Enzymatic conversion of barley carbohydrate into syrup
by Martin J Houle

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University

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By using a portion of the beta amylase naturally present in barley, good syrup yields can be obtained with as little as 0.5% of a wheat bran culture of A. oryzae or by the use of 0.1% commercial enzyme. Preliminary results indicate enzymatically converted barley syrup should compete favorably with corn syrup in Montana.

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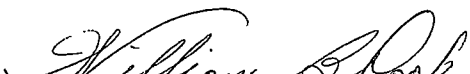

Head, Major Department

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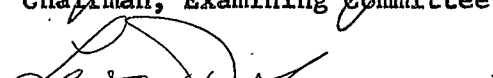

Dean, Graduate Division

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I ABSTRACT

Various combinations of amylolytic enzymes have been used as a means for the conversion of whole barley into syrup. The hydrolysis products have been determined quantitatively and indicate that the sugar composition of the syrup can be varied over wide limits by the proper selection of enzymes and variation of conversion time.

By using a portion of the beta amylase naturally present in barley, good syrup yields can be obtained with as little as 0.5% of a wheat bran culture of A. oryzae or by the use of 0.1% commercial enzyme. Preliminary results indicate enzymatically converted barley syrup should compete favorably with corn syrup in Montana.

II INTRODUCTION

The production of syrup from the carbohydrate fractions of various grains, tubers and fruits, has been periodically investigated for the last 150 years. In 1811, the Russian chemist, Kirchoff, reported the basis for a commercial method of production of starch syrup and crude starch sugars (1). His discovery was accidental for he was not after sugar or syrup but gum arabic for use in porcelain manufacturing. When he subjected his mixture to the action of sulfuric acid for too long a period of time, he obtained a syrup. A short time later, Proust, found a way to produce a sugary substance from grapes (2). The French chemist, de Saussure, determined that both were identical hydrolysis reactions (3). The sugar obtained from these two methods was glucose. A considerable amount of chemical research was concentrated in this field at this time because of a 100,000 franc reward offered by Napoleon I to the first individual to produce sugar in quantity from native plants (4). France was at war with England and her sugar imports had been cut off. However, this young industry collapsed in 1814 when the war was concluded.

The use of starch for syrup and sugar production was introduced into the United States in 1842 but it was not an important industry until 1857. The acid hydrolysis procedure was used as described by earlier investigators. In 1939, Langlois found that the use of molydenum as a catalyst increased the rate of acid hydrolysis (5). The following year Dale and Langlois introduced the use of enzymes with neutralized acid hydrolyzate. This combination further improved the yield of syrup and reduced the time necessary to complete the reaction (6). This process is being used today

for the production of certain kinds of corn syrup. This was not the first time that enzymes had been used in the sugar industry. In 1928, Weichherz used malt to make a high maltose syrup. In order to achieve good conversions, it was found necessary to use high concentration of malt which made the method impractical. In 1944, Baker was able to produce crystalline maltose by using malt with partially acid hydrolyzed starch (7).

The restriction on wheat acreage in the last decade has resulted in increased barley production in Montana because this crop was best suited for these diverted acres. In 1958, Montana produced 48,000,000 bushels of barley on 1,618,000 acres, but was only able to market a fraction of this through the normal grain markets. Montana ranked third in the nation in barley production but last in marketing (8). Due to this limited outlet, a large surplus has accumulated in this state. Nationally, a large amount of barley is used in the malting industry. However, since the major part of the malting industry is located in Minnesota and Wisconsin, its barley is obtained primarily from North Dakota and South Dakota. These two states enjoy a definite freight rate advantage over Montana. The limited usage of Montana barley for malt production has forced most of the barley grown here to be fed directly to livestock, stored as government surplus, or shipped out for feed.

Due to the fact that Montana grain prices are determined by the price at Minneapolis, less freight, Montana barley offers one of the least expensive sources of carbohydrate in the United States.

In searching for outlets to utilize this surplus grain, it appeared

that the conversion into syrup offered the greatest potential since a vast market for this material is available in the Pacific Coast States. It is used for the production of glazed fruit, candy and ice cream as well as for the packing of berries, fruits and for the fortification of wine. In order to compete with corn which is now supplying the syrup demand it would be necessary to have either a less expensive material or one which offered some advantage over corn syrup.

Since the general procedure for making corn syrup involves the acid hydrolysis of corn starch, Goering and Imsande investigated the possibility of producing barley starch for this purpose (9). Although high quality starch was obtained, the yield and processing difficulties did not make this process look too attractive. Therefore the acid hydrolysis of barley starch did not appear practical.

Work by Goering indicated that acid hydrolysis of whole grain gave unsatisfactory yields of sugars and off flavors. Even if flavor difficulties were not encountered, the low sugar yields would make this method impractical (10).

Since barley contains substantial amounts of beta-amylase, it was considered possible to produce a syrup competitive with corn syrup by utilizing this natural enzyme source in combination with other commercial enzymes for the conversion of whole grain. Furthermore, it should be possible to obtain a wide variation in sugar composition by proper selection and variation of the enzyme sources. Recently there has been an increased interest in high maltose syrup because it is less hygroscopic than is glucose and thus better suited for producing hard candy (11).

Since maltose sugar can not be made by acid hydrolysis which yields primarily glucose, the possibility of making such a syrup by direct enzyme conversion seemed worthy of investigation.

If either an economical method of enzyme conversion is found which will allow for direct competition with acid hydrolyzed corn starch, or if an effective procedure is developed for production of maltose syrup, large quantities of Montana barley possibly could be used for this purpose.

III EXPERIMENTAL

The barley varieties used throughout this investigation were of known history and produced by the Agronomy Department at Montana State College. Betzes and Compana varieties were used because of their availability in Montana.

The barley was ground in a laboratory Wiley mill and thoroughly mixed. Whole grain was used instead of flour for reasons of economy.

Both barley varieties were investigated for their proximate feed value and carbohydrate content. The moisture, crude fiber, protein and fat were determined by the official A.O.A.C. methods (12, 13, 14, 15). Total carbohydrate was determined by refluxing with HCl as outlined in Cereal Laboratory Methods (16). The sugar content of the acid hydrolyzate was determined by the colorimetric method of Dubois et.al. and read at 490 mμ in a Beckman Model B Spectrophotometer (17). A standard glucose curve was made by making appropriate dilutions from a glucose solution containing 1000 μg./ml. of the sugar. The readings obtained from the acid hydrolyzate were compared to this curve. The percent of glucose was calculated using the following formula;

$$\frac{A \times \text{dilution factor} \times B \times 100}{\text{Sample weight}} = \% \text{ carbohydrate}$$

Where A = ug. of glucose obtained from the standard curve
B = Total volume of the acid hydrolyzate.

Both strains of barley were checked for beta-amylase contents in the following manner. Exactly 1 gram of barley was added to 100 ml. of distilled water containing 1 gram of papain and the mixture allowed to stand for 3 hours at 20° C. with occasional mixing. It was then filtered, the

filtrate being referred to as a barley infusion. The papain releases bound beta-amylase from the protein moiety and in this way total beta-amylase can be determined. A standard soluble starch solution at a pH of 4.5 was made up by dissolving 20 grams of starch in 100 ml. of boiling water, boiling for 2 minutes, adding 20 ml. of standard acetate buffer and making up to 1 liter. One ml. of barley infusion was thoroughly mixed with 200 ml. of starch solution in a 250 ml. volumetric flask and incubated for exactly 30 minutes at 20° C. At the end of this time 20 ml. of 0.5 N NaOH was rapidly added with thorough mixing and the total volume made up to 250 ml. A blank was made up in the same manner except 0.5 N NaOH was added before the addition of barley infusion. The reducing sugar released in this reaction was determined by the colorimetric method of Bernfeld and read on a Beckman Model B Spectrophotometer at 540 mμ (18). Because the addition of papain would be impractical in the commercial preparation of syrup, a water infusion was made in a similar manner except papain was not added. The activity of the beta-amylase was expressed as degree of Linter (°L) which is an expression of maltose equivalent. By definition, a maltose equivalent is the number of grams of reducing sugar, calculated as maltose, that is produced by 100 grams of malt under certain standard conditions (19). The following formula was used for its calculation;

$$\frac{\mu g \text{ reducing sugar} \times 25{,}000 \text{ ml.}}{\text{Sample weight}} = \mu g \text{ maltose/g barley}$$

$$\frac{\mu g \text{ maltose} \times 100 \text{ g malt}}{1{,}000{,}000} = \text{grams maltose}$$

$$\text{grams maltose}/4 = L$$

In addition to water infusion, the other beta-amylase sources used

were malt and Wiszyme, both produced by the Wisconsin Malting Company. The activity of these two enzymes were determined as described above. All enzyme preparations except the water infusions were checked for alpha-amylase activity by a modified Wohlgemuth method (20). A bacterial alpha-amylase source called HT-44, which has considerable resistance to heat, was used as a starch liquifier. This enzyme is produced by the Takamine Division of Miles Chemical Company.

Two laboratory cultures, Aspergillus oryzae and Aspergillus niger were grown on wheat bran using the method devised by Hao et al (21). In addition to the above analysis these enzymes were analyzed for maltase activity using the procedure of Sumner (19).

Three other commercial enzymes prepared by Rohm and Haas Company were also used and compared to the previously mentioned commercial enzymes.

Barley was "mashed" as follows; 25 g. finely ground whole barley was mixed with 225 ml. water in a 1000 ml. graduated Erlenmeyer flask. Varying amounts of the liquifying enzyme, HT-44, were added to this mixture and heated to 75-80° C., held at this temperature for one or two hours and then autoclaved for 30 minutes at 15 p.s.i. The mash was cooled to 55° C. and varying amounts of the enzyme were added. The mixture was placed in a constant temperature bath and held at 55° C. $\pm$ 1° C. for 16 to 18 hours. After conversion the mash was autoclaved for 30 minutes at 15 p.s.i., centrifuged, suspended in an equal amount of water and centrifuged a second time. The washings were added to the original filtrate and reducing sugars determined on the filtrate using the 3,5, dinitrosalicylic acid reagent (22).

When it was found that pressure cooking did not improve sugar yields, this step was eliminated. The effects of various combinations of commercial and laboratory enzymes and/or water infusion on sugar yields were investigated. In addition, the effects of mash strength, conversion time and certain selected enzyme combinations were investigated.

The enzyme additions were expressed as percent of the total dry barley used in each run. Conversion efficiencies were determined on each and calculated as follows:

$$\frac{A \times \text{dilution factor} \times B \ 100}{\text{Sample weight} \times \% \text{ carbohydrate}} = \% \text{ of total carbohydrate converted}$$

Where A = µg maltose in sample taken as compared to a standard maltose curve

B = Total liquid volume of filtrate from the enzyme extract.

All results were calculated as maltose.

The water infusion was extracted from the barley by mixing the ground barley with cold water and allowing it to stand for 12 hours with occasional stirring. Then various percentages were removed by decantation and used in combinations with other saccharifying enzymes. These solutions were added after the liquifaction step had been completed and the mash cooled to 55° C. since the temperature at which the liquifier operates would denature this crude beta-amylase. Later studies indicated 12 hour extractions were unnecessary and essentially the same enzyme concentration could be obtained in 2 - 4 hours time.

The liquifying enzymes, HT-44 and Rohzyme H-39 were studied to determine the concentrations necessary to give maximum liquifaction. The barley was mixed with different amounts of the two enzymes and held at 75 - 80° C. for two hours. At the end of this time an aliquot was taken

out in a special pipette and flow rate was determined. The viscosity reduction was used as the criteria of maximum liquifaction.

The effect of mash strength was studied by varying the concentration of grain in 250 g. of mash using different enzyme concentrations.

The effect of time of conversion was studied by making up the mash in the normal way and liquifying for the prescribed two hours. The saccharifying enzyme was allowed to act on the carbohydrate for varying lengths of time. The optimum practical time was based on total reducing sugar activity.

The enzymatic hydrolysis of barley produces quite an array of sugars. Some of the more promising enzyme combinations were chromatographed on Whatman No. 1 chromatography paper. Each spot of sugar was spotted on the paper in approximately 4 μmole quantities based on reducing power in the original filtrate. Two spots were made on each strip, one for color development and used for location of a sugar in a undeveloped strip, the other for the elution of the sugar to determine its concentration. The chromatograms were developed in a butanol, pyridine and water system. (6:4:3 v/v). The spots on the chromatogram were produced by the use of the CD-1 spray of Gordan et al (23). From this strip the various sugars could be identified either by a characteristic color or by the Rf values determined by Gordan and his group. If no Rf values were available or if the colors of two spots were the same or if both of these difficulties were encountered, the sugars were subsequently eluted, concentrated, subjected to the action of alpha-amylase and acid hydrolysis and the products rechromatographed. This procedure was used in determination of structure of some of the

disaccharides and trisaccharides. From this data sugar size could be determined and in most cases the linkage could be established. Isomaltose was determined using the starch phosphorylase method devised by French et al (24). The starch phosphorylase procedure is used to determine the number of glucose units in the molecule. The Rf value of isomaltose was also used along with its characteristic CD-1 color.

The quantity of sugar incorporated in each spot was measured by eluting with water and using the method of Dubois et al (17).

The proximate feed values and the total carbohydrate was determined in the grain residue remaining after the enzymatic hydrolysis (13, 14, 15, 16, 17).

IV RESULTS AND DISCUSSION

In order to obtain a semi-complete picture of the Betzes and Compana barley samples used in these analyses, the proximate feed values were determined as well as the total carbohydrate and natural diastatic power. These results are shown in Table I. Compana barley is not considered a good malting variety, while Betzes has met with favorable acceptance in the malting industry. To fulfill malt requirements, barley grain must have certain inherent physical and chemical characteristics which are not required in feeding varieties. The samples selected to represent these two varieties were purposely chosen for low protein, high carbohydrate content. It was felt that a malting barley may produce syrup either in somewhat larger quantities or with a reduced amount of added commercial enzyme than would be the case from the non-malting variety. Although a good malting barley must contain high amounts of natural beta-amylase activity, the Compana variety, which is a non-malting variety, contained slightly more beta-amylase than the Betzes which is the malting barley. Because of the very similar chemical, physical and agronomic characteristic of these two barleys, the results obtained did not show significant differences between malting and non-malting barley. Possibly if greater differences had been shown in the two strains, some basis for selecting barleys would have been established. This factor must be kept in mind in future studies or if this procedure is ever used commercially.

Potential diastatic power is determined by using a proteolytic enzyme, papain, to release more protein than is normally available by

Table I

Preliminary Analysis of the Two Barley Samples Used

Assay	Betzes	Compana
Protein	11.04%	11.57%
Crude fiber	3.70%	5.72%
Moisture	6.30%	7.40%
Extractable fat	1.66%	1.60%
Ash	2.55%	2.60%
Total carbohydrate	73.75%	68.10%
Diastatic activity (Papain)	130.00°L	140.00°L
Diastatic activity (Water)	87.70°L	93.75°L

The proximate feed values and carbohydrate accounts for 99% of the total Betzes and 97% of the Compana.

simple water extraction. In view of the cost of this enzyme it was considered desirable to measure the availability of beta-amylase by a simple water extraction. From the results indicated in Table I it was apparent that a sizable portion of the natural enzyme could be extracted in this manner.

In order to determine the maximum amount of beta-amylase available, a time study was undertaken. Water and sufficient barley were mixed and stirred for various periods of time after which the beta-amylase activity was determined. The results are shown in figure 1. Although the 12 hour extraction appeared to be necessary to obtain the maximum amount of enzyme, spontaneous fermentation was encountered under these conditions. The data presented in figure 1 indicated that 50% of the total beta-amylase was extracted in 2 to 4 hours time.

The enzymatic activities of the laboratory and commercial enzymes used were determined and this information is provided in Table II. The commercial additives were HT-44, malt, Wiszyme, Rhozyme H-39, Rhozyme K-2 and Rhozyme H-33. The two laboratory enzyme sources were Aspergillus oryzae and Aspergillus niger. Malt, Wiszyme, Rhozyme K-2 and Rhozyme H-33 were checked for both alpha- and beta-amylase activity. Malt is a good source of both alpha- and beta-amylase. It is obtained from barley germinated under carefully controlled conditions. Its alpha-amylase operates effectively at 55 - 63° C. Wiszyme is a mixture of beta- and alpha-amylase with a temperature optimum near 55° C. It was found to be far more effective than malt and was used in much lower concentrations. Rhozyme H-33 is also a good combination enzyme. The difference between

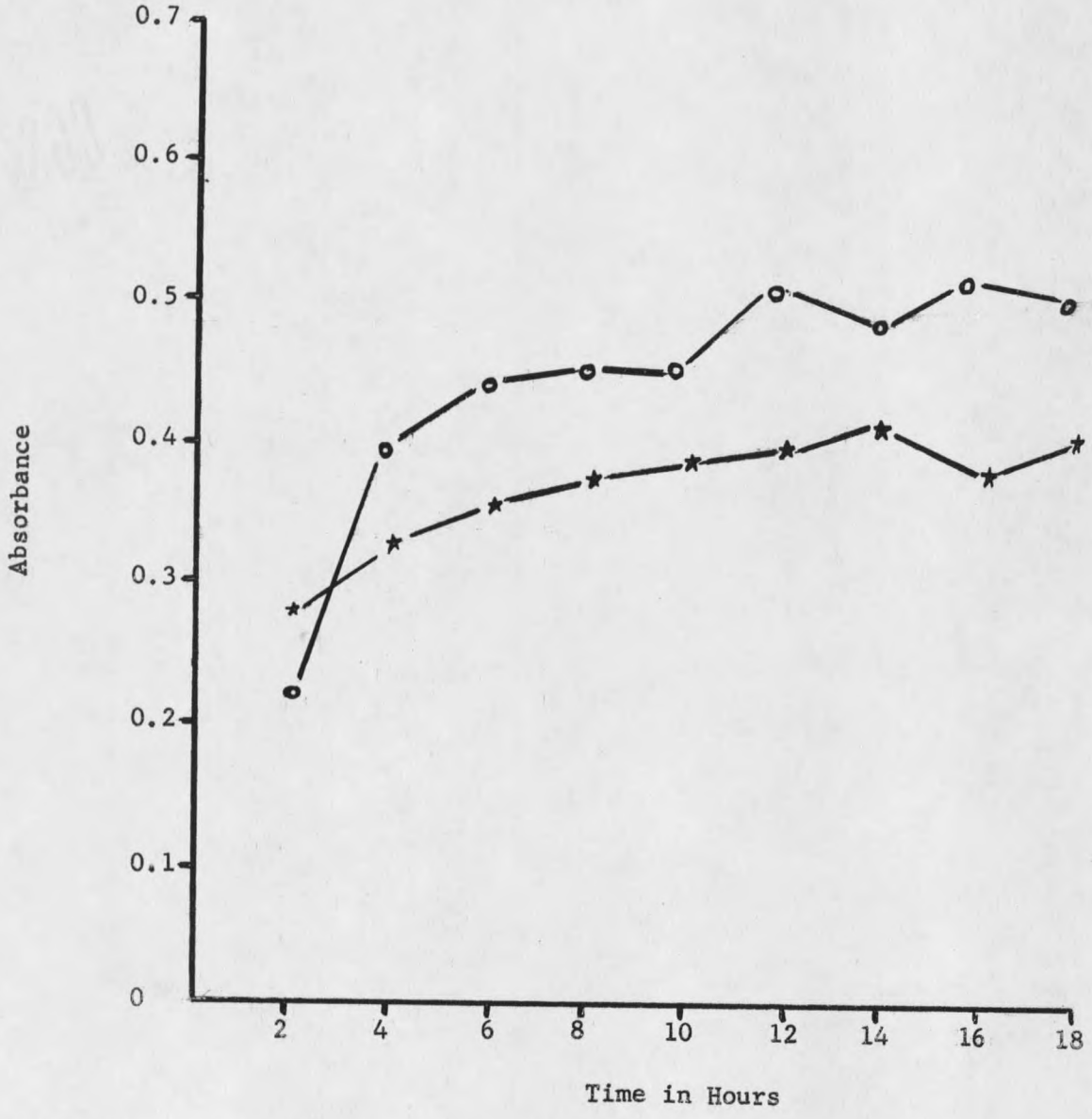

Figure 1. Effect of time on extraction of beta-amylase from barley

Legend: ★ Betzes
o Compana

Table II

Enzyme Activity of Preparations Used

Enzyme source	Alpha units	Beta units[1]	Maltase[2]
Malt	33	75	--
HT-44	105	--	--
Wiszyme	150	208	--
A. oryzae	100	--	28
A. niger	25	--	17
Rhozyme H-39	1920	--	--
Rhozyme K-2	203	400	--
Rhozyme H-33	105	183	--

1 Expressed as mMoles of maltose produced/g. of enzyme.
2 Expressed as time in minutes required for 1 g. or enzyme to hydrolyze 50% of the maltose.

this enzyme and Wiszyme is insignificant. Rhozyme K-2 is a concentrated enzyme source with a very high saccharifying power. Only the Aspergillus oryzae and Aspergillus niger were checked for maltase content because these were the only preparations used in this research that were known to contain substantial amounts of maltase. Although HT-44 had a lower alpha-amylase activity than did Wiszyme, Rhozyme K-2 or Rhozyme H-33 it was essential in this study because it is a high temperature stable alpha-amylase source. This is necessary to reduce the viscosity of the barley starch during its gelatinization. Rhozyme H-39 is extremely high in alpha-amylase activity and is stable at temperatures of 75 - 80° C. The Rhozyme enzymes were not studied as extensively as the other enzymes because they were received when this study was nearly completed. Aspergillus oryzae was used where a good saccharifying alpha-amylase was needed along with some maltase activity. Aspergillus niger was found to be the best source of maltase used in this research and when high glucose syrup was desired, this was used effectively in varying amounts.

The necessary weight of liquifying enzyme needed to produce maximum thinning was determined and these results are expressed in Table III. Rhozyme H-39 can be used in concentrations approximately 1/25 that of HT-44. This advantage is offset however by the cost of this concentrate. For this reason HT-44 was used as the standard liquifier.

Preliminary studies indicated that barley starch was readily converted into sugars. This suggested that pressure cooking, which is routinely used in enzymatic conversions of corn starch, might not be necessary. The validity of this assumption was established by the data presented in

Table III

Effect of Thinning Agent on the Mash Viscosity

Enzyme	% by weight	Flow time[1]
H-39	0.03	7.5
H-39	0.04	7.0
H-39	0.05	7.0
HT-44	0.25	10.8
HT-44	0.50	9.5
HT-44	0.75	9.5
HT-44	1.00	9.0

Mash strength 10%
1 Flow time is expressed in seconds.

Table IV. This was probably due to the continued action of the liquifying enzyme carried over from the liquification step.

The effect of mashing strength and liquifying enzymes were studied together and these results tabulated in Table V. Although the results shown were somewhat lower than normally produced, it would seem that 15% mash strength yields maximum results. Again it was apparent that 0.5% HT-44 was sufficient to produce satisfactory results. It was obvious that mash strengths greater than 15% were undesirable. However, when the HT-44 level was increased to 1%, the maltose equivalents dropped below that obtained with 0.5% HT-44 at the highest mash strength. The cause of this apparent inhibition is unknown but possibly it may be due to an initial rapid production of maltose in the presence of high substrate concentration. It has been reported that maltose in high concentration will inhibit beta-amylase (25). This could account for the observed results. The liquifier will produce more dextrins as its concentration is increased which would allow more non-reducing ends to appear. This in turn will present more sites of hydrolysis for the beta-amylase action causing a more rapid production of maltose. A similar effect was encountered with infusion and reduced amounts of malt and Wiszyme.

After determining the optimum concentration of the liquifying agent and maximum mash strength, the investigation was directed towards the best possible combination of saccharifying enzymes. The first studies did not include water infusion and are reported in Table VI. The use of 10% malt which was used as a control gave slightly better results than 4% Wiszyme. This was expected from the analytical data reported in Table II.

Table IV

Effect of Pressure Cooking Procedure on Sugar Production

Enzyme combination % of barley used					Pressure M.E.[2]	No Pressure M.E.[2]
A. oryzae	A. niger	Malt	Wiszyme	Infusion[1]		
--	--	--	4	--	61	74
0.5	0.5	--	3	--	66	73.1
1.5	0.5	2	--	--	77.5	80.6
0.5	0.5	--	2	30%	75	85
0.5	0.5	2	--	30%	70	82.5

Mash strength was 10% and 0.5% HT-44 was used in all cases as the liquifier enzyme. Barley was Compana.

1. Water extract of ground barley.
2. M.E. is maltose equivalent.

Table V

The Effect of Mash Strength and Liquifying Agent of Syrup Yield

Mash strength %	Syrup yields in M.E. using indicated amounts of HT-44		
	0%	0.5%	1.0%
10	22.7	57.0	61.2
15	26.2	60.6	61.5
20	20.0	55.7	55.7
25	-*	49.0	47.0

Conversion agent 0.5% A. oryzae, 0.5% A. niger and 20% infusion.
Barley was Compana variety.
* Too thick with starch.
M.E. equals maltose equilalent.