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Note

Preparation of L-fructose and D-sorbose by the bacterial oxidation of L-mannitol and L-glucitol, respectively

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As part of a current program concerned with the utilization of unusual carbohydrates by microorganisms to produce industrially significant metabolites, we have reported[1] the purification and properties of a novel polyol dehydrogenase of bacterial origin. The bacterium, as yet unidentified, possesses a constitutive enzyme which was shown to be a nonspecific, inducible, polyol dehydrogenase capable of oxidizing preferentially alditols having the L configuration. In addition to the oxidation of L-glucitol to D-sorbose, the enzyme also oxidizes ribitol, xylitol, and L-arabinitol to ketoses. The present work stemmed from our investigation of the possible further oxidation of the product, D-sorbose, to L-*threo*-2,5-hexodiulose, in a manner analogous to that described for *Trametes sanguina*[2] and *Gluconobacter cerinus*[3]. During the course of this investigation it was found that not only the crude, cell-free extract of our bacterium, designated MD-13, but also its washed-cell suspensions could actively transform L-alditols into the corresponding ketoses. In this Note we describe the adaptation of the system for the preparation of L-fructose and D-sorbose. L-Fructose is potentially of great commercial importance in view of the current intensive and highly competitive, international efforts devoted to the syntheses and utilization of L-sugars as sweetening agents[4].

EXPERIMENTAL

Materials and methods. — (*a*) *Bacterial strain.* The bacterial strain MD-13 (ref. 1) was used in this study.

(*b*) *Media and culture conditions.* The medium[5] was composed of yeast extract (Difco), 0.4 (w/v)%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7\,H_2O$, 0.05%; supplemented with D-glucitol, 2%, and ribitol, 0.5%. Aliquots (50 mL) of medium in 250-mL Erlenmeyer flasks were inoculated with 2-mL portions of a culture grown for 18–20 h on the same medium, and shaken at 175 rev. min^{-1} at 35° for 20–24 h.

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(*c*) *Chemicals.* — NAD, D-glucitol, and ribitol were purchased from Sigma Chemical Co., St. Louis, MO. The samples of L-mannitol, L-glucitol, and L-rhamnitol were prepared by reduction, using sodium borohydride, of L-mannose, L-glucose, and L-rhamnose (Sigma), respectively; the samples of alditols were 98–100% pure as indicated by h.p.l.c. analyses.

(*d*) *Preparation of cell-free extract, and enzyme assay.* The crude cell-free extract was prepared and assayed as described by Dhawale *et al.*[1].

(*e*) *Protein determination.* The protein was determined using a Bio-Rad protein-assay kit[6].

(*f*) *Fermentation.* The cells from the culture [growth (A_{620} = 6.0)] were harvested by centrifugation and washed once with sterile, 0.14M NaCl (40 mL) and then with an equal volume of sterile, distilled water. The resultant cell biomass (~0.5 g wet weight/50 mL of culture) was added to 20 mL of filter-sterilized, 1% aqueous solutions of L-mannitol or L-glucitol, in 250-mL Erlenmeyer flasks and shaken at 175 rev. min^{-1} at 30°. The progress of fermentation was monitored by analysis by h.p.l.c.

(*g*) *Product purification and identification.* At the end of the fermentation period, the bacterial cells were removed by centrifugation at 27,000*g* for 20 min. The supernatant solution was decolorized with activated charcoal and deionized with Amberlite IR-120 (H^+) and Amberlite IRA-400 (OH^-) ion-exchange resins. The resins were removed by filtration. The filtrate was evaporated under vacuum and the residue was extracted with ethanol (L-mannitol fermentation), or water (L-glucitol fermentation). The extract was filtered and evaporated to a syrup under vacuum. The products solidified on being kept in a desiccator in the cold. The products were characterized by optical rotation and thin-layer chromatographic mobility. The solvent systems (v/v) used were: (*A*) 2:1:1 1-butanol–acetic acid–water; (*B*) 7:1:2 1-propanol–ethyl acetate–water. The developed plates (silica gel) were sprayed with *N*-(1-naphthyl)ethylenediamine (0.2%) in 3% methanolic sulfuric acid, or 10% sulfuric acid in methanol, and then heated.

RESULTS AND DISCUSSION

The bacterium MD-13 was cultured in a medium containing ribitol, which had been observed[1] to be an inducer of the polyol dehydrogenase. A crude, cell-free extract was prepared and assayed for the NAD-dependent dehydrogenase activity as described previously[1], using a range of substrates. The data are presented in Table I and show that L-mannitol, L-glucitol, L-rhamnitol, and D-glucitol were the most active substrates, in decreasing order of activity, whereas only minimal activity was observed with D-mannitol, D-sorbose, and L-rhamnose. As already indicated, one of the goals of the work was to investigate the possibility of oxidation of D-sorbose to L-*threo*-2,5-hexodiulose; the inclusion of L-glucitol in the culture medium had no effect on the enzyme activity in the case of D-sorbose as the substrate. In fact, even with prolonged incubation (10–12 days) of L-glucitol with

TABLE I

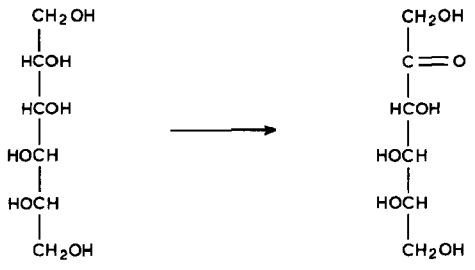
OXIDATION OF VARIOUS SUBSTRATES BY CRUDE, CELL-FREE EXTRACT OF BACTERIUM MD-13

Substrate[a]	*Activity (U/mg protein)*[b]
L-Mannitol	104
L-Glucitol	88
L-Rhamnitol	60
D-Glucitol	29
D-Mannitol[c]	3.4
L-Rhamnose	8.7
D-Sorbose	1.7

[a]Substrate concentration: 25 μmol/mL. [b]1 U = 1 nmol NADH generated per min. [c]Concentration: 50 μmol/mL.

washed-cell suspensions of MD-13, no formation of L-*threo*-2,5-hexodiulose was observed by chromatographic examination, using an authentic sample[7] of the latter compound as a standard.

In the present study, L-mannitol (**1**) and L-glucitol (**3**) were fermented also, using washed-cell suspensions of MD-13. In these cases, ~75% of the substrate was consumed within 24 h; however, 8–10 days were required to attain a 95% conversion level. If an initial substrate concentration of <1% were used, essentially com-



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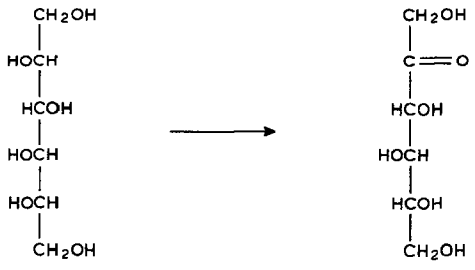

3 **4**

plete conversion was attained in 5–6 days. In contrast, the oxidation of L-alditols by cell-free extracts of MD-13 gave a maximum substrate consumption of 25–30% after incubation for 24 h at 34°; extending the incubation period did not afford improved yields of products.

The product of the fermentation of L-mannitol using washed-cell suspensions of MD-13 was isolated crystalline and identified as L-fructose (**2**, 67%) by comparison of thin-layer chromatographic mobilities (R_F 0.5, solvent *A*; 0.51, solvent *B*) and optical rotation {$[\alpha]_D^{26}$ +86° (*c* 1.0, water); lit.[8] $[\alpha]_D^{21}$ +128° (initial, extrapolated) → +93° (water)} with the corresponding values for authentic L-fructose. The product of the fermentation of L-glucitol (≡D-gulitol) was isolated as a solid and identified as D-sorbose (**4**, 60%) on the basis of thin-layer chromatographic mobilities (R_F 0.51, solvent *A*; 0.53, solvent *B*) and optical rotation {$[\alpha]_D^{26}$ +44.4° (*c* 6.3, water); lit.[9] $[\alpha]_D^{23}$ +43° (*c* 1, water; no significant mutarotation)}.

The fermentation process affords crystalline L-fructose in 67% yield, and is a significant improvement over a published enzymic procedure[10] which provided the target material from L-mannose in an overall yield of only 28–32%.

The conversion of L-gulitol (D-gulitol) into D-sorbose by washed-cell suspensions of MD-13 has been achieved in 60% yield. This yield very greatly exceeds that (~2%) obtained[11] from the alkaline isomerization of D-glucose using strong-base resin, and, although the oxidation of L-glucitol to D-sorbose has been reported[12], using resting cells of *Gluconobacter oxydans*, data pertaining to the yield are unobtainable. Thus, the method described in this Note is potentially of considerable importance as a facile, efficient route to these unusual ketoses.

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