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Production of Bacterial Cellulose from Alternate Feedstocks

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Abstract

Production of bacterial cellulose by *Acetobacter xylinum* ATCC 10821 and 23770 in static cultures was tested from unamended food process effluents. Effluents included low-solids (LS) and high-solids (HS) potato effluents, cheese whey permeate (CW), or sugar beet raffinate (CSB). Strain 23770 produced 10% less cellulose from glucose than did strain 10821 and diverted more glucose to gluconate. Unamended HS, CW, and CSB were unsuitable for cellulose production by either strain, and LS was unsuitable for production by strain 10821. However, strain 23770 produced 17% more cellulose from LS than from glucose, indicating that unamended LS could serve as a feedstock for bacterial cellulose.

Index Entries: Bacterial cellulose; *Acetobacter xylinum*; potato effluent; beet raffinate; whey permeate.

Introduction

Bacterial cellulose has significant advantages over plant cellulose. Bacterial cellulose fibrils are randomly oriented and the product is highly amorphous *(1,2)*. It is generated as a never-dried membrane in a nearly pure form *(3)* that contains 99.1 wt% water, of which 0.3 wt% is bound and 98.8 wt% is free water *(4)*. It has more than 200 times greater surface area than isolated softwood cellulose *(1)* and has a tensile strength similar to that of steel *(5)*. Many potential high-value markets exist for thin film bacterial cellulose, including acoustic diaphragms *(6)*, artificial skin *(7,8)*, artificial blood vessels *(9)*, liquid-loaded medical pads *(10)*, supersorbers *(11)*, and specialty membranes *(12)*. Potential markets for bacterial cellulose produced as pellets in agitated culture include foods and the mining, oil, and pulp and paper industries *(1,2)*.

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The most studied producer of bacterial cellulose is *Acetobacter xylinum (13)*, a Gram-negative, obligately aerobic bacterium *(10,14,15)*. The optimum pH for cellulose production is 5.0 *(13,16)*, and production is associated with and proportional to growth *(17)*. Cellulose production has been demonstrated from glucose, sucrose, fructose, glycerol, mannitol, arabitol, and many other substrates *(5,13,16,18)*. Excess glucose is oxidized to gluconate in wild-type *A. xylinum*, which lowers the pH and inhibits cellulose production *(13)*. Genetically altered strains with substantially reduced ability to form gluconate have been developed *(5)*. Methionine and lactate stimulate cell growth in the early stages, allowing higher rates of cellulose production *(19)*.

Low production rates and high medium costs limit commercial use of bacterial cellulose *(3)*. In this article, we report the testing of unamended food process effluents, which typically represent economical and environmental liabilities to the producers, as substrates for bacterial cellulose production in static culture.

Materials and Methods

Food Process Effluents

Potato effluents *(20,21)*, cheese whey permeate (CW), and concentrated sugar beet raffinate (CSB) were obtained from Idaho processing plants. Two potato process effluents were tested: high-solids (HS) and low-solids (LS) effluents. To lower carbohydrate concentrations to levels similar to that of the control medium (described subsequently), the effluents were diluted with distilled water. HS effluent was diluted 1:10 by weight, whereas LS effluent, CW, and CSB were diluted 1:10 by volume. Each diluted effluent was autoclaved at 121°C for 20 min, and the pH was adjusted to 5.0 with HCl prior to use. Control experiments were conducted using an optimized pH 5.0 glucose medium containing 20 g/L of glucose; 5 g/L of yeast extract; 5 g/L of peptone; 2.7 g/L of Na_2HPO_4; and 1.15 g/L of citrate (Schramm and Hestrin's medium) *(15,22)*. Initial carbohydrate data for the control and the diluted effluents are presented in Table 1.

Cultures and Maintenance

A. xylinum 10821 and 23770 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Several generations were grown in 25 g/L of mannitol; 5 g/L of yeast extract; and 3 g/L of peptone, pH 5.0 *(23)*. Reference cultures were maintained at 4°C on malt agar slants containing Schramm and Hestrin's medium *(15,22)* and 15 g/L of agar (Difco) and were subcultured monthly. Frozen seed stocks were prepared as follows. The cellulose pellicle was removed from the reference slant with sterile distilled water, vortexed for 5 min, and sonicated for 20 min to remove the cells from the pellicle. The pellicle was removed, and the liquid was added to 500 mL of Schramm and Hestrin's medium and cultured with

Table 1
Typical Initial Carbohydrate Data for Control Medium and Diluted HS and LS Potato Effluents, Diluted CSB, and Diluted CW[a]

	Composition (wt%)				
		Effluents diluted 1:10			
Carbohydrate	Control medium	HS	LS	CSB[b]	CW
Glucose	2.0	0.002	0.04	0.04	0.19
Sucrose	—[d]	—	—	2.2	—
Lactose	—	—	—	—	1.3[e]
Soluble starch	—	0.47	1.5	—	—
Insolubles[c]	—	1.6	0.66	—	—

[a]Effluents varied slightly among batches obtained from the food-processing plants.
[b]Although not measured, sugar beet raffinate also contains minute amounts of raffinose and fructose.
[c]Insolubles include starch and nonstarch components. Insoluble starch accounted for up to 85% of the insolubles in both HS and LS, depending on the particular batch from the processor.
[d]None.
[e]This value includes small amounts of galactose.

occasional gentle mixing for 7–14 d at 30°C, until the cell number exceeded 2×10^8 cells/mL. Cells were then removed from the resulting pellicle by vortexing and sonication, and 10-mL aliquots of the culture liquid were frozen at –80°C in 15 vol% glycerol as previously described *(23)*.

Experimental Procedures

Experiments were conducted in static 100-mm storage dishes (cat. no. 08-782; Fisher) containing 100 mL of medium. Abiotic controls contained Schramm and Hestrin's medium and were not inoculated, and biotic controls were prepared as follows. Frozen seed stocks were thawed and added at 10 mL/250 mL of Schramm and Hestrin's medium *(15,22)*. The cultures were grown as described for preparation of seed cultures. When the cell number exceeded 2×10^8 cells/mL, cells were removed from the pellicle and inoculated into a large excess of Schramm and Hestrin's medium to give 1×10^7 cells/mL. The medium was mixed well and transferred in 100-mL aliquots to sterile 100-mm dishes. All controls were incubated at 30°C for 14 d. For each medium, this amounted to 16 separate cultures, all starting at the same conditions and inoculum size. Two of these cultures were sacrificed every second or third day and analyzed for substrate, cell numbers, pH, cellulose weight, and pellicle thickness. This was done because measurement of cellulose weight and cell counts required disruption of the static culture and removal of the cells from the pellicle. Therefore, data at each time point are averages from separate cultures. Experiments utilizing unamended effluents were conducted as described earlier, except the final transfer was completed using the autoclaved, diluted pH 5.0 effluent medium.

Analytical Methods

Cell Numbers and pH

Cells were removed from the pellicle using vortexing and sonication. Liquid samples were then taken for substrate analyses and cell counts. Cell numbers were then determined by direct count of 5 µL of culture fluid on a Petroff-Hauser hemocytometer slide (average of 10 separate counts). pH was measured using a standard pH probe and meter.

Substrate Concentrations

Samples were measured for substrate levels after filtering through a 0.22-µm polysulfone membrane filter to remove cells and cellulose fibers. Glucose, sucrose, and lactose were measured using a YSI Model 6200 Glucose Analyzer (Yellow Springs Instrument, Yellow Springs, OH) fitted with the appropriate membrane(s) (glucose, sucrose, and lactose; cat. no. 2365, 2703, and 2702, respectively), buffers, and standard solutions. Soluble starch was estimated after removal of cells and particulates as previously described *(20)*, using the phenol–sulfuric acid assay for total carbohydrates *(24)*. For potato effluent cultures, an aliquot of well-mixed culture fluid was filtered through a tared cellulose filter paper (Fisher P8, average pore size of 20 µm), and insolubles were estimated by weight difference after drying to constant weight at 105°C.

Pellicle Weight and Thickness

Cellulose pellicle thickness was measured without removal of the absorbed water present in the pellicle at harvest. The pellicle was placed onto a smooth, flat board and spread out. The thickness was measured at three different positions around the periphery to the nearest 0.5 mm, and the values were averaged. The pellicle was then placed into 300 mL of 2 wt% NaOH and autoclaved for 20 min at 121°C. After cooling, the pellicle was neutralized with dilute H_2SO_4 and washed by filtration (Fisher P8 filter paper) until the filtrate pH was 7.0–7.5. The pellicle was then collected by filtration on a tared filter paper and dried to constant weight at 105°C.

Results

Comparison of Strain

Data from 14-d control cultures of *A. xylinum* 10821 and 23770 are shown in Figs. 1 and 2, respectively. Glucose consumption by strain 23770 was much more rapid than that observed for strain 10821, with essentially all the glucose gone in 9 d. Only 37% of the glucose was consumed by strain 10821 in the same period, and only 64% was consumed by 14 d. Growth rates for both strains were essentially the same over the course of the experiment. The pH of the strain 23770 cultures dropped to 3.2 after 4 d of culture, and strain 10821 cultures slowly decreased to pH 4.5. Cellulose production was similar for the two strains over the initial 4 d of culture, at which time

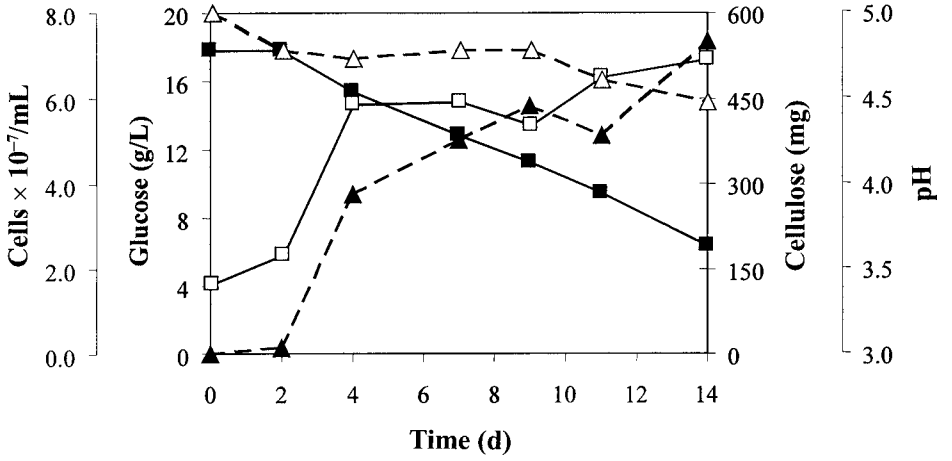

Fig. 1. Culture data for 14-d stationary control cultures of *A. xylinum* 10821. Each data point represents the average of duplicate sacrificed cultures. (□) Cell number; (■) glucose; (▲) dry weight of the cellulose pellicle; (△) pH.

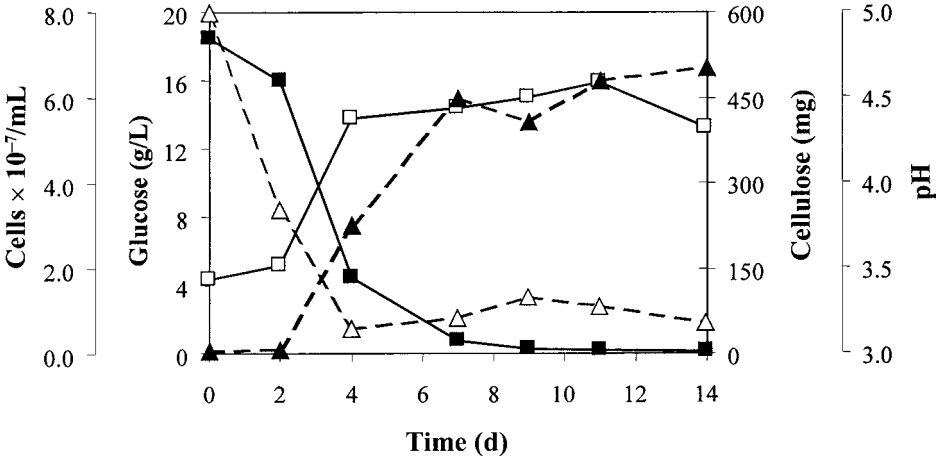

Fig. 2. Culture data for 14-d stationary control cultures of *A. xylinum* 23770. Each data point represents the average of duplicate sacrificed cultures. (□) Cell number; (■) glucose; (▲) dry weight of the cellulose pellicle; (△) pH.

both strains had produced 220–280 mg of cellulose. Linear production was observed after d 4 for strain 10821, to 550 mg on d 14. Strain 23770 reached 450 mg on d 7, after which only 50 mg more of cellulose was produced.

Net cellulose production rates and yields from glucose (g cellulose produced per g glucose consumed) are presented in Table 2. Cellulose production rates were initially high for strain 10821 but decreased slowly over time to a production rate of 5.0 g of cellulose/(d·m^2) of surface and a yield of 48% after 14 d. For strain 23770, the glucose was essentially depleted after 7 d, at which point the net production rate was 8.1 g/(d·m^2) and the

Table 2
Cellulose Production Rates and Yields
for Glucose Control Cultures of *A. xylinum* 10821 and 23770

	A. xylinum 10821		*A. xylinum* 23770	
Day	Net production rate (g/[d·m²])[a]	Yield (%)[b]	Net production rate (g/[d·m²])[a]	Yield (%)[b]
0	0.00	0.0	0.00	0.0
2	0.69	0.0	0.09	0.5
4	8.93[c]	116[c]	7.00	15.8
7	6.84	76.0	8.06	25.1
9	6.19	66.8	5.68	22.1
11	4.45	45.9	5.49	26.0
14	5.00	48.1	4.51	27.1

[a]Net production rates (g of cellulose/[d·m² of surface]) were each calculated from time zero.

[b]Yield is defined as (g of cellulose produced)/(g of glucose consumed) × 100%.

[c]These values are indicative of measurement error in dry weights for samples containing very small amounts of cellulose.

yield was 25%. After 14 d, the net production rate was 4.5 g/(d·m²), with an average production rate of 5.2 g/(d·m²) after d 7. Overall cellulose production in 14 d of culture (volumetric basis) was slightly higher from strain 10821, with 11% more cellulose produced.

Cellulose Production from Effluents

Cell growth data, culture pH, and cellulose production data for the unamended effluents are given in Table 3. Control data are included for comparison. Growth was observed in all media. Cell numbers in the potato effluent cultures were an order of magnitude higher than those observed in the other effluents or in the control, which was found to be from spores in the effluent that survived the autoclaving *(20,21)*. Some contaminating cells that survived the autoclaving step were also seen in the other effluent media, but these cells did not seem to grow as well at pH 5.0 as those in the potato effluents.

There were also differences in the trends of culture pH between the two strains. For strain 10821, there was a general slight decrease in pH to the 4.0–4.5 range for HS and CW, whereas the pH of the LS culture was essentially unchanged. In the CSB culture, the pH increased to about 7.5 over the 14-d period. For strain 23770, the pH increased in all media except CW, for which it did not change significantly. The largest increase was for the LS effluent, to pH 7.3. This is in stark contrast to the glucose control, which decreased to pH 3.2 over the 14-d period.

Comparison of the cellulose production data in Table 3 indicates that neither strain was capable of producing significant bacterial cellulose from unamended HS, CSB, or CW. Values from replicate sacrificed cultures were

Table 3
Culture Data for Production of Bacterial Cellulose
from Diluted Unamended HS and LS Potato Effluents, Diluted Unamended CSB, and Diluted Unamended CW

	A. xylinum 10821					*A. xylinum* 23770				
Day	Control	HS	LS	CSB	CW	Control	HS	LS	CSB	CW
					Cell counts ($\times 10^{-7}$ cells/mL)					
0	1.66	2.19	1.31	1.30	1.78	1.78	2.07	1.26	2.07	1.83
2	2.33	17.8	6.25	3.84	3.88	2.11	22.3	6.48	3.06	4.28
5	5.84	44.6	16.7	4.34	5.09	5.55	32.8	23.3	8.80	7.00
7	5.92	38.7	13.8	5.13	6.70	5.78	15.5	20.7	4.38	6.70
9	5.36	45.4	47.3	8.72	5.08	6.02	43.1	48.9	9.05	8.56
12	6.48	67.8	29.6	5.00	14.4	6.39	58.8	25.1	5.27	17.1
14	6.91	58.9	32.8	15.6	4.02	5.31	70.8	32.3	8.86	6.73
					Culture pH					
0	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
2	4.78	3.87	4.87	5.29	4.15	3.85	3.62	4.59	5.21	3.70
5	4.73	4.02	4.96	5.32	4.18	3.16	4.63	4.85	5.27	4.37
7	4.78	4.13	4.91	5.26	3.96	3.22	5.56	5.10	5.29	4.62
9	4.78	4.64	6.05	5.79	4.03	3.34	5.39	5.62	5.39	4.92
12	4.61	4.76	5.31	7.24	4.01	3.29	5.84	7.14	5.32	4.95
14	4.48	4.65	5.09	7.45	3.93	3.19	5.65	7.27	5.28	4.87
					Mass of cellulose produced (mg)					
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	10.9	19.1	0.00	2.40	0.00	1.40	1.76	17.4	0.00	7.05
5	281	21.5	36.4	1.15	2.15	220	84.4	335	6.00	108
7	376	0.00	28.8	0.00	1.65	443	0.00	553	0.400	97.7
9	437	0.00	94.6	9.55	0.45	401	0.00	498	18.6	17.7
12	385	13.5	35.1	30.3	4.15	474	26.9	483	18.1	17.6
14	550	0.00	48.4	33.0	1.30	496	25.9	581	0.700	113

somewhat scattered, but in all cases, very little cellulose was produced from these unamended effluents. The maximum amounts of cellulose produced in any of the sacrificed cultures utilizing these diluted effluents by strain 10821 were 22, 33, and 4 mg of cellulose from HS, CSB, and CW, respectively. By comparison, strain 23770 produced maximum amounts of 84, 19, and 113 mg of cellulose from the same media. Results with diluted LS effluent were markedly different for the two strains. A maximum 95 mg of cellulose was produced by strain 10821, whereas strain 23770 produced up to 580 mg of cellulose from LS in 14 d. This amounts to an estimated cellulose yield (g cellulose produced per g glucose consumed) from diluted LS effluent of about 27%, which is nearly the same yield as that obtained by strain 23770 from optimized glucose medium.

Discussion

Comparison of Strains

Cell growth on Schramm and Hestrin's medium was similar for the two strains, but strain 23770 consumed the glucose much more quickly. The rapid utilization of glucose and the drop in pH observed for strain 23770 suggest that the excess glucose was converted to gluconate. Excess glucose not used for cellulose synthesis is oxidized by *A. xylinum* to gluconate *(13)*. This allows some accumulation of gluconate, which can lower the pH and inhibit cellulose production at high glucose concentrations *(13)*.

Forng et al. *(25)* demonstrated that glucose concentrations above about 1% give relatively smaller increases in cellulose produced, and Masaoka et al. *(13)* observed no difference in the rate of cellulose production from 0 to 2% glucose, with inhibition observed at 4% glucose. Strain 23770 produced cellulose at a markedly higher initial rate than strain 10821, but at only about half the yield. When initial carbon source concentrations below 2 wt% are used, a production efficiency of 45% (weight of cellulose per carbon source consumed) can be obtained *(25)*. This magnitude was observed for strain 10821 (48% on the same basis), but not for strain 23770 (27%). After the glucose was consumed in strain 23770 cultures, a low rate of cellulose production continued. This production was likely from the citrate in the medium, since it has been shown that citrate is eventually used as a carbon source for growth and cellulose production, but only after the glucose is consumed *(27)*. In the present study, overall cellulose production in 14 d of culture (volumetric basis) by strain 10821 was slightly higher (11%) than that produced by strain 23770.

Cellulose Production from Effluents

All of the unamended food process effluents were able to support growth of both strains of *A. xylinum*, and at least limited cellulose production. Strain 10821 produced 5%, 21%, 7%, and 0.6% normalized yields (weight basis) of bacterial cellulose from HS (insoluble starch), LS (starch), CSB (sucrose), and CW (lactose), respectively, relative to glucose (100%).

Strain 23770 produced 15%, 96%, 3%, and 30% normalized yields (weight basis) of bacterial cellulose from the same effluent media, relative to glucose (100%). For comparison, Masaoka et al. *(13)* showed 18, 33, and 16% normalized yields of bacterial cellulose from starch, sucrose, and lactose, respectively, relative to glucose (100%). The Masaoka et al. experiments *(13)* consisted of 3-d cultures of *A. xylinum* IFO 13693 on a basic medium (pH 6.0) consisting of 2% peptone, 0.5% yeast extract, 0.5% glucose, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.2% ethanol. Glucose was simply replaced by the other carbon sources for the tests.

We cannot make a direct comparison of the HS medium results with those of Masaoka et al. *(13)*, since although it is not specifically stated, we have assumed that their starch substrate was likely to have been soluble. From the LS medium, our results show poorer than expected cellulose yields with strain 10821, and much better than expected cellulose yields with strain 23770. Our results with the CSB effluent are much lower than those reported from sucrose by Masaoka et al. *(13)*, which may be due to the presence of toxic heavy metals in the CSB *(28)*. Finally, cellulose yields from CW effluent were very low from strain 10821 and about twice that expected from strain 23770. Of the unamended, diluted effluents tested, only the LS effluent medium supported substantial cellulose production, and only by strain 23770.

Given the similarities between cell numbers and overall cellulose production by the two strains on Schramm and Hestrin's medium, it is not immediately clear to the authors why strain 10821 was unable to produce substantial cellulose from the LS medium. The most quantifiable differences between the two cultures on LS medium, other than cellulose production, are the different trends in pH. The LS effluent is known to contain indigenous bacteria as received from the processor *(20,21)*. Some of these bacteria survived the autoclaving step and were present in the LS cultures, as evidenced by the much higher cell numbers observed, and by uninoculated controls (data not shown). These bacteria are known to lower medium pH to 4.0 if left unchecked *(20,21)*, suggesting that they are fermentative bacteria. Because they are indigenous to the potato effluents, they would be expected to possess an amylase system and to convert starch to glucose. This would help to increase cellulose yields from the starch in the effluent through supplementation of the ability of *A. xylinum* to convert starch to glucose.

In addition, pH in the strain 23770 culture on LS medium did not drop as it did in pure culture on glucose medium. This suggests that indigenous bacteria consumed the gluconate as it was being produced. Removal of the gluconate would minimize its inhibitory effects on cellulose production. Gluconate, which is not a substrate for cellulose production, may have been degraded by the indigenous bacteria to smaller organic acids, which both stimulate *(29)* and serve as substrates *(13)* for cellulose production. The strain 10821 culture, which did not produce significant amounts of gluconate (as evidenced by the lack of a drop in pH on glucose medium), would not

experience this benefit, because the gluconate would not be present for indigenous bacteria to consume. The observed increase in pH could then be due to consumption of the organic acids in the unbuffered LS medium. Although not a part of the scope of the present study, these possibilities should be examined because they suggest a synergistic mixed culture strategy for production of bacterial cellulose from LS effluent.

Because it has been shown that *A. xylinum* can produce cellulose from a myriad of carbon sources *(13)*, it is likely that additions could be made to the media that would allow higher cellulose production from each of the effluents. Pretreatments of the effluents could be done in order to increase free carbohydrate concentrations, or to remove potentially toxic components such as heavy metals in sugar beet molasses *(28)*. In addition, it is probable that contaminating bacteria already present in the effluents also serve to lower yields, because all the media contained indigenous bacteria that were resistant to heat sterilization by autoclaving.

Because medium costs, among other things such as production rate and process footprint, limit commercial production of bacterial cellulose in static cultures *(3)*, it is important to minimize carbon, nutrient, and energy additions wherever possible. Thus, it is generally undesirable to make medium additions, to use pretreatments, or to use extensive or energy-intensive sterilization procedures beyond the absolute minimum required. The estimated yield of cellulose from unamended, diluted LS effluent by strain 23770 was not significantly different from that on Schramm and Hestrin's medium. This was true even though substantial glucose was diverted to gluconate and the expected yield from starch is only 18% of that from glucose. This suggests that *A. xylinum* 23770 is more suitable than 10821 for the production of bacterial cellulose from LS.

Conclusions

A diluted, unamended LS potato process effluent was found to support substantial production of bacterial cellulose by *A. xylinum* ATCC 23770 in static culture. Diluted, unamended HS potato process effluent, CW, and CSB did not support significant cellulose production by either *A. xylinum* 10821 or 23770. *A. xylinum* 10821, while diverting less glucose to gluconate than 23770, was unable to produce a significant amount of cellulose from the LS effluent medium. Because the yield of cellulose by *A. xylinum* 23770 was equivalent from diluted, unamended LS potato process effluent and from optimized Schramm and Hestrin's medium, this strain is more appropriate to use for cellulose production from this effluent.

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