FUNGAL AMYLASES IN THE ACETONE-BUTANOL FERMENTATION

by

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A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

January 1950

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Acknowledgments

The author of this thesis expresses his great indebtedness to Professor H. D. Reese for the suggestion of the problem for this investigation, and for the helpful advice, criticism, and encouragement which he so freely rendered throughout the course of this study. To Mr. J. F. Cormack the author wishes to express his sincere appreciation for his indispensable assistance in some of the routine preparations and analyses.

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I. INTRODUCTION

Acetone and butanol have become increasingly important chemicals in the past forty years and their future looks even brighter. Butanol was the starting material from which the first commercial synthetic rubber was being produced prior to World War I. The demand for butanol decreased during the war when production of plantation rubber increased enough so that the production of rubber synthetically was no longer feasible. However, the commercial acetone-butanol fermentation continued to expand during World War I, mainly for the by-product acetone which was an essential compound in the manufacture of explosives.

Soon after World War I the demand for butanol again increased, not for synthetic rubber manufacture, however, but for new automobile lacquers, because in the process butyl acetate was found to be superior to amyl acetate.

Originally, corn was used almost exclusively for the fermentative production of acetone and butanol until several strains of organisms were isolated which could utilize molasses as a carbohydrate source, thus decreasing the use of corn. Blackstrap molasses, an imported by-product from the cane sugar industries abroad, was used until late 1941 when lack of shipping facilities made it no longer available to

plants in the United States. During the years of World War II the change from molasses back to grain as a carbohydrate source involved no difficulties, as the techniques for fermenting grain were known before acceptable molasses-fermenting organisms were discovered and isolated.

Today the production of acetone and butanol by fermentation is, on a comparative basis, exceeded only by the industrial production of ethanol. Five fermentation plants in the United States and one plant in Puerto Rico, during the years 1941-1948, produced seventy to eighty per cent of the annual 130,000,000 to 160,000,000 lbs. of butanol (7, 30).

The acetone-butanol fermentation normally takes from forty-eight to seventy-two hours for completion. Only twenty-five to thirty-five per cent of the fermented starch is converted to the solvents, butanol, acetone, and ethanol, while the remaining starch is fermented to acetic and butyric acids, and carbon dioxide and hydrogen. The fermentation gases, composed of approximately sixty percent carbon dioxide and forty per cent hydrogen, are used in the synthesis of methanol. By-product feed from this industry is very valuable because of its high potency of riboflavin.

Formerly seventy-five to eighty per cent of all butanol produced was converted to the acetate derivative which found application as a solvent in preparation of artificial leather, coated paper and textiles, and plastics. Butyl acetate is also used as an extraction solvent for

oils, drugs, and perfumes, and as an ingredient in perfumes and flavors.

Butanol is now being used in increasing amounts in the manufacture of urea-formaldehyde finishing materials and as a solvent for them. Other important uses are in the manufacture of nitrocellulose films and coatings, and in the production of plasticizers and lubricating oil additives.

Although several organisms have been isolated which can convert simple carbohydrates and/or starches to butanol and acetone, greater quantities of starches are being used since they are more readily available and less expensive. The organisms must first hydrolyze the starches to simple carbohydrates before they can be utilized in the formation of solvents. In the industrial production of ethanol the use of fungal amylases in the saccharification of fermentation mashes has proven successful and increased yields noted in comparison to the conventional use of barley malt. In addition a stimulatory effect was noted decreasing the fermentation time from forty-eight to thirty-six hours.

The major objective of this dissertation was to investigate the possibilities of employing fungal amylases in the acetone-butanol fermentation as used in the industrial production of ethanol.

II. REVIEW OF LITERATURE

A. Development and Use of Fungal Amylase Preparations.

Although fungi 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 (28) in his review of the literature regarding the early investigation of Aspergillus oryzae and its important industrial applications gave credit to Hoffman and Korshelt as the first writers on this subject. Korshelt (27) first called this fungus Eurotium oryzae since an amylolytic enzyme developed during its culturing in the preparation of the Japanese alcoholic beverage "sake". Later this fungus was renamed Aspergillus oryzae by other investigators.

It should be noted that in the Orient no attempt was made to utilize pure cultures of molds. A mixture of microorganisms 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 microorganisms made much of the early work of questionable character and its present value is only of a historical nature.

Gayon and Dubourg (18) 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.

Sanguinete (47) 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 (12) 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. Calle (17) and Owen (38) have published articles giving complete details of the Amylo process and reviewing the important developments.

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 the United States, England, and other countries for manufacturing enzymic preparations using Aspergillus oryzae and among the better known preparations are "koji" (56), and "taka-koji diastase" (57). A very comprehensive study of the nature of "koji" amylase has been made by Ito (26).

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 α, Mucor β, and Rhizopus delemar were employed.

In 1914, Takamine (55) 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 Takemine that Ortved (35) tried out the preparation on a plant scale at the Hiram Walker and Sons plant in Canada. Ethanol 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 (11) was able to produce eight per cent more ethanol from the use of taka-diastase as a saccharifying agent than from malt in his experiments investigating the possibility of producing industrial alcohol from cassava.

Aspergillus oryzae and mold forms intermediate between Aspergillus flavus and Aspergillus oryzae were found by Oshima and Church (37) to be the most potent producers of amylase of all the molds isolated from "koji". A noteworthy accomplishment of this 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 many media and wheat bran was found to be the best substrate. Oshima (36) found the activity of the amylolytic enzymes grown on wheat bran using Aspergillus oryzae was the greatest at pH 4.8 to 5.2. The enzymes were also heat labile and became completely inactive when heated one hour at 85° C.

In 1931, Harada (24) studied the cultivation of Aspergillus oryzae paying specific attention to the factors involved in the production of enzymes on wheat bran. He found that the optimum pH for amylolytic activity increased

with increasing temperatures only above 50° C. Below 50° C the pH optimum was 5.2.

In 1935, Takeda (58) made comparisons of the amylolytic activities of twenty-seven 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 consistently higher yields.

In 1939, Underkofler, Fulmer, and Schoene (64) revived the "taka-koji" process of growing molds on wheat bran for use in replacing malt in the saccharification of starchy substances for the ethanol fermentation. A rotating drum technique was used to produce the active amylolytic

preparations by growing mold on wheat bran. Preliminary results gave equal ratings to the Aspergillus oryzae and the Rhizopus molds. Aspergillus oryzae was chosen for further study because of its cultural characteristics, vigorous growth, and consistent high production of amylases. Over ninety per cent conversion of starch to ethanol became common for the Aspergillus oryzae strain, with malt yields about ten per cent lower. It was clearly pointed out that mold bran was less expensive, more quickly prepared, and gave higher yields of ethanol than malt.

Hao, Fulmer, and Underkofler (22) employed a new technique for growing mold 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 twenty-seven different strains of molds selected from the four genera: Aspergillus, Mucor, Rhizopus, and Penicillium. Although Rhizopus cultures gave good amylase production, the best strains of Aspergillus oryzae were judged to be the most suitable because of their superior cultural characteristics, including dense mycelium, better sporulation, and vigorous growth.

Underkofler (60) and Underkofler and Fulmer (63) reviewed the status of microbial amylases for the saccharification of starch in the ethanol fermentation and gave many valuable statistics concerning mold bran and its industrial applications. On a laboratory scale 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, and could be prepared in one-fifth the time required for malt. These results were later substantiated by Roberts, Laufer, Stewart, and Saletan (46), Barinova (3), Pan and Liu (41), Hao and Jump (23), and Schwimmer (50).

In the spring of 1945 tests were begun on the use of mold bran in grain fermentations on a full plant scale basis at the ethanol plant operated by the Farms Crops Processing Corporation at Omaha, Nebraska. A brief report on these tests was given by Boyer and Underkofler (8) and a detailed report by Underkofler, Severson, and Goering (67). 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 or in combination with malt. The fermentation time was reduced from forty-eight hours to thirty-six 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.

Underkofler (61) briefly reviewed and Underkofler, Severson, Goering, and Christensen (68) 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 plant scale production of over ten tons per day at the Mold Bran Company plant at Eagle Grove, Iowa. The

economics of using mold bran were discussed in these papers and are shown to be definitely favorable.

Recently Reese, Fulmer, and Underkofler (43) reported the details of a rapid fermentative method for evaluating the ability of fungal amylase preparations to saccharify grain mashes.

A very recent trend in the use of fungi in the fermentation industry has been outlined by Erb and Hildebrant (14) 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 ethanol fermentation of granular wheat flour mashes. The volume of fungal amylase preparation varied from six to twelve per cent of the total volume of the fermenter mash. Approximately 12.1 proof gallons of ethanol per 100.0 pounds of dry grain were obtained instead of the usual 11.0 proof gallons of ethanol per 100.0 pounds of dry grain obtained by malt alone.

Van Lanen and LeMense (69, 70) 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 only seven samples indicated commercial possibilities, with a strain of

Aspergillus niger being superior. The liquid fungal amylase preparations were used at a rate of ten to fifteen per cent by volume of the corn mash to be saccharified, completely replacing the malt formerly used. The fungal amylase preparations were approximately twelve times as potent as the malt on a comparable basis.

Adam, Balankura, Andreasen, and Stark (1) 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 10 g. ground corn meal per 100.0 ml. They obtained about a five per cent increase in ethanol production over the malt controls, corresponding to an overall fermentation efficiency of nearly ninety-three per cent.

LeMense, Corman, Van Lanen, and Langlykke (31, 32) noted that considerable amounts of dextrinizing enzymes and saccharifying enzymes were produced under submerged aerobic conditions by Aspergillus wentii, Aspergillus oryzae, Aspergillus alliaceus, and Aspergillus niger. Employing the submerged preparations in a continuous process experimental results indicated that saccharification was satisfactory and ethanol yields were comparable to those obtained with malt when mold culture liquor equivalent to six to ten per cent of the final corn mash volume was used. The cost of the amylase was estimated to be 6.06 cents per bushel of grain processed, as compared with 12.1 cents when malt is used.

B. History and Development of the Acetone-Butanol Fermentation.

There are a number of closely associated fermentations brought about by bacteria, which differ in respect to the quantity and nature of the end products and the conditions necessary for their successful termination. The most important of these fermentations, namely, the acetone-butanol, gives as the main end products butanol, acetone, ethanol, acetic acid, butyric acid, carbon dioxide, and hydrogen.

Butanol was discovered by Wurtz in 1852 as a regularly occurring constituent of fusel oil. Pasteur, however, was the first investigator to show that butanol was a direct product of fermentation.

Fitz published a series of articles on bacterial fermentations from 1876 to 1884 and among these he discussed the micro-organism Bacillus butylicus (15). This organism fermented various carbohydrate substrates except starch and lactose, and produced butanol, butyric acid, carbon dioxide, hydrogen, and small amounts of ethanol.

In 1887, Gruber (21) described three strains of organisms under the name of Bacillus amylobacter (Clostridium butyricum). Each of these strains formed butyric acid and butanol from carbohydrate substrates.

Bacillus orthobutylicus, an anaerobe isolated from a calcium tartrate fermentation and described by Grimbert (20)

in 1893, produced butanol; a little isobutyl alcohol; butyric, acetic, and occasionally, formic acids; and carbon dioxide and hydrogen during the fermentation. Grimbert differentiated his organism from Bacillus butylicus of Pasteur, Bacillus amylobacter of Van Tiegham, Bacillus butylicus of Fitz, and Bacille amylozyme of Perdrix.

Duclaux (13) discovered that the use of calcium carbonate in the media when culturing Amylobacter butylicus, an organism isolated from potatoes, caused the production of acids, while its absence favored the production of alcohols. This was confirmed by Reilly, Hickinbottom, Henley and Thaysen (45).

Winogradsky (73) described the morphology and properties of Clostridium pastorianum as a producer of butanol, ethanol, acetic and butyric acids.

Stimulus was given to these early workers when acetone was first discovered and characterized in 1905 by Schardinger (48) using the organism Bacillus macerans on corn mash media. Ethanol, and acetic and formic acids were also produced in the fermentation by this organism. This new source of acetone, whose solvent properties were known and used by several industries, renewed interest in its utilization.

These early developments in the acetone-butanol fermentation were of necessity conducted on a laboratory scale, but definitely indicated some excellent potentialities for

supplying large quantities of inexpensive neutral solvents. The demand for butanol increased as synthetic rubber production began to supplement the diminishing supplies of natural rubber. This need for butanol in synthetic rubber production supplied the impetus that resulted in the first full scale plant process for butanol (16). A crude type of rubber had been synthesized in 1860, but lack of a market had limited research and plant production. Synthetic rubber research, especially in England, Germany, and Russia, was now intensified. The firm of Strange and Graham, Ltd., conducted most of the investigations in England with the aid of Perkin, Weizmann, and Fernbach, who attempted to develop newer processes for the production of butanol. Fernbach isolated a new organism which produced quantities of amyl alcohol, butanol, and acetone. This new organism was soon used in most butanol fermentations and gained widespread usage. In 1912 Weizmann left the firm, and, while working on his own, isolated and named a new organism Bacillus granulobacter pectinovorum, later changed to Clostridium acetobutylicum, which produced nearly four times as much acetone as Fernbach's organism.

World War I halted the synthetic rubber program requiring butanol, but immediately created a tremendous market for the butanol fermentation by-product, acetone, an essential chemical in the manufacture of the explosive, cordite, and "dopes" for treating airplane fabrics. The

available sources, synthetic and fermentative, could not supply the wartime demands until Weizmann's organism was employed exclusively in all fermentative processes producing this solvent.

The termination of World War I curtailed acetone production but postwar developments increased the demand for butanol in the manufacture of automobile lacquers. During 1919 and 1920 several fermentation plants were built for the sole purpose of employing the Weizmann process to produce butanol as its most important recoverable solvent, and acetone was again relegated into the by-product class.

The wartime industrial manufacture of acetone and butanol by fermentation (42) presented many problems which necessitated further investigation. For example, all the equipment with which the mash or butanol organism may come into contact must be adequately sterilized, for contamination is usually detrimental and may involve considerable loss of solvents. Bekhtereva (5) describes these losses when the inoculated mashes were contaminated with an acid forming organism.

It is well known that yields of solvents from acetone-butanol fermentations can be increased by various treatments on certain organisms. Underkofler, Christensen, and Fulmer (62) found that successive growth, transfer, sporulation, and heat-shocking in semi-synthetic carbohydrate media, the cycle being repeated several times, greatly improved the

culture with increased solvent yields.

A wide variety of raw materials such as corn, rice, jawari, bajra, tapioca, and other starches; peanut and oat hulls; corncobs; horse chestnuts; molasses and sirups have been more or less satisfactorily fermented.

Sjolander, Langlykke, and Peterson (53) used Clostridium felsineum and Clostridium butylicum in the fermentation of wood hydrolyzates with almost complete utilization of the available carbohydrates. Leonard and Peterson (33) employed Clostridium butylicum No. 39 to ferment wood hydrolyzates, which were prepared for inoculation by removing the furfural by distillation and neutralizing the liquors to pH 6.5 with lime. More complete hydrolysis of the wood increased the carbohydrate content above three per cent but decreased the efficiency of the fermentation.

Boehm, Hall, and McDonald (6) patented a process for improving wood hydrolyzate utilization by a sulfide precipitation and subsequent activated carbon treatment. Early German and English patents (25, 49, 71) employed sulfite liquor wastes as substrates for acetone-butanol production. Wiley, Johnson, McCoy, and Peterson (72) improved and adapted the methods to the commercial utilization of various types of sulfite liquor wastes.

A newer procedure for more complete utilization of the saccharified liquors from corncobs for acetone-butanol production has been devised by Langlykke, Van Lanen, and

Frazer (29).

Beesch (4) described a revised process utilizing the properties of a new organism Clostridium saccharo acetoperbutylicum which is capable of fermenting black strap molasses and several starches with yields of seventy to seventy-five per cent of the recoverable neutral solvents as butanol instead of the usual sixty per cent.

Carnarius (9) devised a new method and specific equipment which is used to sterilize mashes continuously for full plant scale acetone-butanol production. With only minor changes other commercial fermentations could economically adopt this process.

More recently attention has been given to some of the essential requirements and the effects of additives on the normal fermentation. Oxford, Lampen, and Peterson (39) discovered that two growth factors, biotin and a factor present in yeast extract, are required by Clostridium acetobutylicum in the acetone-butanol fermentation. Tatum, Peterson, and Fred (59) reported that l-asparagine produced a stimulatory action in the fermentation of starch and gave a marked increase in the yield of butanol when certain butyric acid bacteria were grown on sterile corn mashes.

Pan (40) stated that the addition of unheated mold bran to pure carbohydrate mashes seemed to accelerate the acetone-butanol fermentation.

Reilly and Hickinbottom (44), Maravall (34), and Sebek

(51) discussed several aspects of the complex mechanism of the acetone-butanol fermentation. Wood, Brown, and Werkman (74) critically summarized all previous mechanisms, made an attempt to elucidate certain phases by the addition of C^{13} labeled acetone, acetic and butyric acids to an active fermentation, and determined in what products the C^{13} appeared as well as the position in these products. At present the mechanism is still very questionable.

III. MATERIALS

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

Corn meal

The corn meal used in this investigation was hammer mill ground No. 1 yellow corn obtained in a one hundred pound lot from Corvallis Feed and Seed Company, and stored in a galvanized garbage can with a fitted lid. The sample had a moisture content of 12.40 per cent determined by the official methods of the Association of Official Agricultural Chemists (2).

Corn starch

The corn starch used in this investigation was obtained in a one hundred pound lot from the American Maize Company. It had a moisture content of 10.45 per cent determined by the official methods of the Association of Official Agricultural Chemists (2).

Malt extract

The malt extract used to prepare media for the yeast cultures was manufactured and packaged in three pound cans by the Pabst Brewing Company, Peoria, Illinois.

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 amylolytic preparations used in this investigation were of the same strain of Aspergillus oryzae #38 grown at different times in the Biochemistry Laboratories at Oregon State College.

Bacterial organisms

The organism Clostridium acetobutylicum POS, obtained from Iowa State College, was employed for the major portion of this dissertation. Another strain of Clostridium acetobutylicum, NRRL B-527, was obtained from the Northern Regional Research Laboratory and usually employed for confirmatory work.

Chemicals

The chemicals used in the analyses of the various fermentation products were of reagent grade purity.

IV. METHODS

A. Microbiological Procedures

Bacterial culture

A weighed amount of corn equivalent to six per cent by weight of the total mash volume was placed into a flask containing the measured amount of tap water. With constant stirring this mixture was gelatinized by raising the temperature to 90° C. For propagating the cultures 10.0 ml. of the gelatinized mash were placed in each six inch culture tube, and for cultures employed for inoculating experimental mashes, 70.0 ml. of the mash were used in each 125.0 ml. Erlenmeyer flask. The tubes and flasks were plugged with cotton and sterilized for one hour under a steam pressure of twenty pounds.

A new culture was started daily from a stock sand culture of Clostridium acetobutylicum. Approximately 0.1 g. of this spore culture was transfered aseptically with a pipet to one of the test tubes containing six per cent corn mash, heat-shocked for forty-five seconds by immersing in boiling water, removed, quickly cooled to 37°C in a cold water bath, and incubated at 37° C. Aseptic transfers are made every twenty-four hours. The first three transfers of three to five ml. of the active culture were put into another sterile tube, the fourth transfer of three to five

ml. into a 125.0 ml. Erlenmeyer flask, and the fifth transfer of sixty to seventy ml. into 1300 ml. of sterile mash in a two-liter Erlenmeyer flask.

Preparation of amylolytic agents

The laboratory preparations used in this investigation were prepared employing the method described by Hao, Fulmer, and Underkofler (22).

B. Analytical Procedures

Determination of Acetone

At the completion of the fermentation period a 150.0 or 200.0 ml. aliquot from each fermentation flask was transfered into an 800.0 ml. Kjeldahl flask, approximately 0.5 g. of calcium carbonate added to neutralize the acids present, a few drops of corn or mineral oil added to prevent foaming, and 200 to 225 ml. of wash water. Each distillate was collected in a 100.0 ml. volumetric flask until 98 to 99 ml. of distillate had been collected. The volumetric flasks containing the distillates were made up at 25° C to exactly 100.0 ml with distilled water. Ten milliliters were removed from this flask with a 10.0 ml. volumetric pipet and transfered to another 100.0 ml. volumetric flask, the volume brought to exactly 100.0 ml. with distilled water, and, after thorough mixing, aliquots were removed to determine the amount of acetone, butanol, and ethanol.

The acetone was determined by the Goodwin modification of the Messinger method (19). A 20.0 ml. aliquot of the acetone in aqueous solution is pipetted into 50.0 ml. of 1.0 N sodium hydroxide solution in a 500.0 ml. glass stoppered flask. After standing for five minutes, about twenty-five per cent excess of a 0.1 N solution of iodine is added from a buret with continuous rotation of the flask. The

flask is stoppered and allowed to stand for at least ten minutes.

Twenty-five ml. of 2.0 N sulfuric acid is added, 0.3 to 0.4 ml. being added in excess of the amount found necessary to neutralize the 50.0 ml. of sodium hydroxide solution, and the liberated iodine titrated with a 0.05 N solution of sodium thiosulfate until the yellow color is just visible. Freshly prepared starch solution is added and the titration completed. Exactly 10 ml. of 0.1 N iodine is equivalent to 0.96747 mg of acetone. A sample calculation is as follows:

Final volume of fermented mash = 1265.0 ml.

Aliquot distilled = 200.0 ml.

Iodine = 0.0988 N.

Sodium Thiosulfate = 0.0706 N.

Iodine added plus twenty-five per cent excess = 25.0ml. of 0.0988 N.

$\frac{25.0 \times 0.0988}{0.1}$ = 24.7 ml. of 0.1 N iodine added.

9.62 ml. of 0.0706 N sodium thiosulfate require for back titration.

$\frac{9.62 \times 0.0706}{0.1}$ = 6.79 ml. of 0.1 N sodium thiosulfate.

24.7 - 6.79 = 17.91 ml. of 0.1 N iodine used.

17.91 x 0.96747 = 17.33 mg. acetone in 20.0 ml. aliquot.

$17.33 \times \frac{100}{20} \times \frac{100}{10}$ = 867.0 mg. or 0.867 g. acetone in the 100.0 ml. of distillate.

$0.867 \times \frac{1265.0}{200.0}$ = 5.49 g. acetone in the final mash.

Determination of Butanol and Ethanol

The butanol was determined by the method of Christensen and Fulmer (10). A 10.0 ml. aliquot of the diluted distillate is added to a cold mixture of 10.0 ml. of 0.4 N potassium dichromate and 10.0 ml. of concentrated sulfuric acid in a 2.5 by 25.0 cm. test tube. The contents are thoroughly mixed by swirling the tube. A stopper carrying a one mm. capillary tube, the lower end of which is bent at right angles, is inserted and the tube placed in a vigorously boiling water bath. After ten minutes exposure, it is cooled, transfered, and diluted to about 400 ml. in a one-liter Erlenmeyer flask. Fifteen ml. of a 20.0 per cent potassium iodide solution are added, the flask is stoppered, allowed to stand two minutes, and the liberated iodine titrated with 0.1 N sodium thiosulfate. A blank is determined by the same procedure. The difference is designated as M_1, and is expressed in ml. of 0.1000 N dichromate consumed.

Another value is obtained by carrying out the oxidation under different conditions. A five ml. aliquot of the diluted distillate is added to a cold mixture of 25.0 ml. of concentrated sulfuric acid and 10.0 ml. of 0.4 N potassium dichromate in a 2.5 by 25.0 cm. test tube. The above procedure for determining M_1 is followed. A blank is determined by the same method. The difference in ml. of the two titrations is multiplied by two to give the value, N, which

is expressed in ml. of 0.1000 N dichromate consumed.

From the M_1 and N values, ethanol and butanol may be calculated by means of the following equations:

$$B = 0.057 (N - M_1) - 0.96 A$$

$$E = 0.114 M_1 - 0.788 B - 0.0787 A$$

A sample calculation is as follows:

Final volume of mash = 1265.0 ml.

A = acetone (determined above) = 0.867 g.

ml. of 0.1513 N sodium thiosulfate required to back titrate excess dichromate in the determination of M_1 = 16.31 ml.

Blank = 26.35 ml. of 0.1513 N sodium thiosulfate.

26.35 - 16.31 = 10.04 ml. of 0.1513 N sodium thiosulfate which is equivalent to the amount of dichromate used.

$\frac{10.04 \times 0.1513}{0.1}$ = 15.19 ml. of 0.1 N dichromate used = M_1.

B = 0.057 (58.74 - 15.19) - 0.96 (0.867)

B = 1.65 g. butanol in the 100.0 ml. of distillate.

E = 0.114 (15.19) - 0.788 (1.65) - 0.0787 (0.867).

E = 0.36 g. ethanol in the 100.0 ml. of distillate.

$\frac{1.65 \times 1265.0}{200.0}$ = 10.44 g. butanol in the final fermented mash.

$\frac{0.36 \times 1265.0}{200.0}$ = 2.28 g. ethanol in the final fermented mash.

Therefore, the total neutral solvents per fermentation flask are:

Acetone (A)	5.49 g.
Butanol (B)	10.44 g.
Ethanol (E)	2.28 g.
Total Solvents	18.21 g.

C. 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 parallel the procedures a manufacturer may employ.

V. EXPERIMENTAL

A. Production and Evaluation of Mold Bran

The mold bran was grown in special aluminum pots equipped for aeration by the method of Hao, Fulmer, and Underkofler (22). This experiment takes up very little space, requires no special mechanical devices, gives more uniform aeration, and insures no disturbance of the mold mycelium during growth.

The method of culturing is as follows: The bran mash is prepared by taking 750.0 grams of wheat bran and mixing it with an equal weight of 0.3 N hydrochloric acid. The wet bran is packed into the aluminum pot and sterilized in an autoclave at fifteen pounds per square inch steam pressure for thirty minutes. The cooled mash is inoculated with five to ten grams of well sporulated mold culture grown in 250.0 ml. Erlenmeyer flasks on ten grams of wheat bran mash. The inoculated mash is then firmly packed into the aluminum pot and incubated at 30° C until the temperature of the mash rises to 37-40° C. This temperature is reached in about eight hours and indicates rapid growth of the mold. The mass is then aerated by alternately blowing and sucking air through the mash at a pressure of 0.3 to 3.0 inches of water varied to maintain the temperature below 45° C. After twelve to twenty-four hours of aeration the contents of the

pot are removed, spread on paper, and dried at room temperature. The dried product is then ground in a mill or coffee grinder and is designated as "mold bran".

After several samples of mold bran were prepared, using Aspergillus oryzae (Rohm and Haas, No. 38), they were evaluated on the basis of their saccharifying power under actual fermentation conditions using the rapid Standard Evaluation Test method of Reese, Fulmer, and Underkofler (43). Into each one-liter wide-mouthed Erlenmeyer flask are placed 100.0 grams of food grade starch, 5.0 grams of Difco yeast extract, and 250.0 ml. of 0.05 N hydrochloric acid at about 70° C. The contents are well mixed with a glass stirring rod and all flasks are placed in a water bath heated by means of Fischer burners until the temperature of the mashes has risen to about 85° C with occasional stirring. The heated flasks are quickly transfered to a hot autoclave; to prevent irreversible retrogradation of the starch it is important not to let the mashes cool below 80° C. The mashes are cooked for sixty minutes at twenty pounds per square inch steam pressure. The autoclave is then quickly blown down to atmospheric pressure, after which the flasks are steamed continuously in the autoclave, at atmospheric pressure, and removed one at a time for saccharification.

To the hot mash in the flask are quickly added the requisite amount of thirty per cent sodium carbonate solution to adjust the pH within the range of 5.0 to 5.3, and a

slurry of the desired quantity of mold bran in 250.0 ml. of cold water; the original temperature of the mold bran slurry is adjusted so that the temperature of the mixed mash is about 55° C. The contents of the flask are immediately mixed with a high-speed blade mixer for about one minute and then cooled to 30° C in a cold water bath.

When all the mashes for a series have been saccharified and cooled, each is inoculated with 20.0 ml. of an active twenty-four hour culture of yeast grown in twenty per cent malt extract medium. The yeast employed was No. 567 of the Northern Regional Research Laboratory collection. Each flask is fitted with a rubber stopper bearing a water trap containing about thirty ml. of water and the fermentations are incubated at 30° C for twenty-four hours. At the end of this period the water from the trap is added to the flask and the final volume of each fermented mash is measured. An aliquot of 250.0 ml. of each mash is distilled from a Kjeldahl flask after addition of about 0.5 grams of calcium carbonate and 200.0 ml. of wash water. From each flask 98 to 99 ml. of distillate are obtained, made up to 100.0 ml. at 25° C with distilled water, and the specific gravity determined at 25°/25° with a Chain-o-matic 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.

Preparation Mold Bran II was chosen as a reference

agent for comparison because it gave consistently good ethanol yields. Fermentation series, using the Standard Evaluation Test procedures, were run on the other samples, employing triplicate fermentations at various levels of the amylolytic agent in each series. The data obtained for several samples, given as averages for the triplicate fermentations, are presented in Table 1.

Table 1

Standard Evaluation Tests with Samples
MB #II, MB #III, MB #IV, MB #V, and MB #VI

Mold bran lab No.	Mold bran, g. per 100 g. starch	Ethanol g. per flask	Weight of mold bran / Weight of ethanol
MB #II	1.0	21.8	0.0458
	2.0	26.8	0.0746
	3.0	27.6	0.1080
	4.0	29.7	0.1348
MB #III	1.0	19.3	0.0518
	2.0	24.7	0.0810
	3.0	27.2	0.1103
	4.0	29.7	0.1347
MB #IV	2.0	26.1	0.0762
	4.0	30.5	0.1311
MB #V	2.0	26.2	0.0762
	4.0	30.6	0.1308
MB #VI	2.0	22.2	0.0901
	4.0	28.2	0.1429

The data for amylolytic agents tested were plotted as $\frac{\text{Weight of mold bran used}}{\text{Weight of ethanol produced}}$ against weight of mold bran. A straight line is drawn through the points for each sample parallel to the curve for reference Mold Bran #II and extrapolated to the y-axis. These intercept values are either read directly from the graphs or computed by the straight line equation $y = mx + b$. Representative curves are shown in Figure 1, and the intercept values in Table 2.

Table 2

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

Mold bran lab No.	Intercept value, graphical
MB #II	0.019
MB #III	0.024
MB #IV	0.021
MB #V	0.021
MB #VI	0.035

Since the amlolytic activities of the mold bran samples are inversely proportional to their intercept values obtained by plotting data from the Standard Evaluation Tests, the optimal requirements for two different mold brans for maximum

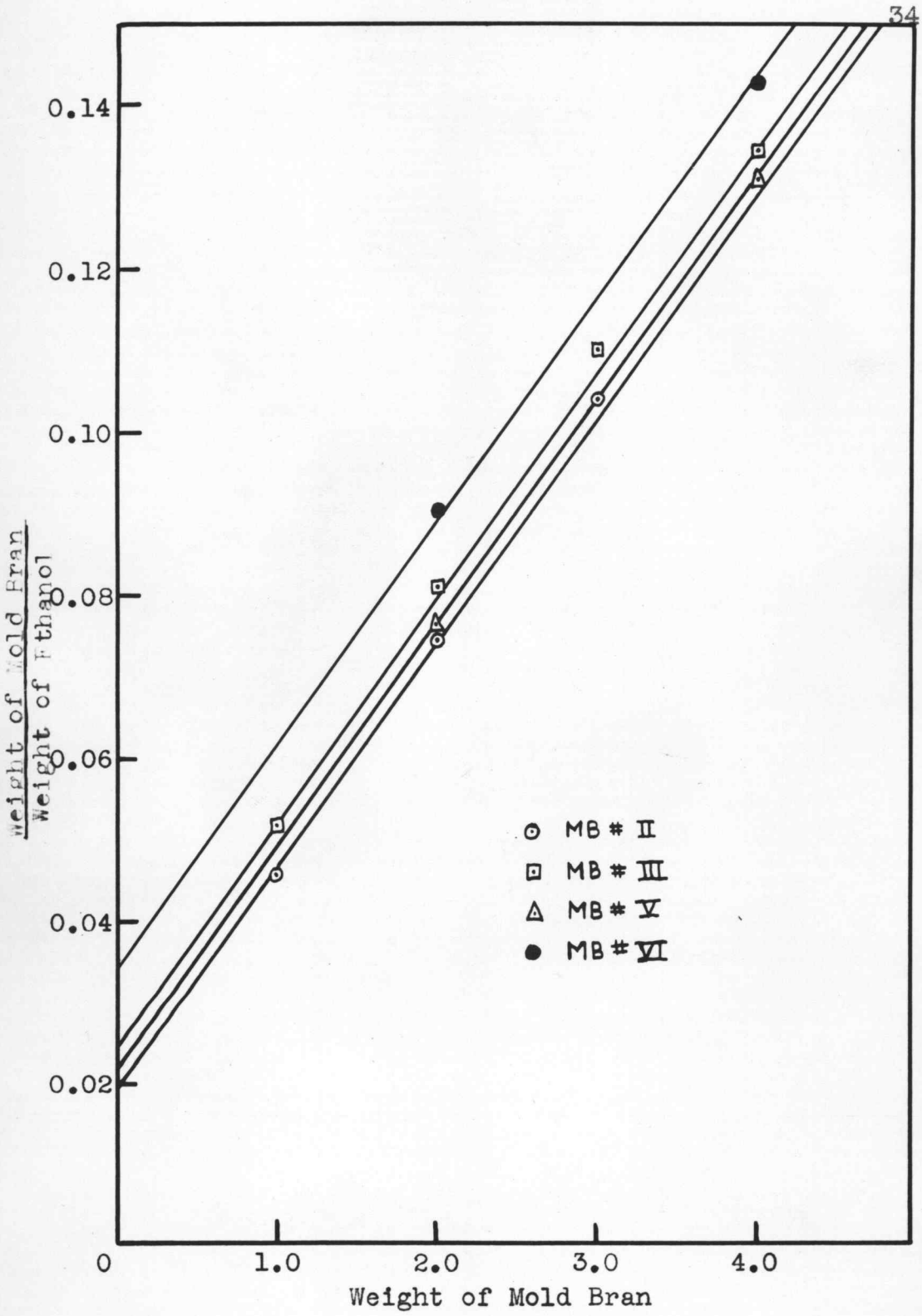

Figure 1. Curves for Standard Evaluation Tests with Samples MB#II, MB#III, MB#V, and MB#VI.

ethanol production would be proportional to the two intercept values. Therefore, if any one good mold bran is selected as a reference standard and conventional seventy-two hour grain fermentations are run, employing various levels of this mold bran to determine the minimum optimal requirements for maximum ethanol production, the minimum optimum levels for other mold brans could be calculated by direct proportion from the intercept values obtained by the Standard Evaluation Test method. The proportion used in such calculations would be:

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

Mold Bran #II was used most often and when any other mold bran was used, for example, Mold Bran #III, the minimum optimal amount was calculated from the above proportion, using the intercept values shown in Table 2. A sample calculation with 1.0 g. Mold Bran #II as the reference standard is:

$$\frac{\text{1.0 g. MB \#II}}{\text{X g. MB \#III}} = \frac{\text{0.019 (intercept MB \#II)}}{\text{0.024 (intercept MB \#III)}}$$

$$X = \frac{0.024}{0.019} = 1.26 \text{ g.}$$

Therefore, every time 1.0 g. Mold Bran #II was employed it could be replaced by 1.26 g. Mold Bran #III, 2.0 g. Mold Bran #II could be replaced by 2.52 g. Mold Bran #III, etc.

When brans other than Mold Bran #II are employed the

weights in grams used will be computed by the Standard Evaluation Test procedure as above, but for direct comparative purposes they will be expressed in weight on the same basis as if Mold Bran #II had been used exclusively.

B. The Effects of Mold Bran and Phosphate Salts, Added at the Time of Inoculation, on Six Per Cent Corn Mash.

Trial fermentations using corn as the substrate for the production of neutral solvents indicated that 1300 ml. of corn mash in a two-liter Erlenmeyer flask could be handled with relative ease and the volume was large enough to give reproducible results. A six per cent corn mash was employed for these fermentations since that concentration is used in a normal industrial fermentation.

Seventy-eight grams of ground corn are weighed into each two-liter Erlenmeyer flask and the volume is made up to 1300 ml. with tap water. These flasks are placed into a water bath which is heated by means of two Fischer burners. Each flask is equipped with a glass stirring rod and, as the temperature of the mash rises, the contents of the flask are occasionally mixed. The corn mash is gelatinized by bringing the temperature of the mash slowly up to 90° C, requiring from twenty to thirty minutes. At this time the stirring rods are removed, the flasks plugged with cotton stoppers, transfered to an autoclave, and the mash sterilized by heating at twenty pounds per square inch steam pressure

for one hour. After cooling to 37° C the six per cent mash is inoculated with approximately seventy ml. of a twenty-four hour culture prepared by the procedure given in the "Methods" section of this thesis.

Several normal seventy-two hour fermentations on six per cent corn mash were run and typical solvent yields with corresponding titrable acidity results are shown in Table 3.

Table 3

Solvent yields and Titrable Acidity
from Six Per Cent Corn Mash at Seventy-two Hours.

Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g. per flask	Titrable Acidity ml. 0.1 N NaOH
5.04	10.48	2.60	18.12	3.66
5.50	9.68	2.28	17.47	3.59
5.63	10.67	2.04	18.34	3.65
5.35	9.92	2.62	17.89	3.30
5.43	10.50	1.88	17.81	3.28
4.99	10.86	1.95	17.80	3.21

It was desirable to establish a complete normal fermentation curve for solvent yields and titrable acidity as a basis for comparison, hence, a time series was run on six per cent corn mash. These data, given in Table 4 and

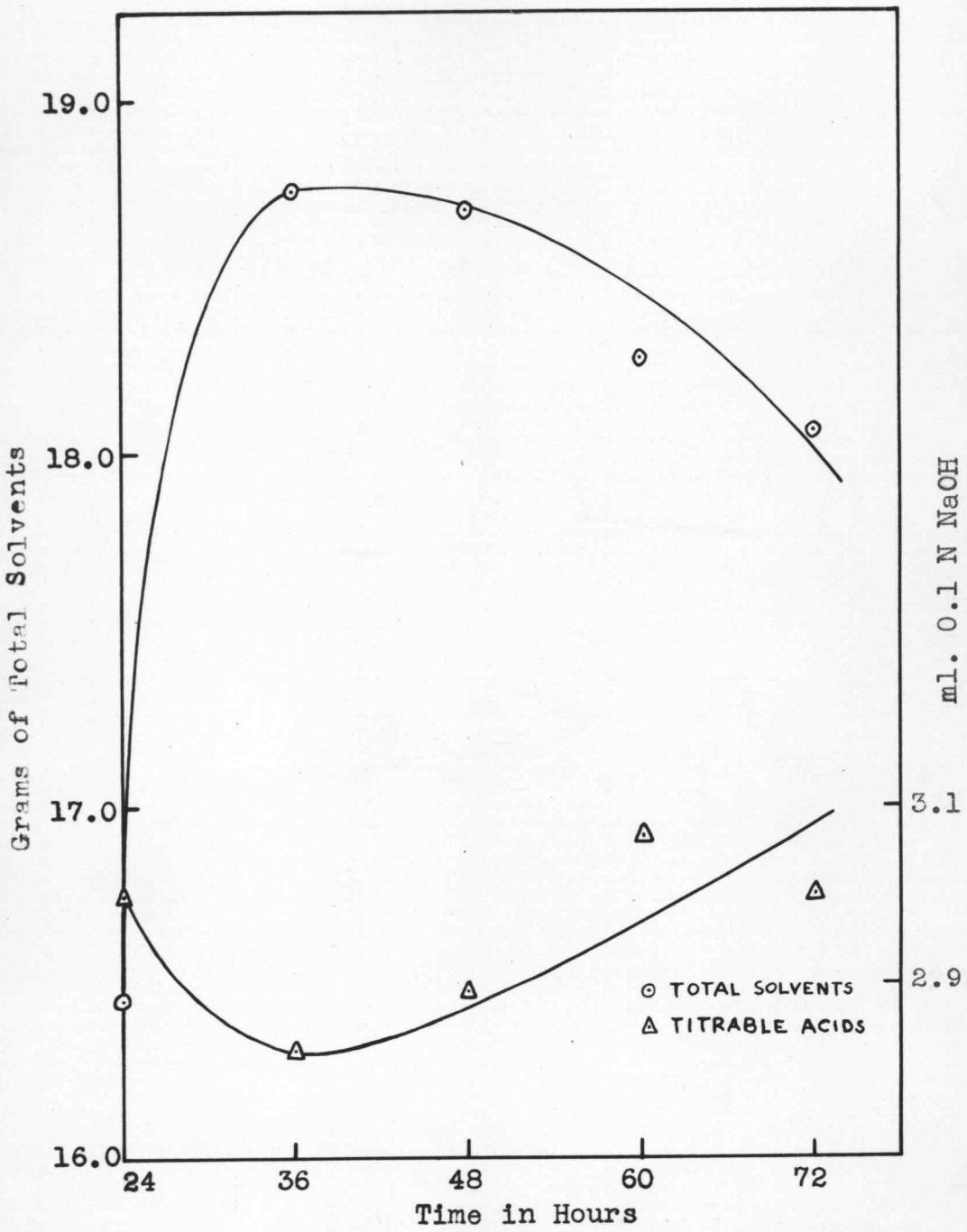

Figure 2. Titrable Acidity and Total Solvent Yields in Six Per Cent Corn Mash.

shown graphically in Figure 2, represent duplicate analyses.

Table 4

Effect of Fermentation Time on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
12	1.11	2.21	0.07	3.39	5.56
24	5.11	9.50	1.85	16.46	3.00
36	5.71	10.99	2.04	18.74	2.82
48	5.62	10.92	2.15	18.69	2.89
60	5.44	10.56	2.27	18.27	3.07
72	5.69	10.16	2.22	18.07	3.00

In the initial phase of the fermentation the titrable acidity decreases as the yield of solvents rapidly increases reaching a maximum at thirty-six hours. The solvent yields level off and drop slightly with a corresponding rise in titrable acidity. The decrease in solvent production is somewhat contrary to the normal fermentation as described by Speakman (54) who divided the fermentation into several phases, the length of each phase being based on the curve for titrable acidity. During the first phase the titrable

acidity increases rapidly to a maximum, usually in thirteen to seventeen hours. The butanol organisms reproduce very rapidly during this period, producing acetic and butyric acids in varying quantities, and hydrogen and carbon dioxide in large quantities. There is a drop in pH, which then tends to remain at a fairly constant level throughout the rest of the fermentation, due to the presence of buffers produced by the hydrolysis of the corn or other proteins.

The quantity of titrable acidity drops sharply during the second phase of the fermentation to a value that is equal to approximately fifty per cent of the maximum. Coincidental with the drop in titrable acidity, there is a rapid conversion of the organic acids to their corresponding solvents, butyric acid being reduced to butanol, and acetic acid is changed to acetone. Butyric acid disappears from the fermentation mash more rapidly than acetic acid. The rate of gas evolution increases quickly to a maximum as the titrable acidity decreases, followed by a gradual diminution in the rate of gas evolution until the end of the fermentation.

The titrable acidity slowly increases in quantity during the third phase of the fermentation with a corresponding decrease in the rate of solvent production until the fermentation ceases.

The particular strain of Clostridium acetobutylicum employed in this investigation apparently has a faster rate

of solvent production reaching the maximum yield in much less time than the organism Speakman studied.

After the normal curve was established, the effects on solvent yields and titrable acidity by various levels of mold bran, added at the time of inoculation, were noted. The six per cent corn mash media was prepared as above. Pertinent data from a seventy-two hour fermentation using Mold Bran #II are shown in Table 5.

Table 5

Effect of Mold Bran Levels on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash at Seventy-two Hours.

Mold Bran Added grams	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
1.0	5.96	10.17	1.30	17.43	4.15
3.0	3.61	6.90	0.60	11.11	4.90
6.0	1.29	3.49	0.34	5.12	7.35
9.0	0.78	0.77	1.09	2.64	8.80

As the levels of mold bran increase there is a marked decrease in the solvent yields with a corresponding increase in titrable acidity; extremely low solvent yields being produced when 9.0 g. Mold Bran #II are added. The 1.0 g. level of Mold Bran #II gave slightly lower solvent

yields and higher titrable acidity values than the normal fermentation, but the data in Table 5 clearly indicated that the solvent yields with 1.0 g. Mold Bran #II was the best level of the four.

For a seventy-two hour fermentation period high levels of mold bran appear to have limited value, but in the beginning phases of the fermentation the organisms visibly show greater activity by an earlier increase in gas production as compared to the normal fermentation. Low levels of mold bran were used in a series in an attempt to determine the significance of this apparent stimulation. Time sequences were run with various mold bran levels and compared to the normal fermentation. These results are tabulated in Tables 6, 7, 8, and 9, and shown graphically in Figures 3 and 4.

Table 6

Effect of 0.5 g. Mold Bran and Fermentation Time on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	4.65	8.65	1.39	14.69	3.73
30	5.45	10.34	1.82	17.61	3.59
36	5.77	10.86	1.91	18.54	3.66
48	5.61	10.82	1.81	18.24	3.88
60	5.52	10.92	1.84	18.28	3.80

Table 7

Effect of 1.0 g. Mold Bran and Fermentation Time on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable acidity ml. 0.1 N NaOH
24	3.55	6.83	0.84	11.22	4.64
30	5.45	8.75	1.83	16.03	2.78
36	5.90	10.39	1.94	18.23	3.27
48	5.91	10.53	1.90	18.34	3.30
60	5.46	10.71	1.73	17.90	3.61

Table 8

Effect of 1.5 g. Mold Bran and Fermentation Time on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	5.54	9.64	1.84	17.02	3.10
36	5.88	10.35	1.91	18.14	3.17
48	5.98	10.34	2.08	18.40	3.52
60	5.58	10.29	1.84	17.71	3.61

Table 9

Effect of 3.0 g. Mold Bran and Fermentation Time on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
12	0.88	1.86	0.93	3.67	6.12
24	4.79	8.64	1.41	14.84	3.83
36	5.54	10.16	1.45	17.15	4.27
48	5.20	9.94	1.46	16.60	4.74
60	5.09	9.77	1.32	16.18	4.54

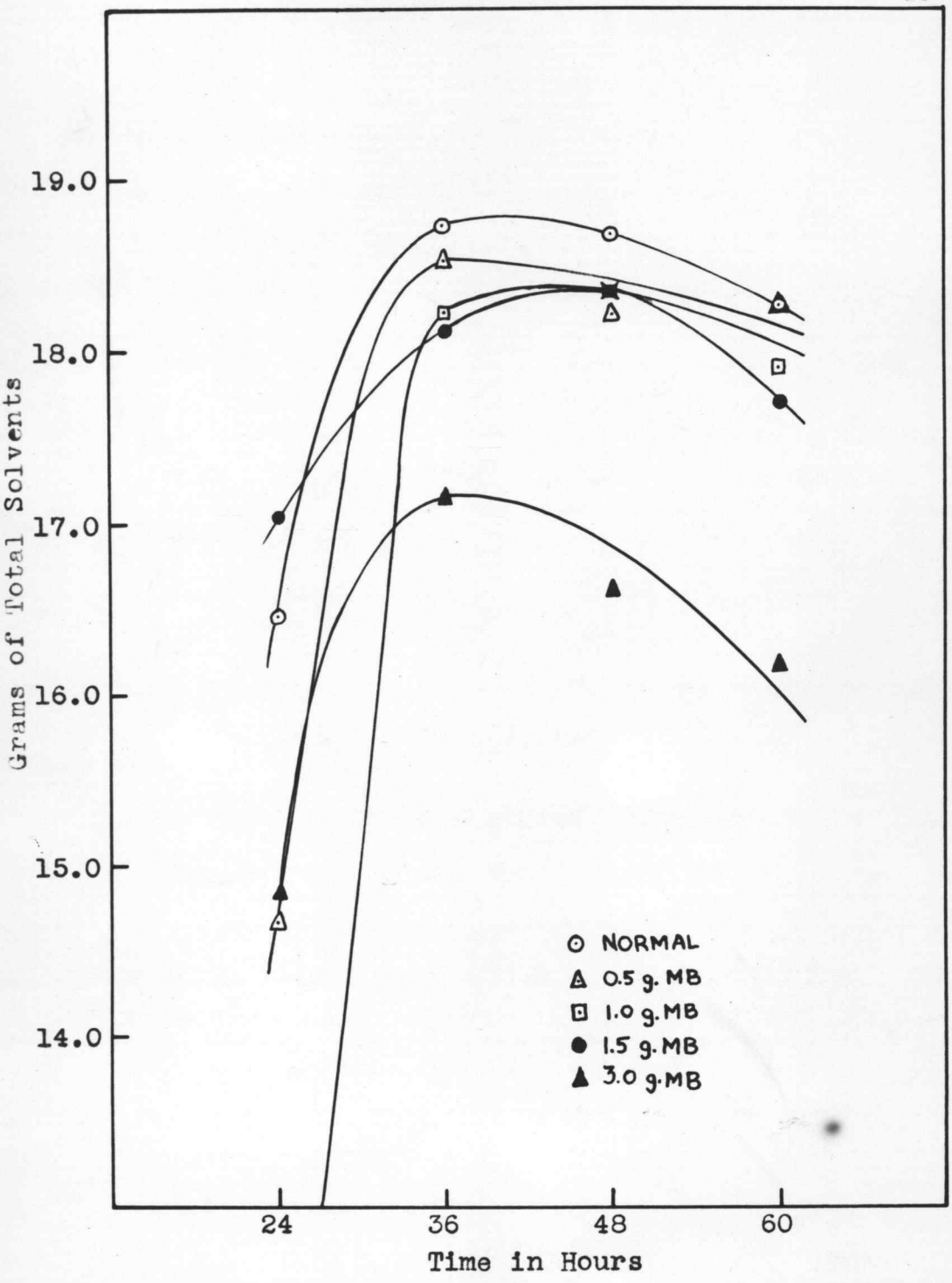

Figure 3. Effect of Mold Bran Levels and Fermentation Time on Total Solvent Yields From Six Per Cent Corn Mash.

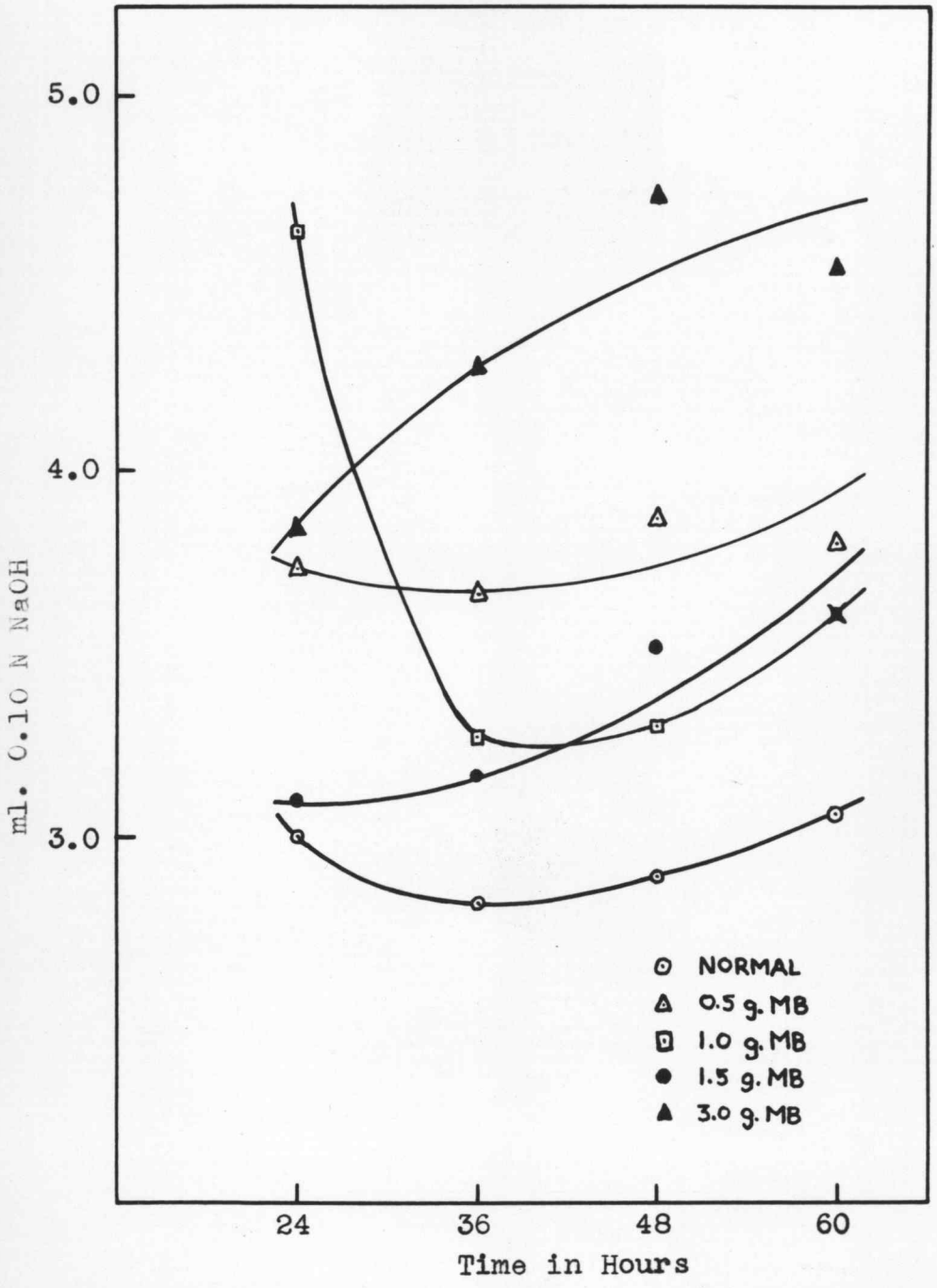

Figure 4. Effect of Mold Bran Levels and Fermentation Time on Titrable Acidity in Six Per Cent Corn Mash.

Data from the above tables and Figure 3 show that the maximum yield of solvents is obtained near thirty-six hours, and the maximum solvent yields are inversely related to the amount of mold bran added. The titrable acidity (Figure 4) is not consistent, but is always higher than the normal for every level of mold bran, and the minimum value for each series occurs near thirty-six hours.

Considerable "bubbling" and "heading up" occurred very early in the fermentations in which mold bran was added at the time of inoculation. This usually occurred from the second to the tenth hour and the activity gradually lessened until about the twenty-fourth hour when the culture almost looked dormant. This initial activity somewhat paralleled the amount of mold bran added and the more added the faster the culture began to ferment, reach maximum activity, and stop the visible gassing common to the normal fermentation. The mold bran appears to increase the metabolism of the organism in some manner enabling it to make acids very rapidly, the concentration of which may become high enough to inhibit the growth of the organisms before the fermentation is complete. Thus, the conversion of these acids into neutral solvents in the latter phases of the process may never be completed. Mold bran may possibly produce an inhibitory effect on the conversion of acids into solvents in the reactions involved although this effect has not been observed in previous fermentations with mold bran. The

potent amylase systems in mold bran are capable of rapidly producing fermentable saccharides whose concentration may become so high that the culture may be unable to tolerate them, although some strains of Clostridium acetobutylicum ferment five to six per cent mono- and disaccharide mashes.

The control of the increased acid formation, which is observed when employing mold bran, might possibly augment the production of solvents. The addition of calcium carbonate, to neutralize the free acids, was eliminated, since several authors (13, 42, 45) found that its addition only increased the formation of acids. Phosphate salts were added to the mashes since they are sometimes employed in other fermentations as a buffering agent as well as a source of inorganic phosphate. One half of one per cent of dipotassium hydrogen phosphate and potassium dihydrogen phosphate, based on the total mash volume, was added to sterilized mashes with 1.0 and 3.0 g. mold bran at the time of inoculation. Only the results for 1.0 g. mold bran are shown in Tables 10 and 11, and graphically in Figures 5 and 6, since preliminary investigations with 3.0 g. mold bran gave such low and erratic results that this level was of little value.

Table 10

Effect of 0.5 Per Cent Dipotassium Hydrogen Phosphate and 1.0 g. Mold Bran on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	0.35	0.51	0.38	1.24	5.80
36	0.76	2.01	0.45	3.22	8.05
48	1.51	4.31	0.59	6.41	8.70
60	1.41	3.93	0.33	5.67	10.26
72	1.43	3.73	0.59	5.75	8.45

Table 11

Effect of 0.5 Per Cent Potassium Dihydrogen Phosphate and 1.0 g. Mold Bran on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	1.00	2.42	0.13	3.55	10.63
36	1.61	3.47	0.13	5.21	10.93
48	1.66	3.76	0.26	5.68	10.31
60	1.95	4.43	0.53	6.91	9.71
72	1.68	3.96	0.26	5.90	10.78

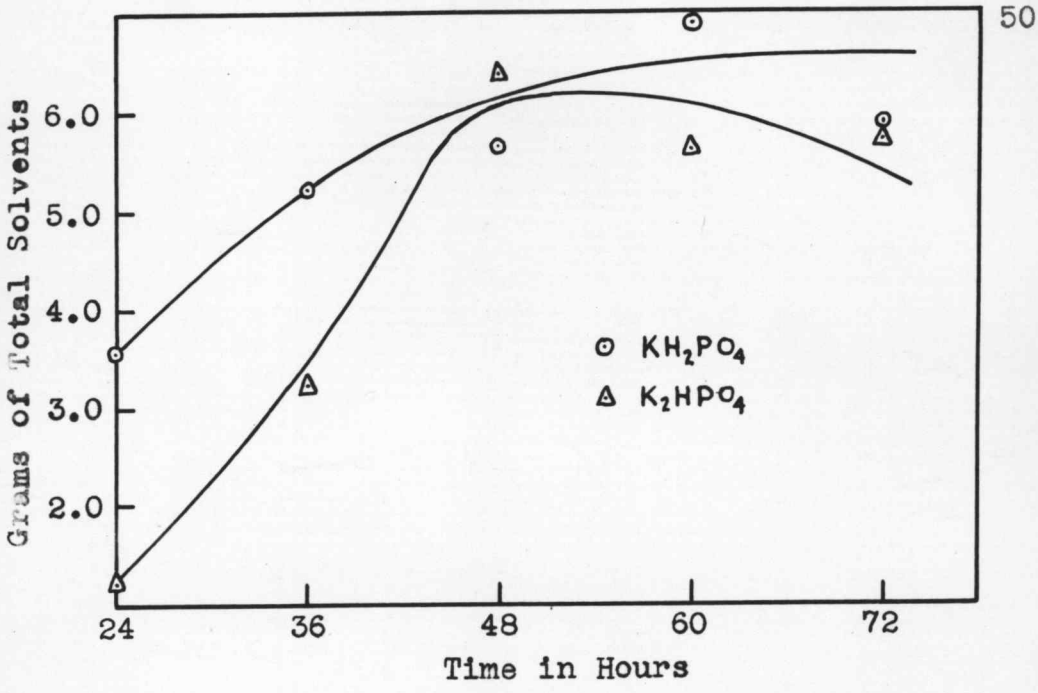

Figure 5. Effect of Phosphate Buffers and 1.0 g. Mold Bran on Total Solvent Yields From Six Per Cent Corn Mash.

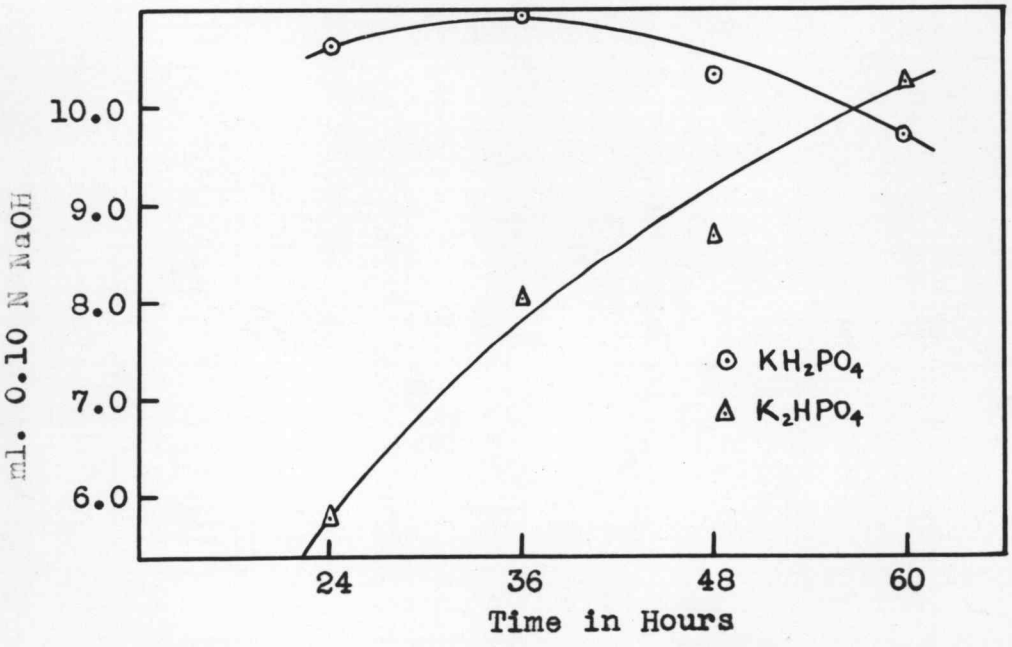

Figure 6. Effect of Phosphate Buffers and 1.0 g. Mold Bran on Titrable Acidity in Six Per Cent Corn Mash.

From the data in Figures 5 and 6, the addition of this concentration of the two phosphate salts only increased the formation of titrable acids and gave a marked decrease in the production of neutral solvents as compared to both the previous normal fermentation and the results using mold bran exclusively. In the early phase of the fermentation the potassium dihydrogen phosphate gave slightly higher solvent yields than did the dipotassium hydrogen phosphate, nevertheless they were considerably below the normal fermentation. The entire fermentation was sluggish and the maximum solvent yields were obtained at sixty to seventy hours as compared to thirty-six hours for the normal fermentation. The titrable acidity increases rapidly for the potassium dihydrogen phosphate but slowly for the dipotassium hydrogen phosphate with maximum values for the former at thirty-six hours but for the latter they continue to rise for the duration of the fermentation. This increased acid formation may have some commercial significance in the production of these organic acids, if there is ever a need for them, but the low solvent yields indicate the addition of phosphates salts to be of limited value.

C. Effects of Mold Bran Added Prior to Gelatinizing on Six Per Cent Corn Mash.

Mold bran has proven to be a stimulating agent as well

as an excellent saccharifying agent in the ethanol fermentation. The addition of low levels of mold bran at the time of inoculation gave slightly lower solvent yields than were obtained from the normal fermentation, but mold bran added before autoclaving might act as a growth or stimulating factor or merely as a pre-thining agent, since heat quickly inactivates the enzyme systems. Preliminary results obtained when three grams of mold bran were added to the media; in one flask before gelatinizing and another after gelatinizing but to both before autoclaving, showed that the yields of total solvents were practically the same. Time sequences were run on six per cent corn mash adding various levels of mold bran before autoclaving with the addition of one gram of mold bran at the time of inoculation to obtain the effects on solvent yields and titrable acidity. The results of this early addition of mold bran are tabulated below in Tables 12 and 13, and compared graphically in Figure 7.

Table 12

Effect of 5.0 g. Mold Bran Added Before Gelatinizing and 1.0 g. Mold Bran at Inoculation on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	2.63	4.39	1.49	9.05	6.60
30	5.86	10.55	1.31	17.72	3.60
36	5.91	9.98	1.67	17.56	3.59
48	5.91	10.71	1.31	17.93	3.93
60	5.79	10.71	1.31	17.81	9.04

Table 13

Effect of 10.0 g. Mold Bran Added Before Gelatinizing and 1.0 g. Mold Bran at Inoculation on Solvent Yields and Titrable Acidity from Six Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N. NaOH
12	0.68	1.52	0.13	2.33	7.42
24	2.50	4.85	0.79	8.14	8.55
36	3.76	7.53	1.08	12.37	6.05
48	2.32	5.23	0.53	8.08	8.43
60	2.66	5.88	0.67	9.21	7.91

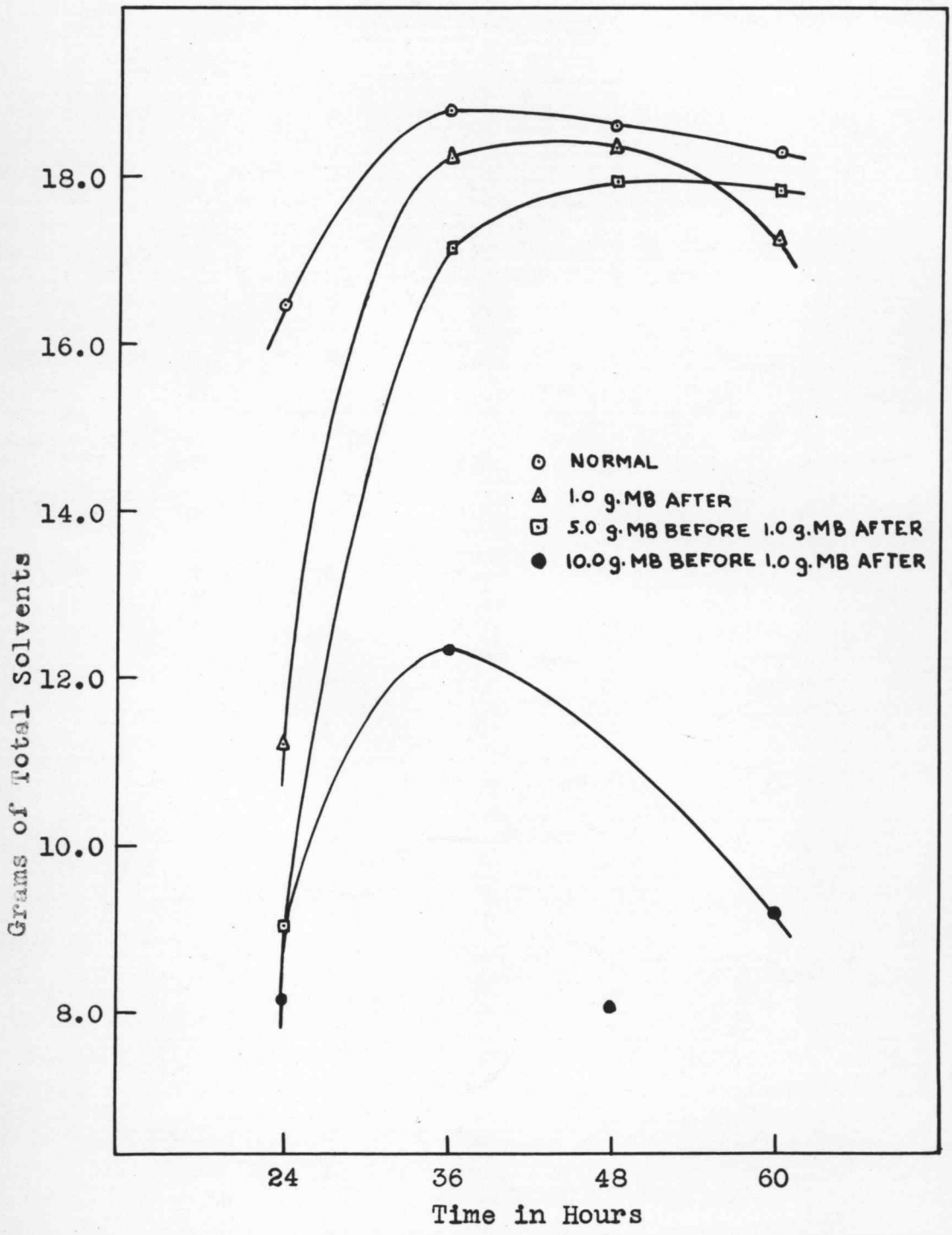

Figure 7. Effect on Total Solvent Yields of Mold Bran Levels Added Before Gelatinizing and 1.0 g. Mold Bran at Inoculation on Six Per Cent Corn Mash.

As the amounts of mold bran added before autoclaving increase the solvent yields decrease as compared to both the normal fermentation and with only 1.0 g. mold bran added at inoculation time. When 10.0 g. mold bran are added prior to autoclaving, a very marked decrease in solvent formation is noted with a corresponding increase in titrable acidity. Probably this high level of mold bran may have enough time to act as a saccharifying agent and produce a high concentration of fermentable sugars which the organism cannot readily tolerate, or the organisms may be able to utilize the fermentable carbohydrates converting them very rapidly to acids, the concentration of which eventually inhibits the fermentation. The same possibilities may to some extent be true when 5.0 g. mold bran are added, although these effects are not as great as observed when using 10.0 g. mold bran.

D. Effects of Mold Bran on Solvent Yields and Titrable Acidity when Added Before or After Autoclaving to Ten Per Cent Corn Mash.

Some of the properties of mold bran indicate that it might find application in higher mash concentrations where the mash is considerably more gelatinous. The preparation of ten per cent corn mash, the highest concentration for normal fermentation, followed the same procedures as given previously for the six per cent corn substrate. The 1300

ml. of sterile mash per flask contained 130.0 g. of ground corn.

A conventional fermentation on ten per cent corn mash was run similarly to the normal fermentation on six per cent corn mash. The solvent yields and titrable acidity results are listed in Table 14, and the solvent yields are shown graphically in Figure 8.

Table 14

Effect of Fermentation Time on Solvent Yields and Titrable Acidity from Ten Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	5.69	9.35	1.41	16.45	3.96
36	8.58	14.85	1.87	25.30	3.20
48	8.89	13.06	3.05	25.00	3.38
60	8.57	14.88	2.67	26.12	3.48
72	9.01	16.15	3.03	28.19	3.41

The values from Table 14 for solvent yields and titrable acidity followed the general trends previously discussed as being normal for the fermentation, although a slightly longer fermentation period is required for maximum solvent yields than for the less concentrated substrate. For comparative purposes the procedures for

employing mold bran were the same as those previously used with the six per cent corn mash. Time sequences were run with various levels of mold bran added at the time of inoculation. These data are tabulated below in Tables 15, 16, and 17, and compared graphically with the normal fermentation curve in Figure 8.

Table 15

Effect of 1.0 g. Mold Bran and Fermentation Time on Solvent Yields and Titrable Acidity from Ten Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	2.51	5.30	0.97	8.78	6.87
36	8.56	14.39	2.40	25.35	3.67
48	9.29	16.30	2.66	28.25	4.12
60	7.84	13.22	1.95	23.01	5.31

Table 16

Effect of 3.0 g. Mold Bran and Fermentation Time on Solvent Yields and Titrable Acidity from Ten Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	1.09	2.62	0.00	3.71	9.00
36	1.26	3.05	0.14	4.45	9.04
48	1.94	4.14	0.41	6.49	8.58
60	3.83	7.38	0.88	12.09	6.32

Table 17

Effect of 5.0 g. Mold Bran and Fermentation Time on Solvent Yields and Titrable Acidity from Ten Per Cent Corn Mash.

Time Fermented Hrs.	Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH
24	1.93	3.55	0.60	6.08	9.00
36	1.52	2.91	0.34	4.77	9.18
48	1.05	2.50	0.00	3.55	9.76
60	0.89	2.23	0.00	3.12	10.37

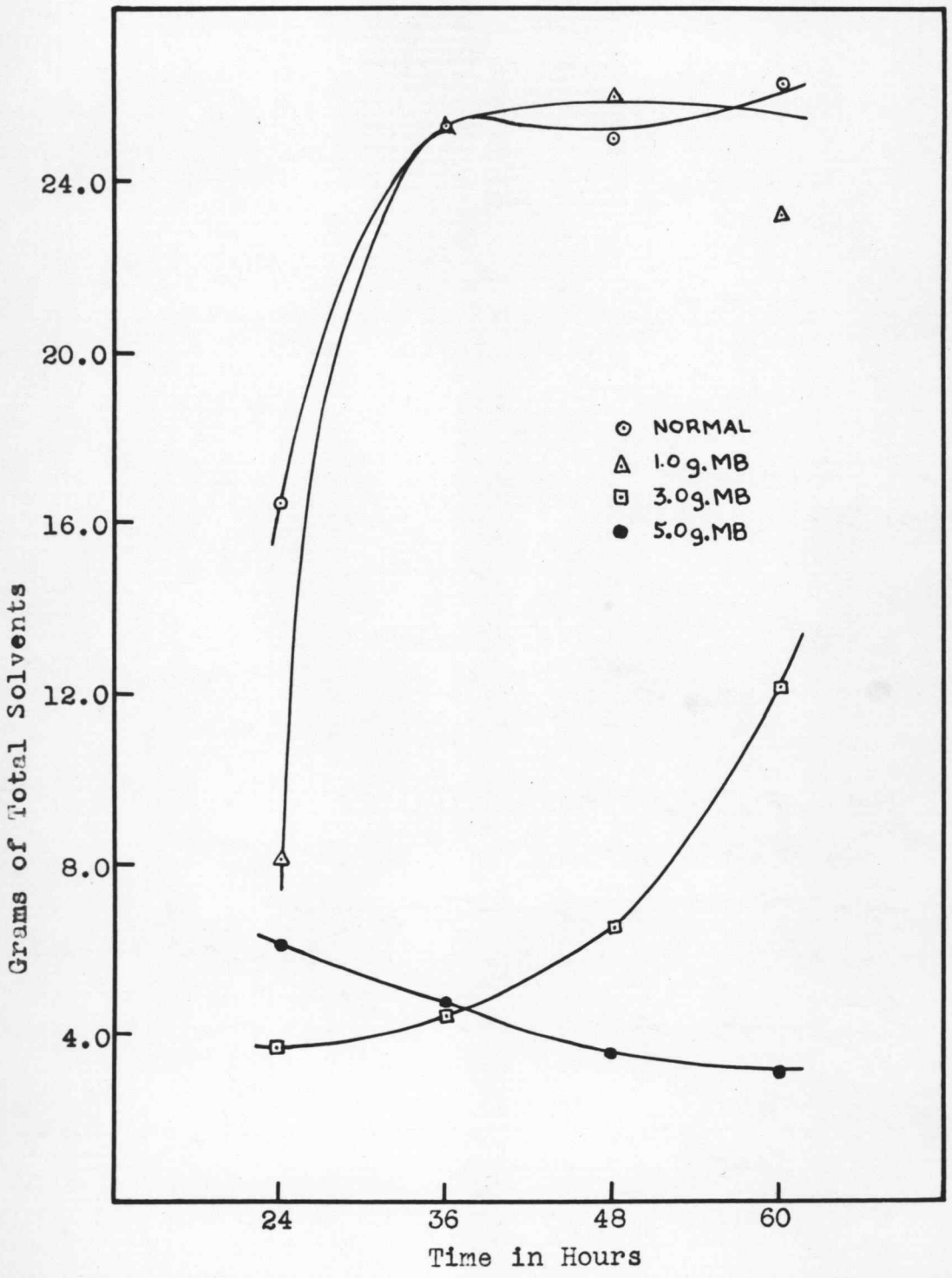

Figure 8. Effect of Mold Bran Levels and Fermentation Time on Total Solvent Yields from Ten Per Cent Corn Mash.

The addition of mold bran to the ten per cent corn mash caused the same initial "bubbling" and "heading up" as observed with the six per cent mashes, but these effects terminated earlier in this higher concentration. The curves in Figure 8 indicated that the lowest level of mold bran gave comparable to slightly higher solvent yields in the early phase of the fermentation as compared to the conventional, but further increase in mold bran levels gave an unusual decrease in solvent yields. These low yields with the high titrable acidity values from Tables 16 and 17 suggests the same actions and effects as previously discussed with the six per cent corn mashes.

Since ten per cent corn mash was observed to be moderately thick and gelatinous the possibility of using mold bran as a prethining agent suggested itself. Different levels of mold bran were added to the corn mash before gelatinizing, and the resultant mixtures were gelatinized, sterilized, cooled, and inoculated, as previously described. The fermentations were run for forty-eight hours since data in Table 14 indicated this fermentation period was very near the minimum time for maximum solvent production. The results obtained are listed in Table 18 and shown graphically in Figure 9.

Table 18

Effect of Mold Bran Levels Added Before Gelatinizing on Solvent Yields and Titrable Acidity from Ten Per Cent Corn Mash at Forty-eight Hours.

Acetone g. per flask	Butanol g. per flask	Ethanol g. per flask	Total Solvents g./flask	Titrable Acidity ml. 0.1 N NaOH	Mold Bran Added g./flask
5.67	9.04	1.97	16.68	4.57	1.0
7.20	10.90	1.60	19.70	6.65	3.0
7.42	12.08	1.76	21.26	4.43	5.0
2.94	5.40	0.71	9.05	7.95	7.0
2.65	4.88	0.71	8.24	8.45	10.0

The above data indicated a direct relationship between solvent yields and levels of mold bran until maximum yields were reached near the 5.0 g. level, after which higher mold bran levels are an inverse relationship with marked solvent decreases as a result. The titrable acidity reached a minimum with 5.0 g. mold bran and increased correspondingly with the addition of higher levels of mold bran. This followed the same pattern previously discussed for fermentations using mold bran.

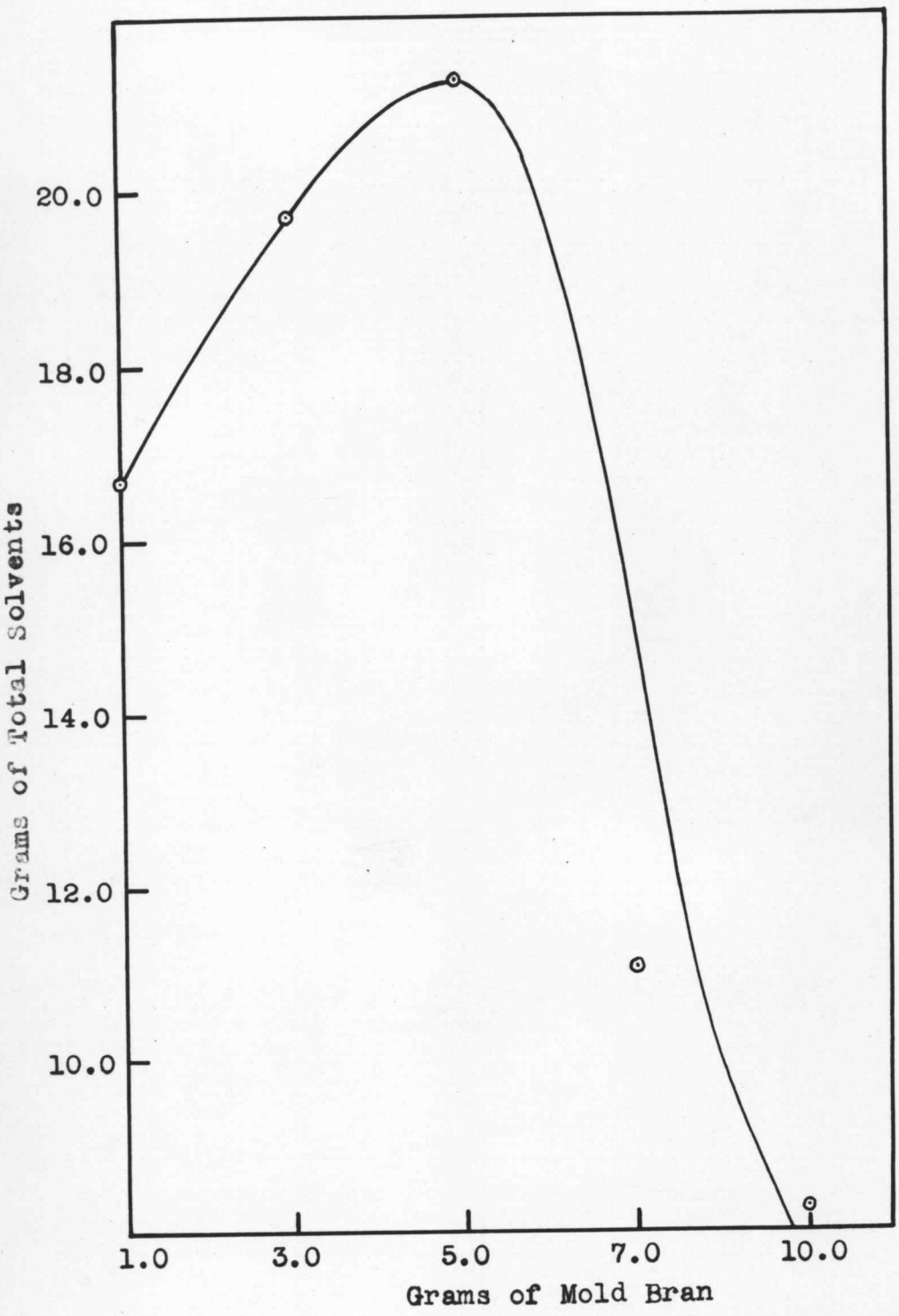

Figure 9. Effect on Total Solvent Yields of Mold Bran Levels Added Before Gelaltinizing on Ten Per Cent Corn Mash.

VI. SUMMARY AND CONCLUSIONS

1. The fungal amylase samples used in this investigation were grown by the method of Hao et al. (22) and tested by the Standard Evaluation Test method of Reese et al. (43). A strain of Aspergillus oryzae was used in the production of the mold bran, and samples which gave high amylolytic activities were employed in the acetone-butanol fermentation.

2. Two strains of Clostridium acetobutylicum, POS and NRRL B-527, were used to ferment all of the corn mashes used for this investigation.

3. Normal solvent yields and titrable acidity were determined on six per cent corn mash to establish a basis for comparison with fermentations involving mold bran. Solvent yields reached a maximum at thirty-six hours and then decreased slightly, which is somewhat contrary to other normal fermentations discussed by Speakman (54). Titrable acidity reached a minimum at thirty-six hours and increased slightly thereafter. The strain of Clostridium acetobutylicum employed may possibly have different growth characteristics at this mash concentration than the one described by Speakman.

4. Series of fermentations were run employing different levels of mold bran, the resultant solvent yields

and titrable acidity values being compared with the normal. An inverse relationship was noted between mold bran and solvent yields while a direct relationship was observed between mold bran and titrable acidity. Although none of the mold bran levels produced solvent yields comparable to the normal, increased stimulation was visibly detected during the initial phase of the fermentation. This stimulation warranted further study.

5. Whenever mold bran was added to the fermentation increased amounts of titrable acids, as compared to the normal fermentation, were obtained. Phosphate salts were added to neutralize or buffer these free acids in an attempt to increase solvent yields. Instead, only marked decreases in solvent production were noted with considerable increases in the formation of free acids. This increased acid formation may have some commercial significance in the production of these organic acids, if there is ever a need for them, but the low solvent yields indicate the addition of phosphate salts to be of limited value.

6. Since mold bran visibly stimulated the fermentation in its early phase, various levels of mold bran were added prior to sterilization to determine its effects as a possible source of growth factors or stimulating compounds, or as a prethining or saccharifying agent. Only

decreased solvent yields and increased titrable acidity were noted with increasing amounts of mold bran, thus this method of using mold bran has limited applications.

7. Normal solvent yields and titrable acidity were determined on ten per cent corn mash to establish a basis for comparison with fermentations that employed mold bran. Solvent yields began to level off at thirty-six hours, but continued to rise slightly for the duration of the fermentation. At this mash concentration the values for both the solvent yields and titrable acidity appear to agree comparatively with those obtained for the conventional fermentation at lower mash concentrations.

8. Ten per cent corn mash, the maximum concentration for normal fermentation, was employed with various amounts of mold bran being added at the time of inoculation. Only the 1.0 g. level of mold bran was found to be stimulatory and gave solvent yields which were equal to or slightly higher than those obtained from a normal fermentation. The higher mold bran levels were stimulatory in the early phase of the fermentation but decreased solvent yields and increased titrable acidity were eventually obtained. Even higher corn mash concentrations may someday be adequately fermented by the use of mold bran and proper thinning techniques.

9. The more concentrated corn mashes, being very gelatinous and viscous, suggested a use for some of the well known amylolytic properties of mold bran. Various levels of mold bran were added prior to gelatinizing the ten per cent corn mash. Solvent yields reached a maximum with 5.0 g. mold bran and decreased rapidly with higher levels of mold bran. The maximum solvent yields obtained were far below the maximum value for the normal fermentation and correspondingly the titrable acidity values were higher than for the normal fermentation at this concentration. Mold bran apparently stimulated the production of organic acids with a reduction in total solvent yields.

10. No significant reduction in the conventional fermentation time for maximum solvent production was noted when using mold bran in either the six or ten per cent corn mash.

11. The mold bran as employed in the six per cent corn mash appeared to be of little value in solvent production even though visible stimulatory effects were noted in the early phase of the fermentation as compared with the conventional fermentation. However, in the ten per cent corn mash meager data indicated low levels of mold bran gave as good as or slightly higher yields of neutral solvents when compared with conventional fermentations.

The proper use of mold bran may make it possible to ferment even higher mash concentrations, which would have important commercial applications.

Titrable acidity values were higher whenever mold bran was added before or with the inoculum. Adding mold bran at a later period when solvents and not acids are being produced in quantity may stimulate and increase the solvent yields. The elucidation of these problems will require further investigations.

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