juice and final molasses were kindly supplied through B. A. Bourne, United States Sugar Corp., Clewiston, Fla.

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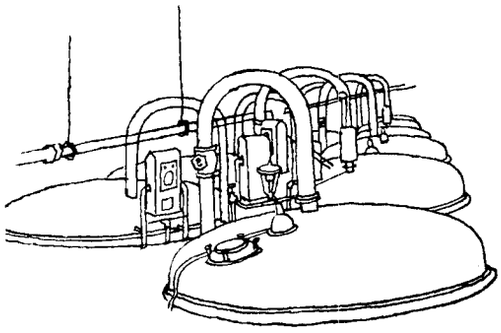

Fermentation and Its Products Have Much to Offer

FUNGAL SACCHARIFYING AGENTS

Amylolytic Factors of Bran Culture and Submerged Culture

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Three saccharifying mold strains have been cultivated on bran and in submerged culture. At optimum levels the alcohol yields from corn mashes saccharified by submerged culture and mold bran from *Aspergillus niger* NRRL 330 were, respectively, 5.44 and 5.38 proof gallons per standard bushel, from *A. niger* NRRL 337 5.28 and 5.30 proof gallons, and from *A. oryzae* ISC 38b 5.13 and 5.28 proof gallons per bushel. Analysis showed submerged and bran cultures of *A. niger* 330 were highest in maltase activity and lowest (very low) in α-amylase. Cultures of *A. oryzae* 38b were highest (very high) in α-amylase and lowest in maltase and limit dextrinase. Cultures of *A. niger* 337 were highest in limit dextrinase and intermediate (high) in α-amylase and maltase. Slight correlation seemed to exist between alcohol yields and maltase activity, but there was no correlation for the other enzymes. However, the optimum level of a saccharifying agent for maximum alcohol yield cannot be predicted from the maltase activity. Although the short fermentation test method of Reese, Fulmer, and Underkofler is applicable for determining the optimum levels of similar fungal saccharifying preparations from the same mold strain, it cannot be used to compare preparations produced by different mold strains nor by the same strain cultivated in different manner.

USE OF FUNGAL saccharifying agents for conversion of starchy mashes for alcoholic fermentation has received much attention during the past two decades. Two successful procedures for cultivating molds to produce active saccharifying agents have been developed. The first was the growth of a selected strain of the mold *Aspergillus oryzae* on moistened wheat bran (*11*). The resulting mold bran may be used in the same manner as malt for conversion of fermentation mashes. More recently amylase-producing molds have been cultivated in submerged culture with vigorous agitation and aeration (*1, 3, 6*).

The media employed for submerged culture have generally been mixtures of thin stillage or distillers' dried solubles and corn, and in most cases the culture employed has been *Aspergillus niger* 337 of the Northern Regional Research Laboratory. The submerged culture preparation is utilized by mixing approxi-

mately 10% by volume with the cooked grain mash to accomplish the saccharification. Tests on both laboratory and commercial scale have conclusively demonstrated that use of either mold bran or submerged fungal culture results in somewhat better alcohol yields than from conventional malt-saccharified mashes (*10*, *12*).

In the present investigation the effectiveness of saccharification was compared, as measured by alcohol yields from corn mashes, by both bran cultures and submerged cultures of selected mold strains. The cultures employed were *A. oryzae* ISC 38b, an organism that has been employed in commercial production of mold bran (*11*), *A. niger* NRRL 337, the organism employed in commercial scale tests of the submerged culture saccharification procedure (*5*, *12*), and *A. niger* NRRL 330, reported to produce high maltase activity (*3*, *9*).

Experimental Methods

Bran cultures of molds were prepared by the method of Hao, Fulmer, and Underkofler (*4*) by growing the molds in perforated aluminum pots with forced aeration. The resulting mold brans were dried and stored in glass bottles until required for use.

Submerged cultures were prepared by shake-flask culture of the molds upon media containing distillers' dried solubles and corn meal. The ingredients were mixed with tap water in the required proportions in flasks. Usually 150 ml. of medium in 500-ml. Erlenmeyer flasks or 75 ml. of medium in 250-ml. Erlenmeyer flasks were employed. The flasks were stoppered with cotton plugs and sterilized at 15 pounds' steam pressure for 15 minutes. After cooling, the medium in each flask was adjusted to the desired pH by means of concentrated sodium hydroxide or sulfuric acid solutions, employing a glass electrode pH meter. The media were then inoculated with the molds and incubated at 30° C. in a reciprocal shaker.

The mold cultures employed as inoculum for both bran and submerged cultures were dried spore cultures on bran medium prepared as described by Underkofler, Severson, Goering, and Christensen (*11*). For inoculum for mold bran preparation the dried spore culture was mixed with the sterilized bran. For shake-flask cultures, dry spores were poured from the bran spore culture flasks into the flasks of sterile medium, or the spores were suspended by mixing the bran culture with sterile 1% saline solution and inoculation was made with the spore suspension by means of a sterile pipet. In some cases serial mycelium transfers were made with submerged cultures. The submerged culture from spore inoculation was incubated for 24 hours and used to inoculate fresh sterile medium which, after incubation for 24 hours, was employed to inoculate the final flasks of medium for submerged culture. No differences in saccharifying effectiveness were observed for submerged cultures inoculated directly from spore cultures or by mycelium transfer. The submerged culture preparations were incubated for 48 hours, and employed immediately after this incubation period.

When each preparation was employed for saccharifying corn mashes, a series of fermentations was run in duplicate using a range of concentrations of the saccharifying agent added to the cooked corn mashes in order to determine the optimum level. The fermentations were carried out in 500-ml. Erlenmeyer flasks equipped with test-tube water traps to decrease the loss of alcohol. In each flask were placed 50 grams of corn meal and 125 ml. of 0.06 *N* sulfuric acid, and the mixtures were cooked for 60 minutes at 20 pounds' steam pressure. The desired amount of saccharifying agent was mixed with tap water to make 125 ml. of slurry at 20° to 25° C. After completion of the cooking period, the autoclave was blown down rapidly to atmospheric pressure, and the flasks were steamed continuously in the autoclave at atmospheric pressure until their removal, one at a time, for saccharification.

To the hot mash was added 0.4 gram of calcium carbonate to bring the pH to approximately 5.0. The cold slurry of the saccharifying agent was then added quickly with vigorous mechanical stirring, resulting in a mash temperature of about 55° C. The mash was then cooled to 30° C. by placing the flask in a cold water bath. When all the flasks in the series had been saccharified and cooled, the mashes were inoculated with 8 to 10 ml. of an active 24-hour culture in malt extract medium of the yeast *Saccharomyces cerevisiae* NRRL 567. The flasks were swirled to distribute the yeast throughout the mash, the stoppers with traps were added, and the flasks were incubated at 30° C. for 72 hours. The fermented mashes were then distilled and alcohol contents of the distillates determined by specific gravity measurements. For comparative purposes the alcohol yields were calculated as proof gallons of alcohol per standard bushel, defined as 56 pounds of total substance of 12% moisture content. The total substance includes grain, saccharifying agent, and inoculum.

Table I. Alcohol Yields from Different Saccharification Levels of Submerged Culture of *A. Niger* 330

Mold Culture, Vol. %	Dry Matter in Saccharifying Volume, G.	Total Dry Matter, G.	Total Alcohol, G.	Alcohol Yield, PG/Bu.
5	1.07	44.38	15.36	5.16
10	2.14	45.45	16.61	5.44
15	3.21	46.52	16.86	5.40
20	4.28	47.59	16.96	5.31
25	5.35	48.66	17.02	5.21

Results and Discussion

Representative data for a typical fermentation series are shown in Table I. From the results of this series it was concluded that the optimum level of the submerged culture of *A. niger* 330 in this experiment was 10% of the volume of the mash. Because in the volume of mash used, 2.14 grams of dry matter was supplied by the saccharifying agent, the optimum level for this sample was $\frac{2.14}{45.45} \times 100 = 4.72\%$, dry basis. Actually, this is the optimun for the 5% increments employed; narrower increments would be necessary to determine the true optimum. The decreases in yield for the higher levels of conversion agent shown in Table I are due to the larger percentages of unfermentable mold solids.

In the first submerged fungal culture preparations the medium recommended by Adams, Balankura, Andreasen, and Stark (*1*), containing 5% distillers' dried solubles (DDS), 1% corn, and 0.8% calcium carbonate was employed. Later, after Tsuchiya, Corman, and Koepsell (*9*) showed that the presence of calcium carbonate in the medium decreased production of maltase and α-amylase by strains of *A. niger*, the medium recommended by these authors for optimum enzyme production, containing 5% distillers' dried solubles and 5% corn, was used. Results shown in Table II indicate that when optimum amounts of the saccharifying agents were employed there were essentially no differences in alcohol yields with mold cultures grown on either medium. Alcohol yields with submerged cultures of *A. oryzae* on these media were very low.

As *A. oryzae* 38b is an excellent culture for mold bran production, attempts were made to improve the saccharifying ability of submerged cultures of this mold by changing the growth medium. Proportions of distillers' dried solubles and corn were varied to change the initial and final pH levels of the media, and all the resulting submerged cultures were tested for saccharifying ability as measured by alcohol yields. The optimum medium contained 2.82% distillers' dried solubles and 2.62% corn, with initial pH adjusted to 5.2. Submerged culture of *A. oryzae* on this medium gave an alcohol yield of 5.13 proof gallons per standard bushel at the optimum level (Table II).

The mold brans prepared from each of

Table II. Alcohol Yields from Corn Mashes Saccharified by Submerged Mold Cultures

Culture	Mold Medium	Alcohol Yield, PG/Bu.
A. niger 337	5% DDS 1% corn 0.8% $CaCO_3$	5.30
A. niger 337	5% DDS 5% corn	5.28
A. niger 330	5% DDS 5% corn	5.44
A. oryzae 38b	5% DDS 1% corn 0.8% $CaCO_3$	4.80
A. oryzae 38b	5% DDS 5% corn	4.78
A. oryzae 38b	2.82% DDS 2.62% corn	5.13

the mold cultures were employed at various levels for the saccharification of fermentation mashes. The results for the optimum level of each are shown in Table III, in which are also given the results with optimum levels of submerged culture of each mold. These data permit a comparison of alcohol yields obtained from corn mashes saccharified by submerged cultures and mold brans of the several mold strains. *A. niger* 330 gave the highest alcohol yields, the submerged culture being slightly superior to the mold bran. Yields with *A. niger* 337 were the same for submerged culture and bran preparations, and essentially the same as for mold bran from *A. oryzae* 38b. The *A. oryzae* is definitely inferior for submerged culture preparations.

The differences in performance of fungal cultures in saccharifying fermentation mashes must be due to differences in enzyme content or ratio of enzymes present. In each preparation therefore α-amylase, maltase, and limit dextrinase were determined. α-Amylase was determined by the method of Sandstedt, Kneen, and Blish (*8*) at 30° C., maltase by the method of Tsuchiya, Corman, and Koepsell (*9*), and limit dextrinase by the procedure of Back, Stark, and Scalf (*2*). The results are shown in Table IV, expressed in the arbitrary units, defined by the respective authors of the analytical procedures, per gram of dry matter.

Both the submerged and bran cultures of *A. niger* 330 were definitely highest in maltase activity and lowest (very low indeed) in α-amylase activity. Cultures of *A. oryzae* 38b were highest (very high) in α-amylase activity and lowest in maltase and limit dextrinase activity. The cultures of *A. niger* 337 were highest in limit dextrinase activity and possessed good maltase and α-amylase activities, intermediate between those for the other two mold cultures.

It is apparent from Table IV that there is no correlation between α-amylase activity and alcohol yield or between limit dextrinase and alcohol yield. When the two classes of saccharifying preparations are considered separately—all submerged cultures or all mold brans—there seems to be a slight positive correlation between maltase activity and alcohol yield.

However, if the comparison is made upon the basis of total enzyme present in the mashes, even this correlation disappears. The product of the optimal level of saccharifying agent and enzyme activity per gram is proportional to the total amount of enzyme added to the mashes, representing the units of enzyme per 100 grams of total dry matter in the mash. In Table V are given data for total enzymes added to the mash for each saccharifying agent at the optimum levels. There is no apparent correlation between alcohol yields and the total enzyme present for any of the three enzymes tested. It may be possible to say, in a qualitative way, that fungal cultures producing high maltase activity are superior for saccharifying fermentation grain mashes, but the measure of the maltase content does not permit prediction of optimum level of preparation that will give maximum alcohol yields.

The lack of correlation between α-amylase and yield reported here confirms the findings of LeMense *et al.* (*5*), although they show a correlation between maltase and yield.

In the use of any saccharifying agent the problem is always presented as to the optimum level for use to achieve maximum alcohol yield. Efforts to measure saccharifying potency by chemical tests for malt as well as fungal agents have led all investigators to conclude that the only entirely reliable method is by actual fermentation tests. Such tests are time-consuming, and most distillers simply use an amount considered to be in excess of the requirement. As saccharifying agents are more costly than grain and the fungal agents contribute little if any alcohol upon fermentation, this procedure is costly and wasteful.

For mold brans prepared with *A. oryzae* 38b, Reese, Fulmer, and Underkofler (*7*) published in 1948 a procedure for a short 24-hour starch fermentation test which permitted evaluation of optimum level of each mold bran preparation to obtain maximum alcohol yields. This method is based upon the discovery that when the ratios of the weight of mold bran employed per weight of alcohol produced are plotted against weight of mold bran used, parallel straight lines are obtained, the intercept values of which are proportional to the required levels of the mold brans.

It was noted in the present investigation that when the fermentation results from the series of fermentations employing the three different mold bran preparations were plotted in the manner of Reese *et al.*, straight lines were obtained, and all coincided as shown in Figure 1. Data from the submerged cultures are plotted in Figure 2. The lines are straight and essentially parallel for *A.*

Table III. Alcohol Yields from Corn Mashes Saccharified by Optimum Levels of Mold Preparations

Culture	Preparation	Alcohol Yield, PG/Bu.
A. niger 337	Submerged	5.30
	Bran	5.30
A. niger 330	Submerged	5.44
	Bran	5.38
A. oryzae	Submerged	5.13
	Bran	5.29
Malt control		5.20

Table IV. Enzyme Activities of Fungal Preparations

Preparation and Culture	Alcohol Yield, PG/Bu.	α-Amylase, Units/G.	Maltase, Units/G.	Limit Dextrinase, Units/G.
Submerged				
330	5.44	21	70	61
337	5.28	192	59	123
38b	5.13	259	41	59
Bran				
330	5.38	25	129	107
337	5.30	113	89	110
38b	5.28	230	82	89

Table V. Total Amounts of Enzymes in Fermentation Mashes

Preparation and Culture	Optimal Level, %	Alcohol Yield, PG/Bu.	Units of Enzyme per 100 G. Total Dry Matter in Mashes		
			α-amylase	Maltase	Limit dextrinase
Submerged					
330	4.72	5.44	98	332	286
337	6.89	5.28	1315	407	848
38b	7.26	5.13	1880	299	426
Bran					
330	3.62	5.38	90	467	386
337	3.67	5.30	415	325	402
38b	4.55	5.28	1047	371	406

oryzae 38b and *A. niger* 330, but not quite parallel for *A. niger* 337.

It was of interest, therefore, to ascertain whether the short fermentation test method could be employed for comparative evaluation of fungal preparations from different molds or of the same mold cultivated on bran or submerged. The 24-hour starch fermentation tests employing different levels of the saccharifying agents were run exactly in accordance with the method of Reese, Fulmer, and Underkofler (7). The data obtained are plotted in Figure 3. The upper line for *A. niger* 330 in submerged culture is off the range of the graph and has been transposed downward (actual intercept 0.298) to illustrate that the line obtained is parallel to that from the mold bran of the same culture. The fact that the data for *A. niger* 330 preparations gave lines of a different slope than those for the other molds suggests that starch saccharification by *A. niger* 330 may be due to different enzymes or at least different ratio of the enzymes present. Actual 72-hour fermentations of corn mashes using fungal preparations of *A. niger* 330 had shown the highest efficiency.

Reese, Fulmer, and Underkofler (7) had found that the graphical intercepts for their mold brans were proportional to the optimal level of saccharifying agent for maximum alcohol production. The intercepts obtained from the graphs of Figure 3 are shown in Table VI. Examination of the data leads to the conclusion that in this series of short starch fermentations there is no correlation between the intercept values and the optimal levels for maximum alcohol production. This applies to all comparisons—that is, over-all, within a group such as all submerged cultures or all mold brans, or between different types of preparation from the same mold.

The problem as to why, in the short starch fermentation tests, the slopes of the lines for *A. niger* 330 are different, and why the intercepts are high is worthy of some speculation. According to the results of Reese *et al.*, a high intercept value in the short starch fermentation test should indicate that the amylolytic agent is inefficient. However, actual fermentations of corn mashes proved preparations of *A. niger* 330 to be very efficient. Identical lines were obtained for 72-hour corn fermentations with all of the mold brans, as shown in Figure 1. When the mold preparations are grouped according to enzyme activity ratios, *A. niger* 330 is found to be very different from the other preparations used in this investigation, having very low α-amylase activity. The combined actions of a group of enzymes upon a substrate must certainly vary as the ratio of the individual enzymes within the group varies. Therefore, since the short starch fermentation test really depends upon the action of the combined enzymes on starch, the different slope must be due to the different enzyme ratio.

It is possible that the method of Reese, Fulmer, and Underkofler may be used for comparative evaluation of preparations of the same type from one mold. These authors showed this to be true for mold brans prepared with *A. oryzae* 38b. This possibility still requires checking for other preparations. However, for comparison and evaluation of fungal preparations of different types and from different molds, the usual laboratory fermentation method must be employed.

Table VI. Comparison of Alcohol Yields, Optimal Levels, and Intercept Values for Fungal Preparations

Culture and Preparation	Alcohol Yield, PG/Bu.	Optimal Level, %	Intercept Value
330 sub.	5.44	4.72	0.289
330 bran	5.38	3.62	0.110
337 sub.	5.28	6.89	0.0202
337 bran	5.30	3.57	0.0202
38b sub.	5.13	7.26	0.0150
38b bran	5.28	4.55	0.0108

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Figure 1. Curves for 72-hour fermentations of corn with varying levels of mold brans

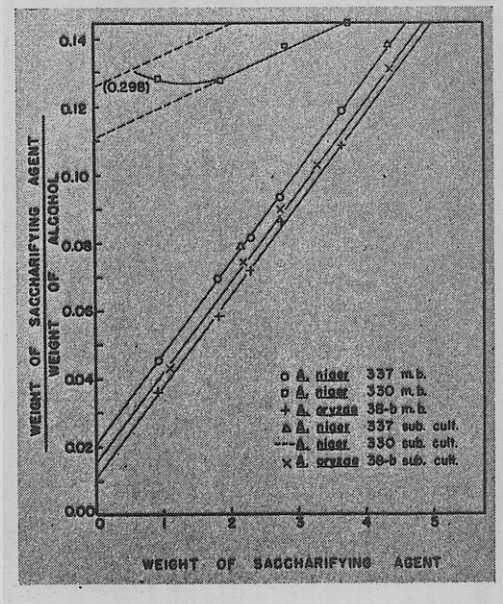

Figure 2. Curves for 72-hour fermentations of corn with varying levels of submerged fungal cultures

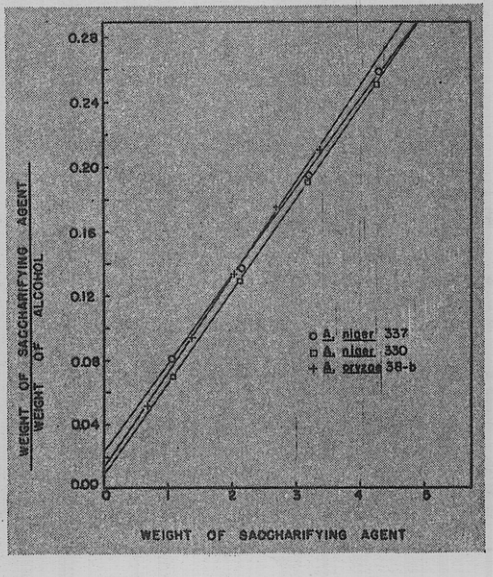

Figure 3. Curves for 24-hour starch fermentation tests with varying levels of fungal saccharifying agents