ANAEROBIC FERMENTATION OF WHEY: ACIDOGENESIS

by

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ABSTRACT

Based on the initial exploratory results of single-phase (acidogenesis and methanogenesis takes place in one vessel) whey biomethanation studies, a two-phase (acidogenesis and methanogenesis takes place in two separated serial vessels) biomethanation process was found to be more suitable for dealing with the current whey utilisation and/or disposal problem. Acidogenesis was found to be less understood in comparison to methanogenesis and therefore acidogenesis became the central problem of this thesis.

Given that 90% of the five-day biochemical oxygen demand in whey is due to lactose, continuous culture (Chemostat) experiments were undertaken to examine the general mechanism of lactose acidogenesis by a mixed undefined culture using ^{14}C-labeled tracers. Also the influence of whey protein (mainly β-lactoglobulin) on the general fermentation scheme was addressed. Experimental factors included a pH range of 4.0 to 6.5, a mesophilic temperature of 35°C and a dilution rate (D) range of 0.05 to 0.65 h^{-1}.

At a fixed pH level, the observed variability in the main acidogenic end products (acetate, propionate, butyrate and lactate) with respect to D were found to be a consequence of the systematic separation of the various microbial groups involved in acidogenesis. Batch incubation of a [$^{14}C(U)$]-lactate tracer with chemostat effluent samples and preparative separation of the end products followed by a liquid scintillation assay of the location of the radio activity demonstrated that a microbial population shift with increasing D was responsible for disabling the conversion of

lactate to other end products and hence the observed increase in lactate concentrations at high D values.

Further use of [^{14}C(U)]-butyrate and [^{14}C(2)]-propionate revealed the predominant carbon flow routes from pyruvate to the various end products. A qualitative lactose acidogenic fermentation model was proposed, in which lactose is converted to pyruvate via the Embden-Meyerhof-Parnas pathway. Pyruvate in a parallel reaction is then converted to lactate and butyrate. In the presence of hydrogen reducing methanogens lactate is converted to acetate in a very fast reaction and not propionate as previously believed. The implications of these findings with regard to optimising the acidogenic phase reactor are discussed.

Acidogenic fermentation of protein together with lactose did not affect the carbon flow scheme. In the D range of 0.05 to 0.15 h^{-1}, low pH (pH < 5.0) was found to favour the butyrate route at the expense of the lactate route and at high pH (pH > 5.5) the lactate route was favoured at the expense of the butyrate route, the pH region of 5.0 to 5.5 being the transition range.

In order to describe the microbial growth, the Monod chemostat model was chosen among the various alternatives, because of its simplicity and its physico-chemical basis. The estimated model parameters are reported.

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NOMENCLATURE AND ABBREVIATIONS

ATP	Adenosine triphosphate
BOD	Biochemical oxygen demand (mg/L)
COD	Chemical oxygen demand (mg/L)
CSTR	Continuous flow stirred tank reactor
D	Dilution rate (h^{-1})
DNA	Deoxyribonucleic acid
EMP	Embden-Meyerhof-Parnas pathway
F	Fermentor feed stream flowrate (mL/h)
k	Constant in Konak's microbial growth model; see equation 2.6
K'	Kinetic constant in Chen and Hashimoto's microbial growth model; see equation 2.19
K_A	Constant in Dabe's microbial growth model; see equation 2.11
K_B	Constant in Dabe's microbial growth model; see equation 2.10
K_d	Microbial decay rate (h^{-1})
K_i	Substrate inhibition constant (μg/mL)
K_p	Product inhibition constant (μg/mL)
K_s	Monod saturation constant (μg/mL)
L	Constant in Powell's microbial growth model; see equation 2.12
M	Parameter in Rogues and co-workers microbial growth model; see equation (μg/mL) or maintenance coefficient
NAD	Electron carrier co-enzyme, nicotinamide adenine dinucleotide
NADH	Reduced form of NAD
OA	Organic acids
p	Constant in Konak's microbial growth model; see equation 2.6

RT Retention time (h)

RNA Ribonucleic acid

S Concentration of limiting nutrient (μg/mL)

S_o S value in feed stream (μg/mL)

t_R Relaxation time (sec)

t_{HR} Hydraulic retention time (h)

V CSTR working volume (mL)

VFA Volatile fatty acids

VSS Volatile suspended solids (g)

WPC Whey protein concentrate

X Organism concentration in the fermentor (μg/mL)

X_m Maximum value of X that may be reached

Y Yield coefficient (g of cell/g of limiting nutrient)

Y_{ij} Yield coefficient for compound j on compound i

Greek Symbols

$\Delta G°$ Free energy of reaction at a temperature of 25°C and pressure of one atmosphere

μ Specific microbial growth rate (h^{-1})

μ_m Maximum μ valve (h^{-1})

λ Constant in Moser's microbial growth model; see equation 2.12

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I. INTRODUCTION

1.1 Whey

Whey is an opaque, greenish-yellow fluid that remains in a cheese vat following the removal of the curd, in the process of converting milk into cheese. Different cheese varieties produce whey with somewhat different characteristics, typical of the cheese process. As an example, the whey obtained from rennet-coagulated milk is referred to as "sweet" to differentiate it from the "acid" whey of cottage cheese. Acid whey forms a very small percentage of the total whey production in North America. For a given quantity of milk used in cheese processing, approximately 10% of the weight ends up as cheese, the balance as fluid whey (Harper and Hall, 1976). As indicated in Table 1.1, whey contains about 5% lactose, 1% protein (predominantly β-lactoglobulin), 0.3% fat and 0.6% ash (Loehr, 1974). Despite the favourable nutrient content, which offers interesting possibilities for by-product recovery, whey is an expensive and often a frustrating disposal problem for the cheese manufacturer. The five-day biochemical oxygen demand (BOD) for whey ranges between 30,000 and 60,000 mg/L, depending on the cheese process, making disposal to streams unacceptable. Generally 90% of the BOD is due to the lactose component (Green and Kramer, 1979), so the question of whey disposal and/or utilization is effectively a question of lactose disposal and/or utilization.

Fluid whey production in the USA and Canada was approximately 27 billion tonnes and 2 million tonnes respectively during 1985 (Tables 1.2 and 1.3). With the increasing cheese demand in North America, fluid whey production is expected to increase.

Table 1.1 Typical Composition of Whey Solids

Component	Composition (g/100 mL)
Carbohydrates	
Lactose	5.00
Proteins	
β-Lactoglobulin	.66
α-Lactoalbumin	.22
Immunoglobulins	.10
Fat	
Triglycerides	.30
Ash	.6
Total % Solids	6.88

Table 1.2 Estimated Quantities of Fluid Whey Produced in the USA.[1]

Cheese Type	Billion Kilograms					
	1980	1981	1982	1983	1984	1985
American(cheddar)	9,700	10,786	11,235	11,953	10,810	11,651
Cottage	6,091	5,801	5,626	5,552	6,388	6,365
Other	6,193	6,250	6,773	7,201	8,271	8,859
Total	21,984	22,837	23,634	24,706	25,470	26,875

[1]Values calculated using annual cheese production figures from, "Dairy Products", published by the US Department of Agriculture, Statistics Reporting Service. It was assumed that for every kg of milk used, 0.1 kg end up as cheese and 0.9 kg as fluid whey.

Table 1.3 Estimated Quantities of Fluid Whey Produced in Canada.[1]

Cheese Type	Million Kilograms					
	1980	1981	1982	1983	1984	1985
Cheddar	956	907	802	895	912	976
Cottage	260	277	286	284	276	-
Other	640	684	727	752	816	882
Total	1,856	1,868	1,815	1,931	2,004	-

[1]Values calculated using annual cheese production figures from, "Dairy Review", published by Statistics Canada. It was assumed that for every kg of milk used, 0.1 kg end up as cheese and 0.9 kg as fluid whey.

1.2 Whey Disposal and/or Utilization

Due to the favourable nutrient content of whey, numerous investigations into developing new schemes of whey treatment, with emphasis on product recovery and new product development, have been carried out. Among these are: fermentation to protein or nitrogen-rich feeds (Reddy et. al., 1976; Gerhardt et. al., 1978); fermentation to ethanol for beverage or gasohol production (Yang et. al., 1976; Palmer, 1978 & 1979; Berstein and Tzeng, 1977; Friend and Shahani, 1979; Everson, 1979); non-alcoholic beverage production; drying to powder which may be used as animal feed or a supplement in human food; feeding directly to livestock (Muller, 1979; Modler et. al., 1980); separation of components by membrane technology (Teixeira et. al., 1982; and land application (Watson et. al., 1977). The permeate from membrane separation schemes is usually high in BOD and therefore necessitates further treatment (Delany, 1981). Most fermentation, membrane separation and non-alcoholic production schemes are limited to large dairy establishments (> 41 million kg fluid whey/year) and, whey being a low value bulky product, transportation to a central processing unit is often uneconomical (Modler et. al., 1980). Drying requires a lot of energy. Also the merits of land application are yet to be established.

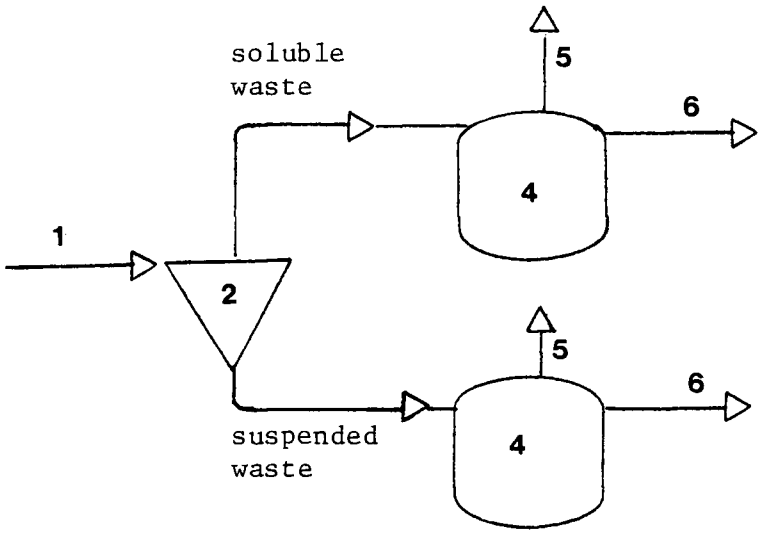
Disposal schemes have taken one or a combination of the following forms: dumping directly into sewers or water systems and aerobic treatment (Muller, 1979). The author is not aware of any full scale anaerobic whey treatment facility in operation at the present time, with the exception of the Millbank Cheese and Butter plant (Bellman, 1986).

1.3 Biomethanation Processes

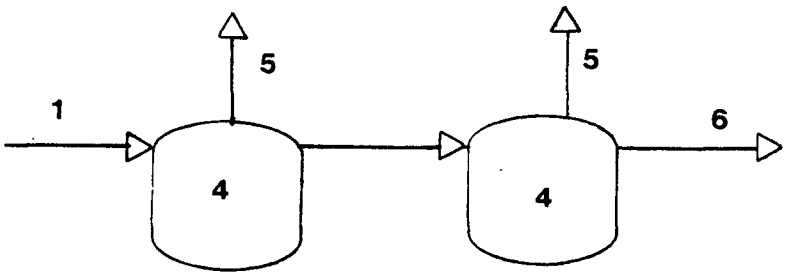
The bioconversion of organic material to methane and carbon dioxide in the absence of molecular oxygen is referred to as the biomethanation process

or anaerobic methane fermentation. Basically this anaerobic degradation of organic compounds is performed by two groups of bacteria, the acid forming and methane forming. Further subdivisions of the two groups is considered in Chapter II. Biomethanation processes for stabilising organic wastes offer several advantages over the conventional aerobic processes (McCarty, 1966), namely: a higher degree of waste stabilisation; lower microbial yield; lower nutrient requirements; no oxygen requirement; and methane production. With regard to whey, these advantages are pronounced since whey constitutes a high strength organic waste and in addition, the methane generated can be consumed by the cheese processing facility itself for various cooking and heating operations. Also the process may be suitable for the medium (14-41 million kg fluid whey/year) and small scale (< 14 million kg fluid whey/year) plants for which the economics of alternative whey utilization processes are not favourable.

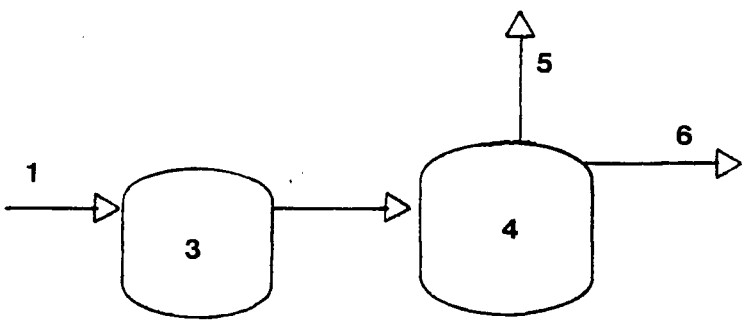
The three most common process layouts are, the parallel, staged and phased processes (Figure 1.1). Generally, the lack of application of biomethanation for this purpose is probably due to the unreliable operation that has often been associated with the process. This unreliability has been attributed to the lack of understanding of the fundamental concepts involved in the process. Other disadvantages historically have been poor process stability, a temperature requirement of 35°C, inability to degrade various substances and large volume requirements because of slow reaction rates (Switzenbaum, 1983). More recently, advances in basic microbiology and biochemistry along with advances in bio-reactor technology, in particular with immobilised bacteria systems, sometimes referred to as fixed film reactors (Young and McCarty, 1969; Lettinga et. al., 1980; Switzenbaum and Jewell, 1980; Switzenbaum and Danskin, 1982; Boeing and Larsen,



A. Parallel biomethanation process



B. Staged biomethanation process



C. Two-phase biomethanation process

Figure 1.1 Three different biomethanation processes: (1) influent stream, (2) solid/liquid separator, (3) acid (only) producing reactor, (4) methane producing reactor, (5) gas, (6) effluent stream.

1982; Bull et. al., 1984; Burgress and Morris, 1984) have helped overcome most of the problems associated with anaerobiosis. It is important to distinguish between reactor types and process layout. In the latter case one or several reactors are included in a total process scheme. Henze and Harremoes (1983) have classfied a number of reactors, which have been investigated or marketed during the last 5-10 years, into eight basic types, shown in Figures 1.2 and 1.3.

1.4 Whey Biomethanation

From Bushwell and Mueller's (1952) empirical formula that predicts methane production from a knowledge of the chemical composition of the degraded material, the following formula can be written for the breakdown of the lactose in whey:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 6CO_2 + 6CH_4 \qquad (1.1)$$

Therefore one gram of lactose (.002775 moles) would yield (6 x .002775 x 22.412) = 0.3722 litres of CH_4 at one atmosphere. For a litre of whey, methane production is approximately 19 litres.

Most of the laboratory experiments conducted to date on the biomethanation of whey or lactose have been designed to evaluate the performance of immobilised bacteria reactor systems (Hickey and Owens, 1981; Yang et. al., 1984; Switzenbaum and Danskin, 1982; Boeing and Larsen, 1982; Dehaast et. al., 1983; Callander and Barford, 1983; Dehaast et. al., 1985). Microbial and biochemical kinetic information on which rudimentary process designs can be based is virtually non-existent. Efforts were made by the present author to experimentally generate the microbial kinetics and

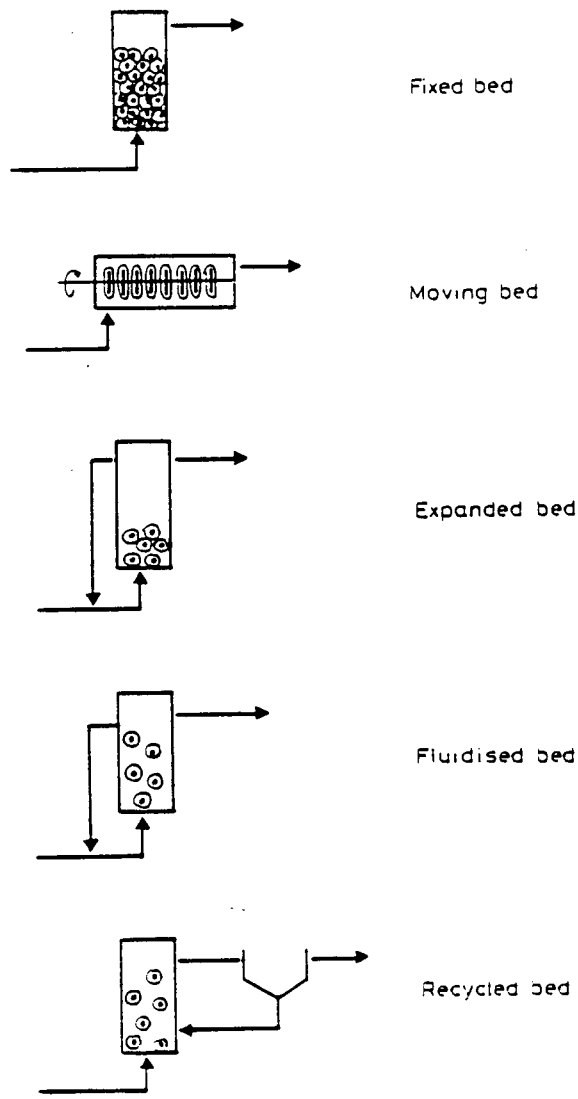


Figure 1.2 Five basic anaerobic fixed film reactor types.

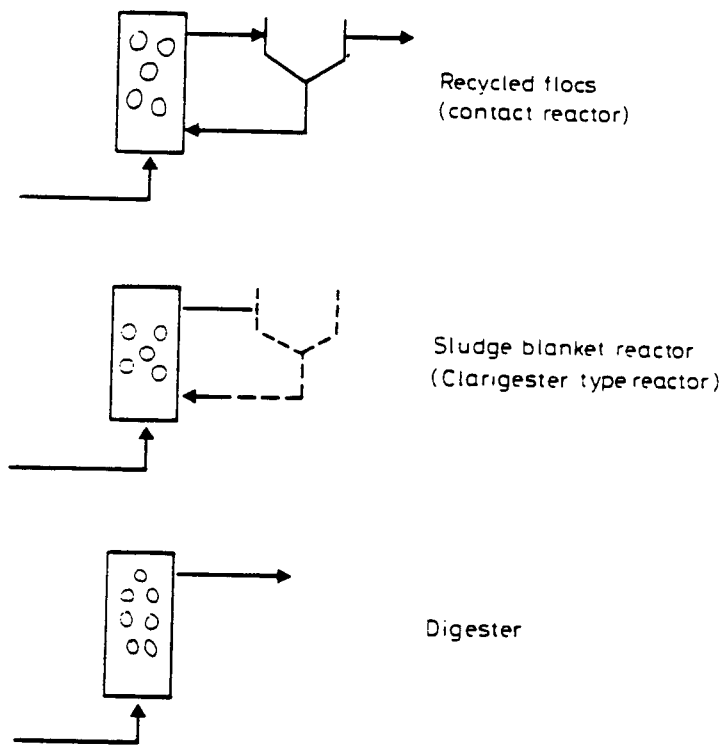


Figure 1.3 Three basic anaerobic flocs/non-attached film reactor types.

biochemical data for lactose in fed-batch, single-phase, one litre reactors, for various dilution rates (D) and influent lactose concentration (unpublished work). At the start of the experiments the substrate was added to the reactors and within a few days the pH fell rapidly and gas production ceased. This was repeated several times, but each experiment resulted in a "sour" reactor. Similar unsuccessfull experiments employing whey or lactose have been reported by a number of investigators (Marshall and Timbers, 1982; Dehaast et. al., 1983; Kelly and Switzenbaum, 1984). The rapid drop in pH was ascribed to the rapid formation of organic acids (OA) from lactose. In order to solve this problem, Dehaast and co-workers prefermented the whey, neutralised the acid, diluted the preferment and used the diluted product as the substrate for biomethanation. Follmann and Maerkl (1979) used a pH-static process in which the control signal for the addition of fresh whey was the pH value. When the pH increased beyond 7.0, a pump was triggered to add substrate automatically until the pH fell to 6.95, at which level the substrate pump was stopped, until the pH again passed the pH = 7.0 level, and this triggered a repeat of the sequence. With this kind of control they were able to achieve 98% chemical oxygen demand (COD) reduction at a residence time (RT) of 12.5 days. The RT of 12.5 days is very long and would necessitate large reactor volumes. However Follmann and Maerkl pointed out that their observed long RT could be reduced approximately in half if immobilised bacteria reactors were employed.

The above observations suggested that perhaps a two-phase process as previously proposed by Babbit and Baumann (1958), Andrews and Pearson (1965) and Pohland and Ghosh (1971) could be more successfull in stabilising whey or whey permeate.

1.5 Two-Phase Process

As already indicated, in a two-phase process, whey or lactose is converted in the first reactor to OA by the acid forming bacteria (acidogenesis). The OA are then reduced in a second serial phase by the methane forming bacteria (methanogenesis). Separation of the different groups of micro-organisms makes it possible to maintain optimal conditions for the different groups of bacteria involved in the process. Also in a two-phase process there is more opportunity to control substrate flow between different groups of bacteria than in one-phase processes, where there is no physical separation of the bacterial group populations. Given these advantages, the two-phase process has been applied at pilot and full-scale levels for several types of high BOD and low suspended solids (SS) industrial wastes (Table 1.4). These two-phase plants consist of a completely mixed acid-phase reactor and an upflow anaerobic sludge-blanket methane reactor. All plants are operated in the mesophilic temperature range and the carbohydrate content of the wastes is predominantly α-glycosidic types such as starch and sucrose.

As indicated in the next chapter, there is a large volume of literature concerning the microbial kinetics and mechanisms for the reduction of OA or methanogenesis and hardly any that is applicable to acidogenesis in a two-phase process. In the few previous laboratory studies on acidogenesis of carbohydrates (Ghosh et. al., 1975; Massey and Pohland, 1979; Cohen et. al. 1979; Zeotemeyer et. al., 1982; Bull et. al. 1984) glucose and starch were employed as the main substrates. The results obtained by the above authors with regard to optimum pH, acidogenic reactor effluent OA distribution, microbial kinetics and overall degradation scheme are valid

Table 1.4 Two-Phase Pilot and Full-Scale Plants

Year	Industry	Location	Plant Type	Capacity (kg COD/day)	Reference
1977	Distillery (enzyme-alcohol)	Belgium	Pilot	180	Ghosh et. al. (1985)
1980	Beet Sugar	West Germany	Pilot	45	"
"	Distillery (yeast-alcohol)	Belgium	Pilot	135	"
1981	Beet Sugar	Belgium	Pilot	170	"
"	Citric Acid	West Germany	Pilot	120	"
"	Beet Sugar	West Germany	Pilot	45	"
1980	Flax Retting	Belgium	Full-Scale	350	"
1982	Starch to Glucose	West Germany	Full-Scale	20,000	"
1983	Yeast-Alcohol	Netherlands	Full-Scale	20,000	Berkovitch (1986)
1984	Yeast	France	Full-Scale	7,000	"

for carbohydrates with α-1, 4 glucosidic bond and may not be valid for lactose (β-1, 4 linkage).

1.6 Research Objectives

The overall objectives of this study were two fold:

1. To determine the microbial kinetics and the general mechanism for the degradation of lactose by a mixed, undefined acidogenic bacteria, using ^{14}C-labeled tracers.
2. To determine the influence of whey protein (β-lactoglobulin) on the degradation mechanism of lactose if degraded together with lactose.

To a limited extent, ^{14}C-labeled tracers have been used to determine the path of carbon and electron flow during anaerobosis of specific matter in diverse ecosystems (Jeris and McCarty, 1965; Weng and Jeris, 1976; Cohen, 1982; Lovley and Klug, 1982; Koch et. al, 1983). With regard to whey biomethanation, Chartrain and Zeikus (1986a and b) have used the technique to propose a carbon and electron flow route for single-phase biomethanation. Physical separation of acidogenic and methanogenic bacteria in a two-phase system may lead to important changes in the composition of bacterial populations as well as in the intermediary routes of substrate degradation (Cohen et. al., 1980). This has been considered a strong enough justification for studying acidogenesis of lactose with ^{14}C tracers. In addition, separation of the two metabolically related groups gives the opportunity to study the particular sub-population in the reactor. To the knowledge of this author, no study using ^{14}C-labeled substrates to elucidate an acidogenic degradation scheme of lactose, has been conducted previously.

All the present experimental work, described in later chapters, was accomplished with mixed, undefined cultures. A microbiologist might have reservations about the use of taxonomically undefined bacterial populations, but the complex nature of the microbial interactions involved in practice, requires an approach which implies a not too drastic simplification of reality. Therefore, when the intention is to gather information with a certain degree of practical applicability, methods which allow a better microbiological description such as that obtained with pure culture studies or studies of mixed and well defined populations might be considered less relevant. On the other hand, as a consequence of wanting to solve engineering problems with specific types of wastes, anaerobiosis has often been treated as a form of heterogeneous catalysis, consisting of reactants (organic matter and nutrients) and a catalyst (the bacteria). This approach has yielded results under proper process conditions, but has ignored biologically important features such as adaptation at the regulatory or population level, eco-physiological interactions and growth conditions. For these reasons it was found useful to direct this study towards an intermediate path between the two extremes mentioned, in order to connect the fields of pure and applied research. The choice of lactose as the sole carbon source was made to avoid the occurrence of side reactions (Elsden and Hilton, 1978; Hirose and Shibai, 1980), so that the lactose degradation scheme could be determined more precisely. The significance of studying the influence of β-lactoglobulin on lactose degradation pathway was two-fold: First organic wastes are frequently composed of carbohydrates, proteins and lipids in various combinations. No study was found in the literature which addressed the anaerobic degradation mechanism of "complex" organic wastes.

Previous work has mainly involved complex organic waste constituents individually and their intermediary metabolities (eg. amino acids, fatty acids, etc.) degraded separately. The importance of separately studying a degradative pathway of an intermediary metabolite like leucine is to highlight the possibility of determining specific metabolic routes which can be used as models for the breakdown of a number of amino acids. However the results of such a study are only useful for a complex waste that contains leucine, if the other constituents and their derived intermediary metabolites do not alter the particular microbial population. Secondly, while a synthetic waste that contains lactose may only serve as an analog of whey permeate from whey membrane processes for protein recovery, a synthetic waste that contains lactose and β-lactoglobulin may serve as a whole fluid whey waste analog.

1.7 Scope of Study

The important factors in the study were identified as substate composition, pH and dilution rate. Specifically, the scope of this study consisted of the following major tasks.

1. With lactose as the growth limiting nutrient, establish the organic acids distribution and the biomass with respect to the dilution rate and pH. The latter independent variable was required for microbial growth modeling and the former was required for initial speculation as to the potential lactose fermentation model.
2. Utilise a radiotracer methodology to confirm the lactose degradation scheme proposed in task 1.

3. With lactose and protein as the growth limiting nutrients utilise a radiotracer methodology to establish the effect on the degradation scheme for lactose established in task 2 of degrading a protein with the lactose.
4. Estimate kinetic parameters for the microbial growth model.

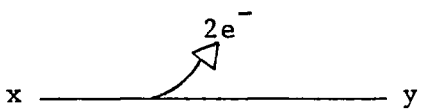
II. PREVIOUS WORK AND THEORETICAL ASPECTS

In this chapter, the theoretical aspects as well as the previous work on energy conservation in microbial systems and microbial growth modeling for anaerobiosis is explored.

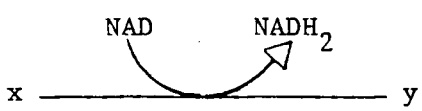
2.1 Energetics and Metabolic Stages in Anaerobiosis

2.1.1 Energetics

The mechanism of energy gain which supports growth of a heterotrophic microbial cell relies upon biological oxidation processes. Chemically bound potential energy is released by a reduced organic compound with a less reduced compound which acts as an oxidiser. By this reaction electrons are transferred from the reduced compound (electron donor) to the more oxidised compound (electron acceptor). Since fermentation is a strictly anaerobic process, oxidative reactions must be anaerobic. Usually in these oxidative reactions two electrons are removed from the substrate molecule (Equations 2.1 and 2.2).



(2.1)



(2.2)

Compound x is oxidised to compound y, and two electrons are transferred to NAD, a common electron carrier coenzyme. Coenzymes are present in cells in limiting amounts. Wolfe (1983) has likened these electron carriers to

trucking systems. Unless there is a point where the cargo (electrons) are unloaded, the trucking system soon becomes saturated. In anaerobiosis, the final electron acceptor is formed in amounts proportional to the substrate being oxidised so that the reduced electron carrier can be unloaded (oxidised) and return to accept another load (pairs of electrons).

This energy which is released by the reaction is stored by the cell in the form of energy rich phosphate esters (ATP) which can be used by the cell for all reactions which support growth. The amount of energy which is released by such an oxidation process (ΔG°) depends on the difference in state of reduction of the compound which acts as the reductant in the reaction. The amount of ATP which is gained by the cell in turn, depends on the energetic efficiency of its metabolism. The majority of anaerobes work at an efficiency of 25-50% (Thauer et. al., 1977). The electron acceptor can be either an inorganic compound (respiration) or an organic compound (fermentation). When an organic compound acts as an electron acceptor, the substrate molecule is normally split into two molecules, of which one acts as the electron donor and the other as an electron acceptor. Some fermentative bacteria possess the ability to use protons as electron acceptors in addition to organic molecules. The reduction of protons in electron transferring reactions gives rise to the production of molecular hydrogen and is of importance for the control of anaerobiosis. Cohen (1982) has pointed out that, in general, fermentative reactions conform to the following principles: (1) From the substrate a proper electron donor and electron acceptor must be formed and the amount of electrons supplied by the donor must equal the amount accepted by the acceptor; (2) Fermentative reactions occur in such a way that an optimal ATP gain is accomplished.

The most important acidogenic fermentation reactions which may occur during anaerobiosis of carbohydrates are shown in Table 2.1, after Thauer et. al. (1977). Although the ΔG° of the different reactions are comparable, the ATP gain varies between 2 and 4. For a more detailed treatise of the energetic aspects, the reader is referred to papers by Thauer et. al. (1977) and Gottschalk and Andreesen (1979).

2.1.2 Metabolic Stages

Effective digestion of organic matter into methane requires the combined and coordinated metabolism of different kinds of carbon catabolising anaerobic bacteria. At least four different trophic types of bacteria have been isolated from either man-made reactors or in nature (eg. gastrointestinal tracts, lake sediments or thermal wells). These bacteria can be distinctly recognised on the basis of substrate fermented and metabolic end products formed (Zeikus, 1980).

The four metabolic groups which function in anaerobiosis include (Figure 2.1): (1) The hydrolytic bacteria which ferment a variety of complex organic molecules (polysaccharides, lipids and proteins) into a broad spectrum of end products (acetate, H_2/CO_2, one carbon compounds and organic acids longer than acetate, and neutral compounds larger than ethanol); (2) The hydrogen producing acetogenic bacteria which include both obligate and facultative species that can ferment organic acids larger than acetate and neutral compounds larger than methanol (ethanol, propanol) to hydrogen and acetate; (3) The homoacetogenic bacteria which can ferment a very wide spectrum of multi or one carbon compounds to acetic acid; and (4) The methanogenic bacteria which ferment H_2/CO_2, one carbon compounds (methanol, CO, methylamine) and acetate to methane.

Table 2.1 Overall Reaction Schemes Of Some Important Acidogenic Fermentation Types

Reaction Type	ΔG° (kJ/reaction)	ATP (mol/reaction)
Acetic acid fermentation $C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3CHOO^- + 4H^+ + 2HCO_3^- + 4H_2$	-206	4
Propionic acid fermentation $C_6H_{12}O_6 \rightarrow \frac{4}{3} CH_3CH_2COO^- + \frac{2}{3} CH_3COO^- + \frac{8}{3} H^+ + \frac{2}{3} HCO_3^-$	-220	3 - 4
Butyric acid fermentation $C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 3H^+ + 2H_2 + 2HCO_3^-$	-255	3
Ethanol fermentation $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2H^+ + 2CHO_3^-$	-226	2
Lactic acid fermentation $C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^- + 2H^+$	-198	2

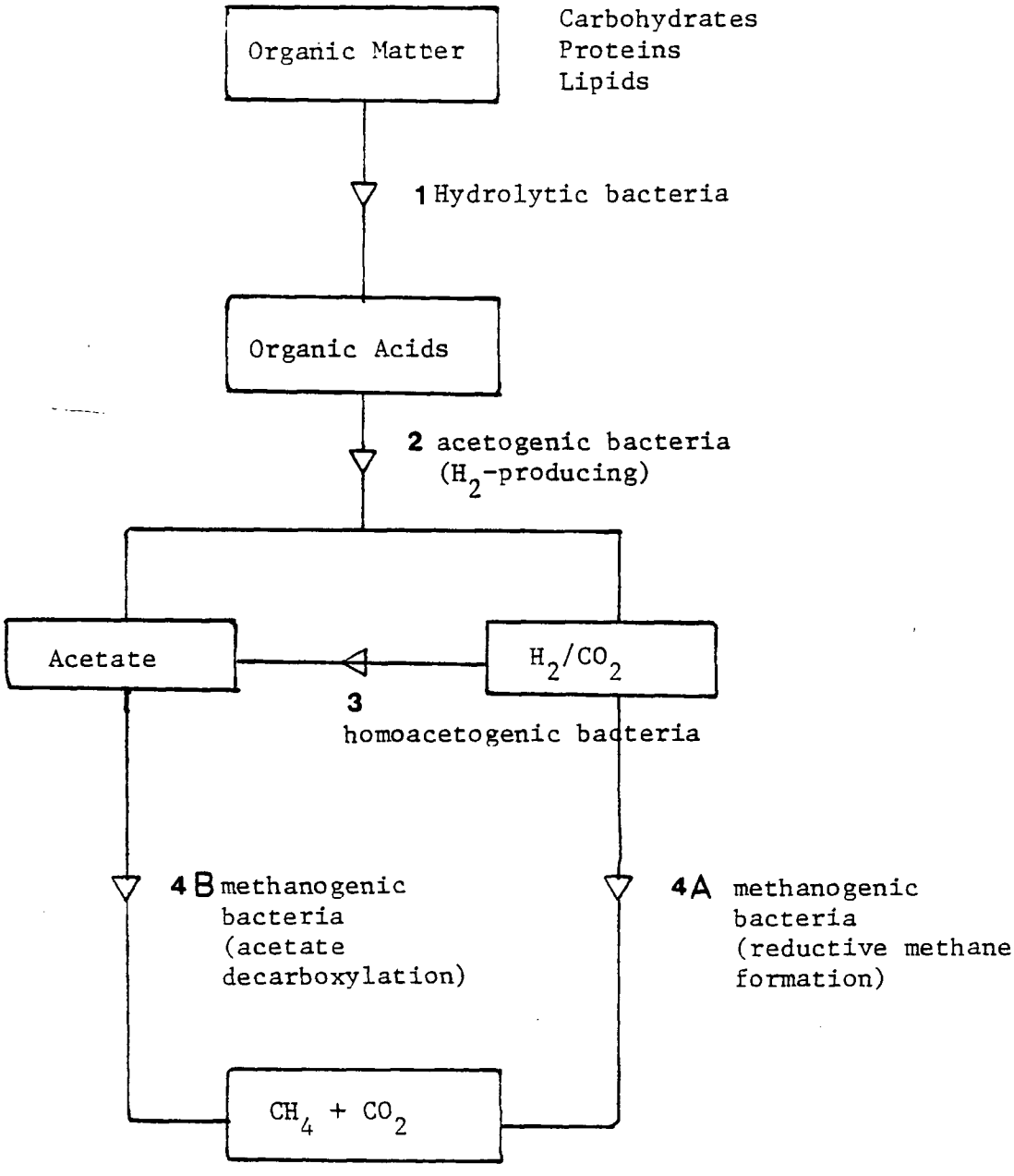


Figure 2.1 Metabolic distinction of microbial populations involved in anaerobiosis.

The methanogenic bacteria perform a pivotal role because their unique metabolism controls the rate of organic degradation and directs the flow of carbon and electrons by removing toxic intermediary metabolites and enhances the thermodynamic efficiency of interspecies intermediary metabolism (Zeikus, 1980). A brief review of the role played by each bacterial group is presented below. For more information the reader is referred to a number of recent detailed reviews (Zeikus, 1977; Mah et. al., 1977; and Batch et. al. 1979).

2.1.2.1 Hydrolytic Bacteria

It has been established that hexoses are mainly fermented via the Embden-Meyerhof-Parnas (EMP) pathway in the rumen (Baldwin et. al., 1963; Wallnofer et. al., 1966) and that other metabolic routes are much less important. Also Wood (1961) showed that the EMP and the hexose monophosphate (HMP) routes are the most important pathways for hexose degradation in anaerobic bacteria up to pyruvate. Pyruvate occupies the control position, from which alcoholic, lactic acid or volatile fatty acid fermentations (VFA) start (Wood, 1961). The lipids are broken down to long chain fatty acids (Novak and Carlson, 1970). The long chain fatty acids are further oxidised by beta-oxidation to propionate and acetate. Amino acids from the degradation of nucleic acids and proteins are deaminated and the corresponding organic acids are formed.

Populations of 10^8-10^9 hydrolytic bacteria per mL of mesophilic sewage sludge have been documented (Zeikus, 1980). The majority of these were unidentified gram-negative rods. To date very few detailed studies or generic identifications of the predominant microbial species have been

reported. Clostridium propionicum, Clostridium acetobutyricum and Eubacterium limosum are examples of organisms whose known metabolic pathways lead to the formation of end products chiefly formed in man-made reactor fermentations, namely, butyrates, propionates and acetates.

2.1.2.2 The H_2-producing Acetogenic Bacteria

The H_2-producing acetogenic bacteria catabolise the products of the first stage of fermentation (propionate, butyrate, long chain fatty acids (VFA), alcohols, aromatics and other organic acids) to acetate and a H_2/CO_2 mixture (Figure 2.1). However these organisms cannot catabolise these substrates to acetate when H_2 in the environment is not at extremely low levels. Figure 2.2 illustrates the relationship that exist between H_2 partial pressure and free energy available to the H_2-producing and H_2-consuming species. For example, in order for energy to be available to organisms oxidising propionate to acetate and H_2, the partial pressure of H_2 can not exceed about 10^{-6} atmospheres (Thauer, et. al., 1977).

Populations of 4.2×10^6 H_2-producing acetogens per mL of sewage sludge have been reported (McInerney et. al., 1978). These organisms have not been either generically identified or physiologically well characterised, despite the belief that VFA's are ecologically much more important as intermediates than lactate and ethanol (Mah, 1981). The isolation of the "S" organism from Methanobacillus omelianskii was the first documentation of a specie in this group. Ethanol was oxidised to acetate and CO_2 reduced to CH_4 by the syntrophic growth of the S organism and Methanobacillus omelianskii. McInerney and Bryant (1981) have recently reported an organism in this group, Syntrophonas wolfeii, that beta-oxidises even-numbered-carbon fatty

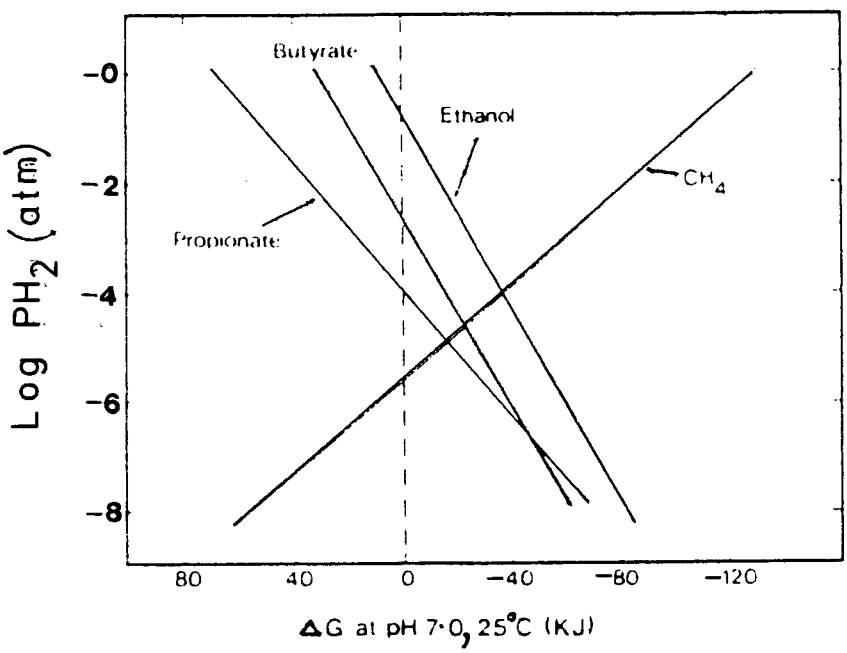


Figure 2.2 Effect of the partial pressure of hydrogen (PH_2) on the free energy charge (ΔG) for the degradation of ethanol, propionate and butyrate with methane formation with H_2 and CO_2. The assumption is that substrate concentrations of fatty acids are 1 mM each and bicarbonate is 50 mM with the partial pressure of
CH_4 at 0.5 atm ($\Delta G^1 = \Delta G^{01} + 1.36$ log ([products]/[reactants]).
Source is McInerney and Bryant (1979).

acids (butyrate, caproate) to acetate and H_2; odd-numbered-carbon fatty acids (valerate) are converted to acetate, propionate and H_2. S. Wolfeii must grow with an H_2-utilising microbe. Boone and Bryant (1980) also reported that Syntrophobacter wolinii oxidises propionate to acetate, H_2 and CO_2.

2.2.1.3 Homoacetogenic Bacteria

Homoacetogenic bacteria possess a high thermodynamic efficiency of metabolism as a consequence of not forming H_2 and CO_2 during growth on multi-carbon compounds. These bacteria can ferment a very wide spectrum of substrates (sugars acids, CO_2, CO, H_2, etc.). The utilization of H_2 by homoacetogens in methane producing reactions is considered to be of little consequence to the overall carbon degradation because the C-2 of acetate and CO_2 generally account for 70% and 30% respectively of the methane carbon in man-made reactors (Jeris and McCarty, 1965). Methanogens appear to successfully out compete homoacetogens for H_2 in the gastrointestinal environment (Prins and Lankhorst, 1977). So far little is known about the functional importance of homoacetogenic metabolism and the acetogenic/methanogenic bacterial interactions (Zeikus, 1980).

Populations of 10^5 - 10^6 per mL of sewage sludge have been reported (Braun et. al. 1979). Clostridium and Acetobacterium are the only recognised genera of H_2 oxidising homoacetogenic bacteria.

2.1.2.4 Methanogenic Bacteria

The methanogens are the key organisms in the production of methane from waste materials. They are the only organisms that are able to break down acetate and hydrogen to gaseous end products. Without the presence of this

group of microorganisms in a methanification process, effective breakdown of the total organic material would stop due to the accumulation of the products of the previously discussed groups. The methanogens are unusual since they are composed of many species with different cell morphology. Their energy yielding mechanisms are not yet known (Zeikus, 1977; Wolfe, 1979). They require a strictly anaerobic environment for their growth.

The methanogens utilize a narrow range of substrates (H_2/CO_2, HCOOH, CH_3NH_2, CH_3COOH, CO). Almost all species use H_2 and CO_2 for growth (Equation 2.3).

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \qquad (2.3)$$
$$(\Delta G^\circ = -135.6 \text{ kJ/reaction})$$

Even though a large amount of hydrogen is produced during anaerobiosis, the methanogens maintain a low concentration of hydrogen. A number of species can degrade acetate (Equation 2.4). Of these only Methanosarsina bakeri, Methanococcus mazei and Methanobacterium soghngenii have been isolated in pure culture.

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad (2.4)$$
$$(\Delta G^\circ = -31.0 \text{ kJ/reaction})$$

The free energy from reaction (2.4) is barely enough to form one mole of ATP (ΔG° = -31.6 kJ/reaction). This may explain the observed slow growth rate of methanogens on acetate.

Methanogenic populations of 10^8 per mL of sewage sludge have been detected (Smith, 1966).

2.1.2.5 Specific Bacterial Species Associated With Whey Biomethanation

In two recent papers (Chartrain and Zeikus, 1986a & b), the organisation and species composition of bacterial groups associated with lactose biomethanation were investigated in a continuous flow, single-phase, whey degrading reactor (pH 7.1, temperature of 37°C and dilution rate of .01 h^{-1}). ^{14}C tracer studies demonstrated that biomethanation occurred in three distinct but simultaneous phases. Lactose was metabolised primarily to lactate, ethanol, acetate, formate and CO_2. These metabolites were transformed into acetate and H_2/CO_2 in a second, acetogenic phase. Finally, the direct methane precursors were transformed during the methanogenic phase with acetate accounting for 81% of the methane formed. A general scheme was proposed for the carbon and electron flow route during lactose biomethanation. Based on the scheme, prevalent microbial populations in the ecosystem were enumerated, isolated, and characterised. The dominant groups were present in the following concentrations (per mL); 10^{10} for hydrolytic bacteria; 10^7 - 10^{10} for acetogenic bacteria; and 10^6 - 10^9 for methanogenic bacteria. The predominant hydrolytic bacteria were identified as Leuconostoc mesenteroides, Klebsiella oxytoca and Clostridium butyricum. Clostridium propionicum and Desulfovibrio vulgaris which were the predominant Lactate utilising, H_2-producing acetogens, while Methanosarcina barkeri and Methanothrix soehngenii were found to be the predominant acetate utilising methanogens. Methanobacterium formicicum was the prevalent H_2-utilising specie.

While Chartrain and Zeikus's work is very useful with regard to the microbial ecology of a single-phase biomethanation process, it is not clear whether their finding will stand for a two-phase process acidogenic reactor

where all the methanogenic bacteria would be washed out due to higher dilution rates (>> .01 h^{-1}). Also it is difficult to assess from their findings what operating the acidogenic reactor at a lower pH (< 7.0) would do to the bacterial population composition reported.

2.1.2.6 Important Process Parameters

Some environmental factors that influence bacterial degradation are pH, alkalinity, temperature, nutrients, organic acids concentration and toxic material. For acidogenic reactions the most important process parameters are temperature, dilution rate and pH. These three variables can be manipulated to favour the predominant production of a particular acidogenic end product. However to date the choice, of the desirable end product is a subject of considerable controversy (Zeotemeyer, et. al., 1982; Pipyn and Vestraete, 1981; Kisaalita et. al., 1986).

2.2 Mathematical Modeling of Microbial Growth

In this section models seldom used in microbiology are considered in a general sense. Emphasis is placed on those that are more relevant to the work reported in this study; with the aim of identifying the most appropriate form which may be used to describe the microbial growth observations reported.

2.2.1 Model Classification

A conceptual framework, classfying models of microbial populations, was first suggested by Tsuchia et. al. (1966). This framework has been retained although there is no universal agreement. Below, a slightly modified framework consisting of three distinct perspectives for cell population

kinetic representation is examined. A perspective suitable for the situation met in this study will also be identified.

Two broad approaches to modeling biological and other systems exist, namely, continuum and corpuscular methods (Harder and Roels, 1982). In the corpuscular method, the distribution of properties among the population is explicitly recognised and therefore the typical behaviour of the system is caused by the concerted action of the population. For a biological system, the cells are considered discrete and heterogeneous. Given our present-day knowledge of matter, the corpuscular approach must be considered the most realistic method of microbal growth modeling. This approach has been used to model microbial systems by Fredrickson et. al. (1967) and Ramkrishna (1979). They used the term "segregated" to describe their approach. Classical microbiology considers the basic unit of all functioning organisms to be the cell. Hence the corpuscular approach lends itself easily to modelling microbial sytems. Despite this fact, the continuum approach, earlier termed "distributive" by Tsuchia et. al. (1966) and sometimes referred to as "unsegregated" is the most commonly encountered method used in descriptions of microbial systems. In the continuum approach, the microbial population is viewed as a lumped solute biomass which interacts as a whole with it's environment. The corpuscular nature of reality is therefore ignored and the system is considered to be continuous in space. The preference for the continuum approach may be attributed to the ease with which mathematical treatment can be employed.

The second perspective distinguishes between the deterministic and stochastic (probablistic) approaches. The difference between these approaches rests on the nature of the predictions about the future behaviour of the microbial system that the model allows. In a deterministic approach,

the knowledge of the state vector of the system (a vector composed of all variables necessary to specify the state of the system at a given moment in time) allows an exact prediction of the future behaviour during an arbitrary period. With the stochastic approach, it is only possible to specify a probability that the state reactor will be in a given region of the state space (state space being a coordinate system of dimensionality of the state vector and each point in state space corresponds to a single value of the state vector). The stochastic approach is often used if the observer is unable to obtain sufficient information about the state of the population and it's subsequent behaviour to allow a deterministic prediction. Shuler (1985) has pointed out that generally a total population greater than 10,000 is sufficient to allow a deterministic treatment of any biological system. Also, Fredrickson (1966) has pointed out that the behaviour of small numbers of organism, as for example during the last stages of sterilization, calls for a probabilistic approach. Since most microbial systems of engineering interest contain populations well in excess of 10,000, deterministic modeling approaches are more popular than stochastic ones.

The third level distinguishes between structured and unstructured models. An unstructured model assumes that a single variable is adequate to describe the population. Typically the single variable is related to the quantity of biomass. Implicit in such models is the idea that biosynthetic capabilities of the population are invariant. A structured model divides the population into subcomponents. With pure cultures the addition of structure is most often achieved by dividing the cells into two or more recognisable chemical subcomponents (eg. DNA, RNA, protein, storage compounds, etc.). One of the earliest two component structured, deterministic and continuum model was proposed by Williams (1967).

Campbell (1957) defined growth over a period of time as being balanced, if during that time interval every extensive property of the growing system increases by the same factor. So in balanced growth the composition of a typical cell is time invariant. Models which ignore the multicomponent nature of cells may be adequate in this situation. Typical balanced growth situations are exponential growth in a batch culture and steady-state conditions in a CSTR (or chemostat).

In Figure 2.3 the possible interactions between two levels (continuum/corpuscular and structured/unstructured) are presented. Predictions by any model from each region could be either stochastic or deterministic. In other words a third cordinate can be added perpendicular to the plane of the page with two regions; above the page representing stochastic model predictions and below the page representing deterministic model predictions, thus giving a total of eight possible regions.

The biological phase in this study is made up of a mixture of species rather than a single specie. Complete structure on the one hand necessitates explicit recognition of each specie involved and on the other hand, the model for each specie must be chemically structured. Given that the number of species in the population is unknown a structured approach would be an almost impossible task. Therefore in this study only models in region one of Figure 2.3 were considered. Also given that large microbial counts were involved only deterministic predictions were entertained. In the next two subsections previously published microbial growth models that belong to the above indicated category are reviewed.

2.2.2 Models For Single Substrate Limiting Growth

In the present day theory of continuous culture there are two opposing schools. In the first school it is assumed that the specific growth rate

	Unstructured	Structured
Continum "Distributive" "Unsegregated"	1. MOST IDEALISED CASE cell population treated as one component solute	2. multicomponent average cell description
Corpuscular "Segregated"	3. single component heterogeneous individual cell	4. ACTUAL CASE multicomponent description of cell to cell heterogenity

Figure 2.3 Possible perspectives of interactions for cell population kinetic representation: Region 1 is an "average cell" approximation of Region 3 and a balanced growth approximation of Region 2. Region 3 is an "average cell" approximation of Region 2.

($\mu = (dX/dt)/X$) is dependent only on the limiting substrate concentration (S). In the second it is assumed that μ is either dependent on the biomass (X) or is some function of both S and X. First let's review models that belong to the former school.

2.2.2.1 Monod Equation

An equation frequently used in kinetic description of growth was proposed by Monod (1942 & 1950) as follows:

$$\mu = (dX/dt)/X = \mu_m S/(K_s + S) \qquad (2.5)$$

in which μ_m is the maximum specific growth rate. S and X are the concentrations of substrate and biomass and K_s is a Monod saturation constant. Equation 2.5 is analogous to the Briggs-Haldane solution of the Michaelis-Menten model for the kinetics of a single enzyme. If microbial growth is considered to be the result of a sequence of enzymatic reactions in which one reaction is much slower than all the others, then the Michealis-Menten equation can be considered as the physical explanation for the good fit that the Monod equation often gives. This reasoning is however by no means unique, for example the Langmuir adsorption isotherm is of the same form as equation 2.5.

2.2.2.2 Other Equations

Monod's equation is by no means the only equation which has been proposed for the substrate concentration dependence of growth. Numerous other proposals have been suggested. Some of these are discussed below.

Konak (1974) assumed that substrate dependence on specific growth rate

is related to the difference between μ_m and μ (Equation 2.6)

$$d\mu/dS = k(\mu_m - \mu)^p \qquad (2.6)$$

in which k and p are constants. The solution of this differential equation leads to the following:

$$\mu = \mu_m(1 - e^{-kS}) \text{ for } p = 1 \qquad (2.7)$$

$$\mu_m^{(1-p)} - (\mu_m - \mu)^{1-p} = (1 - p)kS \text{ for } p \neq 1 \qquad (2.8)$$

For p = 2, Equation 2.8 simplifies to:

$$\mu = \mu_m S/(1/k\mu_m + S) \qquad (2.9)$$

Equation 2.7 is similar to one that was proposed by Teissier (1936) and Equation 2.9 is an analogue of the Monod equation. Therefore the relationship postulated by Konak seems versatile, however, it has no clear relationship with any microbiological mechanism.

Dabes et. al. (1973) developed an equation to describe the kinetics of a series of enzymatic reactions for the case of steady state. The three parameter equation for growth based on their work is:

$$S = \mu(K_A + K_B)/(\mu_m - \mu) \qquad (2.10)$$

Under special conditions (details not presented here) Equation 2.10 reduces to:

$$\mu = \mu_m, \; S > K_A\mu_m$$
$$\mu = S/K_A, \; S \leq K_A\mu_m \qquad (2.11)$$

Equations 2.11 were first proposed by Blackman (1905). Dabes (1970) has indicated that the Monod equation will arise from their general form (not presented here) as a special case. In a paper Dabes and co-workers published in 1973, they analysed a number of published data using Equations 2.5, 2.10 and 2.11, and found that their Equation (2.10) always gave a better fit. This is not surprising since Equation 2.10 includes the Monod and Blackman forms. They also showed Blackman's form to be superior to Monod's. Condrey (1982) has pointed out that Blackmans kinetics have been neglected in microbial growth modeling, perhaps because of the functional form being discontinuous.

Powell (1967) combined mass transfer into and inside the organism (diffusion and permeation) with the Michealis-Menten enzyme kinetics and derived the following expression:

$$\mu_1 = \mu_m(K_s + L + S)/2L$$
$$\mu_2 = 1 - (1 - 4LS/(K_s + L + S)^2)^{.5} \qquad (2.12)$$
$$\mu = \mu_1 \, \mu_2$$

in which $L = q_m/A$; q_m being the maximum specific rate of substrate consumption and A being a constant determined by the transfer resistance inside and outside the cell. Since S, K and L are positive, $(K_s + L + S) > (L + S)^2 = (L - S)^2 + 4SL$. $(L - S)^2$ is necessarily positive, therefore $4LS/(K_s + L + S)^2$ is less than unity and its expansion by binomial theorem

is permissible. If only two terms of the expansion are retained the following expression is obtainable:

$$\mu = \mu_m(K_s + L + S) \qquad (2.13)$$

If L is small compared to K_s the Monod form is retained.

Moser (1958) postulated the following modified form of Monod's equation:

$$\mu = \mu_m S^{\lambda}/(K_s + S^{\lambda}) \qquad (2.14)$$

Unfortunately there is no physical model to support Equation 2.14.

There are a variety of other suggestions, mainly of modified first order forms. These are special cases of the Monod equation ($K_s << S$). Examples of these are those given by Elmaleh and Aim (1976) and Grady and Williams (1975). These types do not predict conditions for maximum biological activity and system failure.

In the next section, attention is turned to the assumption of the second school mentioned at the beginning of this section (2.2.2).

2.2.3 Models With Specific Growth Substrate And/Or Biomass Growth Dependence

In this type of microbial growth modelling, it is assumed that in addition to substrate concentration, μ is also dependent on the biological population density. The first example is the logistic law; written as:

$$\mu = \mu_m(1 - X/X_m) \qquad (2.15)$$

in which X_m is the maximum biomass concentration which can be reached. Equation 2.15 has been used successfully by Constantinides et. al. (1970a & b) and Rai and Constantinides (1974). Its success has been attributed by Roels and Kossen (1978) to the fact that growth curves according to the logistic law bear some resemblence to curves which result from microbial modeling exercises. In all the approaches considered so far the rate of growth of biomass is to some extent dependent upon the growth limiting substrate. This clearly indicates that the logistic law can not be successfully applied in situations where the rate of substrate addition limits the growth rate. This renders it of little value for most microbial processes.

Fujimoto (1963) considered utilization of substrate in three steps; interaction at the surface of the cell, transport into the interior and enzymatic reaction within the cell. For the case where the adsorption rate is proportional to the rate of transport into the cell interior and subsequent enzymatic conversion there, the overall reaction was considered as a reaction between substrate and enzyme

$$X + S \underset{k-1}{\overset{k+1}{\rightleftharpoons}} (X.S) \xrightarrow{k+2} [X.S] + P \qquad (2.16)$$

For growth and substrate consumption where the conversion rate from the substrate into the cell material is constant, Fujimoto solved the kinetics of equation 2.16 and obtained the following solution:

$$\mu = a\mu_m' \, (S/X) \, / \, (K + (S/X)) \qquad (2.17)$$

$$= \mu_m \, S/(KX + S)$$

where $a(0 < a < 1)$ is the activity of the enzyme. Equation 2.17 was first proposed by Contois (1959) without a mathematical justification. For a CSTR at steady state

$$X = Y(S_o - S) \qquad (2.18)$$

where Y is the yield coefficient. Substituting equation 2.18 into equation 2.17 gives,

$$\mu = \mu_m S/(YK(S_o - S) + S) \qquad (2.19)$$
$$= \mu_m S/(K'(S_o - S) + S$$

Equation 2.19 was first proposed by Chen and Hashimoto (1978) and has been used successfully in modeling methane production from agricultural residues (Chen et. al. 1980).

Roques et. al. (1982) has recently proposed the following equation:

$$\mu = \mu_m S/(K(S_o - S) + M + S) \qquad (2.20)$$

in which a third parameter M was introduced. Equations 2.19 and 2.20 are equivalents to Monod's equation with K_s varying as:

$$K_s = K(S_o - S)$$

and (2.21)

$$K_s = K(S_o - S) + M$$

If $M \leq K(S_o - S)$, equation 2.20 becomes analogous to equation 2.19.

2.2.4 Microbial Growth Modelling Recommendation

In order to provide an impression of the variety of possibilities for the modeling of substrate concentration dependence of specific growth rate, some of the equations, considered above are graphically represented for some values of the parameters (Figure 2.4). The equations were scaled in such a way that all curves coincide at $\mu/\mu_m = 1/2$ and a relative S value of unity. It is clear, as pointed out by Roels (1983), that detailed kinetics play a minor role in the fixing of biomass concentration-time relationships. Therefore there is little justification for favouring a particular equation among those discussed above. In this study the Monod equation was favoured for its simplicity and the fact that it is a mathematical homologue of the Michealis-Menten equation, which gives it a physical microbiological interpretation.

There are models that have been proposed that do not fit the above two considered classifications. This is usually a result of the special circumstances surrounding the microbiological process in consideration. A few of these are briefly covered next.

2.2.5 Miscellaneous Special Models

2.2.5.1 Models Of Growth In Presence Of Inhibiting Substrate/Product

Substrate inhibition of growth is a subject of increasing concern. A large number of publications have appeared on this subject. An often used model was proposed by Andrews (1968).

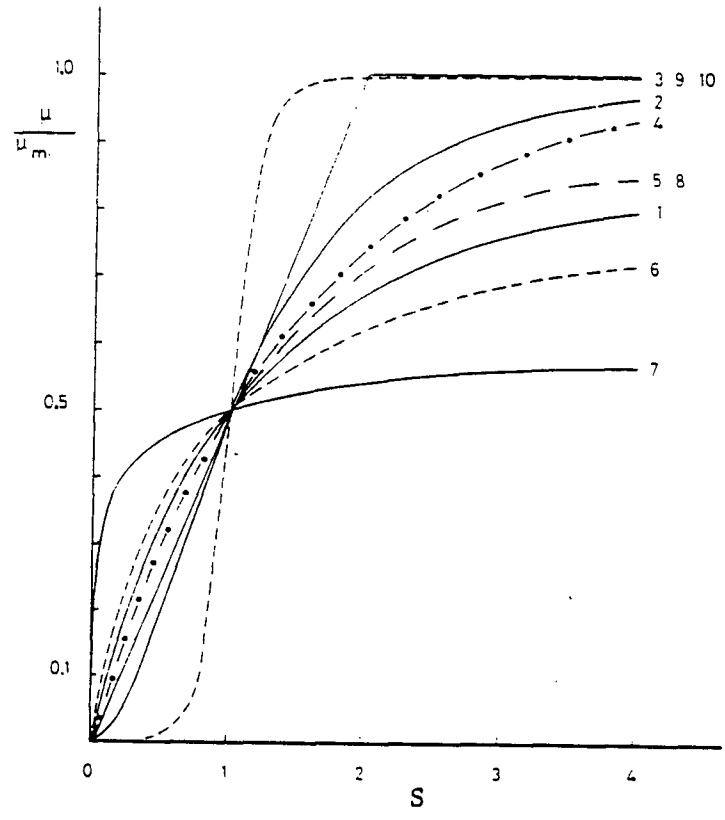


Figure 2.4 A compilation of equations for the substrate and/or biomass concentration dependence of the specific growth rate: (1) Monod; (2) Moser, $\lambda = 2$; (3) Moser, $\lambda = 10$; (4) Teissier; (5) Dabes et. al., $K_A = 1$; (6) Konak, $p = 3$; (7) Konak, $p = 10$; (8) Powell, $L/K_s = 2$; (9) Powell, $L/K_s = \infty$; (10) Blackman.

$$\mu = \mu_m S/(K_s + S + S^2/K_i) \qquad (2.22)$$

in which K_i is an inhibition constant. Like the Monod equation, equation 2.22 is derived from the theory of inhibition of a single enzyme. A number of alternatives to equation 2.22 have been proposed, but distinction between various proposals is impossible due to the limited experimental data (Yano et. al., 1966; Edwards, 1970). The interested reader is referred to Webb (1963), Yamashita et. al. (1969), Wayman and Tseng (1975 & 1976), Yang and Humphry (1975) and DiBiasio et. al. (1981), for the various alternatives to equation 2.22.

Growth can be inhibited by metabolites which are excreted as a direct or indirect consequence of growth. One of the models often used to describe this type of inhibition was proposed by Ierusalimsky (1967):

$$\mu = (S/(K_s + S))\ (K_p/(K_p + P)) \qquad (2.23)$$

where P is the concentration of the product, K_p is an inhibition constant. An example of inhibition that is well known is that of high ethanol concentration inhibition of yeast growth (Hinshelwood, 1946; Aiba and Shoda, 1969). For alternatives to equation 2.23, the reader is referred to Ramkrishna et. al. (1967) and Levenspiel (1980).

2.2.5.2 Models For Growth Limited By More Than One Substrate

A treatment for growth influenced by the concentration of more than one substrate was given by Tsao and Hanson (1975):

$$\mu = \left\{1 + \sum_i \frac{k_i E_i}{K_{E_i} + E_i}\right\} \left\{ \sum_i \frac{\mu_m S_i}{K_{S_i} + S_i}\right\} \qquad (2.24)$$

in which S_i are the concentrations of the essential substrates. E_i are the concentrations of the growth rate enhancing substrates, whose presence is not essential for growth, but growth is enhanced when they are supplied. A special case of equation 2.24 is the double substrate kinetics described by Megee (1970);

$$\mu = (\mu_{m_1}/(K_1 + S_1))\ (\mu_{m_2}/(K_2 + S_2)) \qquad (2.25)$$

Apart from the work of Megee, successful applications of equation 2.25 have been reported by Nagai et. al. (1973) and Ryder and Sinclair (1972). A different approach has also been suggested by Bloomfield et. al. (1973).

2.2.5.3 Models For Product Formation

A number of different groups of product formation processes have been described. The treatment introduced by Gaden (1959), that subdivides these processes into three categories is considered below.

In the first category, the product is a direct result of the primary energy metabolism and is thus strongly associated with growth (eg. ethanol and anaerobic processes without external electron acceptors). For this class modeling is relatively simple. The rate of substrate consumption can be assumed to be represented well by the linear law:

$$dS/dt = (dX/dt)/Y_{sx} + M_s X \qquad (2.26)$$

The rate of product formation is then given by:

$$dP/dt = (dX/dt)/Y_{px} + M_p X \qquad (2.27)$$

In the second category there is no obvious direct or indirect connection between primary metabolism and product formation (examples of these are penicillin and streptomycin production). Modeling is fairly difficult in this category.

The third category includes all those cases that are intermediate between categories one and two. For these the product is indirectly connected to the energy production pathway (examples are production of amino acids and citric acid).

2.3 Mesophilic Microbial Kinetic Modes: Literature Review

2.3.1 Whey and/or Lactose Substrates

Despite a comprehensive search only a handful of papers (Rogers et. al., 1978; Yang, 1984) were uncovered concerning acidogenic microbial kinetic modeling of whey/lactose substrates fermentation. However a number of investigators (Schlottfeldt, 1979; Boening and Larsen, 1982; and Yang, 1984) have reported various microbial kinetic models for defined and undefined single-phase fermentations of lactose limited substrates. Below, the results of each of the above cited works is reviewed.

Rogers et. al. (1978) used a semi-synthetic lactose limited medium to grow Streptococcus cremoris in batch culture at 30°C and a pH of 6.0. S. cremoris is a lactic acid producing micro-organism that has attracted considerable interest as a result of the significant role that it plays as a

starter culture in the dairy industry. Various growth models were tested for S. cremoris and the following growth inhibition and a product formation relations were recommended:

$$\mu = K_1(S/(K_s + S))\ (K_p/(K_p + P)) \tag{2.29}$$

$$dP/dt = K_4(dX/dt) + K_5(S/K'_s + S)) \tag{2.30}$$

$$-dS/dt = (dP/dt)/Y_{sp} \tag{2.31}$$

Estimates of the constants were reported. However on testing the above model with a continuous culture, no washout was observed as predicted by the model. This was attributed to wall growth.

Schlottfeldt (1979) employed an undefined culture (obtained from a methane generating reactor) in fed-batch 4L capacity laboratory single-phase reactors, to treat fresh and reconsitituted whey. The overall first order COD removal rate constant at 35° was determined to be equal to .047 h^{-1}.

Boening and Larsen (1982) also employed an undefined culture (source of inoculum not indicated) in a single-phase fluidised bed reactor with a packing medium of crushed coal particles to degrade whey permeate. Three temperature levels (15, 25 and 35°C) were considered in the study. A model previously proposed by Chen and Hashmoto (1978) of the following form was found to be the best among a number tried.

$$S/S_o = K'(t_{HR}\ \mu_m - 1 + K') \tag{2.32}$$

where t_{HR} is the retention time (=1/D). No values of the constants were reported.

Yang (1984) in an ingenious effort proposed the use of a defined microbial system comprising of a homo-lactic bacteria (Streptococcus lactis), a homoacetogenic bacteria (Clostridium formicoaceticum) and a methanogenic bacteria (Methanococcus mazei).

The following growth kinetic modes for batch fermentation were reported for a temperature of 35°C and pH of 7.0. For S. lactis and C. formicoaceticum, a model similar to the one employed by Rogers et. al. (1978) (Equations 2.29 - 2.31) was used, with the exception that a decay rate (K_d sometimes referred to as the maintenance coefficient) term was incorporated to modify μ (Equation 2.33).

$$dX/dt = (\mu - K_d)\ X \qquad (2.33)$$

For M. mazei, a substrate inhibition model proposed by Andrews (1968) (Equation 2.22) was utilised.

Yang's results seems to suggest that there were serious antagonistic effects between S. lactis and C. formicoaceticum, if the coculture experienced slight pH variations from the optimum value of 7.0. However Yang assumed that each bacteria in the coculture would exhibit the same fermentation kinetics as in the pure culture form. A theoretical model was developed for the three bacteria using the pure culture models. Unfortunately no experimental verification of the model was made. However, it was suggested that a two-phase process with lactic acid as the intermediate was the best for the defined methane fermentation studied.

2.3.2 Other Substrates

The two dominating bacterial steps in anaerobiosis (acidogenesis and methanogenesis) have different microbial kinetic constants. Henze and Herremoes (1983) have compiled for these steps the reported values of μ_m, Y, and K_s. The data includes experimentally determined constants and constants used in modeling, the latter based on more or less extensive literature searches. The generalisations of Henze and Herremoes are presented in Table 2.2.

2.4 Mathematical Analysis of Continuous Cultures

In this study continuous culture methods were favoured over batch culture methods, because pH was an important variable that had to be maintained at a constant level throughout the experimental period. Addition of a pH correcting solution (eg. NaOH) was evaluated for batch culture, but dismissed due to fear of toxic effects caused by an accumulation of Na in the reactor.

Although modeling of continuous cultures is very well documented (Malek and Fencl, 1966; Bailey and Ollis, 1986), the derivation of the so called Monod chemostat model (CSTR) is presented below to illustrate the assumptions made in its development.

The analysis of continuous cultivation of microorganisms starts with the bacterial mass balance equation (refer to Figure 2.5).

Table 2.2 Generalised Growth Constants For Anaerobic Cultures

Parameter / Culture	μ_m Maximum specific growth rate at 35°C (h^{-1})	Y Maximum yield coefficient g VSS[1]/g COD	Maximum substrate removal rate at 35°C (μ_m/Y) g COD/g VSS.h		K_s Monod Saturation constant g COD/mL
			100% active VSS	50% active VSS	
Acetic acid producing bacteria	.0833	.15	.5417	.2917	$.2 \times 10^{-3}$
Methane producing bacteria	.0333	.03	.5417	.2917	$.05 \times 10^{-3}$
Combined	.0333	.18	.0833	.0417	--

[1] VSS - volatile suspended solids - a term used to represent biomass in waste water technology

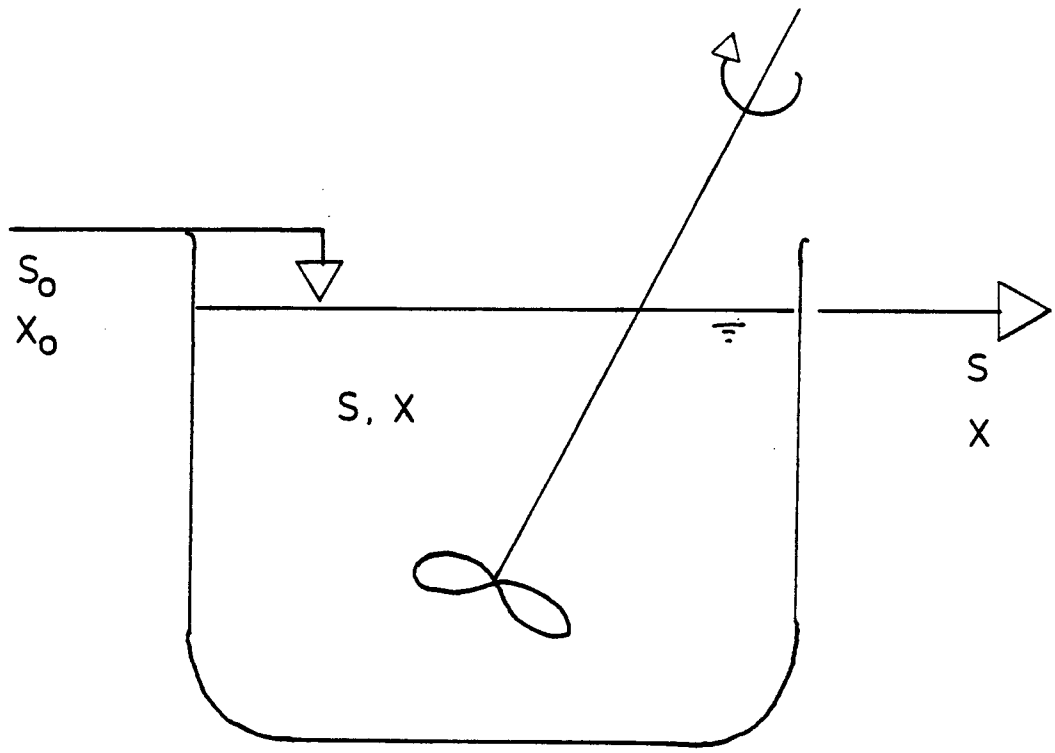


Figure 2.5 The ideal continuous-flow stirred tank reactor (CSTR).

$$\text{rate of change} = \text{input} - \text{output} + \text{reaction} \qquad (2.34)$$

In case of a CSTR, there is a constant rate of feeding (F) of a medium to organisms (X) in the vessel of constant volume (V). This means, of course, that the feed rate equals the overflow rate. The mass balance for the organisms is:

$$(dX/dt)\ V = FX_o - FX + \mu X\ V \qquad (2.35)$$

In most continuous culture systems the feed stream is either freshly made up or is sterile, so $X_o = 0$. A term called dilution rate D which is equal to F/V, is introduced into equation 2.35 to give,

$$(dX/dt) = \mu X - DX \qquad (2.36)$$

The concept of a limiting nutrient is essential to the theory of continuous culture. The ingredient in short supply relative to the other ingredients will be exhausted first and will thus limit cellular or product synthesis. The other ingredients play various roles, such as promoting cellular activities, but they will not be in acute short supply as is the limiting nutrient. A mass balance on the growth limiting nutrient gives,

$$(dS/dt)\ V = FS_o - FS - \mu XV/Y - MXV \qquad (2.37)$$

where Y is the yield coefficient (g of cell per g of limiting nutrient). M is the maintenance coefficient to keep cells alive. Dividing by V yields,

$$dS/dt = DS_o - DS - \mu X/Y - MX \qquad (2.38)$$

A relationship is needed between μ and S. This is where the expressions that have been considered in section 2.2 come in. As previously mentioned, the use of the Monod equation (Equation 2.5) was favoured in this study. At steady state, there is no change, thus the derivatives in the differential equations 2.36 and 2.38 disappear to give,

$$\mu = D \qquad (2.39)$$

and

$$DS_o - DS = \mu X/Y + MX \qquad (2.40)$$

substituting D for μ in equation 2.5 and solving for S gives,

$$S = DK_s/(\mu_m - D) \qquad (2.41)$$

Solving equation 2.40 for X after substituting D for μ gives,

$$X = DY(S_o - S)/(D + MY) \qquad (2.42)$$

Equations 2.41 and 2.42 are often called the "Monod Chemostat Model".

The above analysis applies to any continuous culture that meets the assumption of perfect mixing and constant volume. The equations are fundamental except for the Monod equation which has no time dependence and should be applied with caution to transient states where there may be a lag time as μ responds to the changs S.

2.5 Experimental Plan

Based on the material presented in this chapter it can be concluded that: (1) Effective digestion of organic matter to methane requires the combination of carbon catabolising anaerobic bacterial groups, of which the methanogens play a key role. Therefore, separating the methanogens from the non-methanogens may result in alterations in the intermediary metabolite routes; (2) Due to the complexity of the biochemical processes in anaerobiosis, modeling microbial growth with structure is an almost impossible task. Therefore unstructured models are found to be most suitable; (3) Kinetic data on the acidogenic conversion of lactose is almost nonexistent.

2.5.1 Assumptions

As pointed out in section 2.1.2.1, it is believed that hexoses are mainly fermented via the Embden-Meyerhof-Parnas (EMP) pathway. So it was assumed that lactose is mainly fermented via the EMP pathway to pyruvate. Thus the task of elucidating the degradation pathway using ^{14}C tracers was reduced to the determination of the fate of pyruvate during acidogenesis. It was also assumed that lactose is first broken down to glucose and galactose via β-galactosidase. Glucose enters the EMP pathway and galactose is converted to glucose 6-phosphate before it enters the EMP pathway, a scheme that is well established in E. coli.

2.5.2 Experimental Factors

The experimental factors and levels studied are presented in Table 2.3.

Table 2.3 Factors Investigated

Factor	Levels
Temperature	35°C
Limiting substrate	lactose and lactose + β-lactoglobulin
pH	4.0, 4.5, 5.0, 5.5, 6.0 and 6.5
Dilution rate	.05, .1, .2, .3, .4, .5 and .6 h^{-1}
Radiotracers	Lactate, propionate and butyrate

III. MATERIALS AND METHODS

3.1 Inoculum

The mixed, undefined culture inoculum was sewage sludge. It was obtained from a local two-step anaerobic municipal waste treatment facility at Iona Island. The inoculum samples were drawn from the first stage, at ten or fifteen feet from the surface.

3.2 Media

Two types of growth medium were employed in this study: the first was a chemically defined lactose limited nutrient solution the second was reconstituted whey powder, (comprising of lactose and protein, mainly β-lactoglobulin). The details of these growth medium are given below.

3.2.1 Lactose Limited Growth Sythetic Medium

Since lactose is the major single component in whey, the nutrient medium (shown in Table 3.1) was composed such that lactose is the main carbon source (substrate). The ratios of C:N:P were maintained at approximately 150:5:1, for it has been observed (Speece and McCarty, 1964; Hill, 1979; Pos et. al., 1981; and Pipyn and Verstraete, 1981) that nutritional requirements for single phase anaerobiosis are directly proportional to the sythesis of microbial cells and the indicated ratios are necessary to maintain nutritionally balanced growth. No separate microbial nutritional information for acidogenic bacteria were found. Cations of macro elements, calcium, sodium, potassium and magnesium were kept at 100-200 mg/L for the former two and 200-240 mg/L for the latter two. These

Table 3.1 Lactose Limited Growth Medium

Component	Concentration (g/L)	Supplier
Substrate		
$C_{12}H_{22}O_{11}H_2O$	10.53	BDH
Nutrients (or macro elements)		
NH_4Cl	0.800	BDH
$(NH_4)_2HPO_4$	0.180	FISHER
$MgSO_4.H_2O$	0.150	MCB
KCl	0.740	BDH
$CaCl_2.2H_2O$	0.730	MCB
$NaHCO_3$	0.300	AMACHEM
Trace (or micro) elements		
$Fe(NH_4)_2SO_4$	0.100	MALLINCRODT
$MnCl_2.4H_2O$	0.005	"
$ZnSO_4.7H_2O$	0.005	AMACHEM
$CuSO_4.5H_2O$	0.005	MCB
$NaB_4O_2.10H_2O$	0.005	AMACHEM
$NaMoO_4.2H_2O$	0.005	BAKER
Chelating agent		
Citrate	0.150	BDH

concentration levels were observed by McCarty (1964) to be stimulatory. Micro elements concentrations were kept at levels similar to those used by Speece et. al. (1983). No growth factors were added. All the chemicals were reagent grade unless otherwise indicated.

3.2.2 Lactose/Protein Growth Medium

This medium was made by adding 15.4 g of sweet whey powder (SIGMA) to 500 mL of distilled water. After mixing thoroughly the mixture was made up to one litre. An analysis of the medium is shown in Table 3.2.

3.3 Fermentor Set-Up

A modular bench top fermentor (NEW BRUNSWICK) was modified to allow continuous pumping of influent and effluent. As shown in Figure 3.1, the pH, agitation (rpm) and temperature could be set at desired values and monitored. For pH adjustment a 2N, 4N or 6N NaOH solution was used, depending on the rate of influent flow. With these concentrations of caustic, the pH value could be controlled to better than ±0.1 units of the desired value, while causing only a minimal percentage change in dilution rate (D). The fermentor had a paddle wheel agitator which was run at 400 rpm. A high rpm value was selected to ensure complete mixing (Cholette and Cloutier, 1959) and to minimise attachment of micro-organisms on the fermentor vessel components. The desired temperature was maintained by hot water circulation through stainless steel heat exchanger tubing. The temperature was controlled at 35 ±0.5°C. The fermentor had a working volume from 1.25 to 5.00 L.

Table 3.2 Sweet Whey Growth Medium Analysis

Component	Concentration (mg/L)	Method of Analysis
Substrate		
$C_{12}H_{22}O_{11}$	10,000.0	Colourimetric
Nutrients (macro elements)		
Ammonium nitrogen	9.5	
Total Kjeldahl nitrogen	242.5	
Total protein as		
β-Lactoglobulin	2,600.0	Biuret reaction
Phosphorous	--	
Sulfur	--	
Magnesium	18.0	Atomic absorption
Potassium	334.0	" "
Calcium	83.0	" "
Sodium	134.0	" "
Trace (micro) elements		
Iron	0.7207	" "
Copper	0.2495	" "

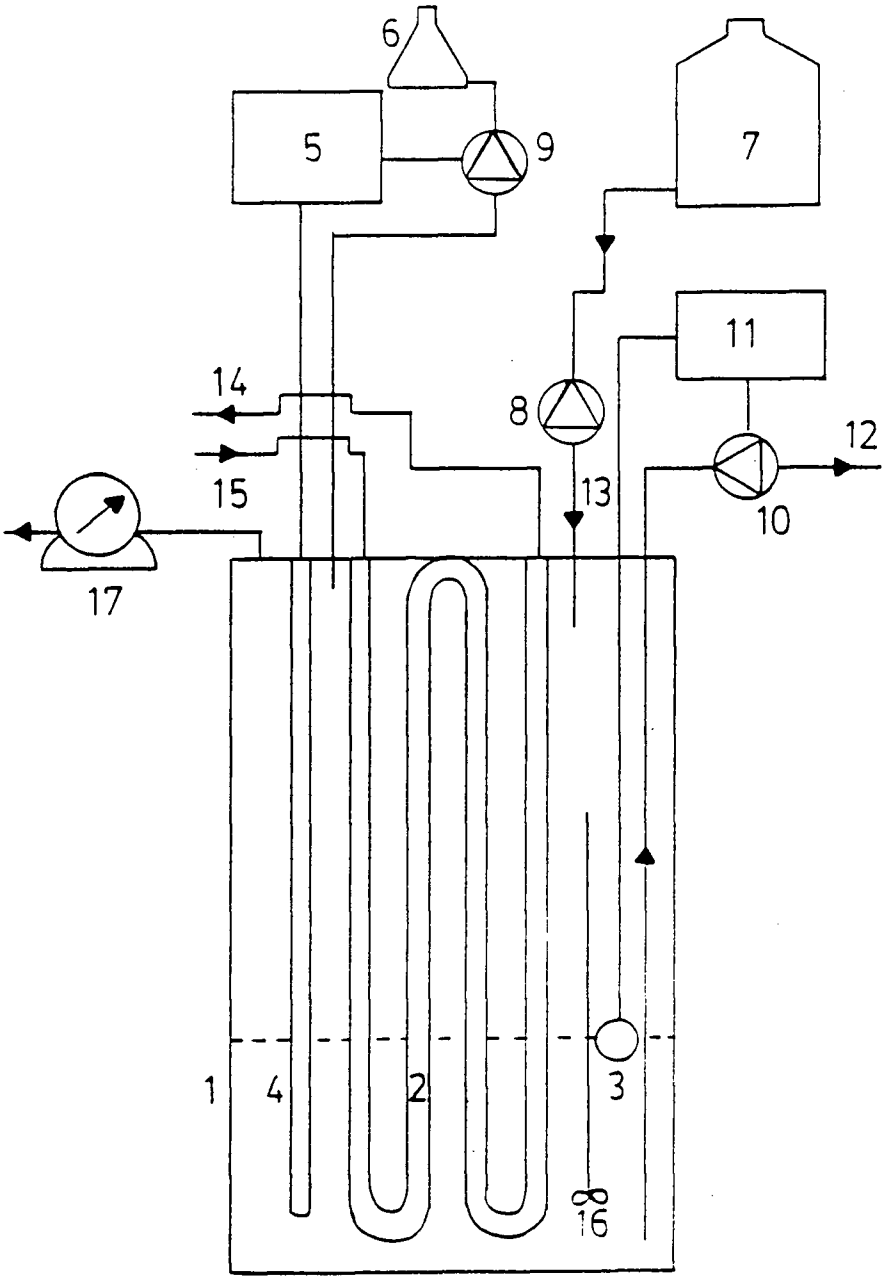


Figure 3.1 The fermentor and auxilliary apparatus: (1) fermentor vessel, (2) heat exchanger tubing, (3) level switch, (4) pH probe, (5) pH controller, (6) 2N NaOH reservoir, (7) substrate reservoir, (8) substrate pump, (9) NaOH pump, (10) effluent pump, (11) level controller, (12) effluent to waste, (13) NaOH to vessel, (14) hot water out, (15) hot water in, (16) stirrer, (17) wet gas meter.

3.4 Fermentor Start-Up And Operation Procedure

For each run at a desired fixed pH value the fermentor, containing 1.5 L of growth medium (diluted three times) was freshly inocculated with 50 mL of screened inoculum. The fermentor was then purged with at least twenty fermentor vessel head space volumes of helium to expel all the oxygen. It was operated in batch mode until all the lactose was used up, after which time the pumps were switched on and set for dilution rate (D) of approximately .05 h^{-1}. This value of D was selected to maintain the specific growth rate (μ) high enough for the methanogenic bacteria to be washed out. This method of acidogenic and methanogenic phase separation has been referred to by Pohland and Ghosh (1971) as phase kinetic control. For experiments conducted at higher dilution rates, D was stepped up in steps of .025 h^{-1} and maintained at each step for a minimum of two dilutions. According to Colin et. al. (1983) it is commonly accepted that a steady state is not reached before at least two or three dilutions under the same running conditions. The fermentor was therefore operated for at least three dilutions, once steady state conditions had been achieved. On average a period ranging between two to three weeks was needed to complete an experiment. Steady state was defined as when fermentor performance, in terms of the rate of consumption of the pH correcting solution of NaOH, showed no significant alteration within a period of at least three dilutions. Analyses of lactose, protein, formate, lactate volatile fatty acids, biomass carbon and dry biomass were made after steady state conditions had been achieved. For most of the runs, these measurements were replicated every two dilutions up to a maximum of four times. The details of the methods used for the above analyses are outlined in Appendix A.

Total gas production was recorded by a wet gas meter (ALEXANDER - 0.25 L per revolution), shown in Figure 3.1. Procedures used for the fermentor head space gas analysis and the calculation of the actual gas volume production are also outlined in Appendix A.

3.5 Set-Up For Radioactive Tracer Incorporation

In a series of experiments, samples from the fermentor were incubated under anaerobic conditions with [^{14}C(U)] - butyrate, [^{14}C(2)] - propionate or [^{14}C(U)] - lactate (NEW ENGLAND NUCLEAR) for degradation mechanism studies. Two types of apparatus were needed for these tests. In the first one it was necessary to monitor and control the pH at a fixed value throughout the period of the experiment. In the second case, pH was neither monitored nor controlled.

3.5.1 Apparatus With pH Control

A small magnetically stirred glass reactor, with a water jacket for temperature control was modified as shown in Figure 3.2 to accommodate a pH probe and a thermometer. The temperature was controlled at 35 ±0.5°C. At the beginning of the experiment, the temperature control, water pump and heater were turned on. Enough time was allowed for steady state to be achieved then the reactor was flushed with N_2. The specific ^{14}C - labeled tracer was injected together with 10 mL of fresh substrate. The fresh substrate was needed for the production of acids that would maintain an initial downward movement of the pH level. Otherwise, the pH would increase necessitating addition of some acid to the reactor to maintain a fixed desired level. Addition of acid could potentially affect the reaction

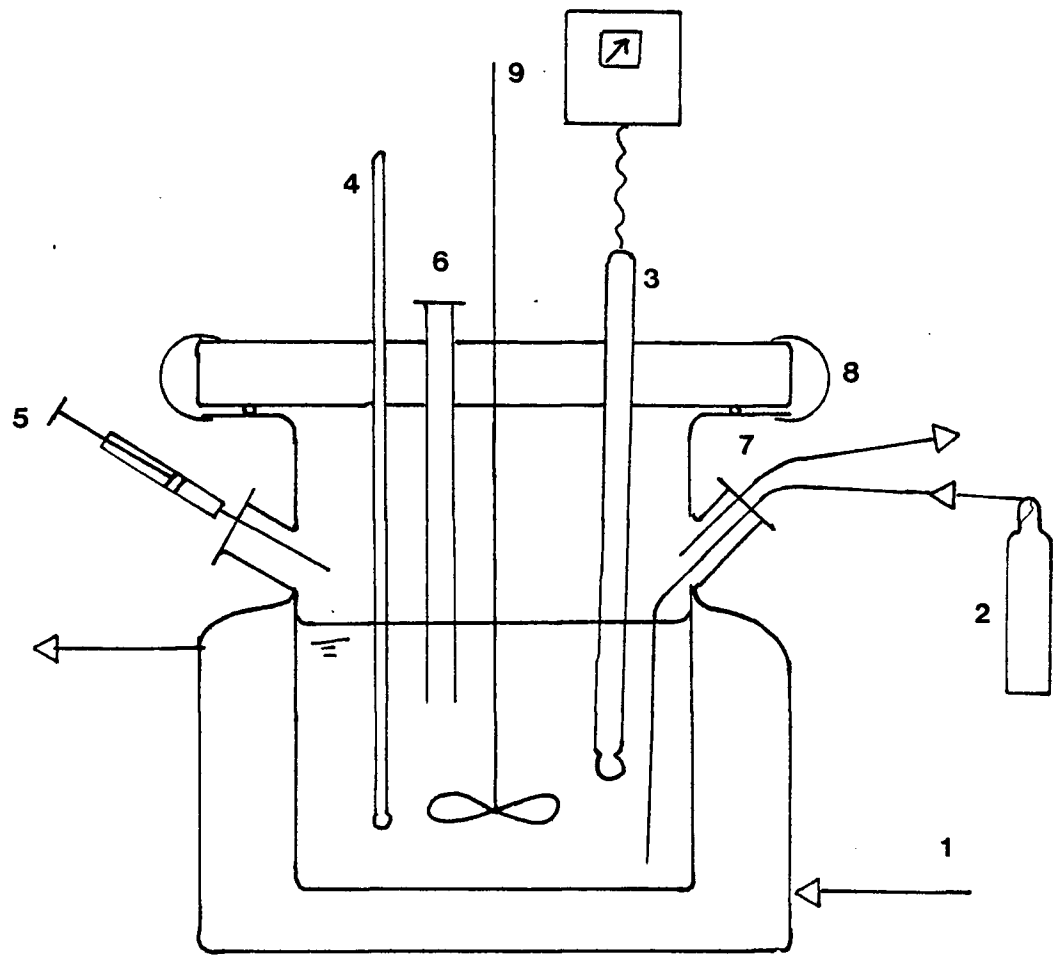


Figure 3.2 Schematic diagram for radio tracer experiments with pH control: (1) temperature control water in, (2) nitrogen, (3) pH probe, (4) thermometer, (5) 2N NaOH syringe for pH corrections, (6) sample port, (7) o-ring seal, (8) clamps, (9) mechanical stirring.

mechanism. After steady state was achieved 80 mL of sample from the fermentor were injected into the reactor. The experiment was run for 30 minutes and during this period the pH was monitored and maintained at 6. ±0.1 manually. At the end of the experiment, 10 mL of sample were removed from the reactor by syringe and immediately introduced into a 24 mL vial and frozen at -40°C. Prior to analysis the samples were defrosted and centrifuged (1h, 4450xg). 1 mL of the supernatant was acidified by adding 1 mL normal sulphuric acid and loaded directly on the liquid chromatography column described in section 3.6.

3.5.2 Apparatus Without pH Control

The cap of a 20 mL vial was modified to allow continuous flow of N_2 in and out (Figure 3.3). The vial was initially flushed with N_2. The specific ^{14}C - labeled tracer was then introduced into the vial by syringe. A 10 mL sample from the fermentor was then immediately injected into the vial. The vial was then maintained at 35 ±.5°C for a specific period. During this period a small amount of N_2 was allowed to flow through the vial to affect complete mixing. At the end of the experiment, the contents of the vial were treated in a manner similar that described in section 3.5.1.

3.6 Preparative Separation Of The Organic Acids

3.6.1 Principle

Single components of a mixture dissolved in one phase show concentration changes at the boundary with a second phase. Often a concentration of components on the surface of the other phase takes place. This phenomenon is referred to as "adsorption" and for single components it

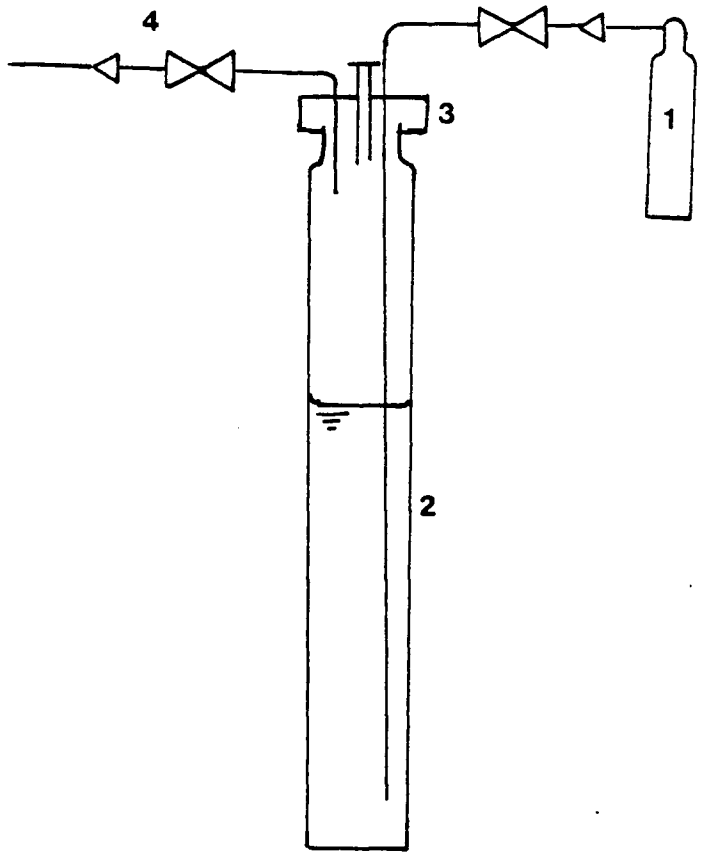


Figure 3.3 Modified vial for radio tracer experiments without pH control: (1) nitrogen source, (2) vial, (3) modified vial cap, (4) nitrogen to fume hood.

is proportional to their adsorption coefficient. The differences in adsorption coefficients determine the differences in concentrations on the phase boundaries. If one phase is moved relative to the other there is a separation of the components which is the basis of chromatographic separation (Mikes, 1979). There are other chromatographic separation methods based on different principles (eg. partition, ion exchange, gel and bioaffinity chromatography) that will not be discussed here.

The oldest known and commonest adsorption chromatography is that which takes place between liquid and solid phases. The particles of a solid (in this study celite coated with sucrose) are placed in a glass tube, a suitable solvent flows around them carrying the components with it and the separation of the components takes place on the adsorbent surface.

Adsorption chromatographic methods may be carried out by three different procedures namely, frontal analysis, displacement and elution chromatography. Frontal analysis is not suitable for preparative purposes. Displacment chromatography is primarily important as a preparative or even a pilot-plant method. However it's requirement of the use of a suitable auxiliary substances with affinities lying between pairs of components being separated, makes it unsuitable for analytical purposes.

In elution chromatography, a small part of the sample solution is introduced into the column, and is then eluted with a solvent whose affinity for the stationary phase is smaller than that of any component. As a result of repeated adsorption the components move slowly down the column. Each component is eluted independent of the others, in order of the components affinities for the solid phase. The component zones are very

often separated by a zone of pure solvent during their movement through the column.

Elution of all components with the same solvent is possible if the separated substances do not differ too much in their affinity towards the stationary phase, so that their zones are eluted without long time intervals. In situations where this is not the case "stepwise elution" may be more suitable. Stepwise elution is carried out by gradual elution of the column by several eluents arranged in order of increasing eluting power. These solvents gradually release individual components of the mixture from the stationary phase and elute them (Figure 3.4). "Gradient elution" uses gradual instead of abrupt changes in composition of solvents.

In this study an adsorption chromatographic techniques that employs both simple and step elution, (first reported by Wiseman and Irvin (1957)) was modified to preparatively separate butyrate, propionate, acetate, formate and lactate. The method employs celite coated with sucrose and hexane-acetone mixtures as eluents. Its distinctive feature is that the acids in aqueous solutions are loaded directly to the column.

3.6.2 Apparatus

A. Chromatographic tube and accessories (Figure 3.5).

B. Tamping rod, consisting of a stainless steel rod 3.2 mm in diameter, 60 cm long, silver-soldered to the centre of a 16 mm in diameter, 24 - gauge wire punched from a 16 - mesh screening.

C. Titration assembly.

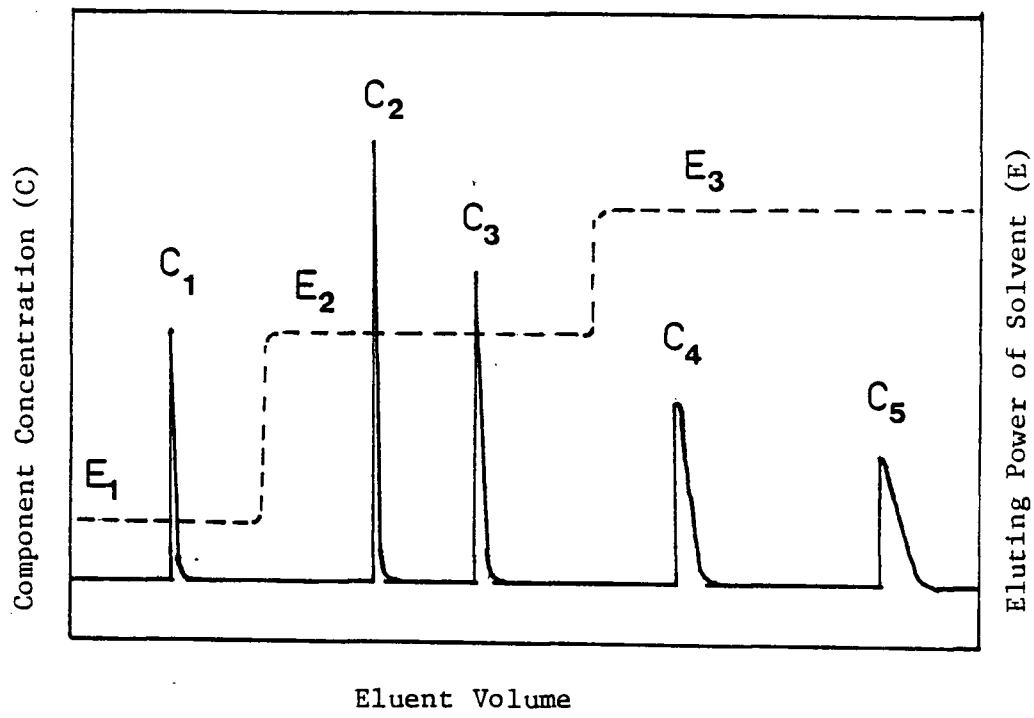


Figure 3.4 Typical chromatogram of a complex mixture separated by a combination of simple and stepwise elution: C_1-C_5 - concentrations of components, E_1-E_3 - eluting power of the eluting solvents.

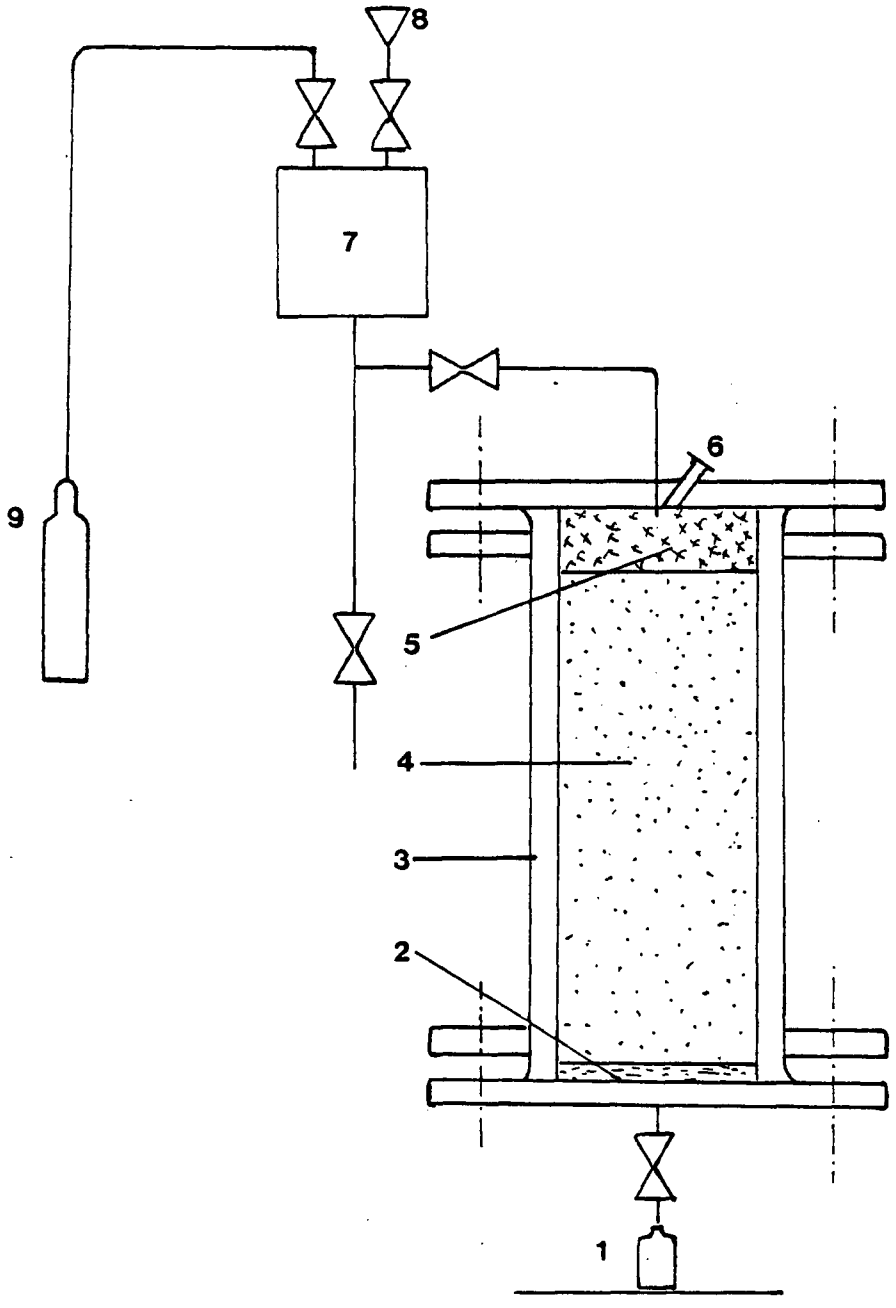


Figure 3.5 Schematic diagram of the liquid chromatography assembly: (1) 20 mL fraction vial, (2) stainless frit , 10-50 μm, (3) QVF glass tube - 1" x 6", (4) adsorbent, (5) cap material, (6) 1/4" swagelook fitting with septum, (7) eluent tank, (8) spout for refilling the tank, (9) nitrogen cylinder.

3.6.3 Materials and Reagents

A. Celite analytical filter aid (SIGMA), fine granulated sugar, anhydrous sodium sulfate and ammonium sulfate.

B. Cresol red indicator: 1.3 mL of 0.1 N NaOH was added to 50 mg of O-cresolsulfonphthalein in 20 mL of alcohol and made to 50 mL with distilled water.

C. Alphamine red-R indicator: 0.4 g were added to 100 mL of distilled water.

D. 0.1 N sulfuric acid.

E. n-Hexane glass distilled (BDH), Acetone, reagent grade (BDH). Various percentages by volume of acetone in n-hexane were made up as follows: 1, 15, 20, 30 and 50%. These were referred to as BA_1, BA_{15} etc. To prevent gradual removal of water from the column by a dry eluent, BA_1 was equilibrated against the stationary phase as follows: Two litres of BA_1 were stirred vigorously with 50 mL of 50% sugar solution to which had been added one mL of saturated barium hydroxide solution and a few drops of cresol red indicator to free the solvent of carbon dioxide and any traces of acids. After settling, the solvent was freed of suspended droplets by passing it through a filter paper.

3.6.4 Procedure

Eight mL of alphamine red-R indicator solution were mixed with 20 mL of sugar solution (2 sugar to 1 water by volume) and 0.1 mL of normal sulfuric acid, resulting in a stationary phase of approximately 50% sugar solution. This mixture was added slowly to a swirling suspension of 50 g of celite in 500 mL of BA_{50} in a blender. Stirring was vigorously continued for 3

minutes. Adsorbent thus prepared was stored in a glass-stoppered flask in a refrigerator until needed.

The column was prepared by flowing the adsorbent slurry from a separatory funnel into two 1" x 6" QVF glass tubes (connected in series, with the bottom end fitted with a stainless steel frit and valve as shown in Figure 3.5) until they were nearly full. A tamping rod was passed lightly through the slurry to dislodge air bubbles. With the bottom valve open a pressure of 10 psig was applied to the chromatographic column, compressing the adsorbent to a fixed volume in a rapidly moving solvent stream. The bottom valve was closed when the solvent had been expressed to the top of the adsorbent. The top QVF glass tube was then removed. BA_1 was added as a fine stream down the side of the tube, isolating the top surface from further turbulent effects. Eight grams of sodium sulfate, celite and ammonium sulfate in weight proportions of 12:8:1, referred to as cap material were added as a slurry in about 25 mL of BA_1. The top flange was bolted on the tube and pressure applied to compress the cap material. Approximately 75 mL of BA_1 were forced through the column to remove the BA_{50}solvent initially present.

For preparative separations, a 2 mL sample from the radio tracer experiments was loaded on the column by a syringe through the septum (Figure 3.5). The bottom valve was adjusted to give a drip rate between 1-2 mL/min. BA_1 was used to elute butyrate, BA_{15} was used to remove propionate and acetate in that order. BA_{30} was used to elute formate ahead of lactate. The fractions were collected by an automatic sample collector (SUPERRAC, KLB) in 20 mL units. In order to test the column a nonradioactive standard sample was initially applied to the column and the

progress of the acids down the column was monitored by titration with 0.005 N barium hydroxide. 10 mL of the 20 mL fraction were pipetted into a titration flask, approximately 30 mL of carbon dioxide free water were added and the solution was stirred magnetically for 3 minutes while a stream of carbon dioxide free nitrogen was bubbled through the solution to remove traces of carbon dioxide. Then the solution was titrated with 0.005 N barium hydroxide to the cresol red end point. A typical chromatogram is shown in Figure 3.6.

3.7 Determination of Radioactivity

Sample radioactivities were determined by liquid scintillation spectrometry.

3.7.1 Principle

Organic compounds called "scintillators" have the property of absorbing radiant energy either in the solid state or in solution. The absorption of this energy by the scintillator results in the formation of excited atoms or molecules that then return rapidly to the normal or ground state, releasing energy as photons (light energy) and heat. These scintillators are transparent to their emitted light, which is in the ultra violet or visible range. The number of photons emitted is approximately linearly related to the radiant energy absorbed. A senstive photomultiplier, a vacuum tube that converts photons into electrical energy, can be used as the detector of the photons.

The term "liquid scintillation" counting is used because these scintillators are usually dissolved in a suitable solvent containing the

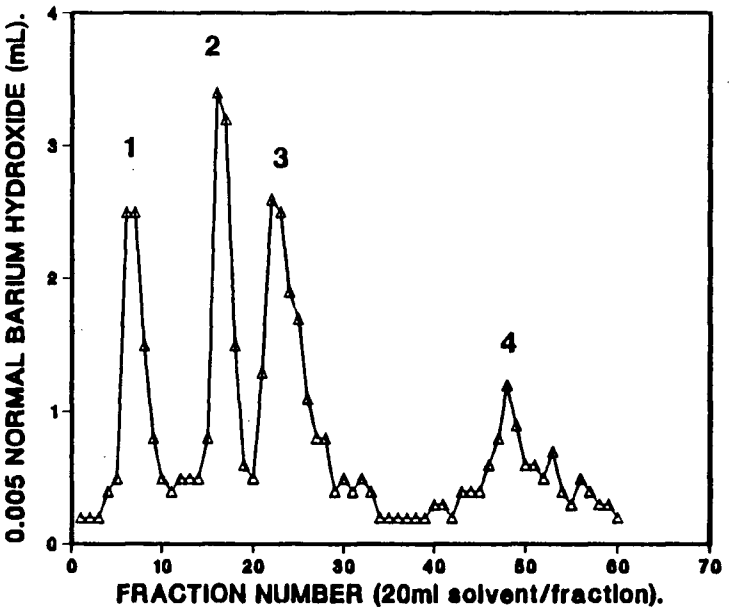


Figure 3.6 Organic acids chromatogram: (1) butyrate, (2) propionate, (3) acetate, (4) lactate.

radioactive material to be assayed. The photon production from this solution results from the following sequence of events. The energy of the β-particle emitted from a radioactive source is first absorbed by the primary solvent molecules (eg. Toluene), causing them to become excited. This excitation, high frequency energy, is propagated within the solvent and transferred to the primary scintillator (eg. 2, 5 - Diphenyloxazole), causing the scintillator molecules to become excited. When they return to their ground state they emit light at frequencies lower than that at which energy is transferred to them. This lower frequency light is propagated by a secondary solvent to a secondary scintillator (eg. 1, 1', 4, 4' - tetra-phenylbutadiene) which emits even lower frequency light that is detectable by a photomultiplier tube. A number of commerical 'scintillation cocktails' (eg. Biofluor (NEW ENGLAND), Instagel (PACKARD)) containing a solvent, a primary and secondary scintillator have been available for some time. For more details the reader is referred to an excellent treatise on the subject by Kobayashi and Maudsley (1974).

Quenching is a term applied to any factor that reduces the light output (photon production) in the system. Quenching can occur in several ways: the sample itself may absorb light given off by the scintillator or some of its radiation; the solvent may not transfer the energy of the β-particle efficiently to the scintillator; the scintillator itself may absorb some of its fluorescence; or chemical interaction of the components contained in the counting solution may result in reduced photon output. The determination of quenching is synonymous with the determination of the sample counting efficiency. The most common methods used to determine sample counting efficiency or the methods used to correct for the loss of detectable

activity by quenching are: the internal standard method; the external standard method; and the channels ratio method. The channels ratio method was employed in this study and its theoretical basis is given below.

3.7.2 Channels Ratio Method

This method is based on the fact that the pulse height spectrum is always displaced when quenching occurs. In a two channel instrument assaying ^{14}C, it is possible to set channel A to include all β-particles having energies from 0 - 156 KeV, which would be equivalent to a counting efficiency of 100% (see Figure 3.7). Channel B can be set to count all β-particles having energies from 0 - 50 KeV, which would be equivalent to counting efficiency of 50%. If the channel ratio is arbtrarily defined as the narrow-window efficiency divided by the wide window efficiency, the ratio is always a number less than one. Non-quenched samples containing various amounts of ^{14}C material would have a channels ration A/B of 0.5. The absolute activity, disintegrations per minute (dpm), of each sample is then obtained by dividing the net counts, counts per minute (cpm) appearing in the narrow window by the counting efficiency determined from the ratio-efficiency curve. An efficiency or quenching curve is usually prepared by counting a series of differently quenched standards whose actual activities are known and then plotting the efficiency against the channels ratio.

3.7.3 Procedure

A Philips PW 4700 liquid scintillation counter (PHILIPS) was used throughout this study. Commercially available quenched standards were used

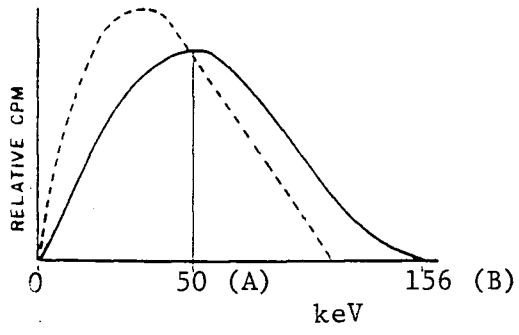


Figure 3.7 ^{14}C spectra: the solid curve represents unquenched ^{14}C, the dashed curve represents quenched ^{14}C, A and B represent various window widths.

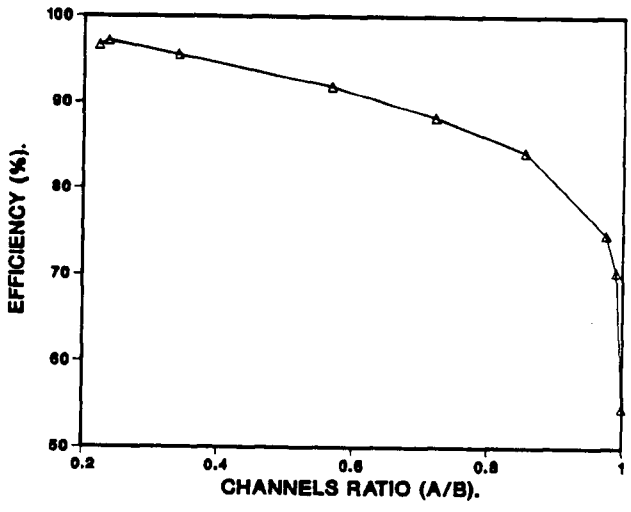


Figure 3.8 Efficiency curve prepared from commercial quenched standards.

to generate the efficiency curve shown in Figure 3.8. One mL from each liquid chromatography fraction was pipetted into a scintillation vial. Five mL of insta-gel (PACKARD) cocktail were added and shaken well. After accumulating a number of vials, counting was done overnight. Each vial was counted for five minutes. Peaks of radioactivity were detected and identified by comparison with the elution of standards.

IV. RESULTS AND DISCUSSION

All the experiments conducted in this study were divided into five categories: (1) A-series experiments were run at an approximately fixed D of 0.05 h^{-1} and temperature, of 35°C. The pH was varied from 4.0 and 6.5 at intervals of 0.5. The results of these experiments were employed to determine two potentially optimal, pH levels (4.5 & 6.0) at which further experimentation was conducted. A paper based on these experiments has been accepted for publication in "Biotechnology and Bioengineering" (Kisaalita, et. al. 1986); (2) B-series experiments were run at a pH of 6.0, but this time D was varied from 0.05 to 0.6 h^{-1} at approximate intervals of 0.05 h^{-1}. The results from these experiments were employed to speculate on various possible paths for carbon flow from pyruvate to the various acidogenic end products; (3) As a result of organic acids distribution found with respect to D; from B-series results, three dilution rate ranges ($D < 0.15$, $0.15 < D < 0.4$, $D > 0.4$) representing different organic acid combinations were studied. C-series experiments, involving the use of a number of radiotracers were conducted in the first two regions to discriminate between the rival degradation patterns proposed with the help of the results of the B-series experiments; (4) D-series experiments were run in a similar fashion to B-series with the exception that the pH was lower (pH 4.5). The results in this experimental phase together with those in A were useful in determining the influence of pH on the lactose degradation model; (5) The substrate used in all the above experimental series was only lactose growth limited (described earlier). In E-series experiments a lactose plus β-lactoglobulin based substrate was employed.

The results from the E-series have helped in determining that apparently degrading protein with lactose had no influence on the lactose fermentation model.

All the tabulated results are presented in Appendix B. The first letter in each run number identifies the experimental phase to which the run belongs. Tables B1, B2, B3 and B4 show the carbon mass balance and Table B5 shows the gas production for all experiments conducted in this study, as a function of experimental cumulative time.

The results are presented in the chapter in an order slightly different from that in which they were conducted. First the influence of D on organic acid and gaseous product distributions is considered. Then the results of the radiotracer studies are employed to determine the operative degradation model for lactose. Next the influence of protein and pH on the fermentation model are considered and finally the acidogenic microbial growth model is presented.

4.1 Influence Of Dilution Rate On Organic Acids (OA) Distribution

The results of the OA analyses for lactose growth limited experiments at a temperature of 35°C and pH of 6.0 are presented in Table B1 (Appendix B). For the seven basic experiments and their replicates, an effort was made to maintain the same temperature and pH, so that the only variable was D. Only one neutral product, ethanol, was found. The organic acids included acetate, propionate, iso- and normal-butyrate, iso- and normal-valerate, caproate, lactate and formate. According to Hobson et. al. (1974), these can be considered normal products of carbohydrate fermentation. Complete acidification of lactose was achieved up to D of

.46 h^{-1}, beyond which, detectable amounts of lactose were observed. A carbon recovery less than 50% was observed at D = .64 h^{-1} which was attributed to bacterial washout. Carbon recoveries for complete lactose conversion were equal to or better than 86%. In two analyses, the recovery was found to be larger than 105%. In the first case the unreasonably large recovery figure was attributed to an error in the measurement of biomass carbon and in the second case a sample dilution error was suspect.

In all experiments, formate, i-butyrate and i-valerate were detected, if at all, in trace amounts. Also ethanol, n-valerate and caproate were found to be minor products. The major products whose concentrations strongly depended on D were acetate, propionate, n-butyrate and lactate. In Figure 4.1, the averaged concentrations of the major OA are presented as functions of D. Three D ranges can be employed to characterise the relationship between OA concentrations and D. The first D range ($D < 0.15$) is characterised by high acetate and no detectable lactate. The second D range ($0.15 < D < 0.4$) is characterised by the fall and rise of acetate and lactate concentrations respectively. The third D range ($D \geq 0.4$) is characterised by less than 100% lactose acidogenesis and a partial bacterial washout, hence the drop in all OA concentrations.

The appearance and subsequent increase of lactate concentration with increasing D in this study is in agreement with the findings of a number of investigations, where pure cultures isolated from gastrointestinal ecosystems were the agents of carbohydrate acidogenesis. For example at low growth rates (equivalent to low D in continuous culture) the rumen bacterium Selenomas ruminatium produces almost entirely acetate and propionate, whereas at high growth rates the fermentation products of glucose are about

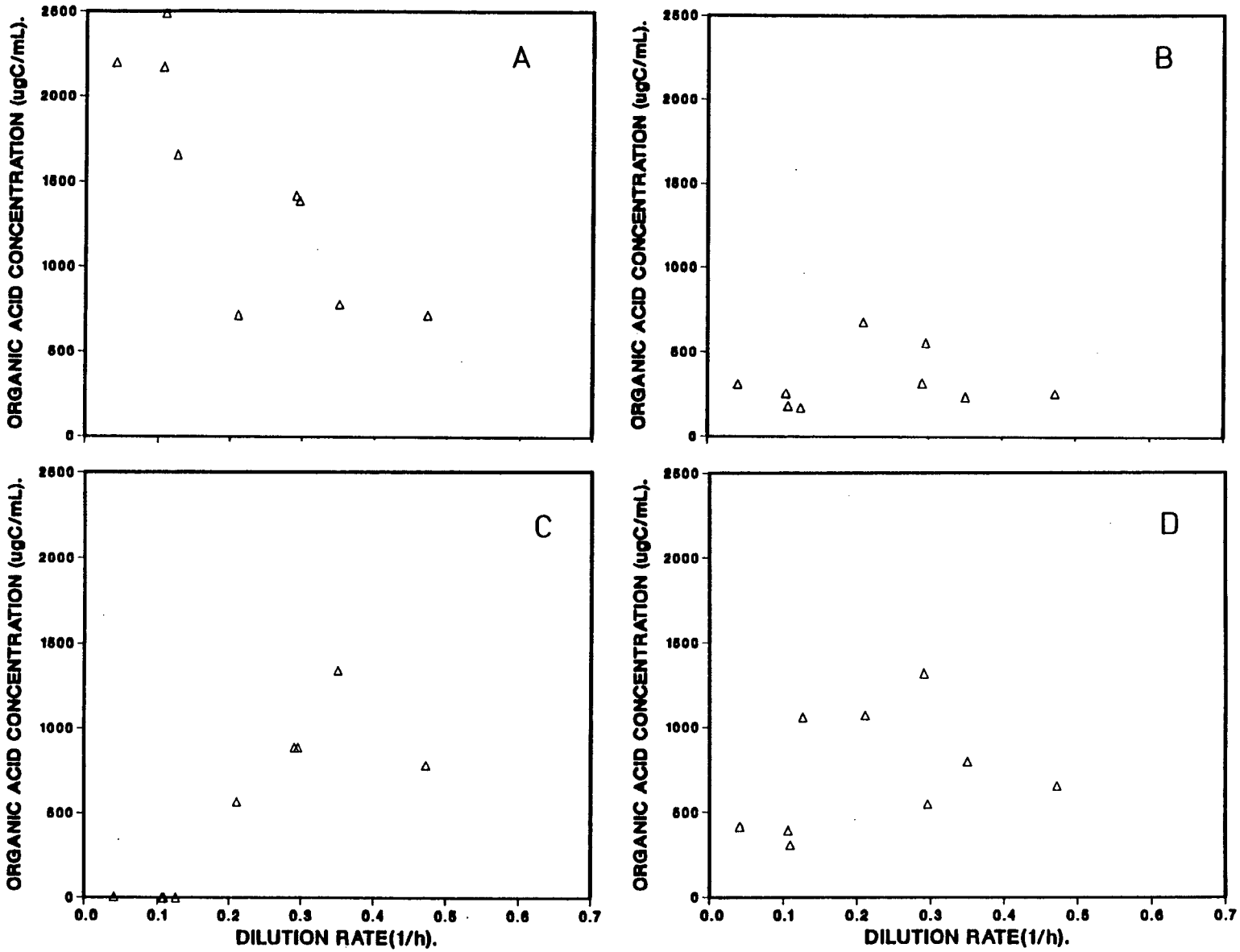


Figure 4.1 Product distribution as a function of dilution rate at a pH of 6.0: (A) acetate, (B) propionate, (C) lactate, (D) butyrate.

50% lactate, and 50% acetate and propionate (Hobson, 1965). Also Hishinuma et. al. (1968) showed that batch cultures of S. ruminantium with high initial glucose concentrations (equivalent to high D in continuous culture) produced a high proportion of lactate and at low glucose concentrations gave low lactate with high acetate and propionate concentrations. Lactobacillus casei in continuous culture produced a high proportion of lactate only at high growth rates (De Vries et. al., 1970).

A number of investigations employing mixed cultures in man-made single or two-phase fermentors have reported findings in quantitative agreement with the results from this study. For example, in municipal and animal waste anaerobiosis, appearance of lactate has been associated with shock loading (Mahr, 1969). Also in a pilot installation (126L) acidifying waste water from a sugar refinery, Zeotemeyer et. al. (1982a) found that at the shortest residence time (high D) the rate of lactate production exceeded that of utilization. In explaining their findings, Zeotemeyer and his collaborators asserted that since the formation of lactate is less favourable from the point of view of energy (2 mol.ATP/mol.glucose), and also since the conversion of pyruvate to lactate is known to proceed via one enzyme (lactate dehydrogenase) and therefore possibly occurs at a higher rate (or it requires a lower biosynthetic activity of the cell), lactate formation offers a solution for removal of excess reduction equivalents. In other words micro-organisms require for growth as much energy as possible per unit time. The highest possible energy conversion is necessary when there is a low supply of substrate, which would translate into formation of VFA. But when there is a large supply of substrate, formation of lactate is

not the best route from the energy view point but the quickest route with a low efficiency.

The production of lactate at high growth rates in pure cultures led Hobson et. al. (1974) to conclude that the growth rate of bacteria can affect fermentation products and specific conditions of low substrate concentration or low growth rate are not conducive to the formation of lactate. However Hobson and his collaborators were cautious in equating the mixed substrate/mixed culture in an anaerobic fermentor, where bacteria may be growing on different substrates at different growth rates and where some bacteria may be dependent on others for substrates or growth factors, with pure cultures under defined laboratory conditions. Lactate in bacterial fermentations is generally believed to be mainly broken down to propionate (Gottschalk, 1979), for lactate is known to be the preferred substrate for propionate-forming bacteria. If this were the case, a high concentration of propionate under low growth conditions would suggest that lactate is produced under all growth conditions, and at low growth conditions it is converted to propionate. On the contrary (Figure 4.1B) propionate was observed to increase with increasing D in the D range up to 0 - 0.15 h^{-1}, which implies that at low growth conditions carbon flow from pyruvate may be directed towards intermediary metabolites other than lactate as suggested by Zeotemeyer and co-workers (1982a). This assertion could easily be confirmed by the use of a uniformly radioactive lactate tracer. Under low growth rate conditions, since no lactate is produced, the bacteria would not be expected to have the capability to degrade lactate into any other metabolite. If a

lactate tracer is administered under batch conditions for a reasonably short period of time, the bacteria would be expected to convert a very small amount of the tracer to propionate.

On the other hand, based on the results plotted in Figures 4.1A and 4.1C, it was considered a possibility that lactate is produced under all growth conditions, but at low bacterial growth rates it is converted mainly to acetate. Independent evidence to support this possibility was published by Nakamura and Takahashi (1971) for anaerobiosis in a gastrointestinal ecosystem. Fresh rumen contents of sheep taken at various periods after feeding, were incubated in vitro with (^{14}C(2))-lactate. The radio-activities of the consumed lactate were predominately found in acetate, in most cases, but also in propionate when the pool size of lactate was large. It was found that the percentage of lactate radioactivity that was found in acetate depended strongly on the type of feed. Also Counotte (1981) indicated that once lactate is formed in mixed rumen contents it can be fermented mainly to acetate if methanogenesis occurs. The evidence supporting the lactate/acetate conversion cited above was generated from gastrointestinal anaerobiosis. No evidence was found from fermentor microbial populations with the exception of the two recent papers by Chatrain and Zeikus (1986a & b) cited in the previous chapter.

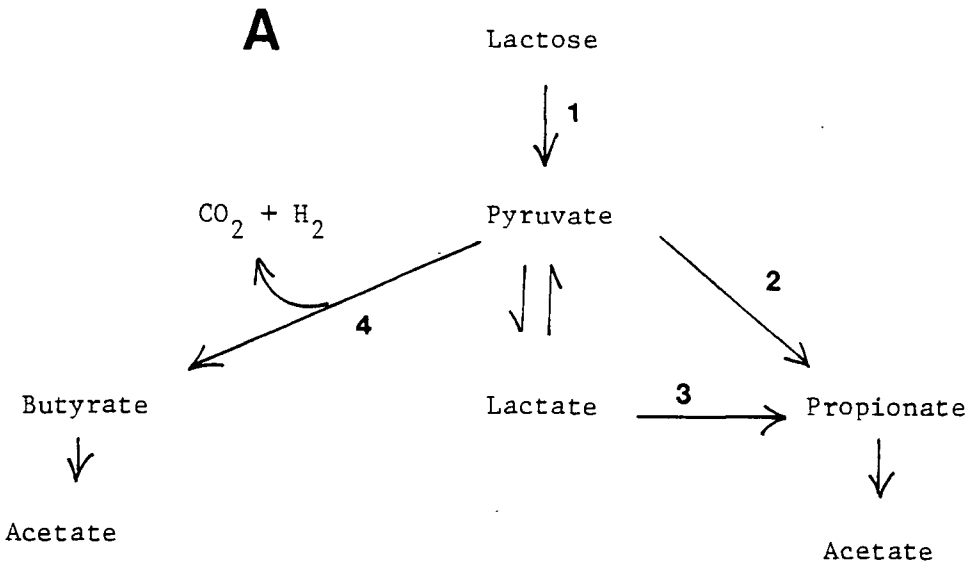
The question of the source of butyrate needed also to be examined before running radio tracer experiments to discriminate between the two rival hypotheses. Generally only obligate anaerobes form butyrate as a main fermentation product. They belong to the four genera, Clostridium, Butyriubrio, Subacterium and Fusobacterium. The mechanism of butyrate formation was not well understood until Baker and his collaborators in 1956

did their pioneering studies on C. kluyveri. The Clostridia are known to employ the EMP pathway for the degradation of hexoses to pyruvate (Gottschalk, 1979). This might suggest that the conversion of pyruvate to butyrate would go on in parallel with that of other organic acids with of course the exception of acetate. On the other hand fermentation of lactate to butyrate has been observed (Zeotemeyer et. al., 1982a; Cohen and de Wit, unpublished results), raising the possibility of lactate being the precursor for butyrate as well.

From the foregoing, under low growth rate conditions ($D < .15\ h^{-1}$) two possible general degradation schemes were postulated (Figure 4.2). The main difference between model A and model B is that, while in B lactate goes to acetate and propionate in A lactate only goes to propionate. A back butyrate to lactate oxidative reaction is included because Zeotemeyer and co-workers (1982a) offered it as an explanation for their observed conversion of butyrate from lactate.

4.2 Influence Of Dilution Rate On Gaseous Products Distribution

Initially, fermentor steady state was defined as when fermentor performance in terms of gas production rate showed no significant alteration (±10% within a period of at least three dilutions). As with previous glucose acidogenic experiments (Zeotemeyer et. al., 1982), CO_2 and H_2 were expected to be the main gaseous products for acidogenic reactions completely separated from methanogenesis. To the surprise of the author, it was found that the manner of pH control during fermentor start-up was a significant factor in determining whether there would be production of H_2 and CO_2.



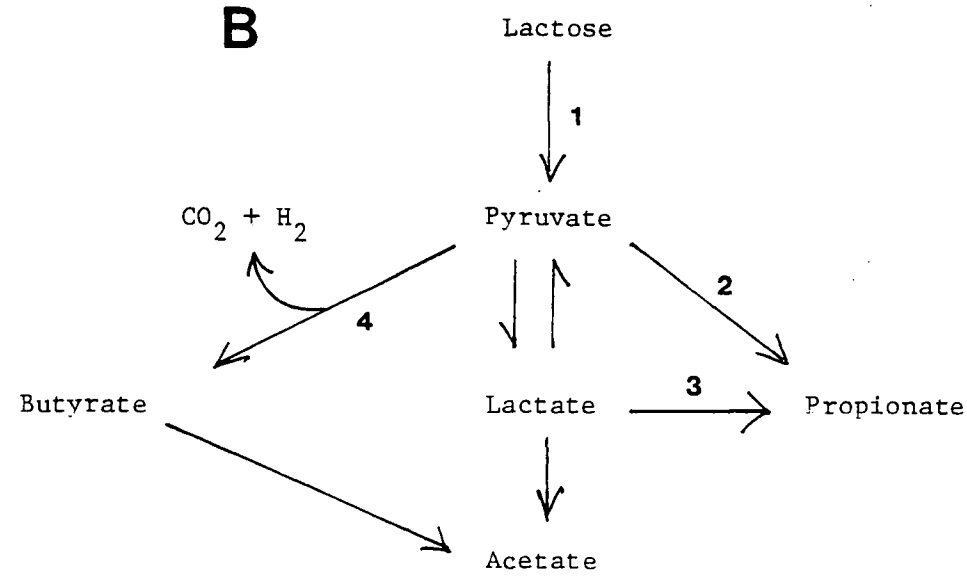


Figure 4.2 Two possible fermentation models of lactose to pyruvate and pyruvate to various end products: (1) EPM pathway, (2) succinate-propionate pathway, (3) acrylate pathway, (4) butyrate fermentation. Known enzyme systems that may be operative in the model are presented in Appedix C.

For a series of experiments run at D = 0.05 h^{-1} (Appendix B, Table B5, A-series) it was found that if the pH fell below the value of 4.5, during start-up, CO_2 and H_2 would be definitely produced even at higher operating pH levels. In Figure 4.3, a few representative plots of total gas produced (25°C and one atmosphere) against cumulative run time are presented. Graph 1 are the results (D = 0.05 h^{-1} and pH = 4.5) for the case when the pH was allowed to drop below the 4.5 level during start-up. Graph 2 is for an identical run in which the pH level was maintained above or equal to 4.5. Graph 3 illustrates the gas production results for a pH of 4.5 and at a higher D of 0.357 h^{-1}. At a higher pH level of 6.0, with control maintained at the desired pH level throughout the experimental period, it was interesting to note that in almost all the experiments at D > .05 h^{-1}, gas production exhibited an irregular cyclic behaviour. One couldn't help being reminded of the classical Lotka and Volterra (Lotka, 1920) mathematical model for predator-prey interactions that produce cycles on population sizes. Figure 4.4 illustrates such gas production cycles. It was noticed that for some experiments the cycles would diminish at an early stage and for others they would persist for longer periods. No single experimental factor was identified as being responsible for the time it took for the cycles to diminish. Coincidentally, Bungay (1985) has indicated that the frequencies and amplitudes of real prey-predator system are erratic. The effect of the gas production pattern on fermentor steady state was negligible, since the carbon fraction in gaseous form in comparison to the total recovered carbon mass was almost always very small (< 3%). As shown above, gas production was found to be an unreliable measure of fermentor steady state. Instead, variation in the consumption rate of the pH

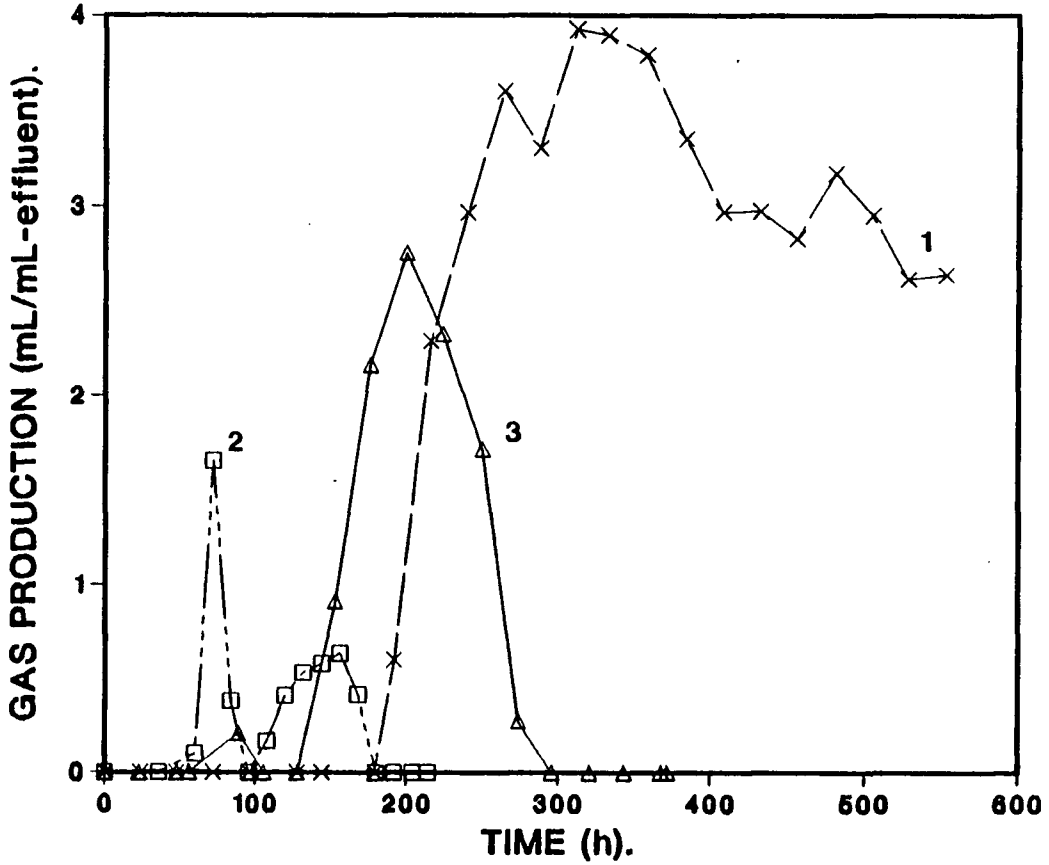


Figure 4.3 Three representative plots of gas production: (1) pH dropped below 4.5 during start-up, run # A2a, (2) pH was maintained at or above a level of 4.5, run # A2b, (3) pH was maintained at or above a level of 4.5, run # D4.

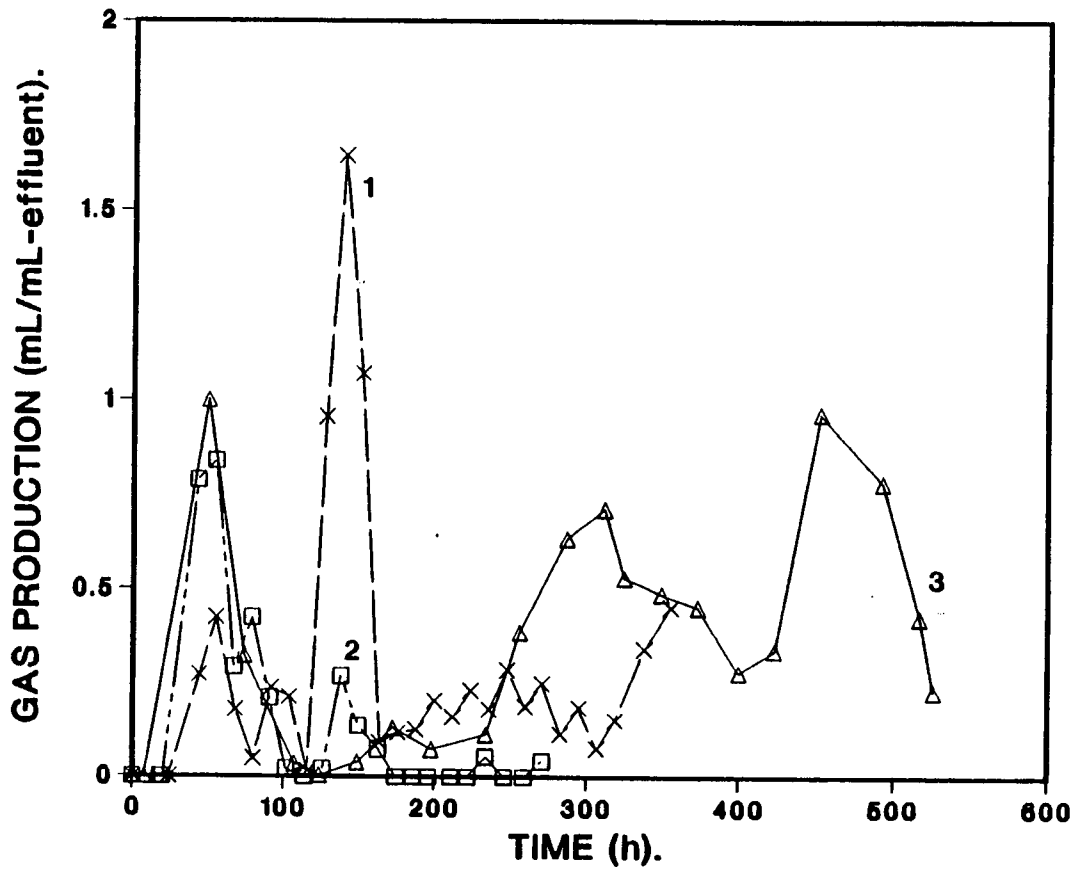


Figure 4.4 Three representative plots for which the pH was maintained at the desired value (pH6.0): (1) D=0.2109 h^{-1}, (2) D = 0.2953 h^{-1}, (3) D = 0.1246 h^{-1}.

correcting NaOH solution was employed for the purpose. It should be pointed out that some authors such as Girard et. al. (1986) have indicated that anaerobic reactors are seldom at steady state, because localised and short inhibitory or stimulating factors cause the microbial population to remain in a "dynamic" steady state. Therefore, the steady states referred to in continuous anaerobiosis have been termed "pseudo-steady states" by many.

For those experiments (at a pH of 6.0) where there was gas production at steady state, Figure 4.5 presents the fermentor head space gas analysis. It can be seen that below D = 0.15 h^{-1}, methane was detected. It is believed that the methane must have been formed via the reductive methane formation route (Figure 2.1) for a number of reasons: (1) Table 4.1 summarises the information presently available on the maximum specific growth rate (μ_m) for the formation of methane from hydrogen and acetate. It is clear that μ_m for all organisms that form methane via acetate decarboxylation is well below 0.05 h^{-1}. However those that reduce H_2 exhibit μ_m values well in the D region of interest (remember for continuous culture D = μ); (2) Above D = 0.15 h^{-1} (Figure 4.5) where no methane was detected relatively higher concentrations of H_2 and CO_2 were observed, obviating the fact that below D = 0.15 h^{-1} they were being converted to CH_4; (3) pH 6.0 is known to be the lowest limit for acetate decarboxylation in most known methanogens, making it unlikely that the observed methane was formed via the acetate route.

The question that comes to mind is what relationship might there be, between the changing gas composition and OA distribution both with respect to D. By comparing Figures 4.1 and 4.5, it was found that the transitions

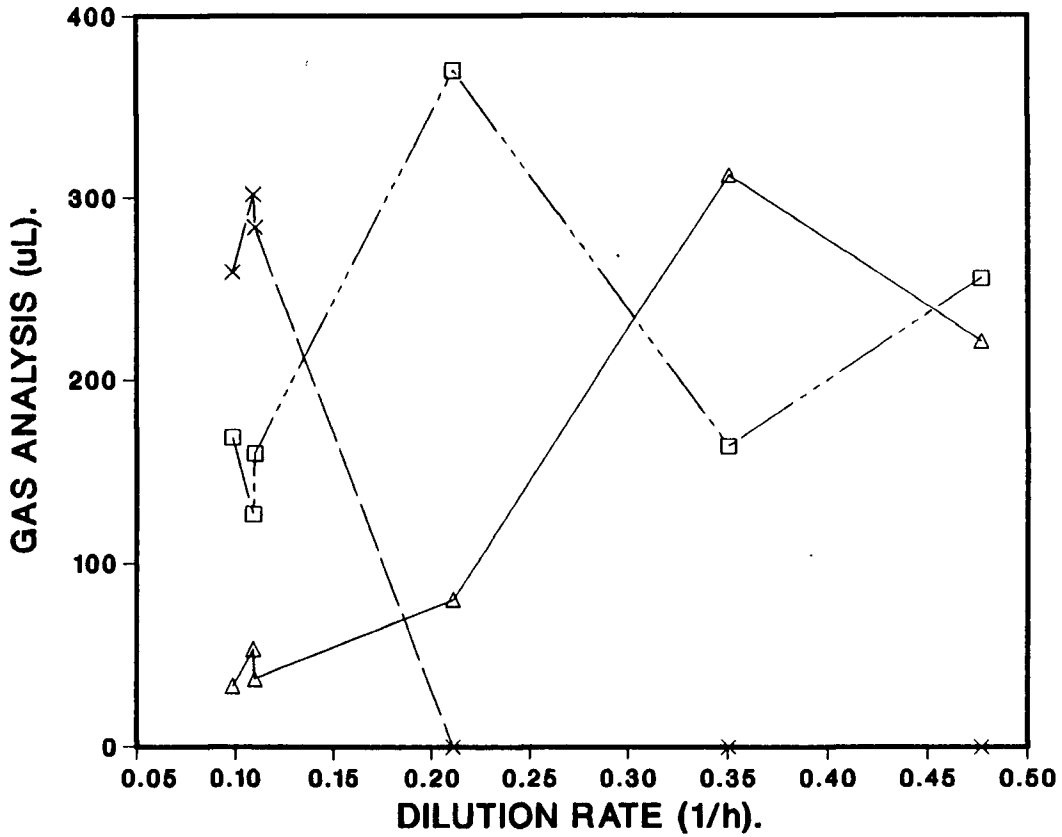


Figure 4.5 Fermentor head space gas analysis: sample size was 500 µL, (□) CO_2, (x) CH_4, (Δ) H_2.

Table 4.1 Maximum Specific Growth Rate Values For Methanogenic Bacteria

Substrate	Organism	$\mu_m(h^{-1})$	Temperature (°C)	Reference
CO_2/H_2	Methanobacterium thermoaceticum	0.69	65	Schonheit et. al. (1980)
"	" "	0.23	65	Zeikus and Wolfe (1972)
"	Methanobrevibacter arboriphilus AZ	0.058	33	Zehnder and Wuhrman (1977)
Acetate	Methanothrix soehngenii	0.0032	33	Zehnder et. al. (1980)
"	Methanosarcina barkeri (227)	0.02-0.03	36	Smith and Mah (1978)
"	" " (TM-1)	0.06	50	Zinder and Mah (1979)
"	" " (227)	0.0192	35-37	Yang (1984)
"	" " (MS)	0.0208	"	Yang (1984)
"	Methanosarcina mazei	0.042	35	Mah (1980)
"	" "	0.0224	35-37	Yang (1984)

in both OA and gas distribution occurred in the same D range. It is therefore suggested that D's in the neighbourhood of 0.15 h^{-1} represent washout conditions for the H_2 utilising bacterial group and consequently the absence of this group results in a totally new bacterial population, hence the shift in OA. However this does not answer the question that arose in the first section of this chapter, whether the shift in population represents the beginning of pyruvate being converted to lactate or just disables the conversion of lactate to other intermediary metabolites, resulting in the higher observed lactate concentration. The answer to this equation is dealt with in the next two sections.

4.3 Radiotracer Studies

The methods employed to incorporate the radiotracers were described in Chapter II. The value of the technique was first appreciated by Zuntz et. al. (1913) and his student, Markoff (1913). They employed it to study rumen microbial functions. Markoff's experiments are particularly noteworthy because of the effort made to conduct in vitro fermentations in such a fashion that quantitative results applicable to the rumen were obtained. The theory behind the technique is that when a sample of the fermentor contents is removed, it continues to function as it did in the fermentor until accumulation of fermentation products, exhaustion of substrate, availability of new substrate or any other factor causes it to change. The onset of the change can be delayed further if the major fermentor conditions such as pH and temperature are maintained at comparable levels in the new environment. The batch radiotracer experiments, the results from which are reported below, were run, at longest, for 1,800 seconds. For biological

systems, Roels (1983) has defined relaxation time, t_R, as the time, which elapses before the difference between a state value of the system and the initial value reaches a fraction (1 - 1/e) of the difference between the old and new steady state values. In Figure 4.6 adaptation mechanisms and the orders of magnitude of their t_R are shown. Since the batch experimental time of 1,800 seconds lies at the lower end of the t_R region for changes in enzymic concentrations, it was considered to be too short a period to have any effect on the fermentation model derived from the radio tracer results.

4.3.1 Results Of The Radiotracer Experiments At High D

A procedure for radiotracer incorporation described in Chapter III was followed to produce a two mL sample that was applied to the OA separation column also described in Chapter III. Figure 4.7 (for Run # C4) is a typical radiochromatogram. Butyrate was eluted within the first ten fractions by BA_1, solvent. A small amount of propionate was eluted by BA_1, as shown by the peak between the 11th and 20th fraction. Complete elution of propionate and acetate were achieved with BA_{15} up to the 40th fraction, after which BA_{30} was administered to elute lactate. The second lactate peak is a result of changing from BA_{30} to BA_{50}, a stronger solvent, at the 80th fraction so that all the lactate remaining on the column, that would otherwise tail badly, was speeded down the column. The radio activity for each product for each of the four experiments done was summed up to calculate the percentage activity recovery and the recovered activity distribution among the various products. The results for the four experiments conducted at high D ($D > 0.15$) are summarised in Table 4.2 All but one of the experiments were carried out in the small (20 mL) batch

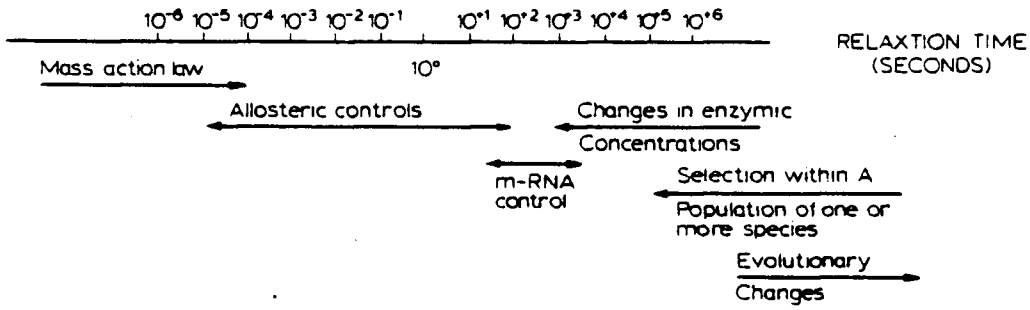


Figure 4.6 Adaptation mechanisms in organisms and the orders of magnitude of their relaxation times.

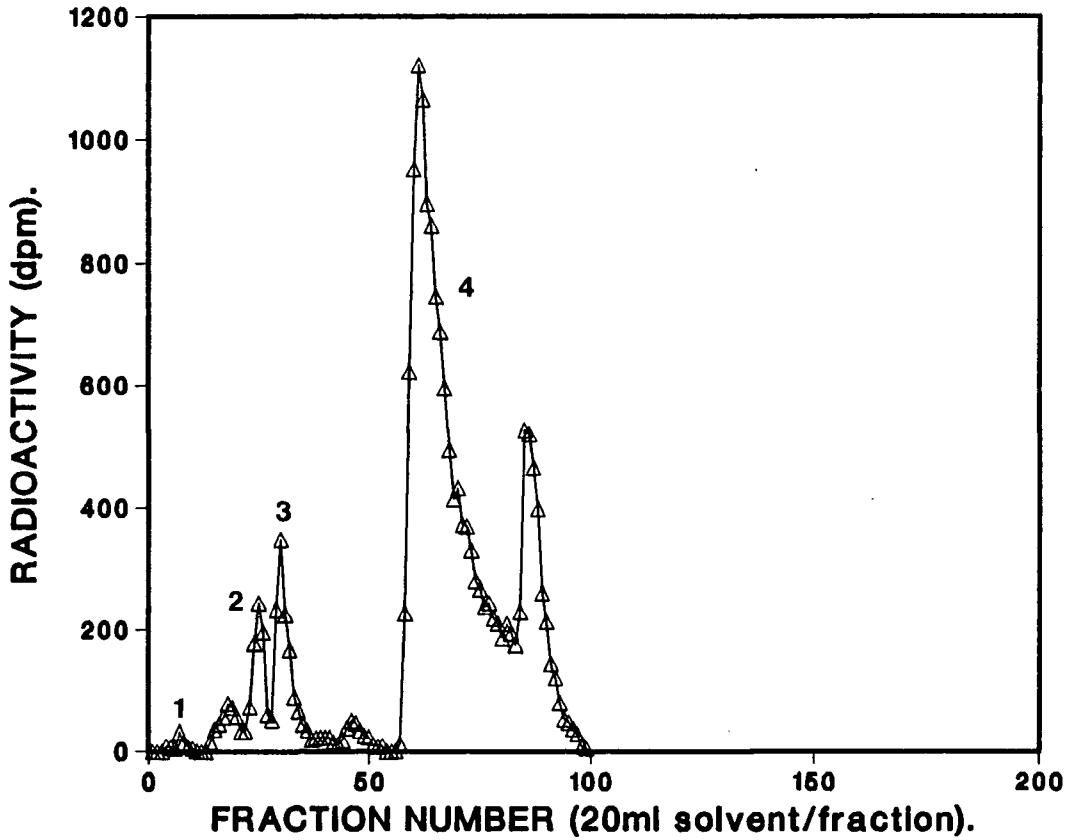


Figure 4.7 Radiochromatogram for run number C4: (1) butyrate, (2) propionate, (3) acetate, (4) lactate. Tracer was [^{14}C(U)]-lactate.

Table 4.2 Radio Activity Distribution for High D Radio Tracer Experiments

RUN #	TRACER	RUN TIME (Min)	REACTOR TYPE	TOTAL TRACER ACTIVITY (dpm)	TOTAL RECOVERED TRACER ACTIVITY (dpm)	RECOVERED ACTIVITY DISTRIBUTION				PARENT RUN #	PARENT RUN D(h^{-1})
						Butyrate	Propionate	Acetate	Lactate		
C7c	[^{14}C(U)]-Butyrate	30	S[1]	394,130	386,576 98.1%	381,420 98.7%	—	5,156 1.3%	--	B3c	0.2075
C2	L-[^{14}C(U)]-Lactate	30	S	359,028	244,048 68%	1,120 0.4%	21,200 8.7%	16,992 7.0%	204,736 83.9%	B3c	0.2075
C3	L-[^{14}C(U)]-Lactate	30	S	319,810	257,880 80.6%	1,960 0.8%	18,640 7.2%	16,520 6.4%	220.760 85.6%	B3c	0.2131
C4	L-[^{14}C(U)]-Lactate	30	L[2]	455,808	369,520 81.1%	1,620 0.4%	23,520 6.4%	33,200 9.0%	311,180 84.2%	B3c	0.2131

[1]Small batch reactor.
[2]Large batch reactor with pH control.

reactor, described in Chapter III, Section 3.5.2. Since pH was known to have an influence on product distribution, it was feared that running a batch reactor without pH control for 30 minutes might affect the results. To investigate the influence of not controlling the pH a larger reactor (60 mL) described in Chapter III, Section 3.5.1 was run in a manner similar to that used with the 20 mL reactor and the pH was monitored but not controlled. Figure 4.8 shows the resultant pH-time relationship. In 30 minutes the pH increased from an initial value of 6.06 to 6.54. A comparison of radioactivity distribution for a run with and without pH control was found necessary to dispell any misgivings about the effects of pH change in the small reactor. Runs number C2 with pH control and C4 without pH control gave results that were considered close enough to make pH control unnecessary. The rest of the radiotracer experiments were carried out in the small batch reactor without pH control. Run number C3 was made to demonstrate the replicability of the experiments. As shown by the combined results of runs number C2, C3 and C4, approximately 15% of the radioactive lactate was converted with a radio- active product distribution of 0.5% butyrate, 7.3% propionate and 7.3% acetate. The low conversion of lactate is not suprising since at high D large concentrations of lactate in the fermentor were observed, indicating the inability of the population to catabolise it. The butyrate tracer was recovered with a negligibly small conversion to acetate (1.3%).

4.3.2 Results Of The Radiotracer Experiments At Low D

Figure 4.9 presents a typical radiochromatogram. Almost all the radioactive lactate ended up as acetate with minimal quantities ending up as butyrate and propionate. The results for all the runs are summarised

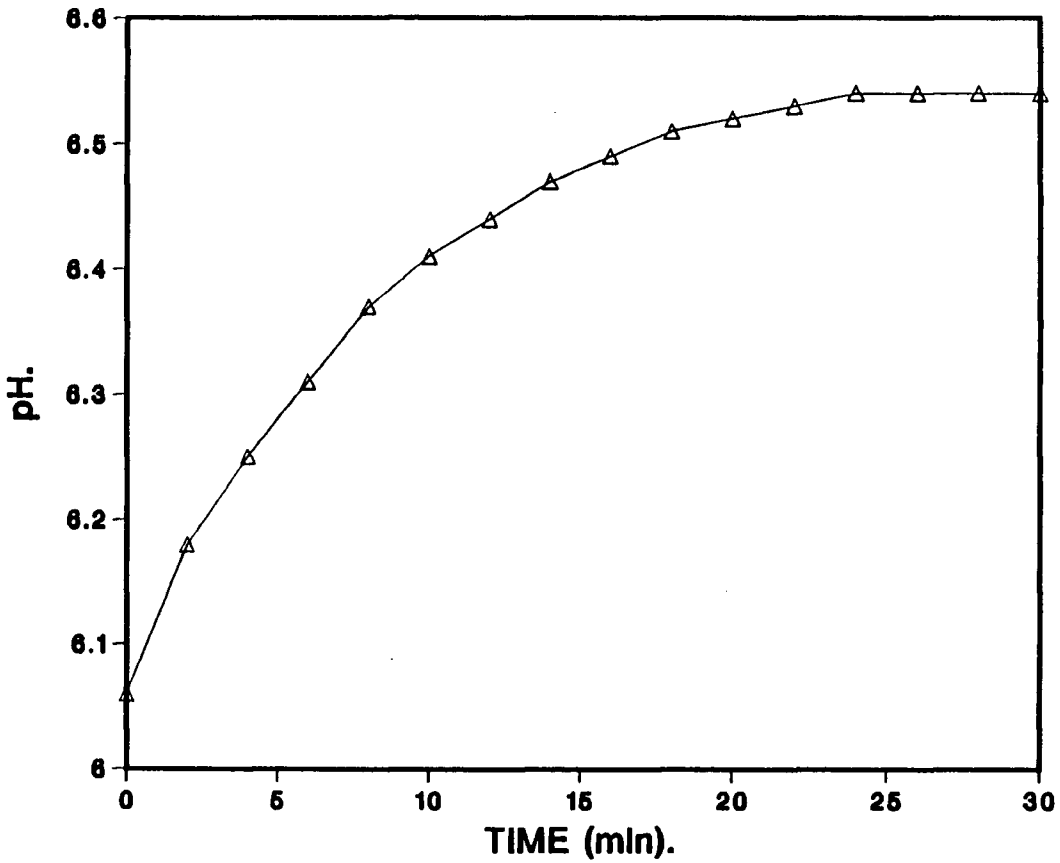


Figure 4.8 pH - time relationship for the batch radiotracer incoporation reactor experiment, without pH control.

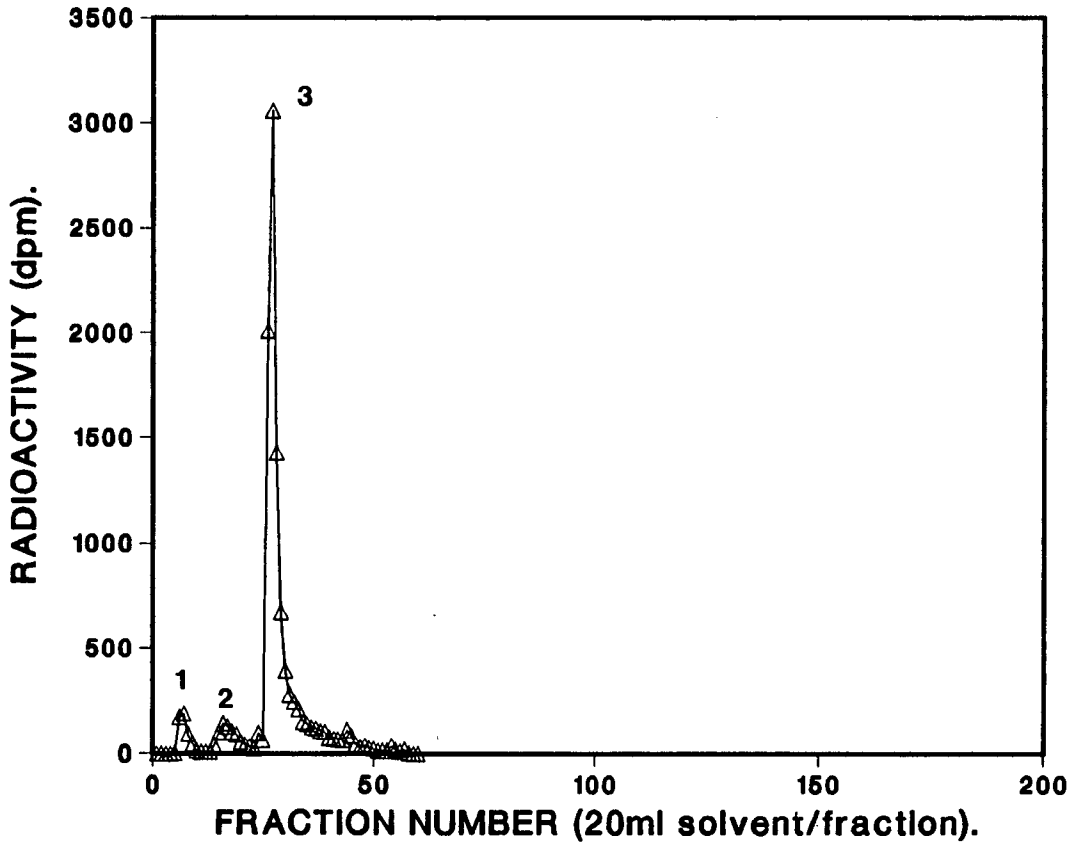


Figure 4.9 Radiochromatogram for run number C11: (1) butyrate, (2) propionate, (3) acetate. Tracer was [^{14}C(U)]-lactate.

in Table 4.3. For the three tracers employed the batch experimental time was varied. In Figure 4.10 the conversion of butyrate to acetate is shown. It was found to be linear over the range of time considered. In a period of 15 minutes only approximately 15% of the butyrate had been converted to acetate. The microbial population at low D was found to possess no capability for converting propionate. Coincidentally, of all the four major OA; propionate was the one found in least amounts. This suggest that both the succinate-propionate and the acrylate pathways (Figure 4.2) probably play minor roles. The fact that nothing happended to the propionate tracer also precluded the possibility of propionate being a major intermediate product whose concentration was observed to be low because it was being catabolised. Approximately 85% of the lactate was converted to acetate in a very rapid reaction, almost too fast for the rate to be measurable by the technique employed in this study (see Figure 4.11). Negligible radioactivity amounts ended up as butyrate (≈3%) and small amounts as propionate (≈12%). This finding not only discriminates between the rival fermentation models proposed (Figure 4.2) in favour of some form of model B, but also puts the question that arose in Section 4.1 to rest. Indeed the shift in OA observed in the neighbourhood of $D = .15\ h^{-1}$, is not a shift in carbon flow via pyruvate, from VFA production to lactate production, but an inability of the bacterial population to catabolise lactate with the concomintant production of acetate. Also, the inability of the bacterial population to catabolise lactate is probably a consequence of a bacterial

Table 4.3 Radio Activity Distribution for Low D Radio Tracer Experiments

RUN #	TRACER	RUN TIME (Min)	REACTOR TYPE	TOTAL TRACER ACTIVITY (dpm)	TOTAL RECOVERED TRACER ACTIVITY (dpm)	RECOVERED ACTIVITY DISTRIBUTION				PARENT RUN #	PARENT RUN D(h^{-1})
						Butyrate	Propionate	Acetate	Lactate		
C14a	[^{14}C(U)]-Butyrate	5	S[1]	452,240	473,300 104.7%	453,080 95.7%	--	20,220 4.3%	--	B2e	0.0947
C15a	[^{14}C(U)]-Butyrate	15	S	447,134	419,060 93.7%	357,500 85.3%	--	61,560 14.7%	--	B2e	0.0947
C16	[^{14}C(2)]-Propionate	5	S	575,880	520,460 90.4%	--	520,460 100%	--	--	B2e	0.1026
C17	[^{14}C(2)]-Propionate	15	S	560,662	561,240 100.1%	--	561,240 100%	--	--	B2e	0.1026
C19	L-[^{14}C(U)]-Lactate	1	S	256,438	202,914 79.1%	20 0%	14,354 7.1%	172,260 84.9%	16,280 8.0%	B2e	0.0947
C11	L-[^{14}C(U)]-Lactate	5	S	348,336	224,640 64.7%	10,660 4.7%	17,120 7.6%	194,900 86.8%	1,960 0.9%	B2d	0.1083
C12	L-[^{14}C(U)]-Lactate	15	S	320,796	274,580 85.6%	8,360 3.0%	46,100 16.8%	220,120 80.2%	--	B2d	0.1083
C13	L-[^{14}C(U)]-Lactate	30	S	311,740	246,436 79.1%	7,920 3.2%	29,100 11.8%	209,416 85.0%	--	B2d	0.1083

[1]Small batch reactor.

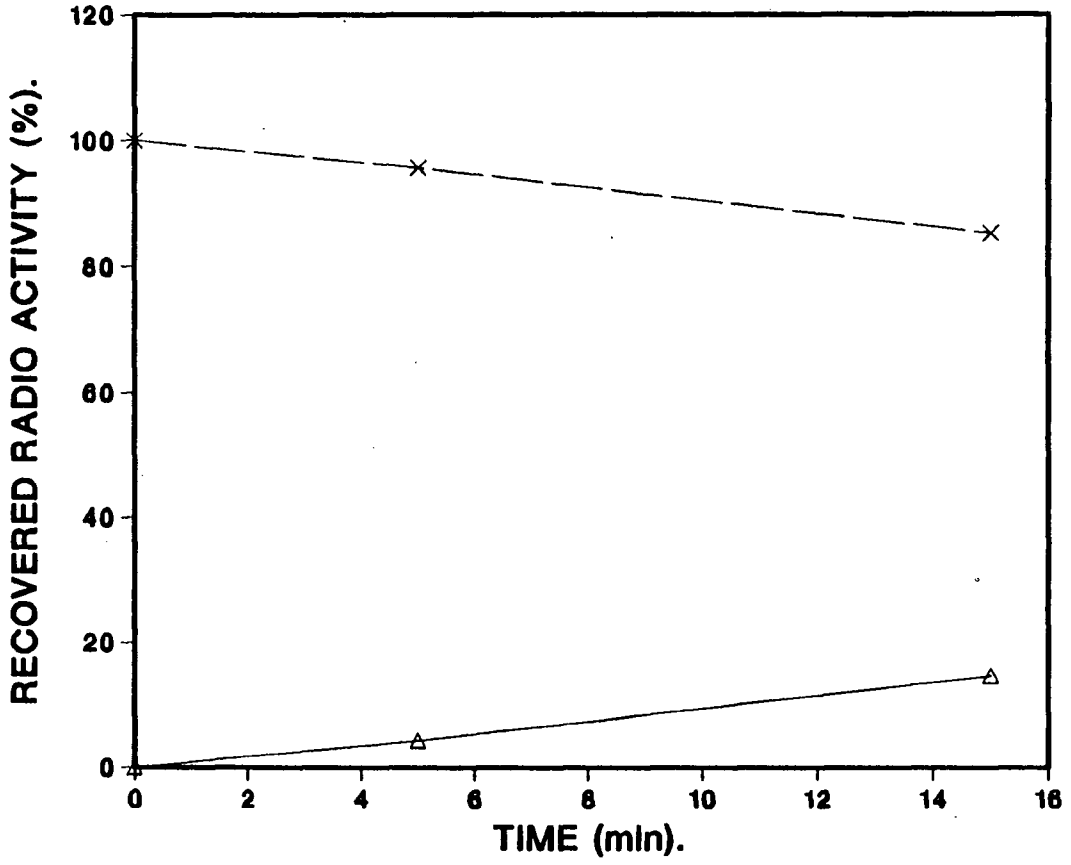


Figure 4.10 Recovered radioactivity distribution from [^{14}C(U)]-butyrate tracer at various batch experiment times: (Δ) acetate, (χ) butyrate.

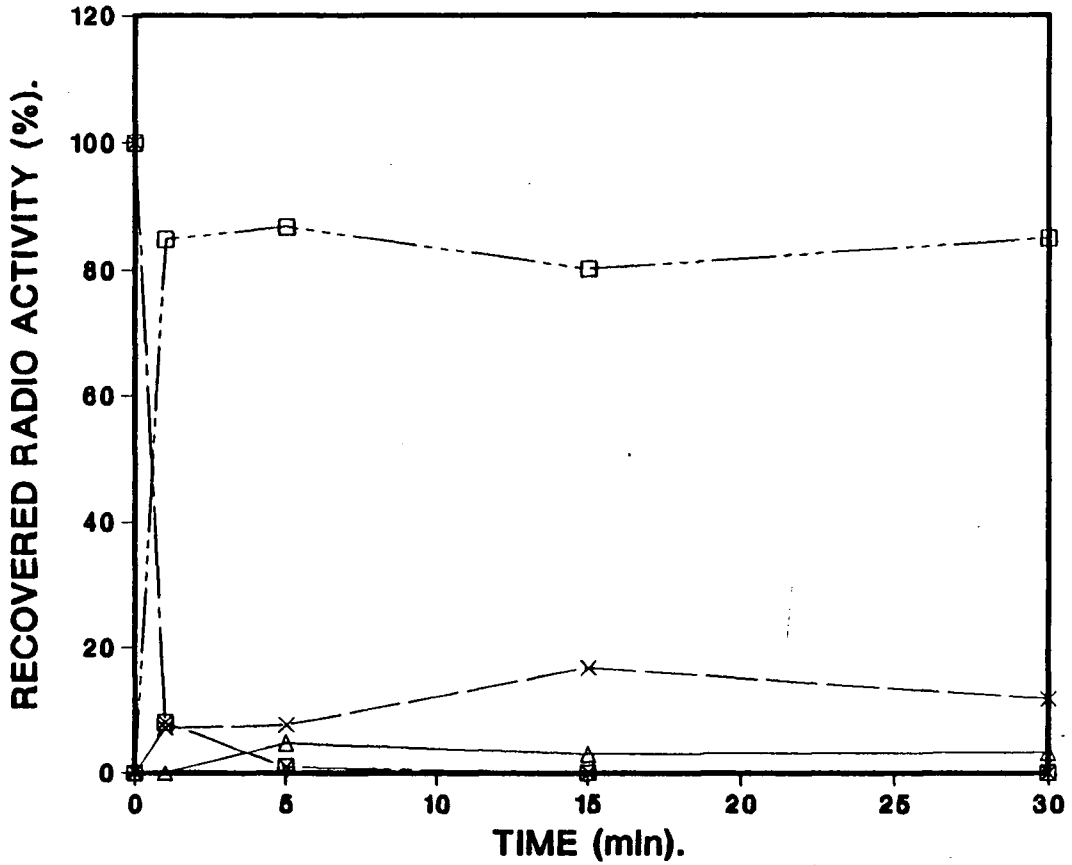


Figure 4.11 Recovered radioactivity distribution from [^{14}C(U)]-lactate tracer at various batch experimet times: (⊠) lactate, (□) acetate, (x) propionate, (Δ) butyrate.

population adjustment due to the wash out of the H_2 utilising methanogens (Figure 4.5, CH_4 concentration dropped to zero between D of 0.1 and .2 h^{-1}).

4.4 Proposed Lactose Acidogenic Fermentation Model

In all radiotracer experiments, where lactate was the radiotracer, a small amount of radioactive butyrate ($\leq$ 4.7% of recovered activity) was found. As intimated by Cohen (1981), this meagre butyrate concentration, whose origin must have been lactate, was attributed to an extremely slow back pyruvate oxidative reaction as shown in Figure 4.2A & B. This precludes the possibility of lactate being the precursor for butyrate and therefore confirms the parallel nature of carbon flow between butyrate and the other organic acids, with of course, the exception of acetate and propionate that is synthesised via the acrylate pathway (see Figure 4.2).

Before a lactose fermentation model is proposed, based on the results in this study it is fitting to put in perspective the relationship between the findings in this study and those of some previous authors and the microbial populations group interactions mentioned in Chapter II. For this task in addition to the results in this study, only the work of Chartrain and Zeikus (1986a & b) was found to be specifically relevant. Using a methodology similar to the one described in this study (with minor variations) for radiolabeled lactate incorporation, Chartrain and Zeikus proposed models for the fate of carbon in samples from a single-phase fermentor run at D = .01 h^{-1}, a controlled pH of 7.1 $\pm$ .01 and a temperature

of 37 ± 1°C. Some of their results are presented in Figure 4.12. On the basis of these results and their microbial enumeration, isolation and general characterization studies a lactose fermentation model (Figure 4.13) was proposed. Chartrain and Zeikus's work has been considered to be valid for a single phase process, and this dictates the range of D over which their findings could be applied (i.e. $D < 0.0333\ h^{-1}$). The value of $.0333\ h^{-1}$ (see Section 2.3.2, Table 2.2) is believed to be the limit, below which acidogenesis and methanogenesis can take place successfully in the same vessel. In this study, three other D ranges of importance with regard to microbial group interactions were disclosed: (1) $.0333 < D < .150$, where bacterial group 4B (Section 2.1.2, Figure 2.1) is washed out; (2) $0.150 < D < .40$, where bacterial group 4A is washed out; and (3) $D > .40$ where general or selective wash out of the remaining bacterial groups commences.

The lactose acidogenic fermentation model, proposed in this study is presented in Figure 4.14. The attention of the reader is drawn to the arrow from CO_2/H_2 to acetate being dotted, to signify lack of experimental evidence to confirm or dispel this possibility. As shown in Appendix C, Figure C2, butyrate formation from glucose can be summarized as:

$$\text{glucose} + 3ADP + 3P_i \rightarrow \text{butyrate} + 2CO_2 + 2H_2 + 3ATP \qquad (4.1)$$

where P_i is inorganic phosphorous.

Therefore as long as butyrate was being detected in the fermentor effluent, concomitant gas production was expected. The irregular gas production, which was observed, has already been reported. Assuming that CO_2/H_2 was

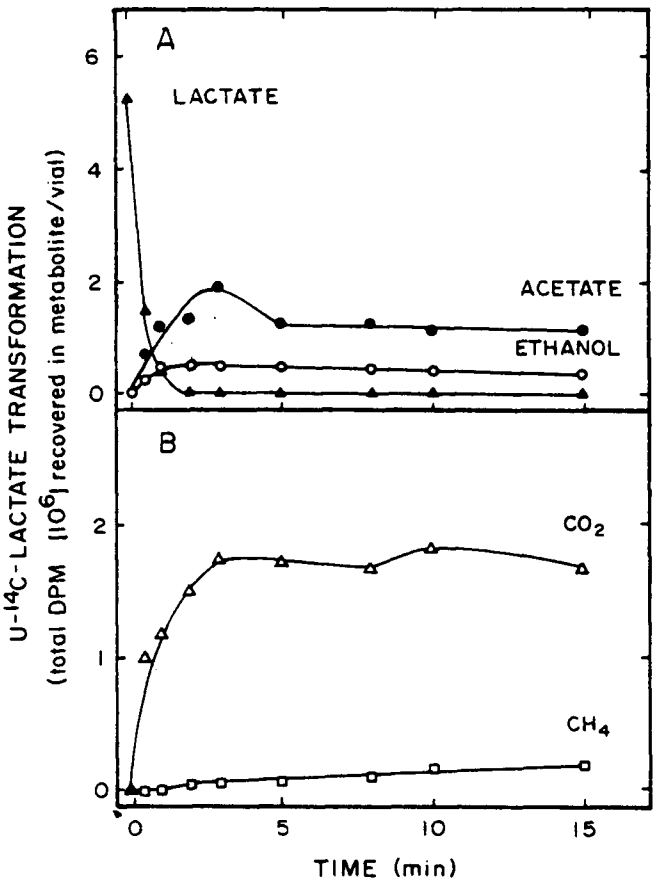


Figure 4.12 Fermentation time course for [^{14}C(U)]-lactate degradation in a single-phase lactose fermentation sample.

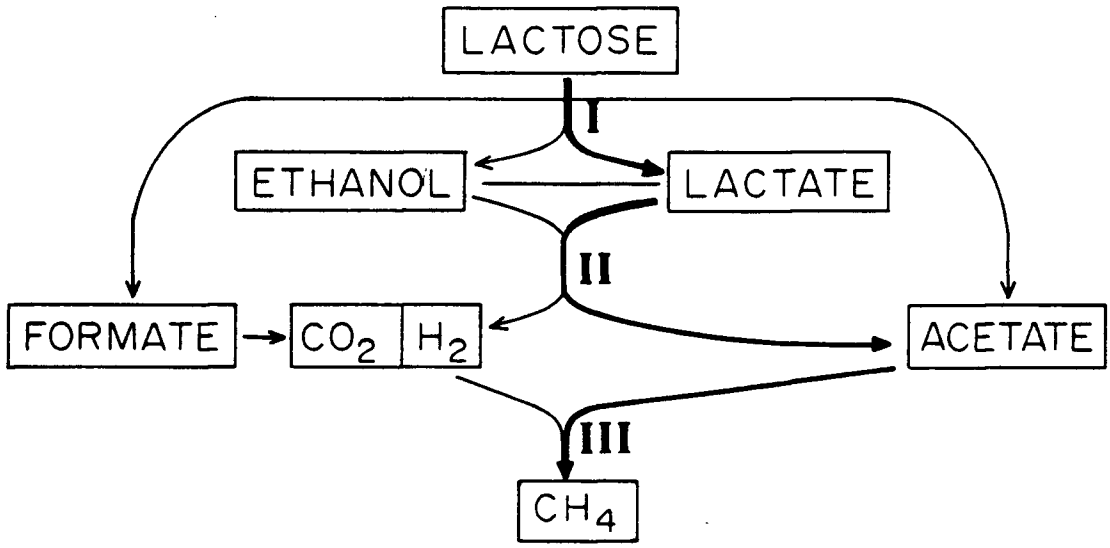


Figure 4.13 The micobial lactose fermentation model in three distinct but simultaneous trophic phases.

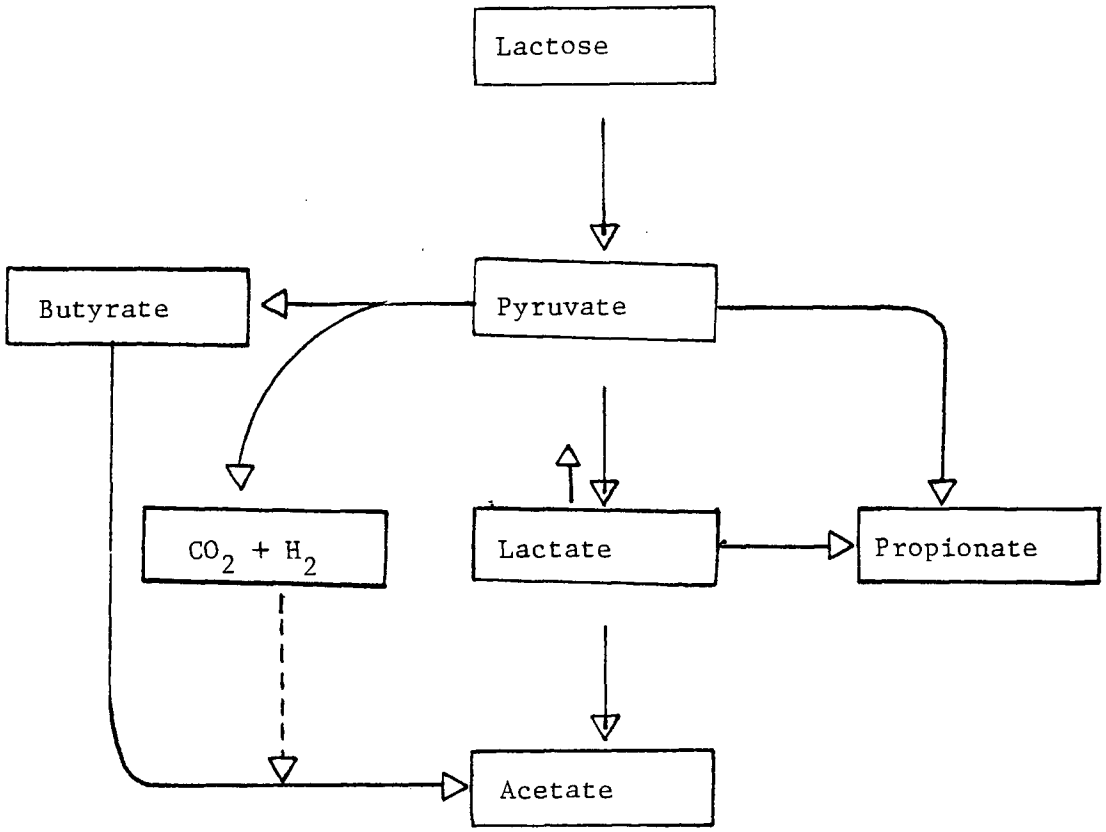


Figure 4.14 The Microbial acidogenic lactose fermentation model.

being produced all through the experimental periods, but being utilised in some way for biosynthetic activities, one would be led to conclude that the most likely product (apart from CH_4) would be acetate and the bacterial group involved would be the hydrogen consuming acetogens otherwise referred to as the homoacetogenic bacterial (Group 3 in Figure 2.1).

In comparing the results of Chartrain and Zeikus with the results in this study, a number of differences are evident. For example, while formate was one of their main intermediate products, hardly any was detected in this study. Also they detected ethanol in substantial quantities. In this study there was no production of ethanol in the fermentor, the ethanol detected under steady state conditions was established to have been produced in the substrate feed line. The most important short fall of their results, that is also reflected in their proposed model is the absence of the role played by butyric acid bacteria. They did not use any butyrate tracers, because they did not detect any butyrate in the fermentor effluent. The reason they did not detect any is because at $D \approx .01\ h^{-1}$, both acidogenesis and methanogenesis were successfully taking place in the same vessel. The production of methane via H_2 reduction kept the partial pressure of hydrogen low enough, to enable butyrate degradation by the acetogens to be energy yielding and thus allow the reaction to go to completion (see Figure 2.2). If they had probed the reaction mechanism with a butyrate radiotracer, complete conversion to acetate would not have been a surprising result. In fact one of the hydrolytic bacteria that they identified was C. butyricum, a well known butyric acid bacteria.

4.5 Significance of Findings

So far the finding with most significant practical implications in this study is the discrimination between rival hypotheses; whether lactate was synthesised at all growth rates or, whether its synthesis was switched on at a particular dilution rate below which the flow of carbon would be directed away from the lactate route. The latter has been confirmed beyond any doubt using a lactate radiotracer.

First this finding is considered not to be limited only to the microbial ecology in this study, but to be valid for all anaerobic microbial habitats were lactate has been detected under conditions of high microbial growth rates. Some of these habitats were cited earlier in this chapter to include, anaerobic municipal waste treatment systems; industrial food processing waste systems and gastrointestinal systems. In other words, all systems that contain carbohydrates as carbon sources.

Secondly, this finding may be useful in resolving the prevalent disagreement about the most suitable organic acid to be used as a marker in the optimization of production by the acidogenic phase in a two-phase process. Pipyn and Verstraete (1981) were the first investigators to address this problem. They devised a framework by which various alternatives could be compared, using the free energy of the reactions both in acidogenesis and methanogenesis. For each of the potential acidogenic end products (acetate, propionate, butyrate, ethanol and lactate), they assumed an acidogenic glucose homofermentative process (ie. all the glucose was assumed to be convertible to only one particular end product). Of course this assumption is unrealistic for mixed culture systems and for many organisms that are known to possess branched biochemical mechanisms.

However it was a necessary approximation to enable them to calculate the free energy for each possibility. The results of their calculation are presented in Table 4.4, in the form of energy quanta available for acidogenesis on the one hand and methanogenesis on the other. Based on the results of their calculation, they concluded that the preferred acidogenic product should be either lactate or ethanol, because the most delicate and rate determining bacterial group (methanogens) would be allocated a maximum of available potential energy.

But from what is known from the present study lactate, butyrate and propionate are precursors of acetate at different levels of reduction. Therefore it does not matter whether, for example, lactate is converted to acetate in the acidogenic reactor (implying that acetate is the predominant acidogenic reactor product) or whether lactate is converted to acetate in the methanogenic reactor (implying that lactate is the predominant acidogenic reactor product), for that conversion in either case will be carried out by the same acidogenic bacteria with the only difference being that in the latter case the acetogenic bacteria will be in the acidogenic reactor, while in the former case they will be in the methanogenic reactor. In both cases the energy available for the methanogenic responsible for decarboxylation of acetate will be the same.

A second group of authors (Zeotermeyer, et. al., 1982; Kisaalita, et. al. 1986) have indicated that the most desirable product distribution from the first phase of the two-phase process would be one in which the concentration of propionate was a minimum. This was based on the well known fact that the methanogenesis of propionate is the slowest of all the reactions in the methanogenic phase (McCarty, 1963; Andrews and Pearson,

Table 4.4 Distribution of Total Free Energy Change (for Growth) of the Two-Phase Anaerobic Process of Glucose to Methane Over Different Microbial Groups.

Acidogenic end product	Free energy change for the acidogenic phase (%)	Free energy change available for methanogenesis phase	
		H_2gas[1] (%)	Otherwise (%)
Acetate	51.1	33.6	15.4
Propionate	88.7	0.0	11.3
Butyrate	63.0	16.8	20.2
Ethanol	55.9	0.0	44.2
Lactate	49.0	0.0	51.1

[1]Potentially subject to loss if head space gases are not reintroduced into the methanogenic phase.

1965; Mahr, 1969). Since lactate had been believed to be the most common precursor for propionate production, it was determined not to be a desirable end product of the acidogenic phase. The present results have demonstrated that conditions can be created where lactate is converted to acetate and not propionate, and at a very fast rate (Figure 4.11). Also since lactate predominates at high D (high D's - reduced t_{HR} - are desirable because of the benefit of smaller reactor volumes), this makes it the optimum product for the acidogenic reactor.

4.6 Influence Of Protein On The Fermentation Model

Initially in this part of the study (E-series experiments) it was intended to use a pure protein, β-lactoglobulin, but the cost was found to be prohibitive. Efforts were made to obtain whey protein concentrates (WPC) from various suppliers, but electrophoretic analyses of some of these WPC revealed that none of them contained β-lactoglobulin only. In fact some of them had substantial quantities of lactose. So it was decided to use reconstituted whey powder, the analysis of which is presented in Chapter II, Table 3.2. A total of three experiments were conducted (E1, E2 and E3). Runs numbered E1 and E2 were started-up in a manner similar to the previously discussed experiments with the lactose - only substrate. Run number E3 was initially started-up with a purely lactose growth limited nutrient medium and once steady state was achieved, the substrate was switched to a lactose/protein growth limited substrate. The purpose of this was to investigate whether bacteria that had been adapted to a lactose-only substrate, if introduced to a lactose/protein substrate would respond differently in any way. The carbon balances for the three experiments are

presented in Appendix B, Table B2. As shown, carbon recoveries for the three experiments were better or equal to 71.5%, with a protein conversions ranging between 65 and 70%, probably being partly responsible for the comparatively lower carbon recoveries. For the three experiments, only trace amounts of lactose were detectable.

The partial conversion of protein and complete conversion of lactose implied that lactose was a better substrate than protein. A similar observation has been recently reported by Breure et. al. (1986a). Breure and co-workers investigated the influence of adaptation procedure on the simultaneous acidogenic fermentation of glucose and gelatin. In one series of experiments glucose dissolved in a mineral salts solution was fed to a mixed population of bacteria in a glucose growth limited chemostat (or CSTR) at 30°C and different pH levels (5.3, 6.3 and 7.0). At steady state, when the glucose substrate was switched to gelatin, growth ceased. However when gelatin was added to a medium as a second carbon substrate (a situation similar to one in this study) the breakdown of the gelatin proceeded to a limited extent ($\leq$ 30%) and the glucose continued to be completely metabolised. In a second series of experiments, Breure and co-workers, adapted bacterial populations to gelatin under comparative conditions to the ones described in their first series of experiments. After reaching steady state, glucose was added to the medium as a second carbon substrate. Following establishment of a new steady state, they found that gelatin was hydrolysed but not degraded any further. All these results demonstrate the preference for carbohydrates over proteins by acidogenic bacteria. In fact Breure et. al. (1986b) in a second paper in which they reported the results of the influence of VFA and carbohydrates on the hydrolysis and acidogenic

fermentation of gelatin, concluded that, "for optimal performance of an anaerobic digestion system purifying waste waters containing carbohydrate/protein mixtures, fermentation of carbohydrates should be partially separated from the hydrolysis and fermentation of protein". The implication of this conclusion with regard to acidogenesis of whey is that it would be more suitable to use whey permeate (deproteinated whey) than whole whey (lactose/protein). However given the high protein conversion observed in this study, it would appear that the preference of lactose over protein resulted in only a slower protein conversion. So the slower total acidogenic conversion of lactose/protein (whole whey) would only result in comparatively larger reactors. The effect of this slightly larger acidogenic reactor volume, to enable total acidogenesis of whey, on the economics of the process is something that deserves further consideration.

The question that this study attempted to address was whether the degradation of lactose together with protein had any influence on the lactose fermentation model. In a preliminary effort to find an answer, the concentration of the two main OA products (acetate and lactate) for lactose/protein acidogenesis were compared to those for lactose acidogenesis at comparable experimental conditions. In Figure 4.15 the comparison is depicted by plotting the lactose/protein OA concentrations on the same graph as the lactose acidogenic products. As shown, acetate concentrations were found to be comparable but lactate was found to be on the high side, possibly a consequence of the specific growth rate being higher for lactose/protein acidogenesis (S_o = 5475.2 μg C/mL) compared to that of lactose acidogenesis (S_o = 4212.5 μg C/mL). The comparison of OA's for the

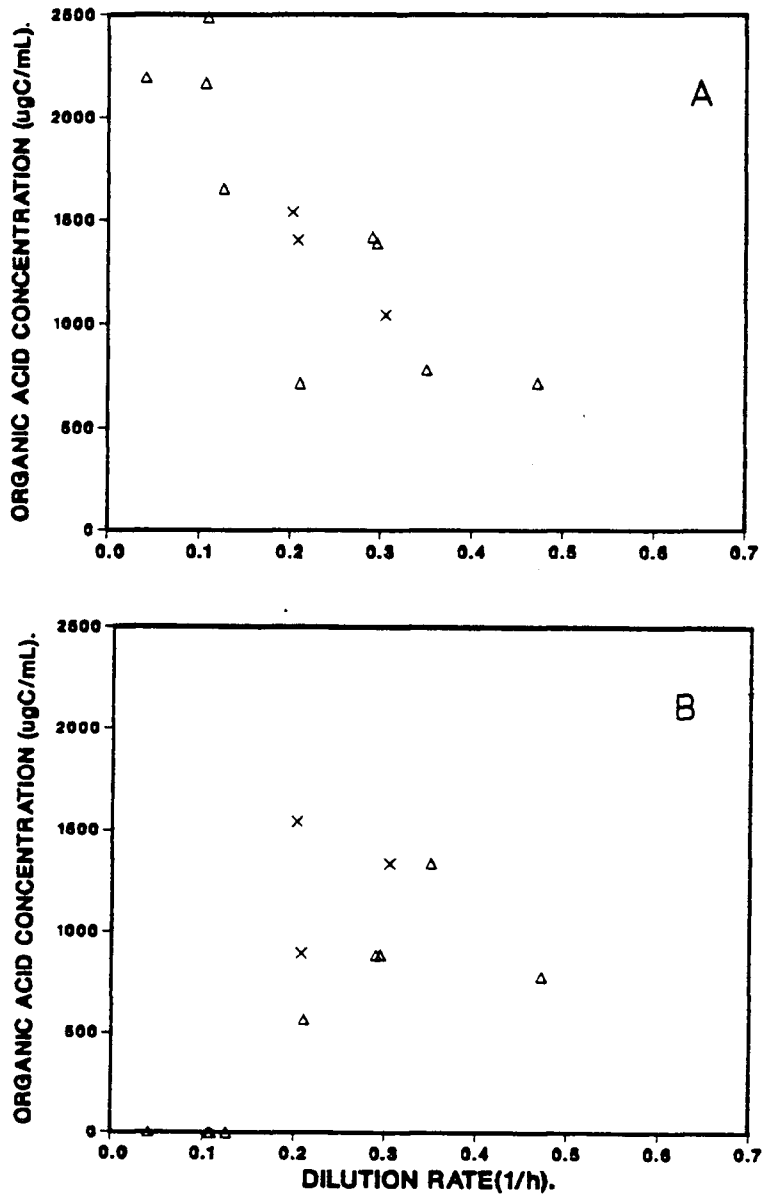


Figure 4.15 Comparison of the main products concentrations for lactose/protein and lactose-only experiments: (A) acetate, (B) lactate, (Δ) lactose-only, (x) lactose/protein.

two substrate type processes revealed no evidence to suggest that the protein had any influence on the lactose breakdown scheme.

At the next investigative level radio tracer experiments were conducted in the same manner as previously described with samples from E-series experiments. The summary of the results are shown in Table 4.5. In general the recovered activity distributions are comparable to those previously presented for high D radio tracer experiments. The conversions here were found to be slightly lower probably due to the higher specific growth rate. Protein apparently does not affect in anyway the pathway for lactose degradation.

The growth of microorganisms on more than one carbon source, usually referred to as diauxic growth is at the moment a subject of interest to a number of research groups around the world. One of these groups (Ramkrishna, 1982; Dhurjati, 1982; and Kompala, 1982) have come up with an innovative method for describing microbial interation termed, "Cybernetics". The cybernetic perspective of microbial growth contends that the cell's internal machinery has the ability to make rational optimal decisions in response to its environment. This has been found to mainfest itself well in situations of diauxic growth (Demain, 1971) so the preferential utilization of a certain substrate over another, although each substrate by itself would have been acceptable to the organism, fits the cybernetic approach since the preference of a particular substrate could well be interpreted as a result of an optimal strategy.

4.7 Influence Of pH On Carbon Flow From Pyruvate

At a dilution rate of approximately 0.05 h^{-1}, the pH was varied from a level of 4.0 to a level of 6.5, at intervals of 0.5 (Run numbers A1 to A6).

Table 4.5 Radio Activity Distribution for Samples from Experiments with Lactose Protein Substrate

RUN #	TRACER	RUN TIME (Min)	REACTOR TYPE	TOTAL TRACER ACTIVITY (dpm)	TOTAL RECOVERED TRACER ACTIVITY (dpm)	RECOVERED ACTIVITY DISTRIBUTION				PARENT RUN #	PARENT RUN D(h^{-1})
						Butyrate	Propionate	Acetate	Lactate		
C6	[^{14}C(U)]-Butyrate	30	S[1]	411,434	400,180 97.3%	375,900 93.9%	--	24,280 6.1%	--	E2	0.2018
C5b	L-[^{14}C(U)]-Lactate	30	S	249,145	235,980 94.8%	--	--	7,220 3.0%	228,760 97.0%	E2	0.2018
C1	L-[^{14}C(U)]-Lactate	30	S	337,414	322,620 95.6%	1,386 .4%	1,224 .4%	12,040 3.7%	307,970 95.5%	E2	0.2007
C9b	[^{14}C(U)]-Butyrate	30	S	372,218	403,000 108.3%	393,800 97.7%	--	9,200 2.3%	—	E2	0.2065
C10	[^{14}C(U)]-Butyrate	30	S	428,836	439,688 102.5%	431,700 98.2%	--	7,988 1.8%	--	E2	0.2065
C8	L-[^{14}C(U)]-Lactate	30	S	233,678	157,018 67.2%	--	1,040 .7%	4,146 2.6%	151,832 96.7%	E2	0.2124

[1]Small (10 ml) batch reactor.

The tabulated results for OA distribution as well as carbon balance are presented in Appendix B, Table B4. In Figure 4.16 are presented the variations of the major OAs against pH. Butyrate predominated below a pH value of 5.0 and acetate predominated above a pH value of 5.5. The region between pH 5.0 and 5.5, being the transition range. It has already been demonstrated, using lactate, butyrate and propionate radiotracers that at low D or low specific growth rate conditions, the microbial population involved in acidogenesis of lactose does not possess the ability to degrade propionate to acetate and that the conversion of butyrate to acetate is slow in comparison to that of lactate. Since lactate was only detected in trace amounts (Figure 4.16), it can be concluded that the source of the observed acetate was lactate. So the influence of the pH on the carbon flow would be to favour the butyrate route at low pH (pH $<$ 5.0) and to favour the lactate route at high pH (pH $>$ 5.5). A similar relative change in OA distribution has been reported by Zeotemeyer et. al. (1982) and Tikka (quoted by Thimann (1963) for glucose acidogenesis. The most plausible explanation for this OA shift is a change in microbial species composition. By varying D at a pH of 4.5 (Figure 4.17), an OA variation similar to one extensively discussed in Section 4.1 (Figure 4.1) was qualitatively reproduced, confirming the validity of the lactose fermentation model that has been proposed in this study.

4.8 Microbial Growth Modeling

Microbial growth modeling approaches were reviewed in Chapter II of this study and the use of the Monod equation was preferred for reasons of

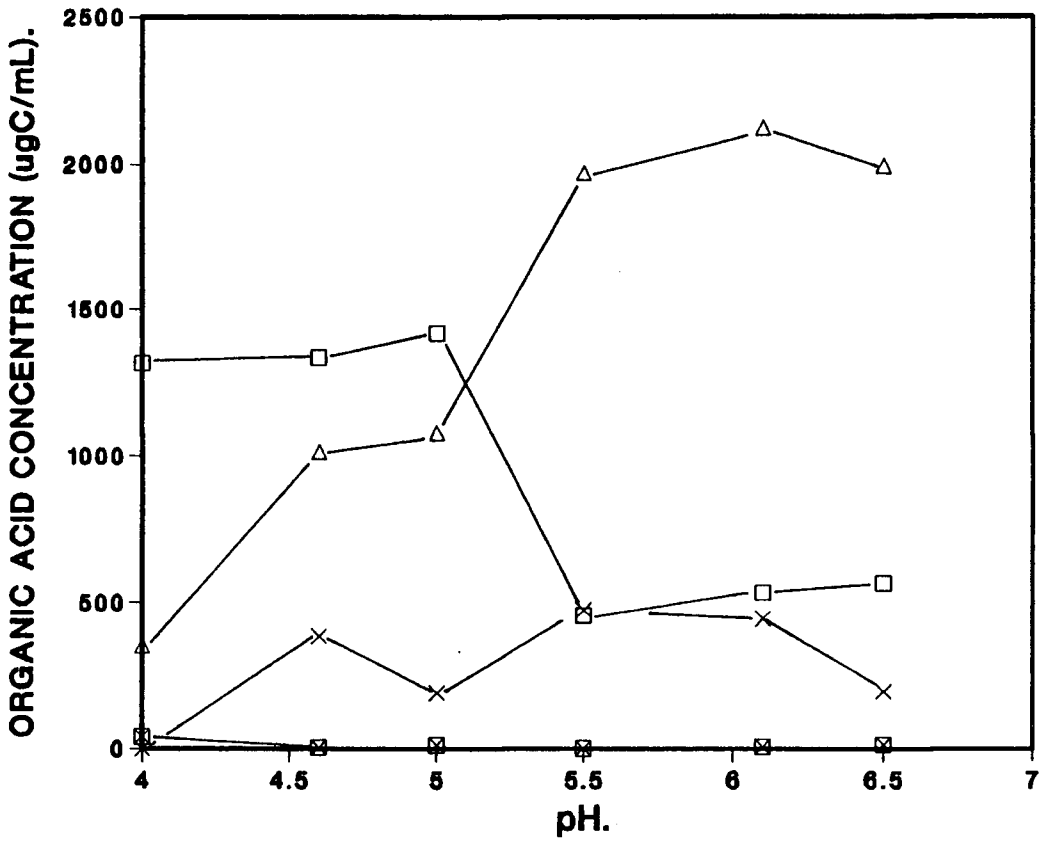


Figure 4.16 Products distribution as a function of pH: (□) butyrate, (Δ) acetate, (x) propionate, (⊠) lactate.
The dilution rate was set at a fixed value of 0.05 h^{-1}.

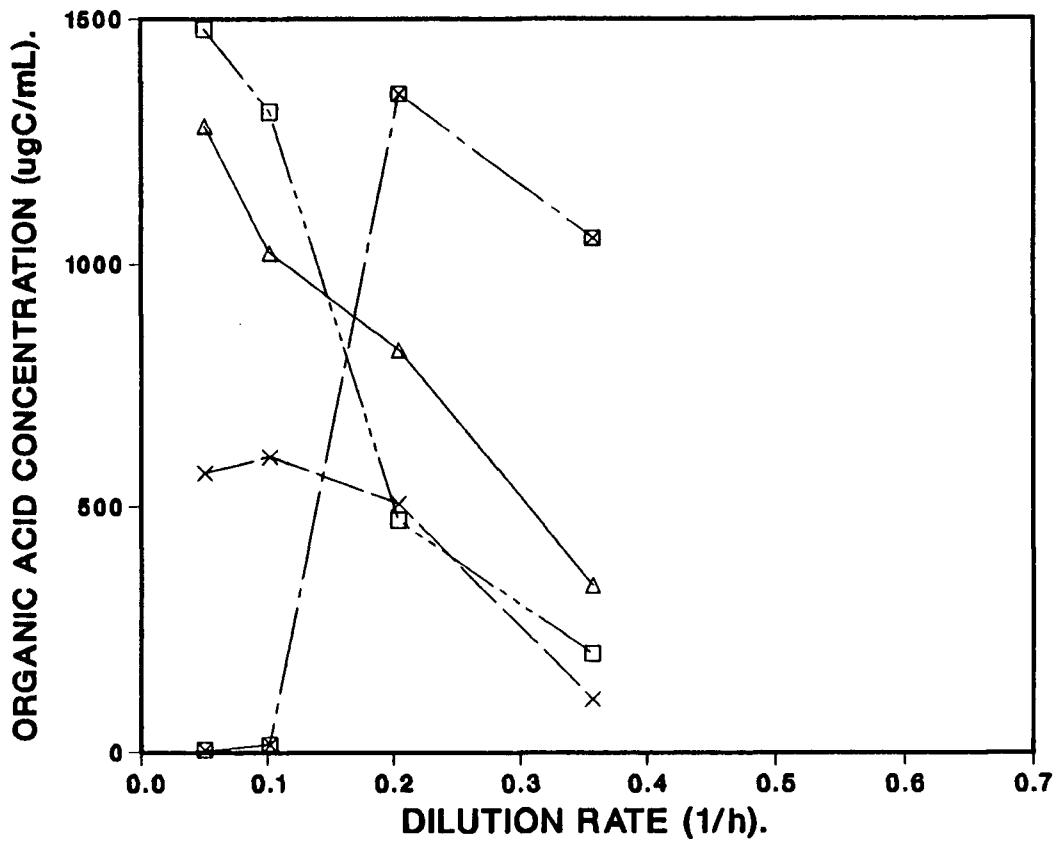


Figure 4.17 Products distribution as a function of dilution rate at a pH of 4.5: (Δ) acetate, (x) propionate, (□) butyrate, (⊠) lactate.

simplicity and inherent physico-chemical meaning. Two experimental examples of continuous culture behaviour from Bailey and Ollis (1986) are shown in Figure 4.18 and 4.19. The data in Figure 4.18 is consistent with the original Monod chemostat model. That is, the observed cell mass and substrate concentrations remain approximately constant over a wider range of conditions than in Figure 4.19. The trend shown in Figure 4.19 which is contrary to the original Monod Chemostat model was first explained by Herbert (1958a & b) who introduced the concept of "endogenous metabolism" in a continuous culture. Endogenous metabolism was interpreted to mean the existence of reactions in cells which consume cell substance. Hence he introduced the maintenance coefficient, M in the limiting nutrient mass balance (Equation 2.37) when developing the now widely accepted more general Monod Chemostat model. Since some microbial systems do not exhibit any detectable endogenous metabolism requirements, it was found necessary to determine whether the experimental results in this study indicated any endogenous metabolism requirements before attempts were made to estimate the model parameters.

In Figures 4.20 and 4.21 are shown the dry biomass concentration data for pH 6.0 and pH 4.5 respectively. With reference to Figure 4.20, between D values of 0.0 and 0.1 h^{-1}, evidence of endogenous metabolism is obvious and is emphasized by the dotted line. However between D values of 0.1 and 0.2, there is a collapse in the biomass concentration that picks up suddenly and does not show any signs of washout from there till a dilution rate of 0.65 h^{-1} is reached. It should be pointed out that for all the experiments run at D values higher than .45 h^{-1} and a pH of 6.0, inspection of the

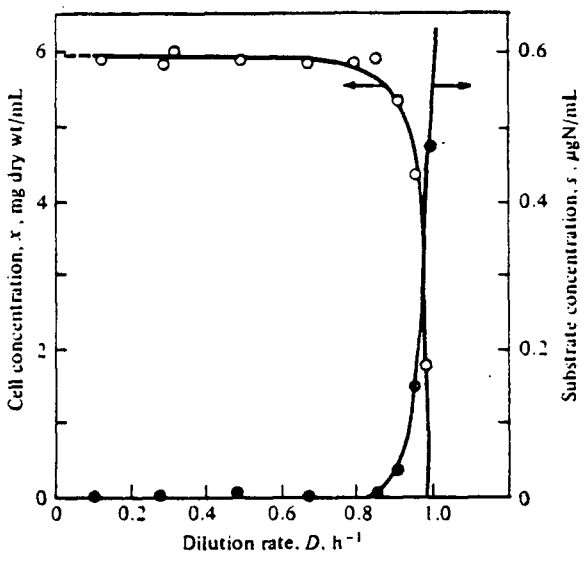


Figure 4.18 Experimental continous culture data qualitatively consistent with the original Monod chemostat model in a pure culture, Aerobacter aerogenes.

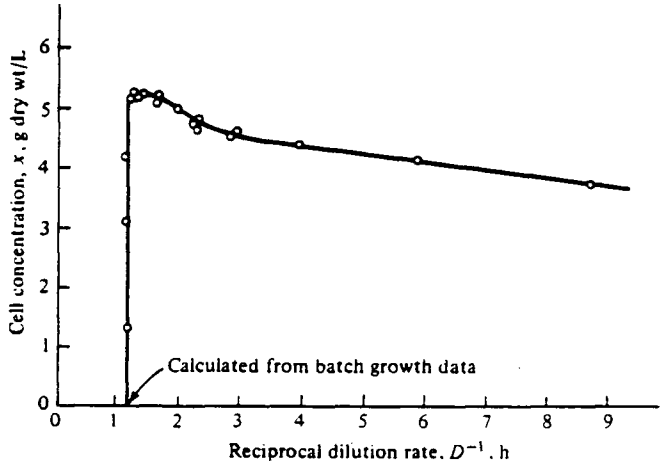


Figure 4.19 Experimental continous culture data with a trend contrary to the original Monod chemostat model in a continous culture of Aerobacter aerogenes in a glycerol medium.

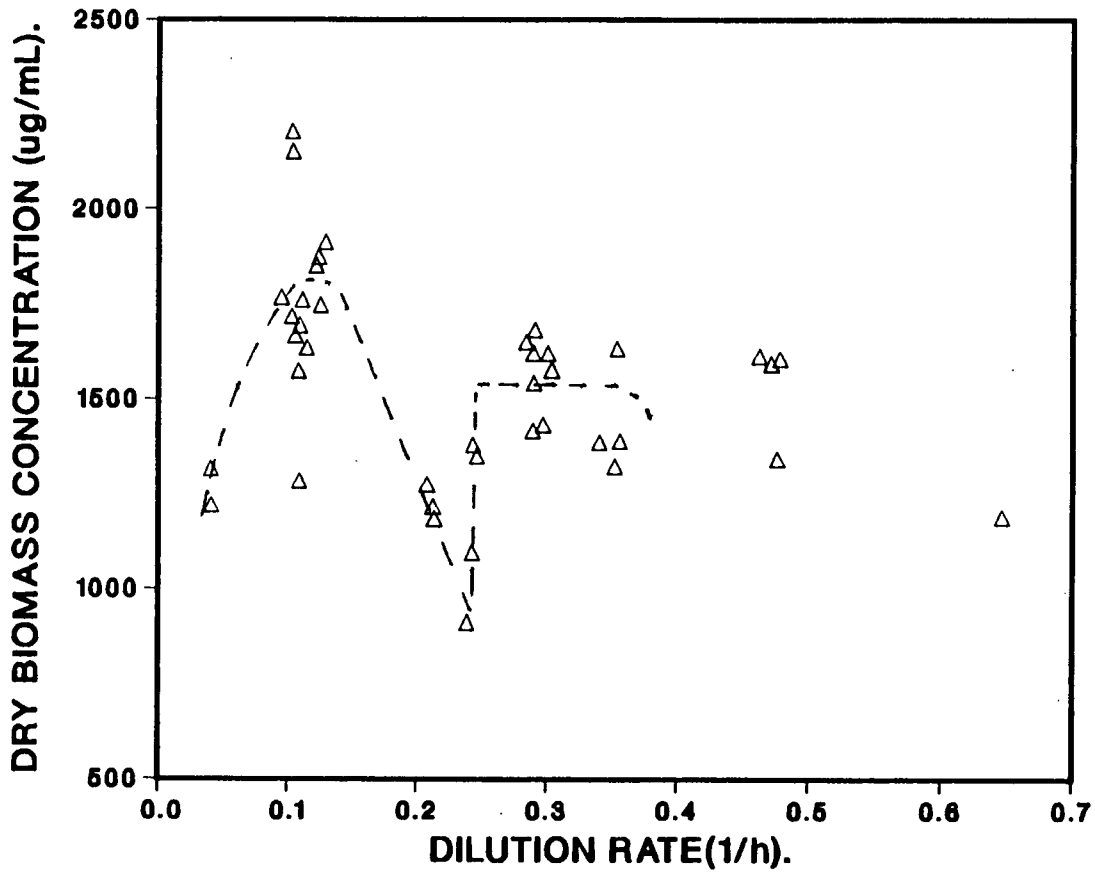


Figure 4.20 Dry biomass concentration at a pH of 6.0.

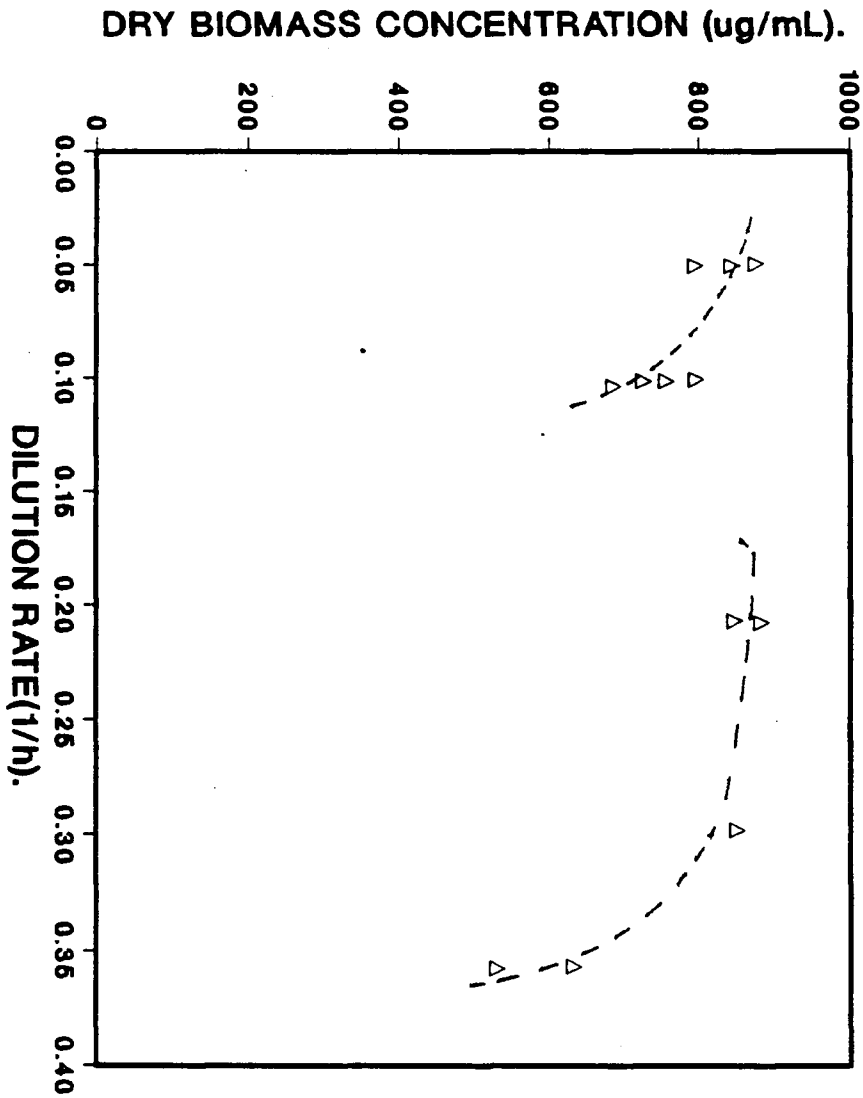


Figure 4.21 Dry biomass concentration at a pH of 4.5.

fermentor vessel at the end of each experiment revealed copious microbial growth adhering to the walls the fermentor vessel (ie. wall growth). So the actual critical dilution rate at which washout should have occurred could not be determined easily. As already pointed out in Chapter II, Rogers et. al. (1978) observed a similar problem with Streptococius cremoris, a lactic acid bacteria, for experiments run at D values above the critical value. The sudden drop in lactate concentration (Figure 4.1) at D = 0.4 h^{-1}, however is evidence of bacterial washout, for during washout concentrations of biomass and other products are known to decrease rapidly if the conditions of complete mixing are met. But the slow biomass decrease like one observed here for D > .4 h^{-1} is characteristic of microbial systems with problems of wall growth (Fiechter, 1984). It is interesting to note that the observed drop in biomass concentration after D = 0.1 h^{-1} coincides with the main OA distribution shift that was considered to be a consequence of microbial population shift. The microbial population shift at a pH of 4.5 is not as obvious as in the previous case (Figure 4.21).

Given that lactate was determined to be the preferred product for the operation of an acidogenic reactor in a two-phase process, the operating D range of interest would have to be above a value of 0.2 h^{-1} (in practice 80 - 90% critical dilution rate value). Therefore the microbial group of interest is that formed after the shift and since, for this group, the initial rise in microbial biomass concentration is sudden (implying a negligible endogenous metabolism requirement), it was decided to drop the M parameter in the Monod chemostat model so equations 2.41 and 2.42 in Chapter II reduce to:

$$S = D\ K_s/(\mu_m - D) \qquad (4.2)$$

$$X = Y(S_o - S) \qquad (4.3)$$

To illustrate the influence of M on the biomass concentration curve, hypothetical parameters in practical ranges were employed to produce Figure 4.22. For curves with shapes similar to curve numbers 4, 5 & 6, M is negligible.

To obtain the parameter estimates in the Monod chemostat model, a subroutine NL2SOL from the University of British Columbia (Moore, 1984) was used. The Fortran program written to define the problem and then call NL2SOL is presented in Appendix D. For the biomass data generated at a pH level of 4.5, the following estimates were obtained:

$$\mu_m = 0.3596 \pm 0.0026\ h^{-1}$$

$$K_s = 8.3258 \pm 3.5216\ \mu g\ C/mL$$

$$Y = 0.2053 \pm 0.0114$$

In Figure 4.23 a good agreement between the model predictions and the experimental data is shown. A high error in K_s is due to the unsteady nature of biomass concentration at or near the critical dilution rate.

Unfortunately, for the biomass data generated at a pH level of 6.0, no sensible solution could be found, because of lack of data of fractional substrate acidogenesis, caused by the wall growth problem, already mentioned. For with wall growth the assumption of complete mixing broke down. However the biomass concentration data is not completely useless. For rudimentary process evaluation purposes, the μ_m may be considered to be

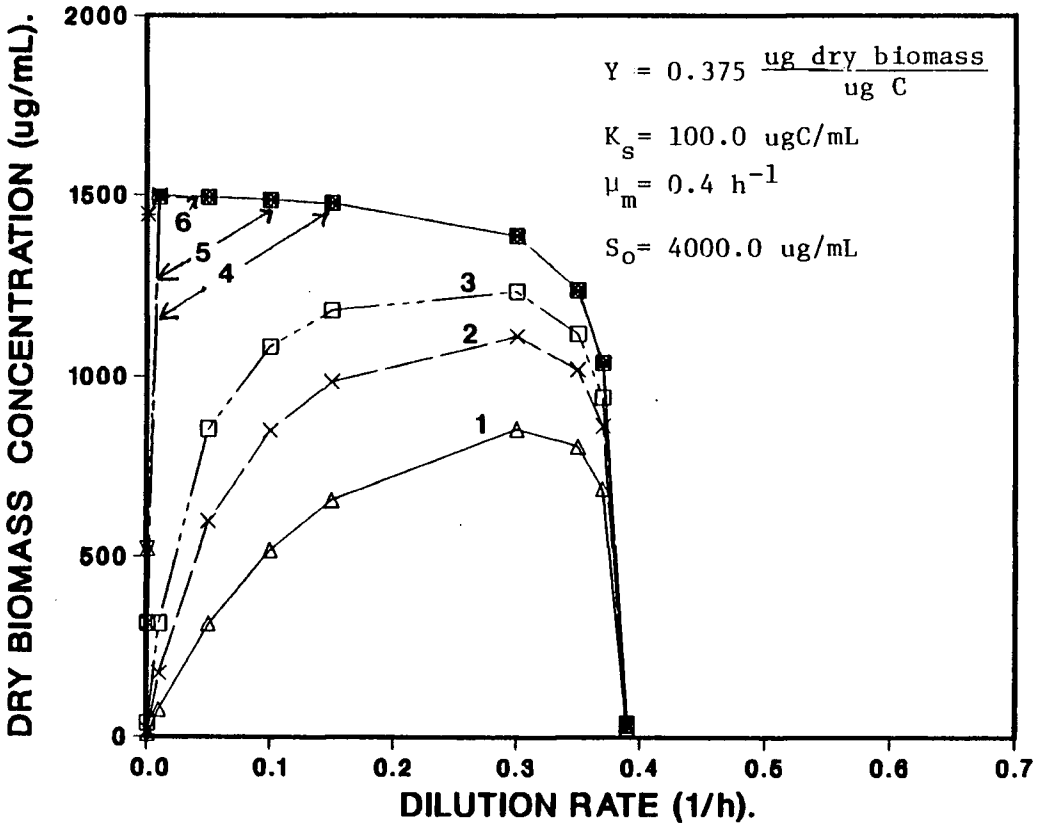


Figure 4.22 Influence of the maintenance coefficient (M) on the shape of the biomass prediction curve: (1) M = 0.5 h^{-1}, (2) M = 0.2 h^{-1}, (3) M = 0.1 h^{-1}, (4) M = 0.01 h^{-1}, (5) M = 0.005 h^{-1}, (6) M = 0.0001 h^{-1}.

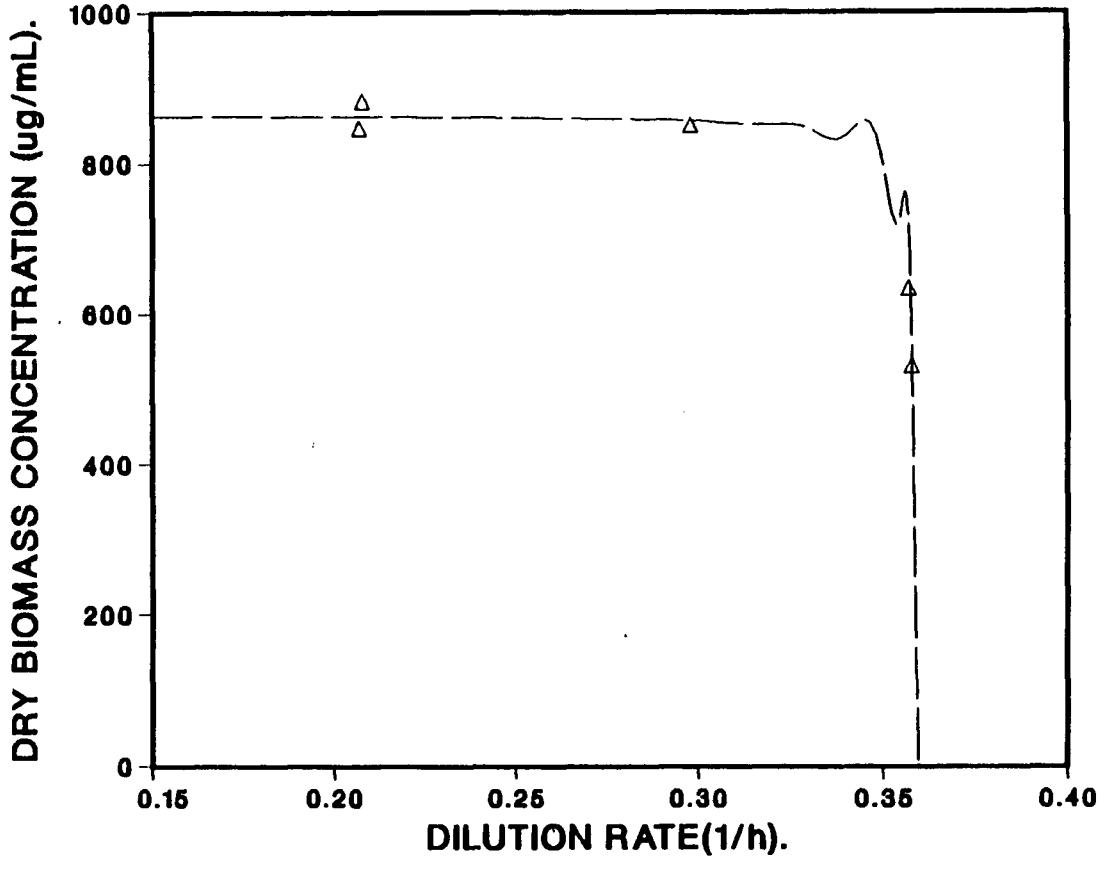


Figure 4.23 Monod chemostat model prediction in comparison with the experimental data for a pH of 4.5: (--) model. (Δ) experimental data.

equal to .41 h^{-1} (bacterial washout commenced at 0 = .4 h^{-1}, based on the variation of lactate with respect to D-Figure 4.1C). Also in the D range of interest, an average dry biomass concentration of 1500 μg/mL, gives a yield coefficient M, of .3561 (S_o = 4212.5 μg C/mL). After fixing μ_m and Y, model predictions at various practical values of K_s were performed and the results are shown in Table 4.6. For K_s values above 3.0 μg C/mL at the critical dilution rate (D = μ_m), the model predicts a negative biomass concentration. At K_s = 2.0 μg C/mL and below, the model predicts a positive biomass concentration. So it can be concluded that an estimate of a K_s value between 2.0 and 3.0 μg C/mL is reasonable. A more accurate determination of K_s is not justifiable given that both Y and μ_m values were approximations. Comparison of microbial growth constants to those previously published (Table 4.7) revealed that the growth of an undefined mixed microbial population predominantly producing lactate on lactose was faster than on glucose. This is shown by the relatively high valve of μ_m. However the μ_m values obtained in this study are not unreasonable, given that they are lower than the μ_m value reported for a pure lactate culture by Rogers et. al. (1978). The μ_m value reported by Ghosh and Poland (1974) is unreasonably high. The high μ_m and extremely low K_s values suggest that probably there were wall growth problems, for the microbial mass curve form predicted by the Monod Chemostat model with the parameters of Ghosh and Poland (1974) is characteristic of those with wall growth. In order to compare K_s values generated in this study to others a conversion factor of 32/12 was used to put them on the same basis as the others. It is clear that the values from this study are an order of magnitude higher. The only explanation that can be offered at this time is that this may be

Table 4.6 Monod Chemostat model predictions for different K_s values based on μ_m and Y approximations for a pH of 6.0

$D(h^{-1})$	Predicated dry biomass concentration				
	K_s(µg C/mL)				
	1.8	2.0	3.0	5.0	10.0
0.2400	1499.1	1499.1	1498.6	1497.6	1495.0
0.2600	1499.0	1498.8	1498.2	1497.0	1493.9
0.2800	1498.7	1498.5	1497.8	1496.2	1492.4
0.3000	1498.3	1498.1	1497.2	1495.2	1490.4
0.3200	1497.8	1497.5	1496.3	1493.7	1487.4
0.3400	1497.0	1496.6	1494.9	1491.4	1482.8
0.3600	1495.5	1494.9	1492.4	1487.3	1474.4
0.3900	1487.6	1486.2	1479.2	1465.4	1430.6
0.4000	1474.4	1471.6	1457.3	1428.9	1357.6
0.4040	1456.9	1452.1	1428.1	1380.2	1260.3
0.4060	1435.0	1427.8	1391.6	1319.4	1138.6
0.4080	1369.3	1357.8	1282.1	1136.8	773.6
0.4090	1237.9	1208.8	1063.1	771.8	43.6
0.4095	975.1	916.8	625.1	41.8	N
0.4098	186.7	40.8	N[1]	N	N

[1]Negative biomass concentration.

Table 4.7 Monod Chemostat Model constants for acidogenesis

Maximum Specific Growth rate	pH	Yield Coefficient		Monod Saturation Constant		Temperature (°C)	Substrate/culture	References
$\mu_m(h^{-1})$		Y	Basis	K_s	Basis			
0.36	4.5	0.21	kg/kg C	22.2	kg COD/m^3	35	lactose/ mixed & undefined	This study
0.41	6.0	0.36	kg/kg C	5.3-8.0	kg COD/m^3	35	lactose/ mixed & undefined	This study
0.33	6.0	0.13	NR[1]	NR		30	glucose/ mixed & undefined	Zeotemeyer et. al. (1982)
0.30	NR	0.14	kg VSS/kg COD	0.37	kg COD/m^3	35	glucose/ mixed & undefined	Ghosh and Klass (1978)
1.25	NR	0.17	kg VSS/kg COD	0.023	kg COD/m^3	37	glucose/ mixed & undefined	Ghosh and Pohland (1974)
0.56	6.0	NA[2]		NA		30	lactose/ S. cremoris	Rogers et. al. (1978)

[1]Not reported.
[2]Not appreciable.

characteristic of the bacterial population that predominated as a consequence of the substrate being lactose.

Lastly a word about the influence of the nature of the carbon source chemical bond is called for. Zeotemeyer et. al. (1982b) pointed out that the hydrolysis of α - 1, 4-glycosidic bonds (as in starch and sucrose) is faster than that of β-1, 4-glycosidic bond substrates (as in cellulose). Since lactose is a disaccharide of galactose and glucose linked by a β-1, 4 linkage, the acidogenesis of lactose was predictably expected to be slower than that of glucose. The higher μ_m values reported in this study suggest that the overall acidogenesis of lactose is faster than that of glucose. Again the only plausible explanation for this at the present time is the type of microbial population that predominates the acidogenic process induced by the presence of lactose as opposed to the one that would predominate in a process in the presence of glucose.

V. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Varying the dilution rate (D) of an acidogenic chemostat or CSTR in the mesophilic temperature range at a pH of 6.0, up to the point of bacterial washout proved to be a very effective tool for separating the microorganisms involved in anaerobiosis into various groups with similar or overlapping specific growth rate ranges. Above a D value or specific growth rate of 0.05 h^{-1}, the methanogens that form methane by the acetate decarboxylation route were successfully eliminated from the ecosystem. Above a D value of 0.15 h^{-1}, the methanogens that form methane via the route of hydrogen reduction were also successfully eliminated from the ecosystem. Above a D value of 0.4 h^{-1}, selective or general microbial washout commenced. At a pH of 4.5, a similar trend was qualitatively reproduced with the acetate decarboxylating and hydrogen reducing methanogens being eliminated above 0.05 and 0.1 h^{-1} respectively. Microbial washout at this low pH commenced at a D value of between 0.3 and 0.35 h^{-1}.

Based on the relative concentrations of the main acidogenic end products (acetate, propionate, butyrate and lactate), produced over the specific dilution rate ranges corresponding to the different microbial groups, two possible hypotheses emerged, namely: (1) The shift in the microbial population in the neighbourhood of a D value of 0.15 and 0.1 h^{-1} for pH levels of 6.0 and 4.5 respectively represented the beginning of pyruvate conversion to lactate; (2) or the microbial shift disabled the conversion of lactate to other intermediary metabolites. Batch incubation of [^{14}C(U)]-lactate with the acidogenic chemostat effluent samples and

preparative separation of the predominant organic acids followed by a liquid scintillation assay of the location of the radioactivity, discriminated beyond any doubt between the two rival hypotheses in favour of the latter hypothesis. This finding is believed not only to be valid for lactose fermentation but for all mixed undefined anaerobic fermentations of carbohydrates.

Further use of [^{14}C(U)]-butyrate and [^{14}C(2)]-propionate at a pH of 6.0 and in the mesophilic temperature range revealed the predominant carbon flow routes. It was assumed that lactose is broken down to pyruvate via the Embden-Meyerhof-Parnas pathway. So the flow routes of concern were from pyruvate to the various observed organic acids. It was found that the flow of carbon from pyruvate to butyrate and lactate was parallel. Also it was found, that in the presence of hydrogen reducing methanogens, lactate was almost completely converted to acetate and not propionate. Butyrate was found to be converted to acetate at a slow rate as long as hydrogen reducing methanogens were present. Further conversion of propionate was not possible under all D ranges considered. This together with the relatively low propionate concentrations observed suggested that the role played by propionibacteria in this lactose acidogenic ecosystem was minor. This knowledge together with the knowledge of the microbial interactions in relation to D, were used to propose a qualitative lactose fermentation model. In this model lactose is converted to pyruvate via the Embden-Meyerhof-Parnas pathway. Pyruvate in a parallel reaction is converted to lactate and butyrate. In the presence of hydrogen reducing methanogens, lactate is converted in a very fast reaction to acetate. Also butyrate is converted to acetate but at a much slower rate. In the light of

this model it was concluded that lactate is the most suitable marker for optimising an acidogenic reactor in a two-phase biomethanation process. For that reason only biomass concentration data in the D range where the production of lactate predominated were used to model microbial growth. The Monod chemostat model was favoured among various alternatives because of its simplicity and the physico-chemical basis. The maintenance coefficient was not included in the model as it was found to be negligible by inspection. At a pH of 4.5 the model parameter estimates were found to be: μ_m = 0.3596 ± 0.0026 h^{-1}; K_s = 8.3258 ± 3.5216 μg C/mL; and Y = 0.2053 ± 0.0114 μg dry biomass/μg C. The large error in K_s was attributed to the difficulty encountered in maintaining a steady biomass concentration as D approached the critical value at which complete bacterial washout occurs. At a pH of 6.0, wall growth was observed at D values above 0.4 h^{-1} and therefore a decrease in biomass as D approached the critical value was not observed. The presence of wall growth invalidated the assumption of complete mixing on which the Monod chemostat model is based. However the critical value of D could be estimated based on the observed sudden drop in the major organic acid concentrations.

Degradation of protein (mainly β-lactoglobulin) together with lactose did not in anyway affect the carbon flow scheme. In the D range of 0.05 to 0.15 h^{-1} low pH (pH < 5.0) was found to favour the butyrate route at the expense of the lactate route and at high pH (pH > 5.5) the lactate route was favoured at the expense of the butyrate pathway, the pH region of 5.0 to 5.5 being the transition range.

5.2 Recommendations

Recommendations for future studies include the following: (1) The problem of erratic gas production needs to be studied further so that the proposed lactose fermentation model can be made more complete. A 2x2x2 experimental protocol (pH = 4.5, 6.0; D = 0.1, 0.3 h^{-1}; start-up procedure = promote gas production, discourage gas production) is hereby proposed. For each of the eight experiments, samples should be withdrawn and incubated with ^{14}C-labeled sodium bicarbonate. Then using the procedures described in this study to assay for the location of the radioactivity among the major organic acids one could confirm or dispell the proposed fate of H_2/CO_2 precisely. (2) Lactate has been proposed as the most desirable acidogenic end product. It can be predicted from the results of this study that in the methanogenic serial reactor of the two-phase process, lactate would be converted to acetate. However, this prediction needs to be experimentally verified together with the methanogenic microbial growth model, since it is necessary for process design. (3) It is also recommended that, microbial population enumeration, characterisation and isolation of the prevalent species for the two D ranges of interest ($.05 < D < 0.15$ and $0.15 < D < 0.4$) should be attempted. Then by studying the behaviour of the isolates in continuous culture for the influence they have on each other a robust mixed and defined culture for a two-phase process could be developed. (4) Finally the economics of the two-phase fermentation process for whey and deproteinated whey (whey permeate) should be established.

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APPENDIX A

ANALYTICAL PROCEDURES

A1 Determination of Lactose

Lactose was assayed by a colorimetric method as total carbohyrates. The method could not discriminate between lactose and its monomers, glucose and galactose.

A1.1 Principle

This procedure sometimes referred to as the phenol-sulfuric acid method was first proposed by Doubis et. al. (1956). Simple sugars, oligosaccharides, polysaccharides and their derivertives including the methyl esters with free or potentially free reducing groups, give an orange-yellow colour when treated with phenol and concentrated sulfuric acid. The reaction is sensitive and the color is stable.

A1.2 Reagents and Chemicals

A. To ten mL reagent grade phenol (MCB), 180 mL of distilled water were added.

B. Concentrated sulfuric acid (BDH).

A1.3 Procedure

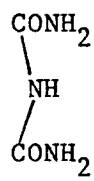
One mL of centrifuged (1 h, 4450 x g) sample, diluted such that it contained between 10 - 70 μg of lactose, was pipetted into a colorimeter tube. One mL of reagent A was added and five mL of concentrated sulfuric acid were added rapidly. The tubes were allowed to stand for ten minutes in air, shaken and placed for ten to twenty minutes in a water bath at twenty to thirty degrees centigrade, before readings were taken at 480 nm using a spectrophotometer, Spectronic 70 (BAUSH & LOMB). Blanks were prepared by

substituting distilled water for the sample. A typical calibration curve is shown in Figure A1.

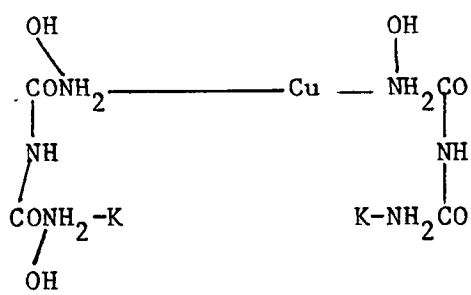
A.2 Determination of Whey Protein

A2.1 Principle

Protein reacts with cupric ion in an alkaline sodium potassium tartrate solution to form a complex colored compound. Any compound that has in its molecular structure pairs of carbamyl groups linked through nitrogen or carbon (or peptide linkages) will show a positive biurent reaction. The name of the test is derived from the simplest of such compounds, biuret.



When biuret is treated with an alkaline potassium copper tartrate solution, two biuret molecules are joined to form the following complex violet-colored compound.



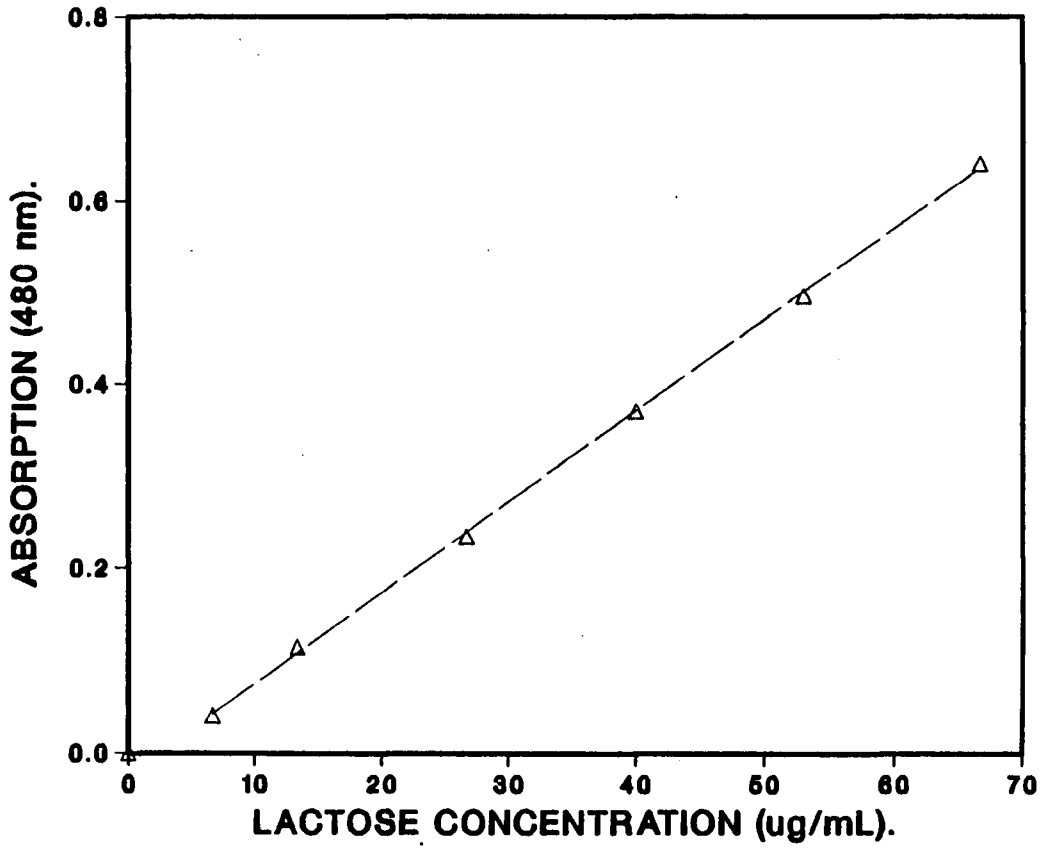


Figure A1 Calibration Curve for Lactose Using the Phenol-Sulfuric Acid Method.

Since proteins contain these linkages, they react to give the characteristic color which is directly proportional to the amount of protein present.

A2.2 Reagents and Chemicals

Biuret reagent (BDH).

A2.3 Procedure

One mL of filtered (.45 μm MILLIPORE membrane) sample was pipetted into a colorimeter tube. Four mL of biuret reagent were added. The solution was throughly mixed and incubated at room temperature for 30 minutes. The absorbance was then determined at a wavelength of 540 nm. Blanks were prepared by substituting distilled water for the sample. A typical calibration curve is shown in Figure A2.

A3 Determination of Formate

A3.1 Principle

This procedure was first described by Lang and Lang (1972). Bright yellow and green-yellow fluorescent reaction products from the formate-citric acid reaction, change to raspberry red in the same medium, at room temperature. The intensity of the color is proportional to the concentration of formate and/or formic acid. It's main light absorption band is at 515 nm.

A3.2 Reagents and Chemicals

A. 0.5 g citric acid and ten g acetamide were dissolved in 100 mL isopropanol.

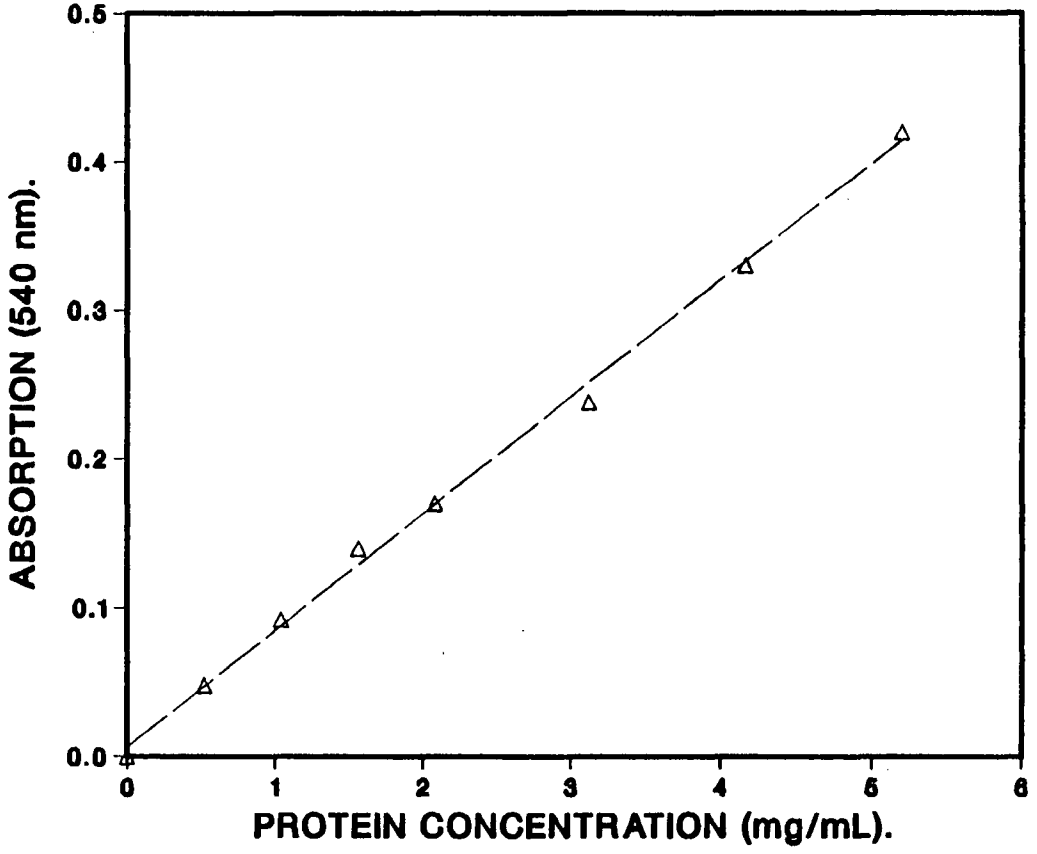


Figure A2 Calibration Curve for Protein Using the Biuret-Reaction Method.

B. A sodium acetate solution, aqueous, 30 g in 100 mL was made up.

C. Aceticanhydride.

A3.3 Procedure

0.5 mL of centrifuged (1 h, 4450 x g) sample, diluted such that it contained less than 200 mg/mL formate was pipetted into a colorimeter tube. 50 µL reagent B to which 3.5 mL aceticanhydride had been added and one mL of reagents A were added to the tube. The mixture was shaken well and incubated at 50°C for thirty minutes to speed up the reaction. The absorbance was then taken at 515 nm. Blanks were prepared by substituting distilled water for the sample. A typical calibration curve is shown in Figure A3.

A4 Determination of Lactate

A4.1 Principle

When a very dilute solution of lactic acid is heated in the presence of a high concentration of sulfuric acid it is coverted to acetaldehyde. The acetaldehyde may then be determined by the sensitive color test employing phydroxydiphenyl. The procedure was taken from a paper by Markus (1950). In the modification of this procedure given below, sufficient heat to convert lactate to acetaldehyde is generated by rapid mixing of sulfuric acid and water.

A4.2 Reagents and Chemicals

A. Four g of $CuSO_4.5H_2O$ were dissolved in water and the volume adjusted to 100 mL.

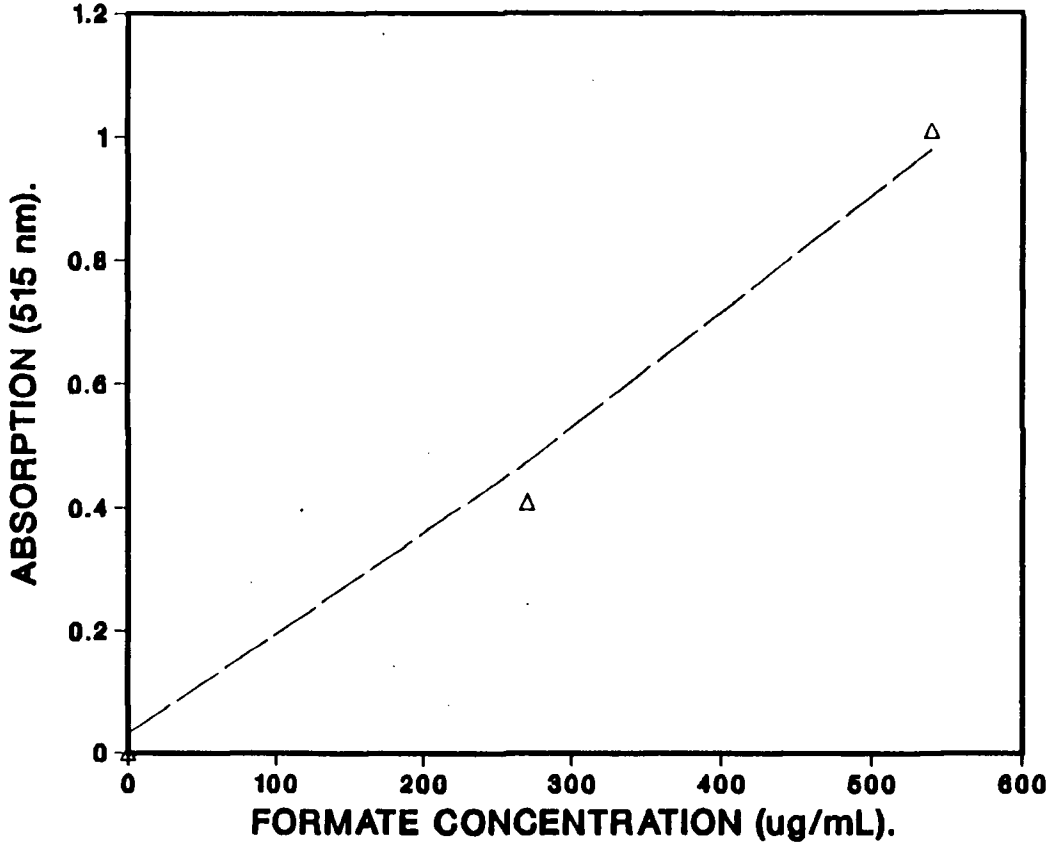


Figure A3 Calibration Curve for Formate Using the Method of Lang and Lang (1972).

B. One g of p-hydroxydiphenyl (SIGMA) was dissolved in 100 mL of 0.08N NaOH and store in a brown bottle in the refrigerator until needed.

C. Concentrated sulfuric acid.

A4.3 Procedure

One mL of centrifuged (1 h, 4450 x g) sample diluted such that it contained less than 10 µg/mL lactate was pipetted into a colorimeter tube. 50 µL of reagent A were added. Six mL of concentrated sulfuric acid was syringed into the tubes, and allowed to stand for five minutes in air, then cooled below 20°C in cold water. 50 µL of reagent B were added without touching the wall of the tube, mixed thoroughly and the tubes allowed to stand for 6 h at room temperature (or overnight). The absorbance was then measured at a wavelength of 570 nm. A blank, prepared by substituting distilled water for the sample, were run with each set of determinations. A typical calibration curve is shown in Figure A4.

A5 Determination of Volatile Fatty Acids (VFA) and Ethanol

VFA concentrations were determined using a gas chromatograph.

A5.1 Apparatus

A. Analytical gas chromatography (GC) - Model 311 (CARLE), equipped with both a Thermal Conductivity Detector (TCD) and a Flame Ionizatin Detector (FID).

B. Gas cylinders of Air, H_2 and Helium (UNION CARBIDE).

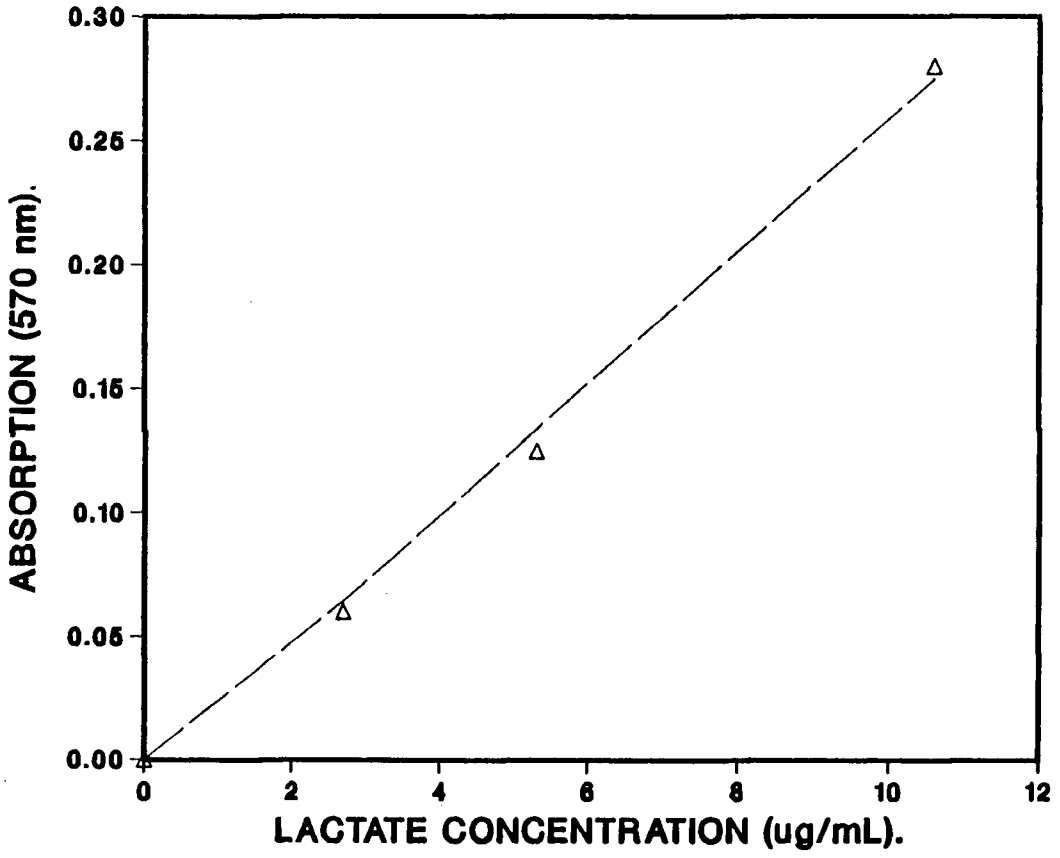


Figure A4 Calibration Curve for Lactate Using a Modified Method of Markus (1950).

C. A 60/80 Carbopack C/0.3 Carbowax 20 M/0.1% H_2PO_4 column, 30" xc 1/8" stainless steel (SUPELCO).

D. A computing integrator - SP4100 (SPECTRA-PHYSICS).

A5.2 Sample Preparation

The VFA can only be detected in free form. It was therefore necessary to acidify the samples. In general:

$$HA \quad H^+ + A^- \qquad \text{(A1)}$$

(free acid)

At equilibrium,

$$K_a = [A^-]\,[H^+]/[HA] \qquad \text{(A2)}$$

where $[A^-]$, $[H^+]$ and [HA] are the molar concentrations of the anion, cation and acid respectively.

If,

$$pK_a = -\log_{10} K_a \qquad \text{(A3)}$$

and

$$pH = -\log_{10}[H^+] \qquad \text{(A4)}$$

then

$$[A^-]/[HA] = 10^{-(pK_a - pH)} \qquad \text{(A5)}$$

For most of the acids (>99%) to be in free form:

$$[A^-]/[HA] < 0.01 \qquad \text{(A6)}$$

or

$$10^{-(pK_a - pH)} < 10^{-2} \qquad (A7)$$

Thus

$$pK_a - pH > 2 \qquad (A8)$$

Since the lowest pK_a, of all the VFA's is that of acetic acid which equals 4.76 (Lehninger, 1970), the pH of the sample had to be less than 2.73. The low pH was achieved by adding a combination of formic acid and phosphoric acids. The samples were centrifuged (1 h, 4450 x g) before adjusting pH.

A5.2 Procedure

Once the GC had been installed, the Helium, Air and H_2 regulators were set at 12, 20 and 23 psig respectively. The oven was set at 120°C.

The system was allowed to stabilise, after which the FID was turned on and 5-10 minutes was allowed for baseline stabilization.

One μL sample was injected and the run started. The run was stopped after 9 minutes and the integrated chromatogram was obtained from the computing integrator. Each sample was analysed at least four times and the mean values reported. A typical chromatogram and some of the standard calibration curves are shown in Figures A5 and A6 respectively.

A6 Gas Volume and Composition Determination

A6.1 Gas Volume

Gas volumes produced over a known period of time were measured by wet gas metres (ALEXANDER - 0.25 L per revolution). The total volume of gas

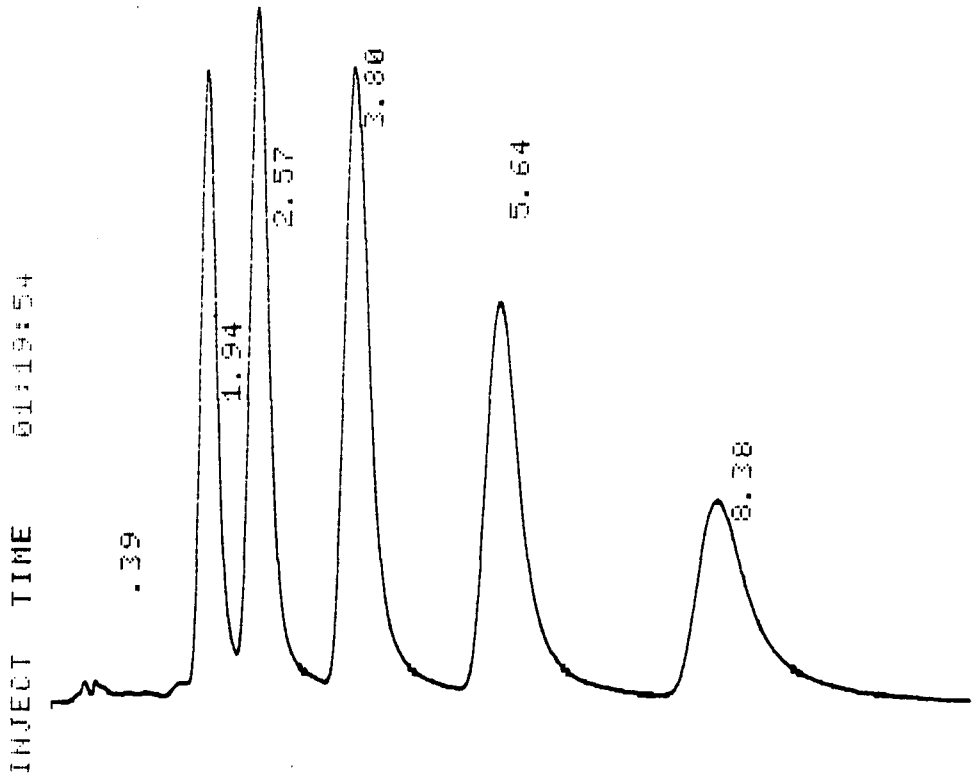


Figure A5 A typical volatile fatty acid chromatogram: (Retention Time (RT) = .39 min) ethanol, (RT - 1.94) acetate, (RT = 2.57) propionate, (RT = 3.80) butyrate, (RT 5.64) valerate, (RT = 8.39) caproate.

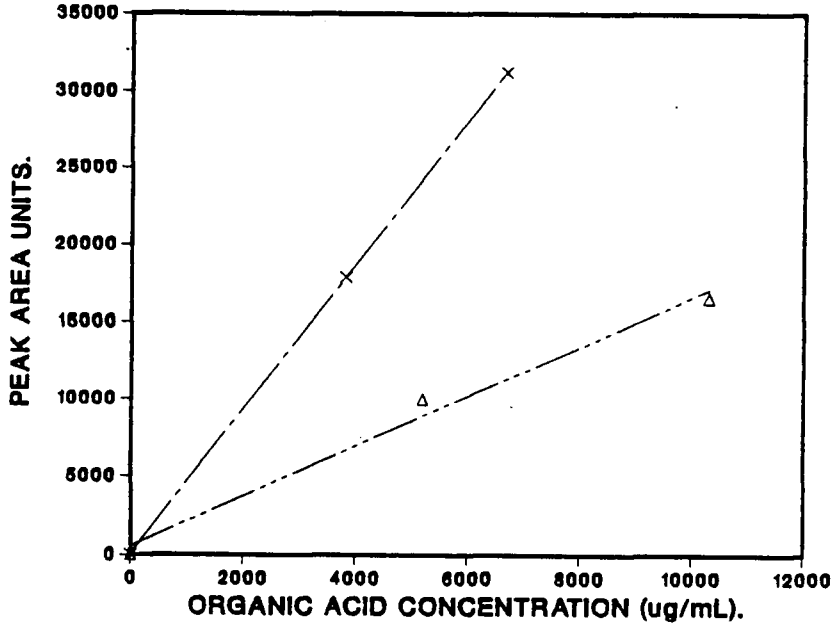


Figure A6 Some volatile fatty acids typical calibration curves: (x) butyrate (Δ) acetate.

recorded (V_{rec}) was larger than the actual volume of gas produced (V_{act}) due to moisture. V_{act} was calculated from the equation:

$$V_{act} = (p_{atm} - p^*) V_{rec}/p_{atm} \qquad (A9)$$

where p^* is the vapour pressure of water at 25°C and one atmosphere (p^* = 23.76 mm Hg) and p_{atm} is the atmospheric pressure.

A6.2 Gas Composition

The GC described in section A5 was used for gas analysis. A 10% carbowax 20 M on chromosorb W-HF 80/100 mesh column, 8' x 1/8" stainless steel (CHROMATOGRAPHIC SPECIALTIES) with Helium (12 psig) as carrier gas was used for the gas separation (N_2; CH_4 and CO_2). Detection was by a thermal conductivity detector (TCD). For the measurement of H_2 the same column and TCD were used with N_2 (15 psig) as the carrier gas. Helium could not be used for H_2 because the thermal conductivities of both gases are very close resulting in a very poor TCD sensitivity.

Once the carrier gas flow rate and oven temperature (35°C) were set, the TCD was turned on and 30 - 45 minutes were allowed for baseline stabilization. A 500 µL sample was taken directly from the fermentor head space using a gas tight syringe and injected into the GC. In figure A7 and A8 are shown typical chromatograms and calibration curves.

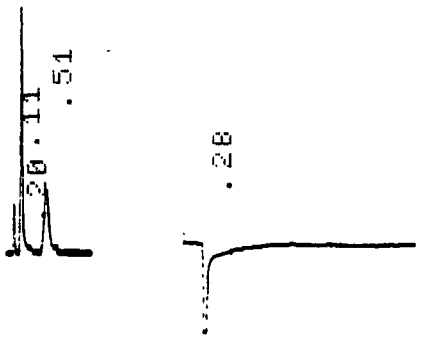


Figure A7 Typical fermentor head space gas chromatograms: (Retention Time = .11 min) nitrogen, (RT = .20) methane, (RT = .51) carbon dioxide, (RT = .28) hydrogen.

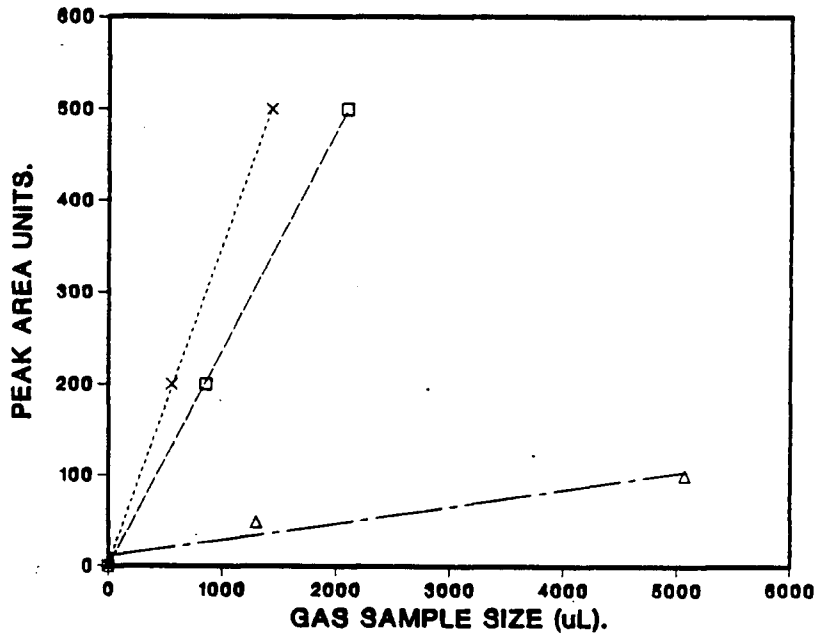


Figure A8 Fementor head space gas calibration curves: (x) carbon dioxide, (□) methane, (Δ) hydrogen.

A7 Determination of Dry Biomass and Biomass Carbon

Dry biomass was determined by gravimetric means. Biomass and substrate carbon were determined by a total carbon analyser. A schematic diagram of a the carbon analyser used in this study is shown in Figure A9.

A7.1 Principle

By examining the spectra of a pure substance a wave length may be found at which absorption is considerably greater than for other compounds present in the mixture. For analysis it is simply necessary to measure absorbance at the selected wavelength. Use is made of this principle in the total carbon analyser. A liquid sample is injected into the reactor with a strong oxidizing agent like sodium persulfate. All carbon containing materials are oxidized to carbon dioxide. The carbon dioxide is carried by a stream of oxygen through an infrared analyser which is specifically designed to measure and record the concentration of carbon dioxide present. Total carbon is measured by this procedure, but if the sample is first prepared by acidification and aeration to remove all the inorganic carbon, then a selective measure of the organic carbon present may be obtained. For more details the reader is referred to Sawyer and McCarty (1978).

A7.2 Apparatus and Reagents

A. Total carbon analyser (ASTRO).

B. 238 g of ultra-pure reagent grade sodium persulfate (ASTRO) were dissolved in distilled water to make one litre of solution.

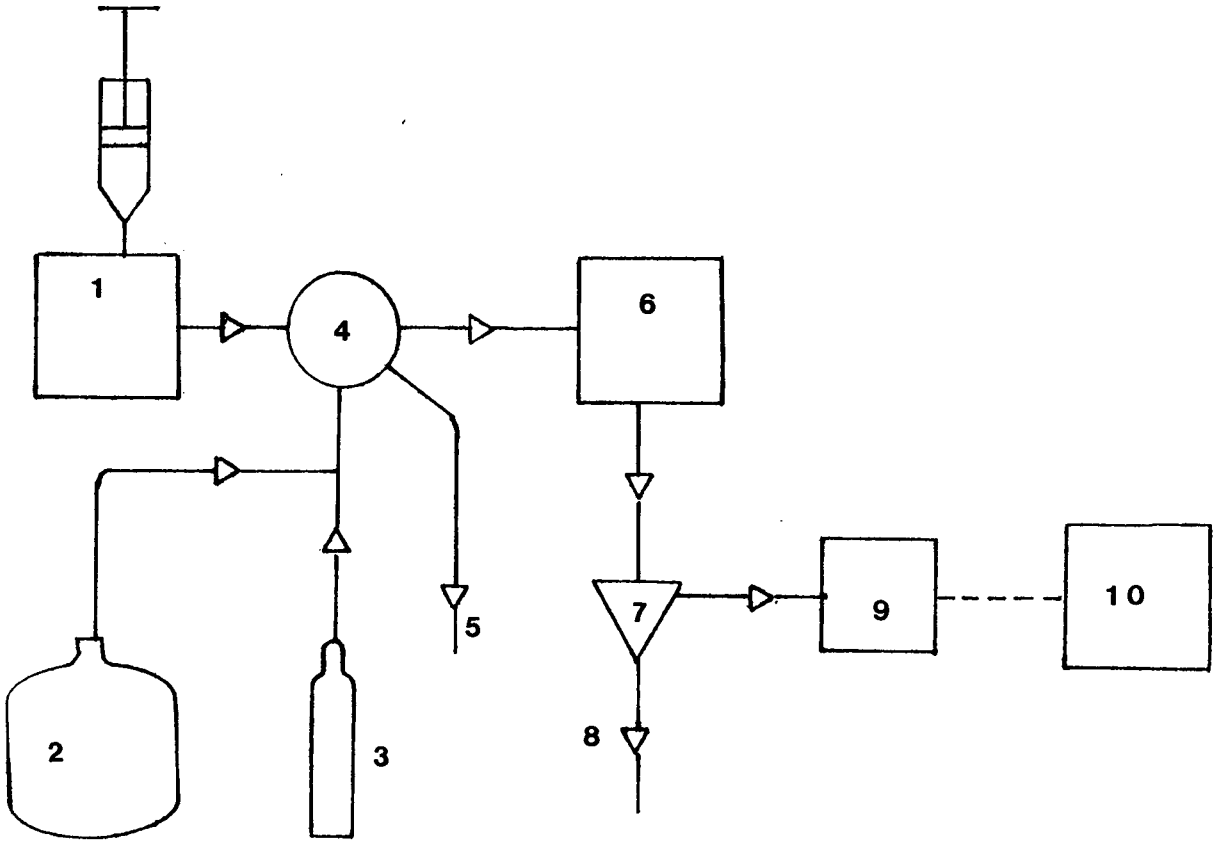


Figure A9 Schematic diagram of the carbon analyser: (1) injection port, (2) persulfate reagent, (3) oxygen supply cylinder, (4) auto injection valve, (5) sample overflow drain, (6) reactor, (7) gas/liquid separation, (8) liquid drain, (9) CO_2 detector, (10) signal processor/printer.

A7.3 Procedure

The total carbon analyser was operated in the total carbon (TC) mode. After following the startup instructions, a syringe with 20 mL of sample direct from the fermenter was loaded into the injection port, leaving the syringe connected to this port. The onboard microprocessor was turned on which assumed the analysis task including the injection of the proper sample volume at the appropriate time and printing out of the sample analysis results. A similar procedure was repeated for a sample from the fermentor, which had first been filtered through a 0.45 um MILLIPORE membrane. The biomass carbon was assumed to be the difference in carbon concentration between the filtered and unfiltered samples. The biomass that remained on the filter was washed, dried overnight at 105 ± 1°C and weighed.

APPENDIX B

TABULATED RESULTS

TABLE B1 - TOTAL CARBON MASS BALANCES (LACTOSE GROWTH LIMITED SUBSTRATE) AT pH = 6.0 AND TEMPERATURE=35°C

RUN #	EXPERIMENTAL FACTORS			SOLUTE PRODUCTS CARBON (ugC/mL)										GASEOUS PRODUCTS CARBON (ugC/mL)				
	TEMP (°C)	pH	DIL'N RATE (h^{-1})	ETHANOL	ACETATE	PROPIONATE	i-BUTYRATE	n-BUTYRATE	i-VALERATE	n-VALERATE	CAPROATE	LACTATE	FORMATE	CARBON DIOXIDE	METHANE	BIOMASS CARBON (ugC/mL)	CARBON RECOVERY (ugC/mL)	CARBON RECOVERY (% OF INFLUENT CARBON)
B1	35.0	6.05	0.0405[1]	197.8	2430.5	478.4	27.9	268.8	T[2]	38.5	ND	NA[4]	NA	ND	ND	NA	3905.3	92.7[5]
	34.5	6.05	0.0408	266.1	2125.0	446.0	71.6	534.1	T	105.1	ND	5.6	ND	ND	ND	496	4049.5	96.1
	34.5	6.08	0.0420	322.5	2002.0	310.8	58.8	496.8	ND[3]	86.5	ND	NA	NA	ND	ND	NA	3741.0	88.8
	34.5	6.10	0.0401	221.4	2230.0	290.6	21.3	368.6	26.9	50.1	ND	NA	NA	ND	ND	420	3634.5	86.3
B2a	34.5	6.00	0.1035	ND	2390.0	340.6	ND	281.9	ND	33.9	ND	NA	NA	6.9	3.6	948	4005.3	95.1
	ND	6.00	0.1091	ND	2325.0	261.9	ND	386.3	ND	41.0	142.9	NA	NA	24.6	12.9	867	4061.6	96.4
	ND	6.00	0.1029	ND	2105.0	166.3	ND	420.4	ND	T	253.3	NA	NA	78.8	41.5	934	3999.3	94.9
	ND	6.00	0.1105	ND	1861.3	156.5	ND	480.6	ND	T	215.0	T	ND	104.4	55.0	NA	3788.8	89.9
B2b	36.5	6.00	0.1108	ND	2533.0	152.4	T	261.3	ND	ND	ND	NA	NA	24.9	59.1	1139	4169.7	99.0
	36.0	6.00	0.1080	ND	2536.1	199.0	T	379.5	ND	ND	ND	NA	NA	42.4	100.6	1634	4791.2	113.7
	36.5	6.00	0.1097	ND	2396.3	194.9	T	382.3	ND	ND	ND	NA	NA	29.2	69.1	NA	4111.0	97.6
	36.0	6.00	0.1088	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	30.0	71.2	939.4	-	-
B2c	34.0	5.95	0.1287	T	1839.5	235.0	ND	100.3	ND	T	T	ND	NA	94.8	163.2	662	3894.8	92.5
	34.5	6.00	0.1249	T	1178.3	139.0	ND	1262.5	ND	T	176.0	NA	NA	271.6	181.1	603	3811.5	90.5
	34.5	6.00	0.1216	T	1370.0	136.9	ND	1006.9	ND	T	240.3	ND	NA	220.1	146.8	550	3671.0	87.1
	34.0	6.00	0.1233	ND	2232.3	181.6	T	961.8	ND	T	131.6	NA	NA	119.5	79.7	481	4187.4	99.4
B2d	35.0	5.90	0.1145	T	2181.9	213.1	ND	362.4	NA	NA	NA	4.4	NA	116.2	205.3	664.5	3747.8	89.0
	34.0	6.00	0.1054	T	2239.3	220.9	ND	391.5	NA	NA	NA	T	NA	91.8	162.1	636.0	3741.3	88.8
B2e	35.0	6.10	0.1026	T	2154.4	211.9	ND	755.6	NA	NA	NA	2.5	NA	171.4	264.4	769.2	4329.6	102.8
	35.0	6.00	0.0947	T	1858.6	288.5	ND	693.8	NA	NA	NA	T	NA	120.9	185.2	872.5	4019.5	95.4
B3b	35.0	6.00	0.2386	135.7	879.4	235.7	ND	695.7	167.7	T	T	NA	NA	NA	NA	798.8	3879.1	92.1
	35.5	6.00	0.2423	130.3	923.5	252.4	ND	876.0	107.9	27.4	T	NA	NA	NA	NA	901.2	4320.5	102.6
	35.5	6.00	0.2459	150.1	1019.3	260.3	ND	939.3	T	T	ND	1101.8	NA	NA	NA	490.4	3961.2	94.0
	35.5	6.00	0.2430	153.4	1008.3	295.3	ND	929.2	T	T	ND	NA	NA	NA	NA	842.8	4177.4	99.2

1. V = 1470 mL; 2. Trace amounts; 3. Not detected; 4. Not analysed; 5. Average influent carbon; S_o = 4212.5 ugC/mL

CON'T TABLE B1 - TOTAL CARBON MASS BALANCES (LACTOSE GROWTH LIMITED SUBSTRATE) AT pH = 6.0 AND TEMPERATURE=35°C

RUN #	EXPERIMENTAL FACTORS			SOLUTE PRODUCTS CARBON (ugC/mL)										GASEOUS PRODUCTS CARBON (ugC/mL)				
	TEMP (°C)	pH	DIL'N RATE (h^{-1})	ETHANOL	ACETATE	PROPIONATE	i-BUTYRATE	n-BUTYRATE	i-VALERATE	n-VALERATE	CAPROATE	LACTATE	FORMATE	CARBON DIOXIDE	METHANE	BIOMASS CARBON (ugC/mL)	CARBON RECOVERY (ugC/mL)	CARBON RECOVE (% OF INFLUE CARBON
B3c	35.0	6.00	0.2075	89.2	585.3	684.6	ND	979.6	T	387.2	ND	637.0	NA	40.8	ND	906.6	4310.3	102.3
	35.0	5.90	0.2131	93.9	843.0	663.1	T	1159.6	T	370.0	ND	497.2	NA	26.6	ND	489.3	4142.1	98.3
	34.5	5.95	0.2121	T	1023.3	727.1	T	1742.6	T	294.8	ND	NA	NA	159.9	ND	796.5	4744.2	112.6
B4a	35.0	6.00	0.2896	112.0	1451.5	342.6	ND	1390.1	ND	T	ND	NA	NA	157.9	T	790.7	4297.8	102.0
	35.5	6.05	0.2840	125.6	1314.4	320.0	ND	1218.0	ND	T	ND	NA	NA	121.3	T	932.4	4084.7	97.0
	35.5	6.00	0.3005	90.9	1334.1	277.1	ND	1265.8	ND	T	ND	NA	NA	150.4	T	856.8	4028.4	95.6
	34.5	6.00	0.2891	101.5	1574.3	335.4	ND	1395.9	ND	T	ND	53.0	ND	112.9	T	705.6	4278.6	101.6
B4b	35.0	6.00	0.2907	142.1	1416.7	588.4	ND	542.8	T	36.9	ND	413.5	NA	ND	ND	834.8	3975.2	94.4
	35.0	6.00	0.3035	196.2	1510.8	629.5	ND	362.0	T	39.4	ND	NA	NA	ND	ND	783.1	4250.1	100.9
	35.5	6.00	0.2971	93.2	1190.5	557.3	ND	615.1	T	104.9	ND	807.5	NA	ND	ND	965.2	4333.7	102.9
	35.5	6.00	0.2898	95.4	1158.7	438.0	ND	699.1	T	122.7	ND	966.3	NA	ND	ND	794.0	4274.2	101.5
B5	34.5	6.00	0.3520	T	659.2	223.9	ND	750.0	T	T	ND	NA	NA	86.6	T	590.6	3651.8	86.7
	35.0	6.00	0.3401	NA	NA	NA	ND	NA	NA	NA	ND	NA	NA	79.3	T	667.2	-	-
	34.0	6.00	0.3537	T	692.1	223.0	ND	667.2	ND	38.0	ND	NA	NA	72.9	T	583.7	3618.4	85.9
	34.0	6.00	0.3560	59.4	983.0	263.6	ND	963.5	T	58.8	ND	1341.5	ND	113.9	T	654.4	4438.1	105.4
B6	35.0	5.93	0.4784	T	592.2	189.6	ND	669.5	T	T	ND	NA	NA	157.3	T	584.4	2975.5	70.6
	35.5	5.98	0.4626	NA	NA	NA	ND	NA	NA	NA	NA	NA	NA	151.1	T	571.8	-	-
	35.0	6.00	0.4716	T	645.5	263.4	ND	597.5	T	27.7	ND	NA	NA	93.1	T	695.2	3015.7	71.6
	35.0	6.00	0.4762	T	910.8	322.0	ND	690.0	T	48.3	ND	782.5	T	83.8	T	NA	2837.4	67.4
B7	35.0	6.00	0.640	T	403.9	238.4	ND	597.1	ND	73.2	ND	702.0	NA	ND	ND	656.4	2014.6	47.8

TABLE B2 - TOTAL CARBON MASS BALANCES (LACTOSE/PROTEIN GROWTH LIMITED SUBSTRATE) OF pH = 6.0 AND TEMPERATURE = 35°C

RUN #	EXPERIMENTAL FACTORS			SOLUTE PRODUCTS CARBON										GASEOUS PRODUCTS CARBON (ugC/mL)				CARBON RECOVERY	
	TEMP (°C)	pH	DIL'N RATE (h^{-1})	ETHANOL	ACETATE	PROPIONATE	i-BUTYRATE	n-BUTYRATE	i-VALERATE	n-VALERATE	CAPROATE	LACTATE	FORMATE	CARBON DIOXIDE	METHANE	BIOMASS CARBON (ugC/mL)	CARBON RECOVERY (ugC/mL)	(% OF INFLUENT CARBON)	% PROTEIN FERMENTED
E1	34.5	6.10	0.3061[1]	81.8	1021.4	274.2	T[2]	207.5	ND[3]	131.7	152.8	NA[4]	NA	22.9	ND	958.4	4311.0	78.7[5]	
	34.5	6.10	0.3005	98.1	1088.1	283.8	T	235.8	ND	113.8	124.0	1940.0	NA	21.9	ND	617.0	4522.5	82.6	
	35.0	6.10	0.3061	84.6	1126.2	408.0	T	290.1	ND	103.9	141.6	1281.5	NA	30.9	ND	876.0	4342.8	79.3	65.1
	34.0	6.10	0.3061	71.4	1107.5	486.8	T	204.2	ND	141.0	181.3	1118.6	NA	17.2	ND	719.2	4047.2	73.9	
	35.0	6.10	0.3061	83.9	861.5	310.4	T	242.4	ND	127.6	187.5	1501.0	NA	21.2	ND	NA	4128.2	75.4	
E2	36.0	6.00	0.2029	69.6	725.3	188.5	T	233.9	ND	89.9	ND	1551.9	NA	19.2	ND	1053.2	3912.3	71.5	
	36.0	6.00	0.2029	69.7	1689.7	212.5	T	284.5	ND	143.5	ND	1720.7	NA	3.1	ND	1356.6	5477.2	100.0	68.7
	35.0	6.00	0.2017	101.0	1386.7	172.3	T	208.5	ND	160.8	ND	1728.9	NA	10.0	ND	NA	4963.1	90.6	
E3	34.5	6.00	0.2029	ND	1699.1	592.7	T	481.8	76.6	65.7	T	962.8	NA	ND	ND	561.0	4439.7	81.1	
	33.7	5.95	0.2097	78.9	1263.1	563.4	T	416.2	76.1	50.8	T	909.4	NA	ND	ND	662.9	4020.8	73.4	69.9
	34.5	5.85	0.2124	100.8	1307.4	656.2	T	461.7	131.6	36.7	T	1182.0	NA	ND	ND	643.1	4519.5	82.5	
	34.5	5.97	0.2071	99.1	1339.8	526.8	T	346.9	154.3	34.9	T	NA	NA	ND	ND	682.8	4202.7	76.8	

1 V = 1470 mL; 2. Trace amounts; 3. Not detected; 4. Not analysed; 5. Influent carbon concentration ; S_o = 5475.2 ugC/mL

TABLE B3 - TOTAL CARBON MASS BALANCE (LACTOSE GROWTH LIMITED SUBSTRATE) AT pH = 4.5 AND TEMPERATURE = 35°C

RUN #	EXPERIMENTAL FACTORS			SOLUTE PRODUCTS CARBON (ugC/mL)										GASEOUS PRODUCTS CARBON (ugC/mL)				CARBON[5] RECOVERY (% OF INFLUENT CARBON)
	TEMP (°C)	pH	DIL'N[1] RATE (h^{-1})	ETHANOL	ACETATE	PROPIONATE	i-BUTYRATE	n-BUTYRATE	i-VALERATE	n-VALERATE	CAPRODATE	LACTATE	FORMATE	CARBON DIOXIDE	METHANE	BIOMASS CARBON (ugC/mL)	CARBON RECOVERY (ugC/mL)	
D1	35.0	4.6	0.0495	261.9	1175.9	711.1	75.0	1391.5	42.0	118.5	361.4	NA[4]	NA	ND	ND	NA	4450.4	105.6
	35.5	4.6	0.0505	257.5	1260.9	641.9	75.3	1382.4	T[2]	112.3	326.3	NA	NA	ND	ND	158	4214.6	100.0
	35.5	4.6	0.0496	250.0	1141.1	434.8	66.9	1503.6	T	90.6	308.4	4.8	ND[3]	ND	ND	396	4196.2	99.6
	36.0	4.5	0.0505	373.1	1554.0	488.0	96.9	1640.0	T	84.1	407.1	NA	NA	ND	ND	371	5019.0	119.5
D2	36.0	4.5	0.1036	ND	1044.1	520.9	T	1369.1	ND	109.3	ND	NA	NA	ND	ND	348	3407.3	80.9
	35.0	4.5	0.1014	ND	960.1	671.3	T	1232.5	ND	73.3	ND	NA	NA	ND	ND	NA	3347.7	79.5
	36.0	4.5	0.1013	ND	990.0	622.6	T	1346.3	ND	T	ND	15.9	ND	ND	ND	410	3385.6	80.4
	35.0	4.5	0.1006	ND	1097.3	594.9	T	1297.4	ND	T	ND	NA	NA	ND	ND	426	3431.5	81.5
D3	35.0	4.5	0.2071	T	824.0	506.5	ND	473.1	ND	44.4	ND	1347.9	NA	ND	ND	692	3887.8	92.3
	35.0	4.5	0.1959	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-	-
	35.0	4.5	0.2080	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	658	-	-
D4	36.0	4.8	0.3571	53.3	268.1	85.8	ND	284.0	ND	T	ND	NA	NA	ND	ND	366	2110.4	50.1
	35.0	4.6	NA	42.1	279.1	105.4	ND	226.6	ND	T	ND	NA	NA	ND	ND	348	2054.4	48.8
	36.5	4.6	0.3560	51.9	379.1	105.4	ND	133.0	ND	T	ND	NA	NA	ND	ND	292	2014.6	47.8
	36.5	4.6	0.3578	42.5	473.9	135.9	ND	162.5	ND	T	ND	1053.4	NA	ND	ND	406	2274.2	54.0

1. V= 1470 mL; 2. Trace amounts; 3. Not detected; 4. Not analysed; 5. Average influent carbon, S_o = 4212.5 ug/mL

TABLE B4 - TOTAL CARBON MASS BALANCE (LACTOSE GROWTH LIMITED SUBSTRATE) AT D = 0.05h^{-1} AND TEMPERATURE = 35°C .

RUN #	EXPERIMENTAL FACTORS			SOLUTE PRODUCTS CARBON (ugC/mL)										GASEOUS PRODUCTS CARBON (ugC/mL)				
	TEMP (°C)	pH	DIL'N[1] RATE (h^{-1})	ETHANOL	ACETATE	PROPIONATE	i-BUTYRATE	n-BUTYRATE	i-VALERATE	n-VALERATE	CAPROATE	LACTATE	FORMATE	CARBON DIOXIDE	METHANE	BIOMASS CARBON (ugC/mL)	CARBON RECOVERY (ugC/mL)	CARBON RECOVERY (% OF INFLUENT CARBON)
A1	34.9	4.0	0.0520	241.9	351.9	ND[3]	ND	1315.0	ND	ND	T[2]	41.6	ND	513.4	ND	308.0	2258.4	53.6[4]
A2a	35.4	4.6	0.0500	222.2	1014.3	386.5	59.4	1336.5	ND	80.5	274.0	4.8	ND	ND	ND	372.0	3750.2	89.0
A3a	35.0	5.0	0.0430	305.6	1078.5	190.4	ND	1418.4	ND	121.4	200.1	12.8	ND	ND	ND	770.0	4097.2	97.3
A4a	34.8	5.6	0.0530	172.4	1635.0	473.8	9.0	456.0	ND	43.5	166.3	2.0	ND	ND	ND	646.0	3938.2	93.5
A5	34.8	6.1	0.0400	265.9	1969.3	446.3	71.3	534.4	ND	105.1	ND	7.0	ND	ND	ND	496.0	4050.5	96.2
A6	34.2	6.5	0.0480	105.6	1993.0	196.4	4.1	564.4	ND	79.4	393.1	14.0	ND	ND	ND	767.0	4117.0	97.7
A2b	35.4	4.6	0.0501	103.9	850.6	110.9	54.8	1863.3	ND	ND	ND	ND	ND	721.8	ND	374.0	4079.3	96.8
A3b	35.0	5.0	0.0430	104.5	622.8	538.1	46.8	1502.2	ND	153.3	ND	5.2	T	415.6	ND	340.6	3729.1	88.5
A4b	35.0	5.5	0.0536	248.6	1635.0	280.9	16.0	604.0	78.4	85.8	120.0	7.2	ND	330.1	177.4	589.0	4172.0	99.0

1. V = 1470 mL; 2. Trace amounts; 3. Not detected; 4. Average influent carbon, S_o = 4212.5 ugC/mL

TABLE B5 - TOTAL GAS PRODUCTION AS A FRACTION OF CUMMULATIVE EXPERIMENT TIME AT 25°C AND ONE ATMOSPHERE

A-SERIES EXPERIMENTS

A1		A2a,D1		A3		A4		A5a,B1		A6		A26		A36		A46	
TIME (h)	GAS[1] (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)
0	-	0	-	0	-	0	-	0	-	0.	-	0	-	0	-	0	
23.	-	23.	-	44.	-	20.	0	24.	-	20.	0	24.	-	24.	-	44.	-
48.	-	48.	-	68.	0	43.	0	34.	0	43.	0	48.	-	48.	-	68.	-
56.	0	56.	0	92.	0	66.	0	48.	0	66.	0.030	72.	0	72.	0	92.	0
89.	0.539	89.	0.2124	116.	0	102.	0	72.	0	102.	0.086	96.	0	96.	5.912	116.	0.639
106.	0.5301	106.	0.0	140.5	0	115.	0	99.	0	115.	0.94	128.	0	128.	4.733	140.5	0.286
128.	1.036	128.	0.0	174.0	0	139.	0	120.	0	139.	0.35	144.	0	144.	4.697	174.	0.708
153.	0.253	153.	0.9115	199.	0	164.	0	143.	0			179.	0	179.	4.485	199.	0.200
176.	0.159	176.	2.163	213.	0	188.	0	167.	0			192.	0.602	192.	3.960	213.	0.368
200.	0.1587	200.	2.756	238.	0	212.	0	191.	0			216.	2.287	216.	2.813	238.	0.490
224.	0.74	224.	2.326	267.	0			215.	0			240.	2.965	240.	2.079	267.	0.174
249.5	1.284	249.5	1.716	285.	0			239.	0			264.	3.601	264.	0.611	285.	0.219
274.0	1.435	274.0	0.2778	308.	0			264.5	0			288.	3.301	288.	0.656	308.	0.290
296.	1.695	296.	0	332.	0							312.	3.924	312.	1.495	332.	0.451
321.	2.164	321.	0	353.	0							333.	3.895	333.	1.812	353.	0.782
344.	2.075	344.	0									358.	3.790	358.	2.805	371.	0.828
368.	2.225	366.	0									384.	3.349	384.	2.706	395.	0.897
372.	2.121	372.	0									407.5	2.969	407.5	1.377	419.	1.315
												431.5	2.973	431.5	1.313	443.	1.285
												455.5	2.830	455.5	1.793	467.	1.313
												480.5	3.167	480.5	1.824	491.	1.116
												505.0	2.953	505.0	2.125	515.	1.116
												527.5	2.615	527.5	1.402	539.	1.199
												553.0	2.638				1.188

1. In litres of gas per litre effluent.

TABLE B5 - TOTAL GAS PRODUCTION AS A FRACTION OF CUMMULATIVE EXPERIMENT TIME AT 25°C AND ONE ATMOSPHERE (CON'T)

B-SERIES EXPERIMENTS

B1,A5a		B2a		B2b		B2c		B2d		B2e		B3b		B3c	
TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)
0	-	0	-	0	-	0	-	0.0	-	0	-	0	-	0	-
24	-	18	-	40	0.170	8	0	20	0	25	0	18.	-	24.	0
34	0	42	0	64	0.305	50	0.001	36	0	37.	0.397	26.	0	44.	0.272
48	0	68.5	0.293	89	0.158	74	0.323	47	0	61.	0	43.	0	55.	0.424
72	0	92.0	0.00	113	0.418	107.5	0.034	71	0	85.	0	55.	0	68.	0.18
99	0	116.0	0.061	135	0.316	124	0.004	95	0	109.	0	67.	0.660	80.	0.048
120	0	140.0	0.218	162	0.609	149	0.038	119	0	133.	0	79.	1.498	92.	0.238
143	0	164.0	0.366	184	0.299	173	0.131	143	0	157.	0	91.	2.877	104.	0.214
167	0	188.0	0.219	208	0.205	198	0.073	167	0.113	181.	0	102.	3.154	116.	0.016
191	0	212.0	0.207	232	0.349	234	0.113	191	0.325	211.	0	114.	0.562	128.	0.958
215	0	236.0	0.027	256	0.240	256	0.386	221	0.473	229.	0.236	126.	0.371	140.	1.644
239	0	260.0	0.096	280	0.247	287	0.634	239	0.257	254.	1.147	138.	0.464	152.	1.070
264.5	0	284.0	0.308			312	0.712	264	0.244	278.	0.825	150.	0.235	164.	0.092
		308.0	0.408			325	0.529	288	0.464	302.	0.859	163.	0	176.	0.118
						349	0.486	312	0.114	326.	1.043	175.	0	188.	0.126
						373	0.452	336	0.703	350.	1.069	186.	0.14	200.	0.204
						400	0.279	360	0.759	374.	0.749	196.	0	212.	0.160
						423	0.337	384	0.600	398.	0.545	211.	0.153	224.	0.231
						453	0.966	408	0.636	422.	0.582	222.	0.169	236.	0.181
						493	0.783	432	0.633	448.	0.768	234.	0.436	248.	0.286
						517	0.425	456	0.769	472.	0.109	246.	0.481	260.	0.188
						526	0.230	480	0.725	496.	0.061			271.	0.250
								504	0.719					283.	0.116
														295.	0.185
														307.	0.075
														319.	0.152
														338.	0.341
														355.	0.451

CON'T TABLE B5 - TOTAL GAS PRODUCTION AS A FRACTION OF CUMMULATIVE EXPERIMENT TIME AT 25°C AND ONE ATMOSPHERE

B-SERIES

B4a		B4b		B5		B6		B7	
TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)
0	-	-	-	0	-	0	-	173.	0.376
36.	0	18.	-	18.	0	18.	0	179.	0.037
60.	1.60	20.	0	32.	0.195	32.	0.211	187.	0.036
72.	0	43.	0.789	42.	0.067	42.	0.213	199.	0
84.	0	55.	0.841	55.	0.031	55.	0.582		
96.	0.171	67.	0.293	67.	0.088	67.	0.446		
108.	0.158	79.	0.424	79.	0.336	79.	0.196		
120.	0.070	91.	0.211	91.	0.592	91.	0.495		
132.	0.156	102.	0.020	102.	0.455	102.	0.767		
144.	0.174	114.	0	114.	0.419	114.	0.922		
156.	0.441	126.	0.020	126.	0.349	126.	1.013		
168.	0.599	138.	0.269	132.	0.320	132.	0.949		
180.	0.652	150.	0.136	139.	0.294	139.	0.667		
192.	0.501	163.	0.073	151.	0.368	151.	0.585		
204.	0.621	175.	0	157.	0.459	157.	0.526		
214.	0.466	186.	0	163.	0.526	163.	0.498		
		196.	0						
		211.	0						
		222.	0						
		234.	0.053						
		246.	0						
		259.	0						
		271.	0.042						

CON'T TABLE B5 - TOTAL GAS PRODUCTION AS A FRACTION OF CUMMULATIVE EXPERIMENT TIME AT 25°C AND ONE ATMOSPHERE

D-SERIES EXPERIMENTS						E-SERIES EXPERIMENTS					
D2		D3		D4		E1		E2		E3	
TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)	TIME (h)	GAS (L/L)
		0	-	0	-	0	-	0	-	383.	0.616
0	-	21.	0	36.	0	21.	0	24.	0	395.	0.467
8.	0	45.	1.993	60.	0.100	32.	0.500	44.	0.043	408.	0.391
50.	1.447	67.	1.066	72.	1.653	44.	0.745	55.	0.061	419.	0.643
74.	1.214	89.	0	84.	0.390	57.	0.214	68.	0.125	431.	0.526
107.5	1.27	113.	0	96.	0	69.	0.121	80.	0.139	443.	0.295
124.	1.527	138.	0.072	108.	0.169	80.	0.016	92.	0.091	455.	0.042
149.	1.672	162.	0	120.	0.409	92.	0.119	104.	0.103	467.	0
173.	1.155	188.	0.056	132.	0.531	105.	0.126	116.	0.045	479.	0
198.	1.194	210.	0.063	144.	0.580	116.	0.147	128.	0.022	494.	0
234.	0.926	235.	0.201	156.	0.633	128.	0.183	140.	0.028	503.	0
256.	0.161	259.	0	168.	0.418	141.	0.249	152.	0.087	515.	0
287.	0			180.	0	152.	0.213	164.	0.1098	527.	0
312.	0.041			192.	0	164.	0.243	176.	0.105	538.	0
325.	0.197			204.	0	176.	0.283	188.	0.086		
349.	0.06			214.	0	188.	0.232	200.	0.078		
373.	0					200.	0.253	212.	0.113		
400.	0					212.	0.238	224.	0.085		
426.	0					224.	0.259	236.	0.201		
453.5	0					236.	0.183	248.	0.263		
493.	0.211					248.	0.185	260.	0		
517.	0.104					260.	0.225	271.	0		
526.	0.050							283.	0.344		
								295.	0.137		
								307.	0.056		
								319.	0.053		
								338.	0.108		
								355.	0.179		

APPENDIX C

ENZYME SYSTEMS

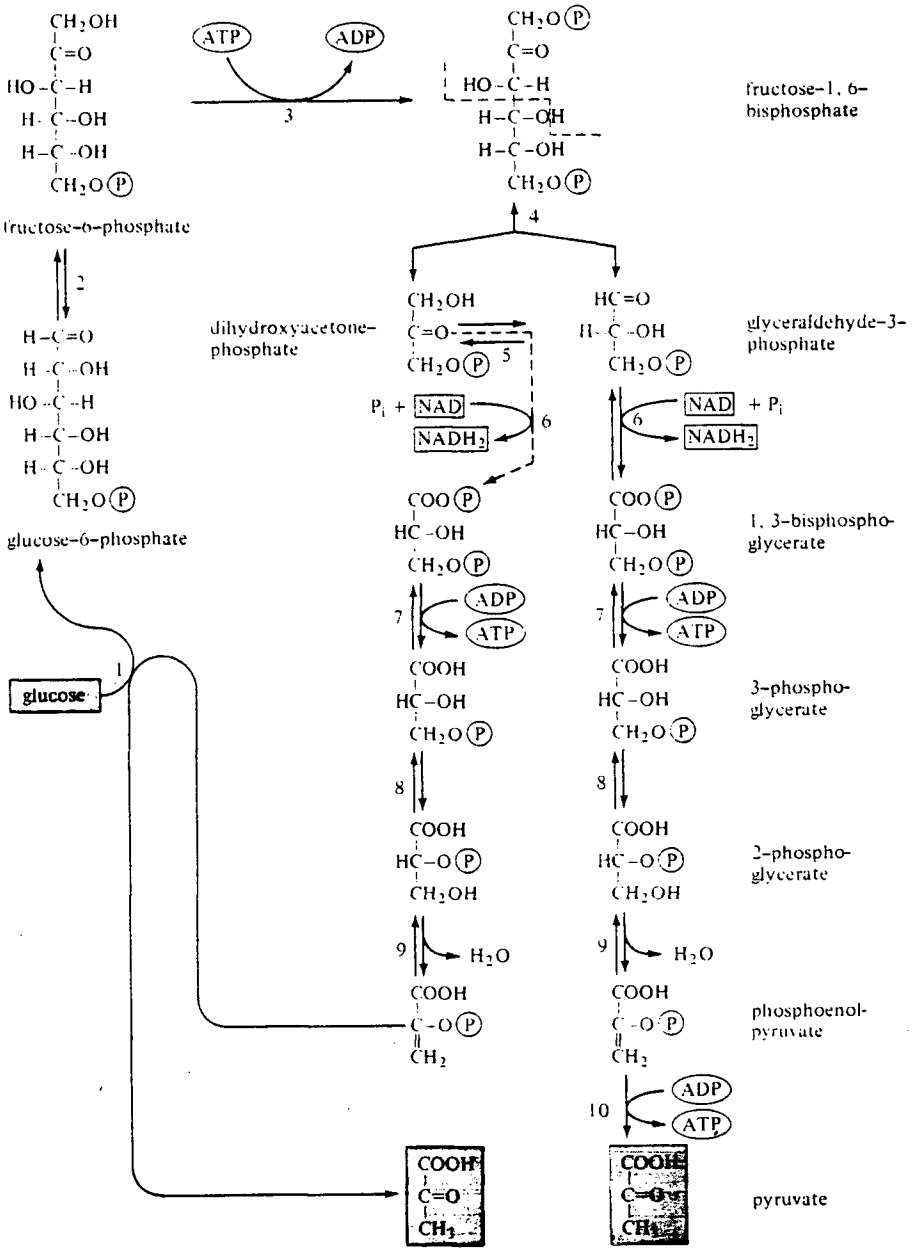


Figure C1 Breakdown of glucose to 2 pyruvate via the Embden-Meyerhof-Parnas pathway: (1) PEP: glucose phosphotransferase; (2) glucose phosphate isomerase; (3) phosphofructokinase; (4) fructose bisphosphate aldolase; (5) triose phosphate isomerase; (6) glyceraldehyde-3-phosphate dehydrogenase; (7) 3-phosphoglycerate kinase; (8) phosphoglycerate mutase; (9) enolase; (10) pyruvate kinase. Source is Gottschalk (1979).

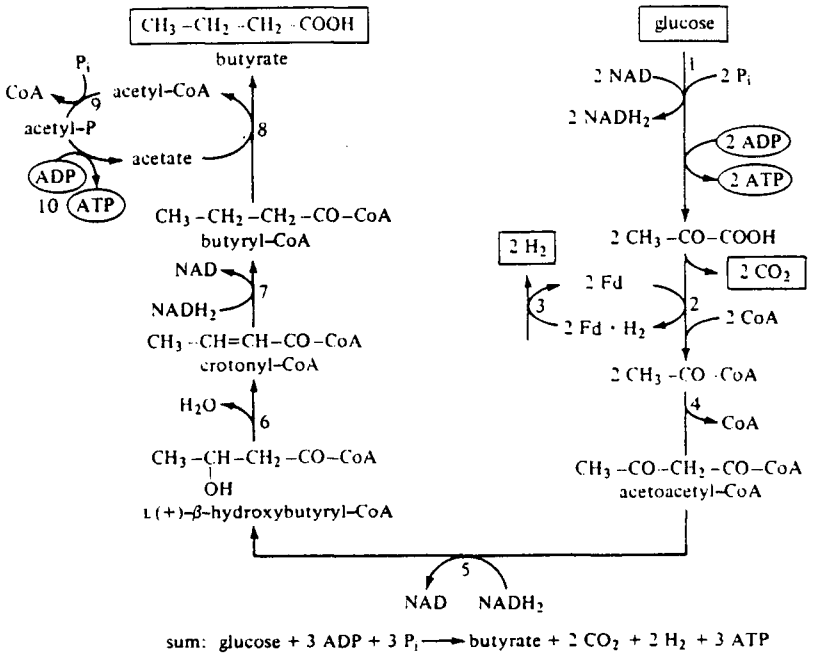


Figure C2 Path of butyrate formation from glucose: (1) phosphotransferase system and Embden-Meyerhof pathway; (2) pyruvate-ferrodoxin oxidoreductase; (3) hydrogenase; (4) acetyl-CoA-acetyltransferase (thiolase); (5) L(+)-β-hydroxybutyryl-CoA dehydrogenase; (6) L-3-hydroxyacyl-CoA hydrolyase (crotonase); (7) butyryl-CoA dehydrogenase; (8) CoA-transferase; (9) phosphotransacetylase; (10) acetate kinase. Source is Gotschalk (1979).

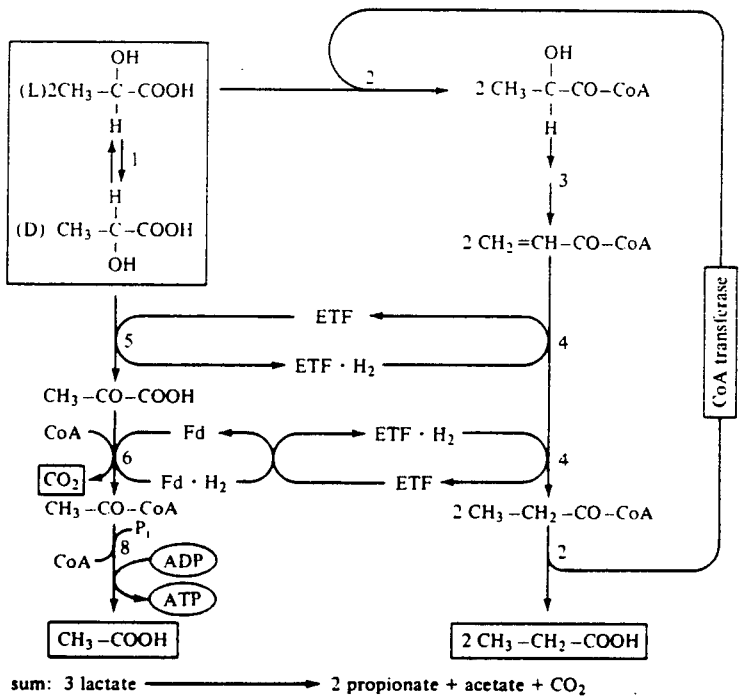


Figure C3 Formation of propionate, acetate, and CO_2 from DL-lactate by Megasphaera elsdenii and Clostridium propionicum: (1) lactate racemase; (2) CoA transferase; (3) reaction not established; (4) dehydrogenase, which employs reduced electron-transferring flavoprotein ($ETF \cdot H_2$) as H-donor; (5) D-lactate dehydrogenase; (6) pyruvate-ferredoxin oxidoreductase; (7) transhydrogenase; (8) phosphotransacetylase + acetate kinase. Source is Gottschalk (1979).

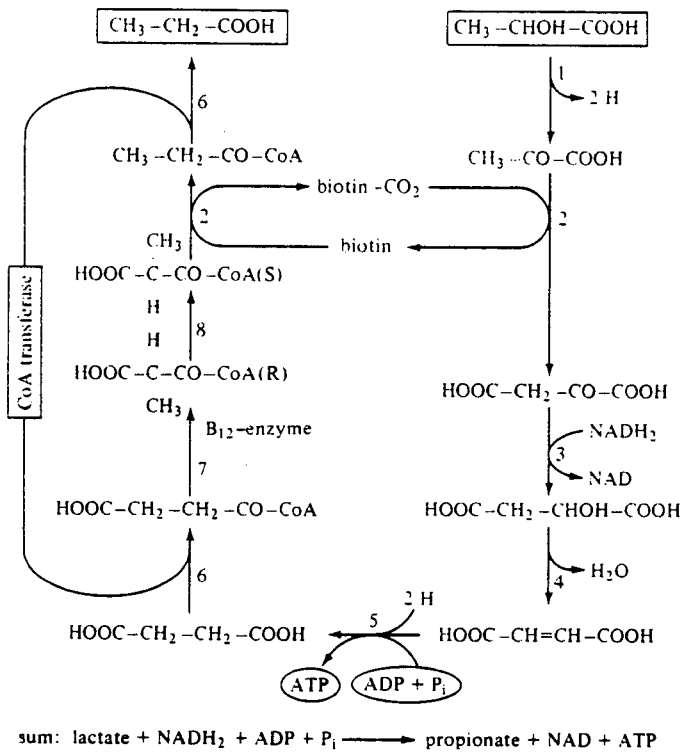


Figure C4 Fermentation of lactate via the succinate-propionate pathway by propionibacteria: (1) lactate dehydrogenase (the H-acceptor is probably a flavoprotein); (2) (S)-methylmalonyl-CoA-pyruvate transcarboxylase; (3) malate dehydrogenase; (4) fumarase; (5) fumarate reductase; (6) CoA transferase; (7) (R)-methylmalonyl-CoA mutase; (8) methylmalonyl-CoA racemase. Source is Gottschalk (1979).

APPENDIX D

A COMPUTER PROGRAM FOR ESTIMATING THE PARAMETERS OF THE MICROBIAL GROWTH MODEL

```
C THIS PROGRAM ESTIMATES THE PARAMETERS OF A NONLINEAR
C MICROBIAL KINETIC MODEL EITHER USING A LEAST SQUARES
C OR A CHERBYCHEV CURVE FITTING METHOD.
C
      IMPLICIT REAL*8 (A-H,O-Z)
      DIMENSION P(3), IV(63), V(282)
      EXTERNAL CALCR, CALCJ
      COMMON X(20), Y(20), SO, EPS

C SETTING INITIAL VALUES AND READING IN DATA

      EPS = 0.001D0
      SO= 4212.5D0
      N=5
      M=3
      P(1)=.200D0
      P(2)=60.D0
      P(3)=.36D0

      DO 1 I = 1,N
      CALL FREAD(5,'2R*8 :', X(I), Y(I))
    1 CONTINUE

C SETTING DEFAULT VALUES IN IV AND V

      CALL DFALT(IV,V)


CC REQUESTING FOR THE PRINTING OF THE COVARIANCE MATRIX
CC AFTER CONVERGENCE HAS OCCURED.

      IV(14)=1
      IV(21)=6

C CALLING THE MAIN SUBROUTINE NL2SOL
      CALL NL2SOL(N,M,P,CALCR,CALCJ,IV,V,IPARM,RPARM,FPARM)
C WRITING THE RETURN CODE AND SOLUTION
      WRITE(6,110) IV(1)
  110 FORMAT('RETURN CODE=', I2)
      WRITE(6,120)(P(I), I=1,M), V(10)
  120 FORMAT(4F16.8)
      STOP
      END

C SUBROUTINE DEFINING THE MODEL
      SUBROUTINE CALCR(N,M,P,NF,R,IPARM,RPARM,FPARM)
      IMPLICIT REAL*8 (A-H, O-Z)
      DIMENSION P(M), R(N)
      COMMON X(20), Y(20), SO, EPS
C
C IMPOSING A CONSTRAINT ON X(I)
      IF (P(3) .LT. (X(N)+EPS)) GO TO 131
C
C PLACING RESIDUALS IN R
      DO 130 I=1,N
      DUMP = P(2)*X(I)/(P(3)-X(I))
      R(I)=P(1)*(SO-DUMP) - Y(I)
  130 CONTINUE
      RETURN
```

```
 131 NF=0
     RETURN
     END

C SUBROUTINE DEFINING THE PARTIAL DELIVERTIVES

     SUBROUTINE CALCJ (N,M,P,NF,D,IPARM,RPARM,FPARM)
     IMPLICIT REAL*8 (A-H, O-Z)
     DIMENSION P(M), D(N,M)
     COMMON X(20), Y(20), SO, EPS
C
C IMPOSING A CONSTRAINT ON X(I)

     IF (P(3) .LT. (X(N)+EPS)) GO TO 131

C PUTTING DERVIATIVES IN D
     DO 130 I=1,N
     D(I,1) = SO-(P(2)*X(I)/(P(3)-X(I)))
     D(I,2) = -P(1)*X(I)/(P(3)-X(I))
     DUMP   = P(3)-X(I)
     D(I,3) = P(1)*P(2)*X(I)/(DUMP*DUMP)
 130 CONTINUE
     RETURN
 131 NF=0
     RETURN
     END
```