UTILIZATION OF ETHANOL BY ACETIC ACID BACTERIA

M. R. RAGHAVENDRA RAO[1] AND J. L. STOKES[2]

Department of Bacteriology, Indiana University, Bloomington, Indiana

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It is well known that the acetic acid bacteria can rapidly oxidize ethanol to acetic acid. But whether ethanol can serve as a source of carbon and energy for these bacteria has not been clearly established. Pasteur (1868) noted that *Mycoderma aceti* which is undoubtedly *Acetobacter aceti* (Frateur, 1950) can grow well in ethanol media only if acetic acid is also present. Addition of one or two per cent acetic acid was required for growth in an ethanol yeast water medium and also in a chemically defined medium consisting of ethanol, mineral salts, and ammonium nitrogen. Both Hoyer (1898) and Beijerinck (1898) confirmed Pasteur's results and showed that *A. aceti* cannot grow in an ethanol-mineral salts-ammonium nitrogen medium unless acetic acid, acetate, or glucose is added. Growth with ethanol alone occurred when tap water was used in place of distilled water, and this was explained by Beijerinck as due to the organic impurities in tap water which stimulated growth.

These results can be interpreted in several ways. It may be that the ethanol is not used for carbon and energy and that these functions are served by the acetic acid or other organic compounds that must be added to ethanol containing media to permit growth. Or the acetic acid or other compounds may stimulate the utilization of ethanol for carbon and energy but are not themselves so used. This seems quite unlikely. Or both ethanol and acetic acid may be the sources of carbon and energy. The fact that tap water may stimulate growth of *A. aceti* with ethanol seems to indicate that under such conditions the ethanol functions as the primary source of energy for growth since it seems unlikely that the organic impurities in tap water would be sufficient for that purpose.

The situation with respect to ethanol utilization is complicated further by the results of Frateur (1950) which show that *A. aceti* can grow with ethanol alone even when the medium is prepared with distilled water if the medium is buffered adequately with phosphates. Moreover, both Hoyer and Beijerinck found that *Acetobacter xylinum* and *Acetobacter rancens* could not grow in an ethanol-acetic acid medium suitable for *A. aceti* but could develop luxuriantly when glucose, sucrose, mannitol, or glycerol was added. Beijerinck interpreted these results as indicating that the additional organic compounds were necessary for the utilization of the ammonium nitrogen in the medium by *A. xylinum* and *A. rancens* but not by *A. aceti*. It will be shown, however, in the present paper that the effect of glucose, sucrose, etc., is not related necessarily to the utilization of inorganic nitrogen since for *Acetobacter suboxydans* and *Acetobacter melanogenum* glucose is necessary for growth and oxidation of ethanol even when amino acids are substituted for inorganic nitrogen.

Our interest in the problem of ethanol utilization stems from our previously reported observation (Rao and Stokes, 1953) that for growth and oxidation of ethanol by many strains of *A. suboxydans* and *A. melanogenum* an unidentified factor in yeast autolysate is essential. The present investigation supplies additional information on the problem of ethanol utilization by acetic acid bacteria.

MATERIALS AND METHODS

A. suboxydans, strain M.A. 8.3, was used in most of the experiments. In many instances, however, other strains of this organism and also *A. melanogenum* were employed and similar results were obtained. The basal medium consisted of ethanol, casein hydrolyzate, minerals, and B vitamins. The exact composition of this medium, the general conditions for growth, and the method for measurement of growth are those described in our previous paper (Rao and Stokes, 1953).

[1] Present address: Department of Bacteriology, University of Illinois, Urbana, Ill.

[2] Present address: Western Regional Research Laboratory, Albany, Calif.

Reducing sugars were determined by essentially the method of Shaffer and Somogyi (1933) after clarification of the solutions with $ZnSO_4$ and $Ba(OH)_2$ (Somogyi, 1945). Ethanol-1-C^{14}, $CH_3C^{14}H_2OH$, was used in the tracer experiments.

EXPERIMENTAL RESULTS

As previously indicated (Rao and Stokes, 1953) many strains of *A. suboxydans* and *A. melanogenum* cannot grow with ethanol as carbon source in media containing $(NH_4)_2SO_4$ or amino acids as the source of nitrogen, but abundant growth occurs, however, if such media are supplemented with small amounts of yeast autolysate, peptone, or other complex materials of biological origin. There is considerable variation in the activity of the different biological materials as shown in table 1. On a dry weight basis, aqueous liver extracts that we prepared were 6 to 8 times as potent as yeast autolysate in stimulating growth with ethanol. Commercial beef liver extracts were poor sources of the stimulating factor. Difco yeast extract, peptone, tryptone, and beef extract also are stimulatory but less so than yeast autolysate.

Yeast autolysate was used in attempts to characterize the growth stimulating factor. Its activity remained unchanged on autoclaving in 0.1 N H_2SO_4 at 15 lb pressure for 15 minutes. Similar treatment of the autolysate in 0.1 N NaOH resulted in the destruction of most of the activity for *A. suboxydans*, strain M.A. 8.3, but the activity for another strain, *A. suboxydans* 3, was unchanged. Most of the activity was removed by dialysis with water and was found in the dialysate. Ether extraction at pH 2 for 48 hr failed to remove any active material, and the residue was fully active. The active fraction appears to be organic in nature since the ash of the yeast is inactive. The stimulatory material was not adsorbed during passage through anion and cation exchange columns which indicates that the activity is associated with nonpolar compounds.

TABLE 1

Stimulatory effect of biological materials on the utilization of ethanol by Acetobacter suboxydans, strain M.A. 8.3

ADDENDUM	DETERMINATION	MG DRY WT PER 10 ML MEDIUM					
		10		100		300	
		−E	+E*	−E	+E	−E	+E
Yeast autolysate	Turbidity†	0	5	30	115	55	167
	Acidity‡	0.1	0.4	0.2	3.1	0.2	4.3
Liver extract	Turbidity	50	120	124	206		
	Acidity	0.1	5.2	0.1	4.5		
Peptone	Turbidity			9	24	16	78
	Acidity			0.2	1.5	0.2	4.0

* Two per cent ethanol.

† Klett values.

‡ Ml of 0.05 N acid per ml of culture: the theoretical value for the oxidation of all of the ethanol to acetic acid is 8.7 ml.

These results suggested that the active material might be a carbohydrate or a related substance. Analyses for reducing materials of a number of biological substances having different activities showed a close relationship between activity and content of reducing sugars (table 2). Furthermore, when the yeast autolysate was incubated with a thick suspension of *Saccharomyces cerevisiae*—a process which may be expected to destroy sugars—it was no longer able to stimulate growth of *A. suboxydans* in ethanol medium.

Although these results do not prove that the stimulatory activity of the biological materials for ethanol utilization is due to the sugars and related substances that they contain, yet the results suggest that this is the case. Moreover, our data with *A. suboxydans* and *A. melanogenum*

parallel those of Hoyer and Beijerinck with *A. rancens* and *A. xylinum* since for these latter organisms sugars also are necessary for growth in ethanol media. And this parallelism, in turn, suggested that glucose and other sugars might replace yeast autolysate in the growth of *A. suboxydans* and *A. melanogenum*. This indeed was found to be the case. Typical data are given in table 3. A tenth of a per cent of glucose, fructose, mannitol, or glycerol is sufficient to enable *A. suboxydans* to grow and to oxidize ethanol to acetic acid. All of these compounds have about the same order of activity. Since none of them gives rise to any appreciable amount of acid in the absence of ethanol, it is clear that when they are used in combination with ethanol the acid formed arises almost entirely from the oxidation of the ethanol. The principal action of the sugars and related compounds, therefore, appears to be to initiate growth of the acetic acid bacteria, and when this has occurred the cells then can proceed to oxidize the ethanol.

TABLE 2

Relation of activity of biological materials to their content of reducing sugars

MATERIAL	REDUCING SUGARS	RELATIVE ACTIVITY*
	mg per g dry wt	
Liver extract	360	600
Yeast autolysate	34	100
Yeast extract	13	25
Tryptone	11	10
Beef extract	8	5
Peptone	5	5

* Compared to yeast autolysate which has been given the value of 100.

TABLE 3

Stimulation of ethanol oxidation by Acetobacter suboxydans with sugars and related substances

DETERMINATION	SUBSTRATES—0.1 PER CENT PRESENT				
	None	Glucose	Fructose	Mannitol	Glycerol
Turbidity*					
No ethanol	3	63	19	83	36
Ethanol†	2	114	73	120	120
Acidity‡					
No ethanol	0.1	0.1	0.1	0.3	0.2
Ethanol	0.1	4.2	4.6	4.3	4.6

* Klett values.
† Two per cent.
‡ Ml of 0.05 N acid per ml of culture.

A marked effect on growth and ethanol oxidation can be obtained with as little as 1 mg of glucose per 10 ml of medium. In addition to the compounds already mentioned, gluconic acid, glyceric acid, and dihydroxyacetone also are active in stimulating growth with ethanol. The following phosphorylated compounds which were tested with the hope of obtaining some insight into the mechanism of the stimulation were completely inactive: Glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate, fructose-1,6-diphosphate, glyceric acid phosphate, and acetyl phosphate. All of these compounds were converted into their sodium salts except glucose-1-phosphate which was a potassium salt and was used as such. They were sterilized by filtration. Sodium acetate also was inactive. The medium of Frateur (1950) in which phosphate buffer is substituted for acetate failed to support growth. In these respects *A. suboxydans* and *A. melanogenum* resemble *A. xylinum* and *A. rancens* rather than *A. aceti*.

The data in table 3 also indicate that in every case the amount of growth obtained with ethanol plus one of the essential growth stimulating compounds is considerably greater than that obtained with the active compound alone. This suggests that the sugar and higher alcohols not only make it possible for the acetic acid bacteria to oxidize ethanol but also to utilize some of the ethanol for growth. Under these conditions, therefore, both organic compounds appear to serve as sources of carbon. It is possible also that the oxidation of the ethanol may give rise to intermediates or available energy which permits greater utilization of the stimulatory compound than is possible in the absence of ethanol oxidation, and, therefore, none of the carbon for cell synthesis comes from the ethanol. To decide between these two possibilities, experiments were made with media containing both radioactive ethanol, $CH_3C^{14}H_2OH$, and unlabeled glucose. If the carbon of ethanol is used for synthesis of cellular substance, then the cells grown with labeled ethanol should be radioactive.

The standard basal medium which contains hydrolyzed casein, salts, and B vitamins and

has a pH of 6.4 was prepared and distributed in 10 ml amounts into 50 ml Erlenmeyer flasks. Four sets of basal medium were prepared, and to these were added, respectively, (a) nothing, (b) 0.25 ml of labeled ethanol containing a total of 50,000 counts per minute, (c) 10 mg of glucose, and (d) 0.25 ml of labeled ethanol and 10 mg of glucose. The media were inoculated with *A. suboxydans* and incubated at 28 C for 4 days. The turbidities of the cultures were measured then, the cells were collected by centrifugation and thoroughly washed 4 times to remove any adhering labeled ethanol or acetic acid, and finally transferred quantitatively to tared planchets. The radioactivity of the cells was determined after the cells had been dried to constant weight at 100 C. The supernatants of the centrifuged cultures were titrated for acid content. The data obtained are given in table 4.

TABLE 4

Uptake of radioactivity by Acetobacter suboxydans when grown with ethanol-1-C^{14} and glucose

ADDENDUM TO 10 ML OF BASAL MEDIUM	TURBIDITY	ACIDITY	CELL CROP	RADIOACTIVITY OF CELLS
			mg dry wt	*counts per minute†*
Nothing	0	0		
Ethanol-1-C^{14}, 0.25	5	0		
ml*	0	0		
Glucose, 10 mg	43	0.1	1.4	
	45	0.15	1.5	
Ethanol-1-C^{14}, 0.25	95	4.7	3.0	422
ml* + glucose, 10	85	4.6	2.6	360
mg	97	5.7	3.0	369

* Equivalent to 50,000 counts per minute.

† Background counts subtracted.

This experiment is typical in that growth and acid formation occurred in the medium containing ethanol and glucose but not in the medium containing only ethanol. The most important result of the experiment is, of course, the finding that the cells from the labeled ethanol-glucose medium are radioactive. Roughly, one per cent of the initial radioactivity of the ethanol appeared in the cells. This quantity is approximately what one would expect to find in the cells if the ethanol supplies only part of the carbon for cell synthesis, the remainder and perhaps the major portion being supplied by the glucose.

The data obtained with labeled ethanol when considered in conjunction with the other data in tables 3 and 4 which show that invariably more growth occurs when ethanol is present than when carbohydrate alone is used lead to the conclusion that, under these conditions, ethanol is used by the acetic acid bacteria as a source of carbon. It appears therefore that the acetic acid bacteria are dependent upon carbohydrates for the initiation of growth, and once this has taken place, the ethanol can be utilized then as a source of carbon and probably also of energy, and this sequence of events finally leads to the rapid and extensive oxidation of the ethanol to acetic acid.

DISCUSSION

It is surprising that the most characteristic metabolic process of the acetic acid bacteria, namely, the oxidation of ethanol to acetic acid cannot, by itself, support the growth of many members of this group. Growth occurs only if some additional organic substance such as acetate or glucose also is provided in the medium. This phenomenon was noted first with *A. aceti* by Pasteur (1868) in his pioneer investigations on the production of vinegar, and it was confirmed later by Hoyer (1898) and Beijerinck (1898) and extended by them to include other acetic acid bacteria such as *A. rancens* and *A. xylinum*. Our results show that *A. suboxydans* and *A. melanogenum* behave in a similar fashion. The requirement for two substrates, therefore, is widespread among the acetic acid bacteria. In fact, the only exceptions so far uncovered are those of Frateur (1950) who was able to grow *A. aceti*, *A. lovaniense*, and *A. peroxydans* with ethanol alone.

It is somewhat difficult to explain Frateur's results. He believes that growth with ethanol alone is due to the phosphate buffer in his medium. This consists of 0.1 g K_2HPO_4 and 0.9 g KH_2PO_4 per liter of medium. But this amount of buffer is not very large especially in view of the relatively large amounts of acetic acid which are formed by the bacteria. It may be that the initial pH of the medium is important for the utilization of ethanol by *A. aceti* and that this was favorable for growth in Frateur's medium but not in the media used by the earlier

investigators. This aspect is not clear and merits further investigation. But in any event, *A. suboxydans* and *A. melanogenum* will not develop in Frateur's medium. They need, in addition to ethanol, small amounts of glucose or other organic compounds.

The requirement for two substrates by the acetic acid bacteria is similar to that of *Clostridium lacto-acetophilum* (Bhat and Barker, 1947) which cannot grow with lactate unless acetate also is provided and of *Clostridium kluyveri* (Bornstein and Barker, 1948) which cannot develop with ethanol unless acetate is present in the medium. Whether the pertinent biochemical mechanisms for growth are the same in all three cases remains to be determined.

SUMMARY

Acetobacter suboxydans and *Acetobacter melanogenum* cannot grow in chemically defined media with ethanol as the sole source of carbon and energy. They will develop, however, when small amounts of yeast autolysate or other complex substances of biological origin are added to the medium. Evidence is presented which suggests that the growth promoting activity of the biological materials is due to the reducing sugars or related substances which they contain. Small amounts of glucose, fructose, mannitol, and glycerol can be substituted successfully for yeast autolysate.

Data obtained with radioactive ethanol, $CH_3C^{14}H_2OH$, indicate that growth in the two-substrate medium is accompanied by the incorporation of the carbon of ethanol into cell material.

All of the results appear to support the view that the sugars and related substances are required by the acetic acid bacteria to initiate growth, and when this has occurred the ethanol is used then as an additional source of carbon and energy and is oxidized extensively to acetic acid.

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