PHYSIOLOGICAL ASPECTS OF THE ACETONE-BUTANOL FERMENTATION

by

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Submitted to the Faculty of Graduate Studies and Research of McGill University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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ABSTRACT

The effect of the key physiological parameters on the production of solvents in the acetone-butanol fermentation using the anaerobic bacterium Clostridium acetobutylicum was examined in this work.

The theoretical solvent yield was calculated based on expressing stoichiometric relationships between the substrate and the products of the process. The maximum theoretical yield under the acceptable process conditions was established ranging from 38.6% to 39.9%.

A linear correlation was established between the production of solvents and gases which varied with the mixing rate in the fermentation system.

Maximum specific rates of solvent production were 5.5, 3.8 and 0.8 mmole/h.gcell for butanol, acetone and ethanol, respectively, corresponding to an impeller Reynolds number of 3.93×10^4. The fermentation became inactive at an impeller Reynolds number of 6.47×10^4.

Elevated hydrogen partial pressure affected the metabolism of C. acetobutylicum resulting in increased butanol and ethanol yields (based on glucose) by an average of 18% and 13%, respectively. These increases were obtained at the expense of acetone and endogenous hydrogen production in the fermentation system.

A mathematical model for the batch acetone-butanol fermentation was formulated using original experimental data for the microbial growth, sugar consumption and metabolite biosynthesis. This model was used for computer process simulations. Parametric sensitivity analysis indicated the importance of the key process parameters.

A method of systems analysis was applied in analysing pronounced physiological differences in the performance of one of the C. acetobutylicum culture strains. The cellular transport mechanism for substrate (glucose), solvents and acids through the cell membrane was established to depend on its permeability and the number of sugar transport "sites". Experimental results obtained from the study of the uptake of 3-O-methyl glucose (0.7mM) by the "normal culture" and the "retarded culture" confirmed the theoretical predictions of a slower transport in the "retarded culture". The initial uptake rate of the substrate in the normal culture was fifteen (15) times higher than that for the retarded culture. Adjustment of the culture pH resulted in a further three fold increase of this parameter.

The theoretical predictions were further confirmed by the cell membrane permeability measurement, as well as by the enhancement of solvent synthesis by addition of 0.2% Tween 80 to the growth medium, and finally by reversing the performance of the retarded culture and producing a normal

fermentation through the use of a small inoculum size of 0.5%.

A mathematical "Physiological State Model" was developed which includes the culture physiological parameters as well as the internal and the external culture conditions. Using this mathematical model the standard and the substandard acetone-butanol fermentations could be simulated.

These results demonstrate the application of the method of systems analysis in elucidation of the role played by the key culture physiological parameters in the fermentation process.

RESUME

La présente étude a porté sur l'effet des paramètres physiologiques clés relatifs à la production de solvents à partir de la bactérie anaérobie Clostridium acétobutylicum.

Le rendement théorique en production de solvants a été calculé à l'aide d'expressions stoechiométriques reliant le substrat aux produits de fermentation.

Les valeurs du rendement théorique maximal obtenues dans les conditions favorables sont de l'ordre de 38.6% à 39.9%.

Une corrélation linéaire a été établie entre la production de solvants et celle des gaz; elle varie avec le taux d'agitation du système de fermentation.

Les taux spécifiques de production de solvants sont respectivement de 5.5, 3.8 et 0.8 mmole/h.g biomasse pour le butanol, l'acétone et l'éthanol, ils correspondent au nombre de Reynolds (ajusté pour une pale) de 3.93×10^4. La fermentation devient inactive pour un nombre de Reynolds (ajusté pour une pale) de 6.47×10^4.

Une pression partielle d'hydrogène plus élevée régularisait le métablisme de C. acétobutylicum et produisit un accroissement respectif de 18% et 13% des rendements de butanol et d'éthanol. Ces augmentations l'ont été au profit

des rendements en acétone et en hydrogène intrinsèque au procédé de fermentation.

Les données expérimentales de croissance microbienne, de consommation du glucose et de biosynthèse des métabolites obtenues de fermentations acétono-butylique en mode discontinu ont permis l'élaboration d'un modèle mathématique qui a servi à des simulations du procédé sur ordinateur. L'analyse de la sensibilité des paramètres a révélé l'importance des paramètres clés du procédé.

Une méthode d'analyse des systèmes a servi à l'étude de l'aspect physiologique et des différences marquées des performances de la culture de C. acétobutylicum. Le mécanisme de transport cellulaire du substrat (glucose), des solvants et des acides à travers la membrane est attribuable à sa perméabilité et à la quantité de "sites" de transport des sucres. Les résultats expérimentaux obtenus lors d'études d'assimilation de 3-O-methyl glucose (0.7 mM) par une culture "normale" et une "culture affaiblie" ont confirmé les prévisions théoriques à l'effet que le transport est ralenti pour les cultures affaiblies. Le taux initial d'assimilation de substrat pour une culture normale est quinze (15) fois plus élevé que celui observé pour une culture affaiblie. Un ajustement du pH a eu pour effet de multiplier par trois cet écart.

Les prévisions théoriques ont également été véri-

fiées à la fois lors de mesures de la perméabilité de la membrane cellulaire, lors d'observations d'augmentation de la production de solvant due à l'addition de 0.2% du Tween 80 au milieu de croissance et lors de l'inversion du rendement de la culture affaiblie en culture normale par l'addition en faible quantité (0.5%) d'inoculum.

Un modèle mathématique de l'état physiologique comprenant aussi bien les paramètres physiologiques que les conditions internes et externes de la culture a éventuellement été développé. Ce modèle mathématique a permis la simulation de fermentations normales aussi bien qu'inférieures à la normale.

Ces résultats illustrent l'usage de la méthode d'analyse des systèmes dans l'élucidation du rôle joué par les paramètres physiologiques clés des cultures dans les procédés de fermentation.

TO

RAFI

for his love and support

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my academic advisor, Dr. B. Volesky for his encouragement, assistance and guidance throughout this work. His support and encouragement particularly during the "unsuccessful" fermentations are deeply appreciated.

I am also grateful to Dr. J. Votruba of the Czechoslovak Academy of Sciences for his concern, attention and his invaluable assistance in the development of the mathematical models. His impressive knowledge and understanding of the biological systems and the naturally occurring phenomena, and the inspiring discussions I had with him have increased my appreciation of Biochemical Engineering.

Thanks are extended to Dr. T. Sczesny for preserving the stock cultures and for his continuous encouragement particularly during the early stages of this work. His assistance in performing the high pressure experiments is gratefully acknowledged.

I would also like to thank Dr. Rose M. Johnstone, Chairperson and Professor of the Biochemistry Department of McGill University, and her kind and friendly research assistants who helped me with the sugar transport studies. Dr. Johnstone allowed me to use the facilities in her laboratory and even stayed overtime to personally help me carry out the experiments. Her constructive criticism and concern have improved the quality of this work.

Finally, I thank my parents who have been sources of encouragement throughout my education. The friendship, concern and companionship of my sister Haydeh and my brother Sassan throughout this work are sincerely appreciated.

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INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The importance of utilization of renewable raw materials for the production of chemicals has been well established and confirmed very forcefully in the past decade. Recent dramatic fluctuations and continuous upward trend in the price as well as uncertainty of a reliable steady supply of hydrocarbon feedstock has sparked an intense interest in biochemical and fermentation technology. The chemicals produced by fermentation routes are usually the end products of the energy metabolism of microorganisms. They include solvents, such as butanol, acetone, ethanol and iso-propanol organic acids, such as citric, fumaric, acetic, butyric and lactic and fuel gases, such as methane and hydrogen.

One of the fermentation processes which has gained increasing renewed attention is the acetone-butanol-ethanol (A-B-E) fermentation by the anaerobic bacterium Clostridium acetobutylicum. This process which is one of the oldest industrial fermentations results in the biosynthesis of three solvents (acetone, butanol and ethanol), two gases (hydrogen and carbon dioxide) and two intermediate metabolites (acetic

and butyric acid). The high and increasing demand for industrial solvents and their future in tertiary oil recovery, applicability of solvents as industrial chemical feedstocks, use of butanol as a fuel and the associated production of hydrogen gas highlight the potential contributions of the A-B-E fermentation process. In addition to the industrial importance of its end products, the acetone-butanol process represents an intriguing microbial model system for study of the regulation of end product formation, evaluation of the process kinetics and interrelationships among a number of variable parameters of the fermentation system. The process features interesting complex biochemistry reflected in its metabolic pathway, inhibitory kinetics mechanisms caused by the end products and fascinating aspects of microbial physiology associated with the microbial culture and its responses to the environmental parameters which have a profound effect on the biosynthesis of the products.

Despite the fact that the acetone-butanol process is one of the oldest industrial fermentations there are serious gaps in the understanding of the basic aspects of this process and the limiting factors controlling the productivity of the process which make proper optimization impossible. A report on the current industrial application of acetone-butanol process implied that very little fundamental change has been made in this process since the early years of its exploitation (Spivey, 1978).

The present work addresses the physiological aspects of the acetone-butanol-ethanol fermentation and attemps to elucidate some of the problems associated with the productivity of the solvents and gases in this process.

Each Chapter of this thesis discusses a self-contained topic which includes sections as Introduction, Materials and Methods, Results and Discussion related to the particular aspect of the acetone-butanol fermentation addressed in the Chapter. These Chapters represent independent papers which are to be or have been already published in scientific journals. The thesis is presented as a compilation of papers maintaining their original formats. Each of the Chapters, therefore, has its own reference section listing the relevant literature citations. Those references marked by an asterisk (*) constitute also a part (Chapter) of this thesis.

1.2 LITERATURE REVIEW

A literature review relevant to each topic dealt with in individual Chapters is presented at the beginning of each Chapter.

A general literature review pertaining to the basic aspects of the acetone-butanol fermentation is presented in this section. This review has been done with respect to the following aspects: 1) Characterization of the microbial culture and its nutritional requirements; 2) Kinetics of the batch acetone-butanol fermentation; 3) Carbohydrate metabolism and biochemistry of C. acetobutylicum.

1.2.1 Characterization of the Microbial Culture and its Nutritional Requirements

Study of the acetone and butanol producing bacteria dates back to 1861 when Pasteur showed direct production of butanol by fermentation. Since then there have been numerous studies on the characterization, morphology, physiology, biochemistry and metabolic pathways, and the nutritional requirements for growth and solvent production by these bacteria (Johnson et al., 1932; Weizmann and Rosenfeld, 1937; Prescott and Dunn, 1959; Taha et al., 1973). The cultures commonly used in this process are members of the genus Clos-

tridium. Species of this genus are divided according to their carbohydrate metabolism and biochemistry, the end products of their energy metabolism, carbon sources, growth requirements and some genetic aspects (Beesch, 1952; 1953; Andreesen et al., 1970; Cummins and Johnson, 1971). Clostridia are anaerobic, spore forming rods, usually motile and occasionally non-motile. They are generally gram positive although the older cultures sometimes stain gram negative. The choice of the microorganism depends on the nature of the raw material being used and the type and concentration of the end products desired (Compere and Griffith, 1978; Soni et al. 1982). A commercial strain of Clostridium acetobutylicum is usually used in the industrial production of acetone and butanol. In addition to these two solvents, small amounts of ethanol, some acetic and butyric acid and large amounts of fermentation gases, hydrogen and carbon dioxide, are also produced in this process. The culture of C. acetobutylicum is capable of growth and solvent production on a variety of substrates of the pentoses and hexoses, mono-, di- and polysaccharide group. The most widely used substrates include: sugar-based materials, such as black strap molasses, sugar cane, sugar beets and sweet sorghum (Beesch, 1952; 1953; Prescott and Dunn, 1959; Mahmoud et al., 1974), starch-based materials, such as corn, rice, wheat, potatoes, rye and cassava (Wilson et al., 1930; Peterson and Fred, 1932; Beesch, 1953; Taha et al., 1973),

hydrolysates of wood or the wood sugars in the form of sulfite waste liquor, as well as corncob hydrolysates (Wiley et al., 1941, Abou-Zeid et al., 1974). Invert or black-strap molasses are the most favored natural media for this process. C. acetobutylicum does not have the ability to effectively ferment cellulose (Compere and Griffith, 1978). The concentration and the type of the carbohydrate used drastically influences production of butanol, acetone and ethanol in both their final yield, based on sugar, and their relative proportions. For example, the highest yield of solvents with an Egyptian strain of C. acetobutylicum was obtained with a concentration of 5% for millet, corn and sweet potato, of 7% for corncobs and 12% for molasses (Taha et al., 1973). Total solvent production by another strain of this culture was the highest at a concentration of 2.5% for cellobiose, lactose, sucrose and dextrin and of 5% for xylose and fructose (Compere and Griffith, 1978). Production of solvents is also dependent on the amount and sources of nitrogen and phosphate and the minerals available in the medium. Inorganic nitrogen in the form of ammonia or ammonium sulfate is usually sufficient when natural media such as black strap molasses or beet molasses is used. However, it is usually preferred to use ammonium salts plus a higher form of nitrogenous material such as yeast water or distillation slop (Langlykke et al., 1934; Beesch, 1952) peanut cake, cotton seed cake, rice bean or corn steep liquor

(Mahmoud et al., 1974; Spivey, 1978) or urea, thiourea, peptone or yeast extract (Abou-Zeid et al., 1978) to secure a higher production rate and a maximum yield of solvents. Phosphate can be supplied in the form of calcium acid phosphate, superphosphate or monoammonium phosphate (Beesch, 1953; Mahmoud et al., 1974), Potassium phosphate (Monot et al., 1982) or many other forms of soluble phosphate. Cell growth and solvent production is also dependent on the presence of Mg, Mn, Fe and K in the medium, although these elements exhibit deleterious effects when in excess (Monot et al., 1982).

Utilization of the synthetic or semi-synthetic media in the acetone-butanol fermentation was reported by Robinson (1922), Speakman (1923), Davies and Stephenson (1941), Ulmer et al. (1981), and Monot et al. (1982). These media provide ease of handling and experimentation and are suitable for optimization purposes. The microbial culture demonstrated requirements for at least two growth factors, namely asparagine and coenzyme(s) available in yeast or maize (Weizman and Rosenfeld, 1937; Davies and Stephenson, 1941) when grown on a synthetic medium.

Monot et al. (1982) reported varying degrees of solvent production in a glucose-salt-vitamin medium depending on the concentration of glucose and the type and concentration of the salts used. A glucose concentration of less than 10 g/L resulted in an acid fermentation and no solvent biosynthesis. The concentration of ammonium acetate, used as

the nitrogen source can also negatively influence the biosynthesis of solvents when used in excess of the optimal concentration of 1.1 g/L.

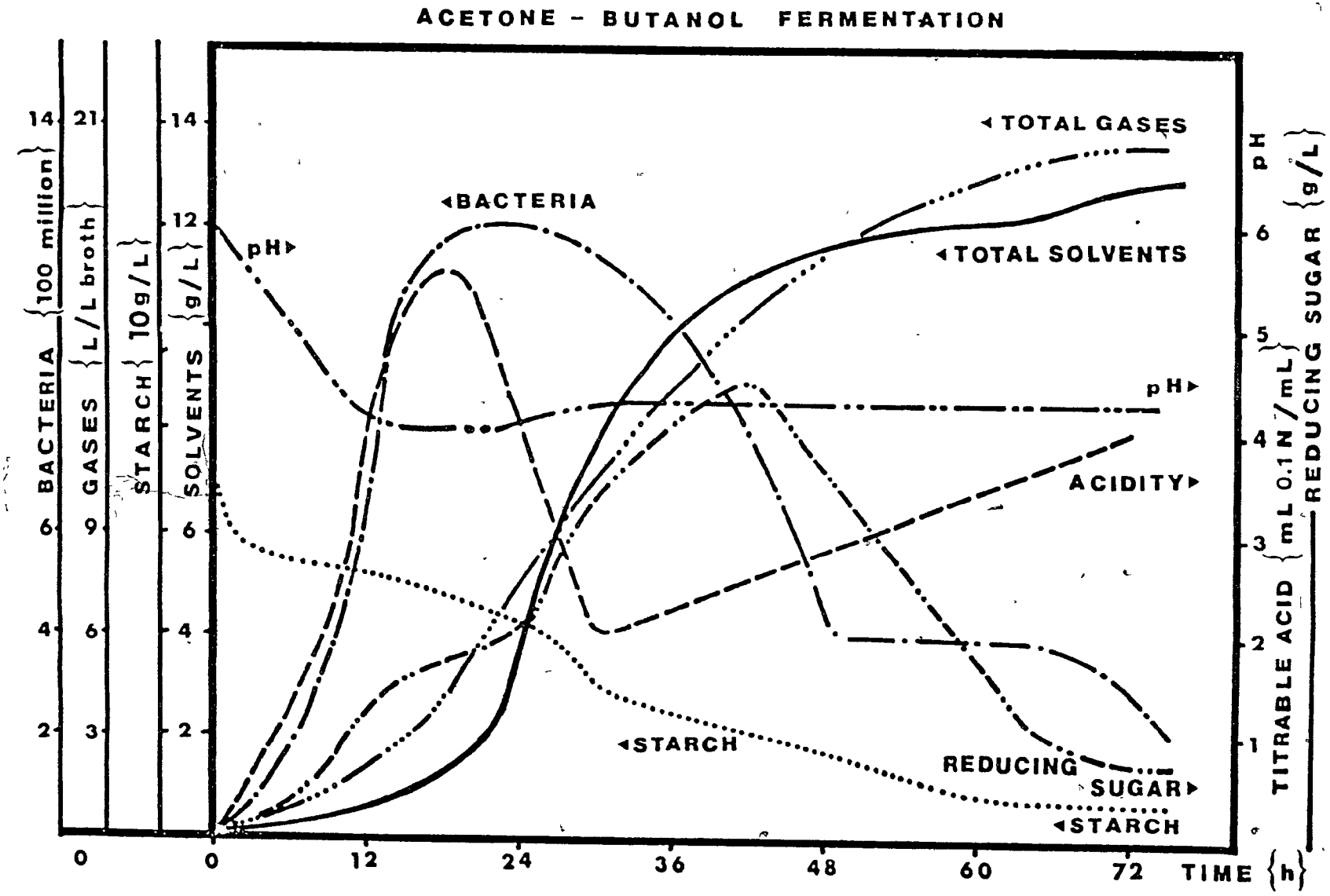
These results demonstrate the great influence of the medium composition on the performance of the microbial culture and indicate that a proper optimization of the medium must be made for the maximum production of the solvents in the A-B-E process.

1.2.2 Kinetics of the Batch Acetone-Butanol Fermentation

The acetone-butanol fermentation process has traditionally been carried out in a batch culture. There have been numerous studies on the product formation kinetics, life cycle of the organism and the chemical changes of the medium during the batch acetone-butanol fermentation (Wilson et al., 1930; Peterson and Fred, 1932; Prescott and Dunn, 1959; Spivey, 1978). An example of the fermentation patterns by C. acetobutylicum is shown in Figure 1. These results provided by Peterson and Fred (1932) illustrate an acetone-butanol fermentation on a 6% corn mash. The variables studied during the entire course of fermentation included the starch consumption, solvent, gas and total acidity formation, reducing sugar content, pH and the morphologicaly changes of the bacterium.

FIGURE 1 The Time Course of a Batch Acetone-Butanol Fermentation on Corn Mash

(Peterson and Fred, 1932)


ACETONE - BUTANOL FERMENTATION
BACTERIA {100 million}
GASES {L/L broth}
STARCH {10g/L}
SOLVENTS {g/L}
pH
TITRABLE ACID {mL 0.1 N/mL}
REDUCING SUGAR {g/L}
TOTAL GASES
BACTERIA
TOTAL SOLVENTS
pH
ACIDITY
STARCH
REDUCING SUGAR
STARCH
TIME {h}
0
12
24
36
48
60
72

The batch A-B-E fermentation is characterized by the growth of the cells, production of the acids which consist mainly of acetic and butyric acid, and formation of the hydrogen and carbon dioxide gases at the beginning of the process. The pH of fermentation broth decreases as a result of the acid production and reaches a value of 3.9 to 4.5 from an initial value of 6.0 - 6.2. When the pH reaches a comparatively low value, formation of the solvents, acetone, butanol and ethanol begins. The acids diminish and exhibit a break in their respective curves. Correspondingly, the pH increases again and formation of the gases continues.

Therefore, the batch acetone-butanol fermentation appears to be a two phase process. The first phase is an acidogenic phase during which the acids accumulate and pH decreases. The second phase is a solventogenic phase. During this phase the acids diminish and the neutral solvents are produced with the resulting increase in the broth pH. Regulation of the metabolic changeover from acidogenic to the solventogenic phase in the acetone-butanol fermentation is still not well understood. There are a number of factors which control the production of solvents and trigger the activity of the enzymes operating in the solventogenic phase. The most important factors include: concentrations of the acetic and butyric acids (Doelle, 1975), pH of the medium (Doelle, 1975; Spivey, 1978), glucose concentration (Davies and Stephenson, 1941), the type and concentration of the

nitrogen source (Wilson et al, 1930), age of the inoculum and motility of the cells (Spivey, 1978). The bacterial culture undergoes conspicuous morphological changes during the course of fermentation and exhibits variable morphology associated with various stages of the fermentation. The morphological variations of the culture depend on the strain of the organism used and the formulation of the medium (Spivey, 1978). Observations of the size, shape, characteristics of grouping and variations in staining of the cells are reported by Peterson and Fred (1932) and Spivey (1978). At the beginning of the fermentation the cells are rod-shaped, motile and short. At the early exponential growth phase, the rod-shaped cells grow longer, vigorous and rapidly dividing. The late exponential growth phase of the culture is characterized by shorter rod-shaped cells and the presence of a large number of large club-shaped cells (Clostridia). Many cells appear in pairs or in longer chains at this stage. As the cells grow older and the fermentation approaches the final hours, the vegetative cells decrease still further in size, less clostridia are apparent and many spores appear. Morphological changes of the culture are relatively constant with time and can be used to assess the age and progress of a fermentation to within one hour (Spivey, 1978).

The fermentation pattern of C. acetobutylicum is usually independent of the type of the raw material used although there may be variations in the rates of biosynthesis

of the products, the overall fermentation time, the final solvent yield, the ratio of the solvents and the maximum or minimum observed concentrations of the fermentation products. For example, in a glucose-liver extract medium (Davies and Stephenson, 1941) the consumption rate of glucose and the production rates of the solvents were much higher than the corn mash fermentation discussed before (Figure 1). The overall fermentation time was almost 40 hours with glucose while it took over 70 hours when corn mash was used as a substrate. The ratios of the solvents were also drastically different in these fermentations. While an acetone to butanol ratio of 1 to 2 was obtained in the corn mash fermentation, the glucose fermentation resulted in a ratio of 1 to 4. The different strains used in these two cases could also have contributed to the observed differences. A solvent yield of almost 30%, based on the sugar consumed, was obtained in these fermentations. This is a normal solvent yield in a typical A-B-E fermentation. The normal ratio of the solvents produced is 6:3:1 of butanol:acetone:ethanol. In the A-B-E fermentation a relatively low concentration of the fermentable sugars in the range of 6% to 7% is usually used. This is due to high toxicity of the solvents produced in the fermentation which inhibit the cellular activities at a concentration of over 2% (w/v) (Leung and Wang, 1981; Ulmer et al., 1981). Butanol has been recognized as the major inhibitory product which limits the specific growth rate of

the culture to one-half of its maximum at concentrations of 11 g/L to 13 g/L. A complete inhibition of growth and cellular activities occurs at butanol concentrations of over 15 g/L. The inhibitory effect of the end products is one of the major limiting factors in improvement of the A-B-E process. The economics of this fermentation process would significantly improve if the solvent yields and concentrations could be increased in the fermentation broth.

1.2.3. Carbohydrate Metabolism and Biochemistry of Clostridium acetobutylicum

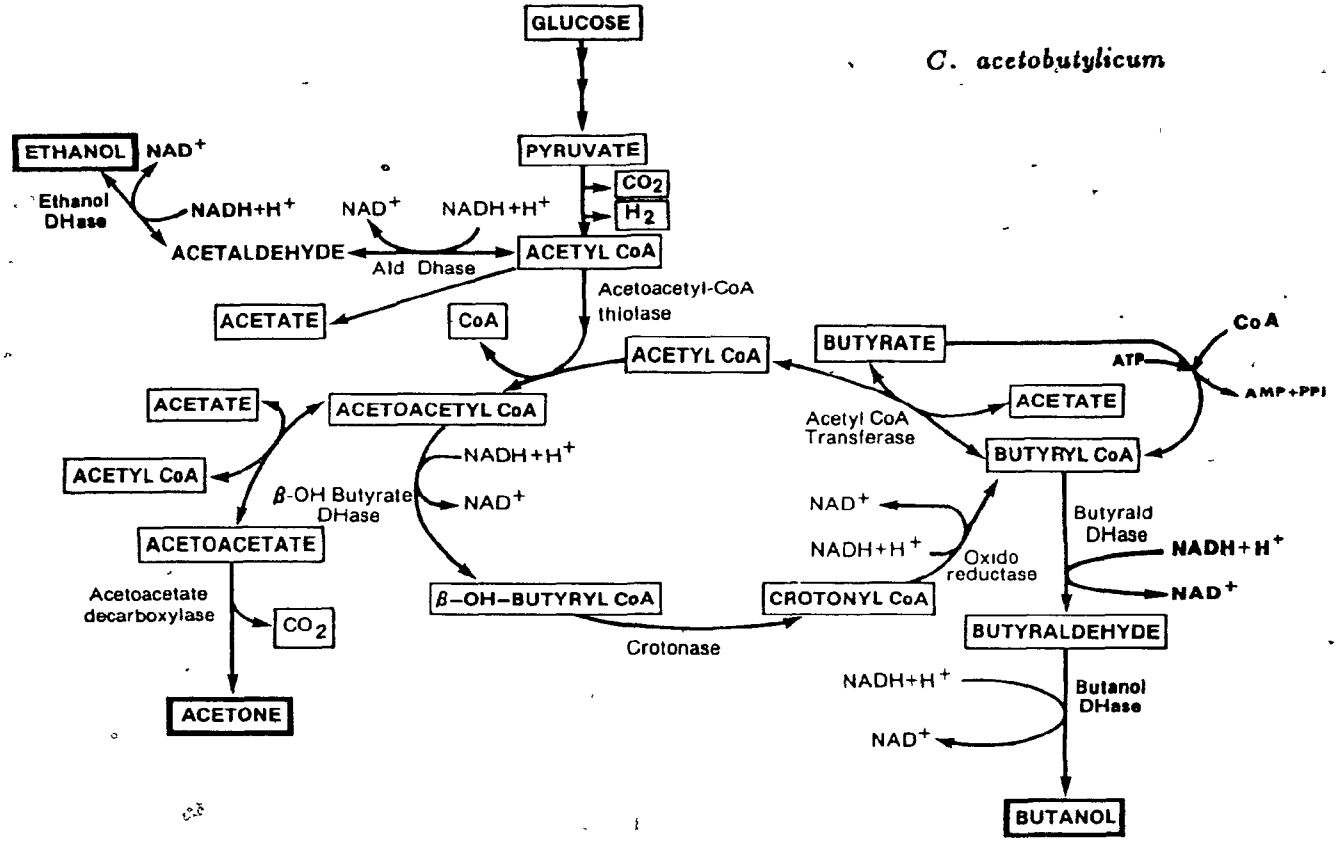
A knowledge of the metabolic features that control the rates, yields and types of fermentation products formed is necessary for a better understanding of the microbial process and its eventual optimization.

The information available in the literature on the acetone-butanol fermentation, its biochemistry and the reactions involved in the production of individual products is sufficient for charting a metabolic pathway for carbohydrate metabolism by C. acetobutylicum. This metabolic pathway has been recently discussed by Doelle (1975) Lenz and Moreira (1980) and Leung (1982). Figure 2 illustrates the overall pathway of the fermentative glucose metabolism by this microorganism. It has been shown that the production of different intermediates in this process is due to the thermodynamic

FIGURE 2 Biochemical Pathway of Glucose Metabolism by Clostridium acetobutylicum

(Doelle, 1975)

C. acetobutylicum



requirements imposed on the cellular energy metabolism and the need for compensation of the hydrogen balance (Thauer et al., 1977).

Glucose is first metabolised by the Embden-Meyerhof-Parnas (EMP) pathway resulting in the formation of pyruvate. This process which is in common with the ethanol fermentation and most true fermentations, yields two moles of NADH (reduced nicotinamide adenine dinucleotide). Pyruvate is converted to acetyl-CoA through the phosphoroclastic reaction accompanied by the formation of gaseous hydrogen and carbon dioxide. In this system pyruvate is first decarboxylated by pyruvate synthase (pyruvate-ferrodoxin oxidoreductase) or pyruvate dehydrogenase with the formation of a thiamine pyrophosphate (TPP) enzyme complex (Doelle, 1975). The electrons are then transferred to ferredoxin. The reduced ferredoxin is reoxidized by hydrogenase and molecular hydrogen is formed. Transfer of the electrons from reduced ferredoxin to molecular hydrogen involves the synthesis of NADH via the action of NADH-ferredoxin oxidoreductase. This enzyme plays a significant role in providing the cells with NADH or NAD^+ and in regulation of the reducing power in the microorganism (Petitdemenage et al., 1976). Through its reversible action, NADH ferredoxin oxidoreductase regulates the ratio $H_2:CO_2$ according to the culture conditions to meet the different cell needs during the acidogenic or synthogenic phases. This is the reason that the ratio

$H_2:CO_2$ greater than 1 or less than 1 is observed in the fermentation of C. acetobutylicum (Blusson et al., 1981).

Acetate is produced in two steps from acetyl-CoA. In the first step acetyl phosphate is produced by the action of phosphotransacetylase:

$$\text{(acetyl-coenzyme A) + phosphate} \xrightleftharpoons{\text{phosphotransacetylase}} \text{acetyl phosphate + coenzyme A}$$

The second step results in the synthesis of acetate and the generation of ATP by acetokinase action:

$$\text{acetyl phosphate + ADP} \xrightleftharpoons{\text{acetokinase}} \text{acetate + ATP}$$

Butyrate is formed to meet the requirements of energy generation and the need for an optimal thermodynamic efficiency of ATP synthesis by the cells (Thauer et al., 1977; Doelle, 1975). From the energetic point of view, production of acetate as the only end product would not be satisfactory because with the decrease of the pH into the acid region it becomes difficult to reoxidize NADH. Therefore butyrate which is much less an acid end product than acetate is formed (Doelle, 1975). The cyclic mechanism involved in the production of butyrate starts with the biosynthesis of the key compound acetoacotyl-coenzyme A from two molecules of acetyl-coenzyme A:

$$2\ CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} \xrightarrow{\text{thiolase}} CH_3\overset{O}{\overset{\|}{C}}\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + HSCoA$$

Acetoacytyl-CoA is then reduced to β-hydroxybutyryl-coenzyme A by the enzyme β-hydroxybutyrate dehydrogenase. During this reation NADH is oxidized to NAD^+ as follows:

$$CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + NADH + H^+ \rightleftharpoons CH_3\text{-CHOH-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + NAD^+$$

(acetoacetyl-CoA) (β-OH-butyryl-CoA)

Dehydration of β-hydroxybutyryl-CoA by the enzyme crotonase yields crotonyl-coenzyme A, which is then reduced by the enzyme butyryl-coenzyme A dehydrogenase to butyryl-coenzyme A:

$$CH_3\text{-CHOH-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} \rightleftharpoons CH_3\text{-CH=CH-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + H_2O$$

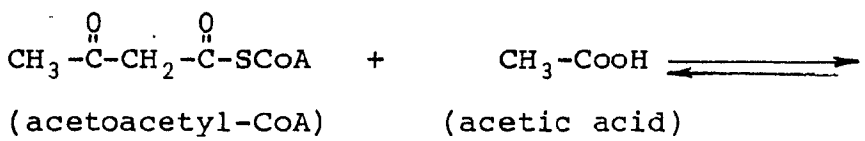
(β-OH-butyryl CoA) (Crotonyl CoA)

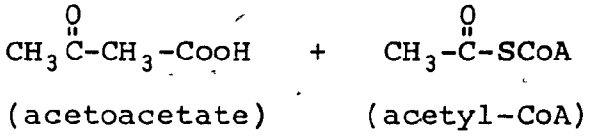
$$CH_3\text{-CH=CH-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + NADH + H^+ \rightleftharpoons CH_3\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + NAD^+$$

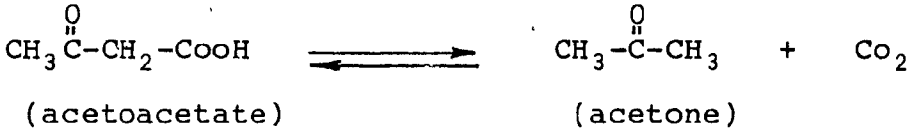
(crotonyl-CoA) (butyryl-CoA)

Biosynthesis of butyryl-CoA from crotonyl-CoA involves the oxidation of NADH. The last step in the cyclic mechanism is a transfer reaction, whereby acetyl-CoA and butyrate are formed through the combined action of acetate and butyryl-CoA with a fatty acid CoA tranferase. A high acetate concentration is required to drive the butyryl CoA-acetyl CoA transferase reaction and produce butyrate. This reaction is also controlled by the regulatory properties of NADH-ferredoxin oxidoreductase and the proton concentration (Zeikus, 1980).

Acetone is produced through the diversion of the cyclic mechanism which results in the conversion of acetoacetyl-CoA to acetoacetate. The irreversible decarboxylation of acetoacetate yields acetone. Diversion of the cyclic mechanism in C. acetobutylicum occurs as soon as the acids (butyric and acetic) lower the pH of the medium to about 4.0. It is believed that a new enzymatic system comes into operation at this low pH value (Doelle, 1975). The enzyme responsible for the decarboxylation of acetoacetate is acetoacetate decarboxylase. The following reactions express the acetone production:







Butanol production occurs through reduction of butyrate. This compound is produced after diversion of the cyclic mechanism and formation of acetone in the system. In addition to the interruption of the cycle, two electron accepting reactions which regenerate the oxidized form of NAD, namely, reduction of acetoacetyl-CoA to β-OH-butyryl-CoA and the reduction of crotonyl-CoA to butyryl-CoA are also eliminated. Continuation of glycolysis and the overall cell metabolism requires that alternative electron accepting reactions be found. Reduction of butyryl-coenzyme A to butyraldehyde and further reduction of butyraldehyde to butanol seems to be a convenient solution. Biosynthesis of butanol takes place in three steps. In the first step the coenzyme from an acetyl group is transferred to the butyryl group and butyryl-CoA is produced by the action of CoA-transferase. Butyryl-CoA may be generated by an alternative

mechanism requiring ATP and CoA if there is a deficiency in the amount of acetyl-CoA available.

The second step involves the reduction of butyryl-CoA to butyraldehyde, catalyzed by aldehyde dehydrogenase. During this reaction NADH is reoxidized and NAD^+ is formed. Reduction of butyraldehyde to butanol is also NAD^+ linked and carried out with the alcohol dehydrogenase:

$$\text{butyryl-CoA} + \text{NADH} + H^+ \rightleftharpoons \text{butyraldehyde} + NAD^+ + \text{HSCoA}$$

$$\text{butyraldehyde} + \text{NADH} + H^+ \rightleftharpoons \text{butanol} + NAD^+$$

Reduction of butyric acid to butanol takes place in the presence of glucose but not pyruvate (Prescott and Dunn, 1959). It it thus probable that glucose or triose-phosphate is the reducing agent for butyric acid (Davies, 1943).

Ethanol production takes place in a side reaction of the major pathway branching off from acetyl-CoA. Reduction of acetyl-CoA to acetaldehyde and ethanol is concomitant with butanol production. It is believed that ethanol production plays a supporting role in the regeneration of NAD^+ when the cyclic mechanism stops and acetone formation starts (Doelle, 1975):

$$CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + NADH + H^+ \underset{\text{dehydrogenase}}{\overset{\text{aldehyde}}{\rightleftharpoons}} CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-H} + NAD^+ + HSCoA$$

(acetyl CoA) (acetaldehyde)

$$CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-H} + NADH + H^+ \underset{\text{dehydrogenase}}{\overset{\text{alcohol}}{\rightleftharpoons}} CH_3\text{-}CH_2OH + NAD^+$$

(acetaldehyde) (ethanol)

C. acetobutylicum uses the same enzymes for the reduction of butyryl-CoA to butanol as for the reduction of acetyl-CoA to ethanol (Doelle, 1975). Fogarty and Ward (1971) also reported that the alcohol dehydrogenase of this microorganism is non-specific with regard to the substrate.

The metabolic pathway of sugar catabolism for C. acetobutylicum discussed here is consistant with the thermodynamic requirements of the cell and with the balances of carbon and electrons in the fermentation system. The roles of butyric acid and acetic acid which are established as intermediate products in the biosynthesis of butanol and acetone in the A-B-E fermentation (Prescott and Dunn, 1959; Doelle, 1975; Wood et al., 1945) and the relationships between these acids and the corresponding solvents are demonstrated in this pathway. Although the factors governing the type and concentration of end products are not clearly known, it seems that the regulation of electron flow and the pH of the fermentation both play significant roles.

1.3 CHOICE OF THE SYSTEM

A review of the literature showed that one of the major problems associated with the acetone-butanol biosynthesis process is the production of a large quantity of the undesirable end products. Butanol is the most valuable and desired end product in the A-B-E fermentation process. However, its production is always associated with the simultaneous production of acetone and, a lesser extent, ethanol. The diversion of substrate carbon greatly influences the final concentration of butanol in the fermentation broth and its yield on glucose.

A logical approach to elucidation of this problem is to determine the theoretical "efficiency" of the fermentative system in terms of solvent production. If the difference between the experimentally observed and the theoretical "efficiency" was too great the existing obstacles could be manipulated or eliminated to improve the conversion efficiency and result in an improved production of the solvent. The production of other end products could be suppressed in order to enhance the biosynthetic production of the desirable product (butanol). The yield coefficient can be used as a criterion to assess the efficiency of the system since it represents the percent recovery of the substrate carbon and energy in the individual products.

A study of the biochemical pathway of sugar metabolism in C. acetobutylicum revealed the biochemical basis of acetone production and the biochemical necessity of its production along with butanol. According to this scheme, a prevention of acetone biosynthesis would result in the halt of butanol production. In spite of this interdependency, it may be feassible to regulate the metabolic pathway in such a way that the production of butanol would be enhanced at the expense of acetone. This could be accomplished by imposing a control on the flow of electrons in the cellular metabolic pathway since the biosynthesis of butanol and acetone is closely related to the balance of electrons in the metabolic system. For this purpose, experimental work has to be carried out in conjunction with an attempt to generalize and quantify the aspects of culture physiology. Based on the biochemical and the physiological response of the culture, a mathematical model capable of adequately describing the culture behavior could be developed which would reflect the interplay of major culture parameters and at the same time indicate the degree of their interdependence.

The second major problem in the acetone-butanol process is the inhibitory effect of the final end products and in particular, butanol. This inhibition limits the initial sugar concentration which can be effectively used in the process and subsequently limits the final concentration of all the end products in the fermentation broth including

butanol. The physical, chemical and biochemical basis of the end product inhibition is still not completely understood. Therefore, any attempt in elucidating the problem and decreasing the end product inhibition should first be directed at developing understanding of the mechanism involved in the inhibition.

Cellular membrane is recognized as the primary site of alcohol inhibition. Due to its controlling role in the mass transport of nutrients and end products in and out of the cell, any inhibition of the membrane functionality will greatly affect the cellular activities. The key physiological interactions in the cellular membrane have to be identified as well as the role quantified of the key parameters in the regulation of solvent biosynthesis.

The experimental and theoretical aspects of this work address the development of methodology as well as insight of the aforementioned problems associated with the microbial process investigated. The culture of C. acetobutylicum and the A-B-E microbial process have been useful in constituting an intriguing fermentation system to be used as a model system for challenging and potentially useful investigations. It is believed that approaches taken as well as some of the conclusions of such work could be useful in studying also other industrially attractive microbial cultures and their activities.

1.4 OBJECTIVES

The main objective of this work was to investigate the effect of the key physiological parameters on the performance of the microbial culture and production of solvents by the fermentation system. The fermentation culture system converting glucose into a mixture of neutral solvents (acetone-butanol-ethanol) by a strain of Clostridium acetobutylicum was selected for the study.

In order to qualify the overall objective, aspects of the general tasks have to be divided into a set of more detailed objectives outlining the main axes of the study undertaken and reported on in this work:

1. Determination of the theoretical solvent yield:

   a) To develop a general method for calculation of the theoretical solvent yield.

   b) To estimate the maximum theoretical solvent yield, under various conditions of cellular growth and metabolite production.

2. Establishment of the effect of mixing rate:

a) To investigate the effect of mixing rate on the production rates of solvents and fermentation gases.

b) To study the possibility of development of a correlation between solvent and gas production in the process.

c) To evaluate the gas-to-solvent ratio for various degrees of mixing in the fermentation system.

3. Elucidation of the effect of hydrogen gas pressure:

a) To study the effect of the hydrogen gas pressure on the overall performance of the microbial culture.

b) To study the regulation of solvent production and in particular butanol biosynthesis under conditions of increased hydrogen gas pressure.

4. Mathematical modelling of the batch acetone-butanol fermentation:

a) To develop a mathematical model based on the biochemical and physiological aspects of the acetone-butanol fermentation.

b) To simulate the acetone-butanol batch fermentation using the developed mathematical model.

c) To determine the key kinetic parameters and their order of significance in control of the culture performance.

5. To develop suitable mathematical models for the transport of sugar, solvents and acids through the cellular membrane.

6. To determine the role of the cellular membrane in regulation of solvent production.

1.5 THEORETICAL ASPECTS

Selected general theoretical aspects considered as an essential background for this work are presented in this Chapter. Included are: 1) Estimation of the medium redox potential, 2) Determination of the carbon, energy and electron balances for the fermentation system.

1.5.1 Estimation of the Medium Redox Potential

The reduction-oxidation or the redox potential (E_h) of the fermentation medium was continuously monitored in the fermentor batch experiments. This parameter which indicates the potential of a medium in donating or accepting electrons is an important parameter particularly in anaerobic fermentation processes. It can be used to regulate the cellular activities and biosynthesis of the fermentation products (Kjaergaard, 1976; 1978). The anaerobic bacteria usually require a low redox-potential for their optimal performance. For C. acetobutylicum as with most clostridial species the optimum redox potential is about - 0.2 volt (Reed and Orr, 1942; Spivey, 1978). In fermentation processes a low redox potential can be attained by addition of reducing agents such

as sodium thioglycollate, sodium formaldehyde sulfuxylate and cysteine or by prevention of oxygen diffusion by the addition of agar to the medium.

In the fermentor batch experiments reported in this work no reducing agent or agar was used. The initial flushing of the system by high purity nitrogen gas, addition of glucose as the substrate and utilization of a strong and active inoculum was sufficient for the establishment of an appropriate anaerobiosis in the fermentation system. Variations of the medium redox potential with the progress of the fermentation was followed and recorded by utilizing a redox-electrode.

The redox-potential (E_h) of a reduction-oxidation process is given by the Nernst equation (Jacob, 1970):

$$E_h = E_o + \frac{RT}{nF} \ln \frac{[ox.]}{[red.]} \qquad (1)$$

where E_h is the potential measured in a solution based on the standard hydrogen electrode, E_o is the standard redox potential of a 50% reduced substrate based on the standard hydrogen electrode, quantities in brackets are the activities of the reactants concerned, R is the gas constant, F is the Faraday number, and n is the number of electrons transferred. Usually the potential is not measured against a standard

hydrogen electrode but rather against a reference electrode. In this case if E_l is the potential measured and E_h^r is the potential of the reference electrode, E_h will be equal to (Pirt, 1975):

$$E_h = E_l + E_h^r \qquad (2)$$

The reference electrode used in this work was an Argenthal system and had a potential of + 200 mV at 38°C with reference to the standard hydrogen electrode.

Redox potential is pH dependent (Jacob, 1970; Pirt, 1975). The E_h values reported in this work are based on the pH of the medium at the time of measurement. Since the pH of the medium was also recorded continuously, it is possible to convert the reported E_h to that based on any pH value. This can be done by considering that a pH decrease of one unit, under conditions of constant activity of the oxidant and reductant and at 38°C, causes the potential to become more positive by 61.8 mV (Jacob, 1970).

1.5.2 Carbon, Energy and Electron Balances in Acetone-Butanol Fermentation

The carbon, energy and electron balances were applied in this work to check the consistency of the experimental results and also to estimate the respective fractions

of the substrate carbon, energy and electrons transferred to the various products of the fermentation process. The following balance equation expressed on one gram-atom carbon of substrate was used to consider the material and energy balances associated with microbial growth and product formation in the A-B-E fermentation:

$$CH_2O + \lambda NH_3 \longrightarrow m\ CH_2N_{0.25}O_{0.5} + q_1\ CH_{2.5}O_{0.25} + q_2\ CH_2O_{0.33} + q_3 CH_3O_{0.5} + q_4\ CH_2O + q_5\ CH_2O_{0.5} + q_6 CO_2 + q_7\ H_2 + SH_2O \qquad (3)$$

In this equation CH_2O represents the elemental composition of glucose;

$CH_2N_{0.25}O_{0.5}$ represents the elemental composition of biomass;

$CH_{2.5}O_{0.25}$ is the elemental composition of butanol;

$CH_2O_{0.33}$ is the elemental composition of acetone;

$CH_3O_{0.5}$ is the elemental composition of ethanol;

CH_2O is the elemental composition of acetic acid and

$CH_2O_{0.5}$ is the elemental composition of

butyric acid. A simple molecular formula of $C_4H_8O_2N$ calculated by Meyberry et al. (1968) for a wide range of bacteria has been used in the present study to represent the biomass concentration.

A carbon balance based on Equation (3) can be written as:

$$m + q_1 + q_2 + q_3 + q_4 + q_5 + q_6 = 1 \qquad (4)$$

where:

- m is the fraction of glucose carbon converted to biomass,
- q_1 is the fraction of glucose carbon converted to butanol,
- q_2 is the fraction of glucose carbon converted to acetone,
- q_3 is the fraction of glucose carbon converted to ethanol,
- q_4 is the fraction of glucose carbon converted to acetic acid,
- q_5 is the fraction of glucose carbon converted to butyric acid, and
- q_6 is the fraction of glucose carbon converted to carbon dioxide.

An energy balance of the system was attempted by utilizing the heat of combustion of the substrate and products (CRC Handbook, 1979; Prochazka et al., 1973) based on the following equation:

$$\sigma = \frac{\Delta H_{C\ product}}{\Delta H_{C\ glucose}} \cdot Y'_p \qquad (5)$$

where σ is the fraction of glucose energy recovered in the products, ΔH_C is the heat of combustion in KCal/mole and Y'_p is the product yield in mole product/mole glucose.

An electron balance concept developed by Erickson (1979) and applied by Yerushalmi and Volesky (1981) was used to determine the fraction of the available electrons in the substrate transferred to the biomass and the fermentation products. This balance is based on the concept of reductance degree for organic substrate, biomass and products, defined as follows (Minkevich and Eroshin, 1973):

$$\gamma_S = 4 + 2\,(+1) - 2 = 4 \qquad \text{Glucose}$$
$$\gamma_X = 4 + 2\,(+1) + 0.25\,(-3) + 0.5\,(-2) = 4.25 \qquad \text{Biomass}$$
$$\gamma_B = 4 + 2.5\,(+1) + 0.25\,(-2) = 6 \qquad \text{Butanol}$$
$$\gamma_A = 4 + 2\,(+1) + 0.33\,(-2) = 5.34 \qquad \text{Acetone} \qquad (6)$$
$$\gamma_E = 4 + 3\,(+1) + 0.5\,(-2) = 6 \qquad \text{Ethanol}$$
$$\gamma_{AA} = 4 + 2\,(+1) + 1\,(-2) = 4 \qquad \text{Acetic Acid}$$
$$\gamma_{BA} + 4 + 2\,(+1) + 0.5\,(-2) = 5 \qquad \text{Butyric Acid}$$
$$\gamma_{H_2} = 2\,(+1) = 2 \qquad \text{Hydrogen}$$

where γ is the number of equivalents of available electrons per gram atom carbon based on carbon = 4, hydrogen = 1, oxygen = -2, and nitrogen = -3. A valence of -3 is used with nitrogen because this is the reductance degree of nitrogen in biomass and in ammonia (Erickson, 1979). Based on Equation (3), an electron balance can be expressed as:

$$\gamma_S = m\gamma_X + q_1\,\gamma_B + q_2\,\gamma_A + q_3\gamma_E + q_4\,\gamma_{AA} + q_5\,\gamma_{BA} + q_6\,\gamma_{H_2} \qquad (7)$$

or

$$m\,\frac{\gamma_X}{\gamma_S} + q_1\,\frac{\gamma_B}{\gamma_S} + q_2\,\frac{\gamma_A}{\gamma_S} + q_3\,\frac{\gamma_E}{\gamma_S} + q_4\,\frac{\gamma_{AA}}{\gamma_S} + q_5\,\frac{\gamma_{BA}}{\gamma_S} + q_6\,\frac{\gamma_{H_2}}{\gamma S} = 1 \qquad (8)$$

The first term in Equation (8) gives the fraction of the available electrons in the organic substrate transferred to biomass, the second term gives the fraction of

available electrons transferred to butanol, the third term gives the fraction of the available electrons transferred to acetone, the fourth term gives the fraction of the available electrons transferred to ethanol, the fifth term gives the fraction of the available electrons transferred to acetic acid, the sixth term gives the fraction of the available electrons transferred to butyric acid and the seventh term gives the fraction of the available electrons transferred to hydrogen. This equation expresses the electron balance for the acetone-butanol fermentation.

1.6 REFERENCES

1. Abou-Zeid, A.A., M. Fouad and M. Yassein. 1978. Microbiological Production of Acetone-Butanol by Clostridium acetobutylicum. 1978. Zbl. Bakt. Abt. II, Bd. 133, 125-134.

2. Andreeson, J.R., G. Gottschalk and H.G. Schlegel. 1970. Clostridium formicoaceticum nov. spec. Isolation, Description and Distinction from C. aceticum and C. thermoaceticum, Arch. Microbiol. 72, 154-174.

3. Beesch, S.C. 1952. Acetone-Butanol Fermentation of Sugars, Ind. Eng. Chem. 44, 1677-1683.

4. Beesch, S.C. 1953. Acetone-Butanol Fermentation of Starches, Appl. Microbiol. 1, 85-95.

5. Blusson, H., H. Petitdemange and R. Gay. 1981. A New, Fast and Sensitive Assay for NADH-Ferredoxin Oxidoreductase Detection in Clostridia, Anal. Biochem. 110, 176-181.

6. Commins, C.S. and J.L. Johnson. 1971. Taxonomy of the Clostridia: Wall Composition and DNA Homologies in

Clostridium butyricum and other Butyric Acid-Producing Clostridia, J. Gen. Microbiol. 67, 33-46.

7. Compere, A.L. and W.L. Griffith. 1978. Evaluation of Substrates for Butanol Production, Proc. 35th General Meeting of the Society of Ind. Microbiol., 509-517.

8. Davies, R. and M. Stephenson. 1941. Studies on the Acetone-Butyl Alcohol Fermentation. 1. Nutritional and Other Factors Involved in the Preparation of Active Suspensions of C. acetobutylicum (Weizmann). Biochem. J. 35, 1320-1331.

9. Davies, R. 1943. Studies on Acetone-Butanol Fermentation. 4. Acetoacetic Acid Decarboxylase of Clostridium acetobutylicum, Biochem. J., 37, 230-238.

10. Doelle, H.W. 1975. Bacterial Metabolism, 2nd ed. Academic Press, N.Y. 574-605.

11. Erickson, L.E. 1979. Energetic Efficiency of Biomass and Product Formation, Biotechnol. Bioeng. 21, 725-743.

12. Fogarty, W.M. and J.A. Ward. 1971. The Alcohol Dehydrogenase of Clostridium acetobutylicum, Proc. Biochem. Society, 19-20.

13. Jacob, H.E. 1970. Redox Potential, In: J.R. Norris and D.W. Ribbons (eds.), Methods in Microbiology, New York, Academic Press, 91-123.

14. Johnson, M.J., W.H. Peterson and E.B. Fred. 1931. Oxidation and Reduction Relations Between Substrate and Products in the Acetone-Butyl Alcohol Fermentation, J. Biol. Chem., 91, 569-591.

15. Langlykk, A.F., W.H. Peterson and E. McCoy. 1934. Products from the Fermentation of Glucose and Arabinose by Butyric Acid Anaerobes, J. Bacteriol. 29, 333-347.

16. Kjaergaard, L. 1976. Influence of Redox Potential on the Glucose Catabolism on Chemostat Grown Bacillus licheniformis, Eur. J. Appl. Microbiol., 2, 215-220.

17. Kjaergaard, L. 1977. The Redox Potential: Its uses and Control in Biotechnology, Adv. Biochem. Eng. 7, 131-150.

18. Lenz, T.G. and A.R. Moreira. 1980. Economic Evaluation of the Acetone-Butanol Fermentation Ind. Eng. Chem., Prod. Res. Dev. 19, 478-483.

19. Leung, J.C.Y. and D.I.C. Wang. 1981. Production of Acetone and Butanol by Clostridium acetobutylicum in

Continuous Culture using Free Cells and Immobilized Cells. Proc. 2nd World Congr. Chem. Eng. 1, 348-355.

20. Leung, J.C.Y. 1982. Production of Acetone and Butanol by Clostridium acetobutylicum using Free and Immobilized Cells, Ph.D. Dissertation, M.I.T.

21. Mahmoud, S.A.Z., S.M. Taha, Y.Z. Ishac, M.El-Sawy and M.E. El-Demerdash. 1974. Acetone-butanol Fermentation in Egypt. 3. Molasses as Raw Material, Egypt. J. Microbiol. 9, 32-43.

22. Meyberry, W.R., G.J. Prochazka and W.J. Payne. 1968. Factors Derived from Studies of Aerobic Growth in Minimal Media, J. Bacteriol. 96, 1424-1426.

23. Minkevich, I.G. and V.K. Eroshin. 1973. Productivity and Heat Generation of Fermentation Under Oxygen Limitation, Folia Microbiol. 18, 376-385.

24. Monot, F., J.R. Martin, H. Petitdemange and R. Gay. 1982. Acetone and Butanol Production by Clostridium acetobutylicum in a Synthetic Medium, Appl. Environ. Microbiol., 44, 1318-1324.

25. Pasteur, L. 1861. Extrait des Procès-verbaux, Soc. Chim. Paris, 52.

26. Peterson, W.H. and E.B. Fred. 1932. Butyl-Acetone Fermentation of Corn Meal: Interrelations of Substrate and Products, Ind. Eng. Chem. 24, 237-242.

27. Petitdemange, H., C. Cherrier, G. Raval and R. Gay. 1976. Regulation of the NADH and NADPH - Ferredoxin Oxidoreductases in Clostridia of the Butyric Group, Biochem. Biophys. Acta, 421, 334-347.

28. Pirt, J. 1975. Oxygen Demand and Supply, In: Principles of Microbe and Cell Cultivation, Oxford: Blackwell Scientific Publications, 81-116.

29. Prescott, S.C. and C.G. Dunn. 1959. The Acetone-Butanol Fermentation. In: Industrial Microbiology, 3rd ed., McGraw Hill, N.Y., 250-284.

30. Reed, G.B. and J.H. Orr. 1943. Cultivation of Anaerobes and Oxidation-Reduction Potentials, J. Bacteriol. 45, 309-320.

31. Robinson, G.C. 1922. A Study of the Acetone and Butyl Alcohol Fermenation of Various Carbohydrates, J. Biol. Chem. 53, 125-154.

32. Soni, B.K., K. Das and T.K. Ghose. 1982. Bioconversion of Agro-Wastes into Acetone-Butanol, Biotechnol. Letters 4, 19-22.

33. Speakman, H.B. 1923. Molecular Configuration in the Sugars and Acid Production by Bacillus granulobacter pectinovorum, J. Biol. Chem., 58, 395-413.

34. Spivey, M.J. 1978. The Acetone-Butanol-Ethanol Fermentation, Process Biochem. 13, 2-4.

35. Taha, S.M., S.A.Z. Mahmoud, Y.Z., Ishac, M. El-Sawy and M.E. El-Demerdash, 1973. Acetone-Butanol Fermentation in Egypt .2. Use of Various Raw Materials, Egypt, J. Microbiol. 8, 15-27.

36. Thauer, R.K., K. Jungermann and K. Decker. 1977. Energy Conservation in Chemotrophic Anaerobic Bacteria, Bacteriol. Rev., 41, 101-180.

37. Ulmer, D.C., J.C. Linden and A.R. Moreira, 1981. The Effect of Ethanol and Butanol on Sugar Transport and Viability of Clostridium species Bacteria, Ann. Report, Dept. Chem. Eng., Colorado State U., 1-77.

38. Weizmann, C. and B. Rosenfeld. 1937. The Activation of the Butanol-Acetone Fermentation of Carbohydrates by Clostridium acetobutylicum (Weizmann) Biochem. J., 31, 619-639.

39. Wiley, A.J., M.J. Johnson, E. McCoy and W.H. Peterson. 1941. Acetone-Butyl Alcohol Fermentation of Waste Sulfite Liquor, Ind. Eng. Chem. 33, 606-610.

40. Wilson, P.W., W.H. Peterson and E.B. Fred. 1930. The Relationship Between the Nitrogen and Carbon Metabolism of Clostridium acetobutylicum, J. Bacteriol. 19, 231-260.

41. Wood, H.G., R.W. Brown and C.H. Werkman. 1945. Mechanism of the Butyl Alchohol Fermentation with Heavy Carbon Acetic and Butyric Acids and Acetone, Arch. Biochem. 6, 243-260.

42. Yerushalmi, L. and B. Volesky. 1981. Experimental Bioenergetics of Saccharomyces cerevisiae in Respiration and Fermentation, Biotechnol. Bioeng. 23, 2373-2390.

43. Zeikus, J.G. 1980. Chemical and Fuel Production by Anaerobic Bacteria, Ann. Rev. Microbiol. 34, 423-464.

2

PRELIMINARY CULTURE STUDIES

2.1 INTRODUCTION

The production of solvents and fermentation gases in the acetone-butanol process is dependent on the type of the culture, formulation of the fermentation medium and the culture conditions.

A number of experiments were performed at the early stages of this work in order to establish optimal medium composition and culture conditions for the maximum production of the solvents in a batch fermentation.

2.2 MATERIAL AND METHODS

2.2.1 Microorganism, Growth Conditions and Medium Composition

Clostridium acetobutylicum, ATCC 824, was used in this study. The culture underwent a selection procedure using butyric acid enriched medium. The selected high-tolerence strain featured decreased residual amounts of butyric acid at the end of the fermentation and good solvent production.

A semi-solid reinforced Clostridium medium (RCM) was used for the maintenance of the culture and activation of the spores. The composition of this medium is presented in Table 1. The spores were stored in 15 cm^3 screw-cap test tubes. Each tube was used for the start of one fermentation. The spores were heat shocked by placing the test tubes in 70°C to 75°C for 20 to 25 minutes. A 3% inoculum was then transferred to 100 mL serum bottles containing 30 mL of the same RCM medium. After twenty-four hours of spore activation, a three percent inoculum was transferred to a soluble glucose medium. After sixteen to twenty hours, a three percent inoculum was transferred to the final fermentation stage (a fermentor or a shake flask). At this stage, the cells were at their exponential growth phase. Three different media with different concentrations of the initial glucose and the other ingredients were used in this study. Table 2 presents the composition of the three glucose media.

For the cheese whey fermentations samples of dry whey were obtained from the KRAFT CO. Ltd. in Montreal which contained 73.0% lactose, 12.5% protein, 8.1% ash, 2.2% Nacl, 1.0% fat and 3.2% moisture on a dry weight basis. A reconstituted solution of whey was prepared containing 4.7% lactose. This solution was supplemented with yeast extract (4.0 g/L), ammonium sulfate (4.6 g/L), calcium phosphate (0.4 g/L), ferrous sulfate (0.5 g/L) and magnesium sulfate (2.0 g/L). pH of the media were adjusted to 6.2 ~ 6.4 before sterilization.

TABLE 1

COMPOSITION OF THE
REINFORCED CLOSTRIDIUM MEDIUM (RCM)

Compound	Concentration (g/L)
Yeast Extract	3.0
Lab-Lemco Powder	10.0
Peptone	10.0
Soluble Starch	1.0
Dextrose	5.0
Cysteine Hydrochloride	0.5
Sodium Chloride	5.0
Agar	0.5

pH after sterilization ~6.8

TABLE 2

COMPOSITION OF THE SOLUBLE GLUCOSE MEDIA USED IN THE ACETONE-BUTANOL FERMENTATION

Compound	Concentration (g/L)		
	Medium A	Medium B	Medium C
Glucose	50.0	30.0	20.0
Yeast Extract	11.0	7.5	5.0
$(NH_4)_2\ SO_4$	9.0	5.0	3.3
K_2HPO_4	0.8	0.5	0.5
KH_2PO_4	0.8	0.5	0.5
$Mg\ SO_4$	0.3	0.2	0.2
$Mn\ SO_4$	0.02	0.01	0.01
$Fe\ SO_4$	0.02	0.01	0.01
NaCl	0.02	0.01	0.01
Cysteine	-	0.5	0.5

2.2.2 Analytical Procedures

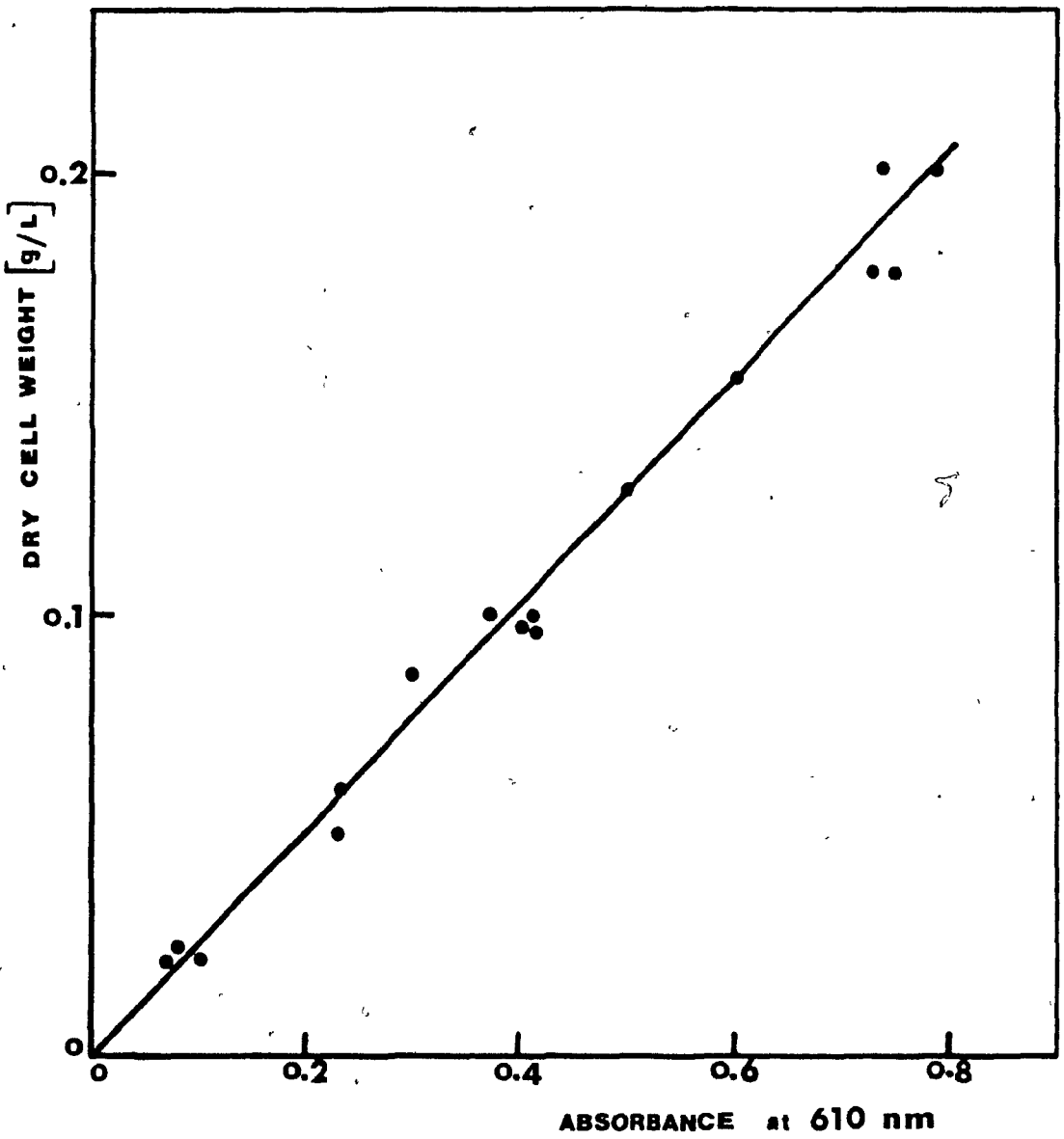
A. Biomass Dry Weight

A 30 mL to 40 mL sample of the fermentation broth was withdrawn whenever required. Cells were separated by centrifugation at 13000 g for 15 minutes at 4°C and the cell-free broth was kept frozen for further analysis. The cells were washed twice with 40 mL of distilled water, placed in a preweighed aluminum dish and dried to constant weight at 90° C. In addition to the dry weight measurement, the optical density (O.D.) of the cells were measured by a spectrophotometer (Baush and Lomb, Model Spectronic 70) at 610 nm. The samples with high cell concentrations were diluted with saline to give appropriate readings. Figure 1 illustrates a calibration curve for the biomass concentration.

B. Glucose Concentration

The concentration of glucose in the medium was estimated enzymatically by a Fisher Diagnostics Glucose Hexokinase (HK) method. This method is based on two coupled enzymatic reactions by hexokinase and Glucose-6-Phosphate dehydrogenase as follows:

FIGURE 1 The Calibration for the Measurement of the Biomass Concentration by Optical Density


DRY CELL WEIGHT [g/L]
0.2
0.1
0
0
0.2
0.4
0.6
0.8
ABSORBANCE at 610 nm

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose-6-Phosphate}$$

$$\text{Glucose-6-Phosphate} + \text{NAD}^+ \xrightarrow{\text{G-6-PDH}} \text{6-Phosphogluconate} + \text{NADH} + \text{H}^+$$

The first reaction involves phosphorylation of glucose by hexokinase (HK) and ATP to yield glucose-6-phosphate. This product is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) with concomittant reduction of NAD^+. Since both reactions are essentially irreversible, the total amount of NADH formed in the second reaction, determined spectrophotometrically at 340 nm, is a direct measure of the glucose concentration.

C. Solvents and Acids Concentration

Analysis of the solvents and acids in the fermentation broth were performed by chromatographic techniques. A gas chromatograph (Carle, Model 311) using a nickel column, 183 cm by 0.32 cm O.D. (6 ft. x 1/8" O.D.), packed with Chromosorb 101, 80-100 mesh, was used in these analyses. The chromatograph operating parameters were:

Injection port temperature	:	210°C
Column temperature	:	210°C
Detector type	:	Flame Ionization
Carrier gas	:	Helium
Flow rate of carrier gas	:	60 mL/min

A Chromatography data station (Perkin-Elmer, Model Sigma 10B) was used for the collection of the chromatographic data, production of the chromatogram and calculation of the respective peak areas. The samples were prepared for the gas chromatrography assay by adding 0.25 mL of a 2% (W/V) sulfuric acid solution to 1 mL of the cell-free samples. The acid was added to ensure that butyrate and acetate were in their acid forms. Concentration of the individual components were found by external standards method using the following equation:

$$C_i = F_i A_i$$

where C_i = concentration of component i in the unknown sample

F_i = response factor for the component i

A_i = area of peak corresponding to component i in the unknown sample

The response factors (F_i) were determined from the slope of the straight lines relating the concentrations of at least three different standard samples to their respective peak areas.

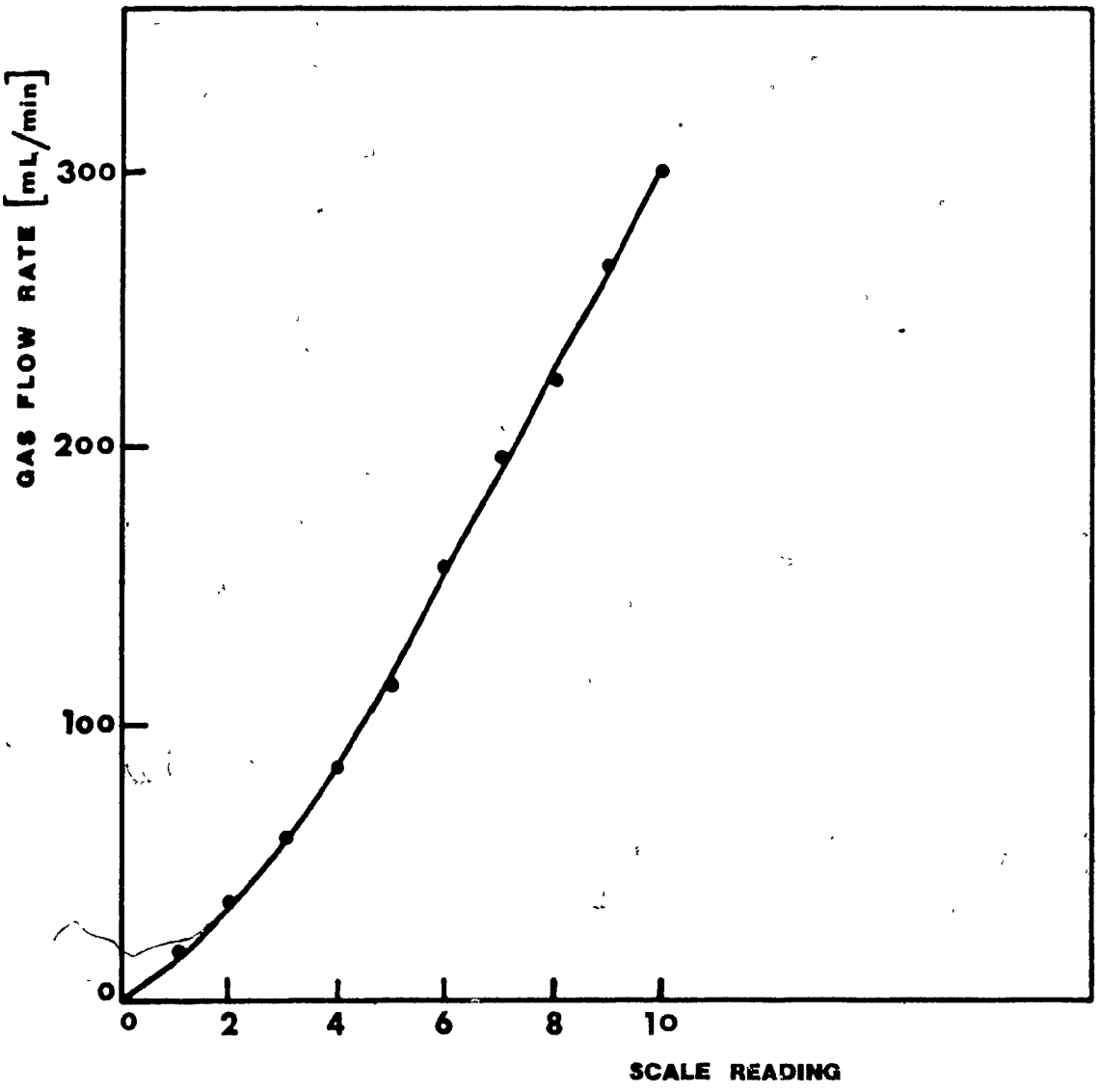
D. Gas Analysis

On-line analysis of the gas phase was performed every 0.5 to one hour by gas chromatography (Hewlett-Packard, Model 700). The gas chromatograph was equipped with a stainless steel column, 366 cm by 0.32 cm O.D. (12 ft. x 1/8" O.D.) packed with (80-100) mesh porapack Q. The chromatographic conditions were:

Column temperature	:	70°C
Injection port temperature	:	130°C
Detector type	:	Thermal Conductivity
Detector temperature	:	210°C
Carrier gas	:	Nitrogen
Carrier gas flow rate	:	60 mL/min

The total fermentation gas flow rate was measured by a rotameter (Model S & K 1875) and the total fermentation gas volume was determined by a wet test meter installed at the end of the gas line. Figure 2 illustrates a calibration curve for the gas rotameter. Concentration of the gases (CO_2 and H_2) in the liquid broth was estimated from their respective partial pressures in the gas phase by utilizing Henry's law and appropriate gas solubilities in aqueuous phase.

FIGURE 2 The Calibration of the Gas Rotameter ($H_2:CO_2$ = 45:55)


GAS FLOW RATE [mL/min]
300
200
100
0
0
2
4
6
8
10
SCALE READING

2.2.3. Fermentation Assembly

A. Fermentation Flasks

The initial culture studies and some of the fermentations were performed in a controlled temperature gyrotory shaker Model G2 (New Brunswick Scientific, N.J.) utilizing 500 mL Erlenmeyer flasks with a working volume of 150 mL to 200 mL. The flasks were modified so that they could be sealed by serum bottle caps. High purity nitrogen gas was used to sparge the sealed flask for 25 minutes before inoculation in order to ensure anaerobiosis.

B. Fermentor

A modified 14-L "Microferm" laboratory fermentor (New Brunswick Scientific Co., Edison, N.J.) with a working volume of 10 L was used in the fermentor batch studies. The temperature of the fermentation broth was controlled at 38°C by a temperature controller which regulated the flow of water through two of the four standard-geometry baffles. Agitation of the fermentation broth was provided by two, six-bladed impellers each, 7.4 centimeters in diameter. pH and redox potential of the medium were continuously monitored by a pH controller (NBS, Model pH 22) and a mV meter (Orion, Model 407A). The pH probe (Ingold, Model 465) and the redox probe (Ingold, Model pt-4865-25) were steam sterilizable with a

stainless steel immersion holder fitted through the fermentor head plate. Before inoculation, the fermentation broth was sparged with high purity nitrogen gas to maintain anaerobiosis. Flushing by the N_2 gas was limited to the head space of the fermentor after inoculation and was terminated as soon as the cells started to produce the fermentation gases.

2.3 RESULTS AND DISCUSSION

2.3.1 Study of the Medium Composition and Culture Conditions

The composition of the medium for the growth and a good production of the solvents and gases is highly dependent on the type of the culture and the culture conditions. Three different media with different initial glucose concentrations (G_o) were used at various stages of this work as mentioned in section 2.2. Medium B with a G_o = 30 g/L was the first medium developed. The composition of this medium is based on the Speakman's medium (1923) with the exception that ammonium sulfate and yeast extract replaced peptone. As indicated in Table 3 this replacement made a dramatic improvement in the overall solvent yield. A maximum solvent yield of 34.8% was observed when ammonium sulfate at a concentration of 5 g/L was used. The ratio of butanol:acetone:ethanol under these conditions was 64:29:7.

TABLE 3

THE EFFECT OF VARIOUS CONCENTRATIONS OF $(NH_4)_2SO_4$ ON THE PRODUCTION OF SOLVENTS By C. acetobutylicum

$(NH4)_2$ SO_4 (g/L)	S/N	Percent of Solvents Based on						
		Weight of sugar				TNVP(1)		
		Butanol	Acetone	Ethanol	TNVP	Butanol	Acetone	Ethanol
2	27.3	15.2	5.9	1.4	22.5	68	26	6
3	22.9	21.2	9.2	2.3	32.7	65	28	7
4	19.7	21.5	10.1	2.3	33.9	63	30	7
5	17.3	22.2	10.1	2.5	34.8	64	29	7
6	15.4	21.3	10.2	2.2	33.7	63	30	7
7	13.9	16.3	5.9	1.6	23.8	68	25	7
8	12.6	16.5	6.0	1.7	24.2	68	25	7
0(2)	13.4	10.0	3.6	1.7	15.3	66	23	11

1 Total Neutral Volatile Products - the rounded off values are reported for the ease of comparison with the literature

2 Peptone with the concentration of 10 g/1 was used

The effect of yeast extract addition is illustrated in Table 4. A constant sugar to nitrogen S/N ratio of 17.3 resulting in the maximum solvent yield (Table 3) was used in all the cases. This ratio has been shown to be an important factor in the production of solvents by C. acetobutylicum (Taha et al., 1973). As indicated in Table 4, under the present experimental conditions, yeast extract was necessary for the production of solvents and attempts to replace the yeast extract with various concentrations of vitamins and amino acids resulted in low solvent production. Solvent yields were directly related to the concentration of yeast extract and the maximum TNVP concentration was obtained with a yeast extract concentration of 7.5 g/L.

Although it contains many amino acids, yeast extract is particularly needed in the acetone-butanol fermentation to supply two compounds i.e. asparagine and a substance of unknown constitution which is referred to as an "activator" (Weizmann and Rosenfeld, 1937). As reported by Weizmann and Rosenfeld (1937), asparagine which plays a role in the nitrogen metabolism of bacteria, acts more like a nitrogen carrier or a coenzyme in the metabolism of C. acetobutylicum. The "activator" also acts as a coenzyme and has a stimulating effect on solvent production. Although these two compounds are not consumed during the course of a fermentation, they are both needed at high concentrations. This is the reason for the requirement of a relatively high concentration of yeast extract in the acetone-butanol

TABLE 4

THE EFFECT OF YEAST EXTRACT CONCENTRATION
ON THE PRODUCTION OF THE SOLVENTS BY C. acetobutylicum

S/N RATIO IS 17.3 IN ALL OF THE CASES

$(NH_4)_2SO_4$ (g/L)	Yeast Extract (g/L)	Percent of Solvents Based on						
		Weight of sugar				TNVP[1]		
		Butanol	Acetone	Ethanol	TNVP	Butanol	Acetone	Ethanol
5	7.5	20.8	8.8	2.3	31.9	65	28	7
5.6	6	17.5	8.9	1.9	28.3	62	31	7
6.5	4	16.3	8.1	1.2	25.6	64	31	5
7.3	2	7.3	3.7	1.2	12.2	60	30	10
8.2	0[2]	2.2	1.2	0.0	3.4	65	35	0
8.2	0[3]	1.0	0.6	0.8	2.4	42	25	33
8.2	0[4]	0.5	0.4	0.0	0.9	56	44	0

1) Total Neutral Volatile Products

2) m-inositol, 0.6 mg/L, Thiamine, 0.6 mg/L; Pyridoxine, 10 µg/L; Ca-Pantothenate, 2 µg/L, Biotin, 1 µg/L, para-aminobenzoic acid, 1 µg/L, L-asparagine, 600 mg/L.

3) m-inositol, 0.6 mg/L, Thiamine, 0.6 mg/L; Pyridoxine, 10 µg/L; Ca-Pantothenate, 20 µg/L, Biotin, 10 µg/L, para-aminobenzoic acid, 10 µg/L, L-asparagine, 600 mg/L.

4) m-inositol, 2 mg/L, Thiamine, 0.6 mg/L; Pyridoxine, 20 µg/L; Ca-Pantothenate, 0.5 mg/L, Biotin, 50 µg/L, para-aminobenzoic acid, 50 µg/L, L-asparagine, 600 mg/L.

fermentation. The obligatory requirement of yeast extract for growth is also reported in the strains of C. lacto-acetophilum and C. butyricum (Cummins and Johnson, 1971).

It is worth noticing that at the early stages of this work, no oxygen scavenger was added to the media since it was established that the initial flushing with nitrogen was sufficient to provide the required anaerobic condition. However, with the introduction of the exogenous hydrogen gas in the high pressure experiments, 0.5 g/L of cysteine was added to the media to ensure a well reduced environment during the entire course of fermentation.

The effects of initial pH on growth and solvent production (Table 5) were evaluated at pH values ranging from 4.6 to 6.3. The results indicated that the initial pH in this range does not have any significant effect on the production of solvents. Good biomass growth and high solvent yields (based on the sugar consumed) were evident in all cases. The solvent yields ranged from 28.8% to 31.6%.

The final concentration of the solvents was shown to be directly proportional to the glucose concentration in batch culture studies with initial glucose concentrations ranging from 20 g/L to 70 g/L (Table 6). Under these conditions, glucose was completely utilized with high solvent yields in all cases.

For an initial glucose concentration of 50 g/L the concentrations of all the salts except for ammonium sulfate

TABLE 5

THE EFFECT OF THE INITIAL pH VALUE
ON THE PRODUCTION OF SOLVENTS
BY C. acetobutylicum

Initial pH	Final pH	Percent of Solvents Based on							Biomass g/L
		Weight of sugar				TNVP(1)			
		Butanol	Acetone	Ethanol	TNVP	Butanol	Acetone	Ethanol	
4.6	4.3	18.5	8.9	1.4	28.8	64	31	5	1.5
4.8	4.4	18.6	9.1	1.5	29.2	64	31	5	1.5
5.1	4.3	19.0	9.0	1.5	29.5	64	31	5	1.6
5.3	4.4	19.1	8.8	1.6	29.5	65	30	5	1.5
5.4	4.3	19.2	10.0	1.6	30.8	62	33	5	1.6
5.5	4.4	20.9	9.0	1.7	31.6	66	29	5	1.6
5.6	4.3	19.3	9.4	1.4	30.1	64	31	5	1.6
5.8	4.3	18.8	9.8	1.5	30.1	62	33	5	1.5
6.0	4.3	18.8	9.7	1.4	29.9	63	32	5	1.5
6.3	4.3	19.0	9.0	1.6	29.6	64	31	5	1.5

1) TNVP = Total Neutral Volatile Products

TABLE 6

THE EFFECT OF THE INITIAL GLUCOSE CONCENTRATION ON THE PRODUCTION OF SOLVENTS BY C. acetobutylicum

Initial glucose g/L	Butanol g/L	Acetone g/L	Ethanol g/L	Total Solvents g/L	Solvent Yield %
20.5	4.2	2.0	0.4	6.6	32.2
30.0	5.8	3.4	0.6	9.8	32.7
41.0	7.4	4.3	0.9	12.6	30.7
50.2	9.6	4.9	1.0	15.5	30.9
60.0	10.6	5.9	1.3	17.8	29.7
70.0	13.0	7.4	1.6	22.0	31.4

and yeast extract were increased proportionally. The results (Table 7) indicated that a higher solvent yield was obtained when the concentration of ammonium sulfate was further increased at the expense of yeast extract. Growth medium A with G_o = 50 g/L was developed containing 9 g/L and 11 g/L of ammonium sulfate and yeast extract, respectively.

Medium C was used for a G_o = 20 g/L. With a G_o of higher than 50 g/L the concentrations of the yeast extract and all other salts were increased in proportion with the G_o.

2.3.2 Fermentor Batch Experiments

A) Glucose Fermentation

Medium A with an initial glucose concentration of 50 g/L was used in these experiments. Values of the key microbial and system parameters were either recorded intermittently or monitored continuously in these experiments.

A number of experiments with glucose were unsuccessful before appropriate culture scale-up conditions in the fermentor were established and good production of solvents achieved. The culture proved to be particularly sensitive to lowering the pH below pH 4.3 which is a natural tendency in the system. In these experiments, the culture started its normal growth, solvent synthesis and gas produc-

TABLE 7

THE EFFECT OF THE CONCENTRATIONS OF YEAST EXTRACT AND AMMONIUM SULFATE ON THE PRODUCTION OF SOLVENTS BY C. acetobutylicum

G_o = 50 g/L

$(NH_4)_2SO_4$ g/L	Yeast Extract g/L	Percent of Solvents Based on						
		Weight of sugar				TNVP(1)		
		Butanol	Acetone	Ethanol	TNVP	Butanol	Acetone	Ethanol
10.5	7.5	20.0	10.4	2.3	32.7	61	32	7
9.0	11.0	20.3	10.8	2.4	33.5	61	32	7
8.3	12.5	19.3	10.2	2.2	31.7	61	32	7

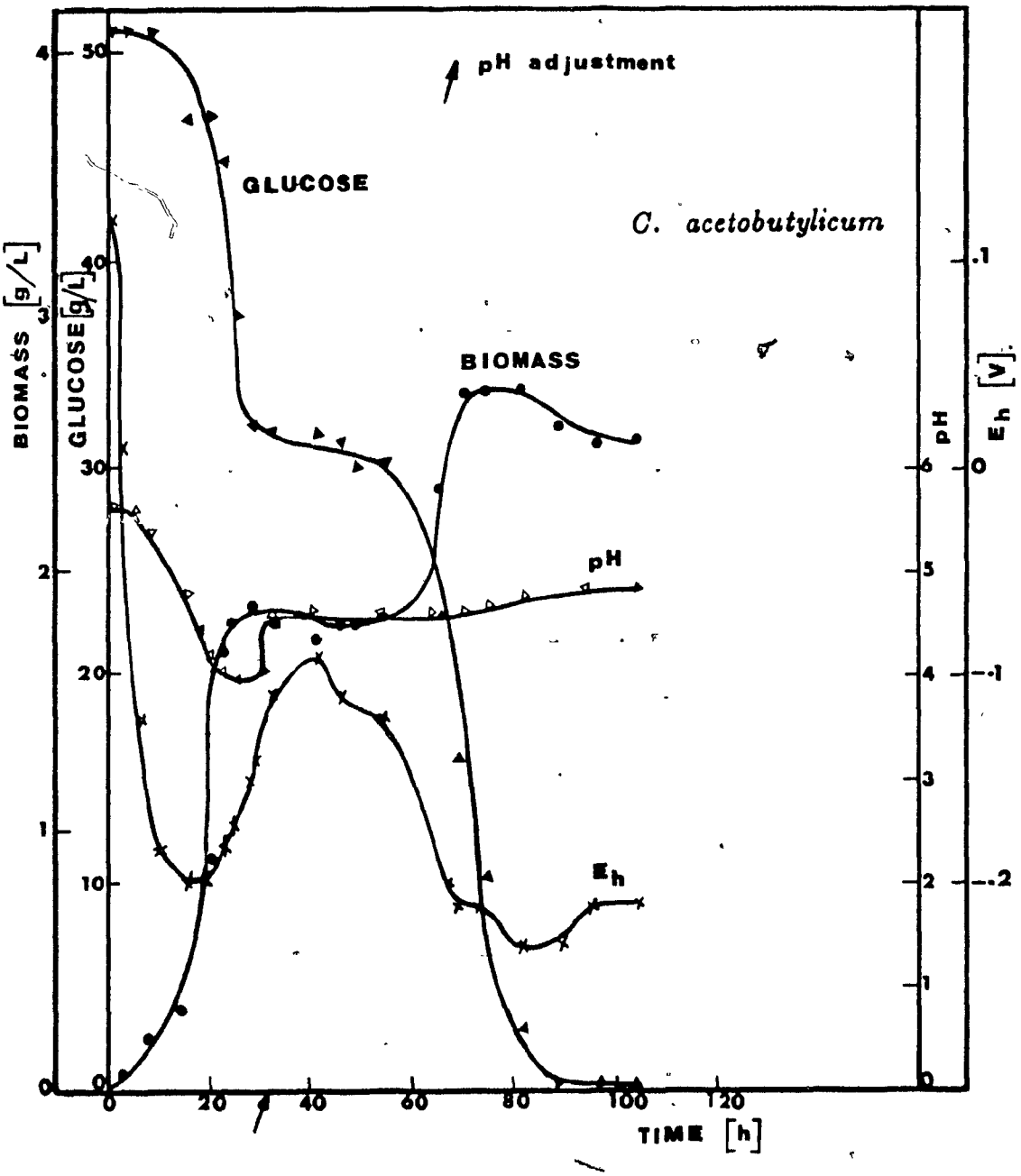
1) TNVP = Total Neutral Volatile Products

tion after an initial lag phase. However, drop of pH to the values of 3.9 to 4.0 resulted in the cessation of the culture growth which was followed by the arrest of solvent synthesis, gas production and virtually all the culture activities. At this stage more than half of the sugar substrate still remained unutilized, most of the acids were unconverted and only small amounts of solvents were produced. A dramatic effect of the spontaneous pH decrease is illustrated in Figures 3(a,b,c). The culture activity following a pH adjustment to the value of 4.6 by addition of 3N NaOH. The fermentation subsequently proceeded to a completion. Glucose was then completely utilized resulting in high solvent production. The overall solvent yield based on the sugar consumed was 31%. A total of 226 liters of gas including 124 liters of carbon dioxide and 102 liters of the hydrogen was produced during the course of this fermentation process.

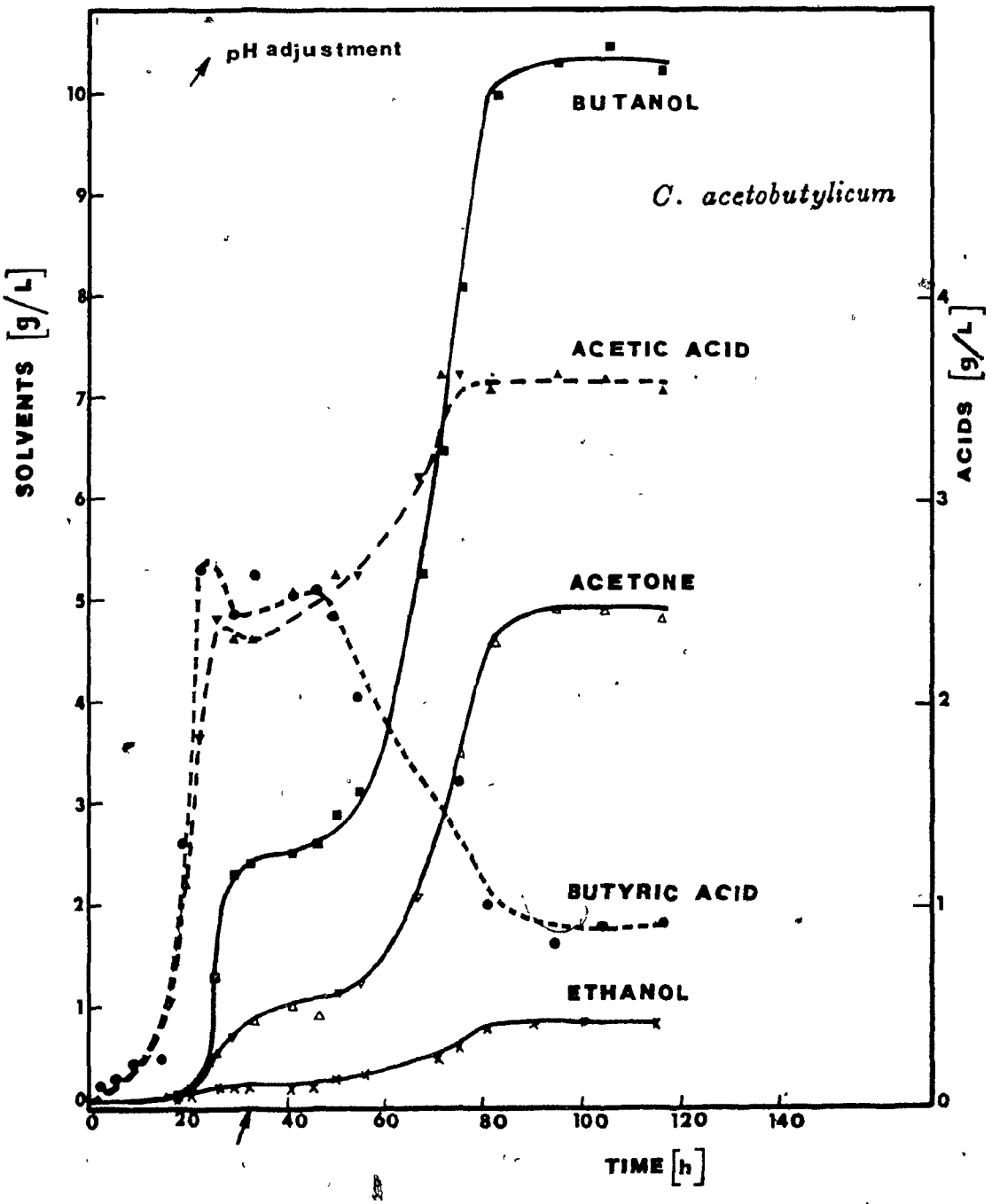
It is interesting to note that during the non-active culture period the redox potential (E_h) of the system increased from -200 mv to in excess of -100 mv. The performance of the culture was renewed upon adjusting pH to 4.6 with subsequent depletion of sugar and solvent accumulation which occurred within another approximately 20 hours following a lag period of several hours during which the redox potential decreased again reaching eventually values of less than -220 mv. The overall time of this interrupted fermen-

FIGURE 3 (a, b, c)
Variations of the Fermentation Parameters in an Interrupted Batch Acetone-Butanol Fermentation on Glucose

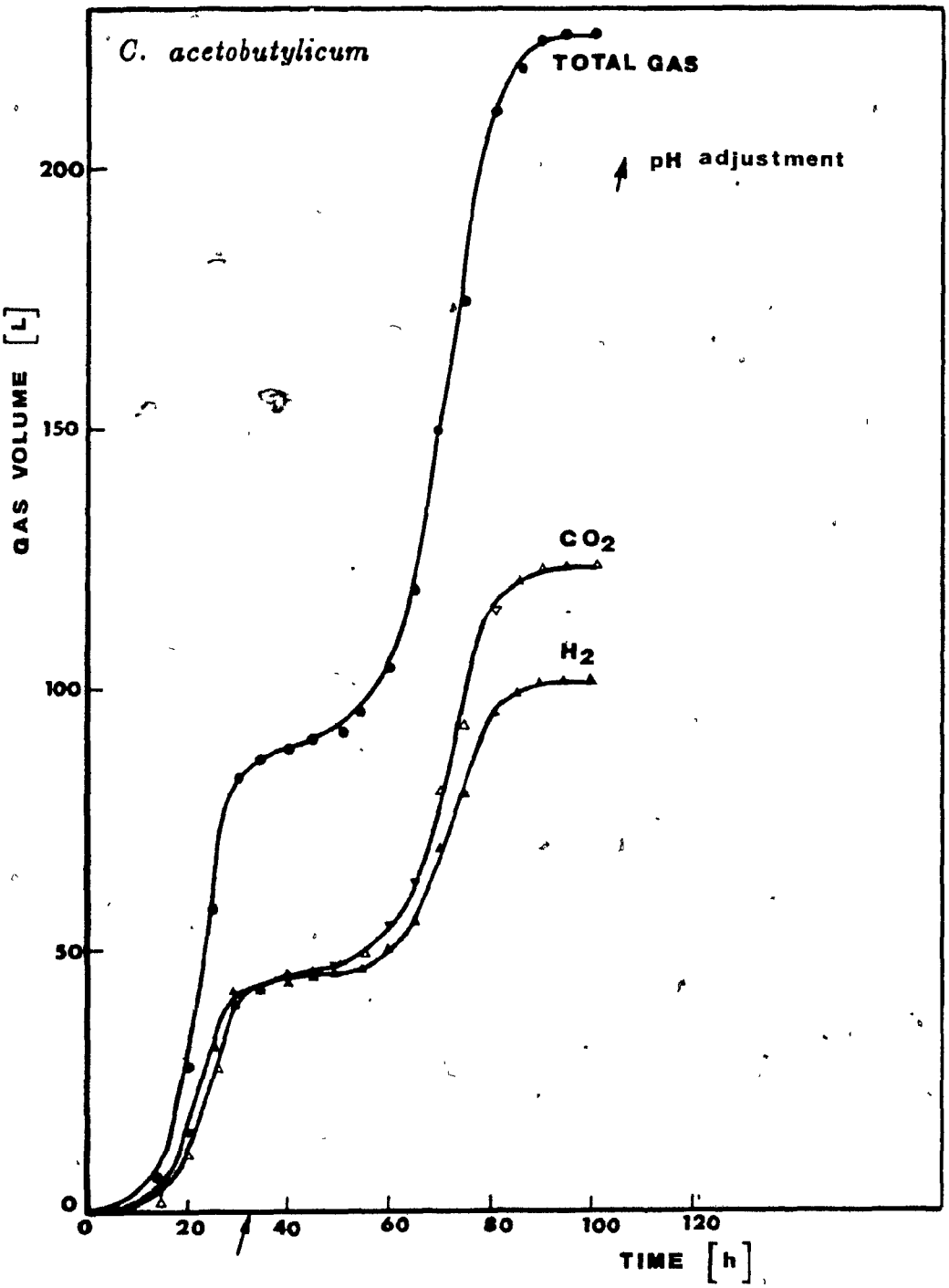
a)


pH adjustment
GLUCOSE
C. acetobutylicum
BIOMASS
pH
Eh
BIOMASS [g/L]
GLUCOSE [g/L]
pH
Eh [V]
TIME [h]
0
20
40
60
80
100
120
10
30
50
1
2
3
4
5
6
-1
-2

b)


pH adjustment
BUTANOL
C. acetobutylicum
ACETIC ACID
ACETONE
BUTYRIC ACID
ETHANOL
SOLVENTS [g/L]
ACIDS [g/L]
TIME [h]
0
20
40
60
80
100
120
140
1
2
3
4
5
6
7
8
9
10

C)


C. acetobutylicum
TOTAL GAS
pH adjustment
CO_2
H_2
GAS VOLUME [L]
TIME [h]
0
50
100
150
200
0
20
40
60
80
100
120

tation experiment was in excess of 80 hours with the intermediate lag of approximately 30 hours.

The efficiencies of solvent and gas production from glucose were evaluated by examining the carbon, energy and electron balances for the experimental system (Table 8). Butanol and carbon dioxide accounted for the highest amount of the glucose carbon incorporated (32.6% and 32.9% respectively). Butanol also accounted for a major portion of the glucose energy (46.6%) and the available electrons (48.9%). Acetone accounted for 14.5% of glucose carbon, 18.5% of its energy and 19% of the available electrons. 15.8% of the glucose energy was incorporated into hydrogen. The harmful effect of lowering the broth pH has also been reported by Ulmer et al. (1981). According to Wynne (1931), this effect is related to the increased toxicity of the fermentation acids (butyric and acetic) at low pH values. In a recent report on the physiology of the A-B-E fermentation by a C. acetobutylicum strain P262, Robson and Jones (1982) pointed out that a low pH of the medium prevents the formation of the swollen, cigar-shaped cells called clostridial forms and results in the little or no solvent production in the system. These results suggest that the hydrogen ion concentration plays an important role in the regulation of solvent production in the A-B-E fermentation.

TABLE 8

FRACTIONAL RECOVERY OF GLUCOSE CARBON, ENERGY AND ELECTRONS IN THE BATCH ACETONE-BUTANOL FERMENTATION ON GLUCOSE

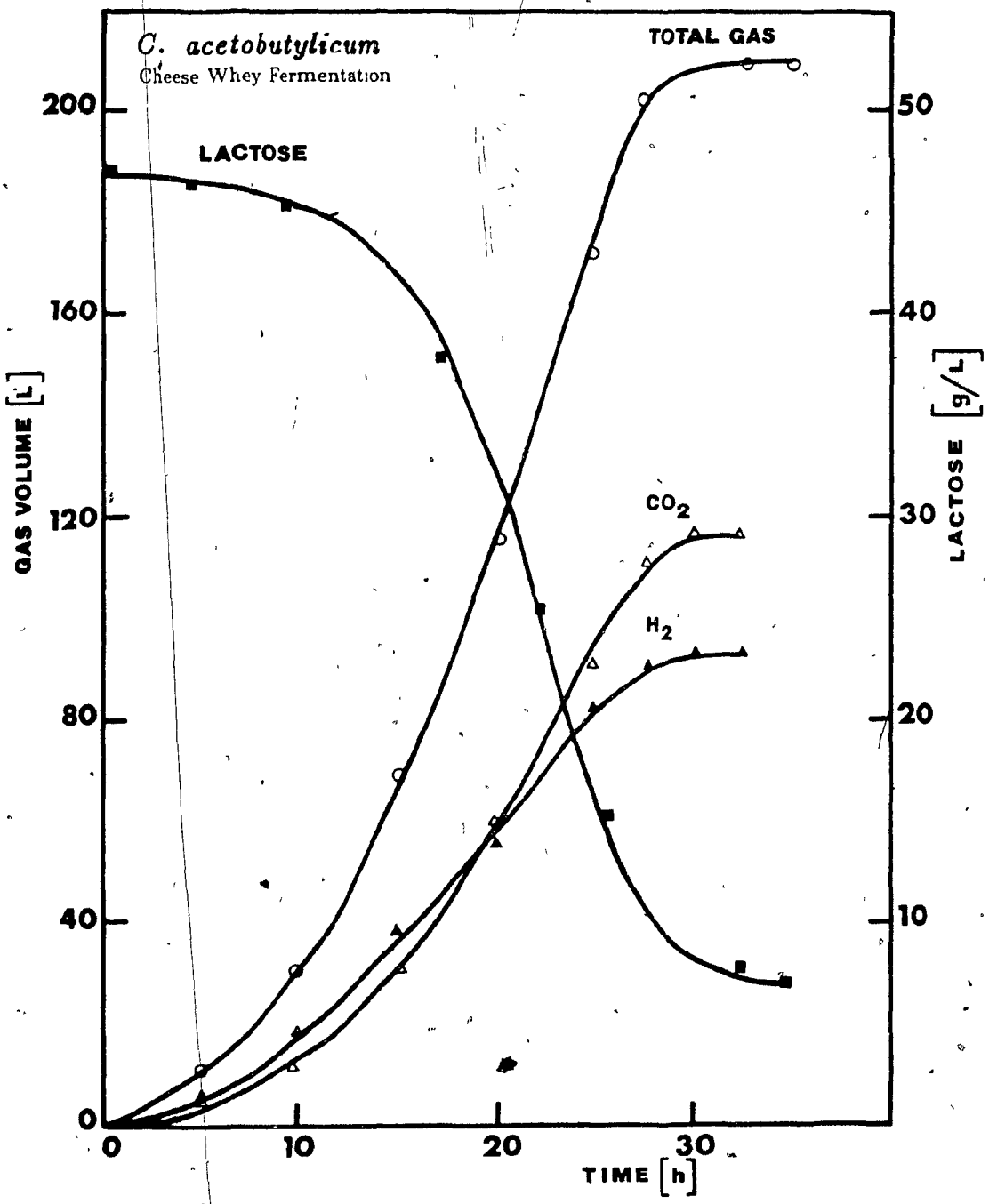
Fermentation Product	Recovered in Product		
	% Total Carbon	% Total Energy	% Total Electrons
Butanol	32.6	46.6	48.9
Acetone	14.5	18.5	19.0
Ethanol	2.0	2.9	3.0
Acetic Acid	6.9	6.5	7.0
Butyric Acid	2.6	3.0	3.2
Biomass	5.8	7.2	6.1
CO_2	32.9	-	-
Hydrogen	-	15.8	12.8
RECOVERED TOTAL:	97.3	100.5	100%

B) Cheese Whey Fermentation

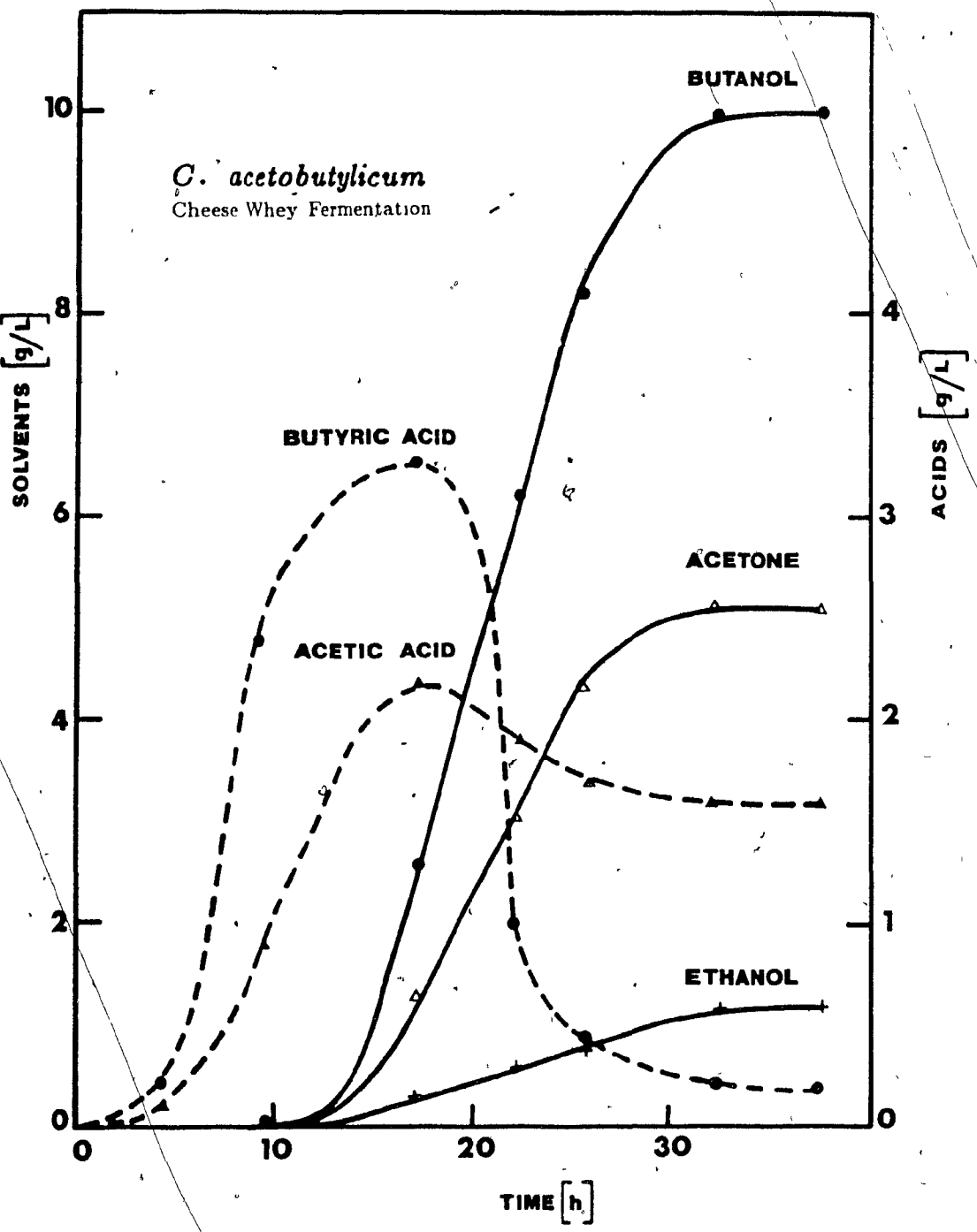
A fermentor batch experiment with cheese whey substrate is illustrated in Figures 4(a,b,c) showing the usual process parameters: concentrations of solvents and acids; volume and composition of the gaseous phase and the concentration of the residual lactose; pH and redox potential, respectively, with the time of fermentation. All of the measured or monitored process variables exhibited a normal pattern of change in their respective values similar to the results of a glucose fermentation. A total of 209 liters of gas and 162.1 grams of solvents were produced in this fermentation process. Almost 7 g/L of lactose remained unutilized at the end of the fermentation after 30 hours. Based on the lactose consumed the overall solvent yield was 39.7%. The pH of the fermentation medium did not fall below a value of pH 4.6 and there was no need for any control on this parameter. With the accumulation of the solvents pH increased and reached a final value of 5.3. Due to the dispersed suspended solids and turbidity of the medium, accurate measurements of the cell density were not possible. Also, due to the proteolytic characteristics of the culture (Doelle, 1975) and a possible contribution of the proteinaceous content of whey in the biomass, a balance of carbon and energy on the experimental system was not attempted. The profile of the gas phase in the whey fermentation was similar

FIGURE 4 (a,b,c)
Variations of the Fermentation Parameters in a Batch Acetone-Butanol Fermentation on KRAFT Whey Substrate

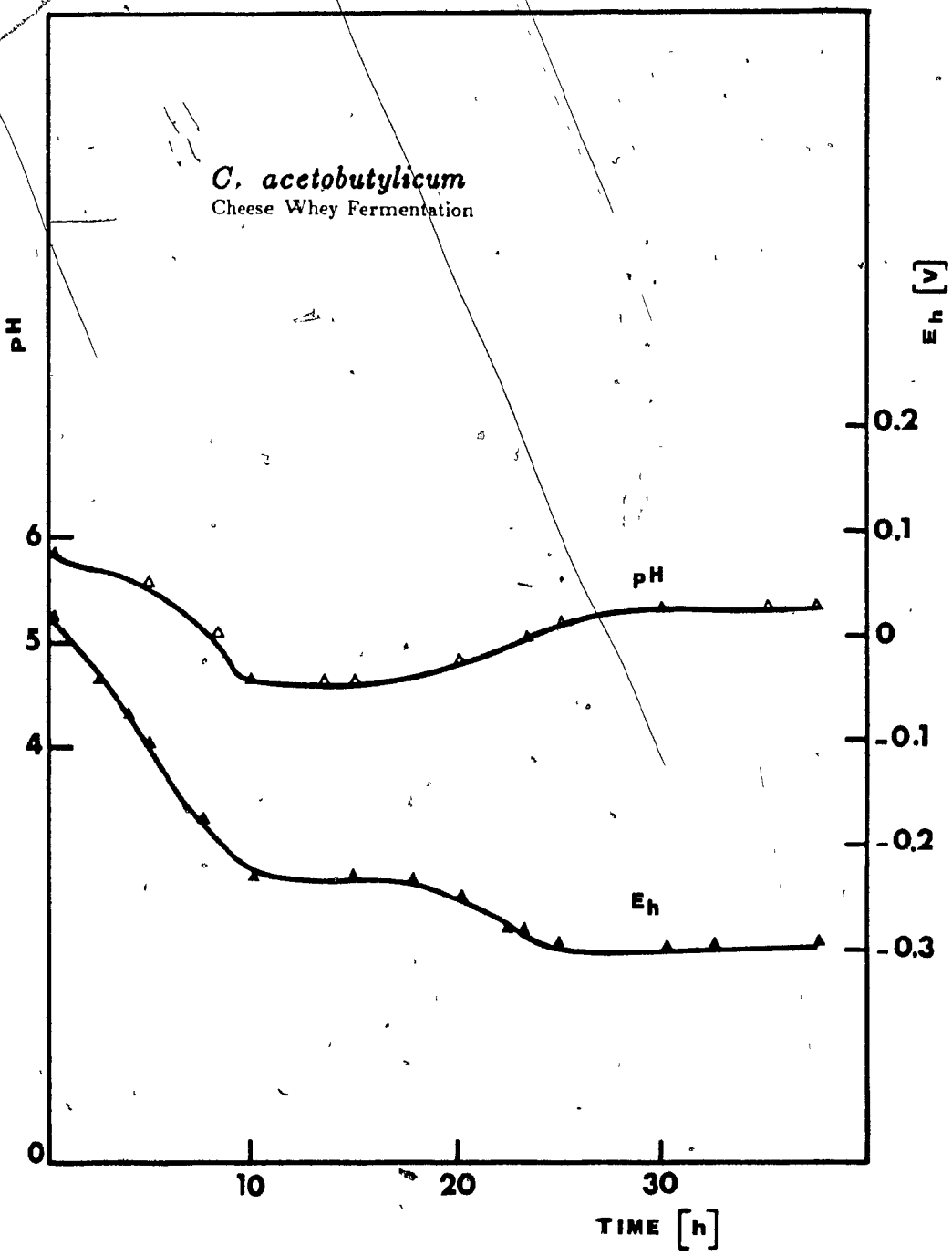
a)


C. acetobutylicum
Cheese Whey Fermentation
TOTAL GAS
LACTOSE
CO2
H2
GAS VOLUME [L]
LACTOSE [g/L]
TIME [h]
0
40
80
120
160
200
10
20
30
40
50
0
10
20
30

b)


C. acetobutylicum
Cheese Whey Fermentation
BUTANOL
BUTYRIC ACID
ACETONE
ACETIC ACID
ETHANOL
SOLVENTS [g/L]
ACIDS [g/L]
TIME [h]
0
2
4
6
8
10
0
1
2
3
4
0
10
20
30

C)


C. acetobutylicum
Cheese Whey Fermentation
pH
Eh [V]
6
5
4
0
0.2
0.1
0
-0.1
-0.2
-0.3
pH
Eh
10
20
30
TIME [h]

to that observed in a glucose fermentation. Initially, the hydrogen fraction in the gas phase was slightly higher. However, with the progress of the fermentation the ratio between Co_2 and H_2 changed in favor of Co_2 and at the end of the fermentation process the gas phase contained 56% of Co_2 and 44% of H_2. The final ratio of butanol:acetone:ethanol was 61:32:7. It is interesting to note that the culture exhibited a high activity and high production rates of the solvents and gases when whey was used as the substrate with the resulting overall fermentation time of less than 30 hours. This is in contrast with the low solvent production rates and the long whey or lactose fermentations reported in the literature (Underkofler and Hunter, 1938; Maddox, 1980) which took from 5 to 7 days.

The application of whey in the acetone-butanol fermentation by C. acetobutylicum may offer an interesting alternative to using the more conventional substrates such as carbohydrate or starch materials for industrial scale bio-synthesis of solvents.

2.4 REFERENCES

1. Cummins, C.S. and J.L. Johnson. 1971. Taxonomy of the Clostridia: Wall Composition and DNA Homologies in Clostridium butyricum and other Butyric Acid Producing Clostridia, J. Gen. Microbiol., 67, 33-46.

2. Doelle, H.W. 1975. Bacterial Metabolism, 2nd ed. Academic Press, N.Y. 574-605.

3. Maddox, I.S. 1980. Production of n-Butanol from Whey Filtrate Using Clostridium acetobutylicum, N.C.I.B. 2951, Biotechnol. Letters, 2, 493-498.

4. Robson, P.M. and Jones, D.T. 1982. Production of Acetone-Butanol by Industrial Fermentation, Proc. Ann. Conf. Société Française de Microbiol. Paris.

5. Speakman, H.B. 1923. Molecular Configuration in the sugars and Acid Production by Bacillus granulobacter pectinovorum, J. Biol. Chem., 58, 395-413.

6. Taha, S.M., S.A.Z. Mahmoud, Y.Z. Ishac, M. El-Sawy and M.E. El-Demerdash. 1973. Acetone-Butanol Fermentations in Egypt. 2. Use of Various Raw Materials, Egypt. J. Microbiol. 8, 15-27.

7. Ulmer, D.C., J.C. Linden and A.R. Moreira: 1981. The Effect of Ethanol and Butanol on Sugar Transport and Viability of Clostridium species Bacteria, Ann. Report, Dept. Chem. Eng., Colorado State U., 1-77.

8. Underkofler, L.A. and J.E. Hunter, Jr. 1938. Butyl-Acetonic Fermentation of Arabinose and Other Sugars, Ind. Eng. Chem., 30, 480-481.

9. Weizmann, C. and B. Rosenfeld. 1937. The Activation of the Butanol-Acetone Fermentation of Carbohydrates by Clostridium acetobutylicum (Weizmann), Biochem. J., 31, 619-639.

10. Wynne, A.M. 1931. Inhibition of the Acetone-Butyl Alcohol Fermentation by Acids, J. Bacteriol. 22, 209-237.

3

VARIATIONS OF SOLVENT YIELD IN THE ACETONE-BUTANOL FERMENTATION

Laleh Yerushalmi, B. Volesky, W.K. Leung and R.J. Neufeld,
Eur. J. Appl., Microbiol. Biotechnol, 18, 279-286 (1983).

3.1 INTRODUCTION

An economic analysis of the acetone-butanol process shows that more than sixty percent of the total production cost is in the raw material (Lenz and Moreira, 1980). The substrate to solvents conversion yield is therefore the most important process parameter. Butanol and acetone are biochemically synthesized from glucose with a yield of approximately 30% based on weight. This value has been reported to be very close to the maximum achievable theoretical yield (Leung and Wang, 1981).

In order to evaluate the efficiency of the biological system, it is essential to establish the theoretical yield value for the total neutral volatile products (TNVP).

A linear stoichiometric relationship has been developed and is presented in this communication between the substrate and the products at 30 different biosynthesis conditions considered in the system. The value of the TNVP yield is calculated at each condition by making certain assumptions based on experimental findings.

3.2 PROCESS ANALYSIS

Calculation of the theoretical yield is based on the information obtained from the fermentation experiments with C. acetobutylicum on glucose in a batch culture. For a glucose fermentation the experimental division of the substrate carbon, energy and the available electrons among the fermentation products are presented in Table 1. Glucose is considered as the sole source of carbon and energy. Almost 99% of the glucose carbon was recovered in the products and the energy and electron balances closed at the total of 102%. The heat of fermentation was neglected since it accounted for less than 5% of the total energy input. The electron balance was attempted by using the concept of the reductance degrees for the reactants and the products developed by Erickson (1979) and applied by Yerushalmi and Volesky (1981). Butanol and acetone accounted for 49.4% of the glucose carbon, 68.3% of its energy and 72.3% of the available electrons in the system. Considering ethanol, the solvents accounted for almost 52.0% of the glucose carbon, 72.2% of its energy and 76.4% of the available electrons.

By incorporating this information, a stoichiometric equation can be written to relate the total glucose consumed to the total solvents, acids and the gases produced. Based on one mole of glucose consumed, the equation would be:

TABLE 1

EXPERIMENTAL BATCH ACETONE-BUTANOL FERMENTATION

DIVISION OF THE GLUCOSE CARBON, ENERGY AND ELECTRONS AMONG THE FERMENTATION PRODUCTS

Fermentation Product	Recovered in the product		
	% total carbon	% total energy	% total available electrons
Butanol	34.0	48.7	51.0
Acetone	15.4	19.6	21.3
Ethanol	2.6	3.9	4.1
Acetic acid	4.5	4.3	4.6
Butyric acid	0.5	0.6	0.6
Carbon dioxide	33.1	-	-
Hydrogen	-	14.9	12.0
Biomass	8.4	10.2	8.9
Total	98.5	102.2	102.5

Glucose → Butanol + Acetone + Ethanol

$$C_6H_{12}O_6 \rightarrow 0.51\ C_4H_9OH + 0.31\ CH_3COCH_3 + 0.08\ C_2H_5OH$$

Acetid acid + Butyric Acid

$$+ 0.13\ CH_3COOH + 0.007\ C_3H_7COOH + 1.98\ CO_2 + 1.43\ H_2$$

$$+ (C,H,O \text{ incorporated in the cell structure}) \qquad (1)$$

An average of almost 9 percent of glucose carbon was incorporated into the cell structure. The ratios between butanol, acetone and ethanol were 64:30:6. TNVP actual yield was 33% (by weight) and total hydrogen to total butanol gave a ratio of 2.80 $\frac{\text{mole } H_2}{\text{mole Butanol}}$

3.3 ANALYTICAL METHODS

Two basically different approaches were taken in expressing the process stoichiometry for the calculation of the theoretical yield.

I) The first approach towards estimation of the theoretical TNVP yield was made by using the stoichiometric relationships expressing the formation of a definite product from glucose (Johnson et al., 1931). The set of equations was as follows:

$$C_6H_{12}O_6 \rightarrow C_4H_9OH + 2\ CO_2 + H_2O \qquad (2)$$

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3COCH_3 + 3\ CO_2 + 4\ H_2 \qquad (3)$$

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 \quad (4)$$

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ H_2 \quad (5)$$

$$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2\ CO_2 + 2\ H_2 \quad (6)$$

These equations however, do not reflect the fermentation process reality well; the underlying metabolic pathway is branched rather than linear and fermentation of the end products follows a dynamic equilibrium rather than a numerical relationship. This set of equations, nevertheless, gives quantitative relationships between the glucose consumed, gases evolved and solvents and acids produced.

In Approach I three different biosynthesis conditions were examined and considered in deriving more meaningful quantitative stoichiometric relationships:

A. Butanol and acetone are the only final products in the liquid phase (i.e. no biomass, ethanol or acids are present).

B. Butanol, acetone and ethanol are the only final products in the liquid phase (i.e. no biomass or acids are present).

C. Butanol, acetone, ethanol, acetic acid and butyric acid are the final products in the liquid phase (i.e. no biomass is present).

The ensuing respective calculations were always based on one mole of glucose consumed and known ratios of butanol:acetone:ethanol in the system.

II) In the second approach a single linear general equation was assumed to relate the consumption of glucose to the formation of all the products as follows:

$$C_6H_{12}O_6 \rightarrow b\ C_4H_9OH + c\ CH_3COCH_3 + d\ C_2H_5OH + e\ CH_3COOH + f\ C_3H_7COOH + g\ CO_2 + h\ H_2 + z\ H_2O \qquad (7)$$

Biomass can also be considered to be stoichiometrically related to the substrate conversion with ammonia providing the nitrogen requirements of the cell. Upon inclusion of biomass with the molecular formula of $C_4H_8O_2N$ (Meyberry et al., 1968), equation (7) will change to the following form:

$$C_6H_{12}O_6 + x\ NH_3 \rightarrow b\ C_4H_9OH + c\ CH_3COCH_3 + d\ C_2H_5OH + e\ CH_3COOH + f\ C_3H_7COOH + g\ CO_2 + h\ H_2 + x\ C_4H_8O_2N + z\ H_2O \qquad (8)$$

Table 2 lists the unknown variables of equation (8). Three independent equations can be written reflecting balances on carbon, hydrogen and oxygen for the calculation of the unknowns in equation (8).

$$\Sigma C = 0 \rightarrow 4b + 3c + 2d + 2e + 4f + g + 4x = 6 \quad (9)$$

$$\Sigma H = 0 \rightarrow 10b + 6c + 6d + 4e + 8f + 2h + 5X + 2z = 12 \quad (10)$$

$$\Sigma O = 0 \rightarrow b + c + d + 2e + 2f + 2g + 2X + z = 6 \quad (11)$$

Six more independent equations or known values are required in order to solve equation (8) for all the unknowns. Three different sets of equations representing three different models can be used. These equations are based on the information obtained from the batch experiments. Model 1 utilizes the real experimentally observed ratios between butanol and acetone, ethanol, acetic acid, butyric acid, biomass and carbon dioxide respectively. Model 2 uses the ratio between carbon dioxide and hydrogen instead of the butanol/CO_2 ratio. The fraction of the glucose carbon incorporated in carbon dioxide was considered to be fixed in Model 3. The summary of the Models is presented in Table 3. A computer program utilizing a "Gauss-Jordan" method for solving a system of equations was used in computation of the unknown variables. The reason for choosing the fixed ratios between

TABLE 2

UNKNOWN VARIABLES OF EQUATION (8)

(PER MOLE OF GLUCOSE CONSUMED)

b = moles of butanol produced

c = moles of acetone produced

d = moles of ethanol produced

e = moles of acetic acid produced

f = moles of butyric acid produced

g = moles of carbon dioxide produced

h = moles of hydrogen produced

x = moles of biomass produced

z = moles of water produced

TABLE 3

EQUATIONS REPRESENTING MODEL 1, 2 AND 3 FOR THE SOLUTION OF EQUATION (8)

BUTANOL:ACETONE:ETHANOL = 64:30:6

Equation No.	Model 1	Model 2	Model 3
9	Σ carbon=0	Σ carbon=0	Σ carbon=0
10	Σ H=0	Σ H=0	Σ H=0
11	Σ O=0	Σ O=0	Σ O=0
12	b = 1.67c	b = 1.67c	b = 1.67c
13	b = 6.37d	b = 6.37d	b = 6.37d
14	b = 3.76e	b = 3.76e	b = 3.76e
15	b = 65.5f	b = 65.5f	b = 65.5f
16	b = 4.01x	b = 4.01x	b = 4.01x
17	b = 0.26g	g = 1.39h	g = 1.98

butanol and the other products as the basis for computations was the interrelated mechanism in the production of all the products in the process and the significance of butanol production in the metabolic pathways. Other equations could also be used in the computations such as the equations based on the ratios between acetone and the other compounds.

Two more biosynthesis conditions are considered in Approach II in addition to those used in Approach I. These conditions are as follows:

D. Butanol, acetone, ethanol and biomass are the only products in the liquid phase (i.e. no acids are present).

E. Butanol, acetone, ethanol, acetic acid, butyric acid and biomass are present in the liquid phase (i.e. a true system is considered).

Depending on the condition considered in the study, a corresponding number of equations were used. For instance, when butanol, acetone and ethanol were the only products considered to be present in the system, equation (8) was reduced to the following form:

$$C_6H_{12}O_6 \rightarrow b\ C_4H_9OH + c\ CH_3COCH_3 + d\ C_2H_5OH + g\ CO_2 + h\ H_2 + z\ H_2$$

There are six unknowns, b, c, d, g, h and z, in this equation. Therefore equations (9), (10), (11), (12),

(13) and (17) were to be solved for the unknown variables (see Tables 3 and 8).

In addition to the foregoing computations, a three-dimensional plot has been developed for each model to reflect the continuous variations of the TNVP yield in response to the variations in the ratios between the solvents. The plots were generated by using the Statistical Analysis System (SAS) facilities of the Computing Center of McGill University (Montreal).

3.4 RESULTS

The results of the calculations based on Approach I are tabulated in Table 4. Since the production of biomass is not related to the glucose consumption by a separate equation, its formation is not included in the calculations.

Tables 5, 6 and 7 present the stoichiometric relationships, the theoretical TNVP yield and the $\frac{\text{mole } H_2}{\text{mole Butanol}}$ ratio when Approach II and Models 1, 2 and 3 were used in computations, respectively. When equation (8) was solved for Condition A, negative values for the gases or unrealistically low values for the solvents resulted. These data are not indicated here. The value of the TNVP yield changed from 31.9% when a fixed molar gas ratio and a true system was considered (Model 2, Condition E, Table 6), to 42.3% when Model 3 was used and no glucose was considered utilized in the production of acids or cells (Condition B, Table 7). The minimum hydrogen to butanol ratio ($0.5 \frac{\text{mole } H_2}{\text{mole Butanol}}$) was obtained when the model resulted in the highest value of the TNVP Yield (Table 7). The maximum hydrogen to butanol molar ratio was 3.22 which occurred at the lowest value of the calculated TNVP yield (Model 2, Condition E, Table 6).

Tables 4 to 7 present the results of the computations related to the actual experimental observations of the process. The ratios between butanol, acetone and ethanol could be different in the fermentations depending on the

TABLE 4

STOICHIOMETRIC COEFFICIENTS, APPROACH I, BUTANOL:ACETONE:ETHANOL = 64:30:6

$$C_6H_{12}O_6 + xNH_3 \rightarrow bC_4H_9OH + cCH_3{-}CO{-}CH_3 + dC_2H_5OH + eCH_3{-}COOH + fC_3H_9{-}COOH + gCO_2 + hH_2 + x\ \text{Biomass} + zH_2O$$

Condition	Coefficient									TNVP yield %	mole H_2 / mole butanol
	b	c	d	e	f	g	h	x	z		
A	0.62	0.37	0	0	0	2.37	1.50	0	0.25	37.5	2.42
B	0.59	0.36	0.10	0	0	2.36	1.43	0	0.24	38.5	2.42
C	0.55	0.33	0.09	0.15	0.008	2.32	1.62	0	0.07	35.6	2.94

TABLE 5

STOICHIOMETRIC COEFFICIENTS, APPROACH II, MODEL 1, BUTANOL:ACETONE:ETHANOL = 64:30:6

$$C_6H_{12}O_6 + xNH_3 \rightarrow bC_4H_9OH + cCH_3{-}CO{-}CH_3 + dC_2H_5OH + eCH_3{-}COOH + fC_3H_7{-}COOH$$
$$+ gCO_2 + hH_2 + x\ \text{Biomass} + zH_2O$$

Condition	Coefficient									TNVP yield %	$\frac{\text{mole } H_2}{\text{mole butanol}}$
	b	c	d	e	f	g	h	x	z		
B	0.60	0.36	0.09	0	0	2.33	1.36	0	0.28	38.6	2.27
C	0.57	0.34	0.09	0.15	0.009	2.20	1.27	0	0.28	36.5	2.23
D	0.54	0.33	0.08	0	0	2.12	1.17	0.14	0.53	35.0	2.17
E	0.52	0.31	0.08	0.14	0.008	2.01	1.09	0.13	0.52	33.5	2.10

TABLE 6

STOICHIOMETRIC COEFFICIENTS, APPROACH II, MODEL 2, BUTANOL:ACETONE:ETHANOL = 64:30:6

$$C_6H_{12}O_6 + xNH_3 \rightarrow bC_4H_9OH + cCH_3{-}CO{-}CH_3 + dC_2H_5OH + eCH_3{-}COOH + fC_3H_7{-}COOH$$
$$+ gCO_2 + hH_2 + x\ \text{Biomass} + zH_2O$$

Condition	Coefficient									TNVP yield %	$\frac{\text{mole } H_2}{\text{mole butanol}}$
	b	c	d	e	f	g	h	x	z		
B	0.57	0.35	0.09	0	0	2.48	1.79	0	0.02	37.1	3.14
C	0.54	0.33	0.08	0.14	0.008	2.35	1.69	0	0.03	34.9	3.13
D	0.52	0.31	0.08	0	0	2.30	1.66	0.13	0.23	33.5	3.19
E	0.49	0.30	0.08	0.13	0.008	2.19	1.58	0.12	0.23	31.9	3.22

TABLE 7

STOICHIOMETRIC COEFFICIENTS, APPROACH II, MODEL 3, BUTANOL:ACETONE:ETHANOL = 64:30:6

$$C_6H_{12}O_6 + xNH_3 \rightarrow bC_4H_9OH + cCH_3{-}CO{-}CH_3 + dC_2H_5OH + eCH_3{-}COOH + fC_3H_7COOH + gCO_2 + hH_2 + x\ \text{Biomass} + zH_2O$$

Condition	Coefficient									TNVP yield %	mole H_2 / mole butanol
	b	c	d	e	f	g	h	x	z		
B	0.66	0.39	0.10	0	0	1.98	0.33	0	0.88	42.3	0.50
C	0.60	0.36	0.09	0.16	0.009	1.98	0.64	0	0.65	38.6	1.07
D	0.56	0.34	0.09	0	0	1.98	0.77	0.14	0.76	36.3	1.37
E	0.52	0.31	0.08	0.14	0.008	1.98	1.00	0.13	0.57	33.5	1.92

culture, fermentation medium and environmental conditions such as pH, temperature and redox potential. Another set of computations was completed whereby the ratio of 60:30:10 for butanol:acetone:ethanol was used. Table 8 presents the three sets of basic equations and Tables 9 to 12 present the outcome of the corresponding computations. Under the new fixed-solvent-ratio assumption, the calculated TNVP yield ranged from 32.4% to 42.8% with corresponding values of the $\frac{H_2}{\text{butanol}}$ ratio from 0.55 $\frac{\text{mole } H_2}{\text{mole butanol}}$ to ratio 3.38 $\frac{\text{mole } H_2}{\text{mole butanol}}$.

The results of the plotting of the TNVP yield versus the weight percents of butanol and ethanol are presented in Figures 1 and 2. Figure 1 reflects the continuous variations in this parameter when no glucose carbon was incorporated into the acids or biomass (Condition B). Figure 2 has been developed by considering Condition D. These plots indicate that increase of the theoretical yield was always accompanied by an unfavourable decrease in the percentage of butanol and a simultaneous increase in the percentage of ethanol in the system. The maximum yield established from the plots occurred at the mutual ratio of solvents 45:35:20 (B:A:E).

TABLE 8

EQUATIONS REPRESENTING MODELS 1, 2 AND 3 FOR THE SOLUTION OF EQUATION (8)

BUTANOL:ACETONE:ETHANOL 60:30:10

Equation No.	Model 1	Model 2	Model 3
9	Σ carbon = 0	Σ carbon = 0	Σ carbon = 0
10	Σ H = 0	Σ H = 0	Σ H = 0
11	Σ O = 0	Σ O = 0	Σ O = 0
12	b = 1.57c	b = 1.57c	b = 1.57c
13	b = 3.73d	b = 3.73d	b = 3.73d
14	b = 3.76e	b = 3.76e	b = 3.76e
15	b = 65.5f	b = 65.5f	b = 65.5f
16	b = 4.01x	b = 4.01x	b = 4.01x
17	b = 0.26g	g = 1.39h	g = 1.98

TABLE 9

STOICHIOMETRIC COEFFICIENTS, APPROACH I, BUTANOL:ACETONE:ETHANOL = 60:30:10

$$C_6H_{12}O_6 + xNH_3 \rightarrow bC_4H_9OH + cCH_3{-}CO{-}CH_3 + dC_2H_5OH + eCH_3{-}COOH + fC_3H_7{-}COOH + gCO_2 + hH_2 + x\ \text{Biomass} + zH_2O$$

Condition	Coefficient									TNVP yield %	$\frac{\text{mole } H_2}{\text{mole butanol}}$
	b	c	d	e	f	g	h	x	z		
A	0.61	0.39	0	0	0	2.39	1.56	0	0.22	37.7	2.56
B	0.56	0.36	0.15	0	0	2.37	1.45	0	0.20	38.5	2.59
C	0.52	0.33	0.14	0.14	0.008	2.33	1.63	0	0.05	35.6	3.13

TABLE 10

STOICHIOMETRIC COEFFICIENTS, APPROACH II, MODEL 1, BUTANOL:ACETONE:ETHANOL = 60:30:10

$$C_6H_{12}O_6 + xNH_3 \rightarrow bC_4H_9OH + cCH_3{-}CO{-}CH_3 + dC_2H_5OH + eCH_3{-}COOH +$$
$$fC_3H_7{-}COOH + gCO_2 + hH_2 + x\ \text{Biomass} + zH_2O$$

Condition	Coefficient									TNVP yield %	$\frac{\text{mole } H_2}{\text{mole butanol}}$
	b	c	d	e	f	g	h	x	z		
B	0.58	0.37	0.16	0	0	2.25	1.13	0	0.38	39.9	1.95
C	0.55	0.35	0.15	0.14	0.008	2.13	1.06	0	0.38	37.8	1.93
D	0.53	0.34	0.14	0	0	2.05	0.96	0.14	0.62	36.4	1.81
E	0.50	0.32	0.13	0.13	0.008	1.95	0.90	0.13	0.60	34.2	1.80

TABLE 11

STOICHIOMETRIC COEFFICIENTS, APPROACH II, MODEL 2, BUTANOL:ACETONE:ETHANOL = 60:30:10

$$C_6H_{12}O_6 + xNH_3 \rightarrow bC_4H_9OH + cCH_3{-}CO{-}CH_3 + dC_2H_5OH + eCH_3{-}COOH +$$
$$fC_3H_7{-}COOH + gCO_2 + hH_2 + x\ \text{Biomass} + zH_2O$$

Condition	Coefficient									TNVP yield %	mole H_2 / mole butanol
	b	c	d	e	f	g	h	x	z		
B	0.54	0.35	0.15	0	0	2.48	1.79	0	0	37.4	3.31
C	0.52	0.33	0.14	0.14	0.008	2.36	1.70	0	0.005	35.6	3.27
D	0.49	0.32	0.13	0	0	2.30	1.66	0.13	0.20	33.8	3.38
E	0.47	0.30	0.13	0.12	0.007	2.20	1.59	0.12	0.20	32.4	3.38

TABLE 12

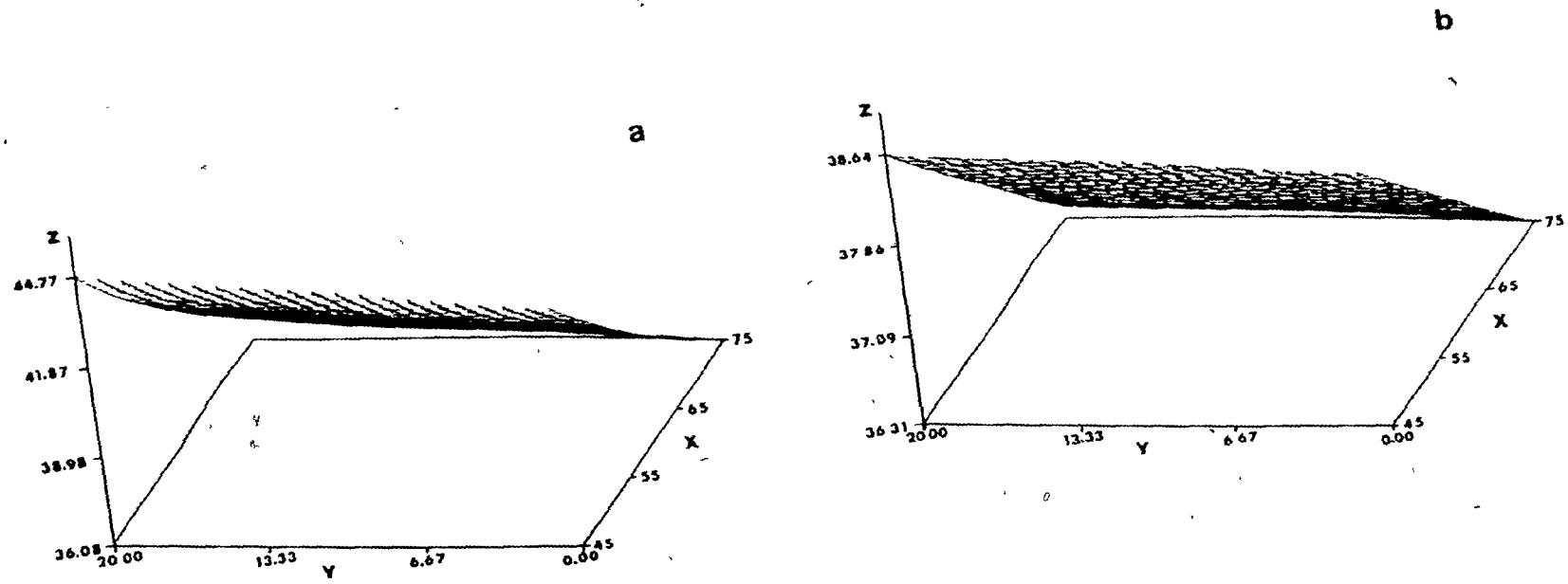
STOICHIOMETRIC COEFFICIENTS, APPROACH II, MODEL 3, BUTANOL:ACETONE:ETHANOL = 60:30:10

$$C_6H_{12}O_6 + xNH_3 \rightarrow bC_4H_9OH + cCH_3{-}CO{-}CH_3 + dC_2H_5OH + eCH_3{-}COOH +$$
$$fC_3H_7{-}COOH + gCO_2 + hH_2 + xC_4H_8O_2N + zH_2O$$

Condition	Coefficient									TNVP yield %	mole H_2 / mole butanol
	b	c	d	e	f	g	h	x	z		
B	0.62	0.40	0.17	0	0	1.98	0.34	0	0.85	42.8	0.55
C	0.60	0.36	0.15	0.15	0.009	1.98	0.63	0	0.63	40.2	1.05
D	0.54	0.34	0.15	0	0	1.98	0.76	0.13	0.74	37.0	1.41
E	0.50	0.32	0.13	0.13	0.008	1.98	0.98	0.12	0.56	34.2	1.96

Figure 1 Continuous Variations of the Total Solvent Yield (z) With the Weight Fractions of Butanol (x) and Ethanol (y) in Cultivation of C. acetobutylicum on Glucose.

a: Model 1, Condition B
b: Model 2, Condition B
c: Model 3, Condition B

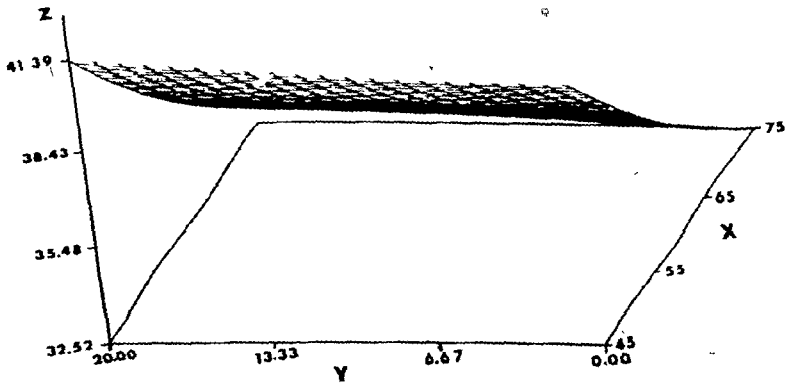


C. acetobutylicum
Theoretical Solvent Yield

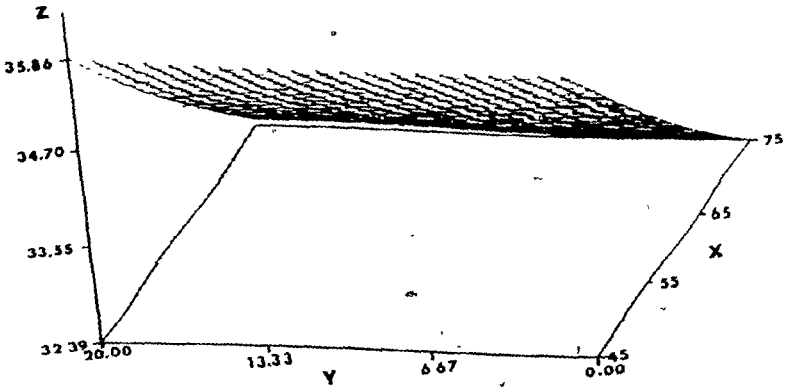
Figure 2 Continuous Variations of the Total Solvent Yield (z) With the Weight Fractions of Butanol (x) and Ethanol (y) in Cultivation of C. acetobutylicum on Glucose.

a: Model 1, Condition D
b: Model 2, Condition D
c: Model 3, Condition D

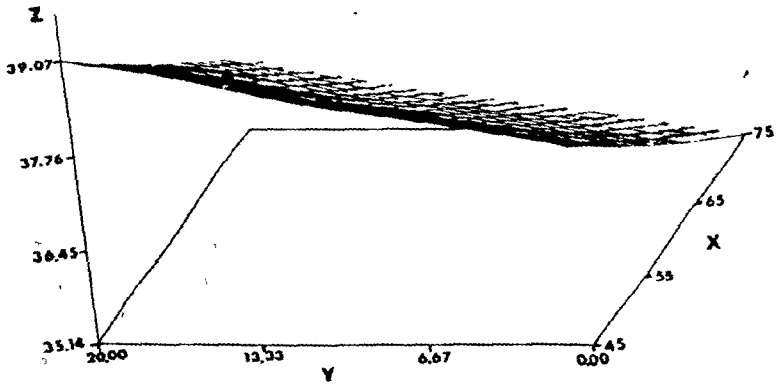
a



b



C



C. acetobutylicum
Theoretical Solvent Yield

3.5 DISCUSSION

An economic evaluation of the butanol-acetone fermentation reveals that most of the production cost in an industrial process is related to the cost of the carbon and energy source. The total production costs for a molasses-based process were found to be slightly higher than the total project annual income even when all the fermentation by-products were taken into account (Lenz and Moreira, 1980). This was mainly due to the cost of the molasses feedstock. It is obvious that the process conversion yield of the products from the carbon and energy source is crucially important for successful exploitation of the fermentation process.

In this paper, experimentally established values of the key fermentation parameters were used in developing models for the estimation of the TNVP yield reflecting the process efficiency of substrate conversion to the solvents. Two different approaches based on simple stoichiometric relationships between the substrate and the products and thirty different combinations of the variables found in equation (8) were considered. The values of the TNVP yield obtained in this study represent the conversion efficiency of glucose to the solvents considering that all the glucose carbon and energy were consumed in the production of the desired compounds and no other product was present in the system.

The actual TNVP yield obtained from the experiments referred to in this paper was ~ 33%. Considering the same ratios for butanol:acetone:ethanol, the models resulted in a maximum stoichiometric calculated TNVP yield of 42.3%. This value was obtained when using the Approach II, Model 3 and Condition B. The maximum stoichiometric calculated TNVP yield was 38.5% when Approach I was used, 38.6% when using Approach II and Model 1, and 37.1% when Approach II and Model 2 were used. Based on these stoichiometric calculated yield values, the efficiencies of 78% to 89% can be calculated for the solvent production in the actual experimental biological system. The range of the calculated yield values of 10.4% is due to the differences in the glucose carbon, energy and electrons divided among the solvents in the three models. It implies that the assumptions made in calculating the theoretical TNVP yield must be defined whenever this parameter is mentioned or used in process "conversion efficiency" calculations. The effect of the ratios between the solvents on the value of the TNVP yield is better presented in the three dimensional plots reflecting the interdependent variations in those parameters. The six plots presented in Figures 1 and 2 are based on the models considered in this paper and can establish a method of predicting the variations in this key fermentation parameter.

Using the figures obtained in the stoichiometric yield computations, a stoichiometric relationship was developed between the glucose consumed, the solvents and acids

produced and the gases evolved. The coefficients of these stoichiometric equations are tabulated in Tables 4 to 12. These relationships do not express the complicated mechanism involved in the production of the intermediates and the solvents. However, they are very important since they give a somewhat simplified picture of the whole process. The molar ratios between the products of the system in gas and liquid phases could also be calculated from these equations. Those equations which describe a real situation whereby all the components are present in the system (Tables 5 to 7 and 9 to 12, Condition E) are of particular practical importance.

Pondering the mechanisms involved in the production of butanol and hydrogen gas, one can see that formation of both of these compounds are convenient ways of disposing of the electrons brought into the bio-system in the substrate. Therefore, there may be a correlation between the production of these two compounds. A ratio of 2.80 $\frac{\text{mole of } H_2}{\text{mole of butanol}}$ resulted from accumulative figures for total hydrogen and butanol respectively produced in the system during the batch experiments with C. acetobutylicum. This ratio ranged from 0.50 $\frac{\text{mole of } H_2}{\text{mole of butanol}}$ to 3.22 $\frac{\text{mole of } H_2}{\text{mole of butanol}}$ in the theoretical computations. The rather large fluctuations of this ratio in the computations were due to the freedom of the movements of the hydrogen atoms between H_2 and H_2O wherever there was no restriction on the molar value of the hydrogen gas in the models. Johnson et al. (1931) applied

equations (2) to (6) in calculating the volumes of carbon dioxide and hydrogen which should evolve during the biosynthesis of the solvents and acids. Their considerations are not quite realistic since they do not account for the amount of carbon incorporated into the cellular structure of the biomass. As a result, higher volumes of the gases produced are obtained from those calculations. Freiberg (1925) presented another set of equations for the formation of acids by considering the cell build-up in the system. Calculating the gases evolved during the fermentation from his equations gives gas volumes closer to the experimental results. However, his equations are unbalanced and mainly hypothetical.

In seeking an answer to the question of the "theoretical yield" in the acetone-butanol fermentation, one should first examine the definition of this parameter and the obstacles in reaching the theoretical value during the actual experimental conditions. The acids (acetic and butyric) have been established to be the precursors of butanol and acetone in the metabolism of glucose by C. acetobutylicum. A complete conversion of these acids would then be necessary for the production of theoretical quantities of the solvents. The carbon, hydrogen and oxygen atoms incorporated in the cell structure should also be minimized. These assumptions will lead to Condition B whereby butanol, acetone and ethanol are assumed to be the only final products of the liquid phase. It is worth noticing that, due to the possible variation in the ratio between the solvents, it is not possible to

establish a certain single value for the maximum theoretical yield which could be considered universal and applicable at any condition. The ratio of the solvents is dependent on the culture and culture conditions. Analysis of the metabolic pathways involved in the production of the solvents also reveals that the ratio between the solvents cannot be a constant fixed value. The energetic metabolism of C. acetobutylicum leading to the production of the solvents is branched. The efficiency of each branch is regulated in such a way that the overall efficiency of the system in the transformation of energy is optimized. This regulation effects the final ratio between the products on the system.

Computations of the TNVP in this work have been based on an experimentally determined ratio of 64:30:6 and on an often literature-quoted ratio of 60:30:10 between butanol, acetone and ethanol. Comparison of the computed TNVP values shows that Approach II, Condition B and Models 1 and 3 give the values of 39.9% and 42.8% respectively, for the ratio of 60:30:10 and 38.6% and 42.3% respectively for the ratio of 64:30:6 which are the highest values of the solvent yield on glucose in the computations. The plots (Figures 1 and 2) indicated an ultimate maximum yield of 44.77% (Model 1, Condition B). However, this value was obtained at a ratio of 45:35:20 for butanol-acetone-ethanol which is rather undesirable. Model 3 also usually resulted in low values of hydrogen gas production and appears unrealistic. Therefore, a TNVP yield of 38.6% to 39.9% resulting

from Approach II, Model 1 and Condition B with a butanol-acetone-ethanol ratio of 64:30:6 to 60:30:10 has been considered in this work as the maximum theoretical yield under "acceptable" conditions. If one neglects the ethanol production in the system, a lower maximum TNVP yield of 38.5% (Approach I, Condition B) will result.

In 1981, Leung and Wang reported a theoretical stoichiometric equation by considering butanol and acetone as the only products of the system in the liquid phase. Their results gave a maximum theoretical solvent yield of 38.0% A ratio of 2.0 $\frac{\text{mole butanol}}{\text{mole acetone}}$ which resulted from their equation is higher than the normally observed ratio for the two compounds. The model reported by Phaff (1981) for the production of butanol and acetone also differs from reality leading to a ratio of 1.0 $\frac{\text{mole butanol}}{\text{mole acetone}}$.

The approach presented in this paper provides a simple method for expressing the stoichiometric relationships between the carbohydrate reactant and the products enabling the calculation of the theoretical TNVP yield without the need for any unrealistic assumptions. In using this approach, however, it should be borne in mind that, despite the validity of all the models considered here, and the perfect balance of the carbon and electrons in all of the equations, the value of the theoretical TNVP yield and the ratios between the gases and the solvents are dependent on the par-

ticular model utilized in the computations and on the ratio of the solvents produced by the microbial culture.

3.6 REFERENCES

1. Doelle, H.W. 1975. Bacterial Metabolism, Academic Press, New York, 574-591.

2. Erickson, L.E. 1979. Energetic Efficiency of Biomass and Product Formation, Biotechnol. Bioeng. 21, 725-743.

3. Freiberg, G.W. 1925. Carbohydrate Metabolism of Acetone-Butyl Alcohol Fermentations, Proc. Soc. Exptl. Biol. Med. 23, 72-73.

4. Johnson, M.J., W.H. Peterson and E.B. Fred. 1931. Oxidation and Reduction Relations Between Substrate and Products in the Acetone-Butyl Alcohol Fermentation, J. Biol. Chem. 91, 569-591.

5. Lenz, T.G and A.R. Moreira. 1980. Economic Evaluation of the Acetone-Butanol Fermentation, Ind. Eng. Chem. Prod. Res. Dev. 19, 478-483.

6. Leung, J.C.Y. and D.I.C. Wang. 1981. Production of Acetone and Butanol by Clostridium acetobutylicum in Continuous Culture Using Free Cells and Immobilized Cells, Proc. 2nd World Congr. Chem. Eng. 1, 348-352.

7. Mayberry, W.R., G.J. Prochazka and W.J. Payne. 1968. Factors Derived from Studies of Aerobic Growth in Minimal Media, J. Bacteriol. 96, 1424-1426.

8. Phaff, H.J. 1981. Industrial Microorganisms, Scientific American 3, 53-65.

9. Prescott, S.C. and C.G. Dunn. 1959. Industrial Microbiology, 3rd Ed., McGraw-Hill, New York, 250-284.

10. Yerushalmi, L. and B. Volesky. 1981. Experimental Bioenergetics of Saccharomyces cerevisiae in Respiration and Fermentations, Biotechnol. Bioeng. 23, 2373-2390.

4

IMPORTANCE OF AGITATION IN THE ACETONE-BUTANOL FERMENTATION

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accepted for publication on January 7, 1985.

4.1 INTRODUCTION

The purpose of aeration and agitation in the fermentation processes is to supply oxygen for the micro-organisms and to mix the fermentation broth in order to make a uniform suspension of the microbes and nutrients in the liquid broth (Aiba, 1965). In aerobic fermentations, agitation increases the interfacial area between the gas and liquid and improves the efficiency of aeration. However, in anaerobic fermentations, since there is no need for oxygen or aeration, agitation is mainly required for maintaining the solid- liquid suspension homogeneous and to ensure good mass transfer in (nutrients) and out (metabolites) of the microbial cell.

The effects of aeration and agitation on the growth of the microbial cultures, enzymatic activities and the production of metabolic products have been subject of numerous studies. Brown and Peterson (1950) found that aeration and agitation were among the main factors affecting the production of penicillin. They showed that increase of the agitation rate up to 600 rpm improved the penicillin forma-

tion. However, further increase of this parameter to 700 rpm, at the optimal level of aeration, decreased the level of penicillin in the fermentor. Similar results were found by Zetelaki and Vas (1968) in a study of the influence of aeration and agitation on growth and production of glucose oxidase by Aspergillus niger. These authors found that the rate of growth and total glucose oxidase activity increased considerably with the increase of the agitation speed from 460 rpm to 700 rpm. However, when the speed of agitation was increased to 940 rpm, both of these parameters decreased.

The agitation effects on hydrogen gas production by Clostridium intermedius were studied by Brosseau and Zajic (1981).

The effects of agitation on the growth and production of solvents and gases by Clostridium acetobutylicum has been studied in this work. The rate of agitation was found to play an important role in controlling the metabolism of C. acetobutylicum. Early studies of this microorganism in shake flasks showed that vigorous shaking could hinder the production of solvents or even completely stop the fermentation. This study was performed to determine the tolerable range of agitation rate by the cells and to evaluate its effects on the kinetic behaviour of the system. The relationship between the gases and the solvents at various levels of agitation has also been evaluated.

4.2 MATERIALS AND METHODS

The fermentation assembly, microbial growth conditions and medium composition, the analytical procedures for the analysis of the solvents, gases and acids and the assay of glucose and biomass are as discussed earlier (section 2.2).

Viscosity of the fermentation broth was measured by a Brookfield Synchro electric visocometer (Model RV) and its density was measured by weighing constant volumes of the broth (25 mL) at a constant temperature.

4.3. RESULTS AND DISCUSSION

4.3.1 Mixing Effect on the Production Rates of Solvents and Gases

Effect of the degree of agitation on the production rates of the solvents and gases was studied by changing the fermentor impeller rotational speed from 190 rpm to 560 rpm. Experimental values of the biomass, sugar, butanol, acetone, ethanol, acetic acid and butyric acid concentrations as well as the total carbon dioxide and hydrogen gas volumes during

the entire course of fermentations are presented in Figure 1 for different agitation rates.

The redox potential (E_h) and pH of the medium were also monitored continuously in these experiments. Values of these two parameters are presented in Figure 2. The medium pH was controlled so as not to decrease below 4.6 by addition of 3N NaOH on demand. During the solvent biosynthesis (phase II), the pH value increased to a final value of 5.0 to 5.3. The redox potential of the fermentation medium decreased to values of as low as -0.3 volts during these fermentation processes. Time required for the completion of fermentations decreased from 27 hours to 24 hours with the impeller rotational speed increasing from 190 rpm to 410 rpm. Termination of the fermentations could be recorded precisely in these experiments by following the gas evolution. Further increase of the impeller rotational speed to 560 rpm inhibited the culture growth and had a general negative effect on the fermentation.

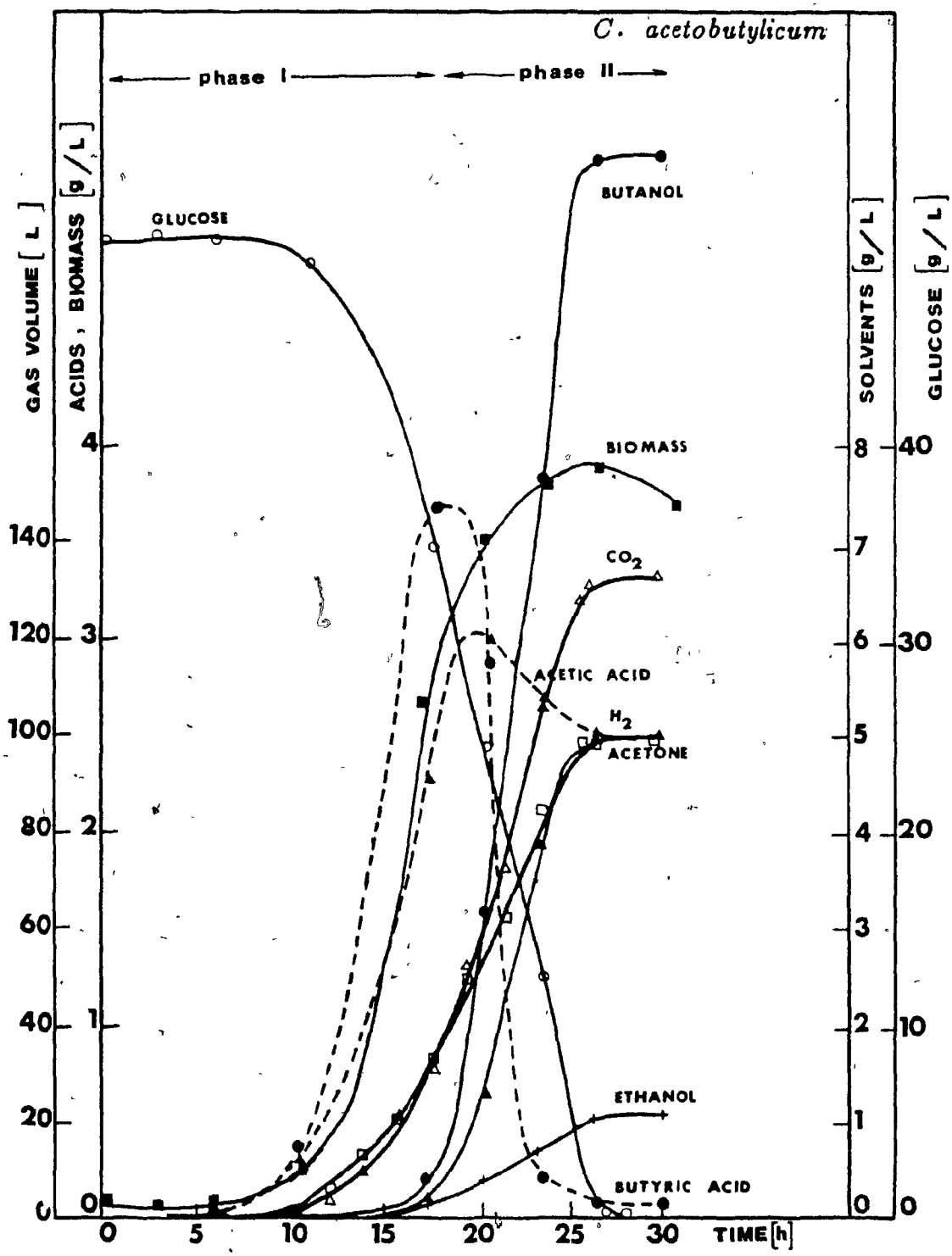
Variation in the agitation rate did not change the general behaviour of the process parameters in these experiments. Phase I of the fermentation was characterized by the growth of the microorganism and accumulation of acetate and butyrate in the medium. There were some gases and only small amounts of solvents produced during this fermentation stage. However, after the break point in the acids curve, the solvents and the gases were produced at a high rate and

FIGURE 1 Variations of the Fermentation Parameters in Batch Cultivation of C. acetobutylicum

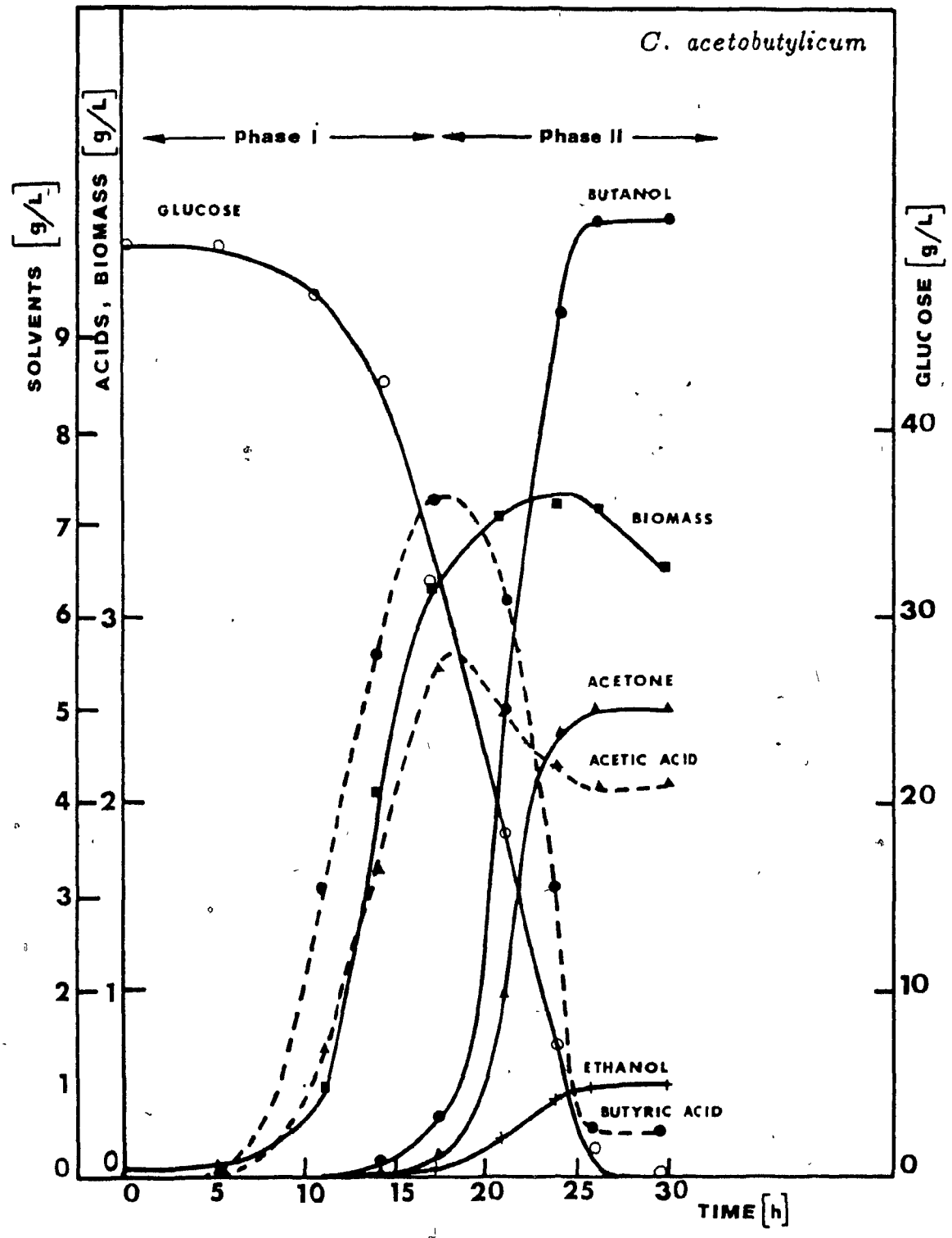
a - agitation rate = 190 rpm
b - agitation rate = 270 rpm
c - agitation rate = 340 rpm
d - agitation rate = 410 rpm

● -	Butanol	(g/L)
▲ -	Acetone	(g/L)
+ -	Ethanol	(g/L)
●---● -	Butyric acid	(g/L)
▲---▲ -	Acetic acid	(g/L)
□ -	Hydrogen	(L)
△ -	Carbon dioxide	(L)
■ -	Biomass	(g/L)
○ -	Sugar	(g/L)

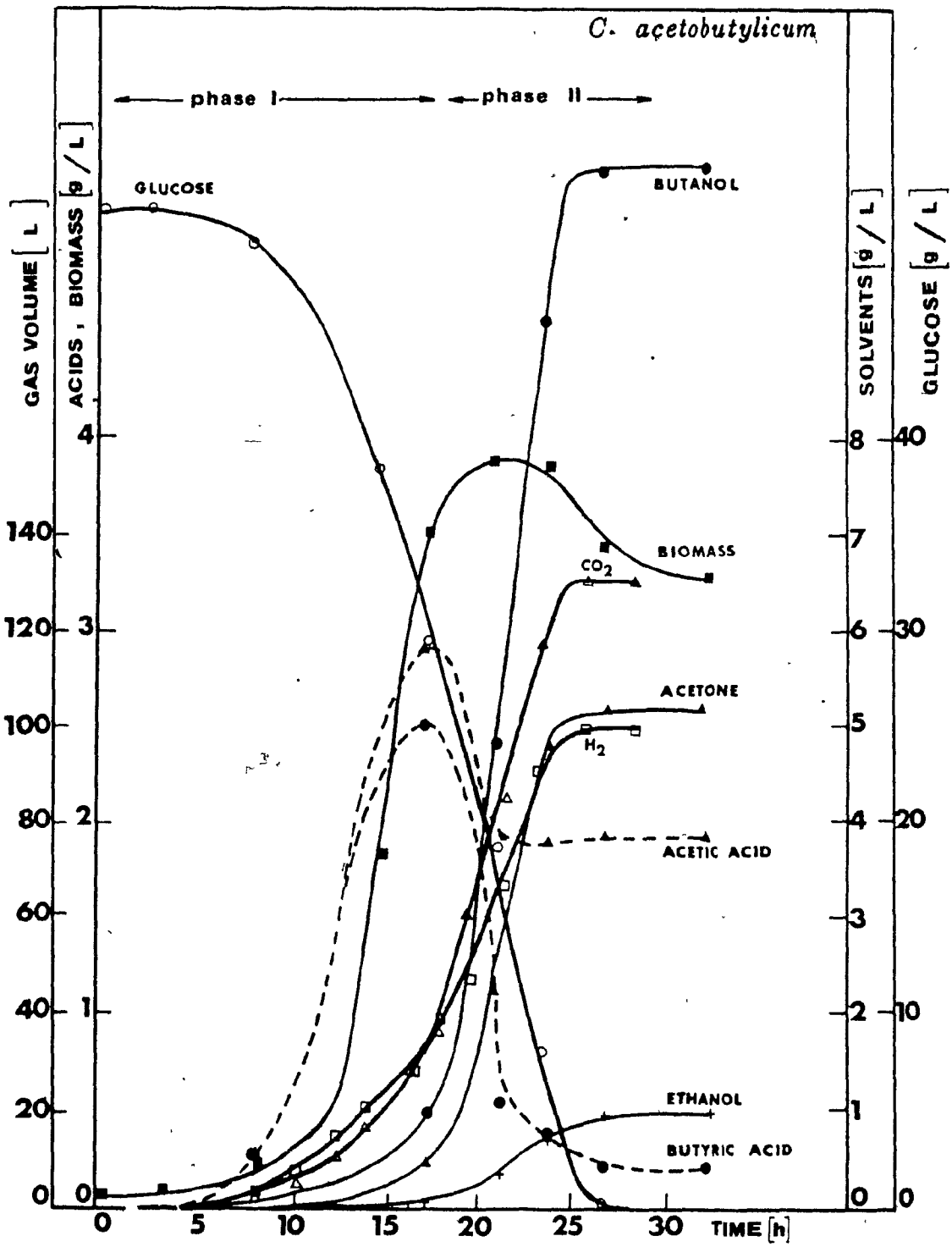
a)


C. acetobutylicum
phase I
phase II
GLUCOSE
BUTANOL
BIOMASS
CO2
ACETIC ACID
H2
ACETONE
ETHANOL
BUTYRIC ACID
GAS VOLUME [L]
ACIDS, BIOMASS [g/L]
SOLVENTS [g/L]
GLUCOSE [g/L]
TIME [h]
0
5
10
15
20
25
30
140
120
100
80
60
40
20
4
3
2
1
8
7
6
5
4
3
2
1
40
30
20
10

b)


C. acetobutylicum
Phase I
Phase II
GLUCOSE
BUTANOL
BIOMASS
ACETONE
ACETIC ACID
ETHANOL
BUTYRIC ACID
SOLVENTS [g/L]
ACIDS, BIOMASS [g/L]
GLUCOSE [g/L]
TIME [h]
0
5
10
15
20
25
30
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40

c)


C. acetobutylicum
phase I
phase II
GLUCOSE
BUTANOL
BIOMASS
CO_2
ACETONE
H_2
ACETIC ACID
ETHANOL
BUTYRIC ACID
GAS VOLUME [L]
ACIDS , BIOMASS [g / L]
SOLVENTS [g / L]
GLUCOSE [g / L]
TIME [h]
0
5
10
15
20
25
30
0
20
40
60
80
100
120
140
1
2
3
4
10
20
30
40
1
2
3
4
5
6
7
8

d)

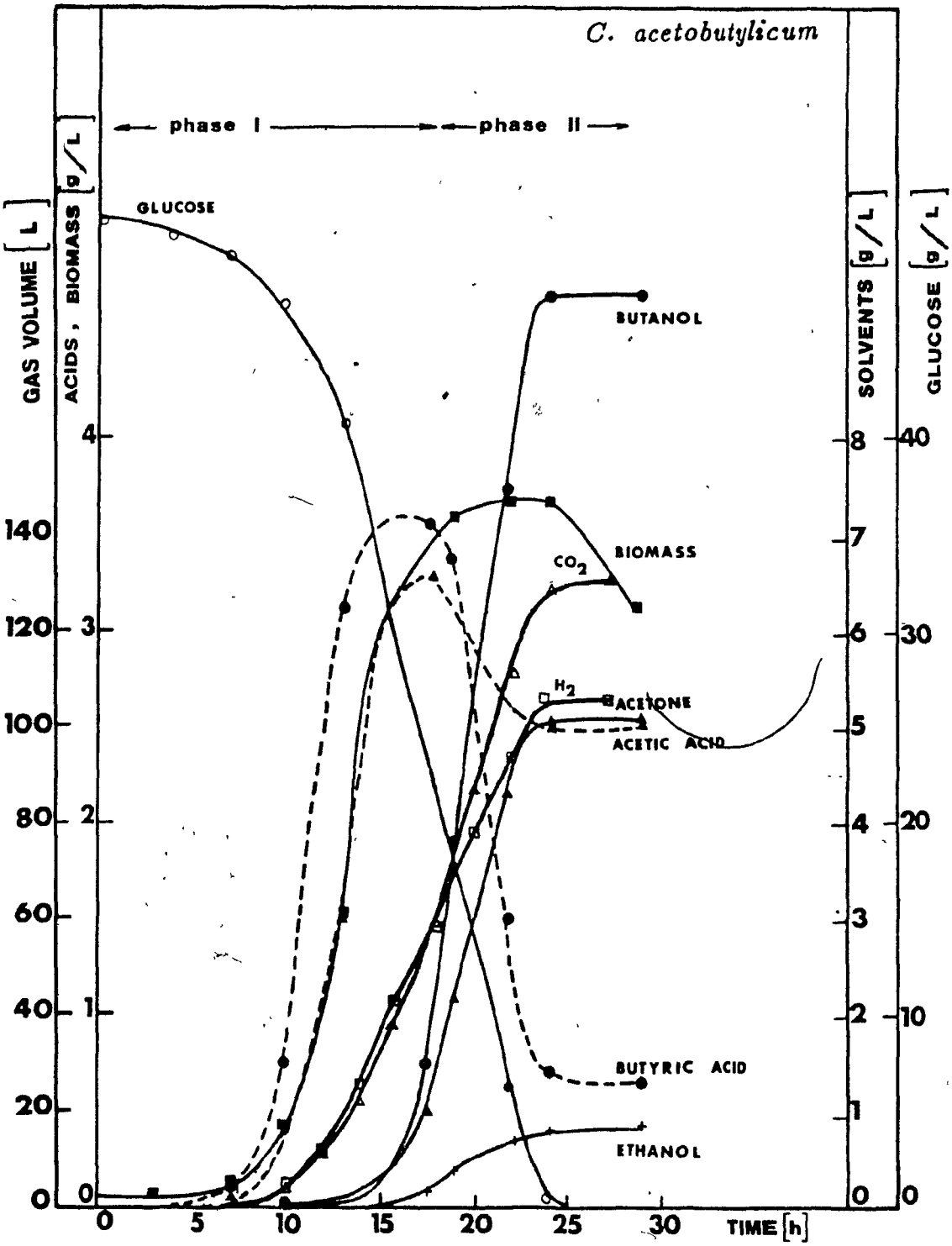
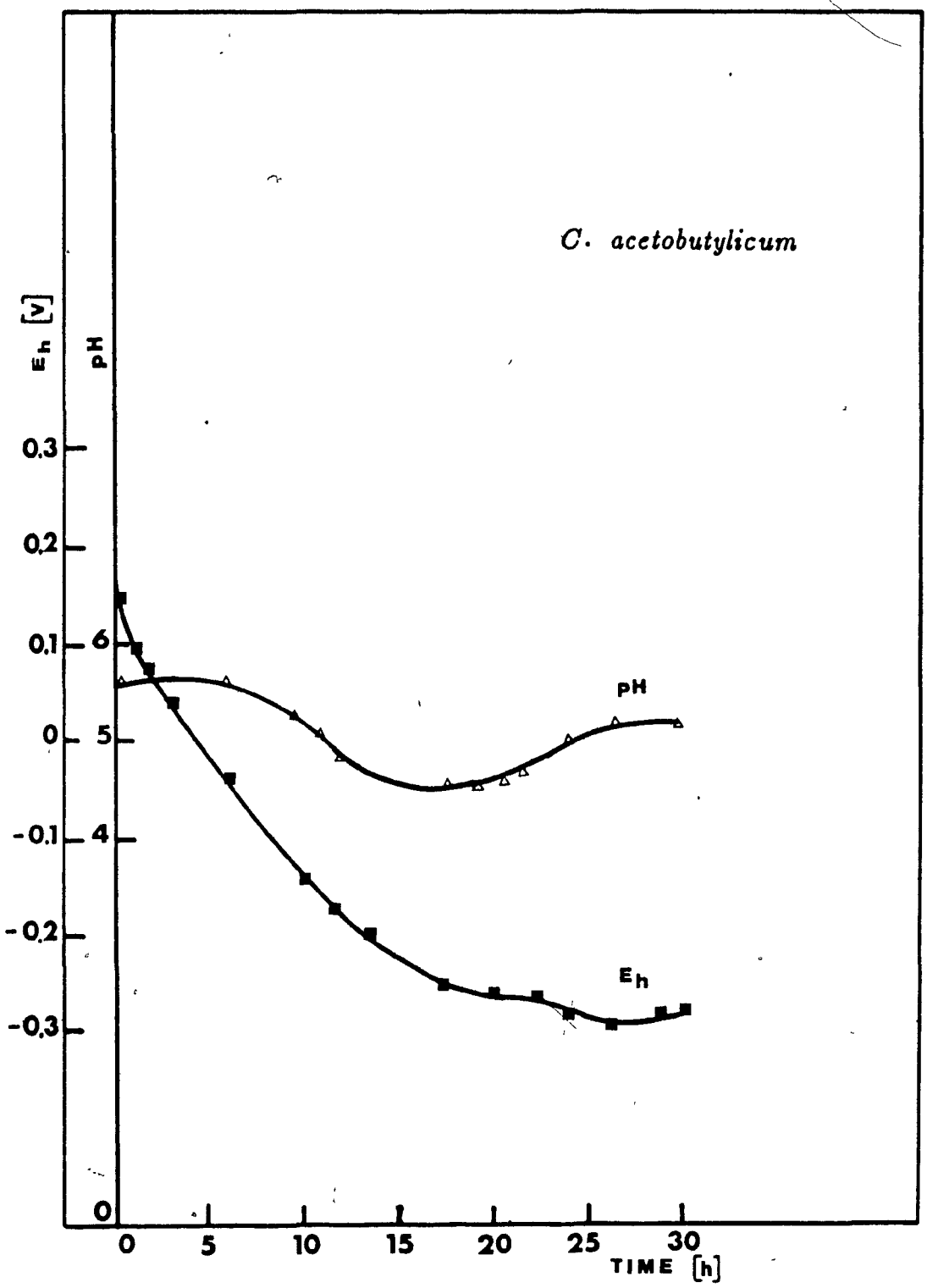

C. acetobutylicum
phase I
phase II
GLUCOSE
BUTANOL
BIOMASS
CO_2
H_2
ACETONE
ACETIC ACID
BUTYRIC ACID
ETHANOL
GAS VOLUME [L]
ACIDS, BIOMASS [g/L]
SOLVENTS [g/L]
GLUCOSE [g/L]
TIME [h]
0
5
10
15
20
25
30
0
20
40
60
80
100
120
140
1
2
3
4
10
20
30
40
1
2
3
4
5
6
7
8

FIGURE 2 Variations of the Fermentation Parameters in Batch Cultivation of C. acetobutylicum

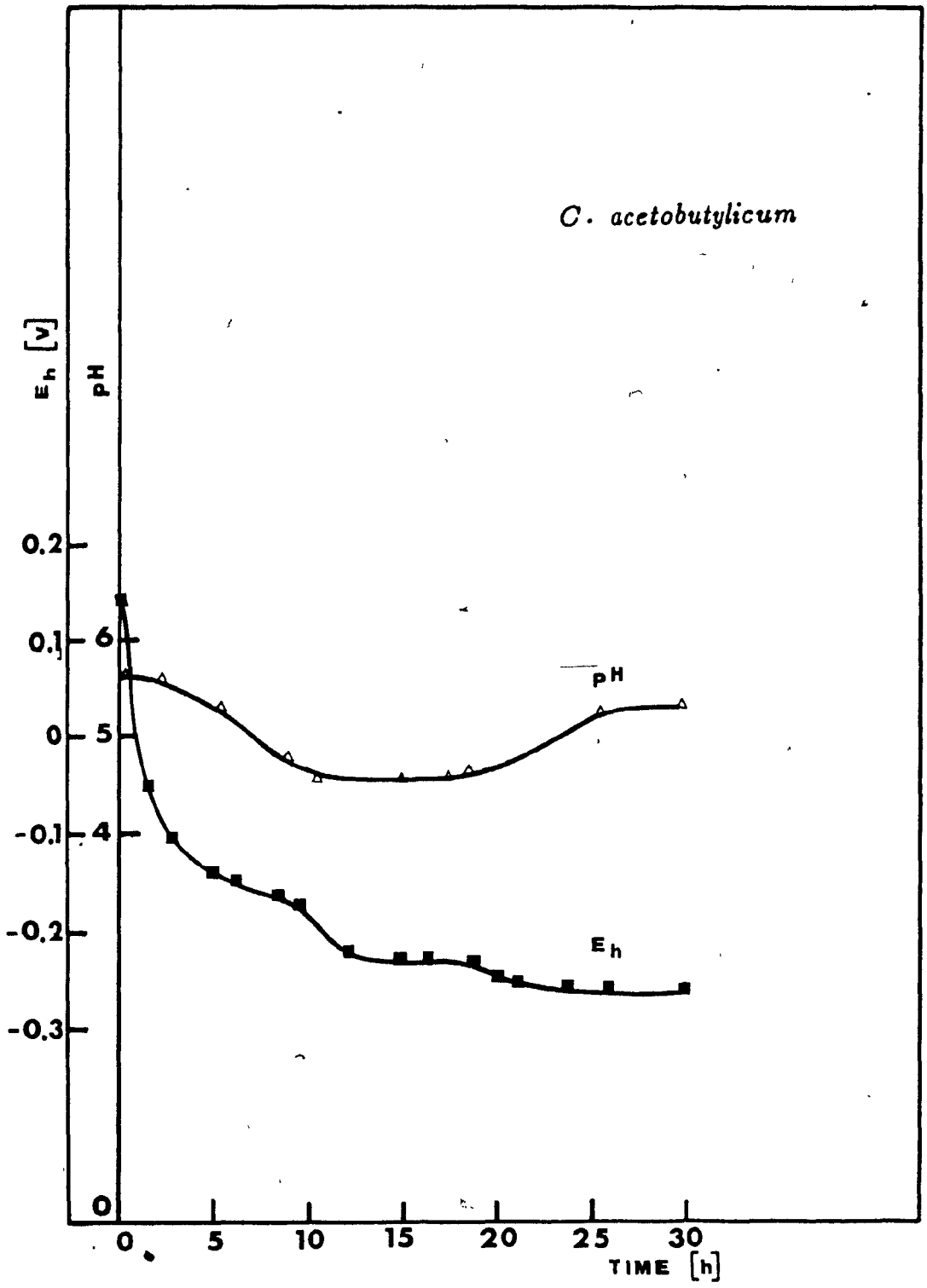
a - agitation rate = 190 rpm
b - agitation rate = 270 rpm
c - agitation rate = 340 rpm
d - agitation rate = 410 rpm

Δ - pH
■ - E_h

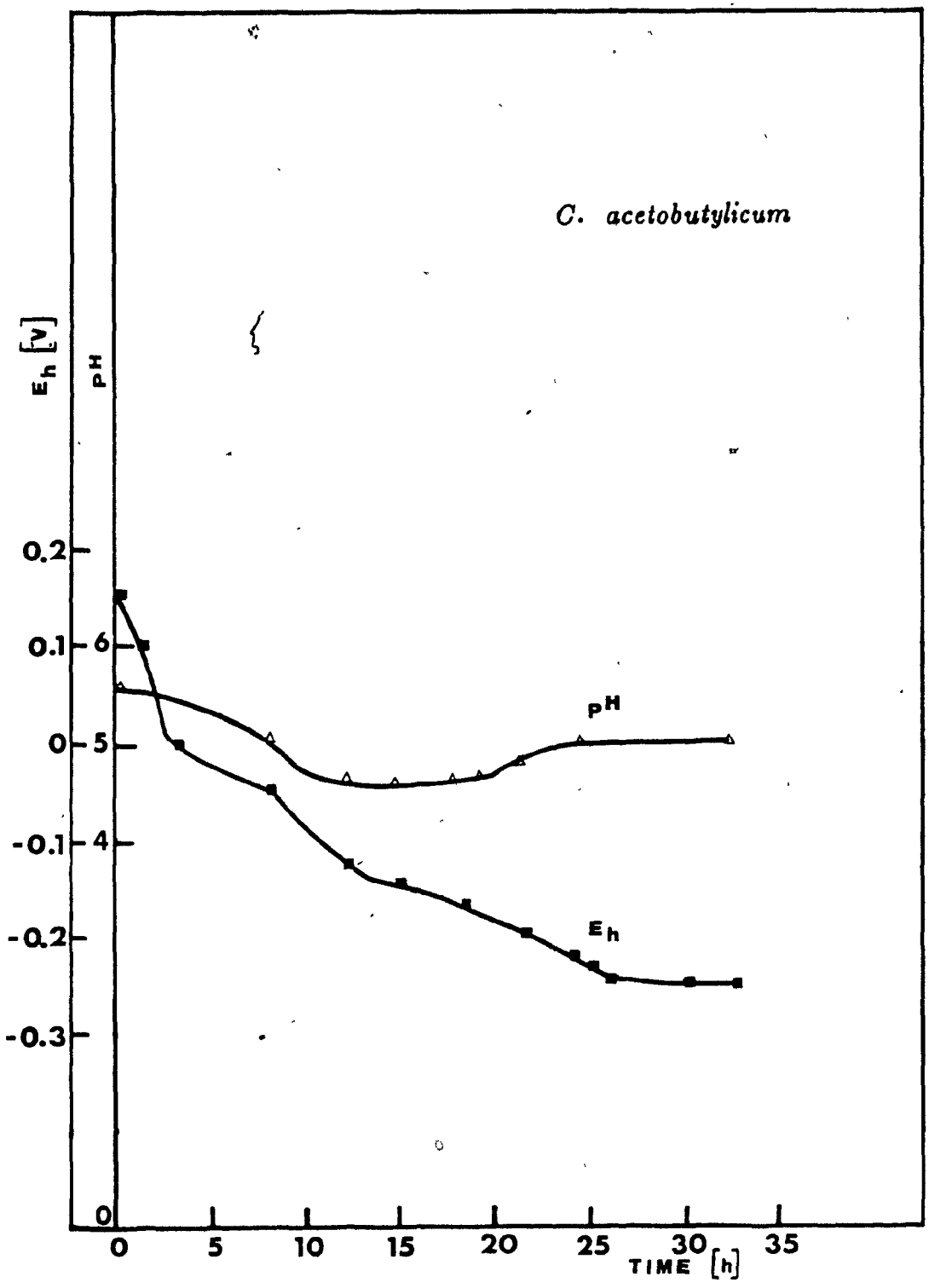
a)


C. acetobutylicum
Eh [V]
pH
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
6
5
4
0
pH
Eh
0
5
10
15
20
25
30
TIME [h]

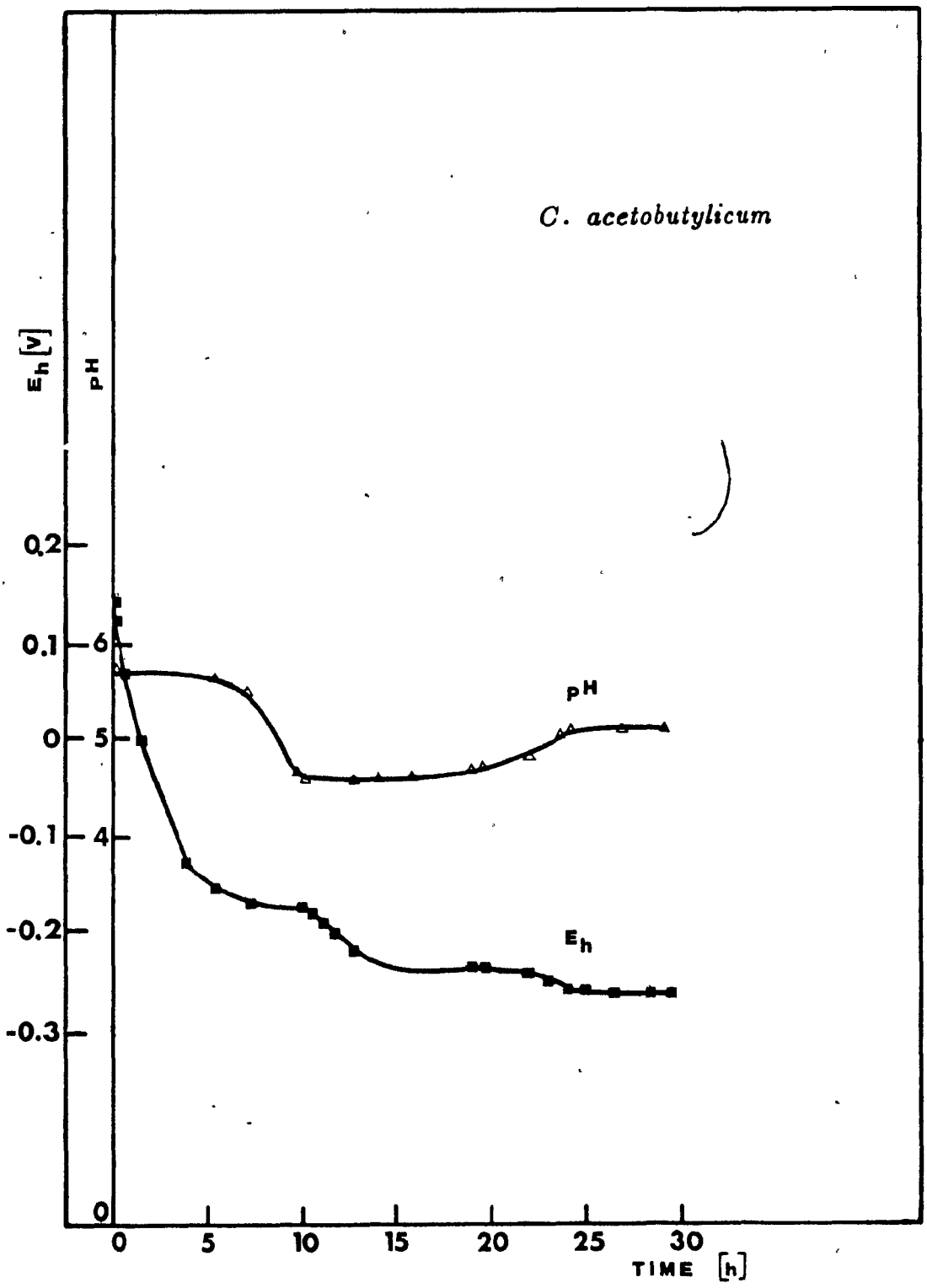
b)


C. acetobutylicum
Eh [V]
pH
0.2
0.1
0
-0.1
-0.2
-0.3
6
5
4
0
0
5
10
15
20
25
30
TIME [h]
pH
Eh

c)


C. acetobutylicum
E_h [V]
pH
0.2
0.1
0
-0.1
-0.2
-0.3
6
5
4
0
0
5
10
15
20
25
30
35
TIME [h]
pH
E_h

d)


C. acetobutylicum
Eh [V]
pH
0.2
0.1
0
-0.1
-0.2
-0.3
6
5
4
0
pH
Eh
0
5
10
15
20
25
30
TIME [h]

reached their final concentrations in less than eight hours. During this stage the culture was at its stationary phase. During Phase I the hydrogen fraction in the gas phase was slightly higher than that of CO_2. However, the CO_2 fraction increased during the solvent production phase (Phase II) and the final gas volume was composed of approximately 57% CO_2 and 43% H_2. Similar results have been reported by other investigators regarding the gas production in the acetone-butanol fermentation (Prescott and Dunn, 1959; Johnson et al., 1931). The total fermentation gases produced during the entire course of fermentation were almost constant at various agitation rates and an average of 24.0 liters of gas was produced per liter of the fermentation medium during these experiments. However, the proportion of gas formation during Phase I to that evolved during Phase II varied with the rate of agitation. The ratio of the total gases produced during Phase I to that produced during Phase II averaged 0.45 for the agitation rates of 190 rpm to 340 rpm. The value of this ratio increased to 0.88 at the high agitation rate of 410 rpm. The effect of the agitation rate on the kinetic behaviour of the system was studied during the period of "High Production Rate". This time period was considered from the break point to the cessation of the gas production. Evaluation of the liquid physical properties showed that the liquid density and viscosity were practically constant throughout the considered period.

The pattern of change in the molar quantities of solvents and the gases produced during this period (mmole/g cell) could be approximated by a straight line which implies a constant value of the specific solvent as well as gas production rates. It is understood that these variables do not exhibit a straight line behaviour in the fermentation processes, however, this assumption was made for comparison purposes. Correlation coefficients close to unity in all cases confirmed the validity of this assumption. Variations in the specific rates of production of butanol, acetone, ethanol, carbon dioxide, hydrogen gas and total gas (mmole/h•g cell) with the impeller Reynolds number during the period of "High Production Rate" are depicted in Figure 3. The impellers Reynolds number was chosen as the criterion for the liquid motion and the degree of mixing in the system. All of the parameters examined exhibited a similar behaviour, increasing with the increase of the impeller Reynolds number up to the $N_{Re} = 3.93 \times 10^4$. Further increase of the Reynolds number resulted in a decrease in the specific rates of product formation. No culture activity was observed when the impeller speed was increased to 560 rpm. No gas phase data were recorded for the experiment with $N_{Re}=3.09 \times 10^4$. The maximum production rates of the gases were 11.6 mmole/h•g cell and 15.9 mmole/h•g cell for H_2 and CO_2, respectively.

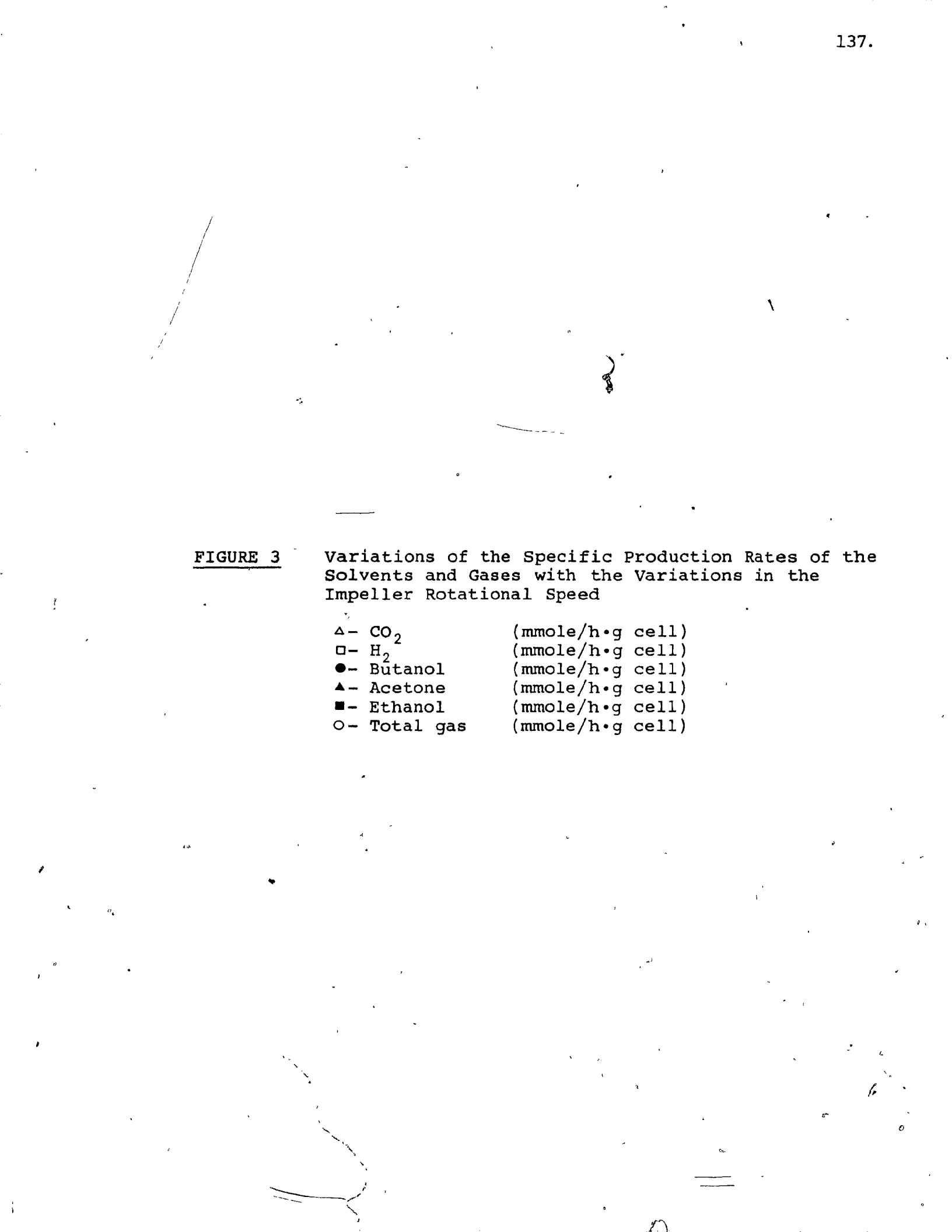
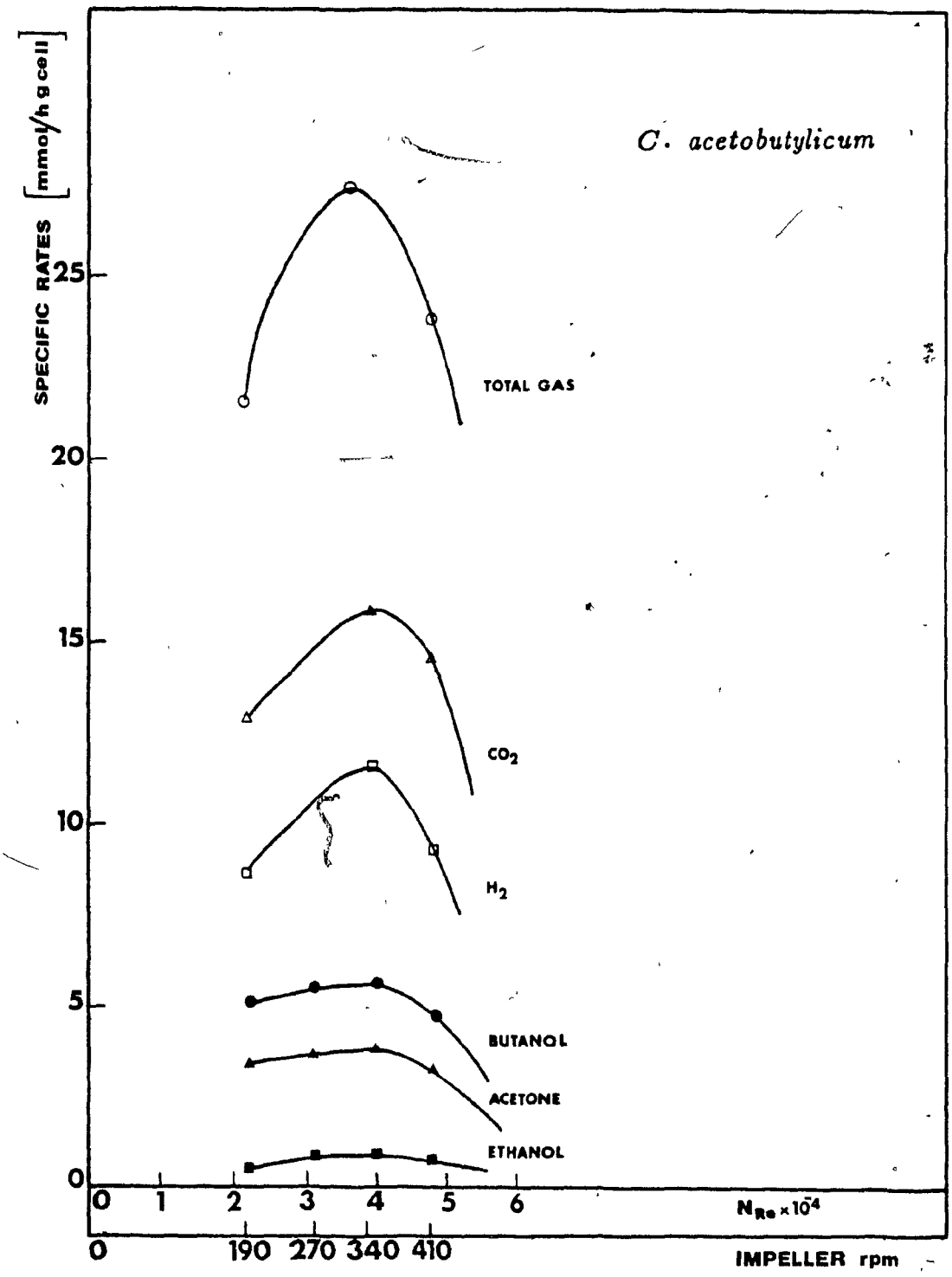


FIGURE 3 Variations of the Specific Production Rates of the Solvents and Gases with the Variations in the Impeller Rotational Speed

△- CO_2 (mmole/h•g cell)
□- H_2 (mmole/h•g cell)
●- Butanol (mmole/h•g cell)
▲- Acetone (mmole/h•g cell)
■- Ethanol (mmole/h•g cell)
○- Total gas (mmole/h•g cell)


C. acetobutylicum
SPECIFIC RATES [mmol/h g cell]
25
20
15
10
5
0
TOTAL GAS
CO2
H2
BUTANOL
ACETONE
ETHANOL
0 1 2 3 4 5 6
NRe x 10^-4
0 190 270 340 410
IMPELLER rpm

The maximum production rates of the solvents were 5.5 mmole butanol/h•g cell, 3.8 mmole acetone/h•g cell and 0.8 mmole ethanol/h•g cell. These values were obtained at the impeller Reynolds number of 3.93×10^4 (340 rpm).

In spite of the eventual decrease in the production rates of the metabolic products with the increase of the impellers speed, the specific growth rate of the culture continued to increase reaching a final value of 0.36 hr^{-1} at the impeller speed of 410 rpm, corresponding to $N_{Re} = 4.79 \times 10^4$.

The final concentration of the solvents and their respective yields on the carbon and energy source are summarized in Table 1. These two parameters slightly decreased with the increase of the impeller rotational speed beyond 340 rpm. The total amount of the gases produced and the overall yield of the gases (CO_2+H_2) on glucose is also presented in Table 1.

Evaluation of the mixing effects on the kinetic behaviour of acetone-butanol fermentation showed that increase of the agitation rate from 190 rpm ($N_{Re} = 2.23 \times 10^4$) to 340 rpm ($N_{Re} = 3.93 \times 10^4$) increased the specific rates of production of the solvents (butanol-acetone-ethanol) and the gases (CO_2 and H_2) during the period of "High Production Rate". This effect is attributed to the enhancement of the extracellular mass transfer of the nutrients and metabolites. Production rates of the solvents and gases were decreased by increase of the agitation speed to 410 rpm (N_{Re}

TABLE 1

EFFECT OF THE IMPELLER SPEED ON THE FINAL TNVP CONCENTRATION AND THE TOTAL GAS PRODUCED

Impeller (rpm)	N_{Re}	Final Solvent Concentration (g/L)	Solvent Yield On glucose ($\frac{g\ solvent}{g\ glucose}$)	Total Gas Produced ($\frac{L\ gas}{L\ liq}$)	Gas Yield on Glucose ($\frac{L\ gas}{g\ glucose}$)
190	2.23×10^4	17.1	33.0	24.1	46.5
270	3.09×10^4	16.2	32.4	-	-
340	3.93×10^4	17.0	32.4	23.8	45.4
410	4.79×10^4	15.4	30.2	24.0	47.1
560		0	0	0	0

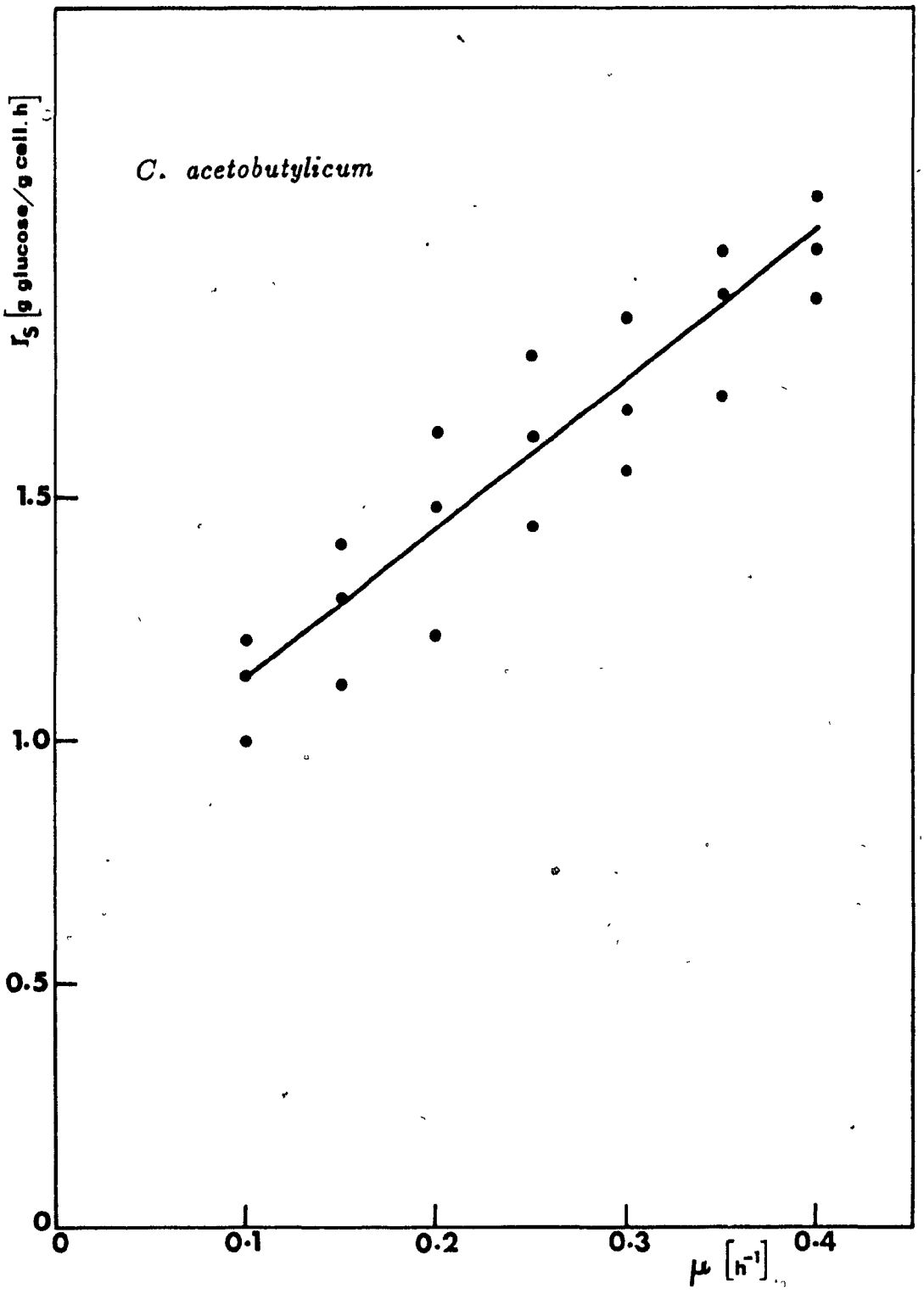
= 4.79×10^4) and the fermentation process stopped at the high agitation speed of 560 rpm.

Similar effects of agitation on hydrogen gas production by C. intermedius (1981) were reported. The maximum rates of gas production were observed at an agitation speed of 500 rpm. At agitation speeds of 750 rpm, gas production decreased sharply and stopped completely at 1000 rpm. These authors pointed out that the observed phenomenon was due to the prevention of the escape of gas (H_2+CO_2) bubbles from the fermentation fluid. This gas phase retention usually resulted in an increase in the production of formic acid, a major substrate for the enzyme complex responsible for H_2 production.

The harmful effects of overagitation have also been found in the antibiotic production (Fortune et al., 1950; Rivett et al., 1950), in the production of glucose oxidase by Aspergillus niger (Zetelaki and Vas, 1968) and in catalase production by Rhodopseudomonas spheroides (Gallili and Mateles, 1977).

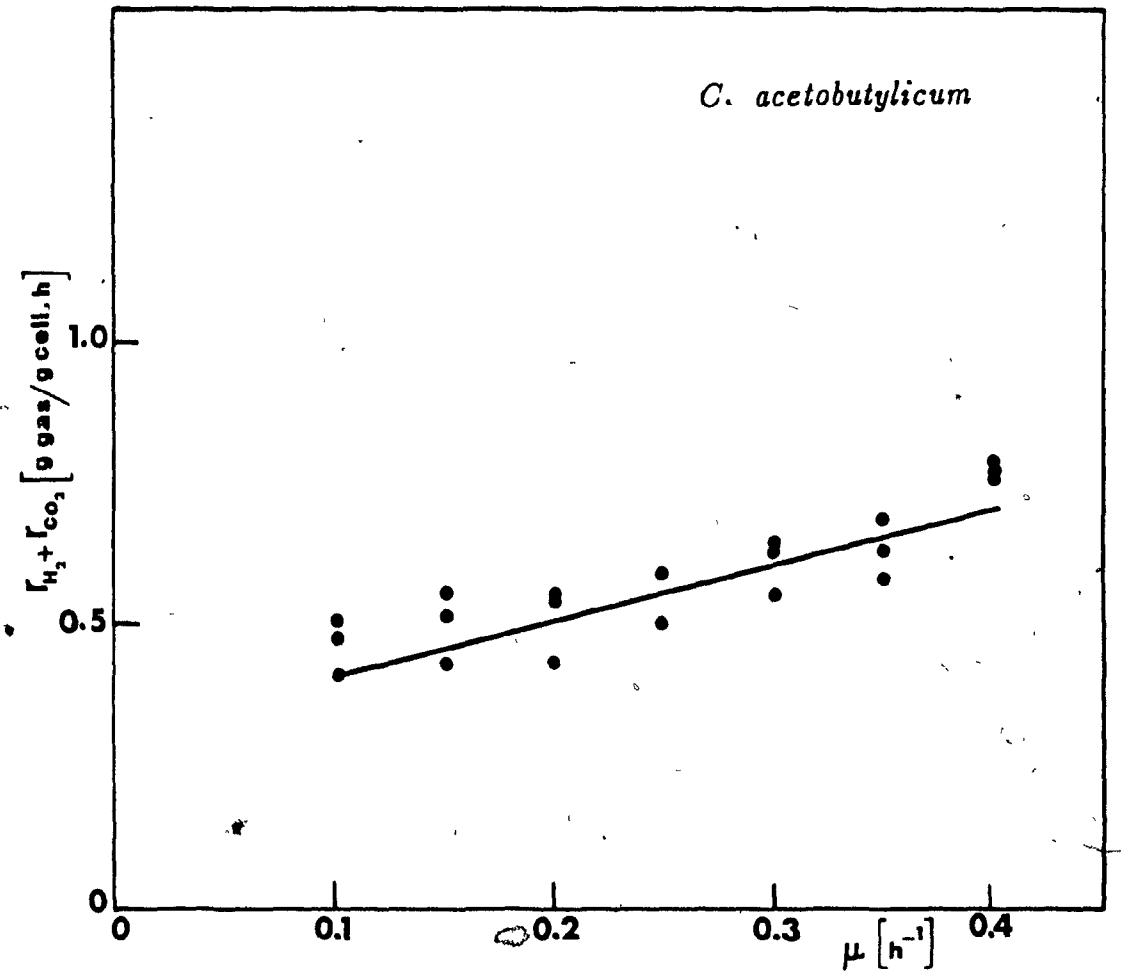
In the present work, evaluation of the process kinetics revealed that the specific consumption rate of the substrate (g glucose/h•g cell) increased with the increase of the culture specific growth rate. This dependence which is graphically shown in Figure 4, implies that the culture becomes more effective in utilizing the substrate when its physiological activity, reflected by its specific growth

FIGURE 4 Dependence of the Specific Consumption Rate of the Substrate on the Specific Growth Rate of the Culture


C. acetobutylicum
r_S [g glucose/g cell. h]
1.5
1.0
0.5
0
0
0.1
0.2
0.3
0.4
μ [h⁻¹]

rate, increases. The specific production rate of the gases also increased with the increase of the specific growth rate (Figure 5). Study of the system performance during the first phase of fermentation (before the acid break point) shows that increase of the impeller rotational speed from 340 rpm to 410 rpm accelerated the microbial activities and resulted in the increased specific growth rate of the culture. The specific rates of substrate consumption and gas production also increased at this level of agitation. The total glucose consumed and the overall gas produced before the break point averaged 36.7 mmole glucose/g cell and 89.5 mmole gas/g cell, respectively, when the impeller rotational speed increased from 190 rpm to 340 rpm. These values changed to 46.7 mmole glucose/ g cell and 129.9 mmole gas/g cell, respectively when the agitation rate was further increased to 410 rpm. It can be concluded that the overall rate of the primary metabolism increased with the increase of the agitation rate. This implies a greater production rate of the intermediate acids and solvents during this fermentation stage. Although the net production of the solvents is very low at this state, intracellular accumulation of these products may occur due to a transient imbalance between the rate of production and the outflux of the organic acids and solvents. Increased levels of particularly butyric acid and butanol will thus result in inhibition of the cellular metabolism. Although these inhibition effects are well known, there have been no studies

FIGURE 5 Dependence of the Specific Production Rate of the Gases on the Specific Growth Rate of the Culture


C. acetobutylicum
$r_{H_2}+r_{CO_2}$ [g gas/g cell.h]
1.0
0.5
0
0
0.1
0.2
0.3
0.4
μ [h^{-1}]

reported on the intracellular accumulation of butanol and butyric acid which are shown to be the major inhibitory metabolites in the acetone-butanol fermentation (Linden and Moreira, 1982; Leung and Wang, 1981). However, there are numerous reports on the accumulation of ethanol in the cell (Navarro and Durand, 1978; Navarro, 1979; Novak et al., 1981) which support the idea of a higher intracellular concentration of ethanol at a higher specific growth rate of the culture. For example, in a study of the inhibitory effect of ethanol in yeast fermentation, Novak et al. (1981) reported that the ratio of the intracellular ethanol to the extracellular ethanol was greater than ten (10) in the early stages of the fermentation and decreased with the progress of the fermentation. The imbalance between the rates of production and the outflux of the products probably result form a lowered permeability of the cellular membrane (Navarro, 1979) perhaps as a result of or as a defense mechanism against high mixing rate and shear stress. Mechanical damage of the cells caused by intensive mixing was also reported by Vrana and Votruba (1982). In a study of the effect of aeration and mixing on the cell cycle of Candida utilis, these authors showed that mechanical strain could be responsible for the prolongation of the cell cycle. Gallili and Mateles (1977) have also reported similar results with catalase production by R. spheroides where vigorous agitation (800 rpm) increased the growth rate of the microbes. However, when the

culture reached the stationary phase, there was a sharp decrease in the concentration of catalase.

The results presented in this communication exhibit the importance of agitation in the acetone-butanol fermentation. It appears that as with many other fermentation processes and microbial cultures, the A-B-E fermentation by C. acetobutylicum is sensitive to the levels of mixing in the liquid broth. The agitation rate should be considered as a controlling factor in the regulation of solvent production, process design or optimization studies of this fermentation process.

4.3.2. Correlation Between the Rates of Solvent Production and Gas Evolution

The metabolic production of solvents and gases by C. acetobutylicum appear to be related. In order to evaluate this relationship, a straight line correlation was developed between the specific production rates of the gases and the solvents. This correlation has the following form:

$$\frac{dA_1}{dt} = \alpha \frac{dA_2}{dt} \qquad (1)$$

where A_1 = mmole gases/g cell

A_2 = mmole solvents/g cell

t = time

Due to the errors encountered in the differentiations of the concentration curves, an integrated form of the above equation was used in this work:

$$\int^t \frac{dA_1}{dt}\, dt = \alpha \int^t \frac{dA_2}{dt}\, dt \qquad (2)$$

A lagrangian interpolation technique was utilized to obtain the values of the solvent concentrations at any given time in the process.

Two different time periods were considered in this evaluation. The first one covered the course of fermentation from the start of the solvent production until the end of the process (Period I). The second time period started after the break point and can be considered as the second phase of fermentation (Period II). Comparison of the correlation coefficients for the straight lines passing through the experimental points showed that there was a linear correlation between the specific molar quantities of the solvents produced and the gases evolved in the system. A better correlation always existed between the two respective parameters after the process passed its break point. The correlation between the gases (H_2 and CO_2) and the solvents (buta-

nol, acetone and ethanol) is graphically presented in Figure 6. Experimental data with the impeller rotational speed of 410 rpm have been used in this figure. The kinetic models representing the gas and solvent production rates are non-linear. In general, it is very difficult to develop a correlation between these parameters during the entire course of fermentation. Division of the course of fermentation into several phases and development of a simple correlation between the parameters during these phases is then a convenient way to analyse and evaluate the relationship between them.

Table 2 presents the molar ratios between the solvents and gases which are the slopes of the lines passing through their respective data points (Equation 2). From the slopes of the lines, it is seen that the ratio of the gases evolved to the solvents synthesized during any considered time period was not constant and varied with the impeller rotational speed. The ratios of the hydrogen gas evolved and the solvents synthesized during the Second Period (Period II) increased with the increase of the impeller Reynolds number up to the value of 3.93×10^4 reaching the maximum values of 2.1 mole H_2/ mole butanol, 3.1 mole H_2/mole acetone and 13.8 mole H_2/ mole ethanol. Further increase in the impeller Reynolds number to 4.79×10^4 decreased the value of this ratio. Considering the entire course of fermentation (Period I), the ratio between the gases evolved (H_2 and CO_2) to the

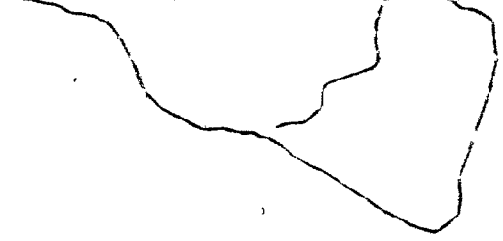


FIGURE 6 Correlation Between the Gases and the Solvents During the Second Phase of Fermentation (rpm = 410)

□- H_2 : Butanol Correlation
●- H_2 : Acetone Correlation
▲- H_2 : Ethanol Correlation
■- CO_2 : Butanol Correlation
○- CO_2 : Acetone Correlation
△- CO_2 : Ethanol Correlation

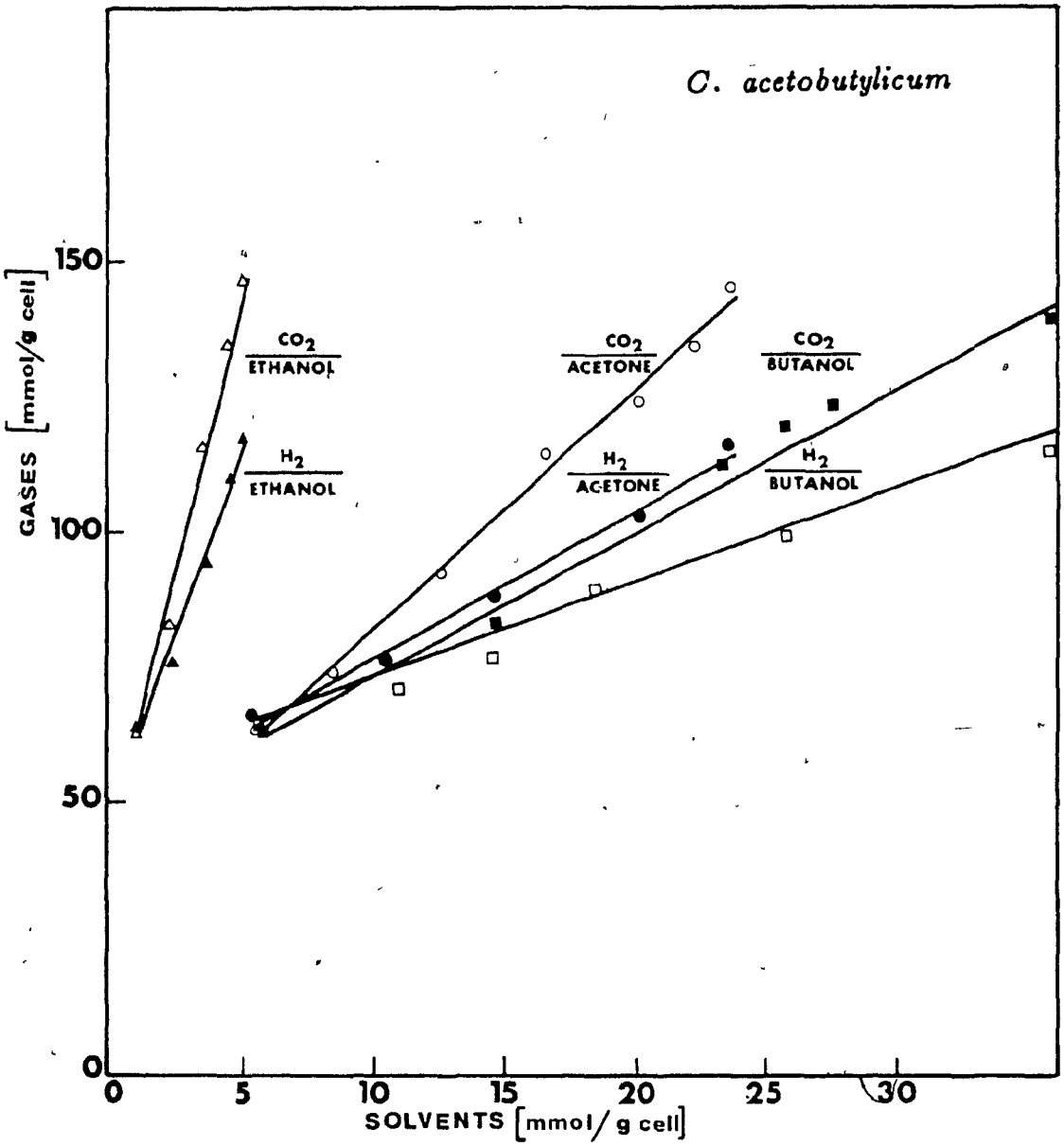

C. acetobutylicum
GASES [mmol/g cell]
150
100
50
0
0
5
10
15
20
25
30
SOLVENTS [mmol/g cell]
CO2 / ETHANOL
H2 / ETHANOL
CO2 / ACETONE
H2 / ACETONE
CO2 / BUTANOL
H2 / BUTANOL

TABLE 2

EFFECT OF THE MIXING RATE ON THE GAS-SOLVENT RATIO

Impeller Reynolds Number / Ratio	2.23×10^4				3.93×10^4				4.79×10^4			
	Period I	Corr. Coef.	Period II	Corr. Coef.	Period I	Corr. Coef.	Period II	Corr. Coef	Period I	Corr. Coef.	Period II	Corr Coef
mole H_2 / mole butanol	1.5	0.98	1.7	0.99	1.6	0.77	2.1	0.99	1.9	0.99	1.9	0.99
mole H_2 / mole acetone	2.3	0.96	2.7	0.99	2.3	0.74	3.1	0.99	2.8	0.99	2.9	0.99
mole H_2 / mole ethanol	10.9	0.98	11.4	0.99	12.2	0.81	13.8	0.99	13.7	0.99	13.5	0.99
mole CO_2 / mole butanol	2.6	0.99	2.7	0.99	2.7	0.96	2.9	0.99	3.0	0.99	2.9	0.99
mole CO_2 / mole acetone	4.2	0.99	4.2	0.98	4.0	0.94	4.5	0.99	4.4	0.99	4.5	0.99
mole CO_2 / mole ethanol	19.2	0.99	18.4	0.99	19.4	0.97	19.9	0.99	21.1	0.99	21.3	0.99

For definition of "Period I" and "Period II" - see the text.
Corr. Coef. - Correlation coefficient of the lines relating the gases and the solvents data points.

solvents synthesized increased with the increase of the impeller Reynolds number to 4.79×10^4 reaching maximum values of 1.9 mole H_2/mole butanol, 2.8 mole H_2/mole acetone, 13.7 mole H_2/mole ethanol, 3.0 mole CO_2/mole butanol, 4.4 mole CO_2/mole acetone and 21.1 mole CO_2/mole ethanol. The values of these ratios are summarized in Table 2. Variations of the gas molar ratios with the impeller Reynolds number (Table 2) represent the responses of the cellular metabolic activity and corresponding mechanisms for regulating the electron transfers to either hydrogen or butanol. When equations (1) and (2) are considered, these gas molar variations mean that although the specific rates of the solvents and gases production are correlated in this process, the proportionality is not constant and depends on the agitation speed. The correlations between the solvents and the gases are particularly important in the anaerobic fermentation of C. acetobutylicum since these compounds are the end-products of the cellular energy metabolism and their formation is necessitated by the need for compensation of the energy and electron balances (Thauer et al., 1977). A balance of energy and of the available electrons on our experimental system (Table 3) showed that almost all of the metabolic energy and the available electrons were preserved within the experimental system. It was also pointed out by Lenz and Moreira (1980) that in the acetone-butanol fermentation less than five percent of the metabolic energy is dissipated as heat and the

TABLE 3

DIVISION OF THE GLUCOSE CARBON, ENERGY AND THE AVAILABLE ELECTRONS AMONG THE FERMENTATION PRODUCTS

	% Total Carbon Recovered in Products				% Total Energy Recovered in Products				% Available Electrons Recovered in Products			
Impeller (rpm)	190	270	340	410	190	270	340	410	190	270	340	410
Ferment. Product												
Butanol	34.7	32.9	33.4	30.0	49.6	47.0	47.8	43.0	52.0	49.5	50.0	45.0
Acetone	15.2	15.1	15.4	15.5	19.4	19.7	19.8	19.7	20.3	20.2	20.7	21.0
Ethanol	2.8	2.6	2.5	2.0	4.1	3.8	3.7	3.0	4.2	3.9	4.0	3.1
Acetic acid	5.1	4.0	3.5	4.9	4.8	3.9	3.3	4.6	5.2	4.0	3.5	4.9
Butyric acid	0.5	0.7	0.6	1.9	0.5	0.8	0.7	2.2	0.6	0.8	0.7	2.4
Hydrogen	-	-	-	-	14.7	a	14.5	15.6	12.0	a	11.8	12.7
Carbon dioxide	33.6	a	32.8	33.0	-	-	-	-	-	-	-	-
Biomass	8.5	7.9	8.5	8.2	10.3	10.0	10.3	10.0	9.0	8.4	9.0	8.7
TOTAL	100.4	-	96.7	95.5	103.4	-	100.1	98.1	103.3	-	99.7	97.8

a - Data not available

rest is recovered in the products of catabolism. This is in contrast with the aerobic fermentations whereby a large amount of the original substrate energy is dissipated as heat and a major fraction of the available electrons are driven out of the metabolic system and transferred to oxygen (Yerushalmi and Volesky, 1981). In aerobic fermentations, particularly when there are no metabolic products except CO_2 and water, there is a correlation between the rates of oxygen uptake and heat generation (Cooney et al., 1969; Luong, 1979). However, in anaerobic fermentations, there is not much heat generation and no oxygen in the system; the accumulating metabolic end-products act as the electron acceptors and the energy pools. Therefore, a correlation between the metabolic product concentrations could be expected. Intriguing appears the possibility of regulating the cellular metabolism leading to the solvent synthesis through controlling the gas production in the system.

4.4 REFERENCES

1. Aiba, S. 1965, Biochemical Engineering, Academic Press, New York 163-216.

2. Brosseau, J.D. 1981. and J.E. Jajic. 1981. Agitation Effects on Hydrogen Gas Production by Citrobacter intermedius, Proc. 2nd World Congr., Chem. Eng. 1, 9, 370-372.

3. Brown, W.E. and W.H. Peterson. 1950. Factors Affecting Production of Penicillin in Semi-Pilot Plant Equipment, Ind. Eng. Chem., 42, 1769-1774.

4. Cooney, C.L. D.I.C. Wang and R.I. Mateles. 1969. Measurement of Heat Evolution and Correlation with oxygen consumption During Microbial Growth, Biotechnol. Bioeng. 11, 269-281.

5. Fortune, W.B., S.L. McCormick, H.W. Rhodehamel, Jr. and J.J. Stefaniak. 1950. Antibiotics Development. Ind. Eng. Chem. 42, 188-190.

6. Gallili, G.E. and R.I. Mateles. 1977. Production of Catalase from Rhodopseudomonas spheroides, J. Appl. Chem. Biotechnol. 29, 110-116.

7. Johnson, M.J. W.H. Peterson and E.B. Fred. 1931. Oxidation and Reduction Relations Between Substrate and Products in the Acetone-Butyl Alcohol Fermentation, J. Biol. Chem., 91, 569-591.

8. Lenz, T.G. and A.R. Moreira. 1980. Economics Evaluation of the Acetone-Butanol Fermentation, Ind. Eng. Chem. Prod. Res. Dev. 19, 478-483.

9. Leung, C.Y. and D.I.C. Wang. 1981. Production of Acetone and Butanol by Clostridium acetobutylicum in Continuous Culture using Free Cells and Immoblized Cells, Proc. 2nd World Congr. Chem. Eng. 1, 348-352.

10. Linden, J.C. and A. Moreira. 1982. In: A. Hollaender, A.I. Laskin and P. Rogers (eds.), Biological Basis for New Developments in Biotechnology, Plenum Press Inc., New York, N.Y., 377-404.

11. Luong, H.T. 1979. Determination of the Heat of Some Aerobic Fermentations, Ph.D. Dissertation, McGill University, 1979.

12. Navarro, J.M. and G. Durand. 1978. Alcoholic Fermentation: Effect of Temperature on Ethanol Accumulation in Yeast Cells, Ann. Microbiol. 129B, 215-224.

13. Navarro, J.M. 1979. Fermentation alcoolique: Influence des conditions de Culture sur L'inhibition par L'ethanol. Cellular and Molecular Biol. 26, 241-246.

14. Novak, M., P. Strehaiano, M. Moreno and G. Goma. 1981. Alcoholic Fermentation: On the Inhibitory Effect of Ethanol, Biotechnol. Bioeng. 23, 201-211.

15. Prescott, S.C. and C.G. Dunn, 1959. Industrial Microbiology 3rd ed. McGraw Hill, N.Y., 250-284.

16. Rivett, R.W., M.J. Johnson and W.H. Peterson. 1950. Laboratory Fermentator for Aerobic Fermentations, Ind. Eng. Chem. 42, 188-190.

17. Thauer, R.K., K. Jungerman and K. Decker. 1977. Energy Conservation in Chemotropic Anaerobic Bacteria, Bacteriol. Rev. 41, 100-180.

18. Vrana, D. and J. Votruba .1982. Effect of Aeration and Mixing in Various Types of Cultivation Devices on the Cell Cycle of Budding Yeasts, , Folia Microbiol. 27, 335-339.

19. Yerushalmi, L. and B. Volesky. 1981. Experimental Bioenergetics of Saccharomyces cerevisiae in Respiration and Fermentation, Biotechnol. Bioeng. 23, 2373-2390.

20. Zetelaki, K. and K. Vas. 1968. The Role of Aeration and Agitation in the Production of Glucose Oxidase in Submerged Culture, Biotechnol. Bioeng. 10, 45-59.

5

EFFECT OF INCREASED HYDROGEN PARTIAL PRESSURE ON THE ACETONE-BUTANOL FERMENTATION BY C. ACETOBUTYLICUM

L. Yerushalmi, B. Volesky and T. Szczesny, J. Appl. Microbiol. Biotechnol., accepted for publication on December 6, 1984.

5.1 INTRODUCTION

The effect of increased hydrogen partial pressure on the growth and metabolism of Clostridium acetobutylicum on glucose was studied in this work.

Regulation of the fermentation pattern and end product formation by H_2 pressure have been frequently shown in ethanologenic anaerobes e.g. Clostridium thermocellum (Su et al., 1981), Clostridium saccharolyticum (Murray and Khan, 1983) and Thermoanaerobium brockii (Lamed and Zeikus, 1980). These studies indicated that increased H_2 concentration in the system inhibited the production of H_2 by the cells and altered the flow of electrons in the biochemical pathway of the microorganism. As a result, the composition of the end products was changed and more ethanol, which is the major reduced end product, was produced in the system.

In the present work the effect of hydrogen on the regulation of the biochemical pathway of C. acetobutylicum was studied in terms of butanol accumulation. Butanol is the major reduced end product in the acetone-butanol (A-B-E)

fermentation process carried out by this bacterium which is an important industrial strain.

5.2 MATERIALS AND METHODS

The high pressure fermentations reported in this work were performed in 300 ml stainless steel "sampling cylinders" Model HS-300 (Hoke, N.J.) with a working broth volume of 60 ml. These cylinders were fitted with pressure gauges for monitoring of the system pressure. The exogenous gas was introduced into the system through sterilized filters and valves. A gyrotory shaker Model G2 (New Brunswick Scientific, N.J.) with modified holding clamps provided shaking of the culture cylinders at 200 rpm throughout the fermentation. Before inoculation, the fermentation broth was sparged with high purity nitrogen gas to ensure anaerobiosis.

Medium C with a glucose concentration of 20 g/l was used in this study. Analysis of solvents and gases and the assay of glucose and biomass are discussed in section 2.2.

5.3 RESULTS AND DISCUSSION

The effect of increased hydrogen partial pressure on the acetone-butanol fermentation was studied by pressurizing the head space of the fermentation vessels at 274 to 1479 KPa absolute using ultra-pure hydrogen gas. One set of experiments was conducted with exogenous hydrogen gas present in the system from the inoculation time (t=0) while in another it was introduced 18 hours after the start of the fermentation (t=18 h). Separate series of experiments served as controls. These experiments used inert helium gas instead of H_2 to pressurize the system under the same culture conditions and over the same pressure range. The results of this study are summarized in Figures 1 and 2. The time of addition of hydrogen gas to the system was not a major factor. Although the yields of butanol and ethanol were relatively constant (0.22 g butanol/g glucose and ~0.01 g ethanol/g glucose) throughout the entire elevated (274 kPa- 1479 kPa) H_2 pressure range examined (Fig. 1), they were respectively higher by an average of 18% and 13% when compared to their corresponding fermentation run controls with no pressure. These increases corresponded to a decrease by almost 40% in the yield of acetone and 30% in the yield of the H_2 produced by the culture. The inert He gas had no major influence on the metabolic system.

Figure 1 Effect of the Exogenous Gas Pressure on the Yields of Solvents and Endogenous H_2

- ● butanol yield with gas pressure from t = 0
- ○ butanol yield with gas pressure from t = 18
- ▲ acetone yield with gas pressure from t = 0
- △ acetone yield with gas pressure from t = 18
- ■ ethanol yield with gas pressure from t = 0
- □ ethanol yield with gas pressure from t = 18
- ⊷ endogenous H_2 yield with gas pressure from t = 0
- ⊸ endogenous H_2 yield with gas pressure from t = 18

only one line could be drawn for the ethanol results

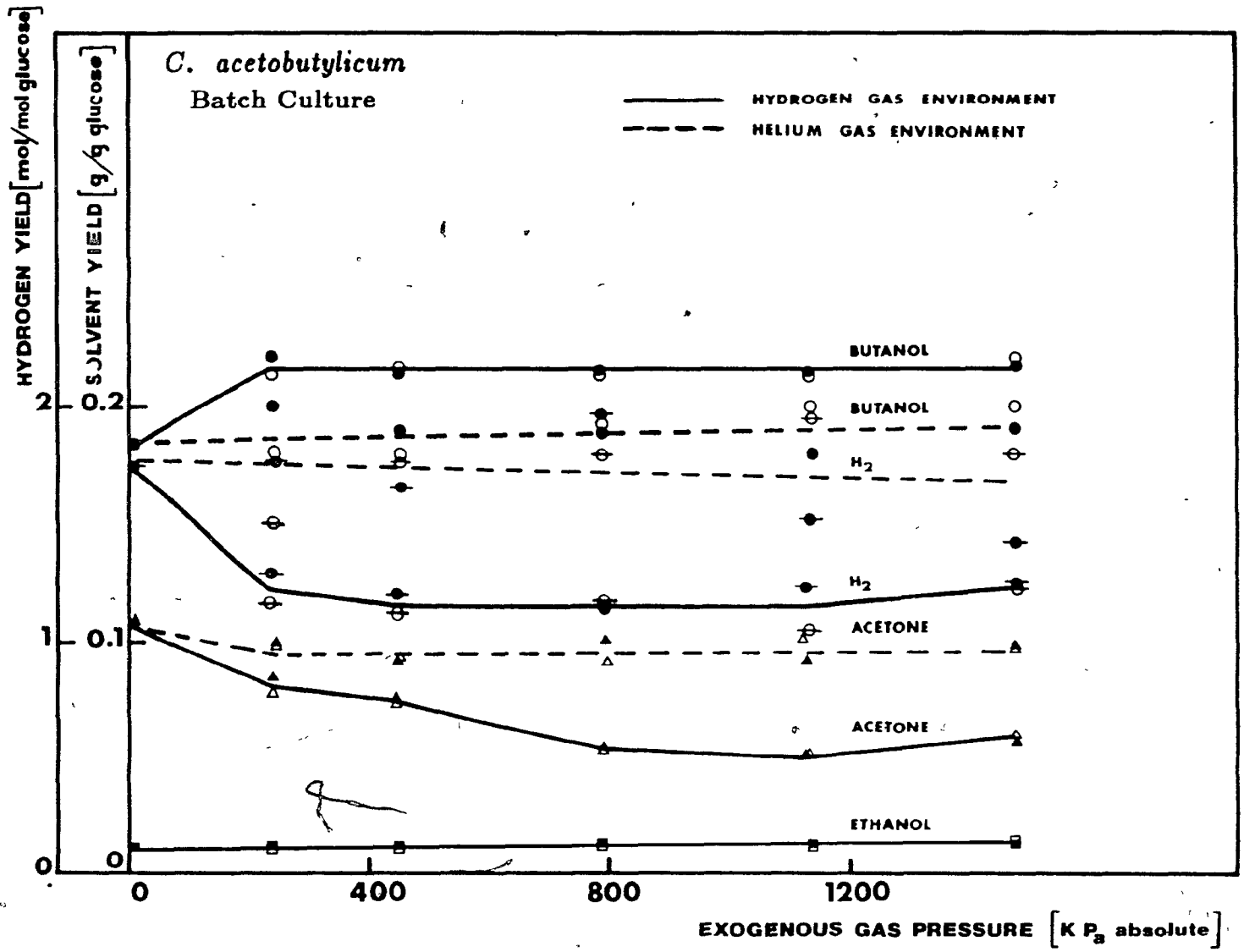
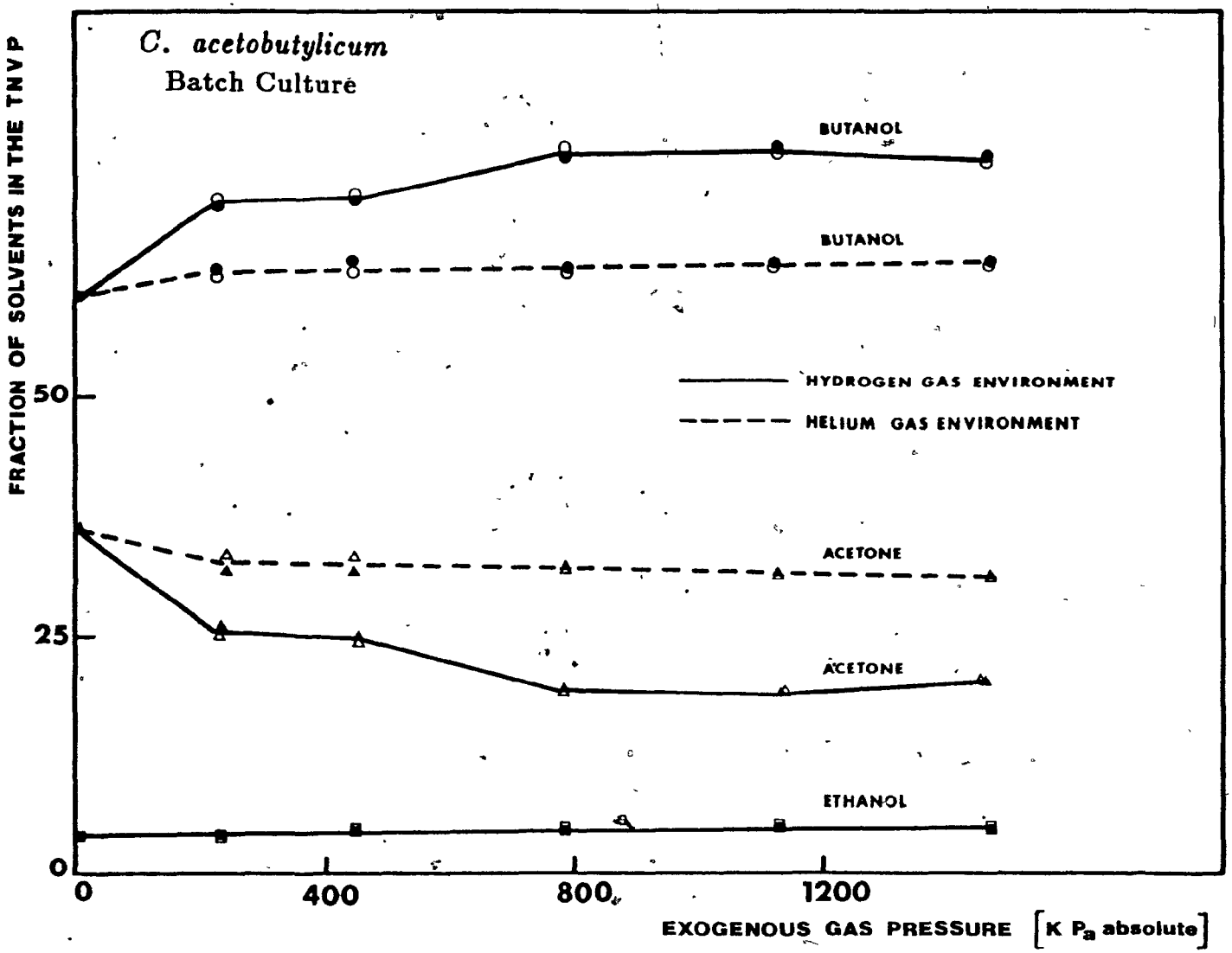

C. acetobutylicum
Batch Culture
HYDROGEN GAS ENVIRONMENT
HELIUM GAS ENVIRONMENT
HYDROGEN YIELD [mol/mol glucose]
SOLVENT YIELD [g/g glucose]
BUTANOL
BUTANOL
H2
H2
ACETONE
ACETONE
ETHANOL
2
1
0
0.2
0.1
0
0
400
800
1200
EXOGENOUS GAS PRESSURE [K Pa absolute]

Figure 2 Fractions of the Solvents in the Total Neutral Volatile Products (TNVP).

only one line could be drawn for the ethanol results.


C. acetobutylicum
Batch Culture
FRACTION OF SOLVENTS IN THE TNVP
50
25
0
BUTANOL
BUTANOL
HYDROGEN GAS ENVIRONMENT
HELIUM GAS ENVIRONMENT
ACETONE
ACETONE
ETHANOL
0
400
800
1200
EXOGENOUS GAS PRESSURE [K Pa absolute]

In spite of a relatively constant yield of butanol on glucose at the elevated H_2 pressure, the proportion of butanol in the total solvents (Fig. 2) increased from an average of 70% to an average of 76.5% with the increase of H_2 gas pressure from 274 to 1135 kPa absolute, and decreased slightly to an average of 75.0% when the H_2 pressure increased to 1479 kPa absolute. Compared with the butanol fraction of 60.5% in a normal fermentation, these values represent a 15.7% to 26.5% increase in the fraction of this solvent. The ethanol portion of the TNVP also increased by 5% to 25% when the H_2 pressure changed from 274 to 1479 kPa absolute. These increases took place at the expense of the acetone fraction in the TNVP which decreased from an average of 25.7% to 18.7% with the change of the H_2 pressure from 274 to 1135 kPa absolute. A slight increase to 20.1% of acetone in the TNVP at the highest test pressure of 1479 kPa absolute was observed. Compared with the normal fermentation control value of 35.5% acetone in TNVP, these results represent a 27.6% to 47.3% decrease in the acetone fraction.

Recovery of glucose carbon and energy in the solvents and in the metabolically produced H_2 are presented in Table 1. The percentage recovery of glucose carbon and energy in butanol increased to averages of 34.8% and 50.1%, respectively. As expected, the recovery of glucose carbon and energy in ethanol also increased while the data for acetone and the endogenous H_2 indicated decreased recovery of

TABLE 1

EFFECT OF H_2 PRESSURE ON THE DISTRIBUTION OF COMPONENTS IN THE A-B-E FERMENTATION

Time of H_2 addition (h)	Initial Pressure (kPa absolute)	% Recovery of glucose carbon in the solvents			% Recovery of glucose energy in the solvents and hydrogen				Butanol / Acetone ratio	Biomass g / g glucose
		Butanol	Acetone	Ethanol	Butanol	Acetone	Ethanol	H_2		
0	274	36.1	13.0	1.8	51.7	16.6	2.6	13.1	2.7	0.09
0	446	34.5	11.7	1.8	49.4	14.9	2.6	12.2	2.8	0.09
0	790	34.9	8.8	1.8	50.0	11.2	2.6	11.7	3.8	0.09
0	1135	34.9	8.1	1.8	49.9	10.3	2.7	12.3	4.1	0.09
0	1479	35.3	9.1	1.8	50.6	11.6	2.7	12.6	3.7	0.09
18	274	34.9	12.0	1.8	49.9	15.7	2.6	11.9	2.8	0.08
18	446	33.3	11.3	1.8	49.8	14.5	2.7	11.2	2.9	0.08
18	790	34.6	8.0	1.8	49.5	10.3	2.7	11.8	4.1	0.09
18	1135	34.1	8.1	1.8	48.9	10.3	2.7	10.6	4.0	0.08
18	1479	35.8	9.2	1.9	51.3	11.8	2.8	12.6	3.7	0.08
0	0	29.8	16.8	1.6	42.7	21.4	2.3	17.7	1.7	0.09

glucose C and energy in these products. As seen from Table 1, although a value of 1.7 was obtained for the butanol to acetone ratio under normal culture conditions, it averaged from 2.7 to 4.0 when the H_2 pressure increased from 274 to 1135 kPa absolute. Subsequently it decreased to 3.7 at a H_2 pressure of 1479 kPa absolute.

The effect of hydrogen on the metabolism and the fermentation pattern of the anaerobic bacteria has been demonstrated previously (Chung, 1976; Lamed and Zeikus, 1980; Su et al. 1981). In the most studied cases ethanol and acetic acid have been the major end products of the fermentation. In a study of the metabolic control of end product formation in Thermoanaerobium brockii (a hydrogen producing anaerobe), Ben-Bassat et al. (1981) showed that glucose fermentation in the presence of exogenous hydrogen (<0.5 atm.) was associated with inhibition of endogenous H_2 production and increased ethanol/acetate product ratio. On the other hand, addition of strong electron acceptors such as acetone, 2-butanone or 2-methylcyclohexanone decreased the amount of the normal reduced end products (i.e. H_2, ethanol and lactate) and increased the metabolic rate and the acetate yield. Similar results are reported by Su et al. (1981) were increased H_2 concentration in the broth either by addition of H_2 to the system or by not stirring the culture inhibited hydrogen and acetic acid formation and increased

the ethanol/acetate ratio in three strains of Clostridium thermocellum. These results indicate that regulation of electron flow in the biochemical pathway of anaerobic bacteria by addition of exogenous electron donors and/or acceptors can alter the composition of the end products. As stated by Murray and Khan (1983), the saccharolytic microbes in pure culture must supply their own electron sink to satisfy the oxidation-reduction balance requirements of the anaerobic fermentation. This is accomplished by increased production of the reduced products, such as ethanol. According to Su et al. (1981) who proposed a mechanism for the H_2 regulation and the control of hexose metabolism to ethanol and acetic acid in C. thermocellum, and Jungermann et al. (1973), under conditions of high H_2 concentration the H^+/H_2 redox potential is lowered and the flow of electrons from reduced ferrodoxin to molecular H_2 via the hydrogenase system is inhibited. Electron flow is then shifted via an oxidoreductase system and more ethanol is produced.

The biochemical pathway and the pattern of electron flow is somewhat different and more complicated in C. acetobutylicum (Doelle, 1975). The major end-products of the glucose metabolism are acetone and butanol which are produced via the intermediate products, acetic and butyric acids respectively. There is also some ethanol produced in the system which accounts for less than five percent of the glucose carbon and energy. In this organism the flow of

electrons from energy sources to end-products is mediated by interconnected oxidoreductases and common electron carriers i.e. ferredoxin and NAD (Thauer et al., 1977; Petitdemange, 1976; Blusson, 1981). According to Blusson et al. (1981) the electron flow in C. acetobutylicum can be regulated by the action of endogenous hydrogenase and the key enzyme NADH - ferredoxin oxidoreductase depending on the culture conditions and the cellular needs. Hydrogen can provide electrons to reduced ferredoxin which will be subsequently reoxidized to form NADH. The reduced power in the form of NADH is required in the synthesis of the other reduced compounds of the cellular metabolic pathway, ethanol and butanol. An inhibition of hydrogen production will shift the flow of electrons to other electron accepting compounds and since butanol is the major electron sink in this process, the effect of H_2 is more pronounced in increasing the production of this compound. Decreased acetone production could be attributed to the inhibition of acetic acid formation which is a metabolic precursor for acetone synthesis in the sugar-metabolizing system.

Although high H_2 concentrations, (up to 1479. kPa absolute) did not inhibit the growth of the culture, and had no major impact on the yield of biomass (Table 1), decrease of the butanol to acetone ratio at the pressures of more than 1135 kPa absolute suggests that the effect of H_2 on the flow of electrons could be concentration dependent. The small

increase of the butanol fraction in the total solvents which was observed with inert pressurized helium environment could have been due to a slower H_2 transfer into the gas phase.

The results presented in this paper show that the metabolic regulation in the Clostridium acetobutylicum fermentation process, like that of many other anaerobes, is affected by the presence of hydrogen gas. The major changes occur at lower than 270 kPa absolute partial pressures of exogenous hydrogen.

5.4 REFERENCES

1. Ben-Bassat A., R. Lamed and J.G. Zeikus. 1981. Ethanol Production by Thermophilic Bacteria: Metabolic Control of End Product Formation in Thermoanaerobium brockii, J. Bacteriol. 146, 92-199.

2. Blusson, H., H. Petitdemange and R. Gay .1981. A New, Fast and Sensitive Assay for NADH-Ferredoxin Oxidoreductase Detection in Clostridia, Anal. Biochem., 110, 176-181.

3. Chung K.T. 1976. Inhibitory Effects of H_2 on Growth of Clostridium cellobioparum, Appl. Environ. Microbiol. 31, 342-348.

4. Doelle, H.W. 1975. Bacterial Metabolism, Academic Press, New York, 574-591.

5. Jungermann K., R.K. Thauer, G. Leimenstoli and K. Decker. 1973. Function of Reduced Pyridine Nucleotide-Ferredoxin Oxidoreductases in Saccharolytic Clostridia, Biochim. Biophys. Acta 305, 268-280.

6. Lamed R. and J.G. Zeikus. 1980. Ethanol Production by Thermophilic Bacteria: Relationship Between Fermentation

Product Yields of and Catabolic Enzyme Activities in Clostridium thermocellum and Thermoanaerobium brockii, J. Bacteriol. 144, 569-578.

7. Murray W.D. and A.W. Khan. 1983. Ethanol Production by a Newly Isolated Anaerobe, Clostridium saccharolyticum: Effects of Culture Medium and Growth Conditions, Can. J. Microbiol 29, 342-347.

8. Petitdemange, H., C. Cherrier, G. Ranal and R. Gay. 1976. Regulation of the NADH and NADPH-Ferredoxin Oxidoreductases in Clostridia of the Butyric Group, Biochim. Biophys. Acta, 421, 334-347.

9. Su T.M., R. Lamed and J.H. Lobos. 1981. Effect of Stirring and H_2 on Ethanol Production by Thermophilic Fermentation, Proc. 2nd World Congr. Chem. Eng. 1, 352-356.

10. Thauer R.K., K. Jungermann and K. Decker. 1977. Energy Conservation in Chemotrophic Anaerobic Bacteria, Bacteriol. Rev. 41, 101-180.

6

DEVELOPMENT OF A MATHEMATICAL MODEL FOR THE BATCH ACETONE-BUTANOL PROCESS

A revised version of this chapter has been submitted for publication as the following paper:

J. Votruba, B. Volesky and L. Yerushalmi, Biotechnol., Bioeng., submitted in January 1985.

6.1 INTRODUCTION

The changes in the physiological activities of C. acetobutylicum were further evaluated by studying the kinetics of the batch acetone-butanol process. The relationships were studied between specific rates and concentrations of the fermentation process parameters. They included the specific growth rate (μ), the specific glucose utilization rate (r_s), specific rates of production of the solvents, acids and gases (r_p) and the corresponding concentrations of these compounds. Evaluation of these relationships was based on graphical plots of these quantities. This study led to expression of suitable mathematical models capable of describing the observed relationships between the key parameters. Mass balances around the bioreactor for the key chemical species were subsequently expressed, using the derived expressions for rates of reacting species. A system of non-linear, first-order ordinary differential equations thus obtained represented a model for the acetone-butanol fermentation which was then used in computer simulation of

the bioreactor performance. The present chapter deals with various aspects of this kinetic study.

6.2 PRELIMINARY DATA ANALYSIS

The results of the four pH-controlled batch fermentations performed at different mixing rates and discussed earlier (Yerushalmi and Volesky, 1985)* were utilized in this study. The fermentation variables used in this study included the concentrations of biomass, sugar, butanol, acetone, ethanol, butyric acid, acetic acid, hydrogen and carbon dioxide. The experimental data were first smoothed by using piecewise polynomial approximating functions also known as the "Spline" functions (Carnahan et al., 1969; de Boor, 1978). This technique is based on passing suitable polynomial functions through various sections of the experimental curve in such a way that there is a continuity throughout the entire range. The criterion for smoothing of the experimental results was that the approximating curve be the closest to the experimental data and the relationship be the smoothest. Equation (1) expresses the relationship between the approximating function $F_i(t)$ and the measured values of the dependent variable y_i :

$$y_i(t) = F_i(t) + \varepsilon_i \qquad (1)$$

Where ε_i is the measuring error. The value of this parameter was in the range of 0.001 - 0.2 of the absolute measurement of individual variables.

The "SMOOTH" complex of programs (deBoor, 1978) was used for smoothing of the experimental values of the dependent variables (y) and for an accurate estimation of the time derivative of these dependent variables ($\frac{dy}{dt}$). Appendix (1) presents a printout of programs used in the smoothing stage and an example of their application. Specific rates of the fermentation variables were also calculated by these programs. The specific rates r_i were expressed by the following equation:

$$r_i = \frac{1}{X} \frac{dy_i}{dt} \qquad (2)$$

where X is the biomass concentration.

6.3 RESULTS AND DISCUSSION

6.3.1 Formulation of the Model

Development of biologically interpretable kinetic relationships between the fermentation variables was based on the information from known metabolic pathways of C. acetobutylicium. This information, which was introduced earlier, established that butanol, acetone, ethanol, biomass, CO_2 and H_2 are the end-products of the metabolism while butyric acid and acetic acid are the intermediate compounds connected with the growth kinetics. Development of the kinetic model reflecting the experimental data was based on the following assumptions:

- There is no process limitation by nitrogen source.
- Functional relationships are valid for the glucose concentration range of 0-50 g/L and the biomass concentration of 0.03 - 10 g/L.
- Product concentrations do not exceed the limits of: butanol 11 g/L, acetone 5 g/L, ethanol 1.5 g/L, organic acids 5 g/L.
- Glucose is the only limiting substrate in batch cultivations.

In the mathematical formulation of growth kinetics, a concept of the "Physiological state" of a microbial culture was used. This concept, discussed by Malek (1976) and

Votruba (1982), made possible a mathematical formulation of growth kinetics during the start up phase (lag phase), which is relatively long in the acetone-butanol process. For a suitable marker of the physiological state the concentration of some intracellular component which significantly changes during the growth can be used (Fredrickson, 1967). Based on the work of Powell (1968) who dealt with the transient states of the culture, the dimensionless concentration of RNA was used in the present work as a marker of the physiological state, as:

$$y = \frac{RNA}{RNA_{min}}$$

where RNA_{min} is the RNA concentration in the cell at $\mu = 0$. This way the specific growth rate of the culture which has been shown to be related to cellular RNA content (Harder and Roels, 1982) can be expressed in the following mathematical way:

$$\mu = a(y - 1) \qquad (3)$$

The numerical values of the coefficient a in Equation (3) have been shown to be constant for most bacterial cultures and equal to 0.56 (Harder and Roels, 1982). Evaluation of the dynamics associated with the marker

of the physiological state was performed by expressing the following dimensionless differential balance:

$$\frac{d(yX)}{dt} = \mu\ (S,B) \cdot y \cdot X \qquad (4)$$

Where the function μ (S,B) is a characteristic of the culture dependent on respective concentrations of the limiting substrate, S, and the inhibitory product, B.

Equation (4) can be mathematically rearranged to assume the following form:

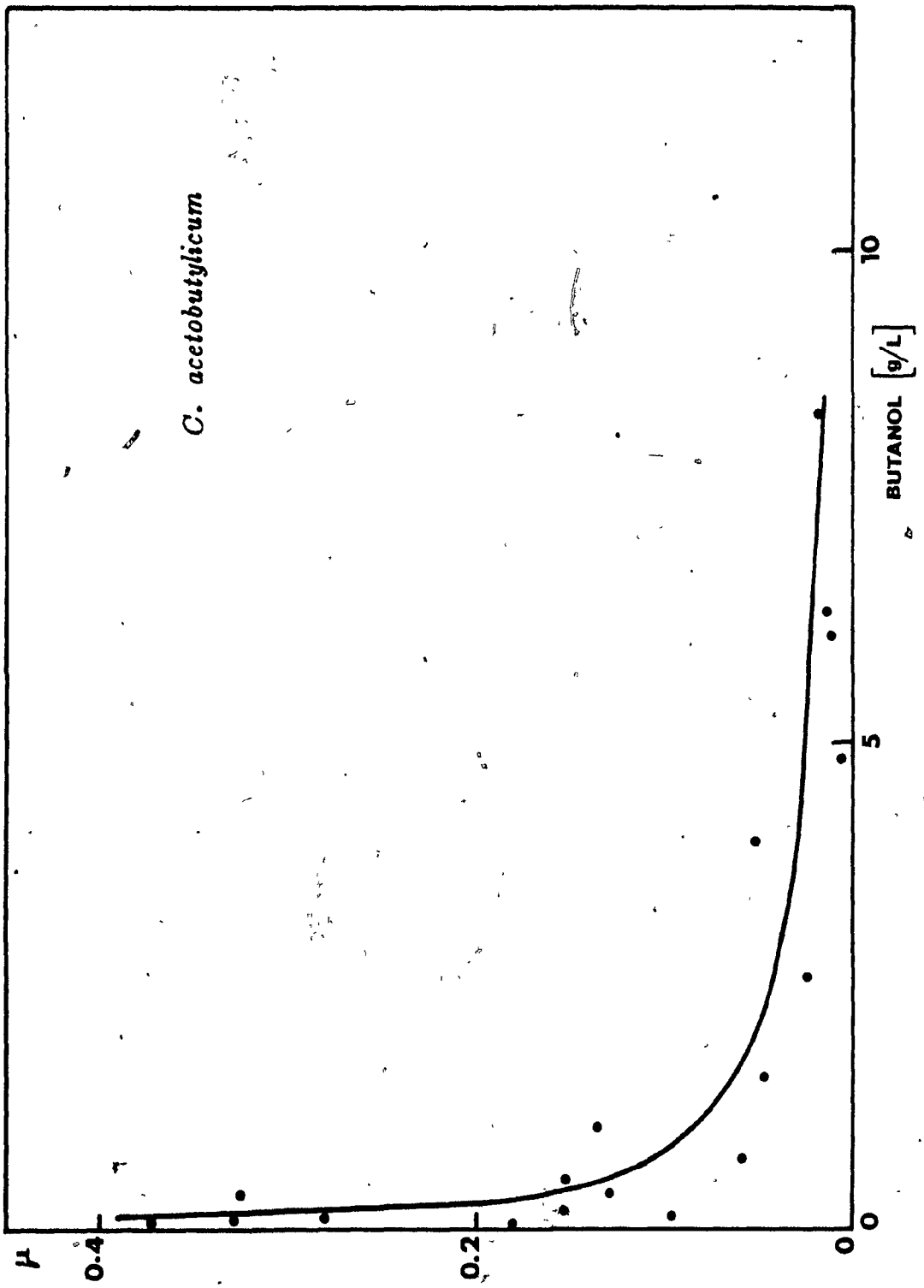
$$\frac{dy}{dt} = \mu\ (S,B)\ y - 0.56\ (y-1)\ y \qquad (5)$$

The initial condition for y is $y(0) = 1$ which characterizes the inoculum in its stationary phase. An inoculum in its exponential phase of growth would be characterized by $y(0) > 1$, while an inoculum in its declining phase when only a small part of biomass is capable of growth would be characterized by $y(0) < 1$.

The function μ (S,B) can be identified from the experimental data. Figure 1 presents the graphical relationship for the function μ (S,B) and the concentration of butanol. A hyperbolic relationship of the form $K_I/(K_I + B)$ can describe the above relationship. A Jerusalimski-Monod

Figure 1

Relationship of the Specific Growth Rate μ (h^{-1}) and Butanol Concentration in the Bioreactor (B) for a Batch Culture of Clostridium acetobutylicum.


C. acetobutylicum
μ
0.4
0.2
0
0
5
10
BUTANOL [g/L]

equation, describing the simultaneous limitation by the substrate and the inhibition by the product, can be used for the mathematical expression of function μ (S,B) with respect to S and B. In the acetone-butanol fermentation it is possible to use a linear relationship with respect to the substrate concentration S, resulting in the following expression for function μ (S,B):

$$\mu\ (S,\ B) = k_1\ S\ \frac{K_I}{K_I + B} \qquad (6)$$

In expressing the differential mass balance for the biomass, the decay process within the cell should be considered. This decay is a result of the cell poisoning due to the increase in the butanol concentration which will result in a loss of viability and cellular activities and eventually in cell lysis. A linear relationship can be used for the description of this phenomenon:

$$\frac{dX}{dt} = 0.56\ (y-1)\ X - k_2\ BX \qquad (7)$$

The use of the marker of the physiological state (y) in Equation (7) enables the use of this model for the initial lag phase while the second term or the right-hand side of this equation describes the final cell decay caused by the product toxicity.

The relationship between the specific substrate utilization rate and the substrate concentration is shown in Figure (2). The following differential equation composed of a linear relationship and a well known Monod-type function (Monod, 1949) is suitable for the mathematical description of this relationship:

$$\frac{dS}{dt} = - \left[k_3 S + k_4 \frac{S}{K_S + S} \right] X \qquad (8)$$

In the metabolic pathway of C. acetobutylicum, there is a mutual interaction between the synthesis of butanol and butyric acid. Therefore, the kinetics of these two products are interrelated. The relationship between the specific rate of butyric acid accumulation (r_{BA}) and the butanol concentration (B) is illustrated in Figure (3).

It can be seen that this profile is similar to that obtained for the specific growth rate (Figure 1) indicating that the growth metabolism and the production of butyric acid are connected. In the expression of the differential balance for the dynamics of butyric acid accumulation, two reaction lines should be considered. In the first one, butyric acid is continuously produced from the limiting substrate with simultaneous inhibition by butanol. The second reaction line is concerning the conversion of butyric acid to butanol. The latter becomes prevalent with increasing concentration of

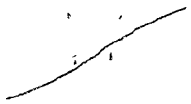
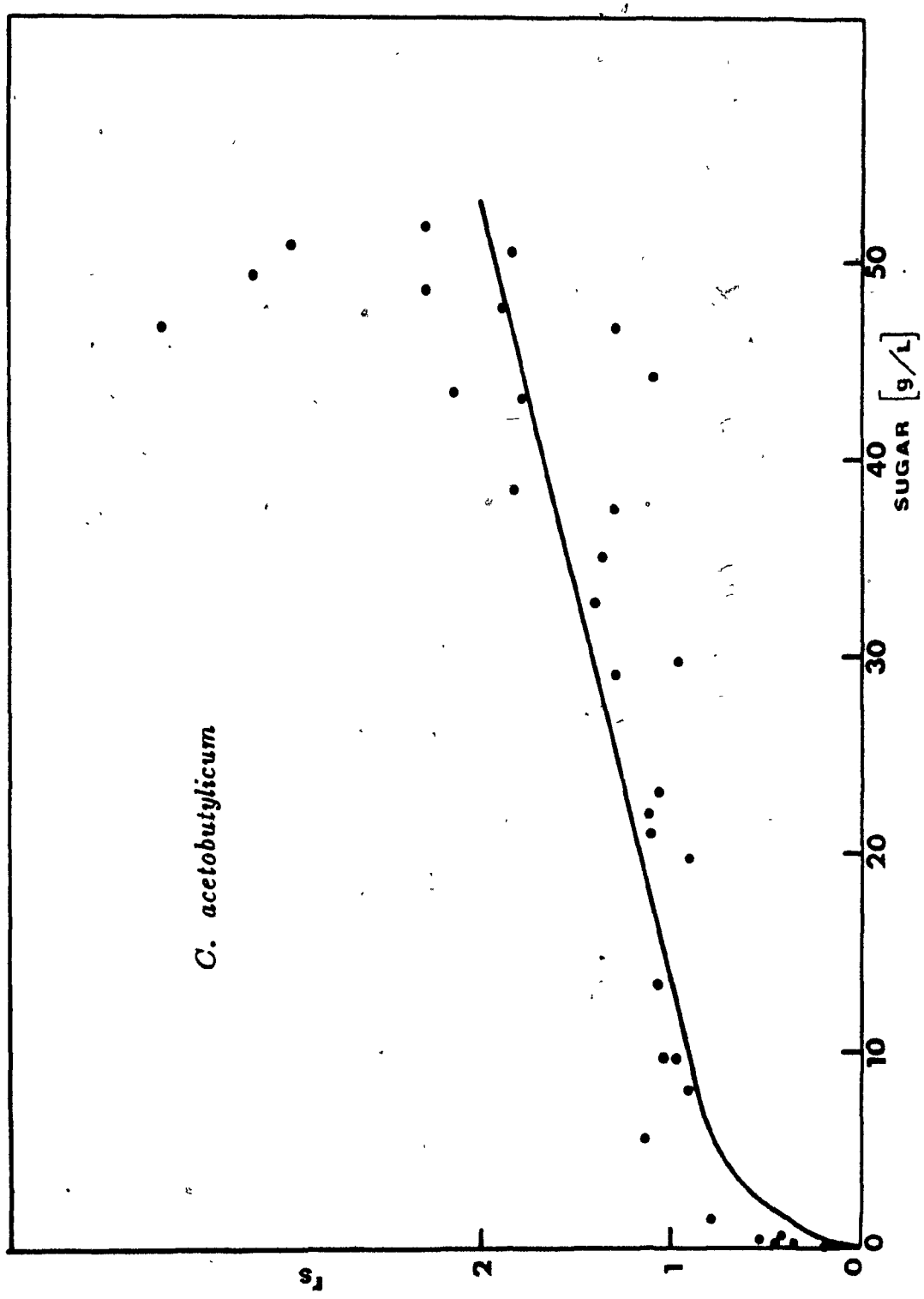


Figure 2

Relationship of the Specific Substrate Utilization Rate r_s [$g_{Glucose}/h \cdot g_{Biomass}$] and the Sugar Concentration (S) for a Batch Culture of *C. acetobutylicum*.


C. acetobutylicum
SUGAR [g/L]
0
10
20
30
40
50
0
1
2
S_1

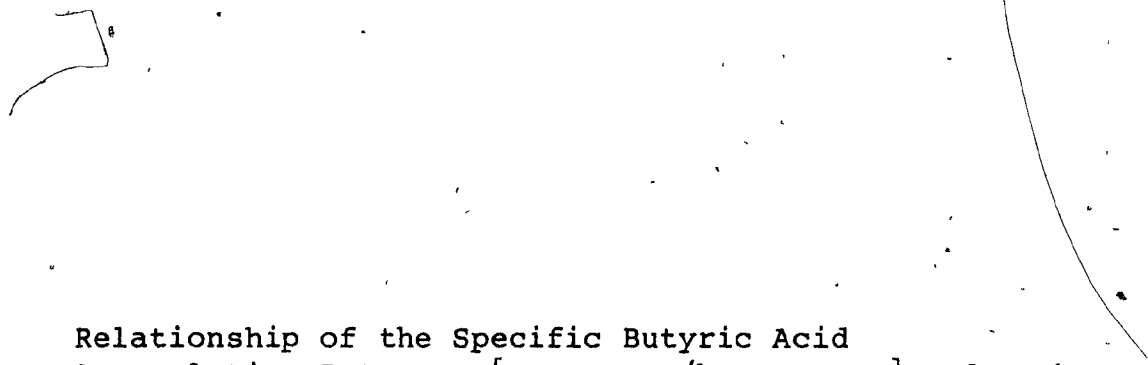
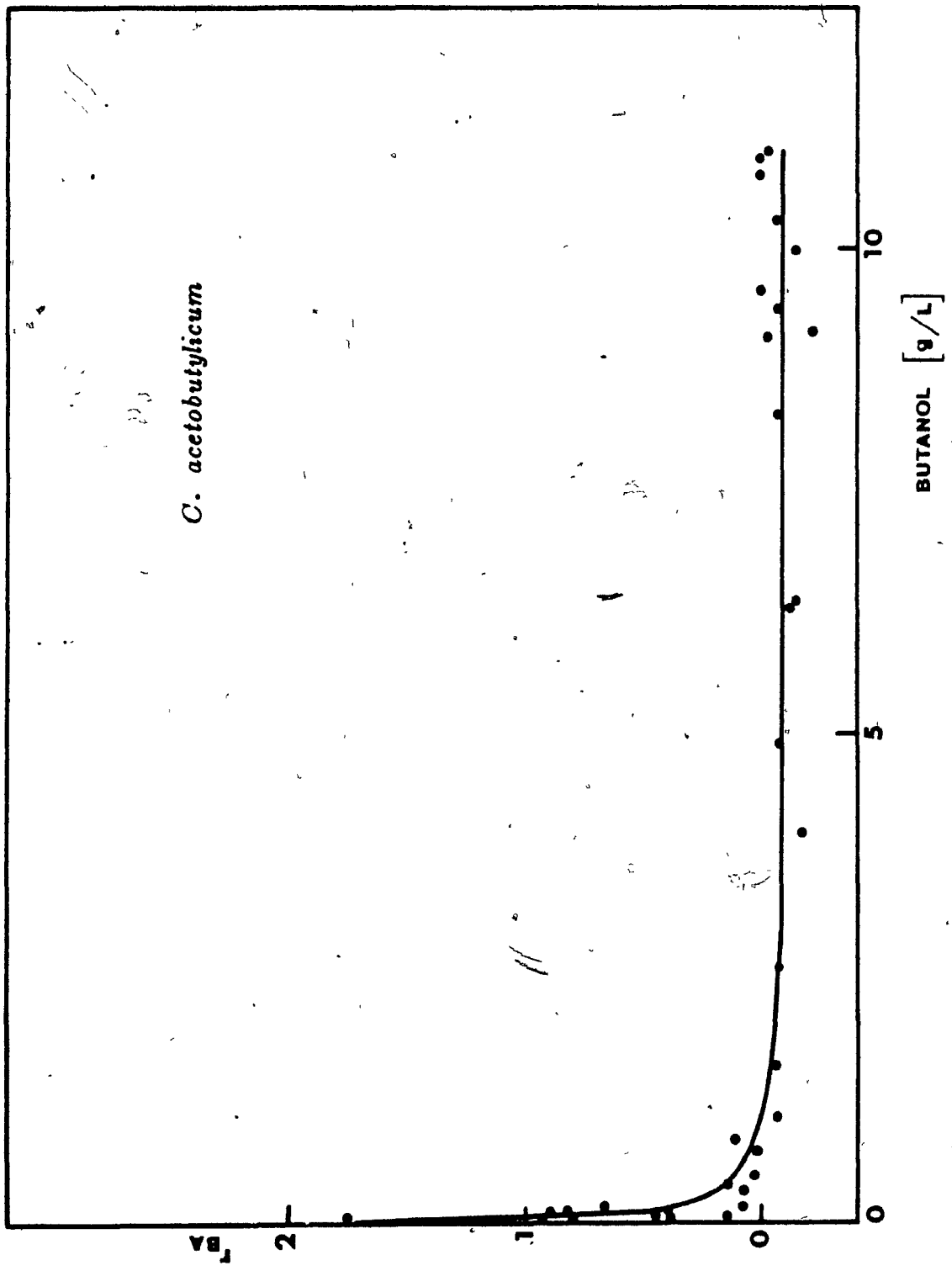


Figure 3

Relationship of the Specific Butyric Acid Accumulation Rate r_{BA} [$g_{But.Acid}/h \cdot g_{Biomass}$] and Butanol Concentration (B) for a Batch Culture of C. acetobutylicum.


C. acetobutylicum
r_{BA}
2
1
0
0
5
10
BUTANOL [g/L]

butanol. The overall expression for the accumulation of butyric acid will thus assume the following form:

$$\frac{dBA}{dt} = (k_5 \; S \frac{K_I}{K_I + B} - k_6 \frac{BA}{K_{BA} + BA}) \; X \qquad (9)$$

where K_{BA} is the reaction saturation constant.

As mentioned earlier, a mutual interaction was considered in the production of butanol and butyric acid since they are synthesized in the same part of the sugar metabolizing system. Correspondingly, a subsystem consisting of butanol and butyric acid was considered for the analysis of butanol production kinetics. When the stoichiometrically converted specific rate of butyric acid production, i.e. $0.841 \; r_{BA}$, is added to the specific butanol production rate r_B, linear relationship is obtained when plotted against the substrate concentration S, as seen from Figure 4. This relationship has the following form:

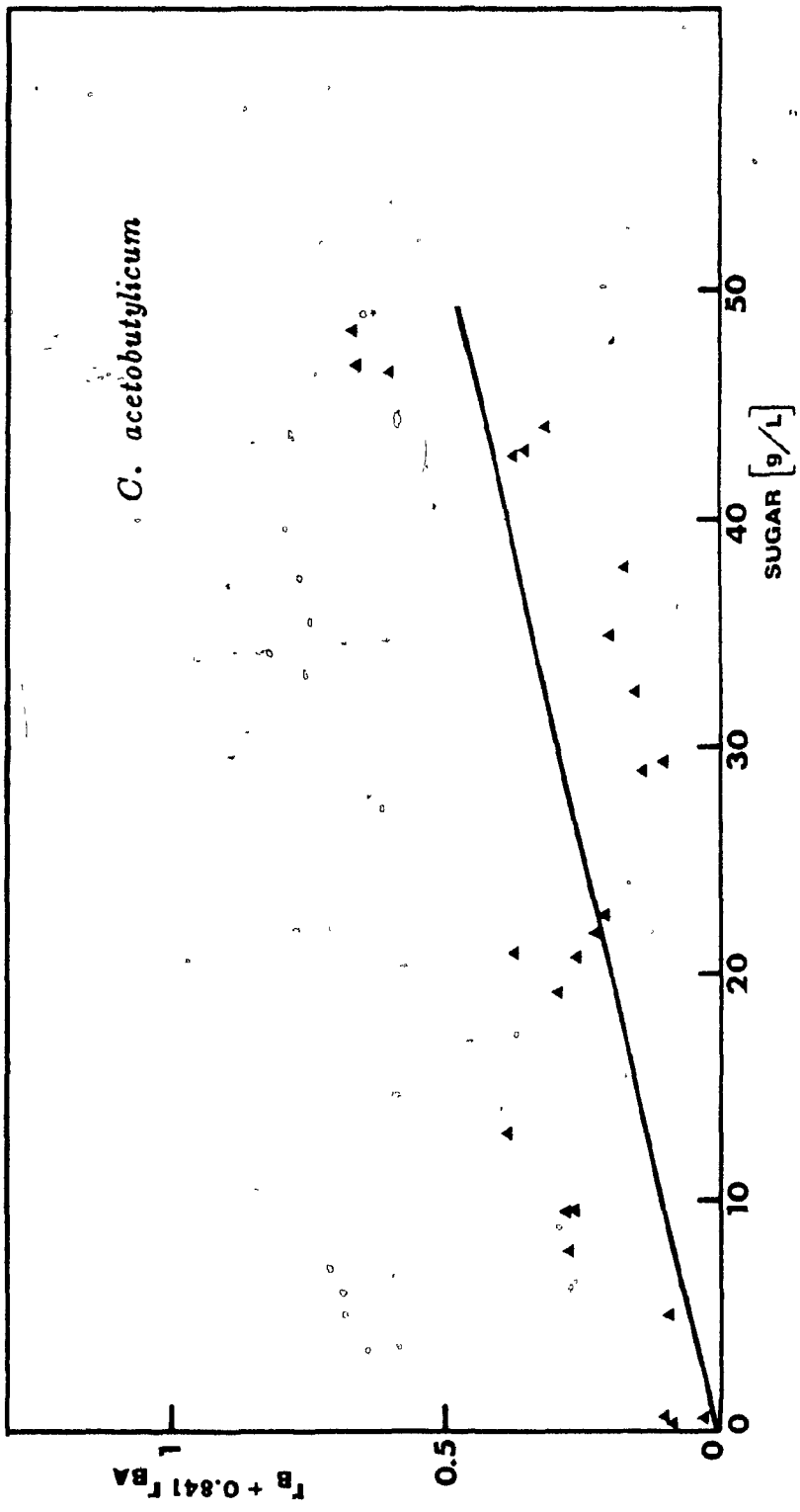
$$k_7 \; S = r_B + 0.841 \; r_{BA} \qquad (10)$$

The dynamics of butanol synthesis is then expressed by the following differential balance:

$$\frac{dB}{dt} = k_7 \; SX - 0.841 \; \frac{dBA}{dt} \qquad (11)$$

Figure 4

Relationship of the Combined Specific Production Rates ($r_B + 0.841\ r_{BA}$) and Sugar Concentration (S) for a Batch Culture of C. acetobutylicum.


C. acetobutylicum
SUGAR [g/L]
0
10
20
30
40
50
0
0.5
1
$r_B + 0.841\, r_{BA}$

Coefficient 0.841 resulted from the stoichiometric conversion considerations as a ratio of molecular weights of butanol and butyric acid.

The differential mass balance for butanol (Equation 11) enables simulation of the initial delay in the butanol production and accumulation caused by the intermediate accumulation of butyric acid.

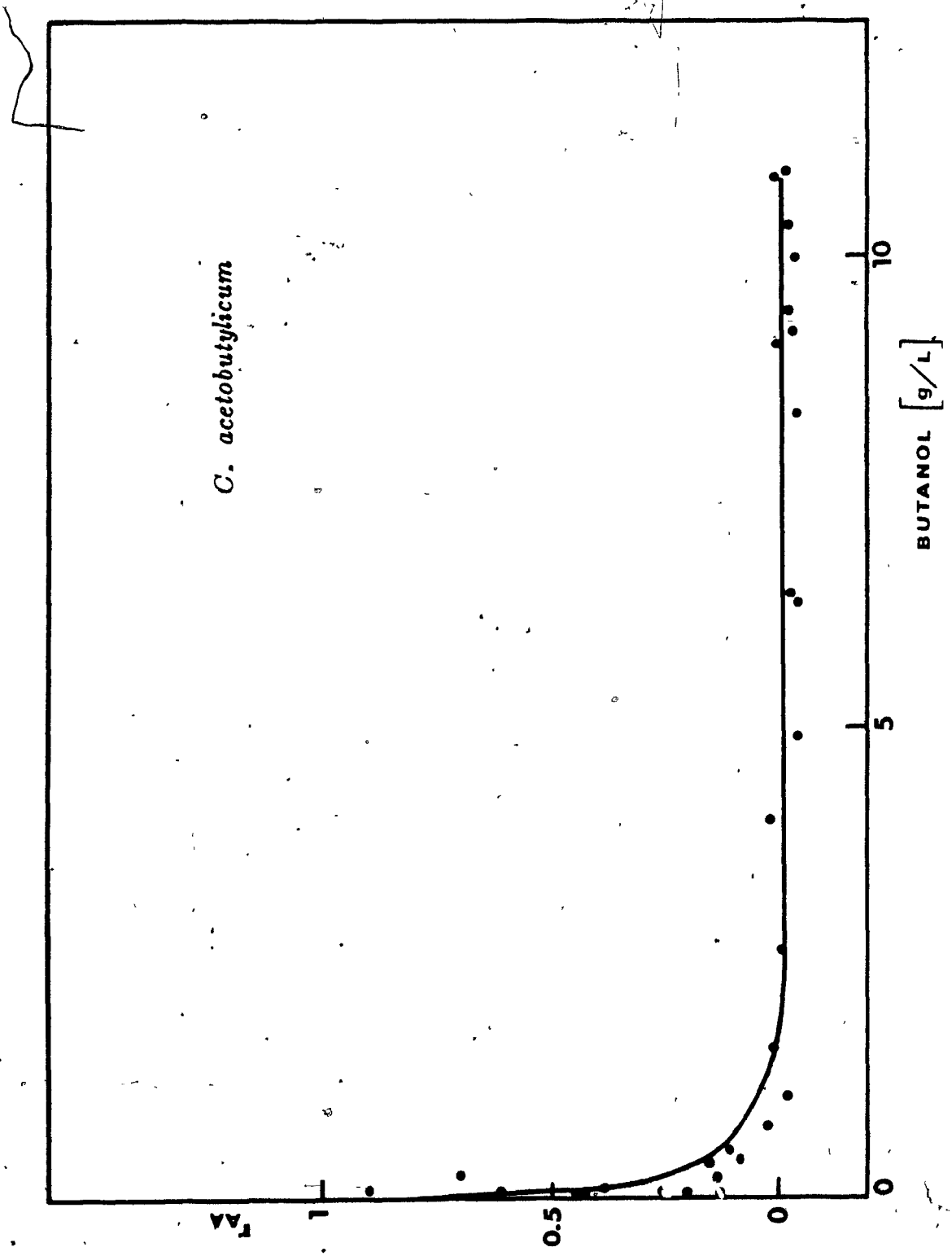
Although acetic acid is not the immediate precussor of acetone in the metabolic pathway of glucose utilization, its production is related to the synthesis of acetone. These two compounds can also be considered to form a sub-system in the acetone-butanol fermentation. The relationship between the specific rate of acetic acid accumulation (r_{AA}) and butanol concentration (B), showing the controlling effect of butanol, is presented in Figure 5. From the model relationship it can be postulated that acetic acid production is connected with the culture growth as the case is with butyric acid. The rate of acetic acid conversion, however, is lower in comparison with that for butryic acid, leading thus to accumulation of this metabolite. The dynamics of acetic acid production can be described by the following differential equation:

$$\frac{dAA}{dt} = X \left(k_8 \frac{S}{K_S+S} \frac{K_I}{K_I+B} - k_9 \frac{AA}{AA + K_{AA}} \frac{S}{K_S + S} \right) \quad (12)$$

where K_{AA} is the saturation constant. The first term on the

Figure 5

Relationship of the Specific Acetic Acid Production rate r_{AA} [$g_{Acet.Acid}/h \cdot g_{Biomass}$] and Butanol Concentration (B) for a Batch Culture of C. acetobutylicum.


C. acetobutylicum
r_{AA}
1
0.5
0
0
5
10
BUTANOL [g/L]

right-hand side of mass balance Equation (12) represents the production of acetic acid from the limiting substrate with simultaneous inhibition by butanol. The second term describes the conversion and disappearance of acetic acid in the biosynthetic pathway.

For the analysis of the dynamics of acetone production based on its dependence on the production of acetic acid, the stoichiometrically converted rate of acetate biosynthesis, i.e. $0.484\ r_{AA}$, was added to the specific rate of acetone production, r_A, and the sum of the two was plotted against the substrate concentration S, as seen in Figure 6. A Monod-type function adequately describes this graphical relationship illustrating utilization of the limiting substrate by the kinetic subsystem of acetone and acetic acid. The following Equation can describe the graphical relationship of Figure 6:

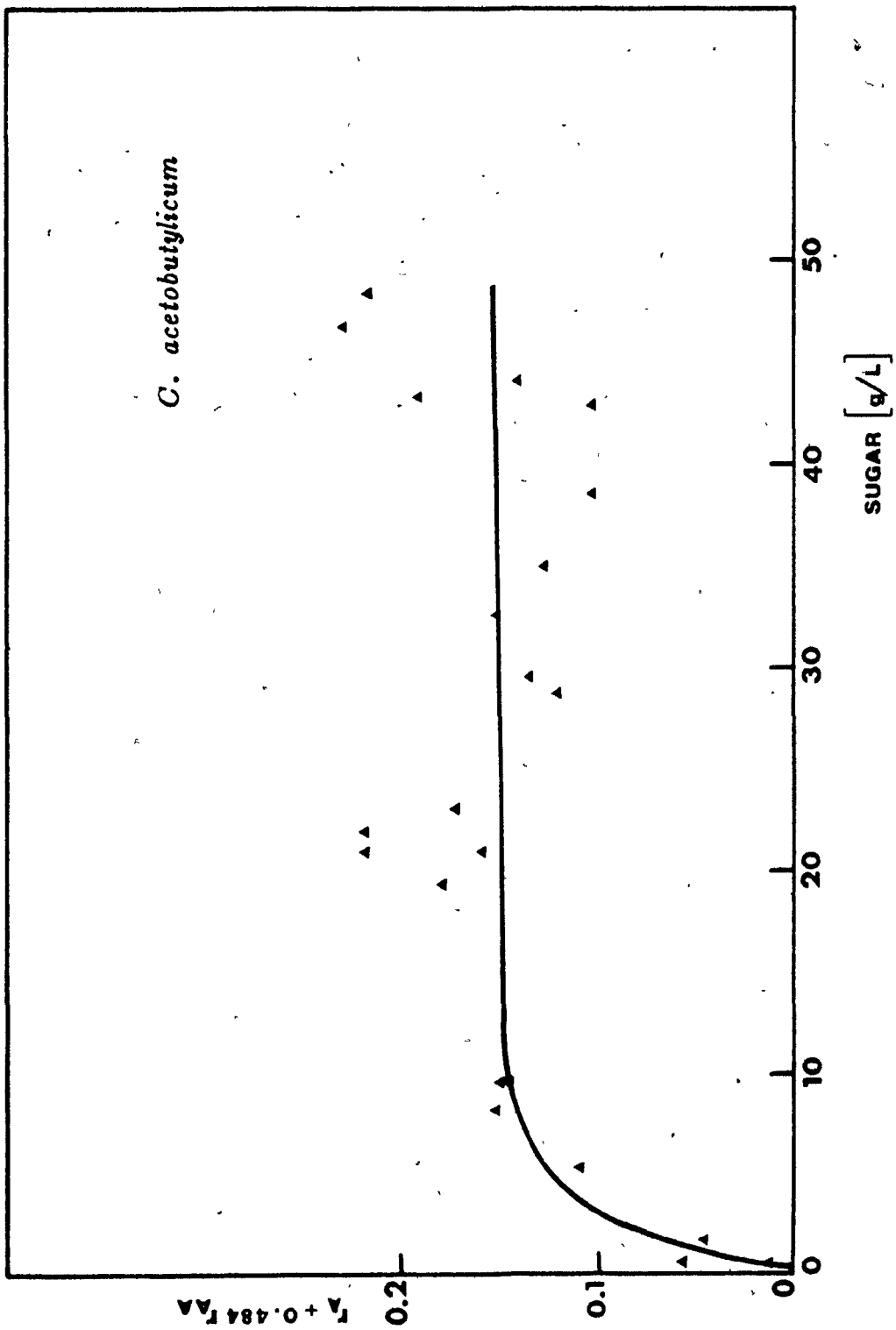
$$k_{10} \frac{S}{K_S + S} = r_A + 0.484\ r_{AA} \qquad (13)$$

A similar function was used also in Equation (12) for the consumption of the limiting substrate. The acetone production rate can thus be expressed by the following equation:

$$\frac{dA}{dt} = k_{10} \frac{S}{K_S + S} X - 0.484 \frac{dAA}{dt} \qquad (14)$$

Figure 6

Relationship of the Combined Specific Production Rates ($r_A + 0.484\ r_{AA}$) and Sugar Concentration (S) for a Batch Culture of C. acetobutylicum.


C. acetobutylicum
$r_A + 0.484\, r_{AA}$
0.2
0.1
0
0
10
20
30
40
50
SUGAR [g/L]

The relationship between the specific production rate of ethanol (r_E) and sugar concentration (S) is presented in Figure 7. A Monod-type function can be used to express the production rate of ethanol as it relates to the sugar concentration:

$$\frac{dE}{dt} = k_{11} \frac{S}{K_S + S} X \qquad (15)$$

Figures 8 and 9 present the kinetics of gaseous product formation. It can be seen that a similar approach can also be taken for modelling the relationship of the rate of evolution of fermentation gases (CO_2 and H_2) and the substrate concentration (S). The following differential relationships describe the dynamics of CO_2 and H_2 evolution, respectively:

$$\frac{dCO_2}{dt} = k_{12} \frac{S}{S + K_S} X \qquad (16)$$

$$\frac{dH_2}{dt} = k_{13} \frac{S}{S + K_S} X + K_{14} SX \qquad (17)$$

Figure 7

Relationship of the Specific Ethanol Production Rate r_E [$g_{Ethanol}/h \cdot g_{Biomass}$] and Sugar Concentration (S) For a Batch Culture of C. acetobutylicum

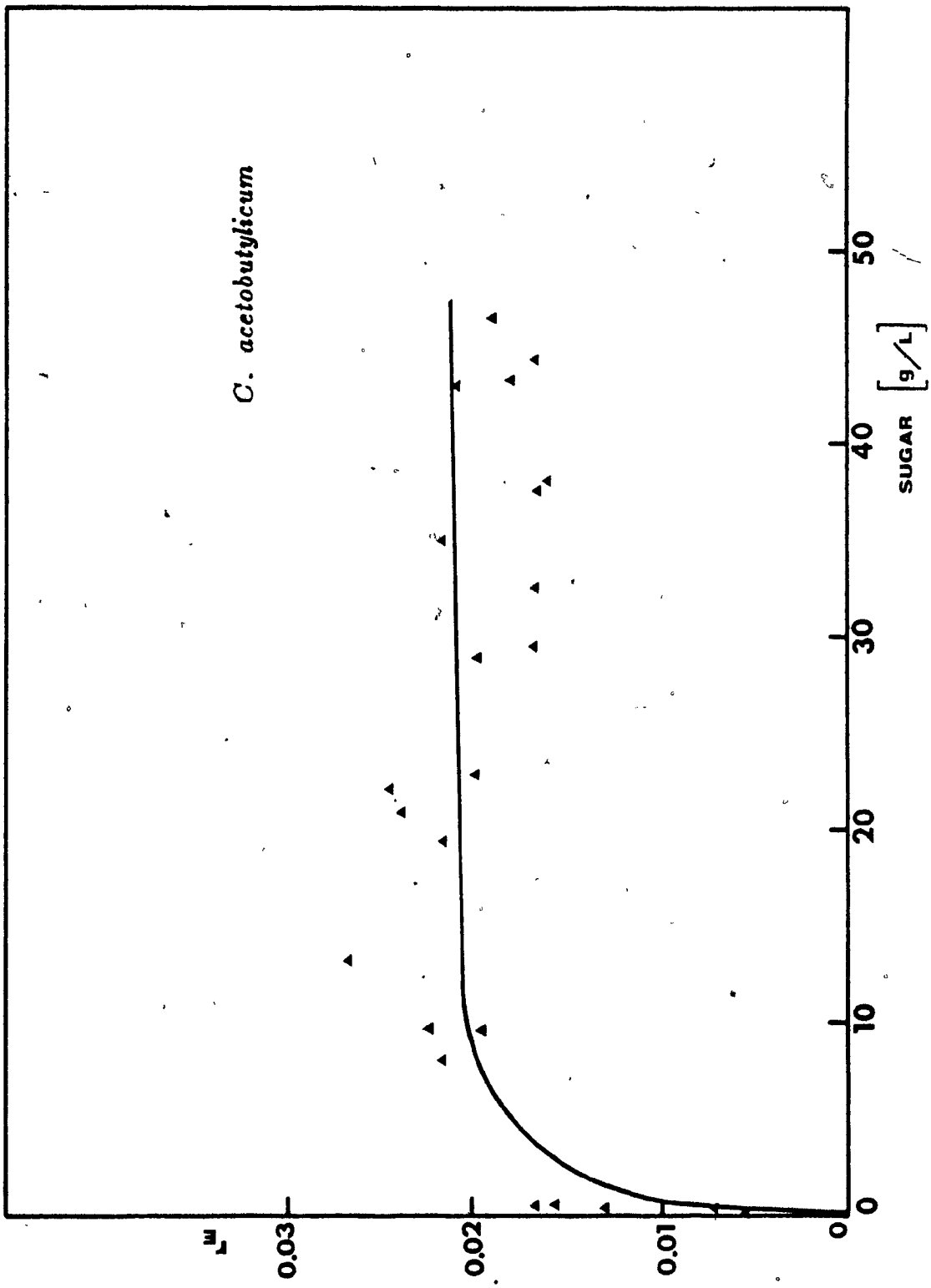

C. acetobutylicum
μ
0,03
0,02
0,01
0
0
10
20
30
40
50
SUGAR [g/L]

Figure 8

Relationship of the Specific Carbon Dioxide Production Rate r_{CO_2} $[g_{Gas}/h \cdot g_{Biomass}]$ and Sugar Concentration (S) for a Batch Culture of *C. acetobutylicum*

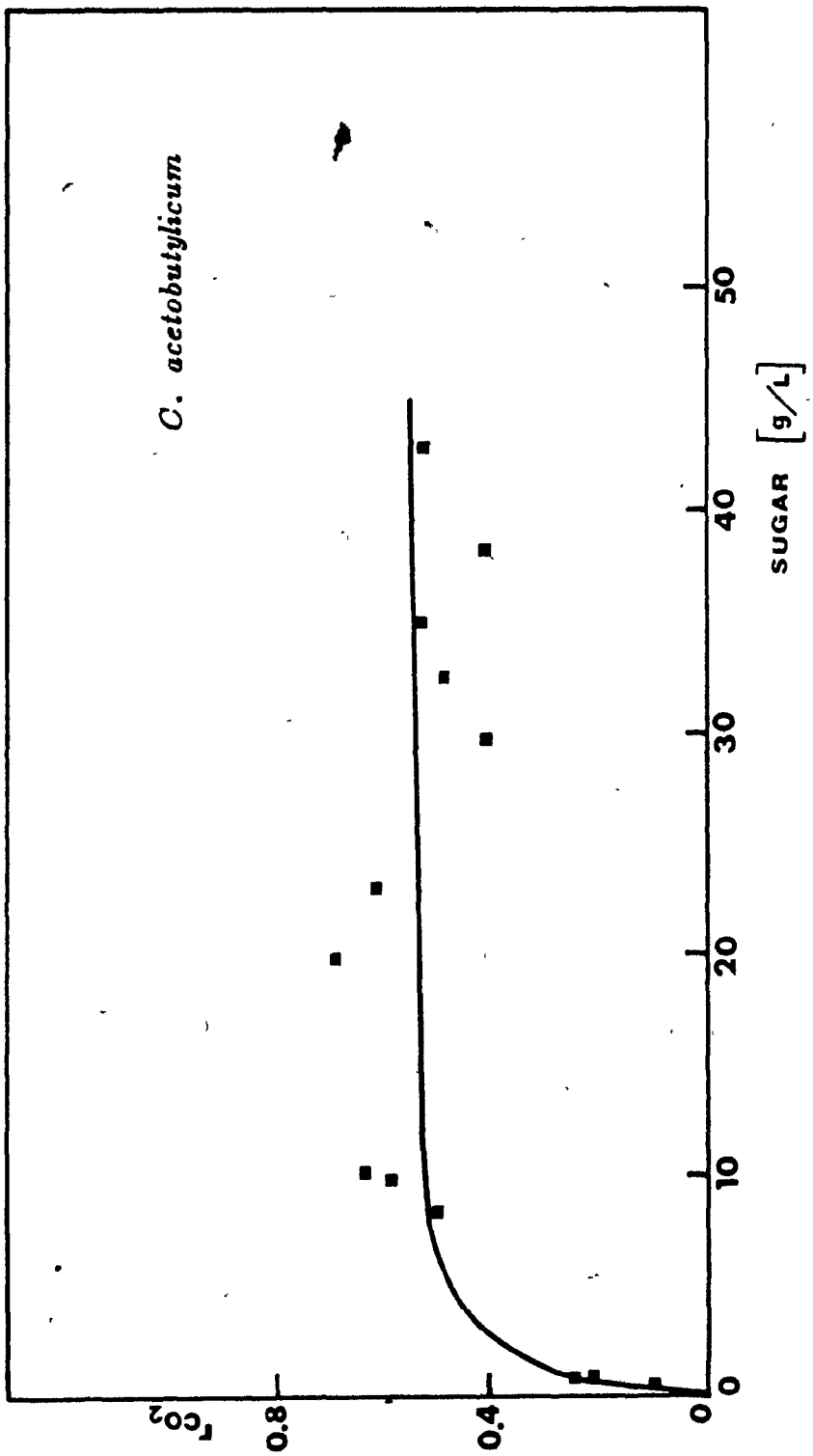
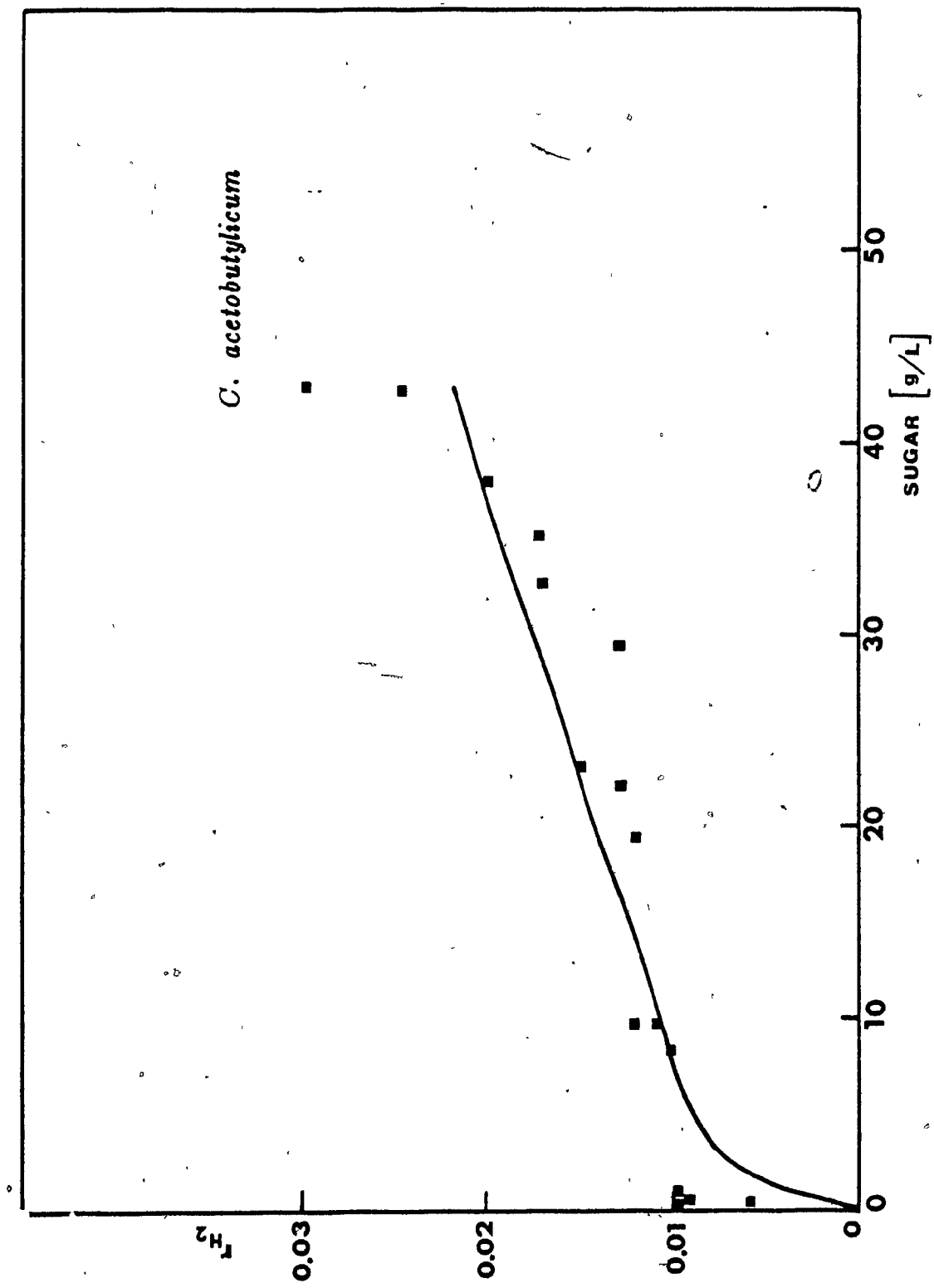

C. acetobutylicum
f_{CO_2}
0.8
0.4
0
0
10
20
30
40
50
SUGAR [g/L]

Figure 9

Relationship of the Specific Hydrogen Gas Production Rate r_{H_2} $[g_{Gas}/h \cdot g_{Biomass}]$ and Sugar Concentration (S) For a Batch Culture of C. acetobutylicum


C. acetobutylicum
r_{H_2}
0.03
0.02
0.01
0
0
10
20
30
40
50
SUGAR [g/L]

6.3.2 Model Parameter Identification and Parametric Sensitivity

The system of ordinary differential equations based on mass balances for the key chemical species in the fermentation process and on the rate analysis of the experimental data represents a kinetic model for the batch acetone-butanol fermentation. These types of models are the most frequently encountered ones in the description of fermentation processes.

While the points in Figures 1 to 9 represent experimental data, the solid lines reflect the mathematical relationships expressed by Equations (6) to (17). The combined treatment of the data from the four different fermentations are responsible for the scatter of the points in these figures. This scatter is particularly remarkable in Figures 4 and 6 which include the specific production rate of the solvents. This is due to the significant effect of the mixing rate on these variables. The mathematical expressions were fitted to the experimental data using a least-square regression. The computer program used provided an estimate for the numerical values of coefficients k_1 to k_{14} used in Equations (6) to (17). This estimate served as an initial approximation of these coefficients in the simulation studies. The use of numerically differentiated data is appropriate only for a semiqualitative evaluation of estimated

kinetic parameters since the error in determining the derivative of a process parameter varying in time can easily exceed 50%, particularly during the initial phase of cultivation. For identification of the kinetic model and for simulation of a given fermentation experiment an accurate estimation of the numerical values of these coefficients is necessary. This was done by minimizing the deviations between the model and the experimental data with regard to the coefficients. An appropriate criterion for the fitting of the model was defined as (Votruba, 1982):

$$SWR = \sum_{i=1}^{m} \sum_{j=1}^{n} W_{ij} r_{ij}^2 \qquad (18)$$

where m is the number of experimental points, n is the number of measured dependent variables, w_{ij} is the weight coefficient used to normalize the magnitude of the measured quantities of the variables and r_{ij} is the difference between the experiment and the model at a given point. For calculation of this difference, the system of ordinary differential equations describing the fermentation system was solved. This was done by an integration program based on the Runge-Kutta method of the fourth order. The Runge-Kutta method was found to be optimal for the simulation of fermentation processes (Votruba, 1982). The optimizing program for the direct search of the minimum of a multivariable, non-linear function

was based on the original method by Rosenbrock (1960). Appendix (2) presents a printout of the programs used in this stage of the modelling.

The numerical values of the model coefficients determined by these programs are presented in Table 1. The degree of significance of the individual model parameters was evaluated by the method of parametric sensitivity analysis. This method is considered as one of the mathematical means for semi-quantitative process optimization because for a given technology it can specify the sequence of importance and the potential impact of individual parameters on the result of the entire process. The absolute parametric sensitivity of the process can be calculated by the following relationship (Votruba, 1982):

$$APS = \frac{\partial f}{\partial k_i} \approx \frac{\Delta f}{\Delta k_i} \qquad (19)$$

where f is the optimized function which, for the acetone-butanol process considered in this study, would be equal to the SWR function introduced earlier (Eq. 18) and k_i is the parameter. The Absolute Parametric Sensitivity characterizes the direction in which the considered parameter is acting. Its positive value leads to increased difference between the model and the data, and vice-versa. For a mutual comparison of the calculated parametric sensitivities and for indication

TABLE 1

VALUES OF KINETIC COEFFICIENTS AND THEIR PARAMETRIC SENSITIVITIES FOR THE MATHEMATICAL MODEL OF THE BATCH ACETONE-BUTANOL-ETHANOL BIOSYNTHESIS

Parameter	Absolute parametric sensitivities	Relative parametric sensitivities
$k_1 = 0.0090$	202827.0	11.5
$k_2 = 0.0008$	2780.9	0.01
$k_3 = 0.0255$	11114.0	1.8
$k_4 = 0.6764$	277.5	1.9
$k_5 = 0.0136$	129034.0	11.1
$k_6 = 0.1170$	-943.8	0.7
$k_7 = 0.0113$	-99973.1	7.1
$k_8 = 0.7150$	30.3	0.1
$k_9 = 0.1350$	-63.2	0.05
$k_{10} = 0.1558$	-32.3	0.03
$k_{11} = 0.0258$	32.2	0.005
$k_{12} = 0.6139$	33.0	0.1
$k_{13} = 0.0185$	7.9	0.0009
$k_{14} = 0.00013$	140.8	0.0001
$K_I = 0.833$	272.4	1.4
$K_S = 2.0$	0.036	0.0004
$K_{BA} = 0.5$	102.9	0.3
$K_{AA} = 0.5$	3.3	0.01

of the significance sequence of individual parameters, the relative parametric sensitivity was used as defined by the following relationship (Votruba, 1982):

$$\text{Relative Parametric sensitivity} = \left| \frac{k_i \partial f}{f \partial k_i} \right| \approx \left| \frac{k_i \Delta f}{f \Delta k_i} \right| \quad (20)$$

A printout of the computer program used in the calculation of the parametric sensitivity of individual kinetic coefficients is presented in Appendix (3). The calculated values of the Absolute Parametric Sensitivity and the Relative Parametric Sensitivity of the model coefficients are also presented in Table (1). It can be seen that coefficients k_1, k_5 and k_7, characterizing the kinetics of biomass growth, butanol and butyric acid production, exhibit the highest sensitivity and can be considered as the most important parameters in the process. This means that in the optimization procedures or for on-line process control, attention should be focused on accurate identification of these parameters.

6.3.3 Process Simulation

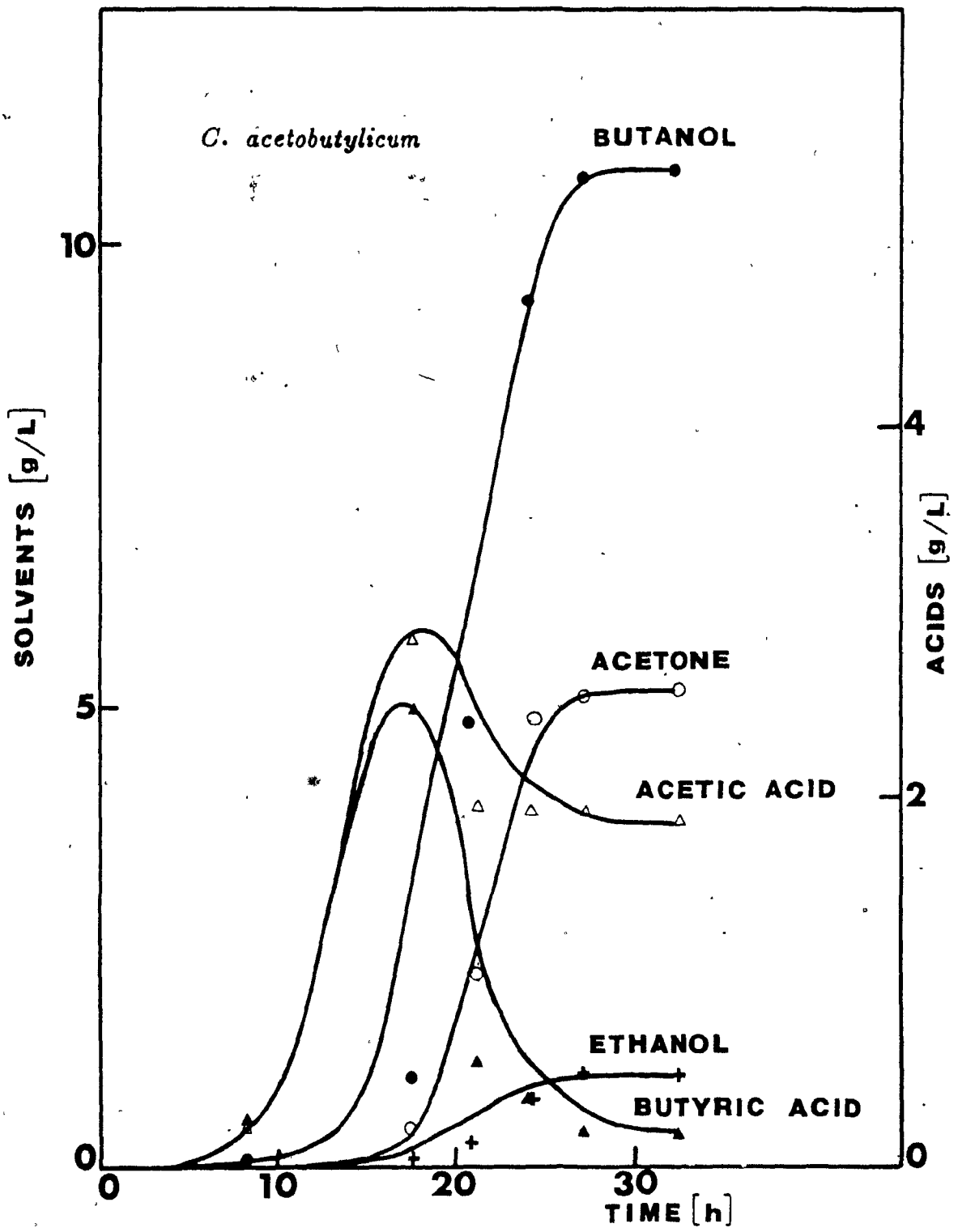
In addition to the estimation of numerical values of model coefficients, the mathematical model identification

process includes determination of validity of the model for a given set of experimental data. This was performed by computer simulation of the fermentation performed at 340 rpm presented earlier (Yerushalmi and Volesky, 1985)* as an example of the batch A-B-E process using programs presented in Appendix (4). Figures 10a, b, c presents the result of this computer simulation for the nine fermentation variables representing the production of solvents, acids, gases, biomass and the consumption of glucose. Although the agreement between the experimental and the simulated results was indicated by visual observations, a statistical F-test was performed to examine this agreement as suggested by Himmelblau (1968). This test is executed by forming the variance ratio S_r^2/S_e^2 where S_r is a measure of deviations about the regression function (the difference between the model and the experimental data) and S_e is a measure of dispersion caused by experimental error. The F-test evaluates the above ratio by comparison with a given numerical value and determines whether the agreement between the experimental and the simulated results is within a required confidence limit. In this study an F-Test for a level of significance $\alpha = 0.05$ was performed on all the nine fermentation variables considered. The results of this test presented in Table 2 indicate an agreement between the experimental and the simulated results for all the nine process variables. This reflects the val-

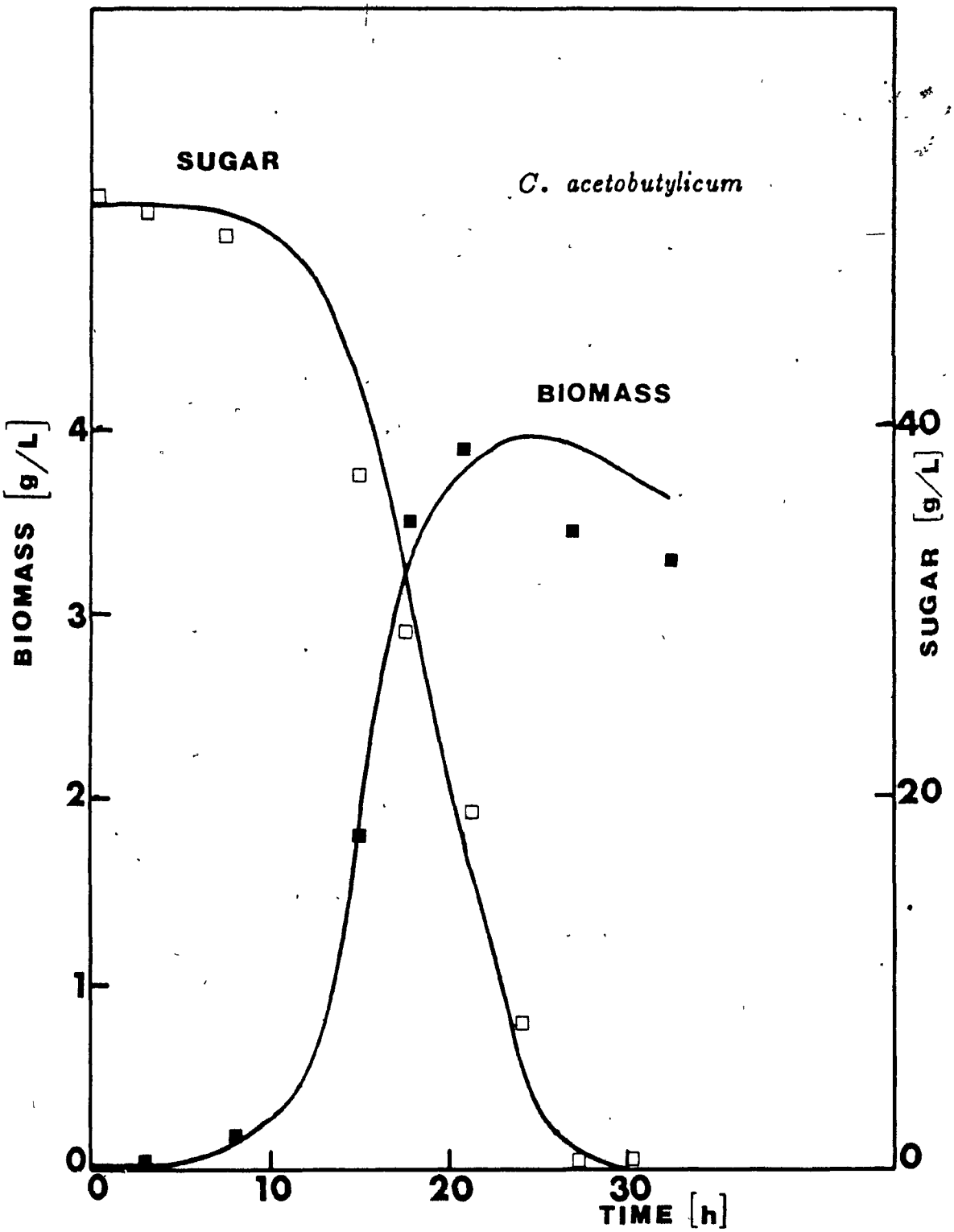
Figure 10 (a,b,c)

Experimental Data and Model Predictions (Full Line) for the main process parameters in the Batch Culture of C. acetobutylicum.

a)


C. acetobutylicum
BUTANOL
ACETONE
ACETIC ACID
ETHANOL
BUTYRIC ACID
SOLVENTS [g/L]
ACIDS [g/L]
TIME [h]
0
5
10
0
2
4
0
10
20
30

b)


SUGAR
C. acetobutylicum
BIOMASS
BIOMASS [g/L]
SUGAR [g/L]
TIME [h]
0
1
2
3
4
0
20
40
0
10
20
30

c)

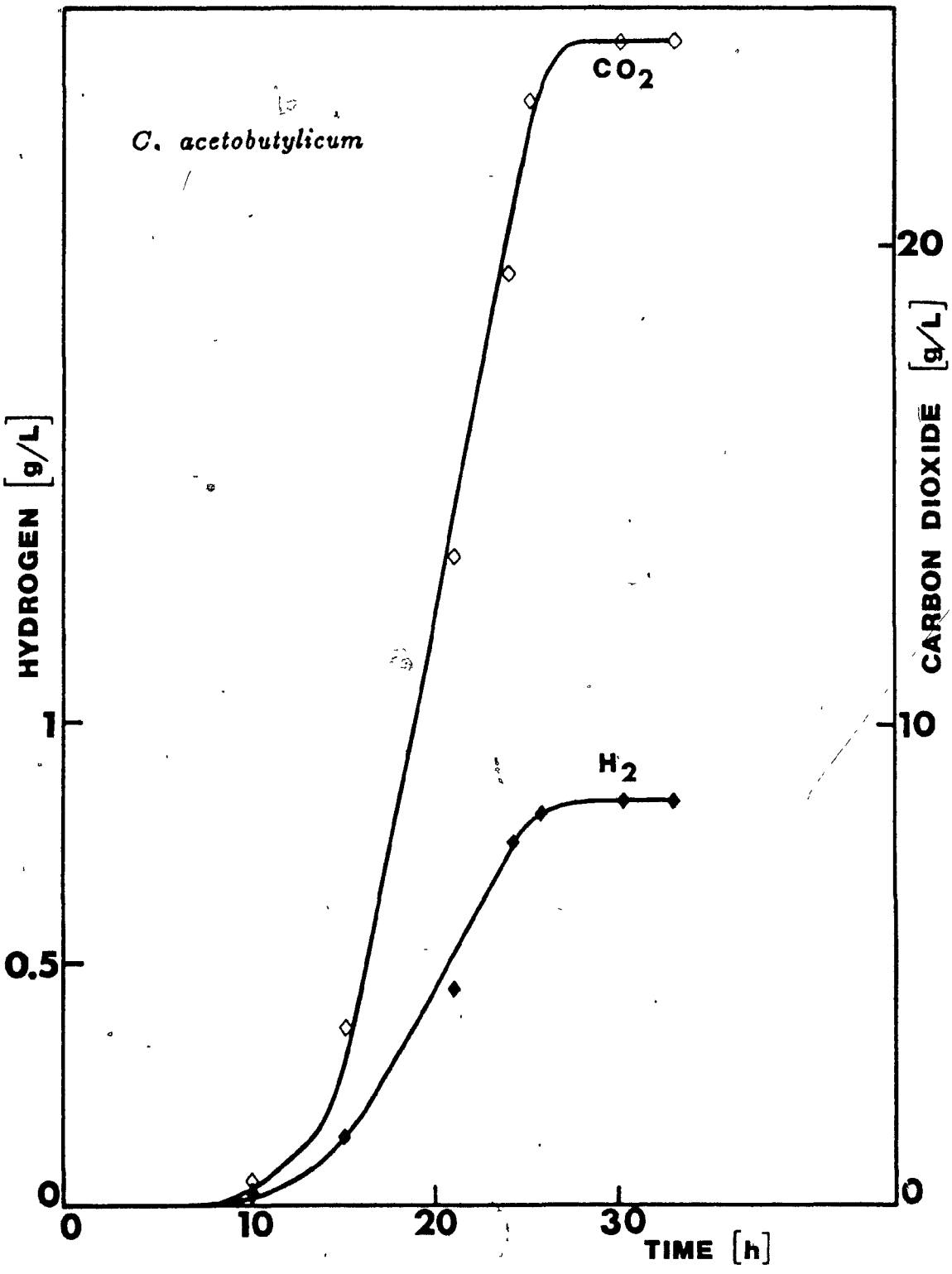

C. acetobutylicum
CO_2
H_2
HYDROGEN [g/L]
CARBON DIOXIDE [g/L]
TIME [h]
0
0.5
1
10
20
30

TABLE 2

THE RESULTS OF THE "F-TEST" WITH THE LEVEL OF THE SIGNIFICANCE (α) OF 0.05 FOR THE AGREEMENT BETWEEN THE EXPERIMENTAL AND THE SIMULATED RESULTS

	s_r^2	s_e^2	s_r^2/s_e^2	
Biomass	0.164	0.077	2.18 < 3.0717 →	agreement between the experimental and the simulated results
Sugar	9.17	5.14	1.78 < 3.0717 →	"
Butanol	1.85	0.65	2.84 < 3.0717 →	"
Acetone	0.137	0.086	1.59 < 3.0717 →	"
Ethanol	0.035	0.012	2.91 < 3.0717 →	"
Butyric acid	0.23	0.102	2.25 < 3.0717 →	"
Acetic Acid	0.114	0.045	2.53 < 3.0717 →	"
Hydrogen	0.0025	0.0018	1.390 < 3.0717 →	"
Carbon Dioxide	1.24	1.39	0.89 < 3.0717 →	"

S_r = Measure of deviations around the regression function.

S_e = Measure of dispersion caused by experimental error.

idity of the kinetic models and an accurate estimation of coefficients in the A-B-E fermentation process model. The slight disagreement observed between the model and the experimental data for butanol at the time interval of 15-21 hours is probably due to the following reasons:

1. The extreme sensitivity of the model to the butanol coefficient (k_7) which made it almost impossible to vary without compromising the quality of the fitness of other models.
2. The possibility of existence of other intermediate products besides those considered in this modelling exercise.

Fermentation models are usually developed considering only the major products of the fermentation. However, it is possible that during the course of fermentation other products were also formed in negligible amounts which were not accounted for.

In spite of the disagreement between the butanol model and the corresponding experimental data at the time interval of 15 hour-21 hour, the simulated butanol results exhibit an overall agreement because of the very good fit at other time intervals which compensates for some partial disagreement.

The cellular autolytic activities at the final stages of the acetone-butanol fermentation results in the loss of viability and cell lysis (Van der Westhuizen et al.,

1982; Webster et al., 1981). This phenomenon is further enhanced by butanol (Westhuizen et al., 1982). Although the decay of cell viability due to butanol inhibition is considered in the model (Equation 7), the complicated lysis phenomenon involved is not fully described and accounts for the disagreement between the biomass experimental and simulated results at the final stages of the fermentation process.

The mathematical kinetic model of the batch acetone-butanol fermentation presented in this work was found to adequately represent the process, and the parametic sensitivity analysis carried out indicated the most important parameters. The model reflects the biochemical and the physiological observations of the culture and was used in further stages of this work.

6.4 NOMENCLATURE

Symbol	Meaning
A	Acetone concentration
AA	Acetic acid concentration
APS	Absolute Parametric Sensitivity
B	Butanol concentration
BA	Butyric acid concentration
E	Ethanol concentration
k_1	Constant in Equation (6)
k_2	Constant in Equation (7)
k_3	Constant in Equation (8)
k_4	Constant in Equation (8)
k_5	Constant in Equation (9)
k_6	Constant in Equation (9)
k_7	Constant in Equation (10)
k_8	Constant in Equation (12)
k_9	Constant in Equation (12)

Symbol	Meaning
k_{10}	Constant in Equation (13)
k_{11}	Constant in Equation (15)
k_{12}	Constant in Equation (16)
k_{13}	Constant in Equation (17)
k_{14}	Constant in Equation (17)
K_{AA}	Acetic acid saturation constant
K_{BA}	Butyric acid saturation constant
K_I	Inhibition constant
K_S	Monod constant
r_A	Specific rate of acetone production
r_{AA}	Specific rate of acetic acid accumulation
r_B	Specific rate of butanol production
r_{BA}	Specific rate of butyric acid accumulation
S	Substrate (glucose) concentration
S_e	Measure of dispersion caused by experimental error
S_r	Measure of deviations around the regression function

SWR	Sum of squares of weighed residues
t	Time
X	Biomass concentration
y	Marker of the physiological state of the culture

Greek Symbols

ε	Measuring error
μ	Specific culture growth rate

6.5 REFERENCES

1. Carnaham, B., H.A. Luther and J.O. Wilkes, 1969. Applied Numerical Methods, John Wiley and Sons, New York, 62-64.

2. De Boor, C. 1978. A Practical Guide to Splines, Springer Verlag, N.Y., 235-245.

3. Fredrickson, A.G., D. Ramkrishna and H.M. Tsuchiya. 1967. Statistics and Dynamics of Procaryotic Cell Populations, Math. Biosci., 1, 327-374.

4. Harder, A. and J.A. Roels. 1982. Application of Simple Structured Models in Bioengineering, Adv. Biochem. Eng. 21, 56-107.

5. Himmelblau, D.M. 1968. Process Analysis by Statistical Methods, Wiley, New York, 112-116.

6. Malek, I. 1976. Physiological State of Continuously Grown Microbial Cultures. In: A.C.R. Dean, D.C. Ellwood and C.G. Evans (eds.), Continuous Culture, Ellis Harwood Ltd., Sussex, England, 31-36.

7. Monod, J. 1949. The Growth of Bacterial Cultures, Ann. Review of Microbiol. 3, 371-394.

8. Powell, E.D. 1968. Transient Changes in the Growth Rate of Microorganisms, Proc. 4th Symposium Cont. Cult. Microorganisms, Prague, 275-284.

9. Rosenbrock, H.H. 1960. An Automatic Method for Finding the Greatest or Least Value of a Function. Computer J. 3, 175-184.

10. Van der Westhuizen, A. D.T. Jones and D.R. Woods, 1982. Autolytic Activity and Butanol Tolerance of Clostridium acetobutylicum, Appl. Environ. Microbiol. 44, 1277-1281.

11. Votruba, J. 1982. Practical Aspects of the Mathematical Modelling of Fermentation Processes as a Method of Descripotion, Simulation, Identification and Optimization, Acta Biotechnol. 3, 119-126.

12. Webster, J.R. S.J. Reid, D.T. Jones and D.R. Woods. 1981. Purification and Characterization of an Autolysin from Clostridium acetobutylicum, Appl. Environ. Microbiol. 41, 371-374.

*13. Yerushalmi, L. and B. Volesky. 1985. Importance of Agitation in the Acetone-Butanol Fermentation, Biotechnol. Bioeng. accepted for publication on January 7, 1985.

7

SYSTEMS ANALYSIS OF THE CULTURE PHYSIOLOGY IN THE ACETONE-BUTANOL FERMENTATION

L. Yerushalmi, B. Volesky and J. Votruba, Biotechnol. Bioeng., submitted for Publication in February 1985.

7.1 INTRODUCTION

In the acetone-butanol biosynthesis process by C. acetobutylicum, the microbial culture sometimes fails to exhibit normal behavior and the fermentation may be considered unsuccessful in terms of yield(s), conversion efficiencies, and/or product accumulation. This failure may not be due to poor experimentation but may be a result of the changes in the physiological conditions of the bacterium itself. These results are usually not discussed or published because of lack of explanation of this abberate behavior. However, a systematic study of this malfunction of the culture may prove very useful in elucidating the relevant microbial phenomena and identifying the potential problems of the fermentation process. Changes in culture physiology are undoubtedly responsible and quantitative assessment of these as well as of their relationships represent an area whose study poses a formidable challenge.

Metabolic changes in Clostridial cultures have been reported in the literature as early as 1893 by Grimbert who described morphological and physiological changes in the

butyric acid bacteria. Kutzenok and Aschner (1952) studied the process of degeneration in C. butylicum and pointed out that their culture would undergo degeneration in a subculture without a heat shock. With the start of degeneration, which usually took place after the 10th transfer, the strain consumed less sugar and produced less butanol and more butyric acid until it died out after the 12th to 15th passage.

Instability of the solvent formation activity in the butyric acid bacteria was also reported by Krouwel et al. (1983). In a study of the degeneration of the solvent production activity by C. beijerinckii in continuous culture, Jobses and Roels (1983) reported that in all of their cultures butanol production took place for a least 20 generations but then the cultures ceased to produce butanol. At this stage, the consumption of sugar decreased and did not improve with the increase of the sugar supply.

A low pH of less than 4.2 (Krouwel et al., 1983), a high pH of more than 6.0 (Jobses and Roels, 1983) or a loss of spore forming ability (Gottschal and Morris, 1981) were found to be among the factors responsible for the degeneration of the culture.

The retarded culture investigated in this work exhibited physiological changes even in its first vegetative transfer. Careful spore activation and preparation of the inoculum and a heat shock treatment which was always practised did not seem to improve the behavior of the culture.

The present work was planned to study the retarded fermentations in a systematic way and to elucidate the key causes responsible for the behavior of the culture during the unsuccessful fermentations. Although it concentrates on the physiological variations of C. acetobutylicum, this work introduces a new method in investigation of the microbial physiology by utilizing the well known physical and physico-chemical principles.

7.1.1 Theoretical Basis for Sugar Transport Mechanism

Transport of sugar through the cellular membrane will be discussed in this paper as one of the major controlling steps in the A-B-E fermentation. Therefore, it seems essential to discuss its mechanism as well as the theoretical basis of the equations used to describe this phenomenon.

Transport of sugar through the cell membrane was considered to be carried out by chemical association through "active sites" in the membrane. This concept considers a chemical interaction of the substance (A) which is to be transported, with a membrane constituent (X) which may be envisaged as a carrier of (A) across the membrane (Snell et al., 1965; Kotyk and Janacek, 1970; Bailey and Ollis, 1979).

Figure 1 presents the schematic diagram of transport through the cell membrane by chemical association.

FIGURE 1 A Schematic Diagram of Transport Through the Cellular Membrane by Chemical Association.

INSIDE
A
2
X
AX
membrane
Energy
X
AX
1
A
OUTSIDE

This type of scheme reflects the transport mechanism for a substance against its concentration gradient which is usually observed in the bacterial sugar transporting systems. In this case, the transport of the solute must be energetically coupled with some energy yielding metabolic reactions since the movement of a component from regions of low to high concentrations would not result unless the transport is coupled to some other "driving" chemical reaction (Snell et al., 1965, Rosen and Kashket, 1978).

It has been assumed that A has a poor distribution coefficient in the membrane and it is unlikely to have unassociated A present in the membrane. It can also be assumed that the total concentration of X, made up of free X, and AX, is constant at each membrane surface. The following equations can be written (Snell, et al., 1965) for the respective concentrations C_T of X at membrane surfaces 1 and 2:

$$C_{T_1} = C_{X_1} + C_{AX_1} \quad \text{and} \quad C_{T_2} = C_{X_2} + C_{AX_2} \tag{1}$$

The affinity of the carrier for the substance may be described in terms of an equilibrium constant in the following form:

$$K = \frac{C_A \cdot C_X}{C_{AX}} \tag{2}$$

Considering that it is a neutral uncharged complex, the flux of AX per unit area of the membrane surface can be directly related to its concentration gradient at the two membrane surfaces through diffusion equations:

$$J_{AX} = -P^m_{AX} \quad \Delta C_{AX} = -P^m_{AX}[C_{AX_2} - C_{AX_1}] \tag{3}$$

where P^m_{AX} is a permeability coefficient for the membrane.

In an active transport system, the concentration of the carrier may be kept very small in the region of surface 2 so that C_{AX_2} would be so small that it may be ignored. This will simplify Equation (3) to the following form:

$$J_{AX} = P^m_{AX}\, C_{AX_1} \tag{4}$$

C_{AX_1} can be expressed in terms of C_{A_1} and C_{T_1} by combining Equations (1) and (2), as follows:

$$C_{AX_1} = \frac{C_{A_1} \cdot C_{T_1}}{C_{A_1} + K} \tag{5}$$

Therefore:

$$J_{AX} = P^m_{AX} \frac{C_{T_1} \cdot C_{A_1}}{C_{A_1} + K} \tag{6}$$

7.2 MATERIALS AND METHODS

The media B and C with the initial glucose concentrations of 30 g/L and 20 g/L, respectively, were used in this study. All of the unsuccessful fermentations and some of the successful fermentations were performed in 500 mL Erlenmeyer flasks with a working volume of 150 mL and at a temperature of 38°C. The results of at least four sets of pH-controlled normal fermentations with an initial glucose concentrations of 50 g/L were also used in this study. The medium composition and the conditions of these fermentation experiments are reported elsewhere (Yerushalmi and Volesky, 1985)*.

The analytical procedures for the analysis of solvents and acids and the assays of glucose and biomass are discussed earlier (Section 2.2).

The cell membrane transport assay used 3-O-methyl-glucose (Sigma Chemical Co., St. Louis, Mo.) where 2.5 mL of its 10 mM solution was mixed with 10μC of its radioactive analogue (New England Nuclear Co.). The cell suspension was prepared by centrifugation of approximately 0.1g of cell at 13,000 g for 10 minutes, washing them twice with the basal medium (medium without glucose, yeast extract and ferrous sulfate), and suspension in 30 mL of the basal medium. 0.5 mL of the 3-O-methyl-glucose solution was added to 6.0 mL of the cell suspension which had been brought to 38°C under an

atmosphere of nitrogen. Samples of 1 mL were drawn at measured intervals and filtered through glass fiber filters (Whatman GF/F). The filters were washed three times with ice cold basal medium, dried and placed in scintillation vials. 10 mL of the scintillation fluid were added and the radioactivity counted. Relating this number to the count of a known amount of the 3-O-methyl-glucose solution and dividing by the cell water volume gave the intracellular sugar concentration.

The intracellular water volume was determined by exclusion of dextran at room temperature and by assuming a density of 1 for the biomass in its wet state. This volume was the difference between the total cell volume, the volume of the cell material and the extracellular cell volume. The intracellular water volume averaged 70.3% $\pm$ 2.0% of the wet cell volume and the extracellular water averaged 28.0% $\pm$ 2.1% of the total cell volume.

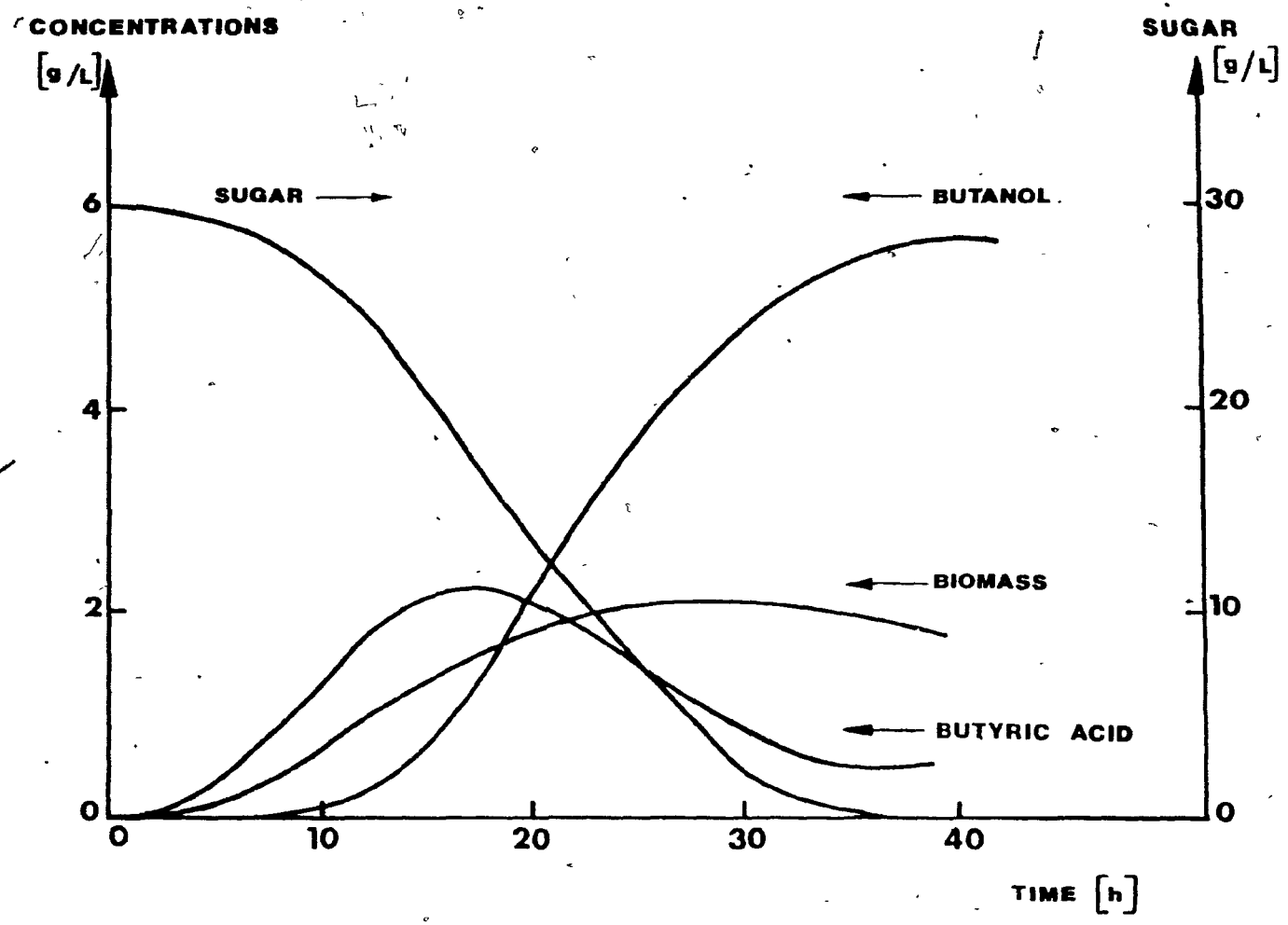
7.3 RESULTS

7.3.1 Identification of the Normal and the Retarded Fermentations

Two types of fermentations are discussed in this paper, "normal" (successful) fermentations and "failed" (retarded) fermentations. Those fermentations with a solvent yield of almost 30% (based on sugar) and a total fermentation time of less than fifty hours were considered as normal. In these fermentations, the "acid break" occurred 18 hours to 20 hours into the fermentation. There was little accumulation of acids at the end of the process which normally took almost 30 hours. The sugar was then completely depleted in the system. Figure 2 presents an example of key parameters in a normal fermentation. A fermentation with a solvent yield of less than 30% and/or a long fermentation of more than 60 hours was considered unsatisfactory (failed) and the culture performing this way was labelled as "retarded". In this case, the acids exhibited a late break (after 25 hours) in their respective concentration curves resulting in high acid accumulation at the end of a prolonged fermentation experiment. Fourteen different sets of experimental data obtained from the "retarded" culture and five different sets of results of "normal" fermentations were used in this work (Appendix 5).

FIGURE 2 An Example of a Normal Acetone-Butanol Batch Fermentation.

C. acetobutylicum



The results of the four pH controlled, normal fermentations used in this study were presented earlier (Yerushalmi and Volesky, 1985)*.

Based on the preliminary observation of the course of fermentations and on the final results of the experiments, the unsuccessful fermentations were divided into three groups. This division, which allowed a more organized evaluation of the process, can be summarized as follows:

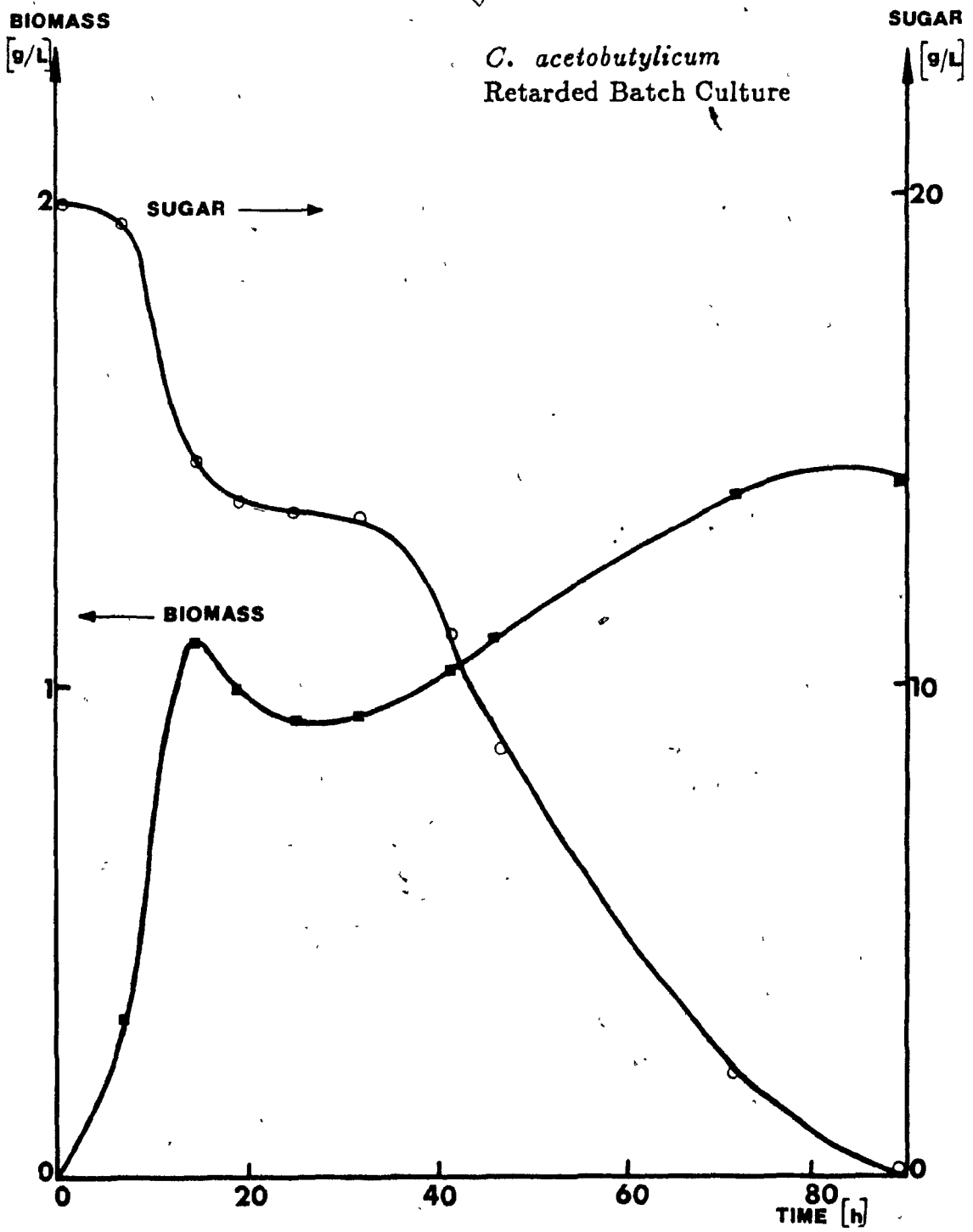
1) Slow fermentation lasting for more than 60 hours exhibiting a break in the activity of the culture in the middle of the process which took approximately up to 20 hours. There were two distinctively different rates of sugar consumption and a late acid break in the system. Although small amounts of solvents were produced before the break in the cellular activities (Phase I) most of the solvents were synthesized after the resumption of cellular activities (Phase II). A complete sugar exhaustion which was usually observed in these experiments did not guarantee a high solvent yield and sometimes a low solvent yield resulted from these experiments. Based on the final solvent yield this group was further sub-divided:

1-a) <u>Complete</u> solvent synthesis (yield ≈ 30%) (Figure 3).

1-b) <u>Incomplete</u> solvent synthesis (yield < 30%) (Figure 4).

FIGURE 3(a,b) An Example of a Retarded Acetone-Butanol Batch Fermentation: Stepwise Prolonged Fermentation with Complete Solvent Synthesis.

a)


BIOMASS
[g/L]
SUGAR
[g/L]
C. acetobutylicum
Retarded Batch Culture
SUGAR
BIOMASS
2
1
0
20
10
0
0
20
40
60
80
TIME [h]

b)

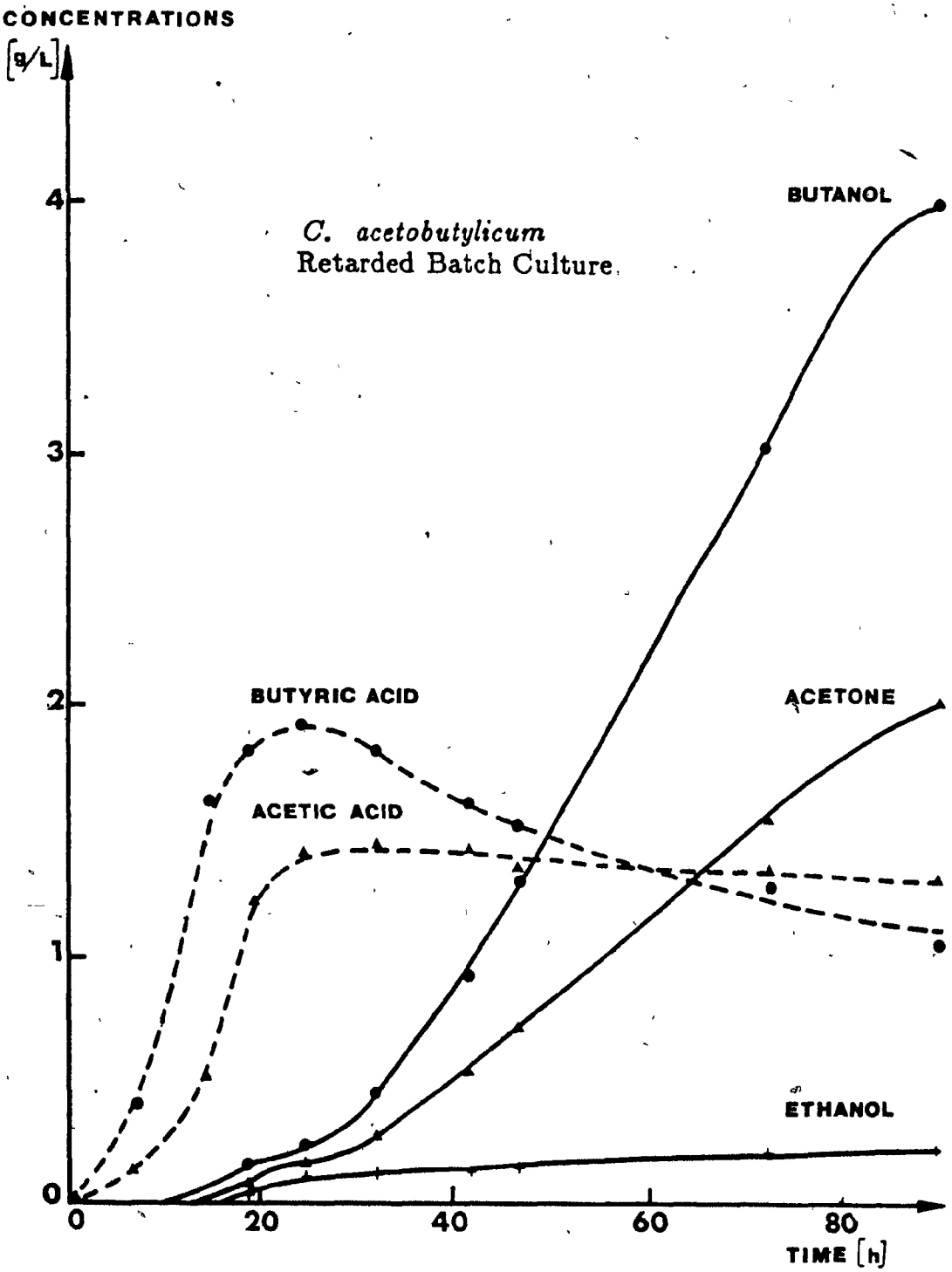
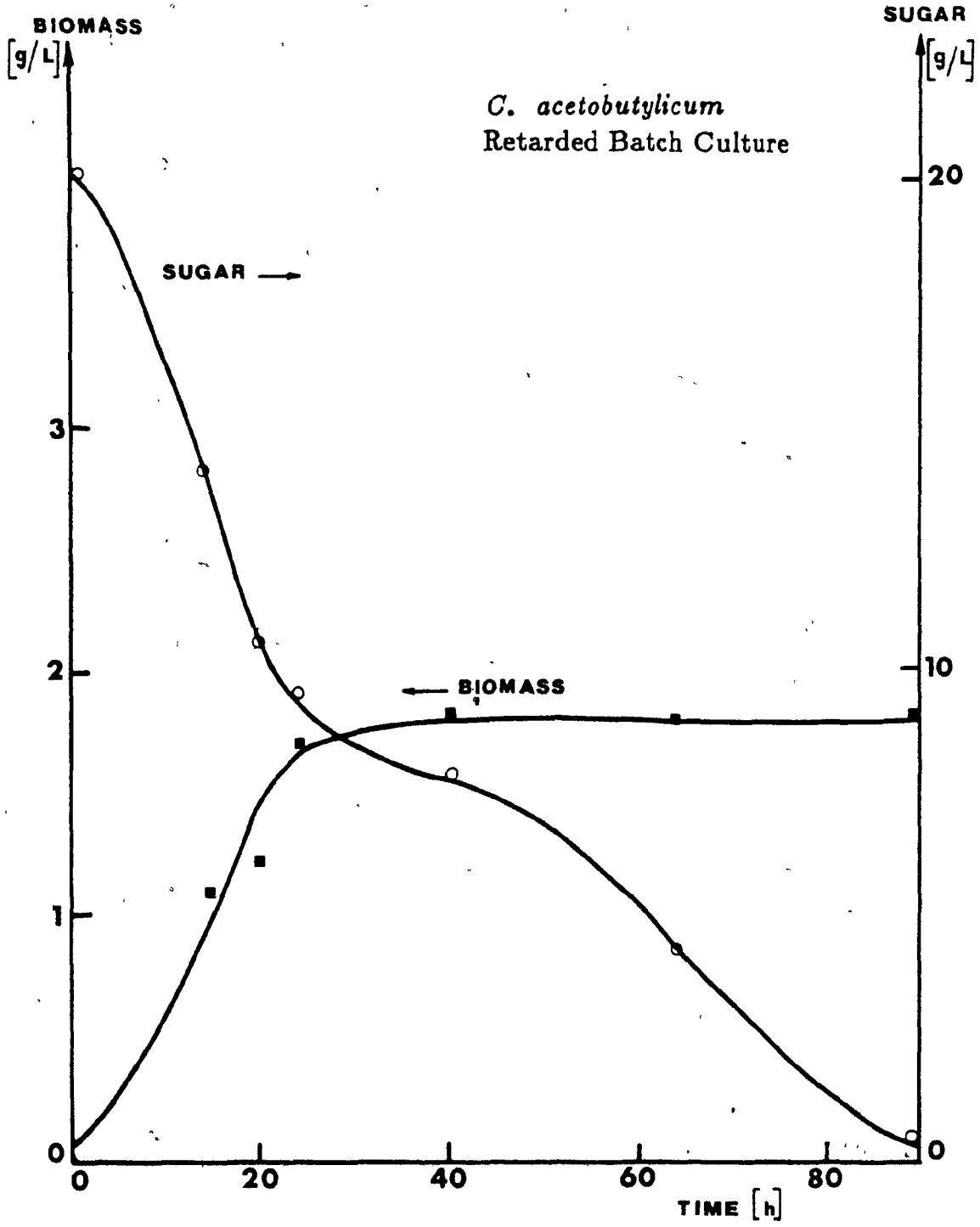
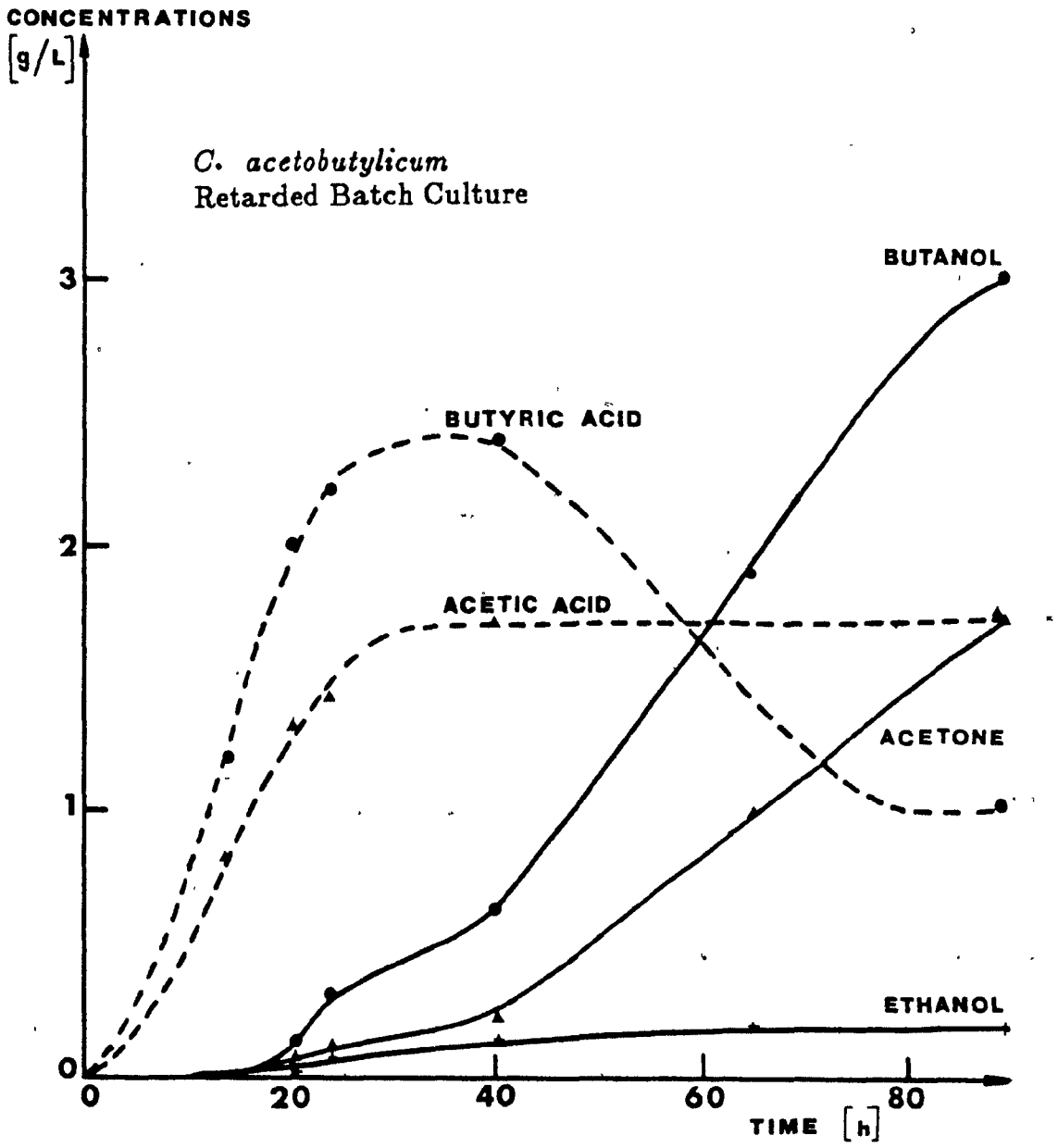

CONCENTRATIONS
[g/L]
4
3
2
1
0
C. acetobutylicum
Retarded Batch Culture
BUTANOL
BUTYRIC ACID
ACETONE
ACETIC ACID
ETHANOL
0
20
40
60
80
TIME [h]

FIGURE 4(a,b) An Example of a Retarded Acetone-Butanol Batch Fermentation: Stepwise Prolonged Fermentation with Incomplete Solvent Synthesis.

a)


BIOMASS
[g/L]
SUGAR
[g/L]
C. acetobutylicum
Retarded Batch Culture
SUGAR
BIOMASS
3
2
1
0
20
10
0
0
20
40
60
80
TIME [h]

b)


CONCENTRATIONS
[g/L]
C. acetobutylicum
Retarded Batch Culture
BUTANOL
BUTYRIC ACID
ACETIC ACID
ACETONE
ETHANOL
3
2
1
0
0
20
40
60
80
TIME [h]

2) Slow fermentation with late acid break and delayed solvent synthesis, incomplete sugar utilization at the end with a total solvent yield of less than 30%. Figure 5 represents an example of this group.

3) Slow fermentation with late acid break and delayed solvent synthesis resulting in a solvent yield of less than 30%. Sugar consumption was relatively fast with its complete exhaustion at the end of the process which took almost 50 hours. Results representative of this type were obtained in experiments where a pH adjustment was made in the early stages of the fermentation (from pH 4.2 to 5.0). An example of this group is presented in Figure 6.

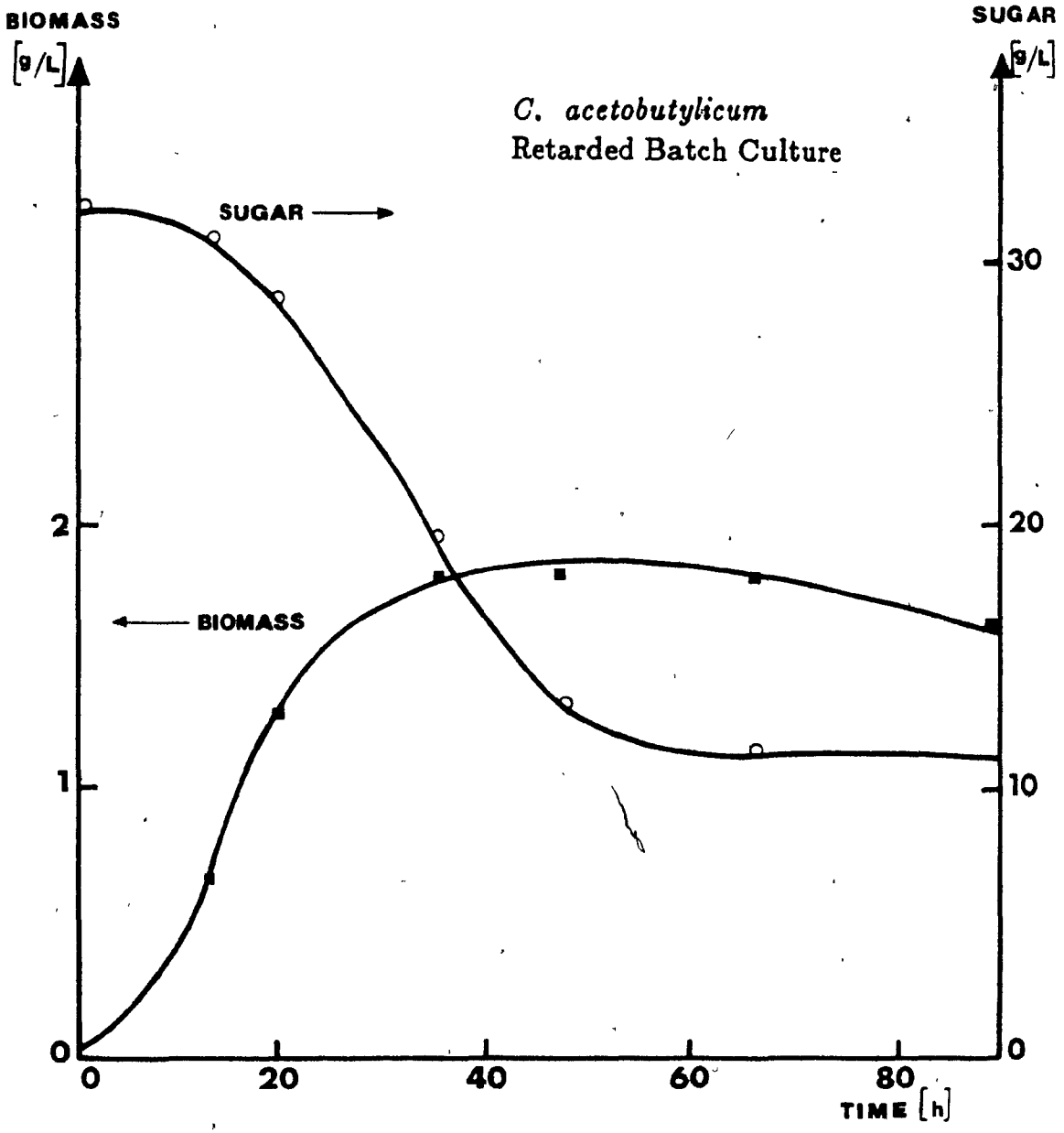
Some conclusions could be drawn from comparing the following relationships for the failed and the normal fermentations:

- specific culture growth rate (μ) vs. butanol concentration (B) (Figure 7)
- specific butyric acid accumulation rate (r_{BA}) vs. butanol concentration (B) (Figure 8)
- specific rate of sugar consumption (r_S) vs. sugar concentration (S) (Figure 9).

These relationships were considered to be the most revealing since butanol is the major product causing inhibi-

FIGURE 5(a,b) An Example of a Retarded Acetone-Butanol Batch Fermentation: Smooth Prolonged Fermentation with Incomplete Solvent Synthesis and Sugar Utilization.

a)


BIOMASS
[g/L]
SUGAR
[g/L]
C. acetobutylicum
Retarded Batch Culture
SUGAR
BIOMASS
2
1
0
30
20
10
0
0
20
40
60
80
TIME [h]

b)

C. acetobutylicum
Retarded Batch Culture

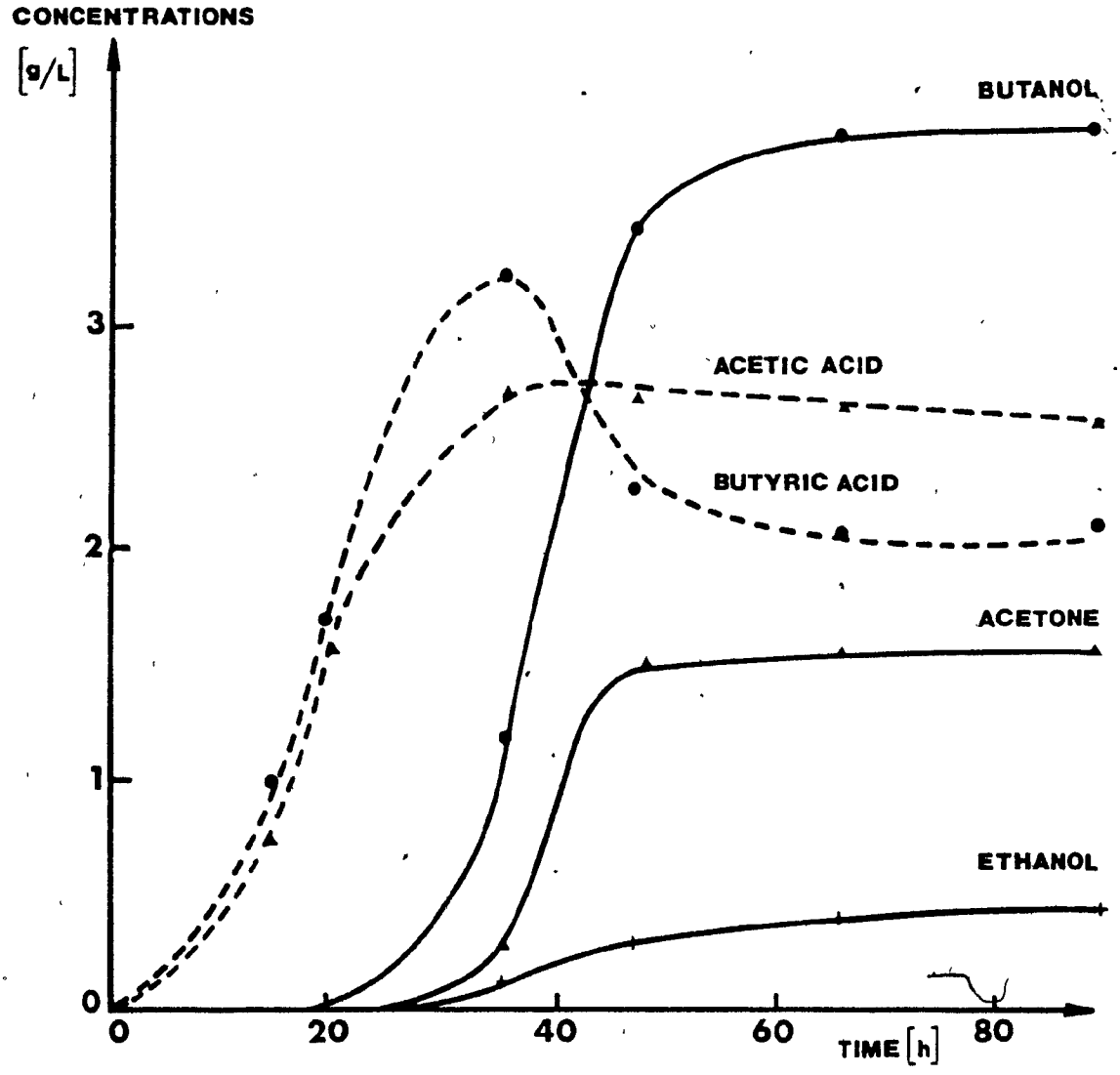
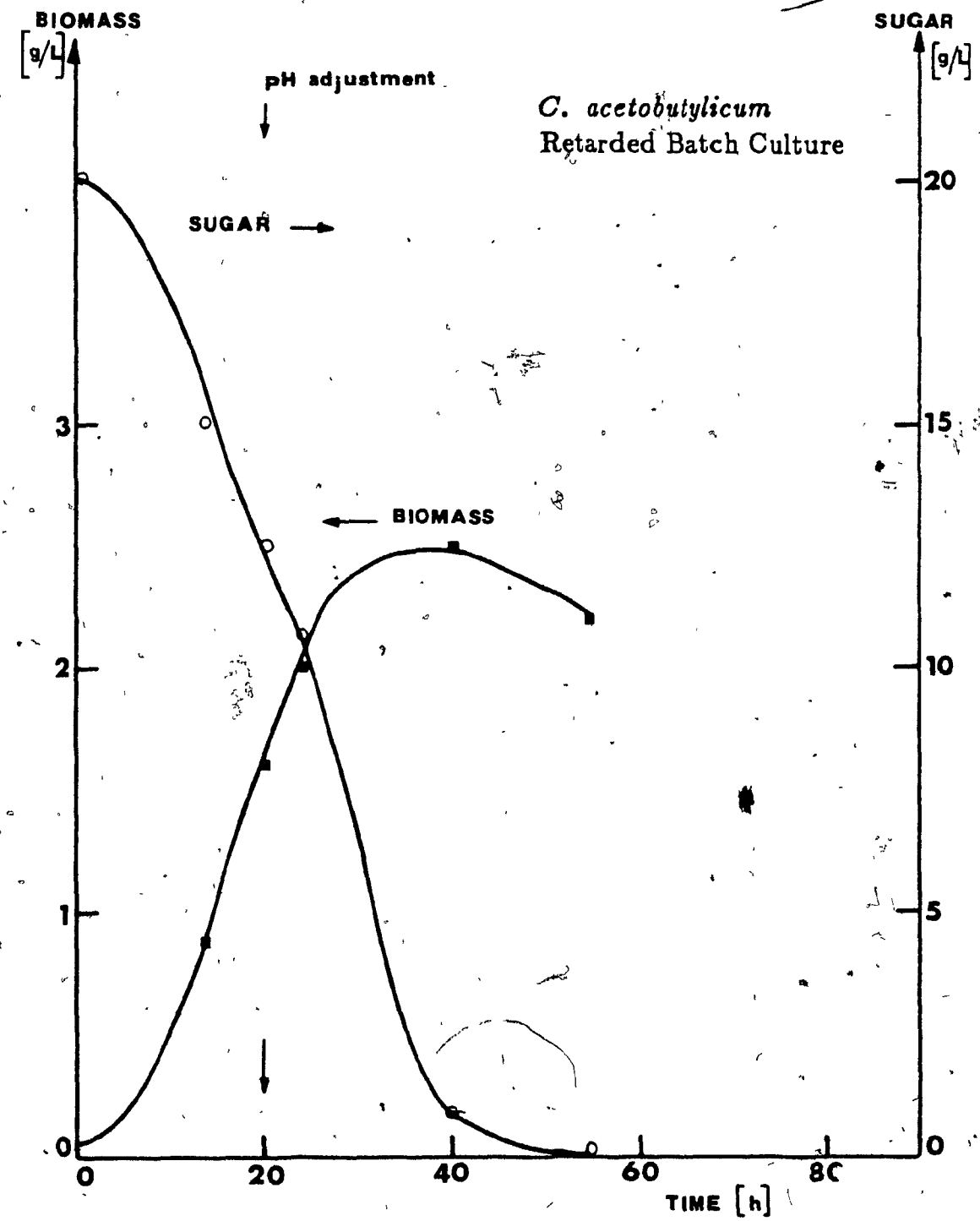


FIGURE 6(a,b) An Example of a Retarded Acetone-Butanol Batch Fermentation: Smooth Fermentation with Incomplete Solvent Synthesis and Complete Sugar Utilization.

a)


BIOMASS
[g/l]
SUGAR
[g/l]
pH adjustment
C. acetobutylicum
Retarded Batch Culture
SUGAR
BIOMASS
0
1
2
3
0
5
10
15
20
0
20
40
60
80
TIME [h]

b)

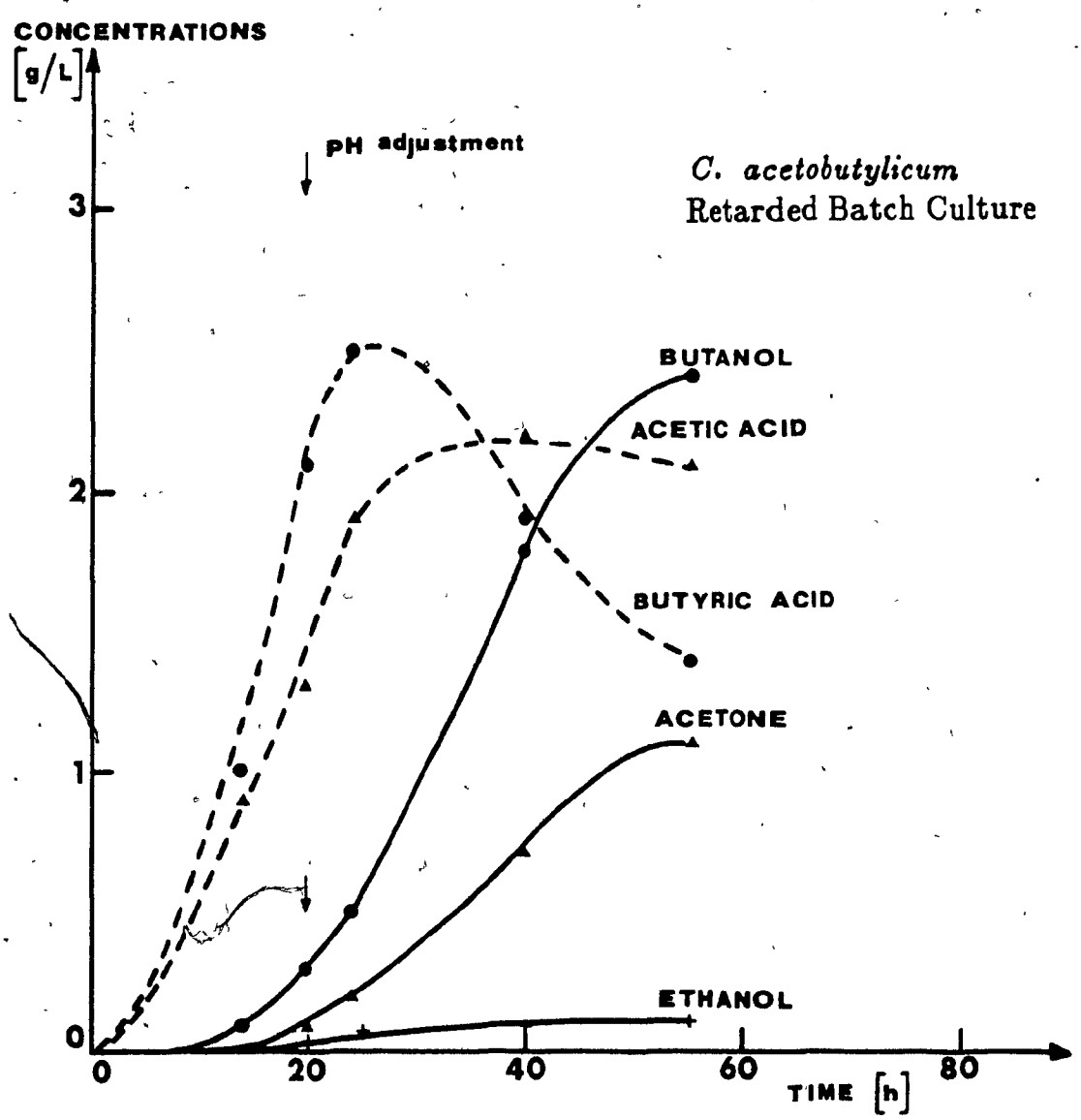

CONCENTRATIONS
[g/L]
pH adjustment
C. acetobutylicum
Retarded Batch Culture
BUTANOL
ACETIC ACID
BUTYRIC ACID
ACETONE
ETHANOL
3
2
1
0
0
20
40
60
80
TIME [h]

FIGURE 7 Relationship of the Culture Specific Growth Rate(μ) and the Butanol Concentration (B) in Normal and Retarded Fermentations.

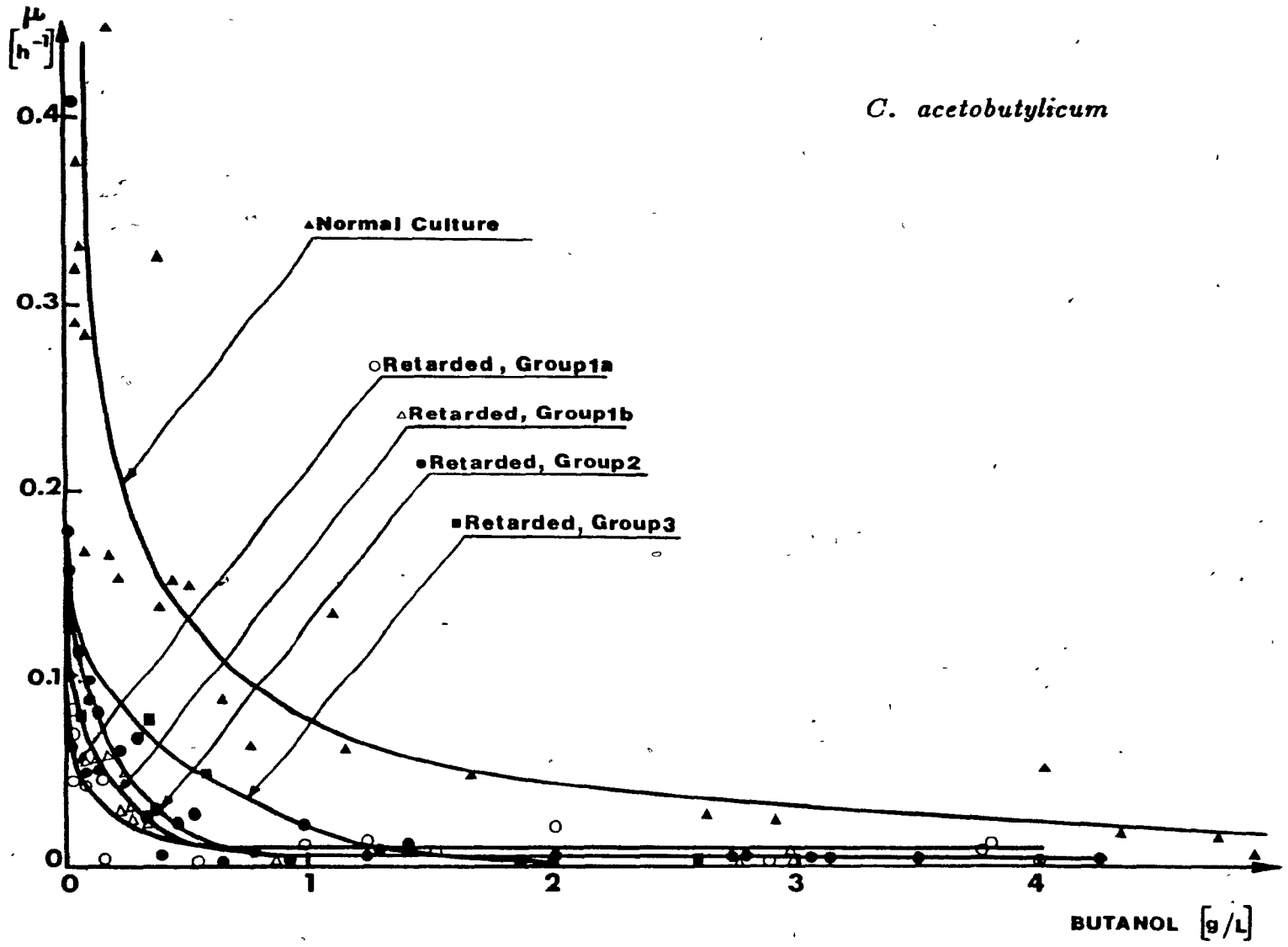

μ
[h⁻¹]
0.4
0.3
0.2
0.1
0
0
1
2
3
4
BUTANOL [g/L]
▲Normal Culture
○Retarded, Group1a
△Retarded, Group1b
●Retarded, Group2
■Retarded, Group3
C. acetobutylicum

FIGURE 8 Relationship of the Specific Butyric Acid Accumulation Rate r_{BA} [g but. acid/h•g biomass] and the Butanol Concentration (B) in Normal and Retarded Fermentations.

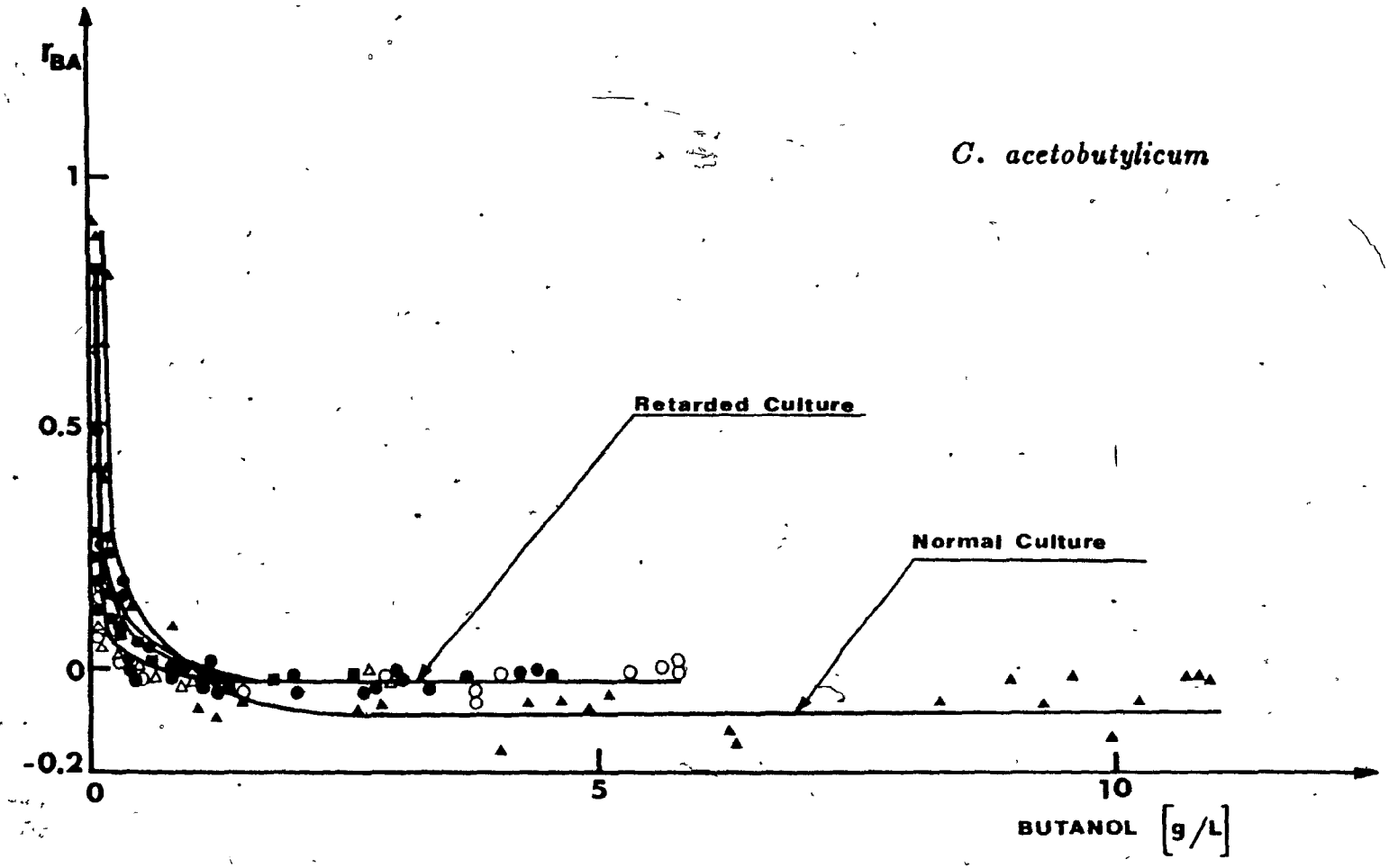
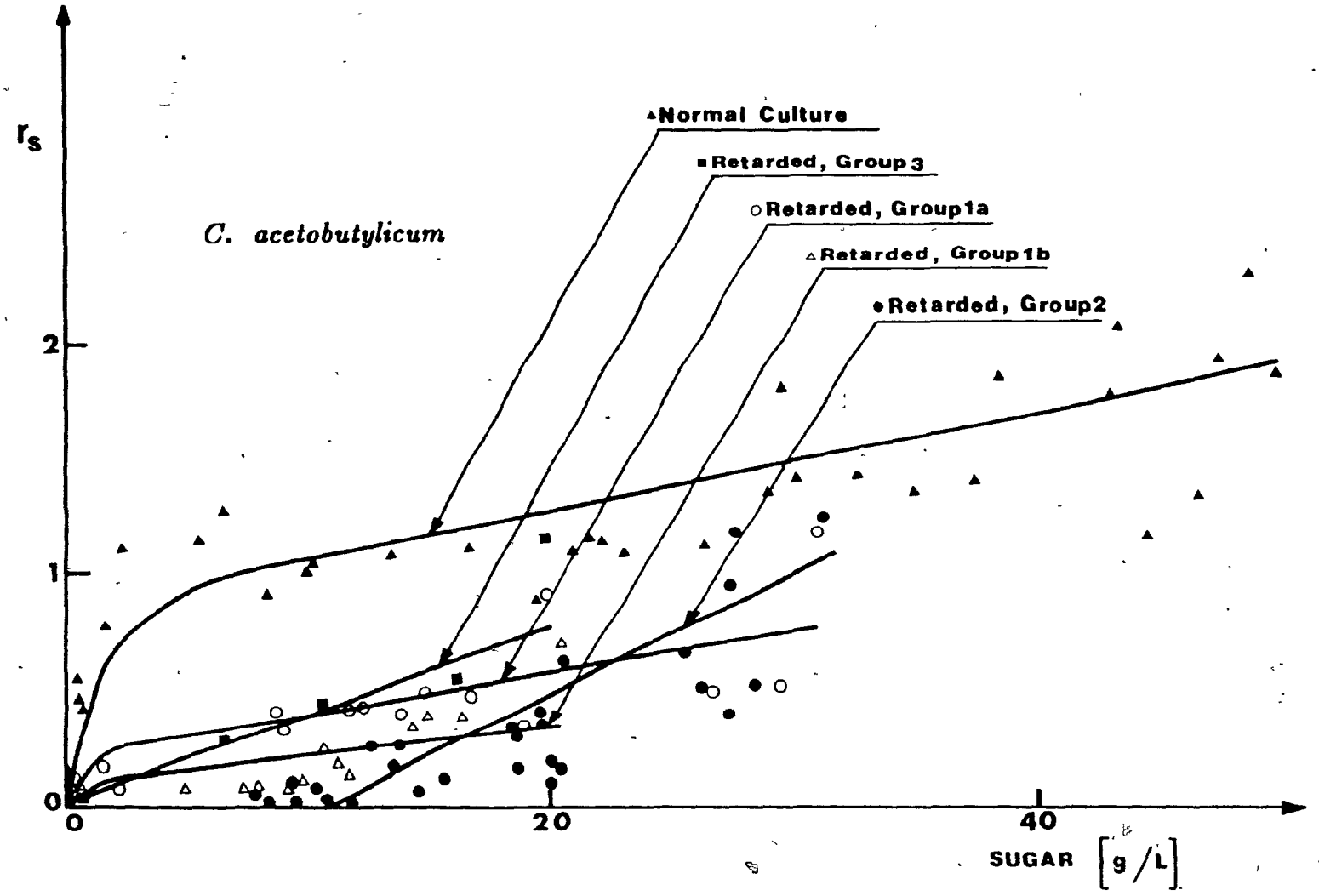

r_{BA}
1
0.5
0
-0.2
0
5
10
BUTANOL [g/L]
C. acetobutylicum
Retarded Culture
Normal Culture

FIGURE 9 Relationship of the Specific Substrate Utilization Rate r_S [g glucose/h·g biomass] and the Sugar Concentration (S) in Normal and Retarded Fermentations.


r_s
Normal Culture
Retarded, Group 3
Retarded, Group 1a
Retarded, Group 1b
Retarded, Group 2
C. acetobutylicum
2
1
0
0
20
40
SUGAR [g/L]

tion in the process and since its production is governed by the conversion of butyric acid. Evaluation of the sugar kinetics were also considered important since sugar transport into the cell controls the overall biosynthesis. The scatter of the points on these figures may be attributed to the fact that the data have been collected from different experiments. The major points to be noticed for the "retarded" culture are:

a) A lower sugar consumption rate. This could have been due to a retarded sugar transport through the cell membrane as a result of its alterations affecting its functionality.

b) Even lower butanol concentrations resulted in a more pronounced inhibition effect and a lower culture growth rate (Figure 7). This would mean a lower apparent inhibition constant (K_I) which can be defined as the inhibition constant based on the extracellular concentration of butanol in the broth. This might have been due to a difference between the extracellular and the intracellular butanol concentration resulting from a possible alteration in the permeability of the cell membrane.

c) Lower conversion of butyric acid to butanol which could have been due to a failure in the function of the enzymatic apparatus controlling this conversion.

The above interpretation of experimental results predicted that the transport phenomena control the biosynthesis process and could be the major factor responsible for the behavior of the retarded culture. Therefore, the respective mechanisms for the transport of sugar, solvents and acids through the cellular membrane were evaluated.

7.3.2. Sugar Transport Mechanism

The theoretical analysis for the active transport of solutes through the cellular membrane resulted in the following equation:

$$J^m_{AX} = P^m_{\Delta X} \frac{C_{T_1} \cdot C_{A_1}}{C_{A_1} + K} \qquad (6)$$

This equation resembles the Monod equation which relates the specific consumption rate of sugar to its concentration in the medium. J_{AX} approaches a constant value at high concentrations of A in side 1 and is thereby independent of C_{A_1}. A plot of J_{AX} vs. C_A gives a similar curve as would result from plotting of r_S vs. S. Based on the above general

theoretical consideration, the specific consumption rate of sugar on n active "sites" can be expressed by the following equation:

$$r_S = n \frac{k_1 S}{S + K_S} \tag{7}$$

or:

$$r_S = \frac{n' S}{S + k_S} \tag{8}$$

where:

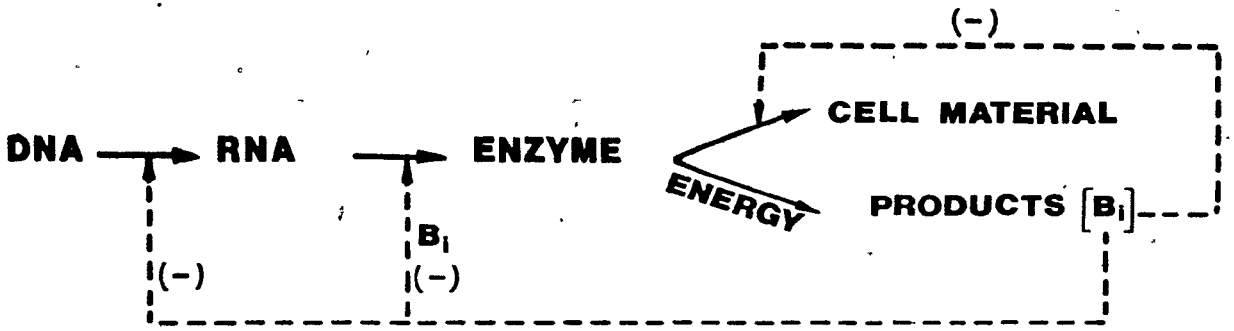
$$n' = k_1 n$$

7.3.3 Dynamics of the Number of Transport "Sites"(n):

For the analysis of the dynamics associated with the cell membrane active "sites" a rather simplified scheme of the sugar metabolism is assumed to represent the metabolic activity inside the cell. As shown in Figure 10, it is considered that the internal butanol has a negative effect on the utilization of sugar and synthesis of cell constituents.

Dynamics of the active "site" generation was also assumed to be dependent on the physiological state of the culture for which (y), dimensionless concentration of RNA,

FIGURE 10 Inhibitory Effect of the Intracellular Butanol on the Sugar Metabolism.



INHIBITORY EFFECT OF BUTANOL ON SUGAR METABOLISM

B_i = INTERNAL BUTANOL CONCENTRATION

could be used as a marker (Votruba et al., 1985)*. Considering these assumptions, n' can be expressed as follows:

$$\frac{d(n'X)}{dt} = f(B_i) \cdot y \cdot X \qquad (9)$$

where $f(B_i)$ is an unknown function of the internal butanol concentration (B_i). Considering this concept, the following material balances could be written:

$$X \frac{dn'}{dt} + n' \frac{dX}{dt} = f(B_i) \cdot y \cdot X \qquad (10)$$

or

$$\frac{dn'}{dt} = f(B_i)\, y - n'\, \mu \qquad (11)$$

The dimensionless marker of the physiological state is related to the specific growth rate by the following equation (Harder and Roels, 1982):

$$y = \frac{\mu}{0.56} + 1 \qquad (12)$$

therefore, equation (11) can be expanded to assume the following form:

$$\frac{dn'}{dt} = f(B_i)\left(\frac{\mu}{0.56} + 1\right) - n'\, \mu \qquad (13)$$

In order to estimate function f (B_i) on the basis of experimental data, a short period of time where n' is essentially constant has been considered. This assumption will result in the left hand side of Equation (13) to equal zero and will establish a steady state condition. Function $f(B_i)$ can then be expressed as:

$$f(B_i) = \frac{n' \mu}{\left(\frac{\mu}{0.56} + 1\right)} \qquad (14)$$

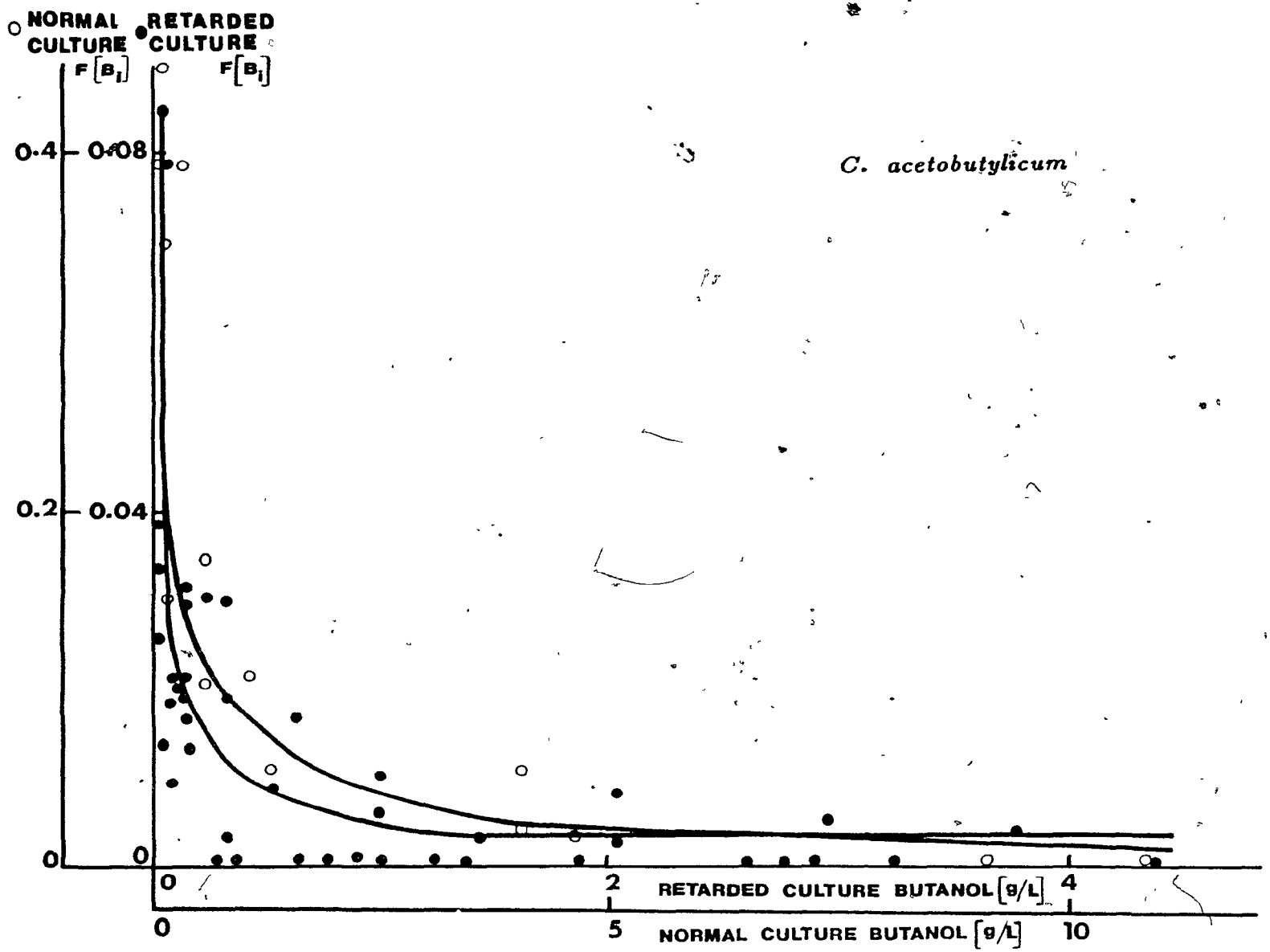
using Equation (8):

$$f(B_i) = \frac{\frac{r_S (S + K_S) \mu}{S}}{\left(\frac{\mu}{0.56} + 1\right)} \qquad (15)$$

A plot of $f(B_i)$, as calculated from Equation (15), vs. the butanol concentration resulted in similar curves for the "normal" and the "retarded" culture (Figure 11). This means that a similar kind of inhibition exists in both cases and a similar hyperbolic function could be used for the expression of $f(B_i)$ function. However, in spite of mathematical similarities, the two curves are at different scales which implies different parameters for the two cultures.

The transport through active "sites" as discussed, accounts for a number of observations. In the failed fermen

FIGURE 11 Variations of the Function $f(B_i)$ with Butanol Concentration.



C. acetobutylicum

tations, lower sugar utilization rates were observed. This could be due to the lower number of the available membrane transport "sites" or due to the deficiencies in their function since the rate of sugar consumption is proportional to the number of active "sites" (Equation 8). Sugar transport controls the synthetic rate of the metabolite production. Therefore, a slow fermentation could be a result of the deficiencies in sugar transport. In other words, slow sugar transport is responsible for slow sugar consumption rate, which in turn is the cause of slow fermentation.

7.3.4 Mechanism of Solvent and Acid Transport

Due to its major role in controlling the microbial metabolism, butanol has been considered as a representative single component in the study of the solvent transport. In this evaluation, it was assumed that the microbial growth is mainly influenced by butanol and that for the same μ (specific growth rate) the intracellular butanol concentration (B_i) is the same for all the different cultivations.

A hypothesis concerning the contribution of active "sites" in the transport of solvents across the cell membrane was first evaluated. According to this hypothesis, the transport rate of butanol out of the cell, described by the

following equations, would be dependent on the number of active "sites" in the membrane:

$$\frac{dB}{dt} = r_B X = PV_B(B_i - B) \qquad (16)$$

or:

$$\frac{r_B X}{PV_B} = B_i - B \qquad (17)$$

where V_B is the wet cell volume and P is the cell membrane permeability which, by accounting for the active "sites", is equal to:

$$P = P' \cdot n \qquad (18)$$

where P' is the permeability of one active "site" and n is the number of active "sites" in one gram of cells.

Thus:

$$\frac{r_B X}{P' n V_B} = B_i - B \qquad (19)$$

or:

$$\frac{r_B}{n} = \frac{P'}{\alpha}(B_i - B) \qquad (20)$$

where $\alpha = X/V_B$.

In order to evaluate the above relationship, n is changed to $n' = k_1 n$ where k_1 is the constant in the sugar transport Equation (8). Consequently:

$$\frac{r_B}{n'} = \frac{P'}{\alpha k_1} (B_i - B) \qquad (21)$$

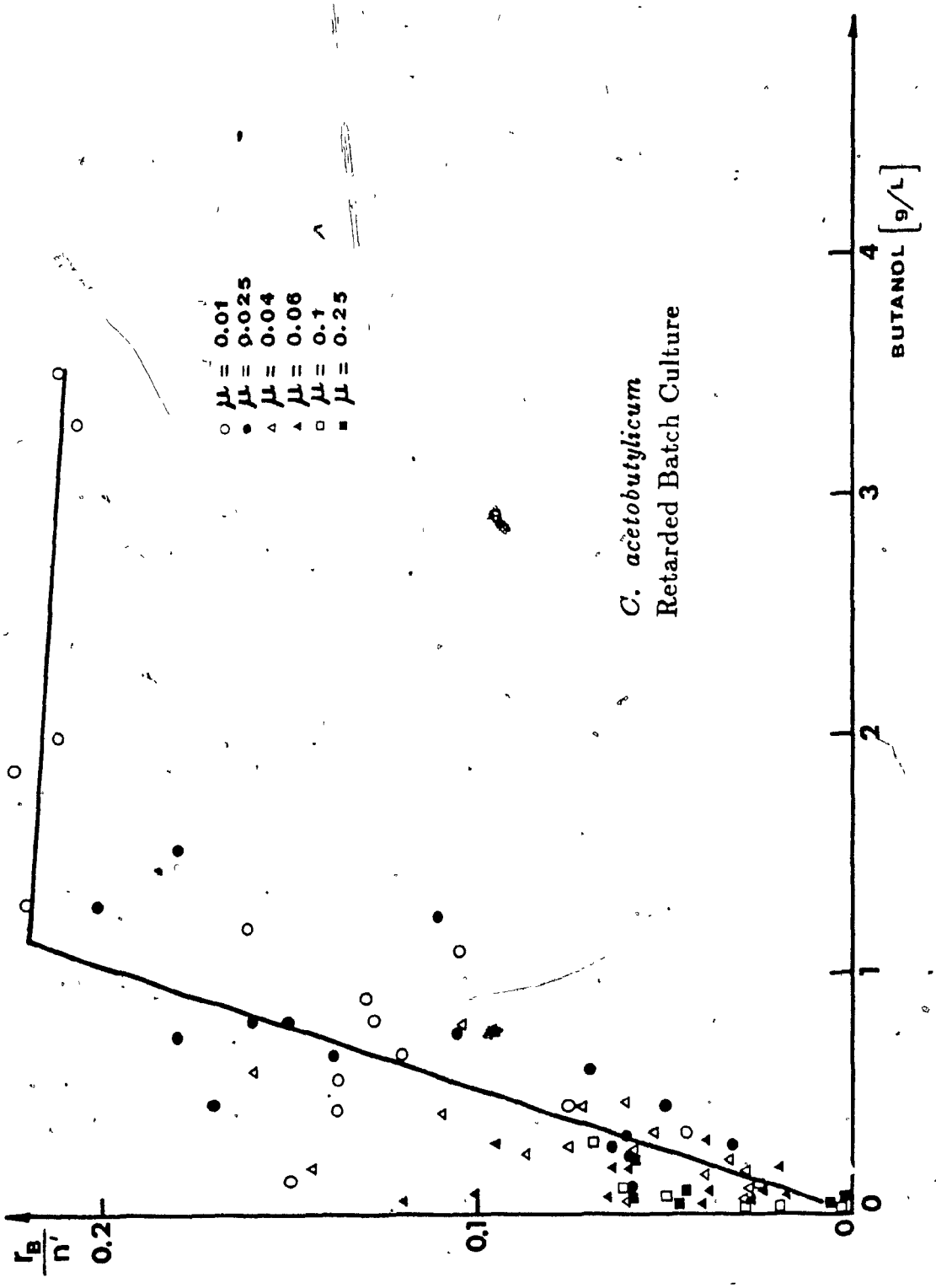
This relationship was studied by plotting the relationship between $\frac{r_B}{n'}$ and B at different μ values for different cultivations. Parameter n' was determined by rearranging Equation (8):

$$n' = \frac{r_S (S + K_S)}{S} \qquad (22)$$

The values of r_B, r_S, B and S at different μ values were found by interpolation.

If the hypothesis regarding the involvement of the active sites in the butanol transport was correct, a graph of $\frac{r_B}{n'}$ vs. B would yield straight lines with a negative slope of $\frac{P'}{\alpha k_1}$ and an intercept of $\frac{P'}{\alpha k_1} B_i$. Figure 12 presents the resulting plots for different values of μ from $0.01h^{-1}$ to $0.25h^{-1}$. The scattered points indicate that the proposed

FIGURE 12 Variations of the Function $\frac{r_B}{\eta'}$ with Butanol Concentration.


$\frac{r_B}{n'}$
0.2
0.1
0
0
1
2
3
4
BUTANOL [g/L]
μ = 0.01
μ = 0.025
μ = 0.04
μ = 0.06
μ = 0.1
μ = 0.25
C. acetobutylicum
Retarded Batch Culture

mechanism is not valid and the transport of solvents is not dependent on the number of active "sites".

Although it is very difficult to make any judgments concerning the mechanism from this graph, it can be seen that at very low values of B the trend is opposite and the lines have a positive slope. This is where butyric acid production takes place. Therefore, it can be concluded that in the region of low butanol concentrations the behaviour of the culture is influenced by the production and transport of butyric acid. However, at higher concentrations of butanol (which occurs at lower μ values) a negative slope was observed.

Based on the fact that the previous hypothesis is not valid and from the shape of the curves in Figure 12, a new hypothesis was developed which considered that transport of butanol out of the cell was mainly due to the chemical concentration gradient across the membrane. This implies that butanol transport across the cell membrane is governed by diffusion. This hypothesis would account for some of the observations in the retarded culture: lower permeability due to the altered cell membrane will cause an accumulation of butanol inside the cell and will result in lower apparent inhibition constant. The plot of $\frac{r_B}{n'}$ vs. B in Figure 12 also

showed that at low values of butanol concentration, the production of butyric acid and its competition for transport is important.

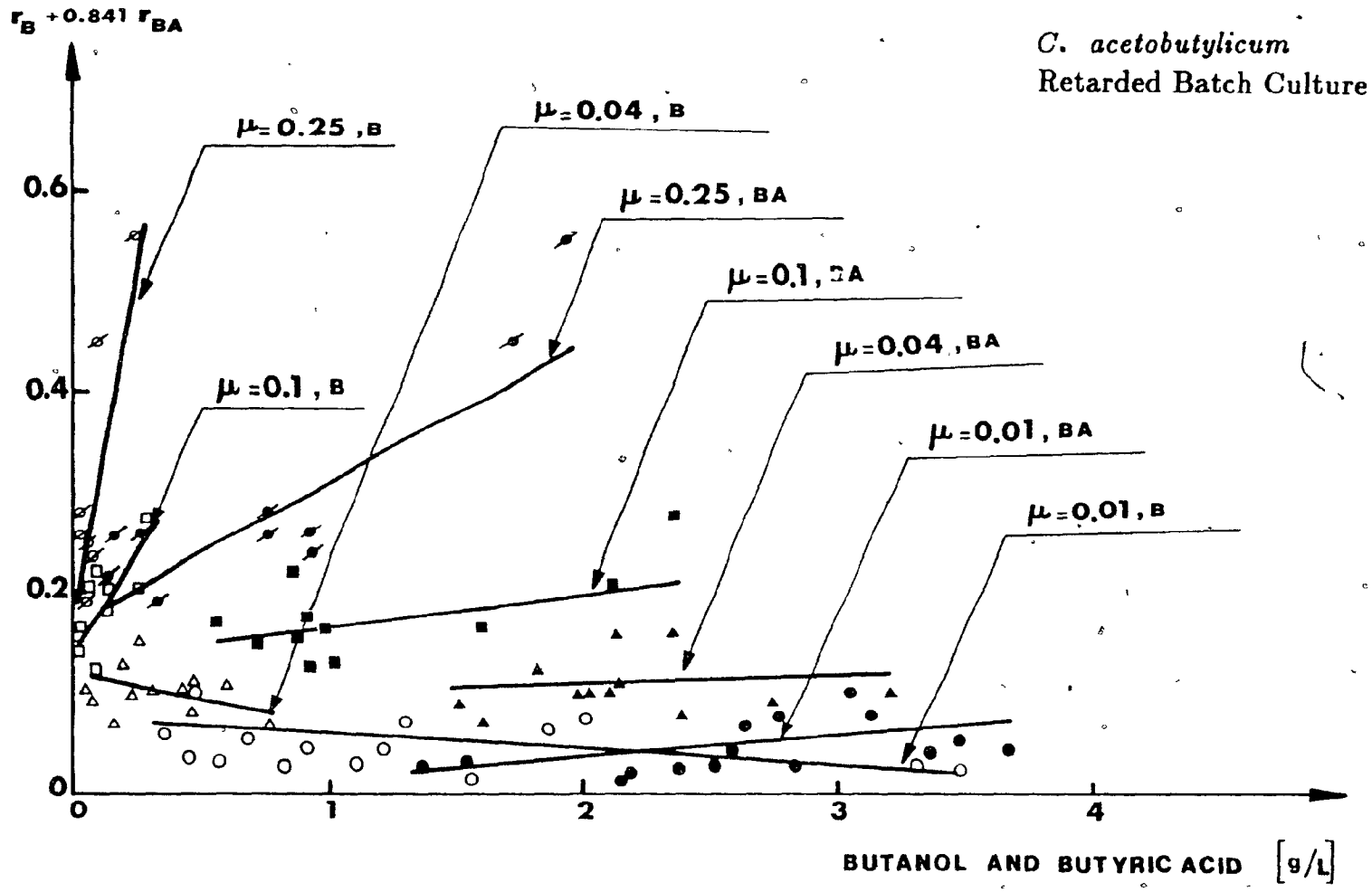
In verification of the new hypothesis, butanol and butyric acid are considered as one subsystem and their overall flux through the cellular membrane can be written as:

$$r_B + 0.841\ r_{BA} = P_1\ (B_i - B) + P_2\ (BA_i - BA) \qquad (23)$$

where P_1 and P_2 are the permeability of the cellular membrane to butanol and butyric acid, respectively, r_B and r_{BA} are the specific rates of change in concentrations of butanol and butyric acid in the system, respectively, and the coefficient 0.841 is the ratio of their respective molecular weights. Subscript i designates the intracellular conditions. This relationship was evaluated by plotting (r_B + 0.841 r_{BA}) *vs.* the concentrations of butanol (B) and butyric acid (BA) for different values of μ ranging from 0.01 h^{-1} to 0.25 h^{-1}, as presented in Figure 13.

The following features can be concluded from the graphical evaluation of the above relationship. At low values of μ (0.01 h^{-1} to 0.06 h^{-1}), where butanol production is important, the lines of (r_B+ 0.841r_{BA}) *vs.* B exhibit negative slopes. This indicates that transport of butanol is carried out by diffusion due to the chemical concentration gradient across the membrane whose permeability is important in this transport. At high values of μ, however, the butanol production is lower with subsequent increase of butyric acid

FIGURE 13 Evaluation of Equation (23) for the Retarded Fermentation.
The Parameter is the Culture Specific Growth rate (μ).



C. acetobutylicum
Retarded Batch Culture

synthesis and its transport importance. The lines for butyric acid exhibit a definite positive slope. This implies that the external butyric acid concentration (BA_{ex}) is larger than the internal butyric acid concentrations (BA_i). However, at this stage of the process, the direction of butyric acid transport is from the inside to the outside of the cell. Its transport is thus against the concentration gradient and simple diffusion due to a chemical potential gradient cannot be the only mechanism responsible for transport of the acid. At this stage of the process, most of the butyric acid in the broth exists in the form of charged ions (butyrate) and the operational force is a combination of both a chemical potential gradient and an electrical potential gradient (Snell et al., 1965).

This concept explains the transport of the acids against the concentration gradients which exist across the cell membrane at this stage of the process.

It can be seen from slopes of the lines in Figure 13 that the membrane potential is proportional to the specific growth rate (μ). At higher μ, the slope is higher. At very low μ, when the physiological activities are low, the electrical potential is low and the physical diffusion plays a more important role. At these low values of μ, the transport of butyric acid is from the outside to the inside of the cell and the positive slopes of the lines predict a higher external butyric acid concentration as compared to the inter-

nal concentration. This hypothesis appears to be valid and Equation (23) can be supplemented to account for the transport by the electrical potential gradient. Considering that this potential is proportional to μ:

$$r_B + 0.841 r_{BA} = P_1(B_i - B) + P_2(BA_i - BA) + K_D \mu \overline{BA} \qquad (24)$$

where $\overline{BA}$ is the mean value of the butyric acid solution concentration.

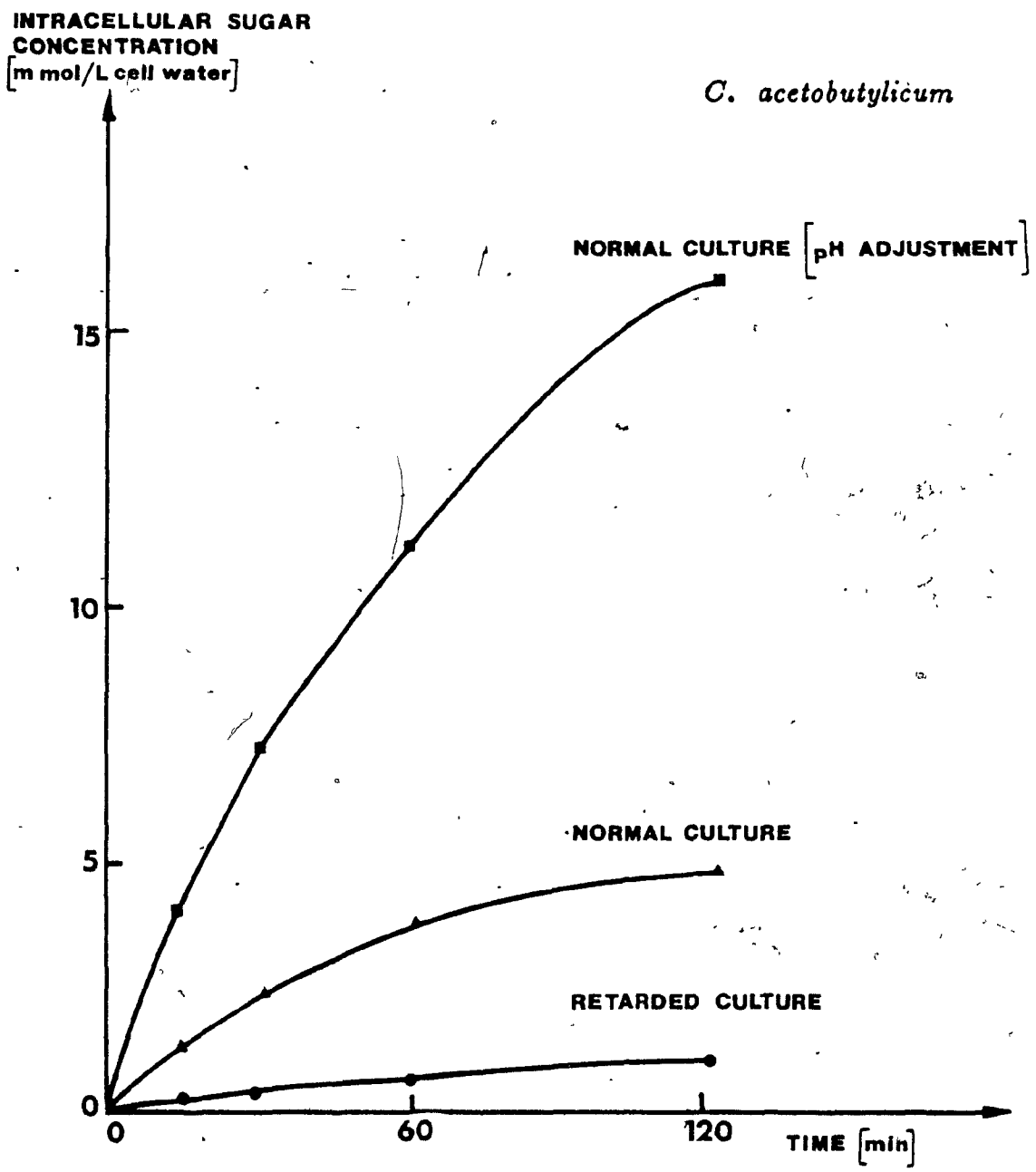
This equation describes the transport of the organic acids across the cellular membrane.

7.3.5 Cell Membrane Permeability Determination

The results of a number of independent experiments supported the hypothesis regarding the responsibility of the cell membrane function for the malfunction of the retarded culture.

One of the most revealing experiments was evaluation of the sugar transport rate through the cell membrane by measuring the uptake of the non-metabolizable 3-0-methyl-glucose (0.7 mM) by the "normal" and the "retarded" culture. The results of this experiment are presented in Figure 14 where the time dependent variations in

FIGURE 14 Time Course of the Uptake of 3-0- Methyl Glucose by the Normal and the Retarded Culture.


INTRACELLULAR SUGAR CONCENTRATION
[m mol/L cell water]
C. acetobutylicum
NORMAL CULTURE [pH ADJUSTMENT]
NORMAL CULTURE
RETARDED CULTURE
15
10
5
0
0
60
120
TIME [min]

the intracellular concentration of non-metabolizable sugar are compared for the two types of cultures.

The accumulation coefficients (concentration inside/concentration outside) and the uptake rates were considerably different for the two cultures. The initial uptake rate of the substrate in the retarded culture was 0.008 mmole/L(cell water)•min. This value changed to 0.12 mmole/L (cell water) •min for the normal culture which exhibited a 15 fold higher sugar uptake rate. An adjustment of pH in the early stages of the culture experiment from pH = 4.2 to pH = 5.0 resulted in further increase in this parameter and a value of 0.36 mmole/L (cell water)•min was obtained for the initial uptake rate of sugar. The value of the accumulation coefficient was 1.6 for the retarded culture as opposed to a value of 6.6 obtained for the normal culture. Adjustment of the medium pH increased the value of this coefficient to 23.0.

This experiment clearly shows the differences in the sugar transport rate for the two cultures and proves the importance of the transport phenomena in the control of the process.

7.4. DISCUSSION

This work demonstrates a use of systems analysis methodology in elucidation of aspects of microbial physiology dealing with a cell membrane transport mechanism. A series of consistently "substandard" fermentation experiments were analyzed by applying the especially developed mathematical models in conjunction with experimental data which would normally be discarded as useless because the abberate culture behavior could not be explained. The approach taken succeeded in locating the culture problem and quantitatively assessing as well as predicting the behavior of the cell membrane which was mainly responsible for the malfunctioning of the retarded culture.

Regulation of the solute transport is a universal cellular phenomenon common to the eucaryotic and procaryotic organisms. Holley (1972) postulated that nutrient transport regulation serves as a primary mode of growth regulation in animal cells. He has also proposed that malfunction of the cellular membrane may account for the loss of growth regulation resulting in uncontrolled proliferative abilities of transformed cells.

Importance of the integrity of the cellular membrane and its major role in the regulation of the cellular activities and viability has been also shown by Hossack et al. (1977), Thomas and Rose (1979), Panchal and Stewart (1981), and by Moreira et al. (1981). Recently, in a study of butanol toxicity in the butylic fermentation, Moreira et

al. (1981) showed that butanol at concentrations of 0.1 M to 0.15 M which is equivalent to those observed in industrial fermentations cause 50% inhibition of both the cell growth and sugar uptake rates. In a later communication Linden and Moreira (1982) pointed out that the cellular membrane is the primary site of the inhibition by butanol in C. acetobutylicum and alterations in the membrane structure and function have been responsible for the observed physiological changes in the culture.

This is consistent with conclusions presented in this work which suggest that the lower rate of sugar transport across the cell membrane is one of the major factors responsible for the substandard behavior of the "retarded culture" of C. acetobutylicum.

Sugar transport in bacteria has been the subject of numerous investigations. However, studies on glucose transport mechanism in C. acetobutylicum are virtually nonexistent. Recently, in a study of the uptake of 3-O-methylglucose in the presence of metabolic inhibitors, Moreira et al. (1981) suggested that energy in the form of ATP is coupled to its transport via the action of ATPase. The intracellular accumulations of maltose (Tuckey and Oujesky, 1976) and α-methylglucoside (Tuckey and Oujesky, 1978) in several Clostridium species have also been shown to depend on an energy-generating mechanism. The activity of the membrane-bound ATPase and the uptake of 3-O-methylglucose was

shown to be inhibited by elevated concentrations of the alcohols (Moreira et al., 1981). This inhibition was shown to be a result of an increase in the activation energy of the membrane-bound ATPase (Moreira et al., 1981; Lenaz et al., 1975) as a consequence of a conformational change of the enzyme protein due to the mobility change of the membrane lipids (Raison, 1973). In addition to the disruption of the enzymatic activities, alcohols inhibit the synthesis and function of the RNA. This effect, which is schematically depicted in Figure 10, has been shown in both procaryotic and eucaryotic organisms (So and Davie, 1964; David et al., 1983; Bohin et al., 1976). It is believed to be interrelated with the alterations in RNA transcription, translation of the genetic information, transport, or overall metabolism of RNA.

In the present work, the number of active "sites" which are responsible for the transport of sugar into the cell was taken as one of the markers of the culture physiological state. Although the knowledge of the cellular membrane active mass transport phenomenon is still vague, it is understood that the "carriers" or the membrane transport "sites" are protein molecules (Kotyk and Janacek, 1970; Leninger, 1975; Wilson and Smith, 1978) capable of reversibly binding specific substrates and transporting them across the membrane by diffusional, oscillational and rotational movements. They may also undergo conformational changes to create a "hole" in

the membrane for the specific substrate transported (Snell et al., 1965; Wilson and Smith, 1978).

The behavior of the membrane carriers and the number of these carriers have been shown to be among the most important factors affecting the function of the active transport systems and the magnitude of the accumulation coefficient of the solute (Maloney et al., 1974; Fox 1969). According to Koch (1963) and Maloney and Wilson (1973), the ratio of the internal to the external lactose concentration showed a high degree of dependence on the number of membrane carriers that were present in the transport system for this particular sugar. For example, in cells with 3% of the normal number of membrane carriers the steady state gradient of thiomethylgalactoside across the membrane was equivalent to only 15% of that found in normal cells.

This is consistent with theoretical predictions made in this work indicating that the changes in the number of membrane carriers or the activity of these carriers could alter the rate of sugar transport.

Uniformity of the experimental practices in preserving the stock cultures and in performing the fermentation experiments precludes the possibility of operational errors or malpractices being responsible for the observed malfunction of the strain of C. acetobutylicum used in this work. However, the retarded culture could have developed an altered membrane possibly as an adaptive response to some

unknown unfavorable environmental conditions. Environmentally induced changes in the lipid structure and composition of the cellular membrane have been frequently reported (Hossack and Rose, 1976; Ingram, 1976; Paterson et al., 1972). These changes may inhibit the activity of the enzymes operating in the transport system by disrupting the critical protein-lipid interactions (Grisham and Barnett, 1972; 1973; Moore et al., 1981). Alteration of the membrane composition and structure could have been enhanced by excessive intracellular accumulation of alcohols, butanol and ethanol, possibly due to a lower permeability of the cellular membrane to these products. Alcohols and other organic solvents have been recognized as major perturbances of the cellular membrane function (Grisham and Barnett, 1972; 1973; Paterson et al., 1972; Rigomier et al., 1980; Moreira et al., 1981). They dissolve in the lipid bilayer and increase the bilayer fluidity, disrupting thus the critical lipid-protein interactions and the energy-generating mechanisms for nutrient transport (Sullivan et al., 1974; Lenaz et al., 1975).

A lower permeability of the cellular membrane to the alcohols and acids, especially butanol and butyric acid, has been suggested by the theoretical analysis in the present work to be another physiological parameter responsible for the behavior of the retarded culture. This concept suggests that an excessive intracellular accumulation of the alcohols and organic acids occurs during the early stages of the

fermentation and disrupts the normal function of the culture.

It has been well established that the pH value of the fermentation medium plays an extremely important role in the acetone-butanol fermentation affecting the solvent synthesis and other cellular activities. A pH increase was shown in this work to increase the rate of sugar uptake and the magnitude of the sugar gradient across the cellular membrane as presented in Figure 14. A low pH value of less than 4.0 can be responsible for a complete arrest of the fermentation (Ulmer et al., 1981). This effect has been shown by Wynne (1931) to be related to the increased toxicity of the fermentation acids, butyric and acetic acid, at low pH values. A too high pH (>5.4) on the other hand, hinders the synthesis of the solvents (Andersch et al., 1982; Gotschalk and Bahl, 1981). This effect has been attributed to the requirements of a critical level of undissociated butyric acid which seems to be an essential factor in the regulation of solvents synthesis (Monot and Engasser, 1983; Bahl et al., 1982). The necessity of pH control and the optimal level of pH in the acetone-butanol fermentation have been frequently reported (Moreia et al., Andresch et al., 1982; Leung and Wang, 1981). The observed effect of pH on the transport of sugar in this work may be related to its respective effect on the activity of the enzymes operating in the sugar transport system. Most enzymes have a characteristic pH at which their

activity is optimal (Leninger, 1978). The adjustment of the extracellular pH might have shifted the intracellular pH to the values closer to the optimal levels facilitating thus a better transport of sugar. pH also affects the lipid composition of the membrane. Study of the effect of acidity of the medium on the phospholipid composition of Staphylococus-aureus showed that the content and composition of membrane phospholipids were greatly dependent on the pH of the medium (Heast et al., 1972). A pH-induced alteration of the lipid composition with a subsequent effect on the activity of the enzymes operating in the sugar transport system may also be responsible for the observed increase of the sugar transport rate in C. acetobutylicum.

Evaluation of the transport processes for solvents and acids, as discussed in this work, showed that the rates for the transport of solvents across the cellular membrane are directly proportional to their respective concentration gradients. For the acids, however, the membrane potential had to be considered as an additional driving force.

Transport of neutral, uncharged species or charged ionic species across membranes has been frequently discussed (Snell et al., 1965; Kotyk and Janacek, 1970).

The force acting upon a neutral constitutent i across the membrane is equal to $\frac{-\Delta\mu_i}{\Delta x}$ where $\Delta\mu_i$ is the chemical potential difference across the membrane and its gradient

in x direction comprises a force acting on this component. In terms of the concentrations on sides 1 and 2 of the membrane, this force can be expressed according to Snell et al. (1965) as:

$$\frac{\Delta \mu_i}{\Delta x} = \frac{RT}{\Delta x} \ln \frac{C_{i_2}}{C_{i_1}} \cong \frac{RT}{\overline{C}_i} \frac{\Delta C_i}{\Delta x} \qquad (25)$$

where $\overline{C}_i$ represents some sort of mean concentration over the interval Δx. The diffusion flux of i across unit area of a membrane m is:

$$J_i^m = \omega_i^m \overline{C}_i^m F_i = -\omega_i^m \overline{C}_i^m \frac{\Delta \mu_i}{\Delta x} = - \frac{\omega_i^m \overline{C}_i^m RT}{\overline{C}_i} \frac{\Delta C_i}{\Delta x} \qquad (26)$$

where ω_i^m is the mobility of substance i within the membrane and $\overline{C}_i^m$ is the mean concentration of i within the membrane phase; $\overline{C}_i$ refers to some mean concentration over the interval Δx derived from the solution concentrations, and would not generally equal $\overline{C}_i^m$.

F is the force operating in x direction. It is assumed that the membrane offers by far the greatest resistance to diffusion. Therefore, at each binding surface there exists an equilibrium distribution of concentrations

between the solution phase and the membrane phase. So, C_i^m would be equal to $K_i C_i$ where K_i is an equilibrium distribution coefficient. The flux of component i across the membrane could then be expressed as:

$$J_i^m = -\omega_i^m K_i RT \frac{\Delta C_i}{\Delta x} = -\Omega_i^m RT \frac{\Delta C_i}{\Delta x} \qquad (27)$$

Ω_i^m stands for the modified mobility defined as $\omega_i^m K_i$.

In biological boundaries the thickness Δx is not a quantity that can be given a precise value or meaning. It is convenient therefore to lump this term together with RT into Ω_i and define the permeability coefficient as $P_i^m = \Omega_i RT/\Delta X$. Equation (27) would then assume the following form:

$$J_i^m = -P_i^m \Delta C_i \qquad (28)$$

In summary, it may be said that the total net flux of a neutral uncharged constituent across a membrane is proportional to the concentration difference across this membrane, the proportionality coefficient being the permeability coefficient (Snell et al., 1965).

Considering the transport of ions across membranes, an additional thermodynamic force of electrical potential difference must be added to the previous concept. In

general, the forces acting on electrolytes are both chemical potential gradients and electrical potential gradients (Snell et al., 1965):

$$F_{KX} = \frac{-d\mu_K}{dx} - Z_K F \frac{d\Psi}{dx} \qquad (29)$$

where F is the Faraday number, Z_K bears the algebraic sign of the charge and the last term represents the gradient of the electrical potential for an ionic species K. The flux of this ion across a membrane would then be:

$$J_K^m = -\omega_K^m C_K^m \left[\frac{\Delta\mu_K}{\Delta x} + Z_K F \frac{\Delta\Psi}{\Delta x}\right] \qquad (30)$$

or

$$J_K^m = -\Omega_K^m RT \left[\frac{\Delta C_K}{\Delta x} + \frac{Z_K \bar{C}_K F}{RT} \frac{\Delta\Psi}{\Delta x}\right] \qquad (31)$$

again the coefficient $\frac{\Omega_K^m RT}{\Delta x}$ can be combined into a single permeability coefficient P_K^m, to give:

$$J_K = -P_K^m \left(\Delta C_K + \frac{Z_K \bar{C}_K F}{RT} \Delta\Psi\right) \qquad (32)$$

This equation describes transport of an ionic species across a membrane which results from a concentration difference as well as an electrical potential difference.

Movement of ions as a response to the electrical potential across biological membranes has been frequently demonstrated. In a study of cation transport in Streptococcus faecalis, Harold and Papineau (1972) showed that the metabolic uptake of ions resulted from the generation of an electric field across the cellular membrane. The uptake of several cations or anions by intact mitochondria (Bakeena et al., 1970) or submitochondrial particles (Grinius et al., 1970) has also been due to the membrane potential.

Equation (32) is similar to equation (24) which was proposed in this work to describe the transport of butanol and butyric acid across the cellular membrane. The membrane potential was considered to be proportional to the specific growth rate of the culture as seen in Figure 13. The proportionality of the membrane electrical potential to the level of cellular activities was also pointed out by Silver (1978) in a study of ion transport in E. coli. Obviously, electrical potential of the cellular membrane plays an important role in the behavior and metabolic performance of the culture.

The novel approach to the analysis of physiological culture phenomena presented in this work is a pioneering

attempt in the use of the method of systems analysis. It demonstrated the use of systems analysis in evaluation of individual reaction steps which related the culture behavior to the basic physical and physiochemical principles. Elucidation of the cellular transport phenomena brings a new quality to the understanding of the culture performance and contributes to better appreciation of the role of key physiological parameters which control the "normal" and "abnormal" behavior of the culture during a fermentation process.

7.5 NOMENCLATURE

Symbol	Meaning
B	Butanol concentration in the fermentation broth
B_i	Intracellular butanol concentration
BA	Butyric acid concentration in the fermentation broth
BA_i	Intracellular butyric acid concentration
C_A	Concentration of the substance (A)
C_{AX}	Concentration of the complex (AX)
C_T	Total concentration of the cellular membrane constituent (X)
C_X	Concentration of the cellular membrane constituent (X)
F	Faraday number
J_{AX}	Flux of AX through the cellular membrane
K	Equilibrium constant defined by Equation (2)
k_1	Constant in Equation (7)
K_S	Mónod constant

n	Number of the active transport "sites" in the cellular membrane
P	The cell membrane permeability
r_B	Specific rate of butanol production
r_{BA}	Specific rate of butyric acid accumulation
r_S	Specific rate of substrate utilization
S	Substrate (glucose) concentration
t	Time
X	Biomass concentration
y	Marker of the physiological state of the culture

Greek Letters

$\Delta\phi$	Electrical potential difference across the membrane
$\Delta\mu$	Chemical potential difference across the membrane
Δx	Membrane thickness
μ	Specific culture growth rate
ω_i	Mobility of substance i within the membrane
Ω_i	Modified mobility

7.6 REFERENCES

1. Andersch, W. 1982. H. Bahl and G. Gottschalk. 1982. Acetone-Butanol Production by Clostridium acetobutylicum, Biotechnol. Letters, 4, 29-32.

2. Bahl, H., W. Andersch, K. Braun and G. Gottschalk. 1982. Effect of pH and Butyrate Concentration on the Production of Acetone and Butanol by Clostridium acetobutylicum grown in Continuous Culture, Eur. J. Appl. Microbiol. Biotechnol. 14, 17-20.

3. Bailey, J.E. and D.F. Ollis. 1977. Biochemical Engineering Fundamentals, McGraw Hill, 267-273.

4. Bakeena, L.E., L.L. Grinius, A.A. Jasaitis, V.V. Kuliene, D.O. Levitsky, E.A. Liberman, I.I. Severina and V.P. Skulachev. 1970. Conversion of Biomembrane-Produced Energy into Electric Form, 2: Intact Mitochondria., Biochim. Biophys. Acta, 216, 13-21.

5. Bohin, J.P., D. Rigomier and P. Schaeffer. 1976. Ethanol Sensitivity of Sporulation in Bacillus subtilis: A New Tool for the Analysis of the Sporulation Process, J. Bacteriol., 127, 934-940.

6. David, E.T., I. Fischer and K. Moldane. 1983. Studies on the Effect of Ethanol on Eukaryotic Protein Synthesis in Vitro, J. Biolog. Chem. 258, 7702-7706.

7. Fox, C.F. 1969. A Lipid Requirement for Induction of Lactose Transport in Escherichia coli, Biochem., 63, 850-855.

8. Ginius, L.L., A.A. Jasaitis, Y.P. Kadjiauskas, E.A. Liberman, V.P. Skulachev, V.P. Topali, L.M. Trofina and M.A. Vladimirova. 1970. Conversion of Biomembrane-Produced Energy into Electric Form, Biochim. Biophys. Acta, 216, 1-12.

9. Gottschal, J.C. and J.G. Morris. 1981. Non-Production of Acetone and Butanol by Clostridium acetobutylicum during Glucose - and Ammonium - Limitation in Continuous Culture, Biotechnol. Letters 3, 525-530.

10. Gottschalk, G. and H. Bahl. 1981. In: A Hollaender (ed.). Trends in Biology of Fermentations for Fuels and Chemicals (Basic Life Sciences 18), Plenum Press, N.Y., 463-471.

11. Grimbert, L. 1893. Fermentation Anaerobic Produite par le Bacillus orthobutylicus, ses Variations sous Certaines Influences Biologiques, Ann. Inst. Pasteur, 7, 353-402.

12. Grisham, C.M. and R.E. Barnett. 1972. The Relationship of Membrane and Protein Structure in the Functioning of the (Na^++ K^+) - Activated ATPase, Biochim. Biophys. Acta, 266, 613-624.

13. Grisham, C.M. and R.E. Barnett, 1973. The Effects of Long-Chain Alcohols on Membrane Lipids and the (NA^++k^+)-ATPase, Biochim. Biophys. Acta, 311, 417-422.

14. Haest, C.W.M., J. DeGier, J.A.F. Op Den Kamp, P. Bartels and L.L.M. Van Deenen. 1972. Changes in Permeability of Staphylococcus aureus and Derived Liposomes with Varying Lipid Composition, Biochim. Biophys. Acta, 255, 720-733.

15. Harder, A. and J.A. Roels. 1982. Application of Simple Structured Model in Bioengineering, Advances Biochem. Eng. 21, 56-103.

16. Harold, F.M. and D. Papineau. 1972. Cation Transport and Electrogenesis by Streptococcus faecalis, 1: The Membrane Potential, J. Membrane Biol. 8, 27-44.

17. Holley, R.W. 1972. A unifying Hypothesis Concerning the Nature of Malignant Growth, Proc. Natl. Acad. Sci. USA, 69, 2840-2841.

18. Hossack, J.A. and A.H. Rose. 1976. Fragility of Plasma Membranes in Saccharomyces cerevisiae Enriched With Different Sterols, J. Bacteriol. 127, 67-75.

19. Hossack, J.A., D.M. Belk and A.H. Rose. 1977. Environmentally-Induced Changes in the Neutral Lipids and Intracellular Vesicles of Saccharomyces cerevisiae and Kluyveromyces fragilis, Arch. Microbiol., 114, 137-142.

20. Ingram, L.O. 1976. Adaptation of Membrane Lipids to Alcohols, J. Bacteriol, 125, 670-678.

21. Jobses, I.M.L. and J.A. Roels. 1983. Experience with Solvent Production by Clostridium beijerinckii in Continuous Culture, Biotechnol. Bioeng. 25, 1187-1194.

22. Koch, A.L. 1964. The Role of Permease in Transport, Biochem. Biophys. Acta, 79, 177-200.

23. Kotyk, A. and K. Janacek. 1970. Cell Membrane Transport, Plenum Press, New York, 183-199.

24. Krouwel, P.G., W.J. Groot and N.W.F. Kossen. 1983. Continuous IBE Fermentation by Immobilized Growing Clostridium beijerinckii Cells in a Stirred-Tank Fermentation, Biotechnol. Bioeng. 25, 281-299.

25. Kutzenok, A. and M. Aschner. 1952. Degenerative Processes in a Strain of Clostridium butylicum, J. Bacteriol, 4, 829-836.

26. Lehninger, A.L. 1978. Biochemistry, Worth, New York, 195-196.

27. Lenaz, G., G. Parenti - Castelli and A.M. Sechi. 1975. Lipid Protein Interactions in Mitochondria, Arch. Biochem. Biophys. 167, 72-79.

28. Leung, J.C.Y. and D.I.C. Wang. 1981. Production of Acetone and Butanol by Clostridium acetobutylicum in Continuous Culture Using Free Cells and Immobilized Cells, Proc. 2nd World Cong. Chem. Eng., 348-354.

29. Linden, J.C. and A. Moreira. 1982. In A. Hollaender, A.I. Laskin and P. Rogers (eds). Biological Basis for New Developments in Biotechnology, Plenum Press Inc., New York, N.Y. 377-404.

30. Maloney, P.C., E.R. Kashket and T.H. Wilson. 1974. Methods in Membrane Biology, 5, 1-49.

31. Maloney, P.C. and T.H. Wilson. 1975. ATP Synthesis Driven by a Protonmotive Force in Streptococcus lactis, J. Membrane Biol. 25, 285-310.

32. Monot, F. and J.M. Engasser. 1983. The Acetone-Butanol Fermentation and Related Topics, J.D. Bu'Lock and A.J. Bu'Lock (eds.) 117-119.

33. Moore, B.M., B.R. Lentz, M. Hoechli and G. Meissner. 1981. Effect of Lipid Membrane Structure on the Adenosine's -Triphosphate, Hydrolyzing Activity of the Calcium-Stimulated ATPhase of Sarcoplasmic Reticulum, Biochem., 20, 6810-6217.

34. Moreira, A.R., D.C. Ulmer and J.C. Linden. 1981. Butanol Toxicity in the Butylic Fermentation, Biotechnol. Bioeng. Symp. No. 11, 567-579.

35. Panchal, C.J. and G.G. Stewart. 1981. Regulatory Factors in Alcohol Fermentations, Proc. Int. Yeast Symp., 9-15.

36. Paterson, S.J., K.W. Butler, P. Huong, J. Labelle, I.C.P. Smith and H. Schneider. 1972. The effects of Alcohols on Lipid Bilayers: A Spin Label Study, Biochim. Biophys. Acta, 266, 597-602.

37. Raison, J.K. 1973. Effect of Temperature-Induced Phase Changes on the Kinetics of Respiratory and Other Membrane-Associated Enzyme Systems, J. Bioenerg. 4, 285-309.

38. Rigomier, D., J.P. Bohin and B. Lubochinsky. 1980. Effects of Ethanol and Methanol on Lipid Metabolism in Bacillus subtilis, J. Gen. Microbiol. 121, 139-149.

39. Rosen, B.P. and E.R. Kashet. 1978. Bacterial Transport, B.P. Rosen (ed.) 559-612.

40. Silver, S. 1978. Bacterial Transport, B.P. Rosen (ed.) 221-308.

41. Snell, F.M., S. Schulman, R.P. Spencer and C. Moos. 1965. Biophysical Principles of Structure and Function, Addison-Wesley Pub. Co. Inc., 288-325.

42. So, A.G. and E.W. Davie. 1964. The Effects of Organic Solvents on Protein Biosynthesis and their Influence on the Amino Acid Code, Biochemistry, 3, 1165-1169.

43. Sullivan, K.H., M.K. Jain and A.L. Koch. 1974. Activation of the β. Galactoside Transport System in Escherichia coli, ML-308 by n-Alkanols, Biochim. Biophys. Acta, 352, 287-297.

44. Thomas, D.S. and A.H. Rose. 1979. Inhibitory Effect of Ethanol on Growth and Solute Accumulation by Saccharomyces cerevisiae as affected by Plasma-Membrane Lipid Composition, Arch. Microbiol., 122, 49-55.

45. Tuckey, J. and H. Oujesky. 1976. Inducible Enzymes Involved in Maltose Metabolism in Five Speices of Clostridium, Dev. Ind. Microbiol., 17, 387-397.

46. Tuckey, J. and H. Oujesky. 1978. Mechanism of Intracellular Accumulation of α - Methylglucoside by Clostridium tetani, Clostridium sphenoides and Clostridium sporogenes, Dev. Ind. Microbiol., 19, 283-280.

47. Ulmer, D.C., J.C. Linden and A.R. Moreira. 1981. The Effect of Ethanol and Butanol on Sugar Transport and Viability of Clostridium Species Bacteria, Ann. Report, Dept., of Chem. Eng., Colorado State U. 1-77.

*48. Votruba, J., B. Volesky and L. Yerushalmi, Development of a Mathematical Model for the Batch Acetone-Butanol Process, Biotechnol. Bioeng. (submitted for publication).

49. Wilson, D.B. and J.B. Smith. 1978. Bacterial Transport, B.P. Rosen (ed.) 520-521.

50. Wynne, A.M. 1931. Inhibition of the Acetone-Butyl Alcohol Fermentation, J. Bacteriol., 22, 209-237.

*51. Yerushalmi, L. and B. Volesky. Importance of Agitation in the Acetone-Butanol Fermentation, Biotechnol. Bioeng., accepted for publication January 7, 1985.

8

FERMENTATION PROCESS DIAGNOSIS USING A MATHEMATICAL MODEL

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8.1 INTRODUCTION

In a previous communication the use of systems analysis was demonstrated in investigation of the behavior of a C. acetobutylicum culture in a fermentative process. A strain of the culture produced consistently atypical fermentations characterized by low solvent production yields and a low sugar conversion efficiency (Yerushalmi et al., 1985*). The systems analysis approach used in analyzing the fermentation results included a study of transport mechanisms for sugar, solvents and acids through the cellular membrane. The problems associated with permeability and a transport function of the cellular membrane were found to be among the major factors affecting the performance of the microbial culture. The present work continues the analysis of the culture performance in the A-B-E fermentation focusing on the formulation of a sophisticated mathematical model capable of describing the process dynamics and the culture behavior during the complete (successful) and the "retarded" fermentations.

Unlike the previous applications of mathematical modelling in fermentation processes which has usually been

for purposes of process control and optimization, or for the development of alternative processes, this model demonstrates a new potential as a "diagnostic tool" for elucidiating process failure conditions. This approach has been taken for the specific purpose of explaining the reason of the incomplete acetone-butanol fermentation. The kinetic model developed in our previous work (Votruba et. al., 1985)* for the batch acetone-butanol fermentation was used in this work as a starting point for developing the new improved model for a microbial culture performance.

8.2 MATERIALS AND METHODS

Experimental conditions, medium composition and the techniques used in assays of glucose, biomass, solvents and acids were as described earlier (Yerushalmi et al., 1985)*.

8.2.1 Cell Membrane Permeability Measurement

0.5 - 1.0 gram of wet cells was harvested in the stationary growth phase, centrifuged at 13,000g and washed twice with basal medium. The cells were then suspended in a 0.6% butanol solution or a 0.4% butyric acid solution. After half an hour they were harvested again by centrifugation and transferred into a syringe equipped with a filter holder and a glass fiber filter (Whatman GF/F) containing 3mL to 4mL of

basal medium. Before transferring the packed cells into the syringe a small and known portion of the cells was taken for the estimation of the initial concentration of butanol or butyric acid in the cell.

The cell suspension in the syringe was vigorously shaken and the sampling was started immediately. The concentrations of butanol or butyric acid in these cell-free samples were estimated by chromatographic analysis. The cell membrane permeability coefficient was calculated based on the following equation:

$$P = \frac{\alpha r}{\Delta C} = \frac{\alpha r}{C_i - C_{ex}}$$

where:

α is the ratio between the dry cell and the wet cell weight,

r is the specific rate of permeation defined as

$$\frac{1}{X} \frac{dC_{ex}}{dt},$$

C_i and C_{ex} are the internal and the external concentrations, respectively.

Since the measurement of internal concentrations at different times was not practical, and also due to the volume change in the syringe, this estimation is based on the initial rate of change in the value of C_{ex}. The extracellular

water volume was assumed to be 28% of the total wet cell weight as defined previously (Yerushalmi et. al., 1985)*.

8.2.2 Measurement of the Cell Size

A Leitz Dialux 20 Microscope equipped with a micrometer, measuring eyepiece, phase contrast optics and a Nikon camera was used in the study of morphology and for the estimation of the cell size. The values reported in this paper are the average lengths of 67 to 277 bacterial cells representing the cell population during different stages of the fermentation. Two independent images were used at each measuring time.

8.3 RESULTS

8.3.1 Formulation of the Mathematical Model

A mathematical model was formulated based on the previous kinetics model developed for the A-B-E fermentation process.

The concept of the physiological state of a microbial culture, outlined and discussed in a previous work (Votruba et al., 1985)* has also been considered as well as knowledge of the transport mechanism in the cellular sys-

tem (Snell et al., 1965; Yerushalmi et al., 1985*). The new model is based on process mass balances around the bioreactor system which are presented in the following paragraphs.

Variations of the physiological state of the culture which is described by the dimensionless RNA concentration (y) can be expressed by the following differential equation:

$$\frac{dy}{dt} = k_{G1} r_S \bar{y} - 0.56\ (y-1)y - k_{G2} B_i \qquad (1)$$

where the first term on the right hand side represents the synthesis of RNA and the last term is the decay of RNA because of the butanol inhibition. The second term describes the change of RNA concentration in the viable phase due to the cell growth.

Since the decay of the cellular activities is considered in the dynamics of y (Equation (1)), the expression for the culture growth can assume a simpler form as follows:

$$\frac{dX}{dt} = 0.56\ (y-1)X \qquad (2)$$

The sugar utilization rate was shown to be dependent on the transport of sugar through the cellular membrane which is characterized by the number of transport "sites" (Yerushalmi et al., 1985)*:

$$\frac{dS}{dt} = \frac{k_1 S}{S+K_{S_1}} \cdot nX = \frac{n'S}{S + K_{S_1}} \cdot X \qquad (3)$$

where n is the number of the active transport sites.

For the dynamics of the number of active sites (n), the following equation can be written:

$$\frac{dn'}{dt} = k_n \frac{K_I}{K_I + B_i} y - 0.56\ (y-1)n' \qquad (4)$$

where the first term on the right hand side represents the rate of butanol inhibited synthesis of active sites during the growth of cells. This rate is proportional to the dimensionless concentration of RNA which has been taken as a marker of the culture physiological state. The second term is the dilution of active sites caused by increasing number of the live phase (wet biomass) in the fermentor.

Ethanol mass balances

(a) A mass balance of the internal ethanol concentration in the volume of wet biomass can be expressed as follows:

$$\frac{d(E_i V)}{dt} = k_E r_S V + P(E_{ex} - E_i)V \qquad (5)$$

where E_i and E_{ex} are the internal and the external ethanol

concentrations, respectively, and V is the wet biomass volume. P is the cell membrane permeability based on the wet volume of the cell. Assuming that $X = \alpha V$ where α is the fraction of dry mass in live cells, Equation (5) can be extended as follows:

$$\frac{X}{\alpha}\frac{dE_i}{dt} + \frac{E_i}{\alpha}\cdot\frac{dX}{dt} = \frac{k_E r_S}{\alpha} X + \frac{P}{\alpha}(E_{ex}-E_i)X \qquad (6)$$

or:

$$\frac{dE_i}{dt} = k_E r_S + P(E_{ex} - E_i) - \mu E_i \qquad (7)$$

The first term in the right-hand side of Equation (7) represents the rate of ethanol production related to the sugar utilization rate. The second term expresses the diffusion of ethanol out of the cell and the third term is a dilution term expressing the change of ethanol concentration with changing volume of biomass during the growth.

b) The external ethanol concentration is a function of the transport of ethanol out of the cell. A mass balance of the external ethanol concentration results in the following equation:

$$\frac{dE_{ex}}{dt} = \frac{PX}{\alpha}(E_i - E_{ex}) \qquad (8)$$

Butyric acid balances

a) Internal concentration of butyric acid in the wet biomass volume can be expressed by:

$$\frac{dBA_i}{dt} = k_{BA_1} r_S \frac{K_I}{K_I + B_i} - k_{BA_2}\left(\frac{BA_i}{BA_i + K_{BA}}\right)\left(\frac{S}{K_{S_2} + S}\right) +$$

$$+ P(BA_{ex} - BA_i) - \mu BA_i - K_D \mu \overline{BA} \qquad (9)$$

The first term is the synthesis rate of butyric acid which is dependent on the sugar utilization rate and is inhibited by butanol, the second term describes the conversion of butyric acid to butanol, the third term is butyric acid transport out of the cell by diffusion, the fourth term is a dilution term (similar to the one in Equation (7)), and the last term describes the transport rate of butyric acid by the electrical potential gradient which, as shown before (Yerushalmi et. al., 1985)*, may be taken as proportional to μ.

(b) External concentration of butyric acid can be described by:

$$\frac{dBA_{ex}}{dt} = \frac{PX}{\alpha'} (BA_i - BA_{ex}) + \frac{K_D}{\alpha} \mu\overline{BA}\ X \qquad (10)$$

which implies that the external BA occurence results solely from the transport of BA_i out of the cell which is controlled by diffusion and the electrical potential force.

Butanol Balances:

(a) Butanol production was considered to occur in a subsystem which consists of butyric acid + butanol. Considering this assumption the internal concentration of butanol in the wet biomass volume can be described as:

$$\frac{dB_i}{dt} = k_B r_S - 0.841 \left(r_S\, k_{BA_1} \frac{K_I}{K_I + B_i} - \right.$$

$$\left. + k_{BA_2} \frac{BA_i}{BA_i + K_{BA}} \frac{S}{K_{S_2} + S}\right) + P(B_{ex} - B_i) - \mu B_i \qquad (11)$$

The first term in this equation describes the total sugar accessible for the (butanol + butyric acid) subsystem which is subsequently divided into two parts. The fraction of sugar which supports butyric acid production is described by the second term. The third term is the transport between the growth medium and the cell interior and the fourth term is the dilution term for increase of the wet cell volume.

(b) The presence of external butanol is a result of transport of the internal butanol out of the cell. Dynamics of the external butanol concentration can be described as:

$$\frac{dB_{ex}}{dt} = \frac{PX}{\alpha} (B_i - B_{ex}) \qquad (12)$$

Acetic Acid Balances:

(a) The subsystem for acetone + acetic acid is similar to the subsystem for butanol + butyric acid synthesis with the exception that the acetic acid synthesis is inhibited by butanol and not by acetone. In such a way this subsystem is coupled with butanol synthesis on the level of inhibition (negative effect of butanol on the subsystem). The transport of acetic acid across the cellular membrane takes place via diffusion due to the concentration gradient and via the electrical potential gradient. A differential mass balance for acetic acid results in the following expression for the internal concentration of acetic acid:

$$\frac{dAA_i}{dt} = k_{AA_1} r_S \frac{K_I}{K_I+B_i} - k_{AA_2} \frac{AA_i}{AA_i+K_{AA}} +$$

$$+ P(AA_{ex}-AA_i) - \mu AA_i - K_D \mu \overline{AA} \qquad (13)$$

(b) External acetic acid concentration is reflected in the following differential mass balance:

$$\frac{dAA_{ex}}{dt} = \frac{PX}{\alpha} (AA_i - AA_{ex}) + \frac{K_D}{\alpha} \mu \overline{AA} X \qquad (14)$$

The model for the dynamics of the external acetic acid concentration is similar to the one for the external butyric acid concentration.

Acetone Balances:

(a) The subsystem for the biosynthesis of acetone is similar to the butanol + butyric acid subsystem. However, the negative (inhibitory) effect is on the level of butanol and not acetone. The internal acetone concentration in wet biomass can be expressed by:

$$\frac{dA_i}{dt} = k_A r_S - 0.484 \left(r_S k_{AA_1} \frac{K_I}{K_I + B_i} - k_{AA_2} \frac{AA_i}{AA_i + K_{AA}}\right) + P(A_{ex} - A_i) - \mu A_i \qquad (15)$$

The biochemical interpretation of all the parameters in this equation is similar to what was described in the butanol model.

(b) The external acetone concentration change can be described by:

$$\frac{dA_{ex}}{dt} = \frac{PX}{\alpha} (A_i - A_{ex}) \qquad (16)$$

The external acetone production rate is described by the transport from the interior of the cell to the external medium.

The system of the ordinary differential equations developed here represents a new mathematical model for the batch acetone-butanol fermentation. This model differs from the previous kinetic model (Votruba et al., 1985)* in that it contains the cellular physiological parameters and the intracellular and the extracellular culture conditions. The new model was termed the "Physiological State" model to be distinguished from the previous "Process Kinetics" model.

The "Physiological State" model was utilized for further investigation of the role of the cellular membrane and the transport phenomena in the performance of the culture. This was done by computer simulation of the "successful" and the "unsuccessful" fermentations and by subsequent evaluation of model parameters under various culture conditions.

8.3.2 Evaluation of Model Parameters

A. Normal Fermentation

In order to solve the model equations the numerical values of the model coefficients had to be determined. For determination of the unknown kinetic coefficients, the method of non-linear regression of a mathematical model was utilized (Pazoutova et al., 1981; Yerushalmi et al., 1985*). The kinetic coefficients were first estimated in such a way that the model could predict the behavior of the culture in a normal fermentation. This estimation used the experimental results of a normal fermentation with an initial sugar concentration of 30 g/L and with no pH control or adjustment during the entire course of fermentation. This particular experiment was chosen since most of the "unsuccessful" fermentations were performed under similar conditions. Table 1 presents the results of this parameter estimation for the normal culture. A method of parametric sensitivity (Votruba, 1982; Votruba et al., 1985*) was utilized to evaluate the degree of significance and the sequence of importance of the individual model parameters. The results of this sensitivity analysis are also presented in Table 1. It can be seen that, as with the Process Kinetics Model (Votruba et al., 1985*), the highest sensitivity in the Pnysiological State Model presented here is exhibited by the coefficients k_B, k_{BA}, and k_{G_1}, characterizing respectively the kinetics of butanol and butyric acid production and biomass growth. Second in the order of sensitivity are the coefficients k_n, k_{BA_2} and P

TABLE 1

PHYSIOLOGICAL STATE MODEL FOR THE BATCH A-B-E PROCESS

Values of Process Parameters (Coefficients) and their Parametric Sensitivities

PARAMETER	PARAMETER VALUE	ABSOLUTE PARAMETRIC SENSITIVITY	RELATIVE PARAMETRIC SENSITIVITY
k_{G1}	0.165	-260.537	2.14
k_{G2}	0.029	49.09	0.071
k_n	0.178	-231.74	2.05
k_E	0.038	-3.21	0.006
k_B	0.607	391.00	11.81
k_{BA_1}	0.78	-181.82	7.06
k_{BA_2}	0.23	72.53	0.83
k_A	0.35	- 1.40	0.02
k_{AA_1}	0.521	7.03	0.18
k_{AA_2}	0.093	- 0.1271	0.06
K_I	1.5	-3.70	0.28
K_{S_1}	2.0	3.02	0.30
K_{BA}	0.5	-7.59	0.19
K_{AA}	0.5	0.78	0.02
P	0.17	-44.60	0.36
K_D	1.56	1.19	0.09
K_{S_2}	0.031	0.20	0.0003

which characterize respectively the dynamics of the number of the membrane active sites, the conversion of butyric acid to butanol, and the membrane permeability. It is important to note that these parameters characterize the phenomena which were found responsible for the unusual performance of the microbial culture in the retarded fermentations.

The results of a computer simulation of a normal A-B-E process using the Physiological State Model is illustrated in Figure 1. The lines represent the simulated process and the points represent the experimental values. A statistical F-test for the level of significance $\alpha = 0.05$ exhibited an excellent agreement between the simulated and the experimental results (Table 2).

B. Retarded Fermentation

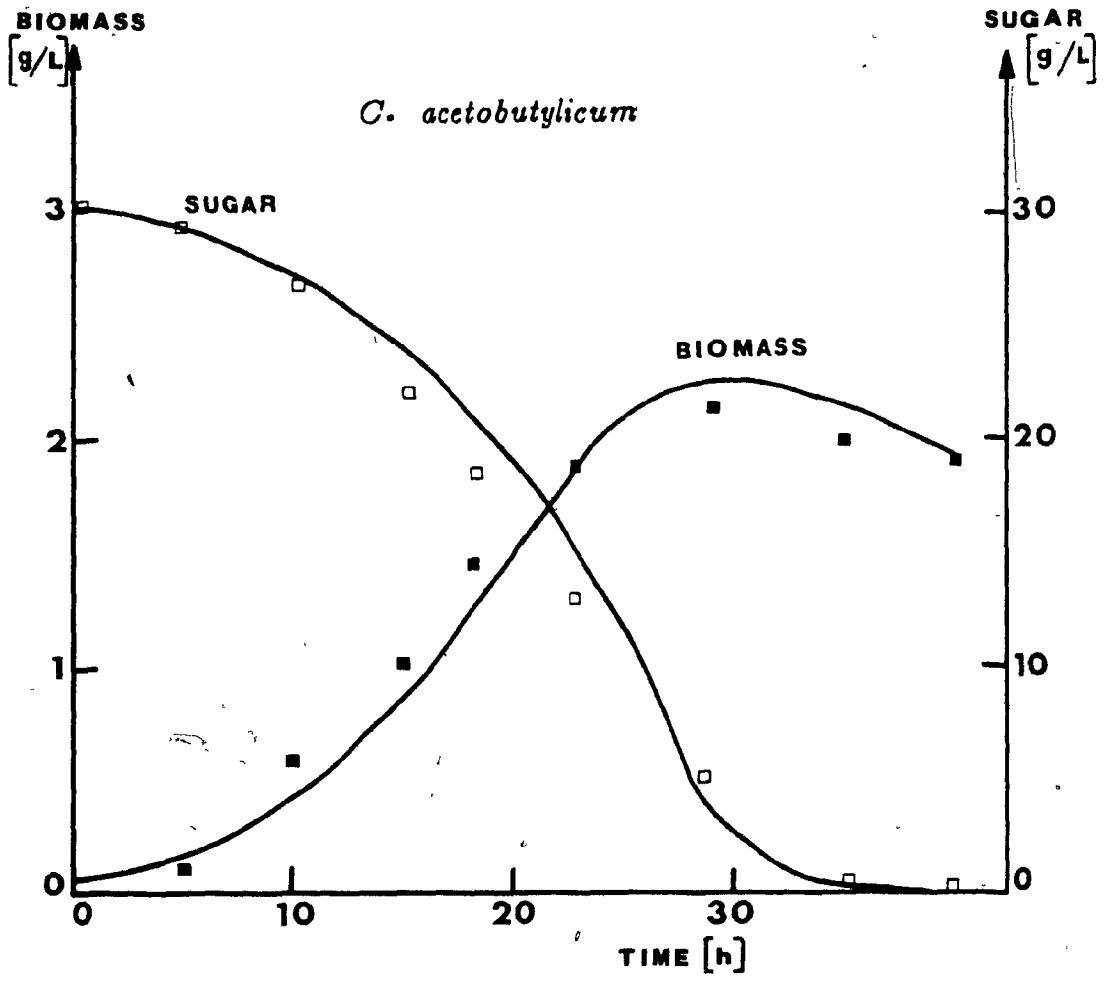
As the main purpose of this investigation, the Physiological State Model was taken, along with the kinetic coefficients estimated for the normal culture, to be used for the simulation of the retarded culture performance. This unconventional approach to the application of a culture performance model could assist in identification of the parameters which governed the atypical and undesirable behavior of the culture as well as in elucidating the quantitative extent to which they play a role in the substandard fermentations.

The retarded culture was simulated by leaving all the model parameters constant except for the following:

FIGURE 1(a, b)

Experimental Data and Model Predictions (full line) for the Process Parameters in a Normal Batch Culture of C. acetobutylicum.

a)


BIOMASS
[g/L]
SUGAR
[g/L]
C. acetobutylicum
SUGAR
BIOMASS
3
2
1
0
30
20
10
0
0
10
20
30
TIME [h]

b)

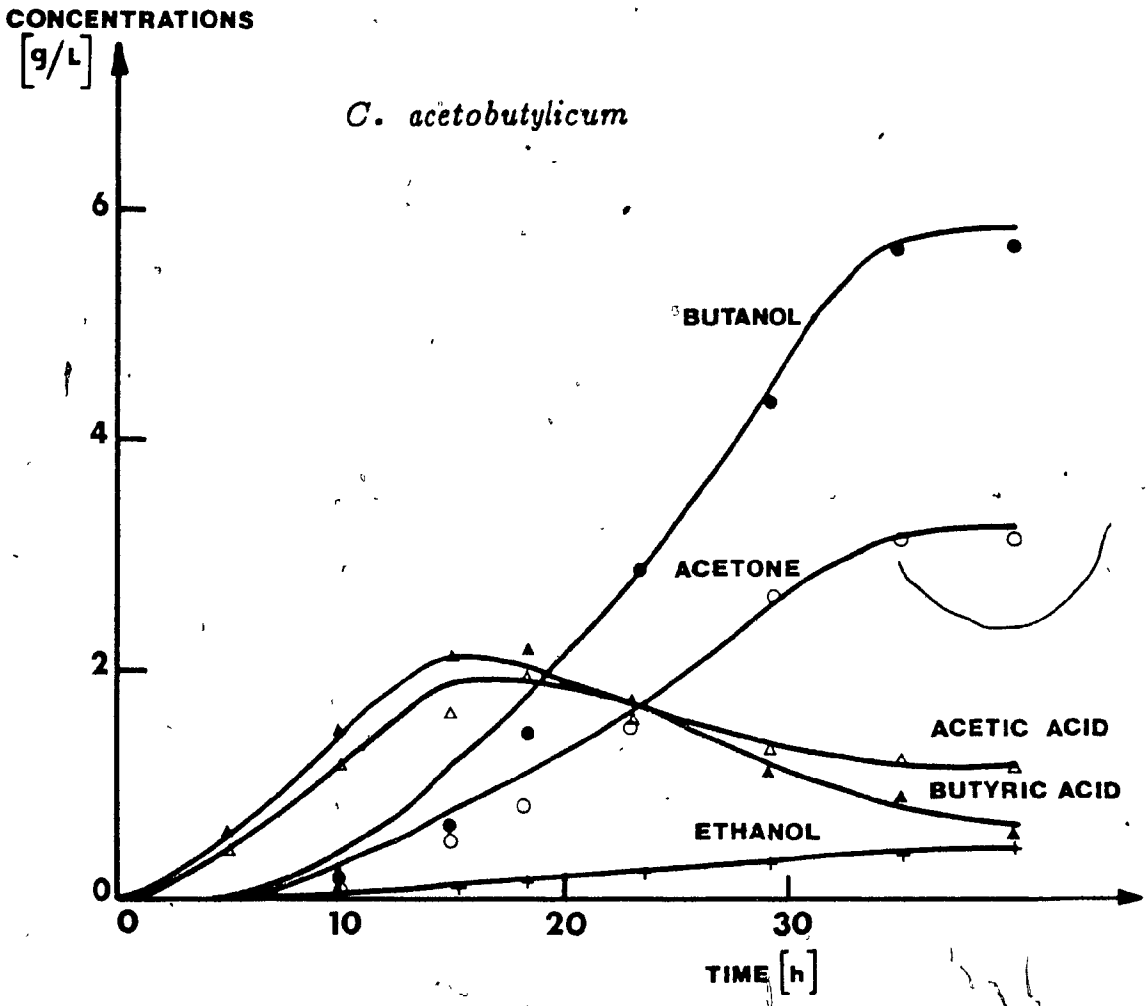

CONCENTRATIONS
[g/L]
C. acetobutylicum
6
4
2
0
BUTANOL
ACETONE
ACETIC ACID
BUTYRIC ACID
ETHANOL
0
10
20
30
TIME [h]

TABLE 2

THE RESULTS OF THE "F-TEST" WITH THE LEVEL OF SIGNIFICANCE (α) OF 0.05 FOR THE AGREEMENT BETWEEN THE EXPERIMENTAL AND THE SIMULATED RESULTS

	S_r^2	S_e^2	S_r^2 / S_e^2	
Biomass	0.18	0.06	$3.0 < 3.29 \rightarrow$	agreement between the experimental and the simulated results
Sugar	6.65	2.57	$2.59 < 3.29 \rightarrow$	"
Butanol	0.17	0.06	$2.83 < 3.29 \rightarrow$	"
Acetone	0.092	0.034	$2.70 < 3.29 \rightarrow$	"
Ethanol	0.004	0.008	$0.5 < 3.29 \rightarrow$	"
Butyric Acid	0.06	0.02	$3.0 < 3.29 \rightarrow$	"
Acetic Acid	0.05	0.03	$1.67 < 3.29 \rightarrow$	"

- Some external parameters such as the inoculum size and the marker of the physiological state (y), since these parameters can change from one culture transfer to another. It was generally observed that the culture had a faster start-up in the retarded fermentations. This implies a better adaptation of the inoculum which was characterized by an increase in the value of y from 1.2 (used in the simulation of standard fermentations) to 1.6. A slightly higher inoculum size was also considered for the retarded fermentations in the range of 0.06 g/L to 0.08 g/L as compared to the value of 0.05 g/L used for the normal fermentation.

- The kinetic parameters which control the transport mechanism of sugar and other metabolites such as the number of active transport "sites" (n) and k_n, and the permeability coefficient P.

- The kinetic parameter controlling the conversion of butyric acid to butanol (k_{BA_2}).

This strategy was particularly important since it was assumed that the major difference between the normal and the retarded culture was at the level of the cellular membrane. The lower conversion of butyric acid to butanol in the substandard fermentations was also indicated earlier (Yerushalmi et al., 1985)*. Simulation of the retarded cul-

ture then proceeded by considering individual groups of experiments separately (Yerushalmi et al., 1985)*.

CULTIVATION GROUP I

This Group exhibited an "activity arrest" in the middle of the fermentation. According to the behavior of the culture before and after the arrest, two phases have been distinguished:

Phase 1

This phase was characterized by a fast biomass production and sugar consumption and very low solvent production. Certain amount of sugar remained unutilized in the system. The culture behavior during Phase I was simulated by considering a low initial number of active transport sites (n), a low permeability (P), and a low k_{BA_2} which represents a low conversion of butyric acid to butanol. A low permeability of the cellular membrane to butanol and butyric acid, which is reflected in a low value of P, implies a higher accumulation of these compounds inside the cell. This results in a higher inhibition and a decay in the RNA concentration and its function. Therefore, a low P should be accompanied by a high k_{G_2}.

Phase 2

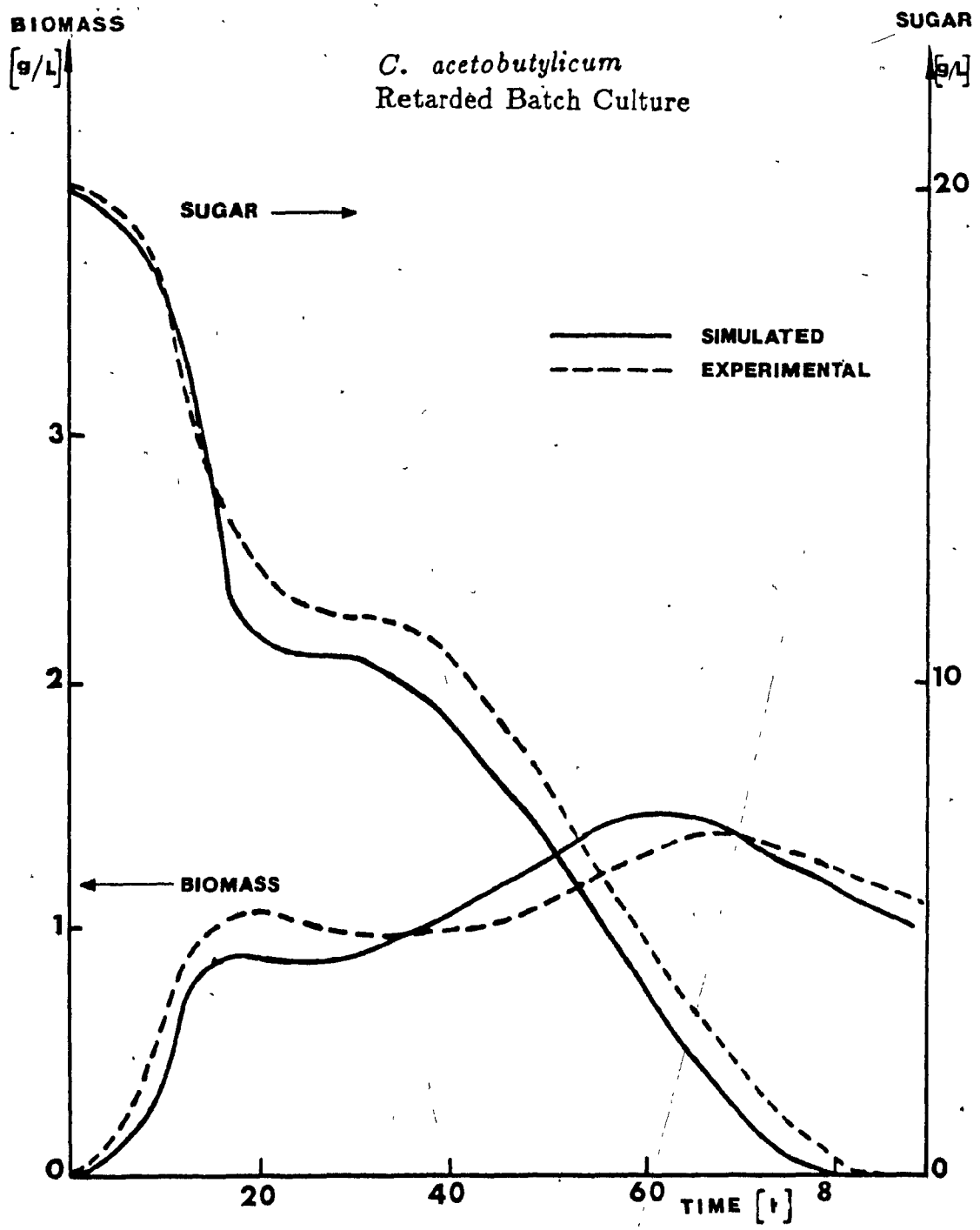
The result of Phase 1 was a low external solvent concentration, incomplete sugar utilization, and increased biomass and acid concentrations. The fermentation system

performance tapered off because the cellular activities ceased. After 10 to 20 hours the culture resumed its activities and the conversion of the acids to solvents picked up. This phase was simulated by starting from an initial condition of higher acids and solvent concentrations which was the terminal state of Phase 1. A larger inoculum was also assumed since it would simulate the biomass concentration obtained from the first phase. The initial number of active sites, and the rate of sugar transport characterized by k_n were low. A low k_{G_2}, characterizing a lower inhibition of the RNA system, was considered for this fermentative phase since it was assumed that the internal concentration of the solvents and acids decreased during the break (between Phase 1 and Phase 2) and an equilibrium betwen the internal and the external concentration of metabolites was attained. An improvement in the permeability of the cellular membrane was also assumed. The simulation for Phase 2 of Group I was slightly more difficult because the initial conditions e.g. the size of the active inoculum was not known. This is because of the undefined changes in the culture during the period of activity arrest. The results of culture simulations for Group I of the retarded culture are presented in Figure 2. Only the concentration profiles for butanol, butyric acid, biomass and sugar are presented since they are the most important and controlling variables characterizing the A-B-E process. The numerical values of the controlling kinetic

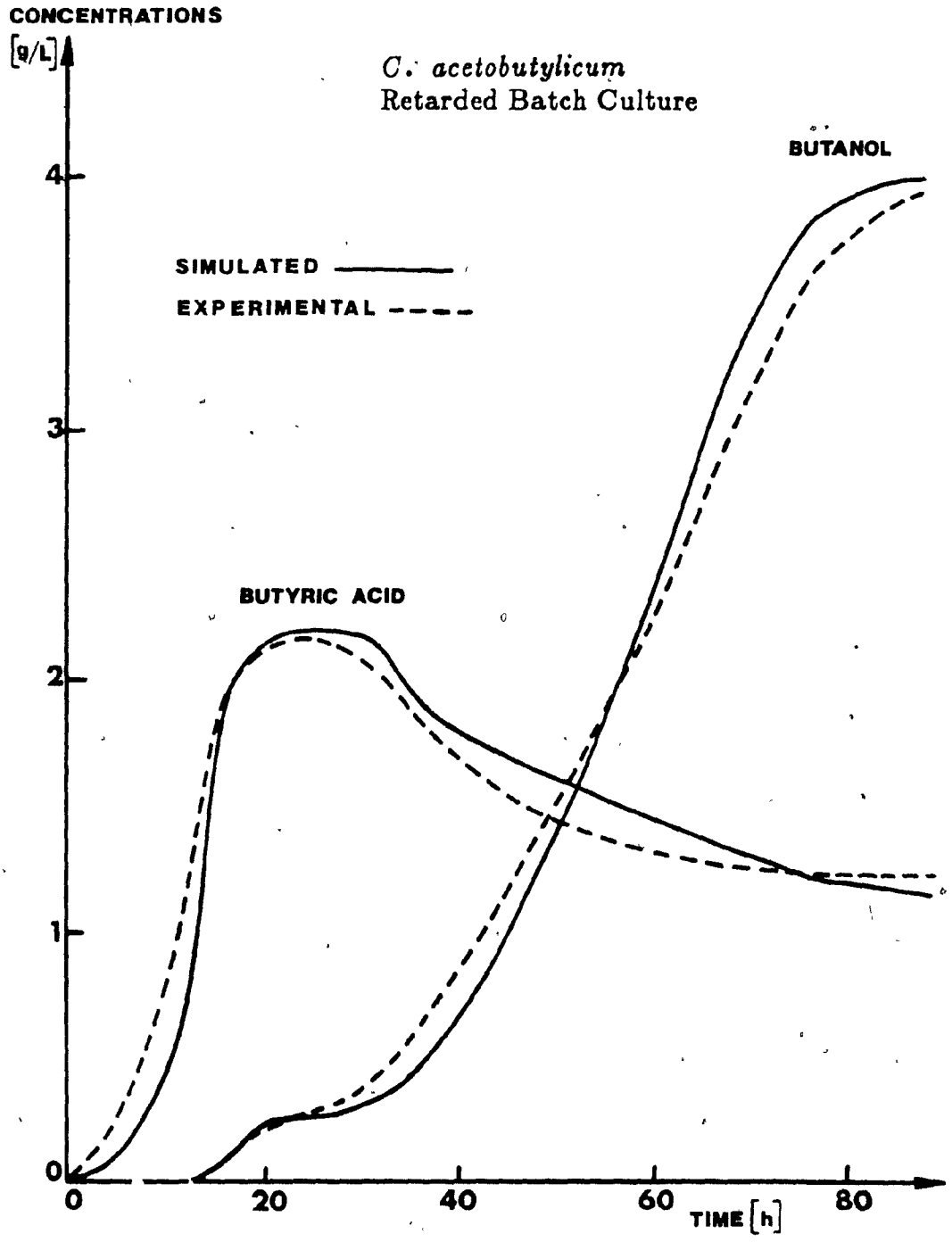
FIGURE 2(a,b)

Experimental Results and Model Predictions (full line) for the Major Process Parameters in a Retarded Batch Culture of C. acetobutylicum, Group 1, Normal Solvent Yield.

a)


BIOMASS
[g/L]
SUGAR
[g/L]
C. acetobutylicum
Retarded Batch Culture
SUGAR
SIMULATED
EXPERIMENTAL
BIOMASS
3
2
1
0
20
10
0
20
40
60
TIME [h]
8

b)


CONCENTRATIONS
[g/L]
C. acetobutylicum
Retarded Batch Culture
BUTANOL
SIMULATED
EXPERIMENTAL
BUTYRIC ACID
4
3
2
1
0
0
20
40
60
80
TIME [h]

parameters for Phase 1 and Phase 2 together with their values corresponding to the normal fermentation are presented in Table 3.

Those fermentations of Group I which resulted in a lower solvent yield of less than 30% were simulated by considering a lower permeability coefficient (P) during Phase 2. An example of the simulation for these fermentations is illustrated in Figure 3. The corresponding values of model parameters are summarized in Table 4. It can be seen that the same type of process parameters governed the behavior of the culture during these fermentations and that the permeability of the cellular membrane was mainly responsible for the lower solvent production.

CULTIVATION GROUP II

The key controlling parameters in this Group of fermentation experiments were the number of "active sites" for the transport of sugar (n) and k_n. Simulations for Group II were performed by considering a slightly lower P and k_{BA_2} and a slightly higher k_{G_2} which reflects a more pronounced inhibition considered in this case. Table 5 presents the numerical values of the key process controlling parameters and Figure 4 illustrates an example of a computer simulation for this group.

TABLE 3

THE KEY CONTROLLING PARAMETERS IN CULTURE GROUP I
(normal solvent yields)

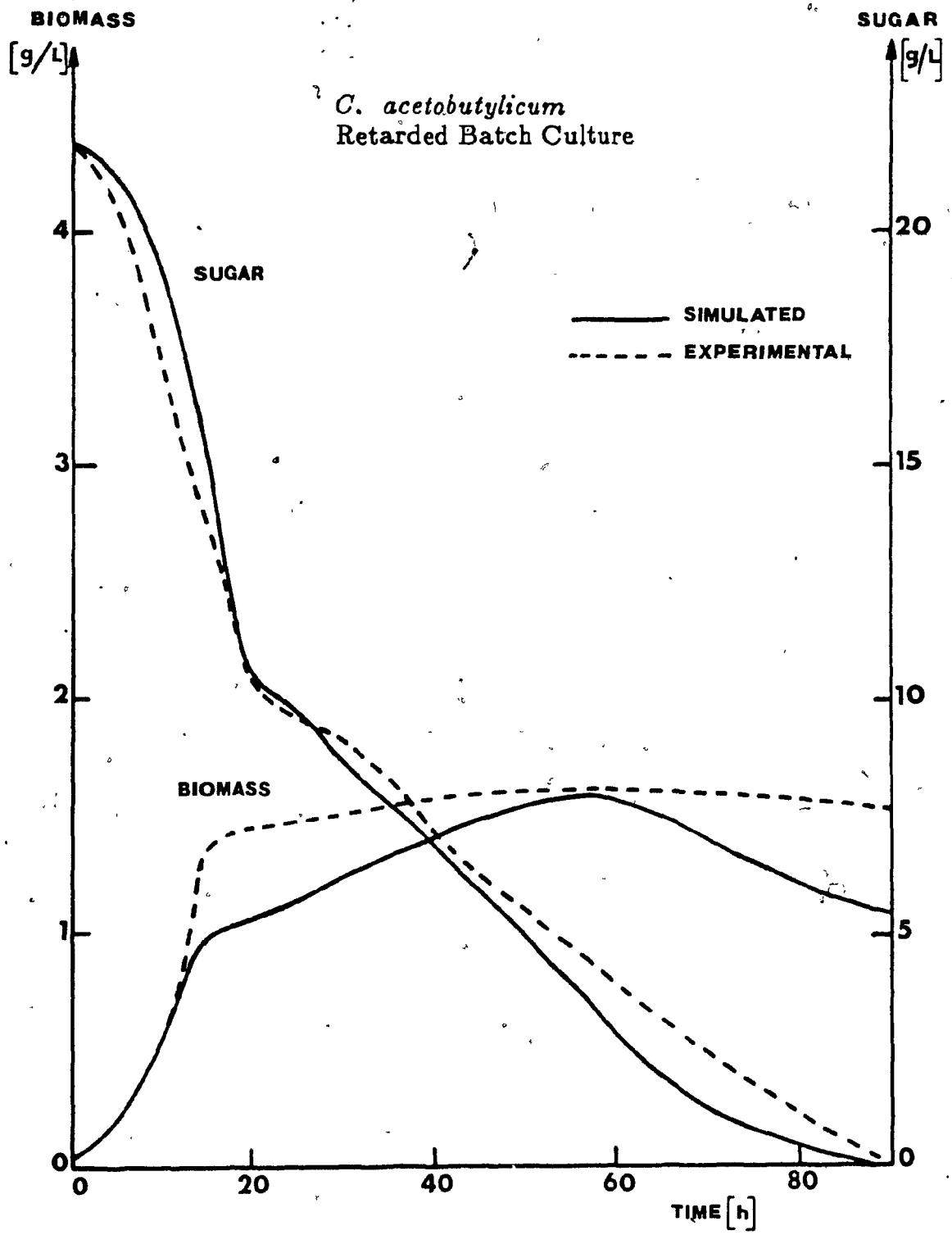
PARAMETER	RETARDED FERMENTATION		NORMAL FERMENTATION
	Phase 1	Phase 2	
n	1.2*	0.1*	2.5*
k_n	0.178	0.02	0.178
P	0.02	0.15	0.17
k_{G_2}	0.495	0.015	0.029
k_{BA_2}	0.1	0.1	0.23

*) Initial value of n

FIGURE 3(a,b)

Experimental Results and Model Predictions (full line) for the Major Process Parameters in a Retarded Batch Culture of C. acetobutylicum, Group 1, Low Solvent Yield.

a)


BIOMASS
[g/L]
SUGAR
[g/L]
C. acetobutylicum
Retarded Batch Culture
SUGAR
SIMULATED
EXPERIMENTAL
BIOMASS
0
1
2
3
4
0
5
10
15
20
0
20
40
60
80
TIME [h]

b)

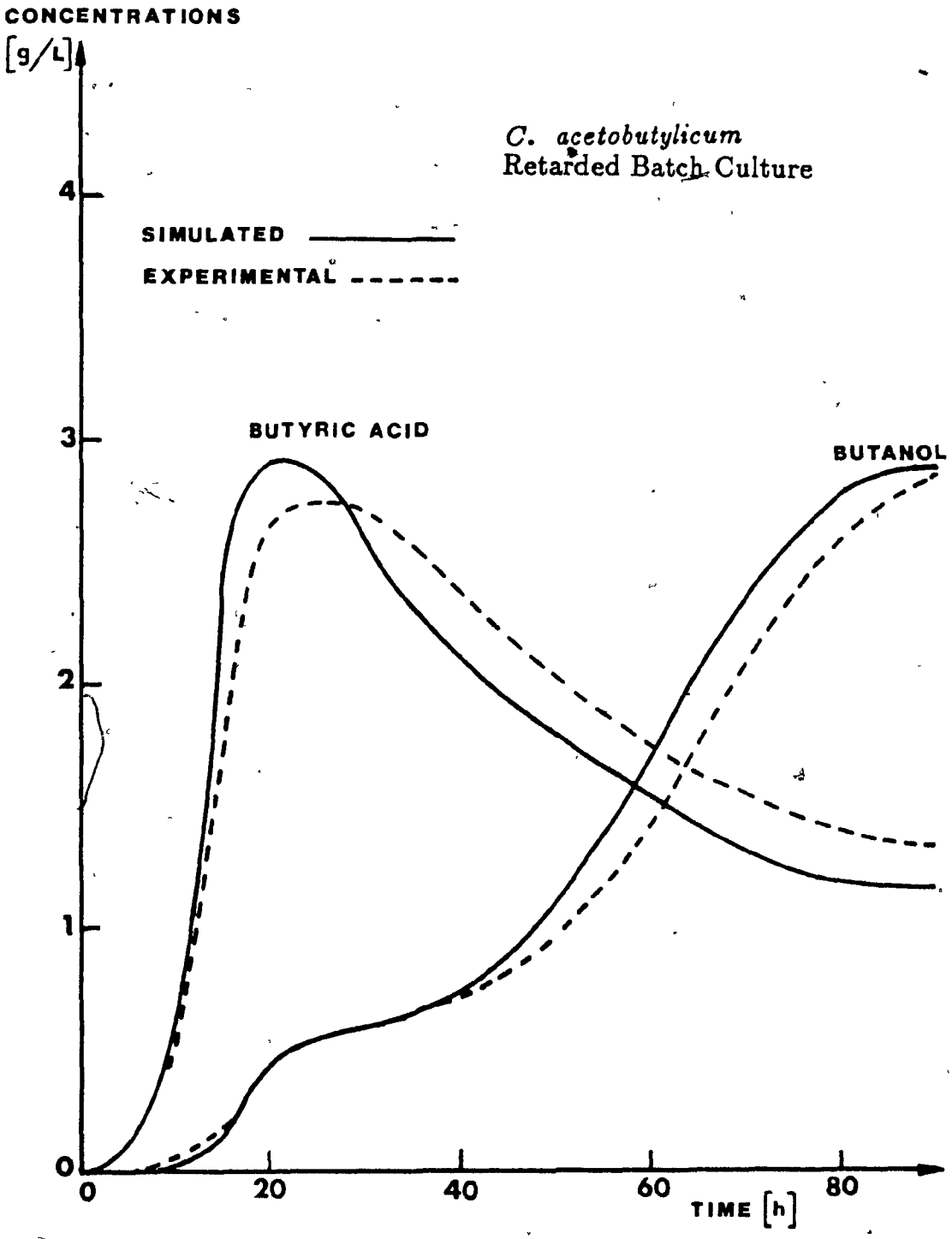

CONCENTRATIONS
[g/L]
C. acetobutylicum
Retarded Batch Culture
SIMULATED
EXPERIMENTAL
BUTYRIC ACID
BUTANOL
4
3
2
1
0
0
20
40
60
80
TIME [h]

TABLE 4

THE KEY CONTROLLING PARAMETERS IN CULTURE GROUP I
(low solvent yields)

PARAMETER	RETARDED FERMENTATION		NORMAL FERMENTATION
	Phase 1	Phase 2	
n	1.2*	0.1*	2.5*
k_n	0.178	0.007	0.178
P	0.025	0.03	0.17
k_{G_2}	0.335	0.012	0.029
k_{BA_2}	0.103	0.08	0.23

*) Initial value of n

TABLE 5

THE KEY CONTROLLING PARAMETERS IN CULTURE GROUP II

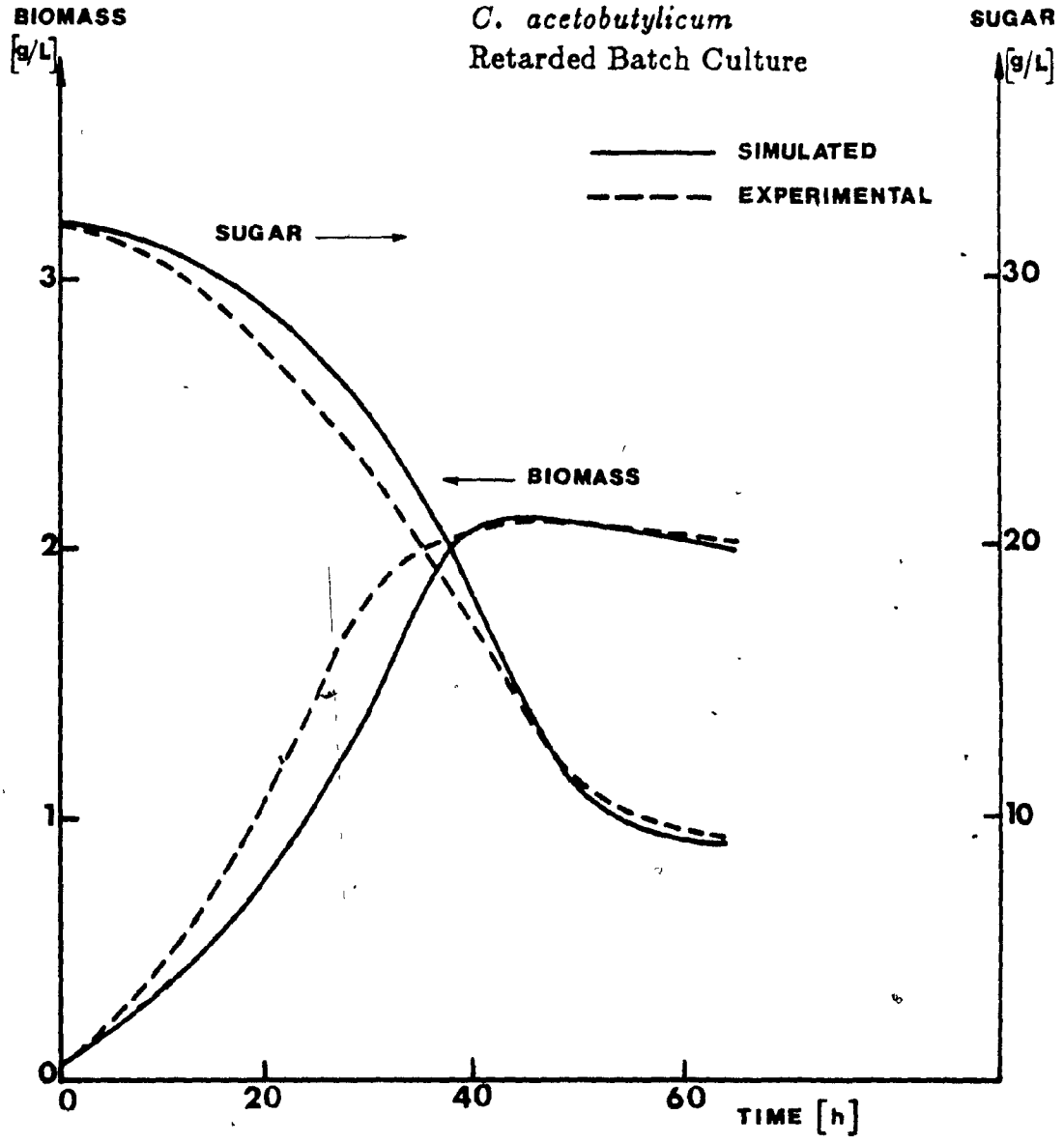
PARAMETER	RETARDED FERMENTATION	NORMAL FERMENTATION
n	1.0*	2.5*
k_n	0.022	0.178
P	0.1	0.17
k_{G_2}	0.04	0.029
k_{BA_2}	0.148	0.23

*) Initial value of n

FIGURE 4(a,b)

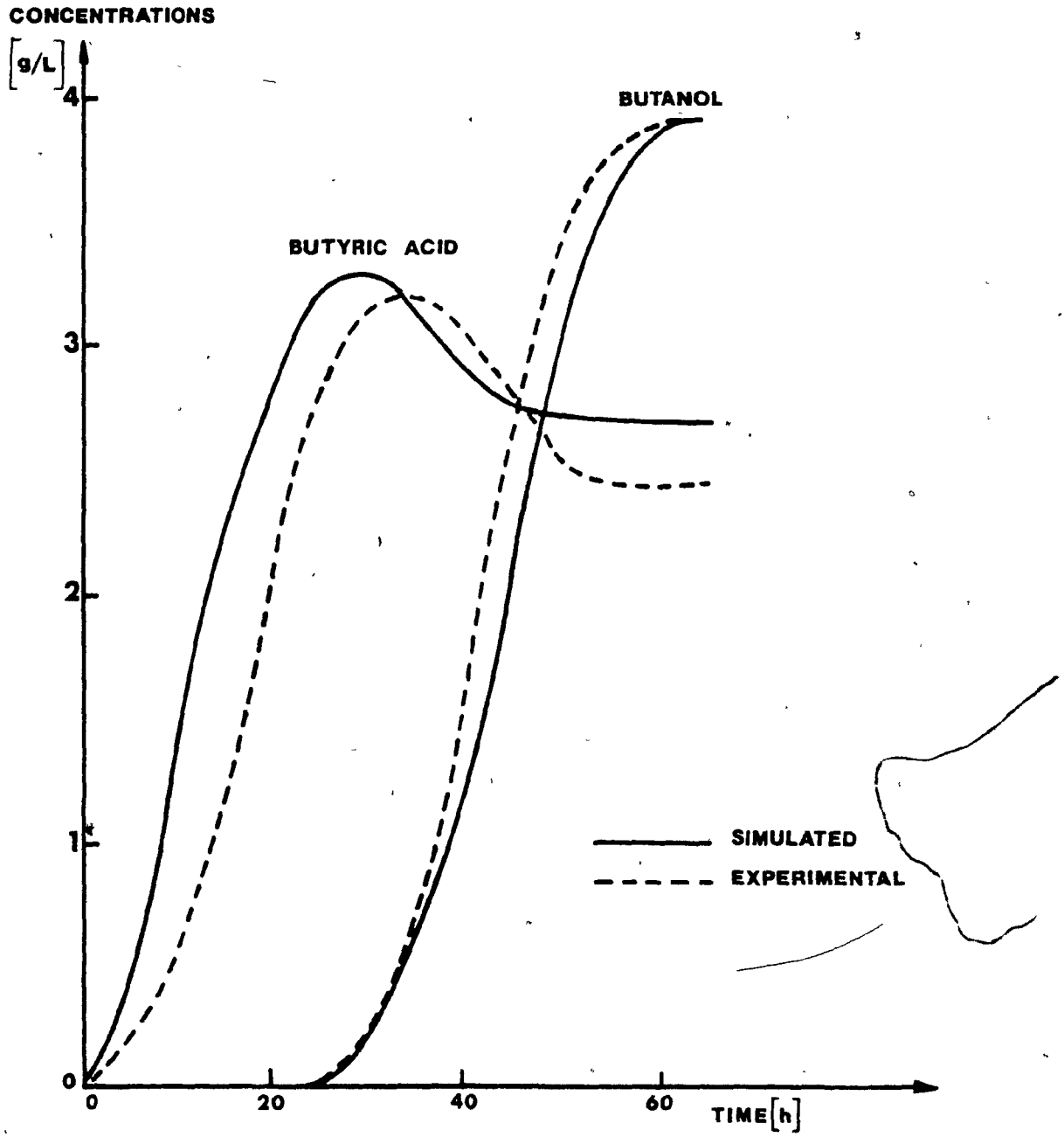
Experimental Results and Model Predictions (full line) for the Major Process Parameters in a Retarded Batch Culture of C. acetobutylicum, Group II.

a)


BIOMASS
[g/L]
C. acetobutylicum
Retarded Batch Culture
SUGAR
[g/L]
SIMULATED
EXPERIMENTAL
SUGAR
BIOMASS
3
2
1
0
30
20
10
0
20
40
60
TIME [h]

b)

C. acetobutylicum
Retarded Batch Culture



CULTIVATION GROUP III

A pH adjustment at t=19h increased the utilization rate of sugar and resulted in its complete exhaustion in almost 50 hours. However, in spite of a complete sugar depletion, a low solvent yield was obtained at the end of the process. Similar fermentations with no pH adjustment resembled the culture behavior characteristic for Group I where a low solvent yield was obtained. The effect of pH adjustment was most remarkable in conjunction with coefficient k_n which controls the rate of sugar transport into the cell. Simulation studies exhibited a 19 fold increase in the value of this coefficient following the pH adjustment. The results of simulations for Group III are presented in Figure 5 while Table 6 presents the numerical values of the key parameters.

8.3.3 EXPERIMENTS

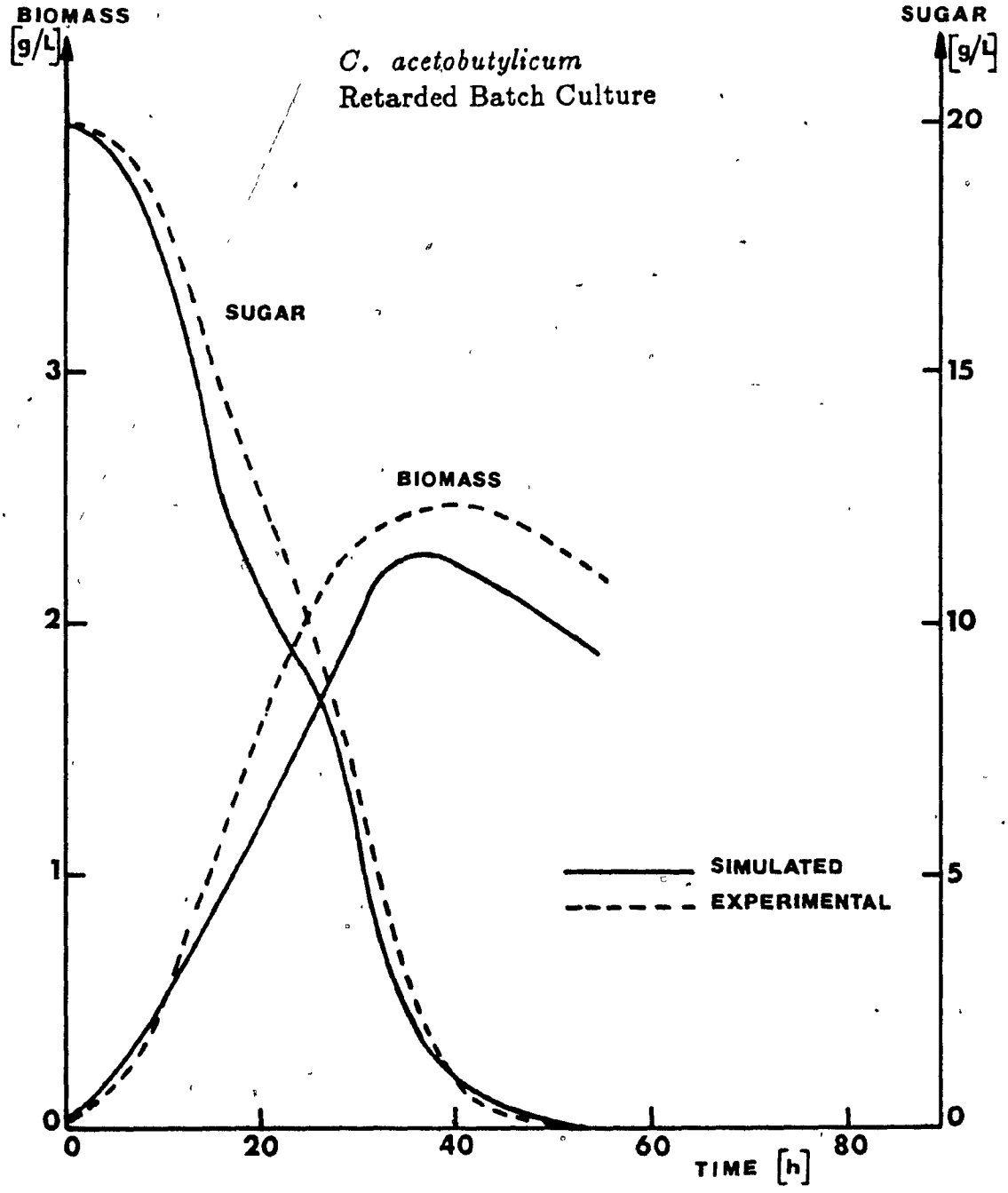
i) Study of Morphology and Size Distribution of C. acetobutylicum Cultures

One of the important physiological characteristics of the clostridial cultures is the variation in the cell morphology associated with different stages of the fermentation (Spivey, 1978; Jones et al., 1983). Figures 6 and 7 present photomicrographs of morphological changes in C. acetobuty-

FIGURE 5(a,b)

Experimental Results and Model Predictions (full line) for the Major Process Parameters in a Retarded Batch Culture of C. acetobutylicum, Group III.

a)


BIOMASS
[g/L]
SUGAR
[g/L]
C. acetobutylicum
Retarded Batch Culture
SUGAR
BIOMASS
SIMULATED
EXPERIMENTAL
TIME [h]
0
1
2
3
0
5
10
15
20
0
20
40
60
80

b)

C. acetobutylicum
Retarded Batch Culture

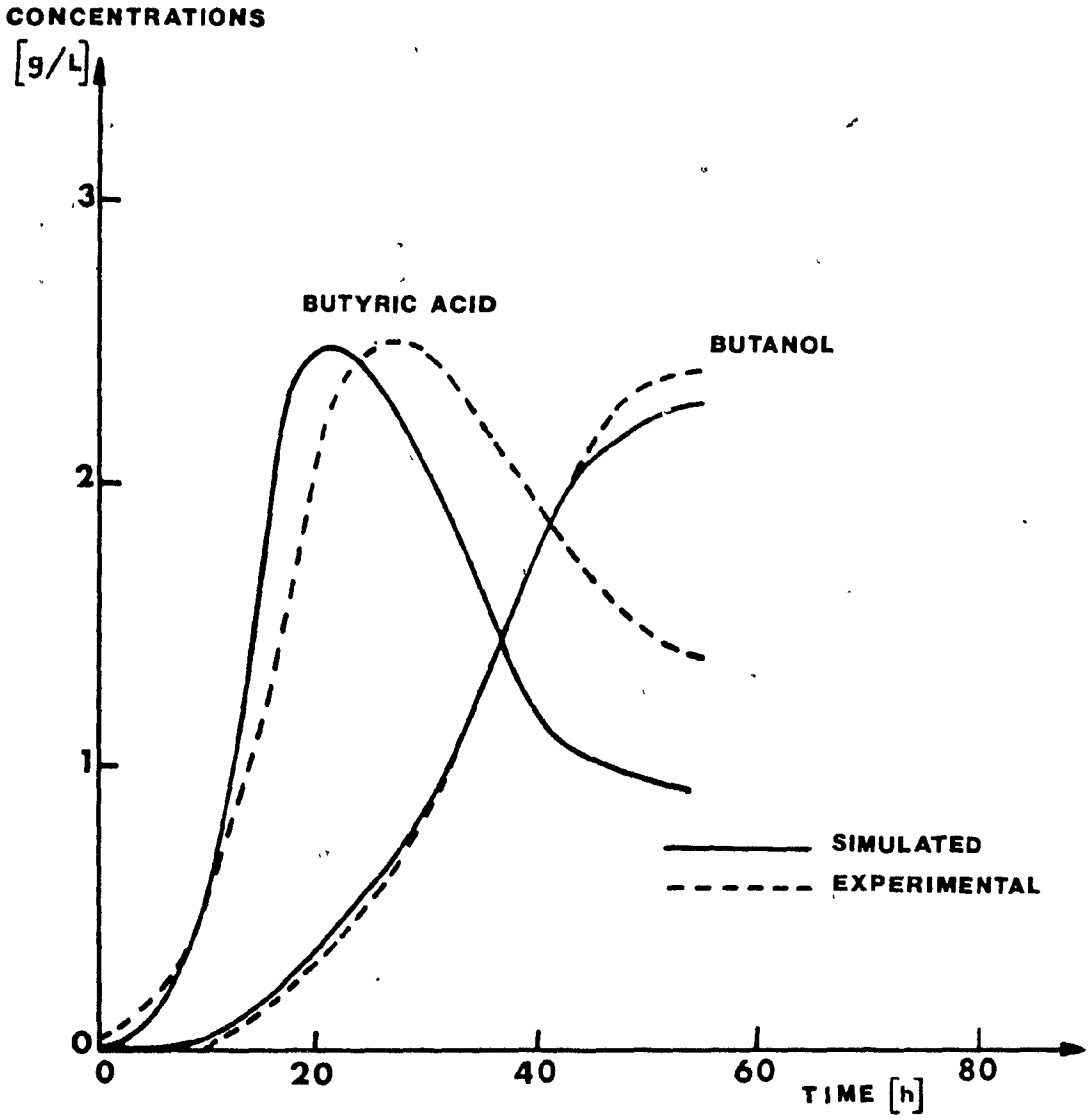


TABLE 6

THE KEY CONTROLLING PARAMETERS IN CULTURE GROUP III

PARAMETER	RETARDED FERMENTATION			NORMAL FERMENTATION
	t=(0-19)h, before pH adjustment	After t=19h, with pH adjustment	After t=19h, without pH adjustment	
n	1.25*	0.5*	0.37*	2.5*
k_n	0.178	0.037	0.002	0.178
P	0.02	0.05	0.05	0.17
k_{G_2}	0.335	0.025	0.015	0.029
k_{BA_2}	0.103	0.173	0.09	0.23

*) Initial value of n

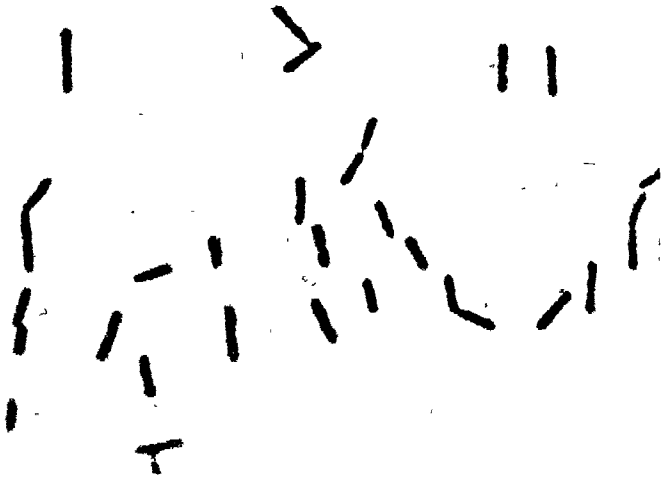
FIGURE 6 Photomicrographs of the Morphological Changes in a Normal Culture of C. acetobutylicum.

a. Actively growing vegetative rods, early exponential growth phase.

b. Cigar-shaped "clostridial" forms, late exponential growth phase.

c. Mostly "clostridial" forms, early stationary growth phase.

a)



b)

c)

FIGURE 7 Photomicrographs of the Morphological Changes in a Retarded Culture of C. acetobutylicum.

a. "Clostridial" forms, some cells appear in pairs, early exponential growth phase.

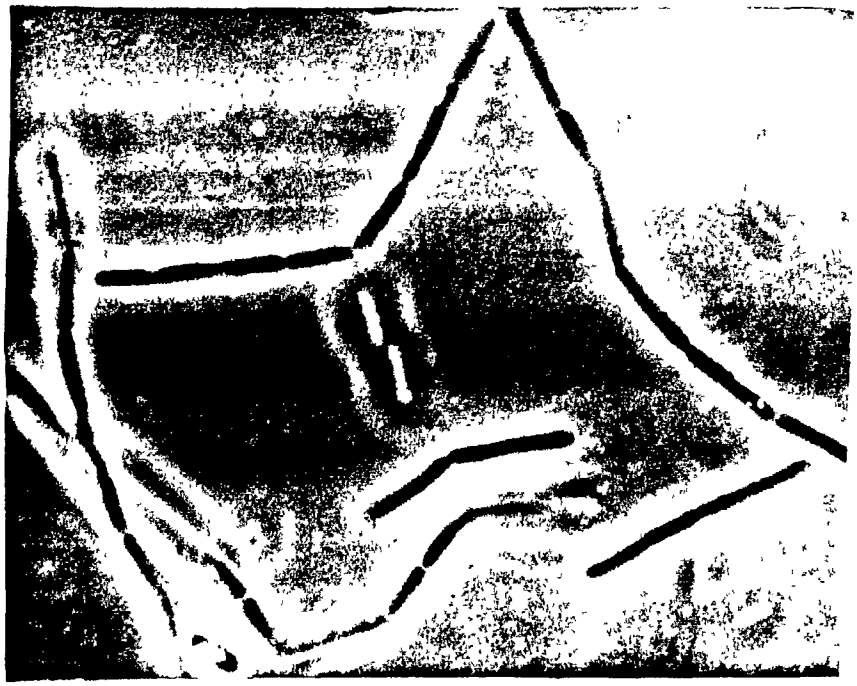
b. Mostly "clostridial" forms, chains of cells appear, late exponential growth phase.

c) A large number of chains of cells, early stationary growth phase.

a)

b)

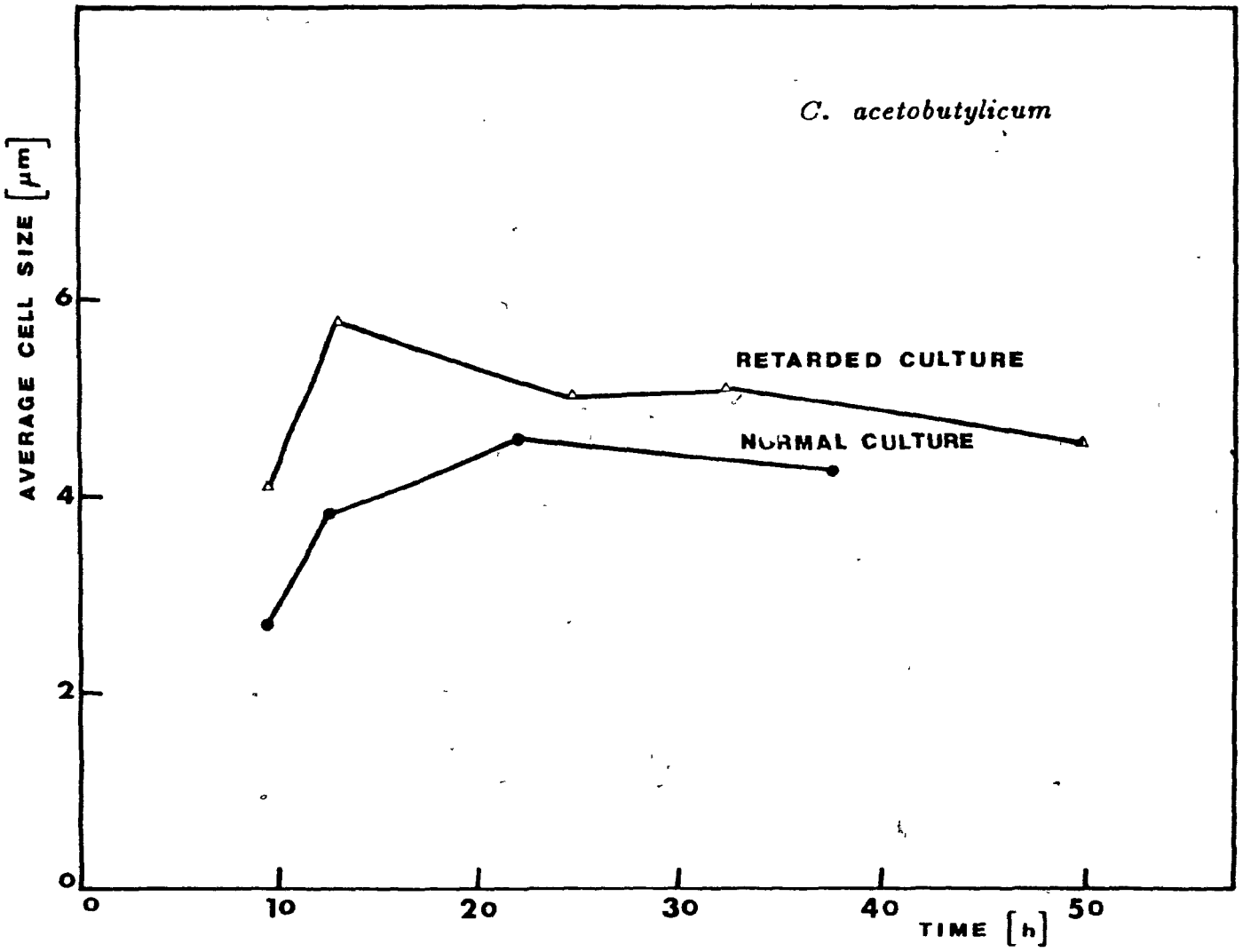
c)



licum during the "successful" and the "substandard" A-B-E fermentations. It is generally believed that the C. acetobutylicum ATCC 824 strain does not exhibit clear morphological changes with the progress of the fermentation as does the P262 strain (Jones et. al., 1983). However, as seen from Figures 6 and 7 the change of the cell from phase-dark vegetative rods to elliptical (cigar shaped) bright forms referred to as the "clostridial" form could be observed in both cultures. In spite of the general morphological similarities, there were distinct differences between the two cultures. The cells of the retarded culture exhibited larger clostridial forms and at an earlier stage coinciding with the beginning of the exponential growth phase, as compared with the normal culture which produced clostridial cells in the middle of the exponential growth phase. Although the cells of the normal culture were mostly free and separated from one another, in the retarded culture the cells were attached to each other and formed chains. These chains were more distinguishable and longer towards the end of the exponential phase.

The average cellular size of the two cultures was also significantly different (Figure 8). The cells in the "substandard" fermentation were generally larger. The cells of the normal culture exhibited a maximum length of 4.6 μm ± 1.0 μm obtained in a late exponential growth phase of the culture. With the retarded culture this value changed to

FIGURE 8 Variations of the Average Cellular Size of the Normal Culture and the Retarded Culture of C. acetobutylicum with the Progress of the Fermentation.


C. acetobutylicum
AVERAGE CELL SIZE [μm]
RETARDED CULTURE
NORMAL CULTURE
0
2
4
6
0
10
20
30
40
50
TIME [h]

5.8 μm ± 1.7 μm as observed in a mid-exponential phase of growth.

ii) Measurement Of The Cellular Permeability

For the measurement of the cellular membrane permeability, butanol and butyric acid were taken as the representative products due to their importance in the A-B-E process and their effect on the physiology of the culture. Table 7 presents the results of this study for the normal and the Retarded Culture. The measured values of the permeability coefficients reported in Table 7 are averages of several independent measurements with standard deviations also listed. Permeability of the membrane was lower for butyric acid than for butanol in both cultures. Also, the cellular membrane had a lower permeability for both of these compounds in the Retarded Culture when compared to the normal culture.

iii) Effect Of The Unsaturated Fatty Acids On The Performance Of The Retarded Culture

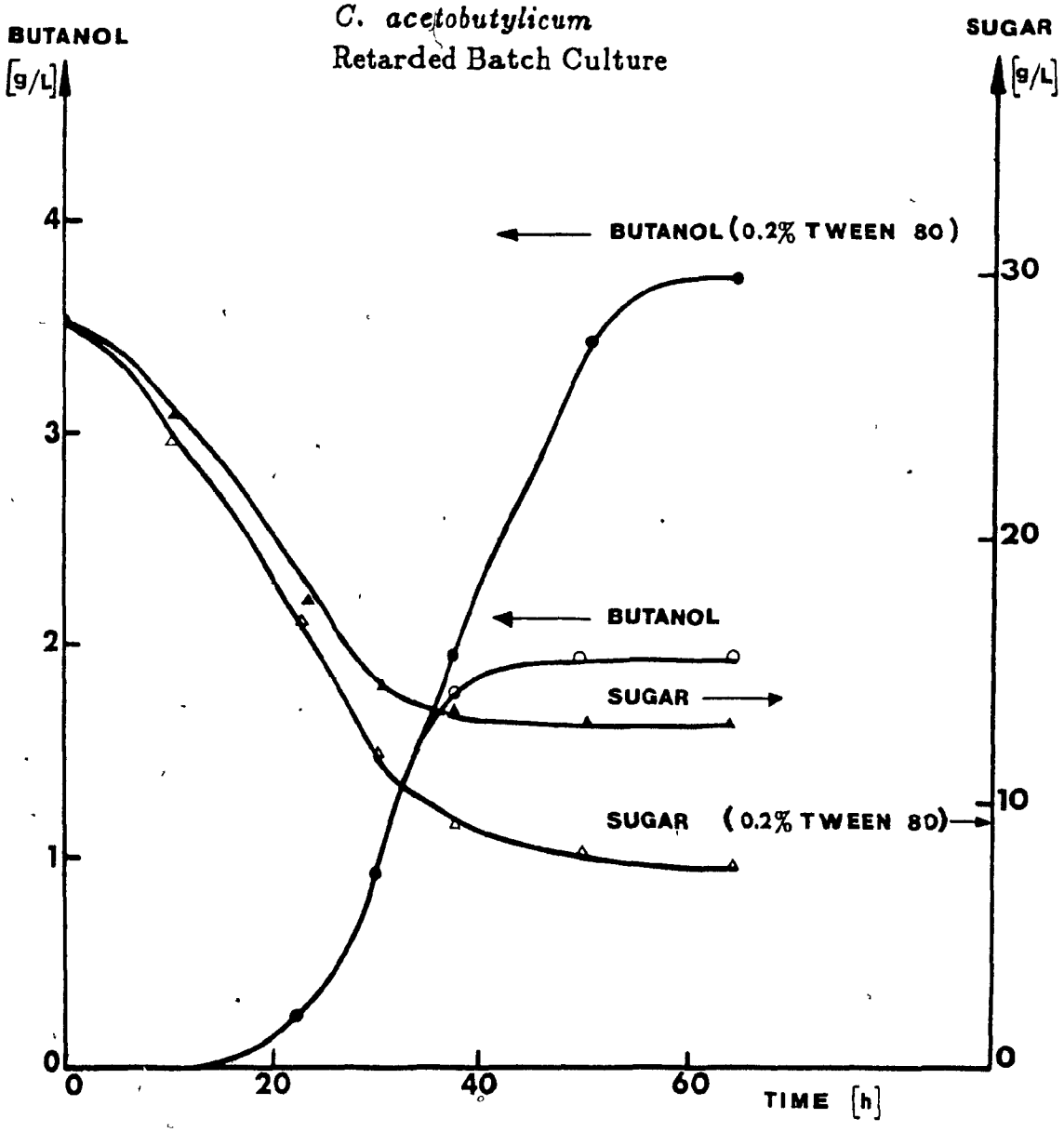
The plasma membrane of bacterial cells can be enriched by unsaturated lipids through direct addition of these compounds to the culture growth medium. In the present study, Tween 80, a water-soluble ester of oleic acid which is a non-toxic source of oleic acid, was used at a concentration of 0.2% (w/v). Figure 9 illustrates the effect of the addition of Tween 80 on the performance of the Retarded Culture. It can be seen that the addition of the oleic acid

TABLE 7

ESTIMATED CELL MEMBRANE PERMEATION COEFFICIENTS
FOR BUTANOL AND BUTYRIC ACID

Compound	*Clostridium acetobutylicum* ATCC824	
	Normal Culture	Retarded Culture
butanol	0.16±0.04	0.07±0.03
butyric acid	0.11±0.04	0.06±0.02

FIGURE 9 The Effect of Tween 80 (0.20% w/v) on the Performance of the Retarded Culture of C. acetobutylicum.

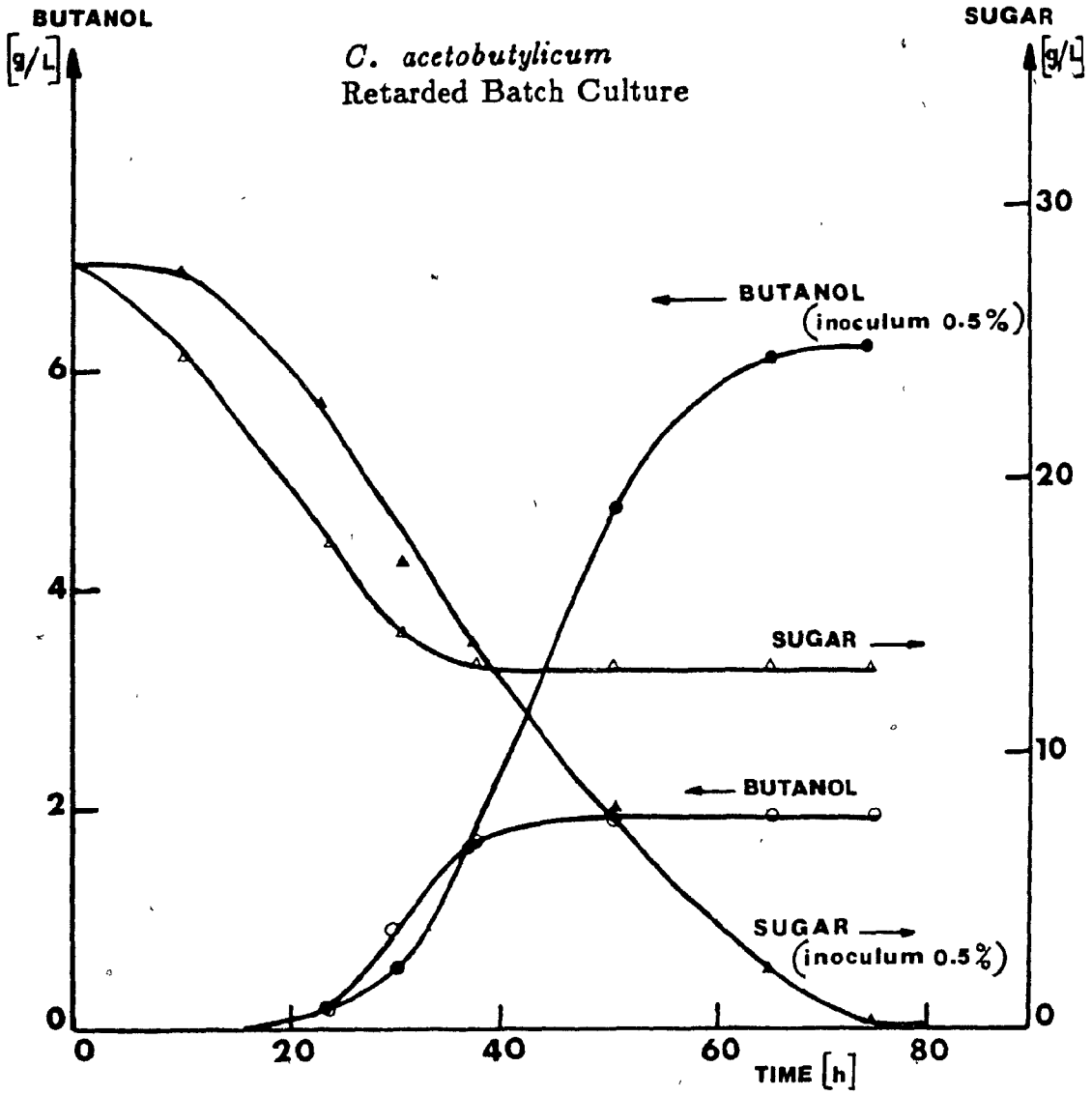

C. acetobutylicum
Retarded Batch Culture
BUTANOL
[g/L]
SUGAR
[g/L]
BUTANOL (0.2% TWEEN 80)
BUTANOL
SUGAR
SUGAR (0.2% TWEEN 80)
TIME [h]
0
1
2
3
4
0
20
40
60
0
10
20
30

based surfactant Tween 80 to growth medium had a significant effect on the production of solvents and increased the final total solvent concentration from 2.9 g/L to 5.7 g/L which represents a 97% increase in the value of this parameter. The consumption of sugar was also enhanced as a result of the Tween 80 addition. Consequently, the total solvent yield based on sugar was increased from 19.3% to 27.4% which represents a 70% increase.

iv) Effect Of A Small Cell Population (Low Inoculum) On The Performance Of The Retarded Culture

A 3% inoculum was used throughout this study in both the normal and the substandard fermentations. Decreasing the inoculum size to (0.5%-0.6%) resulted in a different performance of the culture and greatly enhanced the production of solvents and the uptake of sugar by the Retarded Culture. A rather long lag phase resulting from the use of a small inoculum is seen in Figure 10. However, as soon as the culture reached the exponential phase it exhibited a "normal" fermentation pattern resulting in a complete exhaustion of sugar at the end of the process with a relatively high solvent yield of 35.7%. There was no significant effect on the final concentration of biomass or the maximum concentration of acids, however, the accumulation of acids, particularly butyric acid, in the system at the end of the fermentation was much lower (graphs not presented).

FIGURE 10 The Effect of a Small Inoculum (0.5%) on the Performance of the Retarded Culture of C. acetobutylicum.


BUTANOL
[g/L]
SUGAR
[g/L]
C. acetobutylicum
Retarded Batch Culture
BUTANOL
(inoculum 0.5%)
SUGAR
BUTANOL
SUGAR
(inoculum 0.5%)
0
2
4
6
10
20
30
0
20
40
60
80
TIME [h]

8.4 DISCUSSION

The work reported on in this paper is an expansion of the earlier effort (Yerushalmi et al., 1985)* in applying the method of systems analysis in analyzing atypical performance of a strain of C. acetobutylicum. The method of systems analysis employed in this work was based on the formulation of a mathematical model for the description of dynamics of the acetone-butanol-ethanol (A-B-E) process including the internal and the external cellular conditions and the key physiological parameters of the microbial culture. Among the physiological parameters used for this purpose are the number of "active sites" in the cellular membrane (n) and the cell membrane permeability coefficient (P). This model was later used in the simulation of the substandard fermentations which led to obtaining valuable knowledge regarding the nature of these fermentations and the key controlling parameters in the acetone-butanol process. Unlike the previous A-B-E Kinetic Model (Votruba et al., 1985)*, which was developed by using a non-linear regression for the best estimate of the model parameters, in the present Physiological State Model, the minimization technique was used only for the best estimate of the key parameters. By the theoretical analysis of the system, these parameters were indicated as those mainly responsible for the behavior of the Retarded Culture. All the remaining parameters were considered as fixed variables. Although a very good simulation of the substandard fermenta

tions resulted (Figures 2 to 5), a better simulation and a closer fit would result if all the model parameters were free and found by non-linear regression. This approach was taken because the Physiological State Model was developed to study the mechanism of the process in terms of the transport of components through the cell membrane and to evaluate the impact of the key physiological parameters on the behavior of the culture particularly for the substandard fermentations.

The theoretical and the experimental analysis presented in this paper and in the previous communication (Yerushalmi et al., 1985)* indicate that the quality of the cellular membrane characterized by its permeability and the number of active transport "sites" plays a significant role in the control of the behavior of a microbial culture. In the acetone-butanol fermentation the membrane transport problems were found responsible for a lower solvent yield and substrate conversion efficiency. Deficiencies in the performance of the transport sites and/or their number, and the problems associated with the permeability of the membrane could have been due to the alterations in the composition and structure of the cellular membrane as an adaptive response to some adverse environmental conditions. Although the nature of these possible environmental perturbations are not known to us, it is well established that microorganisms adjust the content and composition of their membrane lipids in response to environmental conditions such as temperature (Ingram, 1976; Goldfine and Johnstone, 1980), carbon source (Kates et

al., 1962), salts (Ingram, 1982), organic solvents (Lenaz et al., 1975; Hill, 1974; Grisham and Barnett, 1973), and detergents (Felix, 1982). Although structural and compositional changes in the cellular membrane might be the logical response of the microorganism to its environment, these changes may interfere with normal cell physiology. Lenaz et al. (1975) reported that the changes occurring in the lipid environment might be transferred to the activity of the membrane-bound ATPase via a conformational change of the enzymic protein, thus increasing the activation energy of this enzyme. Influence of the membrane fatty acid residues on the membrane-bound enzymes and the transport function of the membrane was also reported by Keenan and Rose (1979), Sullivan et al. (1980), Fox (1969), and Lenaz et al. (1976). Apparently, disruption of the lipid bilayer structure and alteration of the lipid composition interferes with the lipid-lipid, lipid-protein and protein-protein interactions and disrupts the transport and permeability function of biological membranes. As stated by Brown and Wolken (1979), the mobility of proteins in the membrane depend on the lipids which surround a specific protein and on the degree of the lipid-protein interactions.

In accordance with the information available in the literature, the computer simulation studies performed in this work pointed out that model parameters n, k_n, P, K_{G_2} and k_{BA_2} were the major parameters responsible for the malfunction

of the Retarded Culture. These parameters respectively characterize the number of active transport "sites" in the cellular membrane (n), the rate of variation of n with time, the permeability coefficient, the decay of cellular RNA due to butanol inhibition, and the conversion of butyric acid to butanol. As mentioned earlier, the effect of the low membrane permeability is characterized by both a low P and a high k_{G_2}. Similarly, improvement of the membrane permeability affects both of these parameters. Although the possibility of existence of other problems, e.g. the lower activity of the enzymes associated with different stages of the microbial metabolic pathway, cannot be eliminated, the results indicate that the factors associated with the cellular membrane and the enzymatic apparatus for the conversion of butyric acid to butanol were the major rate limiting parameters.

Experimental results presented in this work and in the earlier communication (Yerushalmi et al., 1985)* support the theoretical predictions regarding an altered transport and a lower membrane permeability. As stated previously, the behavior of the carriers and the number of membrane carriers are among the most important factors contributing to the magnitude of the concentration gradient generated by an active transport, and the transport rate of a solute across the cellular membrane. This can describe the results of the cellular transport of 3-O-methylglucose by the normal and the retarded cultures indicating a larger accumulation coefficient and a higher initial uptake rate for the normal culture

(Yerushalmi et al., 1985)*. The stimulating effect of pH on the sugar transport which was shown by direct measurement of the transport rate in the same communication was characterized by an increased k_n in the computer simulation of the Retarded Culture in the Culture Group III. In this Group, a lower permeability coefficient (P), a lower initial number of the active sites (n), and a lower conversion of butyric acid to butanol (k_{BA_2}) were observed in spite of a complete sugar utilization. Lowering of the above parameters resulted in a low acid conversion and solvent yield.

The results of measurement of the cellular membrane permeability to butanol and butyric acid (Table 7) confirmed the theoretical predictions regarding a lower cell membrane permeability in the Retarded Culture.

Although constantly lower, the measured values of permeability were in the same range of the predicted values from the modelling and computer simulation studies (Table 7). The lower values obtained by the measurements may be due to the following reasons:

- The errors involved in the approximation of the extracellular water.
- The unavoidable lag time between the exposure of the loaded cells to the external medium and the start of sampling. Although this lag phase did not exceed a few seconds it was enough for the diffusion of the compounds out of the cell and could be a source of error.

- The differences between the measured temperature and that of a real fermentation process. Dependence of the cellular permeability on temperature has been reported by Lieb and Stein (1971). In general, permeability increases with the increase of temperature. Since the comparison between the two cultures was the major objective of this experimental study, the experiments were performed at room temperature which is by perhaps 15°C lower than the temperature at which the fermentation was normally carried out.

It should be noticed that the permeability coefficient used in the mathematical models and in further stages of the work is a lumped parameter based on the wet weight of the cells, with units of L/g (wet cell).h. Its units differ from the usual permeability units of cm/sec.

Study of the morphology and the size distribution of the two cultures revealed differences in their appearance (Figures 6 and 7) a phenomenon which has also been established as an adaptive response of the bacterial cell to environmental changes (Rogers, 1979). The larger size of the Retarded Culture cells as compared with that of the normal culture, accompanied by the frequent mutual adherence of these cells together (Figures 6, 7 and 8), may account, at least to some extent, for the lower permeability coefficient of the cellular membrane determined for the Retarded Culture. This is due to the smaller area available per gram of cells

in this culture. Although the permeability of the membrane to butanol and butyric acid is different (Table 7), the numerical values of these coefficients are close and of the same order of magnitude. This is not surprising considering the structural similarity of these two compounds, their close molecular weights, and the same number of carbon atoms in their molecules all of which results in similar partition coefficients and diffusion through the membrane of the two compounds (Leib and Stein, 1971). Considering this numerical similarity, and for the sake of simplicity, the same permeability coefficient (P) was considered for both butanol and butyric acid in the simulation studies.

The lower permeability of the membrane in the Retarded Culture is particularly important because in the acetone-butanol fermentation it will result in excess intracellular accumulation of the inhibitory products which in turn will further disrupt the cellular membrane and interfere with the cellular metabolic activities.

There are several different ways in which organic solvents, and alcohols in particular, interfere with the normal function of the cell. Cellular membrane is believed to be the primary site of the cell function disruption caused by the alcohols (Ingram, 1976). They can dissolve in the lipid core of the membrane and disrupt the crucial interactions in the membrane lipid bilayer resulting in the compositional, structural and functional alterations of the membrane (Lenaz et al., 1976; Linden and Moreira, 1982; Grisham and

Barnett, 1972). Recently, Linden and Moreira (1982) showed that butanol affects the activity of the membrane-bound ATPase in C. acetobutylicum, thus inhibiting the sugar transport across the cellular membrane. This inhibition was shown to be the result of the increase in the activation energy of this enzyme (Moreira et al., 1981; Lenaz et al., 1976) as a consequence of a conformational change of the enzyme protein due to the mobility change of the lipids (Raison, 1973). Similar effects of alcohol on the permeability of the membrane are reported in Staphylococcus aureus (Heast et al., 1971), in Saccharomyces cerevisiae (Thomas and Rose, 1976) and in E. coli (Sullivan et al., 1974).

The excessive intracellular accumulation of alcohols is probably responsible for the observed growth arrest and break in the middle of the unsuccessful fermentations, exemplified by the Cultivation Group I. There are numerous reports on the intracellular accumulation of alcohols, and in particular ethanol, in fermentation processes (Loureiro and Ferreira, 1983; Novak et al., 1981), which is believed to be due to an imbalance between the rates of production and the outflux of ethanol. This accumulation, particularly in the early stages of fermentation (Novak et al., 1981) or in a rapid fermentation (Nagodawithana and Steinkraus, 1976), results in inhibition of cellular activities and contributes to the cell death rate. Similar alcohol-induced fermentation pattern (fermentations with an arrest of cellular activities in the middle) was obtained in B. subtilis and E. coli

(Regomier et al., 1980) and in Clostridium thermocellum (Herraro et al., 1982; Herraro and Gomez, 1980). In these cultures, addition of n-alkanes stopped the growth and cellular activities for a certain period of time. The cellular activities eventually resumed at an inhibited rate. This behavior is similar to the observations reported in this work (Figure 2) where the culture exhibited a rapid start up followed by a period of arrest in the activity and a lower rate of growth and solvent production during the subsequent phase. Studies on the membrane composition of two C. thermocellum strains challenged with ethanol revealed that the phase of growth arrest resulted in lipid alterations in both strains and an altered membrane composition was observed during the second phase (Herraro et al., 1982). This was explained as an adaptive response of the culture to growth in the presence of alcohols. As concluded by Herraro and Gomez (1980), the duration of the period of growth arrest may be the time required for cells to undergo adaptation in response to membrane alterations caused by ethanol addition. A longer period of arrest observed in the fermentation of Group 1 in the present work may be due to a lower membrane permeability and excess intracellular accumulation of the alcohols which severely inhibited cellular membrane functions.

An approach to enhancement of the permeability of cellular membrane is the use of additives or cofactors such as unsaturated fatty acids which are known to increase the permeability of cellular membranes (Felix, 1982; Panchal and

Stewart, 1981). Addition of linoleic acid at a concentration of 0.1% (w/v) or Tween 80 at a concentration of 0.2% (w/v) to the fermentation medium of Saccharomyces uvarum increased both the production rate of ethanol and the uptake rate of glucose by the yeast cells (Panchal and Stewart, 1981). This is similar to the effect of Tween 80 on the fermentation pattern of the retarded cultures of C. acetobutylicum reported in this work (Figure 9) where the production rate of the solvents, and the uptake rate of glucose were significantly enhanced and a 70% increase in the total solvent yield was obtained. Under these circumstances, addition of the unsaturated lipids to the growth medium enhanced both the efflux of alchols from the cell and the influx of glucose into the cell.

A different approach to the enhancement of cellular membrane permeability and reduction of intracellular alcohol accumulation is the use of a lower-density cell population in the fermentation as suggested by Nagodawithana and Steinkraus (1976). These authors reported that in brewer's yeast, a lower cell population resulted in a facilitated ethanol diffusion from the cells and retained cell viability during fermentation. Influence of a low cell population on the enhancement of cellular permeability was also shown in the present work (Figure 10). The use of a small inoculum resulted in a normal solvent yield of 35.7% and a complete sugar conversion with a Retarded Culture that would otherwise produce a substandard fermentation under normal conditions and

with a regular inoculum size of 3%. This phenomenon has been related to the relatively greater volume of free liquid with the lower cell population and its effect on the membrane permeability. These experiments serve as further evidence for the impact of an impaired cellular membrane permeability and transport on the solvent yield and sugar conversion efficiency in the A-B-E fermentation.

This work utilized the method of systems analysis in analyzing partial reaction steps and biological phenomena based on known physical and physicochemical principles. Coupling of these phenomena to the analysis of the system supported a description of the "normal" and "retarded" behavior of the C. acetobutylicum culture and illustrated the potential for the use of systems analysis in biological processes.

Development of a mathematical model which included the cellular physiological parameters and the internal and external cellular conditions, together with the computer simulation studies which served in elucidation of the key process parameters, demonstrated a new quality in the use of mathematical modelling in microbially catalyzed processes.

8.5 NOMENCLATURE

Symbol	Meaning
A_i	Intracellular acetone concentration
A_{ex}	Extracellular acetone concentration
AA_i	Intracellular acetic acid concentration
AA_{ex}	Extracellular acetic acid concentration
B_i	Intracellular butanol concentration
B_{ex}	Extracellular butanol concentration
BA_i	Intracellular butyric acid concentration
BA_{ex}	Extracellular butyric acid concentration
$\overline{BA}$	Mean butyric acid solution concentration
E_i	Intracellular ethanol concentration
E_{ex}	Extracellular ethanol concentration
k_1	Constant in Equation (3)
k_A	Constant in Equation (15)
K_{AA}	Acetic acid saturation constant

Symbol	Meaning
k_{AA_1}	Constant in Equation (13)
k_{AA_2}	Constant in Equation (13)
k_B	Constant in Equation (11)
K_{BA}	Butyric acid saturation constant
k_{BA_1}	Constant in Equation (9)
k_{BA_2}	Constant in Equation (9)
K_D	Constant in Equation (9)
k_E	Constant in Equation (7)
k_{G_1}	Constant in Equation (1)
k_{G_2}	Constant in Equation (1)
K_I	Inhibition constant
k_n	Constant in Equation (4)
K_S	Monod constant
n	Number of the active transport "sites" in the cellular membrane.
P	Permeability coefficient
r_s	Specific rate of substrate utilization

Symbol	Meaning
t	Time
V	Wet cell volume
X	Biomass concentration
y	Marker of the physiological state of the culture

Greek Letters

α	Ratio of the dry to wet cell weight
μ	Specific culture growth rate

8.6 REFERENCES

1. Brown, G.H. and J.J. Wolken. 1979. Liquid Crystals and Biological Structures, New York, Academic Press, 156-162.

2. Felix, H. 1982. Permeabilized Cells, Anal. Biochem., 120, 211-234.

3. Fox, C.F. 1969. A Lipid Requirement for Induction of Lactose Transport in Escherichia coli, Biochemistry, 63, 850-855.

4. Goldfine, H. and N.C. Jonstone. 1980. In: M. Kates and A. Kuksis (eds.) Membrane Fluidity: Biophysical Techniques and Cellular Regulation, 365-380.

5. Grisham, C.M. and R.E. Barnett. 1973. The Effects of Long-Chain Alcohols on Membrane Lipids and the $(NA^{+}+K^{+})$-ATPase, Biochim. Biophys. Acta, 311, 417-422.

6. Goldfine H., N.C. Johnston and M.C. Phillips. 1981. Phase Behavior of Ether Lipids from Cl. butyricum, Biochemistry, 20, 2908-2916.

7. Heast, C.W.M., J. DeGier, J.A.F. Op Den Kamp, P. Bartels and L.L.M. Van Deenan. 1972. Changes in Permeability of

Staphylococcus aureus and Derived Liposomes with Varying Lipid Composition, Biochim. Biophys. Acta, 255, 720-733.

8. Herraro, A.A. and R.F. Gomez. 1980. Development of Ethanol Tolerance in Clostridium thermocellum: Effect of Growth Temperature, J. Appl. Environ. Microbiol. 40, 571-577.

9. Herraro, A.A., R.F. Gomez and M.F. Roberts. 1982. Ethanol-Induced Changes in the Membrane Liquid Composition of Clostridium thermocellum, Biochim. Biophys. Acta 693, 195-204.

10. Hill, M.W. 1974. The Effect of Anaesthetic-Like Molecules on the Phase Transition in Smectic Mesophases of Dipalmitoyllecithin in the Normal Alcohol up to C=9 and Three Inhalation Anaesthetics, Biochim. Biophys. Acta, 356, 117-124.

11. Ingram, L.O. 1976. Adaptation of Membrane Lipids to Alcohols, J. Bacteriol., 125, 670-678.

12. Ingram, L.O. 1982. Regulation of Fatty Acid Composition in E. coli: A Proposed Common Mechanism for Changes Induced by Ethanol, Chaotropic Agents, and a Reduction of Growth Temperature, J. Bacteriol., 149, 166-172.

13. Kates, M., D.J. Kushner and A.T. James. 1962. The Lipid Composition of Bascillus cereus as Influenced by the Presence of Alcohols in the Culture Medium, Can. J. Biochem. Physiol., 40, 83-94.

14. Keenan, M.H.J. and A.H. Rose. 1979. Plasma-Membrane Lipid Unsaturation Can Affect the Kinetics of Solute Accumulation by Saccharomyces cerevisiae, FEMS Microbiol. Lett. 6, 133-137.

15. Lenaz, G., G. Parenti-Castelli and A.M. Sechi. 1975. Lipid Protein Interactions in Mitochondria, Arch. Biochem. Biophys., 167, 72-79.

16. Lenaz, G., E. Bertoli, G. Curatola, L. Mazzanti and A. Bigi. 1976. Lipid Protein Interactions in Mitochondria, Arch. Biochem. Biophys., 172, 278-288.

17. Leib, W.R. and W.D. Stein. 1970. In: F. Bronner and A. Kleinzeller (eds.), Current Topics in Membranes and Transport, New York, Academic Press, 17-38.

18. Linden, J.C. and A. Moreira. 1982. In: A. Hollaender, A.I. Laskin and P. Rogers (eds.), Biological Basis For New Developments in Biotechnology, Plenum Press Inc., New York, N.Y., 377-404.

19. Long, S., D.T. Jones and D.R. Woods. 1984. The Relationship Between Sporulation and Solvent production in Clostridium acetobutylicum P262, Biotechnol. Lett., 6, 529-534.

20. Loureiro, V. and H.G. Ferreira. 1983. On the Intracellular Accumulation of Ethanol in Yeast, Biotechnol. Bioeng. 25, 2263-2269.

21. Nagodawithana, T.W. and K.H. Steinkraus. 1976. Influence of the Rate of Ethanol Production and Accumulation on the Viability of Saccharomyces cerevisiae in "Rapid Fermentation", Appl. Environ. Microbiol., 31, 158-162.

22. Novak, M., P. Strehaiano, M. Moreno and G. Goma. 1981. Alcoholic Fermentation: On the Inhibitory Effect of Ethanol, Biotechnol. Bioeng. 23, 201-211.

23. Panchal, C.J. and G.G. Stewart. 1981. Regulatory Factors in Alcohol Fermentations, Proc. Int. Yeast Symp. 9-15.

24. Pazoutova, S., J. Votruba and Z. Rehacek. 1981. A Mathematical Model of Growth and Alkaloid Production in the Submerged Culture of Claviceps purpurea, Biotechnol. Bioeng., 23, 2837-2849..

25. Raison, J.K. 1973. Effect of Temperature-Induced Phase Changes on the Kinetics of Respiratory and Other Membrane-Associated Enzyme Systems., J. Bioenerg. 4, 285-309.

26. Rigomier, D., J.P. Bohin and B. Lubochinsky. 1980. Effects of Ethanol and Methanol on Lipid Metabolism in Bacillus subtilis, J. Gen. Microbiol., 121, 139-149.

27. Rogers, H.J. 1979. In: A.H. Rose and J.G. Morris (eds.) Advances In Microbial Physiology, Vol. 19, Academic Press 1-57.

28. Snell, F.M., S. Shulman, R.P. Spencer and C. Moos. 1965. Biophysical Principles of Structure and Function, Addison-Wesley Pub. Co., Inc. 288-321.

29. Sullivan, K.H., M.K. Jain and A.L. Koch. 1974. Activation of the β. Galactoside Transport System in Escherichia coli ML-308 by n-Alkanols, Biochim. Biophys. Acta, 352, 287-297.

30. Thomas, D.S. and A.H. Rose. 1979. Inhibitory Effect of Ethanol on Growth and Solute Accumulation by Saccharomyces cerevisiae as affected by Plasma-Membrane Lipid Composition, Arch. Microbiol., 122, 49-55.

*31. Votruba, J., B. Volesky and L. Yerushalmi. 1985. Mathematical Model of a Batch Acetone-Butanol Fermentation, Biotechnol. Bioeng., submitted for publication.

32. Votruba, J. 1982. Practical Aspects of Mathematical Modelling of Fermentation Processes as a Method of Description, Simulation, Identification and Optimization, Acta Biotechnol., 2, 119-126.

*33. Yerushalmi, L., B. Volesky and J. Votruba. 1985. System Analysis of the Culture Physiology in the Acetone-Butanol Fermentation, Biotechnol. Bioeng. Submitted for publication.

9

CONCLUSIONS AND RECOMMENDATIONS

9.1 CONCLUSIONS

Study of the physiological aspects of acetone-butanol fermentation resulted in the following conclusions:

1. The Process Kinetic Model developed by considering biochemical and physiological aspects of the microbial growth and metabolic biosynthesis adequately represented the experimental system of the batch acetone-butanol fermentation process. This model was capable of describing various batch phases including the initial lag phase and the microbial decay due to the end-product inhibition.

2. A growth model was developed which can describe the growth dynamics depending on the history of the microbial culture.

3. The cellular membrane permeability, the number of sugar transport "sites" and the enzymatic apparatus control-

ling the conversion of butyric acid to butanol were found to be among the major parameters regulating the production of solvents and controlling the performance of C. acetobutylicum.

4. The rate of sugar transport across the cellular membrane is directly proportional to the number of active transport "sites" (n) in the cell membrane.

5. Transport of butanol out of the cell occurs due to the chemical concentration gradient while butyric acid transport is also dependent on the electrical potential gradient across the cellular membrane.

6. pH of the fermentation medium affects the uptake rate of sugar and the sugar accumulation coefficient across the cellular membrane. A pH adjustment from 4.2 to 5.0 in the early stages of the batch process increased the accumulation coefficient of a normal culture of C. acetobutylicum by a factor of 3.5 and its initial sugar uptake rate by a factor of 3.0.

7. Addition of unsaturated lipids to the growth medium can enhance solvent production in the A-B-E fermentation by apparently affecting the mass transport properties of the cell membrane.

8. The parametric sensitivity analysis carried out for the Process Kinetic Model and for the Physiological State Model indicated the importance of the key process parameters. According to this analysis, the kinetic parameters controlling the production rates of butanol, butyric acid and biomass growth as well as those phyiological parameters characterizing the dynamics of the number of active "sites" in the cell membrane, the conversion of butyric acid to butanol and the membrane permeability are the most significant parameters in the acetone-butanol fermentation.

9. The culture of Clostridum acetobutylicum is sensitive to high mixing rates and a control on this operating parameter is necessary for an optimum production of solvents in the A-B-E fermentation. While the "degree of mixing" is difficult to define, the optimum culture agitation under the examined experimental conditions was established at the impeller Reynolds number of 3.93×10^4. Under these conditions the maximum production rates of the solvents were 5.5, 3.8 and 0.8 mmole/h.g cell for butanol, acetone and ethanol respectively. Corresponding rates for the respective gases produced were 11.6 and 15.9 mmole/h.g cell for hydrogen and carbon dioxide.

10. A linear correlation exists between the specific molar quantities of the produced solvents and gases. A better correlation was always obtained during the second phase of fermentation. This correlation has the following form:

$$\frac{dA_1}{dt} = \alpha \frac{dA_2}{dt}$$

where A_1 and A_2 represent mole of gases and solvents, respectively per gram of cells and t is the time. The proportionality constant, α, was not constant and varied with the variations of the mixing rate in the fermentator. The correlation between hydrogen gas and butanol is particularly important since the metabolic production of these two compounds is related. For the impellar speeds of 190 rpm to 410 rpm examined in this work the proportionality constant between the production rates of hydrogen and butanol ranged from 1.5 to 2.1 mole H_2/mole butanol.

11. The results of the theoretical solvent yield calculations depend on the reaction stoichiometry considered

and on the ratio between the solvents. The maximum theoretical solvent yield was established ranging from 38.6% to 39.9%. These values were obtained for an ideal biosynthetic situation when no intermediate acids were left over in the system and no carbon was utilized in the production of biomass.

12. Elevated hydrogen partial pressure regulates the metabolic pathway of C. acetobutylicum and affects the biosynthesis of the solvents. With increasing hydrogen pressure the yields of butanol and ethanol on glucose increased by an average of 18% and 13% respectively. The respective yields of acetone and of the endogenous H_2 decreased by an average of 40% and 30% and almost no effect was observed on the growth of the culture. The major changes occurred at pressures lower than 270 KPa absolute of hydrogen partial pressure. The results suggest that butanol production can be enhanced at the expense of acetone in the A-B-E process.

9.2 ORIGINAL CONTRIBUTIONS

There is a number of aspects in this work which can be considered as an original contribution to the knowledge in biochemical engineering. The major ones have a profound bearing on the process optimization and a corresponding optimal control strategy. The latter part of the thesis is particularly significant in establishing methodology for the development and application of mathematical models including physiological culture parameters.

The following are the major contribution points of this work:

1. Derivation of a correlation between the gas and solvent production is very useful for process control and optimization;

2. Establishment of the theoretical solvent yield and the comprehensive methodology used for its calculation.

Utilization of the three-dimensional plot for the variations of the solvent yield with the weight fractions of solvents is a useful method to demonstrate the effect of the solvent ratio on the TNVP yield.

3. The hypothesis of the solvent biosynthesis regulation by the hydrogen partial pressure in the fermentation system was verified. This approach can be used to maximize the production of solvents and particularly butanol in the acetone-butanol process.

4. Construction of a basic batch culture model capable of describing the behavior of the Clostridium acetobutylicum strain even during the transitional phases of activity. The model, based on biochemical and experimental information, served as a stepping stone for further development of a more sophisticated one.

5. The development of the Physiological State Model for the culture studies. The model includes the cellular physiological parameter as well as the internal and external cellular conditions and is based on the metabolic interrelationship of the biosynthetic products.

6. The culture phenomena, including the transport of sugar, solvents and acids across the cellular membrane, were systematically analyzed. The quantitative assessment of physiological aspects of the microbial culture studies assisted in subsequent demonstration of the membrane function in the regulation of solvent production.

7. The innovative "diagnostic" application of the Physiological State Model in elucidation of the role of cellular membrane parameters in control of the culture behavior establishes aspects of novel methodology and demonstrates a new quality in the use of mathematical modelling in fermentation processes.

9.3 SUGGESTIONS FOR FUTURE STUDY

1. The elevated partial presure of hydrogen gas was shown in this work to regulate to a certain degree the production of solvents in the acetone-butanol fermentation. The biochemical basis of this regulation, the effect of the hydrogen pressure on the metabolic enzymes controlling the flow of electrons and the balance of $NAD/NADH_2$ can be studied.

2. A techno-economic feasibility study can be performed on a modified fermentation process operating under high pressures of hydrogen gas with a resulting increase in the production yield of butanol.

3. The primary site for the inhibitory effect by the end products in the acetone-butanol fermentation is shown to be the cellular membrane of the microbial culture. The possibility of improvement in tolerance of the microbial cellular membrane to the alcohols by incorporation of unsaturated fatty acids, or by alternative

techniques such as genetic manipulation, can be investigated.

4. The effect of a modified and more tolerant cellular membrane on the actual production of solvents by the microbial culture can be examined.

5. The process kinetics model developed in this study can be used as a basis for modelling of the fed-batch and continous-flow culture modes and their subsequent use in the optimization of the corresponding processes.

6. As the continuation of the work presented in this thesis, the method of systems analysis can be employed in evaluation of the culture phenomena and the relationship between the major fermentation parameters of a number of other fermentation processes which show a strong potential of becoming economically attractive for the production of key organic chemicals.

7. The Physiological State Model proved to be a valuable tool in investigating the microbial physiology and in elucidating the role of key culture parameters. This model can be utilized to describe alternative culture modes such as fed batch, continous-flow, and immobilized-cell operations. This approach would be useful in

both evaluating the adequacy of the proposed mathematical model in simulating these cultivation and in studying the influence of the culture physiological parameters on the productivity of the fermentation system..

APPENDIX 1

Listing of the Computer Program for the Smoothing of the Experimental Data.

```
      PRELIMINARY DATA ANALYSIS BY SMOOTHING SPLINES

      DIMENSION LL(30),YP(40,8),T(40),DY(40),V(40,7),A(40,7),
     *YM(40,8),YD(40,8),Y(40)
   0  READ(5,*) NPOINT,NPROF
      IF(NPOINT.LT.1) STOP
      READ(5,99) (LL(I),I=1,30)
   9  FORMAT(30A2)
      WRITE(6,100) (LL(I),I=1,30),NPOINT,NPROF
 100  FORMAT(//30A2/5X,7HNPOINT=,I3,2X,6HNPROF=,I3//)
      DO 1 I=1,NPOINT
      READ(5,*) T(I),(YP(I,J),J=1,NPROF)
      WRITE(6,101) T(I),(YP(I,J),J=1,NPROF)
 101  FORMAT(1X,F4.1,8F7.3)
      S=SQRT(2.*FLOAT(NPOINT))
      DO 10 J=1,NPROF
      DO 11 I=1,NPOINT
      Y(I)=YP(I,J)
   1  DY(I)=0.1
      SFS=SMOOTH(T,Y,DY,NPOINT,S,V,A)
      NPM1=NPOINT-1
      DO 12 I=1,NPM1
      YM(I,J)=A(I,1)
  12  YD(I,J)=A(I,2)
      DX=T(NPOINT)-T(NPM1)
      YM(NPOINT,J)=A(NPM1,1)+A(NPM1,2)*DX+A(NPM1,3)*DX**2/2.
     *+A(NPM1,4)*DX**3/6.
      YD(NPOINT,J)=A(NPM1,2)+A(NPM1,3)*DX+A(NPM1,4)*DX**2/2.
  10  CONTINUE
      WRITE (6,102)
      DO 31 I=1,NPOINT
  31  WRITE(6,103) T(I),(YM(I,J),J=1,NPROF)
 103  FORMAT(1X,F4.1,8F7.3)
 102  FORMAT(//5X,' SMOOTHED VALUES '/)
      CALL TRANSF(NPOINT,T,YM,YD)
      GO TO 50
      END
```

ppendix 1, continued

```
      SUBROUTINE TRANSF(N,X,Y,YD)
      DIMENSION X(40),Y(40,8),YD(40,8),F(20)
      WRITE (6,100)
100   FORMAT(//5X,' TABLE OF RESULTS '/)
      DO 1 I=1,N
      F(1)=YD(I,1)/Y(I,1)
      F(2)=-YD(I,2)/Y(I,1)
      F(3)=YD(I,3)/Y(I,1)
      F(4)=YD(I,4)/Y(I,1)
      F(5)=YD(I,5)/Y(I,1)
      F(6)=YD(I,6)/Y(I,1)
      F(7)=YD(I,7)/Y(I,1)
1     WRITE(6,101) X(I),(F(J),J=1,7)
101   FORMAT(1X,F4.1,7F9.5)
      RETURN
      END

      REAL FUNCTION SMOOTH(X,Y,DY,NPOINT,S,V,A)
      DIMENSION A(40,4),DY(40),V(40,7),X(40),Y(40)
      CALL SETUPQ(X,DY,Y,NPOINT,V,A(1,4))
      IF(S.GT.0.) GO TO 20
10    P=1.
      CALL CHOLID(P,V,A(1,4),NPOINT,1,A(1,3),A(1,1))
      SFP=0.0
      GO TO 60
20    P=0.
      CALL CHOLID(P,V,A(1,4),NPOINT,1,A(1,3),A(1,1))
      SFP=0.
      DO 21 I=1,NPOINT
21    SFP=SFP+(A(I,1)*DY(I))**2
      SFP=SFP*36.
      IF(SFP.LE.S) GO TO 60
      PREVP=0.
      PREVSF=SFP
      UTRU=0.
      DO 25 I=2,NPOINT
25    UTRU=UTRU+V(I-1,4)*(A(I-1,3)*(A(I-1,3)+A(I,3))+A(I,3)**2)
      P=(SFP-S)/(24.*UTRU)
30    CALL CHOLID(P,V,A(1,4),NPOINT,1,A(1,3),A(1,1))
      SFP=0.
      DO 35  I=1,NPOINT
35    SFP=SFP+(A(I,1)*DY(I))**2
      SFP=SFP*36.*(1.-P)**2
      IF(SFP.LE.1.01*S) GO TO 60
      IF(SFP.GE.PREVSF) GO TO 10
      CHANGE=(P-PREVP)/(SFP-PREVSF)*(SFP-S)
      PREVP=P
      P=P-CHANGE
      PREVSF=SFP
      IF(P.LT.1) GO TO 30
      P=1.-SQRT(S/PREVSF)*(1.-PREVP)
      GO TO 30
```

```
60      SMOOTH=SFP
        SIXIMP=6.*(1.-P)
        DO 61 I=1,NPOINT
61      A(I,1)=Y(I)-SIXIMP*DY(I)**2*A(I,1)
        SIXP=6.*P
        DO 62 I=1,NPOINT
62      A(I,3)=A(I,3)*SIXP
        NPM1=NPOINT-1
        DO 63 I=1,NPM1
        A(I,4)=(A(I+1,3)-A(I,3))/V(I,4)
63      A(I,2)=(A(I+1,1)-A(I,1))/V(I,4)
     *  -(A(I,3)+A(I,4)/3.*V(I,4))/2.*V(I,4)
        RETURN
        END
        SUBROUTINE SETUPQ(X,DX,Y,NPOINT,V,QTY)
        DIMENSION DX(40),QTY(40),V(40,7),X(40),Y(40)
        NPM1=NPOINT-1
        V(1,4)=X(2)-X(1)
        DO 11 I=2,NPM1
        V(I,4)=X(I+1)-X(I)
        V(I,1)=DX(I-1)/V(I-1,4)
        V(I,2)=-DX(I)/V(I,4)-DX(I)/V(I-1,4)
11      V(I,3)=DX(I+1)/V(I,4)
        V(NPOINT,1)=0.
        DO 12 I=2,NPM1
12      V(I,5)=V(I,1)**2+V(I,2)**2+V(I,3)**2
        IF(NPM1.LT.3) GO TO 14
        DO 13 I=3,NPM1
13      V(I-1,6)=V(I-1,2)*V(I,1)+V(I-1,3)*V(I,2)
14      V(NPM1,6)=0.
        IF(NPM1.LT.4) GO TO 16
        DO 15 I=4,NPM1
15      V(I-2,7)=V(I-2,3)*V(I,1)
16      V(NPM1-1,7)=0.0
        V(NPM1,7)=0.
        PREV=(Y(2)-Y(1))/V(1,4)
        DO 21 I=2,NPM1
        DIFF=(Y(I+1)-Y(I))/V(I,4)
        QTY(I)=DIFF-PREV
21      PREV=DIFF
        RETURN
        END
```

Appendix 1, continued

```
      SUBROUTINE CHOLID(P,V,QTY,NPOINT,NCOL,U,QU)
      DIMENSION QTY(40),QU(40),U(40),V(40,7)
      NPM1=NPOINT-1
      SIXIMP=6.*(1.-P)
      TWOP=2.*P
      DO 2 I=2,NPM1
      V(I,1)=SIXIMP*V(I,5)+TWOP*(V(I-1,4)+V(I,4))
      V(I,2)=SIXIMP*V(I,6) +P*V(I,4)
2     V(I,3)=SIXIMP*V(I,7)
      NPM2=NPOINT-2
      IF(NPM2.GE.2) GO TO 10
      U(1)=0.
      U(2)=QTY(2)/V(2,1)
      U(3)=0.0
      GO TO 41
10    DO 20 I=2,NPM2
      RATIO=V(I,2)/V(I,1)
      V(I+1,1)=V(I+1,1)-RATIO*V(I,2)
      V(I+1,2)=V(I+1,2)-RATIO*V(I,3)
      V(I,2)=RATIO
      RATIO=V(I,3)/V(I,1)
      V(I+2,1)=V(I+2,1)-RATIO*V(I,3)
20    V(I,3)=RATIO
      U(1)=0.
      V(1,3)=0.0
      U(2)=QTY(2)
      DO 30 I=2,NPM2
30    U(I+1)=QTY(I+1)-V(I,2)*U(I)-V(I-1,3)*U(I-1)
      U(NPOINT)=0.0
      U(NPM1)=U(NPM1)/V(NPM1,1)
      DO 40 II=2,NPM2
      I=NPOINT-II
40    U(I)=U(I)/V(I,1)-U(I+1)*V(I,2)-U(I+2)*V(I,3)
41    PREV=0.0
      DO 50 I=2,NPOINT
      QU(I)=(U(I)-U(I-1))/V(I-1,4)
      QU(I-1)=QU(I)-PREV
50    PREV=QU(I)
      QU(NPOINT)=-QU(NPOINT)
      RETURN
      END
```

Appendix 1, continued

DATA BATCH CULTIVATION NO 9 ACETONE BUTANOL FERMENTATION
NPOINT= 10 NPROF= 7

0.0	0.060	50.500	0.0	0.0	0.0	0.0	0.0
3.0	0.060	50.500	0.0	0.0	0.0	0.0	0.0
8.0	0.100	50.000	0.0	0.0	0.0	0.125	0.125
12.0	0.375	48.500	0.040	0.020	0.025	0.750	0.600
15.0	1.400	43.000	0.150	0.040	0.050	2.350	1.400
17.5	2.700	35.500	0.400	0.150	0.110	3.700	2.300
21.0	3.575	22.000	3.950	1.300	0.400	1.650	3.000
24.0	3.825	10.000	8.430	4.300	0.780	0.200	2.650
27.0	3.875	0.0	11.000	5.000	1.100	0.100	2.500
32.5	3.600	0.0	11.000	5.000	1.100	0.100	2.500

SMOOTHED VALUES

0.0	0.063	50.504	0.000	0.000	-0.001	-0.001	-0.004
3.0	0.044	50.492	-0.001	-0.000	-0.011	-0.000	-0.013
8.0	0.055	50.047	0.002	0.001	-0.020	0.110	0.120
12.0	0.484	48.448	0.032	0.019	0.007	0.786	0.658
15.0	1.491	42.996	0.059	0.028	0.079	2.425	1.467
17.5	2.555	35.486	0.536	0.132	0.199	3.526	2.239
21.0	3.542	22.012	4.017	1.435	0.461	1.716	2.846
24.0	3.849	9.912	8.326	4.146	0.726	0.244	2.737
27.0	3.878	0.166	10.963	5.046	0.950	0.069	2.539
32.5	3.610	-0.063	11.035	5.004	1.177	0.101	2.486

APPENDIX 2

Listing of the Computer Program for the Estimation of the Model Kinetic Constants.

```
C
C     THIS PROGRAM ESTIMATES THE COEFFICIENTS OF THE KINETIC
C    MODEL FOR THE NORMAL BATCH CULTURE OF ACETONE-BUTANOL PROCESS.
C
      DIMENSION YM(20,10),YP(20),T(20),A(20),EPS(20),LL(30),W(10),AA(20)
      COMMON /FCE/ FF
      COMMON /DAT/ NPOINT,NVAR,NEQU,T,W,YP,YM
      COMMON /KON/ A
      READ(5,*) NPOINT,NVAR,NEQU,NPAR,MFC
      READ(5,99) (LL(I),I=1,30)
99    FORMAT(30A2)
      WRITE(6,100) (LL(I),I=1,30)
100   FORMAT(//1X,30A2)
      READ(5,*) T(1)
      READ(5,*) (YP(I),I=1,10)
      WRITE(6,196) (YP(I),I=1,10)
196   FORMAT(1X,10F7.3)
      DO 1 I=1,NPOINT
      READ(5,*) T(I+1),(YM(I,J),J=1,NVAR)
1     WRITE(6,101)  T(I+1),(YM(I,J),J=1,NVAR)
101   FORMAT(1X,F4.1,9F7.3)
      READ(5,*) (A(I),I=1,9)
      READ(5,*) (A(I),I=10,18)
      READ(5,*) (AA(I),I=1,9)
      READ(5,*) (AA(I),I=10,NPAR)
      READ(5,*) (EPS(I),I=1,9)
      READ(5,*) (EPS(I),I=10,NPAR)
      READ(5,*) (W(I),I=1,NVAR)
      WRITE(6,130) (A(I),I=1,9)
      WRITE(6,131) (A(I),I=10,18)
      WRITE(6,136) (AA(I),I=1,9)
      WRITE(6,137) (AA(I),I=10,NPAR)
      WRITE(6,132) (EPS(I),I=1,9)
      WRITE(6,133) (EPS(I),I=10,NPAR)
      WRITE(6,134) (W(I),I=1,NVAR)
130   FORMAT(1X,9F8.4)
131   FORMAT(1X,9F8.4)
132   FORMAT(1X,9F8.4)
```

ppendix 2, continued

```
133   FORMAT(1X,6F8.4)
134   FORMAT(1X,9F8.4)
136   FORMAT(1X,9F8.4)
137   FORMAT(1X,6F8.4)
      FF=1.E+30
      CALL ROS(NPAR,MFC,AA,EPS,0.001)
      STOP
      END
      SUBROUTINE OBJECT(A,SSWR,NPAR)
      DIMENSION AA(20),A(20),W(10),YM(20,10),YP(20),T(20),Y(20),A1(20)
      COMMON /CON/ AA
      COMMON /KON/ A1
      COMMON /FCE/ FF
      COMMON /DAT/ NPOINT,NVAR,NEQU,T,W,YP,YM
120   FORMAT(1X,10F7.4)
      DO 5 I=1,NPAR
5     AA(I)=ABS(A(I))
      SSWR=0.0
      DO 2 I=1,NEQU
2     Y(I)=YP(I)
      Y(10)=AA(15)
      DO 1 I=1,NPOINT
      XB=T(I)
      XF=T(I+1)
      CALL ODE(NEQU,XB,XF,Y,0.01)
      DO 4 J=1,NPROF
4     SSWR=SSWR+((YM(I,J)-Y(J))/W(J))**2
1     CONTINUE
      WRITE(6,120) (A1(I),I=1,18)
      WRITE(6,110) SSWR
      IF(FF.LT.SSWR) RETURN
      FF=SSWR
      WRITE(6,111)
111   FORMAT(1H+,20X,'LAST MINIMUM'/)
110   FORMAT(1X,E12.5)
      RETURN
      END
```

ppendix 2, continued

```
      SUBROUTINE RHS(N,X,YY,F)
      DIMENSION Y(20),F(20),A(20),YY(20),AA(20)
      COMMON /CON/ AA
      COMMON /KON/A
      A(2)=AA(1)
      A(3)=AA(2)
      A(4)=AA(3)
      A(5)=AA(4)
      A(6)=AA(5)
      A(7)=AA(6)
      A(9)=AA(7)
      A(11)=AA(8)
      A(12)=AA(9)
      A(13)=AA(10)
      A(15)=AA(11)
      A(16)=AA(12)
      A(17)=AA(13)
      A(18)=AA(14)
      DO 21 I=1,N
      Y(I)=YY(I)
      IF(YY(I).LT.0.0) Y(I)=0.0
   21 CONTINUE
      FS=Y(2)/(Y(2)+A(8))
      RS=(A(3)*FS+A(4)*Y(2))*Y(1)
      FI=A(1)/(A(1)+Y(3))
      F(2)=-RS
      FBA=(A(5)*Y(2)*FI-A(7)*Y(6)/(Y(6)+A(10)))*Y(1)
      F(6)=FBA
      F(1)=0.56*(Y(10)-1.)*Y(1)-A(9)*Y(1)*Y(3)
      F(3)=A(6)*Y(2)*Y(1)-0.841*FBA
      F(7)=(A(11)*FS*FI-A(15)*Y(7)/(Y(7)+A(14)))*Y(1)
      F(4)=A(12)*FS*Y(1)-F(7)*0.484
      F(5)=A(13)*Y(1)*FS
      F(9)=A(16)*FS*Y(1)
      F(8)=A(17)*FS*Y(1)+A(18)*Y(2)*Y(1)
      F(10)=(A(2)*FI*Y(2)-0.56*(Y(10)-1.))*Y(10)
      RETURN
      END
```

ppendix 2, continued

```
      SUBROUTINE ROS(KM,MAXK,AFK,EPS,EPSY)
      DIMENSION AKE(20),D(20),V(20,20),BL(20,20),BLEN(20),EPS(20),
     1AJ(20),E(20),AL(20,20),AFK(20)
      NO=6
      NSTEP=2
      MKAT=30
      MCYC=50
      ALPHA=2.0
      BETA=0.5
      WRITE(NO,99)
99    FORMAT(1H1,10X,34HROSENBROCK MINIMIZATION PROCEDURE    )
      WRITE(NO,1004) MAXK,MKAT,MCYC,NSTEP,ALPHA,BETA,EPSY
1004  FORMAT(//2X,10HPARAMETERS/2X,7HMAXK = ,I4,4X,7HMKAT = ,I2,4X,
     1 7HMCYC = ,I2,4X,8HNSTEP = ,I2//2X,8HALPHA = ,F5.2,4X,
     2 7HBETA = ,F5.2,4X,7HEPSY = ,1E12.4 )
      KAT=1
      DO 98 II=1,KM
      DO 98 JJ=1,KM
      V(II,JJ)=0.0
      IF(II-JJ) 98,97,98
97    V(II,JJ)=1.
98    CONTINUE
      CALL OBJECT(AFK,SUMN,KM)
      SUMO=SUMN
      DO 812 K=1,KM
      AKE(K)=AFK(K)
812   CONTINUE
      KK1=1
      IF(NSTEP-1) 701,700,701
700   GO TO 1000
701   CONTINUE
      DO 350 I=1,KM
      E(I)=EPS(I)
350   CONTINUE
1000  DO 250 I=1,KM
```

Appendix 2, continued

```
      FBEST=SUMN
      AJ(I)=2.
      IF(NSTEP-1) 702;703,702
  702 GO TO 250
  703 CONTINUE
      E(I)=EPS(I)
  250 D(I)=0.0
      III=0
  397 III=III+1
  258 I=1
  259 DO 251 J=1,KM
  251 AKE(J)=AKE(J)+E(I)*V(I,J)
      CALL OBJECT(AKE,SUMN,KM)
      KAT=KAT+1
      IF(KAT-MAXK) 706,707,707
  707 GO TO 1001
  706 CONTINUE
      IF(SUMN-SUMO) 708,708,709
  708 GO TO 253
  709 CONTINUE
      DO 254 J=1,KM
  254 AKE(J)=AKE(J)-E(I)*V(I,J)
      E(I)=-BETA*E(I)
      IF(AJ(I)-1.5) 710,711,711
  710 AJ(I)=0.0
  711 CONTINUE
      GO TO 255
  253 D(I)=D(I)+E(I)
      E(I)=ALPHA*E(I)
      SUMO=SUMN
      DO 813 K=1,KM
  813 AKK(K)=AKE(K)
      IF(AJ(I) -1.5) 712,712,713
  713 AJ(I)=1.0
  712 CONTINUE
```

Appendix 2, continued

```
255   DO 256 J=1,KM
      IF(AJ(J)-0.5) 256,256,715
715   GO TO 299
256   SUMDIF=FBEST-SUMO
      SUMDIF=SUMDIF/AMAX1(ABS(FBEST),0.001)
      IF(ABS(SUMDIF)-EPSY)1001,1001,257
299   IF(I-KM) 717,716,717
716   GOTO399
717   CONTINUE
      I=I+1
      GO TO 259
399   DO 398 J=1,KM
      IF(AJ(J)-2.) 718,398,398
718   GO TO 258
398   CONTINUE
      IF(III-MCYC) 720,721,721
720   GO TO 397
721   CONTINUE
      GO TO 1001
257   CONTINUE
      DO 290 I=1,KM
      DO 290 J=1,KM
290   AL(I,J)=0.0
      WRITE(NO,280) KK1
280   FORMAT(//2X,13HSTAGE NUMBER  ,I2)
      WRITE(NO,281) SUMO
281   FORMAT(/7X,31HVALUE OF OBJECTIVE FUNCTION =  ,E12.5)
      WRITE(NO,282)
282   FORMAT(/7X,35HVALUES OF THE INDEPENDENT VARIABLES  /)
      DO 284 IX=1,KM
      WRITE(NO,283) IX,AKE(IX)
283   FORMAT(/7X,2HX(,I2,4H) = ,E16.8)
284   CONTINUE
```

```
      DO 260 I=1,KM
      KL=I
      DO 260 J=1,KM
      DO 261 K=KL,KM
261   AL(I,J)=D(K)*V(K,J) + AL(I,J)
260   BL(I,J) = AL(I,J)
      BLEN(1)=0.0
      DO 351 K=1,KM
      BLEN(1)=BLEN(1) +BL(1,K)*BL(1,K)
351   CONTINUE
      BLEN(1)=SQRT(BLEN(1))
      DO 352 J=1,KM
      V(1,J)=BL(1,J)/BLEN(1)
352   CONTINUE
      DO 263 I=2,KM
      II=I-1
      DO 263 J=1,KM
      SUMAVV=0.0
      DO 264 KK=1,II
      SUMAV=0.0
      DO 262 K=1,KM
262   SUMAV = SUMAV + AL(I,K)*V(KK,K)
264   SUMAVV=SUMAV*V(KK,J) +SUMAVV
263   BL(I,J) =AL(I,J) -SUMAVV
      DO 266 I=2,KM
      BLEN(I)=0.0
      DO 267 K=1,KM
267   BLEN(I)=BLEN(I) + BL(I,K)*BL(I,K)
      BLEN(I)=SQRT(BLEN(I))
      DO 266 J=1,KM
266   V(I,J)=BL(I,J)/BLEN(I)
      KK1=KK1+1
      IF(KK1-MKAT) 723,722,722
```

Appendix 2, continued

```
722     GO TO 1001
723     GO TO 1000
1001    WRITE(NO,1002) KK1
1002    FORMAT(///2X,25HTOTAL NUMBER OF STAGES =  ,I2)
        WRITE(NO,1003) KAT
1003    FORMAT(/2X,38HTOTAL NUMBER OF FUNCTION EVALUATION   ,I5)
        WRITE(NO,1005) SUMO
1005    FORMAT(/2X,33HFINAL VALUE OF OBJECT FUNCTION =  ,E12.5)
        DO 1007 IX=1,NM
        WRITE(NO,1006) IX,ANE(IX)
1006    FORMAT(/2X,2HX(,I2,4H) = ,E16.8)
1007    CONTINUE
        RETURN
        END
        SUBROUTINE ODE(N,XB,XE,Y,EPS)
        DIMENSION Y1(20),Y2(20),Y3(20),Y(20)
        X=XB
        H=(XE-X)/10.
C       ADOPTED FROM ALGORITHM 8 C ACM (1960) VOL.3 P.312
        IS=0
        IOUT=0
100     IF((X+2.01*H-XE).LT.0.) GO TO 1
        IOUT=1
        H=(XE-X)/2.
1       CALL RK4(N,X,Y,2.*H,X1,Y1)
101     CALL RK4(N,X,Y,H,X2,Y2)
        CALL RK4(N,X2,Y2,H,X3,Y3)
        ERR=0.0
        DO 2 K=1,N
        Q1=AMAX1(1.E-6,ABS(Y3(K)))
        P=ABS(Y1(K)-Y3(K))/Q1
2       ERR=AMAX1(P,ERR)
        IF(ERR.GT.EPS) GO TO 103
        X=X3
        IF(IOUT.EQ.1) GO TO 104
        DO 3 K=1,N
3       Y(K)=Y3(K)
```

Appendix 2, continued

```
      IF(IS.LT.5) GO TO 4
      H=2.*H
      IS=0
4     IS=IS+1
      GO TO 100
103   H=0.5*H
      IOUT=0
      X1=X2
      DO 5 K=1,N
5     Y1(K)=Y2(K)
      GO TO 101
104   DO 6 K=1,N
6     Y(K)=Y3(K)
      RETURN
      END
      SUBROUTINE RK4(N,X,Y,H,XE,YE)
      DIMENSION Y(20),YE(20),Z(20),W(20),A(5)
      DATA A /0.5,0.5,1.,1.0,0.5/
      XE=X
      DO 1 K=1,N
      YE(K)=Y(K)
1     W(K)=Y(K)
      DO 2 J=1,4
      CALL RHS(N,XE,W,Z)
      XE=A(J)*H+X
      DO 3 K=1,N
      W(K)=Y(K)+A(J)*H*Z(K)
3     YE(K)=YE(K)+A(J+1)*H*Z(K)/3.
2     CONTINUE
      RETURN
      END
```

APPENDIX 3

Listing of the Computer Program for the Parametric Sensitivity Analysis.

```
C       PARAMETER SENSITIVITY OF THE A-B-E PROCESS MODEL
C
        DIMENSION YP(20,10),T(20),A(20),LL(30),W(10)
        COMMON /DAT/ NPOINT,NVAR,T,W,YP
        READ(5,*) NPOINT,NVAR,NPAR
        READ(5,99) (LL(I),I=1,30)
99      FORMAT(30A2)
        WRITE(6,100) (LL(I),I=1,30)
100     FORMAT(//1X,30A2//)
        DO 1 I=1,NPOINT
1       READ(5,*) T(I),(YP(I,J),J=1,NVAR)
        READ(5,*) (A(I),I=1,9)
        READ(5,*) (A(I),I=10,NPAR)
        READ(5,*) (W(I),I=1,NVAR)
        CALL OBJECT(A,SS1,NPAR)
        DO 2 I=1,NPAR
        DA=0.01*A(I)
        A(I)=A(I)+DA
        CALL OBJECT(A,SS2,NPAR)
        APS=(SS2-SS1)/DA
        A(I)=A(I)-DA
        RPS=A(I)*ABS(APS)/SS1
2       WRITE(6,102) A(I),APS,RPS
102     FORMAT(1X,F8.4,2X,2E14.6)
        STOP
        END
        SUBROUTINE OBJECT(A,SSWR,NPAR)
        DIMENSION AA(20),A(20),W(10),YP(20,10),T(20),Y(20)
        COMMON /KON/ AA
        COMMON /DAT/ NPOINT,NVAR,T,W,YP
        DO 5 I=1,NPAR
5       AA(I)=ABS(A(I))
        SSWR=0.0
        NPM1=NPOINT-1
        DO 1 I=1,NPM1
        IF(I.GT.1) GO TO 3
        DO 2 J=1,NVAR
```

Appendix 3, continued

```
2       Y(J)=YP(I,J)
3       XB=T(I)
        XF= T(I+1)
        CALL ODE(NVAR,XB,XF,Y,0.01)
        DO 4 J=1,NVAR
4       SSWR=SSWR+((YP(I+1,J)-Y(J))/W(J))**2
1       CONTINUE
        RETURN
        END
        SUBROUTINE RHS(N,X,Y,F)
        DIMENSION Y(20),F(20),A(20)
        COMMON /KON/A
        FS=Y(2)/(Y(2)+A(8))
        RS=(A(3)*FS+A(4)*Y(2))*Y(1)
        FI=A(1)/(A(1)+Y(3))
        F(2)=-RS
        FBA=(A(5)*Y(2)*FI-A(7)*Y(6)/(Y(6)+A(10)))*Y(1)
        F(6)=FBA
        F(1)=A(2)*Y(2)*FI*Y(1)-A(9)*Y(1)*Y(3)
        F(3)=A(6)*Y(2)*Y(1)-0.841*FBA
        F(7)=(A(11)*FS*FI-A(15)*Y(7)*FS/(Y(7)+A(14)))*Y(1)
        F(4)=A(12)*FS*Y(1)-F(7)*0.484
        F(5)=A(13)*Y(1)*FS
        F(9)=A(16)*FS*Y(1)
        F(8)=A(17)*FS*Y(1)+A(18)*Y(2)*Y(1)
        RETURN
        END
```

APPENDIX 4

Listing of the Computer Program for the Simulation of the Batch Acetone-Butanol Process.

```
C
C
C     SIMULATION OF OF A-B-E PROCESS (BATCH CULTURE)
C
      DIMENSION YP(20),A(20)
      COMMON /DAT/ NPOINT,NVAR,T,YP
      READ(5,*) NPOINT,NVAR,NPAR
      READ(5,*) T,(YP(J),J=1,NVAR)
      READ(5,*) (A(I),I=1,9)
      READ(5,*) (A(I),I=10,NPAR)
      WRITE(6,100) NPOINT,NVAR,NPAR
      WRITE(6,101) T,(YP(J),J=1,NVAR)
      WRITE(6,102) (A(I),I=1,9)
      WRITE(6,103) (A(I),I=10,NPAR)
100   FORMAT(2X,I2,2X,I2,2X,I2)
101   FORMAT(F6.2,10F6.2)
102   FORMAT(9F8.4)
103   FORMAT(9F8.4)
      CALL OBJECT(A,SS2,NPAR)
2     CONTINUE
      STOP
      END
      SUBROUTINE OBJECT(A,SSWR,NPAR)
      DIMENSION AA(20),A(20),YP(20),Y(20)
      COMMON /KON/ AA
      COMMON /DAT/ NPOINT,NVAR,T,YP
      DO 5 I=1,NPAR
5     AA(I)=ABS(A(I))
      NRM1=NVAR-1
      XF=T
      NPM1=NPOINT-1
      DO 1 I=1,NPM1
      IF(I.GT.1) GO TO 3
      DO 2 J=1,NVAR
2     Y(J)=YP(J)
      WRITE(6,100) XF,(Y(JJ),JJ=1,NRM1)
3     XB=XF
      XF=XB+1.0
      CALL ODE(NVAR,XB,XF,Y,0.01)
```

Appendix 4, continued

```
      WRITE(6,100) XF,(Y(JJ),JJ=1,NRM1)
100   FORMAT(1X,F3.0,F5.2,F5.1,F6.2,5F5.2,F5.1)
1     CONTINUE
      RETURN
      END
      SUBROUTINE RHS(N,X,Y,F)
      DIMENSION Y(20),F(20),A(20)
      COMMON /KON/A
      FS=Y(2)/(Y(2)+A(8))
      RS=(A(3)*FS+A(4)*Y(2))*Y(1)
      FI=A(1)/(A(1)+Y(3))
      F(2)=-RS
      FBA=(A(5)*Y(2)*FI-A(7)*Y(6)/(Y(6)+A(10)))*Y(1)
      F(6)=FBA
      F(1)=0.56*(Y(10)-1.)*Y(1)-A(9)*Y(1)*Y(3)
      F(3)=A(6)*Y(2)*Y(1)-0.841*FBA
      F(7)=(A(11)*FS*FI-A(15)*Y(7)*FS/(Y(7)+A(14)))*Y(1)
      F(4)=A(12)*FS*Y(1)-F(7)*0.484
      F(5)=A(13)*Y(1)*FS
      F(9)=A(16)*FS*Y(1)
      F(8)=A(17)*FS*Y(1)+A(18)*Y(2)*Y(1)
      F(10)=(A(2)*FI*Y(2)-0.56*(Y(10)-1.))*Y(10)
      RETURN
      END
      SUBROUTINE ODE(N,XB,XE,Y,EPS)
      DIMENSION Y1(20),Y2(20),Y3(20),Y(20)
      X=XB
      H=(XE-X)/10.
C     ADOPTED FROM ALGORITHM 8 C ACM (1960) VOL.3 P.312
C     BY J.VOTRUBA  INST.MICROBIOL. PRAGUE CZECHOSLOVAKIA
      IS=0
      ICUT=0
100   IF((X+2.01*H-XE).LT.0.) GO TO 1
      ICUT=1
      H=(XE-X)/2.
1     CALL RK4(N,X,Y,2.*H,X1,Y1)
101   CALL RK4(N,X,Y,H,X2,Y2)
      CALL RK4(N,X2,Y2,H,X3,Y3)
      ERR=0.0
      DO 2 K=1,N
      Q1=AMAX1(1.E-6,ABS(Y3(K)))
      P=ABS(Y1(K)-Y3(K))/Q1
2     ERR=AMAX1(P,ERR)
      IF(ERR.GT.EPS) GO TO 103
```

Appendix 4, continued

```
      X=X3
      IF(IOUT.EQ.1) GO TO 104
      DO 3 K=1,N
3     Y(K)=Y3(K)
      IF(IS.LT.5) GO TO 4
      H=2.*H
      IS=0
4     IS=IS+1
      GO TO 100
103   H=0.5*H
      IOUT=0
      X1=X2
      DO 5 K=1,N
5     Y1(K)=Y2(K)
      GO TO 101
104   DO 6 K=1,N
6     Y(K)=Y3(K)
      RETURN
      END

      SUBROUTINE RK4(N,X,Y,H,XE,YE)
      DIMENSION Y(20),YE(20),Z(20),W(20),A(5)
      DATA A /0.5,0.5,1.,1.0,0.5/
      XE=X
      DO 1 K=1,N
      YE(K)=Y(K)
1     W(K)=Y(K)
      DO 2 J=1,4
      CALL RHS(N,XE,W,Z)
      XE=A(J)*H+X
      DO 3 K=1,N
      W(K)=Y(K)+A(J) H*Z(K)
3     YE(K)=YE(K)+A(J+1)*H*Z(K)/3.
2     CONTINUE
      RETURN
      END
```

APPENDIX 5

Process Parameters in the Batch Acetone-Butanol Fermentation on Glucose.

APPENDIX 5.1

Retarded Batch Culture

Experiment No. 1

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	20.0	0.0	0.0	0.0	0.0	0.0
7.0	0.3	19.6	0.0	0.0	0.0	0.4	0.2
14.5	1.1	14.7	0.0	0.0	0.0	1.6	0.5
19.0	1.0	13.9	0.2	0.1	0.1	1.8	1.2
24.5	0.9	13.6	0.3	0.2	0.1	1.9	1.4
32.0	0.9	13.5	0.4	0.3	0.1	1.8	1.4
41.5	1.0	11.1	0.9	0.5	0.1	1.6	1.4
46.5	1.1	8.7	1.3	0.7	0.2	1.5	1.3
72.0	1.4	2.1	3.0	1.5	0.2	1.3	1.3
90.0	1.4	0.0	4.0	2.0	0.2	1.0	1.3

APPENDIX 5.2

Retarded Batch Culture

Experiment No. 2

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	20.0	0.0	0.0	0.0	0.0	0.0
8.0	0.4	18.5	0.0	0.0	0.0	0.6	0.4
15.0	1.0	14.5	0.1	0.0	0.0	1.8	1.2
22.0	1.1	12.0	0.2	0.1	0.0	2.1	1.9
35.0	1.0	11.2	0.5	0.4	0.1	1.9	1.8
52.0	1.1	7.6	1.6	0.9	0.2	1.4	1.7
70.0	1.4	2.2	3.1	1.4	0.2	1.2	1.7
90.0	1.1	0.0	3.9	1.8	0.2	1.2	1.7

APPENDIX 5.3

Retarded Batch Culture

Experiment No. 3

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	20.3	0.0	0.0	0.0	0.0	0.0
14.0	1.1	14.2	0.0	0.0	0.0	1.2	0.8
20.0	1.2	10.6	0.1	0.0	0.0	2.0	1.3
24.0	1.7	9.5	0.3	0.1	0.1	2.2	1.4
40.0	1.8	7.8	0.6	0.2	0.1	2.4	1.7
65.0	1.8	4.2	1.9	1.0	0.2	1.4	1.7
80.0	1.8	0.4	3.0	1.7	0.2	1.0	1.7

APPENDIX 5.4

Retarded Batch Culture

Experiment No. 4

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	21.8	0.0	0.0	0.0	0.0	0.0
12.0	0.9	15.4	0.1	0.0	0.0	0.8	0.7
25.0	1 5	9.6	0.5	0.2	0.0	2.8	1.9
40.0	1.6	7.3	0.8	0.4	0.1	2.4	1.8
70.0	1.6	2.4	2.1	1.0	0.2	1.5	1.6
90.0	1.5	0.0	2.8	1.5	0.2	1.3	1.6

APPENDIX 5.5

Retarded Batch Culture

Experiment No. 5

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	20.8	0.0	0.0	0.0	0.0	0.0
15.0	1.4	16.3	0.0	0.0	0.0	1.1	0.9
22.0	1.5	12.9	0.2	0.1	0.0	2.0	1.2
38.0	1.5	9.1	0.8	0.4	0.1	2.0	1.7
62.0	1.5	5.0	2.1	1.1	0.1	1.5	1.6
90.0	1.5	0.2	3.0	1.7	0.1	1.4	1.6

APPENDIX 5.6

Retarded Batch Culture

Experiment No. 6

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	31.8	0.0	0.0	0.0	0.0	0.0
13.5	0.7	30.6	0.0	0.0	0.0	1.0	0.7
19.5	1.3	28.5	0.1	0.0	0.0	1.7	1.6
35.5	1.8	19.4	1.2	0.3	0.1	3.2	2.7
47.5	1.9	13.1	3.5	1.5	0.2	2.3	2.7
66.0	1.8	11.2	3.9	1.6	0.3	2.1	2.6
90.0	1.6	11.2	3.9	1.6	0.3	2.1	2.6

APPENDIX 5.7

Retarded Batch Culture

Experiment No. 7

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	30.8	0.0	0.0	0.0	0.0	0.0
12.5	0.5	29.8	0.0	0.0	0.0	0.7	0.7
20.0	1.3	26.7	0.0	0.0	0.9	1.9	1.5
35.5	1.7	18.5	1.3	0.7	0.1	2.7	2.5
46.5	1.8	12.4	2.9	1.3	0.2	1.6	1.8
67.0	1.9	7.2	4.2	1.8	0.3	1.4	1.8
82.0	1.9	7.2	4.2	1.8	0.3	1.4	1.8

APPENDIX 5.8

Retarded Batch Culture,

Experiment No. 8

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	31.7	0.0	0.0	0.0	0.0	0.0
10.0	0.4	30.0	0.0	0.0	0.0	0.6	0.5
18.0	0.9	28.3	0.0	0.0	0.0	2.0	1.2
30.0	1.8	22.0	0.2	0.1	0.0	3.1	2.4
37.0	2.0	18.1	1.4	0.7	0.1	3.1	2.4
65.0	2.0	9.4	4.0	2.1	0.4	2.4	2.3
80.0	1.8	9.4	4.0	2.1	0.4	2.4	2.3

APPENDIX 5.9

Retarded Batch Culture

Experiment No. 9

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	20.5	0.0	0.0	0.0	0.0	0.0
8.5	0.3	18.4	0.0	0.0	0.0	0.3	0.1
15.5	0.9	18.0	0.1	0.0	0.0	1.2	0.9
23.5	1.2	11.8	0.5	0.1	0.0	3.3	1.9
32.0	1.2	9.0	0.8	0.3	0.1	3.5	3.8
50.5	1.2	8.5	1.3	0.4	0.1	3.5	2.8
77.0	1.2	8.5	1.3	0.4	0.1	3.8	2.5

APPENDIX 5.10

Retarded Batch Culture

Experiment No. 10

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	20.5	0.0	0.0	0.0	0.0	0.0
8.5	0.2	19.8	0.0	0.0	0.0	0.2	0.4
15.5	0.9	17.9	0.1	0.0	0.0	1.0	0.9
23.5	1.2	15.2	0.2	0.1	0.0	2.9	2.1
32.0	1.3	11.2	0.7	0.2	0.1	3.7	2.3
50.5	1.2	9.5	1.2	0.5	0.1	3.5	2.6
77.0	1.2	9.1	1.3	0.6	0.1	3.1	2.0

APPENDIX 5.11

Retarded Batch Culture

Experiment No. 11

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	30.0	0.0	0.0	0.0	0.0	0.0
9.5	1.3	25.9	0.2	0.1	0.0	2.6	1.5
23.0	1.5	21.0	1.1	0.7	0.2	3.9	3.1
29.0	1.5	19.3	1.1	0.7	0.2	3.6	3.1
45.0	1.4	19.0	1.2	0.7	0.2	3.3	2.4
57.0	1.1	18.8	1.3	0.8	0.2	3.2	2.3

APPENDIX 5.12

Retarded Batch Culture

Experiment No. 12

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	31.0	0.0	0.0	0.0	0.0	0.0
13.5	0.6	29.2	0.0	0.0	0.0	0.8	0.6
21.5	1.5	27.6	0.0	0.0	0.0	1.9	1.7
35.5	1.9	19.8	2.0	0.6	0.1	2.9	1.9
44.5	1.9	13.6	3.6	1.6	0.3	2.2	2.2
57.0	1.9	10.9	4.4	1.9	0.3	1.9	2.5
63.0	1.9	10.6	4.4	1.9	0.3	1.9	3.0
99.0	1.7	10.0	4.5	2.0	0.3	1.7	2.8

APPENDIX 5.13

Retarded Batch Culture

Experiment No. 13

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	20.1	0.0	0.0	0.0	0.0	0.0
10.0	0.3	18.2	0.1	0.0	0.0	0.7	0.4
19.0	2.0	14.4	0.2	0.1	0.0	2.4	1.4
26.5	2.4	5.0	0.8	0.3	0.1	2.8	2.1
42.0	2.4	0.8	2.1	1.0	0.1	2.2	1.9
57.0	2.3	0.0	2.6	1.2	0.1	1.9	1.9

APPENDIX 5.14

Retarded Batch Culture

Experiment No. 14

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	20.0	0.0	0.0	0.0	0.0	0.0
14.0	0.9	15.1	0.2	0.0	0.0	1.0	0.9
20.0	1.6	12.5	0.3	0.1	0.0	2.1	1.3
24.0	2.0	10.7	0.5	0.2	0.1	2.5	1.9
40.0	2.5	1.0	1.8	0.7	0.1	1.9	2.2
55.0	2.2	0.0	2.4	1.1	0.1	1.4	2.1

APPENDIX 5.15

Normal Batch Culture (no pH control)

Time (h)	Biomass (g/L)	Glucose (g/L)	Butanol (g/L)	Acetone (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Acetic Acid (g/L)
0.0	0.1	30.0	0.0	0.0	0.0	0.0	0.0
5.0	0.2	29.5	0.0	0.0	0.0	0.5	0.4
10.0	0.6	27.3	0.2	0.1	0.0	1.4	1.1
15.0	1.0	22.2	0.6	0.5	0.1	2.1	1.6
18.0	1.4	18.0	1.4	0.8	0.2	2.2	1.9
23.0	1.9	13.5	2.8	1.5	0.2	1.7	1.6
28.0	2.1	5.0	4.3	2.1	0.3	1.1	1.3
35.0	2.0	0.4	5.7	3.1	0.5	0.8	1.2
40.0	1.9	0.0	5.7	3.1	0.5	0.6	1.2