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Acetone and Butanol Production by *Clostridium acetobutylicum* in a Synthetic Medium

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The effect of the component concentrations of a synthetic medium on acetone and butanol fermentation by *Clostridium acetobutylicum* ATCC 824 was investigated. Cell growth was dependent on the presence of Mg, Fe, and K in the medium. Mg and Mn had deleterious effects when in excess. Ammonium acetate in excess caused acid fermentation. The metabolism was composed of two phases: an acid phase and a solvent one. Low concentrations of glucose allowed the first phase only. The theoretical ratio of the conversion of glucose to solvents, which was 28 to 33%, was obtained with the following medium: $MgSO_4$, 50 to 200 mg/liter; $MnSO_4$, 0 to 20 mg/liter; KCl, 0.015 to 8 g/liter (an equivalent concentration of K^+ was supplied in the form of KH_2PO_4 and K_2HPO_4); $FeSO_4$, 1 to 50 mg/liter; ammonium acetate, 1.1 to 2.2 g/liter; *para*-aminobenzoic acid, 1 mg/liter; biotin, 0.01 mg/liter; glucose, 20 to 60 g/liter.

Due to higher oil prices, alcohol production by anaerobic bacteria is being examined by a great number of laboratories (20, 24, 25); likewise, acetone-butanol-ethanol fermentation is arousing renewed interest (5, 10, 13). *Clostridium acetobutylicum* is able to metabolize a great variety of carbon sources (12, 18, 22). Depending on the nature of the carbohydrate and the culture conditions, the ratio of conversion to solvents can vary (1, 6, 23). Studies on the production of solvents have always been done on natural media (2, 4, 8, 14); yet, it is well known that *C. acetobutylicum* can grow on a sugar-salt-vitamin medium (9, 11, 19). To determine the nutrients required for maximum solvent production, we investigated the role played by each of the synthetic medium compounds in acetone-butanol fermentation.

MATERIALS AND METHODS

Bacteria and culture maintenance. The organism used was *C. acetobutylicum* ATCC 824. The culture was maintained in a medium containing 5% maize meal in tap water. Before use, sterilized media were regenerated in boiling water and then quickly cooled in an ice batch to the desired temperature. This process permitted us to eliminate dissolved oxygen from the medium and to obtain good anaerobiosis without adding any reducing agent.

Media and test conditions. The reference synthetic medium used for the tests had the following composition: glucose, 20 g/liter; KH_2PO_4, 0.5 g/liter; $K_2HPO_4 \cdot 3H_2O$, 0.5 g/liter; $MgSO_4 \cdot 7H_2O$, 0.2 g/liter; $MnSO_4 \cdot 1H_2O$, 0.01 g/liter; $FeSO_4 \cdot 7H_2O$, 0.01 g/liter; NaCl, 0.01 g/liter; ammonium acetate, 2.2 g/liter; *p*-aminobenzoic acid, 0.001 g/liter; biotin, 0.00001 g/liter. The glucose concentration in the reference medium was 2% (wt/vol), since this concentration was sufficient to obtain the theoretical ratio of conversion of sugars to solvents (28 to 32%).

The effects of the concentration of each nutrient were investigated by studying cell growth, the evolution of the pH, the degradation of the sugar, and the formation of acetate, butyrate, acetone, butanol, and ethanol. A solution of the element, the requirements of which were studied, was initially prepared. Different amounts of this solution were added to tubes containing the other ingredients (at the concentration of the reference medium) to obtain the desired concentrations. At least three transfers were made at each concentration before inoculating the studied series. This operation minimized the errors coming from the first inoculum. The concentrations of every nutritional factor are indicated in Table 1.

Experiments were carried out in tubes containing 50 ml of medium. The volume of the inoculum formed 10% of the total volume.

Preliminary transfers were made after 24 h of culture. The tubes were incubated at 32°C.

Methods of analysis. Cell growth was estimated by the optical density at 600 nm.

The concentration of residual sugars was determined by the method of Miller et al. (15).

The concentrations of solvents (ethanol, acetone, butanol) and acids (acetic and butyric) were determined by injecting acidified and centrifuged samples into a CARLO-ERBA model GB chromatograph equipped with a flame ionization detector. The glass column was 2.10 m in length, with an internal diameter of 3 mm, and was packed with PORAPAK Q (100/120 mesh). The analysis of products was carried out under the following conditions: column temperature, 185°C; injector temperature, 220°C; detector temperature, 220°C; N_2 (carrier gas) flow rate, 13 ml/min; H_2 flow

TABLE 1. Influence of the concentration of compounds on the growth, the production of solvents and acids, the degradation of glucose, and the conversion of glucose to solvents

Ingredient	Concn added	Growth[a]	Final concn of butanol (g/liter)	Final concn of acetone (g/liter)	Final concn of ethanol (g/liter)	Total solvents (g/liter)	Final concn of acetate (g/liter)	Final concn of butyrate (g/liter)	Total acids (g/liter)	Glucose degraded (g/liter)	% Conversion of sugars to solvents
$MgSO_4$[b]	0 (mg/liter)	0.65	2.22	0.87	0.14	3.23	3.18	0.90	4.08	12.3	26
	50–200	2.99	5.28	1.48	0.74	7.50	1.98	Tr	1.98	20.8	36
	350–500	2.64	4.48	1.28	0.61	6.37	2.01	Tr	2.01	20.8	31
$MnSO_4$[c]	0–20	3.44	4.89	1.39	0.41	6.69	2.01	Tr	2.01	20.9	32
	50	3.53	4.00	1.10	0.46	5.56	1.86	Tr	1.86	20.6	27
$FeSO_4$[d]	0	0.86	1.85	0.23	Tr	2.08	0.90	1.76	2.66	8.6	24
	1–50	1.92	4.50	1.04	0.47	6.01	1.44	0.85	2.29	21.1	28
KC1[e]	0	0.18	0	0	0	0	3.48	2.29	5.77	2.2	0
	3	0.59	0.48	Tr	Tr	0.48	3.54	3.35	6.89	8.2	6
	6	0.72	0.78	Tr	Tr	0.78	4.32	4.14	8.46	11.2	7
	9	0.88	4.22	1.39	0.23	5.84	4.14	2.30	6.44	19.7	30
	15	1.28	4.26	1.28	0.25	5.79	2.88	2.99	5.87	21.3	27
	30	1.88	4.26	1.05	0.28	5.59	2.76	0.88	3.64	21.3	26
	60–600	2.60	4.32	1.03	0.50	5.85	1.86	Tr	1.86	20.9	28
	0.6–8 (g/liter)	2.94	4.72	0.81	0.48	6.01	1.69	0.66	2.35	20.7	29
	12	3.10	3.89	0.90	0.37	5.16	1.23	0.35	1.58	20.5	25
Ammonium acetate[f]	1.1	2.14	3.11	0.99	0.12	4.22	1.68	1.67	3.35	15.8	27
	2.2	1.83	2.78	1.10	0.12	4.00	1.83	1.72	3.55	17.7	23
	3.3	1.84	2.04	0.65	0.09	2.78	3.42	2.86	6.28	17.2	16
	4.4	1.80	1.48	0.47	0.09	2.04	4.32	3.44	7.76	16.2	13
	5.5	1.86	1.04	0.35	Tr	1.39	5.52	3.96	9.48	17.5	8
	6.6	1.80	1.04	0.35	Tr	1.39	6.00	4.58	10.58	17.1	8
Glucose[g]	5	1.66	0.45	0.14	0.16	0.75	3.00	1.63	4.63	5.28	14
	10	2.06	1.18	0.41	0.17	1.76	3.18	1.80	4.98	10.65	17
	20	3.12	4.08	1.36	0.32	5.76	2.16	0.44	2.60	21.2	27
	40	5.53	9.19	2.50	1.80	13.49	0.96	Tr	0.96	42.0	32
	80	5.17	13.86	3.31	3.27	20.44	0.60	Tr	0.60	67.0	31

[a] Growth was estimated by the difference between the maximum optical density and the optical density of the inoculum.

[b] The concentrations used were 0, 50, 100, 200, 350, and 500 mg/liter.

[c] The concentrations used were 0, 1, 2, 3, 4, 5, 10, 20, and 50 mg/liter.

[d] The concentrations used were 0, 1, 2, 3, 4, 5, 10, 25, and 50 mg/liter.

[e] The concentrations used were 0, 3, 6, 9, 15, 30, 60, 120, 360, and 600 mg/liter and 0.6, 1.2, 2, 4, 8, and 12 g/liter.

[f] The concentrations used were 1.1, 2.2, 3.3, 4.4, 5.5, and 6.6 g/liter.

[g] The concentrations used were 5, 10, 20, 40, and 80 g/liter.

rate, 20 ml/min; air flow rate, 140 ml/min. Concentrations were measured after 72 h of culture.

RESULTS

The results (Table 1) show that some mineral salts, ammonium acetate, and glucose affected acetone and butanol fermentation by *C. acetobutylicum*.

In Table 1 are indicated the results obtained with factors having a significant influence on fermentation.

$MgSO_4$. It can be seen from Table 1 that cell growth was dependent on the presence of Mg^{2+} in the medium. When no $MgSO_4$ was added, growth was limited, but solvents were released in the medium (3.23 g/liter). When $MgSO_4$ was in excess (350 to 500 mg/liter), growth was better but was accompanied by a slightly decreased production of solvents (6.4 g/liter). A suitable concentration for $MgSO_4$ was between 50 and 200 mg/liter, at which cell growth was best and more solvents were produced (7.5 g/liter) for an equivalent utilization of glucose (36% converted to solvents).

$MnSO_4$. The results show that the presence of $MnSO_4$ in the medium was neither beneficial nor detrimental if not in excess. In the latter case (50 mg/liter), the ratio of conversion of glucose to solvents decreased (27 instead of 32%), although the growth and the degradation of sugars were not affected. With concentrations between 0 and 20 mg/liter, no difference was observed, and the production was optimal (6.7 g/liter) with a complete utilization of glucose. There were only traces of butyrate left in the medium.

$FeSO_4$. Cell growth was dependent on the

presence of Fe^{2+} in the medium. When only 1 mg of $FeSO_4$ per liter was supplied to the bacteria, growth was good, the conversion of glucose was complete, and the production of solvents was normal (5.5 g/liter). No differences were observed with concentrations of $FeSO_4$ between 1 and 50 mg/liter. When no $FeSO_4$ was added to the medium, growth was weak; only 8 g of sugar per liter was degraded, yet the ratio of conversion of glucose to solvents was relatively high (25%). The tubes containing 0, 1, and 2 mg/liter had a greenish coloration, probably due to the synthesis of flavins.

KCl. To investigate the influence of the K^+ concentration on fermentation without affecting the buffering actions of KH_2PO_4 and $K_2HPO_4 \cdot 3H_2O$, these phosphate salts were substituted for by $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_3 \cdot 12H_2O$ to obtain an equivalent buffering effect. K^+ was supplied as KCl. The concentration of 600 mg of KCl per liter was equivalent (for K^+ requirements) to 1 g of potassium phosphates per liter.

The results (Table 1, Fig. 1) show that acetone and butanol fermentation was K^+ dependent. Cell growth increased with concentrations from 0 to 60 mg/liter and then stayed at about the same level. The degradation of glucose regularly increased with increasing concentrations of KCl up to 15 mg/liter, where the utilization of sugar was complete. At higher concentrations, no variations of the residual sugar concentrations were found (about 20 g/liter utilized). Solvent yields became higher and higher with concentrations from 0 to 9 mg/liter, reaching a maximum and then becoming constant. The curve of the production of acids versus the KCl concentration first climbed with concentrations ranging from 0 to 6 mg/liter, then dropped to 60 mg/liter and flattened.

We also studied the influence of high concentrations of KCl. The degradation of glucose became slower upon increasing the concentration of KCl but, nevertheless, was complete. The production of solvents was not changed with concentrations from 0.6 to 8 g/liter (6 g of solvents per liter), but was slightly inhibited at 12 g of KCl per liter (5.16 g of solvents per liter).

Ammonium acetate. The results (Table 1, Fig. 2) show that ammonium acetate greatly affected fermentation. The growth curves (Fig. 2A) were approximately identical at all ammonium acetate concentrations, except at 1.1 g/liter, where growth was a little better. The pH curves (Fig. 2B) show that the pH of the medium became higher and higher as the concentration of ammonium acetate increased. The curves of glucose degradation (Fig. 2C) were the same in each case. The addition of ammonium acetate at concentrations of >2.2 g/liter had deleterious effects on the production of solvents (Fig. 2D). Upon increasing the ammonium acetate concentration, we observed an accumulation of acids (butyric). Thus, there was a good parallelism between the decreasing production of solvents and the increasing production of acids. No growth occurred in the absence of ammonium acetate.

Glucose. Table 1 shows the results obtained with different initial concentrations of glucose (the differences of values between the initial quantities of glucose and the glucose degraded are due to sugars brought by the inoculum). The growth (Fig. 3A) was optimal at 40 g/liter and was a little slower at 80 g/liter. At 5, 10, 20, and 40 g/liter, the generation time was about 4 h.

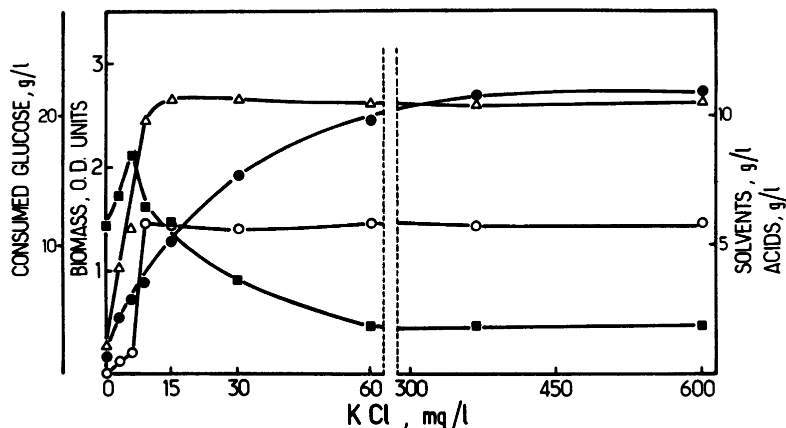

FIG. 1. Influence of the KCl concentration on cell growth (●), the degradation of glucose (△), the production of acids (■), and the production of solvents (○). O.D., Optical density.

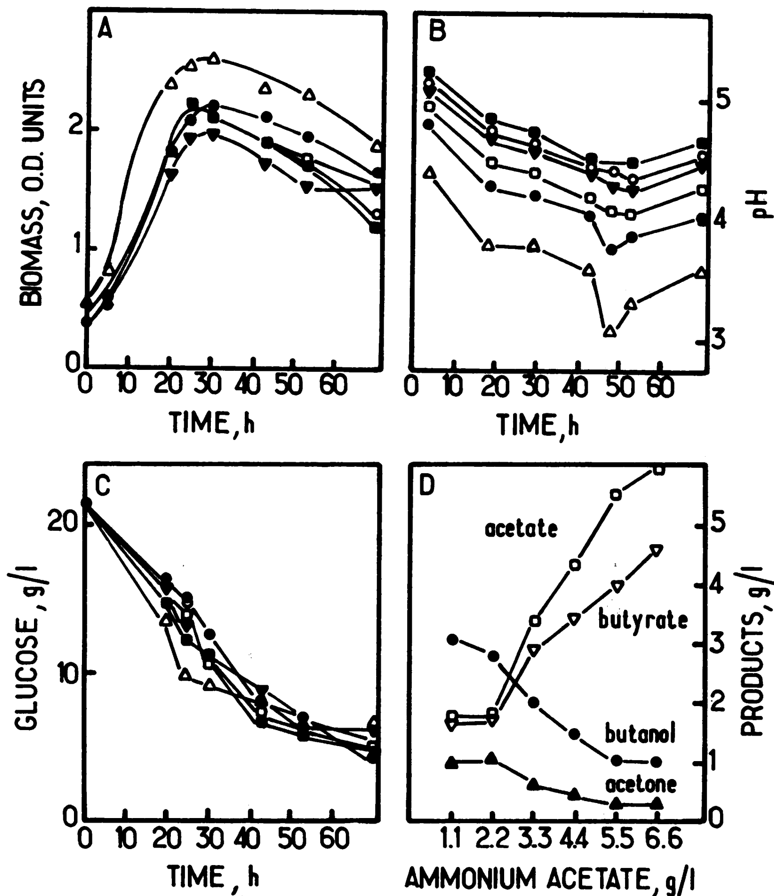

FIG. 2. Influence of the ammonium acetate concentration on the growth (A), the evolution of pH (B), the degradation of glucose (C), and the productions of acetone (▲), butanol (●), acetate (□), and butyrate (▽) (D). The ammonium acetate concentrations (A, B, C) studied were 1.1 g/liter (△); 2.2 g/liter (●); 3.3 g/liter (□); 4.4 g/liter (▼); 5.5 g/liter (○); and 6.6 g/liter (■).

After having reached a maximum, the optical density quickly dropped. This is probably due to a synthesis of autolysins (Fig. 2A). There were no residual sugars except at 80 g/liter, where there remained 20 g/liter (Fig. 3B). The products that accumulated in the medium differed depending on the initial glucose concentrations (Fig. 4). At concentrations of 5 and 10 g/liter, only acetic and butyric acids were produced. At 20 g/liter, two phases could be distinguished: (i) the formation of acids, and (ii) the disappearance of these acids concurrent with the formation of solvents. At 40 and 80 g/liter, the two stages were more pronounced since the conversion of acids to solvents was almost complete and final levels of acetic and butyric acids were very low (traces of butyrate). A total of 2% of solvents were present in the medium at the glucose concentration of 80 g/liter (the conversion of glucose to solvents was 31%).

Other factors. *p*-Aminobenzoic acid and biotin were required for normal cell growth and for a good production of solvents at the respective concentrations of 1 and 0.01 mg/liter. Intensive studies have already been made on the vitamin requirements of *C. acetobutylicum* (11, 19). Likewise, NaCl (from 0 to 25 mg/liter and from 0.5 to 5 g/liter) did not modify fermentation. Moreover, no differences were observed in media containing 0, 10, 20, and 40 mg of ammonium molybdate per liter.

DISCUSSION

First, it should be mentioned that the solvents produced in fermentation are toxic over the 2% level, so that since a 28 to 33% yield on sugars metabolized is normal, only a 6 to 6.5% sugar concentration can be used.

A glucose-salt-vitamin medium allows a good

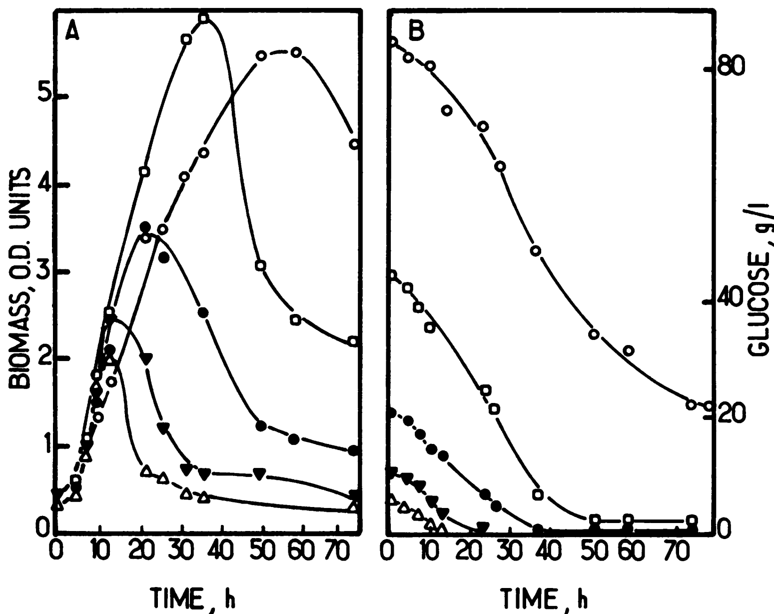

FIG. 3. Influence of the glucose concentration on cell growth (A) and on the degradation of glucose (B). The initial glucose concentrations used were 5 g/liter (△); 10 g/liter (▼); 20 g/liter (●); 40 g/liter (□); and 80 g/liter (○).

growth of *C. acetobutylicum*. On complex media as defined by Davies (7), the average generation time is about 2.5 h; on the synthetic medium, we found that it was 4 h.

As can be observed in Table 1, four cases of fermentation were obtained: (i) media where growth was low and where acetic and butyric acids were accumulated (KCl, 0 to 9 mg/liter; glucose, 5 to 10 g/liter); (ii) media where growth was low and where solvents were accumulated (Fe^{2+}, 0 mg/liter; Mg^{2+}, 0 mg/liter); (iii) media where growth was good and where acids were accumulated (ammonium acetate $\geq$3.3 g/liter); (iv) media where growth was good and where solvents were accumulated—this is the most favorable case.

Moreover, these experiments allowed the determination of the suitable concentration of each nutrient to obtain a maximum acetone and butanol production. The results obtained suggest a medium of the following composition: $MgSO_4$, 50 to 200 mg/liter; $MnSO_4$, 0 to 20 mg/liter; KCl (K is to be supplied as KH_2PO_4 and K_2HPO_4), 0.015 to 8 g/liter; $FeSO_4$, 1 to 50 mg/liter; ammonium acetate, 1.1 to 2.2 g/liter; *p*-aminobenzoic acid, 1 mg/liter; biotin, 0.01 mg/liter; glucose, 20 to 60 g/liter.

It is difficult to compare our results with the literature data where complex media were used. Mahmoud et al. (14) have found that on millet mash, optimum yields of acetone and butanol were obtained on a medium containing 0.2% $MgSO_4$ and 0.05% $FeSO_4$. Our results are in accordance with those of Rosenfeld and Simon (21), who found that Mg and K play an important role in acetone formation by bacterial enzymes. Baghlaf et al. (2) have reported that on a complex medium, K_2HPO_4 is essential, and the optimum concentration is 1 to 2 g/liter. Davies (7) also found that the growth of *C. acetobutylicum* on maize meal is K dependent.

In the case of ammonium acetate, we found that although there was a good utilization of glucose and although growths were approximately equivalent, we had either a butyric fermentation or a butylic one. Thus, an excess of ammonium acetate repressed the reduction of the acids.

The results obtained when no Fe or Mg was added to the medium (bad growth and good conversion of glucose to solvents) can be explained by the fact that the inoculum contributed "ripe cells" which contained an enzymatic system allowing the solvent phase.

Thus, this medium allows a good conversion of sugars to solvents. It must be mentioned that a 6% glucose solution can be converted to solvents on a complex medium, such as that of Davies (7), only if the pH is maintained between 5 and 6 by neutralization with ammonia or NaOH. With the synthetic culture medium used, growth is slow, and the conversion of acetic and butyric acids is good, which avoids a pH breakdown and allows a good production of solvents without neutralization.

At low glucose concentrations (0.5 and 1%),

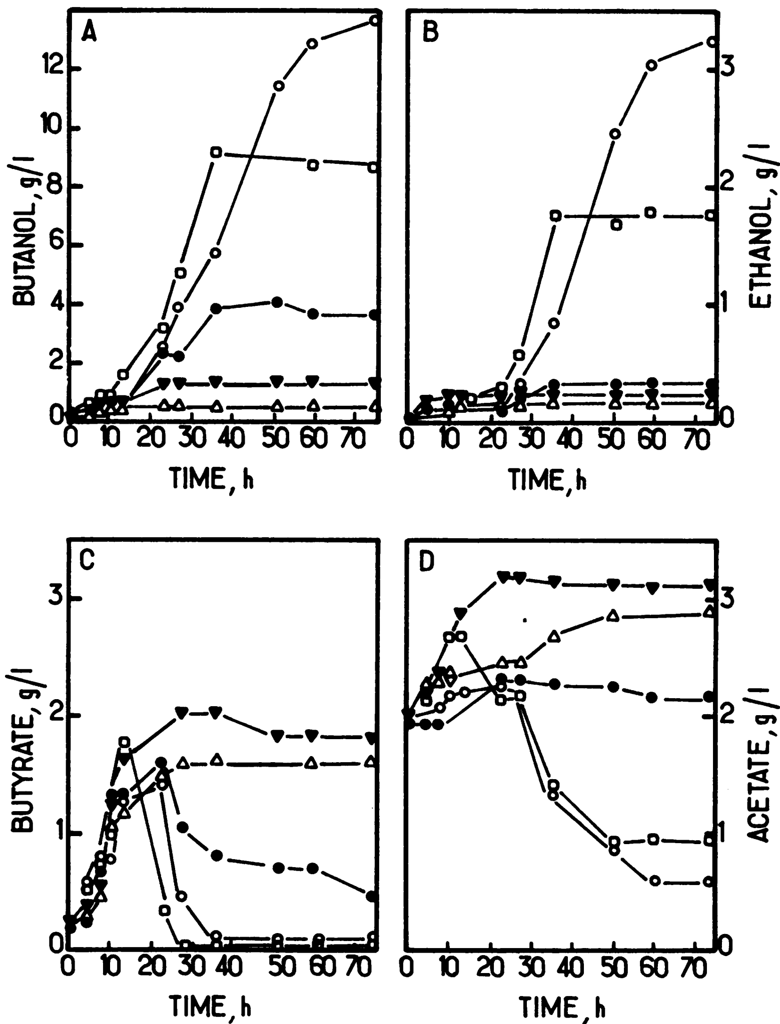

FIG. 4. Influence of the glucose concentration on the kinetics of appearance of butanol (A), ethanol (B), butyrate (C), and acetate (D). The initial glucose concentrations used were 5 g/liter (△); 10 g/liter (▼); 20 g/liter (●); 40 g/liter (□); and 80 g/liter (○).

we observed a butyric fermentation, which is normal since this weak sugar concentration permits only the first phase of the fermentation corresponding to the exponential bacterial growth. This phenomenon is also observed when growth is limited by the absence of a nutritional factor. An acid fermentation allows cell development since the conversion of one molecule of glucose to acetate supplies four ATP to the cell and three ATP to butyrate and acetone. The production of ethanol and butanol supplies only two ATP. It is obvious that the regulations of the enzyme activities responsible for the butyrate and acetate formation were quite different from those implicated in the solvent production. The reducer elements NAD(P)H produced during the catabolism of the carbon source are used for the reduction of acids to solvents correlatively with NAD(P)H-ferredoxin oxidoreductases (16). The biological function of the NADH-rubredoxin oxidoreductase in metabolism remains to be elucidated (17). Nevertheless, the passage from the acid phase to the solvent one is possible only if conditions are favorable since butylic-type metabolism needs energy (ATP) and reducers [NAD(P)H]. The biochemical pathway of glucose utilization by *C. acetobutylicum* is complex, and the mechanisms of fermentation are not well understood. The sugar-salt-vitamin medium used in this paper is a tool to determine the nutrients required for maximum solvent production and to research the biochemical mechanism of fermentation since the culture conditions are well defined.

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