

Growth of *Acinetobacter calcoaceticus* on Ethanol

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A soil microorganism, identified as *Acinetobacter calcoaceticus*, was cultivated on ethanol as a sole source of carbon. This organism grew with a maximum specific growth rate of 0.7/h. The pH optimum for growth was between 6.5 and 7.5, and the temperature optimum was between 32 and 35 C. Ethanol metabolism by this organism was inducible by ethanol, and the presence of acetate led to the repression of ethanol dehydrogenase. At higher cell densities the cessation of growth on ethanol was accompanied by the accumulation of acetate or acetaldehyde, or both. These accumulations were attributed to a reduction in the magnesium or sulfur content of the medium and a lack of feedback inhibition by acetate of alcohol dehydrogenase.

A wide variety of organic compounds has been evaluated as substrates for the production of microbial cells. The eventual goal of many of these studies is to produce a nutritive supplement for animal feed or human food. Gas oil, *n*-paraffins, methane, methanol, and cellulose are often employed in these studies. Each possesses certain advantages, but no single one is superior in all respects. Relatively few studies have appeared on the utilization of ethanol, although its price, availability, and physical properties make it a possible compromise among the other substrates.

Prior reports of ethanol utilization include the cultivation of ethanol-assimilating yeast in continuous culture (11, 12) and the use of ethanol as substrate in the production of lysine (Kyowa Hakko Kogyo Co., Ltd. French patent 2,003,971, 1969) and glutamic acid (T. Oki et al., British patent 1,171,437, 1969, 13). Akiba et al. (1) isolated a strain of *Bacillus cereus* and an *Arthrobacter* strain that grew on C₁ to C₄ primary alcohols, and Harada and Hirabayashi (4) described an ethanol-grown yeast that oxidized C₂ to C₄ diols. Hernandez and Johnson (7) measured yield coefficients (g cells/g substrate) for *Candida utilis* and *Pseudomonas fluorescens* grown on ethanol, and Harada et al. (5) described the production of *o*-ethylhomoserine by a corynebacterium growing in an ethanol medium.

The present study was initiated to determine the maximum growth rate and highest cell

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densities attained by a bacterium capable of utilizing ethanol as a sole carbon source. Subsequently, several factors were identified that were correlated with a cessation of cell growth at high cell populations.

MATERIALS AND METHODS

Organism. The organism used in these studies was isolated from soil by enrichment culture using ethanol as a sole source of carbon (L. Naslund, unpublished data). A fast-growing isolate, designated as strain 4736, was selected for closer study. Stock cultures were maintained on nutrient agar slants and transferred at 2-week intervals. For preservation, cultures were suspended in 10% glycerol and stored in a liquid nitrogen refrigerator.

Taxonomy. All the tests used in the taxonomic studies were carried out according to standard procedures (6, 14, 15).

Cultivation. The medium used to cultivate strain 4736 in fermentors and shake flasks was designated P-1 and consisted of the following: (NH₄)₂HPO₄, 10 g; K₂HPO₄, 5 g; Na₂SO₄, 0.5 g; MgSO₄·7H₂O, 0.4 g; FeSO₄·7H₂O, 0.02 g; MnSO₄·4H₂O, 0.02 g; NaCl, 0.02 g; H₂BO₃, 0.5 mg; CuSO₄·5H₂O, 0.04 mg; Na₂MoO₄·2H₂O, 0.2 mg; ZnSO₄·7H₂O, 8.0 mg; CaCl₂, 50.0 mg; CoCl₂·6H₂O, 0.2 mg; distilled water, to 1 liter. When strain 4736 was cultivated in shake flasks, ethanol was added at 0.1% (wt/vol) concentration, and the medium was sterilized by filtration through a 0.22 μm filter. Growth rates were measured in 300-ml shake flasks that were fitted with cuvette side arms. Each flask received 50 ml of P-1 medium and was inoculated with cells growing exponentially on ethanol to an initial optical density (OD) between 0.05 and 0.10. OD was measured at 660 nm in a spectrophotometer (Spectronic 20, Bausch & Lomb Inc., Rochester, N.Y.). A linear rela-

tionship existed between dry cell mass and optical density up to an OD of about 0.7; each increase of 0.1 OD unit corresponded to an increase of 0.036 mg/ml (dry biomass). Growth rate was expressed as the specific growth rate, μ , where $\mu = \ln X_2/g = \ln X_2 - \ln X_1/t_2 - t_1$ and g is the mean generation time or doubling time; X_1 and X_2 are the biomass concentrations at time t_1 and t_2 , respectively.

Viable cell numbers were determined by plating on Trypticase soy agar after dilution in sterilized tap water.

Inhibition of growth by acetaldehyde (bp 20 C) was measured in sealed shake flasks. The air space in the flask provided enough oxygen to measure μ before oxygen transfer limited growth.

To ensure that limited oxygen transfer did not limit growth at high cell populations, strain 4736 was cultivated in a 5-liter fermentor (Fermentation Design, Allentown, Pa.). The impellor was operated at 700 rpm and the temperature was maintained at 35 C. The pH was automatically controlled at a value of 7 by the addition of ammonia. Dissolved oxygen tension (DO) was monitored continuously by a galvanic membrane probe (Fermentation Design), and air was sparged into the fermentor at 1.0 volume per volume per minute. When the DO declined to 20% of air saturation, the inlet air was enriched with oxygen to prevent an oxygen limitation.

Gas chromatography. Ethanol, acetate and acetaldehyde were detected by flame-ionization gas chromatography. The column was 6 ft by $\frac{1}{4}$ inch (182.88 by 0.64 cm) stainless-steel packed with 50/80 Porapak Q (Applied Science Laboratory, State College, Pa.). It was operated isothermally at 200 C; the carrier gas flow was 30 ml/min of helium. Samples were taken from shake flasks or fermentors, and 5.0 μ liters (cells and supernatant fluid) was injected into the column.

Respirometry. Oxygen uptake was measured by Warburg respirometry according to standard procedures (18). Cells for respirometer experiments were grown in shake flasks on 0.1% (wt/vol) acetate, washed and resuspended in 0.1 M K_2HPO_4 buffer at pH 7.0 to a final cell concentration between 1 and 3 mg/ml (dry wt). Chloramphenicol, when used, was added to achieve a final concentration of 30 μ g/ml.

RESULTS

Taxonomy. Strain 4736 was obligately aerobic, non-sporeforming, and nonmotile, usually appearing as pairs of coccoid and often almost spherical cells. It was gram negative, but under some conditions tended to resist decolorization, sometimes appeared quite mucoid, and did not exhibit fluorescence. The organism was oxidase negative, catalase positive; it did not hydrolyze serum, casein, gelatin, or starch. Also it did not produce H_2S or reduce nitrate. The deoxyribonucleic acid (DNA) contained about 41 mol% G + C. From these characteristics and others listed below, strain 4736 was designed *Acinetobacter calcoaceticus* (3, 9, 17).

In oxidative-fermentative medium with 1%

lactose, or in "Purple broth" with 10% lactose, acid was produced from the following: glucose, maltose (late stage), lactose, mannose, galactose, xylose, and arabinose. From the following no acid was produced: maltose, levulose, sucrose, fructose, glycerol, and sugar alcohols. Growth was positive on media containing: alanine, arginine, histidine, proline, xylose, citrate, and acetate. There was no growth on media supplemented with: aspartic acid, glutamic acid, and glucose. Results of other diagnostic tests using this strain were as follows: litmus milk—slightly reduced, acid reaction; triple sugar-iron—no change; indole, methyl red, and Voges-Proskauer—negative; oxidative-fermentative—oxidative; urease—positive; penicillin sensitivity—resistant; and no growth occurred on Pseudocel agar.

Growth. *A. calcoaceticus* was inoculated into P-1 medium in shake flasks containing 0.1% (wt/vol) ethanol and incubated at various temperatures. The temperature optimum was within a narrow range, somewhat higher than might be anticipated for a soil isolate (Fig. 1).

The pH of P-1 medium in shake flasks was adjusted to values between 5.0 and 8.0 by the addition of H_2PO_4 or NH_4OH . These media, after inoculation, were incubated at 35 C, and the growth rate was determined by OD measurements during the first 6 h of incubation. The pH changes during this period were small (<0.2 units) and did not require periodic adjustments.

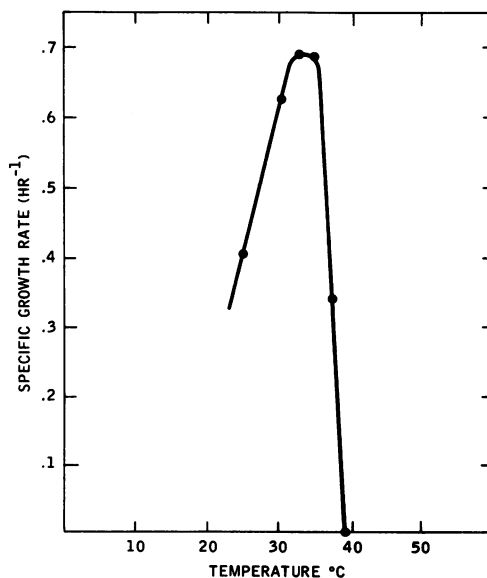


FIG. 1. Effect of temperature on the specific growth rate of strain 4736 growing on ethanol.

A maximum growth rate occurred between pH 6.5 and 7.5; however, the microorganism grew well at pH values as low as 5.5 (Fig. 2). The value of μ at pH 5.5 corresponds to a 2.5-h biomass doubling time. Growth did not occur below pH 5.0 or above pH 8.0.

The temperature and pH optima as determined above were used in fermentor experiments to maximize cell production. In these experiments the initial ethanol concentration was 1.0%, and ethanol was added periodically to maintain a concentration between 0.5 and 1.0% during growth. The highest cell populations obtained varied widely from one experiment to another but were in the range of 2.5 to 12.0 mg/ml. The cessation of growth in each case was abrupt and coincided with a decrease in population viability.

In a previous study acetate and acetaldehyde were found in culture broths of *A. calcoaceticus* (L. Naslund, unpublished data). When these metabolites were measured at various times during cultivation, it was found that their appearance coincided with the cessation of growth (Fig. 3).

Studies were conducted in shake flasks to determine if the amount of acetate or acetaldehyde accumulated was sufficient to inhibit growth. Acetaldehyde concentrations as low as 0.01% (wt/vol) reduced the growth rate of strain 4736 by about 80% (Fig. 4). It was not possible to accurately measure the reduction in growth rate caused by lower acetaldehyde concentrations because acetaldehyde was metabolized before one doubling of the biomass occurred. However, it appeared to be necessary to reduce acetaldehyde to levels below approximately 0.001% (wt/vol) to avoid any growth inhibition.

In similar studies growth rate was measured as a function of ethanol or acetate concentration. Only at ethanol and acetate concentrations greater than 1.0% (wt/vol) and 0.1% (wt/vol), respectively, was the growth rate of *A. calcoaceticus* less than the maximum growth rate. Since the ethanol concentration in fermentor experiments was maintained at or below 1.0% (wt/vol) at all times and acetate did not accumulate beyond 0.1%, the cessation of growth in this experiment appeared to be attributable to the accumulation of an inhibitory amount of acetaldehyde.

In other fermentor experiments higher cell densities were observed and acetate accumulated to inhibitory levels while acetaldehyde levels remained below 0.001%. This variability was eventually traced to the method of medium preparation. The fermentor vessel containing P-1 medium was autoclaved at 121 C for

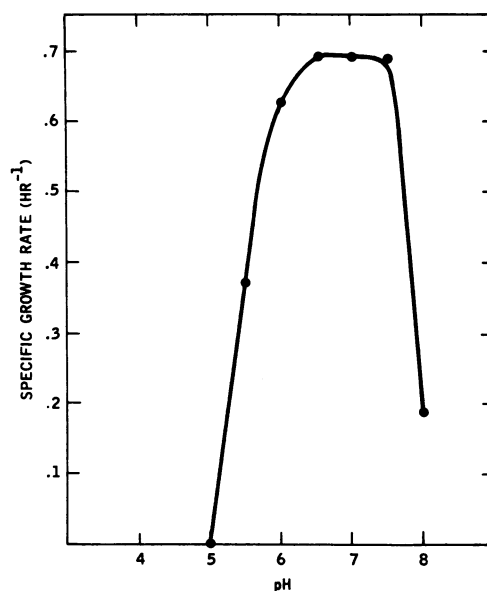


FIG. 2. Effect of pH on the specific growth rate of strain 4736 growing on ethanol at 35 C.

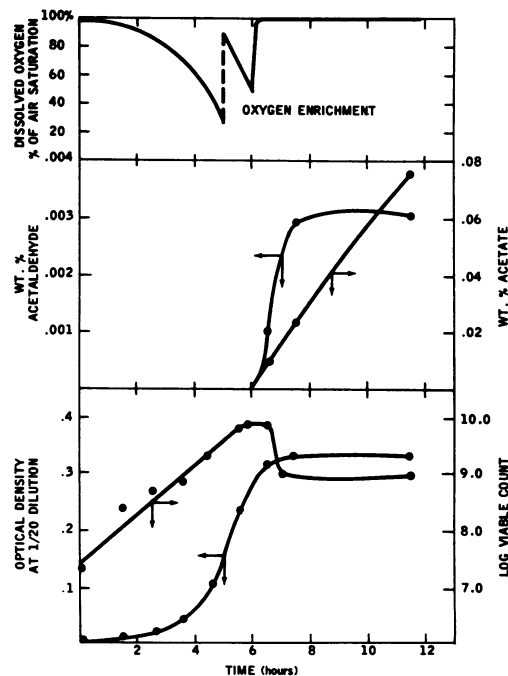


FIG. 3. Time course of growth of strain 4736 growing on ethanol in a fermentor. Dotted line indicates enrichment of the inlet air stream with oxygen to prevent an oxygen limitation. The arrows point to the scales on which the data are plotted.

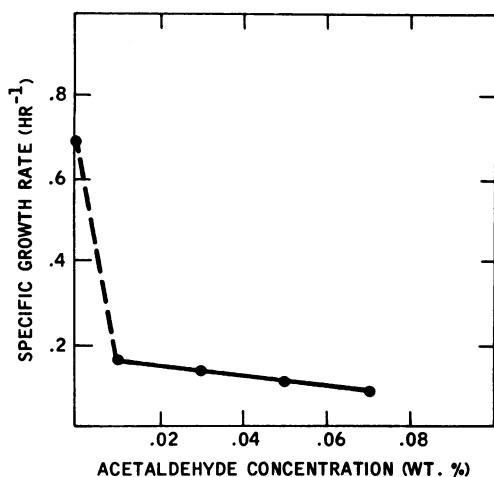


FIG. 4. Effect of acetaldehyde on the specific growth of strain 4736 growing on ethanol. The dotted line refers to the fact it was not possible to measure accurately the reduction in growth rate caused by very low acetaldehyde concentrations, since acetaldehyde was metabolized before one doubling of the biomass occurred.

30 min, cooled, then incubated for different periods of time prior to inoculation. Media inoculated immediately after preparation supported 7 to 10 mg of biomass per ml before the production of large quantities of acetate inhibited growth. Media that were inoculated 24 to 48 h after preparation supported only 2 to 3 mg of biomass per ml, and acetaldehyde, in addition to acetate, usually accumulated. The amount of precipitated salts in P-1 medium appeared to increase during storage of uninoculated media, suggesting that in stored medium insolubilization of an essential nutrient might be responsible for acetate or acetaldehyde accumulation, or both. To test this possibility cells were grown on freshly prepared P-1 medium. When the cell population reached about 7 mg/ml, acetate began to accumulate. Before the acetate concentration exceeded 0.1% (wt/vol), a sample was removed and the cell-free supernatant fluid was recovered, filter-sterilized, and dispensed into shake flasks. Various mineral salts were added to each flask which then were inoculated with the microorganism. Only the flasks that received added magnesium supported growth.

In another experiment the salt components in P-1 medium were deleted individually in a series of flasks and the resultant media were inoculated. After incubation for 12 h, gas chromatography revealed the presence of acetate

only in those media lacking either magnesium or sulfate.

Increasing the magnesium or sulfate content of P-1 medium or making supplemental additions of these ions to growing cultures did not appreciably increase the final cell population. Large additions of magnesium or sulfate were inhibitory, and lower concentrations did not prevent acetate accumulation or increase cell densities beyond 10 to 12 mg/ml. Numerous attempts to favor magnesium solubilization by filter-sterilization of P-1 medium or varying the medium composition were also unsuccessful.

When *A. calcoaceticus* was inoculated into a medium containing a mixture of 0.1% (wt/vol) acetate and 0.1% (wt/vol) ethanol, the acetate was preferentially metabolized and ethanol utilization began after the depletion of acetate. The growth rate on acetate in the presence or absence of ethanol was similar to the growth rate on ethanol alone (Fig. 5). Thus, the pres-

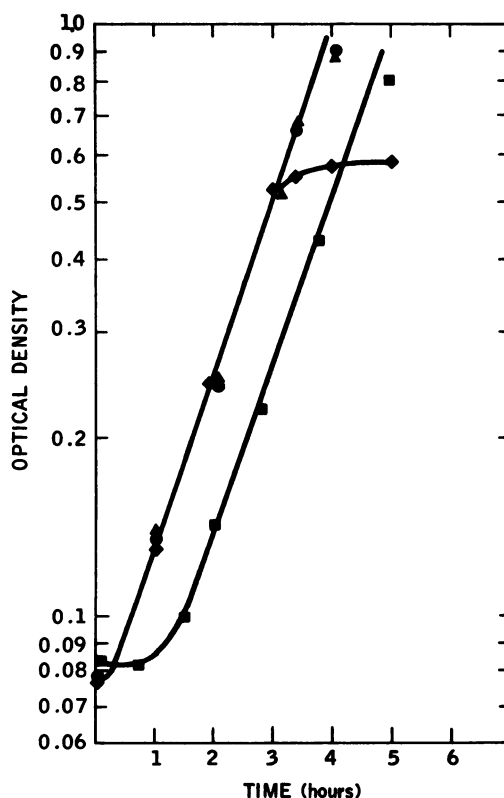


FIG. 5. Growth of strain 4736 in shake flasks. Ethanol-grown cells inoculated into P-1 medium containing 0.1% acetate \blacklozenge , 0.1% ethanol \blacktriangle , or a mixture of 0.1% ethanol and 1.17% acetate \bullet , as the carbon source. Acetate-grown cells inoculated into P-1 medium containing ethanol as a carbon source \blacksquare .

ence of acetate inhibited ethanol utilization but did not prevent cell growth.

Acetate-grown cells readily metabolized ethanol, acetate, or the acetate component of an ethanol-acetate mixture. However, an additional 30-min lag time was observed before growth was initiated on ethanol, suggesting that ethanol oxidation was inducible (Fig. 5). Induction also was indicated by Warburg respirometry which showed that acetate-grown cells in the presence of chloramphenicol consumed oxygen on acetate but not on ethanol (Fig. 6).

DISCUSSION

Many of the isolates from the enrichment procedure were members of the large group of gram-negative, coccoid, nonmotile, oxidase-negative bacteria, the taxonomy of which has been the subject of considerable confusion and controversy (3, 17). These bacteria appear to be ubiquitous and perhaps predominant soil organisms (2). Most recently, the consensus appears to be that they should be placed in the genus *Acinetobacter* (3, 17). The relevant Subcommittee on Taxonomy of the International Committee on Nomenclature of Bacteria considers that this genus contains a single species, *A. calcoaceticus* (9).

Growth of *A. calcoaceticus* on ethanol was terminated by the depletion of magnesium ion causing acetate or acetaldehyde, or both, to accumulate. The deletion of sulfate ion from P-1 medium was also correlated with acetate accumulation. The relationship of these ions to acetate build-up can be seen from the pathway by which ethanol is assimilated. Ethanol is degraded to acetate via acetaldehyde. Acetate is converted to acetyl coenzyme A (CoA) and enters the tricarboxylic acid cycle by condensation with glyoxylate or oxaloacetate. There are two mechanisms for the formation of acetyl CoA from acetate. One occurs in mammalian tissue, yeast, and some bacteria and requires one enzyme, acetyl CoA synthetase (4).

Both reaction sequences require magnesium and sulfur. Magnesium is a cofactor of the

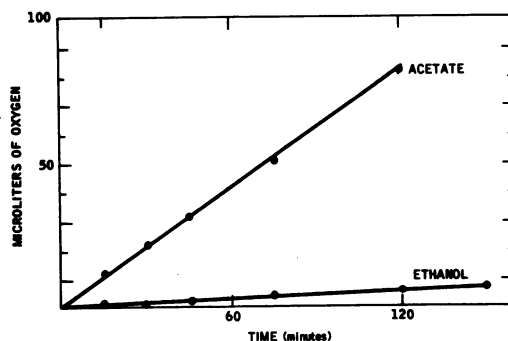
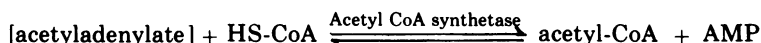
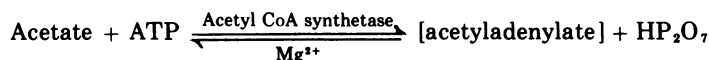


FIG. 6. Oxygen uptake by acetate-grown cells of strain 4736 incubated with chloramphenicol in the presence of either ethanol or acetate.

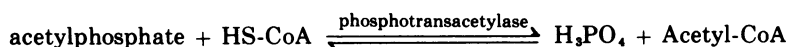
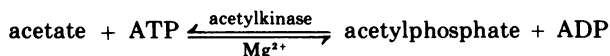
kinase and synthetase enzymes, and sulfur is an integral component of CoA. Thus, a deletion of sulfur or magnesium could cause acetate accumulation by preventing the formation of acetyl CoA.

When cells were inoculated into shake flasks in a medium containing both acetate and ethanol, the presence of acetate prevented ethanol oxidation. In fermentor studies, however, the presence of accumulated acetate did not prevent ethanol oxidation. These results indicate that acetate inhibited ethanol metabolism via a repression mechanism rather than by feedback inhibition. Repression control mechanisms often exhibit a lag period before their effects are observed because preformed enzyme persists until it decays or is diluted out by cell growth. In the fermentor, where the cell population (and hence the enzyme concentration) is high, the lag allows sufficient time for the oxidation of large quantities of ethanol to acetate. Although a similar lag occurs in shake flasks, the low cell population (enzyme concentration) restricts the rate of ethanol oxidation so that only small quantities of ethanol are oxidized to acetate before enzyme decay or inactivation occurs.

Attempts to increase cell concentration beyond 10 to 12 mg/ml by favoring magnesium



The second, found only in bacteria, is a two enzyme system (11, 18).



solubilization were unsuccessful. A possible explanation may be derived from the sequence of events that leads to acetate accumulation. Cells inoculated into P-1 medium that contained ethanol began to grow at a maximum rate of 0.7/h. During this period the amount of ethanol and acetaldehyde dehydrogenase in the cells was sufficient to support this growth rate. When the soluble magnesium concentration fell below a critical level, the rate of acetyl-CoA formation (and hence growth) was reduced. At this time the rate of acetate synthesis exceeded its rate of utilization and acetate rapidly accumulated to an inhibitory level. The accumulating acetate led to the repression of further synthesis of alcohol dehydrogenase; however, the preformed enzyme continued to oxidize ethanol at a high rate until enzyme decay occurred. Before this happened, acetate reached inhibitory levels. If acetate prevented ethanol oxidation by feedback inhibition instead of repression, acetate would not accumulate and cell growth would continue at whatever rate the available magnesium could support. With repression control of ethanol metabolism, growth must continue at its initial rate or acetate will accumulate because of the persistence of the performed enzyme. Thus, sufficient magnesium must be available to support a rapid exponential growth rate. If this explanation is correct, our data indicate that it is very difficult to supply a microorganism with sufficient soluble magnesium to maintain a high exponential growth rate when the cell population exceeds 10 to 12 mg/ml.

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