FODDER YEAST FROM WOOD SUGAR

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FODDER YEAST FROM WOOD SUGAR[1, 2]

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Introduction

The possibility of using hydrolyzed wood as a source of fermentable carbohydrate has been recognized for many years. "Wood sugar," as such hydrolyzates are generally called, may be used for the production of both ethyl alcohol and yeast. The Forest Products Laboratory in 1943 undertook the hydrolysis of various American woods on a pilot-plant scale in an effort to determine the most suitable conditions for the production of wood sugar from many types of wood (7) and to evaluate these wood sugars as sources of ethyl alcohol and fodder yeast.

Production of yeast from wood sugar has been investigated extensively in Germany for reasons of self-sufficiency because of the shortage of protein and relative abundance of cellulosic material in that country. Fink and Lechner have published three papers on the production of fodder yeast from wood hydrolyzates obtained by the Scholler and the Bergius processes (3, 4, 5). Using the so-called "mineral yeast" (Torula utilis), they report utilization of 92 percent of the nitrogen furnished the yeast, as ammonia, in the synthesis of yeast protein. This conversion was obtained on approximately a 1.5 percent reducing sugar wort, and was equivalent to 40 percent yeast dry matter on the total reducing sugar in the wort. The yeast had a high protein content (as calculated from nitrogen content), ranging from 55 to 59 percent of the dry matter. Wood sugar produced by the Bergius process yielded more yeast per 100 grams of reducing sugar; but, since the yeast was of lower protein content, it was no better as a source of protein than that obtained from the Scholler product.

In the second paper of Fink and Lechner (4) data were given comparing wood sugar to molasses for nitrogen content. The wood sugar was only one-tenth to one-fifteenth as rich in assimilable nitrogen as the molasses.

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[2] Presented before the American Chemical Society, New York City, September 11-15, 1944.

[3] Maintained by the U. S. Department of Agriculture at Madison, Wisconsin, in cooperation with the University of Wisconsin, at which institution the senior collaborator is Professor of Biochemistry and the junior collaborator is Professor of Agricultural Bacteriology.

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Fink and Lechner concluded that a continuous subculture process utilizing an 8-hour fermentation cycle was commercially feasible. An over-all yield of about 12 kilograms of yeast protein was obtained from 100 kilograms of dry wood.

There is little doubt concerning the high biological value of Torula utilis protein. Fingerling and Honcamp (2) and Bunger, et al, (1) in Germany have reported excellent results with wood sugar yeast as the protein source in rations of swine and milk cows. English workers (6) describe "trials of nutritive value of dried Food Yeast (Torula utilis major, grown on molasses) made ... on both animals and human subjects. These have demonstrated its twofold value, firstly, as a source of protein of high biological value, and secondly, as a rich source of the "B" group of vitamins..." The value of Torula utilis as a vitamin source has been further supported by the findings of Lewis, et al (9). The yeast was assayed for members of the B group, and found to be roughly comparable in potency to bakers' and brewers' yeasts.

The following discussion deals with the part of the wood sugar investigation conducted by the Forest Products Laboratory that was concerned with the production of fodder yeast.

Fermentation

Successful fermentation of wood sugar required a rather extensive pretreatment of the hydrolyzate. In general, this pretreatment consisted of the following steps: neutralization to about pH 4.5; clarification or detoxification, usually by heating the hydrolyzate with Na_2SO_3, a procedure recommended by the German workers (3, 4, 5); dilution of the hydrolyzate to a fermentable concentration of sugar (which likewise decreased the concentration of "toxic" substances); and finally, addition of phosphorus and nitrogen sources in suitable amounts for the production of cells.

The final medium was fermented in several types of containers but most of the data were obtained from two sizes of fermentation. In one fermentation 90-milliliter portions of the medium were placed in 500-milliliter Erlenmeyer flasks and inoculated with 10 milliliters of a suspension of yeast containing 0.1 gram of cells (dry basis). Thus, the inoculum in the fermentation was equivalent to 1 gram of yeast (as dry matter) per liter of medium. The inoculum was grown on hydrolyzate medium, or molasses-malt sprouts extract medium and will subsequently be described in detail. The flasks of inoculated medium were then placed in a shaker at 30° C. The shaker had a stroke length of 10 centimeters and was operated at 90 cycles per minute. After from 16 to 24 hours, the flasks were removed and analyzed. The second common type of fermentation was carried out on 6,300 milliliters of diluted hydrolyzate medium in 5-gallon Pyrex bottles, into which air was forced at a rate of from 20 to 80 liters per minute through canvas aerators. To this medium, 700 milliliters of inoculum were added. This inoculum gave a concentration of cells in the medium equivalent to 1 gram of dry matter per liter. The bottles were incubated in a constant temperature water bath at 30° C. and were analyzed after from 16 to 24 hours.

Analytical Methods

Yeast Determination

The quantity of yeast was estimated by centrifuging 10 milliliters of homogeneous fermentation liquor in weighed test tubes, washing the cells once with distilled water, recentrifuging, and weighing the tube and yeast after from 18 to 24 hours of drying at 100° C. The method was improved later by washing the cells of the aliquot with 1 milliliter of 6 N HCl before the final centrifugation.

The magnitude of the yield apparently depends upon the time of harvesting. Rapid autolysis seriously affects the maximum yeast yield actually obtained if the fermentation is allowed to proceed longer than the time necessary to utilize the sugar completely.

Reducing Sugar Determination

The sugar method used was the micro method described by Shaffer and Somogyi (10). A 30-minute boiling period with Reagent 50 containing 5 grams of KI was used. Various tests were made on the determination of sugar in the hydrolyzate; no disagreement was detected when aliquots of a given hydrolyzate but of different sugar concentration were analyzed. Added sulfite did not make any detectable difference in the results. Tests showed that the analysis of the supernatant obtained on centrifuging the wort did not differ from the analysis on the whole wort.

Alcohol Determination

A sample of the wort was distilled and a known amount of dichromate added to the distillate and heated. The excess dichromate was reduced with KI and the I_2 liberated was titrated with $Na_2S_2O_3$. A test made on a solution of alcohol and water in a bottle under the same conditions as those used during a fermentation showed a 58 percent loss of alcohol during an aeration period of 24 hours. The rate of aeration was about 20 liters per minute. Under a much higher rate of aeration, 87 percent of the alcohol was lost in 24 hours. Alcohol determinations were made on several of the bottle runs. The range of alcohol found to be present after a regular wood hydrolyzate fermentation varied between approximately 0.01 and 0.1 percent. A higher amount of alcohol was generally found in the beer when the yeast yields were low.

Phosphorus Determination

A colorimetric method described by Holman (8) was used.

Pretreatment of Hydrolyzates

The first problem encountered was the determination of the extent of pretreatment of hydrolyzates required to make them fermentable. The hydrolyzates as received were not fermentable to a reasonable degree of completion. They apparently contained toxic material, which had to be removed or inactivated before good fermentations could be obtained. The various phenomena that indicated the presence of toxic substances may also be explained by assuming the existence of an unsuitable chemical structure of the sugars that was altered favorably by the "detoxifying" treatments.

In the preparation of hydrolyzate for fermentation, the following four pretreatment procedures were developed:

PT-I.--The quantities of the materials added were based on 4 percent reducing sugar calculated as glucose. NH_4OH (28 percent) was added to pH 3.5 and Na_2S added to pH 5 (ca. 0.1 percent); after 12 hours, the solution was filtered through paper with Hy-Flo Supercel; to the clear filtrate, NH_4OH (28 percent) was added to pH 9; H_2SO_4 (concentrated) was added to pH 4.5 and the solution filtered; the filtrate was diluted to twice the volume with distilled water; and to the diluted filtrate, 0.2 percent KH_2PO_4 was added.

This procedure was developed for treatment of hydrolyzates in which considerable concentrations of heavy metals (from the hydrolyzer) were found. It was not used on the majority of hydrolyzates fermented.

PT-II.--The quantities of materials added were based on hydrolyzate containing 4.5 percent reducing sugar. NH_4OH (28 percent) was added to pH 2.5 (ca. 0.63 percent); and $Ca(OH)_2$ added to pH 4.5 (ca. 0.3 percent). The solution was filtered, and the filtrate diluted to 2 percent reducing sugar; to the diluted filtrate, 0.24 percent KH_2PO_4 was added.

This pretreatment was used for preparing a more concentrated solution for fermentation (2 percent reducing material) than was later adopted for routine runs (1 to 1.5 percent reducing material). It was a modification of PT-I due to an observation that adjustment of the pH of the medium to a value above 7 with subsequent heating not only destroyed some sugar but also decreased its fermentability.

PT-III.--The quantities of materials added were based on 5 to 6 percent reducing sugar and were as follows: concentrated NH_4OH, 0.6 percent (by volume); $CaCO_3$, 1.6 percent; and Na_2SO_3, 0.05 percent. The solution was heated to boiling, cooled, and filtered; the filtrate was then diluted to ca. 1 percent reducing sugar; and KH_2PO_4 was added, 0.45 percent.

This was the first regular procedure adopted. The Na_2SO_3 level used was about one-third the level used by Fink and Lechner (4).

PT-IV.--The quantities of materials added were based on 5 to 6 percent reducing sugar. $Ca(OH)_2$ was added to pH 5.0 (about 0.75 gram $Ca(OH)_2$ per 5 grams reducing sugar); followed by the addition of 0.05 percent Na_2SO_3. The solution

was heated to boiling (live steam), cooled, and filtered through a pad of Hy-Flo Supercel. Then 0.06 gram urea and 0.05 gram KH_2PO_4 per gram of reducing sugar were added; and the solution diluted to the concentration desired for fermentation.

This was the most successful procedure and was used for most of the routine fermentations. It differed from the procedure of Fink and Lechner in the following respects: it utilized lower concentration of Na_2SO_3 relative to sugar concentration and involved a heat treatment, which proved definitely beneficial; KH_2PO_4 was used as a source of phosphorus; and urea, rather than NH_4OH, was used as the source of nitrogen.

Choice and Acclimatization of Yeast

Screening experiments were made to find which yeasts grew well on a hydrolyzate medium. Such a yeast should utilize both pentoses and hexoses for cell production, because approximately one-fourth of the reducing sugar in wood hydrolyzate is pentose. Thus, bakers' yeast is unsuitable. The yeast should also be insensitive to the many nonsugar compounds contained in wood hydrolyzates, or at least capable of being acclimatized to such compounds. It should give good yields of cells on wood sugar and be suitable for nutritional purposes.

Typical results indicating comparative fermentation abilities of various yeasts are given in table 1.

Torula utilis No. 3, P-13, and Candida tropicalis were chosen as the three most promising yeasts for further study. In addition to those listed in table 1, a bakers' yeast grown on sulfite liquor (Best Yeast Company, Thorold, Canada) was tested and found to be a poor fermentor of the treated hydrolyzate medium.

Torula utilis No. 3 was tested as a pentose fermenter on various media containing pure xylose (probably the most abundant pentose in wood hydrolyzate) and arabinose. The results indicated that about one-fourth to one-half of the xylose supplied was fermented by this strain under the conditions used. The yield of yeast on xylose fermented ranged from 20 to 36 percent, depending upon presence or absence of corn steep liquor in the medium. Fermentation of arabinose was practically negligible.

The cultures that did well in preliminary trials were subcultured on hydrolyzate medium free from growth factors. Three successive experiments were made to acclimatize Torula utilis No. 3, P-13, and Candida tropicalis. The general procedure was as follows: The yeast was plated on agar containing the hydrolyzate medium either as sole nutrient, or in conjunction with molasses and salts. From these plates, promising colonies were picked and subcultured on hydrolyzate agar through from 7 to 15 stages.

Early experiments failed to produce an acclimatized or improved strain of any of the cultures. All cultures, both stock and acclimatized, gave an average

maximum yield of approximately 30 percent dry yeast (calculated on total reducing sugar).

Later, a batch of Douglas-fir hydrolyzate No. 107 was received that could not be fermented by any of the previously developed techniques. Acclimatization of the yeast strains to this hydrolyzate met with considerable success. Table 2 summarizes the work and indicates the acclimatization possibilities of various organisms on five to ten subcultures. P-13, Candida tropicalis, Hansenula anomala, and certain strains of Torula utilis responded especially well. Five to ten subcultures seemed to induce maximum acclimatization. The strain of Torula utilis seems important in determining potentialities of the yeast for acclimatization. Torula utilis No. 2 and Torula utilis major were subcultured on hydrolyzate, but the effect of the acclimatization on these strains was much less than on Torula utilis No. 3. Although several of the yeasts seemed to give good yields and respond well to acclimatization, Torula utilis No. 3, because of its known nutritive properties, was chosen for all experiments subsequent to those reported in table 2. Acclimatization of yeast in shake flasks was successful, but it was not successful in larger lots (7,000 milliliters). By the third transfer the fermentation proceeded slowly, and the yield often decreased by 50 percent.

Factors Affecting the Preparation and Use of Inocula

The type of inoculum used depended upon several factors: (a) If the strain had been improved through acclimatization, experience showed that the final stage of inoculum had to be grown in hydrolyzate. (b) For practical purposes, if a heavy inoculum was demanded, a concentrated medium in which yeast grew well was used; here, hydrolyzate medium was unsatisfactory. (c) Work indicated that a more vigorous type of cell could be produced on media containing growth factors (Medium 2). Stock strains so developed often produced larger yields on hydrolyzate than did acclimatized strains developed on wood sugar.

The following types of inoculum media have been tried:

1. Two percent glucose, 0.6 percent urea, 0.1 percent $MgSO_4.7H_2O$, 0.1 percent KH_2PO_4, 0.2 percent NaCl, 0.04 percent $CaCl_2$, 0.01 percent $FeSO_4.7H_2O$, trace of $CuSO_4.5H_2O$, pH 5.0. This medium did not produce as heavy an inoculum as a rich medium (Medium 2).

2. Five percent beet molasses, 2.0 percent diastatic malt extract, 0.75 percent (by volume) corn steep liquor, 0.1 percent $(NH_4)_2HPO_4$. This medium produced exceedingly heavy inocula in 16 hours even when started with a small seeding. This medium was modified by the elimination of the diastatic malt extract and use of an extract of 10 percent malt sprouts. No difference in results was obtained. The cells propagated on this medium showed uniformly shorter induction periods in the final fermentation than inocula from any other type of medium.

If inoculum free from its growth medium was desired, the cells were centrifuged and washed before use. Such inoculum did not give perceptibly different yields from those obtained by using uncentrifuged inoculum.

3. Spruce, Douglas-fir, and Southern yellow pine hydrolyzates were used as media for the final stage of inoculum. The sugar concentration had to be below 2 percent in order to give appreciable yields of yeast. The medium was prepared in the same way as the fermentation medium from these woods (Pretreatment IV). Heavy inocula were not obtained with this medium; inocula were used as soon as possible after the 18- to 20-hour growth period expired.

Table 3 summarizes some results indicating the yields obtained with the various types of inocula.

Size of Inoculum

In all the routine fermentations, a standard level of inoculum of 1 gram of cells (dry basis) per 1,000 milliliters of fermentation medium was used. This size gave a suitable rate of fermentation in dilute (1 percent) sugar solutions. The induction period and fermentation time, however, were shortened by using heavier inocula. As high as 4 grams (dry basis) per 1,000 milliliters were tried. A heavy inoculum overcame the effect of residual toxic materials in a shorter period of time.

The size of the inoculum had no perceptible effect on the net total yield of yeast, or upon the completeness of sugar utilization (table 4).

Longevity of Inoculum

An inoculum was not stored in the medium in which it was grown. If the inoculum was to be used uncentrifuged, it was grown and used within 24 hours. Use of older uncentrifuged inoculum occasionally resulted in poor yields of 25 to 30 percent on total sugar. If the cells were centrifuged, washed, and resuspended in distilled water, they served as inoculum without apparent loss in efficiency for as long as 10 days when stored in a cold room at 5° C. This was true only for unacclimatized inocula. If acclimatized inocula were held for periods longer than a day after maximum growth was attained, the advantages of acclimatization were nullified.

In large fermentations under aeration of two volumes of air per minute the most convenient type of inoculum was that grown on a rich medium (Medium 2) for 18 hours and used immediately without centrifugation.

A Typical Procedure for Growing Inoculum

A pure culture of Torula utilis No. 3 from a slant culture was inoculated into two 100-milliliter portions of Medium No. 2 in shake flasks. These were shaken at 30° C. for 18 hours, and both were then poured into 7 liters of similar medium in a 5-gallon bottle. This was aerated at 20 liters per minute via canvas aerators for 18 hours and then used to inoculate the hydrolyzate medium immediately after standardization; an alternative procedure was to centrifuge, resuspend in distilled water, and store in the cold room for use within 10 days.

Aseptic technique was observed only through the shake flask stage. No obvious contamination difficulties occurred in the final stage.

Effect of Hydrolyzate on Yield of Yeast

Considerable variation in hydrolyzates was encountered, due perhaps both to changes in the species of wood hydrolyzed, and to changes in the cooking procedure. This was inevitable since the hydrolyzing procedure was developed concurrently with the fermentation technique.

A change in the hydrolyzate invariably encountered was the separation of an amorphous deposit on the bottom of the container when the hydrolyzate was allowed to stand. This deposit indicates the suspenoid and colloidal nature of the medium, and may be a partial explanation of an increase in fermentability that occurred regularly on allowing the hydrolyzate to age before treatment and fermentation.

Most fermentation work was done on hydrolyzates of spruce, Douglas-fir, and Southern yellow pine. These hydrolyzates differed greatly as fermentation media. Thus, spruce was the most easily fermented of the softwoods. Southern yellow pine hydrolyzates were similar both in fermentation properties and appearance to the spruce hydrolyzates. Douglas-fir, darker in color than either of the others, was the most difficult to ferment. Hardwood hydrolyzates were generally more easily fermented and gave larger yields of yeast than did softwood hydrolyzates. These general remarks may be partially checked by referring to table 5.

Order, Extent, and Agent of Neutralization

Essential steps in the pretreatment of the hydrolyzate were: (1) raising the pH; (2) removing the toxic materials by precipitation procedures, such as heating, addition of Na_2SO_3, etc.; (3) adding nutrients (phosphorus and nitrogen) and (4) diluting to the proper reducing sugar concentration.

The pH of the hydrolyzate as it came from the hydrolyzer was usually between 1.3 and 1.5. Several reagents for raising it were tried. NH_4OH serves both as a basic reagent and as a source of nitrogen. It was suitable for raising the pH and supplying nitrogen simultaneously, but later work indicated that urea was a much better source of nitrogen (table 6).

$CaCO_3$ raised the pH to the proper extent (ca. 5) without careful regulation of concentration. It had the great disadvantage, however, of causing extreme foaming and difficulty in handling the medium.

$Ca(OH)_2$ is the reagent that was adopted. It is cheap, avoids the foaming difficulty, and is easy to handle, but the pH must be watched. Raising the pH higher than 7.0 with subsequent heating caused a destruction of reducing sugar, and also increased the toxicity of the medium. The sugar utilization was from 30 to 35 percent lower in a medium that had been lined to a high pH of 9 or 10

and then lowered for the fermentation. The yeast yields based on sugar fermented, however, did not seem to be appreciably affected by liming.

Removal of Toxic Materials

Sodium sulfite was used by Fink and Lechner in their hydrolyzate pretreatment procedure. It was used regularly in about 0.05 percent concentration just before the heating step. Many experiments, however, have failed to show any real advantage in the use of this reagent. Southern yellow pine hydrolyzate gave slightly better yields in one run but not in four others when sulfite was added, while Douglas-fir hydrolyzate was not improved by sulfite treatment.

Heating Treatments

Although heating the hydrolyzate after addition of the neutralizing agent seemed to be of little advantage in increasing the yeast yield, the medium was much cleaner, and there was less precipitation of debris during the fermentation than if the boiling step were omitted. Several methods of heating were tried: boiling over a free flame for 1 minute; autoclaving at 15 pounds for 15 minutes; and bringing to 100° C. by introduction of live steam directly into the neutralized hydrolyzate. The last method proved to be as good as any, and was more convenient for the preparation of large batches.

Heat treatments were tried at several different hydrogen ion concentrations. The fermentation properties of the resultant media were not significantly different. There was appreciable reducing sugar destruction upon heating at the lower hydrogen ion concentrations (pH 8 to 10).

Lignin, Norit Adsorption Treatments

At pH 5, decrease in the sugar concentration by as much as 10 percent of the original value (ca. 5 percent reducing sugar) resulted from treatment of the hydrolyzate with lignin (1 gram per 100 milliliters). This sugar loss also occurred at higher hydrogen ion concentrations (pH 1.5 to 2).

At equal levels, Norit A did not seem to be better than lignin as an adsorbent for toxic materials, but greater adsorption efficiency per unit of weight was apparent. Concentrations of Norit that improved the fermentability above the best lignin-treated medium, however, are impractical. The decreased toxicity of these lignin and Norit-treated hydrolyzates was apparent in a shortening of the induction period of the fermentation and in a greater yield of yeast (table 7).

Effect of Sugar Concentration and Nutrients

Sugar

The addition of Na_2SO_3 and $Ca(OH)_2$ was made to the undiluted wort. The wort was then diluted before the addition of phosphorus and nitrogen. The sugar concentration adopted for routine trials of different hydrolyzates was 1 percent (reducing material). Concentrations as high as 2 percent gave yields that were no more than 50 percent of that obtained on the more dilute sugar. Noticeable loss of efficiency was noted even at concentrations of 1.25 percent reducing sugar, while concentrations of sugar lower than 1 percent proved to be no better than 1 percent. These effects may be related to the following factors: lack of sufficient aeration to accommodate the eventual yeast population in the more concentrated wort; exceeding the threshold of toxicity for the yeast of the other constituents in the hydrolyzate medium.

Nitrogen

Sources of nitrogen tried were ammonia, diammonium acid phosphate, ammonium sulfate, and urea. Urea proved to give better yields of yeast on the basis of nitrogen supplied than the others. A comparison of yields from urea- and ammonia-containing media is given in table 6. The nitrogen level adopted was that supplied by 0.06 gram of urea per gram of sugar and was about 0.03 gram of nitrogen per gram of sugar (table 8). Analyses of shake flask fermentations indicated that about 90 percent of this level of nitrogen was recovered in the yeast.

Phosphorus

Sources of phosphorus tried were dibasic potassium phosphate, monobasic potassium phosphate, diammonium hydrogen phosphate (as a combined nitrogen-phosphorus source), and superphosphate (20 percent P_2O_5). Most of the results reported in this paper are from fermentations in which monobasic potassium phosphate supplied the phosphorus. Superphosphate (at equivalent phosphorus adopted after studies on optimum requirements (table 9)) was that supplied by 0.05 gram of monopotassium phosphate per gram of reducing sugar.

According to results obtained in an experiment measuring the phosphorus uptake of the yeast in a hydrolyzate medium containing various amounts of phosphorus, the yield of yeast was not critically affected providing there was sufficient phosphorus, although the yeast absorbed higher amounts if supplied.

Growth Factors

Addition of growth factors to any medium developed in these experiments did not result in greater yields than those obtained on media prepared by procedure IV. Addition of corn steep, malt sprouts extract, or Difco yeast extract did not improve yield or fermentation on this medium.

Effect of Other Factors

Temperature

Studies made of optimum fermentation temperatures (table 10) indicated that good yeast production was possible between 25° and 35° C. At the higher temperatures, the fermentation proceeded more rapidly, but favored alcohol production. Hence, a temperature of 30° C. was chosen for routine fermentations.

Aeration

Although aeration studies run in flasks tell little about amounts of air for optimum yeast production in larger fermentation, they indicated the amount required under laboratory conditions. Within limits tested, conversion of carbohydrate to cells increased in efficiency with increasing aeration (table 11).

In 5-gallon bottles the aeration could be controlled better, although not absolutely, because of aerator and diffuser variations. The amount of air to be put through, however, was governed not only by the yeast yield, but by the foaming properties of the medium as well. In the large bottles 2 to 4 volumes per minute were used. The air was filtered through cotton, and diffused into the medium through tightly sewn canvas bags attached to the end of a glass tube. Treated wood hydrolyzate foams profusely on introduction of finely divided air, and the presence of large numbers of yeast cells enhances the foaming problem. To reduce this foaming, trials were made with various antifoam agents, such as Vegifat Y and lard, Vegifat, and various detergents. In all trials, Vegifat appeared to be as good as any and was used freely.

Prevention of foaming in media containing more than 1 percent reducing sugar was still more difficult. On certain slow feed runs where the added sugar amounted to between 2 and 3 percent on the final volume, Vegifat Y failed to hold the wort in the bottle, and the aeration had to be decreased to less than 1 volume per minute.

Time

In bottles, complete fermentation of 1 percent sugar was obtained in 16 hours with the standard inoculum level and a temperature of 30° C. All of the routine flask fermentations were run for 24 hours.

The time could be decreased by increasing the inoculum to higher levels (5 to 6 grams per liter, dry weight) and by raising the temperature of the fermentation to 34° or 35° C. Necessity for immediate harvest of yeast on completion of fermentation was shown by loss in yeast on longer contact with wort.

pH

The production of yeast from wood hydrolyzate apparently occurred efficiently when the initial pH was adjusted anywhere between 4.5 and 5.5

Contamination

Fink and Lechner report no contamination of wood-sugar fermentation in 45 successive transfers. Their pretreatment included no heating step. In all the experiments described here, aseptic precautions were not observed, and contamination of the hydrolyzate fermentation never produced recognizable symptoms; similarly, treated uninoculated medium left in open containers in the Laboratory for several days showed no signs of fermentation.

Standardized Procedure Finally Adopted

On the basis of the experimental results the procedure for a typical fermentation (7,000 milliliters of medium) was as follows:

Six thousand, three hundred milliliters of medium (prepared as described on page 9, pretreatment IV) was placed in a 5-gallon Pyrex bottle. This was inoculated with 700 milliliters (10 percent of total volume) of inoculum containing 1 gram of cells per 100 milliliters. The inoculum was standardized by centrifugation of an aliquot in a graduated centrifuge tube. The inoculum added was diluted so as to contain 7 grams per 100 milliliters.

A canvas aerator was placed in the bottle well below the surface of the medium. Ten milliliters of Vegifat Y were added as antifoam agent. The bottle was placed in a constant temperature bath, or incubator, at 30° C., and from 20 to 40 liters per minute of saturated air were passed through the canvas bag and diffused into the medium. The fermentation was usually complete in from 16 to 18 hours, but was allowed to proceed for 24 hours. The bottle was then removed, and determination of sugar, yeast, alcohol, and pH was made immediately.

Slow Feed Fermentation

Several samples of wood hydrolyzate medium were fermented by slow-feeding processes. Addition of wood sugar was done either continuously or intermittently in such amounts as to maintain the sugar concentration of the fermenting wort between 0.5 and 1.0 percent. In these experiments, an intermittent slow-feed fermentation was not successful unless the initial inoculum was greater than 6 grams of yeast (dry basis) per liter.

The concentration of sugar in the wort never rose above 1 percent, and the addition of nutrient kept the level above 0.3 percent. A gradual addition of nutrient so as to maintain a constant sugar concentration has been carried to a 2 percent sugar level (on final volume), with about 20 percent conversion to yeast on total sugar added.

Production of Yeast on a Pilot Plant Scale

Two fermentations of 300-liter batches of hydrolyzate medium were made in a pilot-plant fermenter of 800-liter capacity. The level of the inoculum and main details of the fermentation were the same as those described for bottle runs except on the larger scale. About a fourth of a liter of air per minute of medium was diffused through carborundum cylinders into the medium. Results of analyses for reducing sugar, alcohol, and yeast are given in table 12.

Summary of Hydrolyzates Fermented and Yields of Yeast

One of the main purposes of this study was to evaluate the fermentability and quantity of yeast obtainable on several types of hydrolyzate from different woods. To this end, a standard procedure of pretreatment was adopted (Pretreatment IV) with the exception that boiling for 1 minute rather than the steam treatment was used. The range of yeast yields obtained from the hydrolyzates of several woods is given in table 5.

From the column headed "number of runs" in table 5, it is apparent that the bulk of the work was done on the three hydrolyzates listed first, Douglas-fir, spruce, and Southern yellow pine. Since the pretreatment procedures were developed especially for these hydrolyzates, the yields given for other hydrolyzates probably do not represent fair tests of their possibilities.

It is to be emphasized that under the best conditions developed in this work, 37 to 40 percent conversion to yeast of the total sugar in the media was obtained repeatedly on Douglas-fir, spruce, and Southern yellow pine hydrolyzates.

Cost of Yeast

The cost of chemicals necessary to process sufficient wood sugar (Pretreatment IV) to make 1 pound of yeast amounts to about 1 cent. This does not include the cost of producing the wood sugar. Insufficient data exist at present for estimating this figure. Plant and labor costs for processing and fermentation of wood hydrolyzate on an industrial scale are unknown, and no estimate has been made. Colonial Food Yeast Limited has estimated a cost of about sixpence per pound of dry Torula utilis major grown on molasses. This price was estimated on the basis of an annual output of 2,500 tons of yeast. If all costs are charged to the protein of the yeast, the surprisingly high figure of about 25 cents per pound is obtained. While this figure seems high, it is doubtful that yeast protein can be produced at a price in the same range as low-cost protein, for example, soy bean protein which sells at approximately 5 cents per pound.

Summary

About 150 fermentations of hydrolyzates from 13 species of wood with 9 types of yeast were run, and the yield of yeast was determined. Pretreatment of the hydrolyzate was necessary to insure good yields. Processing with $Ca(OH)_2$ at pH 5 followed by addition of urea and phosphates produced a suitable fermentation medium. Torula utilis No. 3, Candida tropicalis, and an unidentified yeast, P-13, were satisfactory organisms, but most of the fermentations were run with the first-named yeast. Acclimatization of the yeast to wood sugar increased the yield. Composition and pH of the medium, size of inoculum, rate of aeration, and time of fermentation were other factors that affected the yield.

Yields (calculated as dry yeast) of from 35 to 40 percent of the total reducing sugar in the hydrolyzate were obtained regularly. About 90 percent of the apparent reducing sugar was fermented. The remaining 10 percent was probably unfermented pentoses and nonsugar-reducing substances. Hydrolyzates from spruce and Southern yellow pine gave good fermentations, but those from Douglas-fir were difficult to ferment. Wood sugar from maple, yellow birch, and beech gave results equal to those from the best softwoods.

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Table 1.--Growth of various yeasts in wood hydrolyzate

Organism	Time of fermentation	Initial sugar (as glucose)	Initial sugar fermented	Yeast (dry) on total reducing sugar
	Hours	Percent	Percent	Percent
Series I[1]				
S. cerevisiae	24	1.50	57	4.1
	48	1.50	67	4.7
S. cerevisiae, A.T.C.C. 764	24	1.50	67	7.3
	48	1.50	71	17.2
Torula utilis No. 3	24	1.50	67	24.3
	48	1.50	86	39.1
Series II[2]				
Candida guilliermondi	24	1.58	92	23.5
Candida tropicalis	24	1.58	91	32.9
Candida kruseii	24	1.71	74	22.5
Endomyces magnusii	24	1.71	86	15.0
Hansenula anomala	24	1.71	90	30.4
P-13 (unidentified)	24	1.71	91	40.4
Rhodotorula, sp.	24	1.58	43	10.3
Torula utilis No. 3	24	1.58	81	33.2
Series III[1]				
S. cerevisiae	24	1.50	75	15.3
	48	1.50	83	20.1
S. cerevisiae, A.T.C.C. 764	24	1.50	63	17.2
	48	1.50	73	27.9
Torula utilis No. 3	24	1.50	70	29.8
	48	1.50	89	39.9

[1]Medium: Spruce hydrolyzate 4, pretreatment IV. Shake flask cultures (100 milliliters). Series III contains 0.25 percent corn steep (dry basis) in addition.

[2]Medium: Spruce hydrolyzate 16, pretreatment IV; shake flask cultures (100 milliliters).

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Table 2.—Effect of acclimatization on sugar utilization and yeast yields

Organism	Initial reducing sugar fermented[1]		Yield of yeast (dry weight based on total reducing sugar)	
	First transfer	Twelfth transfer	First transfer	Twelfth transfer
	Percent	Percent	Percent	Percent
Hansenula anomala	75.1	88.5	27.0	40.5
Candida tropicalis	75.1	90.1	29.3	41.3
P-13	77.4	92.3	20.5	43.7
Torula utilis No. 3	70.2	91.8	22.9	38.1

[1]Initial reducing sugar content, ca. 1 percent.

Table 3.--Effect of inoculum medium on fermentation of wood hydrolyzate[1]

Inoculum[2]	Size of inoculum (dry weight)	Initial pH	Time of fermentation	Initial sugar fermented	Yeast yield (dry weight), on total sugar
	Grams per liter		Hours	Percent	Percent
Grown on rich[3] medium, centrifuged	0.828	5.5	24	87.4	36.3
Grown on rich[3] medium, centrifuged	.814	4.5	36	75.9	36.0
Grown on rich medium, uncentrifuged	1.097	5.5	24	87.6	35.9
Grown on rich medium, uncentrifuged	.793	6.0	26	91.2	35.0
Grown on wood hydrolyzate, centrifuged	.72	5.0	22	93.4	28.8
Grown on wood hydrolyzate, centrifuged	.46	5.0	22	88.9	33.5
Grown on wood hydrolyzate, uncentrifuged	.493	6.0	26	87.2	33.6

[1] 7,000 milliliters in 5-gallon Pyrex bottles, Douglas-fir (Hyd. No. 107).

[2] Unacclimatized.

[3] "Rich" medium is Inoculum medium 2, page 6. The hydrolyzate-grown cells are from medium treated by pretreatment IV.

Medium: Douglas-fir (Hyd. No. 107), pretreatment IV, 1 percent reducing sugar; temperature: 30° C.; aeration: 40 liters per minute, through 7 liters medium in 5-gallon bottles.

Table 4.--Effect of concentration of inoculum on sugar utilization and yeast yields

Concentration of inoculum (as dry weight)	Type of hydrolyzate and pretreatment	Volume of fermentation	Initial reducing sugar	Initial sugar fermented	Yeast yield (dry weight) on total sugar
Grams per liter			Percent	Percent	Percent
0.8	Spruce (Hyd. No. 4) untreated	100	1.8	15	0
2.4	Spruce (Hyd. No. 4) untreated	100	1.8	15	0
.8	Spruce (Hyd. No. 4) PT-II	100	1.7	15	0
2.4	Spruce (Hyd. No. 4) PT-II	100	1.7	69.9	24.8
.25	Spruce (composite Hyd. 18-21) PT-IV	7,000	1.6	91.5	39.2
.8	Spruce (composite Hyd. 18-21) PT-IV	7,000	1.6	92.7	40.2
1.14	Douglas-fir (Hyd. 107) PT-IV	7,000	1.0	73.1	27.1
1.56	Douglas-fir (Hyd. 107) PT-IV	7,000	.9	72.1	30.6
.8	Glucose-salts	100	2.0	99.8	34.0
2.4	Glucose-salts	100	2.0	99.8	31.0
.8	Molasses-salts	100	3.6	94.2	33.8
2.4	Molasses-salts	100	3.6	93.8	41.8

Table 5.--Range of yeast yields obtained on various hydrolyzates

Hydrolyzate	Pretreatments	Number of runs of all sizes	Sugars fermented	Yeast yield (dry) on total sugar
			Percent	Percent
Douglas-fir	II, III, IV	83	88 - 92	33 - 37
Spruce	II, III, IV	59	90 - 92	35 - 40
Southern yellow pine	II, III, IV	18	84 - 88	35 - 40
Eastern white pine	III, IV	5	87 - 89	30 - 35
Idaho white pine	III, IV	3	90 - 95	33 - 35
Ponderosa pine	III, IV	3	89 - 91	33 - 35
Sugar pine	III, IV	2	26	5
Redwood	III, IV	2	86 - 89	33 - 35
Western hemlock	III, IV	2	86 - 89	35 - 39
Western larch	III, IV	3	90 - 94	35 - 39
Maple	III, IV	3	90 - 94	39 - 42
Yellow birch	III, IV	2	89 - 93	37 - 42
American beech	III, IV	3	89 - 93	39 - 42

Organism: Torula utilis No. 3 (acclimatized)
Initial reducing sugar: Ca. 1 percent
Fermentation: 100 milliliters in 500-milliliter shake flasks, 30° C.

Table 6.--Comparison of ammonium hydroxide and urea as sources of nitrogen[1]

Nitrogen source		Initial reducing sugar	Sugar fermented	Yeast yield (dry weight) on total sugar
Compound	Concentration			
	Percent	Percent	Percent	Percent
NH_4OH	0.11	1.16	85.0	36.2
NH_4OH	.11	1.16	85.8	34.2
$(NH_2)_2CO$	.05	1.2	81.9	42.2
$(NH_2)_2CO$	.05	1.2	83.1	41.4
$(NH_2)_2CO$	.10	1.2	87.0	43.1
$(NH_2)_2CO$	.10	1.2	86.4	44.3
$(NH_2)_2CO$	.15	1.2	85.8	42.3
$(NH_2)_2CO$	.15	1.2	88.0	43.0

[1]Southern yellow pine Hyd. No. 25 bottle run.

Table 7.--Removal of toxic materials in Douglas-fir by adsorption

Treatment[1]	Volume of fermentation	Number of runs	Initial reducing sugar	Sugar fermented	Yeast yield (dry weight) on total sugar
	Milliliter		Percent	Percent	Percent
Norit (5 percent)	7,000	2	0.96	67.2	44.4
Control	7,000	1	1.06	65.6	29.5
Norit (1 percent)	7,000	2	1.23	75.0	36.5
Control	7,000	1	1.35	75.9	36.0
Norit (1 percent)	7,000	1	1.01	95.1	30.2
Lignin (1 percent)	7,000	1	.99	93.9	29.8
Control	7,000	1	.99	90.1	17.4
Norit (1 percent)	7,000	6	1.04	74.8	39.7
Control	7,000	4	1.09	76.4	30.5

[1]Norit and lignin added on volume basis, to 5 percent hydrolyzate.

Table 8.--Nitrogen uptake of yeast in fermentation[1]

Concentration of urea		Net yeast (dry)	N in yeast	Nitrogen accounted for	Nitrogen recovery in yeast	pH		Initial sugar utilized
Initial	Final					Initial	Final	
Grams per liter	Gram per liter	Grams per liter	Percent	Percent	Percent			Percent
0.34	0.00	3.74	4.18	96.0	96.0	5.0	6.13	90.0
.60	.08	Lost	6.57	(2)	(2)	5.0	6.20	90.0
.90	.11	3.59	8.75	86.1	75.0	5.0	6.50	89.0
1.2	.28	3.75	8.90	82.5	60.0	5.0	6.50	90.7

[1]Douglas-fir Hyd. No. 107, bottle run, 18 hours.

[2]Foamed over.

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Table 9.--Growth and sugar fermentation at various phosphate levels[1]

Phosphorus (from KH_2PO_4)	Volume of fermentation	Number of runs	Initial reducing sugar	Sugar fermented	Yeast yield (dry weight) on total sugar
Milligrams per cubic centimeter	Milliliter		Percent	Percent	Percent
1.02	100	2	1.22	90.2	36.6
.23	100	2	1.21	90.8	34.8
.11	100	2	1.22	91.5	34.8
.023	100	2	1.33	86.9	27.0
.011	100	2	1.22	82.1	24.6
1.02	7,000	1	1.09	88.9	34.3
.56	7,000	1	1.00	93.3	37.6
.34	7,000	1	1.03	87.1	36.8
.23	7,000	1	.93	93.8	40.0
.11	7,000	2	.99	91.4	38.7
.056	7,000	1	1.00	93.4	36.2

[1]Douglas-fir Hyd. No. 107, 24 hours.

Table 10.--Effect of temperature on yield[1]

Temperature of fermentation	Initial reducing sugar	Initial sugar fermented	Yeast yield (dry weight) on total sugar
°C.	Percent	Percent	Percent
25	1.62	92.0	32.4
30	1.66	93.0	36.0
35	1.67	92.0	32.4
40	1.67	87.5	25.8
45	1.65	53.0	1.3

[1]Spruce Hyd. No. 21, bottle run, 24 hours.

Table 11.--Effect of aeration on yield[1]

Volume of medium	Container	Aeration	Initial sugar fermented at 24 hours	Yeast (dry) on total sugar
Milliliters		Liters per minute	Percent	Percent
25	Erlenmeyer, 500 milliliters	(2)	80.6	33.0
50	Erlenmeyer, 500 milliliters	(2)	81.2	30.4
100	Erlenmeyer, 500 milliliters	(2)	76.6	27.9
150	Erlenmeyer, 500 milliliters	(2)	73.1	25.3
7,000	Bottle, 20 liters	50	90.0	35.8
7,000	Bottle, 20 liters	30	90.0	34.5
7,000	Bottle, 20 liters	15	88.3	30.6

[1]Spruce Hyd. No. 21, 24 hours.

[2]Shaken with no measurement of air.

Table 12.--Fermentation in pilot plant[1]

Sugar concentration, untreated hydrolyzate (percent)	5.57
Sugar concentration, treated and diluted hydrolyzate (percent)	1.01
Initial pH of treated hydrolyzate	4.5
pH during fermentation (adjusted with NaOH)	5.0-5.5
Volume of fermentation (liters)	300
Time of fermentation (hours)	18
Temperature of fermentation (° C.)	30-32
Sugar fermented at 18 hours (percent of total)	89.1
Yeast yield (dry) on total sugar (percent)	37.2
Nitrogen in yeast (percent)	6.86
Protein in yeast, N x 6.25 (percent)	42.9
Nitrogen recovered in yeast (percent)	92.2

[1]Wort: (Douglas-fir hydrolyzate No. 107, PT-IV),
Inoculum: Torula utilis No. 3, grown on rich medium, 1 gram (as dry cells) per liter of fermentation.

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