Cellulose Production by *Acetobacter acetigenum* and other *Acetobacter* spp.

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SUMMARY: The synthesis of cellulose by *Acetobacter* on media based on blackstrap molasses was studied. Methods are described for the determination of cellulose, and of growth as cell-N, in static culture. Different carbohydrate substrates were compared for cellulose production by *Acetobacter acetigenum* strain EA-I; hydrolysed molasses was found to give the largest yields. In media based on this substrate growth and cellulose production both reached their maximum values with a sugar concentration of 7.9 % (w/v) after 40 days' growth at 26-29°. The maximum conversion (18 %) of sugar to cellulose, however, was obtained with 1.5 % (w/v) sugar. Cellulose synthesis ceased when growth stopped, even when the sugar in the medium was not exhausted, indicating that cellulose was synthesized only by the actively growing organism. The cellulose produced/mg. cell-N and % cellulose in the pellicles decreased with increased sugar concentration in the medium. Other *Acetobacter* strains were examined in this medium; the cellulose yields varied over a wide range, equivalent to conversions of 1.9-23.5 %.

The synthesis of cellulose by *Acetobacter* species has been the subject of many investigations since the first report by Brown (1886), but apart from the detailed work of Tarr & Hibbert (1931) and Woeber (1954) and the more restricted studies of Kaushal & Walker (1951), Barclay (1951) and Skopek (1955), relatively little attention has been given to the influence of cultural conditions on cellulose yields, and nothing has been published concerning the relationship between cell growth and cellulose synthesis. The present study was undertaken to find the conditions which give maximum yields of cellulose, especially with cheap and readily available substrates, with a view to possible large-scale production of this polysaccharide. The present paper describes experiments to determine the influence of some nutritional factors on cellulose production by *A. acetigenum* in media based on blackstrap molasses. Cellulose yields from three strains of *A. acetigenum*, six strains of *A. xylinum*, two strains of *A. kutzinianum* and one strain of *A. pasteurianum* are reported. Studies of cellulose synthesis in cultures grown statically on defined media and under aerated and agitated conditions will be reported separately.

METHODS

Organisms. *Acetobacter acetigenum*, East African strain, originally sent by Professor T. K. Walker (Manchester University) to the Hankey Culture Collection (HCC) maintained at this Institute, was divided into two variants on the basis of cellulose yields. The culture, maintained for two years on the hydrolysed molasses medium described below, has been designated strain EA-I to distinguish it from the culture maintained on glucose + Marmite + pep-

tone medium, designated strain EA-II; the same strain deposited by Professor Walker at the National Collection of Industrial Bacteria (NCIB) was obtained from the NCIB as strain NCIB 8132. *A. xylinum* strain HCC B-51 was obtained originally from the NCIB. Strains HCC B-150 and HCC B-151 were isolated locally by Miss M. B. Morris (HCC) from stout and from cane juice vinegar, respectively. Strain Hestrin was obtained from Dr S. Hestrin (Hebrew University, Jerusalem). Strain 472 was obtained from the Division of Applied Microbiology, National Research Council, Ottawa. *A. xylinum* strain Walker C (NCIB 8754), *A. kutzingerianum* strains Walker A (NCIB 8752) and Walker D (NCIB 8755) and *A. pasteurianum* strain Walker B (NCIB 8753) were obtained from Professor T. K. Walker; it should be noted that these strains of *A. kutzingerianum* and *A. pasteurianum* are atypical.

The organisms were maintained on the following medium (% w/v): hydrolysed molasses (prepared as described below), 2–3 total sugar; $(\text{NH}_4)_2\text{SO}_4$, 0.3; KH_2PO_4 , 0.5; Marmite, 0.5; adjusted to pH 6. The cultures were grown at tropical room temperature (26–29°) and subcultured at intervals of 4 weeks.

Media. Details of the various media used are given with the results. The blackstrap molasses contained 65 % (w/w) total sugar (42 % sucrose, 23 % reducing sugar) and 0.37 % (w/w) total-N. For hydrolysis the molasses was diluted fivefold with distilled water and treated with one-twentieth of its volume of a crude invertase solution prepared from fresh brewery yeast as described by Hestrin, Feingold & Schramm (1955). Hydrolysis was complete in 24–48 hr. at room temperature, after which bentonite (1 %, w/v) was added and the solution clarified by boiling for 20 min. and filtering while hot.

Inoculation. Culture liquid from well-shaken test-tube cultures, activated by two or three previous transfers at 3-day intervals, was used when less than 10 days old. The yield of cellulose at completion of growth was not sensibly affected by the size of inoculum, over the range 0.2–4 ml./100 ml. of sterile medium.

Culture method. Weighed 250 ml. flasks containing 100 ml. medium were inoculated and allowed to remain undisturbed on the bench at room temperature (26–29°).

Analytical methods. The flasks were weighed before analysis and the decrease in weight, assumed to be loss by evaporation, was taken into account when calculating the residual-sugar results. The flasks were thoroughly shaken and the culture fluid decanted for potentiometric pH measurement and residual sugar determination. When molasses was used the reducing sugars (glucose + fructose) were determined by the method of Somogyi (1952); the total sugar was determined by the anthrone method of Fairbairn (1953); the sucrose present was calculated by difference. When glucose, sucrose, soluble starch or hydrolysed molasses were used, the residual sugar was determined by the anthrone method. The sugar utilized by the cultures was calculated by difference between the residual sugar found and that initially present in the medium.

The pellicles were left in the flasks and washed with repeated changes of distilled water. Mercuric chloride solution (1 ml., 10 %, w/v) was added to

the first few changes of water to prevent the growth of contaminants. Thin pellicles were washed for 1–2 days and thick pellicles for periods up to 10 days, all with several changes of water daily. The final washings were free from sugar and soluble nitrogenous constituents. At this stage it was assumed that the pellicles, referred to as 'crude pellicles', contained only cellulose + organisms.

It was found possible to obtain a measure of growth in the cultures by determining the nitrogen content of washed crude pellicles, on the assumption that all the organisms were trapped in the pellicles. The reproducibility of results from replicate cultures indicated that any error caused by the loss of organisms during washing was small. The use of water to wash the crude pellicles was shown not to affect the nitrogen content of the *Acetobacter* organisms. When samples of a suspension of *Acetobacter acetigenum* were compared after standing at room temperature for 8 days in daily changes of water and physiological saline, respectively, the difference found in the cell-N content was negligible (organisms washed in water: 12.5% N; organisms washed in saline: 13.0% N). Since the wet crude pellicles were highly hydrated (c. 99% water; Schramm & Hestrin, 1954), the weight of the pellicles in grammes was equivalent to their volume in millilitres; it was thus relatively simple to determine the volume of each wet crude pellicle by weighing it. For nitrogen determinations the pellicles were weighed in the flasks and an equal volume of 2N-NaOH added. The flasks were stoppered and put on a rotary shaker overnight to ensure complete dissolution of cell material from the pellicle. Control experiments with thick (1 cm.) pellicles showed that shaking for 10 hr. was sufficient for the purpose. Nitrogen determinations were carried out on 1 ml. samples of the alkaline extracts; from these results the nitrogen content of the crude pellicles was calculated.

To determine cellulose the alkali-extracted pellicles were washed first with dilute acetic acid (c. 1%, w/v) until they remained acid to added methyl red indicator for several hours, and then with 8 changes of distilled water (150 ml.), allowing several hours for each wash, to remove the acid. The cellulose pellicles were hung on tared glass rods, allowed to drain overnight and then dried to constant weight at 105°. The cellulose obtained in this manner generally contained 0.2–0.4% N, equivalent to 1.2–2.5% protein, and was thus more than 95% pure.

Calculation of results. The efficiency of conversion into cellulose was calculated as direct percentages in terms of the total sugar provided in the medium ('conversion of total sugar') and of the sugar utilized by the culture ('conversion of utilized sugar'). The relationship between cellulose yields and amount of organism obtained in the cultures was expressed as the 'cellulose: cell-N ratio' (mg. cellulose produced/mg. cell-N), or as the 'cellulose content of crude pellicle' which was calculated from the fraction

$$\frac{\text{cellulose} \times 100}{\text{cellulose} + \text{dry wt. organism}}$$

The ratio which is obtained directly from the two measurements is independent of any assumptions and is reliable under all conditions, while the second

term depends on the accuracy with which the relationship between cell-N and dry wt. organism is known. The two terms are related by the expression:

$$\text{cellulose content of crude pellicle} = \frac{\frac{\text{cellulose}}{\text{cell-N}} \times 100}{\frac{\text{cellulose}}{\text{cell-N}} + \frac{100}{\%N \text{ in cell}}}$$

In Fig. 1 values for the ratio cellulose:cell-N, ranging from 0 to 30, are related to the cellulose content of crude pellicle calculated on the basis of 14.9 % N in the organisms.

Fig. 1. Relationship between cellulose:cell-N ratio and cellulose content of crude pellicle in cultures of *Acetobacter acetigenum*, calculated for organisms containing 14.9 % N.

RESULTS

Cellulose production on dilute molasses media

Acetobacter acetigenum strain EA-I growing in dilute molasses medium produced the largest cellulose yields in a preliminary experiment in which the cellulose production of a number of strains was compared. This strain was therefore used in the main experiments. The behaviour of strain EA-I in dilute molasses medium is illustrated in Fig. 2. The rate of cellulose production was initially rapid; more than 75 % of the total yield was synthesized in 9 days. Subsequent synthesis was much slower; small increases in yield were detected up to 46 days. Although cellulose synthesis was slow after 9 days, sugar utilization continued at an undiminished rate until the 16th day, by which time most of the reducing sugar had been used. The organism was unable to use the sucrose in the molasses; the slight utilization in the later half of the culture period was probably aided by hydrolysis of some of the sucrose under the mildly acid conditions, as suggested by the slight increase in the residual reducing sugar found toward the end of the cultural period. The pH values of the cultures remained relatively stable throughout. The conversion of sugars

into cellulose on this medium was of a very low order, being only 1.5% of the total sugar and 7.8% of the utilized sugar at the maximum. The nature of the inoculum made little difference to the results.

Fig. 2. Cellulose production, sugar utilization and conversion, and pH changes during growth of *Acetobacter acetigenum* strain EA-I in 100 ml. dilute molasses medium (8%, w/v, total sugar; initial pH 5.6) inoculated with pellicles from 4-day test-tube cultures or with 4 ml. liquid from 24 hr. cultures. Results for cultures inoculated with pellicles shown as filled points (● ▲ ■) and with culture liquid as outline points (○ △ □). Top: cellulose yields (○ ●), pH (△ ▲). Middle: utilization of reducing sugar (○ ●), sucrose (△ ▲), total sugar (□ ■). Bottom: conversion of total sugar (○ ●), utilized sugar (△ ▲).

Inhibition of cellulose synthesis by salts

The inability of *Acetobacter acetigenum* strain EA-I to utilize sucrose (two-thirds of the total sugar in the molasses) indicated the importance of studying the behaviour of the organism in hydrolysed molasses. A preliminary experiment with molasses hydrolysed with hydrochloric acid and neutralized with sodium hydroxide showed that the resulting sodium chloride concentration (2.5%, w/v) in the medium prevented growth. The sensitivity of this strain to sodium chloride and other salts was therefore examined.

Instead of hydrolysing molasses with various acids and neutralizing afterwards it was simpler to add known concentrations of salts directly to the medium. To make the conditions like those which would obtain in an acid hydrolysate of molasses it was desirable to add the salts to hydrolysed as well as unhydrolysed molasses. A sample of molasses was hydrolysed with crude

yeast invertase to avoid the complications of acid hydrolysis. The effect of six salts (representing the products of hydrochloric, sulphuric, nitric, acetic and formic acid neutralized with sodium hydroxide, and of hydrochloric acid with potassium hydroxide) was examined over the range 0.05–2% (w/v) with molasses and hydrolysed molasses (both 2.4%, w/v, total sugar), and in the medium of Schramm & Hestrin (1954) with molasses, hydrolysed molasses and glucose (all 2.4%, w/v) as separate substrates.

Sodium acetate and nitrate showed stimulation, not inhibition, over the range of concentration examined; the other salts were found to be inhibitory at concentrations independent of the medium (sodium formate, 0.037M; sodium sulphate, 0.036M; potassium chloride, 0.137M; sodium chloride, 0.171M). With sodium chloride after 18 days of incubation thick pellicles were found in the media with 0.8%-NaCl (0.137M), and very slight pellicles at 0.9% (0.154M) and 1% (0.171M). Inoculation into fresh salt-free medium from a 1% NaCl culture gave very retarded growth, but on further subculture into fresh medium the growth returned to its normal rate. It was decided to eliminate the complications of the salt effect in further experiments with molasses by using yeast invertase instead of acid for hydrolysis.

Comparison of cellulose yields from hydrolysed molasses, molasses, glucose, sucrose and starch

A comparison was made of cellulose production and sugar utilization by *Acetobacter acetigenum* strain EA-I with different substrates in the following media:

Medium A. Henneberg (1926) medium with the addition of Marmite as used by Professor M. Stacey (1955, personal communication); (% w/v): carbohydrate, 2; $(\text{NH}_4)_2\text{SO}_4$, 0.3; KH_2PO_4 , 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; Marmite, 0.5; adjusted to pH 5; ethanol (1%, v/v) was added after sterilization.

Medium B. (Professor M. Stacey, 1955, personal communication); (% w/v): carbohydrate, 4; KH_2PO_4 , 0.02; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; Marmite, 0.4; glacial acetic acid, 1% (v/v); no pH adjustments; ethanol (2%, v/v) was added after sterilization.

Medium C (Tarr & Hibbert, 1931); (% w/v): carbohydrate, 2; asparagine, 0.1; KH_2PO_4 , 0.5; NaCl, 0.1; adjusted to pH 5; ethanol (1%, v/v) was added after sterilization.

Medium D (Tarr & Hibbert, 1931); medium C + peptone, 0.1% (w/v); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% (w/v); FeSO_4 , trace.

Medium E (Tarr & Hibbert, 1931); medium C + Marmite, 1% (w/v); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% (w/v); FeSO_4 , trace.

Medium F (Schramm & Hestrin, 1954); (% w/v): carbohydrate, 2; peptone, 0.5; Na_2HPO_4 , 0.27; Marmite, 0.5; citric acid, 0.115; adjusted to pH 6.

Each medium was used with five different carbohydrate sources: hydrolysed molasses, molasses, glucose, sucrose and soluble starch. To ensure adaptation to each basal medium + carbohydrate mixture the organism was grown through four serial subcultures in each mixture before the appropriate flasks were

inoculated. Growth on media with glucose, sucrose or soluble starch was slower, requiring 5–7 days between each subculture, than with molasses or hydrolysed molasses, which required 2–3 days. Growth was also relatively slow on basal medium D + glucose, sucrose or starch, showing the need for yeast extract or some growth factor for good growth. Growth was so slow on basal medium C + glucose, sucrose or starch and on medium F + sucrose or starch that they were discontinued. The flasks were each inoculated with 5 ml. adapted culture and incubated for 21 days. The pellicles differed considerably in appearance depending on the medium and added carbohydrate.

The results shown in Table 1 are the average values found for triplicate cultures. Pellicle thickness was found not to be always a reliable indication of cellulose yield. For example the pellicles from media B + glucose, B + sucrose, C + molasses, D + hydrolysed molasses, D + molasses and E + sucrose were all about 0.7 cm. thick but contained 97, 52, 137, 225, 177 and 42 mg. cellulose, respectively. These results suggest that considerable differences in density between pellicles may arise from growth on different media and carbohydrates.

The final pH values of the cultures depended on the nature of the carbohydrate source. All the cultures which contained glucose, sucrose or starch had pH values consistently lower than those containing molasses or hydrolysed molasses: these latter remained closer to the optimum pH range for cellulose synthesis.

The general degree of utilization of each carbohydrate source was independent of the basal medium, and, with three exceptions, reached the following values: hydrolysed molasses, 80–90 %; molasses, 42–65 %; glucose, 14–20 %; soluble starch, 12–15 %; sucrose, 5–10 %. The exceptional utilization results obtained with the cultures grown in medium B + molasses, medium B + hydrolysed molasses and medium F + glucose, are believed to have been due to the presence in these media of sparing adjuncts (ethanol + acetic acid in basal medium B; citrate in basal medium F). The behaviour of these adjuncts is discussed elsewhere (Dudman, 1959). The significance of the utilization figures for reducing sugar and sucrose in molasses media cannot be assessed because their relative final values may have been the direct result of utilization, or more reducing sugar may have been consumed and some replaced by sucrose hydrolysis. The apparent non-utilization of reducing sugar in basal medium B + molasses is believed to have arisen through the fortuitous hydrolysis of enough sucrose completely to replace the reducing sugar used.

Cellulose yields from each medium differed with the carbohydrate substrate. With the exception of medium F from which the highest yield was obtained with glucose, the yields from all the other basal media + carbohydrate sources were found to be in the following order: hydrolysed molasses > molasses > glucose > sucrose > soluble starch. The relatively low yields from molasses and hydrolysed molasses in basal medium F are believed to have been caused by the citrate present, which is also thought to be responsible for the high yield from glucose in this medium (Dudman, 1959).

Table 1. Cellulose production by *Acetobacter acetigenum* strain EA-I grown on hydrolysed molasses (HM), molasses (M), glucose (G), sucrose (S) or soluble starch (SS) media for 21 days at 28–30° under static conditions

Medium	Pellicle thickness (cm.)	Final pH value	Total carbohydrate in medium initially* (g.)	Carbohydrate utilization						Cellulose (mg.)	Carbohydrate conversion	
				Reducing		Sucrose		Total			Total (%)	Used (%)
				(g.)	(%)	(g.)	(%)	(g.)	(%)			
A	HM	1.2	5.75	—	—	—	—	1.79	80.6	268	12.1	15.0
	M	0.9	6.17	0.50	80.6	0.51	29.8	1.01	42.8	168	7.1	16.6
	G	1.1	3.41	—	—	—	—	0.45	19.4	79	3.4	17.6
	S	0.5	3.59	—	—	—	—	0.24	9.5	32	1.8	13.8
	SS	0.2	3.55	—	—	—	—	0.29	12.8	7	0.8	2.4
B	HM	1.2	5.20	—	—	—	—	1.02	24.9	311	7.6	30.5
	M	1.0	4.93	0	0	0.86	28.2	0.86	18.7	247	5.4	28.7
	G	0.7	4.07	—	—	—	—	0.84	19.8	97	2.8	11.5
	S	0.7	4.26	—	—	—	—	0.46	10.2	52	1.2	11.8
	SS	0.2	4.14	—	—	—	—	0.86	18.7	8	0.07	0.8
C	HM	0.9	5.75	—	—	—	—	1.99	90.4	217	9.9	10.9
	M	0.7	5.96	0.75	79.8	0.58	37.2	1.33	53.2	137	5.5	10.8
D	HM	0.6	6.43	—	—	—	—	1.99	86.9	225	9.8	11.8
	M	0.7	6.94	0.50	66.7	0.74	50.7	1.24	56.1	177	8.0	14.8
	G	0.2	3.19	—	—	—	—	0.31	14.6	130	0.6	4.2
	S	0.2	3.40	—	—	—	—	0.11	4.7	6	0.8	5.5
	SS	Very thin	5.07	—	—	—	—	0.25	11.9	—†	—	—
E	HM	0.9	5.74	—	—	—	—	1.83	85.1	268	12.5	14.7
	M	0.8	6.04	0.51	61.7	0.99	66.5	1.50	64.4	162	7.0	10.8
	G	1.4	3.50	—	—	—	—	0.88	19.1	101	5.1	26.6
	S	0.7	4.43	—	—	—	—	0.15	6.1	42	1.7	28.0
	SS	0.2	3.42	—	—	—	—	0.52	24.8	13	0.6	2.5
F	HM	0.8	6.89	—	—	—	—	1.85	86.9	126	5.9	6.8
	M	0.4	6.85	0.37	46.8	0.99	70.7	1.36	61.8	121	5.5	8.9
	G	0.5	4.63	—	—	—	—	1.91	89.2	177	8.3	9.3

* By analysis. † Disintegrated on purification.

Influence of hydrolysed molasses concentration on cellulose production

The effect of hydrolysed molasses concentration on growth and cellulose production by *Acetobacter acetigenum* strain EA-I was examined in a modified form of medium A (above). In addition to hydrolysed molasses the medium contained the following nutrients (% w/v): $(\text{NH}_4)_2\text{SO}_4$, 0.8; KH_2PO_4 , 0.5; Marmite, 0.5; adjusted to pH 6. The hydrolysed molasses used in this experiment was prepared by the action of crude yeast invertase on molasses with the least possible dilution; 1000 ml. molasses was diluted with 500 ml. water and hydrolysed with 200 ml. crude invertase, the action of which was complete in 48 hr. A series of dilutions of the hydrolysed molasses was prepared and sterilized separately from the other nutrients. After the sterile nutrient solutions were combined, the media were analysed for total sugar and the following concentrations found (% w/v): 0.8, 1.5, 4.0, 7.9, 12.0, 15.4 and 23.2. Each flask was inoculated with 4 drops taken from a 4-day culture; after 9, 18, 27 and 40 days cultures at each concentration were analysed.

Although pellicle formation was not observed in the 15.4% sugar cultures until after the 9th day, and in the 23.2% sugar cultures until after the 18th day, sugar utilization (Fig. 3*a*, *b*) was found to have taken place in all the cultures by the 9th day. When expressed as % initial sugar provided in the medium (Fig. 3*a*) the rate of utilization was inversely related to the initial sugar concentration, but when expressed as the weight of sugar utilized (Fig. 3*b*) the rate and final weight of sugar utilized was directly related to the initial sugar concentration, except in the cultures at the two highest concentrations in which sugar was utilized abnormally slowly until the later stages of the culture period. Utilization was complete in 18 days in the 0.8 and 1.5% sugar cultures, and in 27 days in the 4.0% sugar cultures. By the 40th day the 7.9% sugar cultures approached complete utilization. The cultures at the three highest sugar concentrations had not exhausted their sugar supply in 40 days and utilization was still continuing.

The pH values of the cultures at all stages of growth, except those at the two highest sugar concentrations, were related to the initial sugar concentration in the medium (Fig. 3*c*). The 0.8% sugar cultures showed the smallest change from the initial pH value of the medium; the higher sugar concentrations gave progressively lower pH values.

Cellulose production (Fig. 3*d*) in some of these cultures was greater than in the previous experiments. The largest yield was 890 mg. in the 7.9% sugar cultures after 40 days of incubation. Only those cultures which had exhausted their sugar supply reached maximum cellulose production in the 40-day growth period. The rates of cellulose production in the 1.5, 4.0 and 7.9% sugar cultures were similar during the period of active synthesis, showing that sugar concentrations up to 7.9% did not affect the rate of cellulose synthesis. The 12% sugar cultures showed a slower rate of synthesis which remained constant between 9 and 40 days. The inhibitory effect of higher sugar concentrations was more clearly shown at the two highest concentrations; the 15.4% and 23.3% sugar cultures showed no evidence of cellulose produc-

tion until after 9 and 18 days, respectively; after this, slight increases were found.

The highest values for the conversion of total sugar to cellulose (Fig. 8e) were obtained with the 1.5% sugar cultures which had reached their final yield (17–18%) by the 9th day. With the exception of the 0.8% sugar cultures which reached a lower degree of conversion (13–14%) than the 1.5% sugar

Fig. 8. The influence of total sugar concentration on static cultures of *Acetobacter acetigenum* strain EA-I grown in 100 ml. hydrolysed molasses medium: (a) sugar utilization (%); (b) sugar utilization (g.); (c) pH; (d) cellulose yield; (e) conversion of total sugar; (f) cell-N; (g) conversion of utilized sugar; (h) cellulose: cell-N ratio and cellulose content of crude pellicle. The sugar concentrations used were 0.8% (○), 1.5% (●), 4.0% (△), 7.9% (▲), 12.0% (□), 15.4% (■), and 23.2% (×).

cultures all the other cultures showed conversions inversely related to the initial sugar concentration. The conversion of utilized sugar to cellulose (Fig. 3g) varied with the initial sugar concentration in the medium in the same manner as the conversion of total sugar, with the 1.5% sugar cultures giving the highest values. The decrease in the conversion values found in the 0.8 and 1.5% sugar cultures after 9 days was a consequence of the stoppage of cellulose synthesis in these cultures before all the sugar in the medium was exhausted; the subsequent sugar utilization was not accompanied by cellulose synthesis.

Growth (Fig. 3f) in all the cultures was similar in pattern to the cellulose production. During the period of active growth the 1.5, 4.0 and 7.9% sugar cultures showed the same rate of growth, indicating the absence of inhibition by sugar concentrations up to 7.9%. Growth in the cultures with the higher initial sugar concentrations was slower; the 12.0% sugar cultures grew at about half the rate found in the more dilute cultures, while growth in the 15.4 and 23.2% sugar cultures was slower and subject to the same long lag periods as cellulose synthesis.

The cellulose: cell-N ratios (Fig. 3h) showed a decrease with increased sugar concentration, indicating that less cellulose, relative to cell material, was synthesized as sugar concentration increased. To calculate the dry weight of organisms from the cell-N, the growth from two cultures grown in the 1.5% sugar medium for 18 days was harvested following the procedure described by Hestrin & Schramm (1954). The organisms contained 14.9% total-N and 2.1–2.5% total carbohydrate (by micro-Kjeldahl and anthrone methods, respectively). The cell-N content was assumed to be constant at all sugar concentrations and the dry weight of organisms in all the cultures was calculated using this value of 14.9%. The cellulose content of the pellicles varied widely, and decreased with increased sugar concentration in the medium from 71 to 74% in cultures with 0.8% sugar, to 28–35% at the other extreme in the cultures containing 23.2% sugar.

Comparison of growth and cellulose production by various Acetobacter strains grown in hydrolysed molasses medium

The growth and cellulose yields produced by the other *Acetobacter* strains capable of cellulose synthesis was examined on the following medium (% w/v): hydrolysed molasses, 2.8 total sugar; $(\text{NH}_4)_2\text{SO}_4$, 0.3; KH_2PO_4 , 0.5; Marmite, 0.5. The medium was adjusted to pH 6, and after sterilization ethanol (1%, v/v) was added. The cultures were incubated for 80 days before analysis.

The results (Table 2) showed that the cellulose yields varied from 656 mg. (23.5% conversion total sugar) with *Acetobacter xylinum* strain Hestrin, to 53 mg. (1.9% conversion total sugar) produced by *A. acetigenum* strain EA-II, which may be considered to be a low-yielding variant of strain EA-I. *A. pasteurianum* and *A. kutzingianum* produced less cellulose than any of the strains of *A. xylinum* or than *A. acetigenum* strain NCIB 8132. The amount of growth calculated from the cell-N figures (assuming that all strains contained

Table 2. Cellulose production by *Acetobacter* strains grown on hydrolysed molasses medium (100 ml.) for 80 days

<i>Acetobacter</i> spp.	Strain*	Final pH	Sugar utilization (%)	Cell growth			Sugar conversion		Cellulose cell-N ratio	Cellulose in crude pellicle (%)
				N (mg.)	Weight† (mg.)	Cellulose (mg.)	Total (%)	Utilized (%)		
<i>A. xylinum</i>	Hestrin	4.80	94.1	28.5	158	656	28.5	25.0	27.9	81
<i>A. xylinum</i>	HCC B. 150	3.72	90.8	22.1	148	611	21.9	24.1	27.6	80
<i>A. xylinum</i>	472	4.21	85.7	83.6	226	490	17.6	20.5	14.6	68
<i>A. acetigenum</i>	NCIB 8182	4.97	76.1	28.4	157	367	18.2	17.8	15.7	70
<i>A. xylinum</i>	Walker C	4.11	89.2	27.1	182	327	11.7	13.1	12.1	64
<i>A. xylinum</i>	HCC B. 51	4.05	84.9	20.4	137	308	11.0	13.0	15.1	69
<i>A. xylinum</i>	HCC B. 151	3.49	86.5	31.7	213	303	10.9	12.6	9.6	59
<i>A. pasteurianum</i>	Walker B	3.85	91.2	20.6	138	217	7.8	8.6	10.5	61
<i>A. butzingianum</i>	Walker A	3.31	84.2	19.0	128	134	4.8	5.7	7.1	51
<i>A. butzingianum</i>	Walker D	3.63	60.0	18.5	124	132	4.7	7.8	7.1	52
<i>A. acetigenum</i>	EA-II	4.70	96.0	24.5	165	53	1.9	2.0	2.2	24

* The strains are listed in the order of their cellulose yields.

† Calculated from cell-N results assuming cells to contain 14.9% N.

14.9% N) varied between narrower limits (124–226 mg) than the cellulose yields, showing that the degree of growth was relatively uniform throughout the range of strains. There was no connexion between the dry weight of organism produced and the cellulose yields. *A. acetigenum* strain EA-II produced slightly more growth (165 mg.) than *A. xylinum* strain Hestrin (158 mg.), although the former strain produced 12 times less cellulose than the latter.

The cellulose:cell-N ratios (which varied between wide limits: 2.2–27.9) were related to the cellulose yields and showed erratically decreasing values with decreased cellulose production. The % cellulose in the crude pellicles decreased in the same manner, having its highest (81%) and lowest (24%) values with the strains which produced the largest and smallest yields of cellulose, respectively. The intermediate values were graded irregularly and followed the general trend.

DISCUSSION

The relationship between cellulose synthesis and the growth of *Acetobacter* organisms was examined for the first time in the present study. The results show that the rates of cellulose synthesis and growth were closely related and that cellulose was synthesized by *Acetobacter acetigenum* only during the period of active growth. This was clearly shown in the cultures grown in the media with 0.8 and 1.5% sugar, where the early stoppage of cellulose synthesis was linked with the stoppage of growth for which there was no obvious reason, since nitrogen and sugar were still available and the pH value was not unfavourable. The dependence of cellulose synthesis on growth in these cultures is surprising in view of the ease with which washed suspensions of *Acetobacter* synthesized cellulose from glucose under conditions which did not permit growth (e.g. Hestrin & Schramm, 1954). Sugar utilization however continued in these cultures at almost the same rate despite the stoppage of growth and cellulose synthesis, showing that sugar was being converted to products other than cellulose; their identification was not attempted. A similar stoppage of cellulose synthesis followed by undiminished sugar utilization was also observed when the organism was grown in diluted molasses medium.

The amount of cellulose synthesized/unit dry wt. organism was not constant under all cultural conditions. The cellulose:cell-N ratio decreased with increased concentration of sugar. The cellulose content of the crude pellicles, which is related to the cellulose:cell-N ratio, was thus also affected by the sugar concentration in the medium, and ranged from 71 to 74% in the cultures grown in 0.8% sugar medium to 28–35% in cultures grown on 23.2% sugar. Brown (1886) found that the cellulose in the pellicles of *Acetobacter xylinum* varied from 35 to 62% depending on the growth conditions. *A. xylinum* strain Hestrin grown in hydrolysed molasses medium was found in the present study to produce pellicles containing 81% cellulose; Hestrin & Schramm (1954) found c. 38% cellulose in pellicles of the same strain grown on another medium.

The early observation by Brown (1886) that *Acetobacter xylinum* was unable to utilize sucrose was found to apply to *A. acetigenum* with regard to the sucrose in molasses. When grown in diluted molasses medium this organism used

80 % of the available reducing sugar before attacking a small amount of sucrose. In the presence of other nutrients sucrose utilization reached higher values, but total sugar utilization in molasses media never reached the values obtained in hydrolysed molasses media.

Hydrolysed molasses, prepared by the action of yeast invertase, was the best sugar substrate, leading to the highest cellulose yields; molasses was the next best. Fructose, one of the best substrates for cellulose production (Tarr & Hibbert, 1981; Barclay, 1951; Skopek, 1955) and which accounts for *c.* 18 % of the total sugar in molasses, and *c.* 50 % of the total sugar in hydrolysed molasses, was probably the main factor responsible for the good cellulose yields from these substrates.

The observed sensitivity of *Acetobacter acetigenum* strain EA-I to inhibition of cellulose production by low concentrations of simple inorganic salts was in keeping with the finding of Tarr & Hibbert (1981) that cellulose yields from *A. xylinum* in defined medium were maximal when the sodium chloride concentration was 0.1 % (w/v), and decreased to 56 and 31 % of the maximum when the sodium chloride concentration was increased to 0.5 and 1.0 % (w/v), respectively.

The results for the analysis of washed suspensions of *Acetobacter acetigenum* strain EA-I in which the nitrogen content was high (12.5–14.9 %) and the total carbohydrate low (2.1–2.5 %) were of the same order as the values obtained by Hestrin & Schramm (1954) for *A. xylinum* strain Hestrin, which contained 12 % nitrogen and 0.5 % 'apparent cellulose'.

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