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Redox Potential in Acetone–Butanol Fermentations

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ABSTRACT

In batch and continuous cultivations of *Clostridium acetobutylicum* ATCC 824 on lactose, a strong relationship was observed between redox potential of broth and the cellular metabolism. The specific productivity of butanol as well as of butyric acid was found to be maximum at a redox potential of −250 mV. The specific production rate of butyric acid decreased rapidly at redox potentials. For butanol, however, it achieved a lower but stable value. This was true for dynamic as well as steady states. The continuous fermentations involving lactose exhibited sustained oscillations at low dilution rates. These oscillations appear to be related to butanol toxicity to the growth of cells. At higher dilution rates where butanol concentrations were relatively low, no such oscillations were observed. It can be concluded that broth redox potential is an excellent indicator of the resulting fermentation product partitioning.

Index Entries: Acetone-butanol fermentation; *Clostridium acetobutylicum*; redox potential; continuous culture; lactose.

INTRODUCTION

Environmental changes during production of acetone–butanol from *Clostridium* strains have been the subject of several previous investigations (*1–4*). Typical batch fermentations are associated with an initial phase of active growth during which mainly acidic products are released by the cells. This initial phase is followed by a second phase in which

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neutral solvents are produced, generally accompanied by the consumption of some of the acids formed earlier. This shift in cellular metabolism has been a subject of intensive study (*3,5,6*). Pyruvate metabolism, specifically the reactions associated with the formation of acetyl-CoA, appears to be a key to the diversion of carbon skeletons to acids or to solvents (*7–9*). (Fig. 1). These reactions are predominantly concerned with the transfer of electrons between the reduced and oxidized forms of nucleotides and ferredoxin, and their ultimate release as hydrogen. These reduced compounds as well as hydrogen influence the oxidation-reduction potential of the broth (*10,11*). Although several studies have addressed the role of redox potential in anaerobic systems, no quantitative information is available in published literature showing the effect of redox potential on the different metabolic processes in clostridial fermentations.

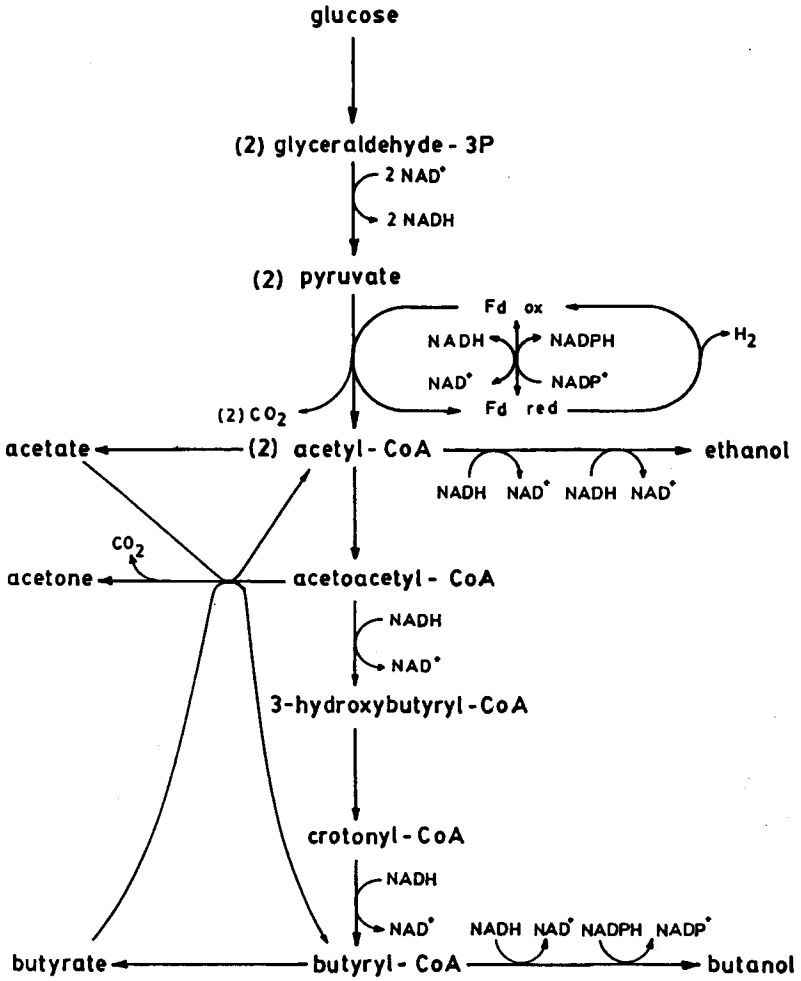

Fig. 1. Schematic biochemical pathways in *Clostridium acetobutylicum*.

This study addresses the relationships between redox potential of broth and the growth and product formation phenomena observed in continuous acetone–butanol fermentations of lactose.

MATERIALS AND METHODS

Microorganism

Clostridium acetobutylicum ATCC 824 was used in this study. Spores were maintained on cornmeal medium under anaerobic conditions at 4°C (*12*).

Medium

A soluble complex medium consisting (in g/L) of lactose, 60; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 0.75; K_2HPO_4, 0.75; $MgSO_4$, 0.02; $MnSO_4 \cdot H_2$), 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 1.0; cystein, 0.5; yeast extract, 5.0; asparagine, 2.0 in distilled water was used for inoculum preparation and for experiments.

Cornmeal medium consisted of 100 g corn meal, 1.5 g agar, and 0.1 mL resazurin/L of distilled water.

Fermentor Assembly

The experiments were conducted in a 2-L Virtis "Omni-Culture" fermentor (1-L working volume) equipped with an overflow tube for chemostat operation. The speed of agitation and temperature was 200 rpm (standard turbine agitator) and 37°C, respectively. pH was measured using an autoclavable Ingold electrode (model 5003-K9) and was controlled at 5.0 in all the experiments by a Horizon pH controller model 5997 using 5N NaOH solution. Redox potential was monitored using an Ingold redox electrode (model Pt. 4865-35-K9). The autoclavable redox electrode was calibrated using Ingold's standard redox buffer (cat. No. 18503) at 25°C and pH 7.0. Foaming was controlled manually by addition of a few drops of 1:10 diluted SAG5693 silicone antifoam agent from Dow Corning. Head space of the fermentor was continuously flushed with oxygen-free carbon dioxide gas in order to ensure an anaerobic environment.

Continuous fermentations were initiated from actively growing batch cultures in the fermentor by starting a continuous flow of feed solution that was also maintained free of oxygen by continuously passing oxygen-free carbon dioxide through the feed reservoir. Dilution rates were varied between 0.02 and 0.05 h^{-1}. For each dilution rate, samples were drawn twice daily after at least three turnovers of fluid in the reactor. Feed medium was changed every 5 d with freshly prepared solutions. At each dilution rate, sampling was continued for a duration of at

least five retention times. In cases where oscillations were observed, several fresh starts were made to ensure absence of any operational reasons for the oscillations.

Sample Analysis

Samples were analyzed for the concentrations of cells, sugar, and products in the broth. Cell concentration was measured by measuring the optical density at 630 nm as well as by dry weight measurements after centrifugation at 10,000 rpm in a refrigerated centrifuge for 10 min and several washes.

Concentrations of solvents (ethanol, acetone, and butanol) and acids (acetic and butyric) were determined using a Varian model 1520 gas chromatograph equipped with a glass column (6 ft long, 2 mm internal diameter; packed with Chromosorb WAW 80/100 having 1% AT1000) and an FID detector. Helium was used as carrier gas and oven temperature was programmed to increase linearly from 70 to 140°C at a rate of 30°C/min. The temperature of the injector and detector were 250 and 22°C, respectively.

Lactose was measured by the Nelson-Somagi method (*13*). Injections of broth into a Perkin Elmer Series 4 liquid chromatograph equipped with a size exclusion Brown Lee column and a Perkin Elmer refractive index detector showed only negligible amounts of monosaccharides, primarily glucose.

RESULTS

A number of experiments were conducted at dilution rates ranging from 0.02 to 0.05 h^{-1}. Higher concentrations of the solvents were obtained at the lower dilution rate, but the steady state showed a characteristic oscillatory behavior in all the measured culture parameters. A typical set of results involving total solvent concentration and the redox potential is presented in Fig. 2. Similar oscillations were obtained with the concentrations of biomass and acids (data not shown). It was established by observations from several independent experiments that these oscillations were not artifacts resulting from experimental procedures. At higher dilution rates, the oscillations were either not present or were marginal (Fig. 2). The solid lines in this and all subsequent figures represent the trends perceived by the authors.

In Fig. 3, the redox potentials at different dilution rates are shown. Under steady-state conditions, the lower the dilution rate, the lower the redox potential in broth. The bars represent the range of redox potentials at each dilution rate (D). The observations under all operating conditions except the one at $D = 0.03\ h^{-1}$ were steady and studied for several residence times. The operation corresponding to 0.03 h^{-1} was an unsteady one during which a malfunction in pH control resulted in system failure.

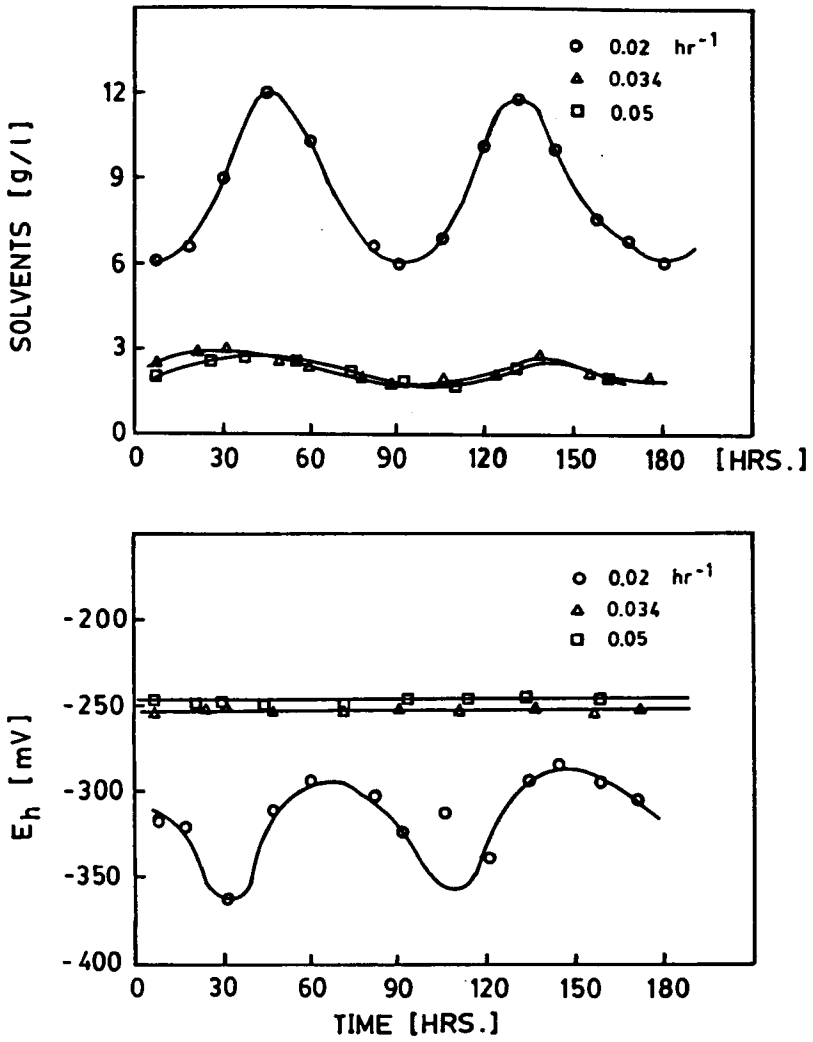

Fig. 2. Concentration of solvents and redox potential in broth during continuous cultivation of *Clostridium acetobutylicum* ATCC 824 on lactose.

The results are included in this analysis since this state corresponded to unusually high redox potential in broth; the cellular metabolism was also slower under these circumstances.

Figures 4 and 5 show the concentrations of various fermentation products as well as the amount of lactose consumed as a function of broth redox potentials for the different dilution rates. Production of cell mass, solvents, and lactose consumption increased at lower redox potential, whereas that of butyric acid decreased. The concentration of acetic acid did not depend upon broth redox potential. At redox values above −200 mV, the metabolism appears to be strongly inhibited. The same phenomenon is expressed by the yield (based upon lactose consumed) of

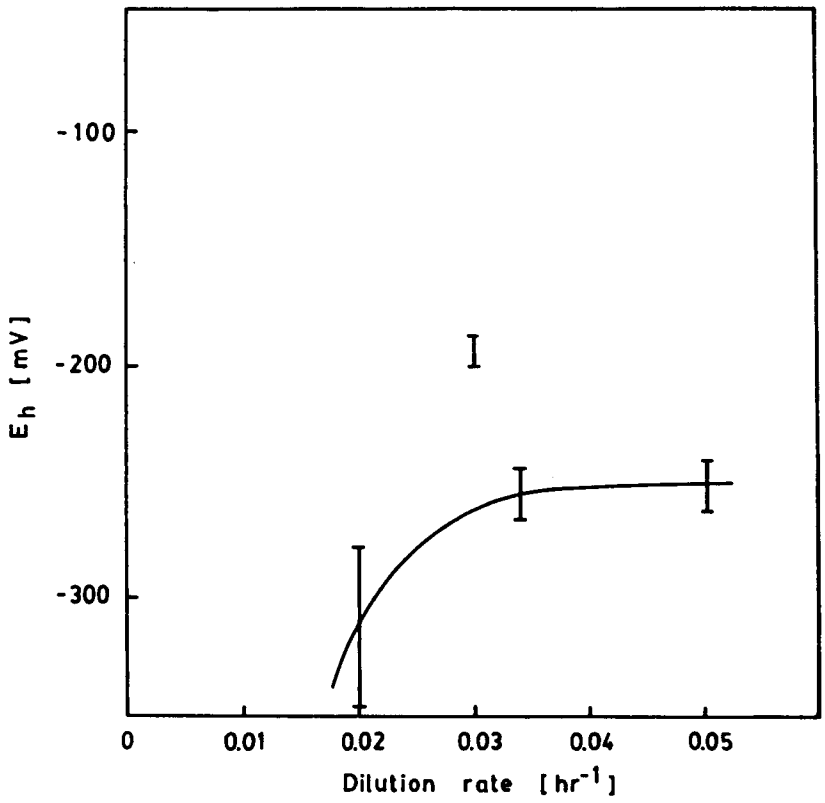

Fig. 3. Broth redox potential as a function of dilution rate.

different products under different conditions, shown in Fig. 6 as function of redox potential.

The specific (net) rates of production of butanol and butyric acids are plotted in Fig. 7 against broth redox potential; both show a maximum at −250 mV. Reduction in these rates at higher redox values confirms slowing down of the metabolism. Decrease of the rates at the lower redox values also points to the possible inhibition of the glycolytic flux caused by increased concentrations of reduced dinucleotides in the cells.

DISCUSSION

The Oscillatory Nature of Steady State

From the theory of continuous operation, one would expect the possibility of oscillating steady states when toxic products are formed in the system. The toxicity of acids and solvents to cellular metabolic phenomena is well documented (*14*). Yet, in the considerable amount of published continuous culture data dealing with acetone–butanol fermentations with glucose, no oscillatory steady states have been reported. In

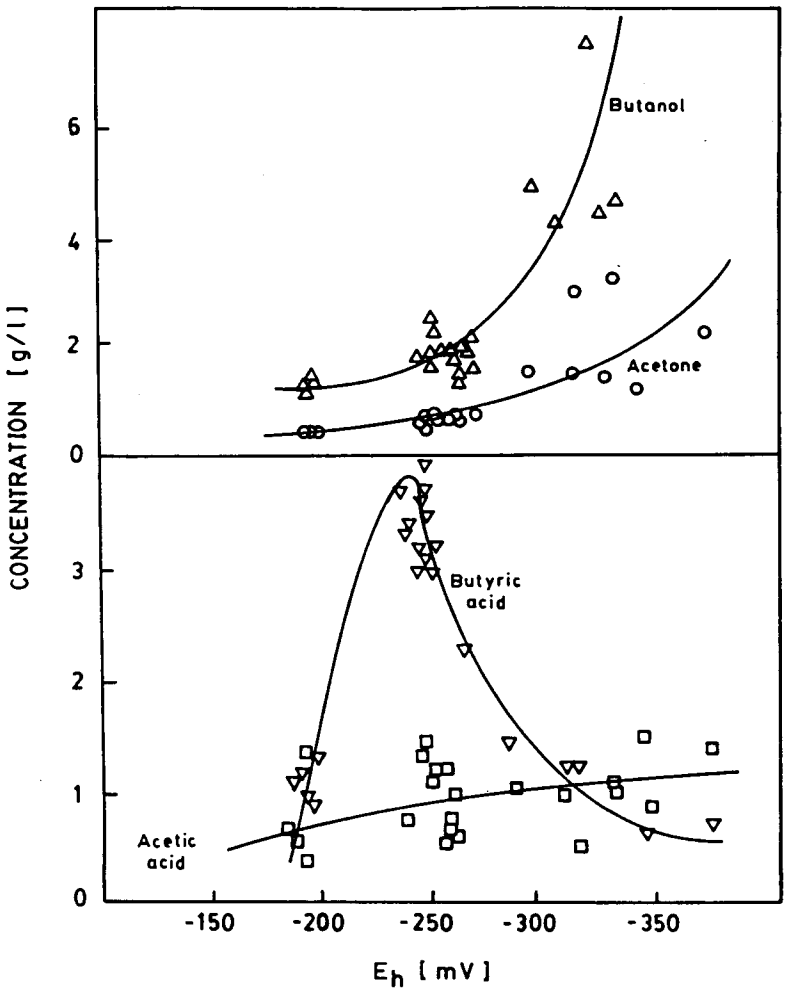

Fig. 4. Concentrations of products as function of redox potential.

our own few experiments involving glucose (data not shown), we also found no oscillations under steady state conditions. However, with lactose as the carbon source, an oscillating steady state was observed (Fig. 2). Considerable precautions were taken to ascertain the validity of these results. Moreover, the level of solvents obtained in these two fermentation systems (glucose and lactose) were similar.

A careful analysis of the observed trends revealed that a minimum in redox potential coincided with total solvent concentrations of 9.0 g/L in broth. At this time, the butanol concentration was 7 g/L. Microscopic examinations of samples drawn at this point also showed changes in cell morphology; simultaneously, cell density started to decrease. The solvent concentrations peaked at 12 g/L and reduced to 6.5 g/L before an increase in cellular activity with an associated decrease in redox potential was observed.

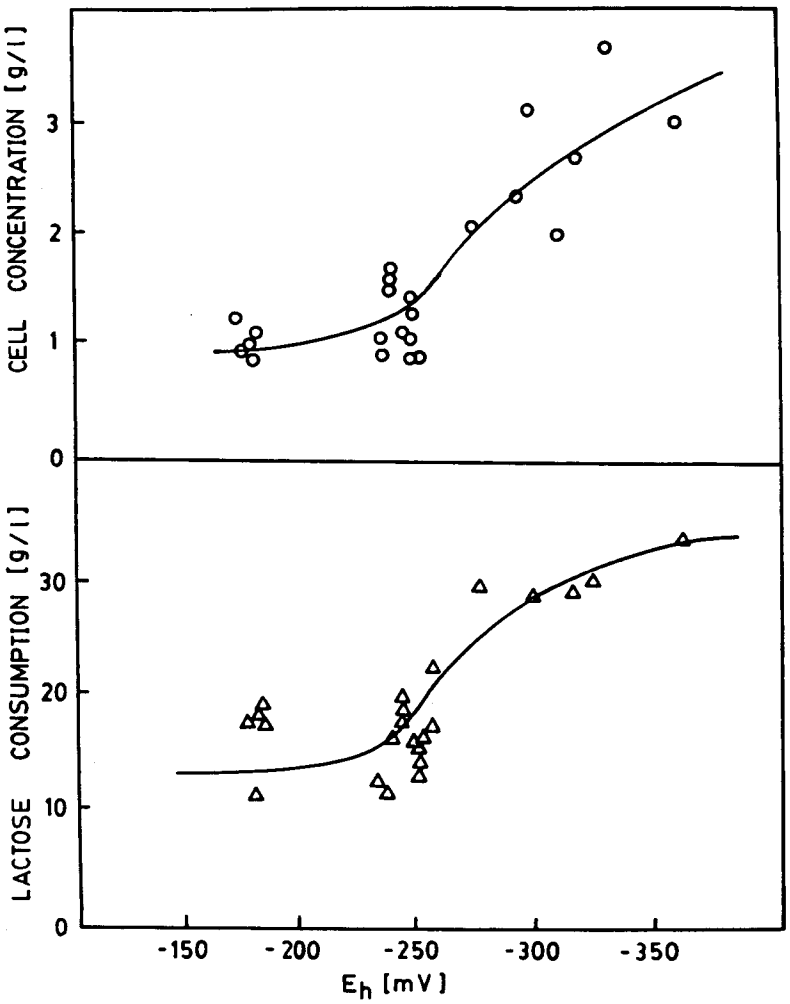

Fig. 5. Changes in cell concentration and lactose consumption as function of redox potential.

The extent of oscillations appears to be related to the levels of solvents produced. At higher dilution rates where relatively low amounts of solvents were formed, the oscillations were also considerably less. Under the unsteady operating condition corresponding to $D = 0.03\ h^{-1}$, the concentrations of products formed as well as the oscillations were the least.

Butanol has been reported to be the most toxic product in this fermentation system (*15*); 7–13 g/L of butanol reduces growth rates of *C. acetobutylicum* cells by 50%. Since no similar oscillations have been found in continuous cultivations on glucose, one may postulate that butanol is more inhibitory to parts of lactose metabolism that are different from that of glucose. This would suggest lactose uptake and hydrolysis as the likely locations of high inhibition. Galactose metabolism is not a likely

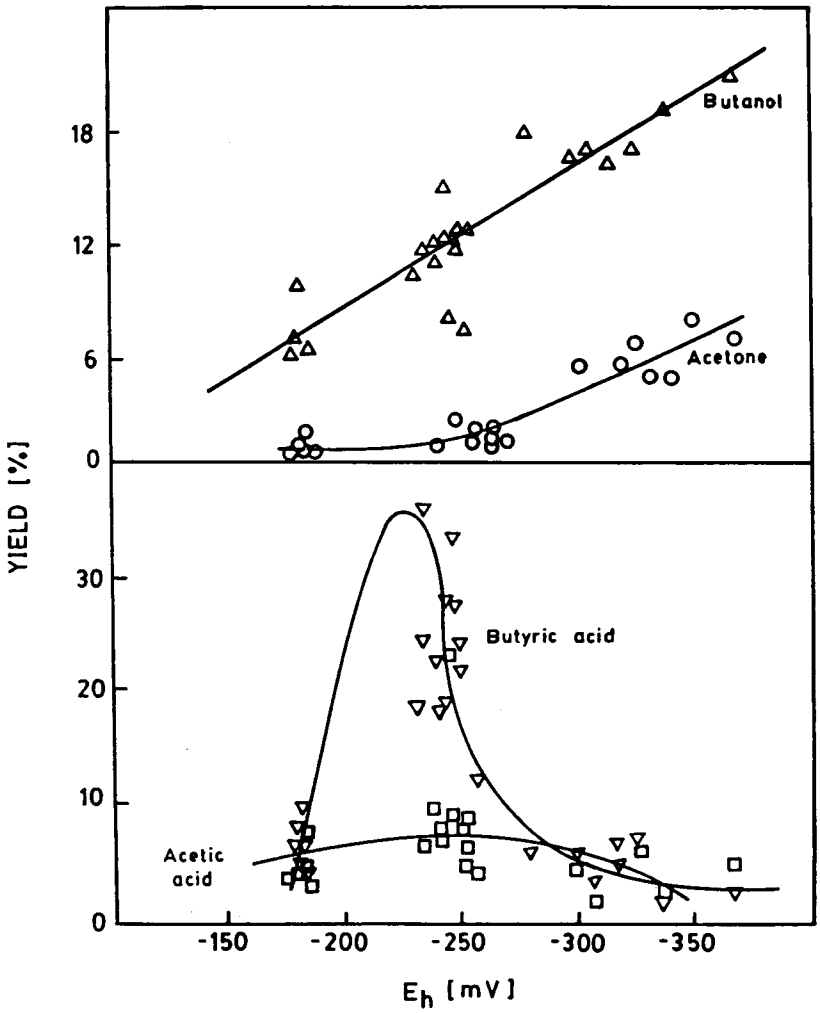

Fig. 6. Variations in yield of products (based upon lactose consumed) with redox potential.

inhibition locale since no extracellular galactose was observed in any experiment. Solvent mediated inhibition of sugar transport system has been reported in *Saccharomyces cerevisiae* cells (*16*). Ounine et al. (*17*) have found that the inhibitory effect of butanol is dependent upon the nature of carbon source.

Relationship of Redox Potential and Metabolism

Interactions between oxidized/reduced forms of ferredoxin and nicotinamide–adenine dinucleotide, and those between reduced ferredoxins and an iron-containing hydrogenase play an important role in determining the flow of carbon skeletons to the acids or to the solvents (*18–20*). Ferredoxin can both accept and donate electrons at a very low

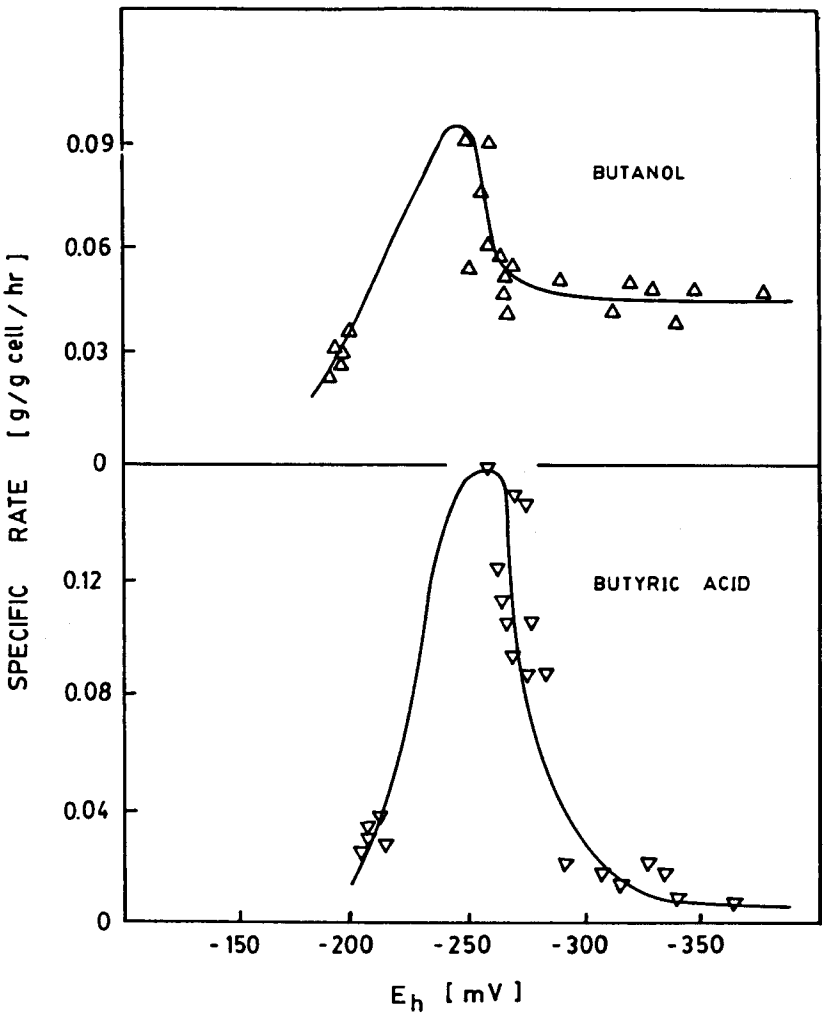

Fig. 7. Specific rates of formation of butanol and butyric acids as functions of redox potential.

potential. Under suitable conditions, reduced ferredoxin can donate electrons with the help of hydrogenase to form hydrogen. In the acid production phase, the flow of electrons takes place primarily from reduced nucleotides to ferredoxins which in turn get rid of the electrons in the form of hydrogen gas. During solvent production, however, the flow is reversed and the terminal transfer of electrons from reduced nucleotides is to butyryl-CoA to produce butanol, and ethanol, if possible (Fig. 1). Since the conversion of pyruvate to acetyl–CoA involves ferredoxin, clostridial metabolism requires a suitably low redox potential before cell growth takes place (*21*). Production of solvents has been related to low activity of hydrogenase in the cells. Reduction of hydrogenase activity by use of inhibitors like carbon monoxide (*8,22,23*) has been shown to improve solvent production. On the other hand, increasing pressures of

hydrogen have also been reported to enhance solvent production and suppress formation of acids (*4,24–26*). This effect may be related to H^+/ Hydrogen redox potential in the cells (*27*).

The experimental data presented here show a strong correlation between the redox potential of broth and the metabolic state of the cells (Figs. 4–6). It is tempting to argue that lowering the redox potential increases electron flux in the direction of NADH/NAD(P)H in the cells, whose abundance permits production of larger amounts of reduced compounds. These reduced compounds are oxidized by reactions forming solvents, thus increasing their production. Alternately, lowering of redox potential may be a purely coincidental phenomenon. Conditions favoring solvent production may also result in lowered hydrogenase activity. This would increase the concentration of reduced nucleotides in the cells, causing their leakage, and thus lowering the oxidation—reduction potential of the broth (Fig. 3). Under the experimental pH of 5.0 used in this study, the intracellular pH in the clostridium cells should be around 6.0 (*28*) at which hydrogenase remains quite active (*18*). Yet, active hydrogenase would siphon off the excess electrons to increased hydrogen production that is not associated with solvent production. Hence, the activity of hydrogenase must have been inhibited. Undissociated acids whose threshold concentrations have been suggested to induce solvent production (*29*), may be inhibitors of hydrogenase activity as suggested by Andersch et al. (*18*). The present continuous culture studies support the postulate that changes in intracellular redox potential should be linked with the changes in electron flow in the cell.

From the observation of decreasing specific butanol productivity with redox potential (Fig. 7), it would appear that too high levels of reduction–oxidation levels in the system reduce the overall flux of carbon through the glycolytic cycle. This is supported by the observations of a maximum in the specific lactose uptake rate (from data shown in Fig. 5). This maximum also corresponds to the E_h level of -250 mV.

CONCLUSIONS

In the continuous cultivation of *Clostridium acetobyutylicum* on lactose, sustained oscillations were obtained under conditions favoring high product concentrations. The specific productivities of butanol and butyric acids were found to be well correlated with the redox potential in the broth.

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