Journal of Industrial Microbiology, 7 (1991) 57–62
Elsevier

SIM 00304

A nonchromatographic assay for sorbitol in chemically complex growth media containing sorbose

Barbara A. Feshami* and G. William Claus

Microbiology and Immunology Section, Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA, U.S.A.

Received 19 February 1990; revised 2 June 1990; accepted 6 June 1990

Key words: Gluconobacter oxydans; Sorbitol assay; Sugar alcohols; Lowry-Lopez interference assay

SUMMARY

Microbiologists who lack gas-chromatographic equipment cannot easily study the microbial conversion of sorbitol to sorbose, because there is no other proven method for quantitatively measuring sorbitol in complex growth media. The purpose of this study was to determine if the polyol assay suggested by Mäkinen in 1980 could be used to measure sorbitol depletion in chemically complex bacteriological-culture media containing sorbose. After we pretreated the culture medium to remove interfering phosphate complexes and slightly modified Mäkinen's assay, we were able to detect 0.1 mg/ml differences in sorbitol concentrations that varied between 0.9 and 1.5 mg of sorbitol/ml. The presence of sorbose in the complex medium did not affect the assay. Growing cultures of *Gluconobacter oxydans* were used to test this assay, because these bacteria reportedly oxidize sorbitol to sorbose and quantitatively release the sorbose into the growth medium. When samples of the culture medium removed during growth were centrifuged to remove cells and precipitated to remove interfering substances then tested with the modified Mäkinen assay, these cultures showed a sorbitol depletion rate of 4.9 ($\pm$ 0.1) mg/ml h^{-1}. The Fehling's assay on the same cultures showed a sorbose accumulation rate of 5.12 ($\pm$ 0.01) mg/ml h^{-1}. We concluded that the modified Mäkinen phosphate-interference assay can be satisfactorily used to quantitatively screen cultures for their ability to oxidize sorbitol.

INTRODUCTION

While growing in a chemically complex medium, the bacterium *Gluconobacter oxydans* rapidly oxidizes mono-, di-, and polyhydroxy compounds by removing one pair of hydrogens from the oxidizable substrate. Typically, more than 90% of the substrate is converted to the corresponding oxidation product which is released into the growth medium [6]. For example, both the oxidation of sorbitol by *G. oxydans* and the recovery of sorbose from the growth medium are so efficient that this process is used as part of the commercial synthesis of vitamin C [6]. We wished to examine gluconobacter cultures for their ability to oxidize sorbitol to sorbose. It was possible to follow both sorbitol depletion and sorbose accumulation with a gas chromatograph [10]; however, our laboratory did not have ready access to this equipment. One could also follow sorbose production using a modified Fehling's test for reducing sugars, but we could find no suitable, published, nonchromatographic method for assaying sorbitol disappearance in chemically complex culture media containing sorbose.

The periodate-oxidation method quantitatively detects sorbitol [1,12], but this method cannot be used when reducing sugars such as sorbose and fructose are present [4]. In 1980, Mäkinen suggested a colorimetric assay for polyhydroxy compounds (polyols) based upon how they interfere with the Lowry-Lopez phosphate assay [9]. Mäkinen demonstrated that polyols containing four or more hydroxyl groups interfere by binding to molybdenum [3,7], and this prevents formation of the blue phosphomolybdenum complex that is typical of the Lowry-Lopez colorimetric orthophosphate assay [8]. Mäkinen demonstrated that polyol interference is directly proportional to the concentration present in solutions, but his reaction mixtures contained only polyols in distilled water, and sorbitol was not used in his study [9]. Other investigators also demonstrated that sorbitol interferes with the Lowry-Lopez orthophosphate interference assay and that reducing sugars, such as glucose and fructose, exert no effect on this assay [5,7]. On the other hand, we found no study that tested the effect of sorbose on this assay, and our preliminary studies showed that common components of chemically complex culture media interfered with this assay. Therefore, the purpose of this study was to determine if Mäkinin's orthophosphate-interference assay

Correspondence: G.W. Claus, Department of Biology, Virginia Tech, Blacksburg, VA 24061-0406, U.S.A.
* Present address: 5228 Kepler Lane, Springfield, VA 22151, U.S.A.

could be modified so that it would detect changes in sorbitol concentrations in cultures of bacteria producing sorbose.

MATERIALS AND METHODS

Reagents. Peptone and yeast extract were obtained from Difco (Detroit, MI); KH_2PO_4 was obtained from J.T. Baker (Phillipsburg, NJ), and sodium acetate was purchased from Mallinckrodt Chemical Works (St. Louis, MO). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO). All reagents were dissolved in water that was passed sequentially through the following systems: (i) Milli-RO-120 reverse osmosis apparatus (Millipore Corporation, Bedford, MA), (ii) 3508 organic removal cartridge LD-5 (Corning Glass Works, Corning, NY), (iii) 3508 mixed-bed (anion/cation) ultra-high removal cartridge (Corning), and (iv) six-liter automatic mega-pure glass distillation apparatus (Corning). Water treated in this way is hereafter referred to as *ultrapure water* (uH_2O). The sorbose solution contained 50 mg of sorbose per ml of uH_2O (5% w/v). The yeast extract and peptone solution contained 10 mg of each per ml of uH_2O (1% w/v).

Culture and growth conditions. The type species for the genus *Gluconobacter* was used in this study. *Gluconobacter oxydans* (ATCC strain 19357) was obtained from the American Type Culture Collection (Rockville, MD), maintained in sorbitol stock cultures at −15 °C as previously described [13], and grown on the chemically complex culture medium which contained the following components (given in percent w/v): 1% yeast extract, 1% peptone, 5% sorbitol, and 2% 2-(*N*-morpholino)-propanesulfonic acid (MOPS buffer) dissolved in uH_2O. Dry-cell weight measurements were made as described by White and Claus [13].

To determine the rates of sorbitol oxidation during growth, cells were grown in 2-l Bellco Nephelometer flasks containing 200 ml of complex medium and incubated with shaking at 28 °C as previously described [13]. Samples were taken from these cultures at half-hour intervals, and cells were removed by centrifugation at 4000 × *g* for 10 min at room temperature. The supernatant fluid was quantitatively assayed for both sorbose and sorbitol.

Precipitation of interfering materials. Bailey's BaOH/$ZnSO_4$-precipitation method [1] was modified by changing the reagent volumes and deleting the phenol-red acid/base indicator once the correct proportion of reagents was determined. The following procedure was used. One ml of a uniform suspension of BaOH (4.7% w/v) was thoroughly mixed with 1 ml of the solution to be tested. This was followed by the stepwise addition of 0.5 ml of 5% (w/v) $ZnSO_4$ and 0.5 ml of 4% (w/v) $MgSO_4$ solutions with thorough mixing after each addition. This mixture was then diluted with uH_2O to bring the sorbitol concentration within the linear portion of the standard curve. The precipitate which formed was removed by centrifugation at 1200 × *g* for 2–3 min. The resulting supernatant fluid was called the "pretreated sample" and was used for the sorbitol assay.

The ratio of BaOH to $ZnSO_4$ may need to be altered when other types of culture media are used. According to Bailey [1], it is necessary to maintain an excess of Ba^+ and OH^- in solution to assure that interfering substances will be precipitated. Bailey used phenol red as an acid-base indicator to determine when BaOH was in excess. However, we found that phenol red caused an inconsistent delay in the formation of the phosphomolybdenum complex in assay mixtures containing only phosphate and uH_2O. We supposed that this was due to a reaction between phenol red and molybdenum [12]. Therefore, we recommend the following procedure when the precipitation method is applied to other chemically complex media. First, set up a series of reaction mixtures containing phenol red, a constant amount of the culture medium, and varying concentrations of BaOH. This will establish the proper ratio of BaOH to $ZnSO_4$, so that the final pH values range from 7 to 8 (excess BaOH). From then on, as long as the medium is the same and the BaOH/$ZnSO_4$ ratio is kept constant, phenol red should not be used.

We also recommend that the precipitated medium be tested for complete removal of interfering materials by adding the same amount of sorbitol to both the precipitated medium and uH_2O, then assaying for sorbitol using the KH_2PO_4 concentration given in Table 1. If interfering materials are not completely removed from the medium, their presence will be detected by an artificially high value for sorbitol in the medium compared to the values obtained in uH_2O for the same sorbitol concentration.

Other materials, such as complex buffers and organic acids, may also interfere somewhat with this assay [3]. These materials are not easily removed from chemically complex growth media. This problem can be overcome (i) by running all sorbitol standard curves in samples of pretreated growth medium and (ii) by using a constant dilution, following the $MgSO_4$ addition, so that each tube, with known or unknown sorbitol concentrations, contains the same amount of pretreated growth medium.

Quantitative determination of sorbitol. One ml of supernatant fluid was diluted with 3 ml of uH_2O and subjected to the colorimetric sorbitol assay suggested by Mäkinen [9], except that we used twice the phosphate concentration to raise the detectable concentration of sorbitol. Solution A contained 1% (weight/volume) of L-ascorbic

acid and 1.47 mM KH_2PO_4. Solution B contained 0.028 M sodium acetate, 2.7 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.0002 N H_2SO_4. Solution B was adjusted to pH 4.0 with concentrated HCl acid before use. One ml of freshly prepared solution A was added to 4 ml of the pretreated or nonpretreated sample and thoroughly mixed. The reaction was started by adding 2 ml of freshly prepared solution B. After thorough mixing, the reaction mixtures were allowed to stand for 60 min at room temperature, after which the absorbance of the blue phosphomolybdenum complex was measured at 700 nm (12.7 mm path length) with a Bausch and Lomb Spectronic-20 spectrophotometer.

Quantitative determination of sorbose. Samples of the culture medium were removed at intervals and tested for sorbose concentration using Fehling's solution [4] and an endpoint titration analysis. Cells were removed from the medium by centrifugation at 4400 × *g* for 15 min, then varying amounts of Fehling's solution were added to 1-ml volumes of spent medium. These reaction mixtures were boiled for 10 min to develop the color then centrifuged at low speed to remove the precipitate. The volumes of Fehling's solution used in these titrations were adjusted so that increasing volumes (placed in separate tubes) gave first a yellow, then a light yellow (the endpoint), and, finally, a pale blue color. Spent media having unknown sorbose concentrations were compared to a standard curve prepared with known concentrations.

RESULTS AND DISCUSSION

Removal of interfering substances

Like Mäkinen, we also found a linear relationship between the concentration of sorbitol dissolved in uH_2O and the extent of interference with the Lowry-Lopez phosphate assay (data not shown). However, we found that this relationship was not linear when sorbitol was dissolved in chemically complex culture media (data not shown). Table 1 shows that solutions containing 1% yeast extract and 1% peptone doubled the absorbance found in the absence of these compounds. This increase in absorbance was eliminated when these solutions were pretreated with Bailey's precipitation method (Table 1). This pretreatment did not adversely affect the Lowry-Lopez phosphate assay used to quantitatively determine sorbitol.

Bailey's precipitation method for protein removal [1] uses $ZnSO_4$ and excess BaOH. According to Cardini and Leloir [2], an excess of Ba^+ at pH 8 combines with inorganic phosphates and phosphate-containing organic compounds to form insoluble barium-phosphate salts. In addition, the excess OH^- allows for ZnOH formation which precipitates proteins at pH 7 to 8 [11]. Therefore, we presumed that the increased absorbance at 700 nm provided by yeast extract and peptone (Table 1) was caused by inorganic- and/or protein-bound phosphates contained in these common medium components.

TABLE 1

Effect of sorbose, sorbitol, medium components, and pretreatment on colorimetric phosphate assay using a constant amount of phosphate [a]

Sample additions [b]	Precipitation pretreatment [c]	Absorbance (700 nm) [d]
None	No	0.64 ± 0.05
1% YE + 1% PE	No	1.20 ± 0.28
1% YE + 1% PE	Yes	0.63 ± 0.06
None	Yes	0.59 ± 0.01
5% Sorbose	No	0.60 ± 0.01
5% Sorbitol	No	0.00

[a] The Lowry-Lopez colorimetric phosphate assay was performed on samples containing ultrapure water (uH_2O) either with or without additions and with or without a precipitation pretreatment as described in MATERIALS AND METHODS. The phosphate concentration in solution A was 0.735 mM KH_2PO_4, so that any enhancement of the color reaction by medium components could be easily detected.

[b] Unless shown as an addition, samples used for quantitative sorbitol determinations contained only uH_2O. Additions were: yeast extract and peptone (YE + PE), 10 mg/ml of each; sorbose, 50 mg/ml; or sorbitol, 50 mg/ml.

[c] Precipitation pretreatment ($BaOH/ZnSO_4$) method is described in MATERIALS AND METHODS.

[d] Reported values are averages of at least two separate experiments. Absorbance values for samples containing no additions reflect the quantity of phosphomolybdenum complex formed by the experimentally added phosphate (in solution A) and the absence of phosphate or the presence of interfering substances in the sample additions.

Effect of sorbose on sorbitol assay

Although reducing sugars such as glucose and fructose reportedly have no depressive effect upon the Lowry-Lopez phosphate assay [5,7], we know of no previous study that included sorbose. Since cultures of *G. oxydans* reportedly oxidize sorbitol quantitatively to sorbose, we needed to determine if sorbose formation in culture media had a depressive effect on the Lowry-Lopez assay. Table 1 shows that the presence of sorbose did not significantly affect the sorbitol assay. Therefore, we concluded that sorbose formation during growth would not significantly interfere with the quantitative assay of sorbitol remaining in the culture medium.

Quantitation of sorbitol in solutions containing yeast extract and peptone

Test media were prepared by adding various quantities of sorbitol to tubes containing a freshly prepared yeast extract and peptone solution. Each tube of test medium was then precipitated to remove interfering phosphate compounds. These pretreated samples were then quantitatively assayed for sorbitol. Fig. 1 shows that the quantity of sorbitol interfered with the Lowry-Lopez phosphate assay in a linear manner at concentrations ranging from 0.9 to 1.5 mg of sorbitol per ml of pretreated sample. In similar experiments, where varying concentrations of sorbitol were placed in uH_2O instead of the yeast-extract/peptone solution and subjected to the precipitation technique, each concentration of sorbitol showed essentially identical adsorption values as those shown in Fig. 1. These data show that changes in sorbitol concentration as small as 0.1 mg/ml of complex medium were detected by this assay method.

Mäkinen [9] showed that distilled-water solutions of perseitol, galactitol, D-mannitol, L-arabitol, ribitol, xylitol, and i-erythritol, in addition to D-sorbitol, all provided various levels of interference with the Lowry-Lopez phosphate assay. Therefore, it is conceivable that polyols, other than sorbitol, can be quantitatively detected in complex culture media supporting bacterial growth. However, our results suggest that these media should first be pretreated, using Bailey's $BaOH/ZnSO_4$-precipitation method, to remove inorganic and organic phosphates present in the culture medium.

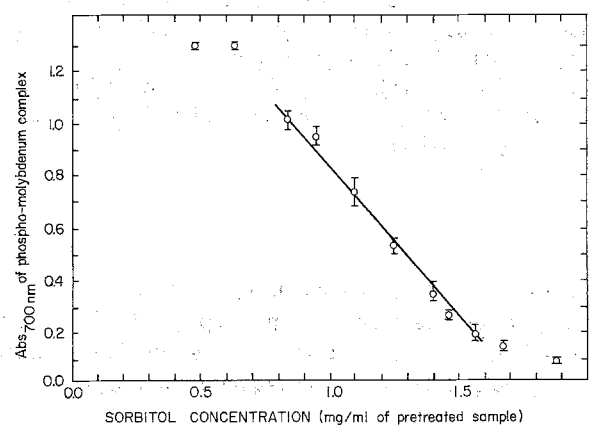

Fig. 1. Effect of sorbitol concentration on interference with the Lowry-Lopez phosphate assay. Various known concentrations of sorbitol were separately added to tubes of a freshly prepared yeast extract and peptone solution. Each sample was pretreated by precipitation to remove interfering substances then assayed for sorbitol as described in MATERIALS AND METHODS. Each data point shows the average of three separate determinations with the range in values indicated by the vertical bar. Linear regression analysis was used to draw the line through these data points to form a standard curve that was later used for analyzing sorbitol utilization in cultures of *G. oxydans*. Lower absorbance values with increasing sorbitol concentrations reflect increasing competition between polyols and phosphate for complexing with molybdenum.

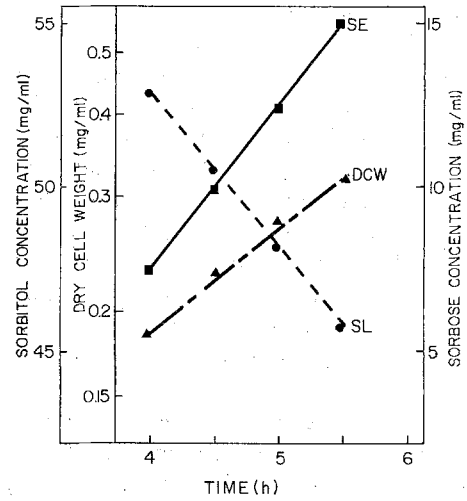

Fig. 2. Rates of sorbitol disappearance and sorbose accumulation in one representative, exponentially growing culture of *G. oxydans*. Sorbose (SE) and sorbitol (SL) concentrations in samples of spent medium, pretreated to remove phosphorous compounds, and dry cell weights (DCW) were determined as described in MATERIALS AND METHODS.

Quantitation of sorbitol and sorbose in inoculated cultures

Since medium used to grow *G. oxydans* contained MOPS buffer (in addition to sorbitol, yeast extract, and peptone), we first determined that MOPS buffer did not interfere with the sorbitol assay (data not shown). Then we sampled exponentially growing cultures of *G. oxydans*, removed the cells with centrifugation, and pretreated the resulting media to remove organically bound phosphates. The resulting samples were then separately assayed for sorbitol and sorbose concentrations, so as to determine the rate of sorbitol utilization and sorbose production. An analysis of one representative culture is shown in Fig. 2. Sorbitol concentration decreased at a rate of 4.9 ($\pm$0.1) mg/ml h^{-1}, and sorbose concentration increased at a rate of 5.12 ($\pm$0.01) mg/ml h^{-1}.

These nearly identical rates of substrate utilization and product accumulation agree with the oxidative characteristics of this bacterium. These results also confirm that sorbitol concentrations in chemically complex microbiological culture media can be determined using the Lowry-Lopez phosphate-interference assay if samples of growth media are first pretreated to remove traces of organically bound phosphate.

ACKNOWLEDGEMENT

The authors wish to thank C.A. Baker (Monsanto, Co., St. Louis, MO) for testing these procedures on other types of media, and with cultures of *G. oxydans* ATCC strain 621, and for critically reviewing the text of the manuscript.

REFERENCES

1 Bailey, J.M. 1959. A microcolorimetric method for the determination of sorbitol, mannitol and glycerol in biologic fluids. J. Lab. Clin. Med. 54: 158–163.

2 Cardini, C.E. and L.F. Leloir. 1957. Acid lability of phosphorous compounds. In: Methods in Enzymology, Vol. 3 (S.P. Colowick and N.O. Kaplan, eds.), pp. 835–840, Academic Press, New York, NY.

3 Chalmers, R.A. and A.G. Sinclair. 1967. Organic molybdate complexes. J. Inorg. Nucl. Chem. 29: 2065–2080.

4 Dawson, R.M.C., D.C. Elliott, W.H. Elliott and K.M. Jones (eds.). 1969. Data for Biochemical Research, 2nd edn., (see p. 618), Oxford Univ. Press, New York, NY.

5 Fedorov, A.A. and A.V. Pavlinova. 1972. Complex formation between molybdenum (VI) and mannitol and sorbitol respectively and its analytical application. J. Anal. Chem. USSR (translation from Zhurnal Analiticheskoi Khimii) 27: 2188–2191.

6 Greenshields, R.N. 1978. Acetic acid; Vinegar. In: Economic Microbiology, Vol. 2, Primary Products of Metabolism (Rose, A.H., ed.), pp. 121–183, Academic Press, New York, NY.

7 Ho, C.H. and S.V. Pande. 1974. Orthophosphate determination: Interference by mannitol and sorbitol. Anal. Biochem. 60: 413–416.

8 Leloir, L.F. and C.E. Cardini. 1957. Acid lability of phosphorous compounds. In: Methods in Enzymology, Vol. 3 (Colowick, S.P. and N.O. Kaplan, eds.), pp. 840–850, Academic Press, New York, NY.

9 Mäkinen, K.K. 1980. Interference of polyols with Lowry-Lopez phosphorous assay: Reactions of polyols with the molybdate ion. Anal. Biochem. 101: 148–153.

10 Manius, G., F.P. Mahn, V.S. Venturella and B.Z. Senkowski. 1972. GLC Determination of sorbitol and mannitol in aqueous solutions. J. Pharm. Sci. 61: 1831–1835.

11 Neish, A.C. 1957. Chemical procedures for separation and determination of alcohols. In: Methods in Enzymology, Vol. 3 (Colowick, S.P. and N.O. Kaplan, eds.), pp. 255–263, Academic Press, New York, NY.

12 Nisli, G. and A. Townshend. 1968. Spectrophotometric determination of some *vic*-diols. Talanta 15: 1377–1384.

13 White, S.A. and G.W. Claus. 1982. Effect of intracytoplasmic membrane development on oxidation of sorbitol and other polyols by *Gluconobacter oxydans*. J. Bacteriol. 150: 934–943.