APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1989, p. 1317–1319
0099-2240/89/051317-03$02.00/0

Vol. 55, No. 5

Synthetic Medium for *Acetobacter xylinum* That Can Be Used for Isolation of Auxotrophic Mutants and Study of Cellulose Biosynthesis

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Received 14 November 1988/Accepted 10 February 1989

***Acetobacter xylinum* is a bacterium that can synthesize cellulose when grown in an undefined medium containing glucose. We developed a defined minimal medium for *A. xylinum* that contains 1% glucose, 0.1% NH_4Cl, 0.115% citric acid, 0.33% Na_2HPO_4, 0.01% KCl, 0.025% $MgSO_4 \cdot 7H_2O$, and 7.5 mg of nicotinamide per liter which both allows cellulose synthesis and can be used to isolate a variety of auxotrophic mutants.**

Acetobacter xylinum is a gram-negative bacterium that synthesizes cellulose when grown without agitation in broth culture (9). The cellulose is chemically identical to that found in eucaryotic plants; therefore, *A. xylinum* is a good model for the study of cellulose synthesis (2, 5). Cellulose is formed from an exogenous substrate, glucose, not from any other polysaccharide, and is secreted and accumulated extracellularly (7, 15). A membrane-bound enzyme complex, cellulose synthase, may be responsible for the synthesis of cellulose (3).

A. xylinum is usually cultured in a chemically undefined medium developed by Hestrin and Schramm (9). However, this medium is not useful for isolating auxotrophic mutants that might serve as good tools for genetic studies. A chemically defined medium can fulfill this purpose. Tepper and Litsky (17) and Dudman (6) have developed synthetic defined media for *Acetobacter* species. However, both media contain compounds (mineral salts, aminobenzoic acid, nucleotide precursors, amino acids, or calcium pantothenate) that we found unnecessary to support growth and cellulose production. We report development of a simpler synthetic minimal medium for *A. xylinum* that permits good growth and cellulose synthesis besides being useful to isolate auxotrophic mutants for genetic studies.

A. xylinum (ATCC 23769) was kindly provided by R. M. Brown, Jr. (University of Texas at Austin). The undefined medium described by Hestrin and Schramm (H&S medium), containing (per liter) 20 g of glucose, 5 g of yeast extract, 5 g of peptone, 2.7 g of sodium phosphate, and 1.15 g of citrate, was used for routine cultivation (9). We assessed several synthetic media for the ability to support the growth of *A. xylinum*, including media developed for *Acetobacter aceti* (12) and *Escherichia coli* (4, 8). A vitamin-complex solution for *Neurospora* strains was also tested (modified from Tatum et al. [16]). The cell inoculum was washed twice by centrifugation at 11,000 × *g* for 20 min each time to remove residual nutrients, and the cell pellet was suspended in buffer (0.035% citrate, 0.14% Na_2HPO_4) and used to inoculate the different media. All incubations were at 26°C.

The synthetic medium that contained the fewest nutrients but maintained growth and cellulose production was prepared as follows. Stock solution A consisted of the following components (for 100 ml): glucose (1% final concentration, 250-g/liter stock solution prepared and sterilized separately), 40 ml; citric acid, 1.15 g; NH_4Cl, 1 g; Na_2HPO_4, 2.7 g; KCl, 0.1 g; and $MgSO_4 \cdot 7H_2O$ (25-g/liter stock solution prepared and sterilized separately), 10 ml. Minimal medium contained the following components: stock solution A, 100 ml; nicotinamide (7.5-mg/ml stock solution, filter sterilized), 1 ml; agar (optional), 15 g; H_2O, 899 ml. This medium was defined by a process of elimination based on the medium for *A. aceti* and the vitamin-complex solution for the *Neurospora* strains. Cultures were assayed for both growth and cellulose synthesis. In addition to four inorganic salts and citric acid, *A. xylinum* required glucose and nicotinamide for growth. To ensure that *A. xylinum* could grow in the minimal medium, the bacterium was subcultured five times in minimal broth for 96 h each time. No loss of bacterial viability was seen, on the basis of colony counts, after each subculturing. Deletion of any one of the specific nutrients from minimal medium resulted in no growth of *A. xylinum*.

Glucose is required for both growth and cellulose production by *A. xylinum*. Growth curves at various glucose concentrations are depicted in Fig. 1. Glucose was not growth limiting even at 0.25%. Growth of *A. xylinum* was slower and produced a lower cell density in minimal synthetic media at all four glucose concentrations compared with that in the undefined complete medium.

Organisms grown in the minimal medium produced cellulose, as determined on the basis of several different criteria. Colonies on agar medium plus 0.01% Tinopal LPW (CIBA-GEIGY Corp.) fluoresced when examined under longwave UV light (Blak-Ray model X-4; Ultra-Violet Products, Inc.). Tinopal is a fluorescent dye that binds to beta-glucans (13). Pellicles formed on the surface of cultures grown in liquid medium and withstood boiling in 2% NaOH for 30 min (14). Microscopic observation of the pellicles confirmed cellulose fibril formation. Cultures grown in the minimal medium produced far less cellulose than did cultures grown in undefined medium. Flasks containing 100 ml of each kind of medium (H&S and minimal medium plus 2%, 1%, 0.5%, and 0.25% glucose) were inoculated with 0.5 ml of washed cells (8×10^8 cells per ml) and incubated without agitation at 26°C. Fifteen days later, cellulose pellicles were removed, dried at 80°C for 60 min, and weighed. The average dry weight of cellulose in complete medium (mean ± standard deviation) was 97.6 ± 7.6 mg; in minimal medium with 2%, 1%, 0.5%, and 0.25% glucose, it was 55.8 ± 2.4, 55.0 ± 5.5,

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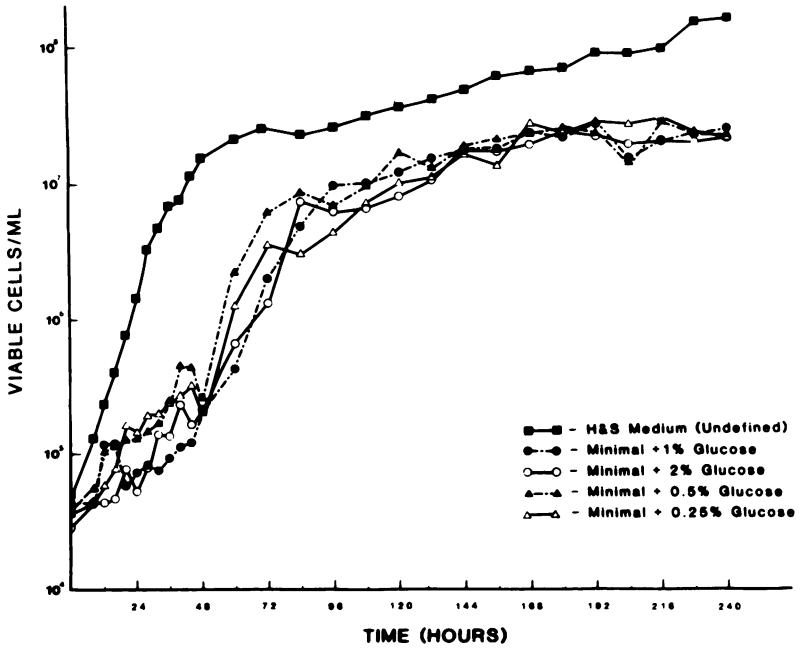

FIG. 1. Growth curves for *A. xylinum* in complete, undefined medium and minimal, synthetic media at four glucose concentrations (2%, 1%, 0.5%, and 0.25%). Washed cells were inoculated into 100 ml of broth medium, and cultures were incubated without agitation at 26°C. Before a 0.5-ml sample was taken at each time point for a plate count on H&S medium, each flask was shaken for 30 s at 150 rpm (New Brunswick Scientific Co. Psychrotherm shaker) to release cells consistently from the cellulose pellicle.

34.8 ± 2.9, and 26.8 ± 6.8 mg, respectively. A 1% glucose concentration seems most appropriate for the minimal defined medium.

To determine whether the new medium was appropriate for isolation of auxotropic mutants of *A. xylinum*, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis was performed (100 μg/ml) (1). After mutagenesis, cells were grown for 48 h in complete medium and then transferred to either minimal or complete medium agar plates. Potential auxotrophs were screened in minimal medium supplemented with 20 essential amino acids at 20 μg/ml or with nucleotides at 10 μg/ml and then characterized by growth on minimal medium or minimal medium plus various combinations of amino acids or nucleotides (10). Glycine, cysteine, proline, and histidine auxotrophs were isolated. Also, a uracil auxotroph and a combined adenine-cytosine auxotroph were identified. Our new medium is simple and contains relatively few ingredients, yet it allows for good growth of *A. xylinum*, even after multiple subcultures. Growth rates in minimal media with different concentrations of glucose were almost identical to that in the complete, undefined medium, though at low glucose concentrations cellulose biosynthesis is reduced. Magnesium is an essential element for growth and cellulose synthesis. It is required for cell wall biogenesis in *Oocystis* spp. (11) and for successful functioning of the *Acetobacter* cellulose synthase that is activated by a guanyl oligonucleotide (14). The remaining ingredients in the synthetic medium provide the essential nutrients for growth with glucose as both a metabolic carbon and energy source and an anabolic source for cellulose biosynthesis.

This simple, chemically defined medium permits not only good growth but also cellulose synthesis by *A. xylinum*. The minimal medium also was useful for the isolation of auxotrophic mutants. The medium will be used to isolate additional auxotrophs, including those with mutations in the uridine pathway, since UDP-glucose has been proposed as a component of the cellulose synthesis pathway (2) in our continuing elucidation of cellulose biosynthesis.

This work was supported by the Research Council of the University of North Carolina at Greensboro.

Pat McCarron and Peg Fete provided able assistance in preparing the manuscript. Special thanks are extended to Janne Cannon for review.

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