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Cellulose production by *Acetobacter acetigenum* in defined medium

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SUMMARY: Growth and cellulose production by *Acetobacter acetigenum* strain EA-I was limited by nitrogen concentrations below 0.01 % (w/v) in glucose defined medium. Ammonium sulphate and asparagine + glutamic acid (50 % of each) were equivalent as nitrogen sources when compared on a nitrogen weight basis over the range 0.001-0.1 %-N; at higher concentrations ammonium sulphate decreased growth and cellulose synthesis, while asparagine + glutamic acid became stimulatory. When used as adjuncts in glucose defined medium, acetate, citrate and succinate at suitable concentrations increased growth and cellulose synthesis, by as much as 20-30-fold under the most favourable conditions. Ethanol stimulated growth but did not increase cellulose synthesis. The addition of calcium or magnesium carbonate to defined medium inhibited growth. The influence of glucose concentration in defined medium on growth and cellulose synthesis was relatively slight in the absence of succinate; in the presence of 0.084 M-succinate the cultures showed a much greater response to glucose concentration, with a maximum cellulose yield that was 7.6 times greater than the maximum in the absence of succinate, and was associated with a 1.9-fold increase in growth. In hydrolysed molasses medium the addition of succinate decreased growth and cellulose synthesis; slightly increased cellulose yields were obtained when ethanol and calcium carbonate were added.

In conjunction with the experiments previously described on cellulose production by *Acetobacter acetigenum* in complex media (Dudman, 1959), a study was made of growth and cellulose production in defined media. The use of defined media, free from complex nitrogenous compounds, was necessary to evaluate the influence of the nitrogen source, about which little is known except the early results of Tarr & Hibbert (1931).

The stimulatory effect of added ethanol on cellulose yields is well known. Ethanol cannot serve as the sole carbon source for growth and cellulose synthesis but when added to media containing suitable carbon substrates it increases the cellulose yields several fold (Tarr & Hibbert, 1931; Woeber, 1954; Minor, Greathouse, Shirk, Schwartz & Harris, 1954*a, b*). Acetate has been found to exert a similar stimulatory effect (Greathouse, Shirk & Minor, 1954). It is not known whether ethanol and acetate act by increasing cellulose synthesis specifically or whether growth is also increased. These adjuncts are believed to act by a sparing mechanism in which they are oxidized in place of part of the sugar substrate; this suggests that other compounds which are readily oxidized, such as the intermediate acids of the tricarboxylic acid cycle, might also act as stimulators of cellulose synthesis. In the present study the influence of ethanol, acetate, citrate and succinate was examined in defined medium. The most stimulatory of these (succinate) was also studied in

hydrolysed molasses medium. The influence of glucose concentration on growth and cellulose synthesis in defined medium, in the absence and presence of succinate, was also investigated.

METHODS

Organism. The organism used, *Acetobacter acetigenum* strain EA-I, has been described (Dudman, 1959).

Media. Details of the various media are given with the results. Three growth factors, *p*-aminobenzoic acid, calcium pantothenate and nicotinic acid were added to all the defined media, in view of the requirement for growth factors of the B group found by Barclay (1951) and Woeber (1954). The factors were added to the media before sterilization in relatively high concentration (10 mg./l.) to allow for loss by heat destruction during sterilization. The trace element solution of Emery, McLeod & Robinson (1946), with the addition of cobalt nitrate (0.02 g./l.), was used to provide trace elements in the defined media.

Cultural conditions. The cultures were grown in 100 ml. medium in 250 ml. flasks, under static conditions at room temperature (26–29°).

Analytical methods. In addition to the methods described previously (Dudman 1959), residual nitrogen was determined by micro-Kjeldahl determinations on samples of culture liquid.

RESULTS

Influence of nitrogen concentration on growth and cellulose production

Two nitrogen sources, asparagine + glutamic acid (50%, w/w, of each) and ammonium sulphate, were compared at the following nitrogen concentrations (% w/v), 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1 and 0.2, in a basal medium containing (% w/v): glucose, 2.0; KH_2PO_4 , 0.5; NaCl, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; *p*-aminobenzoic acid, 0.001; calcium pantothenate, 0.001; nicotinic acid, 0.001; trace element solution, 0.5 ml./l.; adjusted to pH 6. The media were sterilized by steaming for 20 min. on 3 successive days. The cultures were inoculated with 2 drops of a 1/10 dilution in physiological saline of liquid from a 2-day culture grown in hydrolysed molasses medium; this inoculum contained insufficient nitrogen (0.02 mg.) to affect the concentration in the medium. The cultures were incubated for 33 days.

Nitrogen concentrations below 0.01% were limiting for glucose utilization, growth and cellulose synthesis (Fig. 1). The limiting concentration was the same for both the amino acid mixture and ammonium sulphate, showing that these nitrogen sources were equivalent in satisfying growth requirements when compared on an equal nitrogen weight basis. The glucose utilization level (Fig. 1*a*) remained constant at between 94–96% for all the asparagine + glutamic acid concentrations above 0.01%-N; there was no indication of inhibition at the highest concentration used. The cultures containing ammonium sulphate however, showed decreased glucose utilization at 0.2%-N; at higher ammonium sulphate concentrations the organism failed to grow.

The final pH values (Fig. 1*b*) were of the same low order in all the nitrogen deficient cultures; in the ammonium sulphate cultures the final pH values remained low at all nitrogen concentrations. The asparagine + glutamic acid cultures were found to have higher pH values at the non-limiting nitrogen concentrations, reaching pH 6 at the three highest concentrations, presumably as a result of the increased buffering capacity of the media with high amino acid concentrations.

Cellulose yields (Fig. 1*c*) in the ammonium sulphate cultures at non-limiting nitrogen concentrations showed a slight increase from 30 mg. at 0.01 % N to a maximum of 40 mg. at 0.1 % N; the yield decreased to 26 mg. at 0.2 % N. Cellulose yields in the asparagine + glutamic acid cultures remained constant (28–32 mg.) over the range 0.01–0.1 % N, and showed a striking increase to 81 mg. at 0.2 % N. The conversion of glucose to cellulose in all the cultures, however, was of a low order (Fig. 1*c, d*). Growth (Fig. 1*e*) was sharply limited at concentrations below 0.01 % N. At higher nitrogen concentrations the cell-N values remained essentially constant, but showed relatively small changes reflecting the variation in cellulose yields. Assuming the cells grown under these conditions to contain 14.9 % N (Dudman, 1959), the growth level attained (7.2–11.0 mg. cell-N/culture) was of the order of 48–74 mg. total dry weight of organism in the cultures not deficient in nitrogen.

The values of the cellulose:cell-N ratio (Fig. 1*f*) differed considerably between cultures deficient and adequate in nitrogen. The values were highest at 0.001 % N and decreased rapidly with increased nitrogen in the medium to a value of 4.2 for both nitrogen sources at 0.01 % N. At higher nitrogen concentrations the changes in the value of the ratio were relatively small. The cellulose content of the crude pellicles, calculated on the basis of 14.9 % N in the cell dry matter, varied from about 75 % in the cultures with 0.001 % N to about 35 % over the range 0.01–0.1 % N; at 0.2 % N the cellulose content was 52 and 30 % in the asparagine + glutamic acid and ammonium sulphate cultures, respectively.

Nitrogen utilization (Fig. 1*g*) showed a significant difference between the asparagine + glutamic acid and ammonium sulphate cultures. In the ammonium sulphate cultures with excess nitrogen, utilization remained constant (9.5–10.8 mg. N/culture) showing that excess nitrogen was not used though available. In the asparagine + glutamic acid cultures excess nitrogen was used; at nitrogen concentrations above 0.01 %, utilization increased continuously, reaching 34 mg./culture at 0.2 % N. Cell-N, as found, did not account for all the nitrogen utilized (Fig. 1*h*). Results for nitrogen recovery in the cultures with less than 0.01 % N are omitted because of the relatively large experimental error involved in determining these small amounts. The recovery of utilized nitrogen in the ammonium sulphate cultures varied from 75 to 100 % over the range 0.01–0.2 % N; it is likely that the deviations from 100 % recovery indicate the error inherent in the method used for determination of the dry weight of organisms in these cellulose cultures; the method is based on the assumption that all the organisms are trapped in the pellicle. It is to be noted, however, that the recovery increased with increased nitrogen concentration.

In the asparagine+glutamic acid cultures the recovery values decreased progressively from 100% at 0.01%-N to 83% at 0.2%-N, which may be interpreted to mean that a mechanism operated in these cultures for the removal of nitrogen from the medium, in addition to removal by growth.

Fig. 1. Influence of nitrogen concentration in glucose defined medium on 88-day static cultures of *Acetobacter acetigenum* strain EA-I: (a) glucose utilization; (b) final pH value; (c) cellulose yield and conversion of total sugar to cellulose; (d) conversion of utilized sugar to cellulose; (e) cell-N and dry wt. of organism calculated assuming 14.9% N in organisms; (f) cellulose: cell-N ratio and cellulose content of crude pellicle; (g) nitrogen utilization; (h) recovery of utilized-N as cell-N. The nitrogen sources used were asparagine + glutamic acid (50%, w/w of each) (○) and ammonium sulphate (●).

Influence of adjuncts on growth and cellulose production in defined medium

The influence of ethanol, acetate, citrate and succinate on growth and cellulose production was examined at five concentrations (Table 1), equivalent to 0.1, 0.2, 0.4, 0.8, and 1.6 % (w/v) adjunct carbon, in an otherwise complete medium containing excess nitrogen. The defined medium above, containing 1.95 % (w/v) glucose and 0.47 % (w/v) ammonium sulphate (i.e. 0.1 %, w/v, N), and adjusted to pH 5.8, was used. The acetate, citrate and succinate adjuncts, prepared as solutions of the acids and adjusted to pH 5.8 with sodium hydroxide, were added to the medium before sterilization. Ethanol was added to sterile medium. Controls were set up for each adjunct, with 0.4 % (w/v) adjunct carbon in the basal medium without glucose.

Cultures were inoculated with a drop of 2-day culture in hydrolysed molasses medium, and incubated for 28 days. Growth occurred over the whole range of ethanol concentrations used, but the acid adjuncts were found to prevent growth at the following concentrations: citrate, 0.056 M; succinate, 0.333 M; acetate, 0.667 M. Growth was not observed in any medium containing adjuncts without glucose.

Table 1. *Adjunct concentrations required to give 0.1, 0.2, 0.4, 0.8 and 1.6 % (w/v) adjunct carbon in medium*

Adjunct carbon in medium (%)	Concentration			
	Acetic acid (M)	Citric acid (M)	Succinic acid (M)	Ethanol (M)
0.1	0.042	0.014	0.021	0.042
0.2	0.084	0.028	0.042	0.084
0.4	0.167	0.056	0.084	0.167
0.8	0.333	0.111	0.167	0.333
1.6	0.667	0.222	0.333	0.667

All the adjuncts had a marked stimulatory effect on glucose utilization (Fig. 2a). The final pH values (Fig. 2b) found in the cultures were very much dependent on the nature of the adjunct. The cultures with ethanol had the same low final pH values as the cultures grown in the basal medium, while the cultures with acetate, citrate and succinate, all of which are good buffers, were found to have higher pH values. Cellulose yields (Fig. 2c) showed a sharp differentiation between ethanol and the acid adjuncts. Cellulose synthesis was not stimulated by ethanol at any concentration, while very large increases were obtained with acetate, citrate and succinate. Cellulose yields from cultures with these three adjuncts were increased linearly by 0.1 and 0.2 % (w/v) adjunct carbon; at higher concentrations citrate inhibited growth and acetate gave smaller yields. The maximum yields were obtained with 0.4 and 0.8 % (w/v) succinate.

The influence of the adjuncts on growth (Fig. 2f) was not as distinct as in the case of cellulose production. Ethanol stimulated growth to higher levels than in the basal medium. The three acid adjuncts, at 0.1 % (w/v) carbon concentration, increased growth to the same level, but at higher concentrations

the influence of the adjuncts varied. The largest increase in growth was obtained with succinate. The values found for the cellulose:cell-N ratio (Fig. 2g) in the adjunct cultures were all smaller at 0.1% (w/v) adjunct carbon concentration than found in the cultures grown in the basal medium, showing that growth was more stimulated relatively, by the adjuncts at this concentration, than cellulose synthesis. Higher ethanol concentrations in the medium did not affect the value of the ratio. Higher concentrations of the adjuncts however gave increased values of the ratio.

These results suggested that succinate was the best adjunct for use in defined medium to obtain maximum growth and cellulose production.

Fig. 2. Influence of acetate (Δ), succinate (▲), citrate (■) and ethanol (●) adjuncts in glucose defined medium on 28-day static cultures of *Acetobacter acetigenum* strain EA-I: (a) glucose utilization; (b) pH value; (c) cellulose yield, conversion of total sugar; (d) conversion of utilized sugar; (e) conversion of total carbon (i.e. glucose + adjunct carbon); (f) cell-N; (g) cellulose:cell-N ratio, cellulose content of crude pellicle (assuming 14.9% N in organisms).

Attempts were made to examine the effect of ethanol at a more favourable pH by adding calcium carbonate (1–5 g./100 ml.) to the medium as a neutralizing agent, but without success. The use of calcium carbonate in the form of precipitated chalk, crushed marble or limestone, inhibited growth in defined medium even when the material was separately sterilized and added to cold sterile medium. The inhibitory effect was not overcome when additional amounts of the trace element solution and the growth factors were added immediately before inoculation with 1 ml. active 2-day culture. The same negative result was obtained with magnesium carbonate. The cause of inhibition was not investigated further.

Influence of adjuncts on growth and cellulose production in hydrolysed molasses medium

The possibility of increasing cellulose yields from hydrolysed molasses, by the use of suitable adjuncts, was examined in a medium containing (% w/v) hydrolysed molasses, 2.0 total sugar; $(\text{NH}_4)_2\text{SO}_4$, 0.3; KH_2PO_4 , 0.3; Marmite 0.5; adjusted to pH 6. The adjuncts used were: succinate (at the five concentrations shown in Table 1); succinate (0.084 M) + calcium carbonate (5 g./100 ml.); ethanol (0.167 M); ethanol (0.167 M) + calcium carbonate (5 g./100 ml.); calcium carbonate (5 g./100 ml.). The ethanol and sterile calcium carbonate were added to sterile medium. The cultures were inoculated with 2 drops of 3-day culture, and incubated for 49 days.

The addition of succinate to hydrolysed molasses medium had an adverse effect on growth and cellulose production, although growth was not completely inhibited by 0.333 M-succinate as in defined medium. Growth and cellulose yields were progressively reduced by increased succinate concentrations; the cellulose:cell-N ratio was constant however. The conversion of total sugar to cellulose decreased progressively from 28% in the control medium to a value of 15% at 0.333 M-succinate. Sugar utilization was not affected by the succinate adjunct. Of the other adjuncts tested, only ethanol and calcium carbonate, used separately, increased cellulose yields (by 6 and 10%, respectively).

Influence of glucose concentration on growth and cellulose production in defined medium

The influence of glucose concentration in defined medium on growth and cellulose production was examined in the absence and presence of succinate. The basal medium contained (% w/v): $(\text{NH}_4)_2\text{SO}_4$, 0.472 (i.e. 0.1 N); KH_2PO_4 , 0.5; NaCl 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; *p*-aminobenzoic acid, 0.001; calcium pantothenate, 0.001; nicotinic acid 0.001; trace element solution, 0.5 ml./l.; adjusted to pH 6. Glucose was separately sterilized and added to the basal medium to give the following concentrations (% w/v): 0, 1, 2, 5, 10, 15 and 20. Succinate was used at a concentration of 1.165% (w/v), equivalent to 0.4% (w/v) succinate-carbon. The cultures were inoculated with 2 drops of 2-day culture and incubated for 40 days. The results are shown in Fig. 3; results for cultures

grown for 40 days in hydrolysed molasses medium at various sugar concentrations obtained in a previous experiment (Dudman, 1959) are included for comparison.

Glucose utilization (Fig. 3*a, b*) was complete in the 1 and 2% glucose cultures, with and without succinate. At higher concentrations the % utilization decreased progressively with increased glucose concentrations but was always greater in the cultures containing succinate. The final pH values (Fig. 3*c*) were higher in the succinate cultures.

Cellulose production (Fig. 3*d*) was greatly increased at all glucose concentrations when succinate was present in the medium. In the absence of succinate

Fig. 8. Influence of sugar concentration in glucose defined medium on 40-day static cultures of *Acetobacter acetigenum* strain EA-I: (a) sugar utilization (%); (b) sugar utilization (g.); (c) final pH value; (d) cellulose; (e) conversion of total sugar; (f) conversion of utilized sugar; (g) cell-N; (h) cellulose:cell-N ratio; cellulose content of crude pellicle (assuming 14.9% N in organisms). Results are shown for cultures grown in glucose medium with (●) and without (○) succinate adjunct (0.084M), and in hydrolysed molasses medium without adjuncts (Δ).

the yields were low; the maximum was 73 mg. at 2% (w/v) glucose concentration. In the succinate cultures the maximum yield was 553 mg. at 5% (w/v) glucose concentration; yields decreased sharply at lower and higher glucose concentrations. Cellulose yields in the succinate cultures with 1–5% glucose closely paralleled those obtained when the organism was grown in hydrolysed molasses medium at equal sugar concentrations.

The influence of glucose concentration on growth (Fig. 3g) was also affected by succinate. In the medium without succinate, cell-N reached its maximal value (10.4 mg. N) at 2% glucose. In the succinate medium, growth at the 1 and 2% glucose concentrations was approximately the same as in the cultures without succinate, but increased to its maximal value (19.6 mg. N) at 5% glucose. Although the other results for the succinate cultures were similar to those obtained with cultures grown in hydrolysed molasses medium, there was a marked difference in the growth obtained in these two media; growth in the succinate cultures was much less than that obtained in hydrolysed molasses medium except at the lowest and highest sugar concentrations.

The values found for the cellulose:cell-N ratio in the succinate-free cultures ranged from 4.1 to 9.8, equivalent to 38–60% cellulose in the crude pellicles. In the cultures with succinate however the values found for the ratio were very much higher, with a maximum (36.5) at 2% glucose; the cellulose content of the crude pellicles grown in succinate medium ranged from 58 to 85%. The values of the cellulose:cell-N ratio were very much higher in the succinate cultures than in the cultures grown in hydrolysed molasses medium.

DISCUSSION

The synthesis of cellulose by *Acetobacter acetigenum* strain EA-I unlike the synthesis of extracellular polysaccharides by *Aerobacter* and *Escherichia* strains (Duguid & Wilkinson, 1953), was not stimulated when the organism was grown in media containing limited amounts of nitrogen source in the presence of excess glucose. Growth, cellulose yields and glucose utilization were all decreased when *A. acetigenum* was grown under these conditions.

Ammonium sulphate and asparagine + glutamic acid were equivalent on a nitrogen weight basis as nitrogen sources for the growth of *Acetobacter acetigenum*. Similar results were obtained with cultures grown on either nitrogen source over the range 0.001–0.1% (w/v) N, but a difference was found in the effect of these nitrogen sources at higher concentrations. Ammonium sulphate at concentrations equivalent to 0.2% N caused decreased growth and cellulose yields, while the equivalent concentration of asparagine + glutamic acid gave rise to increased growth and cellulose yield. There is no direct evidence to suggest the mechanism by which the amino acid mixture became stimulatory, but it is possible that it may have been the result of deamination, by the organism, of some of the asparagine and glutamic acid to oxalacetic and α -ketoglutaric acids, respectively, which being intermediates in the tricarboxylic acid cycle, would probably act in the same way as acetic, citric and succinic acids. Indirect evidence in support of this was the decreased

recovery in the amino acid cultures, of utilized nitrogen as cell-N, as the nitrogen concentration of the medium increased.

Acetate, citrate and succinate were highly effective in stimulating cellulose synthesis by *Acetobacter acetigenum* in defined medium. The equal yields obtained from the three adjuncts, when used at concentrations equivalent to 0.1–0.2 % (w/v) adjunct carbon, suggests that they act in stimulating cellulose synthesis by the same mechanism, presumably a preferential oxidation of the adjunct to spare the glucose substrate for cellulose synthesis. This is supported by the recent observation of Schramm, Gromet & Hestrin (1957) that acetate, citrate, succinate and other intermediates of the tricarboxylic acid cycle are all readily oxidized to CO₂ by washed suspensions of *A. xylinum*. The buffering action of these adjuncts must also be an important additional factor in producing high cellulose yields by maintaining the cultural pH value within the optimum range for cellulose synthesis. The failure to obtain increased cellulose yields with ethanol added to defined medium may be explained by the low cultural pH values. The values found for the cellulose: cell-N ratio in the cultures with the acid adjuncts showed that the cellulose yield/unit weight of growth varied with the nature of the adjunct and its concentration.

The influence of glucose concentration on cellulose yields in defined medium was relatively slight in the absence of succinate. The addition of 0.4 % (w/v) succinate-carbon to the medium caused the cellulose yields to show a marked response to glucose concentration which paralleled that in hydrolysed molasses medium at sugar concentrations up to 5 %. The increased cellulose yields were associated with much smaller increases in growth, showing that under these conditions the succinate adjunct increased cellulose yields by specifically stimulating cellulose synthesis rather than by increasing growth generally. It had been hoped that cellulose yields from hydrolysed molasses medium, which were already large in the absence of adjuncts, would be increased further by the addition of succinate, but this was found instead to lead to diminished yields. No explanation can be given for the reversal in this medium of the stimulatory action of succinate.

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REFERENCES

- BARCLAY, K. S. (1951). *Studies on bacterial cellulose*. Ph.D. thesis, University of Birmingham.
- DUDMAN, W. F. (1959). Cellulose production by *Acetobacter acetigenum* and other *Acetobacter* spp. *J. gen. Microbiol.* **21**, 812.
- DUGUID, J. P. & WILKINSON, J. F. (1958). The influence of cultural conditions on polysaccharide production by *Aerobacter aerogenes*. *J. gen. Microbiol.* **9**, 174.
- EMERY, W. B., McLEOD, N. & ROBINSON, F. A. (1946). Comparative microbiological assays of members of the vitamin B complex in yeast and liver extracts. *Biochem. J.* **40**, 426.

- GREATHOUSE, G. A., SHIRK, H. G. & MINOR, F. W. (1954). Cellulose production by *Acetobacter xylinum* from unlabelled glucose and C¹⁴-acetate and C¹⁴-ethanol. *J. Amer. chem. Soc.* **76**, 5157.
- MINOR, F. W., GREATHOUSE, G. A., SHIRK, H. G., SCHWARTZ, A. M. & HARRIS, M. (1954*a*). Biosynthesis of C¹⁴-specifically labelled cellulose by *Acetobacter xylinum*. I. From D-glucose-1-C¹⁴ with and without ethanol. *J. Amer. chem. Soc.* **76**, 1658.
- MINOR, F. W., GREATHOUSE, G. A., SHIRK, H. G., SCHWARTZ, A. M. & HARRIS, M. (1954*b*). Biosynthesis of C¹⁴-specifically labelled cellulose from *Acetobacter xylinum*. II. From D-mannitol-1-C¹⁴ with and without ethanol. *J. Amer. chem. Soc.* **76**, 5052.
- SCHRAMM, M., GROMET, Z. & HESTRIN, S. (1957). Synthesis of cellulose by *Acetobacter xylinum*. 3. Substrates and inhibitors. *Biochem. J.* **67**, 669.
- TARR, H. L. A. & HIBBERT, H. (1931). Polysaccharide synthesis by the action of *Acetobacter xylinus* on carbohydrates and related compounds. *Canad. J. Res.* **4**, 372.
- WOEBER, H. H. (1954). *A biochemical study of cellulose synthesis by Acetobacter xylinum*. Ph.D. thesis, University of Florida. Doctoral dissertation series, Publication No. 9722 Ann Arbor, Mich.: University Microfilms.

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