THE PREPARATION OF PERSEULOSE BY OXIDATION OF PERSEITOL WITH ACETOBACTER SUBOXYDANS[1]

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The present work was undertaken at the suggestion of Dr. C. S. Hudson and Dr. Raymond M. Hann for the purpose of improving the method for preparing the rare ketoheptose perseulose, which was needed for chemical studies.[2] The best starting material is unquestionably perseitol, which occurs in the avocado (*Persea gratissima*); a large supply of this crystalline polyhydroxy alcohol was kindly furnished by Dr. A. E. Knauf of this Institute, who had isolated it as a by-product in the preparation of D-mannoheptulose from avocados. Perseulose was discovered by Bertrand (1908) through the oxidation of perseitol by *Acetobacter xylinum*. The experience of Wells, Stubbs, Lockwood and Roe (1937) has indicated that *Acetobacter suboxydans* in submerged growth gives a nearly quantitative conversion of sorbitol to sorbose, and with the coöperation and technical assistance of the above mentioned colleagues of the Industrial Farm Products Research Division of the Bureau of Chemistry and Soils, U. S. Department of Agriculture, conditions have been determined for the quantitative biochemical oxidation of perseitol to perseulose.

Bertrand used 0.5 per cent yeast extract as the only nutrient substance in the culture medium, other than perseitol, the latter being employed in 2 per cent concentration. The oxidation in

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[2] The following publications have been made: Hann, R. M., Tilden, E. B., and Hudson, C. S., J. Am. Chem. Soc., 1938, **60**, 1201. Hann, R. M., and Hudson, C. S., Ibid., 1939, **61**, 336. Richtmyer, N. K., Hann, R. M., and Hudson, C. S., Ibid., 1939, **61**, 340.

these surface cultures required several weeks, and the yield of the sugar was about 45 per cent of the theoretical. Bertrand recovered the sugar by concentrating the culture fluid, after lead treatment, and extracting the syrup with boiling 90 per cent alcohol, which dissolved the sugar and precipitated the unoxidized perseitol. The sugar crystallized in a mass from the concentrated alcoholic solution. After two or more recrystallizations from alcohol, the sugar had an optical rotation at equilibrium of $-81°$.

Bertrand's (1898) sorbose bacterium, which he had obtained from the skin of the berries of the mountain ash (*Sorbus aucuparia*), was later found to be identical with *Acetobacter xylinum*, which had been isolated from vinegar by Adrian Brown (1886). Kluyver and DeLeeuw (1924) found in beer a new member of the group of oxidizing bacteria, which they named *Acetobacter suboxydans*. This organism was found by Fulmer, Dunning, Guymon, and Underkofler (1936) to give better results than *A. xylinum* in the preparation of sorbose, which has become of great importance as the starting point for the synthesis of ascorbic acid, and Wells, Stubbs, Lockwood and Roe (1937), as has been mentioned, used submerged growths of *A. suboxydans* in their studies of the optimum conditions for large scale production of sorbose.

In our preliminary attempts to oxidize perseitol, we were more successful with *A. suboxydans* than *A. xylinum*, and the later work was all done with *A. suboxydans*.[3] We have not made a systematic comparison of the perseitol-oxidizing ability of the two organisms under the most favorable conditions.

EXPERIMENTAL DATA

Perseitol is not very soluble at room temperature, and not more than 3 to 4 per cent could be used without its crystallizing from solution during the early part of the incubation. We used

[3] The culture of *A. suboxydans* was kindly furnished by Dr. L. B. Lockwood of the Division of Industrial Farm Products Research. The *A. xylinum* culture was obtained from the American Type Culture Collection.

3 per cent in most of the experiments. Difco yeast extract, 0.5 per cent, constituted the basic medium. Preliminary experiments with surface cultures were made in 125 ml. Erlenmeyer flasks containing 20 ml. of the medium. *A. suboxydans* was maintained on agar slants containing 0.5 per cent yeast extract and 5 per cent sorbitol, and the flasks were inoculated with 1 ml. of aqueous suspensions of such cultures. Two-milliliter samples of medium were withdrawn at intervals and tested for reducing value by the Shaffer-Hartmann method (1921). Bertrand's (1906) figure (100 mgm. perseulose equivalent to 157.3 mgm. copper) was used for approximate calculation until the pure sugar was obtained for construction of a curve of copper values.

In the simple yeast extract medium, which is adequate for sorbitol oxidation, only 30 to 35 per cent of the perseitol was oxidized. The phosphate content of the medium was increased by adding 0.3 per cent acid potassium phosphate (Underkofler and Fulmer, 1937), leading to considerably increased oxidation. No further increase was obtained by aeration of the submerged culture in a Jena glass gas-washing bottle until the inoculum was varied by using 1 ml. of a 48-hour aerated sorbitol culture, when nearly complete oxidation was obtained. That this result was due to some constituent of the sorbitol culture was evident from the fact that subcultures inoculated from the actively oxidizing perseitol culture showed only partial oxidation. We knew that sorbose was present in the sorbitol culture and also probably some glucose, which is contained in crude sorbitol, and on adding small quantities of either of these to the medium the conditions became so favorable that the perseitol was completely oxidized. These preliminary results are summarized in table 1. Five-hundredths per cent glucose appeared to be slightly more favorable than 0.1 per cent sorbose and was thereafter employed regularly in the medium. Subsequent experiments (Hann, Tilden and Hudson, 1938) showed that the medium containing glucose and phosphate is a favorable one for the oxidation of a variety of sugar alcohols other than perseitol.

TABLE 1

Determination of conditions for oxidation of perseitol

FLASK NUMBER	MEDIUM CONTAINING 3 PER CENT PERSEITOL	VOLUME	INOCULUM	AERATION	TEMPERATURE OF INCUBATION	TIME OF INCUBATION	PER CENT SUGAR IN MEDIUM* AFTER INCUBATION
		ml.			*degrees*	*days*	
1	0.5 per cent Difco yeast extract	20	1 ml. aqueous suspension (20 ml. water from 2 sorbitol agar slants)	None	20	5	0.9
					30	5	1.0
					20	9	1.2
					30	9	1.38
2	Same +0.3 per cent KH_2PO_4	20	Same	None	20	5	1.35
					30	5	1.4
					20	9	1.8
					30	9	1.9
3	Same as 2, +0.0003 M $MgSO_4$	20	Same	None	20	5	1.39
					30	5	1.35
					20	9	1.8
					30	9	2.0
4	Same as 3, +0.05 per cent glucose	20	Same	None	20	5	1.37
					30	5	1.41
					20	9	2.8
					30	9	2.8
BOTTLE NUMBER							
1	0.5 per cent Difco yeast extract +0.3 per cent KH_2PO_4	200	1 ml. from 48-hour aerated fluid sorbitol culture	200 ml. per min. under 8 pounds pressure	30	7	3.0
2	Same	200	1 ml. from 48-hour aerated fluid perseitol culture	Same	Same	Same	1.2
3	Same +0.1 per cent sorbose	200	Same	Same	Same	Same	2.6
4	Same as 2, +0.05 per cent glucose	200	Same	Same	Same	Same	3.3
5	Same as 3	200	1 ml. aqueous suspension, as for flasks 1–4	Same	Same	Same	3.1
6	Same as 4	200		Same	Same	Same	3.4

* Calculated for reducing values by Shaffer-Hartmann test, using Bertrand's figures for copper equivalents. For preincubation values see tables 3 and 4.

Recovery of the sugar

One liter of pooled bottle cultures containing reducing sugar equivalent to 3 per cent was clarified by filtration through decolorizing carbon (Darco G60) and the clear filtrate concentrated *in vacuo* at 35 to 40° to a thick syrup, which crystallized spontaneously after 48 hours. The separated crystals, about 15 grams, were recrystallized from 15 parts of 95 per cent alcohol, and yielded 12 grams of perseulose hemihydrate of melting point 106° and $[\alpha]_D^{20}$ −81.3° in water at equilibrium. This material was used for the determination of copper equivalents by the Shaffer-Hartmann technique (table 2). An additional 7.5 grams was subsequently obtained from the syrup, making the total yield about 65 per cent. An alternative method of crystallizing

TABLE 2

Reduction values of perseulose hemihydrate by the Shaffer-Hartmann method

100 mgm. perseulose hemihydrate reduced	166.4 mgm. copper
70 mgm. perseulose hemihydrate reduced	118.0 mgm. copper
50 mgm. perseulose hemihydrate reduced	83.9 mgm. copper
20 mgm. perseulose hemihydrate reduced	31.2 mgm. copper

the ketose, which gives a higher yield, is described by Hann and Hudson (1939).

Oxidation in rotary drum fermenters

We were fortunate in having put at our disposal on three occasions one or more of the rotary drum fermenters designed by Herrick, Hellbach, and May (1935) and used in the preparation of sorbose and of gluconic acid (Wells, Moyer, Stubbs, Herrick, and May, 1937). Except for the additions to the culture medium already indicated, the conditions employed for sorbitol oxidation were found to be satisfactory for perseitol, though the reaction time was somewhat longer. At the suggestion of Wells and his collaborators,[4] neutralized corn-steep liquor, 0.3 per cent, replaced the yeast extract in these experiments, having been proved satisfactory by bottle culture tests. Three per cent perseitol was used in the first two drums, 4 per cent

[4] Ind. Eng. Chem. In press.

in drums 3 to 6. The volume of culture in each drum was 3200 ml. except in drum 1, in which it was 2000 ml. The inoculum was varied somewhat in the latter four drums, which were run at the same time. Oxidation was complete in all, but much more rapid with a large inoculation (drum 6) than with a relatively small one (drum 5), and more rapid when the inoculum was a yeast-extract culture (drum 4) than one grown on steep medium (drum 3). The bacterial count reached 100 million

TABLE 3

Oxidation of 3 per cent perseitol in rotary drum fermenters

DRUM NUMBER	MEDIUM CONTAINING 3 PER CENT PERSEITOL	VOLUME	INOCULUM	AERATION AND AGITATION	TEMPERATURE OF INCUBATION	TIME OF INCUBATION	pH	PER CENT REDUCING SUBSTANCE CALC. AS PERSEULOSE†	
								Before incubation	After incubation
		ml.			*degrees*	*hours*			
1	0.3 per cent purified corn steep liquor*, 0.3 per cent KH_2PO_4, 0.05 per cent glucose	2,000	Bacteria washed from Petroff flasks (5) and agar slants (6) of 5 per cent sorbitol agar	1200 ml. per min. at 30 pounds pressure 13 R.P.M.	30	0	5.3	0.111	
						16	4.6		0.72
						24	5.2		1.79
						36	5.2		3.10
2	Same	3,200	200 ml. 48-hour aerated fluid culture on steep medium containing 3 per cent perseitol	Same	Same	0	5.6	0.34‡	
						21	5.4		1.70
						27	4.6		2.25
						45	4.9		3.05

* Kindly furnished by the Farm Products Research Laboratory.

† Calculated from curve of copper values constructed from reducing values obtained with known amounts of perseulose (see table 2).

‡ Inoculum contained nearly 6 grams of perseulose.

per ml. in all the drums within 5 hours and remained at that figure throughout; dilutions beyond that point were not made. The pH showed a tendency to fall during oxidation, rising to the initial value at completion of the reaction. The data are summarized in tables 3 and 4 and in the chart. The yield of perseulose in these experiments was found to be quantitative (Hann and Hudson, 1938).

The time necessary for the oxidation in the drums (47 hours), as compared with that of sorbitol (16 hours), suggested that the

TABLE 4

Oxidation of 4 per cent perseitol in rotary drum fermenters

DRUM NUMBER	MEDIUM CONTAINING 4 PER CENT PERSEITOL	VOLUME	INOCULUM	AERATION AND AGITATION	TEMPERATURE OF INCUBATION	TIME OF INCUBATION	pH	PER CENT REDUCING SUBSTANCE CALC. AS PERSEULOSE†	
								Before incubation	After incubation
		ml.			*°C.*	*hours*			
3	0.3 per cent neutralized corn steep liquor*, 0.3 per cent KH_2PO_4, 0.05 per cent glucose	3,200	200 ml. 48-hour aerated culture on steep medium containing 3 per cent perseitol	1200 ml. per minute at 30 pounds pressure 13 R.P.M.	30	0	4.9	0.34‡	
						20	4.4		1.05
						27	4.4		1.80
						42	4.9		3.00
						49	5.0		3.40
						66	5.0		3.90
						72	5.1		3.95
4	Same	Same	200 ml. 48-hour aerated culture on yeast extract medium containing 3 per cent perseitol	Same	Same	0	5.1	0.30‡	
						20	4.9		1.65
						27	4.9		3.15
						42	5.2		4.00
						47	5.1		4.15
5	Same	Same	Bacteria from 2 Kolle flasks solid medium (sorbitol agar) washed into 200 ml. steep medium	Same	Same	0	5.1	0.117	
						20	4.6		1.00
						27	4.6		1.90
						42	5.1		3.10
						49	5.1		3.45
						66	5.2		3.85
						72	5.3		3.95
6	Same	Same	Bacteria from 6 flasks solid med. in 200 ml. steep medium	Same	Same	0	5.0	0.133	
						20	4.9		1.90
						27	4.7		2.95
						42	5.1		3.75
						47	5.0		3.95

* Kindly furnished by the Farm Products Research Laboratory.

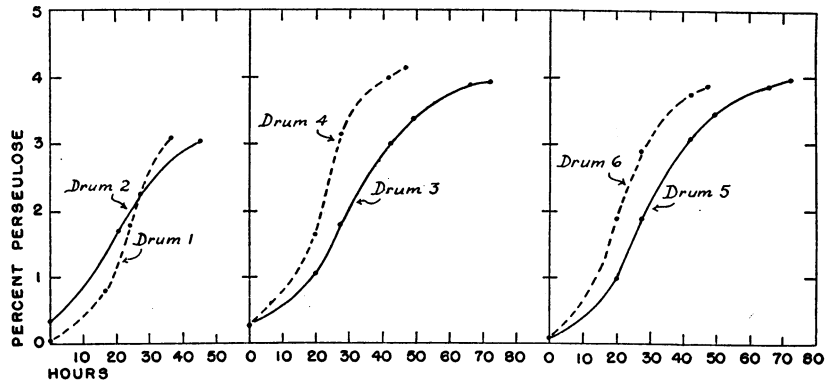
† Calculated from curve of copper values constructed from reducing values obtained with known amounts of perseulose (see table 2).

‡ Inoculum contained nearly 6 grams of perseulose.

conditions might not be ideal, and it was later found that the pH of 5.5 was not the optimum. Surface cultures containing 3 per cent perseitol were set up in 200 ml. lots in 500 ml. Erlenmeyer flasks, in which the potassium phosphate was replaced by various mixtures of dibasic sodium phosphate and monobasic potassium phosphate to give hydrogen ion concentrations ranging from pH 5.9 to 7.3. The salts were made up in M/5 concentration, and 10 ml. of a given mixture were added to each 100 cc. of medium, to give approximately 0.3 per cent phosphate concentration. The pH of the cultures was determined by measure-

CHART 1

OXIDATION OF PERSEITOL IN ROTARY FERMENTERS



ments with the glass electrode. As the figures below show, the optimum pH is 6.4.

Initial pH	*Per cent perseulose calculated from reducing value after 9 and 12 days*
7.3	2.48
6.8	2.89
6.4	2.95
5.9	2.86
5.5	2.54

SUMMARY

The cultural conditions favorable to oxidation of perseitol to perseulose by *Acetobacter suboxydans* are described. A small amount of glucose in the medium (0.05 per cent) was found to promote complete oxidation. Three to four per cent perseitol

is changed quantitatively to perseulose in 36 to 48 hours when the cultures are aerated and agitated. The medium described appears to be favorable for the oxidation of a number of other sugar alcohols.

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