32.00
	5.26	31.70
MB#10	2.70	30.28
	3.20	30.50
	3.70	31.71
	4.20*	32.09
	4.70	32.14
	5.20	32.10

*Computed minimum optimal level.

The data in Table 28 indicated that the computed minimum optimal level for each of the three samples gave the maximum ethanol yield since increasing the levels above the computed minimum optima gave no further increases in ethanol. The curves in Figure 14 were obtained when the ethanol yields were plotted against the weights of mold bran as given in

Table 28. The arrow above each curve indicates the computed minimum optimal level of each particular sample.

The lack of sufficient sample prevented conducting the conventional 72-hour corn fermentations with samples MB#2, MB#3, MB#4, MB#5, and MB#6. They are therefore omitted from Table 29 in which are summarized the minimum optimal levels, computed and experimental, of each sample for maximum ethanol yields from 100.0 g. of this specific sample of ground whole yellow corn.

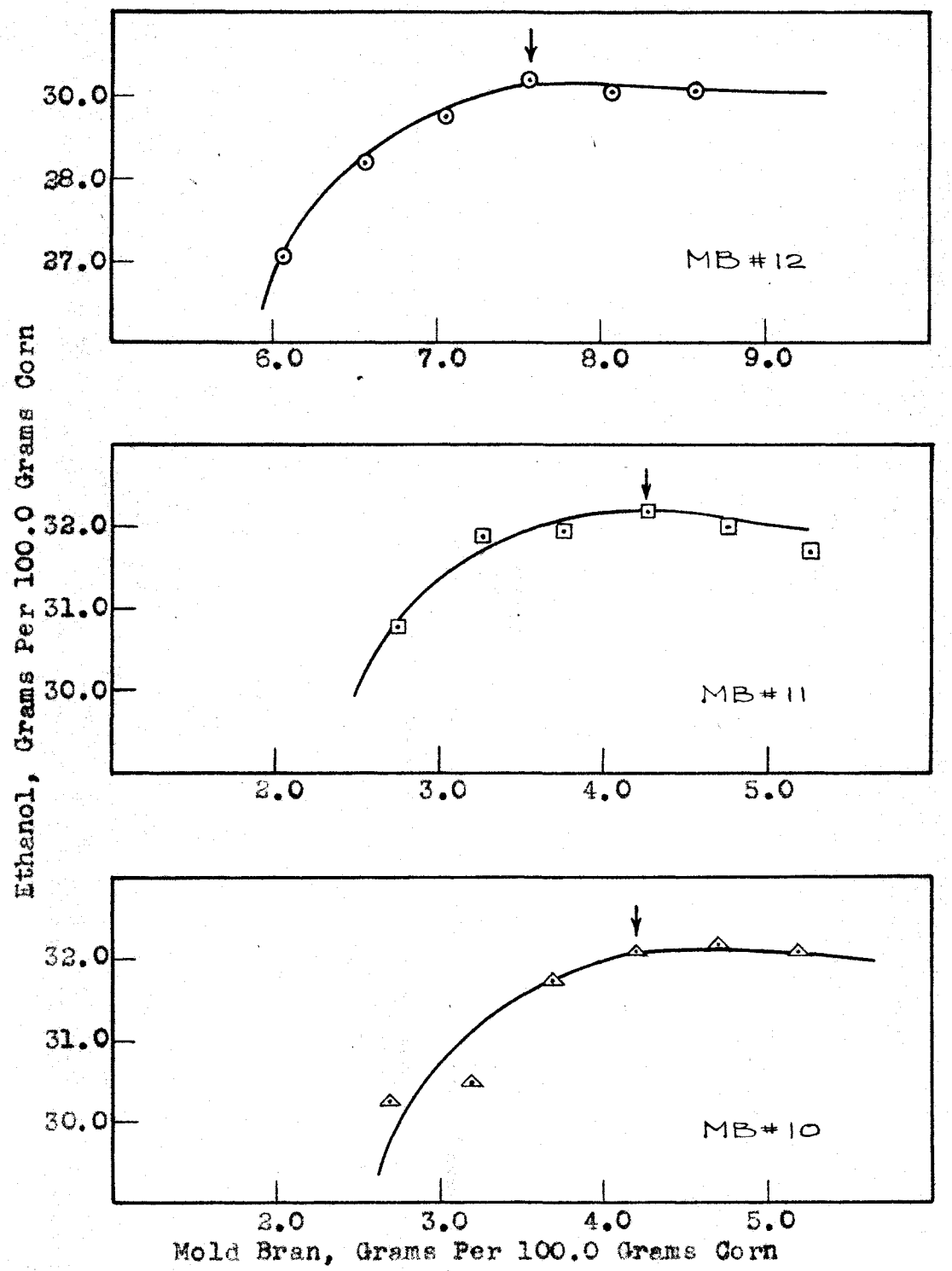

Figure 14. Minimum optimal levels of samples MB#12, MB#11, and MB#10 for maximum ethanol production.

Table 29

Computed Minimum Optimal Levels Compared to Experimental Levels of Amylolytic Agents for Maximum Ethanol Production from Corn

Mold bran, lab No.	Optimum level mold bran, g. per 100 g. corn	
	Calculated	Experimental
FCPC#2 (standard)	----	2.66
FCPC#1	2.00	2.5
MB#9	2.62	2.6
SLI	2.64	2.6
JWC	2.88	3.0 (?)
UN	3.12	3.5 (?)
MB#1	4.00	4.0
MB#10	4.20	4.2
MB#11	4.26	4.3
ISC#2	4.80	4.8
ISC#1	5.28	5.3
MB#12	7.56	7.6

The only samples which gave some deviation from the computed minimum optimal levels were, according to the data in Table 29, FCPC#1, JWC, and UN. The other samples gave very concordant results. To make a more exact determination of

the minimum optimal level of each sample, series could be conducted using very small increments, such as 0.1 g., but for all practical purposes the increments of 0.5 g. were judged sufficiently accurate for this evaluation method.

Thus, the data compiled in Table 29 gave evidence that the relative total enzymic activities of amylolytic agents for the saccharification of fermentation mashes could not only be evaluated by using the intercepts obtained from the Standard Evaluation Test, but also the minimum optimal levels to obtain maximum ethanol production from corn mashes could be calculated from these intercepts. The feasibility of the commercial adoption of this evaluation scheme was suggested immediately. The ethanol manufacturer will no longer have to wait the usual three to four days for the completion of the tedious and time-consuming conventional fermentations to evaluate any new shipment of amylolytic agent, and he will no longer have to employ a considerable excess of the amylolytic agent in plant mashes to insure enough agent being present to saccharify the fermentation mashes for maximum ethanol yields. Just 24 hours from the time he receives his sample, either from the carload lot as it comes to his railroad siding or sent ahead by the manufacturer of the amylolytic material, he will not only know the amylolytic potency in comparison with a standard reference sample, but will also know just the minimum optimal level to employ to produce the maximum ethanol

yield from the chosen substrate.

The alpha-amylase determination of Sandstedt, Kneen, and Blish (1939) has been the most successful chemical test for measuring the enzymic activity of mold bran preparations. It has been pointed out previously that in general if the alpha-amylase value of a material is high there is reason to believe that the preparation will give good ethanol yields when it is used in saccharifying fermentation mashes. The alpha-amylase values for the samples listed in Table 29 are compared in Table 30 with the minimum optimal levels for corn mash fermentations as determined experimentally.

Table 30

Comparison of the Alpha-amylase Values with Experimentally Determined Minimum Optimal Mold Bran Levels

Mold bran, lab No.	Optimum level mold bran, g. per 100 g. corn (experimental)	Alpha-amylase, dextrinizing time, minutes
FCPC#2	2.66	5.25
FCPC#1	2.5	6.25
MB#9	2.6	5.75
SLI	2.6	3.00
JWC	3.0	5.00
UN	3.5	12.25
MB#1	4.0	16.25
MB#10	4.2	8.00
MB#11	4.3	9.25
ISC#2	4.8	21.25
ISC#1	5.3	17.00
MB#12	7.6	15.00

It was apparent from the comparative data in Table 30 that there is no correlation whatsoever between minimum optimal requirements of a mold bran for saccharifying fermentation mashes and the alpha-amylase values, expressed in terms of dextrinizing time. Although the alpha-amylase determination has been devised as a fairly rapid chemical test for just the one component in the amylolytic agent, it gives neither the total evaluation needed to predict the overall amylolytic action produced in the conventional 72-hour fermentation nor the required minimum optimal level for maximum ethanol production from the specified substrate.

It should be pointed out that, in general, the conventional 72-hour ethanol yields indicated that any amylolytic agent which had a greater numerical value for an intercept in the Standard Evaluation Test compared to the standard reference sample should be judged as poor. Ethanol yields for such samples did not quite approach the maximum given by the reference sample or any of the better samples, even though enough mold bran was added to produce the maximum yields for that particular sample. Apparently, when the Standard Evaluation Test indicated a sub-normal amylolytic agent, some part of the enzyme system or other unknown factors were inadequately supplied or developed so that the maximum possibility of ethanol yield from the corn was never quite attained even with an excess of the agent. There was no sharp dif-

ferentiation between a good, medium, or poor amylolytic agent. Generally a good agent required less than 3.0 g. per 100.0 g. of the specified corn sample for maximum ethanol yields, and any agent requiring over 5.0 g. per 100.0 g. of corn was judged a poor material, more because of the increase in cost per unit of ethanol produced which would result due to the necessity of employing higher levels of agent than because of the three to seven per cent decrease in ethanol yields. The latter point is also quite significant.

The data of Tables 25, 26, 27, and 28 indicated a possibility that in commercial practice it might prove more profitable to use 15 to 25 per cent less amylolytic agent than the computed minimum optimal level with an average sacrifice of only three to four per cent in ethanol yields. The economics of such a practice would have to be carefully studied with such items as cost of substrate, cost of amylolytic agent, price of ethanol, price of recovered feeds, etc., being given special statistical analysis to substantiate such an undertaking. More extensive studies should be conducted in this respect before many conclusions can be drawn, but the feasibility of such a procedure is quite possible.

The amylolytic agents employed in this investigation were, as previously mentioned, prepared from a strain or strains of Aspergillus oryzae. This preliminary work sug-

gested that further studies should be conducted applying the same methods and techniques employed in this investigation to other fungal amylase preparations including those produced by strains of the genera Rhizopus, Mucor, and other promising strains of Aspergillus, both on solid media and in submerged culture. Bacterial amylase preparations should also be tested. There is a likely possibility that data from the Standard Evaluation Test for each particular strain might give a slope different from that for the Aspergillus oryzae preparations. However, several preparations from a single strain or species might give parallel curves with intercepts that would afford a means of evaluation. However, if this method did not give concordant results, then another mathematical approach might be forthcoming.

Since barley malt is the most generally employed amylolytic agent in the fermentation industry, a few preliminary investigations of an exploratory nature were tried on barley malt samples. The Standard Evaluation Test data gave straight line curves when plotted, but instead of being parallel and having the same slope as was noted with the fungal amylase preparations, the straight lines for different malt samples all had slightly different slopes but appeared to have the same intercept. Since time did not permit results to be obtained from conventional 72-hour corn fermentations with these malt samples, the relative potencies of the samples

were not ascertained. It might be that they all possessed nearly the same activity for saccharifying fermentation mashes, although the Lintner values differed considerably. It is now well known that Lintner values have almost no meaning in predicting the enzymic action of an amylolytic agent for saccharifying fermentation mashes. More extensive studies will have to be made using barley malt samples of good, medium, and poor amylolytic potency as determined by conventional 72-hour fermentation tests before any definite conclusions can be drawn as to the suitability of the Standard Evaluation Test method for evaluating malts.

VI. SUMMARY AND CONCLUSIONS

1. The evaluation of amylolytic agents employed in the saccharification of fermentation mashes for the production of ethanol was undertaken. The amylolytic activities of the products of the growth of strains of Aspergillus oryzae on wheat bran, called mold brans, were studied in particular, not only because of the commercial importance of fungal amylase preparations as amylolytic agents in the ethanol fermentation but also because of the need for an exact and rapid evaluation method for this new commodity.

2. The 19 amylolytic agents investigated were divided into groups according to their source: one pilot plant sample, one semi-commercial sample, 3 laboratory samples, 4 experimental plant samples, and 10 commercial preparations. Evaluation was made for all but two samples which were of such low enzymic activity that they were considered useless as amylolytic agents.

3. Graphical and mathematical analysis of meager fermentation data covering only limited levels of mold bran and ethanol yields from the conventional 72-hour corn mash fermentations indicated a straight line function. The straight line function was obtained by plotting the weight of mold bran used divided by the weight of ethanol produced against

the weight of mold bran. On this basis a possible fermentative evaluation was postulated, which would have all the merits of the tedious and time consuming 3 to 4 day conventional fermentation, but would require less work and a shorter time period.

4. Although satisfactory in many respects, the conventional corn mash substrate was abandoned for two major reasons: the necessary wide range of ethanol yields for the straight line function was not obtained for the various levels of amylolytic agent until the 36-hour fermentation period, which was judged too long for a rapid evaluation method, and, more important, the lack of uniformity in composition of corn rendered standardization almost impossible.

5. Starch of the pure food grade quality was selected for the substrate not only because of its consistent homogeneity but also for its wide availability. Difco yeast extract, a widely available product of highly uniform quality, was employed to furnish sufficient nutrients and growth factors for yeast growth. The starch was thinned by cooking with dilute mineral acid to produce a mash that could be handled with ease and would ferment well.

6. Three starch mashes were developed with optimum concentration of mineral acid to thin the starch with no appreciable production of reducing substances, optimum cooking time, and an excess of Difco yeast extract to supply a source

of nutrients and growth factors for yeast growth. Only the substrate with 100.0 g. of starch, 5.0 g. of Difco yeast extract, 0.05 normal hydrochloric acid for thinning, and cooking time of 60 minutes at 20 pounds steam pressure was judged satisfactory on the basis of having inappreciable amounts of reducing substances formed in the acid thinning process and more than enough substrate for a rapid 24-hour yeast fermentation when employing the most active amylolytic agent.

7. A standard procedure was developed that gave the necessary wide range of ethanol yields from various levels of amylolytic agent for the straight line function. This method is outlined briefly: to 100.0 g. of starch and 5.0 g. of Difco yeast extract in a wide-mouthed 1-liter Erlenmeyer flask is added 250.0 ml. of 0.05 normal hydrochloric acid previously heated to 70°C, and the contents stirred with a glass rod to facilitate mixing. This is repeated for each flask in the series. The flasks are then heated in a water bath and stirred continuously till the starch is gelatinized. The mashes are then cooked at 20 pounds steam pressure for 60 minutes, blown down to atmospheric pressure, and steamed continuously until removed one at a time for saccharification. To the hot mash is added the requisite amount of standard concentrated sodium carbonate solution to adjust the pH between 5.0 and 5.3 and a slurry containing the amylolytic agent in 250 ml. cold water, and the entire contents mixed

with a high speed stirrer for one minute. The temperature of the cold water slurry is adjusted so that the resultant mash has a final temperature no higher than 55°C after being stirred. A series consists of triplicate flasks for each level of amylolytic agent, namely, 1 g., 2 g., 3 g., and 4 g. per flask. Each flask is cooled immediately in a cold, running water bath till the temperature reaches 30°C, and after the mashes for the entire series have been saccharified and cooled, each flask is inoculated with 20.0 ml. per flask of an active 24-hour yeast culture grown in a malt extract medium. The series is incubated at 30°C for the 24-hour test period. The final volume of each mash is then measured and 250.0 ml. distilled, the distillates being collected in 100.0 ml. volumetric flasks. The distillates are brought to 25°C by immersion in a constant temperature water bath, and the specific gravities determined at 25°/25° with the Chainomatic Westphal balance. Ethanol contents of the distillates are read from an appropriate table and the total weight of ethanol from each test fermentation is calculated. This procedure along with the graphical and mathematical analysis was designated the "Standard Evaluation Test".

8. The Standard Evaluation Test gave curves with the same slopes when the $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$ was plotted against the weight of mold bran for all the samples tested, and upon extrapolating these curves to the Y-axis different numerical

intercepts were obtained that were inversely proportional to the relative amylolytic activity of the various agents employed. In other words, the higher the activity demonstrated by an amylolytic agent the lower was the numerical value of its intercept. An exact fermentation evaluation was thus possible, since, as the value of the intercepts varied between two agents, the minimum optimal level for maximum ethanol production would vary in the same proportion.

9. Since the slopes from all test curves obtained in the Standard Evaluation Test were practically the same, because the curves were essentially parallel, a further simplification in the procedure suggested itself. The starch-yeast extract medium was employed but only a single level of amylolytic agent, namely, 3.0 g. per flask was used, because the lower levels do not fit the curves well due to the poor saccharification under the specified conditions, and the intercept was calculated from the straight line equation, $y = mx + b$. The value for y was the $\frac{\text{weight of mold bran}}{\text{weight of ethanol}}$, m was the slope of 0.0274 from the reference sample FCPC#2 curve, x was the weight of mold bran, and b was the numerical value of the unknown intercept. Although, probably not quite as accurate as the Standard Evaluation Test using four levels of agent, this one-level method was sufficiently accurate for commercial laboratory control work.

10. Correlation of the intercept values from the Stan-

dard Evaluation Test with the minimum optimal requirement of amylolytic agent for maximum ethanol production was made by first carrying out a conventional 72-hour corn fermentation with various levels of standard sample FCPC#2 to ascertain the minimum level for maximum ethanol production. Then the simple proportion

$$\frac{\text{intercept (standard agent)}}{\text{intercept (unknown agent)}} = \frac{\text{optimum level (standard agent)}}{\text{optimum level (unknown agent)}}$$

was employed to compute the minimum optimal level for maximum ethanol production for the 17 amylolytic agents evaluated.

11. Finally to confirm the computed minimum optimal level of each agent, actual maximum ethanol production data were obtained for most samples from the conventional 72-hour corn fermentation employing 6 different levels of each amylolytic agent. The range included the computed minimum optimal obtained by proportion from the 4-level intercept data from the Standard Evaluation Test. The computed minimum optimal levels and the experimentally determined minimum optimal levels for most of the samples gave excellent agreement. Thus the evaluation of the amylolytic activity of the samples investigated by a 24-hour fermentation method was confirmed by checking with the conventional 72-hour grain fermentations.

12. For best correlation of results, the conventional 72-hour fermentation test to determine the minimum optimal

level of the chosen standard amylolytic agent for maximum ethanol production should use the same type of substrate that is employed in the final commercial 72-hour fermentation. For example, if corn is to be the final substrate for ethanol production, then it should be employed in the conventional 72-hour fermentation test, but if wheat, rye, cassava starch, oats, sorghum grains, potatoes, bananas, or eny other suitable starch material is the final substrate, then it should be substituted for the corn in the conventional 72-hour fermentation test.

13. No correlation was found between the minimum optimal requirements of amylolytic agents for saccharifying fermentation mashes for the production of ethanol and the results of the alpha-amylase determination by the method of Sandstedt, Kneen, and Blish (1939), expressed in terms of the dextrinizing time. The meager information supplied by the alpha-amylase determination is that if the dextrinizing time is low it is quite certain that the agent will exhibit good amylolytic activity.

14. In general the good amylolytic agents tested required less than 3.0 g. per 100.0 g. of corn for maximum ethanol production, and the poor ones required over 5.0 g. for maximum ethanol yields. However, a poor agent never appeared to give quite the maximum ethanol yields obtained with the better samples even when more than enough of the former was

employed to produce maximum ethanol yields. Possibly, some unknown enzyme systems or other fermentative factors were improperly developed in the course of the preparation of the poorer agents.

15. The total evaluation scheme for an amylolytic agent as developed during this investigation may be summarized as follows: any good amylolytic agent is selected as a reference standard, and the intercept and the numerical value of the slope, m, are determined by the Standard Evaluation Test. The conventional 72-hour grain fermentation is carried out employing various levels of the same reference agent to determine the minimum optimal requirement for maximum ethanol production. To evaluate any other agent the intercept is obtained, either graphically by plotting data from the regular Standard Evaluation Test employing the four levels of agent or calculated from the straight line equation employing the single level data obtained from the modified Standard Evaluation Test method. From the intercept the minimum optimal requirement of the agent under test may be computed from the proportion:

$$\frac{\text{intercept (standard agent)}}{\text{intercept (unknown agent)}} = \frac{\text{optimum level (standard agent)}}{\text{optimum level (unknown agent)}}.$$

16. As a final conclusion, then, an exact and reliable fermentative method has been developed for evaluating different samples of fungal amylolytic agents, namely, those prepared with strains of Aspergillus oryzae, as to their

relative activity in saccharifying fermentation mashes, and, more importantly, their minimum optimal requirement for maximum ethanol production from grain mashes. This evaluation requires 24 hours instead of the tedious and time consuming three to four day conventional fermentation method previously employed as the only dependable and conclusive method.

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