Bacterial Accumulation of Ribulose and Xylulose

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Several bacteria capable of accumulating large amounts of unknown sugars in culture medium were isolated from natural sources.

These bacteria were identified taxonomically as genera of *Brevibacterium* and *Corynebacterium*, respectively.

The sugars accumulated were isolated by paperchromatography and they were identified as a mixture of D-ribulose and D-xylulose.

Time course of sugar production by one of several strains selected shows that the ketopentoses were accumulated progressively with incubation time and that their maximum yield was approximately 7 to 8 mg per ml of the culture broth.

INTRODUCTION

Both D-ribulose and D-xylulose are ketopentoses, the monophosphate esters of which have been known as intermediates in pentose phosphate pathway.

Saito¹⁾ observed that *Brevibacterium fuscum* accumulated ribulose-like ketopentose in the culture medium, and Suzuki²⁾ reported that *Agrobacter tumefaciens* formed D-ribulose from glucose in the medium.

During the course of investigation on microbial production of sugar, it was observed that several bacteria isolated from natural sources accumulated large amounts of unknown sugars in their culture broth.

These sugars were identified as a mixture of D-ribulose and D-xylulose.

This communication will report the results of identification of these sugars and the taxonomic studies on the sugar-accumulating bacteria.

MATERIALS AND METHODS

Strains. The sugar-accumulating bacteria, strain

Nos. 12-6, 19-12, 22-23 and 34-4, isolated from natural sources were used.

Culture methods. Twenty ml of a seed medium containing 2% of glucose, 1% of yeast extract, 1% of peptone and 0.25% of NaCl were poured into a 250-ml Erlenmeyer flask and the inoculated medium was shaken at 30°C for about 24 hours. Two ml of the grown seed were inoculated to 20 ml of a fermentation medium in a 250-ml Erlenmeyer flask.

Those flasks were shaken at 30°C for 5 days on a rotary shaker, which was operated at 220 r.p.m. and described 5 cm in circle.

The composition of the fermentation medium was as follows: glucose 10%, KH_2PO_4 1%, K_2HPO_4 1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1%, yeast extract 0.5%, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01%, biotin 30 $\mu\text{g/l}$ and urea 0.6%; and its pH was adjusted to 8.2 with 5 N NaOH before sterilization. Urea was autoclaved separately.

Materials. D-Ribulose o-nitrophenyl hydrazone was purchased from California Biochemical Corporation and free D-ribulose was obtained by its hydrolysis.³⁾

D-Xylulose was prepared from the epimerization of D-xylose in hot pyridine.⁴⁾

Paperchromatography. The sugars in the culture

1) N. Saito and S. Sugiyama, This Journal, **28** 48 (1964).

2) T. Suzuki and R. H. Hochster, *Biochem. Biophys. Res. Comm.*, **19**, 637 (1965).

3) O. T. Schmidt and R. Treiber, *Ber.*, **66**, 1765 (1933).

4) L. Hough and R. S. Theobald, "Methods in Carbohydrate Chemistry", Academic Press, 1962, p. 94.

TABLE I. KETOPENTOSE PRODUCTIVITY OF ISOLATED BACTERIA

Strain No.	3-Day				5-Day			
	pH	DCW	Xu	Ru	pH	DCW	Xu	Ru
5-8	5.7	4.3	3.0 mg/ml	2.1 mg/ml	4.9	3.8	1.3 mg/ml	1.6 mg/ml
12-6	5.1	4.9	3.8	1.8	4.7	5.5	3.1	1.6
16-10	6.0	5.4	2.7	2.8	5.6	5.5	2.3	1.5
19-3	6.6	4.6	1.8	1.1	6.0	4.8	0.7	0.9
19-12	5.1	5.4	5.1	1.6	4.8	5.4	2.4	1.3
22-23	5.4	7.1	2.7	2.4	5.0	6.7	1.9	1.0
27-8	5.6	11.4	1.0	0.7	5.1	11.2	0.8	0.6
34-4	5.2	5.3	3.5	0	4.7	5.4	2.5	1.1

DCW: Dried cell weight (mg/ml)

Xu: Xylulose (mg/ml)

Ru: Ribulose (mg/ml)

Fermentation medium: glucose 10%, KH_2PO_4 1.0%, K_2HPO_4 1.0%, $\text{MgSO}_4 \cdot 7\text{aq}$ 1.0%, yeast extract 0.5%, $\text{CaCl}_2 \cdot 2\text{aq}$ 0.01%, biotin 30 γ /l and urea 0.6%. pH 8.2 before sterilization.

broth were separated paperchromatographically. The filter paper (No. 51A type made by Tōyō Roshi Co.) was developed with a solvent system consisting of either *n*-butanol-pyridine-water (3:2:1.5) or of water-saturated phenol.

The sugars were detected by 3% anisidine in acetone. A combination of trichloroacetic acid (TCA)-orcinol⁵⁾ and aniline phthalate⁶⁾ sprays was also used to distinguish between ribulose and xylulose. Namely, paper was first sprayed with TCA-orcinol, dried to heated at 100°C for a few minutes, then cooled and oversprayed with aniline-phthalate but not reheated.⁷⁾ Specific colors were obtained with xylulose (purple) and ribulose (pink).

Quantitative Determinations. After the guide spots of ketopentoses on paperchromatogram were detected by anisidine solution, the adjacent spots which were not treated with the spray reagent were cut off and eluted with water at 100°C for 30 minutes.

The ketopentoses in the eluates were determined by cysteine-carbazole- H_2SO_4 method.⁸⁾ The amounts of ribulose and xylulose were calculated separately, according to Ashwell's method,⁹⁾ from the absorb-

ances using both 15 minutes and 120 minutes heating periods, respectively.

The growth of cell shown as D.C.W. in the Table I and Fig. 4 was estimated from turbidity at 660 $\text{m}\mu$ of cell suspension which was prepared by centrifugation of culture broth.

Taxonomic studies. The taxonomic studies on the sugar-forming bacteria were made by the methods described in "Manual of Microbiological Methods."¹⁰⁾

RESULTS AND DISCUSSION

Selection of strains

Among bacteria isolated from the natural sources, several sugar-accumulating bacteria were observed. These sugars were later identified as a mixture of D-ribulose and D-xylulose.

Therefore, to select strains which accumulate a large amounts of these sugars, they were incubated and their productivities were examined.

As shown in Table I, all of the strains tested accumulated the ketopentoses and strain Nos. 12-6, 19-12, 22-23 and 34-4 were selected as suitable strains for this purpose. They produced dihydroxyacetone-like substance, too.

5) A. Bevenue and K. T. Williams, *Arch. Biochem. Biophys.*, **34**, 225 (1951).

6) S. M. Partridge, *Nature*, **164**, 443 (1949).

7) R. M. Hochster, *Can. J. Microbiol.*, **1**, 346 (1954).

8) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).

9) G. Ashwell and J. Hickman, *ibid.*, **226**, 65 (1957).

10) M. J. Pelczar et al., "Manual of Microbiological Methods" Mc-Graw-Hill Book Co., 1957.

Taxonomic characters of the sugar-accumulating bacteria

The taxonomic characters of two strains, No. 12-6 and No. 22-23, are described as follows:

Nos. 12-6 and 19-12

Rods, 0.6 to 0.8 by 1.5 microns, with round ends. Sometimes club-shaped cells are observed. Occurring singly and occasionally in pairs. Non-motile. Gram-positive. No sporulation.

Gelatin stab: Growth on surface. No liquefaction.

Agar colonies: 1.5 to 2.0 mm in diameter, circular, smooth, convex, entire, amorphous, opaque, glistening, cream color, butyrous.

Agar slant: Abundant growth, filiform, raised, glistening, smooth, opaque, cream, butyrous.

Broth: Moderately turbid, ring on surface, viscid sediment.

Agar stab: Best grown at top, filiform or beaded. No gas formed.

Litmus milk: Litmus reduced. No coagulation.

Potato: Growth moderate.

Indole: Not produced.

No acids from carbohydrates.

Nitrites produced from nitrates.

Aerobic.

Optimum temperature: 30°C, Growth at 37°C, no growth at 45°C.

Acetylmethylcarbinol not produced.

Hydrogen sulfide produced.

Urease positive.

Catalase positive.

Starch is not hydrolyzed.

Source: Feces of birds.

Nos. 22-23 and 34-4

Rods or short rods with round ends, 0.8 to 1.2 by 1.5 to 3.0 microns. Occurring singly and in an angular arrangement due to snapping division of the cells. Sometimes elongated and branched cells are observed. Non-motile. Gram-positive, no sporulation.

Gelatin stab: Growth on surface. No liquefaction.

Agar colonies: 1.5 to 2.0 mm in diameter, circular colony, smooth on surface, convex, entire, amorphous, cream yellow color, opaque, glistening, butyrous.

Agar slant: Abundant growth, filiform, raised, glistening, smooth, opaque, cream yellow, butyrous.

Broth: Moderately turbid, ring on surface, viscid sediment.

Agar stab: Best growth at top, filiform or beaded. No gas formed.

Litmus milk: Litmus reduced.

Potato: Growth moderate.

Indole: Not produced.

No acids from carbohydrates.

Nitrites produced from nitrates.

Aerobic.

Optimum temperature: 30°C, Growth at 37°C, no growth at 45°C.

Acetylmethylcarbinol not produced.

Hydrogen sulfide produced.

Urease positive.

Catalase positive.

Starch is not hydrolyzed.

Source: Feces of birds.

The characters of strains No. 12-6 and No. 19-12 were identical with strains No. 22-23 and No. 34-4, respectively. Both strains were gram-positive rods and did not produce acids from many kinds of carbohydrates tested. Although cell forms of No. 12-6 strain did not vary, those of No. 22-23 strain varied to a branched or to an elongated form on citrate medium.

From the characters described above, it seemed that No. 12-6 strains belongs to the genus "*Brevibacterium*" and No. 22-3 to the genus "*Corynebacterium*." However, type species in characters identical with our strains were not found in Bergey's Manual.¹¹⁾

11) R. S. Breed et al., "Bergey's Manual of Determinative Bacteriology" 7th Ed., The Williams and Wilkins Co., 1957.

TABLE II. R_F VALUES AND COLOURS ON PAPERCHROMATOGRAMS OF UNKNOWN SUGARS AND OF VARIOUS SUGARS

Sugars	R_F value	Spray reagent			
		A	B	C	D
Sample	0.54	reddish brown	purple	green, yellowish green	purple, pink
Ribulose	0.53	"	"	yellowish green	pink
Xylulose	0.56	"	"	green	purple
Ribose	0.49	"	yellow	none	none
Xylose	0.44	"	"	blue	—
Lyxose	0.48	"	"	green	brown
Rhamnose	0.57	yellow	"	yellow	brown orange
Erythrose	0.58	brown	—	brown	brown
Dihydroxy acetone	0.65	reddish brown	none	pale violet	yellowish green

A 3% anisidine in acetone and ethanol

B Urea-phosphate

C Orcinol-TCA

D Orcinol-TCA + Aniline phthalate

Paperchromatography: Solvent system: *n*-butanol: pyridine: H₂O=3:2:1.5

Identification of the sugars

For the purpose of identification of the sugars produced, No. 22-23 strain was incubated for 5 days on the medium described in "Materials and Methods". The supernatant of the cultured broth was treated with cation-exchange resin "SK# 1" (H⁺ form) and then with anion-exchange resin "SA21A" (OH⁻ form). The deionized fluid was concentrated in vacuo and was streaked onto filter paper.

Spots on guide strips of the paperchromatogram were detected with a spray reagent and then untreated portions of the chromatogram that corresponded with those of ketopentoses were cut off and eluted with water at 100°C for 30 minutes. The eluate was concentrated in vacuo and it was streaked again onto paper. Thus, two fractions that corresponded with xylulose and ribulose were obtained paperchromatographically from the mixture of sugars.

The R_F values of the two ketopentoses were identical with those of authentic ribulose and xylulose as shown in Table II and Fig. 1. The former spot was pink and the latter was purple by the treatment of both TCA-orcinol and aniline phthalate sprays.

The absorption spectra of both ketopentoses by cysteine-carbazole-H₂SO₄ reaction and by Bial's reaction were almost identical with those of authentic samples, respectively (Fig. 2 and 3). The maximum absorbance at 540 m μ in cysteine-carbazole-H₂SO₄ reaction was obtained by standing still ribulose-like sugar

FIG. 1. Two Dimensional Paperchromatogram of Unknown Sugars.

A* Xylulose-like spot: purple with a combination of orcinol-TCA and aniline phthalate spray reagents.

B* ribulose-like spot: pink with the same spray reagents.

* R_F values of the spots corresponded to those of authentic xylulose and ribulose, respectively.

and xylulose-like sugar for 15 minutes and for 120 minutes, respectively. The optical rotations of both sugars were shown as D-form. From these results described above, the sugars accumulated in the culture broth of No. 23-22

strain were proved to be identical with the mixture of D-ribulose and D-xylulose.

Chemical changes

Typical chemical changes in D-ribulose and D-xylulose fermentation on the medium described above are given in Fig. 4.

FIG. 2. Absorption Spectra of Ketopentoses by Cysteine-carbazole- H_2SO_4 method.

Ru: Ribulose
Xu: Xylulose

FIG. 3. Absorption Spectra of Ketopentoses by Bial's Reaction.

A Ribulose-like sugar
B Xylulose-like sugar

FIG. 4. Chemical Changes of Ketopentose Fermentation.

Strain: No. 22-23

Fermentation medium: glucose 10.0%, KH_2PO_4 1.0%, K_2HPO_4 1.0%, $\text{MgSO}_4 \cdot 7 \text{ aq.}$ 1.0%, $\text{CaCl}_2 \cdot 2 \text{ aq.}$ 0.01%, yeast extract 0.5%, biotin 30 γ/l and urea 0.6%, pH 8.2 before sterilization, at 30°C, shaking culture.

DCW: Dried cell weight (mg/ml)

Xu: Xylulose (mg/ml)

Ru: Ribulose (mg/ml)

The time course shows that the ketopentoses were accumulated with incubation time and the maximum yield of ketopentoses was approximately 7 to 8 mg per ml of the culture broth.

Although the mechanisms of ribulose and xylulose accumulation in large amounts by these bacteria have been unknown up to the present, such an appreciable accumulation of the sugars seems to be ascribed partly to inability of the organisms to produce acids from many kinds of carbohydrates.