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Response of *Clostridium Acetobutylicum* to the Presence of Mixed Extractants

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ABSTRACT

The volumetric productivity of many fermentations is product-limited. *In situ* removal of these products with liquid organic extractants is limited by either a low product distribution coefficient or toxicity of the extractant. This paper presents results from studies using mixed extractants, namely mixtures of toxic extractants that have high distribution coefficients for the product and nontoxic extractants that have low distribution coefficients. The production of butanol by *Clostridium acetobutylicum* was chosen as a model system for these studies. The mechanisms of toxicity of mixed extractants and the observed responses to their presence are discussed.

Index Entries: *Clostridium acetobutylicum*; fermentation; extraction; toxicity; alcohol.

INTRODUCTION

Most fermentations are limited by the products that are formed. The microorganisms that synthesize these products are limited by various mechanisms. The product can repress the synthesis of certain enzymes in the biochemical pathway leading to the product. The product can directly inhibit the activity of one or more of these enzymes. Additionally, the product may elicit a general toxicity to the microorganism. The net

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result of any of these mechanisms is lower volumetric productivity and final product concentration.

Another major problem associated with industrial fermentations is product recovery. The product is usually present in very dilute concentration in viscous fermentation broths that contain numerous suspended and dissolved solids. Furthermore, some products are prone to biochemical degradation soon after their synthesis has ceased. Thus recovery of a product following a fermentation is often difficult and contributes significantly to the total cost of a product.

In light of these limitations, interest has developed in extractive fermentation, namely, *in situ* removal of a product during the fermentation. Various methods of *in situ* product removal have been proposed including vacuum fermentation, membrane processes, the addition of solid adsorbents to the broth and the use of liquid extractants (*1*). This paper focuses on the use of liquid extractants.

Past investigations of the use of immiscible organic extractants have accumulated data on the toxicity of various compounds (*2–10*). These studies often assess the effects that various compounds have on growth and then use the ones that are not inhibitory for further studies. Little effort has been made to understand the mechanism of toxicity.

Gill and Ratledge (*11*) have shown that the toxicity of *n*-alkanes, *n*-alk-1-enes, *n*-alkan-1-ols and *n*-alkyl-1-bromides toward *Candida tropicalis, Candida* 107, and *Saccharomyces calsbergensis* may be related to the aqueous solubility of these compounds. They showed that toxicity was reduced by adding a nontoxic compound such as hexadecane to the organic phase. The authors hypothesized that the reduction in toxicity was attributable to a reduction in the aqueous concentration of the toxic compound in accordance with equilibrium thermodynamics. In other words, the decrease in the concentration of the toxic compound in the organic phase caused a concomitant decrease in the aqueous phase. However, the growth experiments were completed in shake flasks and thus surface effects were not controlled.

This paper presents studies with an extractant design based upon the concepts presented above. The design involves the use of mixed extractants, namely mixtures of toxic and nontoxic extractants. The reason for such an approach is that most pure, organic compounds are unsuitable for use as extractants. *Clostridium acetobutylicum* was chosen as a model system for this research. *C. acetobutylicum* is an anaerobic microorganism that produces acetate and butyrate during growth and then reduces these compounds to ethanol, acetone and butanol after growth has been completed. Whereas butanol is generally regarded as being the most toxic product in this fermentation (*12,13*), Leung (*10*) found that the inhibitory effects (i.e., to growth) of butyrate and butanol were additive; thus, butyrate may also play an important role in product toxicity to *C. acetobutylicum*.

METHODS

Equilibrium Studies

Butanol distribution coefficients were measured by mixing 900 μL of distilled, deionized water with 900 μL of mixed extractant and with variable volumes of butanol such that the equilibrium, aqueous butanol concentrations ranged from 0 to 12 g/L. Two replicates were completed and their standard deviation was generally less than 5%. Mixtures were placed in 2 mL serum bottles, capped with teflon-lined seals, shaken thoroughly and then incubated for 24 h at 34°C. After the incubation period the phases were separated by centrifugation and assayed for butanol by gas chromatography. A mass balance for butanol generally yielded greater than 90% recovery. For each extractant mixture the equilibrium data for organic versus aqueous butanol concentrations were linearly regressed (least squares) and the distribution coefficient was set equal to the slope. The correlation coefficient (r^2) was generally greater than .99.

The equilibria between *n*-alkanols in organic and aqueous phases were determined in a similar manner to that for the butanol distribution coefficients. However, only the aqueous phase was assayed for *n*-alkanol concentration and these data were plotted against the volume fraction of *n*-alkanol in the organic phase. The volume fraction is defined as the volume of the *n*-alkanol divided by the total volume of the organic phase. The standard deviation for two replicates was generally less than 10%.

Cell Culture and Maintenance

C. acetobutylicum ATCC 4259 was used throughout this study. The culture was maintained as spores. Sporulation was completed in a 5% (wt/v) cooked corn meal medium (*10*). The culture was grown in CAB medium (*14*) with .5 or 6.0% (wt/v) glucose at 34°C. All media were prepared anaerobically as described by Baronofsky et al. (*15*) with the following exception: sparging of medium was done with nitrogen, instead of carbon dioxide, which was passed through heated BASF R3-11 catalyst to remove traces of oxygen. Inocula were prepared by adding 2% (v/v) spore suspension to 10 mL of CAB medium with .5% glucose and .1 mL of 2.5% (wt/v) $Na_2S{\cdot}9H_2O$ as a reductant and heat shocking at 80°C for 2 min. After 24 h this culture was used to inoculate (5% v/v) 50 mL of CAB medium with 6% glucose and no $Na_2S{\cdot}9H_2O$. This culture was grown for 12 h before being used as inocula for an experiment.

Growth and Production Studies

Biphasic fermentations (i.e., fermentations containing a discrete organic phase) were completed using 10 mL each of medium and extractant. Extractants were made anaerobic by repeatedly evacuating serum

bottles containing the extractants and flushing with anaerobic nitrogen. Media and extractants were either mixed in an anaerobic glove box using pipettors and then sterilized or sterilized separately and mixed using sterile syringes. Both methods gave similar results. With either method, the sterile tubes containing both phases were incubated at 34°C overnight prior to inoculation in order to equilibrate the *n*-alkanols in each phase. The tubes were then inoculated (5% v/v), inverted three times to mix the inoculum with the medium, and incubated at 34°C in an upright position without shaking The cultures were harvested after 20 h incubation for growth studies and after 36 h for product studies.

Monophasic fermentations (i.e., fermentations in which *n*-alkanol was present only as dissolved solute in the aqueous phase) were more complicated than biphasic fermentations. The fermentation tubes have butyl rubber stoppers into which decanol can dissolve. Preliminary experiments demonstrated that significant decanol losses from the medium occurred because, even though decanol has a vapor pressure of only .1 mm Hg at 34°C (extrapolated from data in (*16*)), vaporization from the aqueous phase, diffusion through the gas phase and dissolution into the stopper was significant. Therefore, aluminum foil disks were glued with silicone adhesive to the face of these stoppers to hinder decanol losses. Various amounts of decanol (3 replicates each) were added to the fermentation tubes by dispensing different volumes of a decanol solution in methanol and then evaporating the methanol under a stream of nitrogen. These tubes were then transferred to an anaerobic glove box and CAB medium was added and the tubes were sealed and sterilized. Extra tubes were made that were not inoculated but instead were assayed for decanol concentrations (3 replicates each). The standard deviations of the assayed cell and decanol concentrations were generally less than 10%. However, instances arose in which some of the replicates for a given decanol concentration grew and some did not, especially near the completely inhibitory decanol concentration. These data yielded a high standard deviation.

Adaptation and Fatty Acid Studies

Biphasic media were inoculated (1% v/v) and grown to an optical density (600 nm, path length = 1.6 cm) of approximately 1.0. This time corresponded to the early to mid-exponential phase of growth. The tubes were immediately frozen until they were assayed for fatty acid composition. Lag phases were determined by plotting the optical density vs time data on semilogarithmic paper and finding the intersection of the lines that passed through the cell concentrations during the lag phase and during the exponential phase.

Analytical Methods

Cell concentration was measured as optical density by inserting fermentation tubes into a Spectronic 20 spectrophotometer and measuring

the absorbance at 660 nm. If the optical density was greater than .5, the culture was diluted with fresh medium.

Products and *n*-alkanol extractants were measured either on a Chromosorb 101 (80/100, 2 mm × 2 m) or a 10% AT1000 on Chromosorb W-AW (80/100, 2 mm × 2 m) column using a Hewlett-Packard 5840A gas chromatograph. Broth samples were centrifuged to remove cells and then 900 μL of sample supernatant or standard were added to 100 μL of 10 N H_3PO_4 to protonate the volatile fatty acids. The organic phase was also assayed for products and the total product concentration based on the aqueous volume was calculated.

Fatty acids were assayed by the method of Vollherbst-Schneck et al. (*17*) with the exception that a 10% SP-2330 on Chromosorb W-AW (100/120) column (2 mm × 2 m) was used (30 mL/min N_2, 150–230°C at 4°C/min, injector and FID at 250°C).

Glucose was measured by the Somogyi colorimetric method (*18*).

RESULTS AND DISCUSSION

Table 1 presents the toxicity and butanol distribution coefficients of a variety of potential extractants. Toxicity is defined in this particular instance as complete inhibition of growth. A generalization can be made that extractants are classifiable into one of two categories. One, of which the *n*-alkanols are exemplary, is composed of extractants that have good distribution coefficients for the product (i.e., greater than 1.0) but are toxic. The other category, composed of corn oil, hexadecane, and kerosene, describes extractants that are nontoxic but have low distribution coefficients. As is true for any broad generalization, exceptions always exist. Oleyl alcohol is both a good extractant and nontoxic. Unfortunately, compounds such as oleyl alcohol are exceptional rather than typical. Therefore, for extractive fermentation with liquid extractants to become a usable method of *in situ* product recovery, the scope of potential extractants must be widened.

Table 1
Butanol Distribution Coefficients and Toxicities of Pure Extractants

Extractant	Type	Distribution coefficient, 1 s.d.
Hexanol	Toxic	9.91
Octanol	Toxic	7.33
Decanol	Toxic	6.20 (.28)
Undecanol	Toxic	5.55 (.20)
Dodecanol	Toxic	5.14 (.09)
Oleyl alcohol	Nontoxic	3.21
Corn oil	Nontoxic	.653 (.036)
Hexadecane	Nontoxic	.148 (.011)
Kerosene	Nontoxic	.127 (.019)

In this vein, the use of mixtures of these two types of extractants is proposed as a method to obtain a nontoxic extractant that is characterized by a high distribution coefficient. Figure 1 illustrates the butanol distribution coefficient is approximately a linear combination of the individual coextractant distribution coefficients when a mole fraction basis is used. The addition of a small amount, approximately 10% (v/v) in this case, of a toxic coextractant, decanol, to a nontoxic coextractant, hexadecane, yields a mixed extractant with a distribution coefficient of 1.0. Growth experiments with *C. acetobutylicum* (data not shown) in the presence of a decanol-hexadecane mixed extractant demonstrate that growth is possible in mixtures composed of 20 to 30% (v/v) decanol in hexadecane.

Now that mixed extractants have been shown to be capable of demonstrating a satisfactory distribution coefficient, their toxicity was examined. The working hypothesis for these experiments was that the aqueous concentration of the toxic coextractant is the sole determinant of toxicity in a nondispersed system. The first step in testing this hypothesis was to assess the effect of the addition of nontoxic coextractants to the organic phase on the aqueous concentration of the toxic coextractant.

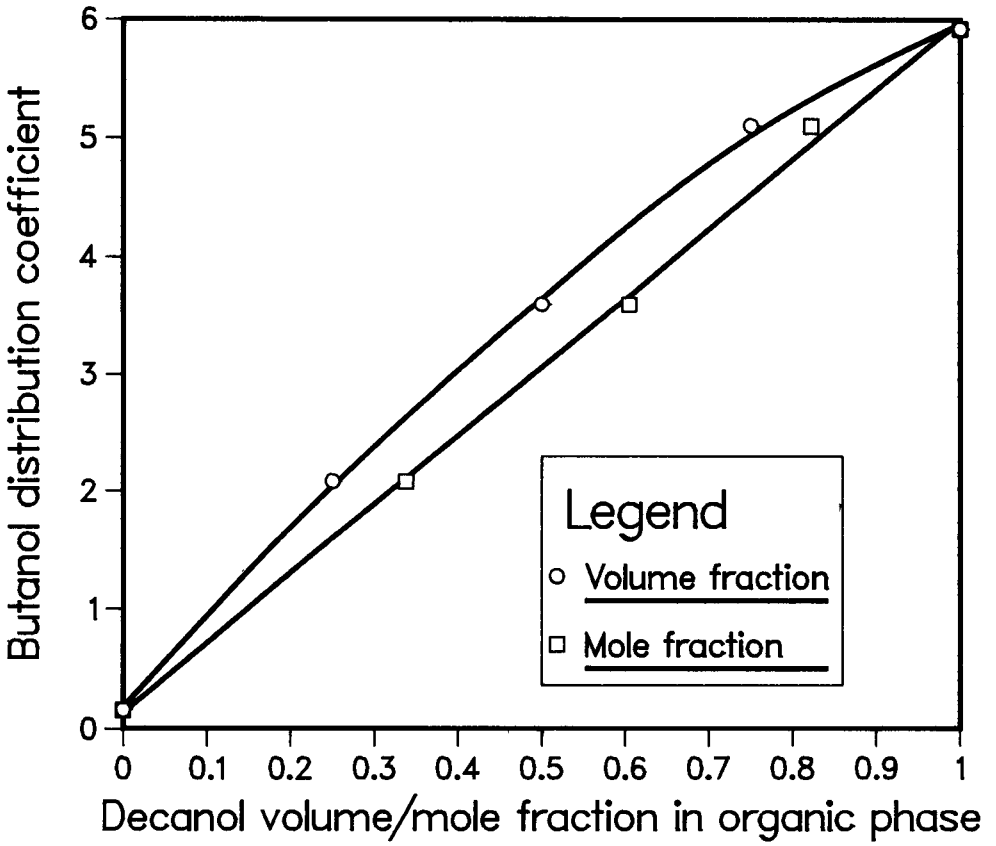

Fig. 1. Butanol distribution coefficient in a decanol-hexadecane mixed extractant.

Figure 2 illustrates such an experiment. The equilibrium, aqueous concentrations of decanol are plotted as a function of the decanol volume fraction in organic phases composed of corn oil, oleyl alcohol (cis 9-octadecen-1-ol), or hexadecane. A volume fraction of 1.0 is representative of pure decanol and thus the aqueous decanol concentration, 35 ppm, is the saturation concentration. This value corresponds well to a value of 34.5 ppm extrapolated from literature data (*19*) on alcohol solubility. Figure 2 shows that while the decanol-oleyl alcohol system is nearly ideal the corn oil and hexadecane systems are nonideal. These differences in ideality lead to different aqueous decanol concentrations at equal volume fractions of decanol in different nontoxic coextractants. For example, at a volume fraction of .2, the corn oil, hexadecane, and oleyl alcohol systems exhibit equilibrium, aqueous decanol concentrations of 24, 21, and 9 ppm, respectively. In order to support the hypothesis that toxicity is dependent on the aqueous decanol concentration, growth studies should demonstrate that, at equal decanol volume fractions above .1, the order of toxicity precedence should be corn oil, hexadecane, and oleyl alcohol, with corn oil being the most toxic.

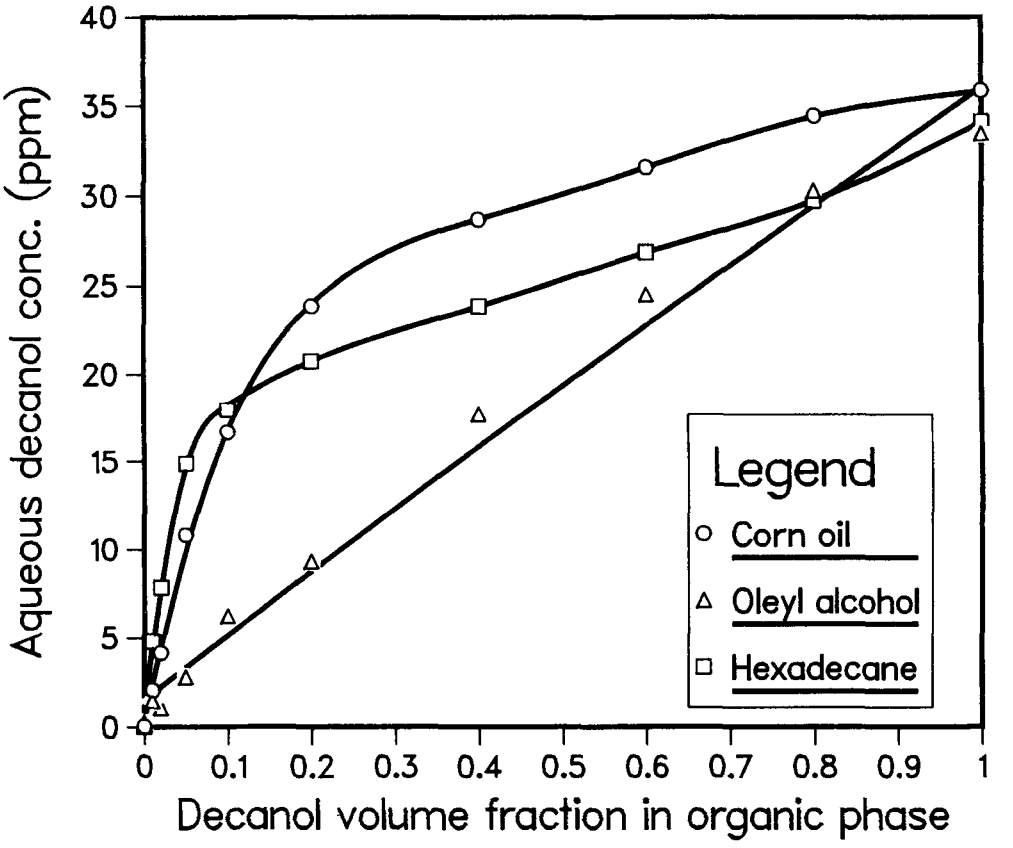

Fig. 2. Equilibrium, aqueous decanol concentrations in the presence of various mixed extractants.

The toxicity of mixed extractants to growth rather than to product formation was investigated initially because solventogenesis is inextricably linked to acidogenesis and growth in a batch fermentation. Therefore, if a mixed extractant is found to cause reduced butanol production, neither toxicity to growth, acidogenesis, or solventogenesis can be concluded.

Figure 3 illustrates the results from a representative study of the toxicity of a mixed extractant to growth. Equal volumes of medium and a mixed extractant of different compositions of decanol and oleyl alcohol were inoculated and incubated for 20 h at 34°C. The optical density of 3.4 achieved in pure oleyl alcohol (volume fraction of 0.0) is equal to that obtained with no organic phase. The optical density of the culture is observed to be inhibited slightly by increasing amounts of decanol up to a volume fraction of .4 and then strongly inhibited until .54, when growth is completely inhibited. The critical volume fraction, 0.54, is defined as the volume fraction of the toxic coextractant that completely inhibits growth. The critical aqueous decanol concentration is obtained from Fig. 2 or similar figures for other coextractants. Therefore, the critical aqueous decanol concentration for the oleyl alcohol coextractant is approximately

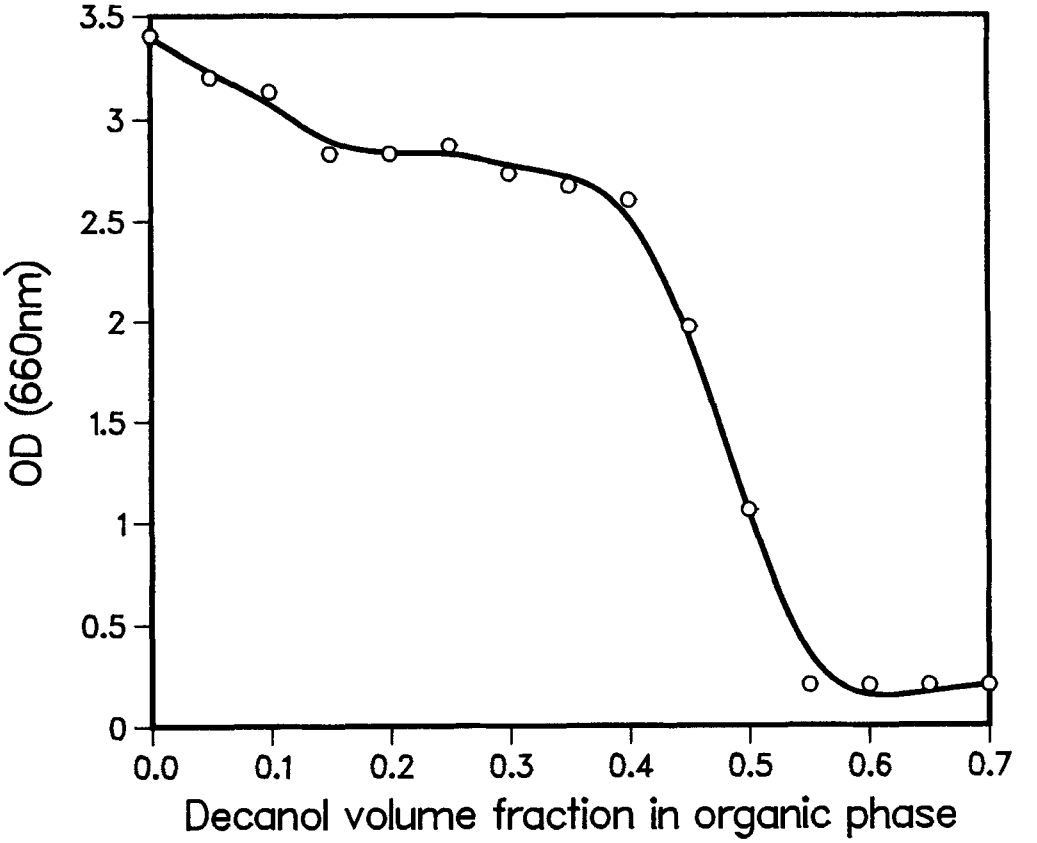

Fig. 3. Inhibition of total growth by the decanol-oleyl alcohol mixed extractant. OD is the optical density of the culture.

22 ppm. The volume fraction of decanol at which growth is inhibited by 50% is .48.

Critical decanol volume fractions and aqueous concentrations for other coextractants were obtained and are compared in Fig. 4. Also presented is the critical aqueous decanol concentration for a monophasic fermentation. This fermentation was completed in the absence of a discrete organic phase. Instead, the aqueous concentration of decanol was varied by adding different amounts of pure decanol to the broth prior to inoculation. Adsorption or absorption of decanol by the cells was found not to alter the aqueous decanol concentration significantly (data not shown).

A number of observations can be made from Fig. 4. First, the critical aqueous decanol concentration is independent of the critical decanol volume fraction for the biphasic fermentations with corn oil, hexadecane, tridecane, and oleyl alcohol. The range of volume fractions from .18 to .54 is not an artifact introduced by differences in molecular weight and density because a significant range across the abscissa is also observed when the data are plotted as a function of the critical decanol mol fraction in the organic phase (data not shown). Second, the order of toxicity precedence, namely corn oil, hexadecane, and oleyl alcohol, is found to cor-

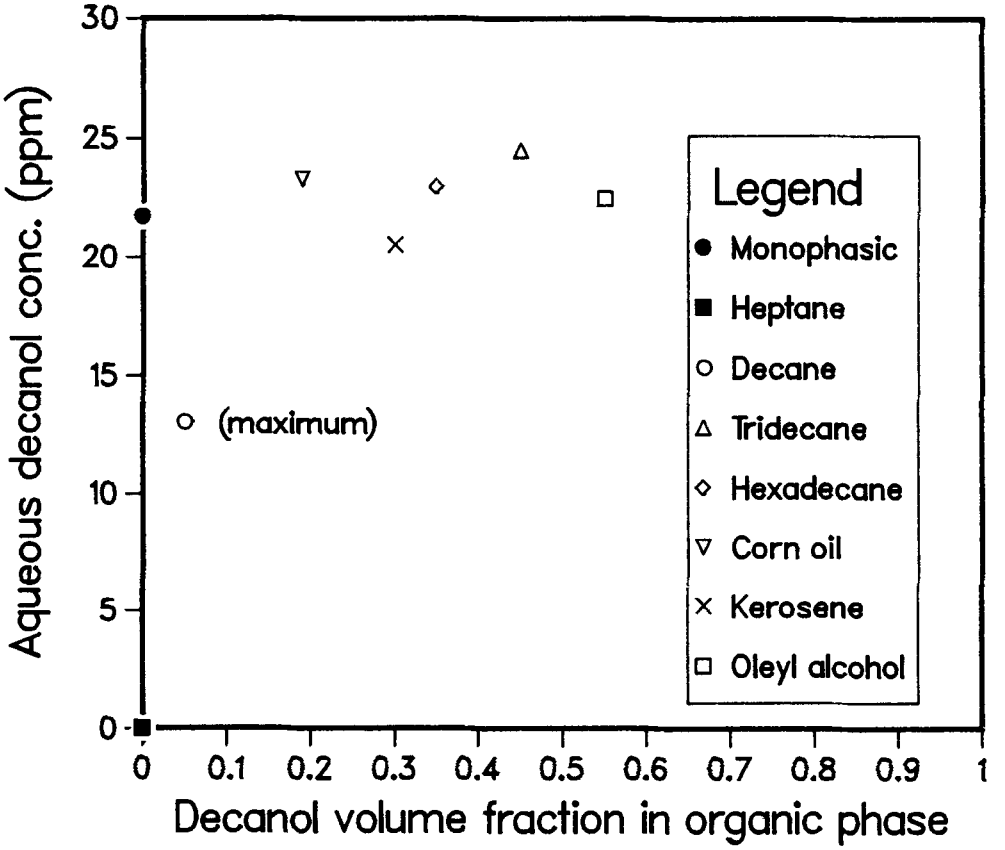

Fig. 4. Critical aqueous decanol concentrations in various monophasic and biphasic systems. The abscissa is meaningless for the monophasic system.

respond to that predicted from Fig. 2. Third, the critical aqueous decanol concentrations for the monophasic and biphasic fermentations are not observed to be significantly different. These observations support the hypothesis that the aqueous concentration of decanol is the sole determinant of toxicity to growth of *C. acetobutylicum* in a nondispersed system.

Another point that can be made about Fig. 4 is that the oleyl alcohol mixed extractant is the least toxic of all of the mixed extractants tested. On the basis of this fact, and on the butanol distribution coefficient (cf Table 1), oleyl alcohol was selected for future studies as the optimal nontoxic coextractant.

The decane–decanol mixed extractant exhibits a critical aqueous decanol concentration that is lower than that of the other systems. This value is probably too high but more accurate estimates were not obtainable because of technical difficulties in performing the experiment. The anomalous behavior of decane may be linked to the following reasoning. Tridecane and hexadecane, longer homologs of the *n*-alkane series, are nontoxic and act as completely nontoxic coextractants. On the other hand, heptane, a smaller homolog, is toxic by itself (cf Fig. 4). These observations are similar to those seen in the anesthetic action of *n*-alkanes or other structurally-homologous series (the analogy between *n*-alkanol toxicity and anesthesia is discussed below). A *cutoff* in anesthetic potency is observed at octane (*20*). In other words, *n*-alkanes longer than octane demonstrate no anesthetic action, unlike shorter *n*-alkanes. A possible reason that decane-decanol mixed extractant is anomalous is that decane is too close to the cutoff. The cutoff may occur because the membrane distribution coefficient of the alkanes may increase with size up to the cutoff and then decrease, as has been observed for *n*-alkan-1-ols (*20,21*). Thus, decanol may increase the membrane distribution coefficient of decane and make it a toxic coextractant. The same argument can be applied to kerosene, which is primarily a mixture of 10- and 11-carbon hydrocarbons (*22*), since it appears to have a slightly lower critical aqueous decanol concentration than the other nontoxic coextractants, and its observed order of toxicity precedence, between corn oil and hexadecane (see Fig. 4), is different from its predicted order, between tridecane and oleyl alcohol.

Next the analogy between *n*-alkanol toxicity and anesthesia was investigated. The *n*-alkanols in a mixed extractant were hypothesized to localize into the plasma membrane of *C. acetobutylicum* and elicit their effect much in the way anesthetics act. Anesthetic potency is directly related to the hydrophobicity of a compound and thus its ability to distribute into a biomembrane (*20,21,23–25*). Further, anesthesia is directly related to the *amount* of a compound that is present in a biomembrane and not the *type* of compound according to the Meyer-Overton hypothesis of anesthesia (*20,23*). Both of these properties are demonstrated by a linear relationship between anesthetic concentration and membrane distribution coefficient for different anesthetics on a full-log plot. The following deri-

vation illustrates this principle. The membrane distribution coefficient, K_{mem}, is defined as

$$K_{mem} = \frac{C_{mem}}{C_{aq}} \quad (1)$$

where C_{mem} and C_{aq} are the membrane and aqueous concentrations that cause anesthesia. Taking the logarithm of Eq. [1] and rearranging

$$\ln C_{aq} = - \ln K_{mem} + \ln C_{mem} \quad (2)$$

Therefore, a linear relationship between ln C_{aq} and ln K_{mem} that has a slope of -1.0 supports the Meyer-Overton hypothesis that C_{mem} is constant for different anesthetics. Figure 5 shows such a linear relationship ($r = .999$) between the aqueous *n*-alkanol (butanol through dodecanol) concentrations that inhibit growth of *C. acetobutylicum* by 50% and their respective dipalmitoyl phosphatidyl choline (DPPC) distribution coefficients. These distribution coefficients were taken from Jain et al. (*26*).

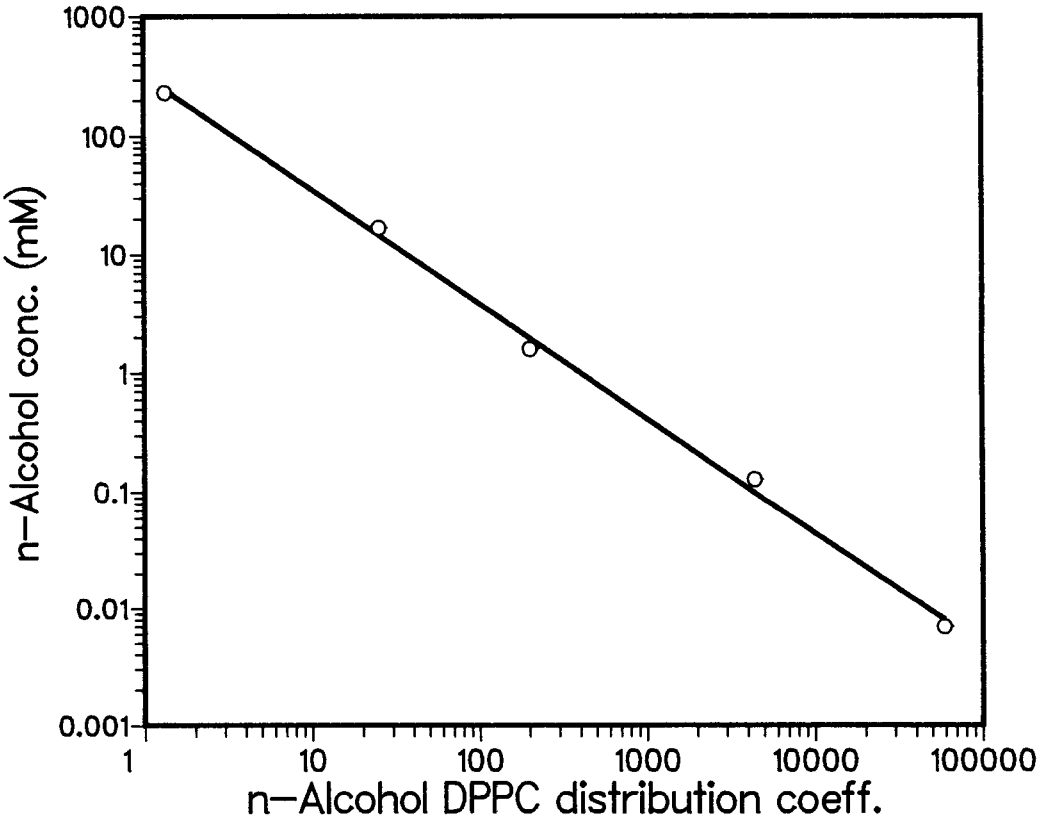

Fig. 5. Dependence of the aqueous *n*-alkanol concentrations that inhibit growth by 50% on their respective dipalmitoyl phosphatidyl choline (DPPC) distribution coefficients. The distribution coefficients were taken from reference (*26*). The *n*-alkanols include the even-carbon compounds butanol (far left) through dodecanol (far right).

Furthermore, the slope is equal to $-.967 \pm .083$ (95% confidence). DPPC was chosen as a model membrane for *C. acetobutylicum* because the plasma membrane of *C. acetobutylicum* is composed of 40–60% palmitoyl residues (*17,27*). While the principal phospholipid base of *C. acetobutylicum* is ethanolamine (*27*) and not choline, the base is not expected to affect the alcohol distribution coefficient as significantly as the acyl chain.

Once the nontoxic coextractant was optimized the optimal toxic coextractant (i.e., *n*-alkanol) was determined. Figure 6 illustrates how two opposing phenomena affected the optimization. Only even-carbon *n*-alkanols were investigated because they have the highest distribution coefficient for butanol of any homologous series and because they are less expensive than the odd-carbon moiety. As the chain length is increased the butanol distribution coefficient of the pure *n*-alkanol decreases making the shorter *n*-alkanols more desirable as toxic coextractants. On the other hand, as the chain length increases, the *n* alkanol volume fraction in oleyl alcohol that inhibits growth by 50% is observed to increase, thus making the longer *n*-alkanols more desirable

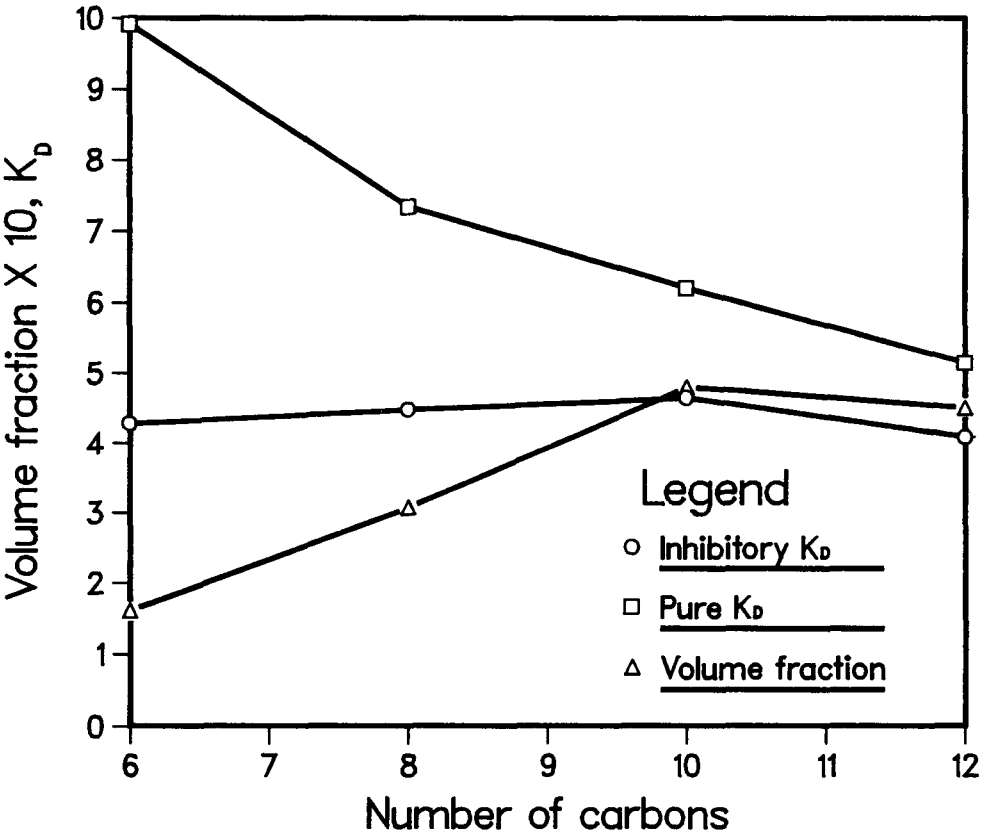

Fig. 6. Optimization of the *n*-alkanol coextractant. Pure K_D is the butanol distribution coefficient for the pure *n*-alkanol and inhibitory K_D is that for the *n*-alkanol/oleyl alcohol mixture that inhibits total growth by 50%. The volume fraction is the decanol volume fraction at which this inhibition occurs.

because more of the *n*-alkanol can be tolerated in the mixed extractant to yield a high distribution coefficient. To optimize these opposing phenomena, the butanol distribution coefficients of the mixed extractants that caused a 50% inhibition of growth were plotted as a function of the chain length of the toxic coextractants in Fig. 6. Whereas decanol appears to be slightly superior to the other *n*-alkanols, the main conclusion from this curve is the chain length of the toxic coextractant is not a significant parameter in optimization of a mixed extractant. The chain length is important, however, when recovery of butanol from the extractant is considered. Nevertheless, decanol was chosen for further studies.

Next the responses of *C. acetobutylicum* to the presence of a decanol-oleyl alcohol mixed extractant were studied. Figure 7 illustrates that increased decanol concentrations led to dramatically greater lag phases prior to growth. The change in decanol volume fraction from .34 to .44 represents an aqueous decanol change of approximately 4 ppm (cf Fig. 2). Ingram (*28*) observed an adaptive lag phase upon addition of 4% (v/v) ethanol to growing cultures of *E. coli*. This adaptation was associated with alteration of the fatty acid composition of the cell. Also, Vollherbst-Schneck et al. (*17*) observed an increase in the degree of saturation of the fatty acid composition of *C. acetobutylicum* ATCC 824 in response to the

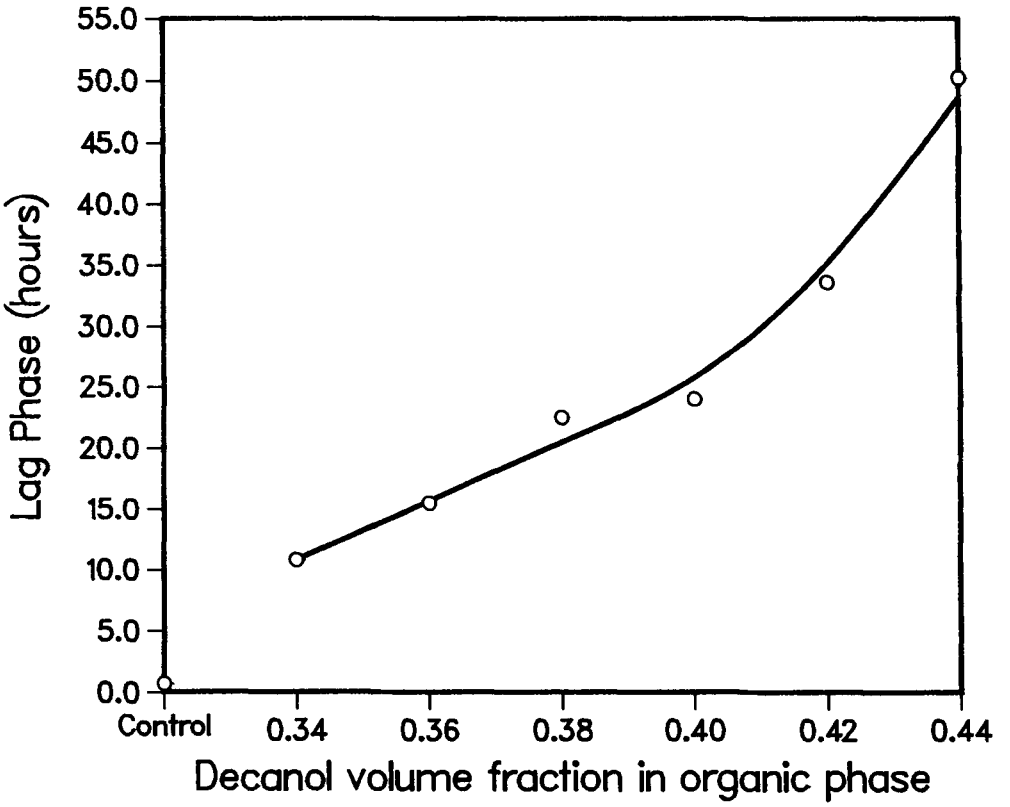

Fig. 7. Lag phase in the presence of decanol-oleyl alcohol mixed extractants. The control has no organic phase.

addition of .5 to 1.0% (v/v) butanol. Figure 8 shows a slight, if any, increase in saturation in response to decanol originating from a decanol-oleyl alcohol mixed extractant. No greater than .4 g/L of endogenous butanol was present in the aqueous phase of any tube, thus eliminating any potential effects of butanol on the fatty acid composition. This slight change in fatty acid composition does not appear to account for adaptive lag phases of up to 50 h greater than the control (viz., no organic phase). Thus another explanation for the lag phase must be sought. Maybe, as has been observed by other investigators (29–34) with various alcohols and cell or lipid systems, decanol disrupts the cellular permeability barrier allowing ions such as Mg^{2+}, Na^{+}, K^{+}, and H^{+}, nucleotides, phosphoenolpyruvate and 2-deoxyglucose-6-phosphate to leak out of or into the cell.

The final part of this study was devoted to studying how product formation is affected by the presence of a decanol-oleyl alcohol mixed extractant. Figures 9 and 10 show the responses of acetate and butyrate that are produced during growth of acetone and butanol, which are pro-

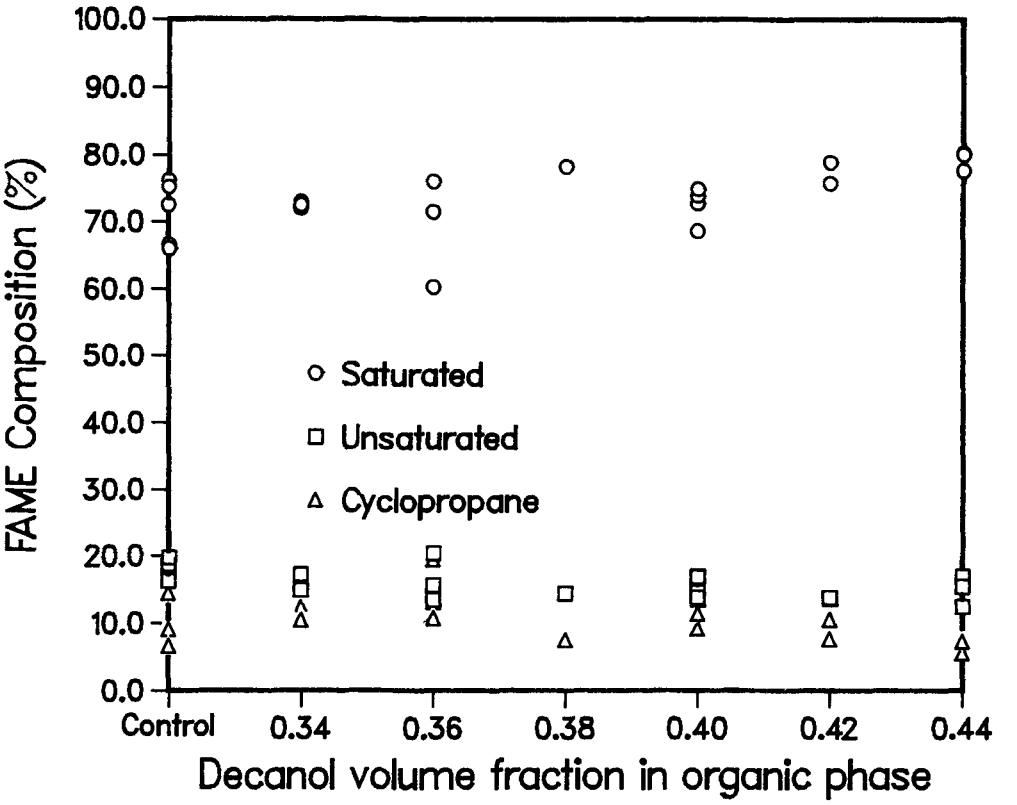

Fig. 8. Fatty acid composition after adaptation to decanol-oleyl alcohol mixtures. FAME is the fatty acid methyl esters. The saturated fatty acids include 14:0, 16:0, and 18:0. The unsaturated fatty acids include 16:1 and 18:1. The cyclopropane fatty acids include $17:0^{\Delta}$ and $19:0^{\Delta}$. The control had no organic phase.

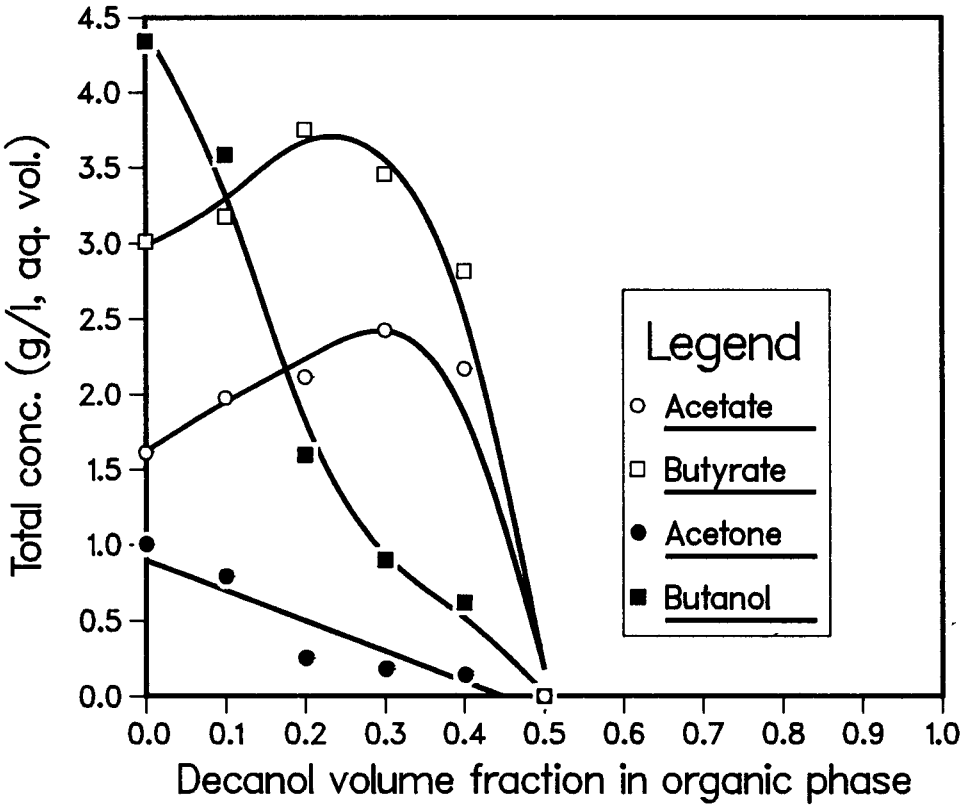

Fig. 9. Total acids and solvents with decanol in oleyl alcohol. The concentrations are the total amounts produced based upon the medium volume.

duced after growth, and of glucose consumption. Ethanol is not presented because it is present as an impurity in decanol. The final concentration of the metabolic acids increase and then decrease, whereas the solvent concentrations decrease monotonically as the volume fraction of decanol increases. On the other hand, the acid yields increase monotonically at the expense of the solvent yields all the while the glucose consumption decreases. The maxima in acid concentrations appear to arise from the initial dominance of the increasing acid yield over the decreasing glucose consumption and then vice versa. A complete understanding of the effects of the mixed extractant is not possible with these data alone, but the increases in acid yields may be a result of the extraction of butyrate—a metabolic uncoupler. The measured distribution coefficients of free butyric acid in oleyl alcohol and decanol are 1.5 and 3.5, respectively. The distribution coefficients at a pH of 4.5 were calculated with the Henderson-Hasselbalch equation (35) to be 1.0 and 2.4, respectively. Thus butyrate extraction may be significant. Since butyrate is reduced by *C. acetobutylicum* to butanol, the decrease in butanol yield may have occurred because the butyrate was extracted before it could be reduced. An increase in acidogenesis at the expense of solventogenesis may also occur if decanol is inhibitory to the reduction of acids to the neutral solvents.

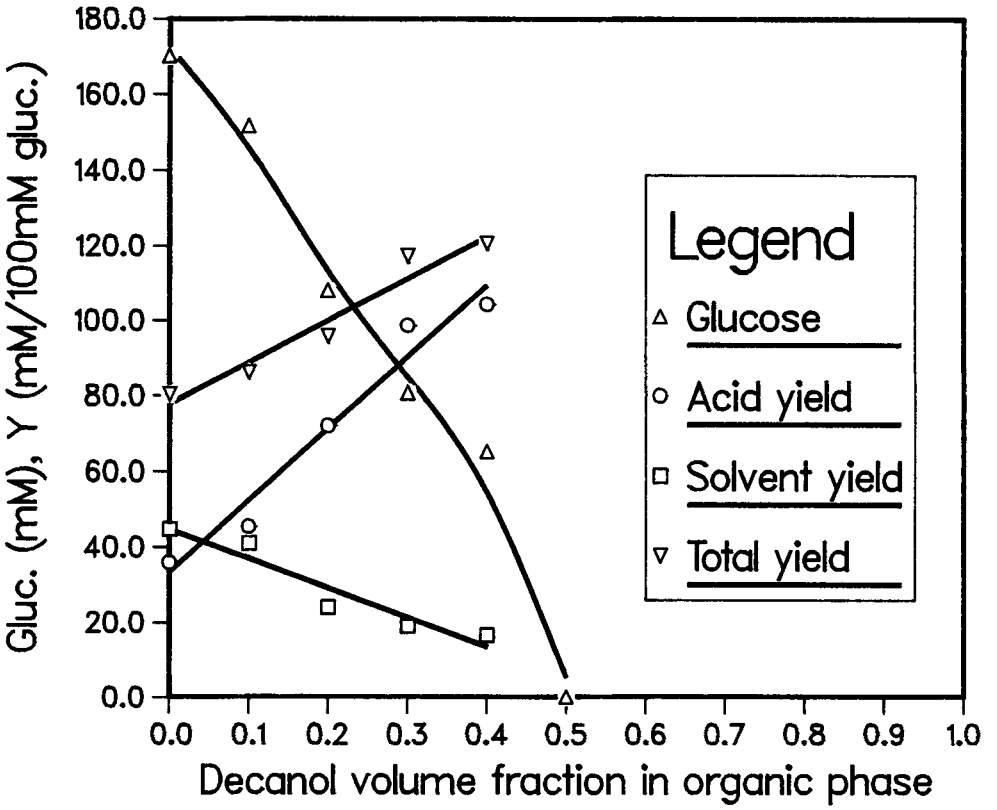

Fig. 10. Glucose consumption and product yield in decanol and oleyl alcohol. *Gluc.* is the glucose consumed. *Y* is the product yield in mmol/100 mmol of glucose consumed. The solvents do not include ethanol.

Which of these or other hypotheses is correct is currently being investigated.

CONCLUSIONS

The addition of a small amount of a toxic coextractant such as decanol to a nontoxic extractant such as hexadecane was shown to increase the butanol distribution coefficient to a value greater than one. The resulting *mixed extractant* is not completely inhibitory to growth, as is pure decanol.

The principal conclusions that can be made from the growth studies are 1) the aqueous concentration of the toxic coextractant solely determines the toxicity of a nondispersed mixed extractant, 2) the ability of the nontoxic coextractant to decrease the aqueous concentration of the toxic coextractant (which is related to the thermodynamic ideality of the mixture) is an important factor in optimizing a mixed extractant; therefore, oleyl alcohol was selected as an optimal nontoxic coextractant, and 3) the chain length of the *n*-alkanol toxic coextractant is not an important pa-

rameter with regard to optimization of the mixed extractant. The homologous *n*-alkanol series is characterized by two opposing phenomena, the butanol distribution coefficient *decreases* and the allowable (i.e., with respect to toxicity) volume fraction in the organic phase *increases* as the chain length increases.

C. acetobutylicum was found to adapt to decanol as illustrated by the increased lag phase with greater volume fractions of decanol in the mixed extractant. Alteration of the fatty acid composition of the plasma membrane does not appear to account for this adaption.

The motivation behind the use of a mixed extractant was to increase the butanol production. Contrarily, the acid yield increased at the expense of the solvent yield. The reason for this is unknown at present and possible explanations include but are not limited to direct toxicity of decanol to solventogenesis, and extraction of metabolic intermediates such as butyrate.

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