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TITLE: Advanced Studies of the Biological Indirect Liquefaction of Coal

PIs: Dr. E. C. Clausen
Dr. J. L. Gaddy

INSTITUTION/ORGANIZATION: University of Arkansas
Department of Chemical Engineering
Fayetteville, AR 72701
(501) 575-4951

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OBJECTIVE:

As an alternative to traditional indirect liquefaction, biological processes may be used to convert the components of coal synthesis gas to liquid fuels. Biological processes offer certain advantages over chemical conversion, including operation at ambient temperatures and pressures, high yields and product specificity. These advantages are partially offset by slower reaction rates and special reactor considerations such as sterility and nutrient provision.

Organisms have been identified in the literature that are capable of converting gaseous mixtures of CO, H_2, and CO_2 to organic acids (such as acetic and butyric acids) and to methane (Clausen and Gaddy, 1986). Although many anaerobic, facultatively anaerobic, and even some strictly aerobic microorganisms are known to form ethanol from glucose (Wiegel, 1980), no organism was known to form ethanol autotrophically from synthesis gas components. In 1987, researchers at the University of Arkansas reported the isolation of a strict anaerobic mesophilic bacterium from animal waste that was capable of converting CO, H_2, and CO_2 to a mixture of acetate and ethanol (Barik *et al.*, 1987). Preliminary identification studies have indicated that the bacterium has a strong possibility of being a new clostridium species (Tanner, 1988). It is likely that in the same manner as with other clostridia growing on sugars, ethanol and acetate are formed from acetyl-CoA by this organism, with the product distribution highly dependent on the regulation of electron flow (Rao *et al.*, 1987).

The purpose of the present research program is to obtain fundamental data to allow optimization of a biological system for the production of liquid fuels from synthesis gas. Basic microbiological procedures are being utilized to determine whether the newly isolated culture is a new Clostridium species on an existing species now capable of producing ethanol from CO, CO_2, and H_2. Studies in batch and continuous culture are being used to manipulate the biological pathway in an effort to eliminate acetate from the product stream at the expense of increased ethanol production. Finally, culture conditions are being optimized to maximize the yield and concentration of ethanol produced from synthesis gas.

TECHNICAL APPROACH:

SUMMARY OF PAST RESEARCH

A comprehensive review of the literature showed that several organisms are capable of converting CO, CO_2 and H_2 in synthesis gas to methane and organic acids such as acetate and butyrate (Lorowitz and Bryant, 1984; Genthner and Bryant, 1982; and Levy et al., 1981). Acetate may, in turn, be used in producing high value chemicals such as citric acid (Yoshinaga et al., 1972; and Tabuchi et al., 1973) and several amino acids (Bedziong et al., 1979). However, no source identified either mixed or pure cultures capable of producing liquid fuels from CO, CO_2, H_2 or acetate. Free energy calculations, however, show that many reactions are energetically feasible if a biological catalyst can be found (Thauer et al., 1977). Thus, bacteria must be isolated from natural sources in order to assess the feasbility of biological conversion.

Natural sources of bacteria such as sewage sludge, animal wastes, and muds serve as a final repository for a consortium of bacteria, capable of converting a wide variety of substrates to many different products. Almost all of the pure culture bacteria found in culture collections today originated from a mixed bacterial population isolated from such natural environments. The natural carbon flow in mixed cultures obtained from sewage sludge and animal rumen is the metabolism of substrate to methane. Thus, methane inhibitors must be added to samples from these sources in order to stop methane production and to allow intermediates to form. Acclimation to CO, CO_2, and H_2 must be achieved over an extended period of time, during which the cultures are supplemented with basal salts, vitamins, and minerals.

Culture isolation studies were carried out in the University of Arkansas laboratories in an effort to find bacteria capable of converting CO, CO_2 and H_2 to liquid fuels. Several natural inocula were used including sewage sludge, animal waste, coal pile runoff and soils. The method of successive transfer was utilized, while simultaneously blocking methanogenesis. Table 1 summarizes the variables and their ranges studied during culture isolation, enrichment and preliminary optimization. The screening medium consisted of an aqueous mixture of vitamins, minerals, yeast extract, and resazurin in addition to the inoculum and carbon source. The result of these experiments was the isolation of a culture capable of producing ethanol and acetate from CO, CO_2 and H_2 when using a chicken waste inoculum at pH 5, and using a BESA inhibitor to block methanogenesis. Based upon preliminary identification studies, the culture was tentatively identified as a member of the Clostridium species.

Batch Fermentation Profiles

Batch fermentation experiments with the newly isolated microorganism in glass bottles under externally controlled conditions did not show a high degree of reproducibility. This behavior is typically found with most clostridia (Finn and Nowrey, 1959), and although the assessment of environmental conditions in an ever-changing substrate and product concentration system is difficult, batch fermentations provide important information in evaluating overall performance. The results presented here summarize the most often encountered patterns during the experimental studies.

The disappearance of CO and H_2 during the batch experiment for various initial pressures is shown in Figures 1 and 2, respectively. As noted, both CO and H_2 were readily consumed at all pressures below 2 atm. Long lag phases occurred, however, which makes repetition of results difficult.

Acetate and ethanol product levels are plotted as a function of time in Figures 3 and 4, respectively. As noted, both acetate and ethanol levels increased roughly with the initial pressure employed. However, a marked difference in ethanol production was observed between the lower pressures of 0.46 and 1.08 atm and the higher pressures of 1.4, 1.42, and 1.99 atm. It seems than ethanol production is not directly related to the amount of substrate consumed, but instead depends more upon other environmental conditions. The utilization of slightly soluble gaseous substrates (such as CO and H_2) in shaken reactors becomes mass transfer limited at a certain level of cell concentration (Vega, 1987; and Vega *et al.*, 1988). Under these conditions, the volumetric rate of carbon monoxide uptake is proportional to the partial pressure of carbon monoxide in the gas phase, as is shown in Figure 5 for the four experiments that fully utilized the substrates. The ratio of the mass transfer coefficient to the Henry's law constant for carbon monoxide, K_La/H, is found to be 4.0 mmol CO/atm L h from the slope of the best fit line through the data. A similar plot can be obtained for hydrogen. The dissolved carbon monoxide and hydrogen concentrations are, therefore, kept at a level very close to zero as soon as the fermentation becomes mass transfer limited. The batch fermentation can be considered to be substrate limited throughout most of the process.

CULTURE IDENTIFICATION

As mentioned previously, preliminary experiments have indicated that there is a strong possibility that the newly isolated culture is a member of the Clostridium species. A subcontract was given to the University of Oklahoma, Department of Botany and Microbiology, to positively identify the bacteria as either a new species or existing species capable of CO, CO_2 and H_2 metabolism to ethanol and acetate. Using established microbiological procedures for culture identification and characterization, Dr. Ralph Tanner of the University of Oklahoma has indicated that there is a very strong likelihood that the isolate is a new Clostridium species. Studies are presently underway to verify the hypothesis.

Evaluation of Reaction Stoichiometry

The stoichiometry for the formation of acetate from carbon monoxide, as well as hydrogen and carbon dioxide, has been well-established for many acetogenic bacteria (Ljungdahl, 1986). These reactions are:

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \qquad (1)$$

$$\Delta G^\circ = -\ 37.8\ \text{kcal/gmole}\ CH_3COOH$$

and

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \qquad (2)$$

$$\Delta G^\circ = -\ 18.6\ \text{kcal/gmole}\ CH_3COOH$$

Similar equations may be written for the formation of ethanol from CO, CO_2 and H_2:

$$6\ CO + 3\ H_2O \rightarrow C_2H_5OH + 4\ CO_2 \qquad (3)$$

$$\Delta G^{\circ} = -59.9\ \text{kcal/gmole}\ C_2H_5OH$$

and

$$2\ CO_2 + 6\ H_2 \rightarrow C_2H_5OH + 3\ H_2O \qquad (4)$$

$$\Delta G^{\circ} = -23.2\ \text{kcal/gmole}\ C_2H_5OH$$

Experiments were first carried out in the University of Arkansas laboratories using a mixture of CO, H_2 and CO_2 in verifying the above stoichiometries. Since both substrates (CO and CO_2/H_2) were utilized in these experiments and both products were formed, it was not possible to directly measure the stoichiometric coefficients for each of equations presented above. However, an indirect indication of the validity of Equations (1-4) was inferred. A combination of Equations (1-4) yields:

$$6\ \Delta N_{ETOH} + 4\ \Delta N_{ACH} = -(\Delta N_{CO} + \Delta N_{H2}) \qquad (5)$$

since 6 moles of CO or 6 moles of H_2 are required to form 1 mole of ethanol, and 4 moles of CO or 4 moles of H_2 are required to form 1 mole of acetate. Thus, a plot of the left-hand side of Equation (5) as a function of the right-hand side should yield a straight line with slope equal to 1.0 if the stoichiometries shown in Equations (1-4) are valid.

Figure 6 shows a plot of the left-hand side of Equation (5) as a function of the right-hand side for various initial pressures. As noted, a single straight line was obtained with a slope of 0.953. Since the slope was very close to 1.0, the validity of Equation (5), and thus Equations (1-4), is demonstrated.

In order to further verify the stoichiometries presented in Equations (2) and (4), experiments were carried out with only CO_2 and H_2 as substrates. Equation (5) is thus tested for CO_2 and H_2 alone in Figure 7 at various initial H_2 partial pressures. As observed, all of the data fell along a single straight line with a slope equal to 0.9 (close to unity). Thus, H_2 and CO_2 not only serve as growth and production substrates for Clostridium sp. without the presence of CO, but also confirm the stoichiometries presented in Equations (2) and (4).

ELIMINATION OF ACETATE

Biochemistry and Energetics of Clostridia

The production of ethanol by Clostridium sp. is accompanied by the production of acetate, an undesirable by-product if a fuel is sought. Although the new isolate is the first organism to show the production of ethanol from CO, H_2 and CO_2, several other clostridial species are known to manufacture ethanol from sugars (Rogers, 1986). C. thermocellum, C. thermohydrosulfuricum and C. saccharolyticum have been shown to produce

ethanol from complex carbohydrates such as cellulose and starch, and from simple sugars such as glucose. In all three cases, however, acetic acid and sometimes lactic acid are always produced as by-products of the fermentation. It is very likely that similar biochemical pathways exist for these bacteria and the new isolate, thus providing information on the methanisms that lead to the production of ethanol and acetate.

The ethanologenic clostridia convert sugars to pyruate via the fructose-biphosphate pathway producing two moles of ATP and two moles of NADH per mole of hexose (Figure 8). The majority of the pyruate is converted to acetyl-CoA with small amounts going to lactate or CO_2 and H_2. Acetyl-CoA can than be reduced to acetaldehyde and then to ethanol, or it can be converted into acetate with stoichiometric production of ATP.

The pathway utilized by clostridial species and other acetogenic bacteria to autotrophically grow on H_2/CO_2 and CO has been recently established by Wood *et al.*, (1982) in studies conducted with C. thermoaceticum, and has been termed "the acetyl-CoA pathway". Evidence is accumulating that this pathway is utilized by other bacteria that grow with CO_2 and H_2 as the sources of carbon and energy. This group includes all of the acetogenic bacteria, the methane producing bacteria and the sulfate-reducing bacteria. A schematic of the acetyl-CoA pathway for autotrophic growth by acetogenic bacteria is shown in Figure 9. The mechanism involves the reduction of one molecule of CO_2 to a methyl group and then its combination with a second molecule of CO_2 (or a molecule of CO) and CoA to form acetyl-CoA. The reduction of CO_2 to a methyl group in the tetrahydrofolate pathway (bottom cycle in Figure 9) requires one molecule of ATP and one molecule of NADH per molelcule of CO_2 reduced. Also, it is important to notice that the conversion of acetyl-CoA to acetate is the only source of substrate level phosphorylation in the acetogenic clostridia during unicarbonotrophic growth (Ljungdahl, 1983). When terminating in acetate, the pathway is balanced in ATP and the production of ethanol or other end-products would result in a net consumption of ATP which would not support growth of the bacteria.

The high growth yields of homoacetogenic bacteria on sugars and the discovery of a large variety of electron transport proteins have provided the first clues to indicate that, in clostridial species, ATP may be generated by a proton gradient mechanism and electron coupled phosphorylation. It has been suggested that the generation of the proton gradient may be caused by the free flow of H_2 through the cyloplasmic membrane and the presence of membrane bound hydrogenase (Ljungdahl, 1983). In view of these findings, it is possible to expect that end products other than acetate (such as ethanol) may be manufactured in microcarbonotrophic growth of clostrial species. The understanding of the basic energetics supporting growth and metabolism is fundamental to the development of products other than acetate in clostridial fermentations.

Elimination of Acetate as a Product

Clostridial fermentations yield a wide variety of end-products which include two to five major acids and/or solvents as well as gaseous products such as H_2 and CO_2. The amount of reduced versus neutral and oxidized products is always balanced with the amount of H_2 and also ATP produced and has the potential of a great deal of natural variation. Several strategies

have been employed in other clostridial fermentations, particularly the acetone-butanol fermentation by *C. acetobutylicum*, which have led to increases in the selectivity of solvents versus acids. Some of these strategies have been investigated with the newly isolated strain and the results are showed below.

1. Control of Growth Rate Parameters

Studies in the acetone-butanol fermentation have shown that high solvent yields depend upon adjusting growth-limiting factors such as phosphate, nitrogen, etc. in a range which allows some growth but not under optimum conditions (Bahl *et al.*, 1982; Bahl and Gottschalk, 1984; Gottschal and Morris, 1981a). Solventogenesis, then is apparently a metabolic response to a condition of unbalanced growth where the utilizable energy source remains in excess but growth is restricted by other limiting factors or growth inhibitors. Still, the molecular changes that trigger the formation of solvents due to growth restriction are unknown.

Early in the studies conducted with *Clostridium* sp., the importance of the yeast extract concentration employed in the medium on the ratio of ethanol to acetate obtained during a fermentation was recognized. In a typical batch fermentation, the production of ethanol occurs simultaneously and a rather constant ratio of products is maintained along the fermentation. The ratio of ethanol to acetate, however, is highly dependent upon the yeast extract concentration as is shown in Figure 10 where the product ratio is plotted for various cases. In accordance with the observations in the acetone-butanol fermentation, the limitation of some growth factor present in yeast extract results in better solvent/acid ratios (a molar ratio of about 1:9 was obtained for yeast extract concentrations of or below 0.05% while a ratio of 1:22 occurred in Figure 10 for higher levels of yeast extract). Unfortunately, the ability of the bacteria to adopt to lower levels of yeast extract has been shown in later experiments. Thus, the effect observed in batch culture has not proven useful in continuous experiments where yeast extract could not regulate growth.

While growth control in continuous culture has not been successful through nutrient limitation strategies, the rate of growth of bacteria in chemostats can be easily controlled by adjusting the dilution rate. At steady-state, in a continuous stirred-tank reactor that is fed a cell-free solution, it is necessary that the specific growth rate equals the dilution rate (Bailey and Ollis, 1977). Therefore, low growth rates can be achieved by using low dilution rates. The molar product ratio of ethanol to acetate achieved at various liquid flow rates in a New Brunswick Bioflo C.30 chemostat specially adapted for anaerobic operation is shown in Figure 11. As is observed, when all other variables are held constant, the lower dilution rates yielded the higher ratios. Values of 0.6-0.7 mol of ethanol/mol of acetate were maintained for over 20 days.

Better product ratios have been obtained in other continuous experimental studies where a combination of factors that are known to improve solventogenesis in other clostridial fermentations are applied. The product ratios obtained in two other runs are shown in Figures 12 and 13. A combination of sudden decreases in yeast extract with decreases in dilution rates led to oscillatory behavior with ratios as high as 1.2 mol ethanol/mol acetate. Even higher ratios (as much as 2.8 mol ethanol/mol acetate) were

achieved by employing a sudden decrease in the operating pH from 5.0 to 4.0, followed by a decrease in the dilution rate. The sudden change in operating pH, however, may have brought about an induction of sporogenesis in the culture and is discussed below.

2. The Use of Reducing Agents as a Method to Improve the Ethanol/Acetate Product Ratio

Several researchers have shown that the presence of reducing agents in the liquid media of *Clostridium* fermentations has brought about an increase in solvent formation in the product stream at the expense of acid formation. Rao and Mutharasan, for example, showed a five-fold increase in acetone yields from glucose at pH 5 when adding methyl viologen to a culture of *C. acetobutylicum* (Rao and Mutharasan, 1986). In a similar study, the addition benzyl viologen to the media in a *C. acetobutylicum* fermentation was responsible for increasing the quantity of butanol in the product stream to over 90% of the total solvents formed. Acid formation decreased significantly in favor of butanol formation. Other reducing agents produced similar results on *Clostridium* strains, including sodium sulfide, cysteine hydrochloride, sodium thioglycolate and electrochemical energy (Rao and Mutharasan, 1988b; Rao *et al.*, 1987; Rao and Mutharasan, 1987; and Kim and Kim, 1988). Reducing agents apparently cause altered electron flow, which direct carbon flow and acid to alcohol production. Reducing equivalents are directed to the formation of NADH which, in turn, resulted in increased alcohol production.

Since the bacteria isolated at the University of Arkansas for the conversion of CO, H_2 and CO_2 to ethanol and acetate is also a *Clostridium*, it was felt that the addition of reducing agents to the medium might also result in increased solvent (ethanol) production at the expense of acid formation. Batch experiments were thus carried out with small quantities of reducing agents (30,50 and 100 ppm) added to the feed in order to assess the feasibility of increasing the ethanol to acetate ratio through reducing agent addition. The experiment carried out with 100 ppm of reducing agents resulted in very limited growth in all cases. On the other hand, 50 ppm and 30 ppm concentrations were successful in improving the ethanol to acetate ratio in some cases as shown in Table 2. The flask containing benzyl-viologen at a concentration of 30 ppm produced 3.7 mmol of ethanol with a ratio of 1.1, the highest ratio observed in batch experiments so far. It is interesting to mention that those reducing agents that improved the product ratio always resulted in slower growth rates of the bacteria, as could be expected from decreased ATP formation. Continuous experiments with the addition of reducing agents will be started soon.

3. Induction of Sporulation

Recently, the connection between sporulation and solventogenesis has been the subject of extensive studies both in batch cultures (Jones *et al.*, 1982; Long *et al.*, 1984a;b) and chemostat cultures (Gottschal and Morris, 1981a;b). Under certain conditions, which are strain-dependent, a shift of the bacteria into a sporulation phase occurs which is accompanied by morphological changes (elongation of the cells) and the production of solvents rather than acids. In some cases, such as *C. thermosaccharolyticum*, an uptake of previously produced acetate takes place during sporulation (Pheil and Ordal, 1967). The selection of two classes of mutants, the *cls* and the *spo* mutants of *C.*

acetobutylicum, has confirmed the relationship between sporulation and solventogenesis. The cls mutants are unable to form a clostridial stage and cannot switch to solventogenesis. The Spo mutants, however, blocked at a later stage in sporogenesis, can all produce solvents (Jones et al., 1982).

The conditions that cause the onset of sporolation and solventogenesis vary among the clostridial strains. In the case of C. thermosaccharolyticum, significant ethanol production and elongated cell formation both require a combined signal of a specific carbon source (L-arabinose or L-xylose), lower pH and a restricted supply rate of the energy source (Landuyt et al., 1983). In the case of C. acetobutylicum, switching to the solventogenic phase and differentiation to the clostridial stage requires the presence of glucose and nitrogen, a low pH and a minimum acetate and butyrate concentration. It has also been observed that, in general, good sporulation can be induced in batch culture using carbon sources that tend to reduce the bacterial growth rate (Hsu and Ordal, 1969).

Experiments have been conducted with Clostridium sp. grown in basal media at pH 5.0 with a 0.01% concentration of various carbohydrates (starch, galactose, rhamnose and cellobiose) in the presence of synthesis gas. Table 3 summarizes the results obtained for each of the nutrients studied, along with the maximum values obtained for cell concentration, ethanol concentration and molar product ratios. As noted, the highest product ratios were obtained for cellobiose with product ratios over 3 times the ratio obtained in the presence of yeast extract. Ethanol and cell concentrations were highest in the presence of cellobiose and galactose, where the ethanol concentrations were over 4 times the value obtained in the presence of yeast extract.

Future Work

The summarized results presented above point towards the neccesity of controlling the growth and the physiological state of the culture in order to achieve higher ethanol to acetate ratios. It has been shown how forcing the culture to grow slowly leads to the production of ethanol in favor of acetate. At the same time, solvent production results in slower growth due to reduced ATP production. The addition of reducing agents such as benzyl viologen that provide electrons to promote the reduction of acetyl-CoA to ethanol have been shown to also result in a higher ratio of ethanol to acetate. Finally, the use of conditions (sudden pH shift, etc.) and chemicals (complex carbohydrates) that may cause the onset of a sporulation stage in the culture have helped increase the ethanol formed from synthesis gas.

The future work in the elimination of acetate as a by-product of the fermentation will concentrate on the optimization of the use of the strategies mentioned above for the particular case of the new Clostridium utilizing synthesis gas. Both separate studies for each strategy will be optimized and a combination of these will be sought that will bring about a maximum ratio of ethanol to acetate. Specifically the following aspects will be subject of study:

a) The use of continuous addition of a reducing agent such as benzyl viologen in a continuous stirred-tank reactor operating at low dilution rates.

b) The search for an optimum sporulation medium in batch experiments, as well as the search for the conditions that trigger the shift to sporulation and the onset of solventogenesis.

c) The separation of an acidogenic phase and a solventogenic phase in a two-stage continuous stirred-tank reactor system in a similar manner as has been successfully accomplished in the acetone butanol fermentation (Bahl *et al.*, 1982). Good conditions for growth in a first stage will be followed by a sudden shift in operating conditions, as well as the addition of chemicals that have shown enhancement in the ethanol to acetate ratio. Eventually, the size of the first stage will be minimized as much as possible and recycle between the stages will be attempted.

CULTURE OPTIMIZATION

The feasibility of converting CO, CO_2 and H_2 in synthesis gas to ethanol by Clostridium sp. must ultimately be demonstrated in batch and continuous laboratory and pilot-scale reactors. Prior to assessing technical feasibility, culture conditions must be optimized to maximize ethanol concentrations and yields, while minimizing the capital and operating costs for fermentation. The optimization studies will include the determination of an inexpensive medium that will enable adequate growth of the culture while at the same time promoting substrate uptake and ethanol formation. Studies directed towards establishing the tolerance of the organism to high product (ethanol and acetate) concentrations are necessary for an adequate design of the fermentation parameters. Also, if the organism shows a low product tolerance, engineering strategies (such as extractive fermentation) or genetic improvement (by mutation/selection studies) may be employed to help improve the fermentation economics. In addition, some genetic studies may be directed towards an optimization of the culture that will result in better ethanol to acetate ratios.

The results of preliminary experiments in culture optimization as well as initial guidelines for future work in this area are summarized below.

Media Optimization

Fermentation studies with Clostridium sp. have, in the past, been carried out in a basal medium containing 0.01% yeast extract, as well as B-vitamins and minerals. Yeast extract has been shown to affect the ratio of products produced by Clostridium sp. (see Figure 10). In addition, yeast extract is a very expensive media constituent and is also expected to interfere with mutation/selection experiments. Experiments were thus carried out in an attempt to remove yeast extract from the medium and, in the process, develop a minimal medium for the growth of Clostridium sp. Eventually the essential amino acids for growth will be found, and an inexpensive source of these amino acids will be substituted into the medium.

The newly isolated Clostridium was first adapted to grow in a medium devoid of yeast extract following strategies similar to those employed by Savage and Drake (1985) with C. thermoautotrophicum. The culture was sequentially transferred into media containing decreasing concentrations of yeast extract (0.01%, 0.005%, 0.001%, 0%) and an enriched formulation in minerals, trace metals, amino acids and B-vitamins. Once the culture was adapted to growth without yeast extract, all solution concentrations were

halved successfully except the trace mineral solution. Next, the removal of the B-vitamins solution was attempted, but prohibited growth. However, the culture was then grown without the amino acid solution. At this point, various combinations of B-vitamins are being examined for possible elimination from the medium composition.

Product Tolerance

A limiting factor in many fermentations is that the organism used as the biocatalyst is not tolerant of low concentrations of its product, so that toxic and inhibitory effects cause growth and substrate uptake to stop. Calculations show that increasing the product butanol concentration in clostridial fermentations from 1.2 to 2% w/v would cut the energy consumption for distillation in half (Linden *et al.*, 1986). Several engineering approaches such as vacuum fermentation and extractive fermentation have been developed to help minimize product inhibition effects by removing the product as it is produced (Phillips and Humphrey, 1983; Griffith *et al.*, 1983; and Mattiasson, 1983). A biological approach is also possible, where the physiclogical and molecular nature of product toxicity and tolerance are determined.

Experiments have been carried out with Clostridium sp. in the presence of various ethanol concentrations to measure the effect of ethanol concentration on growth and CO consumption. These results are summarized in Figures 14 and 15. As noted in Figure 14, neither the rate of cell growth nor the maximum cell concentration was appreciably affected by ethanol concentrations up to 20 g/L. Similarly, Figure 15 shows that CO consumption is unaffected by the presence of ethanol in this concentration range. Future experiments will determine the effects of higher ethanol concentrations on growth and substrate uptake, as well as determining the effects of acetate on culture performance.

Optimization by Mutation/Selection

Genetic studies with various Clostridium species has been the subject of intense research in the 1980s. Several mutants of C. thermocellum, C. acetobutylicum and C. saccharolyticum have been obtained using UV, EMS and NNG mutagenic agents. While some of the genetic studies have been directed towards understanding the genetic characteristics of clostridia, several mutants have been obtained that are more product tolerant (Herrero and Gomezm, 1980; Lin and Blascheck, 1983) as well as better solvent producers (Duong *et al.*, 1983; Jones *et al.*, 1983).

In particular, two types of mutants of the newly isolated Clostridium that will aid the elimination of acetate will be sought: mutants that block the pathway branch that yields acetate from acetyl-CoA and mutants that are deficient in a late sporulation stage. The mutation that results in a strain deficient in acetate production by affecting genes coding for phosphotransacetylase and acetyl kinase (the two enzymes catalyzing the formation of acetate from acetyl-CoA) has been successfully applied to C. thermosaccharolyticum (which can only be done due to the reversibility of the acetyl-CoA to acetate steps) it would be lethal if converted to fluoroacetyl-CoA. In this manner, a mutant producing a ratio of ethanol to acetate from cellobiose 8 times higher than the wild-type parent strain was obtained.

In the case of unicarbonotrophic growth of the Clostridia, however, complete elimination of the acetate branch may lead to an extreme dependency of ATP production on the electron transport phosphorylation mechanism that, as mentioned before, the Clostridia is expected to have. The pcssibility of obtaining such mutants growing on CO or H_2/CO_2 is, therefore, not very clear. Still, if such mutants were obtained, a dramatic increase in the ethanol to acetate ratio is expected.

From preliminary experimentation and information from other genetic studies, mutants that are not able to complete a sporulation cycle are more realistic in the case of growth on synthesis gas components. These mutants are caught in a stage of sporulation from which they cannot proceed or revert and, therefore as mentioned before, continue to produce ethanol for a much longer period of time than the wild type. These mutants may prove to be very useful in the two-stage system described before by allowing a much larger residence time in the solvent stage reactor (2nd stage).

Other mutants that may be more tolerant of higher ethanol or acetate concentrations may also be important to seek if the inhibitory levels of the parental strain are found to be too low for economic exploitation of the culture in an individual scale.

SIGNIFICANT ACCOMPLISHMENTS:

1. Determined overall stoichiometric relationships for the production of ethanol and acetate from CO, CO_2, and H_2 by Clostridium sp.

2. Carried out identification studies which have indicated that the bacteria is a new Clostridium species.

3. Identified the major factors affecting solventogenesis versus acetogenesis for this clostridial fermentation in batch and continuous reactors. It was found that:

a. controlling the rate of growth in continuous culture by simply decreasing the dilution rate increased the ethanol to acetate molar ratio from 0.2 to 0.75;

b. the addition of 30 ppm of benzyl viologen, a known electron donor, in batch culture boosted the ratio from 0.2 to 1.1;

c. the use of cellobiose, a not so readily available carbon source, in place of yeast extract in batch culture increased the ratio from 0.13 to 0.45;

d. an abrupt change in pH and dilution rate in continuous culture brought about an increase in ratio from 0.2 to 2.8.

4. Developed a basal medium for Clostridium sp. which does not include expensive yeast extract to help both the economics of the fermentation and to aid in mutation/selection experiments.

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1. Barik, S., R. E. Corder, J. L. Vega, E. C. Clausen, and J. L. Gaddy, "Biological Production of Liquid Fuels and Chemicals from Coal Synthesis Gas," Proceedings of the Coal-Liquid Fuels Technology Symposium, (October 1985).

2. Clausen, E. C. and J. L. Gaddy, "Production of Liquids from Coal Synthesis Gas," Proceedings Biological Treatment of Coal Workshop, U. S. Department of Energy, (1986).

3. Barik, S., S. Prieto, S. B. Harrison, E. C. Clausen, and J. L. Gaddy, "Biological Production of Ethanol from Coal Synthesis Gas," Biotechnology Applied to Fossil Fuels, CRC PRess, (1987).

4. Gaddy, J. L. and E. C. Clausen, "Biological Conversion of Coal Synthesis Gas," Proceedings of the EPRI Workshop on Biological Coal Conversion, (1987).

5. Barik, S., S. Prieto, S. B. Harrison, E. C. Clausen, and J. L. Gaddy, "Biological Production of Alcohols from Coal Through Indirect Liquefaction," Applied Biochemistry and Biotechnology, 18, 127 (1988).

6. Clausen, E. C. and J. L. Gaddy, "Biological Conversion of Synthesis Gas into Liquid Fuels," Resources, Conservation and Recycling, (1988).

7. Vega, J. L., G. M. Antorrena, E. C. Clausen, and J. L. Gaddy, "Biological Production of Liquid and Gaseous Fuels from Coal Synthesis Gas," ACS Symposium Series, (1988).

Table 1

General Screening Methodology

A. Parameters

- pH: 5.0 7.0
- Temperature: 30°C and 37°C
- Carbon Sources: CO, CO_2 and Acetate
- Nutrient Supplementation
- Methane Inhibitors: Bromo ethane sulfonic acid (BESA) and monensin

B. Enrichment Method

- Anaerobic
- Initial Inoculum: 10% (v/v)
- Incubation and Observation
- Weekly Transfer of 50% Culture Media
- Analytical-Test for Organic Acids and Liquid Fuels

C. Optimization

- Isolation of Bacterial Cultures
- Process and Culture (mixed and pure) Optimization

Table 2

Peak Levels for Ethanol Production and the Molar Ratio (ETOH/ACH at 30 and 50 ppm Reducing Agent Concentrations

Reducing agent	(50 ppm) ETOH(mmol)	ETOH/ACH	(30 ppm) ETOH(mmol)	ETOH/ACH
Control	0.60	0.12	1.40	0.24
Sodium thioglycolate	1.30	0.20	1.30	0.25
Ascorbic acid	1.50	0.24	1.50	0.25
Methyl Viologen	1.90	0.20	2.50	0.40
Benzyl Viologen	1.25	0.21	3.70	1.10

Table 3

Summary of Results with Nutrient Sources
Bringing About Sporulation

Nutrient	Cell Conc (mg/L)	ETOH (mmol)	ETOH/ACH molar ratio
Yeast Extract	140	0.13	0.13
Cellobiose	170	0.56	0.45
Rhamnose	135	0.31	0.31
Galactose	168	0.53	0.36
Starch	130	0.27	0.36

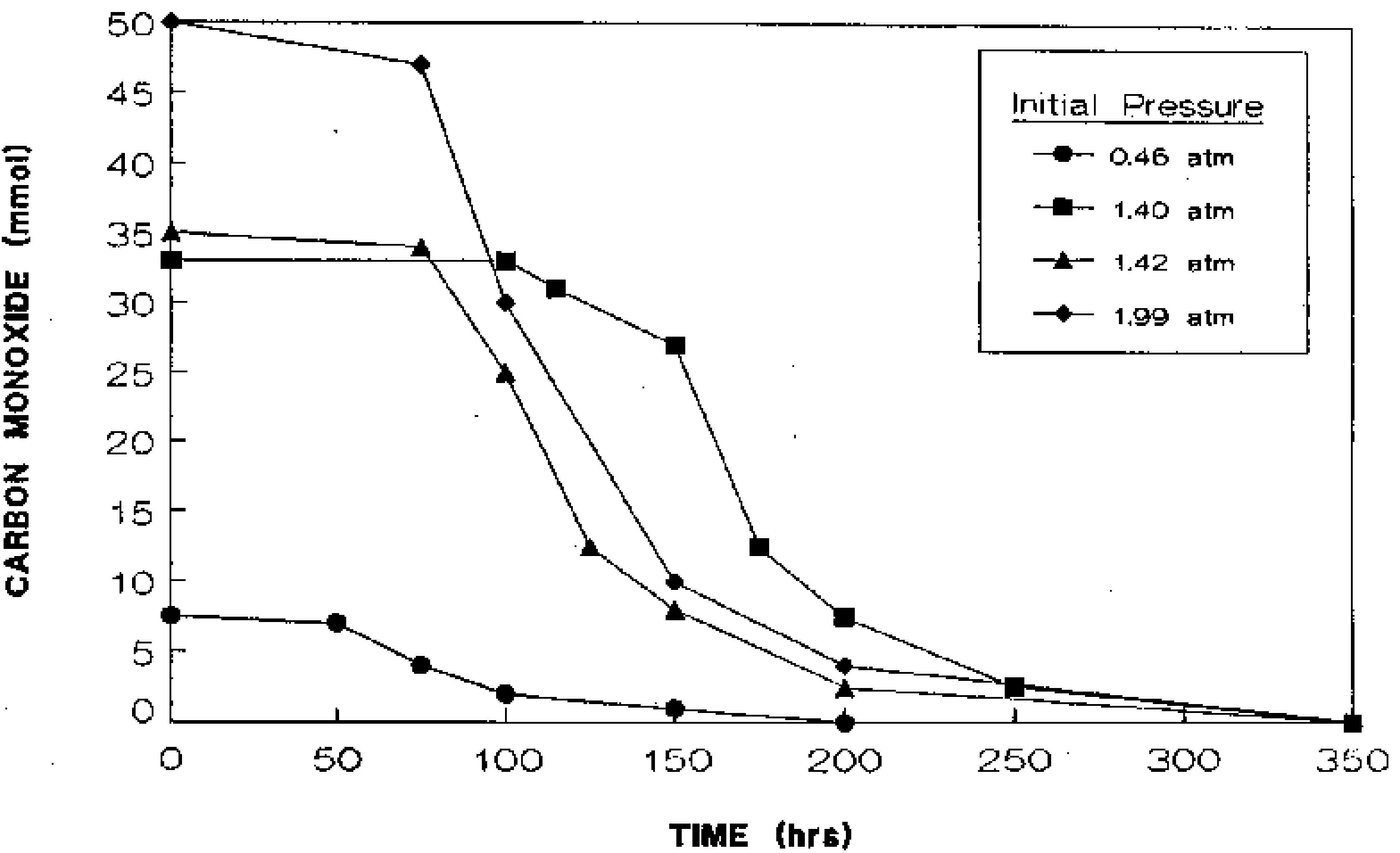

Figure 1. Carbon monoxide disappearance with time for *Clostridium* sp. with 0.01% yeast extract in batch culture.

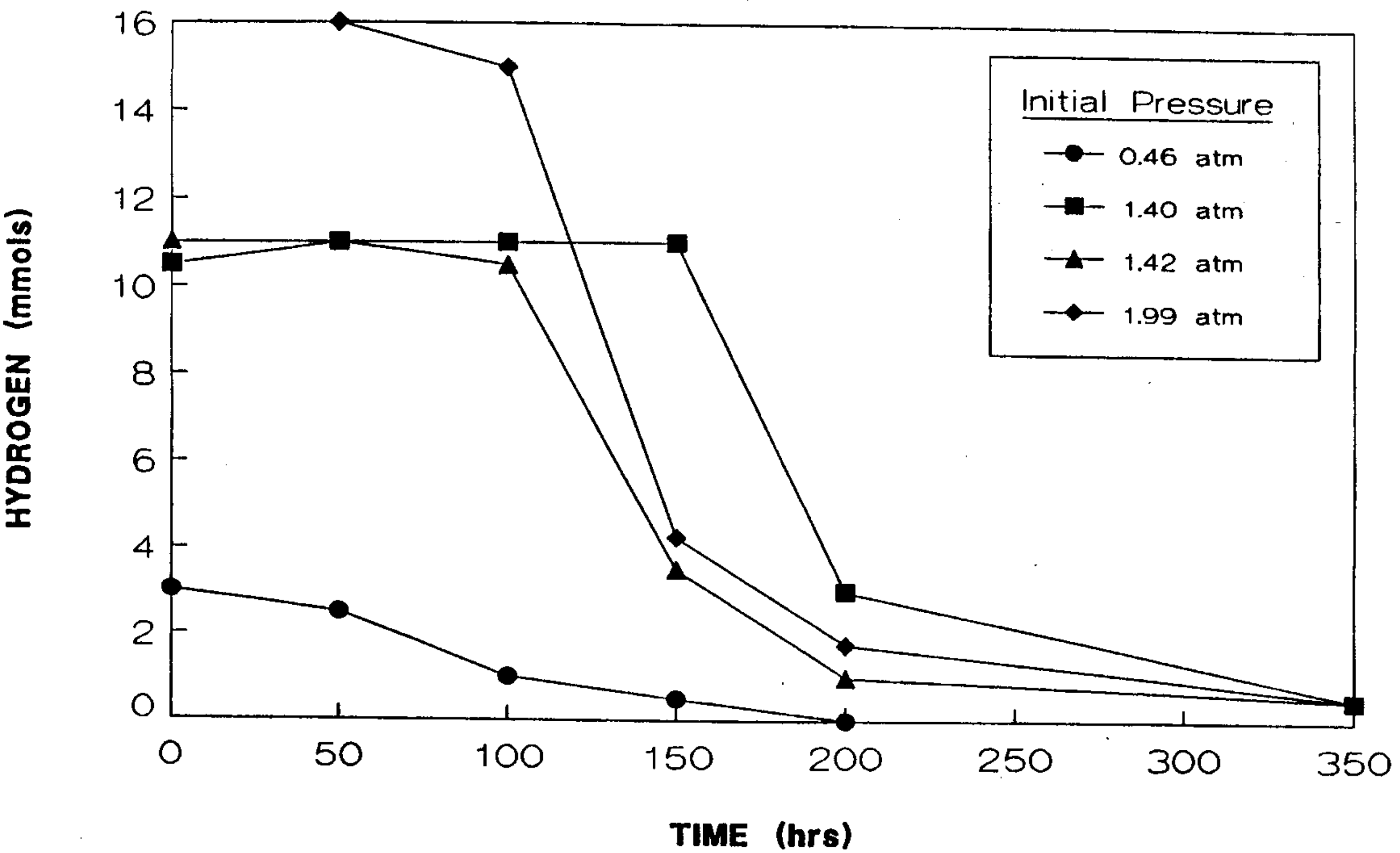

Figure 2. Hydrogen disappearance with time for *Clostridium* sp. with 0.01% yeast extract in batch culture.

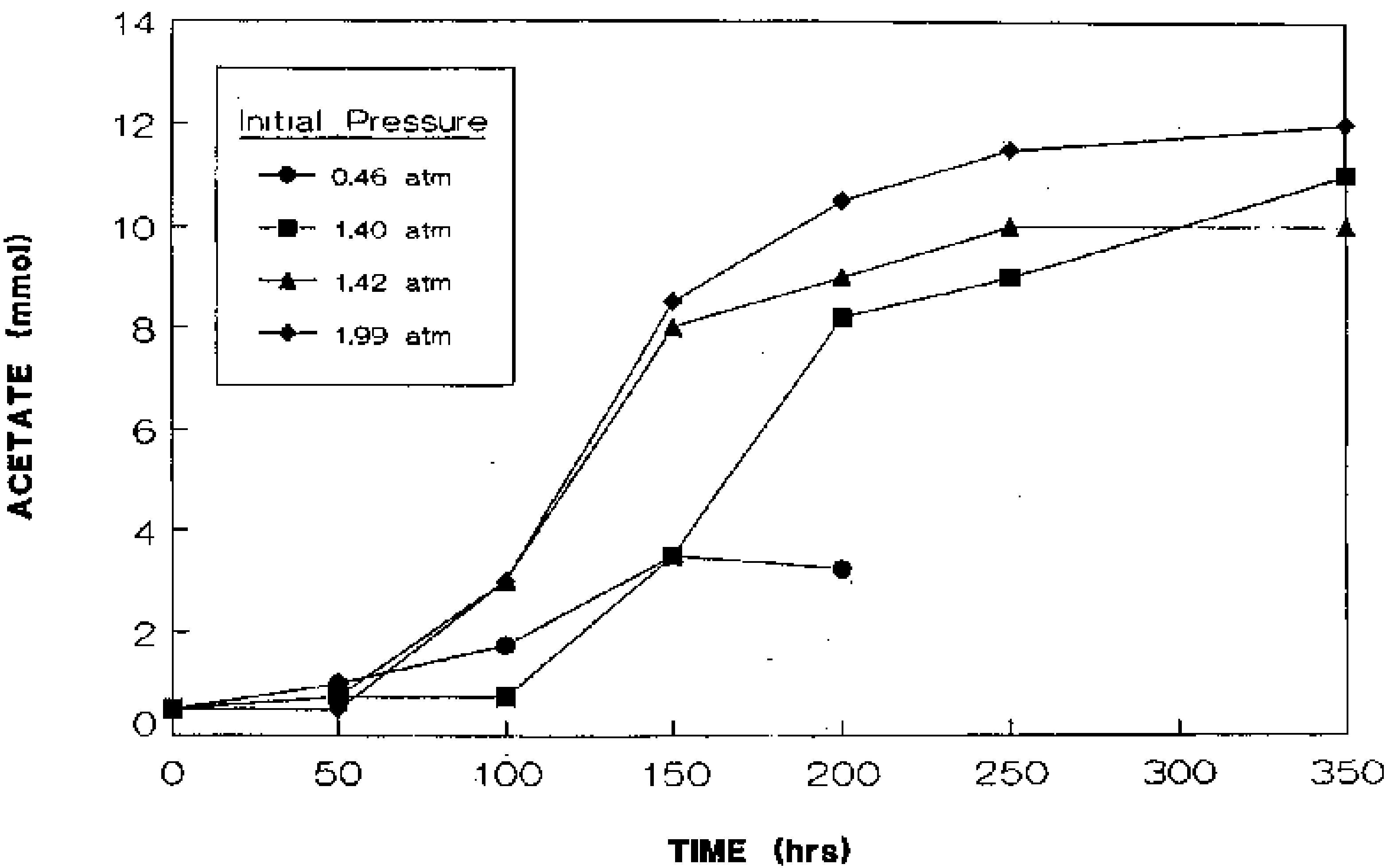

Figure 3. Acetate produced by *Clostridium* sp. as a function of time with 0.01% yeast extract in batch culture.

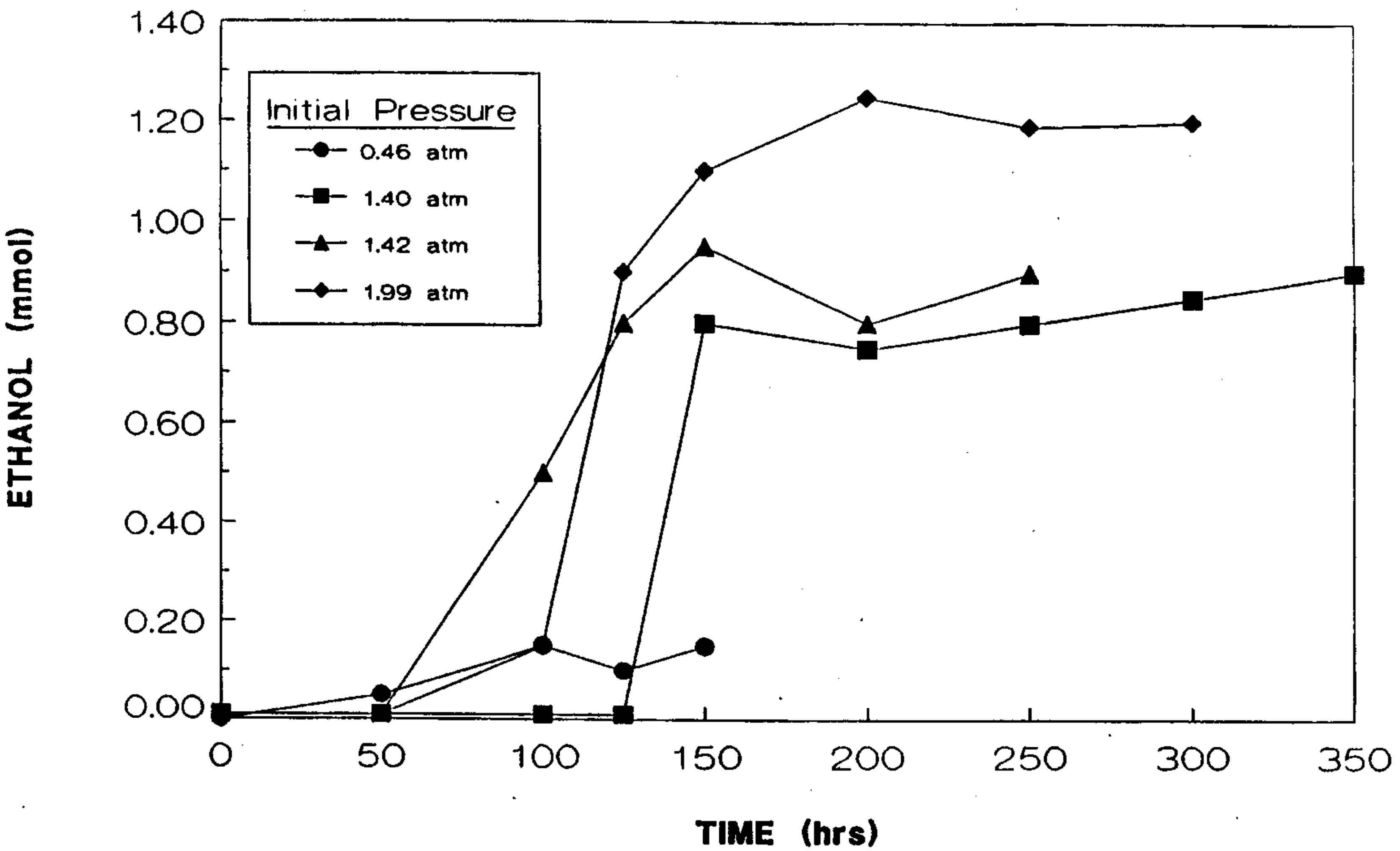

Figure 4. Ethanol produced by *Clostridium* sp. as a function of time with 0.01% yeast extract in batch culture.

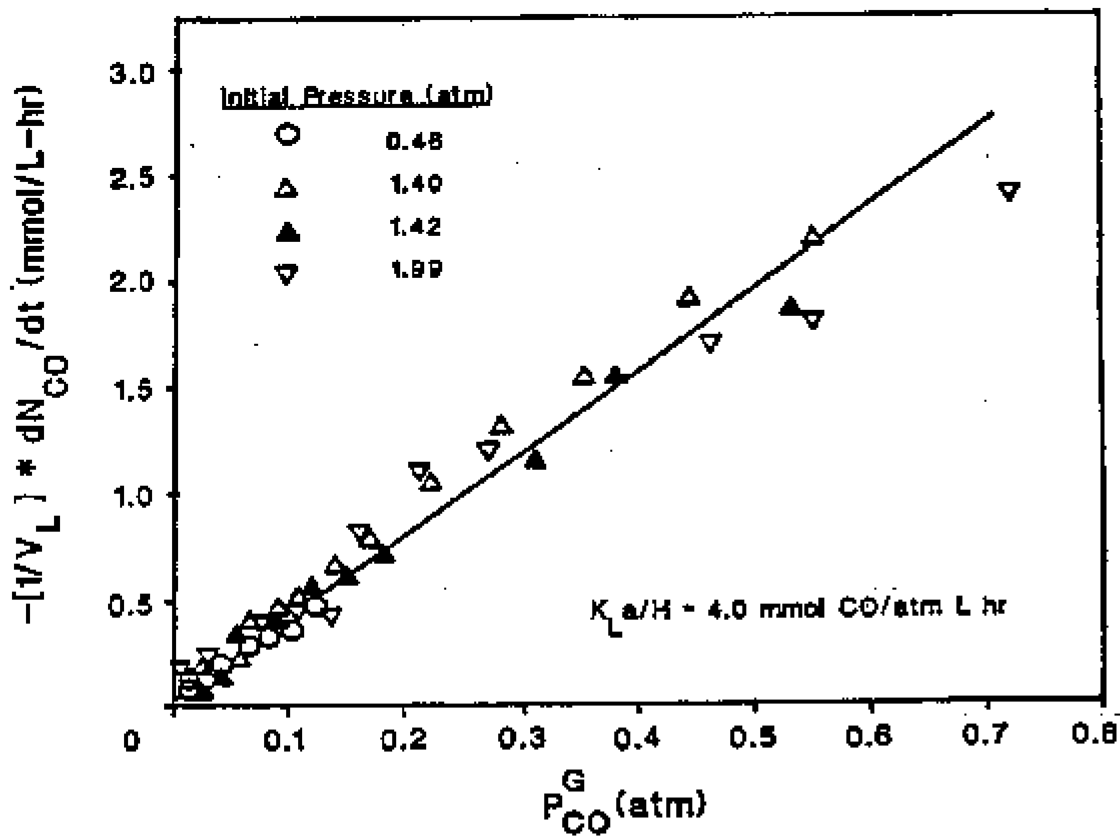

Figure 5. Determination of the volumetric mass transfer coefficient for carbon monoxide.

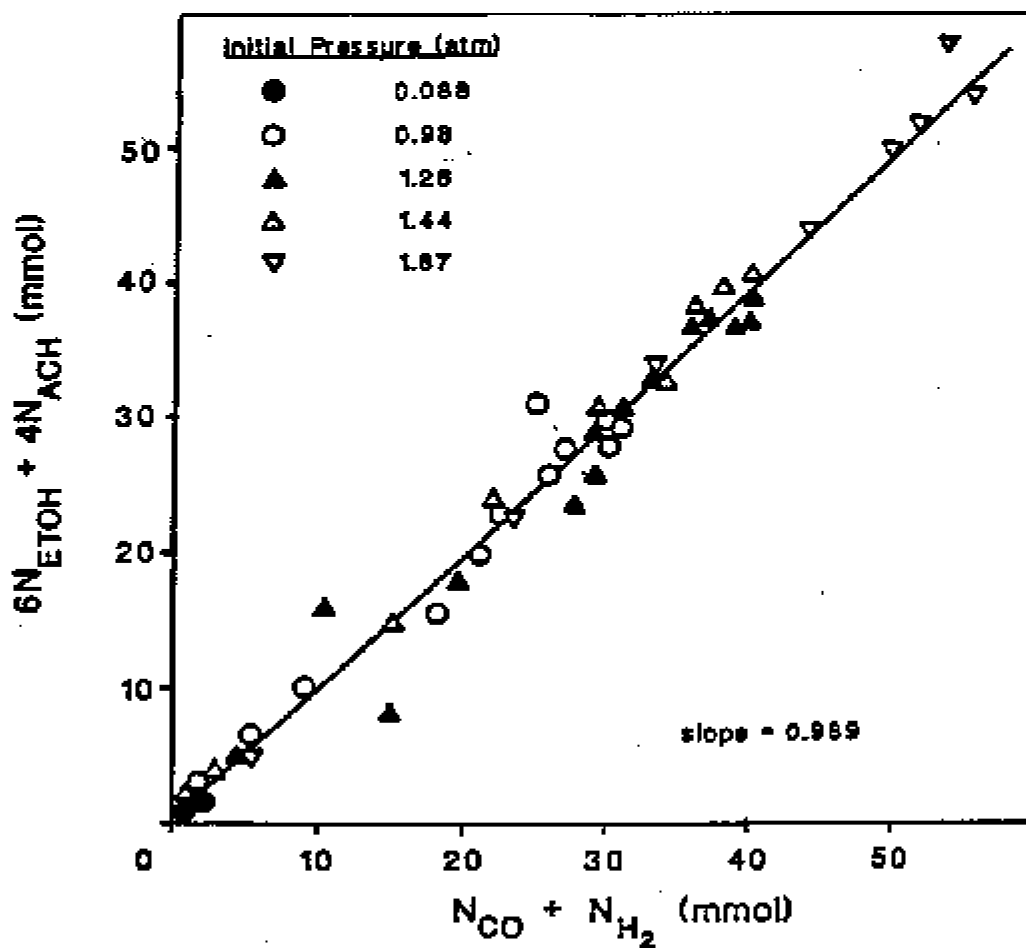

Figure 6. Testing of proposed stoichiometries for mixed substrate conversion to ethanol and acetate by *Clostridium* sp.

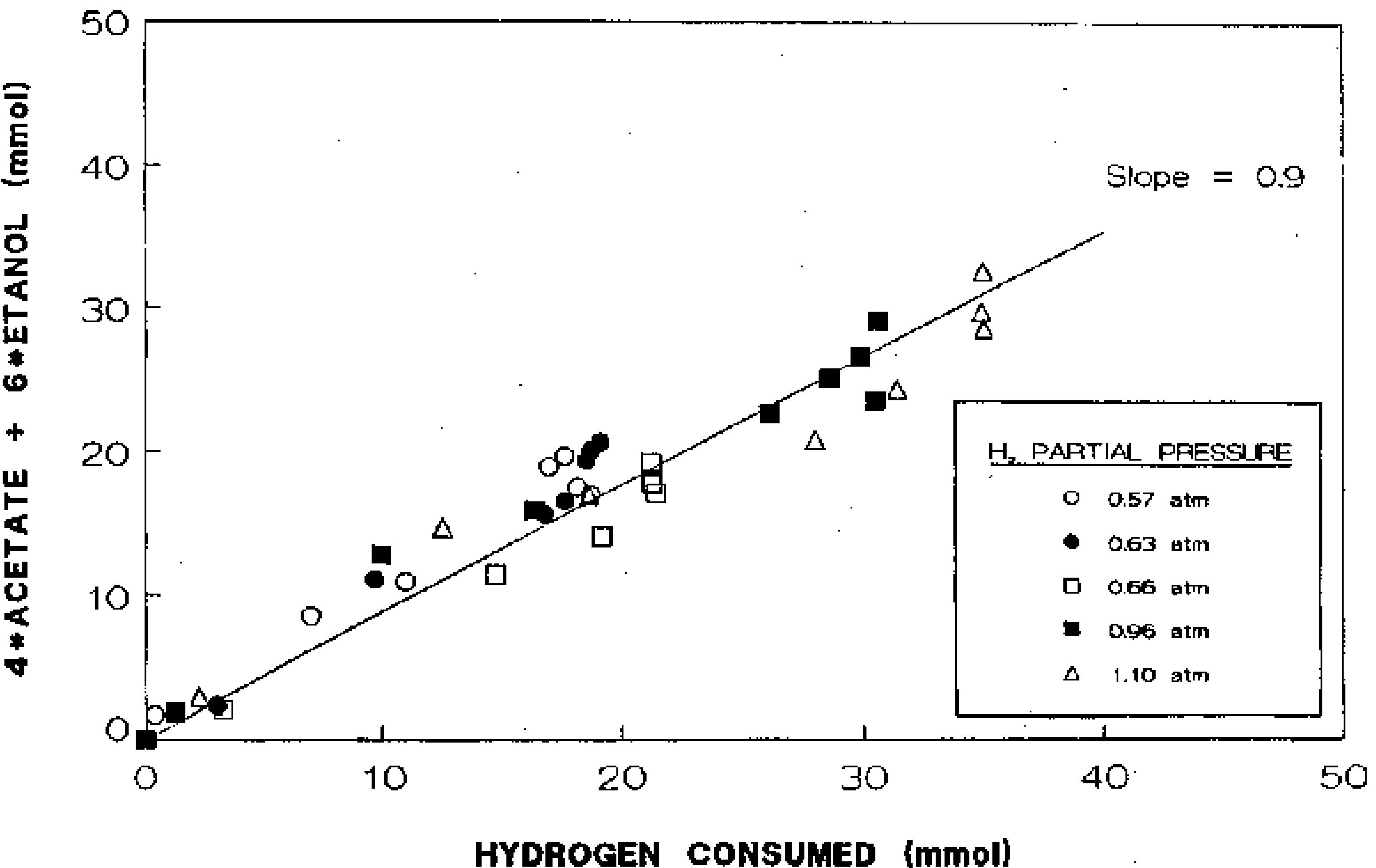

Figure 7. Testing the proposed stoichiometries for ethanol and acetate production from H_2/CO_2 by *Clostridium* sp.

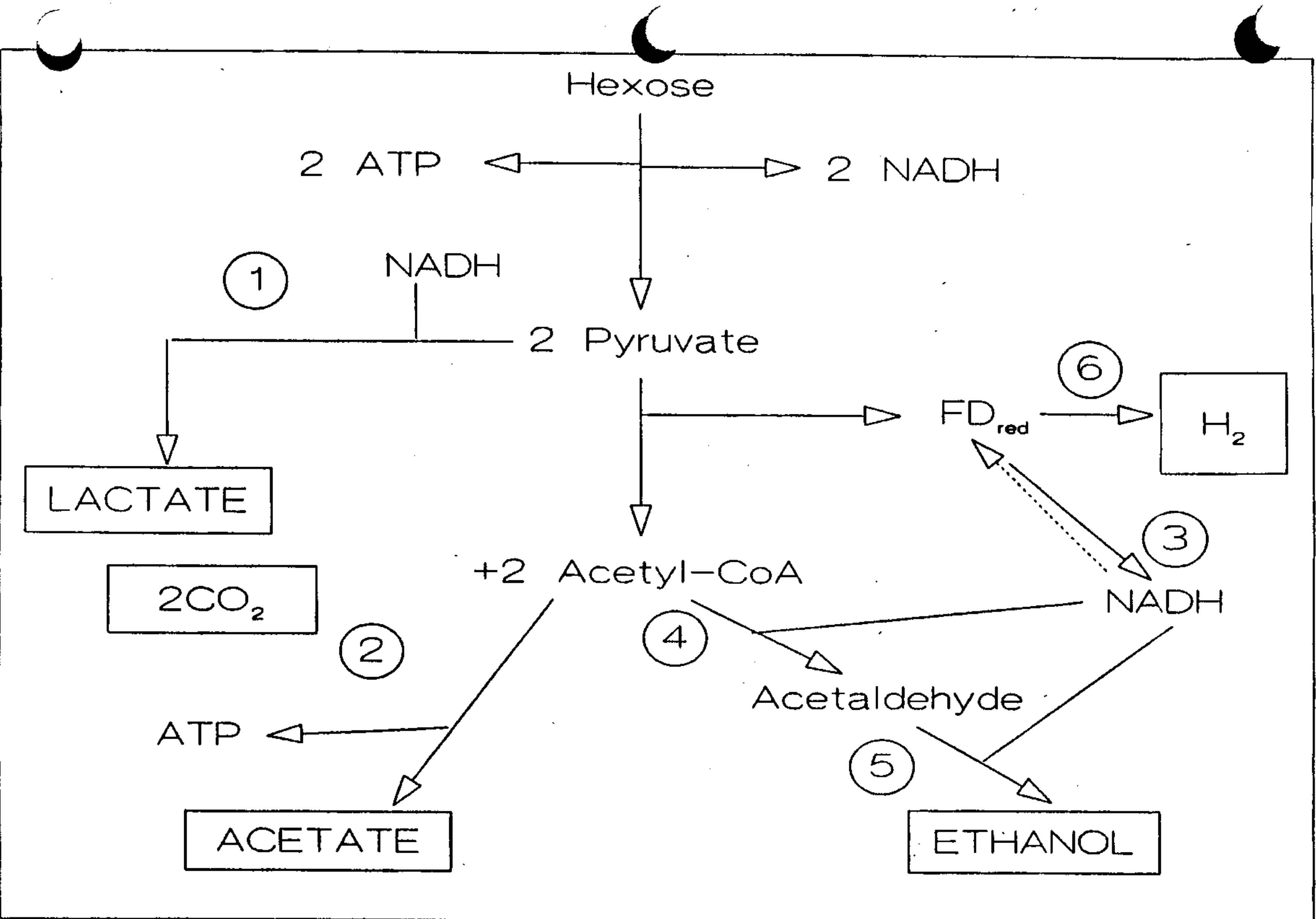

Figure 8. Clostridial ethanolic fermentations. Numbers indicate enzymes: (1) Lactate dehydrogenase; (2) phosphotransacetylase + acetate kinase; (3) NADH:ferredosin oxidoreductase; (4) acetaldehyde dehydrogenase; (5) ethanol dehydrogenase; (6) hydrogenase. *(Rogers, 1981)*

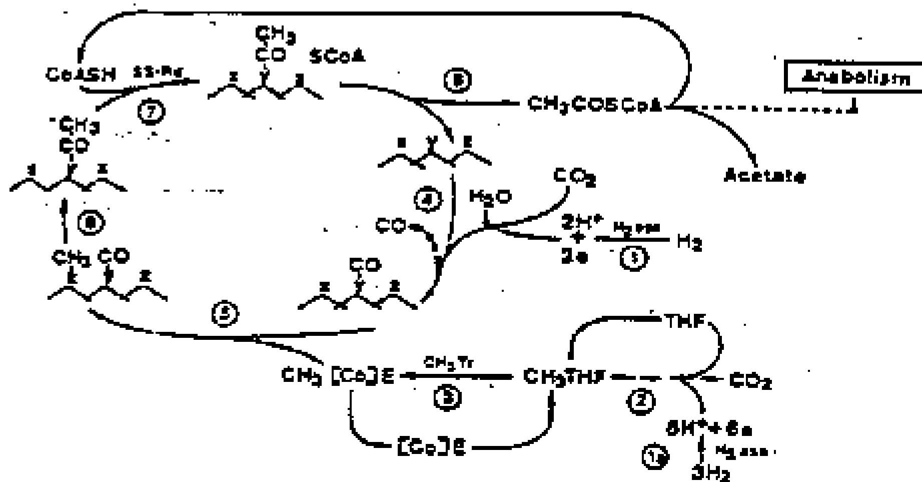

Figure 9. The acetyl-CoA pathway for autotrophic growth by acetogenic bacteria. THF is tetrahydrofolate, CH_3Tr is methyltransferase, ⋀⋀⋀ is CO dehydrogenase with 3 subsites, X, Y, Z. SS-Rd is CO dehydrogenase disulfide reductase and H_2ase is hydrogenase. The broken arrow indicated anabolic reactions. [From Wood *et al.*, 1986].

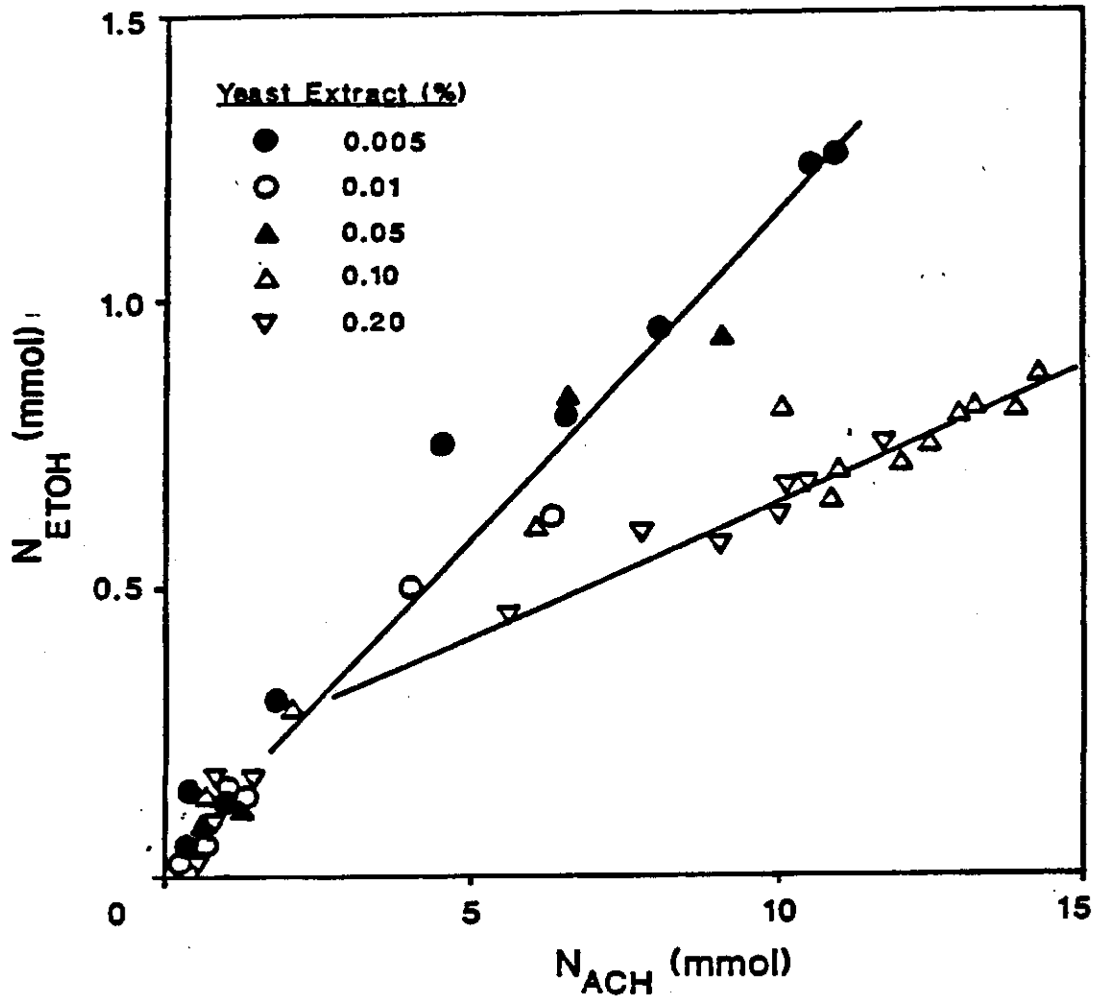

Figure 10. Product ratio for *Clostridium* sp. in batch culture at various initial yeast extract concentrations.

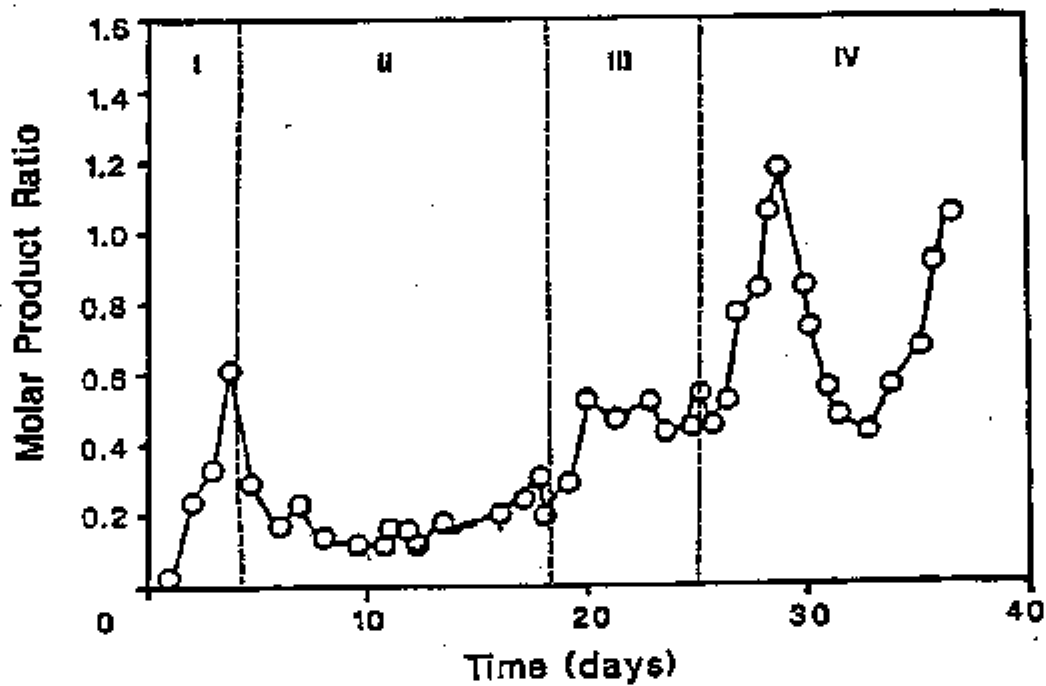

Figure 11. Molar product ratio for Clostridium sp. in continuous culture.

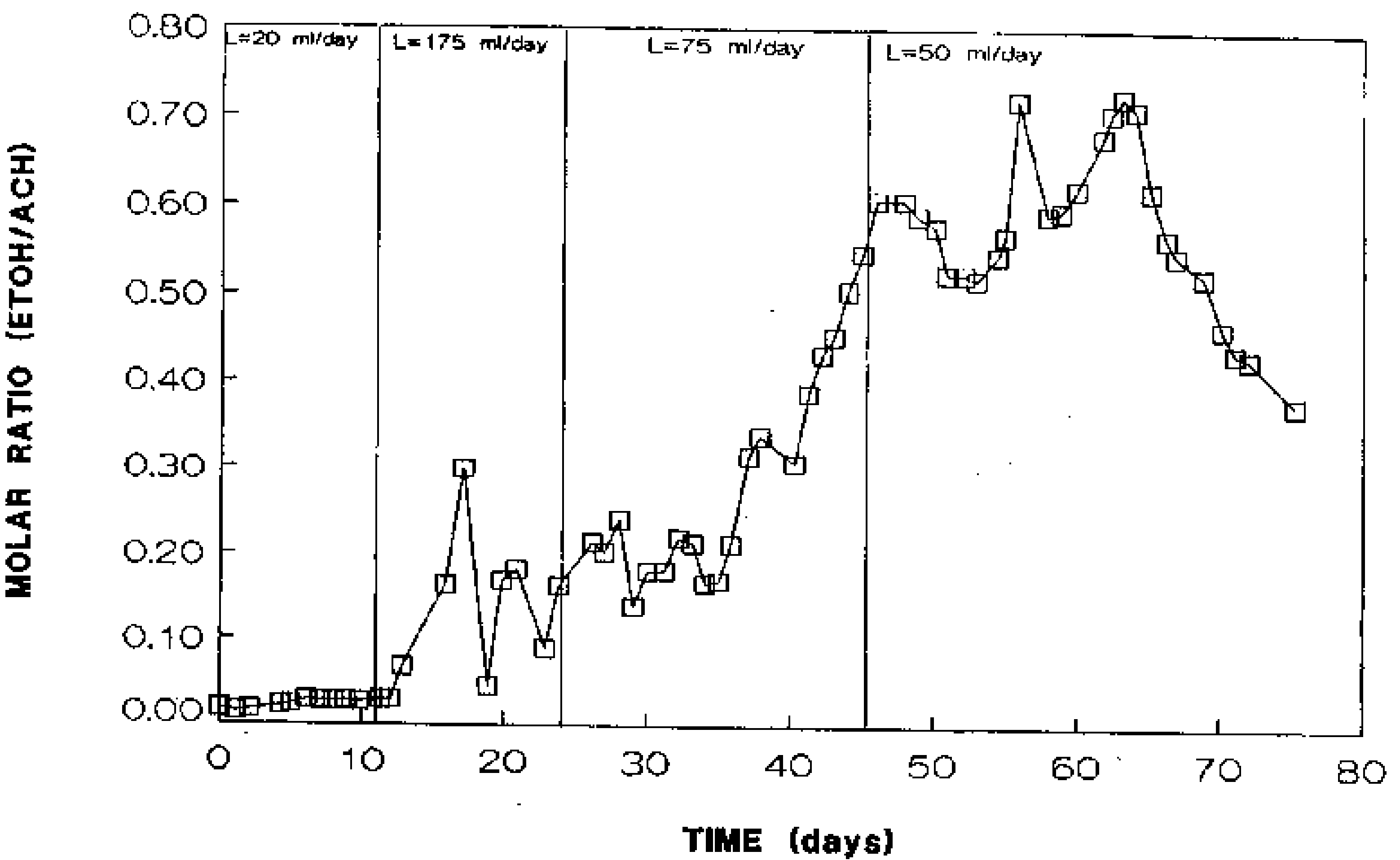

Figure 12. Molar product ratios with time at various liquid flow rates for *Clostridium* sp. in continuous culture (pH 4, T = 37°C, 400 rpm, G = 4.9 ml/min).

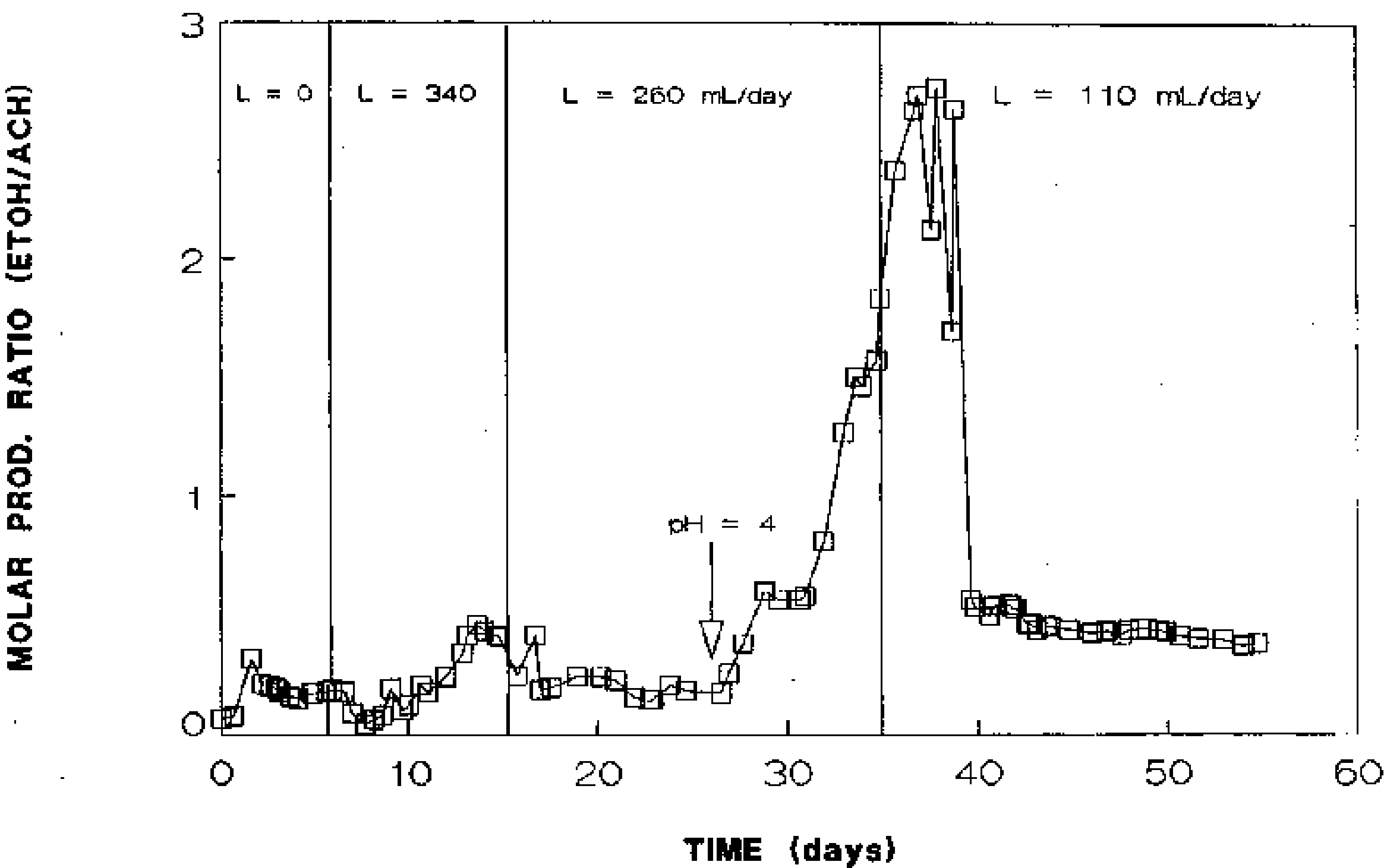

Figure 13. Molar product ratio of ethanol over acetic acid in the CSTR as a function of time.

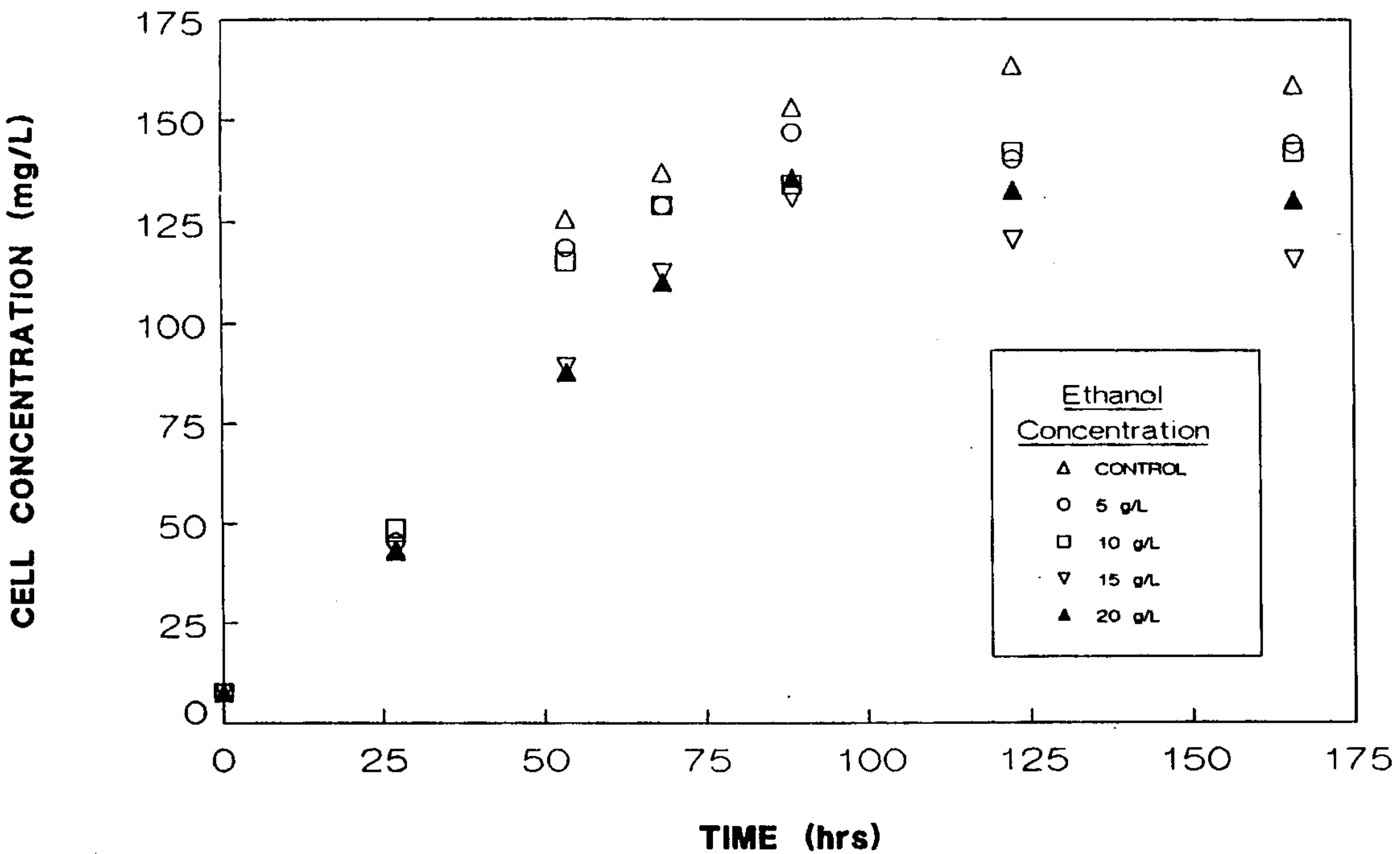

Figure 14. Cell concentration profiles for *Clostridium* sp. in the presence of various concentrations of ethanol.

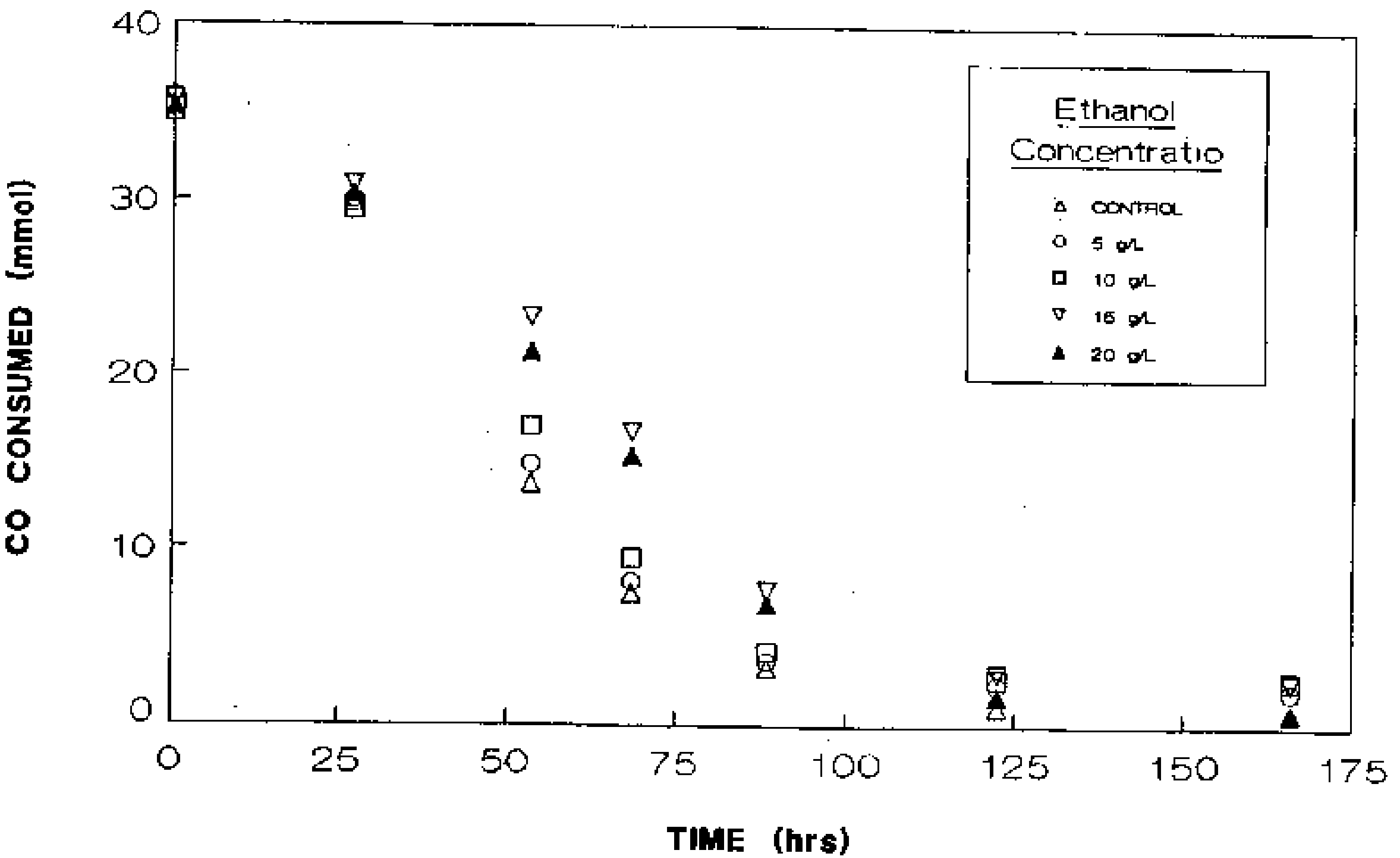

Figure 15. Co consumption profiles for *Clostridium* sp. in the presence of various concentrations of ethanol.