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Production of chemicals by the fermentation of the acid hydrolyzate of oat hulls

Morton Maurice Rayman
Iowa State College

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PRODUCTION OF CHEMICALS BY THE FERMENTATION OF
THE ACID HYDROLYZATE OF OAT HULLS

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MORTON M. RAYMAN

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Signature was redacted for privacy.

Head of Major Department

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Dean of Graduate College

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I INTRODUCTION

Many agricultural wastes or agricultural residues accumulate each year throughout the world. These wastes include straws, stalks, stems, hulls, cobs, shells, and pods. Some may be considered trade wastes, since they are derived from the processing of farm crops which have been transported to the factory and have been separated from the principal product there. Hence at the backyards of national industry there are amassed great heaps of such residues. The accumulation of these bulky residues has presented the industries with a serious problem.

Outstanding among the industries facing these problems are the processors of wood, sugar cane, cottonseeds, peanuts, rice, and oats. In each of these industrial arts there exists the problem of how best dispose of the respective wastes, namely: wood wastes, sugar cane bagasse, cottonseed hulls, peanut shells, rice hulls and oat hulls. It is with the last named raw material that this thesis is concerned.

Statistics on the annual production and availability of certain of the agricultural byproducts have been collected by the United States Department of Agriculture in a recent report on the proposed regional research laboratories (1). For the years 1931-35, inclusive, data are given as follows:

	Average annual production of dry product-tons	Estimated quantity of dry by-product available for industrial use-Tons.
Cottonseed hulls	1,165,000	583,000
Bagasse fiber	3,699,000	3,699,000
Rice hulls	182,000	182,000
Oat hulls	4,651,000	150,000

The question of how to dispose most economically of these residues has not yet been satisfactorily answered. Most frequently in the past these masses of materials have been burned at the factory as a fuel, and in some cases the ash produced could be employed as a fertilizer. Although the cellulosic wastes are not particularly good fuels, a tremendous tonnage can be disposed of by this practice. However, it is believed that these materials have greater potentialities chemically and that other modes of disposal are possible. One possible device for utilizing wastes, that of destructive distillation, has been discussed recently by Jacobs (2). A second channel for disposal of residues has been the technical production

(1) U. S. Senate Document No. 65 (1939) p. 51.
(2) Jacobs, P. B., Ind. Eng. Chem., 32, 214 (1940).

of furfural by a series of hydrolytic and dehydration procedures. Another method of approach is the possible fermentation of the wastes to produce a variety of utilizable chemicals (3).

These vegetable wastes are commonly termed "cellulosic agricultural materials". From the chemical point of view they are composed not entirely of cellulose. Usually they contain more than fifty percent of other substances, chiefly lignin and the hemicelluloses. It is with the hemicellulose fraction that we are here concerned. A typical analysis given for oat hulls by Bryner (4) is as follows:

Moisture	5.60%
Ether extract	2.02
Ash	7.32
Crude fiber	36.04
Lignin	20.69
Hydrolyzable to reducing sugars (calculated as xylose)	39.10

The term "hemicellulose" was introduced by Schulze (5) in 1891 to designate compounds extracted from plant materials which were either pentosans or hexosans or combinations of both. On hydrolysis such substances should yield only the simple sugars, pentoses and hexoses. In 1926 Miss O'Dwyer (6) found that so-called hemicellulose preparations contained

(3) Fulmer, E. I., Ind. Eng. Chem., 28, 206 (1936).
(4) Bryner, L. C., Christensen, L. M., and Fulmer, E. I. Ind. Eng. Chem., 28, 206 (1936).
(5) Schulze, E., Ber., 24, 2277 (1891).
(6) O'Dwyer, M. H., Biochem. J., 20, 656 (1926).

in addition to the pentose and hexose units still another class of substance: namely, uronic acids. The presence of these sugar acids has since been shown to be wide spread in the hemicelluloses from various sources. Some confusion exists in the breadth of the hemicellulose realm, and the members which have been admitted from time to time include the gums, pectins, mucilages, polyuronides, and the polymerized saccharides such as the pentosans and hexosans. The group has been defined by Norman (7) in 1937 as "those cell-wall polysaccharides which may be extracted from plant tissues by treatment with dilute alkalies, either cold or hot, but not with water, and which may be hydrolyzed to constituent sugar and sugar-acid units by boiling with hot dilute mineral acids."

Hemicelluloses were isolated from oat hulls by Anderson and Krznarich (8) in 1935. On hydrolysis they obtained the following cleavage products: d-xylose, l-arabinose, and a uronic acid compound that split into d-galactose and d-glucuronic acid. It was concluded that the hemicelluloses from oat hulls might be considered as a mixture of a xylan and a polyuronide. The polyuronide contained d-glucuronic acid combined with two molecules of d-galactose and with

(7) Norman, A. G., "The Biochemistry of Cellulose, the Polyuronides, Lignin, etc." London, H. Milford, Oxford Univ. Press, 1937.

(8) Anderson, E. and Krznarich, P. W., _J. Biol. Chem._, _111_, 549 (1935).

a series of molecules of l-arabinose.

Examination of xylans in the laboratories of Hirst and Haworth (9, 10) has been in progress for several years. In 1934 the English workers found that xylans contain a fairly constant proportion of l-arabinose units in conjunction with d-xylose units. They showed that one arabofuranose unit was associated with 18 to 20 xylopyranose units in xylans. Evidence was offered to indicate that the xylose units were joined by a _beta_-linkage.

Since oat hulls, when treated with proper concentrations of dilute mineral acids under suitable conditions, produce hydrolyzates which contain reducing sugars, largely pentoses, the idea suggested itself of using the hydrolyzates as media for microbiological growth and for fermentation of the carbohydrates to produce useful chemicals. The purpose of this investigation was to study the methods of preparing hydrolyzates with a view toward improving the media for fermentation. To determine the fermentability of these crude sugar solutions, a comparison could be made with solutions of pure xylose. On the basis of possible industrial application certain bacteria and molds were chosen for study. The procedure should offer promise for application to other material of similar composition and to

(9) Hampton, H. A., Haworth, W. N., and Hirst, E. L., _J. Chem. Soc._, _1929_, 1739.
(10) Haworth, W. N., Hirst, E. L., and Oliver, E., _J. Chem. Soc._, _1934_, 1917.

other micro-organisms.

II HISTORICAL

A. Xylans

Xylan is somewhat of a generic term for a polysaccharide which hydrolyzes to yield chiefly the pentose xylose. Depending to a certain extent on their vegetable origin, various xylan preparations may differ among themselves. Xylan was first isolated in 1846 by Poumarede and Figuier (11) who extracted wood with alkali and precipitated the gum with acid and alcohol. The history of xylan and of xylose has been reviewed by Harding (12). According to Harding, xylese itself was first prepared by Friedrich Koch in 1886. Koch's procedure was essentially the same as the method used today. Modern techniques have since, of course, modified the details in order to secure the greatest possible yield of the sugar from the best possible source. Koch's procedure involved a sulfuric acid hydrolysis of wood gum. This was followed by neutralization with baryta, clarification with charcoal, and concentration to a thick sirup. The filtrate from an alcohol

(11) Poumarède and Figuier, L., J.pharm. chim.,(III) 12, 81, (1847).
(12) Harding, T. S., Sugar, 25, 124 (1923).

precipitation of gums was then concentrated and the crystallization was allowed to proceed during a few days. The new sugar Koch termed "holzzucker" but was soon renamed xylose. In 1891 Bertrand (13) prepared xylose by a direct hydrolysis of oat straw, instead of first going through the intermediate isolation of the xylan by an alkaline extraction. Harding calls attention to this work as the first instance in which a respectable yield, 4% yield of the sugar from the raw material, was obtained, and also the first instance in which a direct hydrolysis was used efficiently.

Xylan is widely distributed in the plant kingdom. It is found in most straws, husks, woods, grains, and seeds. According to Harding (12), xylose has been obtained from the following materials: brewers' grains, corncobs, jute, apricot seeds, beechwood, hay, flax, oat and wheat straws, Psyllium gallicum, the alkaline liquor of the paper industry, oat hulls, and cottonseed hulls. The sugar has also been reported to have been found in the following: sunflower seed husks (14), birch and aspen wood (15), white

(13) Bertrand, M. G., Bull. Soc. Chim., (III), 5, 555 (1891).
(14) Plyushkin, E.Z. and Clutverikov, N.M., J. Applied Chem. (U.S.S.R., 7, 1008 (1934). /C.A. 29, 5418 (1935)/.
(15) Mirlis, D.I. and Gorokholinskaya, M.S., J. Chem. Ind. (Moscow), 12, 56 (1935). /C.A., 29, 1813 (1935)/.

spruce (16), fir and pine woods (17), bamboo (18), bagasse (19), cornstalks (20), the straws of rice (21) and rye (22), peanut hulls (23), pecan shells (24), and recently tobacco stalks (25).

B. Hydrolysis of Hemicellulosic Materials

Because of the ubiquitous presence of xylose in plant tissues, there exists quite an extensive literature on the preparation, and on the occurrence of the sugar in many raw materials. Necessarily the problems of saccharification of cellulose (Bergius process) (26), as well as the pulping of wood for paper have given rise to a closely related literature. While the procedures are, in general,

(16) Sherrard, E. C., and Blanco, G. W., Ind. Eng. Chem., 15, 611 (1923).
(17) Allgeier, R. J., Peterson, W. H., and Fred, E. B., Ind. Eng. Chem., 21, 1039 (1929).
(18) Runkel, R., German patent 576,498 (1933) [C.A., 28, 893 (1934)].
(19) Sherrard, E. C. and Blanco, G. W., Ind. Eng. Chem., 12, 1160 (1920).
(20) Peterson, C. J., Doctoral Dissertation, Iowa State College (1930).
(21) Deshpande, D. D., J. Indian Inst. Sci., 13A, 93 (1930) [C. A., 24, 4890 (1930)] II.
(22) Suida, H., Sadler, H., and Noss, F., Papier. Fabr., 28, 345 (1930) [C. A., 24, 4389 (1930)]
(23) de Belsunce, G., Bull. mat. grasses, 1926, 1 [C. A., 20, 2230 (1926)]
(24) Thor, C. J. B. and Smith, C. L., J. Am. Chem. Soc. 56, 1640 (1934).
(25) Bennett, E., Ind. Eng. Chem., 29, 933 (1937).
(26) Bergius, F., Ind. Eng. Chem., 29, 247 (1937).

quite similar, nevertheless the success of any given developed process is dependent on specific modifications and details. In the case of the Bergius process for the saccharification of cellulose, for example, much of its success has been dependent upon the creation of equipment such as acid-resistant digesters and diffusion batteries.

Among the many factors involved in the hydrolysis of plant tissues are: (1) the type of plant tissue, (2) the choice of hydrolyzing agent (sulfuric acid, sodium hydroxide, etc.), (3) the temperature, (4) the concentration of the hydrolyzant, and (5) the time of hydrolysis.

The sugars from cellulosic materials are commonly obtained by treating the plant tissues with hot dilute mineral acids. Such a treatment will produce a solution of the sugars separable from the cellulose and lignin. Under more drastic conditions it is possible to cleave the cellulose into dextrose units and to leave principally lignin. With this treatment some of the pentoses are degraded, but the increased production of hexoses from cellulose tends to offset this loss. In the alkaline pulping of woods and straws for the manufacture of paper, the hemicelluloses and lignins are extracted while the cellulose is left intact.

The degradation of hemicelluloses by an enzymic cleavage may be considered a valuable aid in the destruction of straws in the soil, but the process of rotting of straws by micro-organisms is usually too slow to be of much industrial importance (except in retting).

The present paper will consider only the mild acid hydrolysis of the hemicelluloses.

One of the limiting factors in the hydrolysis of pentosans is the temperature. Lander (27) showed that xylose solutions were less resistant to destruction than dextrose in dilute sulfuric acid at various temperatures in the range from 120 to 142 degrees centigrade. If a xylose solution (0.1%) were heated with 0.18% sulfuric acid at 142° C., the sugar was destroyed in less than thirty minutes. Under the same conditions dextrose could be exposed safely for three hours. Kressman (28) pursued the subject further to learn some of the limits for dextrose. These solutions in 0.1% sulfuric acid were stable at 150° C. for fifteen minutes, were decomposed but slightly at 175°, but were largely destroyed at 185° C. The relationships existing between the conditions of temperature, time, and concentration of hydrolyzant are all so intimate

(27) Great Britain Dept. of Scientific and Industrial Research. Fuel Research Board. Fuel for motor transport. Fourth Memo. 1927.

(28) Kressman, F. W., U.S.D.A. Bull. 983 (1922).

that a study cannot be interpreted properly if cognizance is not taken of these factors simultaneously.

Kressman (28) in 1922 reported one of the first systematic studies on the mild acid hydrolysis of the hemicelluloses of plant products. His work was concerned with the production of sugars from wood wastes for the purpose of fermentation to alcohol with yeast. He determined conditions which would give optimum yields of sugars and conditions which would give optimum yields of alcohols. The factors which he varied were: the sulfuric acid concentration, the liquid-solid ratio, the temperature, and the time of hydrolysis.

In 1930 Hall, Slater, and Acree (29) of the U. S. Bureau of Standards developed a method for the production of crystalline xylose from cottonseed hulls. This process, conducted on a semi-commercial scale in Alabama, was reported by Schreiber, Geib, Wingfield, and Acree (30). An outstanding feature of their procedure is the fact that no alcohol is needed for the crystallization. The essential steps include: (1) a hot water extraction of the cottonseed hulls to remove the gums, which interfere with the crystallization; (2) a cold dilute sulfuric acid

(29) Hall, W. L., Slater, C. S., and Acree, S. F., Bur. Standards J. Research, 4, 329 (1930).
(30) Schreiber, W. T., Geib, N. V., Wingfield, B., and Acree, S. F., Ind. Eng. Chem., 22, 497 (1930).

extraction to remove the inorganic salts and minimize the ash content; (3) an autoclaving with dilute sulfuric acid and, after neutralization of the hydrolyzate, clarification and concentration. After crystallization a separation from the mother liquor was effected by centrifugation.

Bryner (4) in 1934 conducted a study on the hydrolysis of oat hulls with dilute hydrochloric acid. By systematically varying the temperature, and concentration, he obtained values for the optimum conditions for producing sugars from oat hulls.

Recent papers related to the hemicellulose extractions have been published by Yanovsky (31) in 1939, and by Vilbrandt, Mather, and Dicks (32) in 1940. Yanovsky's work was a study of the course of extraction of hemicelluloses by both dilute acid and alkaline solutions from rice hulls, peanut shells, and beet pulp. The investigation by Vilbrandt and co-workers on peanut hulls included measurements of the rate of hydrolysis with dilute sulfuric acid of varying concentrations at temperatures below 100° C.

(31) Yanovsky, E., Ind. Eng. Chem., 31, 95 (1939).
Ibid., 31., 1245 (1939).
(32) Vilbrandt, F. C., Mather, C. B., and Dicks, R. S., Ibid., 32, 169 (1940).

C. Xylose Fermentations

That little attention prior to 1920 had been paid to the fermentation of xylose is evident from a survey of the literature. A compilation of relationships between substrates, organisms, and fermentation products by Fulmer and Werkman (33) in 1930 lists only five micro-organisms which were employed in quantitative studies on xylose prior to 1920. In Table I are listed these organisms along with others which have since been shown to ferment xylose and for which products have been isolated from the fermentations. In all cases the names of the organisms are those used by the authors cited. Since present-day taxonomy will consider some of the older terms untenable, the possible synonyms used by Bergey's latest manual of determinative bacteriology (34) have been included in the table for convenience.

(33) Fulmer, E. I., and Werkman, C. H., "An index to the chemical action of micro-organisms on the non-nitrogemous organic compounds", The Williams and Wilkins Co., Baltimore. 1930.

(34) Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P., "Bergey's Manual of Determinative Bacteriology", 5th Ed., The Williams and Wilkins Co., Baltimore. 1939.

Table I.

Xylose-fermenting Micro-organisms

Name of organism	Products formed	Reference
Clostridium acetobutylicum	butanol, ethanol, acetone, butyric and acetic acids, carbon dioxide, and hydrogen	(35)
Clostridium thermosaccharolyticum	butyric, acetic, and lactic acids, carbon dioxide and hydrogen	(36)
Bacillus vulgatus * (*Bacillus mesentericus*)	acetone, ethanol, and carbon dioxide	(37)
Bacillus acetoethylicum (*Bacillus macerans*)	acetone, ethanol, formic acid	(38)
Bacillus herbicola aureum (*Pseudomonas trifolii*)	acetone, ethanol, carbon dioxide	(37)
Aerobacillus polymyxa (*Bacillus polymyxa*)	2,3-butylene glycol, ethanol, acetylmethylcarbinol, formic, acetic, succinic, and lactic acids, carbon dioxide, and hydrogen	(39)
Bacillus lactis aerogenes (*Aerobacter aerogenes*)	formic, acetic, butyric, succinic, and lactic acids, ethanol	(40)

* Where the name cited by the author does not coincide with the term used by Bergey, the name proposed is included in parentheses.

Table I continued

Aerobacter faeni (*Aerobacter aerogenes*)	formic, acetic, succinic, and lactic acids, acetylmethylcarbinol, 2,3-butylene glycol, ethanol, carbon dioxide and hydrogen	(41)
Aerobacter indologenes (*Aerobacter cloacae*)	formic, acetic, and succinic acids, 2,3-butylene glycol, ethanol, acetylmethylcarbinol, carbon dioxide, and hydrogen	(42)
Bacillus paratyphoid (*Salmonella schottmuelleri*)	formic, acetic, butyric, lactic, and succinic acids, and ethanol	(40)
Bacillus typhosus (*Eberthella typhosa*)	formic, acetic, butyric, lactic, and succinic acids, and ethanol	(40)
Friedlander's pneumobacillus (*Klebsiella pneumoniae*)	acetic, succinic, and lactic acids, ethanol	(43)
Escherichia coli	formic, acetic, lactic, and succinic acids, ethanol, carbon dioxide, and hydrogen.	(42)
Citrobacter anindolicum (*Escherichia freundii*)	formic, acetic, lactic, and succinic acids, ethanol, carbon dioxide, and hydrogen	(44)
Propionibacterium pentosaceum	propionic and acetic acids, carbon dioxide	(45)
Lactobacillus pentoaceticus (*Lactobacillus brevis*)	lactic and acetic acids	(46)
Lactobacillus pentosus (*Lactobacillus plantarum*)	lactic and acetic acids	(46)
Acetobacter xylinum	acetone, ethanol, and carbon dioxide	(37)

Table I continued

sorbose bacterium (*Acetobacter xylinum*)	xylonic acid	(47)
Bacterium prodigiosus (*Serratia marcescens*)	ascorbic acid	(48)
Oöspora No. 208	ethanol, glycerol, and carbon dioxide	(49)
Saccharomyces cerevisiae	ethanol, carbon dioxide, glyceric aldehyde	(50)
Penicillium luteum purpurogenum	xylonic acid	(51)
Aspergillus flavus	kojic acid	(52)
Aspergillus tamarii	kojic acid	(53)
Aspergillus niger	oxalic acid, citric acid	(54)

Reference List for Table I.

(35) Underkofler, L. A., Christensen, L. M, and Fulmer, E.I., Ind. Eng. Chem., 28, 350 (1936); Underkofler, L. A., Doctoral Dissertation, Iowa State College, 1934.

(36) Sjolander, N. O., McCoy, E., and McClung, L. S., J. Bact., 33, 102 (1937).

(37) Fred, E. B., Peterson, W. H., and Anderson, J. A., Ibid., 8, 277 (1923).

(38) Northrop, J. H., Ashe, L. H., and Senior, J. K., J. Biol. Chem., 39,1, (1919).

(39) Stahly, G. L.,Iowa State Coll. J. Sci., 11, 110 (1936).

(40) Fred, E. B., and Peterson, W. H., J. Infect. Dis., 27, 539 (1920).

(41) Breden, C. R., Fulmer, E. I., Werkman, C. H., and Hixon, R. M., J. Bact., 19, 41 (1930).

(42) Reynolds, H., Doctoral Dissertation, Iowa State College 1935.

(43) Grimbert, M. L., Compt. rend. soc. biol., 48, 192 (1896).

(44) Osburn, O. L., and Werkman, C. H., Proc. Iowa Acad. Sci., 39, 134 (1932).

(45) Werkman, C. H., Hixon, R. M., Fulmer, E. I., and Rayburn, C. H., Ibid., 36, III (1929).

(46) Fred, E. B., Peterson, W. H., and Anderson, J. A., J. Biol. Chem., 48, 385 (1921).

(47) Bertrand, G., Compt. rend., 127, 124 (1898).

(48) Berencsi, G., and Ilenyi, A., Biochem. Z., 298, 298 (1938).

(49) Plevako, E. A., and Altovskaya, N. I., Schriften. zentral. biochem. Forschunginst. Nahr.-Genussmittelind. (Moscow) 2, 212 (1932) /C. A., 27, 5471 (1933)/.

(50) Abbott, O. D., Missouri Agr. Expt. Sta. Res. Bull. 85, 1 (1926).

(51) Angeletti, A., and Cerutti, C. F., Ann. chim. applicata, 21, 491 (1931).

(52) Barham, H. N., and Smits, B. L., Ind. Eng. Chem., 28, 567 (1936).

(53) Gould, B. S., Biochem. J., 32, 797 (1938).

(54) Bernhauer, K., Biochem. Z., 197, 309 (1928).

D. Fermentation of Hydrolyzates Prepared from Hemicellulosic Wastes.

At least three types of fermentations for utilizing the hemicellulose fraction of vegetable wastes have been studied. One of these types produced lactic and acetic acids, another produced acetone and ethanol, and the third produced butyl alcohol and acetone.

For the production of lactic and acetic acid, workers at the University of Wisconsin studied the fermentation by Lactobacillus pentoaceticus of sirups produced from hydrolyzed peanut hulls, oat hulls, corncobs, and sawdusts of fir, spruce, and pine. In 1921 Peterson and Fred (55) obtained 30.9 grams of sugars, principally xylose, by hydrolysis of 100 grams of corncobs. The solutions were fermented almost quantitatively to acetic and lactic acids. When a 2% sugar solution was fermented for two weeks at 30° C., there were produced 13.8 grams of lactic and 12.6 grams of acetic acid. These yields were obtained from a total of 30.9 grams of sugar, which was equivalent to 100 grams of corn cobs.

In 1923 Fred, Peterson, and Anderson, (37) tried, less successfully, the lactic-acetic fermentation with oat hulls and peanut hulls. The fermentations were slow and

(55) Peterson, W. H., and Fred, E. B., Ind. Eng. Chem., 13, 211 (1921).

even after thirty days were incomplete.

Sugar liquors prepared by extractions of sawdust of fir, spruce, and pine using a mild acid hydrolysis, on the one hand, and a Bergius treatment, on the other, were fermented by Allgeier, Peterson, and Fred (17) in 1929. A comparison of the fermentabilities of the two types of sirups was described. They indicated that by the dilute sulfuric acid treatment there could be produced from a ton of wood 400 pounds of sugar, of which 340 pounds would be fermented to yield 316 pounds of lactic and 17 pounds of acetic acids. For the Bergius product a ton of wood would liberate 1200 pounds of sugar, of which 1020 pounds could be fermented to give 950 pounds of lactic acid and 50 pounds of acetic acid.

The use of Bacillus acetoethylicum to convert hydrolyzed hemicelluloses into ethanol and acetone offered some encouragement to industrial application. Employing this organism, Northrop, Ashe, and Morgan (56) developed a process for the fermentation of molasses. Peterson, Fred, and Verhulst (57) investigated the fermentation of sirups produced from corncob hydrolyzates. Approximately 90% of

(56) Northrop, J. H., Ashe, L. H., and Morgan, R. R., Ind. Eng. Chem., 11, 723 (1919).
(57) Peterson, W. H., Fred, E. B., and Verhulst, J. H., Ind. Eng. Chem., 13, 757 (1921).

the sugars in the medium were fermented, and yields of acetone, alcohol, and volatile acids (formic and acetic) were respectively, 12.6, 31.5, and 15.0% of the sugars fermented. Thus, from a hundred pounds of cobs there could be produced 2.7 pounds of acetone, 6.8 pounds of ethanol, and 3.4 pounds of a mixture of formic and acetic acids.

Fred, Peterson, and Anderson (37) examined the hydrolyzates from oat hulls and peanut hulls as possible substrates for _B. acetoethylicum_ and obtained normal yields of the usual products. After ten days of fermentation acetone 3.9%, ethanol 7.2%, and volatile acids 1.4% of the weight of the oat hulls were obtained. The yield of only 7.6% of sugars from peanut hulls compared unfavorably with the 26.5% yield from oat hulls. However, from the standpoint of the sugars available either raw material was equally fermentable.

In India Patwardhan (58) prepared a rice straw sirup which in a 4% sugar concentration was fermented by _B. acetoethylicus_ to yield a mixture of acetone and ethanol. The yield of the mixed solvents amounted to 20.5% of the sugar consumed. From a host of grasses and straws Thaysen

(58) Patwardhan, V. N., _J. Indian Chem. Soc._, _7_, 531 (1930).

and Galloway (59) prepared sirups which they fermented with _B. acetoethylicus_ to give good yields based on the sugar available.

Work in 1933 by Weinstein and Rettger (60) showed that the butyl-acetonic fermentation was applicable to solutions of sugars from hydrolyzed cottonseed hulls, peanut husks, sawdust of several woods, and corncobs. The woods included Douglas fir, white pine, maple, birch, oak, and chestnut. Fermentations of 3% sugar solutions from the hydrolysis of the various natural tissues were studied. In a semi-synthetic medium which contained, in addition to the carbohydrate, both the mono- and the di-potassium phosphates and also 1% peptone, yields of acetone similar to those in the corn mash control were obtained. While the amount of butyl alcohol was normal in the corn mash control, yet in the hydrolyzate medium it was negligible. These investigators showed that by the addition of prolamine or prolamine-allied substances the butanol production was stimulated to a normal level in semi-synthetic media. By supplying zein to solutions containing pure sugars, normal butyl-acetonic fermentations were carried out. The addition of zein or corn,

(59) Thaysen, A. C., and Galloway, L. D., _Ann. Appl. Biol._, **15**, 392 (1928).
(60) Weinstein, L., and Rettger, L. F., _J. Bact._, **25**, 201 (1933).

which contains zein, to the hydrolyzates gave fermentations with normal yields.

After surveying the organisms capable of fermenting xylose, it was possible to select from among the group certain bacteria and molds for a study of their adaptability to fermenting the sugars in oat hull hydrolyzates. For the investigation reported in this thesis two species of bacteria and two of molds were employed.

The activities of the butyl-acetonic organism have occupied a leading place in the fermentation industries. The organism belongs to the group of butyric acid-butyl alcohol bacteria. Brown (61) reviewed the literature and worked out a systematic classification for the group. Studies on the fermentation of pure xylose were described by Underkofler (35) in 1934. Guymon (62) examined the relation of structure of sugars to the chemism of the butyl-acetonic fermentation. Although the nutrition of Clostridium acetobutylicum has been investigated in some detail, the problem remains unfinished. From reports in recent years the presence of certain growth factors necessary in small concentrations seems indicated.

(61) Brown, R. W., Doctoral Dissertation, Iowa State College, 1936.

(62) Guymon, J. F., Doctoral Dissertation, Iowa State College, 1939.

Weinstein and Rettger (60) believed that prolamines or prolamine-allied substances were necessary for the production of butanol. Reynolds, Coile, and Werkman (63) were not able to confirm the report that prolamine was essential for the production of butanol by Cl. acetobutylicum in 3% glucose media. Researches by Weizmann and Rosenfeld (64) and by workers at the University of Wisconsin (65) have emphasized the idea of bacterial growth factors and of their effectiveness in very dilute solutions.

Another bacterium chosen for this investigation was Aerobacter aerogenes. Breden (41) showed that members of this genus produced 2,3-butylene glycol and ethanol in good yields from sugars. The culture used by Breden and termed Aerobacter faeni was isolated from hay infusion and identified by Burkey (66). The organism which Reynolds (42) used for his studies was Aerobacter indologenes. Reynolds investigated the dissimilation of

(63) Reynolds, H., Coile, H. D., and Werkman, C. H., Iowa State Coll. J. Sci., 8, 415 (1932).
(64) Weizmann, C., and Rosenfeld, B., Biochem. J., 31, 619 (1937).
(65) Peterson, W. H., and co-workers, J. Bact., 27, 207 (1934); J. Biol. Chem. 131, 381 (1939).
(66) Burkey, L. A., Iowa State College J. Sci., 3, 57 (1928).

xylose by *Aerobacter indologenes* and quantitatively determined the products formed during the course of the fermentation. A 2% xylose solution was fermented for 7 days; and the main products were 17.1% ethanol, 26.4% 2,3-butylene glycol, 33% carbon dioxide based on the weight of the sugar fermented.

Of the molds which utilize xylose *Aspergillus flavus* offered some promise. Employing *A. flavus* Corbellini and Gregorini (67) first reported the production of kojic acid from xylose. An improved medium to increase the yield of kojic acid was developed by Barham and Smits (52). The literature pertaining to the production of kojic acid has been reviewed in detail by Barham and Smits (68).

Fermentation by *Penicillium chrysogenum* for the production of gluconic acid from glucose, reported by Moyer, May, and Herrick (69) in 1936, indicated that this mold was the most suitable of the molds tested, which included also *P. citrinum*, *P. variablis*, *P. luteum purpurogenum*, and *P. baculatum*. The organism,

(67) Corbellini, A., and Gregorini, B., *Gaz. chim. ital.*, *60*, 244 (1930).
(68) Burham, H. N. and Smits, B. L., *Trans. Kansas Acad. Sci.*, *37*, 91 (1934).
(69) Moyer, A. J., May, O. E., and Herrick, H. T. *Zentr. Bakt. Parasitenk.*, *II* Abt., *95*, 311 (1936).

described by Raistrick et al, (70) was isolated from molded tobacco. Since gluconic acid was produced from glucose by _P_. _chrysogenum_, by analogy it seemed possible that this mold might convert xylose to xylonic acid.

(70) Raistrick, H., et al., _Phil. Trans. Roy. Soc_. _London_, _220_ _B_, 1 (1931).

III METHODS

Oat hulls were hydrolyzed by autoclaving with dilute mineral acids under specific conditions. Since the details of the procedures were modified for the individual experiments these procedures will be described in connection with the experimental work.

The determination of reducing sugars in the hydrolyzates was made by a modification of the Shaffer-Somogyi method (70a), which was developed in this laboratory and described by Guymon (62). The reducing value for pure xylose solutions was measured and from this a calibration curve was prepared.

For each type of fermentation the chemical and bacteriological methods used were different. The particular methods employed will be described along with the description of the experimental work.

(70a) Somogyi, M., J. Biol. Chem., 119, 741 (1937).

IV EXPERIMENTAL RESULTS

A. Preparation of Hydrolyzates

1. General methods.

The air-cleaned oat hulls used were furnished through the kindness of the Quaker Oats Company. The concentrations of acids employed in preparing the hydrolyzates for the fermentation experiments were those which had been found by preliminary experiments to be optimum, at the temperature used, in order to obtain a concentration of about 4 per cent reducing sugars in the hydrolyzates. Although the general procedure has been reported before (71), a typical preparation will be described here.

Into a 6-liter Erlenmeyer flask were placed 600 grams of air-dried oat hulls and 3600 ml. of acid. After standing for 2-4 hours, the flask was heated in an autoclave at 20 pounds per square inch steam pressure for 60 minutes. At the end of the hour the steam was shut off, and compressed air was introduced at the same pressure to prevent the liquid in the flask from boiling over because of a too rapid drop in pressure. The contents of the flask were then cooled below the atmospheric boiling point by

(71) Underkofler, L. A., Fulmer, E. E., and Rayman, M. M. Ind. Eng. Chem., 29, 1290 (1937).

gradually lowering the pressure during the course of 20 minutes and finally, after removal from the autoclave, cooled thoroughly in running water. The hydrolyzate was separated from the solid oat hull residue by straining through cheesecloth supported on a Büchner funnel, and then by squeezing the residue dry in a laboratory press. The combined liquors were filtered through paper and the acid filtrate was neutralized with calcium carbonate. To avoid too vigorous frothing and foaming, the calcium carbonate was added cautiously in small portions with effective stirring. Adjustment of pH to approximately 6.0 was usually achieved by the addition of a sodium hydroxide solution and the pH measurements were made with the aid of a glass electrode. The solution after filtration contained approximately 4 to 5 per cent reducing sugars calculated as xylose. This filtrate was ordinarily concentrated _in vacuo_ at 50-55° C. to a more dense solution from which calcium sulfate could be separated out when hot. In order to ferment the carbohydrates, the sirups were diluted to the proper concentration, and, after the nutrients had been added, the medium was sterilized in the autoclave and was ready for inoculation.

After the work had been in progress for a period of time, attempts were made to improve the medium by preparing the hydrolyzates under somewhat modified conditions. The work reported by Bryner, Christensen and Fulmer (4) had been confined to the use of hydrochloric acid as an hydrolytic agent. Studies with other mineral acids in hydrolysis procedures were made to determine the optimum conditions for each acid in hydrolyzing oat hulls.

2. <u>Rates of hydrolysis of oat hulls with various mineral acids</u>.

The acids selected for comparison with hydrochloric acid were as follows: nitric, sulfuric, and phosphoric. The degree of conversion to sugar was determined for varying concentrations of the various acids by the following method:

Into 150 ml. Erlenmeyer flasks were introduced 5.00 grams of oat hulls and 30.0 ml. of the acid. The mixtures were autoclaved at 20 pounds steam pressure for the chosen times and the same type of cooling under air pressure as described above was employed. The cooled mixtures were diluted somewhat with water, filtered, and the residues were repeatedly extracted with boiling water. The combined filtrate and washings were neutralized to phenolphthalein with sodium hydroxide and diluted to 250 ml. Aliquots were taken for sugar analysis and the yields were calculated as xylose expressed in grams per 100 grams of air-dried hulls. Results for these experiments are shown in Table II.

Table II.

Reducing Sugar Production from Oat Hulls Heated at 20 Lbs. Steam Pressure with Dilute Acids Using a Liquid to Solid Ratio of 6 to 1

Time Heated (min.)	Reducing Sugar Yield, % of Hulls					
	Hydrochloric Acid		Nitric Acid		Sulfuric Acid	
	0.0796 N	0.1050 N	0.0756 N	0.153 N	0.195 N	1.986 N
45	28.5	31.5	26.5	35.5	31.0	32.5
60	28.5	32.0	29.0	34.5	32.5	31.0
90	30.0	33.5	28.5	34.5	32.5	30.5
120	32.5	30.5	32.5	35.0	33.0	25.5

Time Heated (min.)	Reducing Sugar Yield, % of Hulls			
	Phosphoric Acid			
	0.092 N	0.184 N	0.372 N	0.747 N
45	1.50	3.16	9.8	22.0
60	1.75	4.15	13.2	26.0
90	2.00	6.25	17.5	----
120	2.78	8.50	21.5	30.5

3. Attempts at purification of the hydrolyzates.

During the course of the experiments on the crude hydrolyzates it was suspected that a factor toxic or inhibitory to the organism was present. Attempts to remove this factor by physico-chemical methods were considered. Methods attempted involved adsorption, extraction with immiscible solvents, centrifugation, and fractional precipitation by control of pH as described below.

The adsorption agents used were norit and diatomaceous earth. If employed directly on the crude hydrolyzate, neither of these was effective either in decolorizing the liquors or in removing factors which prevented fermentation from proceeding normally.

Attempts at control of pH to allow fractional precipitation of substances in the hydrolyzates were effective in throwing out insoluble substances, but fermentations did not seem improved. The use of basic lead acetate gave somewhat encouraging results from the point of view of producing clear colorless liquors. The lead was removed with hydrogen sulphide after the gums and proteins were precipitated. The hydrogen sulphide was then removed by vacuum evaporation to one-half to one-third the volume. Norit treatment removed color and the sirups were sterilized for preparing the media.

Preliminary experiments with certain organic solvents immiscible with water indicated that chloroform was capable of extracting a soluble material from hydrolyzates, ethyl ether was slightly less efficient, and petroleum ether and benzene were ineffective. Hence, an apparatus similar to one described by Wollner and Matchett (72) in 1938 was devised for extraction of the liquor with chloroform. The syrup was extracted continuously for five days and the dissolved chloroform was removed by evaporating the syrup _in vacuo_ to one-half of its volume. The individual fermentation experiments in which these extracted hydrolyzates were used will be described later.

While standing in the laboratory, the hydrolyzates, originally almost colorless, often turned dark, passing through yellow and red to black. The black color was apparently due to particles of colloidal nature which were difficult to remove. When the solution was repeatedly sent through a Sharples supercentrifuge at 21,000 r.p.m., it became turbid and foamy. However, very little solid material was thrown out. It was thought that perhaps the air whipped into the solution had caused some oxidation, and that dark-colored

(72) Wollner, H. J., and Matchett, J. R., _Ind. Eng. Chem._, _Anal. Ed._, _10_, 31 (1938).

oxidized pigments had been formed.

If this were so, then perhaps by aerating warm solutions of the hydrolyzates the oxidation could be speeded up and the materials thereby removed. Hence experiments were devised in which air was bubbled for several hours through the warmed (60° C.) solutions. A very stable foam was produced which could be readily separated from the liquid. The foam was skimmed off from time to time during the aeration, but little improvement in the fermentability was detected.

B. The Butyl-Acetonic Fermentation of Oat Hull Hydrolyzates

1. Methods.

The culture used was one which had been originally isolated in the laboratories of biophysical chemistry at Iowa State College. It was handled in the manner usual for butyl-acetonic cultures, as outlined by Underkofler, Christensen, and Fulmer (35). After completion of experimental fermentations (3 to 7 days) two 250-ml. aliquots of the beer were measured from each flask and distilled after a little solid calcium carbonate had been added to neutralize the acids. In each case 100 ml.

of distillate were collected, and the distillates were analyzed for solvents by the method of Christensen and Fulmer (73). Volatile acids were estimated by a combination of the methods of Knetemann (74) and of Virtanen and Pulkki (75). The manner of manipulation was the Knetemann modification of the Duclaux distillation; the method of calculation of the relative amounts of volatile acids was that of Virtanen and Pulkki.

2. Fermentation of corn meal-oat hull hydrolyzate mixtures.

Since corn mash is the most favorable medium for development of the butyl-acetone organism, the experimental fermentation media were prepared by mixing oat hull hydrolyzate with corn mash. Ground yellow corn, in quantities calculated to make a total carbohydrate content equivalent to 6.5 per cent corn mash (on the dry basis) when the hydrolyzate liquor was added, was mixed with tap water in 4-liter Erlenmeyer flasks and then steamed for 45 minutes. The amount of water used in each case was sufficient so that when mixed with hydrolyzate

(73) Christensen, L. M., and Fulmer, E. I., Ind. Eng. Chem., Anal. Ed., 7, 180 (1935).
(74) Knetemann, A., Rec. trav. chim., 47, 950 (1928).
(75) Virtanen, A. I., and Pulkki, L., J. Am. Chem. Soc., 50, 3131 (1928).

the final volume of medium in each experimental flask would be about 3000 ml. The flasks were plugged with cotton, capped, and sterilized at 20 pounds steam pressure for 2 hours. After cooling the sterilized mash, it was allowed to come to incubator temperature (37° C.) The requisite amounts of sterile hydrolyzate at 37° C. were added aseptically to the individual flasks of corn mash.

A series of hydrolyzates was prepared by using different acids to determine the effect of different hydrolyzants on the fermentation. The data are presented in Tables III and IV.

Table III.

Solvent Yields from Acid Hydrolyzates

Corn Meal Replaced Per Cent	Total Solvent Yield Wt. % of carbohydrate	Solvent Ratio BuOH	Me_2CO	EtOH
	0.08 N Hydrochloric Acid			
0	33.4	63	25	12
10	34.4	67	27	6
20	31.0	58	33	9
30	30.0	59	32	9
40	30.7	56	33	11
50	19.2	57	33	10
60	2.7	--	--	--
	0.16 N Sulfuric Acid			
0	34.0	58	29	13
10	34.2	56	31	13
20	32.3	60	33	7
30	30.7	60	33	7
40	23.2*	65	34	1
50	30.5	55	35	10
60	5.9	53	28	19
	0.08 N Nitric Acid			
0	34.0	58	29	13
10	32.4	54	31	15
20	32.4	56	31	13
30	18.8	62	27	11
40	17.0	58	29	13
50	4.9	49	24	27

* Abnormal fermentation.

The hydrolyzate prepared with phosphoric acid was used in a medium of slightly different composition. Since the previous series of media were devised to maintain a constant carbohydrate level while the protein concentration was neglected, in this experiment it was deemed feasible to add definite quantities of corn gluten to preserve a constant protein level equivalent to that of standard corn mash. The corn gluten was added to the ground corn before sterilization. For this experiment 2-liter Erlenmeyer flasks were used, and the final volume of the medium was about 1500 ml. The medium was made up in essentially the same manner as described for the series with hydrolyzates of the other acids with the difference noted. Analysis of the corn and gluten showed 7.5% and 31.2%, respectively, of protein on the wet basis. Since standard corn mash contained 7.85 g. of protein in 1500 ml., it was necessary to add gluten to replace ground corn, so that the total protein was 7.85 g. per flask.

To determine whether norit was capable of removing an inhibiting substance, one half the number of flasks contained a medium in which the hydrolyzate had been previously treated with norit; the other half, as a control, contained hydrolyzate which had had no norit treatment.

Half of the acid solution after hydrolysis was treated with 75 g. of norit over night, filtered, neutralized with calcium carbonate and skimmed after each portion of calcium carbonate was added. The pH was adjusted to 4.2 by the addition of sodium hydroxide and again norit (10 g. per L.) was added. Although the first norit treatment gave little or no decolorization, the second treatment at pH 4.2 removed practically all the color. The filtrates were adjusted to pH 6.5 and sterilized. The other half of the acid hydrolyzate was neutralized with calcium carbonate, filtered, the pH adjusted with sodium hydroxide to 6.5, and sterilized. The sterile solutions were inoculated and incubated for 5 days at 37° C.

The data presented indicate that the operations associated with the norit treatment tend to make the medium more fermentable, at least in the higher concentrations of hydrolyzate.

Results of the fermentations are presented in Table IV.

Since experience with similar hydrolyzates had indicated that the solvent ratio was always essentially 60:30:10, analysis for acetone concentration in the distillates from the fermentations would represent approximately 30% of the total solvents. Butanol and ethanol were not directly determined for this series in which the hydrolyzates prepared with phosphoric acid had been used, but calculations from acetone values were used to express total solvents in the table.

Table IV.

Solvent Yields from Phosphoric Acid Hydrolyzates

* Hydrolysis with 0.818 N. Phosphoric Acid

Corn Meal Replaced Per Cent	Acetone Analysis g.	** Total Solvent Yield Wt. % of Carbohydrate
0	6.76	29.8
	Treated with Norit	
20	6.58	29.7
30	5.11	23.2
40	6.10	28.3
50	5.50	25.8
60	4.85	23.2
	Not treated with Norit	
20	6.59	29.8
30	6.43	29.4
40	5.99	27.8
50	1.58	7.44

* The normality of the phosphoric acid solutions was determined by the procedure of Kolthoff and Sandell (76). As a dibasic acid phosphoric acid half-saturated with sodium chloride can be neutralized with sodium hydroxide. Under these conditions the hydrolysis of Na_2HPO_4 will be repressed and phenolphthalein will indicate the theoretical end point.

** Total solvents calculated on basis of acetone = 30% total solvents.

(76) Kolthoff, I. M. and Sandell, E. B., "Textbook of Quantitative Inorganic Analysis", Macmillan, New York 1937, p. 533.

3. Fermentation of pure xylose and of hydrolyzates of xylan and of oat hulls.

A comparison of butyl-acetonic fermentations of pure xylose, xylan hydrolyzates, and oat hull hydrolyzates was undertaken. The media used were as follows: The concentrations of sugars employed were between 3.5 and 5% in a volume of approximately 400 ml. Protein nutrients, corn gluten meal or soybean meal, were added in a final concentration of 2 g. per 100 ml. of medium. The hydrolyzate from oat hulls was prepared with sulfuric acid, neutralized with calcium carbonate, concentrated *in vacuo*, pH adjusted with sodium hydroxide to 7.1, and sterilized.

Xylan from oat hulls, prepared by the method of Hurd and Currie (77), was hydrolyzed with dilute sulphuric acid. Thus 80 g. xylan and 1200 ml. sulphuric acid (0.38 N) were heated to boiling, with continuous stirring, and then autoclaved at 20 pounds steam pressure for 20 minutes. The filtrate was neutralized with calcium carbonate and pH adjusted with sodium hydroxide to pH = 7.0. The media were inoculated and incubated for 5 days at 37° C. The results are shown in Table V, the average values of duplicate fermentations being given in each case.

(77) Hurd, C. D., and Currie, N. R., *J. Am. Chem. Soc.*, **55**, 1521 (1933).

Table V.

Comparison of Solvent Yields from Pure Xylose, Xylan Hydrolyzates, and Oat Hull Hydrolyzates.

	Total Solvent Yield Wt. % of Carbohydrate	Solvent Ratio BuOH	Me_2CO	EtOH
xylose corn gluten	31.2	52.4	29.6	18.0
xylose-soybean meal	27.7	59.6	26.3	14.2
xylan hydrolyzate-corn gluten	33.1	56.0	33.2	11.0
xylan hydrolyzate-soybean meal	30.4	54.3	33.0	12.6
oat hull hydrolyzate-corn gluten	7.2	65.8	25.7	8.5
oat hull hydrolyzate-soybean meal	10.6	62.4	29.0	8.7
corn mash control	34.0	55.9	25.0	19.1

The data presented in Table V show that the culture is capable of converting pure xylose, or xylose obtained by hydrolyzing xylan isolated from oat hulls, into the normal products of fermentation. When either corn gluten or soybean meal were used as the source of the nitrogen nutrient, about the same yields of solvents were obtained. Although the oat hull hydrolyzates fermented poorly the data indicate that soybean meal gave somewhat better results than did corn gluten.

4. Butyl-acetonic fermentation of a phosphoric acid hydrolyzate of oat hulls.

A medium of phosphoric acid hydrolyzate supplemented by varying quantities of corn gluten was prepared for fermentation. Of the hydrolyzate 300 ml. per flask of 4% sugars was mixed with corn gluten in concentrations of 1.5, 2.0, and 2.5% of the medium. The medium was sterilized in 500 ml. Erlenmeyer flasks for 10 minutes at 10 pounds steam pressure and then cooled under air pressure for 30 minutes. After inoculation the cultures were incubated for five days and the beers were distilled. The acetone yield was negligible; however, the titratable acidity was remarkably high. Data are presented in Table VI.

Table VI.

Butyl-acetonic fermentation of Phosphoric Acid Hydrolyzate

Gluten, g./100 ml.	Acidity, ml. 0.10 N per 10 ml. Beer	Acetone Yield, g./100 g. Carbohydrate	Total Solvents *
1.5	21.1	3.2	10.67
2.0	18.3	2.2	7.3
2.5	18.4	2.5	8.3
Corn Mash Control	3.3	13.62	45.4

* Total solvents calculated from acetone yield, assuming acetone = 30%

Acidity peaks in normal butyl-acetonic fermentations rarely rise above 7 ml. of 0.1 N. sodium hydroxide per 10 ml. of culture. In these fermentations of the phosphoric acid hydrolyzate the titratable acidity values were practically triple those of a normal fermentation. Hence, after the neutral solvents had been distilled, one of the abnormal beers was acidified with phosphoric acid and steam distilled until twice the original volume had been collected in the distillate. From this solution volatile acids were estimated by a modified Duclaux distillation. It was found that 0.955 g. of acetic acid and 0.945 g. of butyric acid were present. These values would mean a yield of 7.96% acetic acid and 7.89% butyric acid, based on the original 12.0 g. of xylose present. From the fact that the active fermentation lasted only a few hours it is suspected that very little sugar had actually been utilized; unfortunately no analysis for residual sugar was made. The possible significance of the high acid production will be discussed later in this paper.

5. Effect of possible inhibiting factors.

a. Toxicity of furfural. Since hot acids can convert xylose into furfural, and since certain furanoids are known to be toxic, it was decided to determine the toxicity of furfural to Cl. acetobutylicum in the organism's most favorable medium, corn mash. Furfural was doubly distilled *in vacuo*. The precautions suggested by Adams and Voorhees (78) were followed. Corn mash prepared and sterilized in the usual manner, served as the substrate, and varying concentrations of furfural in sterile water were added to the media before inoculation. The results are presented in Table VII. From the data it is apparent that furfural is not toxic in concentrations of 0.1% or less. There was no fermentation in solutions containing 1.09% or more.

(78) Adams, R. and Voorhees, V., "Organic Syntheses", Coll. Vol. I, p. 274. New York J. Wiley and Sons, 1932.

Table VII.

Toxicity of Furfural to Cl. acetobutylicum

Growing in Corn Mash

Furfural G. per 100 ml. medium	Me_2CO per Flask	*Total Solvent Yield Wt. % of Carbohydrate
5.0	No Fermentation	0
1.0	No Fermentation	0
0.1	11.61	25.4
0.01	10.30	22.8
0. = corn mash control	11.46	25.3

* Total solvents calculated assuming 30% acetone.

b. Effect of chloroform extraction of hydrolyzates on fermentation. It was considered possible that the toxic factor present in oat hull hydrolyzates might be removed by extraction with immiscible solvents. Preliminary experiments indicated that chloroform extracted some substance from the aqueous solution. Fermentation experiments using hydrolyzates which had been subjected to prolonged chloroform extraction in parallel with unextracted material indicated little significant change in fermentability. Data to this effect are presented in Table VIII.

Table VIII.

Effect of $CHCl_3$ Extraction on Fermentability of

Oat Hull Hydrolyzates

	Solvent Yield, Wt. % of Carbohydrate	Solvent Ratio		
		BuOH	Me_2CO	EtOH
corn control	31.2	57.0	25.2	17.8
oat hull hydrolyzate-unextracted	8.9	52.9	28.9	18.2
oat hull hydrolyzate-extracted	3.6	62.0	28.8	9.6

C. The 2,3-Butylene Glycol Fermentation of Oat Hull Hydrolyzates

The organism employed, Aerobacter aerogenes, was obtained from the American Type Culture Collection and designated by them as No. 211. Stock cultures were carried on glycerol-yeast extract agar. The medium used for the fermentation experiments was similar to that reported by Kendall (79) as optimum for sucrose. Expressed in grams per liter, the medium contained the following:

NH_4Cl	3.0
$K_2HPO_4.3H_2O$	1.75
$MgSO_4.7H_2O$	1.75
$CaCl_2$	0.1
Xylose (pure or its equivalent from oat hull sirup)	50.0

After the addition of 0.2 N sulfuric acid to pH = 6.25, the solutions were sterilized at 10 pounds steam pressure for 30 minutes. The media were inoculated with a suspension of the organisms in sterile water and incubated at 37° C. Samples were withdrawn at 24 hour intervals for sugar analysis and the course of the fermentation was followed by thus determining the consumption of carbohydrate.

(79) Kendall, A. R., Doctoral Dissertation, Iowa State College, 1934.

The pH was readjusted to 6.25 by the addition of requisite amounts of sterile sodium carbonate solution (1 M) daily. In the flasks containing oat hull hydrolyzates, the organisms did not grow. On the other hand, the pure xylose solutions fermented readily and gassed vigorously; after 13.5 days the sugar had disappeared completely. The cultures on pure xylose medium were each treated with 3 ml. of a saturated solution of sodium hydroxide, and the gummy precipitates were filtered off. The solutions were then acidified with sulfuric acid, evaporated to 150 ml., and filtered. After the solutions had been made alkaline with sodium hydroxide and saturated with 40. g. of anhydrous potassium carbonate, they were continuously extracted with ether for 94 hours. The ether extract in each case was dried with anhydrous sodium sulfate overnight, and the filtrate was evaporated to 10 ml. before being transferred to a weighing bottle. The solvent was evaporated and the residual 2,3-butylene glycol was dried to a constant weight in a vacuum desiccator over sulfuric acid. From the weights found, it was calculated that 2,3-butylene glycol was produced in yields equivalent to 18.4 and 12.5 per cent of the weight of the xylose in the two individual xylose fermentations. In Table IX is shown the course of the <u>Aerobacter aerogenes</u> fermentation of xylose solutions.

Table IX

The Course of the Fermentation of Xylose by *Aerobacter aerogenes*.

	Flask 1			Flask 2		
Time days	pH before adjustment	Na_2CO_3 ml. added to pH 6.25	Xylose mg./ml.	pH before adjustment	Na_2CO_3 ml. added to pH 6.25	Xylose mg./ml.
0	6.25	0	4.85 ?	6.25	0	4.80
3	4.2	3.0	4.48	4.3	2.0	4.55
4	5.3	1.0	3.80	5.1	1.0	----
5	5.2	1.5	2.55	5.15	1.5	3.23
6	5.3	2.0	----	5.35	2.0	----
7	5.25	2.0	1.28	5.2	2.0	1.79
8	5.5	1.5	0.85	5.6	1.5	1.20
9	5.4	2.0	0.56	5.5	2.0	0.86
10	5.7	1.2	0.33	5.6	2.0	0.55
11	6.05	---	0.18	5.9	---	0.30
12	6.0	1.0	0.11	5.9	1.0	0.20
14	6.5	---	0.03	6.3	---	0.09

D. Kojic Acid Fermentations

The culture used was <u>Aspergillus flavus</u> obtained from Dr. Charles Thom and designated by him as No. 3538. Stock cultures were maintained on glycerol-yeast extract agar. Conditions optimum for good yields of kojic acid from xylose have been developed by Barham and Smits (52). A medium similar to theirs was chosen for kojic acid production from oat hull hydrolyzates.

Analysis of the cultures for kojic acid was undertaken by two methods. For xylose solutions the copper precipitation method of Barham (80) gave good results. Oat hull hydrolyzate cultures seemed not to be as well adapted to this procedure, since copper acetate causes the precipitation of a small amount of substance from the hydrolyzate. Hence continuous extraction of the fermented cultures with ether was used, and the kojic acid in the extract was recrystallized from acetone. An early experiment fairly successfully used the method of evaporating the culture and extracting the residue with several successive portions of boiling acetone.

A preliminary experiment was designed to determine whether an acid or a neutral medium was better for

(80) Barham, H. N., <u>Ind. Eng. Chem.</u>, <u>Anal. Ed.</u>, <u>11</u>, 31 (1939).

kojic acid production from oat hull hydrolyzates. As controls media containing pure xylose were placed under the same conditions.

Two conditions were chosen: pH of 3.5, which Barham and Smits reported as suitable, and pH of 7.0 obtained by addition of an excess of calcium carbonate. In the medium containing pure xylose at pH 3.5 the mold grew well; at pH 7 growth was not so rapid, but sporulation appeared earlier than at pH 3.5. For oat hull sirups the mold did not grow in the acid solutions but grew well in the medium containing calcium carbonate. After the cultures had been incubated for from 15 to 24 days, the contents of the culture flasks were heated to boiling and filtered hot through cheese cloth. The mats of mold mycelium were extracted with boiling water, and the combined solutions were filtered through filter paper and allowed to evaporate slowly at 60° C. in the air. The residues were redissolved in water and made up to 100 ml. for analysis. For the medium containing pure xylose, the yield of kojic acid by the copper kojate precipitation method calculated to be 18.76 g. kojic acid from 100 g. of xylose. Isolation of kojic acid by ether extraction from the media containing oat hull sirup did not give good yields of the crystals. The solutions gave however, a positive ferric chloride test. The ferric chloride color test is said to detect 1 part of kojic acid in 250,000 (67). Pooling of the solutions produced enough crystalline kojic acid to identify satisfactorily by the melting point (152°).

The oat hull hydrolysis was conducted in the usual manner with sulfuric acid. The hydrolyzate was neutralized with powdered calcium oxide while a stream of air was being bubbled through the solution. As the foam rose to the surface, it was repeatedly skimmed off. Basic lead acetate powder was added and the precipitate of proteins and gums was filtered off. Addition of norit removed almost completely the color from the solution. The lead was precipitated from the hot solution with hydrogen sulfide, and the hydrogen sulfide gas was removed by evaporating the solution *in vacuo* at 60° C. to one-third of the original volume. The clear, pale yellow solution showed a blue-green fluorescence when examined under ultraviolet light. The final pH of the sirup was 5.2. On standing for only a few hours, the mixture became dark and turbid. This sirup was used for fermentation experiments with *Aspergillus flavus* and also with *Penicillium chrysogenum*.

The medium employed for *Aspergillus flavus* had the following composition. The values are expressed in grams per liter.

NH_4NO_3 - - - - - - - - - - - - - -	1.00
KH_2PO_4 - - - - - - - - - - - - - -	0.625
$MgSO_4 \cdot 7H_2O$ - - - - - - - - - - -	0.500
Xylose (equivalent from oat hull sirup)- -	128 .0

Of this medium 500 ml. was introduced into a 3-liter Fernbach flask. The medium was sterilized at 10 pounds steam pressure for 20 minutes, and inoculated with a suspension of 5-day old spores. No growth appeared after two weeks of incubation at 30° C. Then 20 grams of sterile calcium carbonate were added to the medium. In two days mold growth was easily visible. After three weeks of incubation subsequent to the introduction of calcium carbonate, the solution was acidified with hydrochloric acid and then decanted from the heavy tough mycelial mat. The mat was repeatedly extracted with boiling water, and the filtrate and washings were combined and neutralized with sodium hydroxide. The solution was made up to 1 liter in a volumetric flask and an aliquot withdrawn for residual sugar analysis. The remainder of the solution was evaporated to a thin sirup and was continuously extracted with ether for a week. The ether extract was warmed, and the residue remaining after the solvent evaporated furnished 1.7 grams of crude kojic acid. The crude product was recrystallized from hot acetone to give a product melting at 151-2° C. with sublimation. This is in good agreement with the values recorded in the literature (68).

E. Xylonic Acid Fermentations

In these experiments a culture of *Penicillium chrysogenum* obtained from Dr. Charles Thom and designated by him as No. 5304.11 was employed. The stock cultures were maintained on a glycerol-yeast extract agar. The medium similar to that reported (69) as optimum for glucose fermentation was employed for the fermentation experiments with xylose. It was recently reported (April 1940) at the Cincinnati meeting of the American Chemical Society that boric acid was of value in the medium. Hence this substance was also included. The method of analysis of these cultures was suggested in a private communication by Dr. Nandor Porges of the Agricultural By-Products Laboratory, U.S.D.A., at Ames, and will be described below.

In a preliminary experiment devised to determine whether an acid or a neutral medium was better for producing xylonic acid from oat hull hydrolyzate, media containing pure xylose as controls were placed under parallel conditions. The same conditions which were chosen for the preliminary experiments with Aspergillus flavus were adopted with Penicillium chrysogenum, a pH of 3.5 for one series and a pH of 7.0 obtained by the addition of an excess of powdered calcium carbonate for the other series. It should be mentioned that the medium for the preliminary experiments did not contain boric acid. It differed from the medium used with Aspergillus flavus in that ferric chloride was included in a concentration of 20 mg. per liter. The growth of mycelium was good on xylose for both acid and neutral media; for the media containing oat hull hydrolyzate growth was obtained only on those containing calcium carbonate and that growth was much slower in starting than was the case with xylose media. Heavy mycelial mats were obtained, however, in 15 days.

The preliminary experiments were conducted with 50 ml. of media contained in 125 ml. Erlenmeyer flasks. For the experiment now to be described a larger volume of medium was fermented and the experiment was performed with the cultures under conditions of aeration. The apparatus employed for the aeration experiment consisted of a

2-liter round-bottomed flask inverted and fitted with a rubber stopper bearing a porous bacterial filter and two accessory tubes. The details of the apparatus are shown in Figure 1. The medium for each flask had a volume of 500 ml. and was sterilized at 10 pounds steam pressure for 20 minutes. The aerator unit was sterilized separately and after inoculation of the medium the assembly was fitted in place. It was securely wired to the neck of the flask in order to withstand the air pressure to which the apparatus was subjected.

The oat hull sirup employed for this experiment was a portion of the same solution whose preparation was described in conjunction with the kojic acid fermentation. The composition of the medium expressed in grams per liter, was as follows:

$NaNO_3$	3.0
KH_2PO_4	0.300
$MgSO_4.7\ H_2O$	0.250
$FeCl_3$	0.100
Boric acid	0.250
$CaCO_3$	40.0
Xylose (pure or its equivalent from oat hull sirup)	200.0

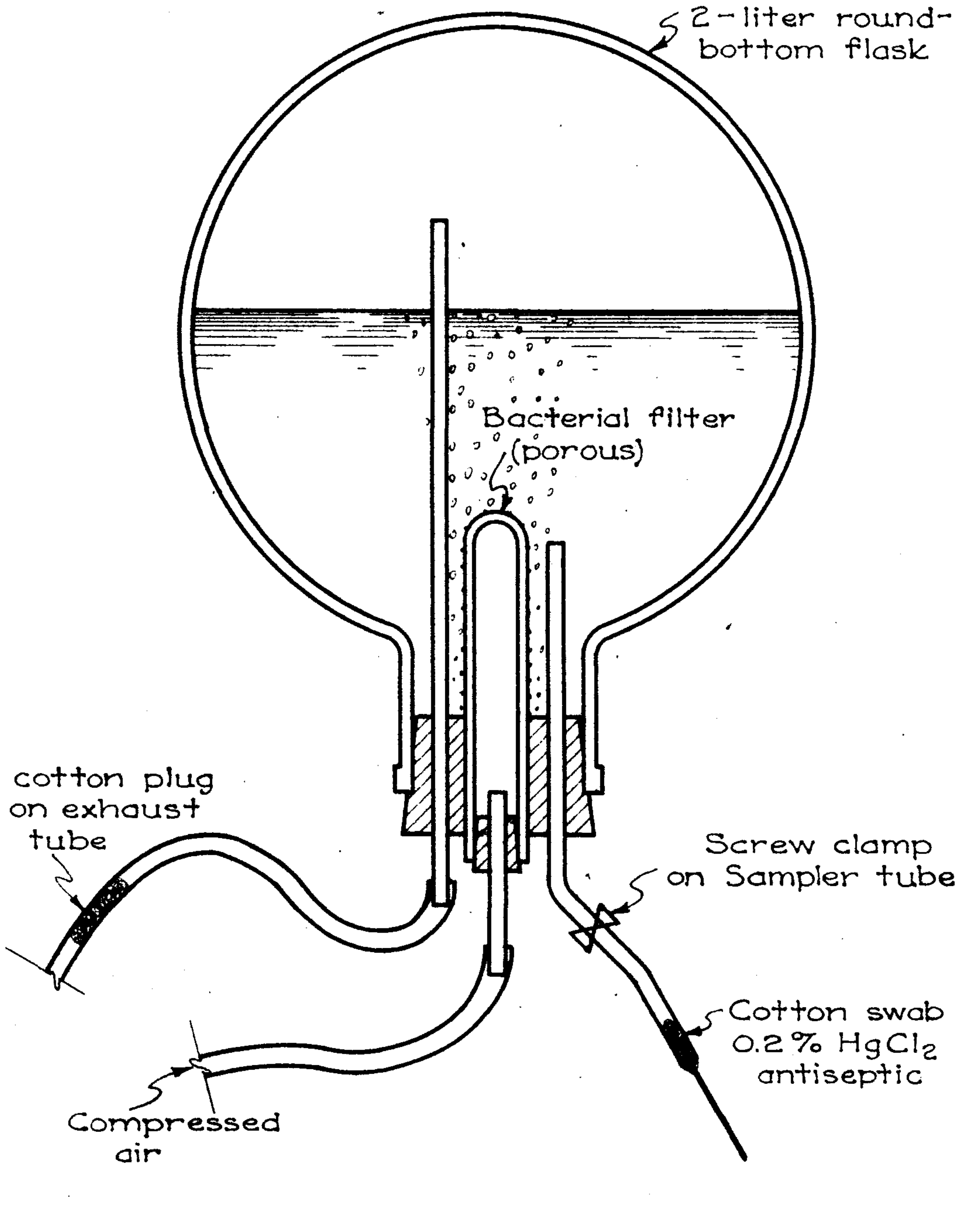

FIG. 1

Apparatus for Aeration of Penicillium Cultures.

Immediately after the medium had been inoculated, the aeration was begun. Sugar analyses were made during the course of the fermentation. After a few days the submerged mycelial growth became dense, especially around the walls of the porous filter. In fact, the growth was so heavy that the pores of the filter became filled and it was necessary to markedly increase the air pressure in order to maintain a continuous stream of tiny bubbles of air through the medium. The air was not humidified, and, consequently, there was considerable evaporation and a corresponding concentration of the sugar solution. It was hoped at the initiation of the experiment that the fermentation would be complete in perhaps four or five days, and the evaporation in that short time would have been negligible. However, it was found that sugar utilization did not warrant a discontinuation of the aeration at 30° C. until 15 days had elapsed. Then, the solutions were heated to boiling, the mycelia were filtered off through cheese cloth, and the excess calcium carbonate was removed by filtering through filter paper. The insoluble matter was thoroughly washed with hot water, the filtrate and washings were combined, acidified with sulfuric acid, and concentrated _in vacuo_ at 60° C. The concentrated filtrate from each culture was then made up to 500 ml. in a volumetric flask. An aliquot was removed for sugar analysis and another sample was taken for the determination of soluble calcium. The remainder of the solution (390 ml.) was evaporated on a hot plate until the appearance of a white gummy precipitate became noticeable. The mixture was then refrigerated for two hours and 725 ml. of ice-cold methanol was added in a fine stream with stirring. The white gummy precipitate settled out, and the mixture was refrigerated for 20 hours before filtering. The precipitate was washed with two successive portions of methanol and then sucked dry on the filter. The weight of the material was 3.5 grams. Evidence for the formation of xylonic acid from xylose by Penicillium chrysogenum was secured by identifying the compound. The brucine derivative, prepared according to the method of Neuberg (81), corresponded to that for xylonic acid. The derivative melted at 170-2°C. Neuberg's preparation melted at 172-4°C.

For the soluble calcium determination, aliquots were taken for analysis. After the organic matter was destroyed by heating the samples with concentrated sulfuric acid for four hours and alternately cooling before adding

(81) Neuberg, C., Ber., 35, 1473 (1902).

concentrated nitric acid and reheating until colorless solutions were obtained, the calcium was precipitated as the oxalate. The precipitate was redissolved and re-precipitated and the oxalate estimated volumetrically with standard potassium permanganate. From the weight of the calcium present in the filtrate, it was calculated that the medium containing pure xylose produced 12.4 grams of xylonic acid; the hydrolyzate medium contained 14.3 grams of xylònic acid. From the difference between initial and final sugar determinations it was computed that 48.0 grams of sugar disappeared from the xylose medium, while only 27.0 grams of xylose disappeared from the oat hull sirup medium. The apparent yield based on the sugar consumed is 25.8 % for the xylose medium and 52.9 % for the hydrolyzate medium. However, it will be noticed that while there were formed practically equal amounts of soluble calcium, nevertheless there was consumed only half as much xylose in the oat hull sirup as in the medium containing crystallized xylose. It may be that the criterion of dissolved calcium is not a good measure, under these conditions, of calcium xylonate, at least from the hydrolyzate.

V DISCUSSION

The nutrition of certain xylose-fermenting bacteria and molds is reasonably well-formulated. The conditions necessary for the growth of the organisms and the dissimilation of carbohydrates have been reported by many workers in the field. The micro-organisms chosen for this work were ones for which an environment had been developed and the composition of an adequate medium had been determined.

The fermentation of xylose by the butyl-acetonic organism was of the normal type reported by Underkofler (35). The usual solvents, butanol, acetone, and ethanol, were obtained in good yields; the sugar was utilized completely. Again, for the <u>Aerobacter aerogenes</u> fermentation a complete conversion of the sugar was obtained and the customary 2,3-butylene glycol was formed in amounts similar to the reports by Breden et. al. (41). The fungus, <u>Aspergillus flavus</u>, was capable of transforming pure xylose into kojic acid. Yields were similar to those found by Barham and Smits (52). The fermentation of xylose by <u>Penicillium chrysogenum</u> and the isolation of xylonic acid from the fermented cultures is reported for the first time.

However, with crude xylose sirups prepared from acid-hydrolyzed oat hulls, the conditions for microbiological growth and for fermentation were not attained to the same degree of proficiency. The butyl-acetonic fermentation was of normal type in media containing oat hull sirup and supplemented by ground corn to the extent of 50% of the total carbohydrate content. At higher concentrations of crude xylose solutions the yield of products was almost always very poor. In the case of the Aerobacter aerogenes experiment the micro-organism showed no signs of growth in the hydrolyzate medium. The extent to which Aspergillus flavus transformed crude xylose into kojic acid was sufficient to isolate the product and to identify it, but the relative amount of final product was small indeed. In the work described with the Penicillium chrysogenum cultures on oat hull sirup medium the consumption of sugar was only half that of the pure xylose control.

The same nutrients were added to media containing pure xylose and oat hull hydrolyzate, and since fermentation occurred in the former but not in the latter solution, it is not reasonable to expect that nutrients were lacking in the crude xylose medium. The fault of poor fermentation cannot be attributed to the omission of essential substances but ought to be associated with the inclusion of some inhibitory substance or substances present in oat hull sirups. These crude sugar solutions may not produce normal fermentations because of one of the following reasons:

(a). The concentration of essential or accessory inorganic nutrients may be too great for fermentation; or

(b). Oat hull hydrolyzates may contain an organic substance or a mixture of organic substances which is toxic to the micro-organisms. It is almost certain that some compound or compounds are present in the crude sirups from oat hulls which prevent fermentations from following the normal pathways.

If the toxic factor were an inorganic ion present in the hydrolyzate, its identity could be established by an experiment in which oat hulls were ashed and the ash included in a favorable medium in varying concentrations. If a normal fermentation could be inhibited by the addition of the inorganic material, it would be fairly easy to establish which fraction of the ash was responsible for the toxic properties. If, on the other hand, the ash could be added to a medium without impeding the fermentation, then clearly, the inhibiting factor could not be inorganic.

The presence in the hydrolyzate of an organic material, rather than an inorganic substance, toxic to the micro-organisms tested is, in the author's opinion, the more probable supposition. Compounds present in small quantities in plant tissues usually escape detection in the ordinary methods of analysis of cellulosic materials.

The empirical procedures employed by the analytical chemist often lump together certain materials which may have a single, common property, for example, insolubility in a definite reagent. A case in point is lignin. Employing one method of analysis wherein an organic substance is first treated with cold 72% sulfuric acid or cold concentrated hydrochloric acid and then the diluted solution is boiled, there results an insoluble residue; this product may be termed "lignin". On the other hand, the theme of Hilpert (82) has been that all "lignin" found by the above method of analysis is not true lignin from the plant tissue, but is, instead, an insoluble resin formed from the sugars present under the conditions of the analysis. In Hilpert's work the pure pentose sugars were subjected to the conditions of a lignin analysis and a black condensation product was produced which, if it were found in an analysis of vegetable tissue, would have been included in the category of "lignin". It is not to be implied here that lignin does not exist in plant materials in some form, but merely, that estimation by the use of the cold concentrated acid method may give results which may be misinterpreted.

(82) Hilpert, R. S., and Litmann, E., Ber., 67B, 1551 (1934);
Hilpert, R. S., and Meybier, H., Ber., 71B, 1962 (1938).

So also the ether-soluble material may not only be fats and oils but also steroids, saponins, etc. In the usual analysis of a vegetable tissue there are found cellulose, lignin, hemicellulose, ash, moisture, and fat; the sum of these values commonly approaches 100%. The point which is to be emphasized here is that in complex tissues there are many other substances present which have escaped the attention of workers.

Possible toxic materials which might have been present and thus have inhibited the fermentations of this investigation include: furfural or related products with the furan structure, lignins or tannins, saponin-like substances, and complex nitrogenous substances.

Furfural, itself, has long been known for its bactericidal and fungicidal properties. It is usually produced by a dehydration of pentoses. The chief commercial source of furfural is oat hulls, which are treated at suitable temperatures with strong mineral acid; the furfural is steam-distilled from the mixture. Under mild hydrolytic conditions only small amounts of furfural are formed in the solution. The compound is quite reactive, however, and commonly tends to condense with other substances to form furanoid resins at moderately elevated temperatures. In oat hull sirups it is to be expected that lignin and furfural react to form condensation products. After sterilization of the hydrolyzate solutions in the autoclave it was found that there was always considerable darkening of the color, and ordinarily the precipitation of some amorphous, insoluble matter. Although a small part of the darkening may have been due to a slight caramelizing of the sugars, nevertheless the greater part resulted from some other constituents in the oat hull hydrolyzates.

The possibility that lignin may be the substance responsible for the failure of the fermentation of these oat hull juices must not be overlooked. Lignin is known to be inhibitory toward certain fermentations. Olson, Peterson, and Sherrard (83) showed that lignin-containing material such as ground wood pulp was not readily fermented by enrichment cultures of thermophilic cellulose-fermenting organisms, and that the non-fermentability of the wood was associated with its lignin content. By submitting the woods to a nitric acid treatment first and then to an alkaline cooking, they were able to reduce the lignin content below 1%. If lignin were less than 1%, these investigators could obtain good fermentations. While lignin is reported to be insoluble in dilute acid solutions, nevertheless certain small amounts are acid-soluble and could be present in oat hull hydrolyzates.

(83) Olson, F. R., Peterson, W. H., and Sherrard, E. C., Ind. Eng. Chem., 29, 1026 (1937).

In fact, Bryner showed that the analysis of oat hulls before and after hydrolysis indicated about 4.5% of the lignin had disappeared from the residue after hydrolysis. Part of this lignin loss may be accounted for as soluble lignin in the hydrolyzate.

Related to lignins are the tannins. These latter substances are quite soluble and may be able to exert their inhibiting influence to a greater degree as a result of this property. Rokusho (84) showed that tannin, extracted with hot water from kaoliang, was toxic in the butyl-acetonic fermentation of kaoliang. By extraction of the bran with dilute sodium hydroxide at 50° C. he obtained a red coloring matter which also retarded the fermentation. Schmidt, Atterer, and Thaler (85) indicated that the humic substances which were produced during the acid hydrolysis of hemicelluloses were toxic in fermentation experiments. However they reported that if a chlorine dioxide treatment were provided, the humic matter was removed and the hexose fermentations with yeast were normal. It appears likely that either aqueous chlorine dioxide, or some similar oxidizing agent destroys the toxicity of lignin-like substances. The nitric acid treatment of the woods reported

(84) Rokusho, B., J. Agr. Chem. Soc., Japan, 10, 1211 (1934) /C. A., 29, 1573 (1935)/

(85) Schmidt, E., Atterer, M., and Thaler, H., Cellulose chem., 10, 153 (1929).

by Olson, Peterson, and Sherrard (83) lends support to this contention.

When the hot acid solutions are removed from the autoclave after the hydrolysis and are cooled and neutralized with calcium carbonate, very stable foams are produced. The appearance of these froths suggested the possibility of the presence of saponins. These compounds are widely distributed among the higher members of the plant kingdom. One of the outstanding properties of the saponin class of compounds is the ready formation of foams in water solutions and stabilization of emulsions of fats and oils. Physiologically, they are capable in very low concentrations of hemolyzing erythrocytes. If saponins can lyse red blood cells, it does not seem too difficult to conceive of their lysing bacterial cells. Their toxic action has been attributed to their ability to extract cholesterol from the walls of the erythrocytes. Bryner's (4) analysis of oat hulls showed 2% ether extract which could have included, besides the oil and fats, also saponins if present. The fact that hydrolyzates after prolonged chloroform extraction did not ferment any better than unextracted sirups does not encourage the postulation that saponins are the toxic agent. On the other hand, Gill (86) also suggests that saponins or compounds related to them produce conditions

(86) Gill, A., J.Soc.Chem.Ind., 38, 411T (1919).

unfavorable for the butyl-acetonic fermentation. The last-named investigator studied this fermentation using horse chestnuts as a substrate. By extracting the nuts with butanol previous to fermentation he presented evidence that horse chestnuts could be fermented almost as well as corn mash. In his experiments the total solvent (butanol and acetone) yield was 24% from corn mash, while it was 18% from horse chestnuts. Gill recommended that the sterilization of the medium be performed at low temperatures (10 pounds steam pressure) and for only a brief period of time (1 hour) in order to extract only small amounts of tannin which he recognized as toxic to the organism.

Another type of substance to which the toxic behavior might be attributed is a complex nitrogenous substance. Only small bits of experimental evidence are submitted for its existence in oat hull sirups. First, when acid solutions of the hydrolyzates are made slightly alkaline with sodium hydroxide, there is liberated a fishy odor not unlike that of methyl amine or one of its homologues. Second, when organic matter is destroyed by digesting the solutions with boiling concentrated sulfuric acid, prolonged severe treatment is necessary. For the calcium analysis after the xylonic acid fermentation had términated, the destruction of organic matter required over 8 hours of boiling with concentrated sulfuric acid with occasional additions of concentrated nitric acid until the solutions became colorless. Certain of the heterocyclic nitrogen compounds are notorious for their resistance to degradation treatment of this type. Substances such as pyridine or histidine are decomposed by boiling sulfuric acid with great difficulty.

Until oat hull hydrolyzates are carefully analyzed and the individual constituents isolated and identified, it will be fruitless to definitely attribute the toxic factor to any one substance. It seems very likely that more than one toxic factor is present; hence to produce a fermentable sirup it may be necessary to resort to several treatments in order to remove the agents of inhibition.

Possible suggestions toward solving the problem of poor fermentation of the hydrolyzate include the following. To prevent furanoids from forming or condensing, sterilization by heat should be avoided. The use of a bacterial filter would produce a sterile medium in which the formation of resins resulting from the heat treatment will have been prevented. It has been shown by Gill (86) that saponins may be removed by a solvent extraction. Hence, either the hulls before hydrolysis or the hydrolyzates after concentration of the sirups could be extracted with solvents such as butanol, for example. Work along these lines is at the present time being conducted by other investigators in these laboratories.

The anomalous behavior of the butyl-acetonic fermentation of phosphoric acid hydrolyzates has perhaps some significance from the point of view of cellular metabolism. It is known that butyric and butylic bacteria both produce butyric acid from carbohydrate supplied to them; the butyric bacteria carry the reaction no further, but the butylic organisms reduce the butyric acid to butanol. If asparagine is provided in the medium for the butyric acid bacteria, they too can convert butyric acid into butanol (60), (87). Perhaps, since butyric bacteria have poor proteolytic powers, they cannot supply themselves with asparagine from the protein of the medium. The butylic organisms are markedly proteolytic; hence they can cleave proteins and obtain asparagine to continue the dissimilation one step further. By incorporating an oat hull hydrolyzate, prepared by the use of phosphoric acid, into the medium the dissimilation by the butyl organism has been arrested at the butyric acid stage. The manner in which

phosphate (or pyrophosphate) inhibits the reduction is a question for speculation.
(1) Does it inhibit the enzyme which cleaves the protein to give asparagine and thus cut off the supply? (2) Or does it tie up, in some manner, the asparagine once it is formed? (3) Or does it phosphorylate the sugar or carbohydrate cleavage products in such a way that they will not supply some substance necessary as an intermediate for proteolytic conversions? To settle the question, further experimentation will be necessary. If (1) is true, then addition of asparagine to the medium should give butanol. If (2) is true, asparagine added should be tied up, and no butanol should result. It is of course possible that phosphate is not the inhibitor, but that the hydrolysis of oat hulls with other acids has not produced the same conditions that are, in some way, an accompaniment of the phosphoric acid treatment.

An example is suggested here of a method for changing the mechanism of a fermentation. Tatum, Peterson, and Fred (87) demonstrated that a butyric acid organism may be made to act as a butanol producer. Evidence is presented here that a butyl alcohol bacterium may be made to produce

(87) Tatum, E. L., and Peterson, W. H., J. Bact., 29, 87 (1935);
Tatum, E. L., Peterson, W. H., and Fred, E. B., Ibid., 29, 563 (1935).

butyric acid and not butanol.

The sluggish fermentation of butyl alcohol bacteria is one in which the acidity increases to a high degree. The agent responsible for sluggish fermentations has been attributed by Starr (88) to have the properties of a filterable virus. It is perhaps possible that this sluggish principle contains, or is, even, an anti-enzyme which prevents proteolysis and cuts off the supply of asparagine, or else a carrier essential for the reduction of butyric acid to butanol is withdrawn from the scene of operations.

Much of the above is purely speculation, and the only answers to the questions will be found by experimentation.

(88) Starr, D. F., Doctoral Dissertation, Iowa State College, 1933.

VI SUMMARY

1. Oat hulls were hydrolyzed with dilute mineral acids, and the pentose sugar solutions were prepared for fermentation.

2. The butyl-acetonic fermentation of mixtures of oat hull hydrolyzates with ground corn gave normal yields of solvents and the ratios of butanol, acetone, and ethanol were essentially 6:3:1. As much as 50% of the total carbohydrate could be derived from the oat hull sugars without diminishing the solvent production.

3. No significant differences in the butyl-acetonic fermentation resulted when hydrochloric, nitric, or sulfuric acids were employed as hydrolyzants. However hydrolyzates prepared with phosphoric acid gave abnormal fermentations in which the activity of the cultures soon ceased and the production of butyric and acetic acids was markedly high. The possible significance of this phenomenon is discussed.

4. <u>Aerobacter</u> <u>aerogenes</u>, under the conditions employed

did not grow in oat hull hydrolyzates, but grew well in a medium containing pure xylose, and gave normal yields of 2,3-butylene glycol.

5. Aspergillus flavus produced kojic acid in small amounts from oat hull hydrolyzates but gave good yields from a medium containing pure xylose.

6. Penicillium chrysogenum produced calcium xylonate in a medium containing either oat hull sirups or pure xylose. The xylonic acid was isolated and identified by the brucine derivative.

7. A discussion of possible inhibiting substances which might be responsible for the poor fermentations of oat hull sirups is presented.