THE PRODUCTION OF LACTIC ACID FROM WHEY BY CONTINUOUS CULTURE AS A POSSIBLE MEANS OF WASTE DISPOSAL

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology at Massey University.

KEVIN RAYMOND MARSHALL

1972

ABSTRACT

THE PRODUCTION OF LACTIC ACID FROM WHEY BY CONTINUOUS CULTURE AS A POSSIBLE MEANS OF WASTE DISPOSAL.

A study was made of the fermentation of lactose in lactic casein whey to lactic acid using a strain of Lactobacillus bulgaricus. Both batch and continuous culture were used.

A culture vessel capable of being operated under controlled conditions was designed and built for this study. Temperature, pH, gas atmosphere, degree of agitation and medium flow rate could be altered and controlled.

A meter was developed for the continuous measurement of lactic acid production. The meter used a capacitance probe to measure the volume of alkali added to the culture to maintain a constant pH.

The kinetics of lactic acid production in a batch culture of whey were characterized by :

$$\frac{dP}{dt} = \left(\alpha \frac{dN}{dt} + \beta N\right) \quad \frac{P_m - P}{K_p + P_m - P}$$

The kinetics of bacterial cell growth were consistent with the normally accepted Monod equation but no direct verification of this was made.

A notable feature of the production of lactic acid in a batch culture was the considerable amount of lactic acid formed by non-dividing bacterial cells. More than 50 percent of the acid produced during a batch culture was synthesised while the cell population was in a stationary growth phase.

The maximum cell number was not limited by the concentration of lactose. Supplementation with tryptophan, casamino acids and a number of vitamins increased the cell population and the rate of acid production and decreased the batch time. Sodium caseinate was a good source of essential and stimulatory nutrients.

The optimum heat treatment of the whey involved heating to 69°C.

In unsupplemented whey the removal of suspended material by centrifuging and filtration prevented the formation of acid. To maintain maximum acid formation rates the impeller Reynolds number had to be greater than 10,000.

The presence of oxygen prevented the growth of the bacterial cell population, but once the maximum cell population had been reached oxygen did not effect the acid synthesis.

In a single stage continuous culture reactor the concentration of lactic acid was given by :

$$P = N\left(\alpha + \frac{\beta}{D}\right)\frac{P_m - P}{K_p + P_m - P}$$

The constants were determined from batch culture data.

A single stage continuous culture is not suitable for the conversion of all the lactose in the whey to lactic acid. If lactic acid production by continuous culture is to be considered as a means of waste disposal it will be necessary to use feed back of cells to a single - stage reactor or multi-stage stirred tanks.

In continuous culture studies it was shown that the optimum temperature for the fermentation of lactic casein whey was 46°C. A pH in the rage 5.4 - 6.0 was best. Outside this range, productivity and yield were decreased.

It can be concluded that though continuous production of lactic acid from whey is feasible, multi-stage continuous reaction systems and/or cell feedback are necessary to reduce the lactose concentration to an acceptable level. The whey should be supplemented with a source of amino acids.

ACKNOWLEDGEMENTS

I wish to thank my supervisory committee and my colleagues in the New Zealand Dairy Research Institute for helpful and stimulating discussions, advice and assistance.

TABLE OF CONTENTS

FIGURES

TABLES

1 INTRODUCTION

Because of the increasing number of large cheese and casein manufacturing units, the New Zealand dairy industry is in a position to consider the economical manufacture of a number of products from whey. Whey, the liquid remaining after milkfat and casein have been separated from whole milk, contains nearly 70 percent of the solids-not-fat portion of whole milk including most of the lactose, salts and serum proteins (Appendix 1). Despite this, whey is a raw material almost entirely wasted in New Zealand.

The high biological oxygen demand (BOD) of whey (35,000 ppm,5 day) renders it a serious pollutant if allowed to discharge to waterways. Increasingly stringent legislation makes it imperative to find an economical method of treatment of the whey before it can be discharged without risk to the environment.

Traditionally in New Zealand casein whey has been discharged to waterways or sprayed onto pasture where some benefit as a fertilizer or for irrigation is obtained. But these sources of disposal are becoming more restricted as dairy companies amalgamate and individual manufacturing units become larger. Cheddar cheese whey in New Zealand is a source of lactose but casein whey with a lower initial lactose concentration is not an economical source of this sugar.

As a feed material for pigs, whey has the disadvantages of being very dilute and not being available in regular supply. Concentration by evaporation and storage alleviates these problems slightly, but the cost of this further processing makes whey a less attractive pig food.

Recent advances in separation techniques (reverse osmosis and ultrafiltration) have given rise to the possibility of producing high quality undenatured whey proteins. These proteins have valuable nutritional and functional properties. Their removal from whey will decrease the BOD level slightly.

The lactose, which causes nearly 75 per cent of the BOD, still remains. One method of approaching the problem of whey disposal is to consider converting this lactose to more marketable products by microbial transformation. Yeast, lactic acid, alcohol, vitamins, amino acids, butanol and acetone are among the many products suggested in the literature.

Lactic acid has potential as a versatile chemical and chemical intermediate (Schopmeyer, 1954; Arnold and Childs, 1960). It is used in the food and beverage industries and in the production of plastics and textiles. As a manufactured chemical lactic acid has the advantage of being marketed in a number of grades and strengths.

Little lactic acid is used in New Zealand. In 1968-69, 143,000 lb of 85 per cent CP grade acid, CIF value $29,000, was imported into New Zealand (Department of Statistics,1970). However if a supply of locally produced acid was available at a reasonable price it is probable that the demand would increase.

In line with many other products having a simple molecular structure, the fermentation route to lactic acid has received increasing competition from direct synthesis. The availability of cheap hydrogen cyanide and acetaldehyde arising from developments in the petrochemical industry, the synthetic fibre industry, and in particular the production of acrylonitrile, have led to an increasing production of synthetic lactic acid. (Thorne, 1969)

New Zealand has little chance of producing synthetic lactic acid because of the lack of suitable chemicals so it is worthwhile to assess the potential of microbial production from indigenous raw materials.

The only local raw material available in large enough quantities at one site and from which lactic acid could be produced is lactic casein whey. Needle and Aries (1949) estimated that a daily supply of 25,000 gal of whey would be necessary for the economical production of lactic acid. An increasing number of factories in New Zealand have average daily productions of whey in excess of this with peak levels in excess of 150,000 gal/day.

The estimated volume of whey produced in New Zealand in 1969/70 was 350 million gallons. The amount of lactic acid which potentially could be produced from this whey (150×10^6 lb) is far in excess of present requirements but a few individual factories could find the manufacture of lactic acid a viable proposition.

Ten years ago an attempt was made to manufacture lactic acid from casein whey in a New Zealand dairy factory but was abandoned as a commercial venture because of difficulties in the purification steps. Iron contamination from the processing plant was a major problem but more resistant materials of construction which are now available could overcome this.

It is difficult to determine the cost of whey as a raw material. In

general the disposal of whey will be a cost to the casein process and the whey can be considered to have a negative value. If it can be pumped to a waterway the cost of pumping and the necessary pipe lines will represent the minimum charge. Spray irrigation will be more expensive. McDowall and Thomas (1961) estimated the capital cost for an irrigation scheme to handle 21,000 gal/day of whey to be $16,000 excluding land. Operating cost depends on pumping charges, but for the example they give is aproximately 0.02 c/gal. If treatment in a conventional sewage plant was used, the cost could be as high as 0.3c/gal based on equivalent BOD levels.

On the other hand, if whey was used as a feed for pigs it is worth up to 1c/gal to the farmer. This is the estimated cost of alternative feeds which would have to be used if whey was unavailable.

In general, however, it can be stated that whey represents a waste disposal cost to the factory. A process which reduces the cost of waste disposal and produces a product capable of being sold for at least the manufacturing cost is a profitable venture for the company.

In line with many other chemicals there is likely to be significant economical advantage in producing lactic acid by a continuous process. This will be particularly so for the purification steps. It is worthwhile, therefore, assessing the possibilities of continuous conversion of lactose to crude lactic acid as the first step towards the production of lactic acid to meet commercial specifications.

The work reported in this thesis was directed towards a study of the most significant variables affecting the production of lactic acid from whey by fermentation with a view to determining the feasibility of continuous operation and evaluating the optimum conditions.

A reactor which would allow the maximum control over the conditions of fermentation was designed and assembled and used for batch and continuous culture experiments.

A statistical technique was used in an attempt to select the most significant variables affecting the continuous fermentation of whey. The optimum levels of pH and temperature were determined. The main aim was to achieve the maximum productivity (rate of acid production per unit of culture volume) consistent with the lowest level of residual lactose. If pollution is to be reduced the lactose level must be reduced as close to zero as possible.

In the single-stage continuous culture experiments the reduction in lactose level was low and a study was made of the kinetics of the batch

culture in an attempt to determine ways of converting more lactose.

An investigation of the effect of supplementary nutrients showed that amino acids and vitamins were essential or stimulatory for acid production. Casein in the whey was a source of essential nutrients.

Because of the large number of nutrients to be studied an attempt was made to use shake flask batch cultures. This gave inconsistent results with low yields. Oxygen concentration and agitation level were studied as possible causes of these low yields.

In the course of these experiments conditions were defined which produced increased yields of lactate. A study of the results obtained leads to kinetic equations which will allow the prediction of optimum conditions for commercial production. The kinetic expressions are similar to those obtained from studies using defined, non-commercial media.

2 A REVIEW OF THE RELEVANT LITERATURE

2.1 Microbial Kinetics

In recent years there have been significant advances in understanding microbial kinetics and their application to continuous systems for fermentation processes, both for the production of microbial cells and the transformation of substrates (Malek and Fencl,1966). In order to make rational use of these advances a knowledge of the kinetics of cell growth, substrate utilization and product formation is essential. Much of the information published in the literature is of little use in practical situations because of the use of idealized systems and raw materials of no commercial significance (Childs and Welsby,1964; Ricica, 1969).

Fermentation kinetics is the quantitative study of the rate of growth of microbes and the consequent rate of substrate utilization and metabolite production. This review is concerned mainly with the kinetics of metabolite production.

The subject has been recently reviewed by Edwards (1967) and Luedeking (1968). The particular topic of product formation ("product" refers to all metabolites other than cells) forms the major part of the proceedings of the 4th symposium on "Continuous Cultivation of Micro-organisms" (Malek, et al 1969) and has been surveyed by Maxon and Chen (1966,1967), and Pirt (1967,1969).

2.1.1. Models of Cell Growth

The most widely used models of microbial cell growth are those based on the principles of chemical kinetics. The models of Monod (1942) and Hinshelwood (1946) form the basis of much of microbial kinetics. These treat cell growth and product formation for the cases limited by a single nutrient or metabolic product. The usual assumptions are that cell growth rate is a function of cell and substrate concentrations. The amount of cell growth is assumed to be proportional to substrate consumption while product formation is proportional to growth. These simplifications limit the usefulness of the models to a restricted number of microbial systems. The simplest situation is the exponential growth phase of the cell's life cycle, where the specific growth rate is assumed to be constant.

Monod's model is expressed by

$$\frac{dX}{dt} = \mu X \qquad (2.1)$$

where μ is the specific growth rate, h^{-1}
(i.e. growth rate of cells per unit of cell concentration)

X is cell mass concentration g/l

and t is time, h.

Monod (1949) proposed that the specific growth rate was related to the concentration of the nutrient which limited growth and he assumed a relationship similar to that of the Michaelis-Menton description of enzyme kinetics.

$$\mu = \frac{\mu_m \quad S}{K_s + S} \qquad (2.2)$$

where μ_m is the maximum specific growth rate
(i.e. the specific growth rate in the exponential phase), h^{-1}

S is the concentration of the limiting nutrient g/l

K_s is the saturation constant for the limiting nutrient. g/l

A number of modifications of equation (2.2) have been proposed and these have been discussed by Powell (1967) who presents a further equation which takes into account the diffusion and permeation of the substrate from the medium into the cell.

Models of the Monod type are of limited value because they do not apply to all phases of the microbial growth cycle. An extension of the application of chemical reaction kinetics has been used to model all phases of the cell growth cycle by the use of the following expression (Kono,1968; Kono and Asai, 1969 a,b)

$$\frac{dX}{dt} = \mu_m \phi X \qquad (2.3)$$

ϕ takes the following values

Induction phase	$\phi = 0$
Transient phase	$0 < \phi < 1$
Exponential growth phase	$\phi = 1$
Constant growth phase	$\phi = \frac{Xd}{X}$
Declining growth phase	$\phi = \frac{Xc}{Xm - Xc} \cdot \frac{(Xm - X)}{X}$

Where Xc is the cell mass concentration at the boundary point between the exponential growth phase and the declining growth phase,

Xd is the cell mass concentration at the boundary point between the exponential growth phase and the constant growth phase;

Xm is the maximum cell mass concentration, predicted by a theoretical procedure.

The equations require the evaluation of a large number of constants from the batch growth curve and take no account of substrate concentration. The constants do have some physical meaning. The equations have been successfully applied to the published results of a number of different types of fermentation including continuous culture. (Kono & Asai, 1969 a,b.)

The Monod models considered previously are known as substrate limited models. Conditions also occur where the metabolism is inhibited by increasing concentration of the product. Hinshelwood (1946) proposed the following equation to describe inhibitory effects:

$$\frac{dX}{dt} = (1 - aP)\mu X \qquad (2.4)$$

where a is a constant, l/g

P is the concentration of product, g/l

In an investigation of the kinetics of product inhibition in the alcohol fermentation (Aiba, Shoda and Nagatani, 1968) it was pointed out that in many industrial fermentations the accumulation of ethanol continues even after the cessation of cell growth. It was shown that alcohol has an inhibitory effect on cell growth at several per cent of ethanol while the fermentative activity of the cells is not impaired until ethanol concentration approaches 20 per cent. The inhibitory effect on cell growth can be formulated by

$$\mu = \mu_o e^{-k_1 P} \frac{S}{K_s + S} \qquad (2.5)$$

where μ_o is the specific growth rate at zero ethanol concentration. The inhibitory effect of ethanol on the fermentative activity of the yeast cells is expressed by

$$\upsilon = \upsilon_o e^{-k_2 P} \frac{S}{K_s + S} \qquad (2.6)$$

Where υ_o is the specific rate of ethanol production at zero ethanol

concentration. By assuming constant values for yield coefficients it was possible to use digital computer techniques to simulate the batch fermentation curves.

A different approach to the modeling of bacterial kinetics, but still based on chemical kinetics, has been the attempts of various authors to use the analogy with heterogeneous catalysis. (Atkinson,Swilley,Busch and Williams,1968; Atkinson and Daoud,1968;). The mass of micro-organisms is likened to catalyst particles and equations derived to describe the mass transfer of nutrients and metabolic products coupled with the enzymic reactions. Experimental verification of the equations has been obtained under conditions where the growth rate is controlled by the rate of diffusion of substrate to the microbial cells. Dummet (1968) and Shore and Royston (1968) have used a similar analogy with heterogeneous catalysis in discussing the production of yeast.

None of the above models attempts to include variables such as the cell age distribution or the cell composition. However, with progress in the understanding of the processes of cell growth proliferation and metabolism, advances have been made with models which include these fundamental variables (Painter and Marr, 1968).

A complex and complete approach has been the attempt by a number of authors to apply principles of engineering analysis to elucidate growth and replication phenomena (Tsuchiya, Fredrickson and Aris,1966; Ramkrishna, Fredrickson and Tsuchiya, 1966,1967). Models of varying complexity have been considered and these successfully predict all the stages of batch culture and many of the observed results in continuous culture. Experimental verification of the models has yet to be obtained.

2.1.2. Models of Metabolite Production

There are three basic types of metabolite production;

1) the rate of production of metabolite parallels the growth of the micro-organism.
2) the rate of production of metabolite is indirectly related to the growth, and
3) production of metabolite is independent of growth, the organisms acting as a carrier for the enzymes which catalyse the desired reaction.

Hinshelwood (1946) assumed the specific rate of product formation to be a constant, r.

$$\frac{dP}{dt} = rX \qquad (2.7)$$

This is the simplest of models and applies to very few real situations.

Luedeking and Piret (1959) empirically derived the following more complex expression for product formation.

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad (2.8)$$

Where α , β are functions of the pH of fermentation. The first term of this equation was said to account for growth associated product formation and the second for non-growth associated product formation.

Pirt (1969) derived an equation of the same form by assuming that the consumption of the energy source by bacteria was represented as the sum of two terms; the consumption of the energy source for growth, and the consumption of the energy source for maintenance. He also assumed that the amount of product was stoichiometrically related to the energy source.

Humphrey (1963) used the same expression to correlate the specific acid production rate in both batch and continuous lactic acid production by Lactobacillus delbrueckii.

An equation similar to (2.8) was shown to represent over 85 per cent of the rate curve of hydrogen sulphide production from sulphate by a strain of Desulfovibrio (Leban, Edwards and Wilke, 1966). It was also said to apply to the alcholic fermentation of glucose by Saccharomyces cerivisiae (Aiyar and Luedeking,1966).

However, Finn (1966) and Holzberg, Finn and Steinbraus (1967) studying the alcholic fermentation of grape juice claimed the Luedeking and Piret equation did not fit their data and they proposed the following;

Exponential growth phase:

$$\frac{dP}{dt} + bP = a\frac{\ln N}{\mu} - C \qquad (2.9)$$

Stationary phase (where N, the cell concentration, is constant):

$$\frac{1}{N}\frac{dP}{dt} = r\,(P_m - P) \qquad (2.10)$$

Where P_m is the maximum amount of alcohol which could be produced.

Terui and Niizu (1969) studied the fermentation production of L-tryptophan and reported batch fermentation results which indicated kinetics of the Luedeking and Piret type, ie product formation was both growth and non-growth associated. However when they extended their work to continuous fermentation the results indicated that tryptophan was produced by growing cells only. They reinvestigated the batch culture and showed that in the "stationary" phase the cell population contained dead and lysing cells mixed with actively growing cells. The latter were probably utilizing metabolic products formed in the early phases of the culture and from the lytic products of the dead cells. It was

these actively growing cells which were responsible for the tryptophan production in the apparent "stationary" phase leading to the assumption of non-growth associated product formation.

Other attempts to model product formation have been those of Shu (1961), Maxon and Chen (1966,1967) Kono (1968), Kono and Asai (1969 a,b) Tsuchiya et al (1966) and Ramkrishna et al (1966,1967). All are extensive and complicated, requiring the evaluation of a large number of constants from the batch curve and/or the use of computers to evaluate and predict the results. All models are of limited value because it is not yet possible to predict the effect of changes in physical variables such as pH, temperature and various nutrients.

2.1.3. Continuous Culture

Continuous culture techniques for the production of microbial products have been extensively studied and there has been much interest in commercial application because of the theoretical advantages of increased productivity and greater control. Despite this interest there has been little commercial application except for the production of yeast and beer, and the activated sludge process for sewage treatment. Some of the reasons for this lack of commercial use are discussed by Evans (1965).

As a research tool for microbiological processes, continuous culture provides a constant chemical and physiological environment in which to investigate the effects of variables on the growth of cells and the products formed. It is also possible to study microbiological processes with the growth rate as an independent variable.

The theory of continuous culture is discussed fully in the literature (Monod,1950; Herbert, Elsworth and Telling,1956; Malek and Fencl,1966; Fencl and Ricica,1968).

There are generally considered to be two modes of control in continuous culture; the chemostat and the turbidostat. In the chemostat the culture is controlled by the concentration of a limiting nutrient in the medium (Monod,1950; Herbert et al,1956), by the concentration of an inhibiting product of metabolism (Luedeking and Piret,1959) or by the concentration of hydrogen ions (pH) (Karush,Iacocca and Harris,1956). In the single stage chemostat the cell specific growth rate is always less than the maximum.

In the turbidostat the culture is controlled "externally" by maintaining the concentration of the cells at the required level by measuring the turbidity (Northrop,1954) or by maintaining some other physical or chemical variable such as pH or oxygen concentration at a constant value by an

external controller which regulates the fresh medium inflow. It is possible to operate cultures at their maximum specific growth rate in one stage. Fundamentally the two modes of operation are the same.

The chemostat consists of a "perfectly mixed" vessel with a constant inflow of fresh medium and outflow of culture at the same rate so the volume in the vessel is constant. It is assumed that the concentrations of all essential nutrients but one, the limiting nutrient, are in excess or at least held constant. Mathematically it can be shown that the specific growth rate of the organisms is equal to the dilution rate, D (the reciprocal of the average residence time).

Thus, at steady state;

$$\mu = D \qquad (2.11)$$

$$\bar{X} = Y\ (S_F - \bar{S}) \qquad (2.12)$$

$\bar{X}$ denotes steady state cell mass concentration.

S_F , $\bar{S}$, concentration of limiting substrate in incoming feed and outgoing culture liquid respectively

Y is the yield of cell mass per unit of substrate utilized.

The maximum value of D is μ_m, the maximum specific growth rate of the organisms. If this value of D is exceeded the organisms "washout" and the concentration of cells in the reactor falls to zero.

The specific growth rate, μ, is a function of the substrate concentration. (See equation (2.2))

Y, the yield coefficient, is often assumed to be a constant, but it is now generally recognized that this is the exception rather than the rule. Modifications to Y which express its dependence on growth rate, and in particular the use of a maintenance coefficient, have been introduced by a number of authors (Powell, 1967).

Luedeking and Piret (1959 b) discussed the case where toxic product accumulation limits the growth of the organism. Toxic product formation was assumed to be described by equation (2.8) and the specific growth rate of the organisms was assumed to decrease as a linear function of product concentration as described by equation (2.4). (This latter assumption is not in agreement with the data of Luedeking and Piret 1959a). For the steady state values of product and cell mass concentration they derived

$$\bar{P} = \frac{(\mu_m - D)}{\alpha} \qquad (2.13)$$

$$\bar{X} = \frac{\bar{P}D}{(\alpha D + \beta)} \qquad (2.14)$$

A number of authors has discussed the desirability of using more than one stage for continuous culture, (Malek & Fencl,1966; Ricica,1969a) particularly for the production of products other than biomass. Qualitatively it can be stated that the first stage is used to produce cells in the optimum condition for product formation in subsequent stages. If it can be shown that the product rate is dependent on the previous stages of growth then the tubular plug-flow reactor with feedback of cells or permanent seeding is desirable. Danckwerts (1954) showed that if the reaction rate increases with concentration the optimum output per unit volume of a completely mixed reactor is superior to that of the tubular type. If the rate decreases with concentration the plug-flow is superior. For a zero-order reaction the efficiency of both types is the same. Because of control problems tubular plug-flow reactors have disadvantages, but they can be approximated by a chain of homogeneous completely mixed fermenters with short individual holding times.

The prediction of conditions in continuous culture from batch data has concerned many authors including Fencl,(1968); Fencl,Machek and Novak,(1969), Herbert et al, (1956); Holzberg et al (1967); Kono and Asai, (1969b); Luedeking and Piret, (1959b); Malek and Fencl,(1966); Pirt, (1967) and Ricica (1969a,b).

Ricica (1969b) in considering the problem of kinetics, particularly as applied to continuous culture applications, concluded that much of the modeling was of limited value. Practical media used in commercial applications are not sufficiently defined and in most cases the limiting factor is unknown. The metabolic pathways are often not known or only partially understood. He suggested that each case would need to be considered individually. The most widely used techniques for considering individual cases have been based on graphical methods which assume that the growth rate of the organism is the variable which provides the link between the two modes of operation.

Pirt (1967) criticised the prediction of continuous culture from batch culture conditions because it is based on the assumption that the organism behaves similarly in batch and continuous culture, an assumption which in some cases can be shown to be not valid. However, provided due recognition is given to possible differences in the two modes of operation it would seem that much progress can be made by the application of these graphical methods.

Luedeking and Piret (1959) used the graphical technique of Adams and Hungate (1950) to predict the continuous culture conditions for lactic

acid production by Lactobacillus delbrueckii. Bischoff (1966) used the analogy between autocatyltic growth and the heat generated in a simple reversible reaction operated under adiabatic conditions and formulated an analytic and graphical technique to simulate even very complex microbial processes.

Ricica (1969 b) illustrated the use of Bischoff's technique to describe the least holding time for the dehydrogenation of D - sorbitol to L - sorbose by Acetobacter suboxydans. A two stage system was shown to give a much shorter holding time than a single stage for the same conversion. He also analysed the sporulation of Bacillus cereus (Ricica,1969a) showing the need for six stages related to the essential physiological stages through which the organism passes. It was pointed out that experimental verification of the conclusions had yet to be obtained.

Fencl (1966) and Fencl, Machek and Novak (1968) defined a specific product formation rate, K, by the equation

$$\frac{dP}{dt} = KX \qquad (2.15)$$

K is a variable, complex function involving at least two factors, i and λ where i is the amount of active enzyme in the cell and λ is the rate of the enzyme reaction controlling the formation of the product, which is a function of the concentrations of substrate and product, temperature, pH, nutrients and etc.

By suitable techniques, K can be determined from batch culture data and applied to predict continuous culture conditions.

2.2 Lactic Acid

Traditionally lactic acid has been produced in commercial quantities by the fermentation of a variety of carbohydrate sources including corn sugar, molasses, whey and starch. The manufacture of lactic acid by fermentation is now being superceded by a synthetic process from petrochemical raw materials (Thorne, 1969). The fermentation methods have been summarized by Peppler (1967) and Prescott and Dunn (1959).

2.2.1 Whey Medium

The commercial fermentation of whey to lactic acid has been described in detail (Anon., 1945; Burton, 1937; Campbell, 1953; Lawrence, 1944; Olive, 1936; Oetiker, 1960; Pont, 1944; Whittier and Webb, 1950; Swaby, 1944). A number of experimental studies has also been reported (Havlatko & Knez, 1959; Jankowska, 1954; Maxova and Maxa, 1958; Nilsson, 1948; Siman & Mergl, 1961; Surazynski, Poznanski, Budslawski, Czerwinski

& Chojnowski, 1967; Swaby, 1945 a, b; and Whittier and Rodgers, 1931).

Normally cheese (cheddar or cottage) whey has been used although Oetiker (1960) used casein whey and Havlatko & Knez (1959) used whey molasses after lactose crystallization. Swaby (1945) found no difference in fermentation with a variety of wheys.

The time to reach completion of the batch fermentation of the lactose in the whey varied between one and six days depending on the organism used and added growth factors. Yields of lactic acid between 85 and 95 per cent based on lactose consumed have been reported by all authors.

2.2.2 Organisms.

The organisms used most frequently have been Lactobacillus bulgaricus and L. acidophilus. In some cases a film yeast (Mycoderma) was grown symbiotically with L. bulgaricus (Whittier and Rodgers, 1931; Olive,1936) but Campbell (1953) found no advantage in using the yeast and Oetiker (1960) claimed that the lower temperatures ($43^{o}C$) which had to be used caused problems from contamination. Swaby (1945) used a mixed culture of L. bulgaricus strains consisting of long- and short-celled variants. Surazynksi et al. (1967) obtained optimum fermentation with a starter consisting of eight pure cultures (three L. lactis, four L. bulgaricus and one Streptococcus thermophilus). Rosell (1949) reported a strain of L. delbrueckii which, by repeated sub-culturing, had been adapted to ferment lactose in milk and whey at 50 - $52^{o}C$.

2.2.3 Growth Factors

Stimulatory growth factors have normally been added to the whey to reduce the fermentation time. Yeast cells, yeast extract, corn steep liquor and malt sprout extract are the most common. An extensive study failed to find the actual stimulatory compounds, (Swaby,1945). Oetiker (1960) claimed the maximum rate of lactic acid production at a lactose level of 20 g/l.

The nutritional requirements of lactic acid bacteria are very complex and this is illustrated by the extensive literature on the subject. Brief surveys are presented by Snell (1952) and Davies (1960).

Numbers of workers have studied the nutritional requirements of particular strains of L. bulgaricus. For example: Snell, Kitay & Hoff-Jorgensen (1948) reported work on the carbohydrate utilization of L. bulgaricus Gere A in a medium containing all the vitamins, amino acids and salts known to be essential for lactic acid bacteria. Rutter & Hansen (1953) also studied the carbohydrate metabolizm of L. bulgaricus Gere A

in a fully defined medium. A strain of L.bulgaricus was shown to require orotic acid or its analogues for growth (see for example Wright et al (1950) and Wieland et al, (1952)). On the other hand Irie, Yano, Morichi and Kembo (1962) showed that orotic acid was not required for the six strains of L. bulgaricus they studied in a chemically defined medium. These workers showed that all six cultures required casein digest and Tween 80. Requirements for pantothenate and pantethine were dependent on the cysteine level.

De Mann, Rogosa and Sharpe (1960) developed a general medium for lactobacilli which gave improved growth of L. bulgaricus in comparison with the medium of Briggs (1953).

Rogosa, Franklin and Perry (1961) described an improved medium for the study of the vitamin requirements of lactobacilli. L. bulgaricus gave erratic results until it had been repeatedly sub-cultured in the medium. Of the vitamins studied only riboflavin was necessary for growth. Folic acid was shown to be inhibitory. This confirmed the results of Rogosa & Sharpe (1959).

Nakanishi and Nakazawa (1961) reported that the number of L. bulgaricus cells during the different phases of growth was related to the concentration of casein in the medium, the numbers increasing greatly over the range 0.0 - 5.0% of casein.

The effect of the presence of gases on lactic acid bacteria has not been extensively reported. Rodgers and Whittier (1928) reported that bubbling air or nitrogen through a culture of S. lactis in milk increased the final cell count. On the other hand Longsworth and Mac Innes (1935, 1936 a,b) showed the presence of oxygen and the absence of carbon dioxide inhibited both growth and acid production when L. acidophilus was cultured in a synthetic medium. Finn, Halvorsen & Piret (1950) found a similar effect when culturing L. delbrueckii in a synthetic medium, as did Keen (1972) using S. lactis and skim milk.

2.2.4 Temperature

L. bulgaricus is described as a thermoduric organism and the temperature of cultivation has usually been reported to be in the range 45 - 49°C. When the Mycoderma is used as a stimulant the temperature has been lowered to 43 °C (Whittier and Webb, 1950). L. acidophilus gives optimum acid production at 37°C.

2.2.5 Heat Treatment of Whey

The high temperature used in the fermentation reduces the possibility

of contamination but it is normal to give the whey some heat treatment. The conditions used have varied. Campbell (1953) heat treated the whey to remove the albumin, but found this destroyed an essential growth factor. Swaby (1945) on the other hand claimed that this treatment had no effect on the time of fermentation. However, he does state that a more severe pasteurization treatment than thirty minutes at 70°C caused some coagulation and the resultant sludge settled on the bottom of the vats harbouring objectionable proteolytic contamination. Jankowska (1954) reported an optimum heat treatment of 85°C for thirty minutes. Surazynski et al (1967) used 68°C for thirty minutes.

2.2.6 pH.

The effect of pH on the growth of organisms and the production of lactic acid from a variety of media have been reported by a number of authors.

The growth of Str. lactis in milk was studied by Rodgers & Whittier (1928). pH control prolonged the exponential growth phase and increased the maximum population. Longsworth and Mac Innes (1935, 1936 a,b) studied the growth of L. acidophilus and the rate of lactic acid production at controlled pH in a synthetic medium. With pH control acid production rate and yield were increased considerably over those obtained without pH control. At controlled pH, the rate of acid production rose to a maximum about the middle of the fermentation period and then declined. The maximum acid production rate correlated with a minimum in the oxidation-reduction potential. The rate of acid production per cell (specific acid production) decreased markedly as growth proceeded.

Krumphazl, Dyr and Kobr (1964) studying the fermentation of a molasses medium by L. delbrueckii found that the highest lactic acid yield was at pH 7.0 although in a sucrose medium the optimum was between pH 5.0 and 6.0. Kempe, Halversen and Piret (1950) found that the yield and rate of lactic acid production in a wheat grit medium were functions of the pH. The pH also affected the requirement for growth factors. They found the average rate of acid production was proportional to the logarithm of the pH.

Finn, Halvorson & Piret (1950) using a glucose, yeast extract and minerals medium found that the increase in pH increased the fermentation rate. Luedeking and Piret (1959 a, b) studied the instantaneous rate of acid formation in batch and continuous cultures of the same medium and found the values of α and β in their equation (2.8) were functions

of the pH.

In commercial fermentation of whey the pH is normally controlled by the periodic addition of calcium carbonate or hydroxide so that the pH is maintained between 5.0 and 6.0. Oetiker (1960) claimed the optimum pH to be 4.2 - 4.6. Swaby (1945) had problems with contamination when the pH was above 5.8.

2.2.7 Continuous Culture

Attempts have been made to use continuous culture to produce lactic acid from whey but not on a commercial scale. Whittier and Rodgers (1931), the earliest report of continuous fermentation of any substrate, described a continuous fermentation with a residence time of one day ($D = 0.042\ h^{-1}$) but Olive (1936) reported a batch process was preferred in the industry because it more readily avoided the side reactions inherent in the continuous process.

Swaby (1945) reported that even when supplemented by yeast, considerable lactose remained unfermented when continuous whey fermentation was attempted. ($D = 0.042\ h^{-1}$).

Siman and Mergl (1961) and Havlatko and Knez (1959) described continuous culture in a five-stage unit. Both found it necessary to replenish the culture in the first stage by inoculating with 1 per cent of fresh culture every 48 hours. The final lactose concentration was 0.1 per cent. Flow rates were low; 1 - 1.25 l/h in 15 l vessels (Havlatko & Knez,1959) and 3.25 l/h in 100 l vessels (Siman & Mergl,1961).

Studies involving lactic acid production from media other than whey are referred to in the review of continuous culture i.e. Luedeking and Piret (1959 a,b) and Humphrey (1963).

2.3 Agitation

In microbial fermentation systems efficient agitation is needed to suspend micro-organisms, to assist heat and mass-transfer, to ensure adequate mixing to avoid local variations in the concentrations of nutrients and products, and, in aerobic systems, to provide sufficient shearing force to ensure a fine dispersion of air bubbles producing maximum area for mass-transfer.

The biological effects of agitation were studied by Dion,Corilli, Sermonti and Chain (1954). As agitation intensity was increased (by alteration of impeller diameter and speed and aeration rate) the morphology of Pencillium chrysogenum changed from thin filamentous

hyphae to short, branched hyphae. At high levels of agitation mechanical damage caused autolysis of the organism. Midler and Finn (1966) concluded that cell damage of a shear-sensitive protozoa was related to the tip speed of the impeller.

For bacterial and yeast cultures it has generally been concluded that, provided agitation is sufficient to ensure suspension of solids, mass transfer to and from the cells is not rate-limiting for growth. (Finn, 1954; Calderbank,1967). The small size of the microbial particles and the low density difference between the cells and the suspending fluid suggest increased agitation will have negligible effect on the transfer of nutrients. Thus with single cells and small chains the rate of transfer is a function only of the properties of the medium. However, if aggregation to form clumps of organisms occurs then a resistance to mass-transfer from liquid to solid may occur.

Kempe and West (1959) found a consistent and reproducible effect of impeller speed on the rate of acid formation by Lactobacillus delbrueckii. The effect was small and varied with the nutrients present. They related the rate of acid formation (dp/dt, g/lh) to impeller speed (n, rpm) by the following expression:

$$\frac{dp}{dt} = a\, n^{0.08} \qquad (2.16)$$

where a is a constant.

West and Gaden (1959) obtained a similar expression for the effect of mechanical agitation on the growth rate of yeast in a well aerated system:

$$dp/dt = bn^{0.15}$$

Calderbank (1967) discussed empirical relationships between the mass-transfer coefficients in solid-liquid suspensions and the agitation intensity. In a given solid-fluid system under conditions of local isotropic turbulence it can be shown from the relationships derived by Calderbank that the mass-transfer coefficient (k_1) is given in a fixed volume system by:

$$k_1 = c\,(P_o)^{0.25} = c_1\, n^{0.75}\, d^{1.50} \qquad (2.17)$$

where c and c_1 are constants. If the rate of transfer of nutrients to the microbial cell (or products away from the cell) limits the growth of the micro-organisms and/or the acid formation then acid formation rate would be expected to follow similar relationships. The value of 0.08 for the exponent of agitator speed obtained by Kempe and West (1959) is less than the value of 0.75 shown in equation (2.17) suggesting that mass-transfer was not limiting acid production in this case.

Keen (1972), in studies of acid production by Streptococcus lactis ML_8 in a skim-milk medium, found, on the contrary, that increased agitation levels caused a decrease in the rate of bacterial growth and acid production. He concluded that in a system from which oxygen was excluded and carbon dioxide was present in adequate concentration the decrease in bacterial growth was the result of the loss of an essential nutrient or enzyme from the immediate vicinity of the cell because of high shear forces. Though his experiments were performed in an essentially unbaffled vessel without pH control, making interpretation of his results difficult, the results do cast some doubts on the validity of the generality of previous work on the effects of agitation in microbial systems.

3. EXPERIMENTAL

3.1 EQUIPMENT

The apparatus designed and built for this study consisted of a culture vessel and control unit (Figs. 3.1, 3.2) similar to that described by Herbert, Phipps and Tempest (1965). In some experiments a Microferm (New Brunswick Scientific Ltd.) was used.

Both batch and continuous culture (chemostat) were possible and provision was made for the continuous measurement and control of temperature and pH. The medium flow-rate and the level of agitation could be varied. By means of a capacitance liquid level meter the volume of alkali required to neutralize the lactic acid formed was recorded, providing a continuous indication of acid production.

3.1.1. Culture vessel

The culture vessel and its associated controls formed a single unit requiring connection to steam, water and electrical services. The tubular steel framework formed three levels, alkali and antifoam reservoirs on the top shelf above the culture vessel, with the medium reservoir, culture receiver and feed pumps on the bottom shelf. All controls were grouped together on an aluminium panel fitted to the framework.

A stock length of industrial Pryex pipeline (Q.V.F.Ltd.) formed the culture vessel (30 cm long, 15 cm dia.). Stainless steel plates (6.3 mm thick) clamped to standard Q.V.F. flanges acted as closures for the vessel. The gaskets were PTFE crescent rings. The top plate carried the stirrer gland and nine ports, the bottom plate a cooling coil, sparge pipe and three ports. The details of the ports are shown in figure 3.3 and the arrangement of the top and bottom plates in figures 3.4 and 3.5.

All flexible connections to the culture vessel ports were made with silicone rubber tubing. Where desired a hose coupling of the type shown in figure 3.3 was inserted.

The culture volume was maintained at 2 l by a 6.3 mm diameter overflow pipe inserted through the bottom plate. Samples of culture fluid were withdrawn directly through a port in the bottom plate. Steam was continuously passed through the sample port.

3.1.2. Agitation

Agitation was provided by a top-entering six-bladed turbine impeller, 51 mm dia., or one of three six-bladed paddle impellers, 25, 51 and 102 mm dia. (Fig.3.6). The design of the stirrer gland is shown in Figure 3.7. The shaft runs in two sealed stainless steel ball races with an air-tight seal

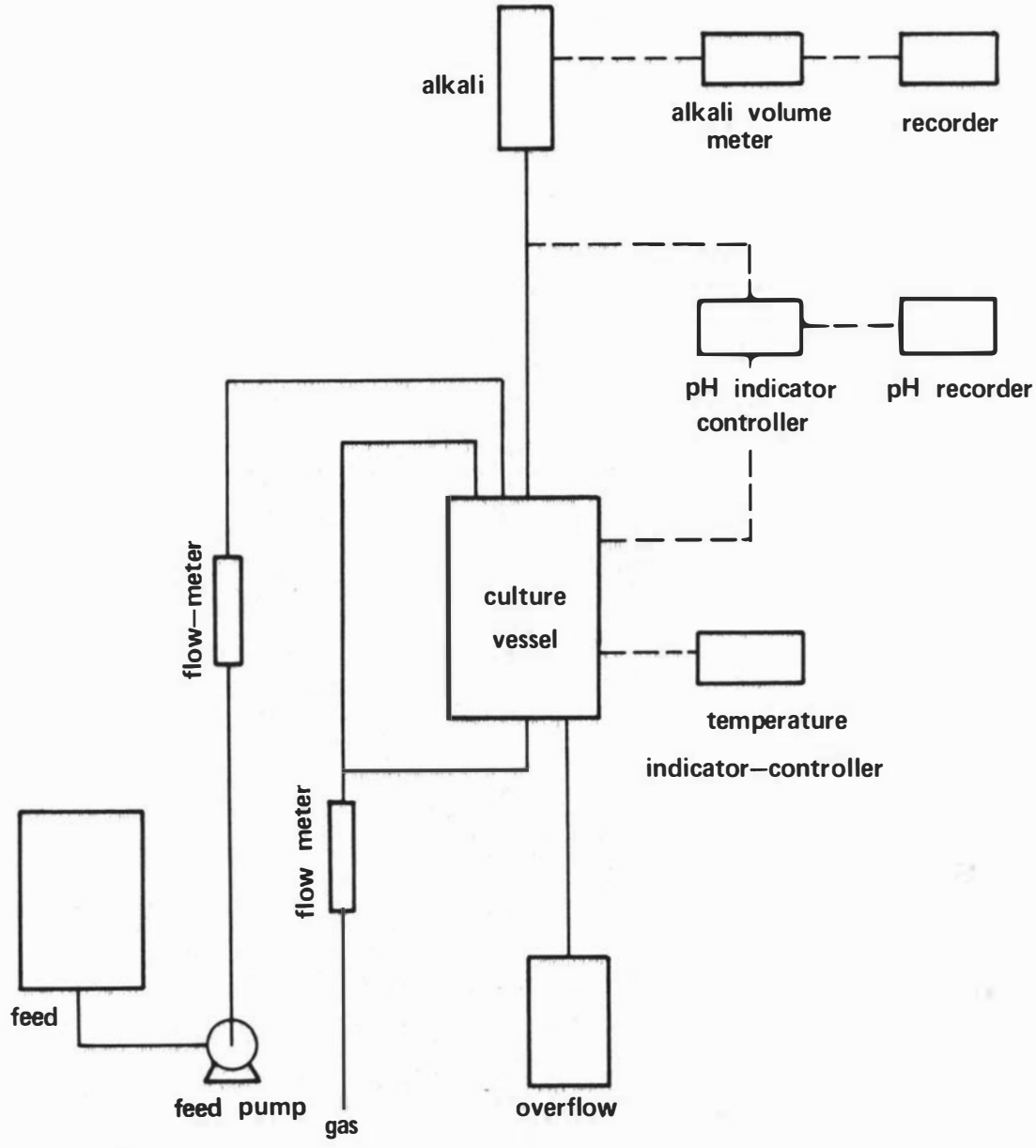


Figure 3.1 Schematic outline of the culture vessel.

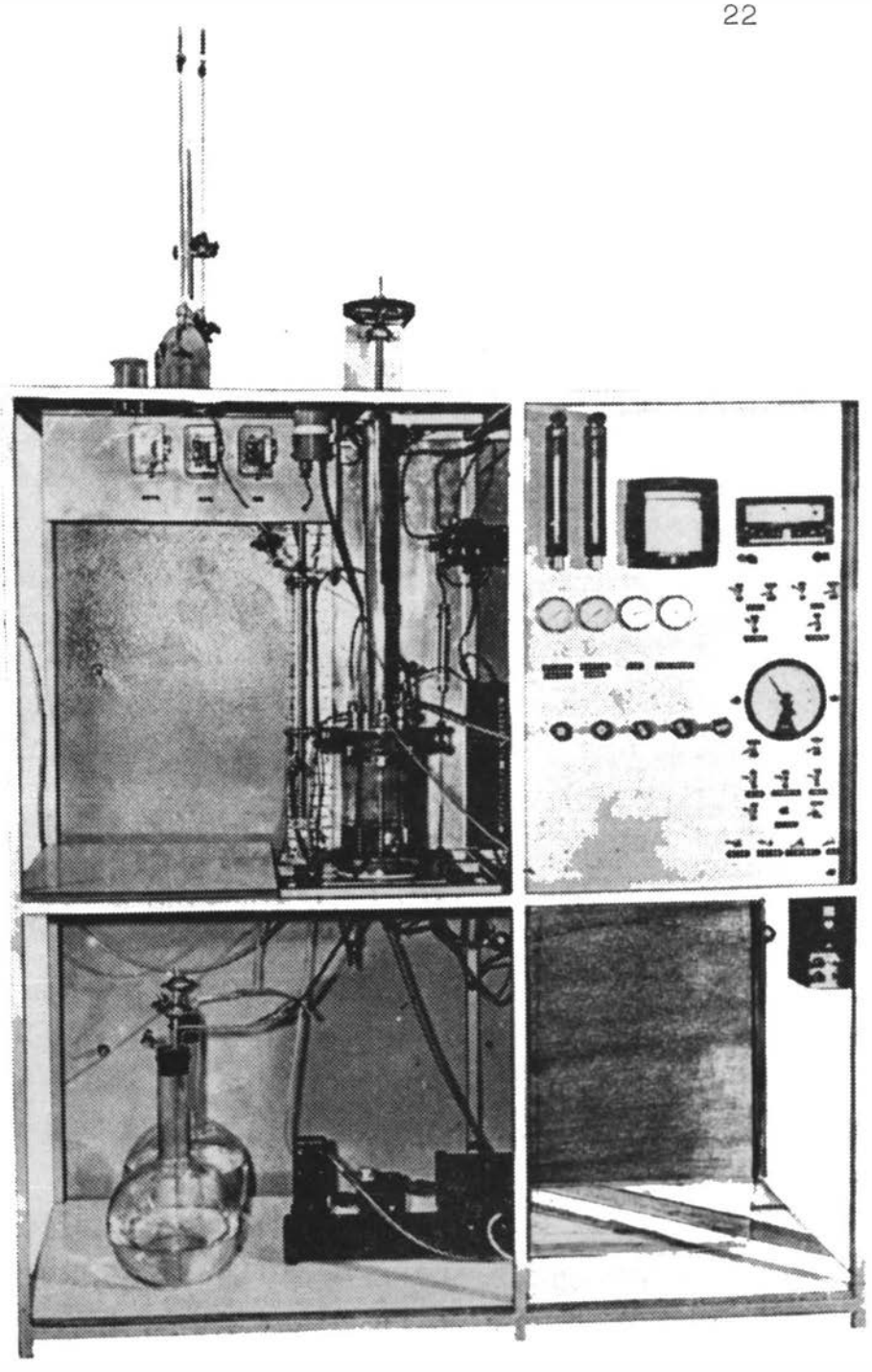


Figure 3.2 The culture vessel and controls.

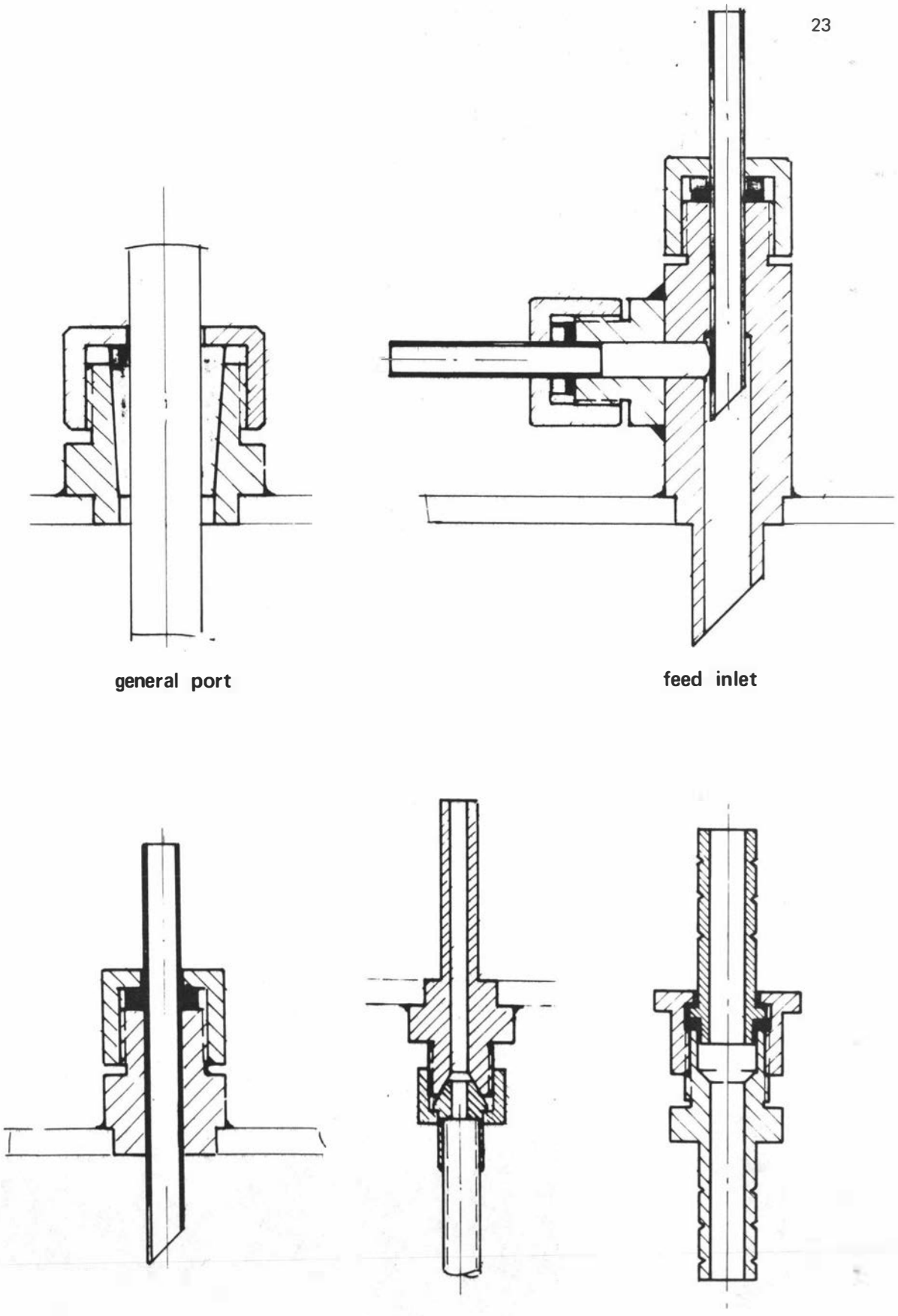


Figure 3.3 Details of the ports in the culture vessel and the coupling used to connect flexible hoses.

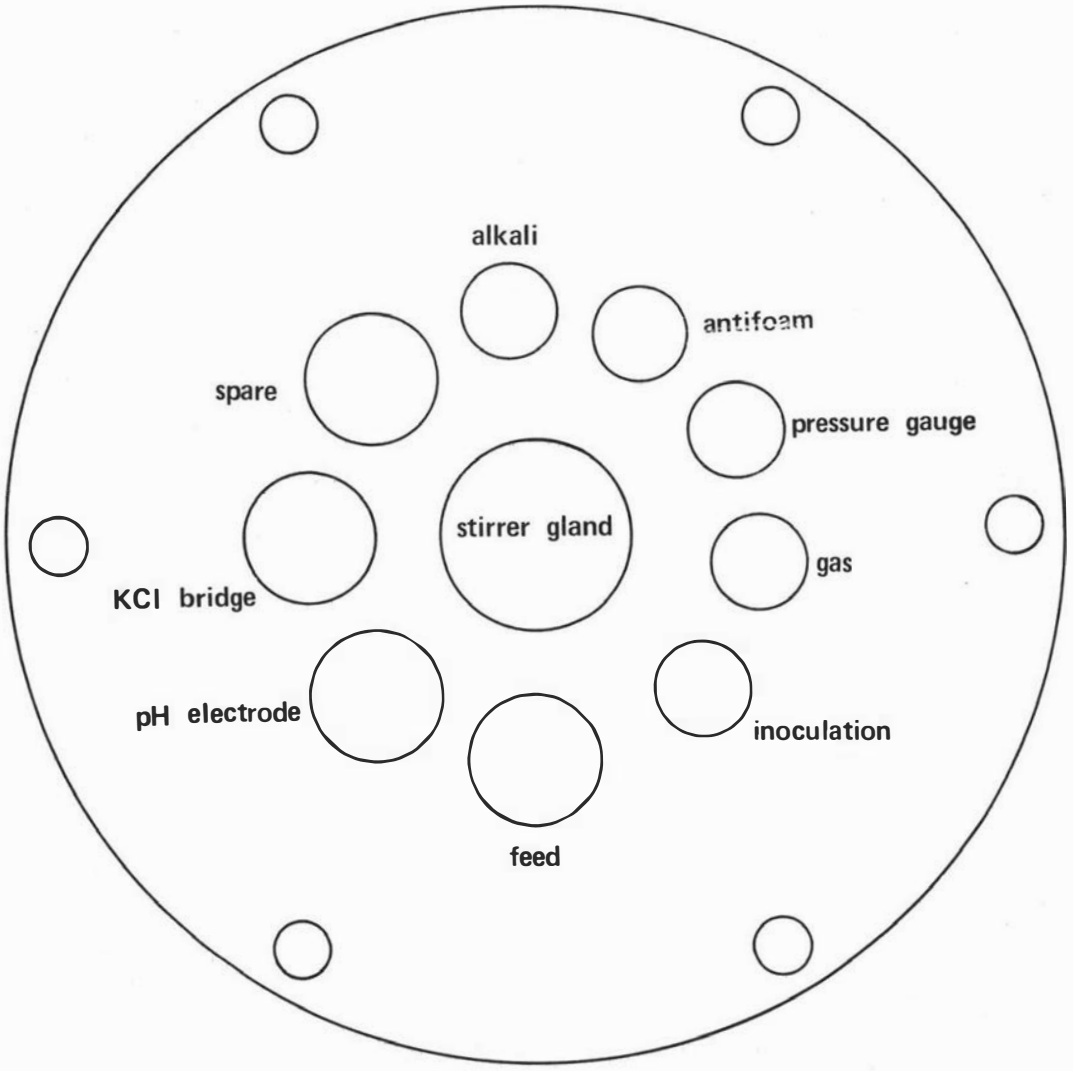


Figure 3.4 Detail of the top plate of the culture vessel.

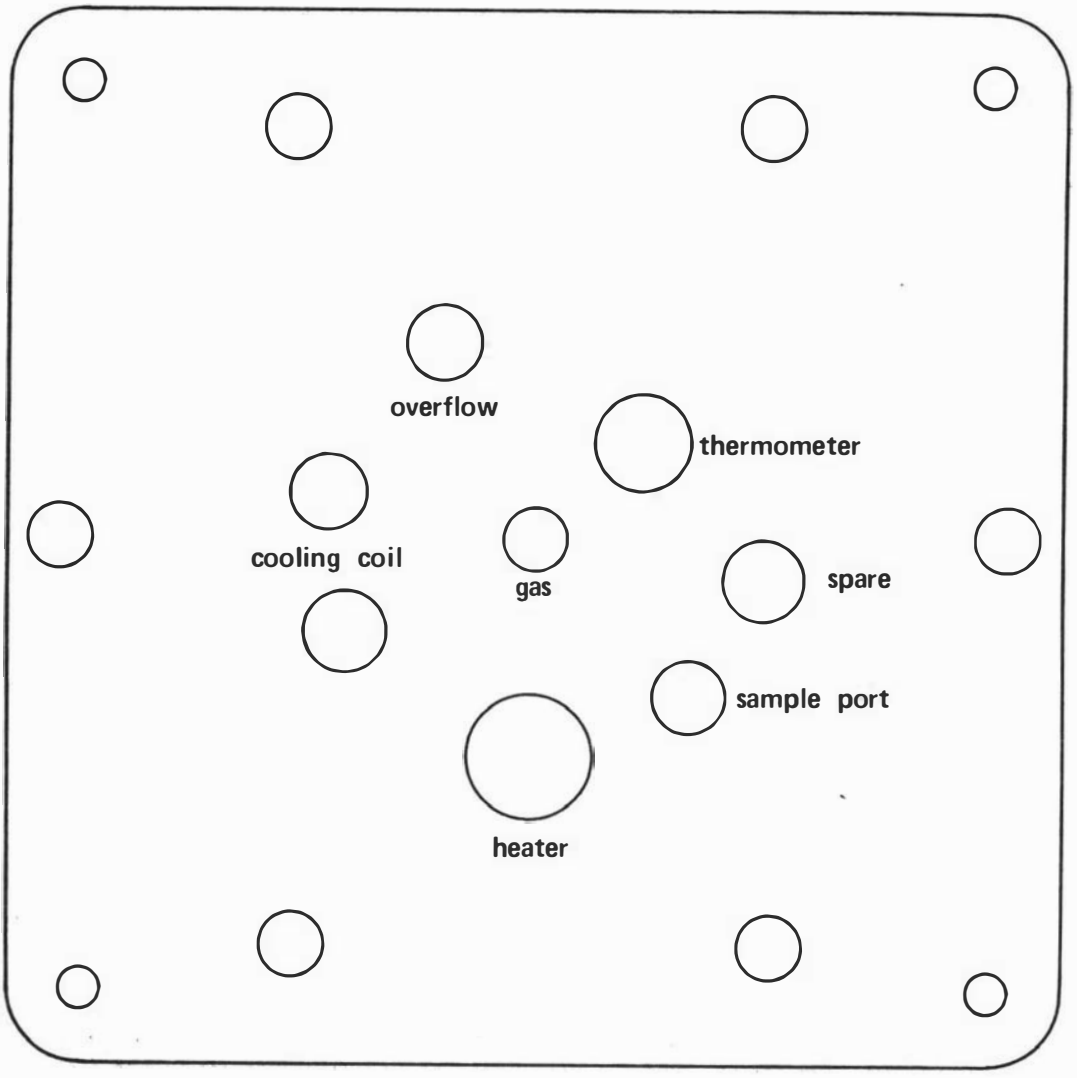
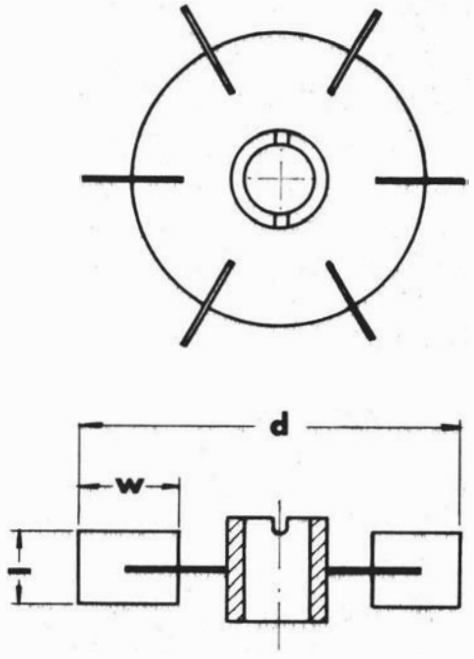


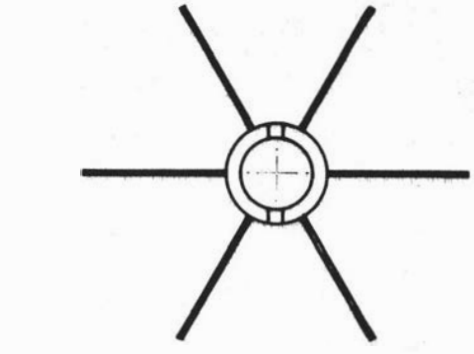
Figure 3.5 Detail of the bottom plate of the culture vessel.

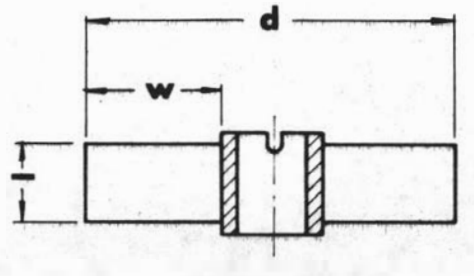


Dimensions mm

d	w	l
paddles		
25	6·4	10
51	19·0	10
102	44·4	10
turbine		
51	12·5	10

turbine impeller





paddle impeller

Figure 3.6 The impellers used for agitating the culture medium.

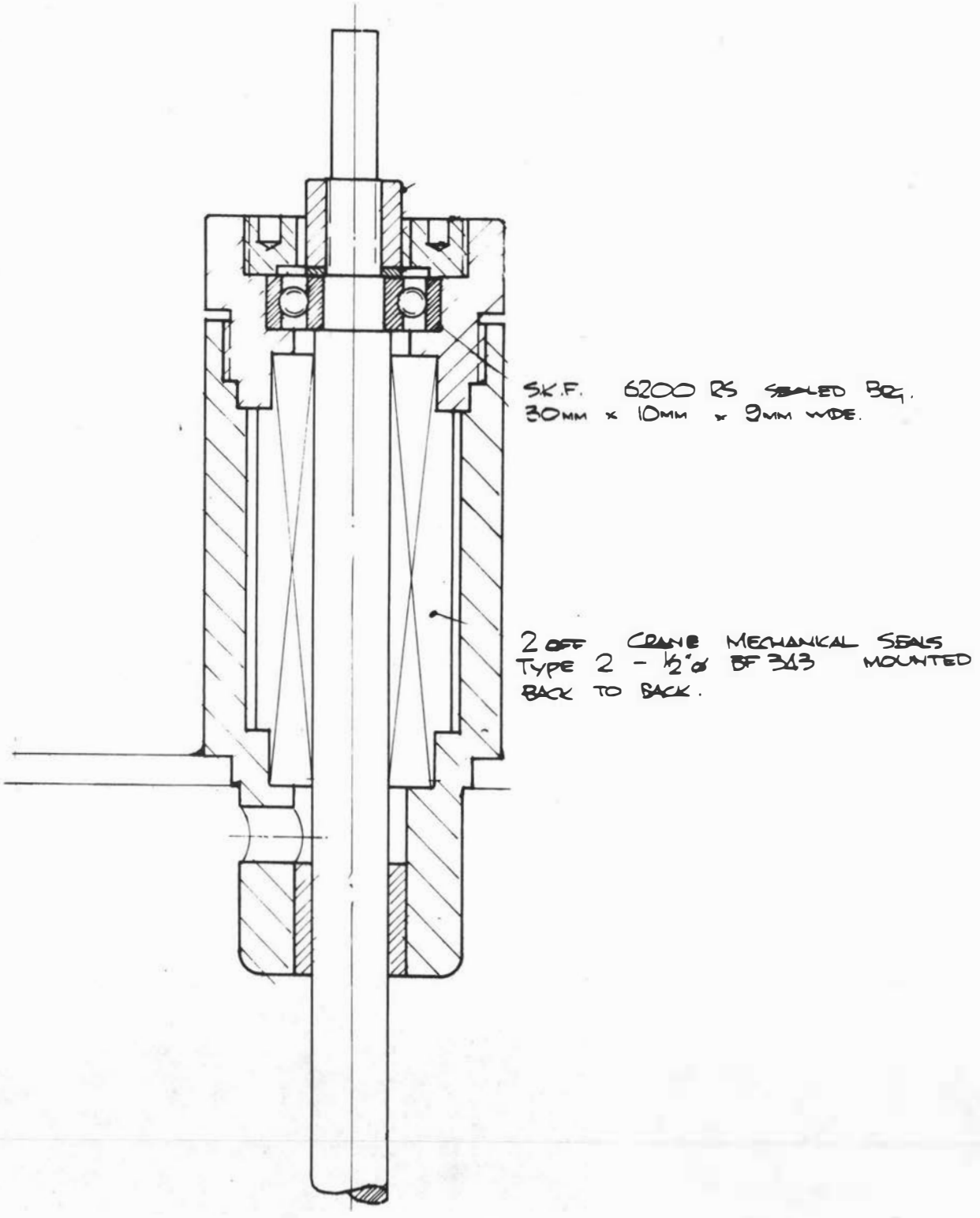


Figure 3.7 **The impeller shaft seal.**

maintained by two mechanical seals "back to back" (John Crane Ltd.,Type T2 BF 343). The seals were lubricated with filtered water.

The impeller was driven,normally at 200 rpm, by a 0.25 hp 1440 rpm motor through a V-belt and pulley. In the mixing experiments this drive was replaced with a continuously variable gearbox (Carter Gears Ltd.).

Four radial baffles (15 mm wide) equally spaced around the periphery of the vessel ensured fully baffled conditions.

3.1.3 Continuous Operation

For continuous culture experiments medium was pumped from a reservoir (5l flat-bottom round Pyrex flask) by a peristaltic pump (Sigma Motor Inc.) controlled by a variable speed gearbox (Revco Zeromax). The flow rate was measured by diverting the liquid to a 10 ml graduated pipette and recording the time for the meniscus to travel between two suitable graduations. The overflow from the vessel was collected in a 20l Pyrex aspirator.

3.1.4 Gas Supply

When required, gas was supplied to the culture vessel through autoclaved tubular glass-wool filters. Air was obtained from the laboratory compressed air system, carbon dioxide was purchased in cylinders from Carbonic Ice Ltd., Palmerston North, and oxygen, nitrogen (dry) and nitrogen (oxygen free) were purchased in cylinders from New Zealand Industrial Gases Ltd.,Palmerston North. The flow of gas was metered by variable area flow meters (Fischer and Porter Flowrators).

3.1.5 Temperature

A platinum resistance thermometer (Sangamo Weston Ltd., Model 110G, Form 4) enclosed in a 6 mm dia. stainless steel sheath in the base of the culture vessel measured the temperature which was indicated and controlled by a Type Tc B2 Electronic Precision Temperature Controller (Fielden Electronics Ltd.). Heating was carried out by a 150 watt heat lamp connected to the control relay. Cooling was provided by water flowing through a coil in the base of the culture vessel controlled by a solenoid valve switched by the controller. The temperature of the culture fluid was maintained within $\pm$ 0.1° C of the set point.

3.1.6 pH

An EIL 91B on-off pH controller (Electronic Instruments Ltd.) indicated and controlled the pH. A steam sterilisable glass electrode (Electronic Instruments Ltd.) and a liquid junction tube, consisting of a ceramic plug sealed into one end of a glass tube, were inserted through the culture vessel lid. The junction tube, filled with saturated KCl, was connected to a

KCl reservoir containing a calomel reference electrode. Both the electrode and the junction tube were protected by stainless steel shields. The pH was continuously recorded on a strip chart recorder (Everett Edgecumbe Ltd.

The controller actuated a solenoid pinch valve to admit alkali from the reservoir. This valve was fabricated from a standard magnetic relay coil. The pH was controlled to ± 0.1 pH units.

3.1.7 Alkali Volume Meter

A unique feature of the design of the apparatus was the device used for recording the rate of acid production. To obtain a full picture of kinetics of fermentation it is desirable to have a measure of the instantaneous rate of acid formation. For homofermentative organisms the alkali additions can be taken as a measure of acid formation. Kempe and West (1959) recorded the volume of alkali required to neutralize lactic acid by means of a float and revolving kymograph drum. Luedeking and Piret (1959 a) abandoned this method because of the greater precision obtained by noting at frequent intervals, the volume of solution retained in the burette which acted as an alkali reservoir.

However, as batch fermentations were likely to last for up to 24 hours and longer, and continuous cultures for possibly weeks, some means of continuously and accurately recording the volume of alkali added was desirable. After considering a number of methods it was concluded that the most suitable was one based on a capacitance level meter, (Davies and Lazenby,1966) using a burette as the reservoir.

An electrode consisting of a vertical stainless steel rod (65 cm long 4.75 mm dia.) sheathed in polytetrafluorethylene (PTFE, 5.20 mm outside dia.) was inserted so that it was concentric with a 100 ml precision burette. A platinum wire sealed through the wall of the burette below the "zero" volume mark earthed the alkali.

The capacitance between the electrode and the alkali was determined by an external measuring circuit connected to an Endress and Hauser Silometer S3:1. The milliamp output, proportional to capacitance, was continuously recorded. Suitable variable capacitances were inserted in the circuit to adjust the capacitance output at "zero" and "100 ml" on the burette to correspond with the 0 and 100 per cent markings on the recorder.

As the level of alkali was reduced in the burette, the change in capacitance was recorded. A calibration curve (Fig.3.8) was determined to convert recorder readings to volume of alkali. Frequent check readings were taken to ensure proper calibration. A typical chart recording is shown in Fig. 3.9.

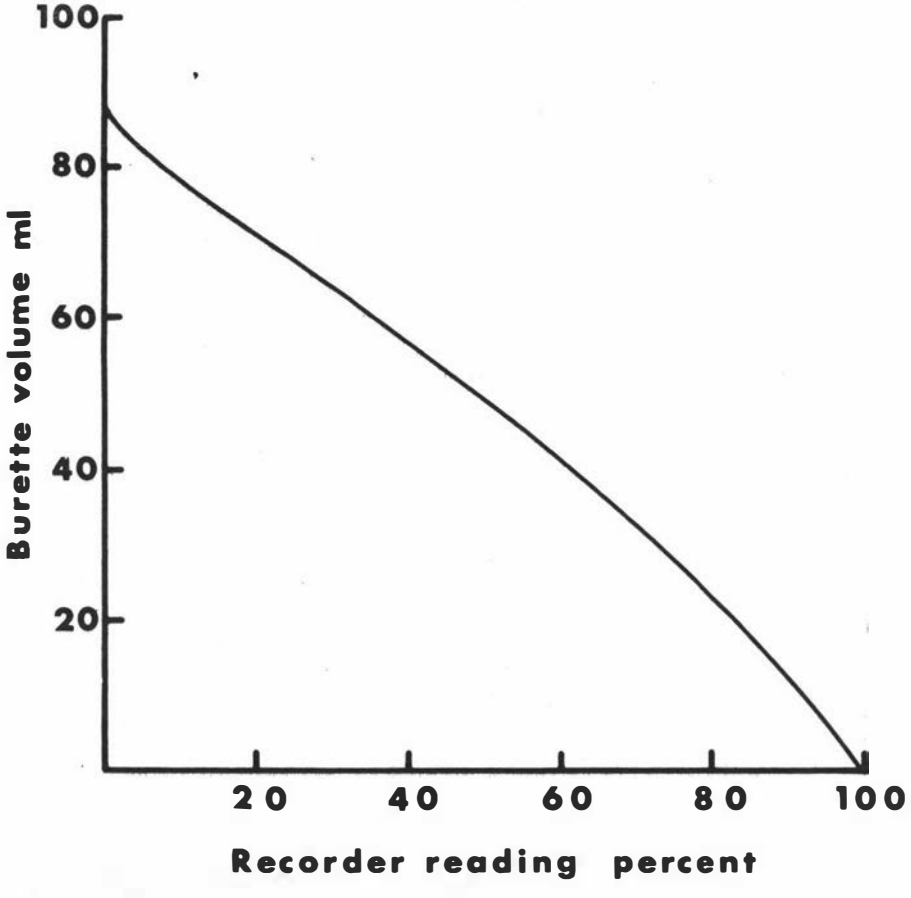


Figure 3.8 The calibration curve for the alkali burette.

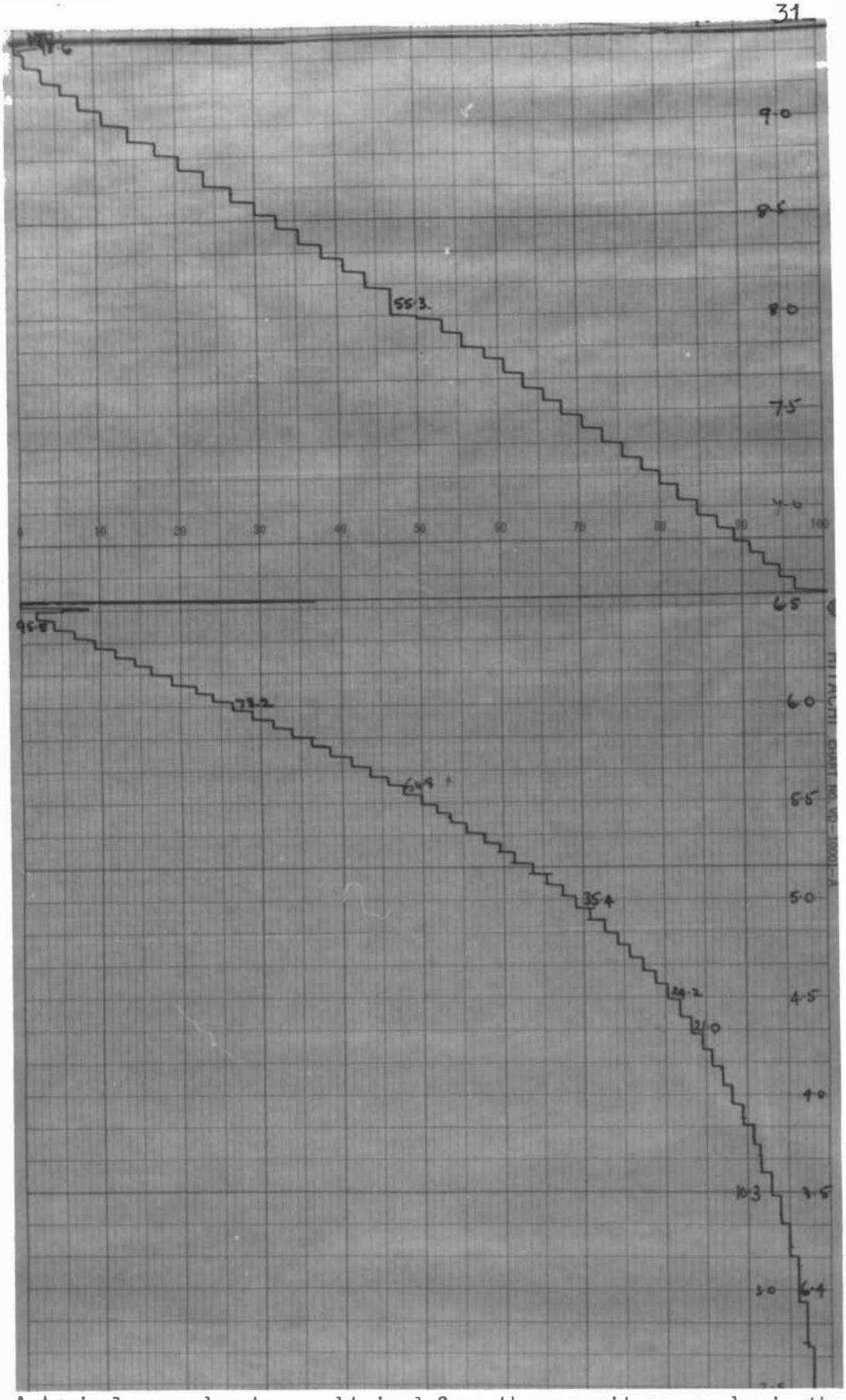


Figure 3.9 A typical recorder trace obtained from the capacitance probe in the alkali burette. Time measured in the vertical direction and volume of alkali in the horizontal. The stepped trace is due to the on-off operation of the pH controller.

When an insulated metallic rod is placed in a conducting liquid its capacitance is a function of the length of immersion and hence differences in capacitance will be a measure of the length of immersion.

For a capacitor in the form of two concentric cylinders, the capacitance is given approximately by (Cotton, 1962):

$$C = \frac{2 \pi K K_o L}{\ln (x/y)} = \frac{55.63 K L}{\ln (x/y)} \quad pF \quad (3.1)$$

Where C is capacitance, farads;

K is the dielectric constant of the material between the plates;

K_o = 8.854×10^{-12} farad/metre

x is the diameter of the outer cylinder, m

y is the diameter of the inner cylinder, m

L is the length of the capacitor, m

The equation (3.1) does not take account of end effects which will affect the linearity of the response.

For the PTFE coated electrode the theoretical capacitance given by equation (3.1) is,

$$C = 1290 L \text{ pF}$$

(K = 2.1. Brydson,1966)

The length of the electrode between the "100ml" mark and the "zero" mark was 56 cm. Thus the total capacitance change for 88 ml (the electrode displaced 12 ml) is

$$C = 723 \text{ pF}$$

The measured values of capacitance are shown in table 3.1

Table 3.1 Measured values of capacitance at different burette volumes for the level meter.

Volume ml	Capacitance pF
100	1000
39.8	623
20	500
0	365

The difference between "full" and "empty" is 635 pF. The difference between the measured value and the theoretical value of 723 pF will be due

to end effects, a value of the dielectric constant different from the assumed value, variations in the diameter of the rod and tubing, and measurement errors.

The first design of an electrode consisted of a length of copper wire (0.69 mm dia.) coated with plasticised polyvinylchloride (PVC. 1.65 mm outside dia.). This displaced a negligible volume of liquid but was abandoned because of slow response and lack of stability.

The slow response was due to the wet conductive skin of alkali which drained slowly from the electrode as the liquid level fell causing the measuring circuit to lag considerably.

The dielectric constant of PVC is relatively high ($K = 3.6$ for PVC *cf* with 2.1 for PTFE at 10^6 c/s, 20° C: Brydson, 1967) but is a function of temperature and frequency.

The dielectric constant of PTFE is virtually independent of temperature and frequency. The nature of the surface considerably reduces the time of drainage of alkali giving a much faster response.

A further advantage of the PTFE coated electrode was the greater sensitivity (change in capacitance from a full burette to an empty burette) as a result of the lower value of the ratio x/y (1.094 *cf* with 2.39 for the PVC electrode). The theoretical sensitivity increased from 129 pF for the PVC electrode to 723 pF for the PTFE electrode.

In practice the PTFE electrode proved very satisfactory and required only minor adjustment once a day.

3.1.8 Cost of the equipment.

The cost of materials and fittings for the culture vessel was $2538. The cost of an equivalent Microferm (New Brunswick Scientific Ltd.) would have been $4,200 excluding the alkali level meter and recorder. Approximately 600 h of design and workshop time were required to build the equipment.

3.1.9 Flask Cultures

A number of experiments were performed in 250 ml or 500 ml conical Erlenmeyer flasks. The flasks were plugged with cotton wool before heat treatment of the culture medium.

For controlled atmosphere experiments the flasks were plugged with rubber bungs through which were inserted two lengths of glass tubing sealed by silicone rubber tubing and screw clamps. After inoculating the medium the flasks were evacuated by a vacuum pump and the air replaced with the required gas mixture introduced through an autoclaved glass-wool filter.

For shake flask experiments a Beckman Rotary Shaker incubator was used (175 rpm, 25 mm throw).

3.2 MATERIALS

3.2.1 Organism

All experiments were conducted using a strain of *Lactobacillus bulgaricus*, designated "LBR". The strain was obtained from a commercial casein factory, and had been derived by X-radiation of a New Zealand Dairy Research Institute rack culture of *L. bulgaricus* (Oetiker,1960). The results of tests carried out by the NZDRI Microbiology Department are consistent with the hypothesis that LBR is a strain of *Lactobacillus bulgaricus* (See Appendix 2). Other organisms held in the NZDRI Culture Collection were tested but produced less acid at a slower rate than LBR.

Lactobacillus bulgaricus is described (Breed, Murray & Smith, 1957; Wheater,1955) as a homofermentative microaerophilic or anaerobic organism fermenting up to 95 per cent of utilized glucose, lactose or galactose to lactic acid by means of the Embden-Meyerhof or glycolytic pathway. It grows as large rods, 2-20 nm long and 1 nm broad, singly or in chains. Older cultures show characteristic granular deposits of volatin. The organism is unable to grow below 20°C and above 51°C. Optimum temperature is 45-48°C. The organism is resistant to acids and can grow at a pH of 3.5. It is considered to be difficult to cultivate, requiring a number of supplementary factors for optimum growth.

To ensure that a pure culture was used in the experiments the following procedure was carried out at six-monthly intervals. MRS agar plates (de Mann *et al.* 1960) were streaked with an actively growing culture of LBR and incubated for 24 hours at 48°C in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide. Up to 50 colonies were picked off into 10 ml quantities of sterile whey and the pH and titratable acidity measured after 6 hours. The fastest acid producers were

microscopically examined to ensure homogeneity and kept as the standard culture.

The culture was maintained in sterile reconstituted skim milk by daily transfer. A 10 per cent inoculum was used and the culture regularly clotted after 2 hours at 48°C. The culture was held at room temperature after it had clotted.

3.2.2. Whey

The original intention had been to use lactic casein whey supplied daily from a commercial dairy company but initial experiments showed that day to day variations in the whey were too great to allow meaningful interpretation of the results.

A quantity of lactic casein whey from Shannon-Tokomaru Co-operative Dairy Company was spray dried at the NZDRI using minimum heat-treatment. The powder was packed in 56 lb multiwall bags and stored at 4°C. An analysis of the powder is shown in Table 3.2.

Table 3.2 Analysis of lactic casein whey powder

Protein	15.8%
Lactose	63.5%
Moisture	2.7%
Ash	11.2%
Fat	0.19%
Titratable acidity, as lactic acid, 7% Solution	0.69%
pH (7% Solution)	4.55
Solubility Index	0.5
Whey Protein Nitrogen Index	4.4

The powder was re-hydrated with distilled water, dispersed in a sufficient quantity of distilled water and agitated with a Polytron Vortex Mixer until the powder had dissolved. The solution was diluted to the required concentration. Normally 70 g/l solids was used, but in some experiments lower concentrations were used to eliminate possible inhibitory effects due to pH or high lactate concentration.

The heat treatment applied to the whey varied throughout the course of the experiments. At first the whey proteins were precipitated by boiling and removed by centrifugation or filtration. The clear solution was then autoclaved. This is called "sterile whey". In later experiments the heat treatment was altered so that the whey proteins were not denatured. The

whey solution was held at 69 ± 1°C for 30 minutes. This is called "pasteurized whey" though it is realised that the treatment described is more severe than normally applied in commercial pasteurization. Tests with skim milk and nutrient broth showed that this pasteurized whey contained no organisms capable of growth or acid production after 48 hours at 45°C.

In a few experiments the whey was sterilized by filtration. The whey powder was hydrated and dissolved as before and made up at twice the required concentration. After centrifuging for ten minutes (Sorvall SS3 9500 rpm, RCF 10,000) the whey was further clarified by filtration through a polishing filter pad (Carlson-Ford grade 6) and finally sterile filtered (Carlson-Ford grade HP/EKS). This is called "sterile-filtered whey".

3.2.3. Chemicals

All chemicals used were of AR or equivalent quality, except where noted.

3.2.3. 1. Supplementary nutrients solutions. Stock solutions of supplementary nutrients were prepared, sterile filtered and stored at 4°C. When required 100 ml of each solution was mixed, made up to 1l with water and sterile filtered again.

The nutrients used, with the quantities per litre in the stock solution and the final whey medium are shown in Table 3.3.

One litre of the stock salt solution was prepared by dissolving the required quantity of each salt in a minimum amount of distilled water, mixing all such solutions, adding a few drops of concentrated hydrochloric acid to dissolve the slight precipitate which formed and making up to one litre.

The amino acid stock solution was prepared by dissolving the acids in hot distilled water adding concentrated hydrochloric acid drop by drop until the solution was clear.

The stock solution of vitamins was prepared by dissolving 2 g L-ascorbic acid, 400 mg of each of choline chloride and inositol, 20 mg of each of niacin (BDH), thiamin hydrochloride (Eastman Kodak) and calcium pantothenate (BDH) and 1 mg of pyridoxal hydrochloride (L.Light & Co.) in 500 ml 20 per cent (v/v) ethanol in water. Riboflavin, 20 mg, was dissolved in acidified 20 per cent (v/v) ethanol in water and added to the above solution. Ten ml quantities of p-amino-benzoic acid (BDH) solution (2 mg in 100 ml distilled water) and D-biotin (L.Light & Co.) solution (2 mg in 100 ml distilled water) were added. Folic acid (BDH), 2 mg, was dissolved in distilled water with a few drops of 10% (w/v) sodium hydroxide added, made up to 100 ml and 1 ml of this added to the stock solution. Vitamin B12

Table 3.3 Supplementary nutrients

a: quantity per litre of stock solution

b: quantity per litre of medium

A	Salts	a	b		a	b
	$NaC_2H_3O_2$ $3H_2O$	40g	2g	$FeSO_4$ $7H_2O$	0.2g	10mg
	$NaC_4H_8O_7$	40g	2g	$MnSO_4$ $4H_2O$	0.2g	10mg
	NH_4Cl	40g	2g	NaCl	0.2g	10mg
	KH_2PO_4	10g	0.5g	$CoCl_3$	0.1g	5mg
	K_2H PO_4	10g	0.5g	Zn SO_4	0.1g	5mg
	$MgSO_4$ $7H_2O$	4g	0.2g	Na_2Mo O_4	0.1g	5mg
	$CaCl_2$ $6H_2O$	2g	0.1g	$CuSO_4$ $5H_2O$	0.1g	5mg
B	Vitamins	a	b		a	b
	Niacin	20mg	1mg	L-ascorbic acid	2g	100mg
	Thiamin HCl	20mg	1mg	Pyridoxal HCl	1mg	50µg
	Ca.pantothenate	20mg	1mg	PAB	200µg	10µg
	Riboflavin	20mg	1mg	Biotin	200µg	10µg
	Choline chloride	400mg	20mg	Folic acid	20µg	1µg
	Inositol	400mg	20mg	Vitamin B12	20µg	1µg
C	Amino acids				a	b
	Vitamin free casamino acids				20g	1g
	D-L-tryptophan				1g	50mg
	L-cysteine HCl				1g	50mg
	L-cystine				0.5g	25mg
D	Nucleic acid compounds				a	b
	Adenine				0.2g	10mg
	Guanine				0.2g	10mg
	Uracil				0.2g	10mg
	Xanthine				0.2g	10mg

(Glaxo Co.) was prepared by making one ampoule containing 250µg up to 25 ml with distilled water and adding 2 ml of this to the stock solution. One ml of glacial acetic acid was added to the stock solution, and the volume made up to 1 l with distilled water.

To prepare the nucleic acid compound stock solutions 0.2 g of each

adenine (L.Light & Co.), guanine (BDH) and uracil was dissolved in boiling distilled water acidified with just sufficient concentrated hydrochloric acid to effect solution. Xanthine, 0.2g, was dissolved in boiling water, solution being aided by a few drops of 10% (w/v) sodium hydroxide. Each of these two solutions was made up to 500 ml and stored separately.

3.2.3.2 Sodium Caseinate (NaCas)

Sodium caseinate was obtained from Rangitaiki Plains Dairy Co. and where appropriate the production batch is identified. Solutions were prepared by dissolving the sodium caseinate in hot water and autoclaving (15 psi, 20 minutes). The pH of whey solutions was adjusted to above 5.5 before adding sodium caseinate solutions.

3.3 MEASUREMENTS

Routine determinations were made of cell concentration, lactose concentration, and lactate concentration and a record was kept of the volume of alkali added to maintain the pH at a constant value.

3.3.1 Cell Concentration

In a study of bacterial kinetics the most appropriate measurement is "bacterial density" rather than "cell concentration"(Monod,1949). The former is determined by dry mass measurements, or indirectly by turbidimetric methods or by cell volume after centrifuging. The latter is measured by cell counting or plate counting techniques.

Bacterial density is the more appropriate measurement because it is more closely related to the quantity of enzyme present.

The bacterial density could also be determined indirectly by the measurement of some cellular components such as nitrogen, RNA,DNA,etc. However these can be shown to vary with growth rate although DNA per unit of bacterial protoplasm mass varies only slightly. The measurement of products of metabolism, another technique used, is of limited value as an indication of growth.

It was found to be difficult to use dry mass, turbidimetric or centrifugation techniques in the whey medium used in these experiemnts. The medium was opaque and the amount of centrifugable solids varied with time,temperature and pH.

Therefore it was necessary to use a cell counting technique as an indication of growth, despite the limitations of this type of method. Plate counting was rejected because of the time involved to obtain results and the difficulty of allowing for the variable chain length. On a few occasions the concentration of DNA was measured using the method of Burton

(1956) but this was not used routinely because of the time involved.

The method adopted for cell counting was a slight modification of the direct microscopic method of Duitschaever & Leggat (1965). The stain was prepared by mixing 0.6 g methylene blue in 52 ml 95 per cent ethanol and 44 ml tetrachloroethane and holding at 45°C until the methylene blue was dissolved. After cooling, 4 ml glacial acetic acid was added and the solution filtered. Two ml saturated alcoholic solution of basic fuchsin (1 g in 15 ml 95 per cent ethanol) was then added.

Suitable dilutions of the culture were made in 1 per cent sterile reconstituted skim milk (the skim milk was necessary to prevent lifting of the smears when washed) and 0.01 ml spread over a 1 cm square on a clean glass slide. The film was dried at 45°C and the stain applied for 1 minute. After drying, the stained smear was washed by gentle agitation in warm water. The dried smear was microscopically examined using an oil immersion lens. The cells were stained blue against a pink background and normally the divisions between cells in the chain were clearly discernible. A total of 15-30 randomly selected fields was counted and record kept of cell concentration and chain number.

3.3.2 Lactose Concentration

The reducing sugar concentration of the culture medium was routinely determined by the method of Wahba (1965). In some experiments the method of Lane & Eynon (McDowell & Dolby, 1941) was used. The latter is the standard method used by the NZDRI Analytical Chemistry Section but the former has the advantage of requiring smaller volumes of culture medium for each analysis.

The method of Wahba involves the reaction of lactose with excess phenylhydrazine to form the yellow coloured lactosozone which is soluble in acetic acid. The concentration of lactosozone is determined from the optical density at 370 nm.

The stock solution of phenylhydrazine was prepared by dissolving 2.5 g phenylhydrazine hydrochloride (recrystallized from ethanol) in 200 ml distilled water, adding 12.5 g sodium metabisulphite and diluting to 250 ml. The stock solution was stored in a brown glass stoppered bottle and renewed each month. When required 10 ml of stock phenylhydrazine solution was diluted to 100 ml with glacial acetic acid.

All solutions for analysis were deproteinized with a 2 per cent (w/v) aqueous solution of zinc acetate.

Stock standard solutions containing 1, 3 and 5 per cent lactose monohydrate were prepared weekly and stored at 3°C.

All glassware was cleaned with chromic acid followed by hot detergent solution and well rinsed with distilled water.

One or two ml (so that final solution was 50-250 μg/ml lactose monohydrate) of the solution to be analysed was pipetted into a 200 ml calibrated flask, 1 ml of zinc acetate solution added, mixed and made up to the mark with distilled water. Ten ml quantities were centrifuged (RCF 3100) for 10 minutes. Ten ml of phenylhydrazine working solution was added to 1 ml of centrifugate in a Pyrex boiling tube, the contents of the tube mixed, tube stoppered and placed in a boiling water bath for 1 hour. The tube contents were mixed and cooled to room temperature. The optical density was measured at 370 nm (Bausch and Loumb, Spectronic 20, 1 cm square cuvettes). Lactose standards and a distilled water blank were treated in the same way. The results, expressed as concentration of lactose monohydrate, were calculated by reference to the standards included in each batch of analyses. All analyses were performed in duplicate. The standard deviation, determined by 20 determinations of the same whey sample, was 2.55×10^{-2} g/l.

Figure 3.10 is a typical calibration line.

The phenylhydrazine determination was compared with the Lane & Eynon method by having a number of analyses performed on three solutions; (a) whey with 35 g/l solids, (b) solution a with 2.5 g/l of lactose added, (c) solution a which had been partially fermented.

The results are shown in Table 3.4.

Table 3.4 Lactose determinations by the method of Wahba (A) and Lane & Eynon (B) on three whey solutions.

Mean ± 95% limits (No. of determinations) g/l

Solution	Method A	Method B
(a) 35 g/l whey solids	23.9 ± 0.3 (12)	23.1 ± 0.2 (18)
(b) 35 g/l whey + 2.5 g/l lactose	26.1 ± 0.5 (12)	25.7 ± 0.2 (18)
(c) Partially fermented whey	15.1 ± 0.4 (12)	13.6 ± 0.2 (18)

The variance-ratio test shows that the method of Wahba has a significantly higher variance and gives a mean lactose concentration 5 per cent higher than the Lane & Eynon method. However, it was considered that the advantages of small sample volumes outweighed the slightly higher results and variability of the Wahba method.

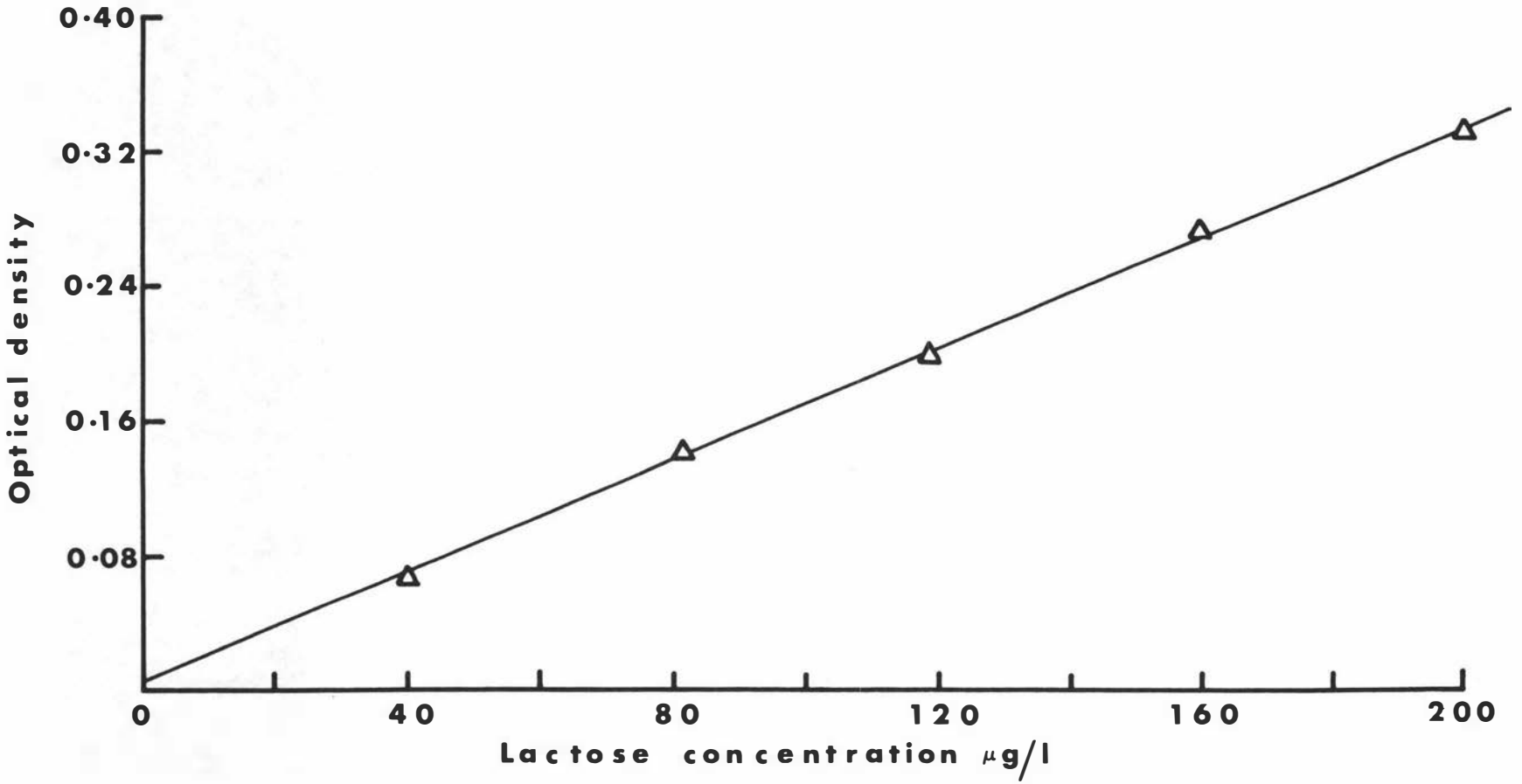


Figure 3.10 A typical lactose calibration. Optical density at 370 nm of the lactosozone solution formed by reaction of lactose with phenylhydrazine as a function of lactose concentration.

3.3.3 Lactate Concentration

The lactic acid was determined as it was formed by recording the additions of an alkali solution (normally 2-N sodium carbonate containing 5 g/l sodium hexametaphosphate, but in the early experiments 10 - N sodium hydroxide) required to maintain the pH constant. In flask cultures without pH control, the change in titratable acidity (to pH 8.5) was taken as a measure of lactic acid synthesis.

The amount of lactic acid formed as determined by the amount of alkali added was adjusted to correct for undissociated acid. In batch cultures at controlled pH the amount of acid was corrected for dilution by the neutralizing liquid and for removal of samples using the procedure of Longsworth and MacInnes (1936b). In continuous cultures the alkali added was corrected for the amount required to adjust the pH of the feed to the pH of the fermentation. The amount of the correction was determined by titrating aliquots of the feed to the required pH using 0.1-N sodium hydroxide.

The validity of measuring lactic acid synthesis from the volume of alkali added was confirmed a number of times by analysing the culture fluid specifically for lactate by the colourmetric method of Steinsholt and Calbert (1960). This method uses ferric chloride to develope a colour in a deproteinated culture fluid. Lithium lactate solutions were used as standards.

For 31 comparitive determinations (ranging from 0.1 to 41.8 g/l lactate) the mean difference between the observed lactate (from alkali determination) and expected lactate (from analytical method) was 0.1 g/l with a standard deviation of 0.97 g/l.

Student's t - test was used to compare the mean difference with zero

$$t = \frac{0.10 \times \sqrt{31}}{0.97} = 0.59$$

$$t_{(30,0.5)} = 2.04 \qquad \text{(Davies,1963)}$$

Thus the hypothesis that the difference in readings does not differ from zero can be accepted.

3.4 METHODS

3.4.1 Batch Culture

For batch cultures in the culture vessel whey was prepared by one of the methods described in 3.2.2. The culture vessel and attached lines which could not be autoclaved, were soaked overnight where possible, but for at least one hour, in an iodophor solution containing 100 mg/l iodine. After draining the iodophor solution from the vessel,steam at atmospheric pressure was

passed through the attached lines and vessel for at least one hour. When the steam was turned off a flow of sterile gas (air or CO_2 - N_2 mixture) was passed into the vessel at 0.25 l/h to maintain a low positive pressure.

The whey and required nutrients were added aseptically to the vessel. When the temperature, pH and agitation conditions had been established at their required level and the alkali meter and recorder correctly zeroed the medium was inoculated.

Two types of inoculum were used. Normally 20 - 100 ml of a freshly clotted skim-milk culture of LBR was added. The skim-milk was prepared by autoclaving (160°C, 5 minutes) a 10 per cent solution of spray-dried skim-milk powder. When it was desired to reduce the amount of skim-milk solids added to the culture, a "whey inoculum" was used. A conical flask containing the required volume of a 70 g/l solution of reconstituted whey powder was inoculated with 1 per cent of a clotted skim milk culture of LBR and incubated overnight at 48°C. This was used to inoculate the contents of the culture vessel. The pH of this inoculum was below 4.0.

Throughout the course of the batch culture the amount of alkali added to retain the pH constant was continuously recorded. Periodically the volume remaining in the alkali burette was compared with the chart recording and the latter adjusted if necessary. Samples of the culture fluid were taken for analysis of cell concentration, lactose, and lactate if required and for checking the pH of the solution using a laboratory pH-meter. The volume of the samples withdrawn was recorded.

3.4.2 Continuous Culture

The initial stages of a continuous culture experiments were as for a batch culture. The feed vessel, the attached flow metering device and the overflow receiver were separately autoclaved.

When the required conditions in the batch culture were reached, the flow of medium to the vessel was started. If nutrients were to be added to the whey a second feed stream, containing the required nutrients was pumped into the vessel at the appropriate rate. The volume of liquid collected in the overflow receiver was measured as a check that the flow-rate had remained constant. Samples for analysis of steady-state conditions were not taken until a time equivalent to at least five residence times had elapsed from the time of changing the conditions or the chart recording of alkali addition indicated a steady-state had been achieved, which ever was the longer time.

3.4.3 Flask Cultures

When sterile whey was required the whey was added to the flasks and autoclaved *in situ*. Sterile-filtered whey was added aseptically to previously autoclaved flasks. To prepare pasteurized whey, the reconstituted whey was added to previously autoclaved flasks and the flasks placed in a water-bath at 69°C for 30 minutes.

The pH of the whey was adjusted to 6.5 before heat treatment by the addition of 2-N sodium carbonate. If necessary the pH was aseptically readjusted after heat treatment.

The inoculum was prepared by either of the methods described above and a volume equivalent to 1 per cent of the volume of medium in the flask was added to each flask.

3.5 Method of expressing results

In considering the commercial production of lactic acid from whey the important criteria are the concentration of lactate in the spent medium, and the productivity of the culture equipment. The productivity is the amount of lactate produced in a unit of culture vessel volume in unit time. If this is high the equipment and hence capital expenditure is being used to best effect. The concentration of lactate in the spent medium should also be high to facilitate subsequent recovery operations.

If the lactate concentration is high then the remaining lactose level will be low, an advantage when the process is being considered as a means of reducing pollution.

Throughout this work the aim has been to increase lactate concentration, to improve productivity and to obtain a yield as high as possible. Yield is defined as the ratio of the amount of lactate obtained from the culture vessel to the amount of lactose added.

The three criteria of productivity, lactate or acid concentration and yield have been considered as dependent variables throughout this work.

4.1 Selection of Variables

A series of continuous culture experiments was carried out to select the major variables to be studied. For a number of reasons the results are thought to be not reliable. For completeness and to illustrate the techniques used a report of the experiments is included as appendix 6.

4.2 BATCH CULTURE

4.2.1 Typical batch culture*

The results from a number of batch cultures are shown in Tables 4.1 and 4.2 and figures 4.1 - 4.5 and are typical of all batch experiments carried out. The rate of acid production (dP/dt) as listed in Table 4.1 was measured by visual determination of the slope of a large scale plot of figure 4.1.

As shown in figure 4.2 cell numbers, after an initial phase of approximately 1.5 h increased exponentially from 2×10^{10} to 8×10^{11} cells/l in 3 h. The specific growth rate, calculated from "the line of best fit" is 1.17 h^{-1}. The acid concentration increased by essentially the same exponential rate in this time (1.22 h^{-1}).

The bacterial population then entered a phase of declining growth rate and reached an apparent stationary phase approximately 7 h after inoculation.

The rate of acid synthesis was a maximum aproximately 5 h after inoculation. It then fell to a steady value of 0.97 g/lh as the bacteria entered the stationary growth phase (fig.4.3). This linear increase in acid concentration was maintained for more than 9 h during which over half of the total acid produced was synthesised. The acid production rate then dropped, reducing to zero as the lactose was depleted. (See fig.4.4)

To confirm that the cell numbers, as measured,were representative of the changes in bacterial concentration a number of determinations was made of the increase in DNA concentration. Figure 4.5 shows that DNA followed essentially the same pattern as the cell numbers.

The constant linear increase in acid concentration was so typical of all batch fermentations and represented a significant proportion of the total acid produced that it has been used to characterize many of the results in this work.Where necessary the exponential rate of acid increase is also reported.

*Note: Though the significance of the type of batch curve obtained was not realised until many of the experiments reported had been performed a brief description is presented here to facilitate the presentation of the results.

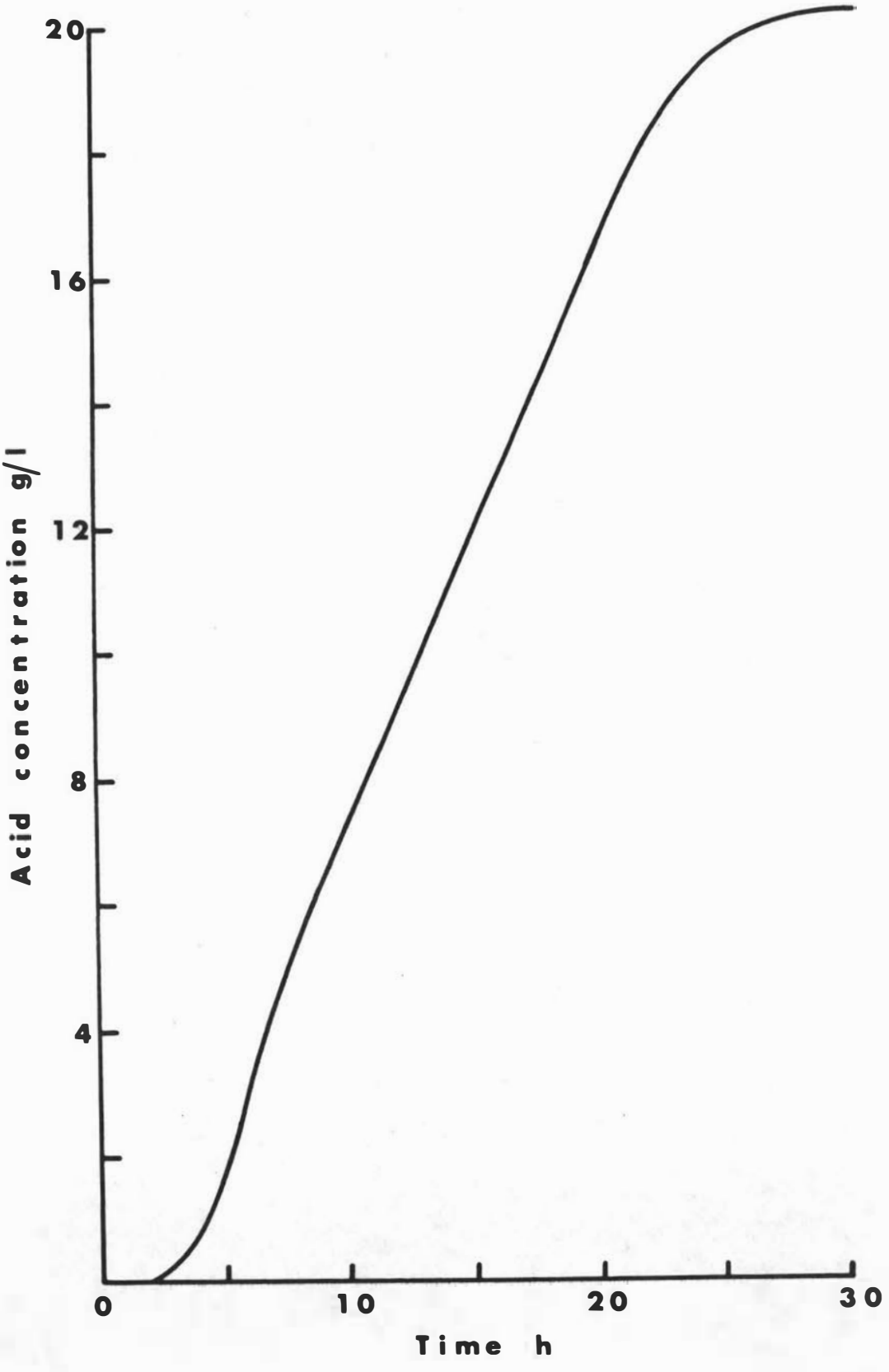


Figure 4.1 A typical batch culture. B9.1.5. Acid concentration as a function of time. Whey 35 g/l, pH6.0, 46°C.

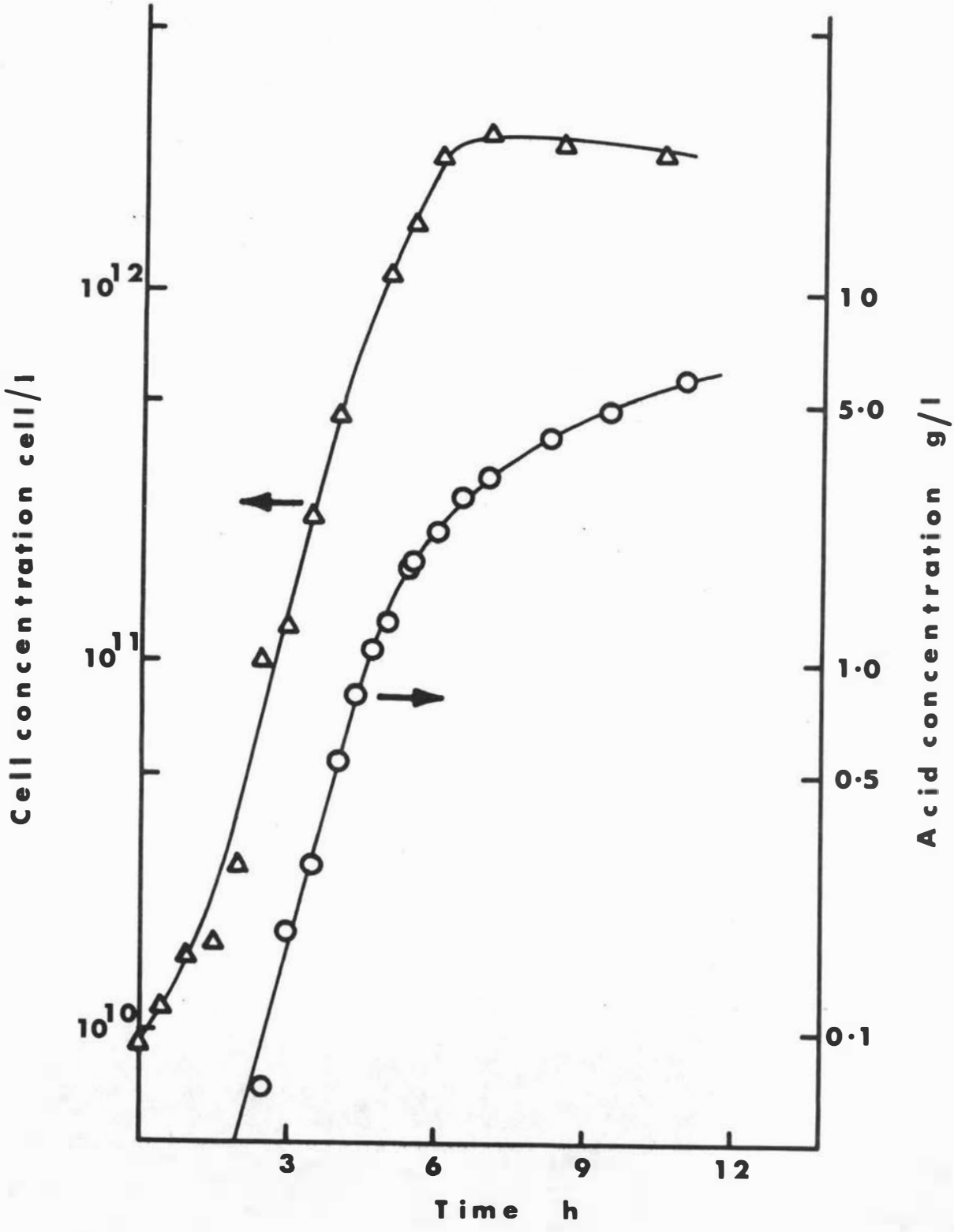


Figure 4.2 A typical batch culture; B9.1.5; ln cell number concentration and ln acid concentration as functions of time. Whey 35 g/l, pH6.0, 46°C.

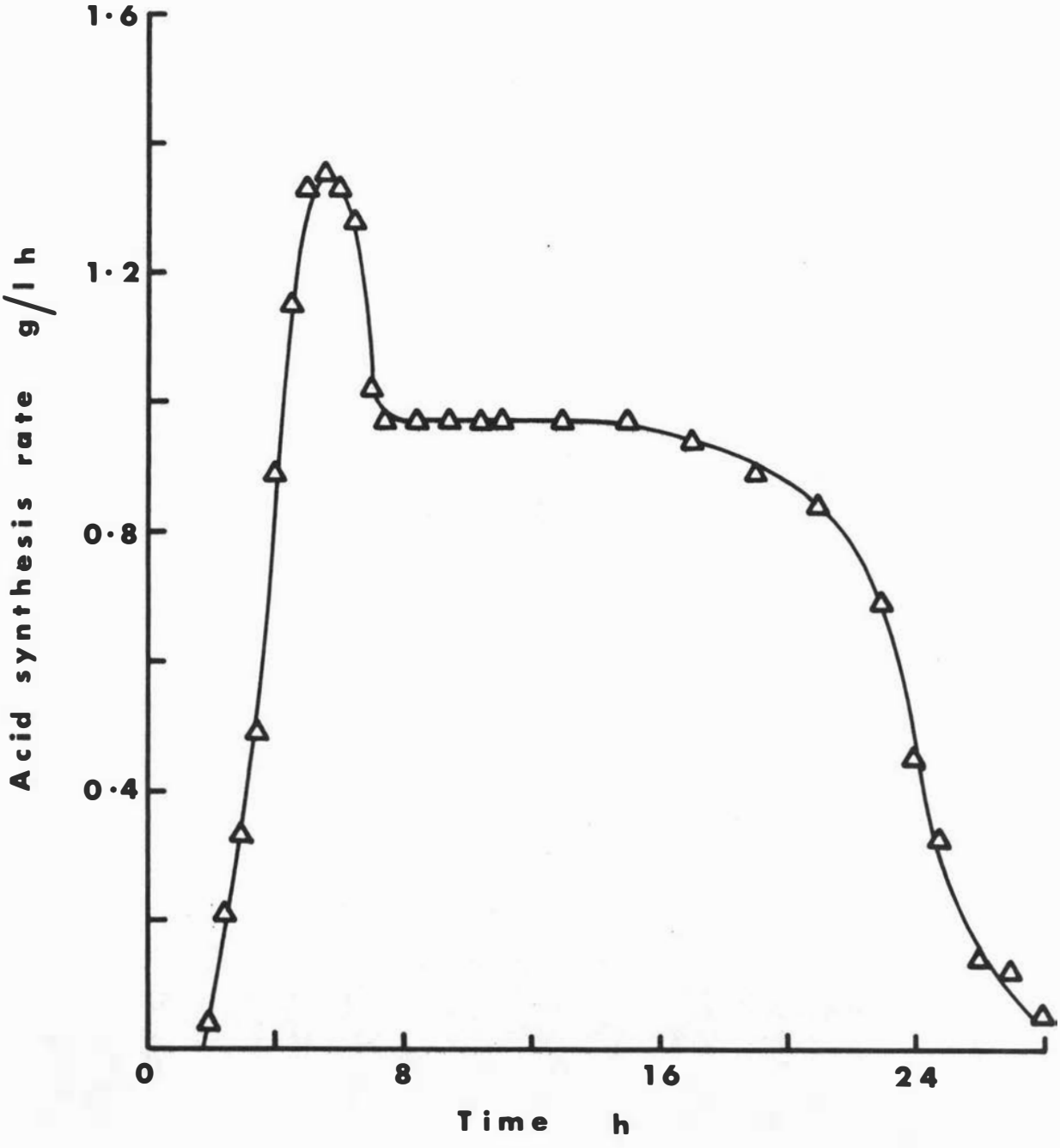


Figure 4.3 A typical batch culture; B9.1.5; the rate of acid formation as a function of time. Whey 35 g/l, pH6.0, 46°C.

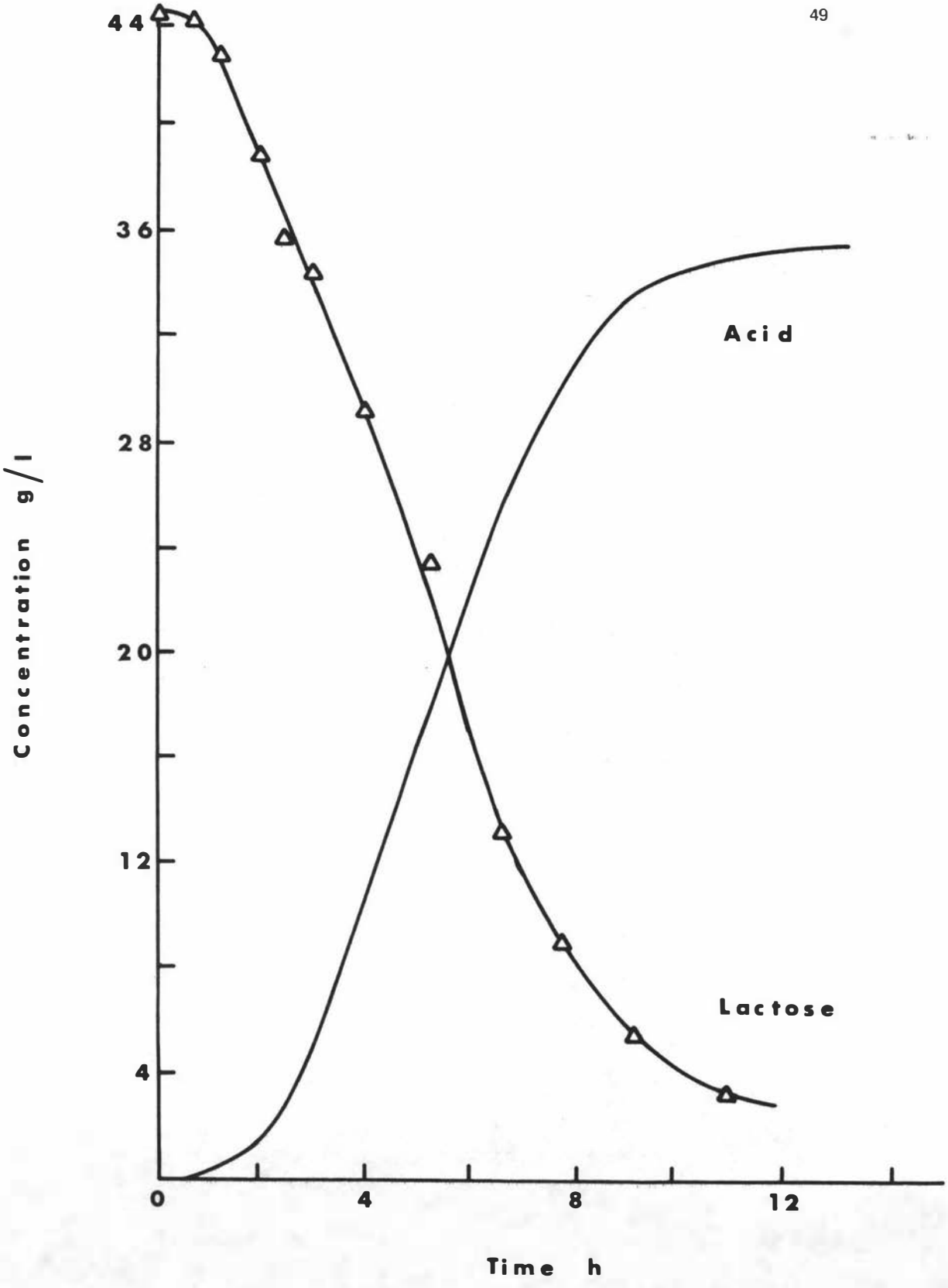


Figure 4.4 Acid and lactose concentrations during the batch culture of whey. Whey 75 g/l with supplementary nutrients, pH5.5, 46°C.

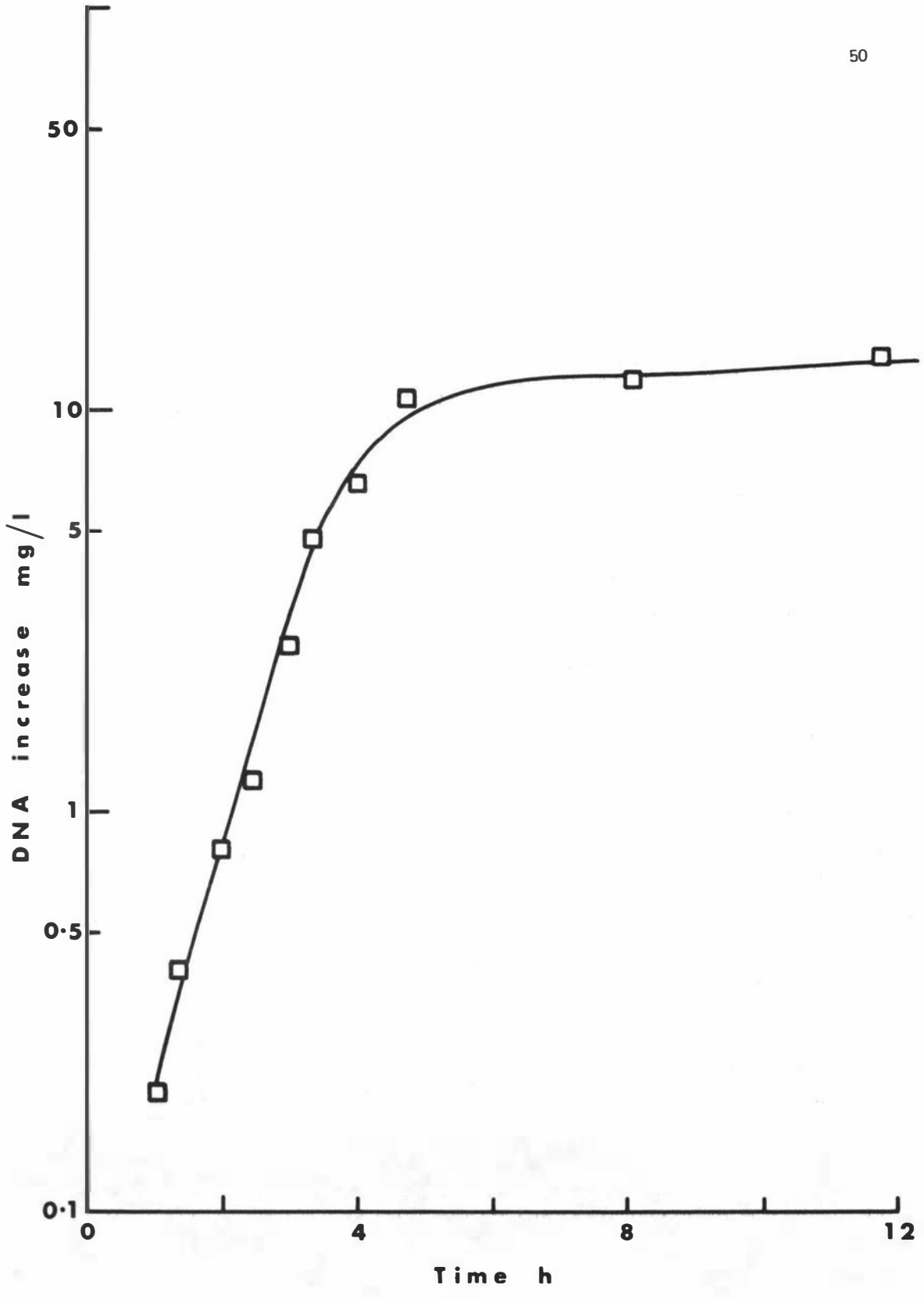


Figure 4.5 Ln deoxyribonucleic acid (DNA) concentration as a function of time during the batch culture of whey. Whey 35 g/l, pH6.0, 46°C.

Table 4.1 A typical batch culture (B9.1.5)

Time h	Acid Conc.(P) g/l	Cell conc.(N) Cells/ l x 10^{-10}	$\frac{dP}{dt}$ g/l h	$\frac{dN}{dt}$ cells/l h x 10^{-10}
0	0.0	0.9		
0.5		1.2		0.53
1.0		1.6		1.12
1.5		1.7		1.64
2.0	0.03	2.8	0.04	3.17
2.5	0.10	10.0	0.21	8.90
3.0	0.26	12.4	0.33	15.6
3.5	0.40	24.6	0.49	29.8
4.0	0.75	45.7	0.89	50.7
4.5	1.26		1.15	72.8
5.0	1.78	113	1.33	89.5
5.5	2.57	152	1.35	103.2
6.0	3.17	229	1.33	70.0
6.5	3.91		1.28	42.1
7.0	4.40	266	1.02	1.0
7.5	5.05		0.97	
8.5	5.73	242	0.97	
9.5	6.66		0.97	
10.5	7.60	235	0.97	
11.0	8.18		0.97	
13.0	10.11		0.97	
15.0	11.94		0.97	
17.0	13.71		0.94	
19.0	15.42		0.89	
21.0	17.11		0.84	
23.0	18.80		0.69	
24.0	19.30		0.45	
25.0	19.69		0.33	
26.0	19.88		0.14	
27.0	20.00		0.12	
28.0	20.10		0.05	
29.0	20.15			
30.0	20.18			

Whey - 35 g/l solids; pH - 6.0; Temp. - 46° C.

Inoculum - 1 per cent clotted skim milk culture of LBR.

Table 4.2 Changes in acid and lactose concentrations in a typical batch culture (B 8.2.6) (see fig. 4.4)

Time	Acid conc. g/l	Lactose conc. g/l	Time	Acid conc. g/l	Lactose conc. g/l
0	0	42.2	5.92	22.14	
0.63	0.12	41.9	6.60	25.58	13.2
0.92	0.23		6.89	26.94	
1.13	0.35	41.5	7.33	28.68	
1.30	0.47		7.75	30.24	9.1
1.53	0.77		7.99	31.02	
1.75	1.01		8.39	32.19	
1.92	1.28	38.9	9.17	33.72	5.5
2.40	2.58	35.7	9.78	34.41	
2.94	4.47	34.4	10.35	34.76	
3.26	6.62		10.92	35.00	3.7
3.56	7.87		11.31	35.20	
3.97	10.64	29.2	12.80	35.60	
4.50	13.12				
5.20	18.20	23.4			

Whey - 75 g/l solids with supplementary nutrients; pH - 5.5; Temp - 46°C. Inoculum - 5 per cent clotted skim milk culture of LBR

4.2.2 Whey Concentration

Whey with a solids concentration of 75 g/l gave a specific acid production rate of 1.28 h^{-1} in the phase of exponential cell number increase and an acid production rate of 2.37 g/l h in the apparent stationary phase. When the whey solids were reduced to 35 g/l the corresponding results were 1.20 h^{-1} and 1.08 g/l h. The exponential phase acid production rate is not altered but the stationary phase acid production rate is reduced approximately in proportion to the total solids of the whey.

The final yield of acid with respect to the initial lactose concentration was 95 and 93 per cent respectively.

4.2.3 Supplementary nutrients

It is possible to group the nutrients used by others in studies of L.bulgaricus into a number of distinct classifications :-

1) Salts
2) Vitamins
3) Amino acids
4) Nucleic acid compounds
5) General purpose sources of growth factors

(i.e. yeast extract, peptone, corn steep liquor,etc.)

The four groups of nutrients considered to be chemically defined (1 - 4) were prepared as described in EXPERIMENTAL (3.2.3.1) and considered to be the four variables (A- salts, B- vitamins, C- amino acids, D- nucleic acid compounds) of a 2^4 factorial experiment with absence of the nutrient considered to be the lower level and its presence at the level designated in table 3.3 considered to be the upper level. Sterile-filtered whey, 35 g/l solids, was used. In experiments a,b,c,d, abc,abd, acd and bcd* the whey was also pasteurized before the nutrients were added.

The fermentations were carried out at 46°C and pH 6.0. The inoculum was 1 per cent of a clotted skim milk culture of LBR. The experiments were performed in random order and the results are shown in figure 4.6. The rate of acid production over the linear portion of the acid concentration vs time curve, or the maximum rate where a linear portion was not present, are tabulated in table 4.3a.

An analysis of variance based on the results is shown in table 4.3.b The third and fourth order interactions are unlikely to be significant and can be combined to give an estimate of the error variance. The hypothesis that the mean squares for the third and fourth order interactions are in fact estimates of the same variance and may be used as an estimate of error can be tested by Bartlett's criterion, (Davies,1963 p.287). The value for M obtained from the above results is 2.3 and a value of 12.0 is required for significance; therefore there is no evidence of heterogeneity.

(* lower case letter indicates the nutrient was added to the whey ie abc indicates salts, vitamins and amino acids were added).

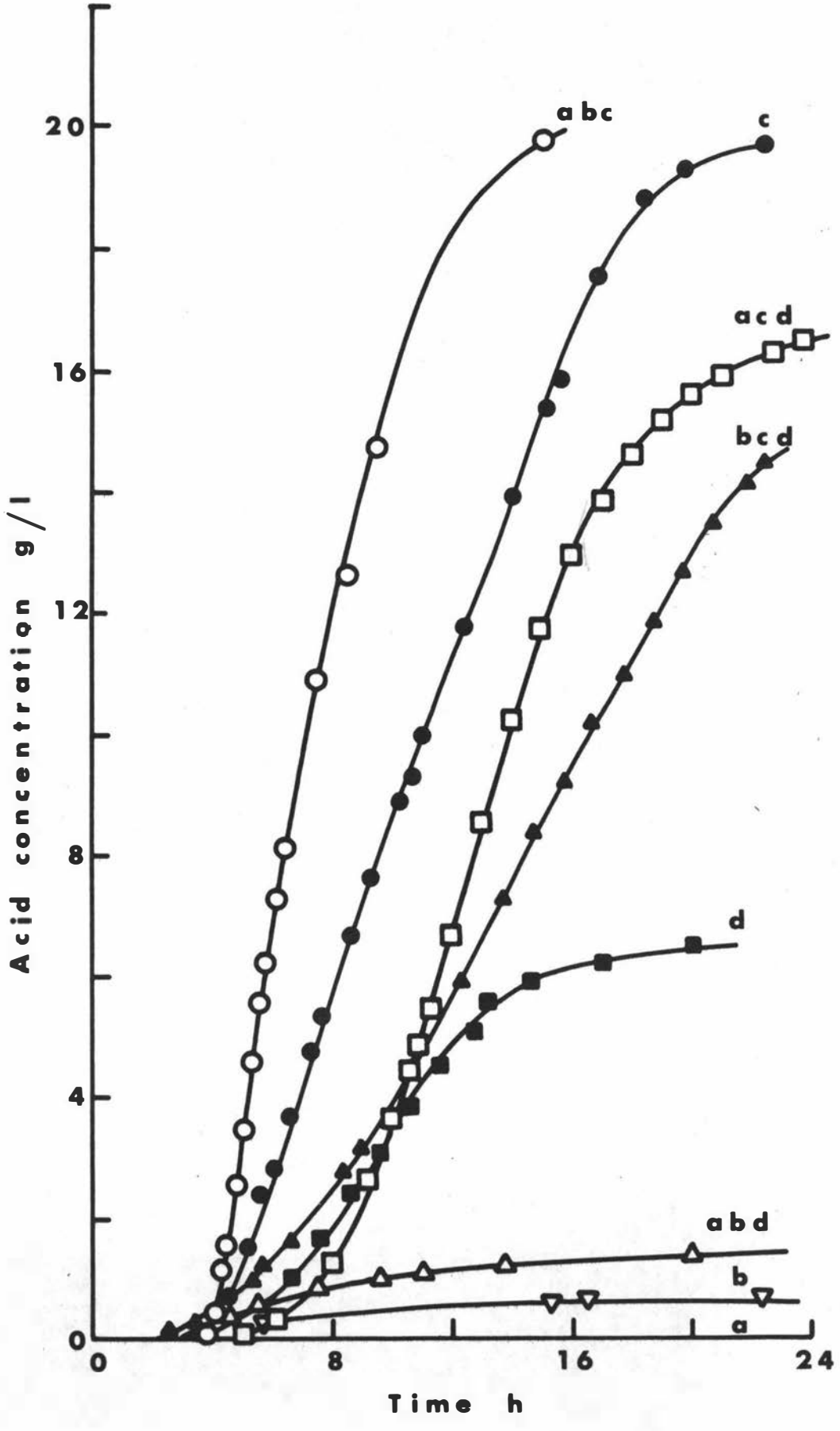


Figure 4.6a Acid concentration as a function of time during the batch culture of pasteurized sterile-filtered whey with supplementary nutrients.
a - salts, b - vitamins, c - amino acids, d - nucleic acid compounds.
Whey 35 g/l, pH6.0, 46^{o}C.

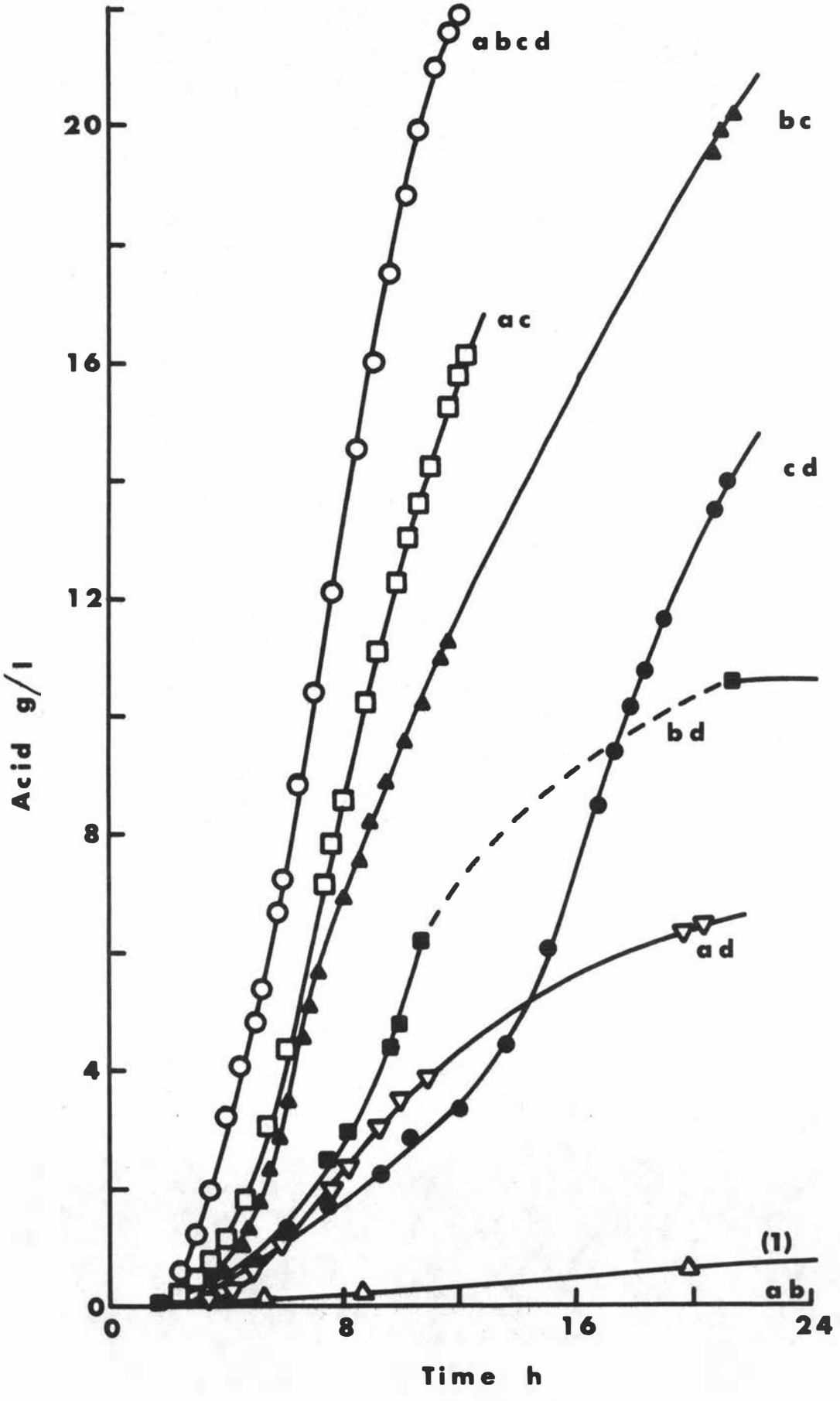


Figure 4.6b Acid concentration as a function of time during the batch culture of sterile-filtered whey with supplementary nutrients. a - salts, b - vitamins, c - amino acids, d - nucleic acid compounds. Whey 35 g/l, pH6.0, 46^{o}C.

Table 4.3.a The effect of nutrients on the rate of acid production in batch fermentation of whey.

Nutrients	$\frac{dP}{dt}$ g/l h.	Nutrients	$\frac{dP}{dt}$ g/l h.
(1)	0.10	d	0.70
a	0.00	ad	0.38
b	0.04	bd	0.59
ab	0.00	abd	0.07
c	1.36	cd	1.40
ac	2.29	acd	1.76
bc	1.19	bcd	0.97
abc	2.55	abcd	2.69

Sterile-filtered whey, 35 g/l solids; pH 6.0; 46°C, inoculum 1% clotted skim-milk culture of LBR

A - salts, B - vitamins, C - amino acids, D - nucleic acid compounds

Table 4.3b The effect of supplementary nutrients on the rate of lactate increase in batch fermentation of whey, Analysis of variance.

Source of Variation.		Effect.	Mean Square.
Salts	A	0.424	0.7180
Vitamins	B	0.014	0.0008
Amino acids	C	1.541	9.5018 **
Nucleic acids	D	0.129	0.0663
Interactions	AB	0.206	0.1702
	AC	0.669	1.7889 **
	AD	-0.114	0.0518
	BC	0.134	0.0716
	BD	0.006	0.0002
	CD	-0.271	0.2943
	ABC	0.241	0.2328
	ABD	0.084	0.0281
	ACD	0.061	0.0150
	BCD	0.096	0.0371
	ABCD	0.149	0.0885

Note: Estimate of error variance is 0.0802 with five degress of freedom

The estimate of the error variance is 0.0802 with five degrees of freedom. A mean square as great as 0.530 is significant at the 5 per cent probability level and a mean square as great as 1.310 is significant at the 1 per cent probability level.

Vitamins and nucleic acid compounds do not appear to have any effect on the rate of acid production. When the results are analysed as a replicated factorial experiment with salts and amino acid as the two variables the amino acids are seen to have a highly significant positive effect with the effect being increased when the salts are present (AC interaction is highly significant) Salts on their own have no effect.

Pasteurizing the sterile-filtered whey had no effect (the ABCD interaction is insignificant).

Sterile-filtering of whey appeared to remove some compound essential for growth. In all experiments in which amino acids were absent there was essentially no cell growth and no acid production.

Bacteriophage were not involved. A 1 ml sample of the culture always caused 10 ml of sterile skim-milk to clot after a few hours incubation at 48°C and the organisms in these cultures had the typical appearance of LBR.

Sterile-filtration did not appear to introduce a toxic compound because at least half of the cultures produced normal amounts of acid. This effect was studied further (see 4.2.4)

Some individual nutrients were screened for their effect on cell growth in batch cultures without pH control. The whey (7.5 g/l) was pasteurized in conical flasks, and one of the nutrients listed in table 4.4 added. The medium was inoculated and incubated for 7 h at 46°C. The results are shown in table 4.5

Table 4.4 Nutrients added to whey
Quantities in g/l of final whey solution.

	Nutrient	g/l		Nutrient	g/l
1	no additives		9	niacin	0.002
2	adenine	0.01	10	Ca-pantothenate	0.002
	guanine	0.01	11	p-amino benzoic acid	0.002
	uracil	0.01	12	pyrodoxine	0.04
	xanthine	0.01	13	riboflavin	0.001
3	DL-methionine	0.004	14	thiamine HCl	0.02
	L-cysteine HCl	0.004	15	tryptophan	0.08
4	L-ascorbic acid	0.04	16	Vitamin B-12	0.001
5	biotin	0.001	17	$MgSO_4\ 7H_2O$	0.8
6	cas-amino acids	0.08		Na Cl	0.04
7	folic acid	0.002		$FeSO_4\ 7H_2O$	0.04
8	inositol	0.1		$MnSO_4$	0.04

Note: Adenine, biotin and pyrodoxine were purchased from L.Light & Co.; casamino acids (Vitamin free), Difco; tryptophan , Sigma Chemicals; thiamin HCl, Eastman Kodak; Vitamin B-12, Glaxo Lab.Ltd.; and the remainder from BDH.

Table 4.5 The effect of supplementary nutrients on cell concentration after 7 h incubation at 46°C. Whey 7.5 g/l, phosphate buffer 0.25 molar, initial pH 6.3

Sample No.	Cell concentration x 10^{-11} / l			Mean cell concentration x 10^{-11} / l	pH		
1	2.14,	1.84,	2.49	2.08	4.85,	4.70,	4.75
	2.28,	1.84,	1.92		4.70,	4.80,	4.75
2	3.00,	2.68,	2.70	2.79	4.90,	4.90,	5.60
3	2.48,	2.88,	2.64	2.67	4.65,	4.50,	4.69
4	0.1 ,	0.24,	0.1	0.1	5.70,	5.69,	5.95
5	2.25,	2.32,	3.08	2.55	4.62,	4.60,	5.15
6	3.68,	3.28,	3.48	3.48	5.35,	4.52,	5.40
7	3.00,	4.04,	4.44	3.83	4.65,	4.55,	4.65
8	3.64,	3.64,	3.60	3.63	5.15,	4.60,	5.10
9	4.92,	3.12,	4.84	4.32	4.70,	4.70,	5.09
10	3.76,	3.28,	4.08	3.71	4.79,	4.70,	4.89
11	3.84,	4.24,	3.80	3.96	4.49,	4.45,	4.85
12	0.14,	0.14,	-	0.14	5.90,	5.90,	6.05
13	3.20,	3.32,	3.08	3.20	4.80,	4.75,	4.95
14	4.04,	4.16,	4.48	4.23	5.05,	4.79,	5.10
15	4.72,	4.72,	-	4.72	4.80,	4.55,	4.80
16	2.72,	3.40,	3.08	3.07	4.57,	4.55,	4.65
17	0.44,	2.24,	0.45	1.04	5.05,	4.50,	5.10

The pH variation within samples shows less variation than between samples. In general a low pH corresponds to a high cell concentration, although there are some exceptions, illustrating a possible buffering effect from the added nutrients. All replicates have been used to calculate the variance of the results. The value obtained is 0.3209×10^{11} cells/l with 34 degrees of freedom. Hence a cell concentration of 3.24×10^{11} cells/l can be considered to be a real increase on 2.08×10^{11} cells/l obtained in unsupplemented whey. A cell concentration as low as 0.92×10^{11} cells/l can be assumed to be an indication of inhibitory effects.

From these results the following can be considered to stimulate cell growth. (Number in brackets is average cell concentration per litre after 7 h): tryptophan (4.72×10^{11}), niacin (4.32×10^{11}),

thiamine (4.23×10^{11}), p-amino-benzoic acid (3.90×10^{11}), folic acid (3.83×10^{11}), Ca pantothenate (3.71×10^{11}), inositol (3.63×10^{11}) and cas-amino acids (3.48×10^{11}).

The following appear to be inhibitory: L-ascorbic acid (0.01×10^{11}) and pyrodoxine (0.14×10^{11}).

These results confirm the stimulatory effect of tryptophan and cas-amino acids shown to occur in the acid production experiments at controlled pH. However contrary to the results obtained in the latter experiment, some of the vitamins are shown to have a stimulatory effect. It is likely that the inhibitory effect if L-ascorbic acid and pyrodoxine cancelled out the effect of the other vitamins in the mixture. Neither the salt solution nor the nucleic acid compounds had any effect confirming the results obtained at controlled pH.

4.2.4 Sterile- filtration

Figure 4.7 shows the acid concentration during fermentations of wheys treated in the following manner.

1) Whey, 35 g/l solids, pasteurized
2) Whey, 35 g/l solids, centrifuged, pasteurized
3) Duplicate of 2
4) Whey, 35 g/l solids, centrifuged, sterile-filtered and pasteurized.

The duplicated experiments agree very closely. Whey which had been sterile-filtered produced a small amount of acid but only after it had been re-inoculated.

The rate of acid production was greatest in whey which had not been centrifuged; the linear rate of acid production was 1.15 g/l h in pasteurized whey compared with 0.83 g/l h in the pasteurized centrifuged whey. Centrifuging and sterile-filtration remove from the whey compounds essential for acid production.

4.2.5 Casein as a supplementary nutrient

Centrifuging and sterile-filtration removed from the reconstituted whey some compound or compounds essential for the production of lactic acid by LBR. (4.2.4) Suspended insoluble matter in whey comprises whey proteins denatured during drying, casein fines not removed from the whey before drying, fat not removed as cream or in the casein and debris from the starter bacteria used to produce acid in the casein making process.

Of these the simplest to check for stimulating acid production is the casein which can be added in the form of sodium caseinate. The fact that acid is readily produced in reconstituted skim-milk which has a much lower

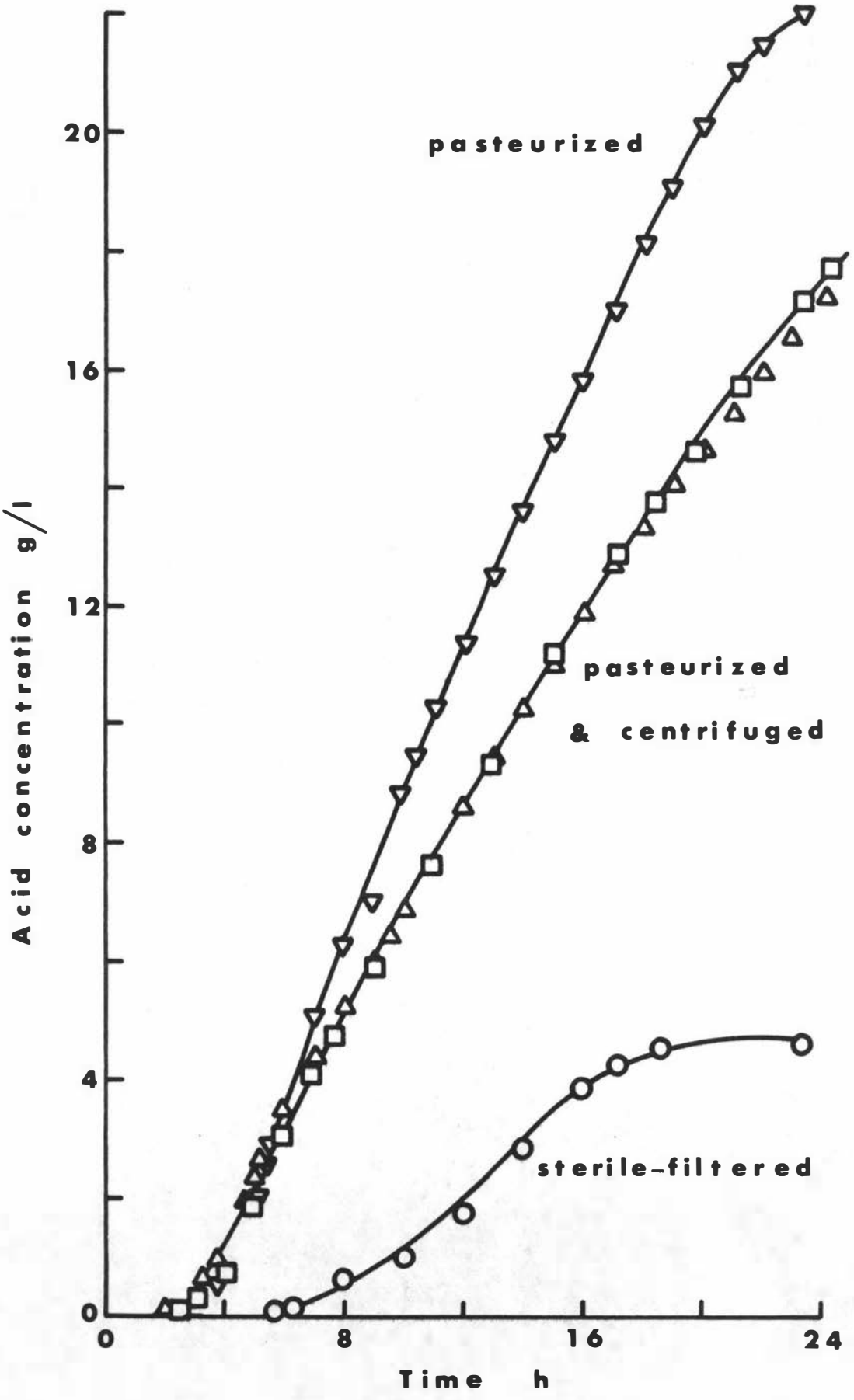


Figure 4.7 The reduction of acid synthesis caused by centrifuging or sterile filtering whey. Whey 35 g/l, pH6.0, 46°C.

level of bacterial debris would tend to rule out this as a possible cause. Experiments with supplementary nutrients have shown that casamino-acids and tryptophan stimulate cell growth and acid production, a further reason for trying sodium caseinate as a nutrient.

Figure 4.8 shows the acid concentration increase in sterile-filtered whey, with 5 g/l sodium caseinate (AD74) added. The sodium caseinate stimulated the rate of acid production - linear acid production rate 1.5 g/l h with NaCas present cf with 1.15 g/l h in pasteurized whey and zero in sterile-filtered whey.

Different batches of sodium caseinate do not have the same effect. Figure 4.9 compares batches AD74 and HF145. However, as shown in figure 4.10 when the concentration of the sodium caseinate HF145 is increased so too does the acid production rate.

Table 4.6 and figure 4.11 show the effect of increasing the concentration of sodium caseinate (mixed 134-136, 1968) on the linear rate of acid production using pasteurized whey.

Table 4.6 Batch fermentation of whey with additional sodium caseinate (134-136). (B9.4.1- 8) "Stationary phase" acid synthesis rates g/l h

Sodium Caseinate added g/l	Acid synthesis rate g/l h
0	0.85, 0.93
2.5	1.30
5.0	1.54
10.0	2.41, 2.48
20.0	2.54
40.0	2.78

Whey 35 g/l, pasteurized. pH 6.0, 46°C inoculum 1 per cent skim-milk culture.

Attempts to determine why some batches of sodium caseinate were more effective than others were not successful. All batches used stimulated acid production provided the concentration of sodium caseinate was high enough, hence an inhibitory compound was not responsible. There was no correlation between the age of sodium caseinate and its effectiveness.

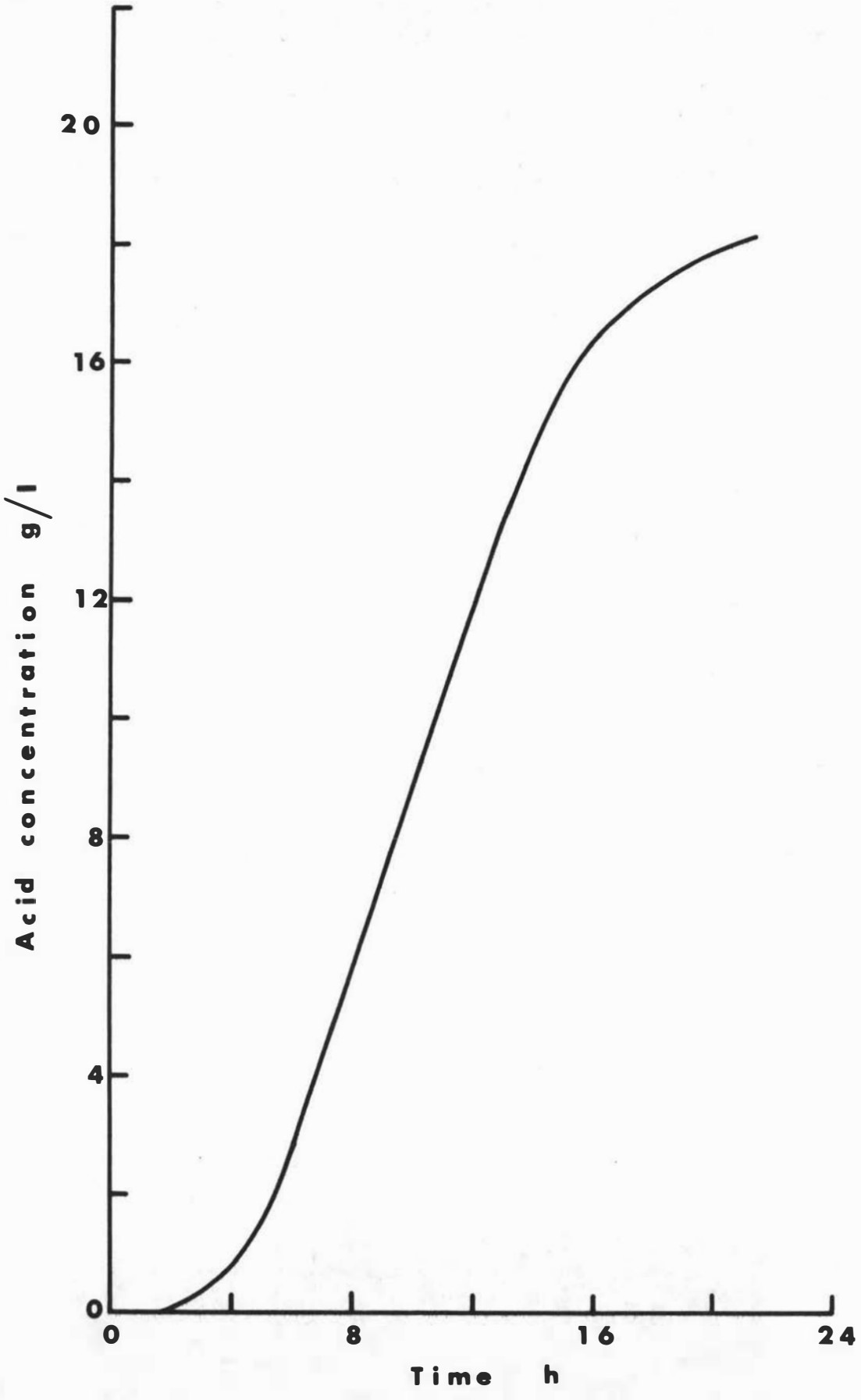


Figure 4.8 The effect on the rate of acid synthesis of adding 5 g/l sodium caseinate (AD 74) to sterile filtered whey (B9.3.12) cf Fig. 4.7. Whey 35 g/l, pH6.0, 46°C.

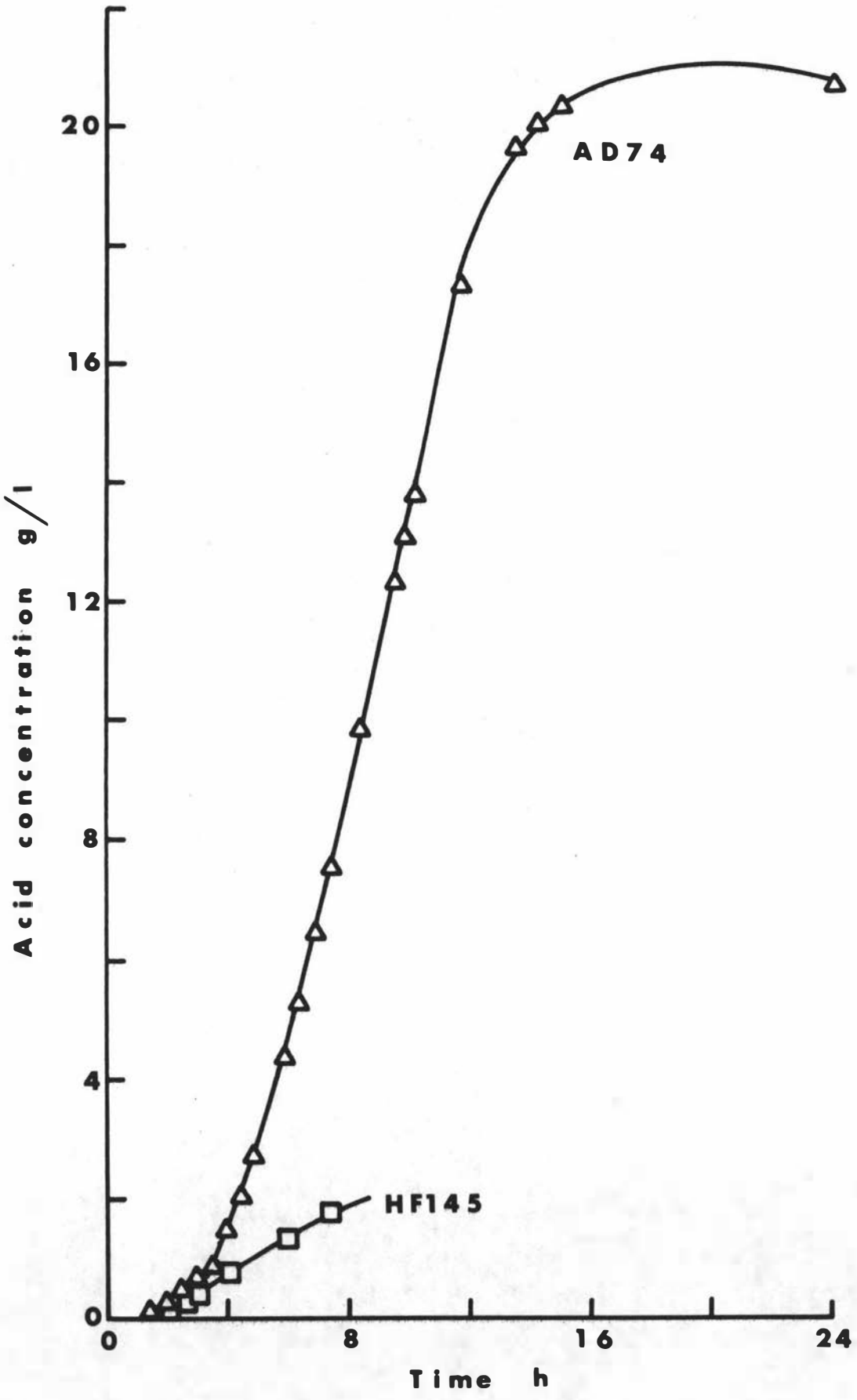


Figure 4.9 Different batches of sodium caseinate added to sterile filtered whey. 10 g/l Na Cas. Whey 35 g/l, pH6.0, 46°C.

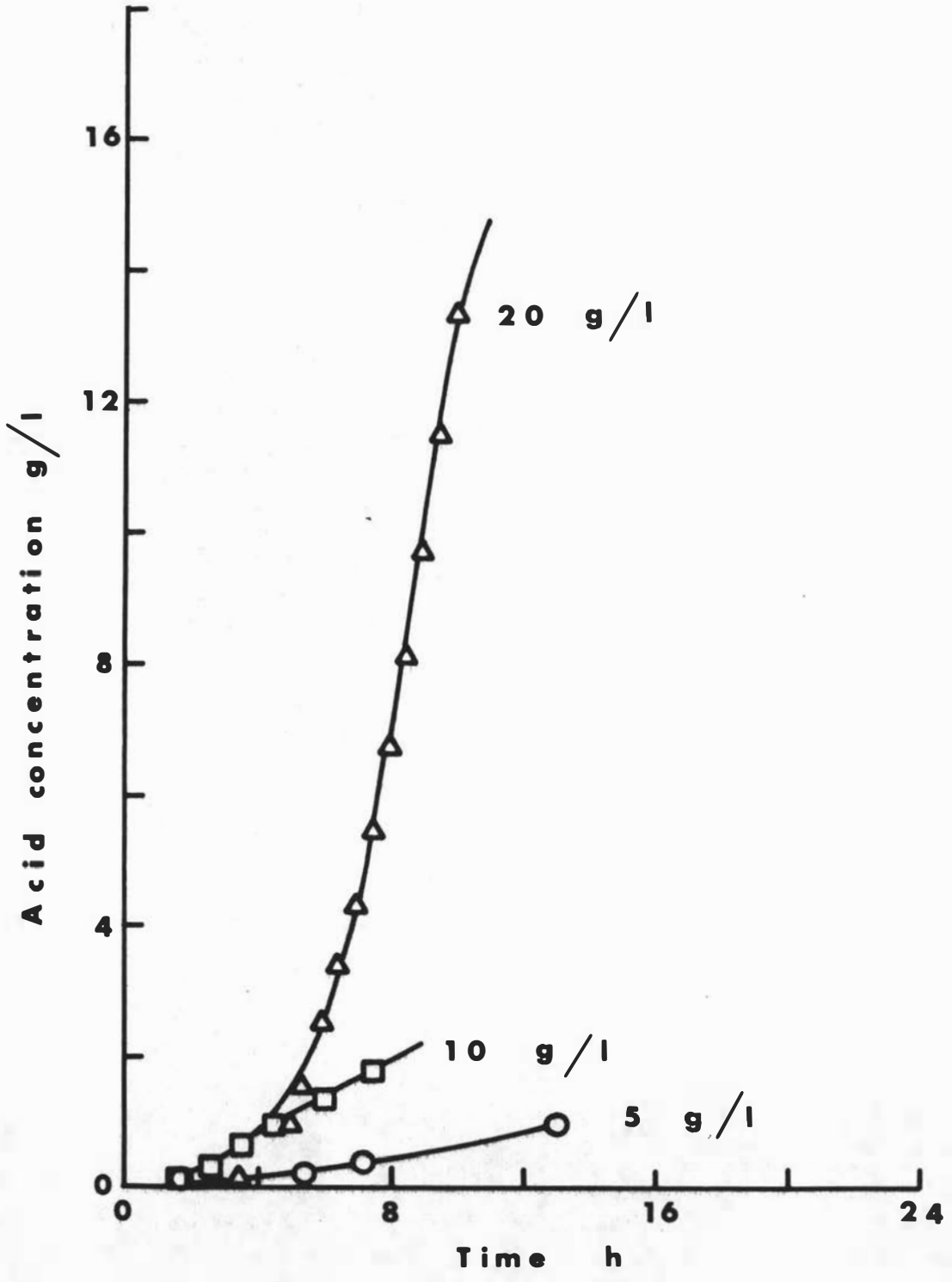


Figure 4.10 Acid concentration during batch culture of whey as a function of sodium caseinate (HF 145) concentration. Whey 35 g/l, pH6.0, 46°C.

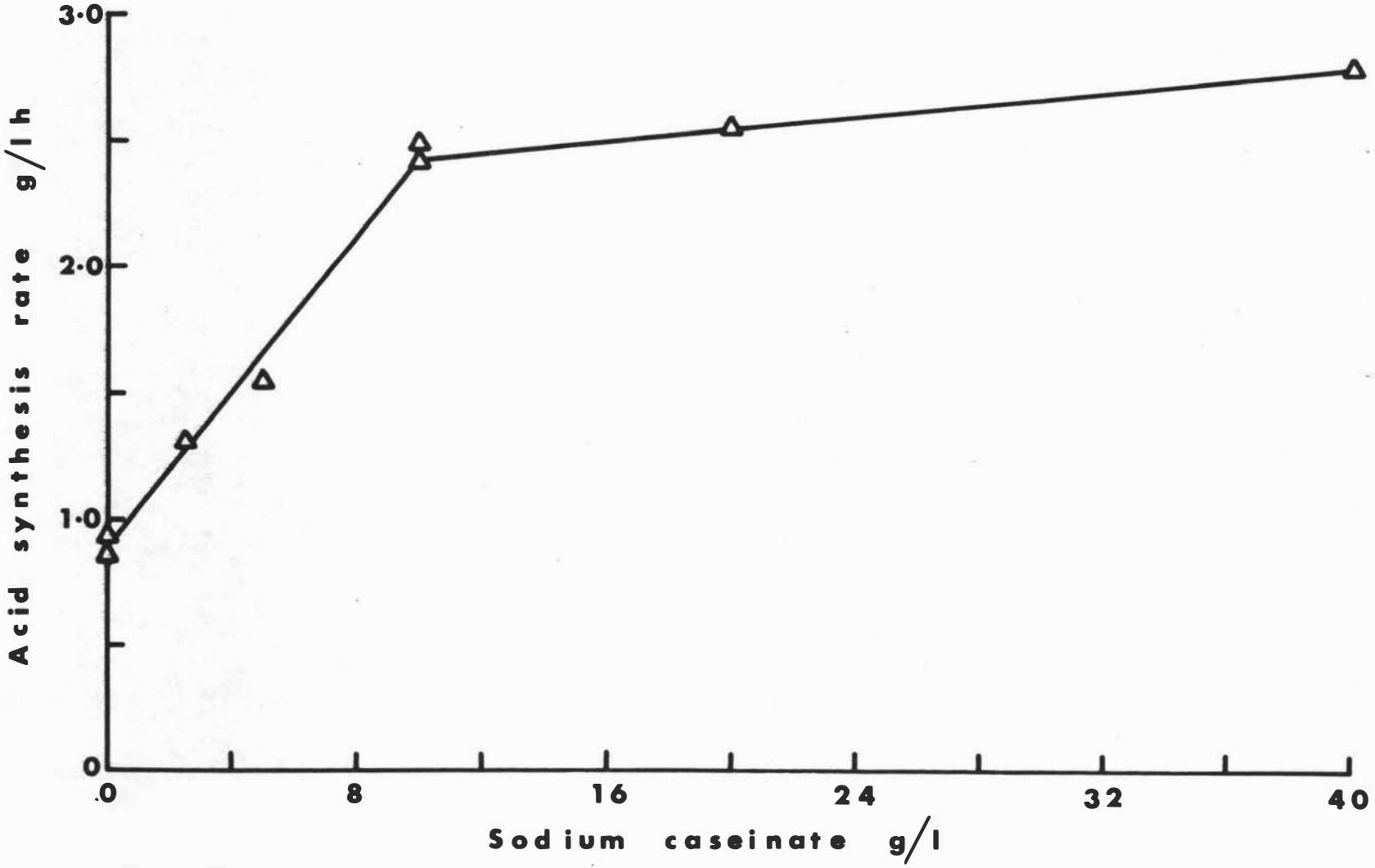


Figure 4.11 Acid synthesis rate during the phase of stationary cell population as a function of the concentration of sodium caseinate (134–136).
Whey 35 g/l, pH5.0, 46°C.

4.2.6 Controlled Atmosphere in Shaken Flasks

Table 4.7 and figure 4.12 show the effect of shaking in conical flasks plugged with cotton-wool on the production of acid from whey and whey supplemented with casein.

Table 4.7 Batch fermentation of whey in conical flasks B8.12.20 - the effect of shaking; pH and lactate concentration after 7 hrs incubation

Medium	pH			Increase lactate conc. g/l	
	Control	Shaken	Not Shaken	Shaken	Not Shaken
Whey*	5.92	5.25	4.22	0.75	6.8
Whey + 20 g/l skim-milk solids	6.35	5.95	4.15	0.3	7.9
Whey + 20 g/l sodium caseinate	6.50	6.12	3.82	1.0	13.2
Whey + 50 g/l sodium caseinate	6.70	6.27	3.82	0.8	15.1

*Mean of 4 experiments

175 rev/min, 2.5 cm throw.

The results indicate that in shaken flasks acid production is almost completely depressed. The addition of skim-milk solids or sodium caseinate, while increasing the amount of acid formed in the unshaken flasks, had no effect on the culture in the shaken flasks. The inhibition could be caused by agitation itself or because some inhibitory substance is being absorbed from the air.

A further experiment was performed in which the air was replaced by various gas mixtures. The gas mixtures used are shown in table 4.8 and results in table 4.9 and figure 4.13.

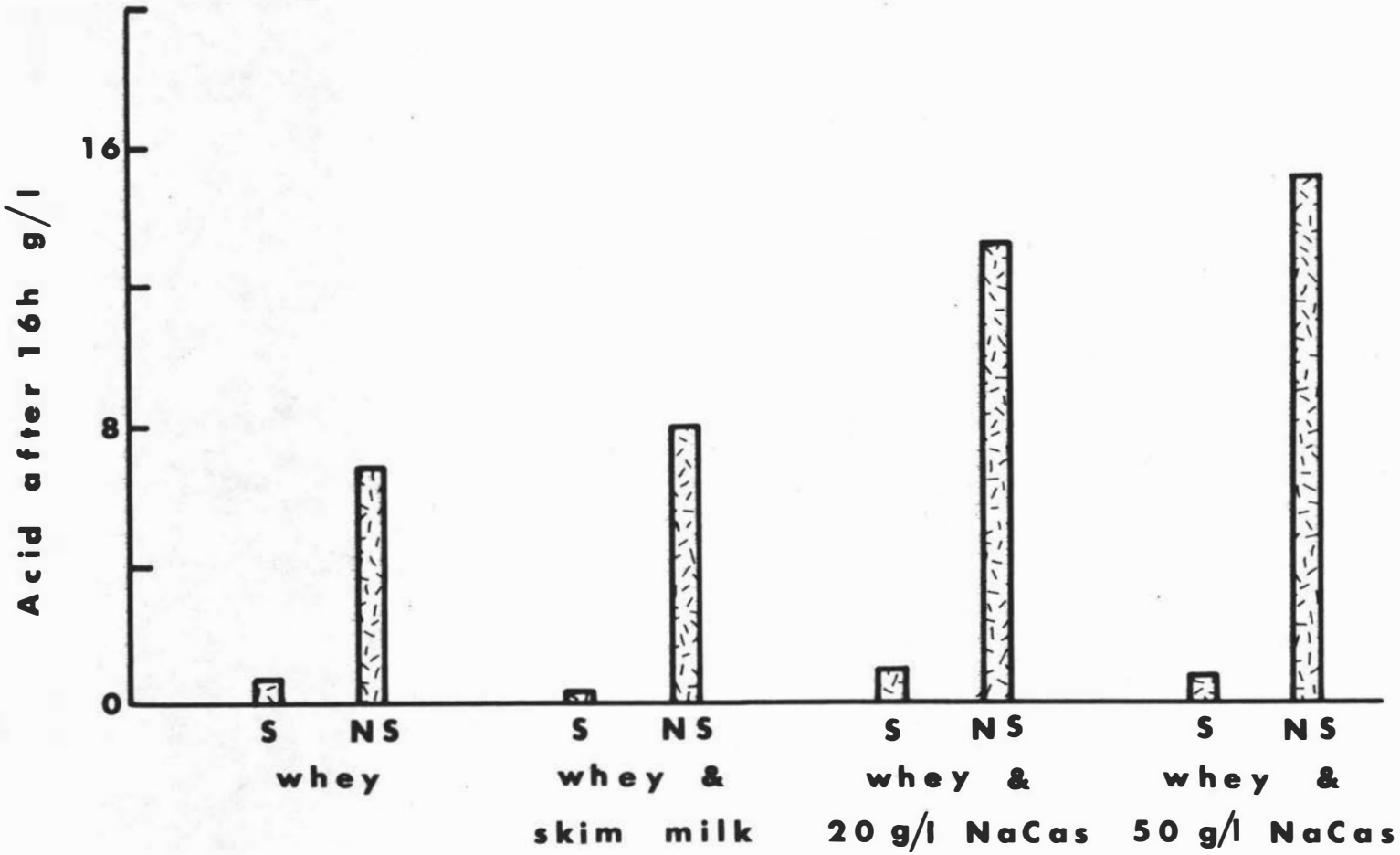


Figure 4.12 The effect of shaking various whey cultures in conical flasks. Acid concentration after 16 h incubation at 46°C without pH control. S - shaken; NS - not shaken. Whey 75 g/l.

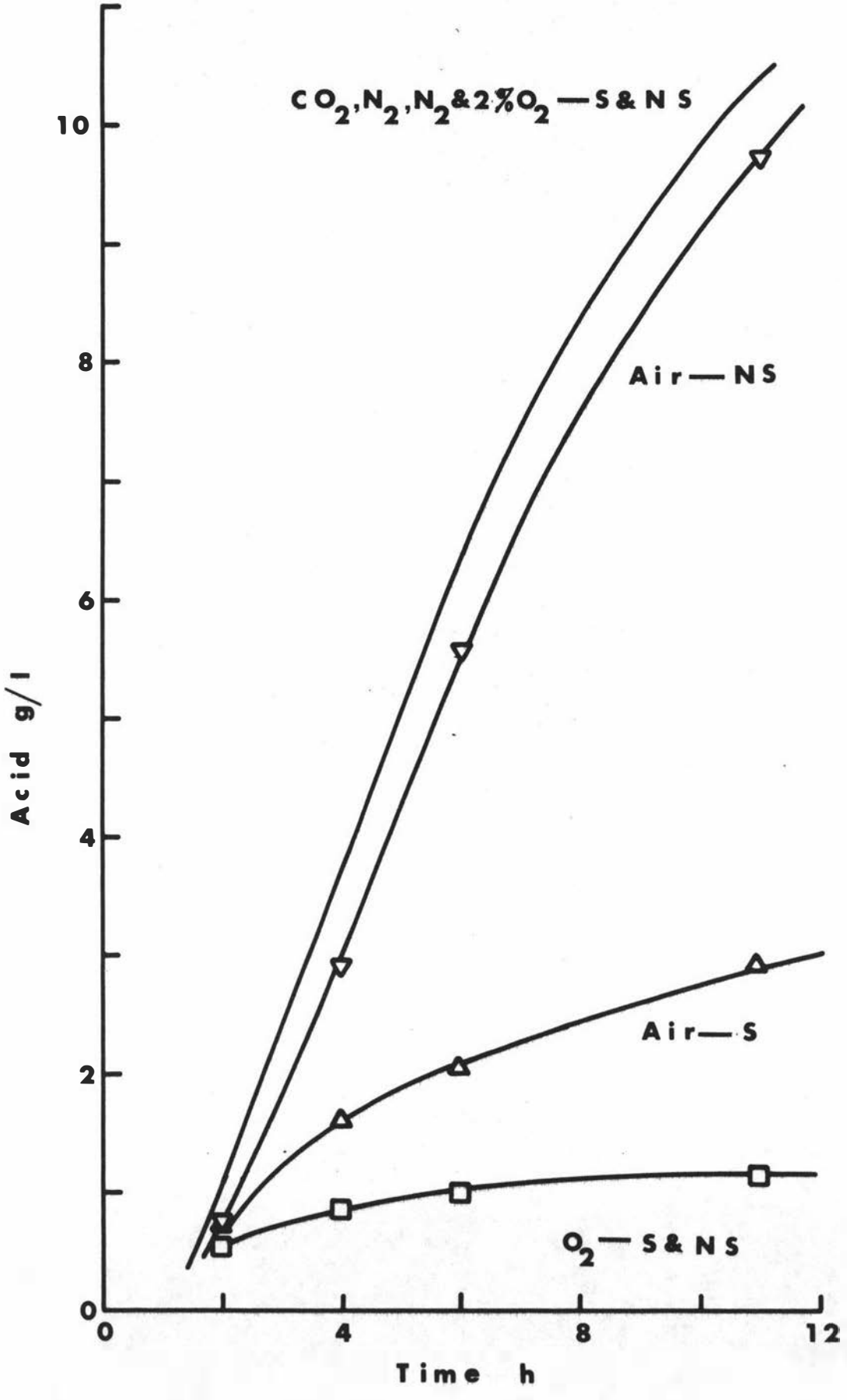


Figure 4.13 Acid concentration after 7 h incubation at 46°C in whey cultures shaken and unshaken with different gases in the head space. Whey 75 g/l.

Table 4.8 Head-space gas mixtures

A.	Nitrogen (oxygen free)
B.	98 per cent nitrogen + 2 per cent oxygen
C.	Air
D.	Oxygen
E.	95 per cent nitrogen + 5 per cent carbon dioxide
F.	Carbon dioxide

Table 4.9 The effect of headspace gas on acid production (g/l) in shaken (S) and not shaken (NS) cultures of whey.

Gas in headspace		Time h 2	4	6	11
A	S*	0.73	3.01	5.84	10.49
	NS*	0.62	3.14	5.88	10.11
B	S	0.93	3.62	6.51	10.43
	NS	0.89	3.94	6.43	10.38
C	S	0.72	1.59	2.04	2.90
	NS*	0.74	2.91	5.58	9.73
D	S	0.41	0.59	0.60	1.08
	NS	0.56	0.85	1.00	1.14
E	S	1.13	3.59	6.59	10.42
	NS	0.95	4.16	6.53	11.26
F	S	1.69	4.41	6.66	9.90
	NS	1.62	4.14	6.66	10.22

* Average of two results from separate cultures.
75 g/l solids. 45°C. Inoculum 2 per cent clotted skim-milk culture LBR

From the duplicate experiments performed, the variance of the acid concentration was determined to be 0.11 g/l. The 95 per cent confidence limits for each result are therefore $\pm$ 0.60 g/l.

Oxygen (D) suppresses acid formation whether the flasks are shaken or not. Acid synthesis is also inhibited in flasks shaken with air in the head-space. With air in the headspace of a stationary culture flask, acid synthesis is slightly less than that with all other gases in both shaken and stationary culture flasks. There is an indication

that carbon dioxide (E,F) is stimulatory in the early phases of acid production, but further experiments will be needed to prove this.

It can be concluded that oxygen inhibits acid synthesis when it is in solution at a high enough concentration. When oxygen is present at less than a particular concentration in the gas phase (between 2 and 5 per cent; cf B and C) or is unable to diffuse into solution at a fast enough rate (cf C unshaken and shaken) then it does not inhibit acid production.

Two further experiments carried out in the 2 l culture vessel with pH control, also illustrate the effect of air in inhibiting acid production. (Figure 4.14) Air was sparged into the culture medium (pasteurized whey, 35 g/l solids) at 120 l/h. After 28h, 0.39 g/l of acid had been produced.

The experiment was repeated, but after 19.5 h, (0.48 g/l acid) the air was turned off. No more acid was produced for 3 h. A further 20 ml of LBR culture was added. Twenty-one hours after reinoculation 10.6 g/l of acid had been produced. Acid production was almost totally inhibited by the sparged air, but the inhibition was removed once air sparging ceased, despite the fact no attempt was made to remove air from the headspace.

In the other experiment, the same conditions were used, except that the whey had a solids content of 75 g/l, and air was not sparged into the vessel for 9 h after inoculation. At this stage the air flow was turned on to the sparger at 120 l/h. The acid production rate followed a pattern similar to that obtained in cultures blanketed with nitrogen. Air effects the growth of cells, but not the acid synthesis.

(Note: The apparent difference in the acid production rate in the two reported experiments is due to the solids concentration of the whey. In the first reported experiment the average acid production rate in the "stationary" phase is 0.74 g/l h, while in the second it is 1.61 g/l h; i.e. a ratio of 2.18:1. The total solids are in the ratio of 2.14:1.)

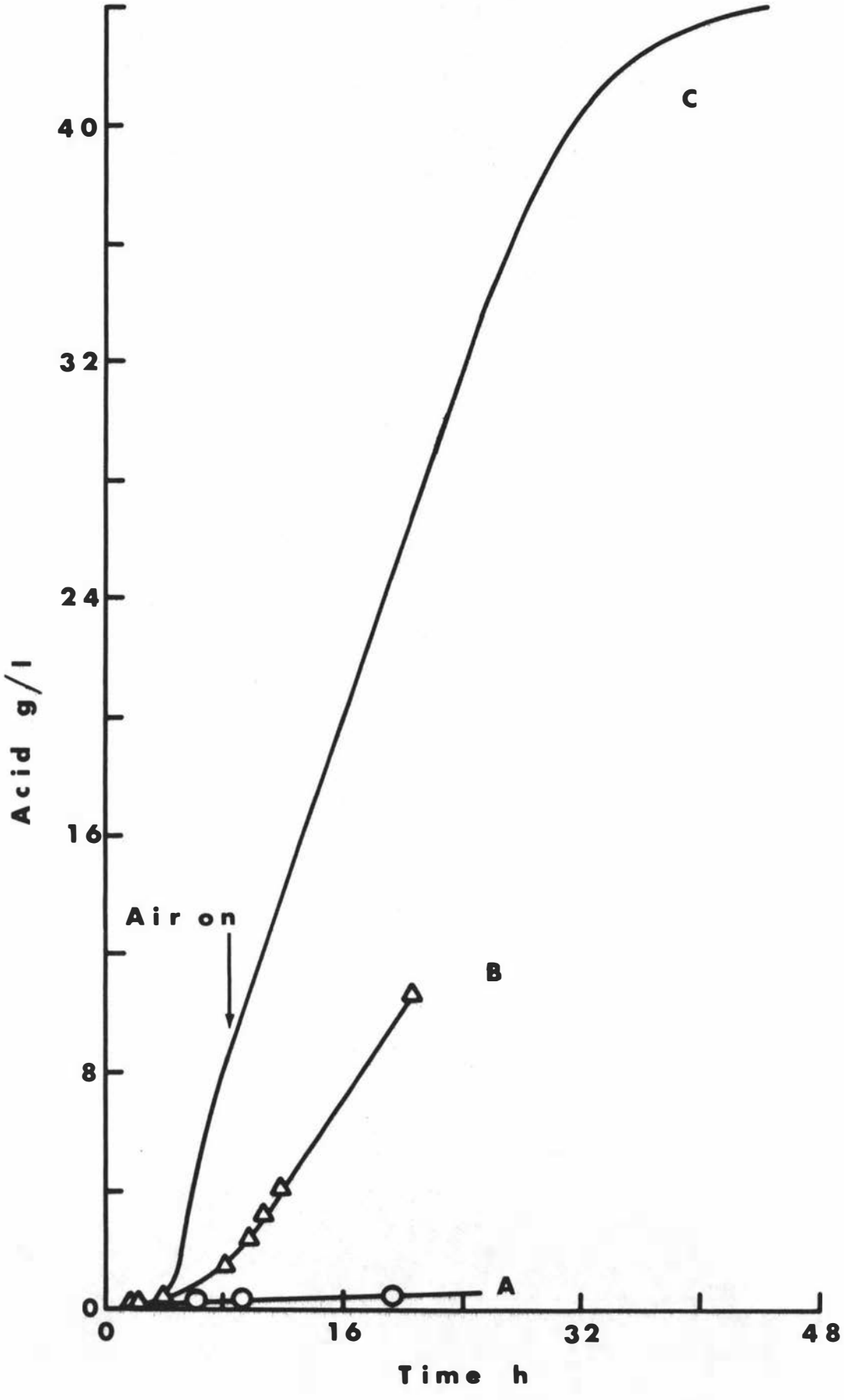


Figure 4.14 The effect of air on acid production at pH6.0, 46°C. A – air sparged at 120 l/h from the time of inoculation, pasteurized whey, 35 g/l. B – air sparged at 120 l/h for 19 h after inoculation; air flow stopped and culture reinoculated with a fresh inoculum of LBR. Whey 35 g/l. C – air sparge at 120 l/h commenced after 8 h incubation. Whey 70 g/l.

4.2.7 Agitation

The effect of agitation was studied in detail in the culture vessel using the three paddle impellers.

The agitation conditions used are shown in Table 4-10. The impeller Reynolds Number ($Re = \frac{nd^2\rho}{\mu}$) was calculated assuming the density was 1023 kg/m^3 and viscosity was 0.7cP. The lowest value of Re was 3145 and hence in all experiments fully developed turbulence was attained (Bates, Fondy & Corpstein, 1963).

The power consumption by the paddle may be related to the impeller blade dimensions by the following expression derived by Calderbank and Moo-Young (1961) :

$$\frac{P_o g}{n^3 d^5 \rho} = \frac{kwl\ (d - w)}{d^3} \qquad (4.1)$$

where P_o is the power dissipated in mixing and k is a constant. For the present case equation 4.1 reduces to equation 4.2 which is defined as the power factor, P_f.

Table 4.10 Paddle impeller diameter and speed used to determine the effect of the agitation intensity on the rate of acid production.

Run No.	n rpm	d mm	Re $\frac{nd^2\rho}{\mu}$	P_f Power Factor
1	200	25	3,145	5.625×10^5
2	635	25	9,980	1.800×10^7
3	1270	25	19,900	1.440×10^8
4	100	51	6,290	1.406×10^6
5	200	51	12,580	1.125×10^7
6	400	51	25,160	9.000×10^7
7	800	51	50,320	7.200×10^8
8	20	102	5,032	1.890×10^5
9	50	102	12,580	2.953×10^6
10	63	102	15,760	5.907×10^6
11	100	102	25,160	2.363×10^7
12	126	102	31,520	4.726×10^7
13	200	102	50,320	1.890×10^8

$$P_f = wl\ (d - w)n^3d^2. \qquad (4.2)$$

The small effect on power dissipated by the impeller introduced by alteration of the ratio of impeller diameter to tank diameter has been ignored (Bates et al. 1963).

All runs were at pH 6.0, 46°C using pasteurized whey, 70 g/l solids. A 0.5 per cent whey inoculum was used and the headspace was sparged with carbon-dioxide.

The results of a typical experiment are shown in Table 4.11. The cell counts were determined with a precision of ± 13 per cent. The average cells per chain has a standard deviation of 0.35 and within any one sample the chain lengths follow a Poisson Distribution, i.e. the mean chain length is equal to the variance. Thus quoting the average cells per chain gives as complete a picture as is possible of the conditon of the chains.

The "exponential" rate for acid production (i.e. the exponential rate of acid production corresponding to the exponential phase of cell growth) was determined by means of an exponential least squares regression on the available data of acid produced after a given time. A minimum of four data points was used in each determination. Acid concentrations in the first hour or two were ignored. The regression coefficients were greater than 0.99 in all but run 1 when it was 0.97.

The "stationary phase" rate of acid production was determined by a linear least-square regression of acid concentration on time. Typically eight data points over about 14 h were used in each determination. All regression coefficients were greater than 0.997.

The results are shown in table 4.12.

Table 4.11 Typical result for batch fermentation of whey - effect of agitation

Run B 9.12.13: n = 200 rpm, d = 102 mm

Time h	Acid increase g/l	Lactose g/l	cells/ l x 10^{-10}	cells/ chain
0	0.00	43.7	0.72	4.0
1	0.07	43.6	2.04	3.8
2	0.21	43.9	2.28	2.9
3	0.42			
4	0.98	43.6	7.37	3.0
5	2.13			
6	4.32	41.6	33.7	3.7
7	5.50	39.5	46.4	3.2
9	8.64			
11	11.50			
13.25	15.75	31.9	65.6	2.7
15	17.95			
17	21.08			
19	23.96			
21	26.51			
24	30.28	17.2	59.0	2.6
26	32.47			
27.5	33.88	13.4	69.1	2.5

Table 4.12 The effect of agitation intensity on the rate of acid production

Run No.	n rpm	d mm	"Exponential Rate" h^{-1}	"Stationary Rate" g/l h
1	200	25	0.56	0.84
2	635	25	0.71	1.41
3	1270	25	0.79	1.69
4	100	51	0.65	1.21
5	200	51	0.76	1.39
6	400	51	0.80	1.58
7	800	51	0.80	1.65
8	20	102	0.54	1.10
9	50	102	0.85	1.37
10	63	102	0.75	1.52
11	100	102	0.77	1.50
12	126	102	0.73	1.44
13	200	102	0.82	1.52

In Figures 4.15 and 4.16 the rate of acid synthesis is shown as a function of impeller speed and diameter on logarithmic co-ordinates. As the speed of agitation is increased, the rate of acid formation increases, but at high levels of agitation the effect is small. Even at low paddle speeds, the rate of acid formation does not increase as the 0.75 power of the speed of rotation as predicted by equation 2.17 for a reaction limited by the rate of transfer of lactose (or other nutrients) and lactic acid.

Figures 4.17 and 4.18 show the rate of acid formation as a function of the agitator Reynolds Number. The results form two distinct regions with a transition at Re = 10,000.

The two distinct regions are also shown when rate of acid formation is plotted against power (Figures 4.19 and 4.20).

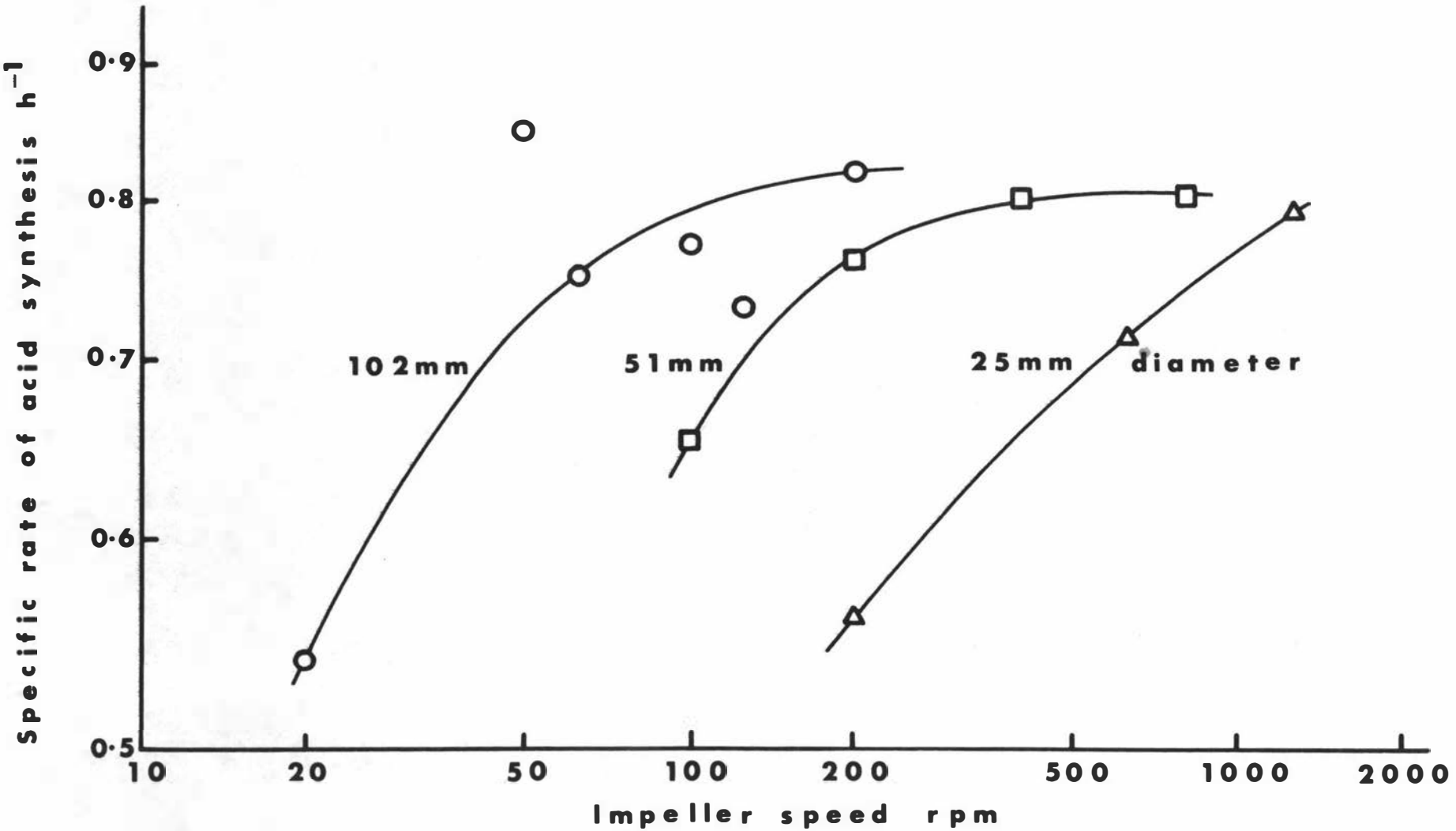


Figure 4.15 Acid production in the bacterial exponential growth phase as a function of impeller speed and diameter. (In x In). Pasteurized whey 70 g/l, pH6.0, 46°C.

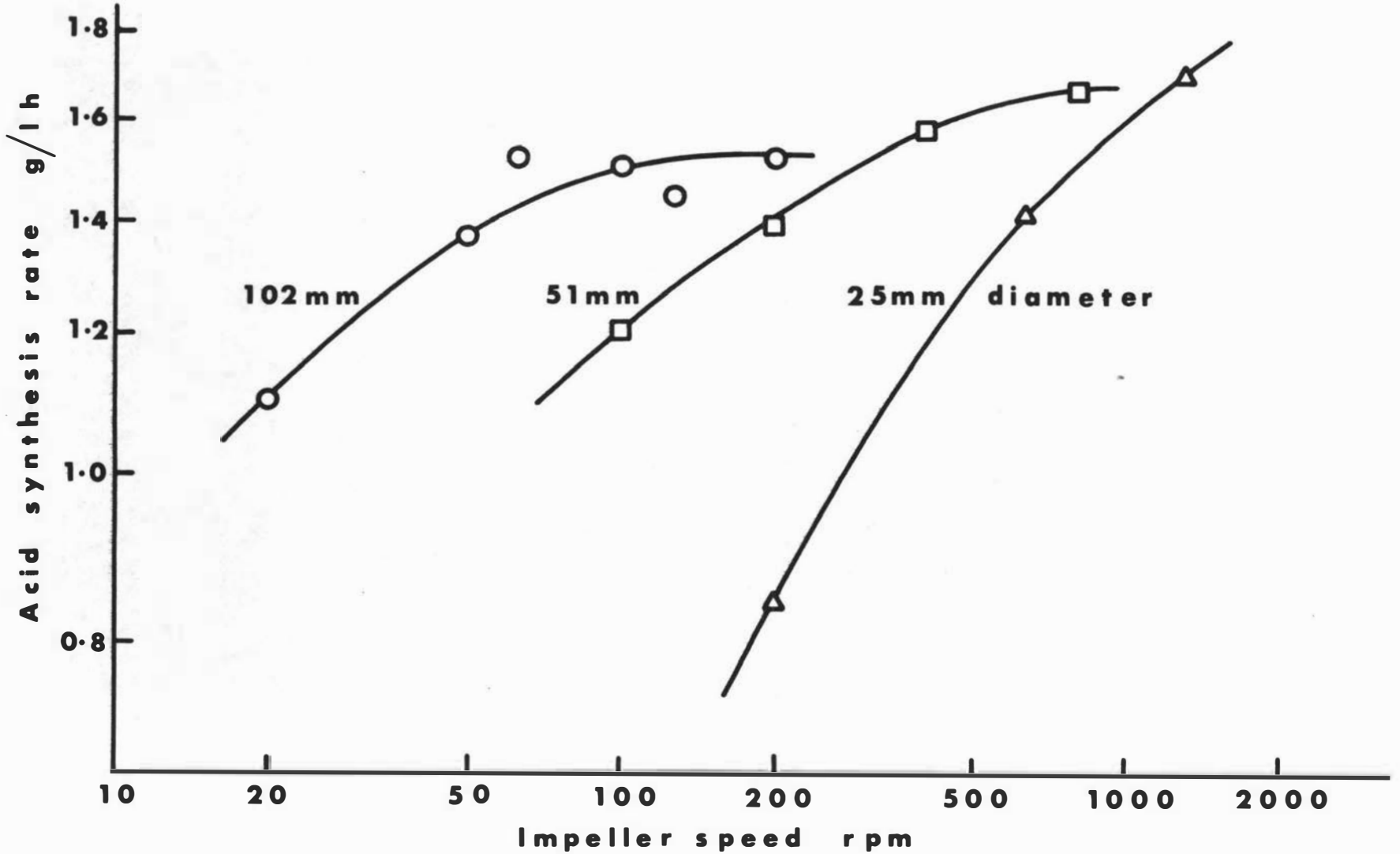


Figure 4.16 Acid production rate in the bacterial stationary growth phase as a function of impeller speed and diameter. (In x In). Pasteurized whey 70 g/l, pH6.0, 46°C.

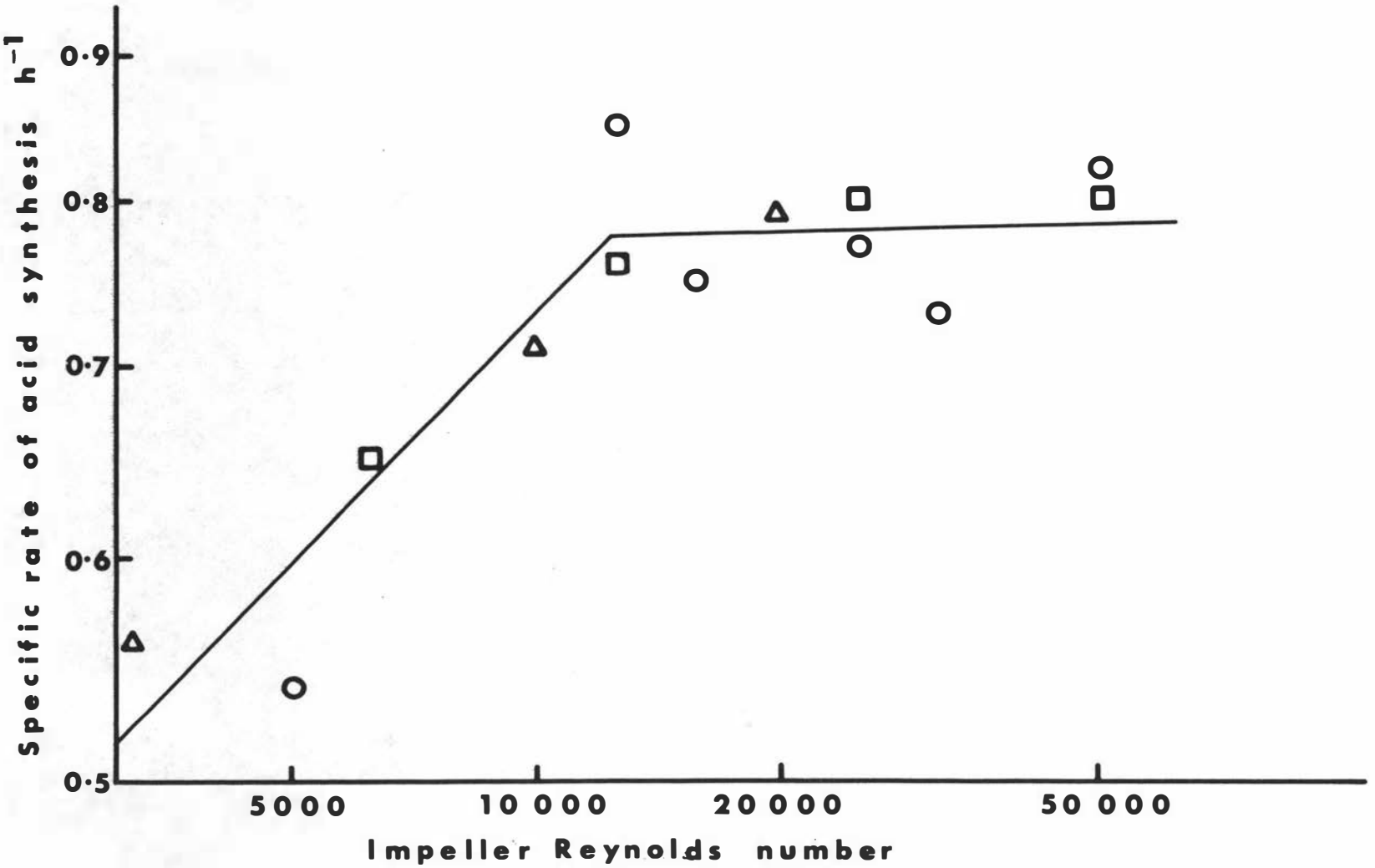


Figure 4.17 Acid production in the bacterial exponential growth phase as a function of the impeller Reynolds Number. (ln x ln). △ - 25 mm paddle, □ - 51 mm paddle, ○ - 102 mm paddle. Pasteurized whey 70 g/l, pH6.0, 46°C.

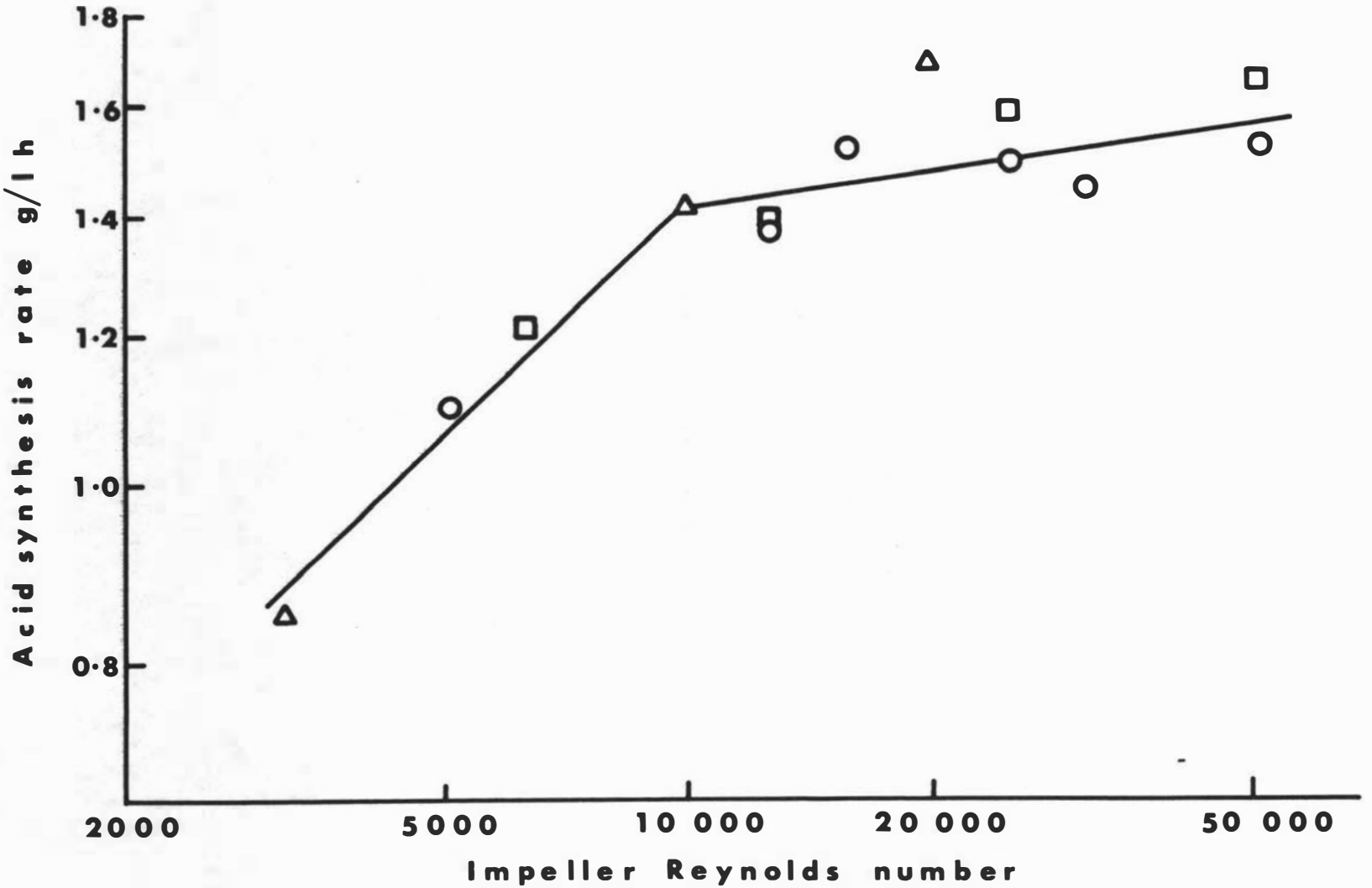


Figure 4.18 Acid production rate in the bacterial stationary growth phase as a function of the impeller Reynolds Number. (ln x ln). △ - 25 mm paddle, □ - 51 mm paddle, ○ - 102 mm paddle. Pasteurized whey 70 g/l, pH6.0, 46°C.

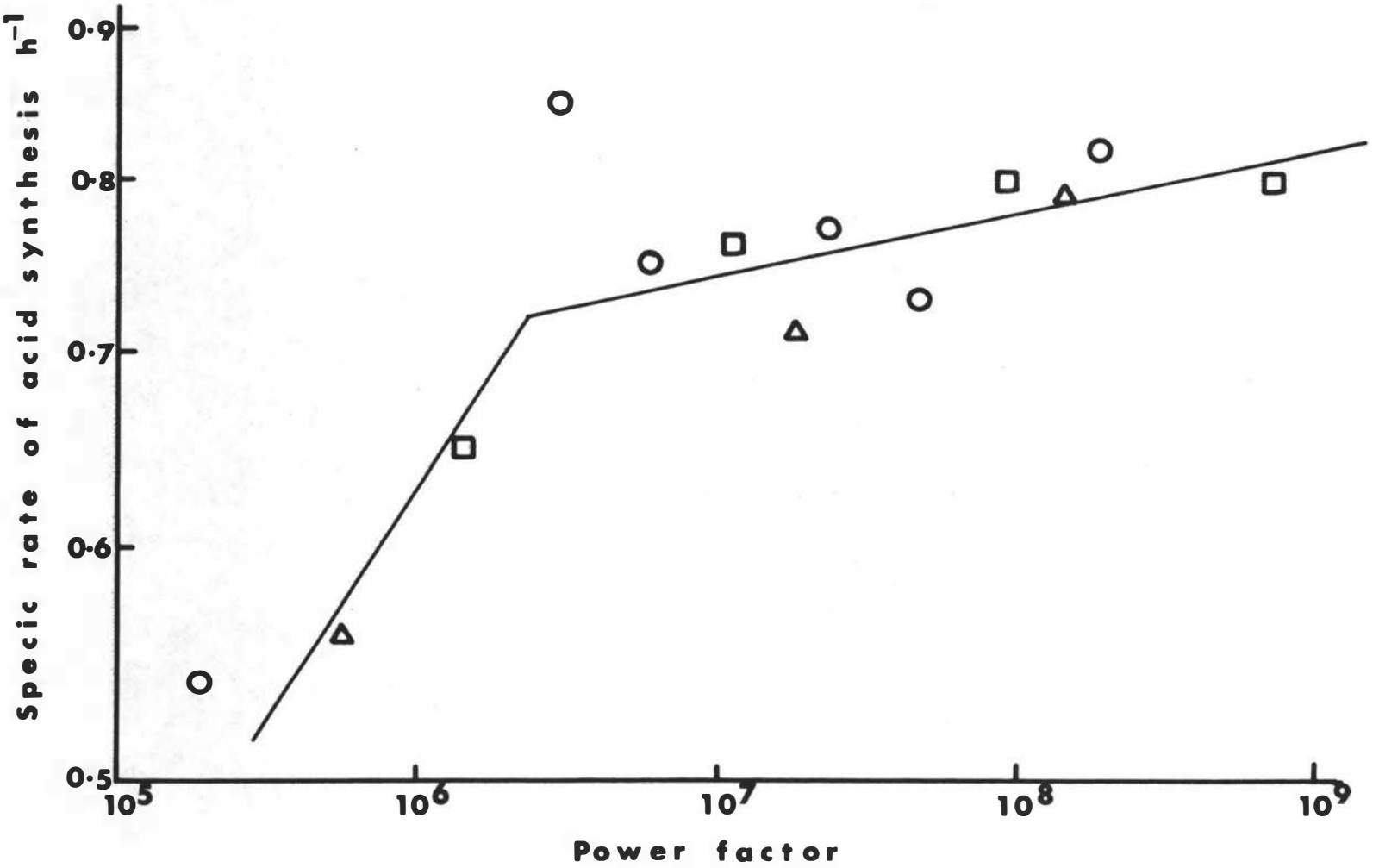


Figure 4.19 Acid production in the bacterial exponential phase as a function of P_f (= wl(d - w)n^3 d^2), the power dissipated by the paddle. (ln x ln) △ - 25 mm paddle, □ - 51 mm paddle, ○ - 102 mm paddle. Pasteurized whey 70 g/l, pH6.0, 46°C.

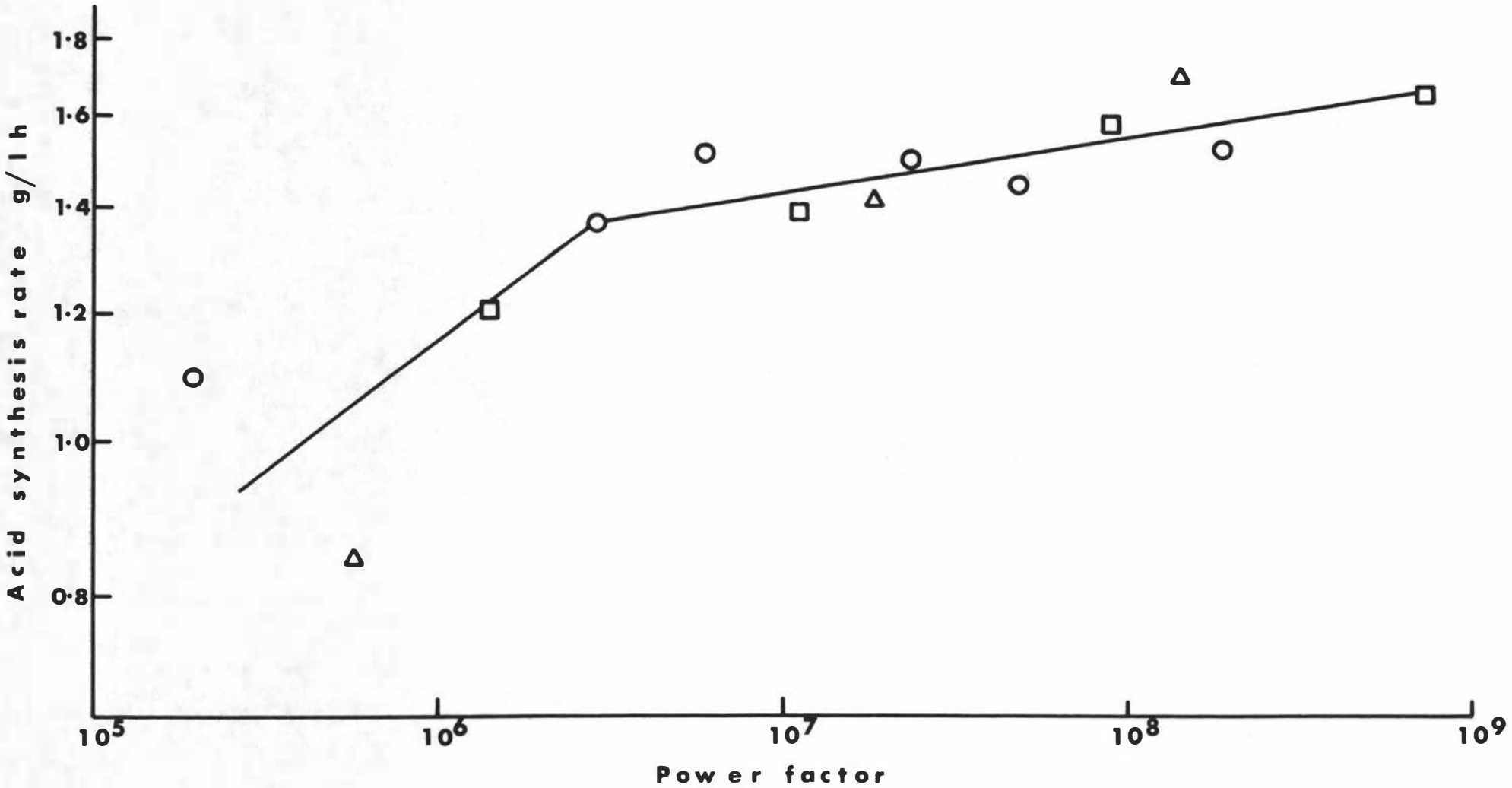


Figure 4.20 Acid production rate in the bacterial stationary growth phase as a function of P_f (= wl(d - w) n^3 d^2), the power dissipated by the paddle. (ln x ln). △ - 25 mm paddle, □ - 51 mm paddle, ○ - 102 mm paddle. Pasteurized whey 70 g/l, pH6.0, 46°C.

For $5 \times 10^5 \leq P_f \leq 3 \times 10^6$

$$\frac{1}{N}\frac{dP}{dt} = 0.07\ P_f^{0.159}$$

(correlation coefficient = 0.915, significant at 0.05 probability level)

$$\frac{dP}{dt} = 0.27\ P_f^{0.103}$$

(correlation coefficient= 0.58 which is not significant)

For $3 \times 10^6 \leq P_f \leq 8 \times 10^8$

$$\frac{1}{N}\frac{dP}{dt} = 0.53\ P_f^{0.021}$$

(correlation coefficient= 0.687 significant at a 0.02 probability level.)

$$\frac{dP}{dt} = 0.86\ P_f^{0.032}$$

(correlation coefficient= 0.77 significant at a 0.001 probability level.)

4.2.8 Cell Recycle

To simulate the effect of recycling cells two litres of whey (35 g/l) in an Erlenmeyer flask was inoculated with a clotted skim milk culture of LBR. The culture was incubated at 46°C without agitation with periodic additions of 3N-Na_2CO_3 to maintain the pH at approximately 6.0. Two hours after the flask was inoculated a normal batch culture was commenced in the fermenter. (B9.1.6)

Seven hours after the flask was inoculated the culture was cooled by placing the flask in crushed ice. The cells were recovered by 15 min centrifuging at 10,000 x g (refrigerated Sorvall SS3,1°C). The cells were washed with cold sterile phosphate buffer, (pH 6.0) centrifuged again, resuspended in approximately 60 ml of buffer and inoculated into the fermenter. Cell recovery took approximately 1.5 h and hence the culture in the fermenter was in the stationary phase of the growth cycle.

To check that washing of the cells and the addition of phosphate buffer did not affect the production of acid a further experiment was performed in which the culture was removed from the fermenter seven hours after inoculation, cells recovered and washed as above and reinoculated into the centrifugate. The results are plotted in Fig. 4.21 with the

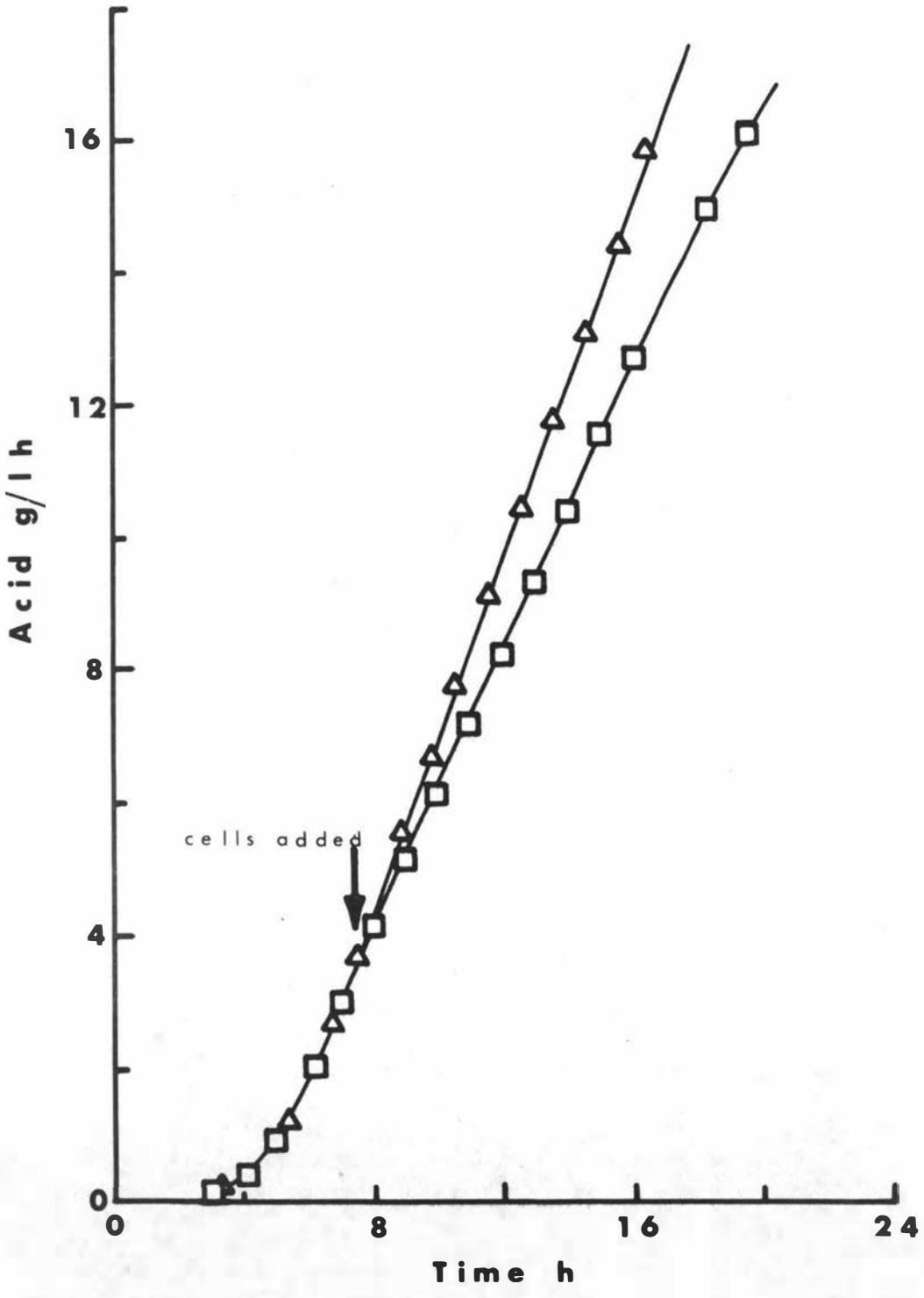


Figure 4.21 Increase in the acid production rate by adding stationary phase cells of LBR to a batch culture at 7 h. □ - control △ - cells added at time shown by the arrow. Whey 35 g/l, pH6.0, 46°C.

time adjusted to produce correspondence of the acid concentration curves in the exponential growth phase (this simply adjusts for the inevitable differences in lag time).

The results show that recycling cells causes an increase in acid production rate. Washing and resuspending in buffer does depress the production rate. The rate of acid production remains essentially constant, but at a different value. The expected stationary phase acid production rate is 1.00 g/l h. With cells resuspended in the centrifugate a value of 0.8 g/l h is obtained, a 20 per cent decrease. When the washed cells are inoculated into a normal fermentation a rate of 1.34 g/l h is achieved, a 34 per cent increase.

As shown by the control, cell recovery by the method used causes some loss in activity possibly because of some loss in cell viability, or because some suspended slightly soluble component of the whey is being removed by the washing process. The cells were washed in this experiment to reduce the possibility of suspended casein being concentrated and added to the fermentation, causing an increase in acid production.

On a larger scale it would be simpler to continuously recover the cells, washing would not be necessary and from the above result it can be concluded that the higher bacterial concentration achieved in doing this would have a significant beneficial effect on the productivity of the fermentation.

4.3 CONTINUOUS CULTURE

4.3.1 Determination of the optimum conditions of pH, temperature and dilution rate for a single stage continuous culture.

Using sterilized whey as the feed for a single stage continuous culture vessel the effects of pH, temperature and dilution rate on the productivity, acid concentration and yield were studied using a central composite rotatable experimental design (Cochran and Cox, 1962).

The experimental conditions are shown in table 4.13. The method of calculation of results is shown in appendix 4 and the results are given in table 4.14.

Using these results it is possible to depict the response surface by an equation of the form;

Table 4.13 Experimental conditions in continuous culture experiments to determine the effect of pH, temperature and dilution rate on productivity, acid concentration and yield.

Run No.	Experimental Order	Code X_1	Code X_2	Code X_3	pH	Temp ^{o}C	Dilution rate h^{-1}
1	1	-1	-1	-1	5.5	45	0.103
2	3	1	-1	-1	5.8	45	0.103
3	4	-1	1	-1	5.5	47	0.103
4	2	1	1	-1	5.8	47	0.103
5	8	-1	-1	1	5.5	45	0.309
6	7	1	-1	1	5.8	45	0.309
7	5	-1	1	1	5.5	47	0.309
8	6	1	1	1	5.8	47	0.309
9	14	-1.68	0	0	5.4	46	0.206
10	17	1.68	0	0	5.9	46	0.206
11	15	0	-1.68	0	5.65	44.3	0.206
12	16	0	1.68	0	5.65	47.7	0.206
13	12	0	0	-1.68	5.65	46	0.031
14	11	0	0	1.68	5.65	46	0.380
15	9	0	0	0	5.65	46	0.206
16	19	0	0	0	5.65	46	0.206
17	13	0	0	0	5.65	46	0.206
18	10	0	0	0	5.65	46	0.206
19	20	0	0	0	5.65	46	0.206
20	18	0	0	0	5.65	46	0.206

Sterilized whey 75 g/l total solids (Average lactose in,45.7 g/l); agitation 200 rpm, headspace gas,95% N_2, 5% CO_2

Code determined from equations for x_i

ie (-1, -1, -1) x_1 : -1 = $\frac{pH - 5.65}{0.15}$ $\therefore$ pH = 5.5

x_2 : -1 = Temp -46 $\therefore$ Temp= 45

x_3 : -1 = $\frac{D - 0.206}{0.103}$: D = 0.103

Table 4.14 The effect of pH, temperature and dilution rate on lactic acid production (See table 4.13 for experimental conditions)

Run No.	Code X_1	X_2	X_3	Cell conc No/l x 10^{-12}	$\frac{dP}{dt}$ g/l h	P g/l h	Y
1	-1	-1	-1	1.22	1.63	16.00	0.36
2	1	-1	-1	2.52	1.95	19.10	0.43
3	-1	1	-1	1.60	1.94	19.00	0.42
4	1	1	-1	3.44	1.75	17.20	0.38
5	-1	-1	1		3.33	10.80	0.23
6	1	-1	1		3.44	11.13	0.24
7	-1	1	1	1.24	3.31	10.70	0.24
8	1	1	1	1.30	3.39	10.95	0.24
9	-1.68	0	0	7.59	2.93	14.20	0.31
10	1.68	0	0	1.62	3.06	14.85	0.34
11	0	-1.68	0	6.96	2.37	11.50	0.24
12	0	1.68	0	7.64	2.66	12.93	0.28
13	0	0	-1.68	2.02	0.85	27.38	0.58
14	0	0	1.68	1.44	3.40	8.94	0.19
15	0	0	0	2.67	2.87	13.90	0.30
16	0	0	0	2.52	3.55	17.22	0.35
17	0	0	0	4.30	2.79	13.52	0.30
18	0	0	0	2.04	2.71	13.18	0.29
19	0	0	0	2.20	3.26	15.82	0.36
20	0	0	0	1.70	3.41	16.55	0.38

$$F = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3$$

Where F is the dependent variable (productivity, $\frac{dP}{dt}$; acid concentration, P ; or yield, Y);

x is given by

$$x_1 = \frac{pH - 5.65}{0.15}$$

$$x_2 = \text{Temperature} - 46\ ^{o}C$$

$$x_3 = \frac{D - 0.206}{0.103} \qquad h^{-1}$$

and b_{ij} are the regression coefficients.

The calculations required to calculate b_{ij} are described by Cochran and Cox (1962) and an example is given in appendix 5.

The values of b_{ij} for the three equations relating the dependent variables with pH, temperature and dilution rate are shown in table 4.15 and an analysis of variance in table 4.16.

Table 4.15 Regression coefficients (± standard error) for the equations relating productivity, increase in acid concentration and yield with pH, temperature and dilution rate :

	$\frac{dP}{dt}$	P	Y
b_0	3.10 ± 0.35	15.1 ± 1.7	0.330 ± 0.038
b_1	0.04 ± 0.13	0.22 ± 0.61	0.007 ± 0.013
b_2	0.08 ± 0.13	0.24 ± 0.61	0.006 ± 0.013
b_3	0.77 ± 0.13	-4.30 ± 0.61	-0.095 ± 0.013
b_{11}	-0.02 ± 0.14	-0.31 ± 0.66	-0.003 ± 0.015
b_{22}	-0.19 ± 0.14	-1.12 ± 0.66	-0.026 ± 0.015
b_{33}	-0.33 ± 0.14	0.98 ± 0.66	0.018 ± 0.015
b_{12}	-0.07 ± 0.18	-0.62 ± 0.87	0.015 ± 0.020
b_{13}	0.01 ± 0.18	-0.09 ± 0.87	-0.003 ± 0.020
b_{23}	-0.02 ± 0.18	-0.17 ± 0.87	0.000 ± 0.020

Note: The equation representing the response surface for the effect of pH (x_1), temperature (x_2) and dilution rate (x_3) on productivity is therefore

$$\frac{dP}{dt} = 3.10 + 0.04\,x_1 + 0.08\,x_2 + 0.77\,x_3 - 0.02\,x_1^2 - 0.19\,x_2^2 - 0.33\,x_3^2 - 0.07\,x_1x_2 + 0.01\,x_1x_3 - 0.02\,x_2x_3$$

Table 4.16 Analysis of variance for the equations relating productivity increase in acid concentration, and yield with pH, temperature, and dilution rate.

Source of Variation	Degrees of Freedom	Mean Squares $\frac{dP}{dt}$	Mean Squares P	Mean Squares Y
First order terms	3	2.721	84.68	0.0414
Second order terms	6	0.324	6.66	0.0030
Lack of fit	5	0.002	3.01	0.0012
Error	5	0.125	2.94	0.0014

In each case the "lack of fit" mean square, which is a measure of the deviation of the experimental values from the fitted equation, is about the same size as the error mean square indicating that the second order equation is an adequate representation of the response surface. The first order terms mean square is highly significant but the second order terms mean square is not significant at the 10 per cent probability level. The F ratios of the second order mean squares to the error mean squares are 2.6, 2.4 and 2.1 for the productivity, increase in acid concentration and yield respectively. The tabulated value of the variance ratio at the 10 per cent probability level is 3.40 (Davies,1963). Thus a linear equation would be an adequate representation of the experimental results.

However, the regression coefficients indicate that in each case the responses to changes in temperature in the experimental range are not linear as is shown by comparing b_2 with b_{22}. The same holds for the dilution-rate, but to a lesser extent. The other second order regression coefficients appear negligible in comparison with their standard deviations but there is no particular reason to assume that they are zero.

Though it is realised that a linear model may well be an adequate description of the results the second order equations are retained as the best estimate of the response surface available. The equations obtained have been used to plot figures 4.22 - 4.25 illustrating the salient points of the results.

Figures 4.22 - 4.24 show the changes predicted in the productivity, acid concentration and yield as temperature, pH and dilution-rate are varied.

The response to temperature is peaked with a maximum about 46°C. The pH response shows little effect of pH over the range 5.4 - 5.9.

Of the three variables, dilution-rate shows the greatest effect. As the dilution rate is increased the productivity increases but the acid concentration and yield decrease. The productivity is predicted to have a maximum at a dilution-rate of aproximately 0.33 h^{-1}. However, at this dilution-rate the yield is predicted to be only 0.23 and acid concentration 11 g/l. High productivity gives low yield and acid concentration.

By differentiating the derived equations and equating the differentials to zero the maximum productivity (3.58 g/l h) is predicted to occur at 45.9 °C, pH 5.9 at a dilution-rate of 0.33 h^{-1}. This point occurs within the experimental region studied and hence is a reasonable estimate of the optimum (Cochran and Cox,1962).

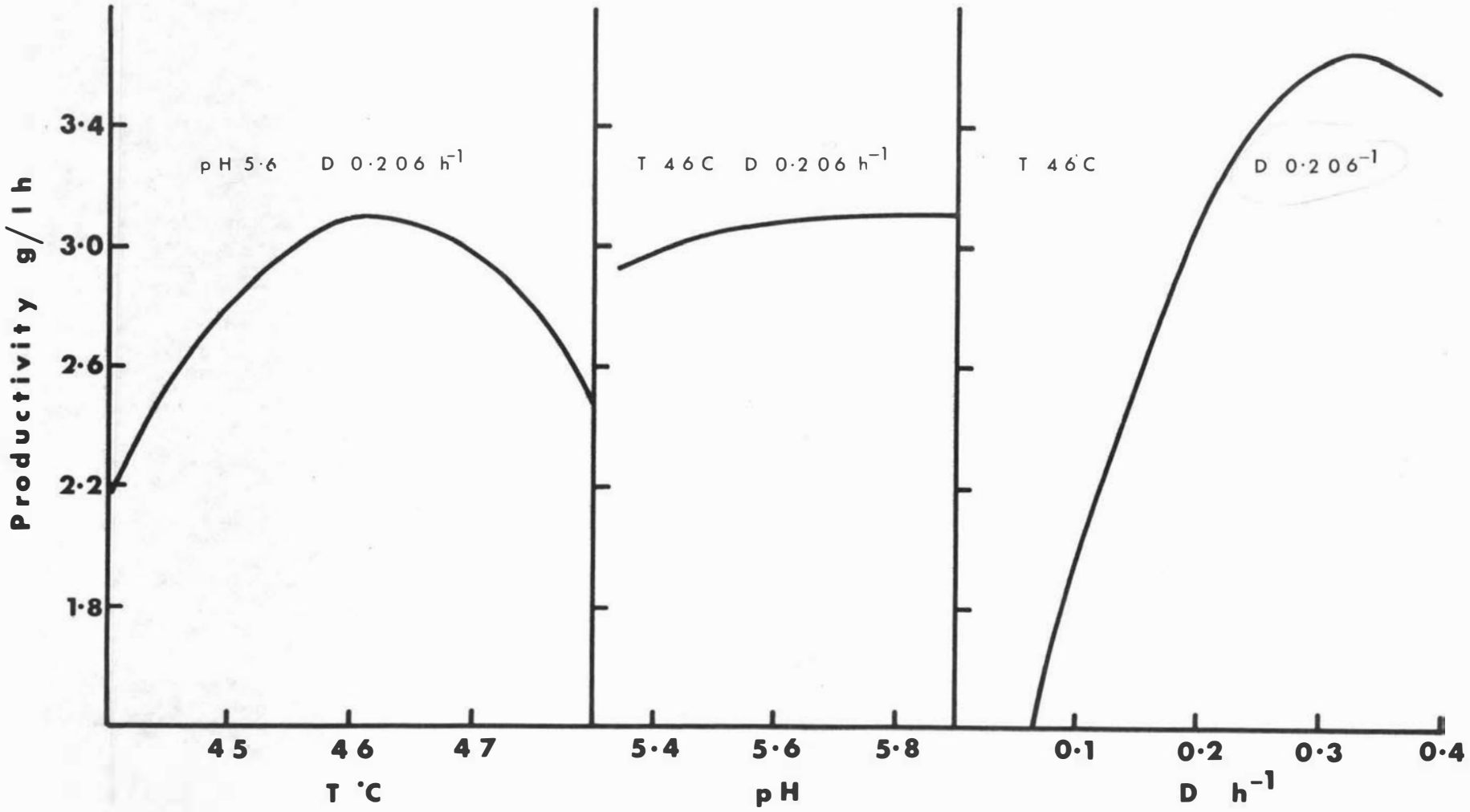


Figure 4.22 The productivity of lactic acid $\left\{\frac{dP}{dt}\right\}$ as a function of temperature, pH and dilution rate in a single stage continuous culture. Sterilized whey 75 g/l.

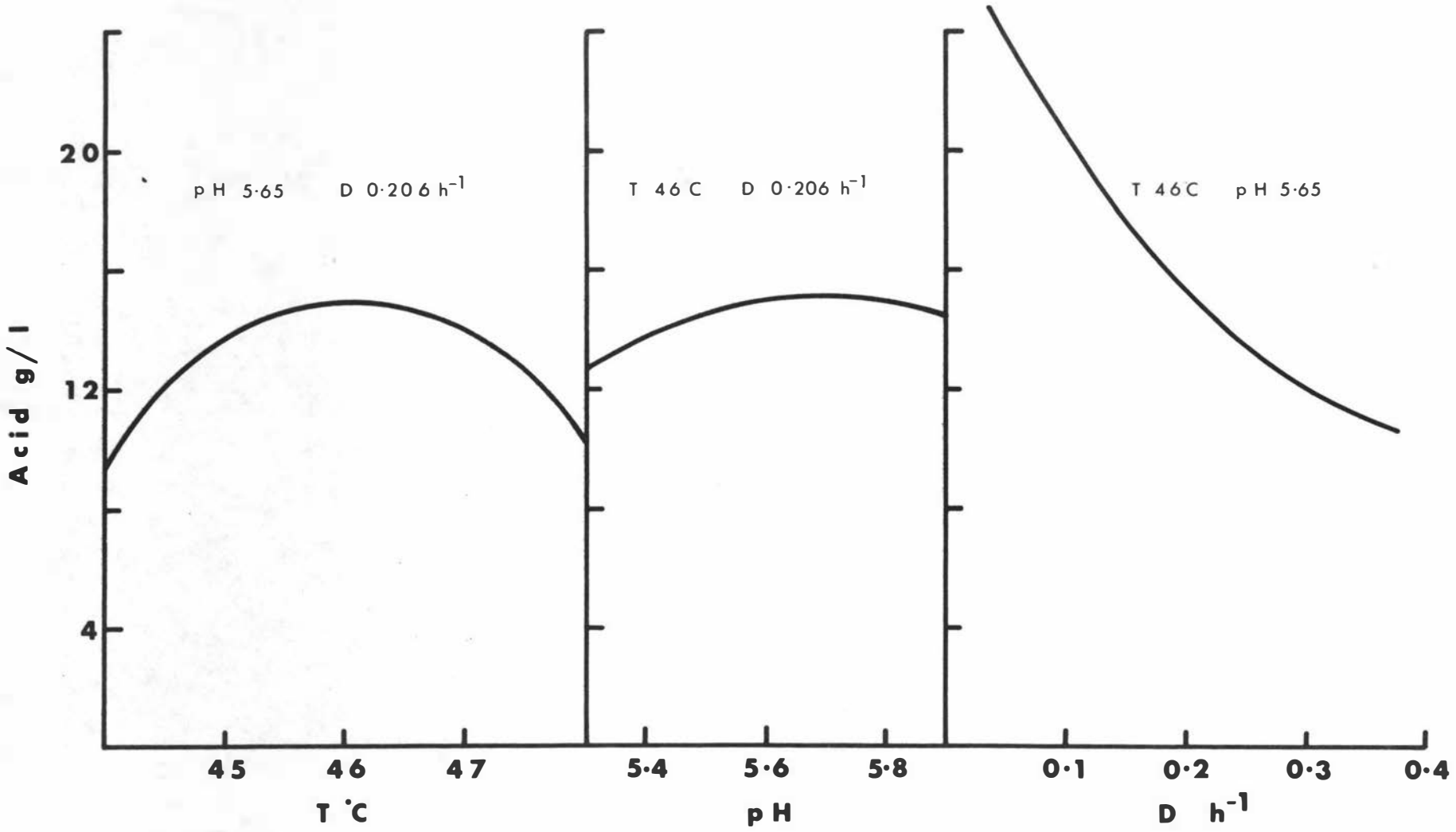


Figure 4.23 Acid concentration (P) as a function of temperature, pH and dilution rate in a single stage continuous culture. Sterilized whey 75 g/l.

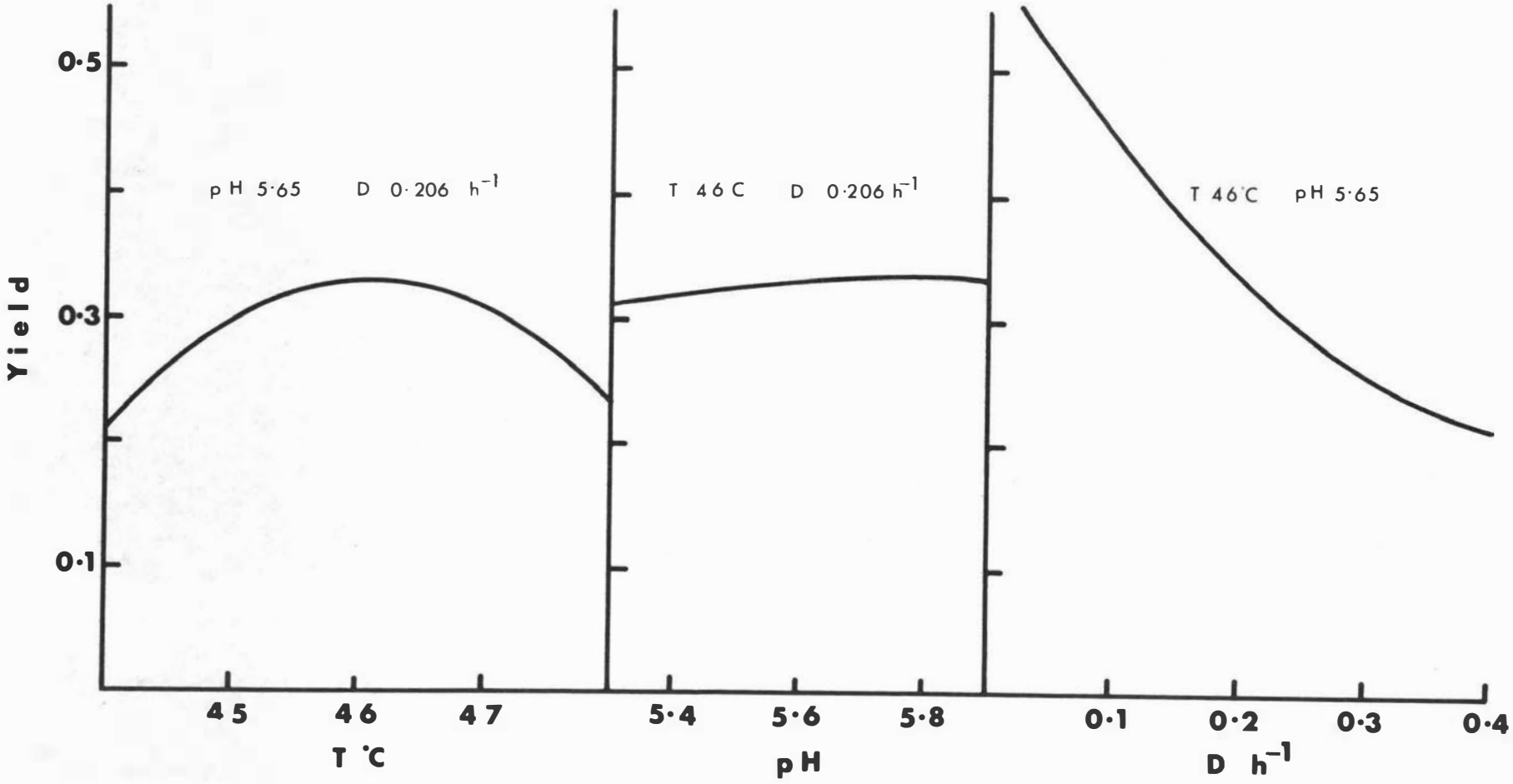


Figure 4.24 Yield of lactic acid from lactose (Y) as a function of temperature, pH and dilution rate in a single stage continuous culture. Sterilized whey 75 g/l.

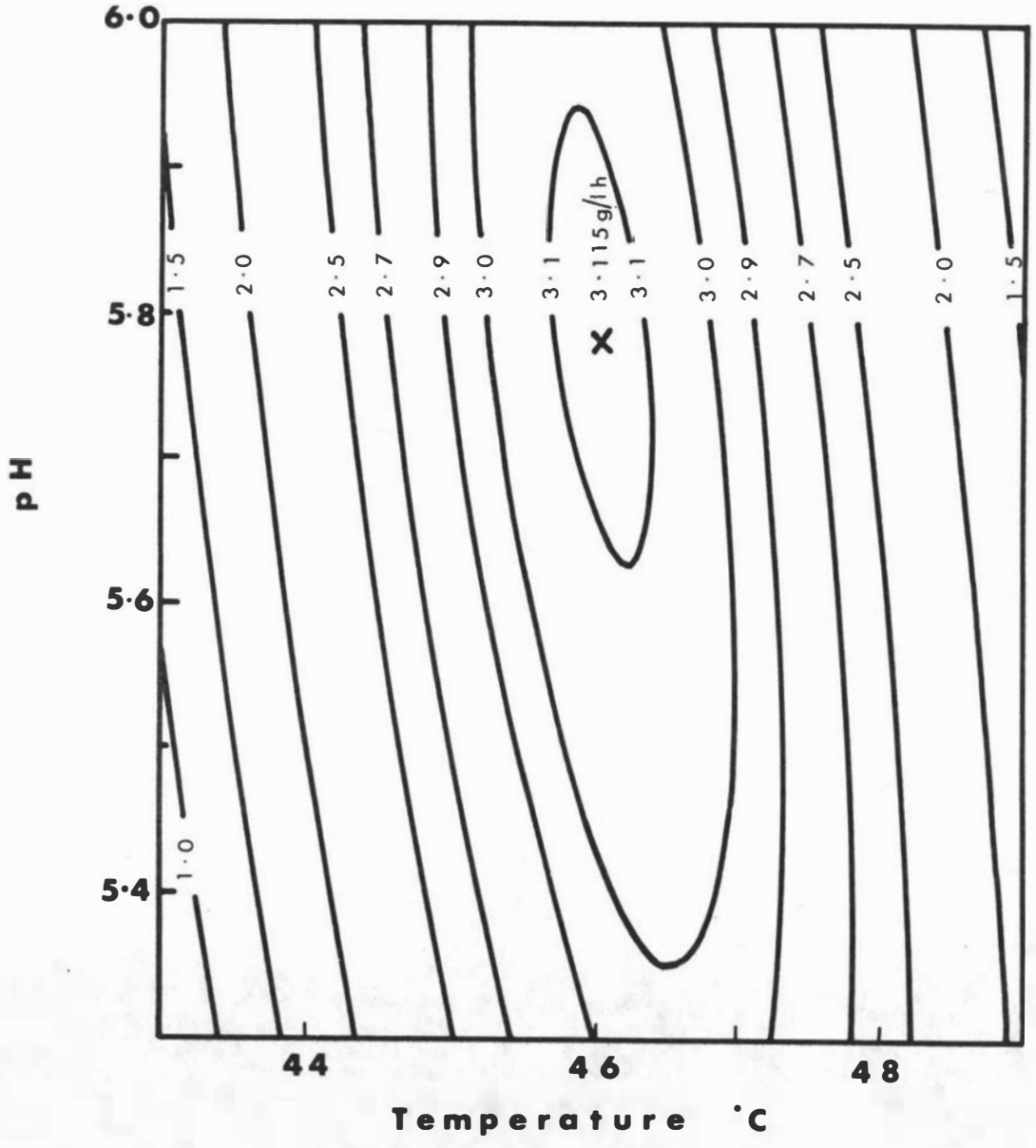


Figure 4.25 The rate of acid production $\left(\frac{dP}{dt}\right)$ as a function of pH and temperature at a dilution rate of 0.2 h^{-1}. Sterilized whey 75 g/l.

The contour-plot in Fig. 4.25 illustrates for the productivity the slight interaction between pH and temperature, the relatively small effect changes in pH have, and the peaked nature of the temperature response.

4.3.2 Continuous culture of sterilized whey with added sodium-caseinate

Table 4.17 shows the results obtained when sterilized whey was supplemented with sodium caseinate at two dilution-rates.

Table 4.17 Continuous culture of whey with added sodium caseinate (AD74, 10 g/l)

Dilution-rate h^{-1}	0.22	0.12
Lactose in feed g/l	44.2	43.9
Acid production g/h	9.16	6.88
Acid concentration g/l	21.9	30.4
Productivity g/l h	4.81	3.65
Yield	0.50	0.69
Cell concentration /l		2.76×10^{12}

Sterilized whey 70 g/l ; 46 °C ; pH 5.65 ; Agitation 200 rpm.

The results in table 4.17 can be compared with Figs. 4. 22 and 4.24. In unsupplemented whey the predicted productivities are 3.24 and 2.20 g/l h at dilution-rates 0.22 and 0.12 h^{-1} respectively with predicted yields of 0.31 and 0.42 respectively. The productivity and yield are increased by aproximately 60 per cent by the addition of 10 g/l of sodium caseinate.

4.3.3 Continuous culture of pasteurized whey.

Table 4.18 shows the experimental conditions used and results obtained in continuous culture of pasteurized whey, with a fresh whey feed solution being prepared every 48 - 72 h. Despite long hours of operation there was no evidence of contamination.

Table 4.18 Continuous culture with pasteurized whey

No	$D\ h^{-1}$	pH	Temp ^{o}C	Inlet Lactose g/l	Acid conc. g/l	Cell conc. cells/ l $x10^{-11}$	Product-ivity g/l h	Yield
1	0.048	6.0	46	25.2	17.9	-	0.86	0.71
2	0.061	6.0	46	18.2	13.1	6.7	0.80	0.72
3	0.061	6.5	44	18.5	5.4	5.2	0.33	0.30
4	0.061	6.0	46	18.5	12.6	6.3	0.77	0.68
5	0.061	5.5	48	24.4	11.1	5.4	0.68	0.45
6	0.061	6.5	48	18.2	4.0	4.2	0.25	0.22
7	0.061	5.5	44	18.2	12.9	-	0.79	0.71
8	0.153	6.0	46	22.8	9.5	-	1.45	0.41
9	0.061	6.0	46	38.8	27.6	21.1	1.69	0.71
10	0.061	6.0	46	18.4	13.6	-	0.83	0.74

Experiments 2, 4 and 10, at the same conditions, give a measure of the reproducibility of the results. The mean and standard deviation of the values are given in table 4.19. The experiments were carried out over a period of three months.

Table 4.19 Reproducibility in continuous culture experiments using pasteurized whey. (Two degrees of freedom)

	Mean $\pm$ std error
Acid concentration g/l	13.10 ± 0.05
Productivity g/l h	0.80 ± 0.03
Yield	0.71 ± 0.03
Cell concentration / l	$(6.5 \pm 0.3) \times 10^{11}$

Experiments 2 and 8 illustrate the effect of changing the dilution-rate. As with sterilized whey increasing the dilution-rate increases the productivity and decreases the yield and acid concentration.

The effect of changing the temperature and pH is shown by experiments 2 to 7. As the temperature is shifted away from 46 °C or the pH is shifted away from 6.0 the productivity and yield decrease in general agreement with the results for sterilized whey.

In the batch culture experiments the rate of acid production was shown to be proportional to the concentration of whey solids. (4.2.2). The same relationship can be shown to hold for continuous culture by comparing experiment 9 with experiment 2. Increasing the solids concentration by a factor of 2.13 increased the productivity and acid concentration by a factor of 2.11. Yield is independent of the solids concentration.

From the equations in table 4.14, the predicted values of productivity, acid concentration and yield for sterile whey are 1.12 g/l h, 18.9 g/l and 0.51 respectively (adjusted to 38.8 g/l lactose concentration). These are considerably lower than the values for experiment 9, illustrating the increase as a result of using pasteurized whey.

To illustrate the stability of the continuous culture the results obtained during experiment 10 over a period of 11 days (residence time x 16) are plotted in figure 4.26. The mean productivity calculated from the data is 0.83 g/l h with a standard deviation of 0.05 g/l h. Stable continuous culture with regular acid production is thus possible.

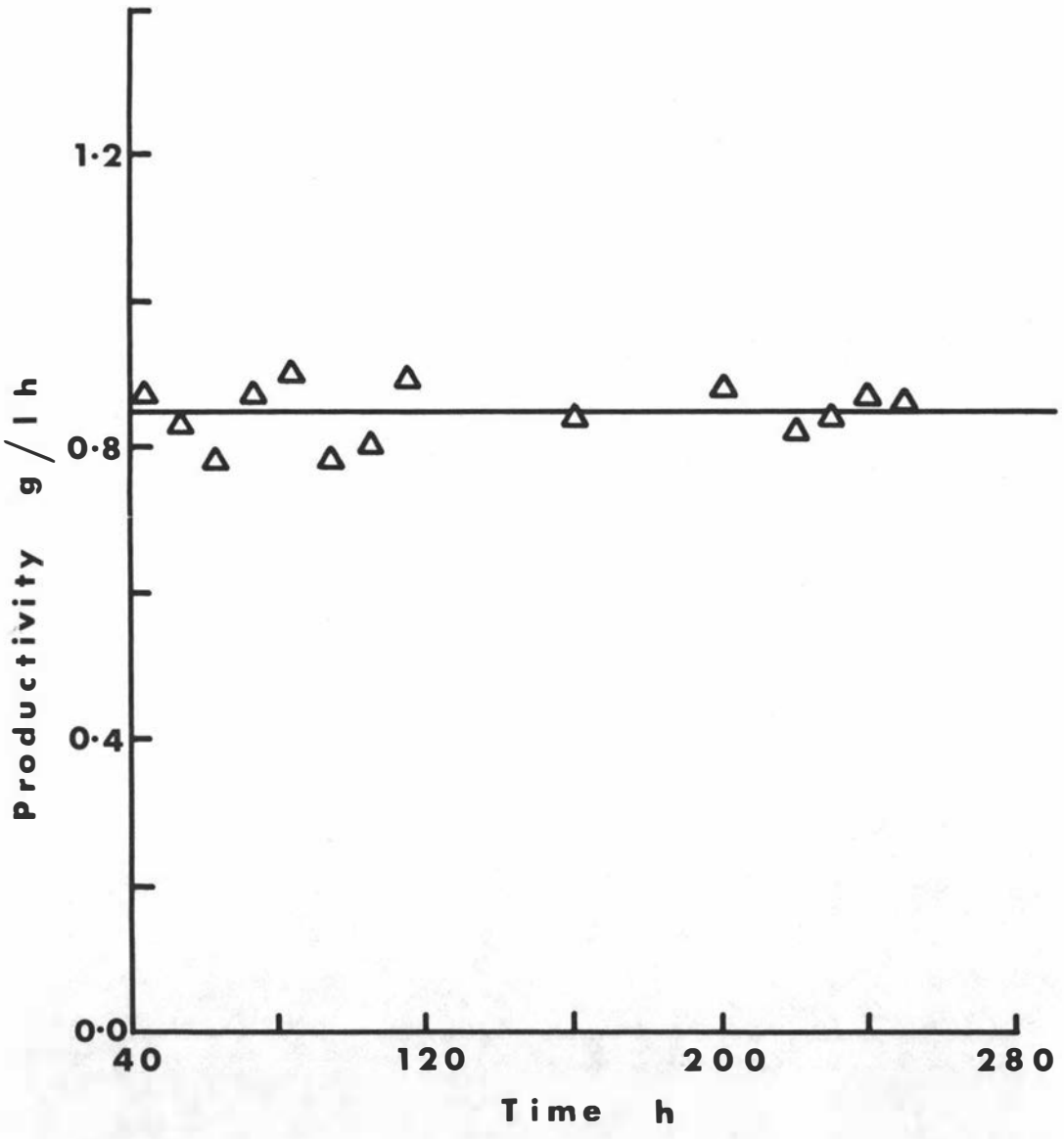


Figure 4.26 The steady state value of the productivity of lactic acid from a single stage continuous culture of whey. Pasteurized whey 35 g/l, D 0.061 h^{-1}, pH6.0, 46°C.

5 KINETICS OF LACTIC ACID PRODUCTION FROM CASEIN WHEY

An understanding of the basic kinetics of microbial growth, substrate utilization and production formation forms an integral part of the successful commercial exploitation of a biological synthetic process. Pirt (1969) has pointed out the lack of real knowledge of the kinetics of traditional fermentations such as the production of alcohol and lactic acid. Childs and Welsby (1964) and Ricica (1969) discussed the difficulties of extrapolating from the results of fermentations carried out with defined media to those where practical media are used in industrial practice. Often the nature of essential and limiting nutrients is unknown or at best only partially understood. Product engineers have to use empirical techniques to obtain the maximum benefit from a commercial fermentation.

From the studies reported in this work it is possible to gain some insight into the kinetics of lactic acid production from whey and to make predictions regarding the optimum values of some of the variables affecting the fermentation.

5.1 Batch culture

One of the most significant results to emerge from this study is the pattern of the batch production of acid.

Cell numbers follow a normal growth pattern from inoculation. After a slight lag phase the cell concentration increases exponentially to a maximum of 1-5 x 10^{12} cells per l (value depends on the conditions and supplementary nutrients used). Cell numbers then remain virtually constant in an apparent stationary or quiescent phase until exhaustion of the lactose. Acid synthesis, as indicated both by the amount of alkali added to maintain a constant pH and by analysis for lactate, increases exponentially at the same specific rate as the cell concentration. The rate of acid production reaches a peak towards the end of the bacterial growth phase and then decreases slightly. In unsupplemented whey the rate of acid production is maintained at a constant value for a considerable period of the growth cycle. As the lactose is depleted the rate of acid production eventually falls to zero. This pattern is illustrated in figures 4.1-4.6 and 5.1. Figure 5.1 is plotted from data in table 4.11.

Figure 5.2 (from Fig.4.6 ac) shows a somewhat different pattern in that there is no discernible period of constant rate of acid production.

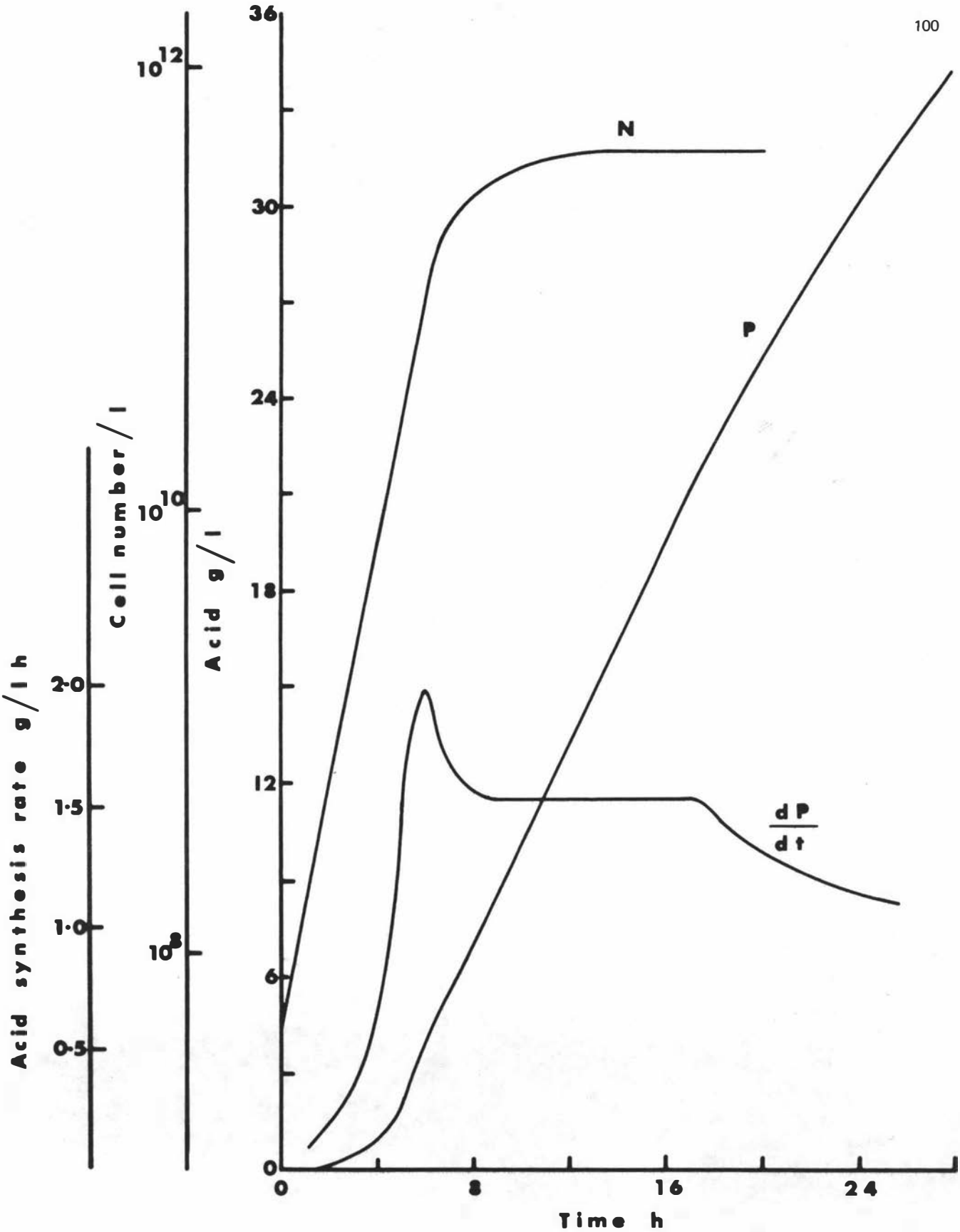


Figure 5.1 A typical batch culture (B9.12.13). Ln cell number concentration, acid concentration and rate of acid synthesis as functions of time. Pasteurized whey 70 g/l, pH6.0, 46°C.

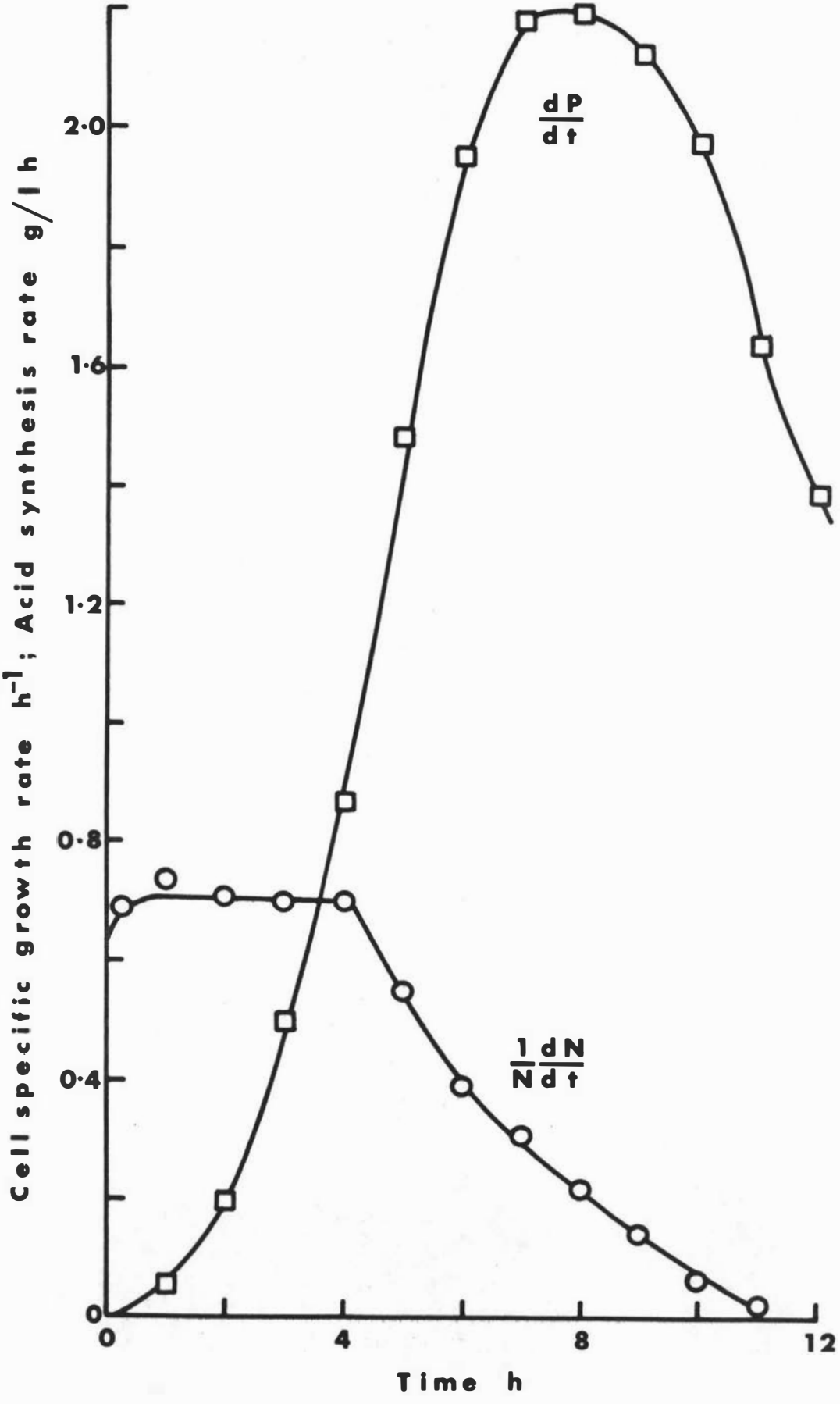


Figure 5.2 A typical batch culture with supplementary amino acids and salts added (B9.2.1). Rate of acid synthesis and specific rate of cell growth as functions of time. Sterile–filtered whey 35 g/l, pH6.0, 46°C.

This was in a culture supplemented with amino acids and mineral salts.

In batch cultures in which it was measured the decline in lactose concentration paralleled the acid production. The average yield of acid, based on initial lactose concentration, was 90 per cent. At the completion of a batch fermentation a residual level of "lactose" was invariably measured, values varying from 2 to 5 g/l. This is assumed not to be utilizable by LBR. If this residual is ignored the yield of acid based on true "lactose" is increased to 95 per cent.

5.1.1 Comparison with the kinetic equation derived by Pirt.

Qualitatively, the acid production as a function of time is similar to the growth and non-growth associated product formation derived by Pirt (1969) for the utilization of the substrate for two purposes, growth and maintenance. He derived the following expression for the time course of product formation in a batch culture:

$$\frac{1}{N}\frac{dP}{dt} = a\,\frac{1}{N}\frac{dN}{dt} + b \qquad (5.1)$$

If this expression is a valid description of the kinetics of acid production then the following relationships hold:

(a) in the bacterial exponential growth phase $\frac{1}{N}\frac{dN}{dt}$ is a constant $= \mu_m$. Therefore $\frac{1}{N}\frac{dP}{dt}$ is a constant.

(b) in the declining phase of the bacterial growth cycle $\frac{1}{N}\frac{dP}{dt}$ is a linear function of $\frac{1}{N}\frac{dN}{dt}$

(c) in the stationary phase, N is a constant, $\frac{dN}{dt}$ is zero and therefore $\frac{1}{N}\frac{dP}{dt}$ is again a constant.

The data obtained from experiments B9.1.5 (Figs.4.1-4.3), B.9.2.1 (Fig.5.2) and B9.12.13 (Fig.5.1) have been used to illustrate these points. These experiments cover a range of conditions - B9.1.5,35 g/l pasteurized whey, skim-milk inoculum, 200 rpm; B9.2.1, 35 g/l sterile-filtered whey supplemented with amino acids and minerals; skim milk inoculun, 200 rpm; B9.12.13, 70 g/l pasteurized whey, whey inoculum, 200 rpm with 105 mm paddle. All three runs were conducted at pH 6.0, 46°C using sodium carbonate to control the pH.

5.1.2 Exponential growth phase

The maximum specific growth rates are in table 5.1

Table 5.1 Maximum specific growth rate ($\frac{1}{N}\frac{dN}{dt} = \mu_m$) for batch culture

	h^{-1}
B9.1.5	1.17
B9.2.1	0.70
B9.12.13	0.65

In the exponential growth phase where $\frac{1}{N}\frac{dN}{dt}$ is a constant ($= \mu_m$) equation 5.1 becomes:

$$\frac{dP}{dt} = (a \mu_m + b) N \qquad (5.2)$$

ie the rate of acid production, is proportional to cell concentration. The linear relationship between $\frac{dP}{dt}$ and N in the exponential growth phase is shown in fig.5.3. The "line of best fit" was determined by a linear least squares regression on the data. The correlation coefficients are greater than 0.99. At $N = 0$, $\frac{dP}{dt}$ is insignificantly different from zero as predicted by equation 5.2.

5.1.3 Declining growth phase

As shown in fig.5.4, $\frac{1}{N}\frac{dP}{dt}$ is a linear function of $\frac{1}{N}\frac{dN}{dt}$ in the declining growth phase. The correlation coefficients are greater than 0.98.

The values of the constants, a and b, are given in table 5.2.

Table 5.2 Batch culture kinetics: value of constants in Equation 5.1

Run	a ($g\ cell^{-1}$) x 10^{12}	b ($g\ cell^{-1} h^{-1}$) x 10^{12}
B9.2.1	8.93	1.26
B9.12.13	6.36	2.21
B9.1.5	1.04	0.31

The "constants" for equation 5.1 vary with the conditions of fermentation. In the runs illustrated whey concentration and the levels of supplementary nutrients were different. In B9.12.13 a whey inoculum was used and therefore the medium contained little casein whereas in B9.1.5 the medium contained casein introduced with the inoculum. Run B9.2.1 contained casein introduced with the inoculum and was supplemented with amino acids and mineral salts. In B9.12.13 whey concentration was 70 g/l compared with 35 g/l in the others. Luedeking and Piret (1959a) found a and b to be functions of the pH of the fermentation. From the above

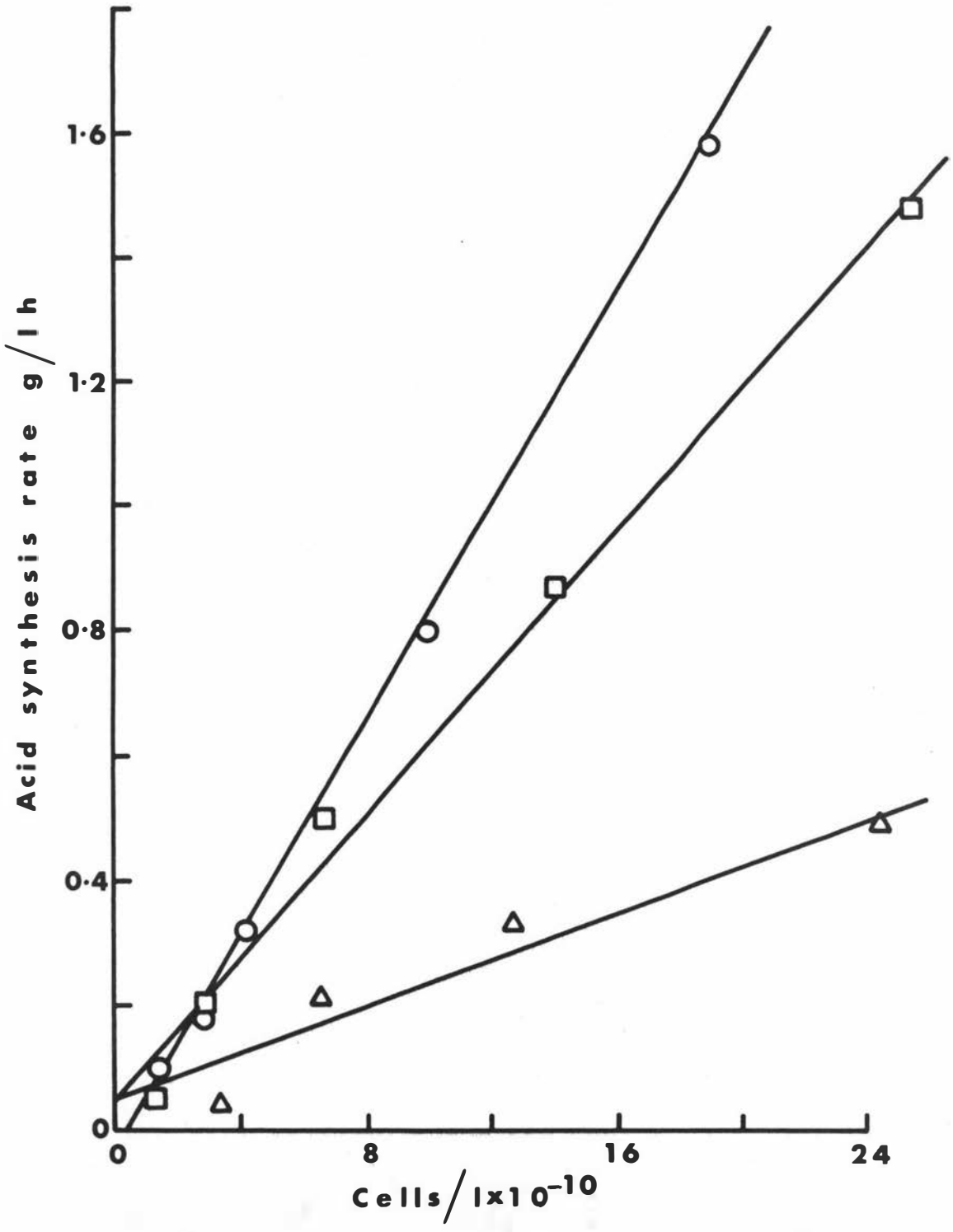


Figure 5.3 The rate of acid synthesis as a function of cell number concentration in the exponential growth phase. ○ - B9.12.13. □ - B9.2.1. △ - B9.1.5.

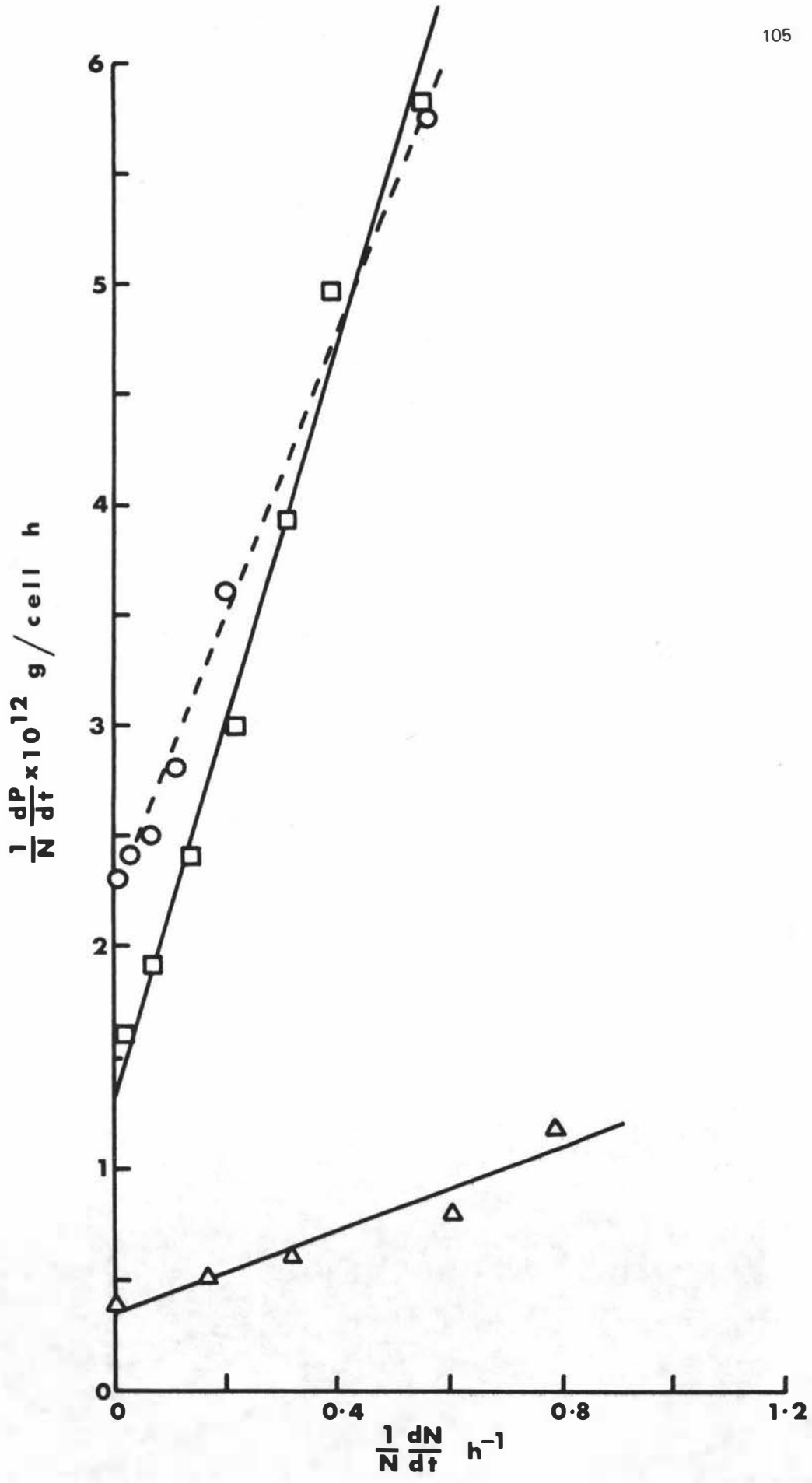


Figure 5.4 The specific rate of acid production is a linear function of the specific rate of cell growth in the bacterial declining growth phase.
○ - B9.12.13. □ - B9.2.1. △ - B9.1.5.

results it appears that variables other than pH also affect a and b.

5.1.4 Stationary phase In the apparent stationary growth phase $\frac{dP}{dt}$ is predicted by equation 5.1 to be a constant. As shown in figures 4.3 and 5.1 a constant value is recorded. On a few occasions (e.g. fig.5.2) a period of constant rate of acid formation was not clearly discernable.

5.1.5 Enzyme Kinetics Equation 5.1 does not describe the complete time course of a batch culture as it does not predict the fall-off in rate of acid formation depicted by the data in figures 4.3, 5.1 and 5.2.

If it is accepted that the bacterial cells are in a stationary or "quiescent" phase then they can be considered to form a constant concentration of glycolytic enzymes. The enzymes act as a catalyst converting lactose to lactic acid. If this is the case the reaction kinetics should be capable of being described by the usual Michaelis-Menten equation for enzyme reactions for constant enzyme concentration and activity

$$v = \frac{V_m Z}{K_z + Z} \qquad (5.3)$$

or

$$\frac{1}{v} = \frac{K_z}{V_m}\frac{1}{Z} + \frac{1}{V_m} \qquad (5.4)$$

where v is the reaction-rate ($= \frac{dP}{dt}$) V_m and K_z are constants and Z is the concentration of substrate (lactose). Assuming that the enzyme concentration and activity of the bacterial cells of LBR are not affected by time and increasing lactate concentration then the reciprocal of the rate of acid synthesis should be proportional to the reciprocal of the lactose concentration. The data from run B8.2.6 (Table 4.2) are tabulated in table 5.3 and shown in figure 5.5. It has been assumed that a final "lactose" level of 3.1 g/l is not utilizable by the organism and this value has been subtracted from the recorded lactose concentrations.

Table 5.3 Data from B8.2.6 (Table 4.2) as a test of equation 5.4

Time h	$\frac{dP}{dt}$ = v g/l h	Z g/l	$\frac{1}{v}$	$\frac{1}{Z}$
3.97	6.36	26.1	0.1572	0.0383
5.20	5.97	20.3	0.1675	0.0493
6.60	4.71	10.1	0.2123	0.0990
7.75	3.55	6.0	0.2817	0.1667
9.17	1.47	2.4	0.6803	0.4167
10.92	0.46	0.6	2.1739	1.6667

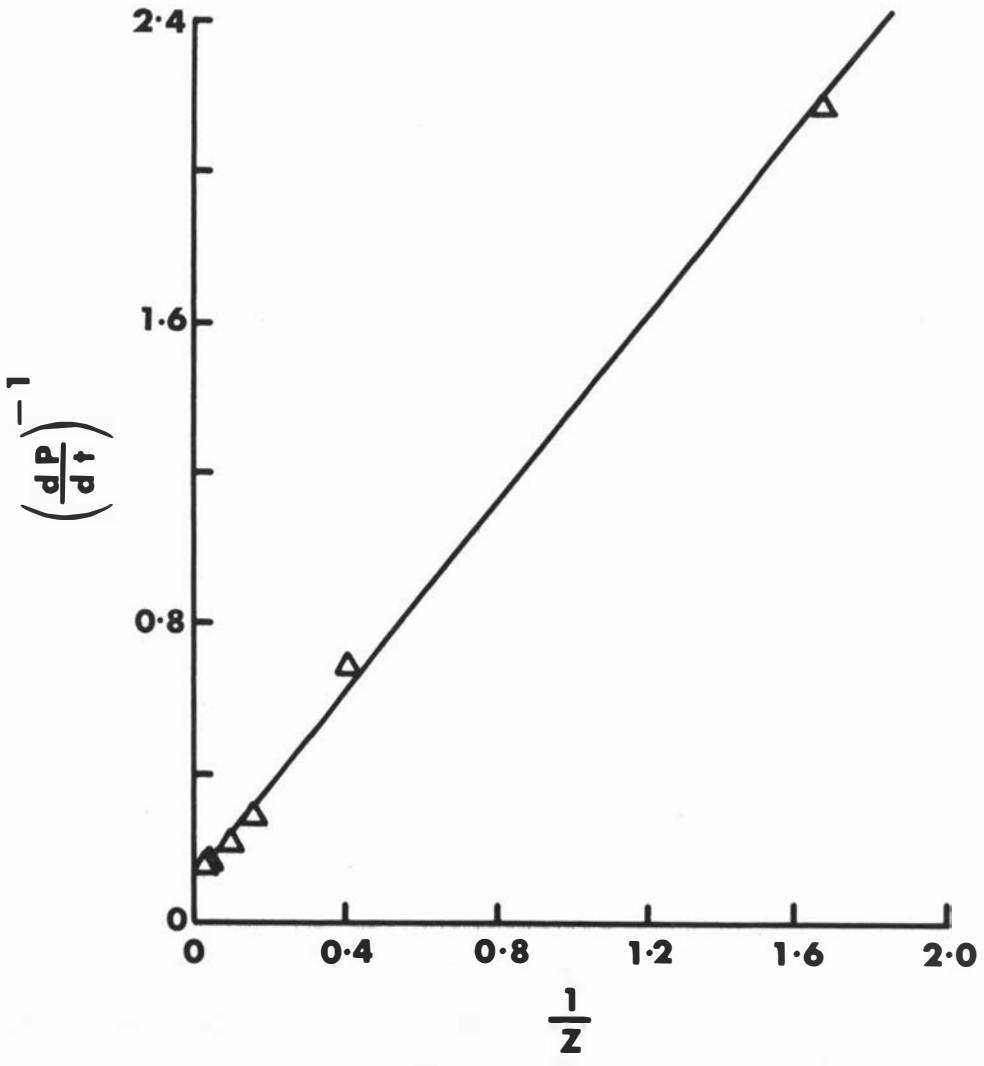


Figure 5.5 The reciprocal of the rate of acid synthesis as a function of the reciprocal of the lactose concentration, as a test of equation 5.4 (B8.2.6.)

A linear-least squares regression analysis gives the following equation:

$$\frac{1}{v} = 1.25 \frac{1}{Z} + 0.11 \qquad (5.5)$$

with a correlation coefficient of 0.99. V_m = 9.1 and K_z = 11.4 g/l. Vm is a "constant" which includes the concentration of the enzyme and the maximum rate of reaction.

It has been shown that in a batch culture the yield of acid relative to lactose utilized is a constant. Hence it should be possible to replace Z, the concentration of lactose, in equation 5.4 by (Pm-P), where P is the concentration of acid and Pm is the maximum attainable concentration of acid. This will be approximately 90 per cent of the initial lactose concentration. Thus

$$\frac{1}{v} = \frac{K_z Y}{V_m} \frac{1}{P_m - P} + \frac{1}{V_m} \qquad (5.6)$$

where Y is the yield of acid from lactose.

The data from B9.12.13 are shown in figure 5.6.

The calculated regression equations are

B9.12.13 $$\frac{1}{v} = \frac{2.98}{39.3-P} + 0.52$$

Correlation coefficient 0.971.

B9.1.5 $$\frac{1}{v} = \frac{1.52}{20.2-P} + 0.89$$

Correlation coefficient 0.996.

The data follow equation (5.6) closely. Table 5.4 gives the values of V_m and K_z.

Table 5.4 Values of Vm and Kz for equation 5.6

	Vm	Kz
B9.12.13	1.9	6.4
B9. 1. 5	1.1	1.9

5.1.6 Batch culture kinetics

The rate of acid production as a function of time in a batch culture of whey can thus be expressed over the complete batch cycle, excluding the lag phase, by an equation of the form:

$$\frac{dP}{dt} = \left(\alpha \frac{dN}{dt} + \beta N \right) \frac{P_m - P}{K_p + (P_m - P)} \qquad (5.7)$$

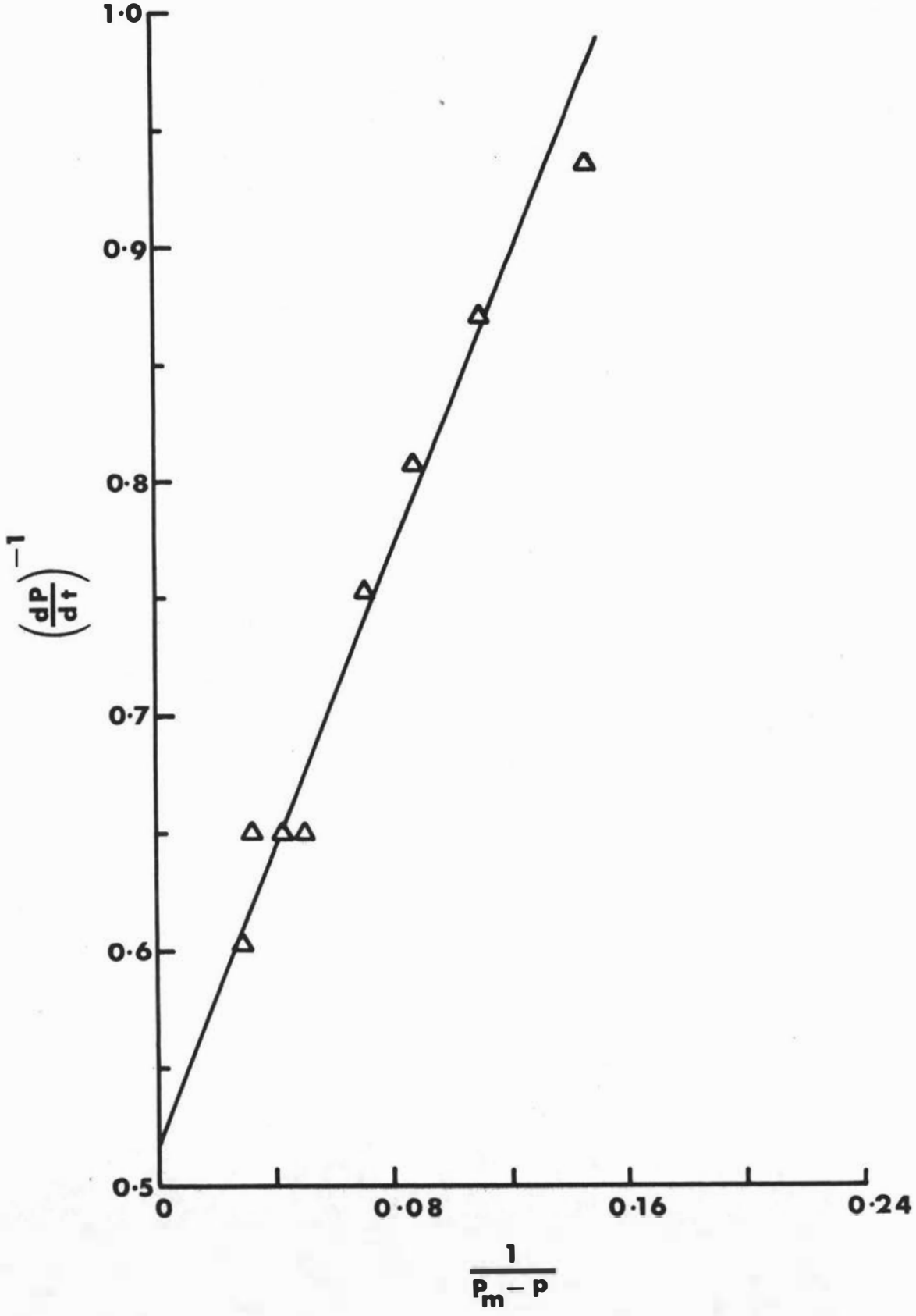


Figure 5.6 The reciprocal of the rate of acid synthesis as a function of the reciprocal of $(P_m - P)$, where P_m is the maximum potential acid concentration, as a test of equation 5.6 (B9.12.13.)

In (5.7) α replaces a Vm and β replaces b Vm from equations 5.1 and 5.6 and Kp = Y Kz.

The bacterial cell growth is consistent with the normal Monod equation

$$\frac{1}{N}\frac{dN}{dt} = \frac{\mu_m S}{K_s + S} \qquad (5.8)$$

where S is the concentration of the limiting nutrient. Because the nature of the limiting substrate is not known it is not, as yet, possible to confirm this equation. Simultaneous solution of equations 5.7 and 5.8 would fully characterize the production of lactic acid from whey.

The following procedure can be followed to determine the aproximate value of the constants in equation 5.7.

(1) P_m is aproximately given by 0.90 Z_i where Z_i is the initial lactose concentration.

(2) In the stationary phase of the bacterial growth cycle and the subsequent phase of declining rate of acid production $dN/dt = 0$

$$\frac{dP}{dt} = \frac{\beta N_m (P_m - P)}{K_p + (P_m - P)}$$

or

$$\left(\frac{dP}{dt}\right)^{-1} = \frac{K_p}{\beta N_m}\frac{1}{P_m - P} + \frac{1}{\beta N_m}$$

A plot of $\left(\frac{dP}{dt}\right)^{-1}$ vs $\frac{1}{Pm - P}$ should be a straight line with slope $\frac{K_p}{\beta N_m}$ and intercept $\frac{1}{\beta N_m}$ from which K_p and βN_m can be determined.

(3) In the phase of declining growth rate

$$\frac{1}{N}\frac{dP}{dt}\frac{K_p + (P_m - P)}{P_m - P} = \frac{\alpha}{N}\frac{dN}{dt} + \beta$$

and a plot of

$$\frac{1}{N}\frac{dP}{dt}\frac{Kp + (Pm - P)}{Pm - P} \quad \text{vs} \quad \frac{1}{N}\frac{dN}{dt}$$

allows α and β to be estimated.

Using this procedure the following constants have been estimated for B9.12.13

Pm = 39.3 g/l

Kp = 5.7 g/l

α = 7×10^{-12} g/cell

β = 2.8×10^{-12} g/cell h

Table 5.5 compares the values of $\frac{dP}{dt}$ calculated from equation 5.7 with those determined by graphical differentation of the experimental results of P vs t.

Table 5.5 The rate of acid production from experimental results (B9.12.13) and calculated from equation 5.7

Time h	$\frac{dP}{dt}$ exp. g/l h	$\frac{dP}{dt}$ calc. g/l h	Time h	$\frac{dP}{dt}$ exp. g/l h	$\frac{dP}{dt}$ calc. g/l h
1	0.10	0.09	12	1.60	1.51
2	0.18	0.18	14	1.54	1.48
3	0.32	0.27	16	1.60	1.43
4	0.80	0.64	18	1.54	1.38
5	1.58	1.22	20	1.33	1.31
6	1.96	1.95	22	1.24	1.23
7	1.66	1.67	24	1.15	1.19
8	1.54	1.66	26	1.07	1.02
9	1.54	1.69	28	0.90	0.90
10	1.54	1.61			

5.2 Continuous culture

In a single stage continuous culture :

$$D = \mu \qquad (2.17)$$

A mass balance with respect to product formation in a single stage continuous culture gives

$$\frac{dP}{dt} V = P_i F + \frac{dP}{dt}_G V - PF \qquad (5.9)$$

where V is volume of culture

F is medium flow rate

P_i is inlet acid concentration

and $\frac{dP}{dt}_G$ is the rate of acid production within the vessel.

Assuming conditions in the batch and continuous culture are the same, $\frac{dP}{dt}_G$ is given by equation 5.7 Assuming steady state $\frac{dP}{dt} = 0$ and substituting $P = P_i - P$ and $\mu = D = \frac{F}{V}$

$$P = N\left(\alpha + \frac{\beta}{D} \right) \frac{Pm - P}{K_p + P_m - P} \qquad (5.10)$$

Equation 5.10 predicts a linear relationship between the reciprocal of the dilution-rate and the expression $\frac{P}{N} \quad \frac{K_p + P_m - P}{P_m - P}$

Figure 5.7 is a plot of data from the continucus culture experiments with sterilized whey (Table 4.14) assuming K_p = 6 g/l and P_m = 40 g/l. The data show reasonable consistency with equation 5.10 (correlation coefficient = 0.989)

5.3 Prediction of continuous operation from batch data

If equation 5.10 is an adequate description of the kinetics of lactic acid production in continuous culture of whey it should be possible to predict the conditions in continuous culture from constants established in batch culture.

The conditions most closely aproximating those of batch experiment B9.12.13 are those in the continuous culture experiment number 9 in table 4.18. Using the constants from B9.12.13 it can be predicted that the acid concentration at a dilution-rate of 0.061 h^{-1} would be 32 g/l. This is in moderate agreement with the experimental value of 27.6 g/l.

This can not be taken as proof of the validity of equation 5.10 but does give some confidence that it can be used to predict continuous culture operation.

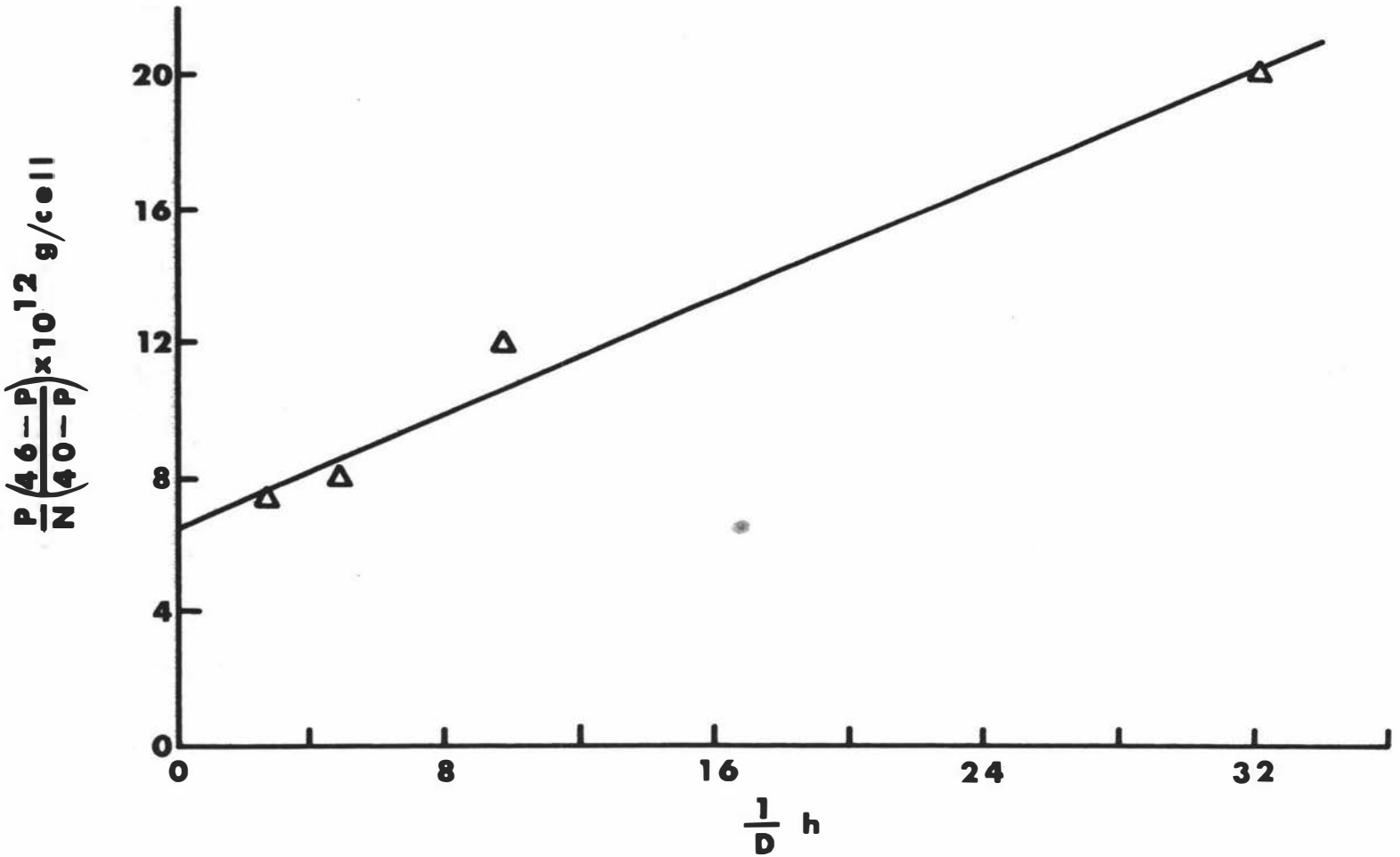


Figure 5.7 The factor $\frac{P}{N} \frac{K_p + P_m - P}{P_m - P}$ as a function of the reciprocal of the dilution rate as a test of equation 5.10. Sterilized whey 70 g/l.

6 DISCUSSION

The commercial exploitation of continuous culture techniques is not widespread (Evans,1965) and one of the main reasons for this is a lack of understanding of the kinetics of product formation by micro-organisms using media suitable for industrial use (Pirt,1969; Ricica,1969). Though the fermentation of whey to lactic acid has been used commercially for many years (Burton 1937; Whittier and Webb,1950) there are no reports of a continuous process being successful. Experimental studies showed a single stage culture could not convert all of the available lactose (Clive,1936; Swaby,1945). Five stages were necessary to reduce the final lactose level to 0.1 per cent (Havlatko and Knez, 1959; Siman and Mergl,1961). These findings are confirmed in this study but some of the reasons are now clearer. Though this is the first reported study of the continuous production of lactic acid from lactic acid casein whey it was expected that results would be comparable with those reported for other types of whey.

The reasons for the incomplete conversion of lactose to lactic acid in a single-stage continuous culture can be found from a study of the kinetics of bacterial growth and acid production in a batch culture.

In a batch culture of whey with LBR the bacterial cell numbers show the expected exponential increase from an initial level of approximately 10^9 cells/ l up to 2×10^{12} cells/l. During this time acid concentration also increases exponentially at the same specific growth rate as the cells (about 1.2 h^{-1}). The cell concentration then remains apparently stationary while more than half of the total acid synthesised in a batch culture is produced. The acid production rate shows a maximum as the cell population enters the phase of declining growth rate. In the stationary or quiescent phase acid production rate becomes linear at a rate dependent on the whey concentration and the presence of supplementary nutrients.

The rate of acid production remains linear with time until the lactose concentration is reduced to a level which causes the rate of conversion to fall. Product inhibition did not appear to be a factor. When the concentration of whey solids was increased to 70 g/l the rate of acid production in the stationary phase of the bacterial population's growth cycle was increased in proportion to the increase in total solids. It is possible that higher levels of lactose could produce product inhibition, but this would only occur in practice if the whey was preconcentrated before fermentation.

The type of production pattern obtained in unsupplemented whey has been mentioned in the literature but not extensively studied (Adams and Hungate,1950; Finn et al. 1950; Holzberg et al. 1967; Terui and Niizu, 1969). Rodgers and Whittier (1928) found pH control prolonged the exponential growth phase of Streptococcus lactis in skim milk and caused a prolonged fermentation phase after growth had ceased. They described the cells as being "quiescent".

It is unexpected that such large quantities of acid should be produced without apparent bacterial growth. Lactose dissimilation, normally by glycolysis, is the main source of carbon and energy for Lactobacilli organisms. To continue to utilize the energy source without growth is unusual and implies an uncoupling of energy production from growth. The organism used in this work is a mutant obtained by X-radiation of a strain of Lactobacillus bulgaricus, and possibly the normal feedback inhibition of glycolysis caused by an increase in the concentration of ATP has been rendered inoperative.

However, the most likely explanation is the energy produced from the metabolism of lactose is being used in the synthesis of some other product being formed as a reserve compound as a result of the nutrient limitation in the medium. This would cause the cell mass to increase but the cells would not necessarily divide. It was not possible to measure the cell mass in this study. It was observed in the stationary growth phase that the cells of LBR accumulated metachromatic granules. This is typical of physiologically old cells of L. bulgaricus (Wilson and Miles,1964). Metachromatic or volatin granules are composed of an inorganic metaphosphate in a polymer of high molecular weight (Mandelstam and Mc Quillen,1968). Another possible storage compound is glycogen, which tends to accumulate under conditions of nitrogen starvation when the cell has a source of carbon and lipid materials.

Another possibility is that acid is in fact being produced by growing cells only. Using a defined medium Terui and Niizu(1969) found the production of tryptophan by Hansenula anomala to be associated with growth. Their batch culture results indicated a growth and non-growth associated product formation similar to the pattern described by Luedeking and Piret (1959a). However, when tryptophan was produced in continuous culture the specific rate of tryptophan production correlated linearly with the specific bacterial growth rate and non-growth associated tryptophan production was zero. Investigation of the stationary growth phase cell

population showed a mixture of dead and growing cells. They reasoned that the dead cells lysed and released back into the medium an essential nutrient.

Terui and Niizu (1969) plotted the specific rate of tryptophan production against the dilution rate (equivalent to the specific rate of cell growth in a continuous culture) and obtained a straight line correlation with a zero intercept at D = 0. They therefore reasoned when the specific rate of cell growth was zero no tryptophan was formed. This is equivalent to putting $\beta = 0$ in equation 5.10.

From the data in fig.5.7 it can be shown that there is a significant linear correlation between $\frac{PD}{N} \frac{K_p + P_m - P}{P_m - P}$ and the dilution rate D (correlation coefficient 0.995). At D = 0, the factor $\frac{PD}{N} \frac{K_P + P_m - P}{P_m - P}$ has a value of $(0.5 \pm 0.3) \times 10^{-12}$ g/h cell (95 per cent confidence limits). Thus the specific rate of acid formation at zero specific cell growth rate can be assumed to be significantly different from zero. Unlike the situation described by Terui and Niizu (1969) acid appears to be formed by non-growing as well as growing cells.

Herbert (1961) showed the DNA content per unit cell (or nucleus) remained aproximately constant throughout the growth cycle. The DNA analysis throughout a batch cycle (fig.4.5) showed the same trends as cell concentration tending to confirm the long stationary phase shown by the latter. This cannot be taken as proof that the population did not contain growing cells. If cells were lysing it is possible the DNA would be rapidly degraded by catabolic enzymes.

An indirect proof that the population of cells in the stationary growth phase contained few dividing cells contributing to acid production is obtained from the experiments with air sparging into the culture medium. Relatively small amounts of oxygen depressed acid production in shake flasks (Table 4.9). In the culture vessel with pH control, virtually no acid was formed when air was sparged into the medium at the rate of 2 l/min from the time of inoculation. However if the flow of air was turned off from the time of inoculation until the cell population entered the stationary growth phase, and then turned on, acid formation continued at its normal rate (fig.4.14).

The first experiments described above show the oxygen suppresses cell growth and hence acid production. But in the apparent stationary

phase acid production is not altered implying the absence of significant acid formation by growing cells.

It seems reasonable to assume therefore that acid production by LBR from lactose in whey is by means of both dividing and non-dividing cells. In fact, in a batch culture over half of the acid synthesized is produced by non-dividing cells. The phenomenon of apparent uncoupling of energy production from growth requires further biochemical study.

Equation 5.10 shows the steady state concentration of acid is proportional to the cell concentration. In a single stage continuous culture maximum steady state cell concentration is approached as the dilution-rate is reduced to zero. Thus to achieve maximum acid concentration in the effluent from the continuous culture a low dilution-rate must be used. However as productivity is proportional to dilution-rate this mode of operation will be expensive in terms of capital utilization.

Because the maximum cell numbers achievable in a single stage continuous culture is less than the maximum obtained in a batch culture a single stage continuous culture will not be the most efficient for a system which is dependent on cell concentration as well as cell growth rate. Such a large proportion of the acid is produced by non-dividing cells acting as a source of enzymes it is not surprising that the production of lactic acid by LBR growing in whey is not efficient in a single stage continuous culture.

The kinetic equations derived to describe acid production in batch and continuous culture are similar to those derived by Pirt (1969) and Luedeking and Piret (1959a,b) except for the addition of the term $\frac{Pm - P}{Kp + Pm - P}$ to describe the fall off in rate caused by the change in lactose concentration. This term can only be applied because of the direct stoichiometric relationship between lactose and lactic acid. The yield of lactic acid was 95 per cent of metabolized lactose.

In the derivation of an equation to describe steady-state conditions in continuous culture Luedeking and Piret used a specific growth rate of cells which was modified to account for inhibition of growth by lactate in the medium. They assumed the specific growth rate was described by an equation of the form of 2.4. However an investigation of their data shows the specific growth rate is not in fact a linear function of the acid concentration. In the present work it has not been necessary to assume product inhibition of cell growth.

Holzberg et al (1967) studying the alcoholic fermentation of grape juice claimed the Luedeking and Piret equation did not fit their data. They proposed the following:

Exponential growth phase:

$$\frac{dP}{dt} + bp = a \ln N / \mu \quad - C \qquad (2.9)$$

Stationary phase:

$$\frac{dP}{dt} = N_m r \ (P_m - P) \qquad (2.10)$$

where P, in this case, is the alcohol concentration.

Equation 2.9 does not apply to the lactic acid fermentation from whey. Equation 2.10 can be compared with equation 5.7 when $\frac{dN}{dt} = 0$ and $N = Nm$ equation 5.7 becomes

$$\frac{dP}{dt} = \beta \ Nm \ \frac{Pm - P}{Kp + Pm - P}$$

If $Kp >> Pm - P$, this equation reduces to the same form as that deduced by Holzberg et al (1967).

Equation 5.7 is a more accurate description of the kinetics of lactic acid production from whey than those of Luedeking and Piret or Holzberg et al.

The kinetic equations derived from the results allow a number of predictions to be made regarding the best mode of operation of commercial fermentations of whey. A full description cannot be given because no attempt has been made to determine the kinetic equations describing cell growth. It can be stated that the cell growth pattern is in general agreement with the normal kinetics as described by Monod (1942). For the prediction of continuous culture cell concentration the graphical techniques of Luedeking and Piret (1959b), Bischoff (1966) or Ricica (1969b) allow at least a first estimate to be made from the batch data. These techniques can be shown to apply to results presented earlier.

From the equation describing cell concentration (Herbert et al, 1956)

$$N = (S_o - K_s \ \frac{D}{\mu_m - D}) \qquad (6.1)$$

and equation 5.10

$$P = N \left(a + \frac{\beta}{D}\right) \ \frac{Pm - P}{Kp + Pm - P} \qquad (6.2)$$

a number of predictions can be made about operation in continuous culture.

As the dilution rate increases the cell and acid concentrations decrease. Because lactose utilization is stoichiometrically related to acid production this means lactose concentration in the effluent is increased as the dilution rate is increased, an undesirable feature if the intention is to reduce the BOD level of the whey. The results obtained in continuous culture experiments show that despite very low dilution rates ($0.031\ h^{-1}$, an average residence time of over 32h) it is not possible to

achieve high enough cell concentrations to obtain total utilization of the lactose. The productivity (PD) of the equipment is also reduced to levels unacceptable economically.

One means of increasing the concentration of cells, and hence of acid, is to increase the concentration of the limiting nutrient (or nutrients) i.e. the value of S_o in equation (6.1).

Lactose is not the limiting nutrient. When cell growth has ceased in batch culture only 20-40 per cent of the lactose has been assimilated.

When supplementary organic nutrients were added to whey an increase in cell concentration and acid production rate were observed. Peptone, yeast extract and beef-extract showed definite stimulatory effects. Commercially these nutrients are of limited value because of their high cost and some effort was made to define more precisely the stimulatory nutrients.

Many compounds have been suggested as being essential or stimulatory to Lactobacillus bulgaricus though the nutrient requirements of LBR have not been previously reported. The study of nutrient requirements is difficult. Experiments carried out without pH control are confused by varying buffering capacities of the nutrients and the high level of buffering required. Only a limited number of experiments can be carried out with pH control in the culture vessel because of the time involved.

To overcome these problems the nutrients were divided into related groups and some pH controlled experiments carried out. A small number of test-tube cultivations was also performed.

In the culture vessel it was shown that amino acids (casamino acids, tryptophan and cysteine) were stimulatory to acid production. The addition of the mineral salts increased the effect of the amino acids, but alone they had no measurable influence. Vitamins and nucleic acid compounds had no apparent effect.

The test-tube experiments confirmed the stimulatory effect of the amino acids, with tryptophan having the greatest effect. These experiments showed some of the vitamins also stimulated cell growth, and acid production, but ascorbic acid was toxic. This is probably why the experiments in the culture vessel did not show that vitamins had an effect- the stimulation of most of the vitamins was counteracted by the toxic effect of ascorbic acid.

These results are in general agreement with those of other workers on the nutrition of L. bulgaricus (Snell,1952; Davies,1960). However, Rogosa et al. (1961) found that folic acid was inhibitory, an observation

contrary to that made with LBR. Also, there have been no reports of the inhibitory effects of pyrodoxine or ascorbic acid. Rogosa et al. (1960) found riboflavin was the only one of the vitamins they studied which was essential for growth of L. bulgaricus. Riboflavin added to whey has only a slight stimulatory effect on cell growth of LBR, but there is probably sufficient riboflavin naturally present in the whey (Whittier and Webb,1950).

Sodium caseinate has been shown by a number of experiments in this study to provide essential growth nutrients. Nakanishi& Nakazawa (1961) reported a similar finding for Lactobacillus spp in nutrient medium.

Sodium caseinate added to whey markedly stimulates the amount of acid production, although not all sodium caseinates have the same effect. Skim milk solids also increase the rate of acid synthesis, presumably because of the addition of casein. Attempts to find a reason for the differences between caseinates were not successful. Age of the casein did not correlate with activity, nor was the activity appreciably altered by treatment of the caseinate solution with activated charcoal.

It was shown that up to 10 g/l of added sodium caseinate the stationary rate synthesis of acid was a linear function of added sodium caseinate. Further added sodium caseinate had little effect. These results are consistent with the following hypothesis.

If it is assumed that the sodium caseinate is a source of an essential nutrient for cell synthesis, then the final cell concentration will be proportional to the sodium caseinate concentration;

$$N_m \quad \alpha \quad C \qquad (6.3)$$

Where C is the concentration of the added sodium caseinate. Now when $\frac{dN}{dt} = 0$;

$$\frac{dP}{dt} \alpha N_m \qquad (6.4)$$

(see equation 5.7).

Hence combining equations 6.3 and 6.4

$$\frac{dP}{dt} \alpha C \qquad (6.5)$$

Equation (6.5) will hold unless other nutrients or cell reactions become limiting. From figure 4.11 the response predicted by equation 6.5 is seen to hold up to a level of 10 g/l added sodium caseinate. At 20 and 40 g/l sodium caseinate there is only a small increase in acid production, presumably because of some other limitation to the reaction.

Table 4.17 shows that when sodium caseinate (10 g/l) is added to the

whey in continuous culture there is an increase of aproximately 60 per cent in the productivity and yield compared with unsupplemented sterilized whey at the same dilution rate confirming the general predictions made from the batch culture data.

It can be concluded that whey is not a complete medium for the growth of LBR and production of lactic acid. Supplementary sources of amino acids and vitamins are required for maximum rates of acid production. The supplement of choice will depend on the economics but probably small levels of sodium caseinate would be feasible.

Referring to table 4.17 and figure 4.23 it can be seen that at a dilution rate of 0.12 h^{-1} the addition of 10 g/l sodium caseinate increases the lactic acid concentration in the effluent cut of a single-stage continuous culture from 19.2 g/l to 30.4 g/l. Assuming the sodium caseinate to be used for this process costs 10 c/kg the increased cost if it is added will be 3.3 c/kg lactic acid. This is small compared with the New Zealand selling price of 70 c/kg of 100% lactic acid (edible grade). The improved enconomies resulting from the higher concentration of lactic acid in the feed to the purification process and the decreased lactose level of the effluent are further justifications for the addition of some sodium caseinate.

Supplementary sources of amino acids and vitamins which increase the cell concentration will increase the acid concentration in continuous culture.

Another possible means of increasing the lactic acid concentration and the productivity in continuous culture predicted by equation (6.2) is to increase the cell concentration by recycle of the bacteria. The effluent from the reactor would pass through a centrifuge and a concentrated cell stream returned to the reactor while the product with reduced cells would flow onto the purification process. Fencl (1966) discusses the theoretical aspects of feed back of cells showing the dilution rate can be greater than the specific growth rate of the bacteria and equation 5.10 must be expressed as follows:

$$P = \frac{N}{D} \left(\alpha \mu + \beta \right) \frac{P_m - P}{K_p + P_m - P} \qquad (6.6)$$

The concentration of cells (N) in a single stage culture is limited by the concentration of a limiting nutrient (So). But it has been shown that non-dividing cells are capable of producing lactic acid from lactose. Hence increasing the concentration of cells by recycle of cells from the

effluent back to the culture vessel will result in an increase in the amount of acid formed. This will result in an increased productivity and a decrease in the amount of lactose in the effluent.

For feedback of bacterial cells to be an advantage, the activity of the culture must not be lowered by recirculation or infection. Continuous separation of bacteria on a small scale is difficult and it was not feasible to assess the effects in this work.

However, bacterial cells were recovered from a batch fermentation and added to an active batch culture. The rate of acid production was increased in approximate proportion to the number of added cells. A control experiment showed a slight loss in activity, but this was probably due to the relatively long time taken to recover the cells and also to losses in the centrifugate.

Infection by contaminating bacteria would not be as great a problem as in some other fermentations. The high temperature of cultivation considerably limits the range of infecting organism which will be able to reproduce at a rate fast enough to affect the fermentation.

A further method of increasing the utilization of lactose in continuous culture would be the use of multi-stage continuous culture reactors (Fencl,1966). Qualitatively this can be considered as consisting of a first stage from which cells are obtained in the best condition to act as an enzyme catalyst for the second and subsequent stages.

The mathmatical relationships described by Fencl (1966) can be applied. They show that the overall dilution rate can be increased above the maximum specific growth rate, thus increasing the productivity of the equipment. The lactose utilization and the acid concentration in the effluent stream from the final reactor will be increased in comparison with the equivalent single stage unit.

Because the relationships between the concentration of the limiting substrate (S_o) and the cell number concentration were not determined in this work the application of the mathmatical relationships is difficult. However the use of a graphical estimating technique will provide at least an indication of the trends in a multi-stage continuous culture.

The method developed by Bishoff (1966) and illustrated by Ricica (1969b)has been applied to the batch data from B9.3.13 (Fig.6.1).

From the plot of $(\frac{dN}{dt})^{-1}$ vs N the maximum cell production rate would be obtained in a homogeneous reactor with $D = 0.94\ h^{-1}$.

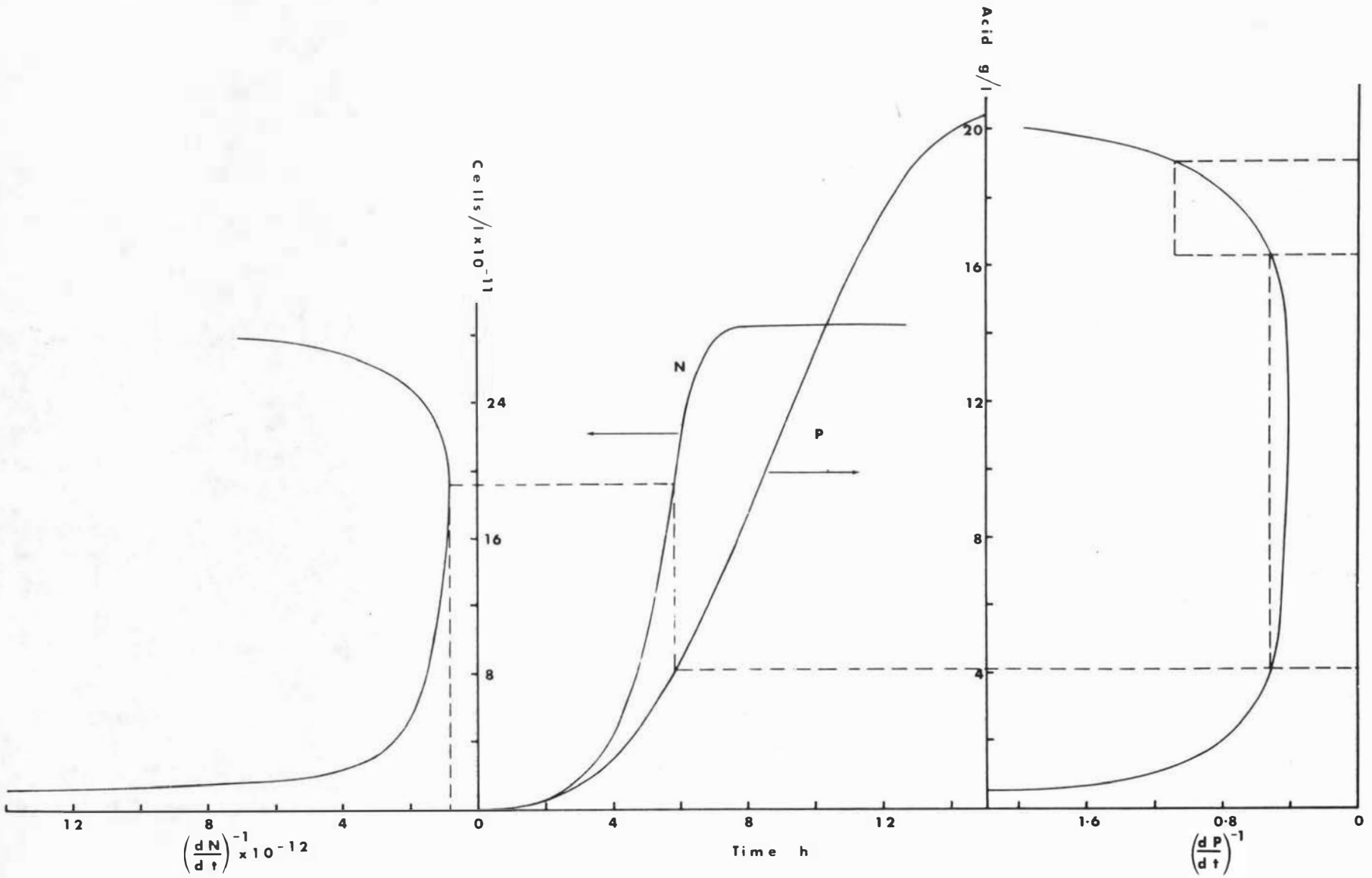


Figure 6.1 Graphical estimation of the operation of a multistage continuous culture. (Ricica, 1969b) Data from B9.3.13. Whey 35 g/l, Na Cas, 10 g/l, pH6.0, 46°C.

The acid concentration in the overflow would be 4 g/l. A second stage with a volume such that D = 0.158 h^{-1} would give an overflow acid concentration of 16.3 g/l. A third stage such that D = 0.34 h^{-1} would give an overflow with 19 g/l lactic acid, 95 per cent of the potential yield. The overall dilution rate would be 0.096 h^{-1} with almost total utilization of the lactose.

A further system suggested for use in continuous fermentation reactions is the tubular flow reactor. Danckwerts (1954) showed the ideal piston flow tubular reactor to be more advantageous than the homogeneous reactor when the reaction rate decreases with the concentration of the reactants. Equation 5.3 shows the rate of acid production is reduced at low levels of lactose and there could be advantages in using such a system.

In the early stages of the fermentation the conversion of lactose to lactic acid is essentially zero-order and it is irrelevant whether a homogeneous or tubular reactor is used. However as the lactose content decreases the reaction rate approaches first-order and a tubular reactor could be advantageous. For maximum utilization of substrate a tubular "finishing" reactor appears attractive.

There is a number of practical difficulties however. The tubular reactor would need to be quite large because of the relatively slow rate of the reaction. The major difficulty is one of pH control. Though the fermentation rate is not too greatly affected by changes of pH in the range 5.5-6.0, outside this range the rate falls off quite markedly. The use of a buffer such as calcium carbonate is possible, but the flow rates required to keep the particles in suspension would make the tubular reactor even bigger and cause considerable deviations from the ideal piston flow.

A tubular reactor can be readily approximated by a multi-stage homogeneous reactor, particularly if recycle is used (Fencl,1966) and this is a more practical method to attain maximum substrate utilization.

Continuous culture of whey to produce lactic acid is feasible, but to attain maximum utilization of the lactose the whey must be supplemented with a source of amino acids and vitamins. Multi-stage continuous culture (maximum of three stages would be satisfactory) with cell recycle is needed to give a lactose concentration low enough to reduce the pollution to an acceptable level.

The advantages of operating with multi-stage continuous culture would be enhanced by using the optimum fermentation conditions.

On the small scale of operation used in this study pasteurization of the whey was more satisfactory than sterilization. The whey proteins or

other heat labile compounds present apparently act as a source of supplementary nutrients but their effectiveness is reduced by severe heat treatment.

From an economic viewpoint there is a considerable advantage in being able to use pasteurized whey rather than aiming for complete sterility. The relatively high temperature of fermentation makes this feasible for this application whereas in fermentations at lower temperatures the risk of contamination from organisms in the pasteurized medium would be too great for operation in continuous culture. High heat treatments will also cause precipitation of sludge with its resultant problems (Swaby,1945). It has been shown that a continuous culture can be operated for more than ten days without any apparent effects due to contamination despite the use of pasteurized whey. As would be the case in commmercial operation this whey was prepared and heat-treated every 24-48 h. The savings in heat treatment costs will be worthwhile and a high pressure system is not required for pasteurizing. The heat treatment proposed is similar to that advocated by others using cheese whey (Campbell,1953; Jankowska,1954; Surazynski et al,1967, and Swaby,1945).

The pH of fermentation has been shown to be an optimum for acid production in the range of 5.4-6.0. From the experiments in continuous culture the pH in the range 5.4 - 6.0 was shown to have little effect on acid concentration, productivity or yield. As the pH increases above 6.0 however, these parameters fall quite sharply. At pH 6.5 the productivity for example is reduced to 0.29 g/l h (35 g/l whey solids, 46°C, D = 0.061 h^{-1}). In a few batch experiments in which the pH was uncontrolled, acid production slowed down as the pH fell below 5.3 and virtually ceased as the pH fell below 4.0.

As has been found by other workers, pH control markedly increases the maximum cell population and the rate of acid production in batch culture (Rodgers and Whittier,1928; Longsworth and MacInnes,1935,1936). Without pH control it took 24 h to produce 10 g/l of acid.

The observation made by Oetiker (1960) of an optimum pH between 4.2 and 4.6 using LBR in whey is not confirmed. Swaby (1945) had problems with contamination at pH's above 5.8, but the only time in this work that difficulties were noticed with contamination was in a continuous culture experiment after 3 days operation at pH 6.5, temperature 45°C.

One interesting observation made in a number of batch experiments cannot be explained but warrants further investigation. Some of the acid production rates were found to be much greater than would be expected

from previous results. A study of the records of alkali addition and pH of the culture showed in most of these cases the high rates of acid production followed a failure in the pH control system. The pH fell to 4.5-5.5 before the control system was repaired. After the pH of the medium was adjusted to the correct set point acid production was at a rate almost twice as fast as expected. A similar observation has since been made by Hansen and Tsao (1972) in a study of lactic acid production from a glucose and yeast extract medium. Possibly the lower pH releases some nutrient which had previously been bound in a way which made it inaccessible to the bacteria or the pH causes some alteration to the permease system used to transport lactose into the cell. There was no evidence of changes in the appearance or concentration of the bacterial cells.

If this increased activity due to pH "shock" is a real effect a three-stage continuous system could be envisaged; in the first stage cells would be produced in the maximum possible numbers, in the second the cells would be subjected to a low pH, and in the third stage the remainder of the lactose would be converted to lactic acid by the non-dividing cells with an increased glycolytic activity.

From the results obtained it is obviously necessary for maximum acid production, by batch or continuous fermentation, to control the pH at a level between 5.4 and 6.0.

The differences between neutralizers have not been studied. In commercial practice chalk or limestone has been the main choice but there are some difficulties in using these materials where precise pH control is desired. Sodium carbonate was used in most of the reported work because the evolved carbon dioxide assisted in maintaining an anaerobic environment. The use of concentrated sodium hydroxide would cause less dilution of the final product and this could have benefits in the subsequent processing stages.

The temperature of incubation has a marked effect on production of lactic acid from whey. At a given pH and dilution rate, the productivity, acid concentration and yield have optimum values at 46^{o}- $46.2^{o}C$. At a dilution rate of 0.21 h^{-1}, pH 5.65, the productivity is 3.1 g/l h at $46.2^{o}C$ and decreases to 2.2 g/l h if the temperature is lowered to $44^{o}C$ or increased to $48.4^{o}C$. The temperature optimum is similar to the values reported for L. bulgaricus strains growing in other media.

Because of the precise temperature control required it is questionable whether the open fermentation vats reported as being used in the commercial production of lactic acid are desirable (Campbell,1953; Cetiker,1960; and Olive,1936).

Another reason for not using open vats is the inhibitory effect of oxygen on the growth of LBR. Air in the headspace above the whey, particularly if the culture surface was not quiescent, caused the growth of the LBR culture to cease due to the adverse effect of oxygen. When the air was replaced with nitrogen or carbon dioxide cell growth proceeded normally. Once a culture of LBR had entered the stationary growth phase air had no effect on acid production, even when bubbled through the culture medium. Acid was being produced by non-dividing cells and oxygen appeared to be toxic only to cells which were growing and dividing.

The adverse effect of oxygen on cell growth is consistent with the description of L.bulgaricus as an anaerobic or microaerophilic organism (Breed et al,1957).One possible cause of the toxic effect of oxygen is the lack of the enzyme peroxidase in LBR. This enzyme reduces hydrogen peroxide formed by cell metabolism. If the hydrogen peroxide is not removed from the medium it causes death of the cell. LBR was in fact killed when air was sparged into the medium. The organism was incapable of reproducing after air sparging ceased. However no permanent toxic compound accumulated as was shown by normal growth after reinoculation (4.2.6). Catalase added to the medium would have no effect because this enzyme is inactivated at 45°C.

Experiments on the effect of agitation show an increase in acid production rate as the level of agitation is increased to a particular level, but above that level further increases in agitation offer no advantages. The decrease in acid production at high impeller speeds observed by Keen (1972) was not observed.

For each impeller there was a distinct increase in acid production rate with increased speed of rotation (Fig.4.15, 4.16). The average impeller shear rate is a function of the impeller speed and is important if coalescence of particles in parts of the vessel remote from the impeller causes reaction rates to be limited. As discussed later, at low impeller speeds the agitation was insufficient to suspend all solid particles and this is reflected in Figs. 4.15 and 4.16.

The maximum impeller shear rate is a function of tip speed and would be important if the bacterial system was sensitive to shearing forces. There is no discernible trend in acid production as the tip speed is in-

creased. This is also reflected by the small changes in the average chain length as agitation was increased. It can therefore be concluded that this bacterial system is not sensitive to shear forces.

The correlation of Reynolds Number with acid production rate (Fig.4.17, 4.18) is similar in shape to that obtained by Kneule (1956) in an investigation of the effect of agitation intensity on mass-transfer coefficients for solid particles in agitated liquids. Kneule correlated the mass-transfer coefficient with the impeller Reynolds Number. He showed that two distinct regions exist and concluded that up to a particular agitation intensity the particles were incompletely suspended. Once the particles were completely suspended, increased mixing resulted in only a small increase in the mass-transfer rate.

For the system of Lactobacillus bulgaricus fermenting lactose in whey to lactic acid a similar explanation is likely. Below a Reynolds Number of 10,000 solids in the medium are probably incompletely suspended. In fact, in runs 1 and 8 (Re < 5,000 table 4.12) a layer of settled solids was observed on the base of the fermenter. It is unlikely the lack of suspension of bacterial cells is the cause of reduced acid production rate. Bacterial cells have a specific gravity of approximately 1.03 and hence will be readily suspended even at low levels of agitation.

However there are other solids in the whey which consist of casein fines, bacterial debris and insoluble, denatured whey proteins. When whey was centrifuged at 10,000 x g to remove some of the suspended solids, total acid production and the acid synthesis rate were reduced compared with whey at the same lactose concentration. When the whey was sterile-filtered, thus removing essentially all the suspended solids, cell growth and acid production were reduced almost to zero. (Fig.4.7).

No toxic component was being introduced by the procedure because a supply of amino acids allowed cell growth and acid production to proceed normally. The amino acids could be added in a defined form (tryptophan and casamino acids, fig.4.6) or as sodium caseinate (fig.4.8). Heating to 69°C after sterile-filtration did not increase the acid production rate.

Hence, when agitation of the whey culture medium is insufficient to fully suspend the insoluble or colloidal material in the whey the reduced availability of some essential nutrient causes the acid production rate to be considerably reduced. As shown by Kneule (1956) an increase of the impeller Reynolds Number up to 10,000 causes an increase in the mass transfer rate from the suspended solids. Once the solids are completely

suspended there is only a minor increase in the rate of conversion of lactose to lactic acid.

Kempe and West (1959) and Keen (1972) claimed a possible increase in the rate of mass transfer between the bacterial cell wall and the medium as the agitation intensity was increased. If the rate of reaction was limited by the rate of mass-transfer then it would be expected from the empirical correlation derived by Calderbank (1967) that the rate of acid formation would increase as the 0.25 power of the power input. The value of 0.032 (0.065 if a single straight line is drawn through all experimental points fig.4.20) obtained with whey is significantly less than 0.25, showing that mass-transfer between solid and liquid is not the rate limiting step in the reaction sequence.

Kempe and West (1959) obtained a value of 0.08 as the exponent for agitator speed as a function of acid production rate. Their experiments were performed with a single turbine and if it is assumed that power is proportional to n^3 then their results can be expressed as:

$$\frac{dP}{dt} = aP_o^{0.027}$$

which is very close to the expression obtained in the work reported here.

Keen (1972) reported a decrease in the rate of change of pH and hence presumably acid synthesis rate as the level of agitation was increased in a culture of Streptococcus lactis growing in skim-milk. With LBR growing in pasteurized whey the rate of acid synthesis is increased as the agitation level is increased.

From the results obtained in this work it can be concluded that the main requirement of agitation in the whey-LBR system is to maintain insoluble or colloidal material in suspension ensuring maximum availability of nutrients for the bacterial cells, allowing the maximum rate of cell growth and acid production. The impeller Reynolds Number should be greater than 10,000.

The production of lactic acid from lactic casein whey is obviously technically feasible. The economic viability will depend on a number of factors including the cost of the fermentation, the cost of recovery and purification of the lactic acid from the fermented whey and the available markets. The latter aspects are outside the scope of this study.

Successful operation in continuous culture is possible with productivities improved compared with batch culture. However yields are reduced unless multi-stage cultures are used.

In batch culture the best overall productivity obtained was 2.0 g/l h in whey supplemented with amino acids, salts and vitamins (abcd fig.4.6b). In a typical batch culture the productivity was 0.8 g/l h. This includes only the fermentation time and not the time to prepare the vessel and the medium. In continuous culture, productivities varied with the dilution rate. The maximum attained with 35 g/l whey solids was 1.8 g/l h but the yield was only 0.23. At lower dilution rates so that the productivity approached 0.8 g/l h (0.061 h^{-1} with pasteurized whey) the yield was increased to 70 per cent compared with 95 percent in the batch culture. The estimated productivity from the three stage continuous unit would be 1.8 g/l h but the yield would be increased to 95 percent. Productivity is proportional to the concentration of whey solids.

It is interesting to note that with the IBR organism, the optimum conditions of fermentation and the addition of sodium caseinate to the whey, the time to completion of a batch culture of whey was about 15 h compared with the literature reported values of from one to six days (Whittier and Webb,1950).

The production of lactic acid from lactic casein whey as a means of waste disposal will be successful only if multi-stage continuous culture with cell feed-back is used. A single-stage continuous culture will not give an effluent with a lactose concentration low enough.

CONCLUSIONS

Though the lactose in lactic casein whey can be fermented to lactic acid with yields in batch culture of 90-95 percent, in a single stage continuous culture fermentation the yield of lactic acid and the utilization of lactose are unacceptably low. If pollution abatement is the main aim the lactose in the effluent from the fermenter must be reduced to as low a level as possible.

In continuous culture the acid concentration and decrease in lactose concentration are limited by the low level of essential nutrients in whey for the optimum growth of LBR, a strain of L.bulgaricus. The cell concentration and the rate of acid formation can be increased by the addition of supplementary nutrients in the form of tryptophan and casamino acids, or sodium caseinate.

The rate of acid synthesis is also increased by the use of pasteurized rather than sterile whey, by operating at the optimum temperature of 46°C and pH 5.4 - 6.0, providing sufficient agitation to fully suspend insoluble or colloidal compounds in the whey and restricting the access of oxygen to the growing cells.

Kinetic equations derived from batch culture data can be used to predict the operation of various types of continuous culture apparatus. These equations show that the use of a multi-stage continuous culture system with feedback of cells will be necessary to reduce the lactose concentration in the effluent whey low enough to give an acceptably low BOD. The use of such a system is possible because non-dividing cells of LBR are capable of converting lactose to lactic acid, acting as a source of glycolytic enzymes to catalyse the reaction.

The two biochemical problems, production of lactic acid by non-dividing organisms and the increased rate of acid synthesis after the culture has been exposed to a low pH, are worthy of further study.

An economic assessment of the cost of the multi-stage continuous culture operation compared with batch culture operation will need to be carried out before a firm decision can be made as to the best system for commercial operation. Such an assessment would be part of an overall study which would include the recovery of the lactic acid from the fermented whey, an aspect not studied in this work.

NOMENCLATURE

a	constant.
A	constant.
b	constant.
C	capacitance.
d	impeller diameter.
D	dilution rate.
g	gravitational constant.
i	amount of active enzyme.
K	constant.
K	dielectric constant.
K	Specific product formation rate.
Kp	Saturation constant for lactic acid.
Ks	Saturation constant for the limiting nutrient.
Kz	Saturation constant for lactose.
l	impeller blade width.
L	length of capacitor.
n	impeller speed.
N	cell number concentration.
Nm	maximum cell number concentration.
P	product or lactic acid concentration.
P_f	power factor (equation 4.2).
P_m	maximum lactic acid concentration.
P_o	power.
r	Specific rate of product formation.
R	rate of acid production $(\frac{dP}{dt})$.
S	concentration of limiting substrate.
t	time.
t	"student's t".
v	rate of enzyme reaction
V	variance
x	outer diameter of capacitor.
X	cell mass concentration.
y	inner diameter of capacitor.
Y	yield
Z	lactose concentration.

α constant in kinetic equation.

β constant in kinetic equation.

γ rate of enzyme reaction.

μ specific bacterial growth rate.

μ_m maximum specific bacterial growth rate.

μ viscosity.

υ specific growth rate.

ρ density.

ϕ factor in equation 2.3.

REFERENCES

Adams,S.L. & Hungate,R.E. (1950). I.E.C. 42, 1815.

Aiba,S., Shoda,M. & Nagatini,M. (1968). Biotechnol. Bioeng. 10, 845.

Aiyar,A.S. & Luedeking,R. (1966). CEP Symp. Ser. 69, 62, 55.

Anon. (1945). Canadian Dairy Ice Cr. J. 24, 54.

Arnold, N.H.M. & Childs,C.G. (1960). Manufact.Chemist, Aug.,333.

Atkinson,B. & Daoud,I.S. (1968). Trans. Inst. Chem. Engrs. 46, T19.

Atkinson,B., Swilley,E.L., Busch,A.W. & Williams,D.A. (1967). Trans. Instn. Chem. Engr. 45, T257.

Bates,R.L., Fondy,P.L. & Corpstein,R.R. (1963). I.E.C. Process Design & Development, 2, 310.

Bischoff,K.B. (1966). Can. J. Chem. Eng. 44, 281.

Box,G.E.P. & Hunter,J.S. (1961). Technometrics, 3, 311.

Breed,R.S.,Murray,E.G.D. & Smith,N.R. (1957). Bergey's Manual of Determinative Bacteriology, 7th. Ed. p546, Williams & Wilkins Co. Baltimore.

Briggs, M.J. (1953). J. Gen. Microbiol. 9, 234.

Brydson,J.A. (1966). Plastic Materials, p88, Iliffe Books,London

Burton,K., (1956). Biochem. J., 62, 315.

Burton,L.V. (1937). Food Industries, 9, 571, 617, 634.

Calderbank,P.H. (1967). Biochemical & Biological Engineering Science. p101 Ed. N.Blakebrough,Academic Press,London.

Calderbank,P.H. & Moo-Young,M.B. (1961). Trans. Inst. Chem. Eng. 39, 337.

Campbell,L.A. (1953). Can. Dairy Ice Cream Jour. 32, 29.

Childs,C.G. & Welsby,B. (1964). 2nd. Int. Ferm. Symp., London

Cochran,W.G. & Cox,Gertrude,M. (1962). Experimental Designs, 2nd. Ed. p350. John Wiley & Sons. Inc., N.Y.

Cotton,H. (1962). Applied Electricity 5th. Ed. p89. Cleaver Hume Press, London.

Danckwerts,P.V. (1954). Ind. Chemist, 30, 102.

Davies,J.G. (1960). Progress in Industrial Microbiology, 2, 1.

Davies,M.A.S. & Lazenby,B.D. (1966). Process Biochem. 1, 145.

Davies,O.L. (1963). The Design & Analysis of Industrial Experiments. Oliver & Boyd, London.

de Mann, J.C. Rogosa,M. & Sharpe,M.E. (1960). J. Appl. Bact. 23, 130.

Dept. of Statistics (1970) "N.Z. Imports, Part A, 'Commodity by Country' ", Wgtn.

Dion,W.M., Carilli,A., Sermonti,G. & Chain,E.B. (1954). Rc. 1st. Sup. Sanita (English Ed) 17, 187.

Duitschaever,C.L. & Leggat,A.C. (1965). J.Milk & Food Technol. 28, 97.

Dummet, G.A. (1968). Aust. J. Dairy Tech. 23, 110.

Edwards,V.H. (1967). Analytical Methods in Bacterial Kinetics. Ph.D. Univ. of Calif. Berkeley; University Microfilms Inc., Ann Arbor, Michigan.

Evans,C.G.T. (1965). Lab. Pract. 14, 1168.

Fencl,Z. (1968). Theoretical & Methodological Basis of Continuous Culture of Micro-organisms, p.133. Ed. I.Malek & Z.Fencl, Czech. Acad. Sci. Prague.

Fencl,Z., Machek,F. & Novak,M. (1969). Fermentation Advances. p301, Ed. D.Perlman, Academic Press, N.Y.

Fencl,Z. & Ricica,R. (1968). Process Biochem., 3, 41.

Finn,R.K. (1954). Bact. Rev. 18, 254.

Finn,R.K. (1966). J. Ferm. Tech. 44, 305.

Finn,R.K. Halvorsen,H.C. & Piret,E.L. (1950). I.E.C. Industr. 42, 1857.

Hanson,T.P. & Tsao,G.T. (1972). Biotech. & Bioeng., 14, 233.

Havlatko,F. & Knez,W. (1959). XI International Dairy Congress,2, Sect.3, p.1235.

Herbert,D. (1961). XI Symp. Soc. Gen. Micro., P.391, Cambridge University Press.

Herbert,D., Elsworth,R. & Telling,R.C. (1956). J. Gen. Microbiol, 14, 601.

Herbert,D., Phipps,P.J. & Tempest,D.W. (1965). Lab. Prac., 14, 1150.

Hinshelwood,C. (1946). The Chemical Kinetics of the Bacterial Cell. The Clarendon Press, Oxford.

Holzberg,I., Finn,R.K. & Steinbraus,K.H. (1967). Biotech. & Bioeng. 9, 413.

Humphrey,A.E. (1963). I.A.M. Symposia on Microbiology. No.5. Biochemical Eng. p.215 Proc. of Symp., Univ. of Tokyo.

Irie,R., Yano,N. Morichi,T. & Kembo,H. (1962). Jap. J. Bact. 17, 360. Dairy Sci. Abst. (1964). 26, 1348.

Jankowska,H. (1957). Dairy Sci. Abst. 19, 23d.

Karush,F. Iacocca,V.I. & Harris,T.N. (1956). J.Bacteriol. 72, 283.

Keen,A.R. (1972). J. Dairy Res. 39, 141.

Kempe,L.L., Halvorsen,H.O. & Piret,E.L. (1950). I.E.C., 42, 1852.

Kempe,L.L. & West,R.E. (1959). J. Biochem. Microbiol. Tech & Eng., 1, 335.

Kneule,F. (1956). Chem. Ing. Tech., 28, 221.

Kono,T. (1968). Biotech. & Bioeng., 10, 105.

Kono,T. & Asai,T. (1969a). Biotech. & Bioeng., 11, 19.

Kono,T. & Asai,T. (1969b). Biotech. & Bioeng., 11, 293.

Krumphazl,V., Dyr,J. & Kobr,V. (1964). Chemical Abstracts 61, 6343.

Lawrence,A. & Co.Ltd. (1944). Aust. Food Manuf. & Distrib., 13, 2.

Leban,M., Edwards,V.H. & Wilke ,C.R. (1966). J. Ferm. Technol., 44, 334.

Longsworth,L.G. & MacInnes,D.A. (1935). J. Bact., 29, 595.

Longsworth,L.G. & MacInnes,D.A. (1936a). J. Bact., 31, 287.

Longsworth,L.G. & MacInnes,D.A. (1936b). J. Bact., 32, 567.

Luedeking,R. (1968). Biochemical & Biological Engineering Science, Vol.1, ed. N. Blakebrough. Academic Press, London.

Luedeking,R. & Piret,E.L. (1959a). J. Biochem. Microbiol. Tech. Eng., 1, 393.

Luedeking,R. & Piret,E.L. (1959b). J. Biochem. Microbiol. Tech. Eng., 1, 431.

McDowall,F.H. & Thomas,R.H. (1961). Disposal of Dairy Wastes by Spray Irrigation of Pasture Land, Pollution Advisory Council Publication No.8. Wellington.

McDowell,A.K.R. & Dolby,R.M. (1935). J. Dairy Res., 6, 243.

Malek,I., Beran,K., Fencl,Z., Munk,V., Ricica,J. & Smrokova,H. (1969). Continuous Cultivation of Micro-organisms, 4th. Symp. Academia, Prague.

Malek,I. & Fencl,Z. (1966). Theoretical & Methodological Basis of Continuous Culture of Micro-organisms. Czech. Acad. Sci. Prague.

Mandelstam,J. & McQuillen,K. (1968). Biochemistry of Bacterial Growth, Blackwell, Oxford.

Martin,A.W. & Tartar,H.V. (1937) J. Am. Chem. Soc., 59, 2672.

Maxon,W.D. & Chen,J.W. (1966). J. Ferment. Technol., 44, 255.

Maxon,W.D. & Chen,J.W. (1967). Microbial Physiology & Cont. Cult. Third Intern. Symp., p.155, HMSO.

Maxova,Milena & Maxa,V. (1959). Dairy Sci. Abst., 1067.

Midler,M. & Finn,R.K. (1966). Biotech. & Bioeng. 8, 71.

Monod,J. (1942). Recherches sur la croissance des cultures bacteriennes., Masson et Cie, Paris.

Monod,J. (1949).Microbiol., 3, 371.

Monod,J. (1950).Ann. Inst. Pasteur. 79, 390.

Nakanishi,T. & Nakazawa,Y. (1961). Jap. J. Dairy Sci., 13, A117. Dairy Sci. Abst. (1965), 27, 1169.

Needle,H.C. & Aries,R.S. (1949). Sugar, 44 (12), 32.

Nilsson,G. (1950). Dairy Sci. Abst. 11, 197.

Northrop,J.H.(1954). J. Gen. Physiol. 38, 105.

Oetiker,N. (1960). Aust.J. Dairy Tech., 15, 69.

Olive,T.R. (1936). Chem. & Met. Engr., 43, 480.

Painter,P.R. & Marr,A.G. (1968). Annual Review Microbiology, 22, 519.

Peppler,H.J. (1967). Microbial Technology, p.407. Reinhold,N.Y.

Pirt,S.J. (1967). Microbial Physiology & Continuous Culture, 3rd.Internat. Symp., p.162,HMSO.

Pirt,S.J. (1969). Microbial Growth, 19th Symp. Soc.Gen. Microbiology,p.199, Cambridge University Press.

Plackett,R.L. & Burman,J.P. (1946). Biometrika, 33, 305.

Powell,E.O. (1966). J. Gen. Microbiol., 45, 11.

Powell,E.O. (1967). Microbial Physiology & Continuous Culture, 3rd. Internat. Symp., p.34, HMSO.

Pont,E.G. (1944). Aust. Milk & Dairy Products Journal, 11, 11.

Prescott,S.C. & Dunn,C.G. (1959). Industrial Microbiology, 3rd Ed. McGraw-Hill Book Co. Ltd. N.Y.

Ramkrishna,D., Fredrickson,A.G. & Tsuchiya,H.M. (1966). J. Ferment. Technol., 44, 203.

Ramkrishna,D., Fredrickson,A.G. & Tsuchiya,H.M. (1967). Biotech. & Bioeng., 9, 129.

Ricica,J. (1969a). Fermentation Advances. p.427 Ed. D.Perlman. Academic Press, London.

Ricica,J. (1969b). Fourth Symp. Cont. Cult., p.163, ed. I.Malek, Academia, Prague.

Rogers,L.A. & Whittier,E.O. (1928). J. Bacteriol., 16, 211.

Rogosa,M., Franklin,J.G. & Perry,K.D. (1961). J. Gen. Microbiol., 25, 473.

Rogosa,M. & Sharpe,M.E. (1959). J. Appl. Bact., 22, 329.

Rosell,J. (1949). Milchwissenschaft, 4, 280.

Rutter,W.H. & Hansen,R.G. (1953). J. Biol. Chem., 202, 311.

Schopmeyer,H.H. (1954). Chapt.12 in "Industrial Fermentations" Vol.1, ed. L.A. Underkofler & R.J.Hickey, Chemical Publishing Co. Inc., N.Y.

Sharpe,M.E. (1962). Dairy Sci. Abst., 24, 110.

Shore,D.T. & Royston,M.G. (1968). The Chemical Engineer, 218, CE 99.

Shu,P. (1961). Biotech. & Bioeng., 3, 95.

Siman,J. & Mergl,M. (1961). Prumysl Potravin, 12, 42.

Snell,E.E. (1952). Bact. Rev. 16, 235.

Snell,E.E., Kitay,E. & Hoff-Jorgensen,E. (1948). Arch. Biochem. & Biophys., 18, 495.

Steinsholt,K. & Calbert,H.E. (1960). Milchwissen schaft, 15, 7.

Stowe,R.A. & Mayer,R.P. (1966). I.E.C. 58, 36.

Surazynski,A., Pozanski,S., Budalowski,J., Czerwinski,S. & Choynowski,W. (1967). Dairy Sci. Abst. (1968), 30, 3029.

Swaby,R.J. (1944). Agric. Gazette of NSW, 55, 211 & 257.

Swaby,R.J. (1945a). J. Aust. Inst. Agric. Sci., 11, 179.

Swaby,R.J. (1945b). J. Aust. Inst. Agric. Sci., 11, 191.

Terui,G. & Niizu,H. (1969). Global Impacts of Applied Microbiology II, Ed. Elmer L. Gaden Jr. Interscience,N.Y.

Thorne,J.G.M. (1969), Chem. Proc. 32, 10.

Tsuchiya,H.M., Fredrickson,A.G. & Aris,R. (1966). Adv. Chem. Eng., 6, 125.

Wahba,N. (1965). Analyst, 90, 432.

West,J.M. & Gaden,E.L. Jr. (1959). J. Biochem. Microbiol. Technol. Eng., 1, 163.

Wheater,Dorothy M. (1955). J. Gen. Microbiol., 12, 123.

Whittier,E.O. & Rodgers,L.A. (1931). I.E.C., 23, 532.

Whittier,E.O. & Webb,B.G. (1950). Byproducts from Milk. p.34 Reinhold,N.Y.

Wieland,O.P., Avener,J., Boggiane,E.M. Bohonos,N., Hutchings,B.L. & Williams,J.H. (1952). J. Biol. Chem., 186, 737.

Williams,K.R. (1963). I.E.C., 55, 29.

Wilson,G.S. & Miles,A.A. (1964). Topley & Wilson's Principles of Bacteriology and Immunity, 5th. Ed., Vol.1, Chapt.30, Edward Arnold Ltd.London.

Wright,L.D., Huff,J.W., Skeggs,H.R., Valentik,K.A. & Bosshardt,D.K. (1950). J. Amer. Chem. Soc., 72, 2312.

APPENDIX 1.

LACTIC CASEIN WHEY

In New Zealand, casein is normally produced by inoculating skim milk with a mixed strain culture of lactic acid producing bacteria. The acid formed as the bacteria reproduce causes the pH of the skim milk to fall to the isoelectric point of the casein. The coagulated casein is removed from the liquid by heating and draining. The liquid remaining is known as whey. Of the whole milk processed in a casein factory, 76 per cent appears as whey (McDowall and Thomas; 1961). The estimated volume of lactic casein whey produced in New Zealand in 1969/70 was 356 million gallons.

The composition of whey is variable depending on the stage of lactation of the cows, the season and the processing conditions. A typical lactic casein whey composition is shown in table A1.1. The composition given can only be used as a guide. The seasonal or lactational variation can be very large so that lactose can be as high as 54 g/l in October and as low as 38 g/l in June. Processing also has an effect. Eight samples of whey from a commercial casein factory taken over a period of one month had total solids concentrations from 44 to 60 g/l with average 57 g/l.

Some physical properties of whey are given in table A1.2.

Table A1.1 Typical compositions of whole milk and lactic casein whey

	Whole Milk g/l	Whey g/l
Total protein (N x 6.38)	37.3	9.5
Non-protein nitrogen	0.25	0.48
Ash	7.0	7.2
Lactose	49	44.7
Fat	46	0.5
Acidity (as lactic acid)	2.1	6.4
Water	870	932
pH	6.5	4.5

Table A1.2 Some physical properties of lactic casein whey

Temperature ^{o}C	Viscosity cP (Ostwald)	Density k g/l
20	1.24	1.0209
35	0.86	1.0163
50	0.63	1.0097

APPENDIX 2

Classification of LBR

Tests were carried out by Mr.H.A.Heap, NZDRI Microbiology Dept. to confirm that the organism used in the study of the production of lactic acid from whey was a strain of Lactobacillus bulgaricus.

The strain, designated LBR, had been derived by X-radiation of an NZDRI rack culture of Lactobacillus bulgaricus and was characterised by fast acid production. It had been used for some years by the Rangitaiki Plains Dairy Company to produce lactic acid for casein making (Cetiker,1960).

The tests used are outlined by Sharpe (1962).

L.bulgaricus and L.helveticus grow at 45°C but not at 15°C. They do not produce NH_3 from arginine. They ferment galactose and glucose. L.helveticus ferments maltose and trehalose but L.bulgaricus does not. L. helveticus produces up to 2.7 per cent acid in milk and L.bulgaricus 1.7 per cent. Metachromatic or volutin granules can be detected in L.bulgaricus, but not L.helveticus.

Other tests which can be used to differentiate Lactobacillus spp. are based on lactic acid configuration, nutritional requirements and serological typing but these were not carried out.

Control cultures used in the tests were strains of L.bulgaricus and L.helveticus from the NZDRI culture collection. Cultures tested are shown in table A2.1 and results in tables A2.2 - A2.5.

The results obtained though not conclusive, are consistent with the hypothesis that LBR is a strain of Lactobacillus bulgaricus.

Table A2.1 Lactobacilli classification - test cultures

Key	Culture	Source
A	LBR	NZDRI rack culture
B	L. bulgaricus	NZDRI rack culture
C	L. helveticus	NZDRI rack culture
D	LBR	Strain being tested
E	LBR	From continuous culture of LBR on whey

RESULTS

Table A2.2 Growth in MRS Broth. Optical density at 580 nm after 48 h.

Culture	15°C	45°C
A	.005	.68
B	.000	.83
C	.010	1.08
D	.000	1.23
E	.005	1.23

The test organism grew at 45°C but not at 15°C. Tests for lactic acid production in skim milk confirmed this.

Table A2.3 Microscopic appearance of Lactobacilli cultures

A :	long slender rods, occuring mainly in chains with round ends. Volatin particles.
B :	as for A
C :	rods, occuring singly and in chains.
D :	long slender rods, occuring mainly in chains. Volatin particles.
E :	as for D.

Table A2.4 Acid production in sterile reconstituted skim milk

Culture	per cent lactic acid after 24 hr	per cent lactic acid after 48 hr
A	1.15	1.66
B	1.53	1.88
C	0.96	1.74
D	1.18	2.06
E	1.08	1.92

Table A2.5 Sugar fermentations in modified MRS broth after 48

Culture	NH_3 from arginine	Cellobiose	Galactose	Lactose	Maltose	Mannitol	Melibiose	Salicin	Sorbitol	Sucrose	Trehalose
A	-	-	+	+	±	-	-	-	-	-	-
B	-	-	+	+	±	-	-	-	-	±	-
C	-	-	+	+	+	-	-	-	-	-	-
D	-	-	+	+	-	-	-	-	-	-	-
E	-	-	+	+	-	-	-	-	-	-	-

\+ Positive

\- Negative

± Variable

APPENDIX 3

SAMPLE CALCULATION FOR DETERMINATION OF RESULTS

The following are the details of the calculation used to determine the responses in the experiments to select the variables. (Appendix 6)

Experiment 1. Run No. 8

Temperature 48°C; pH 6.0; Agitator 300 rpm; Flow rate 0.375 l/h; Gas 15 l/h; EDTA, lactose inorganic nutrients and organic nutrients added.

Results

Feed pH = 5.0; 10 ml of feed required 1.32 ml of 0.1N NaOH to adjust pH to 6.0

Lactose = 52.0 g/l

Lactate (P_i) = 14.5 g/l

Flow rate (Fi) = 400 ml/h

Alkali

Normality = 11.7

Flow rate at "steady state" = 6.35 ml/h

Culture medium

Lactate (Po) = 28.5 g/l

Cell number - not recorded (smear washed off)

Calculation

Overflow rate (Fo) = 400 + 6.35

= 406 ml/h

(Adjusts the feed flow-rate for the alkali added to neutralize the acid formed).

Lactate concentration increase = Po - Pi

= 28.5 - 14.5

= 14.0 g/l

Lactate production rate = Fo Po - Fi Pi

= 0.406 x 28.5 - 0.400 x 14.5

= 5.8 g/h

Volume of 11.7N NaOH to change the pH of the feed from 5.0 to 6.0 = $\frac{400 \times 1.32 \times 0.1}{10 \times 11.7}$

= 0.451 ml/h

∴ Volume of 11.7N NaOH to neutralize the lactic acid formed = 6.35 - 0.45

= 5.90 ml/h

The lactate measured analytically includes dissociated and undissociated acid, whereas the alkali is a measure of the dissociated acid only. Hence a correction is required. This is significant at low pH values. The dissociation constant of lactic acid at 45°C is 1.309×10^{-4} (Martin and Tartar, 1937) The equation for the dissociation constant is

$$K = \frac{(H^+)\ (LA^-)}{(HLA)}$$

where (H^+) = concentration of hydrogen ions

(LA^-) = concentration of lactate ions

(HLA) = concentration of undissociated lactic acid

K = dissociation constant

Total lactic acid formed, P, is

$$P = (HLA) + (LA^-)$$

$$\therefore\ P = (LA^-)\left(1 + \frac{(H^+)}{K}\right)$$

(H^+) is fixed by the pH.

If the acid were completely dissociated each ml of alkali would be equivalent to $\left(\frac{89.08 \times 11.7}{1000}\right)$ g of lactate. Hence with the correction to allow for undissociated acid each ml of alkali is equivalent to $\frac{89.08 \times 11.7}{1000}\left(1 + \frac{10^{-6}}{1.309 \times 10^{-4}}\right)$ g of lactate at pH 6.0.

$\therefore$ Amount of lactate formed

$$= 5.90 \times \frac{89.08 \times 11.7}{1000}\left(1 + \frac{10^{-6}}{1.309 \times 10^{-4}}\right)$$

$$= 6.20 \text{ g/h}$$

$$\frac{\text{Lactate by analysis}}{\text{lactate equivalent to alkali}} = \frac{5.80}{6.20}$$

$$= 0.94$$

$$\text{Yield} = \frac{5.8}{0.400 \times 52.0} = 0.28$$

$$\text{Productivity} = \frac{5.8}{2} = 2.9 \text{ g/l h}$$

Sample calculation of the effect of a variable

Effect of variable A (temperature) on the productivity.

$$\text{Effect} = \tfrac{1}{8}(2.9 + 0.6 + 2.2 + 0.2 + 0.6 + 1.6 + 4.4 + 0.5) - \tfrac{1}{8}(0.8 + 0.5 + 1.2 + 3.0 + 2.3 + 4.0 + 3.2 + 0.9)$$

$$= -0.36$$

$$t = \frac{\text{Effect}}{SD} = -\frac{0.36}{0.49} = -0.73$$

The probability of obtaining a t-value this high by chance is 0.48. Hence temperature can be assumed to have no effect over the range of values investigated.

APPENDIX 4

YIELD OF LACTATE FROM LACTOSE

The following figures were taken from a series of batch experiments in which lactose and lactate concentrations were recorded as functions of time.

	Time h	$\frac{\text{lactate}}{\text{lactose}}$
B8.2.1	2.20	1.05
	4.36	1.11
	6.86	1.03
	10.50	1.02
B8.2.2	1.00	0.26
	1.50	0.27
	2.27	0.67
	3.38	0.91
	5.42	0.94
	7.00	0.92
	10.01	0.87
	22.75	0.86
B8.2.3	9.35	0.99
B8.2.4	14.23	0.90
B8.2.5	6.82	0.97
B8.2.6	0.63	0.33
	1.13	0.57
	1.92	0.39
	2.40	0.37
	2.94	0.53
	3.97	0.79
	5.20	1.02
	6.60	0.91
	7.75	0.92
	9.17	0.96
	10.92	0.93
B8.2.7	17.58	1.01

Average yield taking all times (mean $\pm$ sd)

$$\bar{x} = 0.80 \pm 0.27$$

Average yield for a batch run

$$\bar{x} = 0.95 = 0.06$$

APPENDIX 5

The Analysis of a Central Composite Rotatable Design: The effect of pH, temperature and dilution-rate on the increase in acid concentration. (Cochran and Cox, 1962)

The relationships between the coded x- scales and the original scales are shown by equations 1 - 3.

$$x_1 = \frac{pH - 5.65}{0.15} \qquad (1)$$

$$x_2 = \text{Temperature} - 46\ ^{o}C \qquad (2)$$

$$x_3 = \frac{\text{Dilution-rate} - 0.206\ h^{-1}}{0.103} \qquad (3)$$

Table A5.1 shows the design, the X matrix, and the increase in lactate concentration (P) in the Y column.

Each column of the X matrix is multiplied in turn by the Y column, giving the sums of products (0y), (1y), etc. shown in Table A5.2. The auxiliary total $\sum(iiy)$ is computed and the regression coefficients b_o, b_i, etc. are found from the following equations:

$$b_o = 0.166338\ (0y) - 0.056791 \sum(iiy)$$

$$b_i = 0.073224\ (iy)$$

$$b_{ii} = 0.062500\ (iiy) + 0.006889 \sum(iiy) - 0.056791\ (0y)$$

$$b_{ij} = 0.125000\ (ijy)$$

The values of b_{ij} are given in Table A5.2.

The analysis of variance is shown in Table A5.3 and is obtained as follows :

The sums of squares due to first order terms:

$$\sum_{i=1}^{3} b_i\ (iy)$$

The sums of squares due to second order terms:

$$= b_o\ (0y) + \sum_{i=1}^{3} b_{ii}\ (iiy) + \sum_{i<j} b_{ij}\ (ijy) - G^2/20$$

where G is the grand total.

The total sums of squares is calculated in the normal way. The error sums of squares is computed from the 6 central points. The sums of squares for lack of fit is obtained by subtraction.

If S is the standard error per observation (computed from the six central points) then the standard errors of the regression coefficients are given by:

$$\text{s.e.}\ (b_i) = 0.354\ S$$

$$\text{s.e.}\ (b_{ii}) = 0.379\ S$$

$$\text{s.e.}\ (b_{ij}) = 0.5\ S$$

The standard error per observation for this experiment is 1.734 and the standard errors for the regression coefficients are shown in Table A5.2.

Table A 5.1 Central Composite Rotatable Design for 3 Variables

X = Matrix of x-variables										Y
x_0	x_1	x_2	x_3	x_1^2	x_2^2	x_3^2	x_1x_2	x_1x_3	x_2x_3	y
1	-1	-1	-1	1	1	1	1	1	1	16.00
1	1	-1	-1	1	1	1	-1	-1	1	19.10
1	-1	1	-1	1	1	1	-1	1	-1	19.00
1	1	1	-1	1	1	1	1	-1	-1	17.20
1	-1	-1	1	1	1	1	1	-1	-1	10.80
1	1	-1	1	1	1	1	-1	1	-1	11.13
1	-1	1	1	1	1	1	-1	-1	1	10.70
1	1	1	1	1	1	1	1	1	1	10.95
1	-1.682	0	0	2.828	0	0	0	0	0	14.20
1	1.682	0	0	2.828	0	0	0	0	0	14.85
1	0	-1.682	0	0	2.828	0	0	0	0	11.50
1	0	1.682	0	0	2.828	0	0	0	0	12.93
1	0	0	-1.682	0	0	2.828	0	0	0	27.38
1	0	0	1.682	0	0	2.828	0	0	0	8.94
1	0	0	0	0	0	0	0	0	0	13.90
1	0	0	0	0	0	0	0	0	0	13.18
1	0	0	0	0	0	0	0	0	0	17.22
1	0	0	0	0	0	0	0	0	0	13.52
1	0	0	0	0	0	0	0	0	0	15.82
1	0	0	0	0	0	0	0	0	0	16.55

Table A 5.2 Regression coefficients for the equation showing the effect of pH, temperature and dilution-rate on acid concentration.

				Std.error
(oy)	294.87	b_o	15.05	1.73
(1y)	2.9733	b_1	0.22	0.61
(2y)	3.22526	b_2	0.24	0.61
(3y)	-58.73608	b_3	-4.30	0.61
(11y)	197.03340	b_{11}	-0.31	0.66
(22y)	183.96804	b_{22}	-1.12	0.66
(33y)	217.59296	b_{33}	0.98	0.66
(12y)	-4.98	b_{12}	-0.62	0.87
(13y)	-0.72	b_{13}	-0.09	0.87
(23y)	-1.38	b_{23}	-0.17	0.87
$\sum$(iiy)	598.59440			

Table A 5.3 Analysis of variance for the equation showing the effect of pH, temperature and dilution-rate on acid concentration.

	Degrees of Freedom	Sum of squares	Mean squares
First order terms	3	254.027	84.676
Second order terms	6	39.967	6.661
Lack of fit	5	18.041	3.007
Error	5	14.710	2.942
Total	19	326.745	

APPENDIX 6

The work reported in this appendix was carried out in an attempt to determine the major variables affecting the production of lactic acid from whey. For a number of reasons the results are not considered to be reliable but the work is presented for completeness.

EXPERIMENTAL DESIGNS

The development of new processes or the improvement of existing ones requires a consideration of the effects of a large number of variables. For the proper utilization of resources a screening technique which efficiently determines those variables which merit most investigation is essential.

The experimental designs most commonly used to investigate the variables in process systems are the factorial and fractional-factorial designs. A special case of fractional factorial designs, known as the saturated fractional factorials can be used to investigate (n-1) variables in n experiments. The designs are known as the "Plackett-Burman designs" (Plackett and Burman,1946) or the "2^{k-p} fractional factorials of resolution III" (Box and Hunter,1961).

Plackett and Burman (1946) determined designs for screening (n-1) variables, each at two levels, in n experiments where n is a multiple of four. They presented the design matrices for 4 up to 100 runs (except n = 92). Where n is a power of two the designs are identical with the 2^{k-p} fractional factorial designs of Box & Hunter (1961). Plackett & Burman (1946) also presented designs for screening variables at 3,4,5 and 7 levels.

Stowe & Mayer (1966) illustrated the use of the Plackett-Burman experimental design for screening the effect of twelve variables on catalyst activity. Williams (1963) compared three designs for screening variables in the development of an epoxide adhesive. A Plackett- Burman design in 28 experiments was shown to be more efficient than a fractional-factorial design in 32 experiments or a random balance design in 28 experiments.

The method is as follows. After choosing the independent variables to be studied, each is assigned two values or levels; a low level designated (.) or (-) and a high level designated (+). The number of variables determines the experimental plan to be used. Up to (n-1) variables can be studied in n experiments, but it is desirable to include a number of additional experiments for estimating the variance due to experimental errors. The variables so included are called "dummy variables" and no changes are associated with them in the design.

The design matrix is set up by writing down the first row of the given design (Plackett and Burman,1946) then shifting this first row cyclically to the left one place (n-2) times to generate (n-1) rows. The n^{th} row is a row of (.)'s. Thus the design consists of n rows designating the experimental runs and (n-1) columns designating the variables.

Thus for n = 16 the first row of the design is (Plackett and Burman,1946)

+ + + + . + . + + . . + . . .

The full design matrix is shown in table A6.1

Table A6.1 Matrix for 16 runs for a Plackett and Burman design

Experiment No.	VARIABLE														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	+	+	+	+	.	+	.	+	+	.	.	+	.	.	.
2	+	+	+	.	+	.	+	+	.	.	+	.	.	.	+
3	+	+	.	+	.	+	+	.	.	+	.	.	.	+	+
4	+	.	+	.	+	+	.	.	+	.	.	.	+	+	+
5	.	+	.	+	+	.	.	+	.	.	.	+	+	+	+
6	+	.	+	+	.	.	+	.	.	.	+	+	+	+	.
7	.	+	+	.	.	+	.	.	.	+	+	+	+	.	+
8	+	+	.	.	+	.	.	.	+	+	+	+	.	+	.
9	+	.	.	+	.	.	.	+	+	+	+	.	+	.	+
10	.	.	+	.	.	.	+	+	+	+	.	+	.	+	+
11	.	+	.	.	.	+	+	+	+	.	+	.	+	+	.
12	+	.	.	.	+	+	+	+	.	+	.	+	+	.	.
13	.	.	.	+	+	+	+	.	+	.	+	+	.	.	+
14	.	.	+	+	+	+	.	+	.	+	+	.	.	+	.
15	.	+	+	+	+	.	+	.	+	+	.	.	+	.	.
16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

The experiments are performed in random order and the response for each experiment determined.

The effect on the response of changing each independent variable from its low level to its high level is the difference between the average of the values of the response obtained at the high level of the variable and the average of the values of the response obtained at the low level. Thus if E_A is the effect of A, and R is the response of one experiment then

$$E_A = \frac{\sum(R \text{ at } (+))}{\text{No. of } (+) \text{ values}} - \frac{\sum(R \text{ at } (.))}{\text{No. of } (.) \text{ values}}$$

The effects of the dummy variables are determined in the same way. If there are no interactions or experimental errors, the effect shown by the dummy variable should be zero.

The variance V, is equal to the average of the squares of the dummy effects;

$$V \text{ (effects)} = \frac{\sum(E \text{ Dummy})^2}{\text{Number of Dummy Variables}}$$

$$\text{The Standard Deviation (S D)} = \sqrt{V \text{ (effects)}}$$

The significance of each effect is determined by the use of the standard t-test with the degrees of freedom equal to the number of dummy variables.

$$t = \frac{\text{Effect}}{\text{SD (effect)}}$$

The t-test for each effect is an evaluation of the probability of finding an effect this large by chance when in fact no effect really exists. If this probability is small enough it means that in going from the low level to the high level of that variable the change observed in the response is due to changing the variable and not to random error. The confidence level of being correct in accepting that the effect was caused by the change in level is 100 x (1-probability due to chance).

Selection of variables in whey fermentation

The number of factors which could influence the production of lactic acid using casein whey as a substrate is large. The organism, the physical variables of temperature, pH, degree of agitation and residence time in the culture vessel and the presence of stimulatory and inhibitory substances present a bewildering array of variables which could be studied.

The experimental design techniques of Plackett and Burman were chosen as a means of screening some of the variables in a series of continuous culture experiments. The organism was not considered as a variable because initial experiments had shown that LBR produced more lactic acid faster than any other organism available in the NZDRI culture collection.

The physical variables temperature, pH, the degree of agitation and the residence time could be readily studied in the available equipment. The degree of agitation could be varied either by altering the diameter of the impeller or by altering its rotational speed. The latter was more convenient. The dilution rate (reciprocal of residence time) could be altered by adjusting the volume of the vessel contents or by adjusting the flow-rate of the incoming medium. Alterations in volume of the vessel contents would affect the degree of agitation and hence the flow rate of the medium was considered to be the independent variable.

The supplementary nutrient factors were considered in groups: a) Chemically defined nutrients, acetate, citrate, magnesium, manganese, calcium and iron; b) undefined nutrients, peptone, beef extract, yeast extract and tween 80; c) phosphate; d) EDTA (added to complex any copper which might be present in the whey. Rogosa and Sharp (1959) showed copper to have a delcterious effect on L.bulgaricus) e) lactose; and f) Carbon dioxide.

This gave a total of ten variables to be considered. The Plackett and Burman design shown in table A 6.1 allows for 15 variables. This design was chosen in preference to the standard design for 11 variables because it allowed a greater number of dummy variables to be inserted and because of the possibility of forming a table of two-factor and main factor interactions (Stowe and Mayer,1966). The variables studied and the high and low levels used are shown in table A6.2. The levels were chosen to cover the range of values mentioned in the various literature references and such that the effects obtained would be measurable but not too great.

The runs were carried out in random order. As an example of the conditions used in an experiment in run 1 a (+) in table A6.1 indicates the high level of the variable and (.) the low level; ie temperature was 48°C, EDTA, chemically defined nutrients, gas and undefined nutrients were added, pH was 6.0 the lactose concentration was 50 g/l, agitation was set at 300 rpm and the feed rate of incoming medium was maintained at 0.375 l/h.

The results obtained are shown in table A6.3. A sample calculation is shown in appendix 3.

The effects of the variables on the increase in acid concentration, productivity, and yield are shown in tables A6.4 - A6.6 together with the calculated values of Student's t and the tabulated values of the probability of obtaining a value of t that high by chance.

Table A 6.2 Levels of the independent variables in the Plackett and Burman screening experiments.

Code	Variable	Level +	Level -
A	Temperature ^{o}C	48	45
B	EDTA g/l	0.3	0
C	Dummy		
D	Chemically defined nutrients g/l		
	$Na\ C_2\ H_3\ O_2\ 3H_2O$	5	0
	$(NH_4)_2\ HC_6\ H_5\ O_7$	2	0
	$Mg\ SO_4\ 7H_2O$	0.2	0
	$Mn\ SO_4\ 4H_2O$	0.05	0
	$Ca\ Cl_2\ 6H_2O$	0.05	0
	$Fe\ SO_4\ 7H_2O$	0.05	0
E	Dummy		
F	Gas (95 per cent N_2 5 per cent CO_2) l/h	15	0
G	Dummy		
H	pH	6.0	5.0
I	Undefined nutrients g/l		
	Peptone (Oxoid)	5	0
	beef extract (Oxoid)	5	0
	yeast extract (BBL powder)	2.5	0
	Tween 80 (Atlas Powder Co)	1	0
J	Dummy		
K	Phosphate $K_2\ HPO_4$ g/l	2	0
L	Lactose concentration g/l	50	40
M	Agitation rpm	450	300
N	Flow rate of feed l/h	0.75	0.375
O	Dummy		

Table A6.3 Selection of variables; experimental results

Experiment No.	Run No.	Increase acid conc. g/l.	Productivity g/l h	Yield
1	C.7.12.8	14.0	2.9	0.28
2	15	2.8	0.6	0.07
3	13	5.6	2.2	0.14
4	6	0.3	0.2	0.01
5	7	2.1	0.8	0.05
6	5	1.5	0.6	0.03
7	14	2.4	0.5	0.05
8	10	4.2	1.6	0.09
9	9	21.2	4.4	0.54
10	1	3.6	1.2	0.06
11	11	8.0	3.1	0.21
12	2	2.4	0.5	0.04
13	16	11.6	2.3	0.26
14	4	10.0	4.0	0.25
15	3	16.3	3.2	0.42
16	12	4.8	0.9	0.06

Table A6.4 Screening of variables: increase in acid concentration

		Effect g/l	t	Probability
A	Temperature	0.9	0.56	0.60
B	EDTA	0.0	-	-
C	Dummy	0.1		
D	Chemically defined nutrients	6.7	4.40	0.01
E	Dummy	1.4		
F	Gas	0.3	0.18	0.85
G	Dummy	0.9		
H	pH	2.2	1.42	0.22
I	Undefined nutrients	6.0	3.91	0.01
J	Dummy	2.6		
K	Phosphate	1.6	1.03	0.35
L	Lactose concentration	-3.4	2.22	0.08
M	Agitation	-0.3	0.20	0.85
N	Flow-rate	5.0	3.29	0.02
O	Dummy	1.5		

Note: - The standard deviation of an effect = 1.5 g/l

As an example of interpretation of these results the addition of the chemically defined nutrients (d) increases the lactic acid concentration by an average of 6.7 g/l, the productivity by 1.5 g/l h(A6.5) and yield from the ingoing lactose by 17.3 per cent (A6.6).

Table A6.5 Screening experiment: productivity.

		Effect g/l h	t	Probability
A	Temperature	-0.4	0.73	0.48
B	EDTA	0.1	0.20	0.85
C	Dummy	-0.3		
D	Chemically defined nutrients	1.5	2.99	0.03
E	Dummy	-0.3		
F	Gas	0.3	0.55	0.60
G	Dummy	0.2		
H	pH	0.8	1.55	0.18
I	Undefined nutrients	1.1	2.30	0.07
J	Dummy	0.8		
K	Phosphate	0.6	1.25	0.27
L	Lactose concentration	-1.0	2.08	0.10
M	Agitation	0.3	0.65	0.55
N	Flow-rate	-0.2	0.41	0.70
O	Dummy	0.6		

Note: - The standard deviation of an effect = 0.5 g/l h

Table A6.6 Screening experiment : yield

		Effect per cent	t	Probability
A	Temperature	-2.0	0.52	0.60
B	EDTA	0.5	0.14	0.90
C	Dummy	-2.6		
D	Chemically defined nutrients	17.3	4.33	0.01
E	Dummy	-2.0		
F	Gas	-1.1	0.27	0.82
G	Dummy	-1.3		
H	pH	5.7	1.42	0.21
I	Undefined nutrients	14.6	3.65	0.01
J	Dummy	7.8		
K	Phosphate	5.4	1.35	0.24
L	Lactose concentration	-10.5	2.62	0.04
M	Agitation	1.8	0.46	0.70
N	Flow-rate	-11.2	2.79	0.04
O	Dummy	- 2.5		

Note: The standard deviation of an effect = 4.0 per cent

Discussion

The maximum value of the probability which is taken to be significant is arbitary. For the purposes of this experiment 0.3 (Stowe & Mayer (1966)) was taken to be significant. Thus any variable for which the probability of obtaining the calculated value of t by chance is greater than 0.3 can be rejected as having no effect on the response. Where the probability is less than 0.3 it can be assumed that the variable has a significant effect on the response.

From the tabulated values of the probabilities the responses all increased when the defined nutrients, undefined nutrients and phosphate were added. Increase in pH also increased all responses. Increasing the lactose concentration caused all responses to decrease. The flow-rate had an adverse effect on the lactate concentration and the yield but did not effect the productivity. In the range studied the other variables had no effect on any of the responses. It is surprising that the temperature had little effect. It is possible that the values used were not far enough apart to produce a significant effect, or the values chosen are on either side of the optimum value.

From the tabulated results (tables A6.4 - A6.6) it can be seen that the dummy variables all had fairly high values. The expected value is zero. There are three possible explanations;

(1) In Plackett-Burman designs the main effects are not confounded with each other, but because the designs are highly fractionated factorials (the design used is a 2^{-11} fraction of a 2^{15} factorial) each main effect is confounded with large numbers of two-factor and higher order interactions. Hence the dummy variable with an effect greater than zero may be confounded with a significant interaction.

(2) Considerable error may have existed in the reproduction of the level of some of the variables or in the measurements.

(3) Some unknown independent variable which was not controlled may have had a significant effect.

Table A6.7 shows the primary and two-factor effect confounding which exists in this design (Stowe & Mayer,1966).

Table A6.7 Primary and two-factor effect confounding in a 16 experiment Plackett and Burman design

A	Temperature	BM	CJ	DE	FK	GI	HN	LO
B	EDTA	AM	CN	DK	EF	GL	HJ	IO
C	Dummy	AJ	BN	DO	EL	FG	HM	IK
D	Chemically defined nutrients	AE	BK	CO	FM	GH	IN	JL
E	Dummy	AD	BF	CL	GN	HI	JO	KM
F	Gas	AK	BE	CG	DM	HO	IJ	LN
G	Dummy	AI	BL	CF	DH	EN	JK	MO
H	pH	AN	BJ	CM	DG	EI	FO	KL
I	Undefined nutrients	AG	BO	CK	DN	EH	FJ	LM
J	Dummy	AC	BH	DL	EO	FI	GK	MN
K	Phosphate	AF	BD	CI	EM	GJ	HL	NO
L	Lactose Concentration	AO	BG	CE	DJ	FN	HK	IM
M	Agitation	AB	CH	DF	EK	GO	IL	JN
N	Flow-rate	AH	BD	DI	EG	FL	JM	KC
O	Dummy	AL	BI	CD	EJ	FH	GM	KN

Dummy variable C is confounded with IK, variable E with HI, variable G with DH and variable J with DL all of which could be expected from the results to be significant second order effects, variables D,H,I,K and L being those with the highest effects.

Thus it is not too surprising that the dummy variables have high values. It however highlights a problem of using this type of experimental design when a number of variables expected to have a large effect on the experimental results is being investigated. The inevitable significant second- and higher-order interactions being confounded with main effects may possibly lead to erroneous conclusions. It would be better to confine this type of experimental design to situations where only one or two factors are thought to be significant but a large number of apparently minor factors need to be screened for possible effects. The experiment can then be considered to be a repeated factorial experiment of the major factors with the minor variables confounded with the interactions.

Subsequent experimental work did not show that a major variable was uncontrolled.

As a result of this series of experiments the factors most likely to improve the economics of continuously processing whey to produce lactic acid are the supply of supplementary nutrients, and the determination of optimum levels of pH and medium flow rate. Subsequent experiments were directed towards determining which specific compounds were most useful. The lactose level was not studied further except indirectly as it varied during fermentation because of the difficulty of adjusting it in commercial practice. Phosphate, shown to have a marginal effect on the responses was not studied further, but was added to the alkali used for neutralizing (Luedeking and Piret 1959a). Temperature was also studied as a variable in some experiments to determine if it in fact had no effect over the range studied or whether the values chosen in the selection of variables experiments were on either side of an optimum temperature.

Subsequent experimental work showed in fact that the temperature effect did give a curved response surface with an optimum value close to 46° C (Section 4.3.1). The same results showed pH had very little effect, a response contrary to expectation from the screening experiment. One possible explanation for this was the observation in batch culture experiments that fermentation at low pH for a period of time (due to a failure of the controller) gave an increased rate of acid production when the pH was adjusted up to its desired value. A similar observation has since been made by Hanson and Tsao (1972). Thus in the continuous experiments in which conditions were altered without re-inoculation it is possible there was a bias introduced to the results as a consequence of operation at a previous pH.

Subsequent work also showed that agitation and the gases oxygen and carbon dioxide affected the rate of acid production. These were not shown to be significant variables in the screening experiments because the agitation level was above that required for complete suspension of insoluble particles and the use of a baffled vessel meant the medium surface was stationary and air was not entrained in those experiments in which carbon dioxide was absent.