Proceedings of the Iowa Academy of Science

Volume 48 | Annual Issue Article 39

1941

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SACCHARIFICATION OF STARCHY GRAIN MASHES FOR ETHANOL FERMENTATION.

USE OF MOLD-AMYLASE PREPARATIONS.

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Ethyl alcohol has been listed by scientists as one of the six most useful substances; in fact it has been said that, next to water, ethanol is the most useful chemical compound known to mankind. At the present time approximately 85 per cent of all industrial alcohol is made by fermentation, the balance by synthesis from ethylene derived from the cracking of petroleum. Of the fermentation alcohol about nine-tenths is produced from blackstrap molasses, one-tenth from grains. However, by-product molasses is limited in amount and there is at the present time virtually no surplus. This is shown by the statistics of molasses production (4) and by the fact that the price of molasses has recently risen from 5c to 7c per gallon. It is likely to rise still higher as alcohol production expands to meet the needs of defense industries. Hence, other raw materials will become increasingly important. Of these, corn is the only one now available in adequate quantities in the United States. The present price of molasses at 7c per gallon is equivalent to about 60c per bushel for corn as an alcohol source.

The overall chemistry of alcoholic fermentation is quite simple. The zymase system of yeast converts fermentable sugars to ethanol and carbon dioxide in equimolar ratio. However, before yeasts can act upon starch this carbohydrate must first be hydrolyzed. The starch may be saccharified by means of dilute mineral acids or by the enzyme called diastase or amylase. This saccharification makes maltose or dextrose available to the yeasts.

Malt, which is sprouted barley, is universally employed in this country for the saccharification of starchy fermentation mashes. There are several disadvantages to the industrial use of malt, but as yet a better method of of saccharification has not been introduced into practice. The disadvantages are: (1) The high cost of the malt; (2) the time and careful control required; (3) the relatively low yields of alcohol obtained; and (4) the threatened shortage of supply of suitable malting barley. In view of the deficiencies of the malting procedure, other more efficient saccharifying methods are being sought in our laboratories.

In the orient fungi have been employed as a source of amylase for alcoholic fermentations for centuries. Takamine (5) deserves chief credit for the introduction into the United States of commercial fungal enzyme preparations. He suggested the use of mold products to replace malt in the fermentation industry, but in spite of successful large-scale trials in 1913 his suggestion has not found favor due to the production of slight off-flavors or odors in the alcohol. For manufacture of industrial alcohol this would not be an important factor, and recently new reports (6) have come from our laboratories suggesting the use of mold preparations for saccharifying starchy fermentation mashes.

EXPERIMENTAL

In the course of the present investigation mold-amylase preparations were obtained by growing several strains of molds on various substrates. The molds were grown in the manner described by Underkofler, Fulmer and Schoene (6). Several fibrous materials were tried as substrates for the growth of a strain of the mold *Aspergillus oryzae,* including wheat bran, corn bran, oat hulls, cottonseed hulls, corn cobs, sawdust, peanut hulls, and rice hulls. Only wheat bran and dry-milled corn bran supported the growth of the mold adequately although some growth was also obtained with oat hulls and cottonseed hulls when additional nutrients were added to the mixtures. Since wheat bran was the best substrate, most of the mold-amylase preparations were produced by growing the molds on this material. The dried products, hereafter referred to as "mold-brans," were ground in a Wiley mill and stored in stoppered bottles until used. The several mold strains used, with their sources, are listed in Table I.

A comparison was made of the effectiveness of the various mold- amylase preparations for saccharifying fermentation mashes. Two commercial mold-diastase products were included in these tests as well as samples of malt for controls. The effectiveness of the materials was measured by using them to saccharify corn mashes and then determining the ethanol formed on fermenting the saccharified mashes with yeast. The procedure for conducting these fermentation tests was as follows: In a 500-ml. Erlenmeyer flask 32 g. of corn meal were thoroughly mixed with .225 ml. of distilled water, the starch was gelatinized by heating over a burner, and the mixture cooled to about 60° C. To bring about preliminary liquefaction of the starch an aqueous suspension containing 0.16 g. of mold-bran was added and the mixture allowed

to stand for 30 min.; at the end of this period the mash was sufficiently liquid to mix easily by shaking. The mash was then cooked in the autoclave for 30 min. at 20 lbs. steam pressure. The mash was cooled at 60° C. and maintained at this temperature by placing the flask in a constant-temperature water bath. The mold-amylase preparation to be tested (the amount used in each case was 5 per cent of the weight of corn meal) was weighed into a small beaker containing a little distilled water. The thick slurry resulting was added quantitatively to the flask of mash with the aid of a little water from a wash bottle. After saccharification at 60° C. for an hour the mash was cooled to 30° and then inoculated with 20 ml. of an active yeast culture in 10 per cent beer wort. All mashes were prepared in duplicate, and a series of a dozen or more fermentations was conducted simultaneously. After incubation for 72 hours at 30° C. the fermentations were distilled, the distillates collected in 100-ml. volumetric flasks, and the alcohol yields determined by measuring the specific gravities with a Westphal balance. The alcohol yields were calculated as per cent of the theoretical yield on the basis of the starch present.

Typical comparative data for the mold-amylase preparations tested are presented in Tables I and II. It is quite apparent that the mold-amylase preparations are definitely superior to malt in their ability to saccharify starch for fermentation, under the conditions of test employed. Two strains of *A. oryzae,* the two *Rhizopus* species, and one of the commercial mold-diastase products were especially effective as measured by the alcohol yields, and gave approximately 10 per cent higher yields of ethanol than did malt. Using the various combinations of five mold-amylase preparations and five different yeast strains some variation in effectiveness of saccharification or of fermentation was found as shown in Table II. In general yeast No. 43, which is the stock culture employed for most of the work on alcoholic fermentations reported from our laboratories, gave the best results.

Obviously, the method of comparing the saccharifying ability of different amylase preparations by running fermentation tests is very laborious and time consuming. It is customary to compare the saccharifying power of different malts on the basis of chemical analyses of degrees Lintner. In general, the Lintner number represents the number of grams of a standard starch saccharified under carefully controlled and standardized conditions by one

gram of the malt. An attempt was made to compare the saccharifying powers of malt and of mold-amylase products on the basis of Lintner values.

Since the official method (1) of determining the Lintner values is very time consuming, the rapid electrometric method recently developed by Burkhart (3) was employed in this work. The malt and mold-bran infusions, and the buffered starch solution were prepared according to the official method of the American Society of Brewing Chemists (1). The actual diastasis was carried out in the manner of Anderson and Sallans (2). The reducing power of the saccharified starch mixture was determined by Burkhart's (3) method; this involved measurement at 25° C. of the potential difference between a reference electrode containing 10 ml. of ferricyanide reagent with 5 ml. of water and an electrode containing 10 ml. of ferricyanide reagent which had been heated with 5 ml. of the digested starch mixture in a boiling-water bath for exactly 20 min. The reading in millivolts for the digested sample minus the reading of a starch blank which had been treated in the same manner gave the E. M. F. value which was substituted into Burkhart's equation

$$Y = 2.734X - 25.49,$$

where Y represents the degrees Lintner and X is the E. M. F. Burkhart has shown that this equation gives values for malts of varying diastatic activity which compare favorably with those obtained by the official method of determining Lintner values. Fermentation tests were also run, in the manner described above, using the same mold preparations which were analyzed for Lintner values. The results of these experiments are presented in Table III. The values given represent duplicate determinations in all cases. Several preparations were made at different times with each mold strain employed so that the results obtained could be attributed to the mold in question, and not to either good or poor experimental conditions.

From the data it can be concluded that the Burkhart method for determining Lintner values is satisfactory for malt, but that such Lintner values are meaningless with regard to mold-amylase preparations. Although the malt gave a much higher Lintner value than did any of the mold products, less alcohol was produced than with many of the mold preparations. The negative Lintner values obtained with some of the mold materials is due to the fact that the potential obtained depends directly on the amount of reducing sugar produced during diastasis, which is much small-

Table I. Ethanol Yields from Corn Mash Saccharified by Means of Several Mold-Bran Preparations

Lab. No.	Mold Species	Source	Ethanol Yield, % of Theory	
			Expt. 1	Expt. 2
2	*Aspergillus oryzae*	A.T.C.C.,[a] No. 4184	(82.2 (84.9	90.2
38	*Aspergillus oryzae*	Röhm and Haas, No. 38	80.8	——
40	*Aspergillus oryzae*	Röhm and Haas, No. 40	83.3	——
42	*Aspergillus oryzae*	Röhm and Haas, No. 42	78.0	——
14	*Rhizopus oryzae*	Lockwood,[b] No. 649	83.3	86.9
19	*Rhizopus tritici*	Lockwood, No. 654	84.1	86.9
21	*Mucor javanicus*	Lockwood, No. 718	80.0	82.8
22	Yellow mold	Isolated from oat hulls	72.9	——
45	Yellow mold	Isolated from silage	80.7	——
	Comml. mold preparation A		——	88.6
	Comml. mold preparation B		——	82.1
	Malt (control)		——	73.2

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

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

Table II. Ethanol Yields with Five Yeast Cultures from Corn Mash Saccharified by Means of Five Mold-Bran Preparations.

Mold Species	Ethanol Yield % of Theory, from the Yeasts				
	No. 43[a]	No. 16[b]	No. 21[c]	No. 2[d]	No. 35[e]
A. oryzae No. 2	86	83	84	81	83
R. oryzae No. 14	87	83	83	83	80
R. tritici No. 19	86	84	83	85	85
A. oryzae No. 40	85	84	83	83	83
Mold No. 45	83	77	80	69	83

a *Saccharomyces cerevisiae* No. 43
b *Saccharomyces cerevisiae* No. 16
c *Saccharomyces cerevisiae* No. 21
d *Saccharomyces anamensis* No. 2
e *Schizosaccharomyces pombe* No. 35

Table III. Amyloytic Activity of Several Materials As Measured by Lintner Values and by Ethanol Yields from Corn Mash Fermentations.

Material	Degrees Lintner (avg. of 2 detns.)	Ethanol Yield, % of Theory
	107.1	
Malt	107.1	68.4
	105.7	
Comm'l. mold-diastase prepn. A.	78.4	
Comm'l mold-diastase prepn. B.	22.4	
Mold No. 2 on wheat bran	46.7	77.6
	41.5	78.2
	45.6	82.2
Mold No. 2 on corn bran	− 8.4	72.9
	−11.8	70.0
Mold No. 2 on oat hulls	1.9	61.5
	− 3.6	63.2
	−10.5	62.7
Mold No. 2 on cottonseed hulls	−13.2	51.5
	−23.4	51.5
Mold No. 22 on wheat bran	−15.2	64.8
	−15.9	72.9
Mold No. 38 on wheat bran	15.6	78.5
	27.8	80.8
Mold No. 40 on wheat bran	47.0	82.3
	66.1	83.3
Mold No. 42 on wheat bran	25.8	73.9
	51.1	78.0

er in the case of mold-amylase than for malt-amylase. Hence, the potential is so small that the Burkhart equation gives negative values. An attempt was made to vary the time of diastasis and thus improve the results, but this proved entirely unsatisfactory.

An explanation of the reason that mold preparations show greater saccharifying power with regard to alcohol production than do malts, even though the Lintner values would indicate that the opposite should be true, probably lies in the fact that equilibrium is reached by saccharification with mold-amylase at lower sugar concentrations than in saccharification with malt-amylase. However, the mold-amylase has greater activity at fermentation temperature than does the malt-amylase. Hence, as the fermentable sugars are utilized by the yeast, eventually the total starch present in the mash is more completely saccharified during the course of fermentation with mold-amylase than with malt-amylase.

It may be concluded that Lintner determinations give poor indications of the ability of mold preparations to saccharify starch for fermentation. Fermentation tests still must be relied upon for accurate information in comparing the amylolytic activity of various materials from different sources.

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