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Effect of Gluconic Acid on the Production of Cellulose in *Acetobacter xylinum* BRC5

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Abstract Four mutants of *Acetobacter xylinum* BRC5 defective in gluconic acid production were isolated from UV-irradiated cells. The gluconic acid-negative mutants did not show glucose oxidase activity. The mutants were also defective in cellulose production. A randomly selected mutant grown in the Hestrin-Schramm medium (pH 6.0) supplemented with gluconic acid, however, was found to synthesize cellulose. The mutant grown in Hestrin-Schramm medium whose pH was adjusted to 5.0 with HCl and contained no gluconic acid also produced cellulose. Wild-type cells grown under the same condition synthesized cellulose more rapidly than those grown in the pH 6.0 medium.

Key words: *Acetobacter xylinum* BRC5, cellulose, cellulose-negative mutant, glucose oxidase, gluconate-negative mutant

Microbial polysaccharides and its derivatives have been studied extensively, since they have many useful properties such as being biodegradable and low in toxicity [1, 11, 15]. Among the polysaccharides produced by bacteria, cellulose has received special attention since it is produced in pure form from various substrates [1, 2, 11, 14, 15], easily modified to a variety of derivatives [1, 11, 15], and excellent in water absorption, and it shows high crystal and mechanical strength [1, 11, 15].

Acetobacter xylinum, a cellulose-producing bacterium, is also known to excrete gluconic acid along with cellulose during growth on glucose [5, 6]. Schramm *et al.* [16] reported that gluconic acid accumulated in the cultivation medium inhibited cellulose production of *A. xylinum* by lowering the pH of the medium. According to Masaoka *et al.* [12], however, gluconic acid produced from glucose not using for cellulose biosynthesis in *A. xylinum* IFO 13693 did not show any effect on the cellulose production, and it was suggested to be metabolized to other substances. In the case of *A. xylinum* BRC5 (KCCM 10100), most of the glucose added into the medium was metabolized to gluconic acid and little cellulose was produced during the exponential growth phase [17]. A part of gluconic acid accumulated then disappeared, and both cell growth and cellulose production ceased, even though over two-thirds of the gluconic acid still remained. From these reports, we assumed that the conversion of glucose to gluconic acid is not beneficial for overall cellulose productivity in *A. xylinum*. The onset of active synthesis of cellulose in *A. xylinum* BRC5 after accumulation of a large amount of gluconic acid in the medium, on the other hand, implied that gluconic acid has to be produced prior to the production of cellulose in *A. xylinum* growing on glucose as a source of carbon and energy.

We, therefore, undertook the present study to find out the effect of gluconic acid on the production of cellulose in *A. xylinum* BRC5 and found that gluconic acid production is necessary for cellulose biosynthesis in this bacterium.

A. xylinum BRC5 (KCCM 10100) is a cellulose-producing bacterium isolated from cellulose pellicles formed as contaminants in conventional vinegar plants in Korea [18]. Wild-type cells and mutants defective in gluconic acid production (gluconate-negative mutant) were cultivated at 30°C using Hestrin-Schramm (HS) medium (pH 6.0 [2, 8]) containing 2% (wt/vol) glucose. To obtain liquid culture, cells were grown at an agitation speed of 200 rpm either in 7-liter jar fermenters containing 5 l of fresh HS medium and 250 ml of seed culture under aeration of 0.6 vvm or in 1,000-ml Erlenmeyer flasks containing 200 ml of the same medium and 10 ml of seed culture. The medium was supplemented with 0.3% (wt/vol) cellulase (Celluclast, Novo Nordisk Co., Dittingen, Denmark) to prevent cells from clumping, thereby inhibiting production of intact cellulose fibrils, except otherwise stated. To prepare seed culture, a colony selected from solid HS medium was cultivated in a flask for 36 h. Growth was measured by turbidity determined at 540 nm, using a spectrophotometer (U-2000, Hitachi, Tokyo, Japan).

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The ability of *A. xylinum* BRC5 to produce cellulose was assessed by exposing colonies formed on solid HS medium containing 0.01% (wt/vol) Calcofluor WT (Sigma Chemical Co., St. Louis, MO, U.S.A.) (HSC medium, pH 6.0) to a short wave (254 nm) UV hand lamp (UVGL-55, Ultra Violet Products Limited, Upland, CA, U.S.A.) and measuring fluorescence [4, 7].

Glucose oxidase in the cell-free extract was assayed at 35°C by measuring the increase of glucose-dependent absorbancy at 500 nm in the presence of horseradish peroxidase (Sigma) and *o*-dianisidine (Sigma) by the method described by Sigma Chemical Co. To prepare cell-free extract, cells were harvested at the mid-exponential growth phase, suspended in 0.1 M sodium acetate buffer (pH 5.1), and disrupted by sonic treatment (25 s/ml; VCX-6000, Sonics & Materials, Newtown, CT, U.S.A.). The suspension was then centrifuged at 27,000 ×g for 30 min, and the resulting supernatant was used as cell-free extract. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of glucose to gluconic acid and hydrogen peroxide per min. Protein was determined by the method of Bradford [3], using bovine serum albumin as a standard.

Gluconic acid in the medium was detected and estimated by absorbancy at 210 nm using a high pressure liquid chromatography (HPLC; Delta Prep 400, Waters Co., Milford, OH, U.S.A.) equipped with Aminex HPX-87H column (7.8 by 300 mm; Bio-Rad Co., Richmond, VA, U.S.A.) and an UV monitor (model 486, Waters Co.). The mobile phase contained 0.002 N H_2SO_4 and the flow rate was 0.6 ml per min.

To isolate gluconate-negative mutants, cells in 20-ml culture growing at the mid-exponential growth phase (A_{540}= 0.4 to 0.5) in 1,000-ml flask were harvested and suspended in 200 ml of 0.1 M $MgSO_4$. A 10-ml of the suspension was then transferred to a Petri plate (85 mm in diameter), and irradiated with a short wave UV (254 nm; UVGL-55, Ultra Violet Products Limited) for 2 min at a distance of 22 cm. The UV-irradiated cells were spread onto solid HS medium containing 0.2% bromothymol blue (wt/vol) (HSB medium), a pH indicator [13], and then incubated for 36 h at 30°C. The pH of the HSB medium was adjusted to 7.2 using disodium phosphate. Gluconate-negative mutants of *A. xylinum* BRC5 were screened based on the property that mutant cells were unable to change the dark greenish blue color of the medium to a greenish yellow color.

It has been generally known that the ability to produce cellulose in *A. xylinum* strains is highly sensitive to shearing force [10]. *A. xylinum* BRC5, however, did not lose this ability even when it was cultivated at an agitation of 200 rpm in a flask or jar fermenter, indicating that the cellulose-producing ability of this bacterium is resistant to shearing force.

When *A. xylinum* BRC5 was cultivated in a jar fermenter, the bacterium excreted a large amount of gluconic acid during the early exponential growth phase (specific growth rate=0.25 h^{-1}). The gluconic acid concentration was maximal (19 g/l) at 3 h after inoculation and then decreased thereafter. No gluconic acid was detected in the culture media in which cells were grown for over 24 h. The activity of glucose oxidase (0.22 units/mg), however, did not fluctuate significantly for 66 h. The pH of the 3 h-grown culture was 5.0.

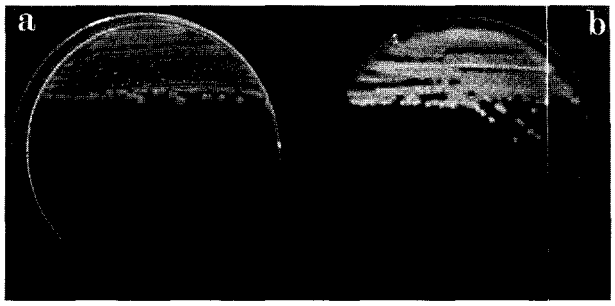

Fig. 1. Wild-type and gluconate-negative mutant cells of *A. xylinum* BRC5 on HSC medium under UV.
To assess the ability to produce cellulose of gluconate-negative mutants, gluconate-negative mutant #23 (a) and wild-type cells (b) grown on HSC medium (pH 6.0) for 36 h were examined for the ability to fluoresce under UV (254 nm) as described in the text.

The survival rate of *A. xylinum* BRC5 irradiated with UV under the experimental condition was 0.1%. Four gluconate-negative mutants (#10, #22, #23, and #24) were isolated from 3×10^5 UV-irradiated cells. As expected, the mutants did not exhibit glucose oxidase activity. When the mutant cells from the HSB medium were transferred to solid HSC medium and cultivated for 36 h, they did not exhibit fluorescence under UV lamp (Fig. 1). This result together with an observation that the mutant cells did not form cellulose pellicles under the static cultivation condition indicated that the mutants of *A. xylinum* BRC5 defective in gluconate production were also defective in cellulose biosynthesis. Colonies of wild-type were small (1.5-2.0 mm in diameter after 36 h at 30°C), convex, and compact, while those of the mutants were larger than the wild-type (2.5-3.0 mm in diameter after 36 h at 30°C), simply raised, and relatively loose on solid HSC medium (pH 6.0) (Fig. 2). The mutants grew faster than the wild-type cell (specific growth rate=0.47 h^{-1}) under the conditions used for the wild-type, which may be due to the fact that the mutants did not have to consume glucose for biosynthesis of gluconic acid and cellulose.

Since the gluconate-negative mutants of *A. xylinum* BRC5 did not synthesize cellulose, a randomly selected mutant #23 was examined for the effect of gluconate on the cellulose biosynthesis in this bacterium. The mutant #23 was first cultivated for 36 h in a flask in the presence of various concentrations of sodium gluconate (Sigma) but in the absence of cellulase. Cells were then transferred to solid HSC medium supplemented with 2% (wt/vol) sodium

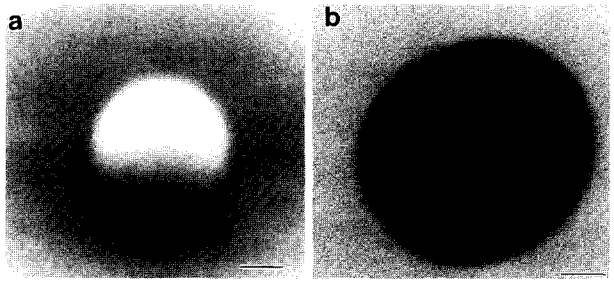

Fig. 2. Colonies of wild-type and gluconate-negative mutant cells of *A. xylinum* BRC5 on HSC medium under UV.
The photomicrographs of colonies of wild-type cell (a) and gluconate-negative mutant #23 (b) grown on HSC medium (pH 6.0) for 36 h were taken under UV (254 nm) using an Olympus SZ40 microscope (Olympus Optical Co., Tokyo, Japan). The bar represents 0.5 mm.

gluconate, incubated for 36 h, and examined for the ability to exhibit UV-dependent fluorescence.

It was found that the mutant #23 grown in the presence of gluconic acid restored the ability to produce cellulose. The mutant grown in the absence of gluconic acid did not show fluorescence, while that grown in the presence of 0.1 and 2% sodium gluconate exhibited weak and strong fluorescence, respectively (Fig. 3). This result indicates that gluconic acid has a positive effect on the production of cellulose in *A. xylinum* BRC5, which is different from the report that gluconic acid inhibited cellulose production in other strains of *A. xylinum* [16]. The present result also indicates that gluconic acid production is necessary for cellulose biosynthesis in *A. xylinum* BRC5 growing on glucose. The mutant grown in the presence of 5% sodium gluconate exhibited a similar level of fluorescence to that of the cells grown in the presence of 2% sodium gluconate, indicating that 2% sodium gluconate is sufficient for the mutant #23 to achieve an optimal level of cellulose production in HS medium. The 2% sodium gluconate solution contains 18 g of gluconic acid per liter, which coincides with the maximal concentration of gluconic acid (18 to 19 g/l) measured in the culture supernatants of the wild-type *A. xylinum* BRC5 in the present and previous studies [18].

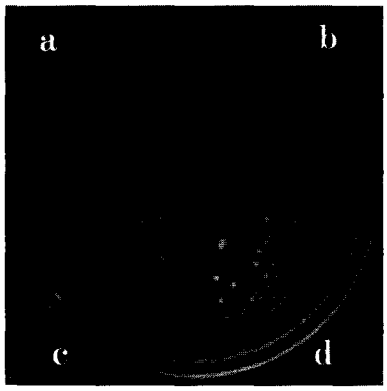

Fig. 3. Effect of gluconate on the cellulose biosynthesis.
To test the effect of gluconic acid on the cellulose biosynthesis of *A. xylinum* BRC5, a gluconate-negative mutant #23 grown in cellulase-free HS medium in the absence (a) and presence of 0.1 (b), 2 (c), and 5% (wt/vol) (d) sodium gluconate were streaked onto HSC medium (pH 6.0) supplemented with 2% sodium gluconate, cultivated for 36 h, and analyzed for the intensity of fluorescence under UV (254 nm) as described in the text.

The present results that gluconic acid concentration was maximal in the 3 h-cultivated medium, the pH of the culture medium dropped sharply during the first 3 h of cultivation, and gluconic acid induced cellulose synthesis in a gluconate-negative mutant suggest that gluconic acid may induce cellulose biosynthesis in *A. xylinum* BRC5 by lowering the pH of the medium. This suggestion is further supported by a previous observation that accumulation of a large amount of gluconic acid in the medium resulted in a sharp drop in pH followed by triggering of active synthesis of cellulose in the same bacterium [17]. To test this possibility, the mutant #23 was first cultivated for 36 h in a flask containing cellulase-free HS medium whose pH was adjusted to 5.0 with HCl. The cultivated cells were then streaked on solid HSC medium whose pH was adjusted to 5.0 with HCl and were incubated for 36 h.

It was found that the mutant #23 grown at pH 5.0 (the pH of the 3-h cultivated medium) in the absence of added gluconic acid exhibited weak fluorescence on HSC medium (data not shown), implying that acidic environment induces formation of cellulosic fibers in the gluconate-negative mutant. Wild-type cells grown under the same low pH condition were found to synthesize cellulose more rapidly than those grown in the pH 6.0 medium (data not shown), suggesting that acidic condition also accelerates the rate of cellulose biosynthesis in *A. xylinum* BRC5.

The present study clearly indicates that gluconic acid production has positive effects on cellulose biosynthesis in *A. xylinum* BRC5. Gluconic acid may affect cellulose production in this bacterium first by lowering the environmental pH and then possibly the internal pH, resulting in stabilization and/or activation of key enzyme(s) for cellulose biosynthesis, similar to the situation where acetic acid and butyric acid accumulated in the medium during acetogenic phase triggering solventogenesis in *Clostridium acetobutylicum* [9]. The decrease in the amount of gluconic acid after peak accumulation also coincides with that of acetic acid and butyric acid observed during metabolic transition from acetogenesis to solventogenesis in *C. acetobutylicum* [9].

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