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George Warren Buckaloo
Iowa State College

THE USE OF VARIOUS MOLD AMYLASES IN THE SACCHARIFICATION OF CORN MASH FOR ETHANOL FERMENTATION

by

GEORGE WARREN BUCKALOO

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I. INTRODUCTION

Prior to 1897 it was believed that vital processes of minute living organisms were associated with fermentation, and when these organisms were destroyed the fermentations were brought to a standstill. At this time Buchner showed that a non-living cell-free juice (extracted from yeast) was capable of converting glucose into alcohol and carbon dioxide in just the same way as the living yeast. It is as a result of this work that the term "enzyme" (Greek: en = in; zyme = leaven, or yeast) has come into being as the general term for all such substances. Enzymes then, can be regarded as biochemical, organic catalysts which are produced by living organisms (Anderson, 1938). In general, a catalyst is a substance which changes the speed of a chemical reaction, or to use a clever analogy which someone has suggested, "it is the traffic cop of a chemical reaction".

The conversion of starch into ethyl alcohol and carbon dioxide is accomplished by the action of certain enzyme systems. Amylase converts starch into maltose, this process being called saccharification.

Maltose is converted to glucose by the enzyme maltase. Zymase changes the simple sugar into the final products, ethanol and carbon dioxide. Yeasts produce the maltase and zymase required, but the amylase must be furnished from some other source.

Amylase is found in small amounts in most cereals and in larger quantities in soybeans and sprouting grains. Malt, the name applied to sprouted grains, has long been used as a saccharifying agent. At present malt prepared from barley is most commonly used in the beverage alcohol industry.

Amylase is also produced by many molds and by a few bacteria. It has been shown by Takamine (1914) and others that higher yields of ethanol are obtained when Aspergillus oryzae mold preparations are substituted for malt in the saccharification of starch for alcoholic fermentation.

At present most of the industrial alcohol used in this country is produced from molasses. However, in the event of an increased demand for ethanol, it would probably be produced from grain or other starchy materials. The role of mold amylases as saccharifying agents, therefore, may be increasingly important in the future. The ultimate objective of the research

in this field conducted in the Chemistry Department of Iowa State College is to contribute to increased utilization of farm crops by industry.

The object of the work presented in this thesis was to select the most promising strains of molds for preparation of amylase to be used in the saccharification of corn mash for ethanol fermentation. The investigation, however, included certain related aspects such as, simplifying procedures for estimating degree of saccharification and methods of growing the various molds on wheat bran medium.

II. HISTORICAL

The scientific literature of the last hundred years has included numerous papers devoted to the study of amylases. Walton (1927) has published the titles, and in many cases an abstract, of over twelve hundred articles dealing with amylase. In 1939 Schoene gave an excellent historical account of amylase with particular emphasis on the applications of this enzyme in the fermentation industry. Therefore, the historical review given below was not intended to be comprehensive and it is confined to studies on mold amylases, more especially the amylases of Aspergillus oryzae.

In the Orient, the enzymes of fungi have been utilized as saccharifying agents for centuries. The use of mold amylases in the saccharification of starchy materials for the alcoholic fermentation was introduced in the United States by Dr. Jokichi Takamine, a prominent Japanese scientist.

Dr. Takamine first came to this country in 1884 as one of the Japanese Commissioners to the New Orleans Exposition, and it was at that time that he met his

future wife. On the invitation of his father-in-law he returned to the United States in 1890 to superintend the manufacture of alcohol by his patented method (Kawakami, 1929).

From 1891 to the present time numerous patents have been issued to Dr. Takamine in this country and also in Canada, England, France, Belgium and Austria-Hungary. These patents cover the methods of preparation and various uses of mold amylase products.

Takamine's process for the manufacture of alcohol differed from the conventional method in that mold preparations were used in place of malt as the saccharifying agent. The mold, Aspergillus oryzae, was grown on steamed rice (1898) and the resulting preparation was called "Koji". Similarly, "Taka-koji" was the product obtained by growing this mold on wheat bran. The chief advantages of Taka-koji over malt were given as follows: (1) Malt was more subject to price fluctuations and (2) Taka-koji was prepared in 48 hours, while the preparation of malt required from six to eight days (1914). However, in spite of these advantages, Taka-koji has not been used to a very great extent in the fermentation industry up to the present time. An objection to the use of this material in

the manufacture of beverage alcohol is that disagreeable tastes and odors may be imparted to the finished product. Since most of the industrial alcohol not used for beverage purposes is at present produced by the fermentation of black strap molasses, no saccharifying agent is required.

Although Takamine's product did not find significant application in our fermentation industries, extensive use has been made of his products in the desizing of textiles. Also a similar product "Taka-diastase" has been used medicinally as an aid in digestion.

In 1914 Takamine published an article entitled "Enzymes of Aspergillus oryzae and the Application of Its Amyloclastic Enzyme to the Fermentation Industry". In this publication he gave an historical account of the use of this mold in Japan and other countries. Also he reported success in the acclimatization of Aspergillus oryzae toward formaldehyde, and stated that the corresponding Taka-koji prepared with the acclimatized mold was 100% higher in amyloclastic power.

His next successful experiment was in growing the acclimatized fungus in a rotating drum apparatus. Regarding this investigation with an apparatus

consisting of a Mason jar and a clock mechanism for rotating, he made the following observation,

> "I noticed a very interesting fact, namely, one side of the bran particle is covered with the fungus. It is the inner side of the bran. This side is coarse in structure and rich in starch and protein matter. If the bran is moistened and steamed, this particular side shows a tendency to curl inward and assumes the shape of pericarp previous to its severing from the kernel. When each particle of the bran is curled the inner side is naturally protected from the friction between particles when they are put in cylinders and motion is imparted to the mass by revolution."

After numerous experiments with larger drums, a suitable apparatus was devised for commercial production (i.e., in 4800 pound batches) of Taka-koji and similar products. This drum apparatus consisted of a huge, plain iron cylinder provided with an inlet on one side and an outlet on the other through which air could be passed by means of a suction fan located on the outlet side. The drum was rotated at one revolution per minute. A separate pipe with branching spray nozzles extended through the drum.

Takamine's procedure for preparing Taka-koji in the drum apparatus was as follows:

Wheat bran and water were dumped in and the mass was steamed, the drum being turned constantly. After cooling, first formaldehyde and then the mold spores

suspended in water were sprayed in. The drum was brought to a standstill and left for about twelve hours until the temperature started to rise, indicating incipient germination of the mold. Rotation of the drum was then begun, and the temperature was controlled by passing in sterile, cool, moist air. In forty-eight hours at 30 ^{o} C. the process was concluded. The Taka-koji produced in this manner was more compact and glossy than that prepared by other methods. The labor needed for this process was approximately one-sixth of that required by the older method of culturing in trays or on a cement floor and fewer contaminating organisms were introduced.

Oshima and Church (1923) studied growth and enzyme production of four strains of Aspergillus flavus-oryzae on the following media: wheat bran, wheat middlings, corn meal, cocoanut meal, peanut meal, soybean meal, crushed soybeans, dried yeast, ground dried codfish, and casein. They found wheat bran to be the best substrate. Also, the presence of starch in the substrate was desirable for maximum amylase production.

The relation between temperature and amylolytic activity of preparations similar to Taka-koji was

studied by Taichi Harada (1931). He concluded that the amylolytic activity of the preparation underwent little change when heated at low temperatures, such as 30 ° and 37 ° C., for five and two and one-half hours. At relatively high temperatures (above 45 ° C.) the activity was adversely affected when heated for only thirty minutes. The optimum pH was 5.4 within the temperature range of 55 ° to 60 ° C.

Since the work of Märcker (1878), later verified by Ohlsson (1922) and by Kuhn (1925), malt amylase has been known to be a mixture of two components, i.e., an amyloclastic or liquifying fraction and a maltose forming or saccharogenic fraction. Oshima (1928) studied the liquifying and saccharifying properties in Taka-koji and found that the amylase showed optimal amyloclastic and saccharogenic activities at pH 4.8 to 5.2 and was most stable to heat at pH 6.4. It was completely inactivated by heating to 65 ° C. for one hour but retained its activity at neutrality and below 40 ° C.

Underkofler, Fulmer and Schoene (1939) studied the growth of Aspergillus oryzae on moist wheat bran and employed the resulting moldy bran as a saccharifying agent for ethanol fermentation mashes. Substi-

tution of this bran preparation for malt gave ethanol yields of more than 90 per cent of the theoretical. Fermentation tests also indicated that the moldy brans gave higher ethanol yields after grinding to a coarse powder, either while moist or after air drying.

Schoene (1939) and Schoene, Fulmer and Underkofler (1940) studied the effect of using binary combinations of moldy bran (from Aspergillus oryzae), malt, and soybean meal. They found that in each case the combinations resulted in higher yields than were obtained from the individual substances used alone. The malt-soybean meal combinations were not as efficient, however, as moldy bran, either singly or in combinations. The effect of combinations was less when a preliminary acid saccharification was employed. A 50 to 70 per cent saving in moldy bran was obtained with the use of acid-saccharified mashes in place of normal mashes (i.e. corn meal-water mixtures). This effect of moldy bran in increasing ethanol yields from acid-saccharified mashes was shown to be due to its amylolytic activity rather than to yeast growth stimulants. The substitution of thick mashes for normal mashes was also found to give a 20 per cent saving in the amylolytic materials needed for maximum

ethanol production.

No attempt has been made to review the literature on the alcoholic fermentation. For this material the reader is referred to the books written by the following authors: Mathews (1901), Allen (1926), Smyth and Obold (1930), Thaysen and Galloway (1930) and Harden (1932).

III. METHODS

A. Microbiological Procedures

1. Production of mold amylase preparations.

Various molds were grown on wheat bran moistened with an equal weight of 0.5 normal sulfuric acid. Sterilization of the bran mash was made with live steam at 20 pounds pressure for 2 hours. Stock mold cultures were carried on wort-agar, malt extract-agar, or glycerol yeast extract-agar slants. Transfers were made from slants to 50 grams of the sterile bran medium (i.e. 25 g. bran and 25 g. dilute sulfuric acid) in 500 ml. Erlenmeyer flasks. These flasks were used both for carrying the cultures and for inoculating drum batches containing 1600 grams (i.e. 800 g. bran and 800 g. dilute acid) of the bran medium.

The apparatus described and used by Underkofler, Fulmer and Schoene (1939) for growing molds aerobically in a rotating drum was employed throughout this investigation. A five gallon pyrex bottle was mounted horizontally on a system of rollers. The rollers

served as a support as well as a rotating device. A current of sterile air, saturated with moisture, was passed into the drum by means of a glass tube reaching through the stopper nearly to the bottom of the bottle. The air outlet also passed through the stopper. The air was sterilized by passing it through a flask containing concentrated sulfuric acid and glass wool. The sterile air was saturated with moisture by passing it through a second flask containing sterile distilled water and glass wool.

After inoculating the drum of bran medium with a well sporulated culture the contents were thoroughly mixed by shaking or rotating. During the germination period the charge was rotated or mixed only enough to prevent lumping. After about sixteen hours the mold was actively growing and the drum was then rotated continuously for the next twenty to thirty hours. The aeration was regulated to prevent overheating during the active growth period. In some cases, especially with Rhizopus molds, intermittent instead of continuous rotation seemed preferable. After the bran medium was well covered with vegetative mycelia, usually after about forty hours, the charge was removed from the drum and then dried in a well ventilated

incubator. Some growth took place while drying; however this method of drying was sufficiently rapid to prevent excessive sporulation. The dried moldy bran was ground to a coarse powder by means of a Wiley mill, and as such was used for fermentation tests. The method of Takamine (1914), i.e., the use of the mold amylolytic substances in the same manner as malt is used, was employed.

2. The saccharification of corn mash by means of mold amylase preparations.

Thirty-two grams of corn meal were placed in each 500 ml. flask to which 225 ml. of boiling water were added, thus bringing about a preliminary gelatinization of the starch. As soon as the mixture had cooled to about 60 ° C., a weak suspension of mold amylase, containing 0.16 grams (0.5% of weight of corn) was added for the purpose of "premalting". After premalting for one-half hour the mash was sufficiently liquid to mix by shaking. The corn mash was then cooked for one-half hour in the autoclave at 20 pounds steam pressure. After cooling somewhat the flasks of mash were placed in a constant temperature water bath held at 60 ° C. The mold amylase preparations

to be tested were weighed out into small beakers containing a little distilled water. The thick slurry resulting was then added quantitatively to the appropriate corn mash flask with the aid of a little water from a wash bottle. Saccharification was carried out at 60 ° C. for one hour.

3. Estimation of extent of saccharification by fermentation methods.

After cooling the saccharified corn mash to 30 ° C. a 10% inoculation was made with an active yeast culture (designated as Saccharomyces cerevisiae No. 43 in this laboratory) growing in beer wort. The fermentations were usually completed in about 70 hours.

When ethanol is to be produced from corn, undoubtedly the yield of ethanol is the best criterion for evaluating various saccharifying agents. However, a brief study, which is described in the experimental results below, indicated that the loss in weight of the corn mash due to evolution of carbon dioxide during fermentation may also be used as a criterion. In the present investigation most of the data were based on the yields of ethanol, and the method used

is specified in each case.

B. Analytical Procedures

1. Determination of ethanol.

The volume of the fermented mash, or beer, in each flask was around 275 ml. Acidity was neutralized by adding an excess of finely powdered calcium carbonate and then the beer was distilled, the distillate being collected in 100 ml. volumetric flasks. These flasks were kept stoppered until the specific gravities of the contents could be determined. The specific gravity (d. 25 °/25 ° C.) determinations were made with a Chainomatic Westphal Balance, and the alcohol concentrations, in grams per 100 ml. of solution, were read from an appropriate table.

2. Determination of carbon dioxide.

During the alcoholic fermentation carbon dioxide and ethanol are produced in equimolecular amounts according to the equation

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 .$$

Immediately after inoculation the flasks were weighed to within 0.1 gram by means of a good trip

balance. The carbon dioxide produced was estimated by determining the loss in weight of the flasks during fermentation, appropriate corrections being made for loss of water by evaporation. A brief study of the correlation of ethanol yield and loss in weight due to evolution of carbon dioxide is given below in the section on experimental results.

3. Determination of starches and sugars.

A starch determination (or its dextrose equivalent) was made on the yellow corn meal used during the course of this investigation according to the official direct acid hydrolysis method of the Association of Official Agricultural Chemists (1935). The reducing substances formed were estimated by the method of Shaffer and Hartmann (1921) and calculated as dextrose. The Shaffer-Hartmann reagents were standardized against a sample of pure dextrose.

4. Calculation of results.

The experimental results were calculated as per cent of the theoretical conversion of carbohydrate in the corn to alcohol (or carbon dioxide) according to the equation:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 .$$

Except when otherwise specified, all the data given in the tables represent the average values for duplicate fermentations, and all yields were corrected for the amount of alcohol derived from the inoculum and amylolytic materials. The correction used for the alcohol produced from 10% beerwort was 0.03 gram per ml. The correction used for the mold amylase preparations was 0.032 gram of alcohol per gram of the preparation and the correction for malt was 0.334 gram of alcohol per gram of malt. A sample calculation would be as follows:

The dextrose equivalent of the corn was found to be 74.66%. If 32 grams of corn produced 9 grams of alcohol, the % of theoretical yield is

$$\frac{(9)}{(32)\ (.7466)\ (2C_2H_5OH/C_6H_{12}O_6)}(100) = 73.8\%$$

IV. EXPERIMENTAL RESULTS

A. Preliminary Studies

1. Isolation of amylase-producing fungi from normal habitats.

Practically any material containing starch may be the normal habitat of amylase-producing fungi. In this study four samples were taken from silage and corn meal. The material in each case was mixed with 10 ml. of sterile water to suspend the micro-organisms present. Three one-to-ten dilutions were made and these diluted suspensions together with the original suspension were used for streaking starch-agar medium in petri dishes. After incubation at 30 ° C. for two days certain colonies were characterised by a clear zone in the starch-agar medium, indicating production of amylase. Transfers were then made to beerwort agar slants from the amylase-producing colonies. One of the mold cultures (No. 45), isolated in this way from a kernel of corn in silage, was grown in the rotating drum apparatus and the resulting preparation

used in subsequent studies.

2. Determination of the amount of alcohol distillate to be collected.

The object of this study was to determine the minimum amount of distillate from fermentation beers in which the alcohol could be collected quantitatively. The volume of fermentation mash, or beer, distilled and the corresponding amount of alcohol collected in successive 50 ml. of distillate is given in Table 1. The extreme values (i.e., the highest and lowest percentage of ethanol in the distillates) among 28 fermentations are given. It was concluded that 100 ml. of distillate should be collected for analysis of alcohol in subsequent studies when the volume of beer was around 300 ml.

TABLE 1.

Ethanol Recovered in Three 50 ml. Fractions of Distillates from Fermentation Beers.

Volume of beer, ml.	Ethanol in first 50 ml of distillate, %	Ethanol in second 50 ml. of distillate, %	Ethanol in third 50 ml. of distillate, %
200	99.8	0.2	0
200	100.0	0	0
300	89.4	10.6	0
300	97.2	2.8	0

3. Correlation between ethanol and carbon dioxide yields as Criteria for comparing various saccharifying agents.

The yield of ethanol has been used for the quantitative estimation of the degree of saccharification of starch in the alcoholic fermentation of corn mash. Although this may not give an absolute value for the per cent of saccharification it enables one to make a true comparison of the effect of various saccharifying agents on the yields of ethanol.

Since carbon dioxide and ethanol are produced in equimolecular amounts in fermentations by yeast it was thought that the weight of the carbon dioxide liberated might also be used as a measure of the amount of saccharification. The conversion of starch to ethanol and carbon dioxide is represented by the following equation:

$$\underset{\text{starch}}{\frac{2}{x}(C_6H_{10}O_5)x} + \frac{2}{x}H_2O \xrightarrow{\text{amylase}} \underset{\text{maltose}}{C_{12}H_{22}O_{11}} \xrightarrow{\text{maltase, zymase}} \underset{\text{ethanol}}{4C_2H_5OH} + \underset{\text{carbon dioxide.}}{4CO_2}$$

There are two general methods for measuring the

amount of carbon dioxide: (1) by absorption of the gas in alkali and (2) by noting the loss in weight of the media during fermentation. The latter method was studied with the object of facilitating the work involved in comparing a large number of saccharifying preparations.

In the first series of this study the fermentations were carried out in one liter flasks and an attempt was made to correlate the ethanol and carbon dioxide yields at various stages in the fermentation. The carbon dioxide was determined by the loss in weight during the fermentation. Two blanks (i.e., sterile saccharified mash) were run in order to correct for evaporation. The data, given in Table 2, indicate that the carbon dioxide yield is low during the first part of the fermentation. This may be accounted for, however, by the solubility of carbon dioxide in the medium. Gassing was found to be very vigorous even after considerable shaking, hence the medium was probably supersaturated with carbon dioxide.

TABLE 2.

Yields of Carbon Dioxide Produced at Various Stages in the Yeast Fermentation of Saccharified Corn Mash (1) Determined by Loss in Weight of Fermenting Medium and (2) Calculated from Ethanol Analysis.

Time after inoculation, hours	(1) Carbon Dioxide loss, grams	(2) Carbon Dioxide calculated from ethanol analysis, grams
3	0.4	1.0
5	1.8	2.6
7	3.2	4.6
10	5.1	6.1
28	13.1	13.7
56	17.0	16.9
80	20.2	18.8

In the next series of this study the fermentations were carried out in 500 ml. flasks. In Table 3 the values for the amounts of carbon dioxide evolved (after correction for evaporation), determined by weighing, and by calculation from ethanol yields are given for each fermentation. The method involving weight loss checked with the ethanol analysis in nine fermentations out of ten. It was concluded that this method may well be used for preliminary comperison studies and should, of course, be run in duplicate or in triplicate. A subsequent ethanol analysis would be recommended in cases of high carbon dioxide yields and also if there is considerable variation (i.e., over 5%) in the results of duplicate fermentations.

TABLE 3.

Yields of Carbon Dioxide Produced in the Yeast Fermentation of Saccharified Corn Mash (1) Determined by Loss in Weight of Fermenting Medium and (2) Calculated from Ethanol Analysis

Time after inoculation, hours	(1) Carbon Dioxide loss, grams	(2) Carbon Dioxide calculated from ethanol analysis, grams
23	6.7	6.6
33	8.0	8.1
39	8.3	8.4
69	9.8	9.6
82	9.7	9.7
82	9.8	9.9
82	11.0	9.8
82	9.9	9.9
82	9.7	9.9
82	10.0	9.8

B. Amylase Production by Various Molds

Twenty-one moldy bran preparations were made by growing various molds on moistened wheat bran medium in the rotating drum apparatus.

The molds studied are listed in Table 4, and henceforth reference to the individual molds will be made by number. The drum preparation from each mold is designated by the laboratory number of the mold plus an alphabetic suffix. For example Drum Nos. 2a, 2b, 2c etc. were prepared from mold No. 2.

TABLE 4

Molds Tested for Amylase Production.

Lab. No.	Name	Source	
2	Aspergillus oryzae	A. T. C. C.,[1]	No. 4184
38	Aspergillus oryzae	Röhm and Haas	No. 38
40	Aspergillus oryzae	Röhm and Haas	No. 40
42	Aspergillus oryzae	Röhm and Haas	No. 42
22	Unidentified yellow mold	Isolated from Oat Hulls	
45	Unidentified yellow mold	Isolated from Silage	
14	Rhizopus oryzae	Lockwood,[2]	No. 649
19	Rhizopus tritici	Lockwood,	No. 654
21	Mucor javanicus	Lockwood,	No. 718

(1) American Type Culture Collection, Georgetown University Medical School, 3900 Reservoir Road, Washington, D.C.

(2) Lockwood, L. B., U. S. Department of Agriculture, Bureau of Chemistry and Soils, Washington, D.C.

C. Comparison of Drum Preparations from Various Molds

The saccharifying power of preparations from Drum Nos. 2a, 2b, 2c and 2d (prepared from mold No. 2) was compared using different concentrations of the amylolytic materials. The corn mash was made up, saccharified, and the ethanol fermentation carried out as previously described under Methods. The selection of the best drum preparation in this group, No. 2c, was based on the yields of ethanol as given in Table 5. It was interesting to note that the moldy bran from Drum No. 2c was dried before sporulation took place, while that from Drum No. 2d was allowed to sporulate before drying. The inoculum for Drum No. 2b consisted mainly of vegetative mycelia with relatively few spores, and poorer growth was evident in this preparation.

Since the moldy bran from Drum No. 2c gave highest yields, it was used as the reference amylase preparation throughout the subsequent investigation.

TABLE 5

Ethanol Yields from Corn Mash Saccharified by Moldy Brans of Drum Nos. 2a, 2b, 2c and 2d.

Amylolytic material	Proportion of Amylolytic material, g./100 g. corn	Ethanol from corn, grams
2a	2.5	9.84
	5.0	11.36
	10.0	11.90
2b	2.5	9.52
	5.0	11.51
	10.0	11.64
2c	2.5	10.69
	5.0	11.71
	10.0	12.00
2d	2.5	10.06
	5.0	11.26
	10.0	11.47

In order to select the most effective amylase-producing strain of Aspergillus oryzae, fermentation mashes were saccharified using the following preparations:

Drum Nos. 2b, 2c, 2d (A. oryzae No. 2)

Drum Nos. 38a, 38b, 38c (A. oryzae No. 38)

Drum Nos. 40a, 40b, 40c, 40d (A. oryzae No. 40)

Drum Nos. 42a, 42b (A. oryzae No. 42)

Drum Nos. 22a, 22b (Unidentified yellow mold No. 22, probably belongs to A. flavus-oryzae group)

In this selection, however, laboratory facilities did not allow parallel fermentations employing all the A. oryzae preparations in a single series. Therefore, separate fermentations were run for each concentration of the amylolytic material used (i.e. 2.5%, 5.0% and 10.0%). Also the duplicates for each concentration were carried out as separate fermentation series. The yields of ethanol for each of the above concentrations are given in Table 6, Table 7 and Table 8, respectively. The yields in per cent of theoretical were calculated as described previously in the section on Methods.

In Table 6 the yields of ethanol using 2.5% concentration of amylolytic materials are given. With

this concentration the data indicated that the preparation from Drum Nos. 40a and 2c were the most effective saccharifying agents of this A. oryzae group.

TABLE 6

Ethanol Yields from Corn Mash Saccharified by 2.5% Concentration of Various A. oryzae Preparations

Amylolytic material	Proportion of Amylolytic material, g./100 g. corn	Ethanol from corn, grams	Yield, %
2b	2.5	7.02	57.4
2c	"	7.63	62.4
2d	"	7.11	58.2
22a	"	5.52	45.2
22b	"	6.88	56.3
38a	"	6.60	54.0
38b	"	6.99	57.2
38c	"	6.95	56.9
40a	"	7.75	63.5
40b	"	7.40	60.6
40c	"	6.72	55.1
40d	"	6.13	50.2
42a	"	6.56	53.7
42b	"	- -	- -

Theoretical ethanol yield = 12.20

Table 7 shows the yields of ethanol using 5.0% concentration of each amylolytic preparation. At this concentration moldy bran from Drum Nos. 40a, 40b and 2c gave the highest yields of ethanol.

TABLE 7

Ethanol Yields from Corn Mash Saccharified by 5.0% Concentration of Various A. oryzae Preparations.

Amylolytic material	Proportion of amylolytic material, g./100 g. corn	Ethanol from corn, grams	Yield, %
2c	5.0	10.02	82.2
2d	"	9.43	77.2
22a	"	7.73	63.3
22b	"	8.89	72.9
38a	"	9.20	75.3
38b	"	9.59	78.5
38c	"	9.87	80.8
40a	"	10.17	83.3
40b	"	10.05	82.3
40c	"	9.88	80.9
42a	"	9.53	78.0
42b	"	9.03	73.9

Theoretical ethanol yield = 12.20

Table 8 shows the yields of ethanol using 10% concentrations of the Aspergillus oryzae preparations. The differences in ethanol yields were rather small in this series. The following preparations gave yields between 76 and 78 per cent of theoretical: Drum Nos. 40b, 2c, 40c, 38b, 22b, 38c, 40a, 40d and 2d. The fact that the yields were low in this particular series cannot be satisfactorily explained. All of the fermentations, the ethanol yields for which are given in Table 8, were incubated at the same time. It is believed that overheating in the incubator toward the end of the fermentation may have caused the low values, due to evaporation of the alcohol.

Table 8

Ethanol Yields from Corn Mash Saccharified by 10% Concentration of Various A. oryzae Preparations.

Amylolytic material	Proportion of amylolytic material, g./100 g. corn	Ethanol from corn, grams	Yield %
2b	10.0	9.22	75.5
2c	"	9.44	77.4
2d	"	9.32	76.3
22a	"	8.85	72.5
22b	"	9.38	76.8
38a	"	9.05	74.1
38b	"	9.42	77.1
38c	"	9.38	76.8
40a	"	9.36	76.6
40b	"	9.52	77.9
40c	"	9.42	77.1
40d	"	9.32	76.3
42a	"	8.62	70.6
42b	"	8.90	72.8

Theoretical ethanol yield = 12.20

In the comparisons to this point the preparations of Drum Nos. 2c and 40a were found to be definitely superior as saccharifying agents in the alcoholic fermentation of corn mashes.

The results given in Table 9 serve to compare the following drum preparations:

Drum No. 2c (*A. oryzae* No. 2)

Drum No. 14a (*Rhizopus oryzae* No. 14)

Drum No. 19a (*Rhizopus tritici* No. 19)

Drum No. 21a (*Mucor javanicus* No. 21)

Drum No. 45a (unidentified silage mold No. 45).

The carbon dioxide loss method was used instead of the ethanol yield for this series of fermentations. Preparations from Drum Nos. 14a and 19a compared quite favorable with that of Drum No. 2c.

TABLE 9

Carbon Dioxide Yields from Yeast Fermentation of Corn Mash Saccharified by 5% Concentration of Various Mold Preparations.

Amylolytic material	Proportion of amylolytic materials, g./100 g. corn	Carbon dioxide from corn, grams
2c	5.0	10.2
14a	5.0	10.0
19a	5.0	10.1
21a	5.0	9.6
45a	5.0	9.7

D. Comparison of Mold Amylases Produced in Three Different Kinds of Apparatus

The main advantage of growing the molds in comparatively large batches in the rotating drum apparatus is to obtain consistent amylase preparations. Matting of the media and uneven growth was characteristic of the mold amylase preparations produced in ordinary flasks. To overcome this difficulty each flask was equiped with a stirrer, i.e. a heavy glass rod inserted through the cotton plug to the bottom of the flask. Also it was observed that much better growth occurred if the flasks were placed on their side instead of in the regular upright position.

The object of the first part of this study was to compare amylolytic material produced in the rotating drum with the saccharifying material produced by the technique using the special flasks. If consistent results could be obtained with preparations grown in these flasks, investigations to determine the effect of pH, temperature, salts, inhibitors etc. would be greatly facilitated.

The carbon dioxide loss method was used as the criterion for comparing drum and flask preparations

from one strain of Aspergillus oryzae and from one strain of Rhizopus oryzae. The data in Table 10 indicate that the flask preparations and drum preparations checked in their saccharifying ability.

TABLE 10

Carbon Dioxide Yields from Yeast Fermentation of Corn Mash Saccharified by 5% Concentration of Mold Preparations Produced in Special Flasks and in Rotating Drum Apparatus.

Amylolytic material	Proportion of amylolytic material, g./100 g. corn	Carbon dioxide from corn, grams
Drum 40f	5.0	10.4
Flask 40f	5.0	10.3
Drum 14a	5.0	10.0
Flask 14a	5.0	10.1
Drum 40e	5.0	9.6
Flask 40e	5.0	9.8

Two samples of mold amylase preparations, which had been grown in an aerated aluminum pot, were obtained through the courtesy of Dr. Leo M. Christianson of the University of Idaho. These preparations were produced in 750 gram batches by growing A. oryzae No. 2 on wheat bran medium. The above two samples were designated as LMC #3 and LMC #7 and a comparison was made with moldy bran of Drum No. 2c. The data, given in Table 11, indicate that the material from Drum No. 2c was the most effective; however the differences between the three saccharifying preparations were small.

TABLE 11

Ethanol Yields from Corn Mash Saccharified by Mold Preparations Produced in an Aerated Aluminum Pot and in the Rotating Drum Apparatus.

Amylolytic material	Proportion of amylolytic material, g./100 g. corn	Ethanol from corn, grams	Yield, %
LMC #3	2.5	9.41	77.0
	5.0	9.45	77.3
	10.0	9.68	79.2
LMC #7	2.5	9.28	75.9
	5.0	9.46	77.4
	10.0	9.58	78.3
Drum No. 2c	5.0	9.52	77.7
	10.0	9.77	80.0
Theoretical ethanol yield = 12.20			

E. Comparison of Drum Preparations with Two Commercial Diastase Products and Malt

Two commercial mold-diastase products were compared with two mold preparations made during the present investigation and with two malts. One of the commercial preparations was "Taka-koji" obtained from Takamine Laboratory, Inc., and the other was "Diastase DC" obtained from Röhm and Haas Company, Inc. The reference mold preparations employed were those previously designated as from Drum Nos. 2c and 40a. One sample of malt was obtained in 1938, the other in 1939. The data for the two fermentation series are given in Table 12 and in Table 13. The commercial mold-diastase products are designated as Preparation "A" and Preparation "B". Comparison of amylase activity is based on ethanol yields from the fermentation of the saccharified corn mash.

Moldy bran of Drum No. 2c, Preparation "A" and 1938 malt were compared in one series of fermentations and the results are given in Table 12. Preparation "A" was superior in concentrations of 2.5%, while at the higher concentrations, the material from Drum No. 2c was the most effective saccharifying agent in this group.

TABLE 12

Yields of Ethanol from Corn Mash Saccharified by Moldy Bran from Drum No. 2c, Malt and a Commercial Diastase Preparation.

Amylolytic material	Proportion of amylolytic material, g./100 g. corn	Ethanol from corn, grams
Preparation "A"	2.5	10.83
	5.0	11.37
	10.0	11.82
1938 Malt	2.5	9.42
	5.0	10.51
	10.0	11.21
Drum No. 2c	2.5	10.69
	5.0	11.71
	10.0	12.00

Mold preparations from Drum Nos. 2c and 40a, Preparation "B", 1938 Malt and 1939 Malt were compared as saccharifying agents in a second fermentation series, the data for which are given in Table 13. Preparations from Drum Nos. 2c and 40a gave the highest yields of ethanol. The lowest yield of ethanol was obtained using 1938 Malt.

TABLE 13

Yields of Ethanol from Corn Mash Saccharified by Moldy Bran from Drum Nos. 2c and 40a, 1938 Malt, 1939 Malt and a Commercial Diastase Preparation.

Amylolytic material	Proportion of amylolytic material, g./100 g. corn	Ethanol from corn, grams	Yield, %
Preparation "B"	2.5	6.26	51.2
	5.0	9.09	74.4
Drum No. 2c	2.5	7.63	62.4
	5.0	10.02	82.2
Drum No. 40a	2.5	7.75	63.5
	5.0	10.17	83.3
1938 Malt	5.0	8.51	69.7
1939 Malt	5.0	8.94	73.2

Theoretical ethanol yield = 12.20

F. Comparison of Yeast Strains in the Fermentation of Corn Mashes Saccharified by Various Mold Amylase Preparations.

In this study five high alcohol-producing strains of yeasts were used in the fermentation of corn mashes which had been saccharified by various high amylase preparations. The strains of yeasts employed in the fermentation are designated in this laboratory as follows: Saccharomyces cerevisiae No. 43, Saccharomyces cerevisiae No. 16, Saccharomyces cerevisiae No. 21, Saccharomyces anamensis No. 2, and Schizosaccharomyces pombe No. 35. The moldy bran preparations used in this series were prepared from the following molds: A. oryzae No. 2, A. oryzae No. 40, Rhizopus oryzae No. 14, Rhizopus tritici No. 19, and the unidentified silage mold No. 45.

The amylase preparation from Rhizopus tritici No. 19 gave consistently high yields with all of the strains of yeasts employed. Also, the strain of yeast Saccharomyces cerevisiae No. 43 gave high yields with all of the saccharifying agents employed. The data are given in Table 14.

TABLE 14

Carbon Dioxide Produced by Five Yeast Strains Grown on Corn Mash Saccharified by Five Mold Amylase Preparations.

Amylolytic material	Saccharomyces cerevisiae No. 43 Carbon Dioxide Produced, grams	Yield, %	Saccharomyces cerevisiae No. 16 Carbon Dioxide Produced, grams	Yield, %	Saccharomyces cerevisiae No. 21 Carbon Dioxide Produced, grams	Yield, %	Saccharomyces anamensis No. 2 Carbon Dioxide Produced, grams	Yield, %	Schizosaccharomyces pombe No. 35 Carbon Dioxide Produced, grams	Yield %
No. 2c, (A. oryzae No.2)	10.3	86	9.9	83	10.1	84	9.7	81	10.0	83
No.14a (R oryzae No.14)	10.5	87	9.9	83	10.0	83	10.0	83	9.6	80
No.19a (R tritici No19)	10.3	86	10.1	84	10.0	83	10.2	85	10.2	85
No.40f (A oryzae No.40)	10.2	85	10.1	84	10.0	83	9.9	83	9.9	83
No. 45a (silage mold#45)	9.9	83	9.2	77	9.6	80	8.3	69	9.9	83

Theoretical Carbon Dioxide Yield = 12.0

V SUMMARY AND CONCLUSIONS

1. A high amylase producing mold was isolated from corn silage by the method employing starch agar medium.
2. A preliminary study on distillation indicated that 100 ml. of distillate should be collected in order to obtain the ethanol, quantitatively, from 300 ml. of fermented corn mash, or beer, of such concentration as employed during this investigation.
3. Twenty—one moldy bran preparations were made by growing various molds on moistened wheat bran medium in a rotating drum apparatus. These molds included four strains of A. oryzae, two strains of Rhizopus species, a Mucor, and two unidentified molds.
4. Moldy bran preparations from Drum No. 40a and Drum No. 2c gave the best yields of ethanol in the comparative studies of fifteen drum preparations produced by growing A. oryzae strains on the wheat bran medium.
5. Two strains of Rhizopus species were found to be high amylase producers, being about equal to the reference mold A. oryzae No. 2.
6. The main advantage of growing molds in comparatively large batches in the rotating drum apparatus is to obtain consistent amylase preparations. If consistent

products could be obtained by growing molds in flasks it would facilitate studies on the many variable factors. A simple technique for growing molds on moistened wheat bran medium in Erlenmeyer flasks was devised. It was found that corresponding flask and drum preparations (i.e. preparations made by growing the same mold in special flasks and in the rotating drum apparatus) checked in their saccharifying ability.

7. Two samples of amylase preparations produced by growing A. oryzae No. 2 on wheat bran medium in an aerated aluminum pot were compared with the reference product from Drum No. 2c. The latter, however, gave slightly higher yields upon fermentation of the saccharified corn mash.

8. Amylase preparations from Drum No. 40a and Drum No. 2c and two commercial mold diastase products (Preparation "A" and Preparation "B") were found to be superior to malt as saccharifying agents for the alcoholic fermentation of corn mash.

9. Saccharification and fermentation studies were made using the various combinations of five high amylase preparations and five different yeast strains. The amylase preparation from the mold Rhizopus tritici No. 19 gave high yields (83 to 86% of theory) with all

of the strains of yeasts employed. The best strain of yeast was Saccharomyces cerevisiae No. 43, the yields being from 83 to 87% of theory.

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