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Production of Acetaldehyde by *Zymomonas mobilis*

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Mutants of *Zymomonas mobilis* were selected for decreased alcohol dehydrogenase activity by using consecutively higher concentrations of allyl alcohol. A mutant selected by using 100 mM allyl alcohol produced acetaldehyde at a level of 4.08 g/liter when the organism was grown in aerated batch cultures on a medium containing 4.0% (wt/wt) glucose. On the basis of the amount of glucose utilized, this level of acetaldehyde production represents nearly 40% of the maximum theoretical yield. Acetaldehyde produced during growth was continuously air stripped from the reactor. Acetaldehyde present in the exhaust stream was then trapped as the acetaldehyde-bisulfite addition product in an aqueous solution of sodium bisulfite and released by treatment with base. Acetaldehyde was found to inhibit growth of *Z. mobilis* at concentrations as low as 0.05% (wt/wt) acetaldehyde. An acetaldehyde-tolerant mutant of *Z. mobilis* was isolated after both mutagenesis with nitrosoguanidine and selection in the presence of vapor-phase acetaldehyde. The production of acetaldehyde has potential advantages over that of ethanol: lower energy requirements for product separation, efficient separation of product from dilute feed streams, continuous separation of product from the reactor, and a higher marketplace value.

In the fermentation of ethanol, the ultimate step in the pathway is the reduction of acetaldehyde to ethanol by the enzyme alcohol dehydrogenase. The product ethanol must then be separated from the reaction mixture, an energy-intensive process which may account for 63% of the total energy use involved in the fermentation process (7). However, if the reaction pathway is altered to inhibit the reduction of acetaldehyde to ethanol, the acetaldehyde produced might be collected as an attractive alternative to ethanol.

Pure acetaldehyde has a boiling point of 20.8°C, whereas ethanol boils at 78.5°C. Thus, at temperatures typical for mesophilic fermentation, acetaldehyde may be more readily stripped from solution than ethanol is. In addition to the advantage of having greater volatility than ethanol, acetaldehyde does not form an azeotrope with water. In January 1987, 99% acetaldehyde had a marketplace value nearly 1.5 times that of synthetic 100% ethanol and a value approximately twice that of fermentation ethanol (5). Acetaldehyde is quite reactive and can be used to produce a variety of compounds such as acetic acid, acetic anhydride, ethyl acetate, butanol, and pyridines. Acetaldehyde also has uses in the food industry as a flavor additive. It is listed as a GRAS (Generally Recognized As Safe) substance by the U.S. Food and Drug Administration and delivers a fresh or fruity flavor to food such as meats, fruits, breads, spices, vegetables, and dairy products, as well as to candies and chewing gums (5). Since acetaldehyde is both volatile and reactive, dry acetaldehyde delivery systems are currently being developed for use in dry flavors and instant foods (4).

Previous research on the commercial production of acetaldehyde by biological means has been limited to the reoxidation of biologically produced ethanol back to acetaldehyde. Kierstan (8) conducted a preliminary study on the feasibility of using a free enzyme system to oxidize aqueous solutions of ethanol to acetaldehyde. This enzyme system consisted of the alcohol oxidase from *Candida boidinii* and a catalase. Similarly, a patent was issued for the production of acetaldehyde from ethanol using an enzyme complex containing alcohol dehydrogenase, NADH, flavine mononucleotide, and a catalase (W. R. Raymond, U.S. patent 4,481,292, 1984). By using this system, 10 to 20% of the ethanol was converted; after 9 h, acetaldehyde was produced at a level of 2.5 g/liter of solution. Armstrong et al. (1) have researched the use of whole cells of *Candida utilis* for the conversion of ethanol to acetaldehyde. The maximum accumulation of acetaldehyde occurred at a level of 6.5% ethanol in solution, with 3.5 g of acetaldehyde per liter accumulating in batch cultures after 5 h of growth. No increase in acetaldehyde was noted with additional incubation. Production of acetaldehyde by this method, however, must be carefully regulated to limit the conversion of acetaldehyde to acetic acid. The Electrohol process (T. M. Meshbesher, U.S. patent 4,347,109, 1982) electrochemically converts fermentation alcohol to acetaldehyde. In an assessment of the process, Trevino (10) determined that yields of 93% or greater must be obtained for it to be competitive with the current ethylene-based technology of acetaldehyde manufacture. The Electrohol process is most efficient with a feed stream of 95% ethanol. As the ethanol concentration in the feed stream is reduced, the efficiency of this process drops considerably.

The present research was structured to evaluate a system which uses *Zymomonas mobilis* for the production of acetaldehyde from glucose. *Z. mobilis* is known to produce acetaldehyde in the presence of oxygen (9). This is due to increased NADH oxidase activity resulting in the decreased availability of NADH for the reduction of acetaldehyde to ethanol by alcohol dehydrogenase. In addition, *Z. mobilis* apparently does not have an aldehyde dehydrogenase to oxidize acetaldehyde to acetic acid (3). Alcohol dehydrogenase mutants of *Z. mobilis* showing increased levels of acetaldehyde production have previously been isolated by using allyl alcohol as a selective agent (13). Figure 1 depicts

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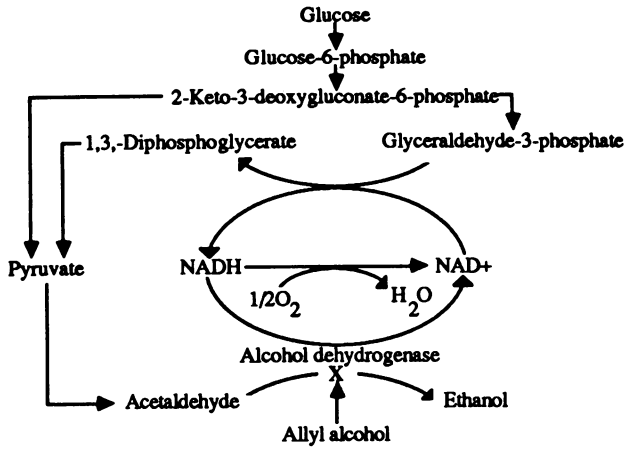

FIG. 1. Entner-Doudoroff pathway for glucose metabolism in *Z. mobilis*; the effects of oxygen and allyl alcohol.

the effects of oxygen and allyl alcohol on glucose metabolism in *Z. mobilis*.

In the present study, acetaldehyde was produced by a stripped-acetaldehyde respiration. The term stripped-acetaldehyde refers to the separation and collection of acetaldehyde as it is formed; the term respiration is used since oxygen acts as the terminal electron acceptor. Four areas of research are germane to this study: the use of a method for selecting alcohol dehydrogenase-negative mutants; the development of a plate screening method for microorganisms that produce increased levels of acetaldehyde; the use of a method for selecting acetaldehyde-tolerant mutants; and the development of a reactor system that supplies oxygen to the growing culture, strips acetaldehyde from the growth medium, and allows the acetaldehyde present in the exhaust gases to be collected and quantified.

MATERIALS AND METHODS

Strain and cultivation. The organism used in this study was *Z. mobilis* subsp. *mobilis* (ATCC 10988). Cultures were maintained by culturing them in either 4% glucose standard medium (4GSM) or 4% sucrose standard medium (4SSM). The components of the standard medium were (in g/100 ml of distilled H_2O): sucrose or glucose, 4.0; Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 2.0; Bacto-Yeast Extract, 1.0; KH_2PO_4, 0.1; $(NH_4)_2SO_4$, 0.1; and $MgSO_4 \cdot 7H_2O$, 0.05. For solidification, Bacto-Agar was added at 1.5 g/100 ml unless otherwise indicated. The pH was adjusted to 5.5 before steam sterilization at 121°C and 15 lb/in^2 for 15 min. Cultures were maintained in screw-cap tubes by transferring every 2 days with 1.0% inocula. All cultures were incubated aerobically at 30°C to select for those organisms most suited to function under aerobic conditions. Since the prolonged growth of *Z. mobilis* on sucrose results in the loss of the *Zymomonas* acetaldehyde dehydrogenase II enzyme (13), the wild type was maintained in both 4GSM and 4SSM.

Analytical methods. Acetaldehyde and ethanol concentrations in cell cultures were measured with a Varian 6000 gas chromatograph (Varian Instrument Group, Walnut Creek, Calif.) fitted with a flame ionization detector. A Varian 4290 integrator was used for peak integration. A 1-m column was used with Porapak Q-S 80/100 mesh as the packing material. The following gas chromatograph settings were used: carrier nitrogen, 30 ml/min; air, 300 ml/min; hydrogen, 30 ml/min; column, 150°C; injector, 180°C; detector, 190°C. Culture samples were centrifuged for 10 min at 4°C and 3,000 rpm before analysis. Isopropanol was added at a level of 0.1% (wt/wt) to each sample as an internal standard. An external standard of 0.1% (wt/wt) acetaldehyde (Fisher Scientific Co., Pittsburgh, Pa.), 0.1% (wt/wt) ethanol, and 0.1% (wt/wt) isopropanol was prepared daily. All solutions to which acetaldehyde was added were cooled to approximately 0°C before the addition. Glucose concentration was determined enzymatically by using the Sigma Glucose (HK)10 single reagent system (Sigma Chemical Co., St. Louis, Mo.). All analyses were done in triplicate.

Differentiation of strains with the use of acid fuchsin. The amount of acetaldehyde produced by plate cultures was qualitatively assessed at 76 h of growth by using a 0.5-cm agar overlay containing acid fuchsin (Sigma) as an indicator. The overlay medium was buffered to pH 5.5 to limit the false-positive reaction of acid fuchsin with acid production by the colonies. The overlay medium consisted of the following (in g/100 ml of distilled H_2O): acid fuchsin, 0.02; KH_2PO_4, 3.18; $Na_2HPO_4 \cdot 7H_2O$, 0.88; and agar, 2.0. Molten acid fuchsin medium at 45°C was decolorized with SO_2 before it was used as an overlay. To increase the resolution of acetaldehyde production by colonies, the inoculum was diluted to limit the plate count to approximately 30 colonies. Diffusion of the acetaldehyde was limited both by cooling the plates to 4°C before applying the overlay and by developing the plates at 15°C.

Selection of strains by allyl alcohol. Allyl alcohol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was used to select for those microorganisms with altered alcohol dehydrogenase activity. The initial level of allyl alcohol was 0.2 mM (13.6 μl/liter), as given by Wills et al. (13). A 3-day-old, sucrose-grown, wild-type culture of 2.4×10^9 cells per ml was inoculated onto each of 10 4SSM–0.2 mM allyl alcohol plates without dilution. Twenty-eight strains were isolated from these plates and screened for acetaldehyde production by using acid fuchsin overlays. Seven strains representing the entire range of color reaction to acid fuchsin were then grown on 2% glucose standard medium in shake-flask cultures to compare levels of acetaldehyde production. The strain which showed both the strongest acid fuchsin reaction and the highest level of acetaldehyde production was then consecutively selected on 4SSM agar with allyl alcohol concentrations of 2.0 mM, 10.0 mM, 20.0 mM, and 100.0 mM. The selection of mutants on 4SSM with allyl alcohol was carried out over 6 months.

Acetaldehyde production with shake flasks. Each culture used for shake-flask culturing was transferred every 24 h for 3 consecutive days before the final inoculation. Each transfer was made with a 1.0% inoculum into 125-ml, cotton-plugged Erlenmeyer flasks containing 50 ml of either 2% glucose standard medium or 4GSM. Cultures were incubated in a waterbath shaker at 100 rpm. All trials were carried out in triplicate.

Acetaldehyde production with direct aeration. Cultures were transferred every 24 h for 3 days before the final inoculation. The final transfers were made by using a 1% inoculum in 50 ml of 4GSM in 125-ml Erlenmeyer flasks. The flasks were allowed to incubate for 6 h before direct aeration. Cultures were aerated at either 23 or 11 ml/min. All trials were done in triplicate. Filtered air was distributed to each culture flask and released through syringes fitted with 26-gauge needles. Acetaldehyde was collected from the exhaust gas of each aerated-culture flask by sparging the exhaust stream through two consecutive 25-by-200-mm screw-cap

tubes, each of which contained 50 ml of 0.25M $NaHSO_3$. The total yield of acetaldehyde from each flask was computed as the sum of the concentrations of acetaldehyde from the two collection tubes plus any residual concentration of acetaldehyde remaining in the culture medium. Acetaldehyde concentrations were corrected for any loss in fluid volume in the flasks or tubes due to aeration.

Quantification of acetaldehyde in solutions of $NaHSO_3$. The acetaldehyde trapped in solutions of $NaHSO_3$ was quantified by using a modification of the method of Jones et al. (6). $NaHSO_3$ solutions were diluted to 0.05M before the analysis. All chemistry was carried out at room temperature with constant stirring. A 1-ml sample of 20% HCl was added to 10 ml of the $NaHSO_3$ solution and allowed to react for 1 min. At this time, 2 ml of Na_3BO_3 solution (10 g of H_3BO_3 and 17 g of NaOH per 100 ml of H_2O) was added and titrated with a KI-I_2 solution (49.8 g of KI and 24.9 g of I_2 per 100 ml of H_2O) to give a lasting yellow color. The mixture was then brought to pH 9.00. After 5 min, 20% HCl was added to bring the pH of the mixture to 7.00. This mixture was immediately transferred to a tared 100-ml serum bottle, isopropanol was added to 0.1% by weight, and the bottles were capped. Standards were made by adding 0.1 or 0.5% (wt/wt) acetaldehyde to the original-strength $NaHSO_3$ solution. These standards were allowed to equilibrate while the other $NaHSO_3$ solutions were sparged with gas from the aerated cultures. The standards were then chemically treated in a manner identical to that of the test samples. All samples were allowed to equilibrate at 30°C before the acetaldehyde concentration was determined by gas chromatography. Gas chromatography was conducted under conditions identical to those given for the determination of acetaldehyde in solution, although injections were made with 0.5 ml of the headspace gas. All gas chromatograph sampling was done in triplicate.

Vapor-phase selection for acetaldehyde-tolerant mutants. Strains of *Z. mobilis* were selected for tolerance to acetaldehyde by growing the cultures in the presence of a mutagen in an atmosphere containing acetaldehyde. 4GSM agar plates were inoculated with 0.1 ml of an inoculum containing 10^5 cells per ml and were incubated for 3.5 h before the addition of the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Sigma). The NTG was applied to the center of each plate by using a 5-μl sample of a 5 mg/ml solution of NTG in 0.1M sodium citrate buffer. This level of NTG resulted in a 4- to 5-cm diameter of kill. The NTG-treated plates were then allowed to incubate for an additional 8.5 h before they were subjected to acetaldehyde vapor within GasPak System 100 chambers (BBL Microbiology Systems, Cockeysville, Md.). The chambers were supported horizontally and supplied with support racks for the petri plates. No catalyst was used with the chambers. A glass petri dish was placed at the bottom of each chamber and filled with 10 ml of a solution of given acetaldehyde concentration. A range of between 0.0 and 10.0% (wt/wt) acetaldehyde was used within the chambers. The plates were incubated in the GasPak chambers for 48 h and then removed and incubated aerobically for 76 h. Colonies were selected both on the basis of size and for growth near the approximate periphery of NTG kill.

Evaluating tolerance of strains to acetaldehyde. Mother cultures of 50 ml of 4GSM in 125-ml Erlenmeyer flasks were transferred with a 1% inoculum every 24 h for 3 days before the start of the experiment. Screw-cap tubes (16 by 150 mm) containing 8.9 ml of 4GSM were used for growth analysis at various concentrations of acetaldehyde. Each tube was brought to 10.0 ml by using transfers of sterile H_2O, acetaldehyde, and a 0.1-ml inoculum. All trials were done in triplicate. All incubations were carried out in a water bath shaker at 100 rpm. A Bausch and Lomb Spectronic 710 at 550 nm was used for the absorption studies in the growth curve analysis. The percentage of acetaldehyde in solution was measured both before and after growth to check for any change in acetaldehyde concentration due to growth. Tubes of media containing acetaldehyde at the given levels were left uninoculated as controls and were incubated at both 4 and 30°C for the duration of the experiment.

TABLE 1. Acetaldehyde and ethanol production by allyl alcohol mutants of *Z. mobilis*[a]

Strain	Concn of allyl alcohol (mM)	% Acetaldehyde produced	% Ethanol produced
Wild type	0.0	0.078	1.32
RZ1A	0.2	0.071	1.38
RZ1B	2.0	0.087	1.37
RZ1C	10.0	0.139	1.11
RZ1E	100.0	0.248	0.14

[a] 4GSM shake-flask culture without direct aeration.

RESULTS

Selection and differentiation of strains with increased acetaldehyde production. After 5 days of aerobic growth on 0.2 mM allyl alcohol agar plates, 28 colonies of *Z. mobilis* subsp. *mobilis* were obtained with a survivorship of approximately 1 in 10^8 cells. After 10 days of growth, the survivorship increased to about 1 in 10^5 cells. When clones of the 28 strains were overlaid with decolorized acid fuchsin agar, the degree of reaction by the colonies corresponded to the level of acetaldehyde produced during shake-flask culturing with 2% glucose standard medium. Acetaldehyde production in these shake-flask trials ranged from 0.02% (wt/wt) acetaldehyde by the wild type to 0.08% (wt/wt) acetaldehyde by a strain designated RZ1A. When RZ1A was consecutively selected by using higher concentrations of allyl alcohol, there was an overall increase in acetaldehyde production and a decrease in ethanol production (Table 1). The combined yield of acetaldehyde and ethanol, however, decreased for those strains selected by using higher levels of allyl alcohol. This is in part related to a decrease in the amount of glucose utilized, for when the wild-type strain and strain RZ1E (selected on 100 mM allyl alcohol) were grown in shake-flask cultures, they used, respectively, 97 and 52% of the available glucose. When the wild-type and allyl alcohol-selected strains were grown in screw-cap tubes, the growth curves for these strains were quite similar (Fig. 2). When these same strains were grown in shake-flask cultures, however, decreased growth was observed for those strains selected by using allyl alcohol (Fig. 3). After 41 days of subculturing, strain RZ1E showed a 38% decrease in acetaldehyde production when it was grown in 4GSM shake-flask cultures without direct aeration.

Acetaldehyde production with direct aeration. The level of acetaldehyde in solution decreased nearly exponentially with time when 50 ml of a 0.3% (wt/wt) solution of acetaldehyde in 4GSM was sparged with 250 ml of air per min (data not shown). Therefore, acetaldehyde may be readily stripped from the growth medium during culturing. When air is used to strip acetaldehyde from the culture medium, oxygen is delivered to the growing cells and acetaldehyde production by *Z. mobilis* increases. Table 2 shows the

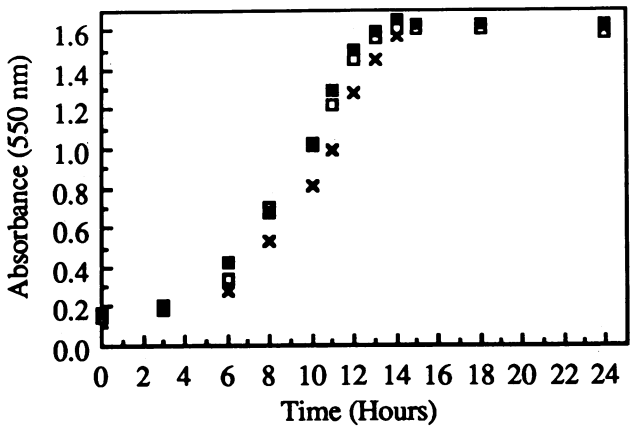

FIG. 2. Growth curves of wild-type and allyl alcohol-selected strains of *Z. mobilis*. Symbols: □, wild type; ×, strain RZ1A; ■, strain RZ1E. Screw-cap tube culture, 4GSM.

amount of acetaldehyde produced by both the wild-type strain and strain RZ1E when the cultures are grown under aeration, with 23 ml of air per min. The allyl alcohol-selected strain produced nearly twice as much acetaldehyde as that of the wild type. The amount of glucose utilized by strain RZ1E when it was grown with direct aeration, however, was only approximately 60% of that used by the wild-type strain grown with direct aeration. On the basis of the amount of glucose utilized, strain RZ1E produced nearly 40% of the theoretical yield of acetaldehyde, three times that of the wild type. In a comparison of data from Tables 1 and 2, there was an approximate twofold increase in acetaldehyde production when strain RZ1E was grown with direct aeration as opposed to when the strain was grown in shake-flask cultures without direct aeration. When the flow of air was decreased to 11 ml/min, strain RZ1E produced only 2.1 g of acetaldehyde per liter. With either flow rate, the amount of acetaldehyde remaining in the medium after 24 h was at a level of 0.11% (wt/wt).

The acetaldehyde levels shown in Table 2 are a total of that stripped from the medium and that remaining in the medium after 24 h of growth. The method used for trapping

TABLE 2. Acetaldehyde production by directly aerated cultures of the wild-type and RZ1E strains of *Z. mobilis*[a]

Strain	Acetaldehyde production (g/liter)	Glucose (g/liter)	% Theoretical yield of acetaldehyde
Wild type	2.20	34.5	13.0
RZ1E	4.08	21.0	39.7

[a] 4GSM (50 ml) in 125-ml Erlenmeyer flasks, under aeration, 23 ml of aeration per min, 24 h.

the stripped acetaldehyde was approximately 79% efficient. Any loss of acetaldehyde due to this method of collection was not accounted for in the data of Table 2.

Vapor-phase selection for acetaldehyde-tolerant mutants. The growth rate of *Z. mobilis* was inhibited by as little as 0.05% (wt/wt) acetaldehyde (Fig. 4). Figures 5A and B show growth curves for both wild-type *Z. mobilis* and strain RZt1.0, which had been selected for tolerance to acetaldehyde in a 1.0% acetaldehyde chamber. When grown with 0.0% (wt/wt) acetaldehyde, strain RZt1.0 showed a growth curve similar to that of the wild type (Fig. 5A). With 0.1% acetaldehyde, strain RZt1.0 showed a higher rate of growth than that of the wild type (Fig. 5B). With 0.2% acetaldehyde, however, there was no apparent difference in growth rate between the wild type and RZt1.0 (data not shown). When cultured in screw-cap tubes with 0.1% acetaldehyde, the level of acetaldehyde in solution decreased by 64% for the wild-type strain and 73% for strain RZt1.0, as compared to a sterile control. In addition, when strain RZ1C (selected on 50 mM allyl alcohol) was selected for tolerance to acetaldehyde by using a 1.6% acetaldehyde chamber, the resultant strain, RZ1Ct1.6, produced approximately 53% less acetaldehyde than strain RZ1C did and 16% less acetaldehyde than the wild type did during shake-flask culturing.

DISCUSSION

The range of acetaldehyde production by *Z. mobilis* strains selected by using 0.2 mM allyl alcohol is similar to that reported by Wills et al. (13). The acid fuchsin overlay technique proved to be useful for the differentiation of acetaldehyde production by those strains selected by using allyl alcohol. The diffusion of the acetaldehyde, however, limited the resolution of the overlay to about 30 colonies per plate. Therefore, the overlay technique was impractical for

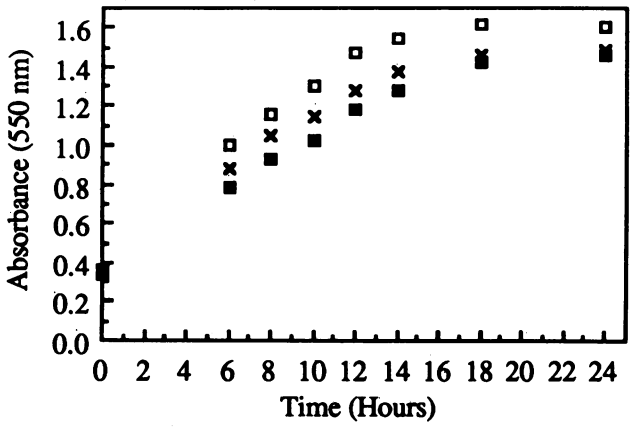

FIG. 3. Growth curves of wild-type and allyl alcohol-selected strains of *Z. mobilis*. Symbols: □, wild type; ×, strain RZ1A; ■, strain RZ1E. Shake-flask culture, 4GSM.

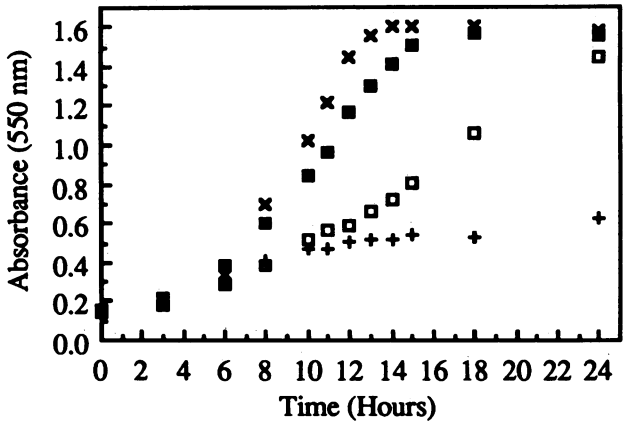

FIG. 4. Growth inhibition of wild-type *Z. mobilis* by 0.0 (×), 0.05 (■), 0.1 (□), and 0.2% (wt/wt) (+) acetaldehyde. Screw-cap tube culture, 4GSM.

use with a mutagen alone. The range of mutants was thereby limited to those acquired through allyl alcohol selection. This is a disadvantage since two allyl alcohol-selected mutants have been shown to have single amino acid substitutions (12) and as such would be more likely to revert than mutants produced by using a deletion mutagen.

The decrease in the combined yield of ethanol and acetaldehyde for strains selected at increased levels of allyl alcohol was potentially due to decreased growth for the allyl alcohol-selected strains, an increase in side-reaction product formation, or both factors. A decrease in growth was noted for strain RZ1E when it was grown under shake-flask conditions. However, little difference was noted between the growth curves of the wild-type and RZ1E strains during screw-cap tube culture. Since the growth rate of the wild type was shown to be inhibited by 0.05% (wt/wt) acetaldehyde, the difference in growth during shake-flask culture of the two strains may be related to differences in the level of acetaldehyde production by the two strains when they are grown under increased oxygen tension. No difference in final pH was observed between the wild-type and RZ1E strains grown in shake-flask cultures. This suggests that little to no excess acetic acid or lactic acid was produced in response to the decrease in alcohol dehydrogenase activity. Tests have not yet been conducted to determine whether there was any increase in sorbitol production by strain RZ1E over that of the wild type. Sorbitol is produced by *Z. mobilis* (2, 11); the greater reducing power of the increased NADH levels within the cell might produce polyhydroxylated compounds such as sorbitol or glycerol.

Approximately twice as much acetaldehyde was recovered by culturing strain RZ1E under batch stripped-acetaldehyde respiration as opposed to culturing under shake-flask conditions. This increase in the level of acetaldehyde produced may be attributed to the decreased inhibition of growth due to the separation of acetaldehyde from solution, the loss of acetaldehyde from the nonaerated shake flasks due to volatility of the acetaldehyde, and the limitation of alcohol dehydrogenase activity due to the increased level of oxygen available to the cells. Previous work has indicated that *Z. mobilis* does not show an increase in growth yield in the presence of oxygen; this may be the result of the formation of toxic products, specifically acetaldehyde (3).

In this study, 4.08 g of acetaldehyde per liter was produced from 4.0% (wt/wt) glucose when strain RZ1E was grown with batch stripped-acetaldehyde respiration. This level of acetaldehyde production compares favorably to the accumulation of 1.3 g of acetaldehyde per liter in an aerated continuous culture of wild-type *Z. mobilis* with a feed stream of 14% glucose, as reported by Bringer et al. (3). In addition, the level of acetaldehyde production achieved in this study is competitive with the 3.5 g of acetaldehyde per liter formed from the oxidation of 65 g of ethanol per liter by whole cells of *C. utilis* (1).

The growth rate of *Z. mobilis* is greatly decreased in the presence of acetaldehyde. In addition, the efficiency of stripping acetaldehyde from solution decreases with the decreasing concentration of acetaldehyde in solution. Therefore, the selection of acetaldehyde-tolerant mutants is of critical concern to the overall feasibility of stripped-acetaldehyde respiration. It is not clear what changes in genotype might be responsible for the increased acetaldehyde tolerance of the tolerance-selected strain. Since *Z. mobilis* does not appear to have an aldehyde dehydrogenase to convert acetaldehyde to acetate, the decreased level of acetaldehyde in solution with the growth of both the wild-type and

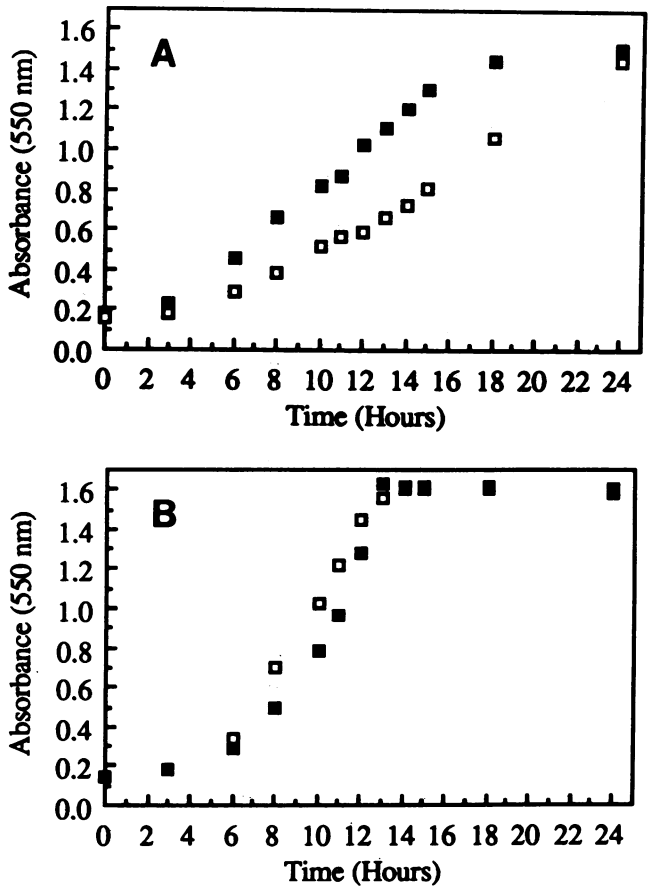

FIG. 5. Growth curves for wild-type and acetaldehyde-tolerant strains of *Z. mobilis* with 0.0 (A) and 0.1% (B) (wt/wt) acetaldehyde. Symbols: □, wild type; ■, strain RZt1.0. Screw-cap test tube culture, 4GSM.

acetaldehyde-tolerant strains may be due to the formation of complexes between acetaldehyde and protein or other cellular material. The acetaldehyde-tolerant strains have a more translucent milky appearance in colony morphology, although no difference in cellular morphology could be ascertained microscopically at ×1,000 magnification. Although the data obtained indicate that it is possible to select for strains of *Z. mobilis* which are tolerant to acetaldehyde, it is important to determine whether this phenotype can be compatible with increased acetaldehyde production by the organism.

Stripping acetaldehyde from the growing culture is advantageous in that it both decreases growth inhibition due to product buildup and represents a potentially economical means of product recovery. If the acetaldehyde is efficiently recovered from the exhaust gases, the stripped-acetaldehyde respiration represents an important advantage over the fermentation of ethanol; the product is continuously separated from solution without the need for a distinct distillation step. To increase the efficiency of acetaldehyde production and to further understand the commercial feasibility of this production method, a number of factors must be addressed: the techniques for the selection of mutants with increased tolerance to acetaldehyde and increased levels of acetaldehyde production, the stabilization of the selected mutants, the

optimization of both oxygen delivery and gas stripping, and the method of trapping acetaldehyde from the exhaust gases.

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