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THE PHYSIOLOGY OF LACTIC ACID PRODUCTION BY *LACTOBACILLUS DELBREUCKII* IN A CELL RECYCLE FERMENTER

A thesis submitted to the University of Kent for the degree of Ph.D. in the Faculty of Natural Sciences.

Nicolas Charles Major,

Biological Laboratory,

July 1987.

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.

N.C. Major 31-7-87.

N.C. Major

To Melanie, with love

and thanks.

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The electron micrographs of hollow fibres were prepared by Dr. Eleanor Linton and Mr. Gary Higton, CHN analyses were performed by Mr. Tony Fassam, the manuscript was prepared by Mrs. Val Heap and Ms Sue Davies.

Finally, I offer my most sincere thanks to my wife and our families for their continuing love, support and encouragement which provided the incentive for my efforts.

Abbreviations used in this thesis

A	Optical absorbance
ADH	Alcohol dehydrogenase
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
CFE	Cell free extract
C_oA	Coenzyme A
CRF	Cell recycle fermentation (or fermenter)
FDP	Fructose-1, 6-diphosphate
GTP	Guanosine 5'-triphosphate
ID	Inner diameter
LDH	Lactate dehydrogenase
MF	Microfiltration
MRS	Growth medium of de Man *et al.* (1960)
NAD	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
OD	Outer diameter
PDH	Pyruvate dehydrogenase
PFL	Pyruvate formate lyase
ppGpp	Guanosine 3'diphosphate 5'diphosphate
pppGpp	Guanosine 3'diphosphate 5'triphosphate
P_i	Inorganic phosphate
pmf	Proton motive force
PTS	Phosphotransferase system
RNA	Ribonucleic acid
UF	Ultrafiltration
VR	Growth medium based on Vick Roy *et al.* (1982, 1983).

Symbols used in this thesis

C_b	Solute concentration in bulk phase
C_g	Solute concentration in gel layer
D_{crit}	Critical dilution rate
F	Flow rate
J	Filtrate flux rate
K	Mass transfer coefficient
K_m	Michaelis constant
K_s	Monod (saturation) constant
P	Statistical probability
p	Lactic acid concentration
P_f	Filtrate back pressure
P_i	Pressure at culture inlet
P_o	Pressure at culture outlet
ΔP	Pressure differential along filter surface
ΔPTM	Transmembrane pressure
$Q_{glucose}$	Volumetric rate of glucose consumption
$Q_{lactate}$	Volumetric rate of lactic acid formation
q_{ATP}	Specific rate of ATP formation
$q_{glucose}$	Specific rate of glucose consumption
$q_{lactate}$	Specific rate of lactic acid formation
q_m	Specific maintenance rate of glucose consumption
R	Recycle ratio
Rg	Hydraulic resistance of gel layer
Rm	Hydraulic resistance of filter membrane
R_m	Maximum rate of biomass output
r_o	Rate of acid production at time zero
r_t	Rate of acid production at time t
s	Substrate concentration in culture
s_o	Substrate concentration in feed stream

t_a	Batch culture lag/down time
t_d	Culture doubling time
μ	Specific growth rate
μ_{max} (or μ_m)	Maximum specific growth rate
V	Culture volume or culture circulation velocity
V_{max}	Maximum rate of enzymic reaction
x	Biomass concentration
x_m	Maximum attainable biomass concentration
x_o	Initial biomass concentration
Y(or Yx/s)	Growth yield (i.e. biomass from substrate)
Y_{ATP}	Yield of biomass from ATP
Y^{max}	Maximum growth yield
Yp/s	Yield of lactic acid from substrate
Yp/x	Yield of lactic acid from biomass

N.B. A tilde (e.g. $\bar{x}$) denotes a steady state value.

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The Physiology of Lactic Acid Production by

Lactobacillus delbreuckii in a Cell Recycle Fermenter

Nicolas C. Major, Ph.D. Thesis, University of Kent, July 1987.

Abstract

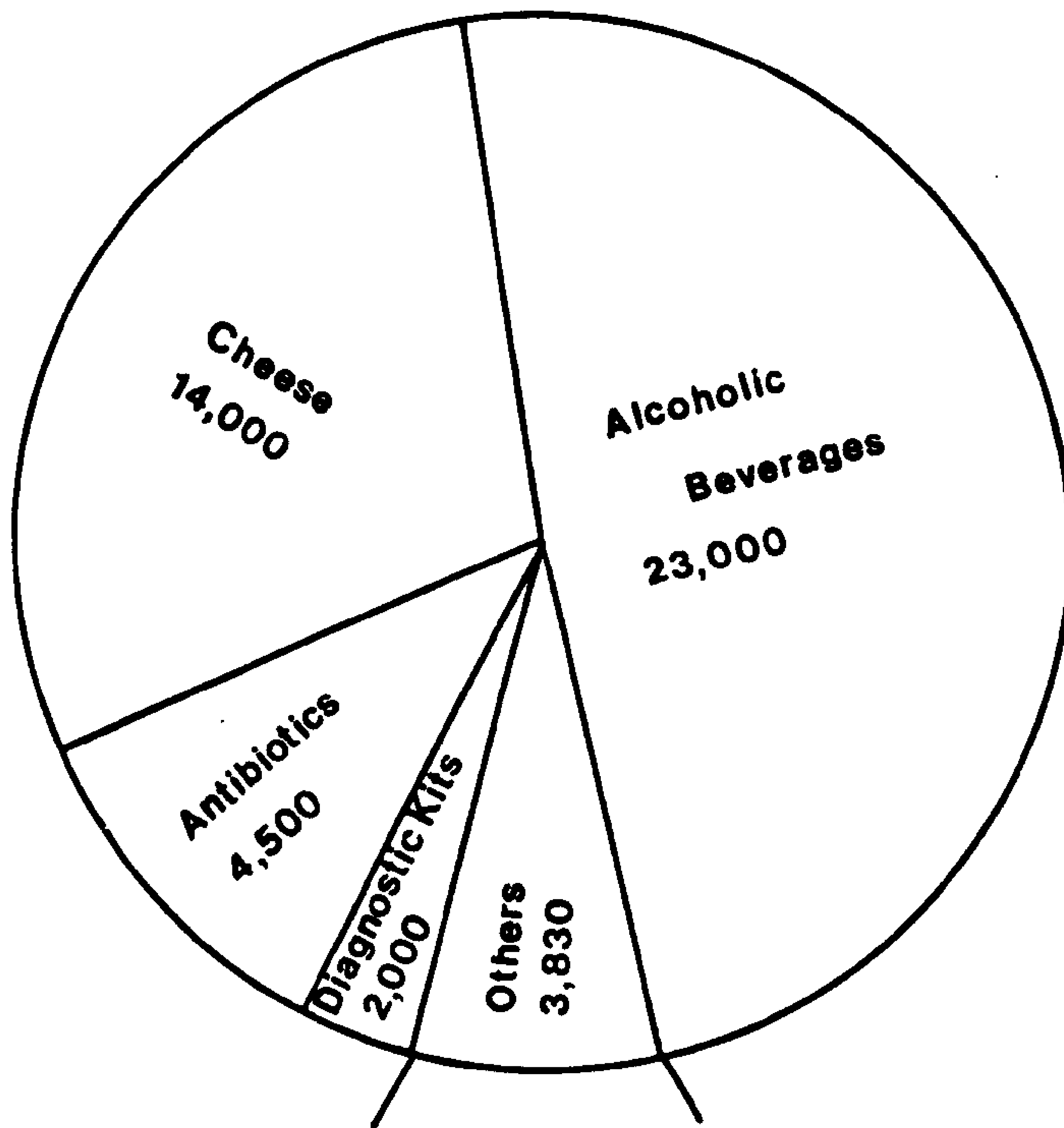
The physiology of lactic acid production by *Lactobacillus delbreuckii* NRRL B-445 in a continuous fermenter with partial cell recycle has been studied and compared with that observed in a conventional chemostat. Increased formation of ethanol and acetate occurred in the recycle fermenter although lactic acid remained the major product. The yield of lactic acid from biomass and the molar product ratio, lactate: ethanol + acetate, decreased with increasing recycle ratio. The volumetric productivity of lactic acid was enhanced in the recycle fermenter due to the complete utilization of glucose, the greatest productivity (12.1 gL^{-1} h^{-1} at D = 0.3 h^{-1}) being achieved using the lowest recycle ratio (0.5). The change in product profile was phenotypic in nature and due to glucose limitation. The specific activity of lactate dehydrogenase and the specific rate of ATP formation were maintained during cell recycle fermentation. The cellular content of polysaccharide, protein, carbon and nitrogen also remained constant and culture viability was maintained. The biomass growth yield was reduced with increasing recycle ratio. A simple mathematical model was derived which described the biomass concentration produced in the recycle fermenter at steady state over a five-fold range of concentrating effect. The derived model included a constant specific rate of glucose consumption for non-anabolic (e.g. maintenance) functions. The results suggest that catabolism and anabolism were less closely coupled in the cell recycle fermenter than in the conventional chemostat.

CHAPTER 1

INTRODUCTION

1.1 Impetus for Process Intensification

Current commercial processes which produce a product by the mass culture of microorganisms are diverse in product range and considerable in terms of world trade. Stanbury & Whitaker (1984) classified these microbial processes into four groups: (a) those which produce microbial cells (biomass) as the product; (b) those which produce microbial enzymes; (c) those which produce microbial metabolites; and (d) those which involve specific microbial transformations. Bull, Holt & Lilly (1982) classified microbial processes on the basis of the volume and value of the process. High volume, low value processes included the production of methane, ethanol and biomass (single-cell protein). High volume, intermediate value processes included the production of amino and organic acids, acetone and butanol, bakers' yeast, and polymers. Low volume, high value processes included the production of antibiotics, steroids, enzymes, vitamins, vaccines and other fine biochemicals. The current commercial value of these processes has recently been outlined by Bull *et al.* (1982) and Dunnill & Rudd (1984). In the latter report, the world market for biological products in 1981 was estimated to be £47,330 million. The market distribution of these products is shown in Figure 1.1. The latter authors estimated the total sales of U.K. fermentation products



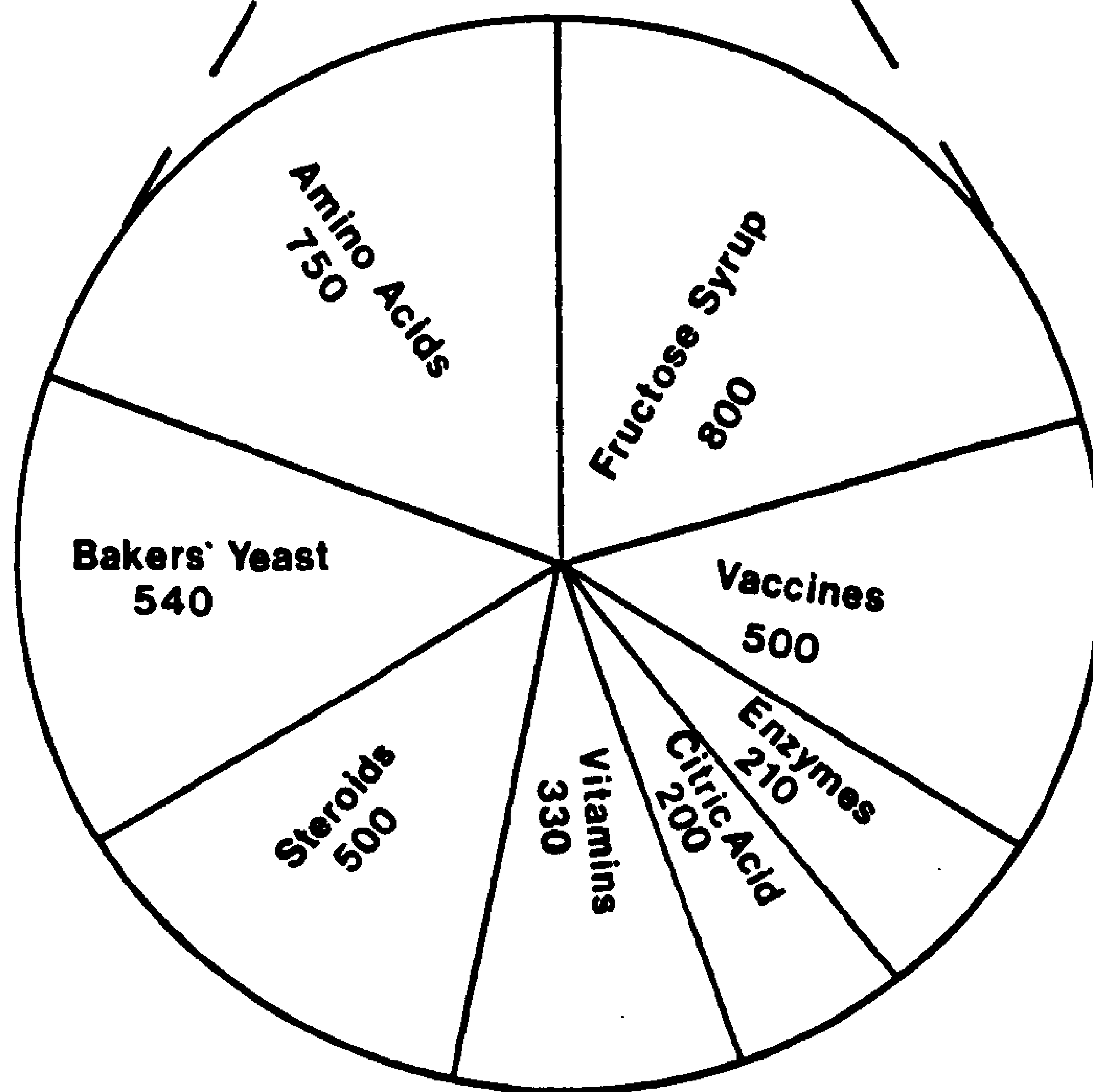


Figure 1.1 World markets for biological products (£ million): Data from Dunnill & Rudd (1984).

in 1982 to be £5,000 million with a net surplus of trade in these products of £550 million. Bull *et al.* (1982) estimated the U.S.A. fine biochemicals-via-biotechnology market to be of the order of $8,000 million of which antibiotics comprised greater than 50 per cent.

The current commercial interest in these processes is reflected in the number of small, new companies recently established in the broad field of biotechnology. These companies number about 40 in the U.K. and include independent enterprises and joint ventures between universities and industry. The number of these small, new companies in the U.S.A. is estimated to be at least 200 (Dunnill & Rudd, 1984). The activities of these companies are diverse and include general consultancy, molecular biology, protein technology and fermentation plant sales and service.

Another reflection of current commercial interest in microbial product-forming processes may be found in an analysis of the patent literature (Bull *et al.*, 1982). The number of patents relating to all aspects of the production of microbial products has risen sharply over the last two decades with major upswings in those concerning enzymes, antibiotics, pharmaceuticals and fine biochemicals in the years 1974 to 1975. Patenting activity was especially high in amino acids, peptides and proteins, carbohydrates, and organic acids in the years 1975 to 1979.

Despite the great commercial interest in microbial product forming processes, these processes remain much less reaction intensive than many comparable commercial chemical reactions (Bull *et al.*, 1982; Cooney, 1983). The reasons for this include the following: (a) concentrations of biocatalyst tend to be low and restricted by mass and heat transfer limitations; (b) products may be growth inhibitory;and (c) substrates may be inhibitory to product formation

at high concentrations. In addition to low reaction intensity, most commercial processes endure the economic penalties of high product recovery costs and discontinuous production. Product recovery costs vary greatly depending on the nature of the process involved and the scale of production but in general there are two main components to the cost; removal of microbial biomass and the handling of large volumes of dilute aqueous product solution.

Large centrifugation plant is often used to remove cells from fermentation broths but this approach is expensive in terms of plant depreciation, return on capital, maintenance, and energy consumption. Atkinson & Mavituna (1983) estimated that depreciation, maintenance and poor return on capital can account for over 80% of the overall cost of a large scale centrifugation plant. Tutunjian (1984) estimated that, for the industrial harvesting of bacterial cells, the capital and annual operating costs of a continuous disc-type centrifuge were of the order of $150,000 and $34,000 respectively. The latter author concluded that for bacterial harvesting, hollow-fibre ultrafiltration required 30% of the capital cost and 70% of the operating cost of a continuous centrifuge system but that for harvesting larger yeast cells, centrifugation was more cost effective. Thus, although separation costs may be reduced by using filtration, the reduction possible depends on the nature of the process to which it is applied.

In all commercial microbial processes the concentration of product in the harvested broth is low, of the order of 0.2 to 20% (w/v) (Cooney, 1983). Vitamins and enzymes are produced at the lower end of this range, ethanol and organic acids at the upper end (Atkinson & Mavituna, 1983). This is a serious problem as the cost of water removal is high, being roughly proportional to the quantity of water present (Bull *et al.*, 1982; Cooney, 1983). This cost is endured in several forms: unused reagents and substrates, although

dilute in spent broth, constitute a large loss as broth volumes are great; drying to reduce process liquid volume is energy intensive, a fuel cost of about £13m^{-3} water evaporated in a spray drier (Atkinson & Mavituna, 1983); the cost of transporting a large volume of dilute product (Bull *et al.*, 1982).

In addition to the relatively low reaction intensity and high product recovery costs, most commercial microbial processes incur the penalties of discontinuous operation. The industrial potential of continuous culture processes has been outlined regularly for at least 25 years and, with the same regularity, it has been concluded that these processes have not found wide acceptance. Gerhardt & Bartlett (1959) reported that continuous culture processes were to be found only in traditional bulk manufacture such as vinegar and bakers' yeast and in waste treatment. Righelato & Elsworth (1970) concluded, "It is evident that continuous culture has not gained a foothold in pharmaceutical or organic chemical production". Most recently, Bull (1982) concluded that although continuous culture has been deployed in the waste treatment, bakers' yeast, and brewing industries, "The impact on the production of pharmaceutical and other chemicals has been negligible".

The value of continuous culture to the study of microbial physiology and process development is widely recognized and the impact of the technique in these areas has been extensively reviewed (e.g. Tempest, 1970; Pirt, 1975; Herbert, 1976; Bull & Brown, 1979). In brief, this value lies in the ability to maintain steady state cultures at defined sub-maximal growth rates with defined growth limiting substrates. Thus, the effect of the physico-chemical environment can be systematically evaluated independently of growth rate and the manipulation of growth rate is possible without varying the growth medium composition or growth conditions. In terms of production processes however, the value of continuous culture lies in

the ability to maintain highly productive cultures over long periods of time. Mathematical treatments comparing the output of biomass from batch and continuous cultures have been presented by Bull (1982) and Pirt (1975). Continuous culture is generally advantageous as biomass can be produced at close to the maximum growth rate without the growth lag and 'down time' associated with a batch culture. Pirt (1975) defined the ratio of the maximum output rate of biomass from a continuous culture to that from a batch culture (R_m(chemostat)/R_m(batch)) in terms of the maximum attainable biomass concentration (x_m), the initial biomass concentration (x_o), the culture doubling time at maximum specific growth rate (t_d) and the batch lag/down time (t_a) as shown in Equation 1.1.

$$R_m(\text{chemostat})/R_m(\text{batch}) = \ln x_m/x_o + 0.693 t_a/t_d$$

Equation 1.1

Bull (1982) outlined some of the real and perceived disadvantages of continuous culture in production processes. These disadvantages centred on anticipation of problems concerning the necessary capital investment, possible contamination, unreliability of equipment, organism stability, and product quality. Cooney (1983) highlighted the concern that although the volumetric productivity of a continuous culture may be greater than that of a batch process, the product concentration is often lower being inversely proportional to the flow rate of the system. This reduction in product concentration serves to enhance the product recovery problems outlined earlier in this Section. This difficulty must be overcome by increasing the specific activity and concentration of the microorganisms in the reactor system.

In summary, it is proposed that the impetus towards the intensification of commercial microbial product-forming processes exists in

the following terms: (a) current and potential commercial interest in microbial processes is great; (b) current processes are of low reaction intensity compared with competing chemical processes; (c) product recovery is problematic in terms of biomass harvesting and handling of bulk dilute product; (d) the deployment of continuous processes remains very limited with consequent under-utilization of process capacity due to batch lag and down times; and (e) the disfavour of continuous processes is partly due to the low product concentrations resulting from biocatalysts inadequate in activity, stability and concentration.

1.2 Cell Recycle Fermentation

1.2.1 Theory

Cell recycle fermentation (CRF) is an approach to the production of microbial metabolites which has been developed in response to the impetus for microbial process intensification outlined in Section 1.1. In essence, a CRF is one in which all, or a proportion of, the cells are retained internally or are recycled back to the fermentation vessel. The result is an accumulation of cells in the fermenter and the generation of a stream of cell-free or cell-depleted culture broth leaving the system. The concept and theory of CRF is not new. A chemostat with cell recycling was first represented by Herbert (1961) and this initial treatment was subsequently developed by Powell & Lowe (1962) and Pirt (1969). The theory was further extended and experimentally tested for the first time by Pirt & Kurowski (1970). In the latter study, theoretical studies were presented for four possible cases of CRF. These latter encompassed both the internal retention and the external recycling of cells and allowed for cell-depleted and cell-free outflow streams. The studies of Pirt &

Kurowski (1970) remain the most comprehensive applied to CRF. Bull & Young (1981) presented a theoretical treatment of a CRF in which there were two outflow streams from the fermenter, one which was completely cell-free, the other containing the same concentration of cells as the culture in the fermentation vessel. This latter type of system has been analysed in the present study.

The cell recycle fermenter is shown schematically in Figure 1.2. The ratio of the flow rate of the cell-free stream to the total outflow rate is a measure of the fraction of the cells in the combined outflow streams that is returned to the fermentation vessel. This ratio of the cell-free to the total outflow is called the recycle ratio, R. The range of possible values of R is $0 < R \leqslant 1$. When $R = 0$, the total outflow of the system contains cells and the fermenter is a conventional chemostat. When $R = 1$, the total outflow is cell-free, no cells leave the fermenter, and recycle is total. The behaviour of the specific growth rate (μ), growth limiting substrate concentration (s), biomass concentration (x), critical dilution rate (D_{crit}), and volumetric biomass production rate (μx) for this particular CRF system are modelled as follows (Bull & Young, 1981; Pirt, 1975).

(i) Specific growth rate (μ)

The biomass balance for the fermenter may be written as:

net increase in biomass = Growth - Output

For the whole culture (volume, V) in an infinitely small time interval, dt, this becomes:

$$V.dx = V.\mu x.dt - (1-R)F.x.dt \qquad \text{Equation 1.2}$$

Dividing throughout by V.dt, and as $D = F/V$, equation 1.2 becomes:

$$dx/_{dt} = [\mu - (1-R)D]x \qquad \text{Equation 1.3}$$

At steady state $^{dx}/_{dt} = 0$ and so

$$\mu = (1-R)D \qquad \text{Equation 1.4}$$

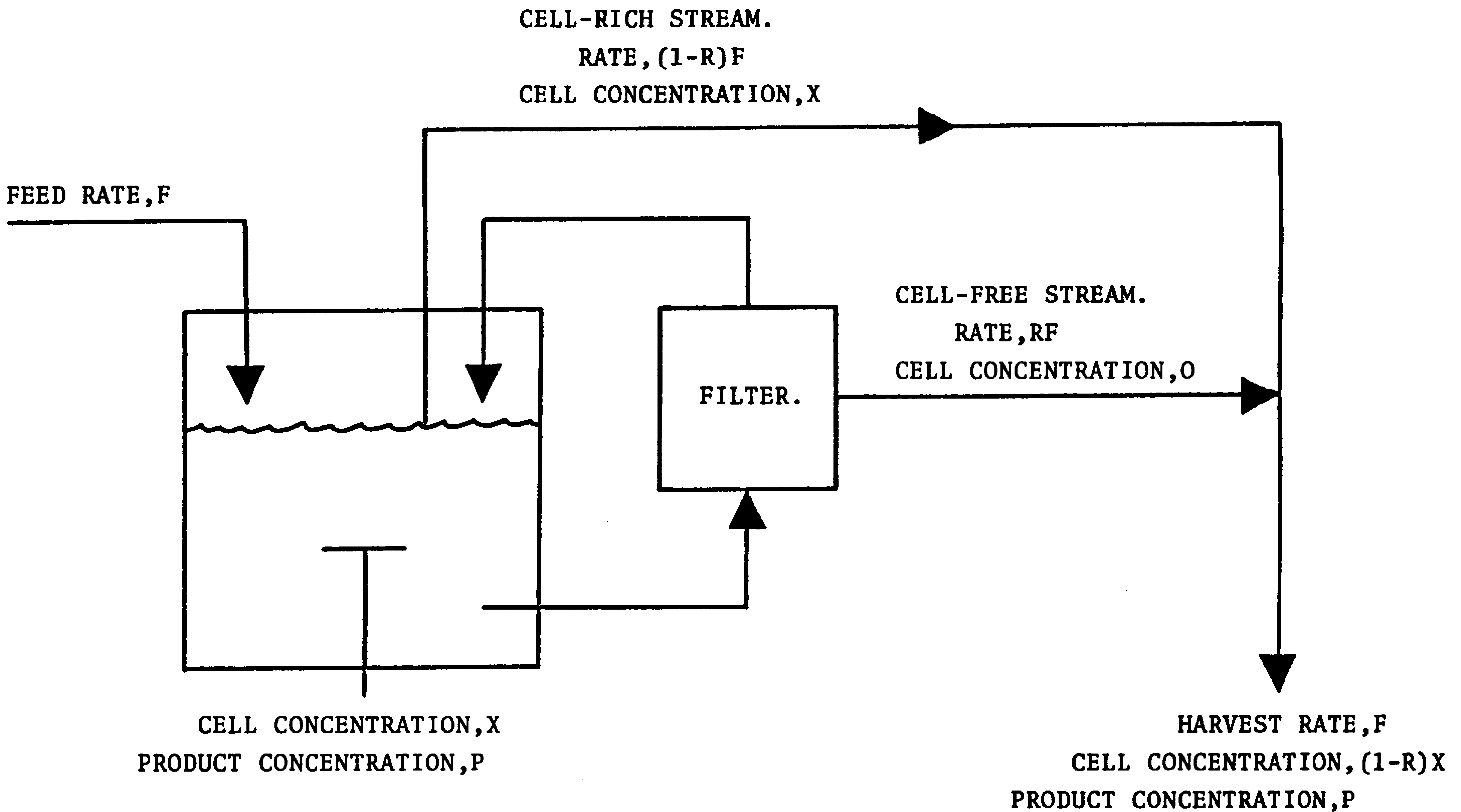


Figure 1.2 Schematic Diagram of a Cell Recycle Fermenter

It follows from equation 1.4 that when R = 1, $\mu = 0$. The biomass production at R=1 is a measure of maintenance energy (Beyeler, Rogers & Fiechter, 1984).

(ii) Growth limiting substrate concentration (s)

If the relationship between specific growth rate and growth limiting substrate concentration can be taken as being represented by the Monod equation then:

$$s = K_s \mu (\mu_m - \mu) \qquad \text{Equation 1.5}$$

where K_s is a saturation constant and μ_m is the maximum specific growth rate. At steady state, substituting for μ from equation 1.4, equation 1.5 becomes:

$$\bar{s} = K_s (1-R)D/[\mu_m - (1-R)D] \qquad \text{Equation 1.6}$$

Equation 1.6 predicts that for a given value of D, $\bar{s}$ will be lower in a cell recycle culture than in a conventional chemostat.

(iii) Biomass concentration (x)

The biomass concentration is the product of the substrate consumed and the growth yield, Y. The concentration of biomass in the fermenter is equal to the concentration in the combined outflow streams divided by (1-R). Thus, at steady state:

$$\bar{x} = Y (s_o - \bar{s}) / (1 - R) \qquad \text{Equation 1.7}$$

where s_o is the concentration of growth limiting substrate in the feed stream. When s_o is very much greater than $\bar{s}$, equation 1.7 approximates to:

$$\bar{x} = Ys_o / (1-R) \qquad \text{Equation 1.8}$$

Equation 1.8 may be used to estimate predicted biomass concentrations in the cell recycle fermenter.

(iv) Critical dilution rate (D_{crit})

D_{crit} is defined as the dilution rate at which s becomes equal to s_o and culture washout occurs. Substituting for μ with D from equation 1.4, the Monod equation becomes:

$$D = (\mu_m s/K_s + s) / (1-R) \qquad \text{Equation 1.9}$$

D_{crit} is given by substituting s for s_o in equation 1.9:

$$D_{crit} = (\mu_m s_o / K_s + s_o) / (1-R) \qquad \text{Equation 1.10}$$

When s_o is very much greater than K_s, equation 1.10 approximates to:

$$D_{crit} = \mu_m / (1-R) \qquad \text{Equation 1.11}$$

Equation 1.11 states that for any value of R above zero, D_{crit} will exceed μ_m. At values of R approaching unity, the culture may be operated at dilution rates considerably in excess of μ_m without approaching D_{crit}; and when R = 1, D_{crit} equals infinity and the culture cannot be washed out.

(v) Volumetric cell growth rate ($\mu\bar{x}$)

$\mu\bar{x}$ is given by multiplying equation 1.7 (giving $\bar{x}$) by equation 1.4 (giving μ):

$$\mu\bar{x} = DY(s_o - s) \qquad \text{Equation 1.12}$$

The magnitude of $\mu\bar{x}$ for a given value of R is given by substituting for s in equation 1.12 by equation 1.6:

$$\mu\bar{x} = DY(s_o - [K_s(1-R)D/\mu_m - (1-R)D]) \qquad \text{Equation 1.13}$$

Equation 1.13 states that at values of D below μ_m, the increase in $\mu\bar{x}$ achievable by cell recycling is marginal, being due to the reduction in the value of s, the degree of which is determined by the value of K_s. However if D exceeds μ_m, as allowed for by equation 1.11, then $\mu\bar{x}$ may be greatly increased beyond that achievable in a conventional

chemostat.

Throughout this theoretical treatment, the primary assumption made is that the Monod relationship applies. In addition, the following secondary assumptions are made: (a) the retention time of culture in the external recycling loop is negligible; (b) there is no loss of culture viability in the recycle loop; (c) growth limitation is the same in the recycle fermenter as in a chemostat; (d) maintenance effects are negligible; and (e) product limitation of biomass production is negligible.

In summary, the theoretical treatment of the Bull & Young (1981) model of CRF predicts: (a) reduced specific growth rate (equation 1.4); (b) reduced growth limiting substrate concentration (equation 1.6); (c) increased biomass concentration (equation 1.8); (d) increased critical dilution rate (equation 1.11); and (e) increased volumetric cell production rate, particularly at dilution rates in excess of μ_m (equation 1.13). The potential benefit inferred from this model is that any metabolite, the rate of production of which is a strong positive function of biomass concentration, rate of biomass production or both, should be produced faster in a cell recycle fermenter than in a conventional batch or continuous culture, all other factors being equal. Other potential benefits of CRF, outlined recently by Bull (1982), Hamer (1982), and Rogers *et al.* (1982), arise from the extreme resistance of the system to washout and may be listed as follows: (a) transformation of, or growth in the presence of, inhibitory materials may be achieved; (b) unavoidably dilute substrate streams may be utilized at high flow rates (e.g. brewing and waste water treatment); and (c) growth inhibitory products, such as ethanol, may be produced in high concentration but rapidly washed from the culture. Perhaps the most important potential benefit of CRF is that

at high recycle ratios a large proportion of the total fermenter outflow is cell-free thereby reducing costs of downstream processing.

1.2.2 Applications

An examination of reports describing the application of CRF to the intensification of microbial processes reveals a literature which is diverse in approach, procedure and terminology yet comparatively restricted in terms of products formed and substrates and organisms utilized. Since the concept of CRF was first realized experimentally by Pirt & Kurowski (1970), interest in cell recycling has increased steadily. The majority of applications by CRF have been reported in the current decade with interest gradually increasing such that at least four different applications were reported in the first six months of 1986. An overview of reports of CRF applications is presented in Table 1.1.

1.2.2.1 Approaches and Procedures

An examination of the literature outlined in Table 1.1 reveals a wide range of different approaches taken and procedures followed to achieve CRF. A detailed analysis of this breadth of approach is hampered by a lack of common terminology and by inconsistency in the precision with which the procedures are described. However, it is possible to classify the approaches taken and procedures followed into four broad groups.

(A) Chemostats with partial cell recycle

Examples of studies following this approach are those by Pirt & Kurowski (1970), Bull & Young (1981), Limtong *et al.* (1984), Damiano *et al.* (1985), Kuriyama *et al.* (1985) and Pierrot *et al.* (1986). These six studies are characterized by the maintenance of defined dilution rates, statements of defined degrees of partial cell recycle, and by

Reference	Organism	Substrate	Product	Method of Concentration	Degree of recycle varied?
Pirt & Kurowski (1970)	*Saccharomyces cerevisiae*	Glucose	Biomass	Internal filter on outflow	Yes
Cysewski & Wilke (1977)	*Saccharomyces cerevisiae*	Glucose	Ethanol	Settling tank & vacuum ferment-ation	Yes
Rogers, Lee & Tribe (1980)	*Zymomonas mobilis*	Glucose	Ethanol	Flat membrane ultrafiltration	No
Lee *et al.* (1980)	*Zymomonas mobilis*	Glucose	Ethanol	Flat membrane ultrafiltration	No
Bull & Young (1981)	*Gluconobacter oxydans*	Sorbitol	Sorbose	Hollow fibre ultrafiltration	Yes
Bull & Young (1981)	*Serratia marcesens*	Glucose	2-keto-gluconic acid	Hollow fibre ultrafiltration	Yes
Nishizawa *et al.* (1983)	*Saccharomyces cerevisiae*	Glucose	Ethanol	Hollow fibre ultrafiltration	No
Cheryan & Mehaia (1983)	*Kluyveromyces fragilis*	Lactose (whey waste)	Ethanol	Hollow fibre ultrafiltration	No
Charley *et al.* (1983)	*Zymomonas mobilis*	Glucose	Ethanol	Hollow fibre ultrafiltration	No
Vick Roy *et al.* (1983)	*Lactobacillus delbreuckii*	Glucose	Lactic Acid	Flat membrane ultrafiltration	No
Cheryan & Mehaia (1984)	*Saccharomyces cerevisiae*	Glucose	Ethanol	Hollow fibre ultrafiltration	Yes
Limtong *et al.* (1984)	*Saccharomyces cerevisiae*	Glucose	Ethanol	Settling tank	Yes
Janssens, Bernard & Bailey (1984)	*Kluyveromyces fragilis*	Lactose	Ethanol	Flat membrane ultrafiltration	No
Kuriyama *et al.* (1985)	*Saccharomyces cerevisiae*	Glucose (simulated sugar cane juice)	Ethanol	Settling tank	No
Damiano *et al.* (1985)	*Saccharomyces cerevisiae*	Glucose	Ethanol	Hollow fibre ultrafiltration	Yes
Afschar *et al.* (1985)	*Clostridium acetobutylicum*	Glucose	Acetone-butanol	Hollow fibre ultrafiltration	Yes
Holst *et al.* (1985)	*Streptococcus lactis*	Galactose	Biomass (superoxide dismutase)	Hollow fibre ultrafiltration	No
Schlote & Gottschalk (1986)	*Clostridium acetobutylicum*	Glucose	Acetone-butanol	Flat membrane ultrafiltration	No
Enziminger & Asenjo (1986)	*Saccharomyces lipolytica*	Glucose	Citric acid	Hollow fibre ultrafiltration	No
Pierrot, Fick & Engasser (1986)	*Clostridium acetobutylicum*	Glucose	Acetone-butanol	Hollow fibre ultrafiltration	No
Mehaia & Cheryan (1986)	*Lactobacillus bulgaricus*	Lactose (whey permeate)	Lactic acid	Hollow fibre ultrafiltration	No

Table 1.1 Survey of Reports of Cell Recycle Fermentation

biomass concentrations which were allowed to respond to the regime of dilution rate and recycle imposed. Even within this broad group, differences in terminology and procedure are apparent. In five of the six studies, the degree of recycle imposed was defined and expressed in a different way. Bull & Young (1981), Limtong *et al.* (1984) and Damiano *et al.* (1985) each described the recycle imposed in terms of a 'recycle ratio' but these ratios were each calculated differently and were not directly comparable with each other. Three different procedures for establishing and controlling the degree of partial cell recycle were used. Pirt & Kurowski (1970) controlled the rate of cell-free flow; Bull & Young (1981), Damiano *et al.* (1985), and Pierrot *et al.* (1986) set the cell-free flow to a constant rate and established the degree of recycle by controlling the rate of cell-rich flow; Limtong *et al.* (1984) and Kuriyama *et al.* (1985), used settler tanks to concentrate flocculating yeast and established the degree of recycle by controlling the extent of the separation taking place in the settler. The studies of Pirt & Kurowski (1970), Bull & Young (1981) and Limtong *et al.* (1984) are distinguished as being the only studies within the literature outlined in Table 1.1 in which experimental data were compared with those predicted by stated theory. The general lack of comparison of experiment with theory is one of the criticisms which may be made of the CRF literature as a whole. The study of Damiano *et al.* (1985) addressed the physiological considerations of an ethanol-producing fermentation under cell recycle conditions. Physiological aspects of CRF are discussed in Section 1.2.3 of this thesis.

(B) Chemostats with total cell recycle

Examples of studies following this approach are those by Vick Roy *et al.* (1983) and Holst *et al.* (1985). In both studies the dilution rate was defined and it was stated that recycle was complete and that

no cells left the fermenter. The biomass concentration was allowed to respond to the dilution rate and cell recycle imposed.

(C) Recycle fermenters with constant biomass; 'Recycle turbidostats'.

Examples of studies following this approach are those by Nishizawa *et al.* (1983), Cheryan & Mehaia (1984), Afschar *et al.* (1985) and Mehaia & Cheryan (1986). These four studies were characterized by the maintenance of defined biomass concentrations. The control of biomass concentration was not achieved by manipulation of the growth medium feed rate as is a conventional turbidostat, but by manipulation of either the initial inoculum size or the rate of removal of cells. Nishizawa *et al.* (1983) established particular concentrations of biomass by controlling the initial inoculum size and performing total recycle of non-growing cells in a nitrogen deficient medium. Cheryan & Mehaia (1984) operated a fermenter in which the substrate feed and cell-free flow rates were equal and periodically adjusted the biomass concentration to defined values by controlled bleeding of cells from the recycle loop. Afschar *et al.* (1985) were the only authors to describe their fermenter in terms of turbidostatic control, with control of the biomass concentration being achieved via continuous photometric measurement of cell concentration and the regulated bleeding of culture. No such continuous measurement of cell concentration was described by Cheryan & Mehaia (1984).

(D) Other approaches

This group of approaches to CRF consists of those which cannot be classified into any of the other three groups and includes the studies of Cysewski & Wilke (1977), Lee *et al.* (1980) and Charley *et al.* (1983). Cysewski & Wilke (1977) controlled the concentration of cells by the combination of an external settling tank and vacuum fermentation. This latter feature provided the benefit of relief from

product (ethanol) inhibition. Lee *et al.* (1980) applied cell recycle to fed-batch cultures. Neither the biomass concentration nor the degree of recycle were set to defined values and the rate of flow of cell-free filtrate was allowed to fall continuously throughout the course of the cultures. Charley *et al.* (1983) applied cell recycle to a twin-stage fermenter in order to produce large concentrations of cells in the second stage vessel.

A review of the methods used for concentrating cells for CRF as outlined in Table 1.1 emphasises the dominance of cross-flow filtration as the method of choice. Apart from the study of Pirt & Kurowski (1970), in which the restrictions of internal cell retention were described, all the applications of CRF have involved external recycle loops. Those studies which did not use cross-flow filtration to concentrate cells (Cysewski & Wilke, 1977; Limtong *et al.*, 1984; Kuriyama *et al.*, 1985) used settling tanks for the separation of cultures of *Saccharomyces cerevisiae*, an approach which proved especially effective with selected flocculating strains used in the latter two studies. Amongst the studies in which cross-flow filtration was used to concentrate cells, hollow fibre membrane systems have been more widely used than flat membrane systems. The hollow fibre filters used included novel laboratory-made units (e.g. Bull & Young, 1981) and commercially produced units (e.g. Amicon HIP100-20; Charley *et al.* 1983). The scale of the units has ranged from that used by Holst *et al.* (1985), 220µm ID; to the twin in-series filters used by Damiano *et al.* (1985), each filter being 2.5cm ID and 1.8m in length.

1.2.2.2 Organisms and Substrates

Table 1.1 shows that the range of organisms and substrates utilized in cell recycle fermentations has been somewhat restricted. The majority of the applications of CRF have been to anaerobic

fermentations performed by obligately or facultatively anaerobic organisms. Exceptions to this trend were the studies of Bull & Young (1981) and Enziminger & Asenjo (1986). In the study of Damiano *et al.* (1985), the fermenter was well aerated but the authors reported a shift to anaerobiosis with high recycle rates. The bacterium *Zymomonas mobilis* and the yeasts *Saccharomyces cerevisiae* and *Klugveromyces fragilis* have each been used to produce ethanol in cell recycle fermentations. Cell recycling has also been applied to the culture of another yeast, *Saccharomycopsis lipolytica*, for the production of citric acid (Enziminger & Asenjo, 1986). Other bacteria which have been utilized in cell recycling fermentations are *Clostridium acetobutylicum* and the lactic acid bacteria *Lactobacillus (L.)delbreuckii*, *L. bulgaricus* and *Streptococcus lactis*.

The substrate of choice for the majority of CRF applications has been glucose contained in salts media, often supplemented with complex nitrogen sources. Interestingly, a number of authors (Cheryan & Mehaia, 1983; Janssens *et al.*, 1984; Kuriyama *et al.*, 1984; Mehaia & Cheryan, 1986) have applied CRF to processes utilizing actual or simulated waste materials as the feedstock. Cheryan & Mehaia (1983) and Janssens *et al.* (1984) used CRF to ferment lactose in whey waste to ethanol; Mehaia & Cheryan (1986) used a similar feedstock for a lactic acid producing culture. Kuriyama *et al.* (1985) used glucose contained in a simulated waste sugar cane juice as the feedstock for ethanol production by CRF.

1.2.2.3 Products and Productivities

The comparatively restricted range of substrates and organisms utilized for cell recycle fermentations is reflected in a similar lack of diversity in the products. The majority of the products which have been investigated are primary metabolites classified as high volume, low/intermediate value by Bull *et al.* (1982).

Exceptions to this trend include the studies of Bull & Young (1981) and Holst *et al.* (1985). The latter study was especially interesting as an example of the application of CRF to the production of a non-bulk product of high added value, the intracellular enzyme superoxide dismutase.CRF has most regularly been applied to the production of ethanol but interest has been shown in applying the technique to the production of lactic acid and butanol/acetone, products in which the fermentative route of production competes with chemical synthesis (Vick Roy *et al.*, 1983 ;Pierrot *et al.* 1986).

There have been no reports of CRF being applied to processes on a pilot plant or production scale **except for waste water treatment.** Nevertheless, the improvements in fermenter performance which have been reported on a laboratory scale have been impressive. As stated in Section 1.2.1, the theory of CRF predicts that the greatest increases in productivity result from the operation of fermenters at high dilution rates and especially in excess of the maximum specific growth rate of the organism under culture. This prediction has been confirmed in practice as the studies which operated at high dilution rates also reported the greatest increases in fermenter productivity.

As the majority of applications of CRF have been to ethanol production, a body of literature exists describing improvements in performance of ethanol producing fermentations under cell recycling conditions. In a review of this literature, Cheryan & Mehaia (1984) compared the ethanol production from batch, conventional continuous, and immobilized microbial cell cultures with those from CRF cultures. This latter review included data obtained from *Zymomonas mobilis*, *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*, each grown at a range of feed concentrations of glucose or lactose. The conclusion drawn was that although CRF did not significantly increase ethanol concentrations, the volumetric productivity of high dilution rate

($2.0h^{-1}$) CRF cultures was far greater than that of other systems for each organism and feed concentration quoted. On average, the volumetric productivity of the CRF cultures was 30 times greater than batch culture, 14 times greater than conventional continuous culture, and 3 times greater than cultures of immobilized cells. Cysewski & Wilke (1977) and Charley *et al.* (1983), in studies not reported by Cheryan & Mehaia (1984), presented data showing significantly increased ethanol concentrations (> $110gL^{-1}$) from CRF linked, in the former study to vacuum fermentation and, in the latter study to a twin-stage fermentation. Despite the high concentrations of ethanol produced in the latter two studies, the volumetric productivities were not as great as those reported by Cheryan & Mehaia (1984) due to the relatively low dilution rates used.

The production of lactic acid in different fermenter systems has been reviewed by Vick Roy *et al.* (1983) and Mehaia & Cheryan (1986), and is discussed in Section 1.4.3. of the present study. In brief, both the latter authors reported volumetric productivities of lactic acid from high dilution rate ($\cong 2h^{-1}$) CRF to be 10 to 40 times greater than from previously reported batch, continuous and immobilized cell cultures. Butanol/acetone production has not been widely evaluated by CRF and dilution rates analysed have been moderate ($< 0.7h^{-1}$). However, Schlote & Gottschalk (1986) reported a four-fold increase in productivity of butanol whilst operating CRF at recycle ratios between 0.8 and 0.9 (estimated from latter report) at a dilution rate of $0.1h^{-1}$.

In conclusion, it may be stated that the data presented in the literature strongly suggest that, for a comparatively limited range of products, substrates, and organisms, on a laboratory scale, the potential of cell recycle fermentation as an approach to microbial process intensification has been confirmedin practice.

1.2.3 Incentive for a Physiological Study

Microbial physiology has been described as the relationship between the metabolic capability of a microorganism and the environment within which it exists (Bull, 1982). Phenotypic changes in physiology in response to changes in environment are recognised as widespread phenomena within microbial cultures. Virtually every aspect of microbial structure and function has been shown to be variable in response to changes in such environmental parameters as nutrient status, temperature, pH, redox potential and growth rate. Amongst the variations reported in response to such changes are alterations in substrate uptake affinities, cell wall and membrane structure and permeability, the regulation of carbon source metabolism and cofactor recycling, the efficiency of coupling of ATP generation to anabolic processes, the excretion of extracellular macromolecules, and the content of intracellular amino acid pools. Continuous culture techniques have been instrumental in the illumination of the relationship between microorganism and environment and much of this work has been reviewed by Dean, Pirt & Tempest, (1972); Dean, *et al.*, (1976); Dean, Ellwood & Evans (1984) and Neijssel & Tempest, (1979). In a biotechnological context, changes in physiology in response to changes in microbial environment may be a potential source of exploitation in the fine tuning of process parameters to produce the desired physiological result. Equally, such variability may become a source of difficulty. Alterations of process parameters during, for instance, scaling-up of laboratory experiments to pilot or production scale may result in the depletion or total loss of the desired activity or product due to the alteration of regulatory or excretory mechanisms promoted by changes in the growth environment (Bull, 1982).

In Section 1.2.2.3 of the present study it was suggested that, on a laboratory scale, cell recycle fermentation has displayed promise

as an approach to the intensification of commercial microbial processes. However, the growth environment within a CRF is likely to be very different from that within a conventional batch or continuous culture. In addition, further changes in growth environment may result from alteration in the degree of recycle imposed. Theoretical treatments of CRF may be used to predict some of the changes in growth environment which may be experienced by microorganisms under cell recycle conditions.

The most striking effect of CRF is to substantially increase the concentration of biomass present in a culture. The magnitude of this increase is described by Equation 1.7 (Section 1.2.1). It is intuitive that unless the concentration of the growth limiting substrate in the feed stream is increased by the same proportion as that by which the biomass has been concentrated, the amount of substrate available per cell must decrease as the biomass accumulates. In the model of Bull & Young (1981), the concentration of the growth limiting substrate decreases under recycle conditions according to Equation 1.6. Thus, one environmental change brought about by the recycling of cells is a reduction in the nutrient status of the culture. Furthermore, it follows from Equation 1.7 that the concentrating effect experienced by the culture is equal to $^{1}/(1-R)$ (where R is the recycle ratio) and thus as recycle approaches totality (i.e. as R tends to 1), very small changes in the recycle ratio result in disproportionately large changes in the concentrating effect and hence in the nutrient status of the culture. In addition to enhancing biomass and reducing substrate concentrations, Bull & Young's theoretical treatment of CRF also predicts that the growth rate should be reduced in such cultures. As described by Equation 1.4, the growth rate tends to a value equal to the dilution rate multiplied by the factor (1-R). Thus at degrees of recycle approaching totality, the

growth rate tends to very low values and tends to zero under total cell recycle.

In theory therefore, cell recycle fermentations are likely to produce growth environments very different from those produced by conventional cultures. Specifically, the CRF environment will be of extremely low nutrient status and will impose very low growth rates. In addition, large changes in the growth environment may be produced by small changes in the degree of recycle imposed, especially when the degree of recycle is near totality. Given the capacity of microorganisms for phenotypic change of structure and function in response to the growth environment and the importance of physiological stability to commercial microbial processes, it is pertinent to investigate the effect of the particular growth environment produced under CRF on the physiology of microbial product forming processes. A review of the applications of CRF shows that such physiological considerations have not been addressed in a systematic or comprehensive manner. The following criticisms may be made of the literature in this regard:

(a) Whilst most reports have quoted yield coefficients and specific rates of product formation and/or sugar consumption, very little mention has been made of the significance of these terms as indicators of the underlying metabolism. In many cases yield and specific rate values have not been compared with those obtained from batch and chemostat experiments using the same organism and growth conditions.

(b) Very few studies have compared product profiles in CRF and conventional culture. Previous studies of a wide range of organisms have shown product profiles to be powerful indicators of physiological state (e.g. DeVries *et al.*, 1970; Neijssel & Tempest, 1975; Tempest & Neijssel, 1978).

(c) No studies have been made of the activity or stability of

enzymes of importance to product formation in microbial cells under recycle conditions.

(d) No studies have been made of the chemical composition of microbial cells under recycle conditions.

Of the studies listed in Table 1.1 (Section 1.2.2), two are worthy of comment with regard to physiological study. Damiano *et al.* (1985) addressed the physiological considerations of ethanol production by recycled *Saccharomyces cerevisiae* cells. The latter study covered aspects of product inhibition, substrate utilization and apparent maintenance requirements under different recycle ratios. However, despite the thorough approach adopted by the latter authors, a question may remain as to the validity of some of the mathematical treatments used. It was stated that the concentration of glucose in the feed stream was varied with the recycle ratio so that the concentration of the substrate would not become "too low to be limiting"; yet subsequent treatments of data (calculation of true growth yields, ATP yields, maintenance requirement) were made on the basis that glucose was the growth limiting substrate. Schlote & Gottschalk (1986) applied cell recycle to cultures of *Clostridium acetobutylicum*. These authors reported changes in product profile and substrate utilization in cell recycle culture compared with chemostat culture and reported further changes in product profile on changing the dilution rate of the cell recycle culture. Unfortunately, they did not discuss the possible physiological implications of their observations and, surprisingly, concluded that no change in activity of the cells was observed under recycle conditions.

In conclusion, there is an incentive for the study of the physiology of microbial product formation under cell recycle conditions, for such an understanding is essential if microbial processes are to be optimized as CRF is developed towards a production scale.

1.3 Cross-Flow Filtration

As described in Section 1.2.2.1, cross-flow filtration has been the method of choice for the separation of non-flocculating microbial cultures in previous studies of cell recycle fermentation. This Section outlines some of the principles of cross-flow filtration and the apparatus which has been developed for the operation of the process. The potential and realized applications of cross-flow filtration within the field of biotechnology have been recently reviewed by Tanny, Mirelman & Pistole, (1980); Short (1983); Kroner *et al.* (1984); Gabler (1984); Tutunjian (1984, 1985); and Strathman (1985). These applications include the harvesting and washing of cells, the harvesting of cell debris following lysis, and the concentration of recovered products. The present discussion focuses on the application of the technique to the separation of microbial cultures for use in cell recycle fermentation systems.

1.3.1 Principles

Cross-flow filtration is a relatively new approach to the separation of suspensions and solutions which has been developed to overcome the restrictions of conventional 'dead-end' filtration. In dead-end filtration, the direction of suspension flow is perpendicular to the filter medium which results in the accumulation of retained material (filter cake) at the filter surface. The cake acts as a secondary filter which has properties that are independent of those of the filter medium and leads to a reduction in filtrate flow. In cross-flow filtration, the flow of suspension is parallel to the filter medium and the retained material is carried along with the flow. The parallel flow greatly reduces the accumulation of retained material at the filter surface, resulting in higher filtrate

flow rates and overall throughput, with more efficient harvesting of retained material (Gabler, 1984).

Several theoretical models exist for characterizing the performance of a cross-flow filtration operation including models of gel polarization, fluid dynamics, and dynamic cake formation. It has been claimed that these models do not fit experimental results very closely and that optimum conditions for cross-flow filtration are usually determined empirically (Kroner *et al.*, 1984). Despite these considerations, the gel polarization model has found a measure of acceptance as a description of cross-flow filtration processes (Gabler, 1984; Tutunjian, 1984, 1985). This latter model may be outlined as follows (Tutunjian, 1984, 1985). The rate of filtrate flux (filtrate flow per unit area) is largely determined by the development of a solute gradient with the maximum solute concentration at the filter surface. This phenomenon is known as gel polarization and the layer of maximum solute concentration is called the gel layer. Such a concentration gradient is shown diagramatically in Figure 1.3. During cross-flow filtration, gel polarization occurs to a certain degree at all but the most dilute solute concentrations and, in most operating conditions, flux rates are determined by gel polarization. Under such conditions the flux rate (J) is defined as

$$J = K \ln \frac{Cg}{Cb} \qquad \text{Equation 1.14}$$

where Cg is the solute concentration in the gel layer, Cb is the bulk solute concentration (see Figure 1.3), and K is a mass transfer coefficient. K has been shown to be a function of the nature of the flow (i.e. laminar or turbulent), the velocity of the suspension across the filter surface (V), and the viscosity of the suspension. K has been found to increase with the square root of V under laminar flow conditions, and to increase linearly with V under turbulent flow

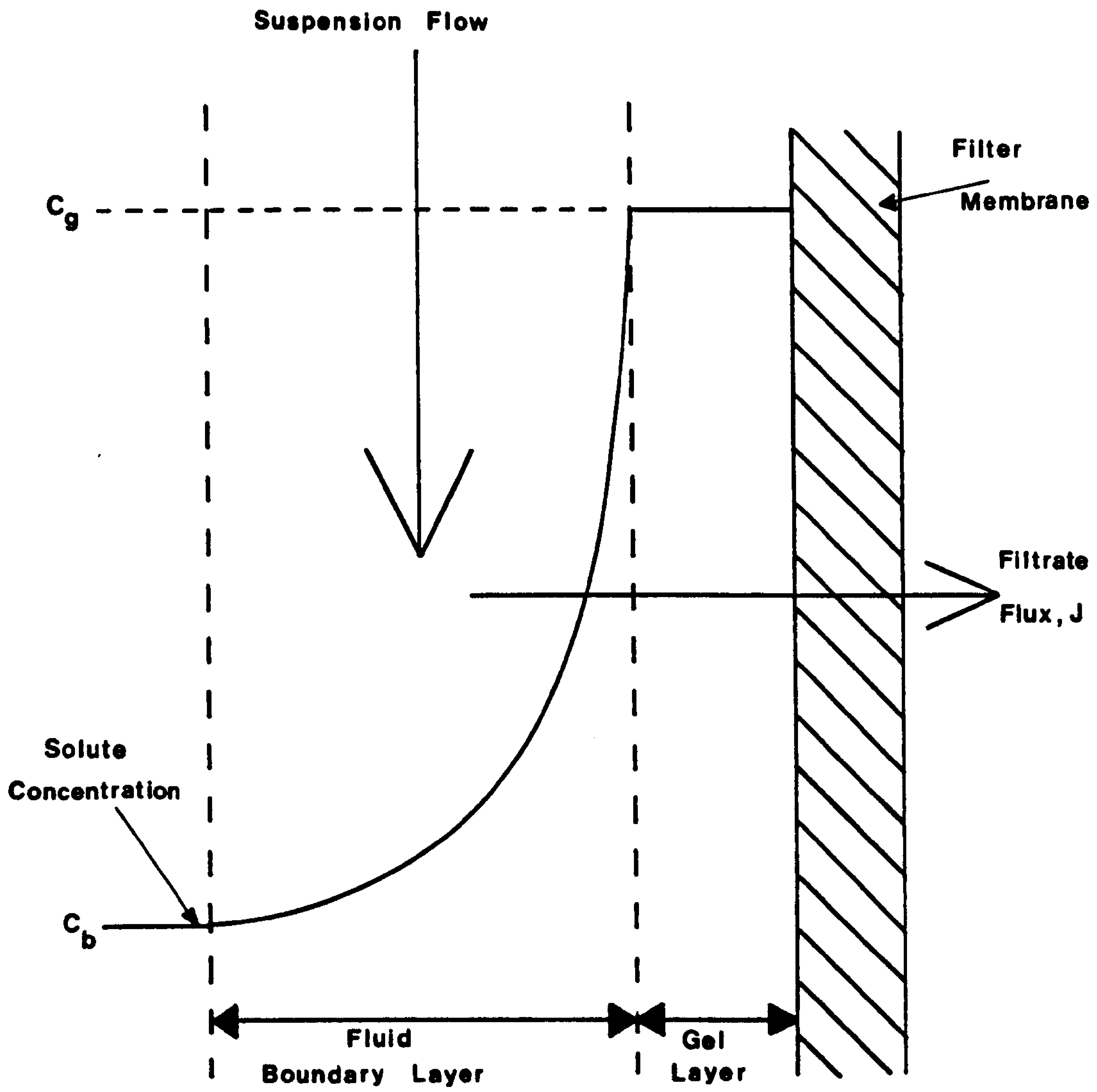


Figure 1.3 Solute concentration gradient during gel polarization.

conditions. Under laminar flow conditions, the flux during the separation of cell and particle suspensions has been found to be even more dependent on V than during the processing of solutes of molecular size. This has been attributed to the tubular pinch effect (Segre & Silberberg, 1961), which enhances the movement of particles away from the fluid boundary layer and reduces polarization. The rate of particle movement away from the wall is dependent on the square of the velocity of circulation. As a result of the tubular pinch effect, flux remains relatively constant up to moderately high cell densities, at which point culture viscosity increases sharply and becomes a major factor in determining filtrate flux. As viscosity increases, V must be reduced if operating pressure is not to exceed the maximum allowed for the system. This reduction in V causes the flux to fall due to the close relationship between V and K stated above. Furthermore, since most highly concentrated cell suspensions exhibit pseudoplastic behaviour, reduction in V will give rise to a further increase in viscosity. The overall result of high culture viscosity is thus a substantial fall in filtrate flux.

Another key factor in determining the flux is the transmembrane pressure (ΔPTM) which is defined by the pressure differential along the filter and the back pressure of the filtrate. A diagram showing fluid pressures during cross-flow filtration is presented in Figure 1.4. The pressure differential (ΔP) along the filter surface is defined as

$$\Delta P = P_i - P_o \qquad \text{Equation 1.15}$$

and the average ΔPTM as

$$\Delta PTM = P_i - (P_o - P_f)/_2 \qquad \text{Equation 1.16}$$

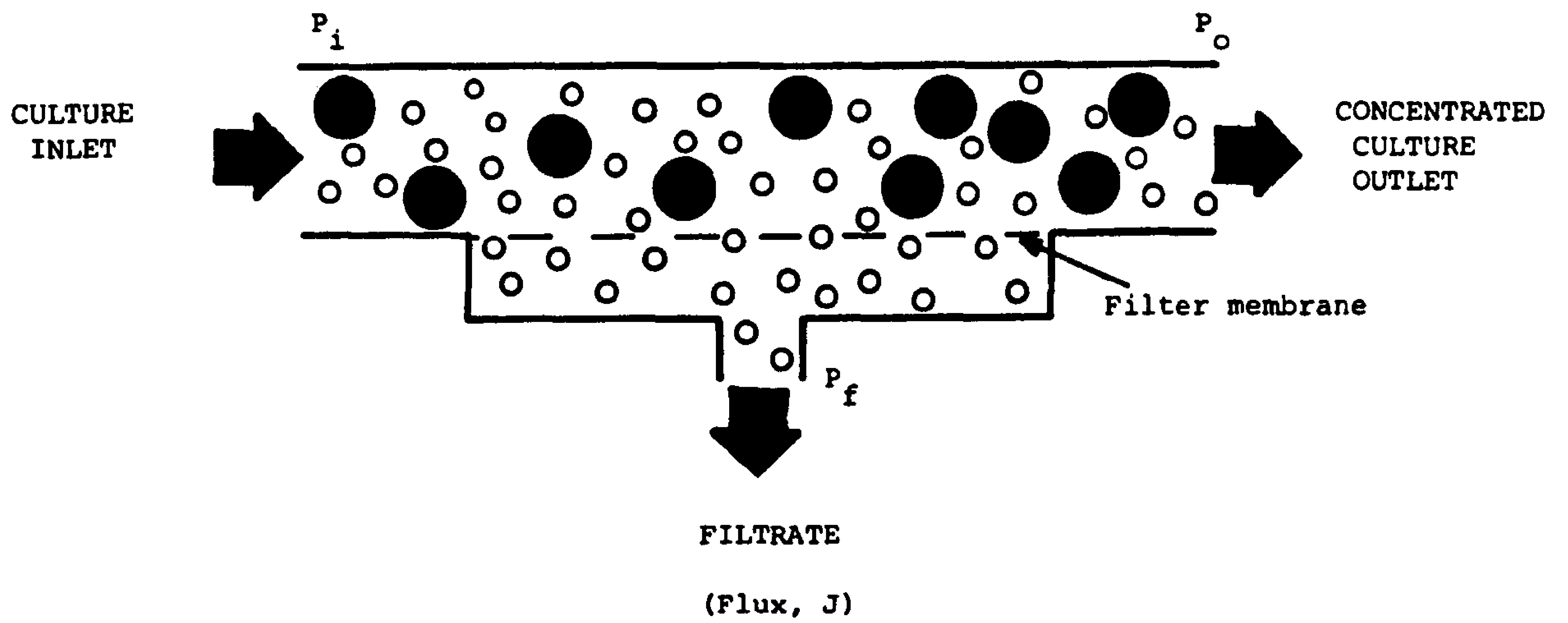


Figure 1.4 Cross-flow filtration pressures.

The filtrate flux (J) is determined by ΔPTM as follows

$$J = \frac{\Delta PTM}{Rm + Rg} \qquad \text{Equation 1.17}$$

where Rm is the hydraulic resistance of the filter membrane (considered to be constant except where filter compaction occurs at very high pressures); and Rg is the hydraulic resistance of the gel layer, determined by the degree of gel polarization. It follows from Equation 1.17 that so long as Rg remains constant, J increases linearly with ΔPTM. However, high ΔPTM favours the formation of a gel layer thus increasing Rg and the resistance to flow. Thus, above a certain value of ΔPTM, further increases in pressure do not increase J and the circulation velocity must be increased to reduce polarization.

1.3.2 Filtration Equipment

Two types of filter membrane have been used for the cross-flow filtration of microbial cultures; microfiltration or microporous (MF) membranes, and ultrafiltration (UF) membranes. The two types of membrane are similar in that they both separate particles and cells and the basis of size exclusion and thus all retained particles are separated with the same efficiency and throughput (Strathman, 1985). Filter membranes are classified as being either MF or UF primarily on the basis ofpore size. The pore sizes of MF membranes are of the order of 0.1 to 10μm, whilst those of UF membranes are of the order of 1 to 50nm in size. The pore size of a UF membrane is usually described in terms of the maximum molecular weight of a globular protein which may be retained (Gabler, 1984). UF membranes are commercially available with nominal molecular weight cutoff (MWCO) values in the range 5×10^2 to 3×10^5 and thus may be used for the retention of macrosolutes as well as cells and particles.

MF and UF membranes generally differ in structure. Most MF membranes possess an open tortuous structure which is symetrical from top to bottom (isotropic) and made of one continuous interlocking polymer. MF membranes, made in a variety of materials such as polypropylene,cellulose esters, polyvinylidine fluoride, and polycarbonate, are between 100 and 200µm in thickness (Gabler, 1984) and act as depth filters retaining particles within the structure (Strathman, 1985). By contrast, UF membranes are anisotropic in structure. Figures 1.5 and 1.6 show magnified cross-sections of UF membranes in a hollow fibre configuration (Romicon PM100,X 50 and 200 respectively), in which the structure may be seen. The surface of the membrane at which separation takes place (the inner surface of the hollow fibre) is a very thin, smooth layer which carries pores of a size which determines the nominal MWCO of the membrane. The filter surface is supported by a very open structure of polymer with a large proportion of spaces or 'macrovoids'. The outer surface of the membrane is a very thin layer which carries pores which are very much larger than those on the inner membrane surface. Due to the anisotropic structure of the UF membranes, the separation of suspensions by size exclusion occurs solely at the inner membrane surface and particles are not trapped or retained by the membrane. The size of the macrovoids and the pores in the outer layer allows the free flow of permeate away from the inner filter surface. UF membranes are manufactured in a range of materials including polysulfone, cellulosics, fluoropolymers, polyamide and vinyl/acrylic copolymers and are generally hydrophillic and susceptible to organic chemical attack. Fluoropolymer UF membranes are hydrophobic in nature and offer greater organic resistance. Some newer MF membranes (e.g. MP01 membrane, 0.1µm pore; Amicon Ltd., Stonehouse, Glous.) also possess an anisotropic structure and, like UF membranes, do not retain

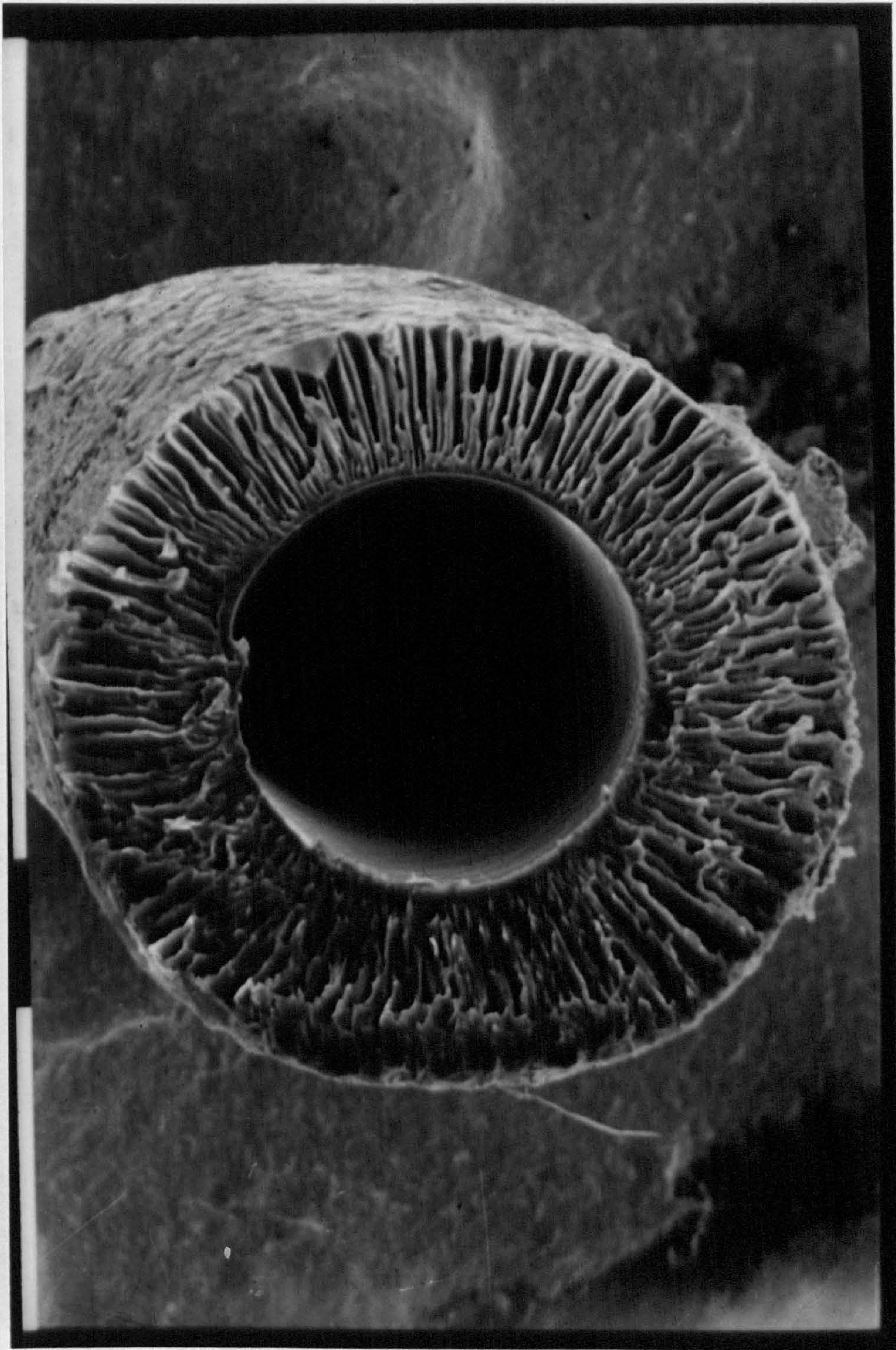


Figure 1.5 Romicon PM100 UF hollow fibre x 50 magnification

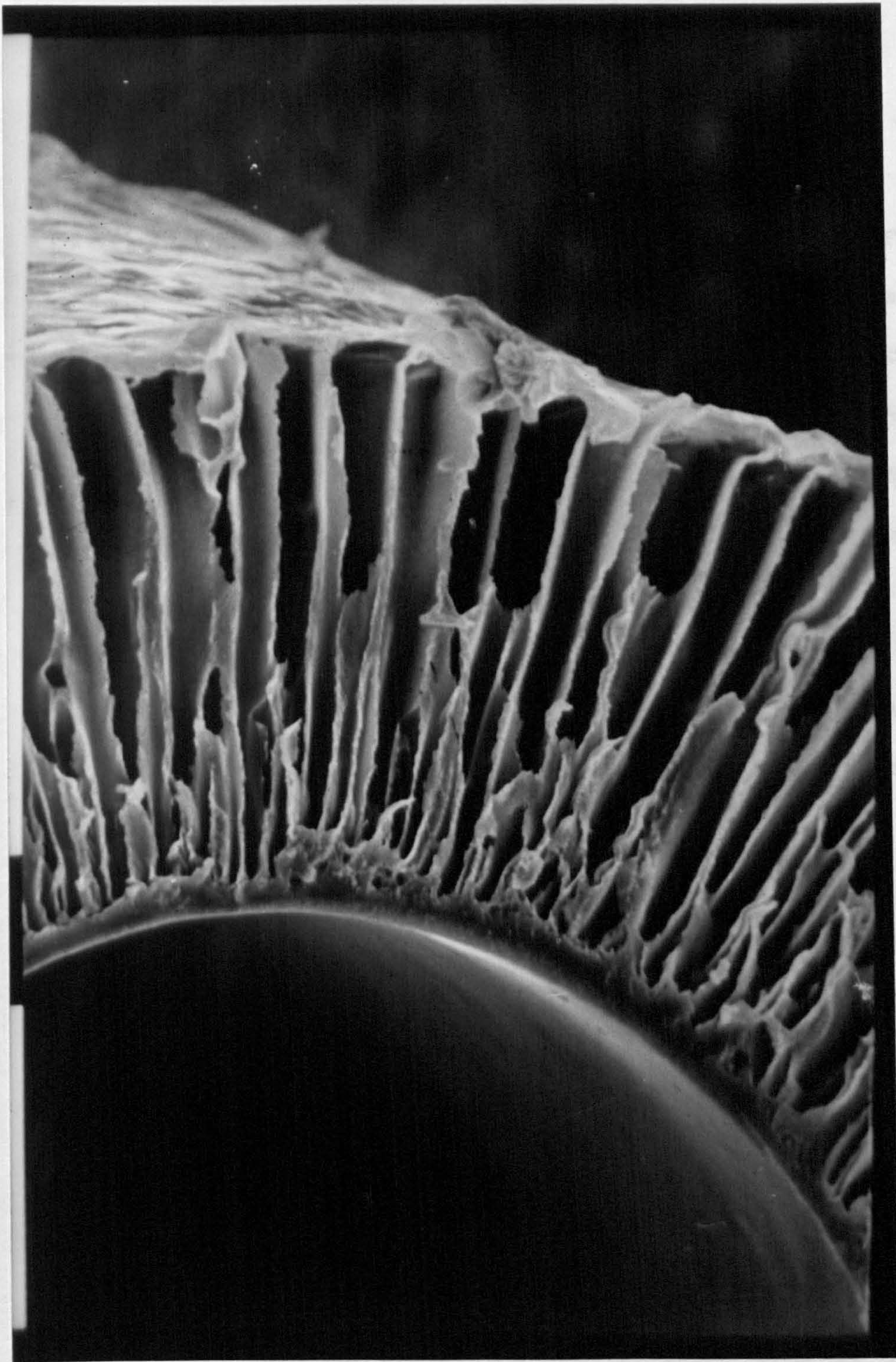


Figure 1.6 Romicon PM100 UF hollow fibre x 200 magnification

particles within the structure of the membrane.

Both MF and UF membranes are commercially available in a variety of configurations and arranged in a variety of modules. The two main configurations are flat sheets and tubes or hollow fibres. Flat sheet membranes have been arranged in different ways to form filtration modules. One arrangement is a simple stack of square or circular membranes, usually separated by spacer screens, held in a frame. Up to $4.6m^2$ filter area may be accommodated in a single unit. Newer high performance systems, designed for large scale cell harvesting, do not have spacer screens and offer less resistance to flow (Gallantree & Docksey, 1983). Flat membranes have also been arranged in modules in which the membranes, separated by spacer screens, are pleated or rolled into a spiral. Pleated and spiral modules form cylindrical units of relatively high filter area density.

The other common configuration for MF and UF membranes is as tubes or hollow fibres. Whole culture is circulated along the lumen of the membrane tubes and filtrate is collected from around the outside of the tubes. The distinction between the two tubular types is largely on the basis of lumen diameter. The lumen diameters of 'tube' filters are of the order of 1 to 3 cm whereas those of 'hollow fibres' are of the order of 0.5 to 1.5mm. The two types of tubular membrane are usually arranged into different filtration modules. The tube filters are larger and rigid in structure and are arranged in series (Strathman, 1985). Hollow fibres are more delicate in structure and are not self supporting. The fibres are arranged in parallel in bundles of 50 to 1000 fibres sealed into a rigid outer casing made of polymer or stainless steel. Falling between the tube and hollow fibre types are anisotropic, ceramic MF tubular filters (e.g. 'Ceraflo' filters; Norton Co., distributed by Amicon Ltd.) These latter filters are of 3mm lumen diameter, and are rigid in structure

(like tube-type filters) but are arranged in bundles of 10 to 200 filters within a stainless steel casing (like hollow fibre-type filters).

Most of the configurations of MF and UF membranes as filter modules are commercially available over a wide range of scale. The smallest modules are flat sheet units with as little as $60cm^2$ filter area ('Minitar'; Millipore (UK) Ltd., Harrow, Middx). Modules offering approximately $0.5m^2$ filter area include hollow fibre UF units (HIP100-20 cartridge; Amicon Ltd.) and stacked flat sheet units ('Pellicon' cassette with single filter; Millipore (U.K.) Ltd.). The smallest pleated sheet module designed for harvesting microbial cells provides approximately $1m^2$ filter area ('Acroflux Capsule', Gelman Sciences Ltd., Brackmills, Northants). Increasing the scale of filter area usually proceeds in two stages: firstly, larger individual moduels are used; and secondly, these modules are arranged in series, parallel or cascade patterns to create very large filter areas. Amongst the larger individual modules are hollow fibre UF units ($5\ m^2$, H35P cartridge, Amicon Ltd.) and flat sheet units with or without spacerscreens ($4.65m^2$, 'Pellicon' cassette or 'Prostak'; Millipore (U.K.) Ltd). Most manufacturers of large filtration modules offer complete pilot and production scale units which carry 2 to 10 modules, complete with pumping, pipework, and automatic cleaning facilities, which may be monitored and controlled by a wide range of instrumentation and control systems. Filtration systems incorporating even larger membrane areas may usually be manufactured to meet particular user requirements.

1.3.3 The Selection of Filter Systems

The choice of a filtration system for use in a cell recycle fermenter is in part governed by the anticipated scale of the

process. If cell recycle fermentation is to be performed on a commercial scale, the selection of a filtration system will be affected by consideration of the volume of cell-free filtrate flow, per unit of total process cost, per unit of total process time, which may be expected from a particular filtration system under particular operational conditions. A filtration system should be selected to maximize the filtrate volume, per unit cost, per unit time, over a complete operational lifetime which includes periods of cell recycling and filter cleaning. Factors which may affect such a selection are listed in Table 1.2. Other factors which may be important in filtration system selection on a commercial scale include the plant space occupied by the filtration system (i.e. filter area/ volume density), and the suitability of the filtration system for connection to on-line utilities, such as on-line steam for sterilization. The considerations listed in Table 1.2 may not be of direct relevance to the selection of filtration systems for laboratory scale studies of cell recycle fermentation. On the laboratory scale, factors such as the ease and flexibility of operation and the cost of filter area per unit of total fermenter volume may be of greater importance in selecting a filter system. However, Tutunjian (1985) identified several key parameters which must remain constant throughout development from laboratory experiments to final production scale if test results are to form accurate projections of the performance of larger scale cross-flow filtration systems. These parameters included: the diameter of hollow fibre lumens and width of flat sheet flow channels; the tangential velocity of culture circulation across the filter membrane; inlet and outlet pressures; culture content and concentration; and overall process time. Therefore, factors important in a final scale filtration system, such as those listed in Table 1.2, should be

1. Maximum filtrate flow per unit filter area under recycling conditions

2. Capital cost per unit filter area

3. Length of operating time under recycling conditions before cleaning is required

4. Cost of operating under recycling conditions, including energy and labour costs

5. Length of down-time whilst cleaning

6. Cost of cleaning, including energy, materials, and labour costs

7. Fraction of maximum filtrate flux restored with each cleaning cycle

8. Total lifetime of filter area under optimal cleaning/recycling regime

Table 1.2 Factors affecting filtrate volume, per unit filtrate cost, per unit process time; includes periods of cell recycling and filter cleaning

considered during the selection of a laboratory scale filtration system in order that the parameters identified by Tutunjian (1985) should remain constant throughout scale-up.

An early consideration in filter system selection is the choice of the type of membrane to be used. Isotropic MF membranes possess an open, tortuous structure which retains cells within the membrane and may cause irreversible blockage. By contrast, anisotropic MF and UF membranes do not retain material within the membrane as separation occurs at the thin outer layer of the membrane surface (Tutunjian, 1984). As a result, anisotropic membranes are generally more resistant to fouling, easier to clean, and may have a longer operational life (Strathman, 1985). It has been suggested that MF membranes are better suited to the separation of microbial cultures than UF membranes since the latter may yield lower filtration rates due to their tight pore structure and low surface porosity compared with MF membranes (Lasky & Grant, 1985). UF membranes are not sterilizable by exposure to on-line steam, unlike ceramic and some polymeric MF membranes, and must be chemically sterilized. This restriction has been claimed to be a disadvantage by manufacturers of steamable MF membranes (Millipore (U.K.) Ltd; Norton Co.). Despite these drawbacks, UF membranes have found wide application in cell recycle fermenters since MF membranes with anisotropic structures, and in hollow fibre configurations, have been comparatively recent additions to the commercial market.

Both MF and UF filter membranes are commercially available as filtration modules in a variety of configurations. Some of the properties of different filtration modules are compared in Table 1.3. One conclusion which may be drawn from such a comparison is that spiral sheet, pleated sheet, and tubular modules are less suitable for use in cell recycle fermenters than are the flat sheet and hollow fibre types. The spiral sheet modules offer high filter area density at moderate

Filtration module	Membrane types	Filter area per module volume	Average filtrate flow per m² filter area	Initial cost	Control of gel formation and fouling	Resistance to pressure	Sterilizable with on-line steam?
Flat sheet (with spacer screens)	MF/UF	High	Moderate	High	Good	Low	No
Flat sheet (without screens)	MF/UF	High	High	High	Very Good	High	Yes (MF only)
Spiral sheet	UF	High	Low	Moderate	Poor	Low	No
Pleated sheet	MF	High	Moderate	Low	Fair	Low	No
Tubular	MF/UF	Low	High	Very High	Very Good	High	Yes (MF only
Hollow Fibres	UF	High	High	Moderate	Good	Low	No
	Ceramic MF	Moderate	High	High	Very Good	High	Yes

Table 1.3 Properties of Commercial Filtration Modules
from Stratham (1985) and manufacturers' literature

cost but are unsuitable for use with high concentrations of particulate material as membrane fouling may be poorly controlled with resultant low filtrate flow rates. Spiral modules are much more suited to the ultrafiltration of non-particulate solutions. Pleated sheet modules offer the highest filter density per unit of initial cost but operational difficulties may make such modules unsuitable for extended periods of cell recycling. Pleated sheet membranes are only available with isotropic MF membranes which are separated by spacer screens, properties which have been shown to create resistance to flow and enhance fouling (Tutunjian, 1984), although to a certain extent the modules may be cleaned by backflushing of filtrate to partially clear the membrane surface. Such modules may be more suitable for the batchwise harvesting of microbial cells over relatively short periods of time, an application in which the modules may form a highly economical alternative to centrifugation (Tanny, Mirelman & Pistole, 1980). Tubular filter modules may be operated at very high volumetric rates of culture circulation due to the large lumen diameter and structural rigidity of the filters. As a result, turbulent flow conditions ensure that polarization and fouling are very well controlled. In addition, the modules are sterilizable by exposure to on-line steam. However, due to the large lumen diameter, tubular filters are of very low filter area density and high cost per module, resulting in very high initial costs per unit of filter area. Another consequence of the large lumen diameter is that the hold-up volume of the filters is great compared with the filter area available (Strathman, 1985). This latter consideration combines with the high cost of filter area to make tubular filter modules unsuitable for all but the largest scale systems.

Flat sheet and hollow fibre modules offer several advantages

over the spiral, pleated and tubular modules already discussed. The former modules are of moderate to high filter area density, yield moderate to high filtrate flows per unit of filter area, and offer good resistance to gel polarization and fouling due to the high rates of shear produced across the membrane surfaces (Tutunjian, 1985; Gabler, 1984). The hold-up volume and initial cost of these modules is much lower than for tubular modules and a wide range of sizes of module are available. Thus flat sheet and hollow fibre filters are quite suitable for use in cell recycle fermenters over a wide range of scale and accurate scaling-up is possible.

In drawing comparisons between flat sheet and hollow fibre modules, it may be most useful to compare, firstly, flat sheet modules using spacer screens against polymeric UF hollow fibre modules, and secondly, flat sheet modules without screens against ceramic MF hollow fibres. Flat sheet modules and polymeric UF hollow fibre modules have both been used in cell recycle fermenters on a laboratory scale (Section 1.2.2.1). The flat sheet modules offer great flexibility in filter area since different numbers of filter membrane cartridges may be held within the same module. The main disadvantages of such modules are that the spacer screens may offer great resistance to highly concentrated cultures and the modules cannot be backflushed for *in situ* cleaning. However, the flat sheet modules can be completely disassembled to gain access to the membrane surfaces for thorough post-run cleaning. By contrast, polymeric UF hollow fibre modules are much less flexible in filter area as extra area can only be added by purchasing an entire new module, partially offsetting the advantage in initial cost possessed by the hollow fibres. UF hollow fibres offer no resistance to the flow of culture and may be cleaned *in situ* by backflushing of filtrate or cleaning solution, although the modules cannot be disassembled for cleaning. Whilst both types of module have

found application in cell recycling, their ultimate usefulness may be limited. Neither type is steamable and thus both must be chemically sterilized. This feature may prove to be merely an inconvenience in some applications of cell recycle fermentation, but may rule out the use of these modules in applications linked to food or pharmaceutical production in which the adulteration of product by sterilizing solutions must be scrupulously avoided. In addition, both module types are only resistant to low pressures, up to approximately 30 psi. This may restrict their usefulness with highly concentrated, and hence highly viscous, cultures since if, as outlined in Section 1.3.1, the upper limit of module pressure is reached due to high viscosity, the recirculation velocity must be reduced with a resultant loss of filter performance.

Flat sheet modules without spacer screens and ceramic MF hollow fibre modules are comparatively recent additions to the market. Both module types combine excellent control of gel polarization and fouling with very high filtrate flow rates. This performance is achieved by operation at very high shear rates and overall pressures, up to 100 to 200 psi. Also, both module types are repeatedly sterilizable by exposure to on-line steam (except when using UF membranes in the flat sheet modules) and thus would be acceptable for all possible applications of cell recycle fermentation. The comparison between the two module types is similar to that between flat sheet screen modules and polymeric UF modules; the flat sheet modules offer greater flexibility of filter area, but the hollow fibre modules may be backflushed for effective cleaning *in situ*. In comparing the MF ceramic hollow fibre modules with those containing polymeric UF hollow fibres, manufacturers of the former modules ('Ceraflo', Norton Co.) claim that, in addition to the operating pressure and sterilization advantages, the ceramic MF modules require backflushing much less

frequently and therefore yield higher overall filtrate flow rates than the UF modules. Although both hollow fibre types share the benefit of anisotropic membrane structure, the MF filters provide greater porosity per unit of filter area than the UF filters which carry much smaller pores. In a comparison of initial cost, the UF hollow fibre modules offer an advantage as the ceramic MF filters are of comparatively low filter area density and high initial cost. Therefore, although the ceramic MF hollow fibre modules may be superior in operation, cost considerations may make their application marginal for small scale systems.

The filter system selected for use in the present study was a single polymeric UF hollow fibre module (H1P100-20 cartridge, Amicon Ltd.). The reasons for this choice were as follows: (a) the hollow fibre membranes were anisotropic; (b) the hollow fibre configuration offered reasonable resistance to gel polarization and fouling combined with potential for backflushing; (c) the module offered a good compromise on initial cost between a cheaper pleated sheet module and a more expensive flat sheet module with spacer screens; (d) flat sheet modules without screens and ceramic MF hollow fibre modules were not available at the time of choice and would in any case have been too expensive; (e) being the smallest in the extensive range of UF hollow fibre modules offered by Amicon Ltd., the module had great potential for extrapolation of performance to larger systems. The extent to which the choice of filtration system was justified in practice is discussed in Chapter 7.

1.4 Lactic Acid Production as a Model System

The model system chosen for the present study was the production of lactic acid by a homofermentative bacterium *Lactobacillus delbreuckii*. Lactic acid production was a useful model system since: (a) Lactic acid is a traditional fermentation product for which a commercial demand still exists, and for which the fermentative route of production competes with chemical synthesis; (b) Lactic acid production has been well characterized in conventional cultures; and (c) several novel approaches have been taken to the fermentative production of lactic acid including dialysis, electrodialysis, immobilization, and cell recycle cultures.

1.4.1 Lactic Acid as an Industrial Fermentation Product

The fermentation of sugar to lactic acid is a process rooted in antiquity. Since earliest times man has used lactic acid formation as a means of preserving milk as cheese and yoghurt, and preserving vegetables as pickles and sauerkraut (Demain & Solomon, 1981). In 1780 the Swedish chemist Scheele isolated lactic acid from sour milk and in 1847 Bloudeau identified lactic acid as the final product of a microbial fermentation (Buchta, 1983). The microbiology of fermented lactic acid-containing foods and beverages has been comprehensively discussed in Carr, Cutting & Whiting (1975) and Steinkraus (1983). Whilst such indigenous lactic acid production remains important in certain foods and beverages, lactic acid has been produced for use as a feedstock for industrial processes and as an additive to non-fermented foods since the beginning of the present century. The current levels and methods of production, uses, and markets for lactic acid have recently been reviewed by Vick Roy (1985). The latter author stressed that economic information about lactic acid is not

widely available as much production data is proprietry and it is difficult to follow the flow of lactic acid from manufacturers through importers, distributors and sub-distributors to the final users. However, Vick Roy (1985) estimated the total world production of lactic acid to have been 24 to 28 x 10^6Kg in 1982, of which 48 to 56% was provided by fermentation. More than half of all lactic acid consumed is used as a direct additive to food products as a preservative and acidulent; about one fifth is used for the manufacture of stearoyl-2-lactylate which acts as a texture enhancer in dough products. The rest of the lactic acid consumption is in pharmaceutical and technical applications; for adjusting the pH of preparations, as a topical treatment for warts, and in the manufacture of bio-degradable polymers for surgical use and for the controlled release of pesticides.

The fermentative production of lactic acid on an industrial scale has traditionally been made in batch fermenters. One particular process which has been described in detail by Atkinson & Mavituna (1983) and mentioned by Miall (1978) and Vick Roy (1985) involved the growth of *Lactobacillus delbreuckii* in 25 or 114 m^3 stainless steel fermenters on a medium containing corn starch glucose (150 g L^{-1}), malt sprouts (3.75 g L^{-1}), and diammonium hydrogen phosphate (2.5 g L^{-1}). Solid calcium carbonate was added to neutralize the acid formed, maintaining the pH at 5.8 to 6.0 at a temperature of 49°C. The culture was gently stirred to hold the carbonate in suspension but was not aerated. Conversion of glucose (93 to 95% w/w) was achieved in 4 to 6 days. Other processes have used different fermentable carbohydrates (Miall, 1978; Atkinson & Mavituna, 1983; Vick Roy, 1985). The most commonly used substrate has been sucrose refined from sugar cane or beet.

The fermentation

of pentose-containing sulphite waste liquor has been performed using *L. pentosus* after the substrate has been steam stripped and alkali treated to remove sulphur dioxide and lignins. Lactic acid has been produced from the fermentation of starch by *L. amylophilus* or by mixed cultures of Lactobacilli including *L. delbreuckii*.

Continuous lactic acid fermentations have been reported on a pilot scale by Whittier & Rogers in 1931 and by Childs & Welsby in 1966 (Miall, 1978; Vick Roy, 1985). In the former study, sweet whey was fermented over a two-week period in a 2 m^3 fermenter at a pH maintained between 5.0 and 5.8. The use of a long residence time (24 h) resulted in a 90% yield of lactic acid from lactose, a volumetric productivity of 2 to 2.5 g L^{-1} h^{-1}. A later evaluation of the process of Whittier & Rogers indicated that 'side reactions', possibly caused by contamination of the non-sterile fermentation, made the process unattractive on a commercial scale. In the process of Childs & Welsby (Miall, 1978), starch hydrolysate (total sugar 97 g L^{-1}) was fermented by *L. delbreuckii* at 50°C with pH maintained between 4.9 and 5.8 by the addition of solid sodium carbonate or ground limestone. At high residence times (e.g. 16 h; estimated from Miall, 1978) the highest volumetric productivity obtained was 3.7 g L^{-1} h^{-1}. There was no subsequent report of this process being extended to a manufacturing scale.

Lactic acid bacteria have complex nutritional requirements, especially for amino acids and B vitamins. Crude nitrogenous sources such as malt sprouts, malt extract, corn-steep liquor, barley, undenatured milk, and yeast extract have been used to supplement carbohydrates to promote rapid and heavy growth (Vick Roy, 1985). However, such crude extracts present great problems in isolating the lactic acid in sufficiently high purity for certain applications. The

fermentation processes employed in lactic acid production have not been complex and it is in the recovery and purification processes that problems lie, forming a large part of the total cost of manufacture.

Vick Roy (1985) described and evaluated several different recovery methods which are, or have been, used commercially. The first method, also described by Atkinson & Mavituna (1983), involved precipitation of lactic acid as calcium lactate followed by repeated steps of filtration, bleaching with activated vegetable carbon, recovery of free acid by treatment with sulphuric acid, and evaporation to concentrate the product. Different grades of lactic acid are subjected to different degrees of activated carbon treatment. This method relies on relatively pure sugars with minimal amounts of nitrogenous material being used as the fermentation feedstock. The second method is initially similar to the first but the product is further purified by recrystallisation and is low in unfermented sugars but may contain some ash which is mainly calcium sulphate. The method may be applied to fermentations using cruder raw materials such as whey or mollases. Disadvantages of the recrystallisation method are that the crystals tend to form clusters which can be difficult to wash, and the wash water and mother liquor contain high concentrations of calcium lactate and must be recycled. The third method is one of liquid-liquid extraction. In this process, the acid is first extracted from the crude liquor into an immiscible solvent phase and then recovered from the solvent by back extraction into water or distillation of the solvent - lactic acid mixture. Lactic acid refined by liquid-liquid extraction is substantially free from ash but may contain impurities from raw materials and require additional treatment by activated carbon or oxidation. A fourth method involves the esterification of lactic acid with a low molecular weight alcohol, distillation of the lactate ester,

hydrolysis of the distilled ester back to lactic acid and alcohol, and distillation of the alcohol from the regenerated lactic acid. The lactic acid produced by this method is of the highest purity obtainable by fermentation and is suitable for the manufacture of stearyol-2-lactylates.

The purity and heat stability of lactic acid is of great importance in the manufacture of stearoyl-2-lactylates. Lactic acid purified by methods other than lactate ester distillation produces unacceptable colours, odours, and flavours during the manufacture of stearoyl-2-lactylates. This particular requirement for lactic acid of high purity and heat stability was a major driving force in the development of synthetic chemical processes for the manufacture of lactic acid. The main synthetic route is based on the hydrolysis of lactonitrile by a strong acid to yield a mixture of lactic acid and ammonium lactate. The lactic acid formed is recovered by a methyllactate ester distillation process. The earliest synthetic processes used lactonitrile formed as a by-product of acrylonitrile synthesis but presently lactonitrile has been formed from hydrogen cyanide and acetaldehyde (Vick Roy, 1985).

Today, synthetic processes compete successfully with fermentation, each providing approximately half of the world's supply of lactic acid. The outlook for the production of lactic acid by fermentation was viewed as unfavourable by Lockwood (1979) but Vick Roy (1985) stated that, with alternative raw materials, intensified fermentation processes, and improved recovery processes, the fermentative production of lactic acid could compete favourably with the synthetic route.

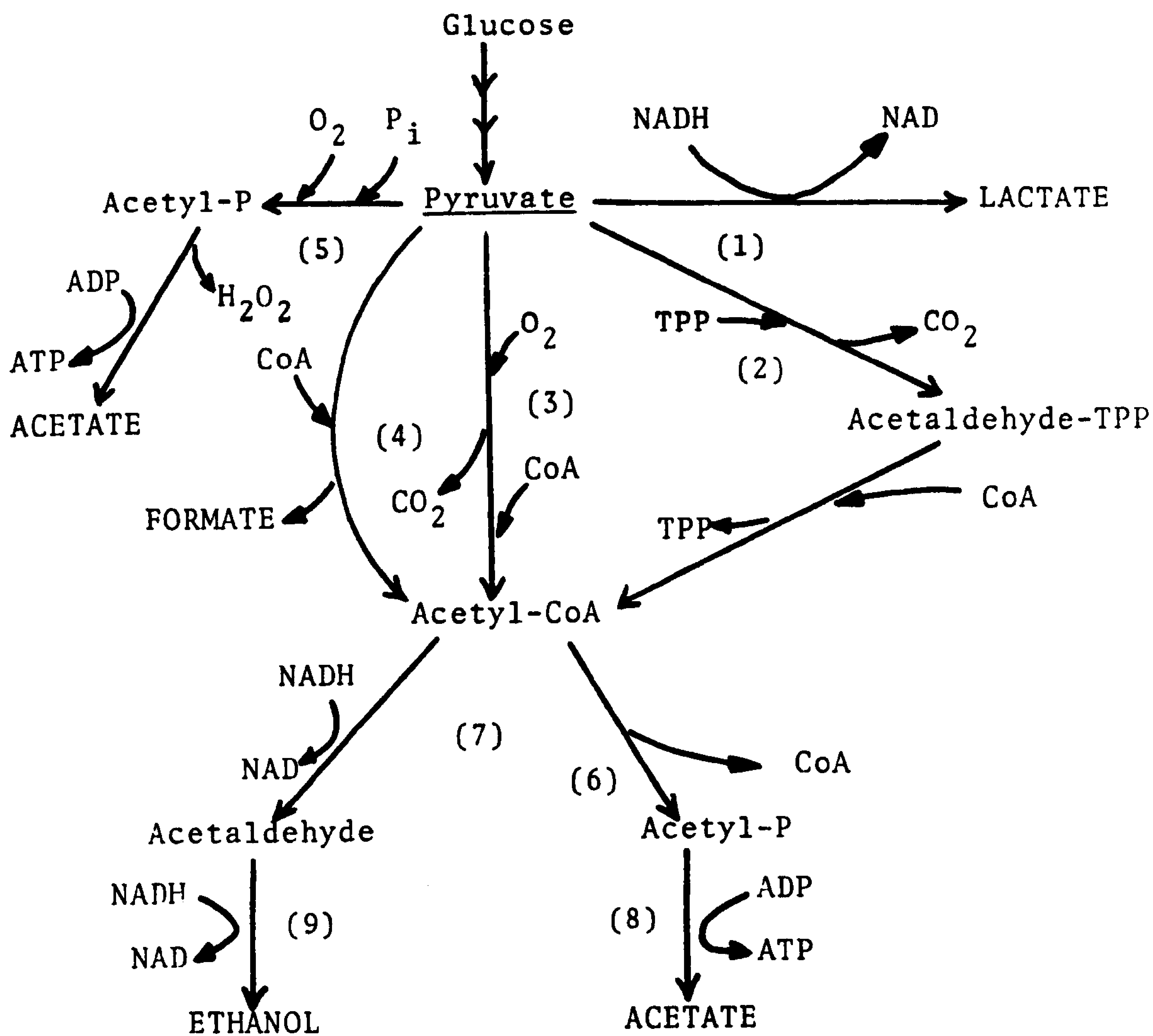
1.4.2 The Biochemistry of Lactic Acid Production

The broad taxonomic group of the lactic acid bacteria includes species from the genera *Lactobacillus*, *Streptococcus*, *Pediococcus*,

Bifidobacterium and *Leuconostoc*. The metabolism of carbohydrates by these organisms produces at least 50% lactic acid (Kandler, 1983). Those species producing almost exclusively lactic acid (homofermentative) are of particular importance to the industrial production of lactic acid and their metabolism of sugars has been well characterized.

The uptake of hexoses and disaccharides can proceed via an inducible, membrane-associated phosphoenolypyruvate phosphotransferase system (PTS), and a chemiosmotically driven uptake system. The former mechanism accumulates the 6-phosphorylated derivative whilst the latter accumulates the native sugars (Keevil, Marsh & Ellwood, 1984). Lactose accumulated by the PTS system is hydrolysed by an inducible phospho-ß galacosidase to yield a glucose moiety and galactose-6-phosphate. Glucose, fructose and their phosphorylated derivatives are metabolized via the Embden-Meyerhof-Parnas pathway whilst galactose-6-phosphate is metabolized via the tagatose-6-phosphate pathway (Hamilton & Lebtag, 1979). In each case the common product is pyruvate with a net gain of 1mol adenosine 5'-triphosphate (ATP) per mol pyruvate formed. During the conversion of hexoses to pyruvate, nicotinamide adenine dinucleoide (NAD^+) is reduced forming NADH. In order to maintain the NAD^+ supply within the cell, NADH may be reoxidised by several routes each of which involve the conversion of pyruvate, some of which result in additional ATP formation (Dawes & Sutherland, 1976) (summarized in Figure 1.6).

The classical homofermentative route involves the oxidation of NADH as a cofactor in the reduction of pyruvate to lactate. This latter reaction is catalysed by the enzyme lactate dehydrogenase (LDH) and it has been shown to be reversible *in vitro* (Garvie, 1980). NAD-dependant LDHs display pH optima in the range 4.5 to 8.0, may be inhibited by



Key to enzymes:

(1) Lactate dehydrogenase*

(2) Pyruvate decarboxylase

(3) Pyruvate dehydrogenase*

(4) Pyruvate-formate lyase*

(5) Pyruvate oxidase

(6) Phosphotransacetylase*

(7) Acetaldehyde dehydrogenase*

(8) Acetate kinase*

(9) Alcohol dehydrogenase*

(*: Active in anaerobic cultures)

Figure 1.7 Alternative fates of pyruvate.

phosphate, ATP and ADP, and may be stimulated by fructose-1, 6-diphosphate (FDP) and manganese ions (Garvie, 1980).

Other metabolic fates of pyruvate are known which result in a heterofermentative pattern of products (acetate, formate, ethanol, carbon dioxide) by 'homofermentative' organisms. Factors which govern pyruvate metabolism include the oxidation-reduction balance of the fermentation and the availability of glucose. Three reactions have been identified by which pyruvate may be oxidised to acetate, ethanol and CO_2 under aerobic conditions (Hager, Geller & Lipmann, 1954; Lloyd *et al.*, 1978; Kandler, 1983). The first is via the pyruvate dehydrogenase complex (EC 1.2.4.1) and requires Coenzyme A (HSCoA) and lipoic acid. The initial reaction is:

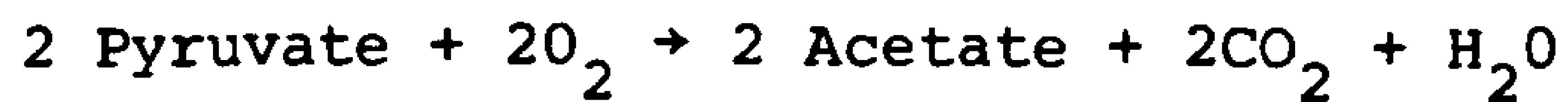
$$\text{Pyruvate} + \tfrac{1}{2}O_2 + \text{HSCoA} \rightarrow \text{AcetylCoA} + CO_2$$

The acetylCoA formed may be converted to acetyl phosphate by a phosphotransacetylation reaction and thence to acetate by acetate kinase with the formation of 1 mol ATP per mol acetate formed. Ethanol may also be formed from the acetylCoA intermediate, reoxidising NADH. The second route of pyruvate oxidation under aerobic conditions is via pyruvate oxidase (EC 1.2.3.3). In this latter sequence lipoic acid or coenzyme A are not involved but thiamine pyrophosphate flavin adenine dinucleotide, inorganic phosphate (P_i), and a divalent cation (Mn^{2+}, Mg^{2+} or Co^{2+}) are required. The initial reaction is:

$$\text{Pyruvate} + P_i + O_2 \rightarrow \text{Acetylphosphate} + CO_2 + H_2O_2$$

The acetylphosphate is converted to acetate via acetate kinase and the peroxide condenses with a second molecule of pyruvate to form further

acetate and CO_2. The overall reaction is:



$$2\ \text{Pyruvate} + 2O_2 \rightarrow 2\ \text{Acetate} + 2CO_2 + H_2O$$

The third route of oxidation involves pyruvate decarboxylase (EC 4.1.1.1) and the generation of acetaldehyde as an intermediate.

Under aerobic conditions further acetate may be produced by the oxidation of lactate to pyruvate via the NAD-independant flavoprotein, lactate oxidase. In this latter reaction lactate is oxidised by molecular oxygen to yield pyruvate and hydrogen peroxide. The peroxide causes the spontaneous oxidative splitting of pyruvate to acetate and CO_2 (Kandler, 1983). It has been suggested that the latter lactate oxidation can yield metabolic energy since *Sreptococcus faecium* has been grown in a defined medium with lactate as the sole carbon and energy source. However, it was not clear if lactate oxidase was coupled to energy production (London, 1968). The pyruvate formed may be metabolized via the ATP-yielding pyruvate oxidase system previously mentioned in the p resent section (Murphy *et al.*, 1985).

Under anaerobic conditions, glucose limitation has been shown to cause a channelling of pyruvate from LDH to other enzymes leading to a more heterofermentative pattern of product formation (De Vries *et al.*, 1970; Thomas *et al.*, 1979). Two enzymes have been implicated in such channelling: pyruvate dehydrogenase (PDH) which is also active in aerobic cultures; pyruvate formate-lyase (PFL), which is oxygen sensitive and is also known as the 'phosphoroclastic enzyme'. Both the latter enzymes require coenzyme A (CoA) and form acetyl-CoA with the loss of a C_1 fragment. The fragment released by PDH is CO_2 with the formation of one molecule of NADH whilst that released by PFL is

formate, without NADH formation. The fate of the acetyl CoA formed by both enzymes is the same: equimolar conversion to ethanol and acetate, via acetaldehyde and acetylphosphate respectively, with a net gain of 0.5 mol ATP per mol pyruvate utilized. The PFL route yields net 2 mol NAD^+ whilst the PDH route yields only 1 mol net (Fordyce, Crow & Thomas, 1984). De Vries *et al.* (1970) suggested that only PFL was active in glucose-limited anaerobic cultures of lactobacilli whilst Thomas *et al.* (1979) and Fordyce *et al.* (1984) suggested that both PDH and PFL were active in similar cultures of lactic streptococci.

1.4.3 Novel Fermenter Developments Applied to Lactic Acid Production

Lactic acid production has frequently been used as a model system for developing novel fermentation technology, e.g. dialysis culture, cell immobilization and cell recycle fermentation.

1.4.3.1 Dialysis Culture

The principle of dialysis culture is that microorganisms are grown on one side of a permeable membrane, on the other side of which water, a buffer solution, or a solution of nutrients is circulated. Soluble products and/or nutrients diffuse across the membrane in response to concentration gradients thus reducing the concentration of product in the fermentation vessel. As a result, growth inhibition by toxic products may be alleviated (Landwall, 1977). Lactic acid has long been held to be growth inhibitory thereby providing the impetus for the study of lactic acid production by dialysis culture. Friedman & Gaden (1970) applied dialysis to batch cultures of *L. delbreuckii* and reported a reduction in the concentration of lactic acid in the fermenter compared with conventional batch cultures. Dialysis did not lengthen the exponential phase of growth nor increase the maximum

specific growth rate, but it extended the production of biomass following the onset of the deceleration phase. Stieber, Coulman & Gerhardt (1977) and Stieber & Gerhardt (1979, 1981) presented detailed mathematical treatments of dialysis culture for lactic acid production from sweet whey waste. Experiments were reported of *L. bulgaricus* in continuous dialysis cultures with a conventional feed stream, and continuous dialysate-feed systems in which the transfer of both nutrients and products occurred across the dialysis membrane. Each of the latter studies reported increased lactose conversion efficiency and enhanced lactic acid productivity. However, in these novel fermenters the concentration of lactic acid in the fermentation vessel was enhanced rather than reduced. Therefore, it may be that these fermenters were not acting as true dialysis cultures but rather as forms of cell recycle fermenter, the observed improvements in lactose utilization being due to an overall reduction in growth rate within the system.

Hongo *et al.* (1986) grew *L. delbreuckii* in a novel batch fermenter which used electrodialysis as a means of pH control and lactate removal. The fermentation broth was circulated around a copper cathode in a compartment bounded by anion-exchange membranes. When a direct current was applied lactate ions were drawn towards twin anodes, passed through the anion-exchange membranes, and were trapped within concentration compartments by cation-exchange membranes isolating the anodes. A flow of electrolyte solution was passed through the concentration compartments ensuring the continuous removal of lactate. Electrodialysis resulted in a reduction in the concentration of lactic acid in the fermentation broth and an extended exponential phase of growth.

1.4.3.2 Cell Immobilization

The immobilization of bacterial cells is a method of process

intensification which aims to develop biocatalysts having enhanced biomass density, activity and stability, and yielding cell-free product streams at high volumetric productivity. Three approaches to cell immobilization have been applied to lactic acid production: attachment to solid supports by a crosslinked polymer; entrappment within a gel matrix; and, dense packing around microporous hollow fibres.

Compere & Griffith (1976) and Griffith & Compere (1977) immobilized mixed cultures of lactobacilli and lactose-fermenting yeasts in gelatin crosslinked with 5% glutaraldehyde coating inert ceramic particles. The resultant microbial film reactor was operated in plug flow mode using sweet whey (Compere & Griffith, 1976) and wood pulp waste (Griffith & Compere, 1977) as feedstocks. In the former study the lactic acid content of the whey was increased from 1.4% to 2.1% (w/v) in a single passage through the fermenter. In both studies the immobilized biocatalyst was operated continuously for periods in excess of one month.

Stenroos, Linko & Linko (1982) entrapped *L. delbreuckii* within beads of calcium alginate gel and operated packed bed reactors in batch and continuous modes. The immobilized biocatalyst had a half life of about 100 days in continuous operation, and could be stored for about five months at $7^{o}C$ with only 10% loss in activity. Tipayang & Kozaki (1982) isolated a novel species, *L. vaccinostercus* for use in immobilized cell fermenters producing lactic acid from xylose. The alginate-immobilized biocatalyst was more productive than washed free cells in the same medium. Tuli *et al.* (1985) used agar and polyacrylamide for the entrappment of *L. casei* and the production of lactic acid from whey permeate. These latter authors reported that agar was the superior gel matrix, producing

biocatalysts of enhanced yields and extended activity.

Vick Roy *et al.* (1982) immobilized *L. delbreuckii* in polypropylene hollow fibres. Growth medium was circulated throughout the lumen of the fibres and returned to a holding vessel in which the temperature and pH were maintained at constant levels. The reactor was operated using 60, 108 or 300 hollow fibres and in each case the volumetric productivity of lactic acid was enhanced by comparison with conventional batch culture. However, mass-transfer limitations were apparent, caused by dense packing of biomass and resulting in a reduction of the specific productivity to 10 to 15% of that observed in the exponential phase of conventional batch culture. Distortion and penetration of the hollow fibres by the growing cell mass was reported.

1.4.3.3. Cell Recycle Fermentation

Lactic acid production by cell recycle fermentation has been reported by Vick Roy *et al.* (1983) and Mehaia & Cheryan (1986). Both of these studies are discussed elsewhere in this Chapter (Section 1.2.2). Briefly, total cell recycle was applied to cultures of lactobacilli operated at high dilution rates resulting in enhanced volumetric productivity compared with other fermentation systems. Both studies emphasised the potential usefulness of cell recycle fermentation for lactic acid production but physiological considerations were not addressed.

Recently, Boyaval, Corre & Terre (1987) have combined cell recycling and electrodialysis in a single fermenter for the production of sodium lactate by *L. helveticus* on a feed-stock of sweet whey. Fermentation broth was passed through filters consisting of a carbon tube with an ultrafiltering zirconium oxide coating.

All the cells were returned to the culture vessel and the cell-free filtrate was passed to the electrodialysis unit. Electrodialysis generated a lactic acid-depleted stream which was recycled back to the culture vessel and a sodium lactate-rich stream which was discharged from the system. High volumetric productivity (88 g L^{-1} h^{-1}) was reported but the specific productivity declined during cell recycling. The physiological basis of the latter decline was not investigated.

1.5 Summary and Objectives

Within the present Chapter it has been argued that: (1) an impetus exists for the intensification of microbial processes; (2) the theory of cell recycle fermentation has predicted that the technique may be of value as an approach to process intensification; (3) the few laboratory studies so far reported have confirmed the latter potential value in practice; (4) cross-flow filtration has been the method of choice for the generation of cell-free outflow streams in cell recycle fermenters; (5) considerations of microbial physiology have not been adequately addressed under cell recycling conditions; (6) mathematical treatments of cell recycle fermentation have not been independently tested; and (7) lactic acid production forms a useful model system for a physiological analysis of cell recycle fermentation because the product is in commercial demand; fermentative routes of production compete with chemical synthesis; the metabolism of lactic acid production has been well characterized in conventional cultures; and, lactic acid production has been used as a model system for other novel fermenter developments.

The main objectives of the present study were:

(1) to characterize lactic acid production in conventional cultures;

(2) to develop a fermenter which would allow the maintenance of defined degrees of partial cell recycle over extended periods of operation; (3) to provide independent testing of a previously published mathematical treatment of cell recycle fermentation (Bull & Young, 1981); and (4) to observe the effect of different degrees of partial cell recycle on accepted indicators of physiological state (growth, yield, product profile, cellular composition, and the activity of a key enzyme, lactate dehydrogenase).

CHAPTER 2

METHODS: ANALYSIS AND CONVENTIONAL CULTURE

2.1 Bacterial Strains Used

The organism used throughout the present study was *Lactobacillus* (*L.*) *delbreuckii*, a homofermentative lactic acid-producing bacterium. The background to the choice of this organism for the study is outlined in Chapter 1. The strains of *L. delbreuckii* which were screened for use were: NCIB 8130 (National Collection of Industrial and Marine Bacteria, Torrey Research Station, Aberdeen); CN960 (Wellcome Research Laboratories, Beckenham, Kent); 7473 (Glaxo Group Research Ltd, Greenford, Middx) and NRRL B-445(Northern Regional Research Centre, Peoria, Illinois, USA).

During the study cell suspensions of these strains were maintained at -20°C in nutrient broth (Oxoid Ltd., Basingstoke, Hants) containing 80% v/v glycerol. The cultures were revived by adding the contents of a vial of cell suspension to a 500mL conical flask containing 200 mL MRS broth (Section 2.2). The inoculated broth was incubated with shaking at 42°C until turbid.

2.2 Growth Media

The growth medium used to revive stored cultures and to grow inocula for fermenter experiments was that of DeMan, Rogasa & Sharpe (1960) (MRS broth) obtained in dried form from Oxoid Ltd.,

Basingstoke, Hants. Bacteriological agar (Agar No. 1, Oxoid) was added to MRS broth at a concentration of 1% w/v when a solid plating medium was required (MRS agar). MRS broth and MRS agar were sterilized by autoclaving at 121°C for 15 min.

The growth medium used for shake flask screening of strains and for all fermenter experiments was essentially that of Vick Roy *et al.* (1982, 1983) (VR medium). The composition of VR medium is shown in Table 2.1.

The glucose monohydrate ('Meritose') was obtained from Tunnel Refineries, Greenwich, London and the yeast extract powder was obtained from London Analytical and Bacteriological Media Ltd. (Lab M), Salford, Manchester. The salts included in the medium were all of AnalaR grade and obtained from various suppliers.

VR medium was sterilized by autoclaving at 121°C. Volumes of 1L or less were autoclaved for 15 min, volumes of 10 and 20L were autoclaved for 30 and 60 min respectively. To avoid caramelization and phosphate salt precipitation in the medium during autoclaving, solutions of glucose monohydrate and the phosphate salts were made up and autoclaved separately. These sterile solutions were then aseptically added to the sterilized bulk medium.

Table 2.1 Composition of VR Medium

	gL^{-1}
Glucose monohydrate ('Meritose')	50
Yeast extract powder	30
Sodium succinate hexahydrate	2
Na_2SO_4	2
$MgSO_4.7H_2O$	0.6
K_2HPO_4	0.2
KH_2PO_4	0.2
$MnSO_4.4H_2O$	0.03
$FeSO_4.7H_2O$	0.03
Silicone antifoam (Sigma)	0.5mL L^{-1}

pH adjusted to 6.0 with 10M HCl.

2.3 Analysis of Cultures

2.3.1 Biomass Dry Weight

Culture biomass dry weight concentrations were measured by weighing the oven-dried biomass from a known aliquot of concentrated washed cell suspension.

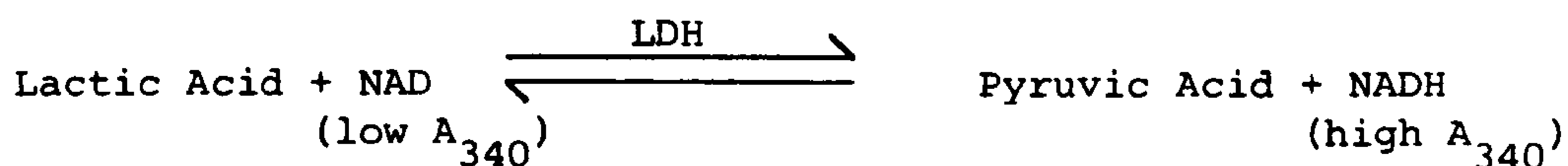
A 20mL volume of culture was collected on ice. Culture (10mL) was introduced into each of two 50mL polycarbonate centrifuge tubes (Nalgene, Rochester, N.Y, USA). The tubes were capped and spun at 18,500g for 10min at 4°C in a Beckman J2.21 centrifuge. The culture supernatants were drawn off and stored at -20°C for subsequent chemical analyses. The cell pellets were washed by adding 10mL water to each tube and resuspending the cells by vortex mixing. The washed cell suspensions were spun down as before and the supernatant water was discarded. The cell pellets were once more washed and spun down in the same way and the final washed cell pellets were resuspended by adding 4mL water and vortex mixing. These concentrated washed cell suspensions were each introduced into one of two aluminium boiling tube caps (Oxoid) which had previously been dried at 105°C overnight and weighed when cool. The centrifuge tubes were then washed out with 3 to 4 mL water to ensure that no cells remained, the washings being added to the suspensions in the boiling tube caps. The caps containing the washed cell suspensions were then placed in an oven at 105°C and dried overnight. After drying, the caps were placed in a dessicator and allowed to cool for 15 to 20min before being weighed. The caps were handled with forceps at all times to avoid contamination with residues from the skin. The culture biomass dry weight concentration was calculated from the difference in weight of the dried caps before and after the addition of cells and based on the

known volume of culture originally taken. The mean of the two values thus obtained was taken to be the biomass concentration of the culture for that sample.

During cell recycle experiments when very large concentrations of biomass were generated the procedure for biomass dry weight measurement was different in detail. Cell suspensions required centrifugation at 51,500g for 20min to give a clear supernatant and the final washed pellets were resuspended with 10mL water and the tubes washed with 5mL water. The suspensions and washings were introduced into pre-dried and weighed 25mL glass beakers rather than boiling tube caps. The suspensions were dried and the dry weight calculated in the same way.

2.3.2 Lactic Acid

The concentration of lactic acid in culture supernatants was measured by an enzymic method involving the reduction of nicotinamide adenine dinucleotide (NAD) which is catalysed by the enzyme lactate dehydrogenase (EC 1.1.1.27/28) (LDH) according to the following reversible reaction:



To measure lactic acid the reaction was carried out from lactate to pyruvate with excess NAD. To force the reaction to completion in this direction, the formed pyruvic acid was trapped with hydrazine. The increased absorbance at 340nm (A_{340}) due to NADH formation was a measure of the lactic acid originally present.

For the measurement of the L(+)- isomer of lactic acid the assay

was performed using an analytical kit obtained from Sigma Chemical Company, Poole, Dorset (product no. 826 A/B) and the method is described in detail in Sigma Technical Bulletin No. 726-UV/826-UV. Standard curves were constructed using solutions of L(+) lactic acid (Sigma product no. L1750). These curves were used as a rough guide to aid in the dilution of culture supernatants to within the useful range of the assays. The concentrations of lactic acid in the supernatants were calculated by use of standard solutions freshly made and diluted alongside the supernatants. Fresh growth medium was included in the assay as a negative control. The lactic acid concentrations in the test samples were calculated by comparison of test A_{340} values with those obtained using the standard solution. Assays were performed in triplicate for each supernatant sample.

2.3.3 Glucose

2.3.3.1 Rapid Qualitative Assay

The rapid qualitative analysis of glucose in whole culture and supernatant samples was performed using 'Clinistix' enzymatic reagent strips obtained from Ames Division, Miles Laboratories, Ltd., Stoke Poges, Bucks. The reagent strips are based on the principle established by Keston (1956) and carry test areas of cellulose impregnated with a buffered mixture of glucose oxidase, peroxidase and a chromogen system. In the presence of glucose atwo-stage coupled reaction takes place:

1. Glucose in solution is oxidised by atmospheric oxygen in the presence of glucose oxidase to gluconic acid and hydrogen peroxide.

2. The hydrogen peroxide in the presence of peroxidase oxidises the chromogen system to a shade of purple.

This assay was used simply as a rapid indicator of the presence or absence of residual glucose in culture samples and was performed as follows. A test strip was dipped into a sample of whole culture or culture supernatant and removed immediately. Ten seconds after wetting, the colour of the test area was compared with a colour chart provided with the test strips. Any change in colour of the test area from the initial pink towards purple was taken as an indication of the presence of residual glucose in the culture. In those supernatant samples which gave a negative reaction with this assay, no glucose could be detected by the quantitative assay described below (Section 2.3.3.2).

2.3.3.2 Quantitative Assay

The quantitative assay was also based on the method of Keston (1956) but included the modifications of Raabo and Terkildsen (1960). The assay involved similar coupled reactions to those in the rapid qualitative test (Section 2.3.3.1) except that a different chromogen, O-dianisidine, was used giving a change from colourless to brown in the oxidised form. The intensity of the brown colour measured at 450nm (A_{450}) was proportional to the original glucose concentration. The assay was performed using an analytical kit from Sigma Chemical Co (product no. 510) following the procedure described in Sigma Technical Bulletin No. 510. Culture supernatants were diluted in water to give approximately 1mg mL^{-1} glucose and were assayed in triplicate. Supernatant glucose concentrations were calculated by comparison of A_{450} values of test samples with those obtained from a concurrently assayed standard glucose solution (Sigma, product no.635-100).

2.3.4 Ethanol

Culture ethanol concentrations were measured by a procedure which was a modification of that described by Bucher and Redetzki (1951) and which involved the use of alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD). In the assay ADH catalyses the conversion of ethanol to acetaldehyde with concomitant reduction of NAD to NADH. Alkaline conditions (pH 9.0) and a trapping agent to remove the acetaldehyde formed ensure that the reaction proceeds nearly to completion (Lundquist, 1957). The increase in absorbance at 340nm (A_{340}) which occurs when NAD is reduced to NADH is proportional to the amount of ethanol present in the assay mixture. The assay was performed using an analytical kit (Sigma; product no. 332-UV) following the procedure described in Sigma Technical Bulletin no. 332-UV. Culture supernatants were assayed in triplicate. Culture ethanol concentrations were calculated directly from A_{340} values and the absorption coefficient of NADH at 340nm ($6.22mM^{-1}$ cm^{-1}, Sigma Tech. Bull 332-UV).

2.3.5 Acetic Acid

The concentration of acetic acid (acetate) in culture supernatants was measured by a method which involved the following three enzymic reactions:

1. In the presence of acetyl-CoA synthase acetate is converted with adenosine-5'-triphosphate (ATP) and coenzyme A (CoA) to acetyl-CoA.

2. Acetyl-CoA reacts with oxaloacetate (OAA) to form citrate in the presence of citrate synthase.

3. The OAA required for reaction 2. is formed from malate and NAD in the presence of malate dehydrogenase. In this reaction NAD is reduced to NADH.

The determination is based on the formation of NADH measured by the increase in absorbance at 340nm. This procedure was a simplification of the method described by Bergmeyer and Möllering (1974). The assay was performed using an analytical kit obtained from Boehringer Corp. Ltd., Lewes, E. Sussex (product no. 148261). Since a preceding indicator reaction is used (reaction 3.), the amount of NADH formed in the assay is not linearly proportional to the initial acetate concentration. Therefore, the concentration of acetate was calculated in the manner described by Bergmeyer (1974) which is generally applicable to preceding indicator reactions. Supernatant samples were analysed in triplicate.

2.3.6 Phosphate

Phosphate concentrations in supernatant samples were measured by a modification of the method of Chen, Toribara and Warner (1956). The method involved the formation of phosphomolybdate by reaction of the phosphate present with ammonium molybdate followed by the reduction of the molybdate with ascorbic acid to form a blue colour which was measured photometrically. To measure total (inorganic plus organic) phosphate concentrations, supernatant samples were heated with an acid digestion mixture. The details of the reagents were as follows:

1. Digestion Mixture

100% sulphuric acid and 60% perchloric acid mixed 3:2 (v/v).

2. Colour Reagent

3M sulphuric acid (10mL), 2.5% (w/v) ammonium molybdate solution (10mL), water (30mL) and ascorbic acid (1g). This reagent was mixed freshly each day and kept cool until used.

3. Standard Phosphate Solution

KH_2PO_4 (87.8mg) dissolved in water and made up to 100mL. 5mL of this concentrated solution was diluted to 100mL to give a solution containing $10\mu gmL^{-1}$ phosphorous.

All glassware used for this assay was washed with concentrated nitric acid, rinsed first in deionised water, then in distilled water and then oven dried.

2.3.6.1 Inorganic Phosphate

Supernatant samples containing 1 to 5 μg phosphorous were adjusted to 4mL with water. Colour reagent (4mL) was added and the mixtures were incubated at 37°C for 1.5 to 2h. The solutions were cooled to room temperature and their absorbances at 820nm (A_{820}) were measured. The concentrations of phosphate were calculated by comparison of the test A_{820} values with those obtained using dilutions of the standard phosphate solution.

2.3.6.2 Total Phosphate

Supernatant samples containing 1 to 5μg phosphorous were evaporated to dryness using an electrically heated plate and two 276W infra-red lamps. Digestion mixture (0.1mL) was added and the samples were heated for a further 20min. After digestion the samples were allowed to cool to room temperature at 4.0mL colour reagent was added to each sample. The samples were incubated, the A_{820} measured, and phosphate concentration determined as in 2.3.6.1.

Organic phosphate concentration was defined as being equal to the total minus the inorganic phosphate concentration.

2.3.7 Total Cellular Protein

The total protein content of cells was measured by the method described by Herbert, Phipps and Strange (1971) which was a modification of the method of Lowry *et al.* (1951). The method used the 'phenol reagent' of Folin and Ciocalteu (1927) and the NaOH-Na_2CO_3-$CuSO_4$-tartrate reagent of Lowry *et al.* (1951) to measure photometrically proteins solubilised by heating of cells at 100°C in 0.5M NaOH solution.

Twice-washed suspensions of cells were prepared as described in Section 2.3.1 and diluted to a concentration of approximately 200µg dry weight mL^{-1}. The protein content of these cells was measured by following the procedure of Herbert *et al.* (1971) with solutions of bovine serum albumin (Sigma Chemical Co., Poole, Dorset) containing 50 to 200µg protein assayed in parallel to construct a calibration curve for each assay. The Folin-Ciocalteu reagent was obtained from BDH Chemicals Ltd., Poole, Dorset (product no. 19058). The cellular protein content was expressed as a percentage of biomass dry weight.

2.3.8 Total Cellular Polysaccharide

The total polysaccharide content of cells was determined using an acid-anthrone method following the procedure of Herbert, Phipps and Strange (1971). This method is based on the formation of a green colour when anthrone is heated with polysaccharide derivatives in concentrated sulphuric acid.

A twice-washed suspension of cells was prepared as described in Section 2.3.1 and diluted in water to approx. 0.5mg dry weight mL^{-1}. The analysis was performed as described by Herbert *et al.* (1971), the results obtained being compared with those obtained with standard glucose solutions assayed in parallel. The total poly-

saccharide content of cells was expressed as the weight of total glucose equivalents as a percentage of biomass dry weight.

2.3.9 Supernatant C, H and N

Total carbon, hydrogen and nitrogen concentrations in culture supernatants were determined using a Carlo-Erba model 1106 simultaneous CHN analyser. Known aliquots (0.1 to 1.0mL) of supernatants were introduced into vials which had been dried and weighed. The supernatants were lyophilized in an Edwards Modulyo freeze drier and the vials were reweighed to determine the dried weight of supernatant per unit volume of liquid. The dried supernatants (1 to 2mg samples) were introduced into tin crucibles and subjected to controlled oxidation and then reduction to form carbon dioxide, water and nitrogen. These gases were separated by gas chromatography on the basis of polarity and detected by thermal conductivity. Acid digested standards were periodically analysed to calibrate the system. The C, H and N concentrations were expressed as percentages of the supernatant dry weight and calculated back to $mmolesL^{-1}$ supernatant.

2.3.10 Lactate Dehydrogenase

Lactate dehydrogenase (LDH) (E.C.1.1.27/28) catalyses the interconversion of lactate and pyruvate as outlined in Section 2.3.2. The LDH activity of cells from different cultures was determined in cell free extract (CFE) preparations by following the oxidation of NADH in the presence of pyruvate as the substrate. The requirement of the enzyme for manganese ions (Mn^{2+}) and fructose, 1-6,diphosphate (FDP) was investigated as was the inhibitory effect of phosphate ions. The buffer used to wash cells, prepare CFE and assay mixtures (histidine 0.08M, pH 6.0) was that of De Vries

et al. (1970).

2.3.10.1 Cell Free Extract Preparation

A sample of culture (10 to 20mL) was collected on ice. An aliquot containing approximately 100mg dry weight of cells was introduced into a 50mL polycarbonate centrifuge tube (Nalgene, Rochester, N.Y., USA). The cells were separated by centrifugation at 18,500g for 10 min at 4°C and then resuspended in 10mL ice-cold histidine buffer. The cells were then twice washed by centrifugation and resuspension in histidine buffer in the same way. The final cell pellet was stored at -20°C for 24h before breaking as Garvie (1980) suggested that thawed cells break more readily than fresh cells.

The frozen pellet was thawed by the addition of 10mL histidine buffer at 4°C and resuspended by vortex mixing. The cells were broken by a single passage of the suspension through a French press (Aminco Co.) at 8 to 10 te pressure. The broken cell preparation was clarified by centrifugation at 29,000g for 45min at 4°C. The cell free extract (CFE) was drawn off the pellet of broken cells, stored on ice and assayed for LDH activity within 1 to 2h.

2.3.10.2 CFE Protein Assay

The method used to measure the protein concentration of the CFE was based on that of Bradford (1976) which is considerably more rapid than Folin-Ciocalteu type determination such as Lowry *et al.* (1951) and thus requires less delay in the assay of LDH activity. The method is based on the colour change from red to blue which occurs on binding of Coomassie Brilliant Blue dye to protein. The binding is a very rapid process (approximately 2mins) and the protein-dye complex remains dispersed in solution for a relatively long time

(approximately 1h). Thus the procedure is very rapid and does not require critical timing (Bradford, 1976). The details of the procedure were as follows:

1. Preparation of protein reagent

Polyacrylamide gel electrophoresis (PAGE) Blue G90 dye (100mg) (BDH Chemicals Ltd., Poole, Dorset; product no. 44248) was dissolved in 100mL absolute ethanol. To this solution 100mL 88% (w/v) phosphoric acid was added. The resulting solution was diluted to 1L with water. Final concentrations in the reagent were (w/v):

PAGE Blue G90, 0.01%; ethanol, 4.9%; phosphoric acid, 8.8%. The protein reagent was stored at 4°C and made freshly each month.

2. Protein Assay

The CFE was diluted in histidine buffer to give 0.1 to 1.0mg protein mL^{-1}. Diluted CFE (0.1mL) was introduced into a testtube and 5mL protein reagent was added. After vortex mixing, the tube was allowed to stand at room temperature for 5 to 10min before the absorbance of the solution at 595nm (A_{595}) was measured against a reagent blank containing 0.1mL histidine buffer in 5mL protein reagent. The assay was performed in triplicate for each CFE prepared. The weight of protein in the assays was calculated by comparison of the test A_{595} values with a calibration curve freshly constructed using solutions of bovine serum albumin (0.1 to 1.0mg mL^{-1}).

2.3.10.3 LDH Assay and Kinetic Studies

The assay was based on that of DeVries *et al.* (1970) with changes inspired by Garvie (1980) and Holland and Pritchard (1979). No attempt was made to establish optimum conditions for the assay; instead the objective was a consistent system with which to compare the LDH activity of cells from different cultures. The pH of the assay

was 6.0 as this was the pH at which all cultures were grown. Manganese ions and fructose, 1-6,diphosphate (FDP) were included in the complete assay mixture at concentrations similar to those of DeVries *et al.* (1970). However, pyruvate was included at a concentration approximately double that of DeVries *et al.* (1970). The full composition of the LDH assay mixture is shown in Table 2.2. The NADH was obtained in preweighed vials (1,2 and 5mg; Sigma Chemical Co., Poole, Dorset; product nos. 340-101/102/105). The pyruvate and FDP were also obtained from Sigma (product nos. P-2256 and 752-1).

The details of the procedure were as follows: The assay was conducted at 25°C in quartz cuvettes (volume 3ml). A_{340} was continuously recorded against a blank containing the assay mixture excluding NADH. The background rate of NADH oxidation in the absence of the substrate, pyruvate, was measured first. NADH solution, diluted CFE, FDP and Mn^{2+} solutions were introduced into a cuvette and the fall in A_{340} recorded. Pyruvate solution was then introduced and the fall in A_{340} measured further. Following the experiments, the rates of fall of A_{340} were measured and the rate due to LDH activity was calculated as the rate following pyruvate addition minus the background rate. To determine the requirement of the system for FDP and Mn^{2+}, the assay was performed in the presence and absence of these substances. Kinetic constants for pyruvate in this system were estimated by measuring the corrected rate of NADH oxidation in the presence of a range of pyruvate concentrations (0.39 to 7.7mM) and derivation by Lineweaver-Burk plots. A range of concentrations of potassium phosphate (0 to 37mM) was introduced into the assay to determine if phosphate was inhibitory to the system.

The specific activity of LDH was calculated from the rate of fall of A_{340} corrected for background oxidation, the molar absorption

Table 2.2 LDH Assay Mixture

Solution	Vol. Added (mL)	Conc. in Assay (mM)
βNADH, 0.3mM	2.2	0.3
FDP, 30mM	0.1	1.2
Pyruvate, 200mM	0.1	7.7
$MnCl_2.4H_2O$,100mM	0.1	3.9
Diluted CFE (80 to 160μg protein mL^{-1})	0.1	3 to 6μg mL^{-1}

In histidine buffer 0.08M, pH 6.0, 25°C

coefficient of NADH at 340nm (6.22mM^{-1}cm^{-1}) and the weight of protein in the CFE added to the assay.

2.3.11 Culture Viability

Two approaches to the determination of culture viability were taken. The first was a direct approach involving slide culture (after Postgate, 1969); the second was an indirect approach involving a comparison of viable counts with total counts.

2.3.11.1 Slide Culture

The method used was that of Postgate (1969) which is a development of that of Postgate *et al.* (1961). Four slides were prepared with MRS agar as described by Postgate (1969) and inoculated with a loop of culture diluted in MRS broth to a concentration of 5×10^7 to 5×10^8 cells mL^{-1}. The slides were incubated at 42°C. At hourly intervals one slide was removed, allowed to cool and examined under phase contrast microscopy at x650 magnification with the coverslip removed. The aim of this examination was to count the number of single cells (one dead unit) and microcolonies (one viable unit). The extent to which this was possible is outlined in Chapter 6 (Section 6.1.2). The viability of the culture is given by ratio of single cells to microcolonies expressed as a percentage.

2.3.11.2 Total Count

Total counts were made by use of a Helber slide with Thoma ruling observed under phase contrast microscopy. A sample of fresh culture was diluted in water to a concentration of 1×10^8 to 1×10^9 cells mL^{-1}. A small drop (10µL) of diluted culture was

placed on the grid of the Helber slide and covered with a coverslip of 1mm thickness. Firm pressure was applied to the coverslip until 'Newton's rings' could be seen around the edges of the counting area. The slide was observed under phase contrast microscopy (x650 magnification) and the number of cells per ruled square counted. Squares were counted in a diagonal pattern so that no two squares sharing common boundaries were counted. Enough squares were observed to give a total of at least 600 cells counted. The mean number of cells per square was calculated, divided by the volume above each square in the Thoma grid (5×10^{-8}mL) and multiplied by the dilution factor used to give a mean total count mL^{-1} for the original culture.

The mean length of chains of cells in the Helber slide preparation was calculated by counting the number of cells per chain for at least 50 chains. This mean chain length value was combined with the viable count as outliend below (Section 2.3.11.3) in the calculation of culture viability.

2.3.11.3 Viable Count

Viable organisms were counted by a method involving a two-stage dilution as outlined by Postgate (1969) followed by a drop-plating technique based on that of Miles and Misra (1938) using semiautomatic pipettes as outlined by Blousefield, Smith and Trueman (1973). The procedure was as follows:

1. Dilution ($\times 10^{-6}$).

(a) MRS broth (100mL) was aseptically dispersed into each of two sterile 250mL conical flasks.

(b) Fresh culture (1 µL) was taken up into a sterile tip of a Gilson pipette and introduced into the first 100mL batch of broth diluent. The conical flask was swirled to mix the contents thoroughly.

(c) Diluent (1mL) was aseptically removed from the second batch of diluent and discarded. This volume was replaced by 1mL of the first dilution. The flask was swirled to mix.

2. Plating.

(a) Each of two plates of MRS agar was divided into six segments by ruled lines on the base. The plates were placed on a horizontal surface.

(b) An aliquot (20μL) of the second dilution was taken up into a sterile tip using a Gilson pipette.

(c) The Gilson pipette was held vertically over one of the ruled segments on one of the agar plates and the diluted culture was expelled to form a drop on the surface of the solid medium.

(d) This plating was repeated on each of the other 11 segments on the plates using a fresh tip each time.

(e) The drops were allowed to dry into the surface of the solid medium and the plates were incubated at 42°C for 24h.

(f) Colonies were counted from drops giving rise to 20 to 100 colonies with a minimum of 200 colonies being counted in total.

A mean value for colonies per drop was calculated. The overall count of viable units was calculated from this figure and the dilution factor. This count, in terms of viable units mL^{-1}, was converted to viable cells mL^{-1} by multiplication by the previously determined mean chain length for that culture (Section 2.3.11.2). This step of the calculation was to allow for the chain forming nature of this organism (Chapter 6, Section 6.1.2). The viability of the culture was expressed as:

$$\text{total cells mL}^{-1} / \text{Viable cells mL}^{-1} \times 100\%$$

2.3.12 Spectrophotometry

All spectrophotometric measurements were made using a narrow-waveband, single-beam Perkin Elmer Lambda One spectrophotometer. Unless stated otherwise, all measurements were performed using disposable polycarbonate cuvettes of 3mL volume. The light path was 1cm. Absorbance values for all assays except LDH (Section 2.3.11.3) were obtained as integrated values over a period of 4s. In the LDH assay, absorbance was continuously recorded on a chart trace.

2.3.13 Statistical Treatment of Data

The results of all analyses reported in the present study were expressed as mean values obtained from between 3 and 12 replicate points. Confidence limits about the mean were calculated from the number of replicate points (n), the standard deviation and the Student's t value for a two-tailed test at n-1 degrees of freedom and P = 0.05 (unless otherwise stated). Linear regression was performed by a least-squares procedure permanently programmed into a Texas TI-51 III calculator. Coefficients of correlation were calculated to be equal to the slope of the regression line multiplied by the ratio of standard deviation of the x and y coordinates.

2.4 Strain Selection

The strains of *Lactobacillus delbreuckii* which had been received (Section 2.1) were screened for lactic acid production in shake flask culture. Aliquots of MRS medium (Section 2.2) (100mL) in 250mL conical flasks were inoculated by loop transfer from MRS agar plates of these strains which had been incubated for 24h at 42°C. The flasks were incubated with shaking at 42°C overnight and formed the inocula for the screening experiments.

Three aliquots of inoculum culture (10mL) were removed from each flask and introduced into triplicate 90mL aliquots of fresh MRS broth in 250mL conical flasks. These flasks were then incubated with shaking at 42°C for 24h. After incubation, samples of culture were removed for measurement of biomass dry weight and lactic acid concentrations. Mean values and the yield of lactic acid g^{-1} biomass were calculated for each strain from the triplicate flasks of each inoculated.

2.5 pH Controlled Batch Culture

2.5.1 Apparatus

All fermentations were performed using an MBR 'Mini' bioreactor with an MBR MCS1 control cabinet (MBR Bioreactor Ltd., Widmer End, High Wycombe, Bucks.). The bioreactor consisted of a glass fermentation vessel of capacity 2L with stainless steel end plates which was mounted above a stirrer motor. The stirrer shaft passed through a mechanical seal in the centre of the bottom end plate and carried two disc turbine impellers. A seal was made between the glass vessel and the end plates by PTFE seatings for the plates and a rubber O-ring at each end of the vessel. Probes for the measurement of pH and dissolved oxygen tension (Ingold; MBR Ltd., High Wycombe, Bucks) were mounted in ports in the top end plate. The pH probe had previously been calibrated against buffers at pH 4.0 and pH 10.0. Probes for measuring temperature, two heater coils (400W each) and a cooling coil were mounted in ports in the bottom end plate. A stainless steel condenser was mounted in the central part of the top end plate and was topped by a stainless steel exhaust air filter assembly. The filter assembly consisted of a candle filter within a steel tube; gas flow through the filter was controlled by a diaphragm valve mounted at the top of the

assembly. A stainless steel pressure safety valve was mounted in a port in the top plate.

The bioreactor was sterilizable *in situ* as follows. All empty ports in the bottom end plate were sealed by silicone rubber septa held in cups which were then sealed with blind plugs. The fermentation vessel was partially filled with approximately 1.5L distilled water and the remaining ports in the top plate were sealed with rubber septa and blind plugs. The stirrer speed was set to 1000rpm and the fermentation vessel was enclosed in a stainless steel protective jacket. The temperature control was set to 121°C and the temperature in the vessel was allowed to rise. At 100 to 105°C, steam flowed freely from the exhaust air filter assembly. The diaphragm valve was partially closed until only a trickle of steam was escaping. The vessel temperature continued to rise until being maintained automatically at the set level. The sterilizing temperature was maintained for 30min and then the temperature control was switched to manual and the vessel was allowed to cool slowly to room temperature, usually overnight.

Apparatus ancillary to the bioreactor included: a line for filling the fermentation vessel with growth medium; a supply of oxygen-free nitrogen via a rotameter and a 0.2μm PTFE filter (Acro 50; Gelman Sciences Ltd., Northampton); a line supplying 2M NaOH solution for pH control via a multi-roller peristaltic pump (Eyela; MBR Bioreactor Ltd., High Wycombe, Bucks) activated by the bioreactor MSC1 unit; and a line to a sampling hood with Universal screw thread for removing samples of culture and emptying the fermentation vessel. All lines were sterilized by autoclaving at 121°C for 20min before being aseptically connected to the sterilized bioreactor by needle connectors which pierced the rubber septa held in ports in

the end plates and screwed into the septum holding cups.

2.5.2 Batch Culture

Following *in situ* sterilization of the bioreactor, the water in the fermentation vessel was drained into 500mL medical flat bottles via the sampling line. Fresh VR medium (Section 2.2)(1.5L) was pumped into the fermentation vessel by a twin-roller peristaltic pump (Watson-Marlow, Falmouth, Cornwall). The medium supply line was then clamped off. The stirrer speed was set to 550rpm and the culturing temperature to 42°C. Filter sterilized oxygen-free nitrogen was passed over the medium at 0.2L min^{-1}. A sample (approximately 10mL) of growth medium was removed and the pH measured with a separate electrode and meter. The pH reading shown by the bioreactor control cabinet was adjusted to read the same value. The culturing pH was set to 6.0. The bioreactor was left in this state for 24 to 36h to ensure no contamination and to allow dissolved oxygen to leave the growth medium.

The culture was inoculated by first removing 150mL growth medium from the fermentation vessel via the sampling line. This volume was replaced with overnight shake flask culture of *L. delbreuckii* NRRL B-445 grown in MRS broth and introduced via a sterile syringe and hypodermic needle inserted through the septum of one of the vacant ports in the top end plate. The culture was immediately sampled by the removal of approximately 20mL culture into a sterile bottle on ice. After the sample had been removed

by exchanging the bottle with a fresh sterile bottle, the sampling line was voided with air from a syringe applied to the sterile filter of the sampling hood. This sample marked the start of the batch fermentation. Further samples of the culture were taken in the same way at hourly intervals until 12h had elapsed. A further sample was taken after 24h culturing. The culture samples were immediately analysed for biomass dry weight concentration (Section 2.3.1) and the supernatants stored at -20°C for subsequent analysis for glucose and lactic acid concentrations (Sections 2.3.2 and 2.3.3).

2.6 Chemostat Culture

2.6.1 Apparatus

The bioreactor and ancillary apparatus used for chemostat experiments were as described in Section 2.5.1. Additional ancillary apparatus included a growth medium line for continuous feed and a culture outflow line with a vertical weir above the culture level.

Growth medium (VR medium; Section 2.2) was pumped through silicone tubing from a reservoir by a triple-roller peristaltic pump (Watson-Marlow, Falmouth, Cornwall) acting on silicone tubing of either 1.6 or 3.2mm ID depending on the desired range of medium flow rate. A 10mL glass pipette topped by a 0.2µm filter (Acro 50; Gelman Sciences Ltd., Northampton) was included on a branch of the line and was used to measure the flow rate of growth medium as described in Section 2.6.2. The feed line was connected to the bioreactor by a glass anti-backgrowth chamber, blown with nitrogen, connected to the top end plate by a short length of PTFE tubing and a needle connector. The purpose of the PTFE tubing was to raise the anti-backgrowth chamber above the level of the top plate and to minimise cell adhesion with the aim of reducing backgrowth from the

fermentation vessel to the growth medium feed line. The anti-backgrowth chamber was siliconized by treatment with SurfaSil liquid (Pierce UK Ltd., Cambridge) for the same reason. The feed line was joined to the growth medium reservoir by stainless steel connectors with screw-threaded collars. Fresh medium was connected by aseptic transfer of connection from the used to the fresh reservoir.

The culture outflow line from the fermentation vessel consisted of a vertical weir above the culture level connected to a waste receiver reservoir via a needle connector and silicone tubing. Culture was pumped by a twin-roller peristaltic pump (Watson-Marlow) acting on silicone tubing of 4.8mm ID. The length of the weir tube was adjusted to give a working culture volume of 1.4L at a stirrer speed of 550rpm.

2.6.2 General Points

The growth medium, culture conditions and bioreactor sterilization procedure used were as described for batch culture (Section 2.5). All continuous cultures were inoculated and established as batch cultures as described in Section 2.5.2 and grown in this way for 12 to 18h before the continuous feed of growth medium was switched on. The flow rate of growth medium was measured by the rate of filling of a 10mL glass pipette on a branch of the line downstream of the medium pump. In this way the flow rate could be set to a particular value. Hence, the dilution rate (D) was defined as the growth medium flow rate, F(Lh^{-1}), divided by the working culture volume, V (1.4L). Sampling of the culture was performed as described for batch culture (Section 2.5.2).

2.6.3 Dilution Rate Profiles

The aim of these experiments was to construct a profile of the production of biomass and lactic acid and the consumption of glucose over a wide range of dilution rate, D. A more detailed profile of end-products, lactate dehydrogenase (LDH) activity and nutrient status at a number of dilution rates within this range was also constructed.

The profiles were constructed from three continuous cultures covering a range of D from $0.05h^{-1}$ to $0.50h^{-1}$ (approximately $0.1\mu max$ to approximately μmax from batch data). Biomass, lactic acid and glucose concentration data were obtained from steady state cultures at D = 0.05, 0.064, 0.10, 0.15, 0.20, 0.30, 0.35, 0.40, 0.45 and $0.50h^{-1}$; ethanol and acetate data at D = 0.05, 0.064, 0.10, 0.20, 0.30, 0.40 and $0.50h^{-1}$; LDH activity data at D = 0.064 and $0.35h^{-1}$; nutrient status data (supernatant carbon, nitrogen and phosphate) at D = 0.30 and $0.40h^{-1}$. Analyses were performed as described in Section 2.3.

On switching from batch to continuous culture and on changing D the culture was left for between 6 and 10 residence times before samples were taken. Sampling continued over two consecutive days or until a steady state was established. Steady state was defined as by Jin *et al.* (1983), that is less than 10% variation in biomass and lactic acid concentration over the sampling period.

2.6.4 Investigation of Growth Limitation

Steady states established at D = $0.15h^{-1}$ to $0.50h^{-1}$ were found to be glucose-excess (Chapter 5, Section 5.1). Attempts were made to clarify the nature of the growth limiting substrate in cultures within this range of dilution rates. Two approaches were taken; the first involved direct injection of concentrated nutrient solutions

into growing cultures, the second involved alterations in the composition of the growth medium with the culture allowed to establish a steady state after each alteration. The common aim of these approaches was to elicit growth stimulation by the addition of particular groups of nutrients. The nutrients used were: hydrolysates of casein, trace elements, vitamins, nucleosides, purines and pyrimidines. The effect of varying the feed concentration of yeast extract was also investigated. The concentrations of the nutrients used were chosen with reference to the basal medium of Ikawa and O'Barr (1956) and were 2 to 3 times those quoted in the latter study.

2.4.6.1 Direct Injection of Nutrients

The method used was based on that of Mateles & Battat (1974). Four groups of nutrients were used; two complex mixtures of amino acids from tryptic and acid hydrolysates of casein (acid hydrolysate was used at two concentrations), and two defined mixtures of vitamins.

The details of the procedure were as follows. A steady state culture was established at $D = 0.20h^{-1}$. Nutrient solutions were freshly prepared and sterilized as shown in Tables 2.3 and 2.4. One solution was aseptically taken up into a sterile syringe. The solution was then rapidly expelled into the culture via a hypodermic needle inserted through the rubber septum of a vacant port in the top end plate of the bioreactor. This point marked the start of the timing of the experiment. The culture was sampled after 2min and at approximately 8 to 10min intervals thereafter over a time course of 70 to 80min. At each sampling point, culture (10mL) was withdrawn via the sampling line and 0.1mL was introduced into triplicate polycarbonate cuvettes each containing 3.9mL phosphate buffer pH 7.0.

Table 2.3 Complex Amino Acid Sources Added in Direct Injection Experiments

Nutrient Solution	Concentration in Fermentation Vessel (gL^{-1})
Tryptone (Oxoid)	10
Casein Hydrolysate (Oxoid)	10
Casein Hydrolysate (Oxoid)	40

100mL solutions of each amino acid source were made and autoclaved at 121°C for 15min before injection

Table 2.4 Defined Vitamin Mixtures Added in Direct Injection Experiments

Mixture 1 (20mL volume)

Vitamin	Conc. in vessel (mg L^{-1})
Ca-Pantothenate	1.5
Riboflavin	1.5
Nicotinamide	1.5

Mixture 2 (10mL volume)

Vitamin	Conc.in vessel (mg L^{-1})
Ascorbic acid	600
Nicotinic acid	1.6
Riboflavin	1.6
Thiamine	0.8
p-amino Benzoic acid	0.8
Ca-Pantothenate	0.06
Folic acid	0.032
Cyanocobalamin	0.01
Pyridoxamine-5-PO_4	0.008
Biotin	0.004

Both mixtures were filter sterilized before injection

After mixing, the absorbance of the diluted culture at 640nm (A_{640}) was measured against a phosphate buffer blank.

Mean A_{640} values were calculated for each sample and plotted against time. This procedure was repeated for the other nutrient solutions with a 20 to 24h pause between injections to allow the effects of the previous injection to wash out.

2.6.4.2 Growth Medium Supplementation

These experiments involved the modification of the VR growth medium (Section 2.2) fed to a continuous culture at D = $0.20h^{-1}$. Steady state conditions were established with each modified medium and the biomass and lactic acid concentrations produced were compared with those from normal VR medium.

The first experiment investigated the effect of yeast extract feed concentration on steady state biomass and lactic acid concentrations. The yeast extract concentrations used were 7.5, 15, 25, 30, 35, 40 and $60gL^{-1}$ in otherwise normal VR medium. These media were fed to the bioreactor in turn and the culture allowed to come to steady state over 5 to 9 residence times. The baseline state of normal VR medium which contained $30gL^{-1}$ yeast extract was returned to several times. Culture samples were taken at each steady state and analysed for biomass and lactic acid concentration.

The second set of experiments involved the supplementation of VR medium with a wide range of nutrients. These supplementations are outlined in Table 2.5. The VR medium for experiment 1 (Table 2.5) was supplemented with tryptone powder (Oxoid Ltd., Basingstoke, Hants). before sterilization and then autoclaved in the usual way (Section 2.2). The VR medium for the other experiments was made up and sterilized in the usual way and then the supplement solution was filter sterilized and

Table 2.5 Supplementations to VR Medium

Expt. No.	Nutrients	Conc. in Medium Reservoir
1	Tryptone	$30gL^{-1}$
2	Cu^{2+}	4μM
	Ca^{2+}	20μM
	Co^{2+}	0.7μM
	MoO_4^{2-}	1.0μM
	Zn^{2+}	0.3μM
	I^-	0.6μM
	BO_3^{3-}	3.0μM
3	Thiamine	$1.2mgL^{-1}$
	Riboflavin	$1.5mgL^{-1}$
4	Uridine	$20mgL^{-1}$
	Thymidine	$20mgL^{-1}$
5	Adenine SO_4	$10mgL^{-1}$
	Uracil HCl	$10mgL^{-1}$
	Guanine HCl	$10mgL^{-1}$
6	Mn^{2+}	130μM

injected directly into the cooled medium through a rubber septum. Due to the lability of riboflavin in light (Pirt, 1975), the medium reservoir for experiment 3 was covered with black polythene and the medium line was wrapped in aluminium foil. The supplemented media were fed to the bioreactor in turn and the culture was monitored in the same way as in the yeast extract feed concentration experiment.

CHAPTER 3

DEVELOPMENT AND OPERATION OF THE CELL RECYCLE FERMENTER

This Chapter describes the objectives and stages of development of the partial cell recycle fermenter system, the design and operation of the developed system, and experiments performed using the latter system. A fermenter system for total cell recycle (R = 1.0) is also described.

3.1 Objectives and General Points

The objectives which guided the development of the cell recycle fermenter are presented in Table 3.1. Two developmental stages led to the final fermenter system. During this development most of the objectives listed in Table 3.1 were achieved successfully.

Several features were common to the preliminary and final systems. All cell recycle fermenter developments were based on the continuous culture fermenter described in Chapter 2 (Section 2.6). The cell separation device used throughout was an H1P100-20 hollow fibre ultrafiltration cartridge with a DH4 adaptor and Bourdon-type pressure gauges (Amicon Ltd., Stonehouse, Glos.). The H1P100-20 cartridge is shown in Figure 3.1. The cartridge consisted of 250 polysulfone hollow fibre filter elements of 0.5 mm ID having a nominal molecular weight cutoff of 100,000 Daltons. The fibres were mounted in a polysulfone cartridge shell and sealed in place at either end of the shell with epoxy resin. The cartridge was 20.2 cm in length and offered a total area for filtration of 550 cm^2. The hollow fibre cartridge operated on the principle of cross-flow filtration (Chapter 1, Section 1.3). Whole culture was passed down the lumen of the fibres generating a stream of cell-free filtrate

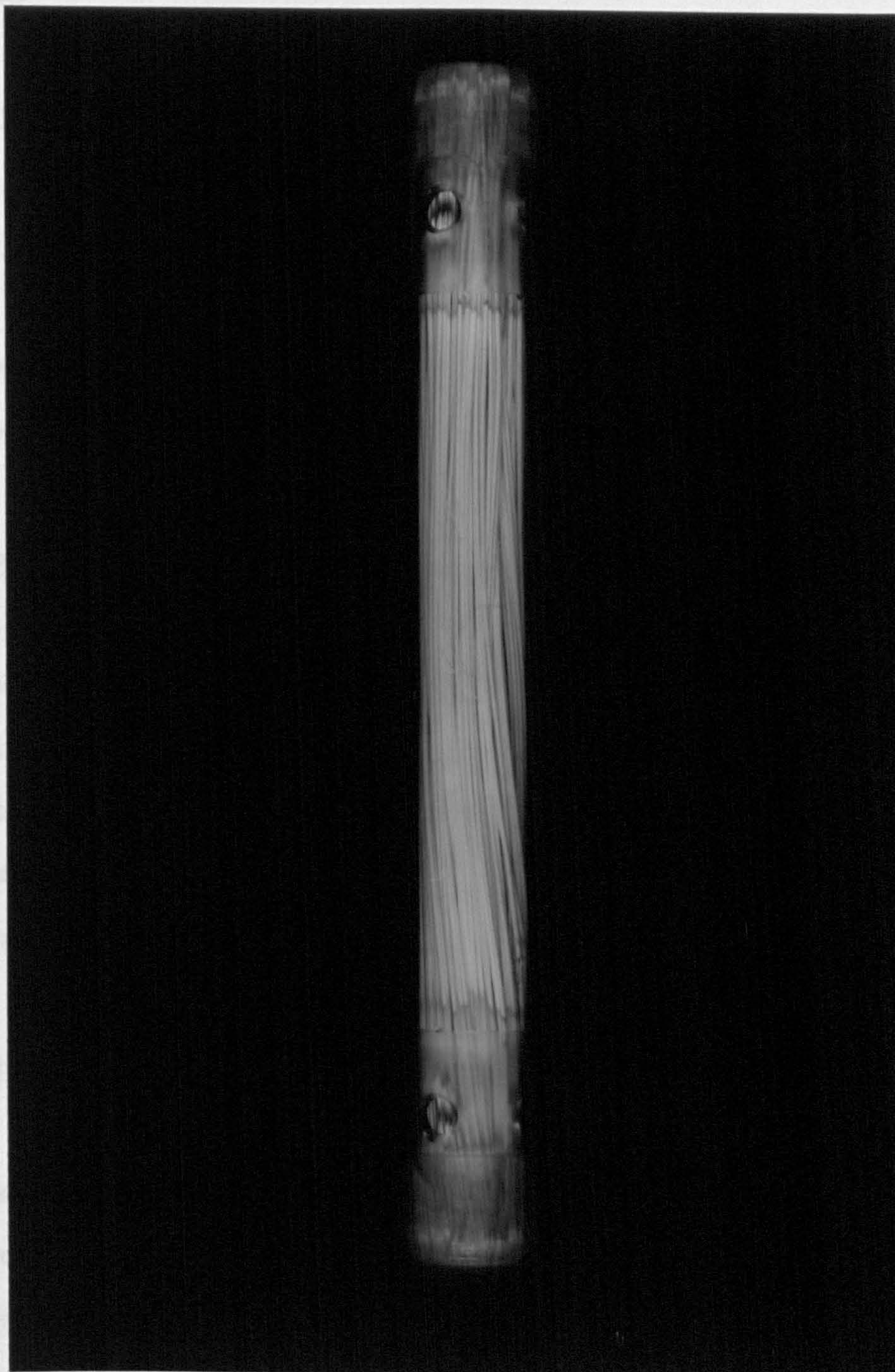


Figure 3.1 Amicon HIP100-20 hollow fibre filter cartridge (Length 20.2 cm)

Table 3.1

Objectives of Cell Recycle Fermenter System Development

(1) To achieve stable, defined recycle ratios

(a) To accurately measure filtrate flow rate.

(b) To accurately control filtrate flow rate.

(2) To reduce trauma to bacteria

(a) To reduce overall volume of recycle circuit.

(b) To reduce residence time in recycle circuit.

(c) To reduce pressure throughout system.

(d) To reduce pressure pulsing.

(e) To ensure efficient rinsing following chemical sterilization.

(3) To allow long-term operation

(a) To ensure efficient pre-run sterilization.

(b) To allow rapid mid-run cleaning if necessary.

(c) To reduce circulation pump cavitation and pulsing.

(d) To increase circulation pump tube life.

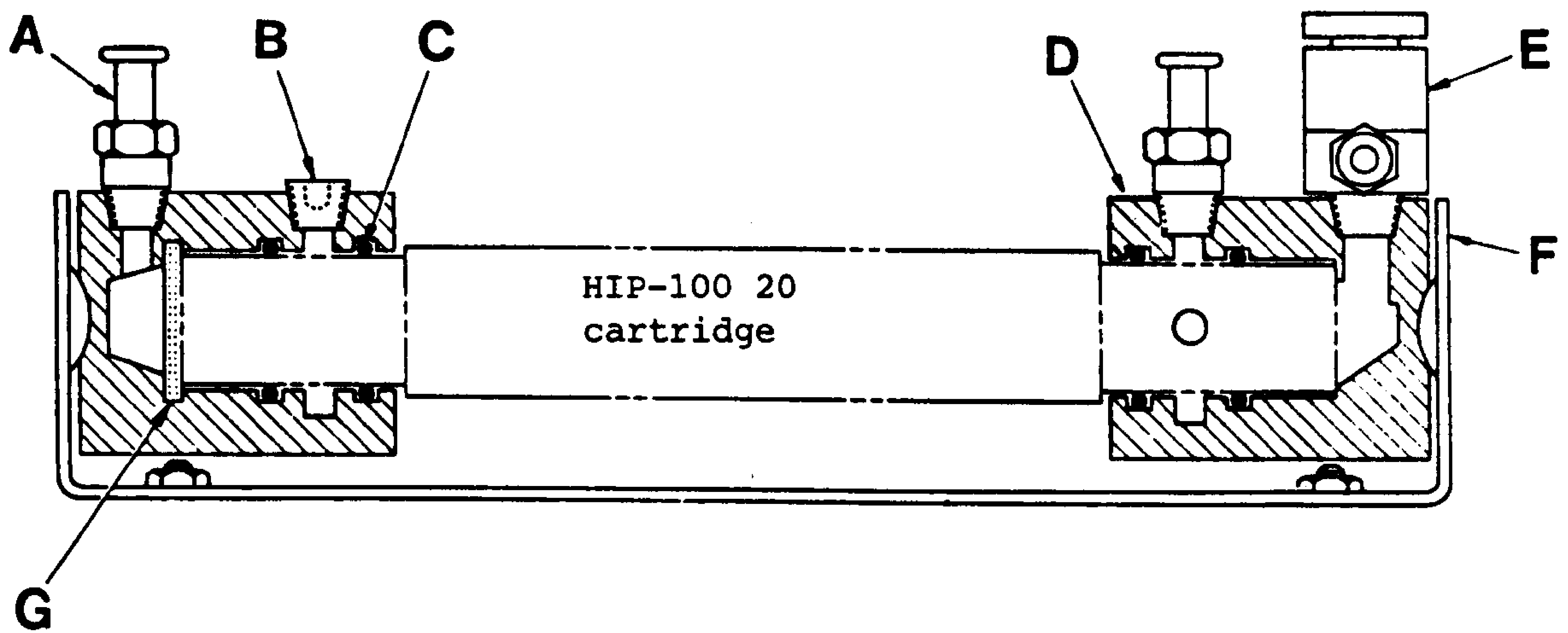
(e) To reduce foaming in fermentation vessel.

(f) To allow easy changing of prefilters.

which filled the space between the fibres and the cartridge shell and was drawn off via the holes at either end of the shell. The DH4 adaptor end pieces were constructed of PTFE and had threaded ports for tubing adaptors, diaphragm flow control valves, and gauges to measure culture inlet and filtrate outlet pressure (0 to 2 bar). The arrangement of the adaptors, valves and gauges on the end pieces was varied in the different systems. The H1P100-20 cartridge fitted into the DH4 adaptor as shown in Figure 3.2 and was held in place by clipping the H1p100-20/DH4 unit into a steel mounting bracket.

The H1P100-20 cartridge was autoclavable but in order to achieve greater operational flexibility, all systems were designed for *in situ* chemical sterilization and rinsing of the filter unit. The sterilizing solution used was 200 ppm sodium hypochlorite. This solution was circulated through the filter unit and ancilliary pipework before being returned to the reservoir from which it had been drawn. In this way, sterilizing solution could be continuously recirculated through the system. Rinsing of the system with sterile distilled water was operated on a total-loss basis; after being passed through the filter unit, the water was discharged into a separate waste receiver. In this way, the filter unit was continuously rinsed with fresh water and all traces of hypochlorite were washed to waste. The details of the sterilizing and rinsing procedures were varied as described below (Sections 3.2 and 3.3).

In each of the cell recycle fermenter systems developed, the total circulation was maintained by a single peristaltic pump. The particular mode of circulation (e.g. cell recycling, sterilizing, rinsing) being operated was defined by the opening and closing of particular screw clips acting on silicone tubing at junctions in the lines. This approach allowed great operational flexibility and the



A: Culture inlet

B: Blind plug

C: O-ring

D: DH4 end piece

E: Diaphragm valve on culture outlet

F: Steel mounting bracket

G: Polyethylene prefilter

Figure 3.2 Diagram of HIP-100 20 hollow fibre cartridge mounted in DH4 end pieces.

finally developed system could be operated in eight different modes of circulation. With the exception of the first stage development, the systems also used a peristaltic pump on the filtrate line to control accurately the rate of filtrate flow and hence the degree of recycle imposed.

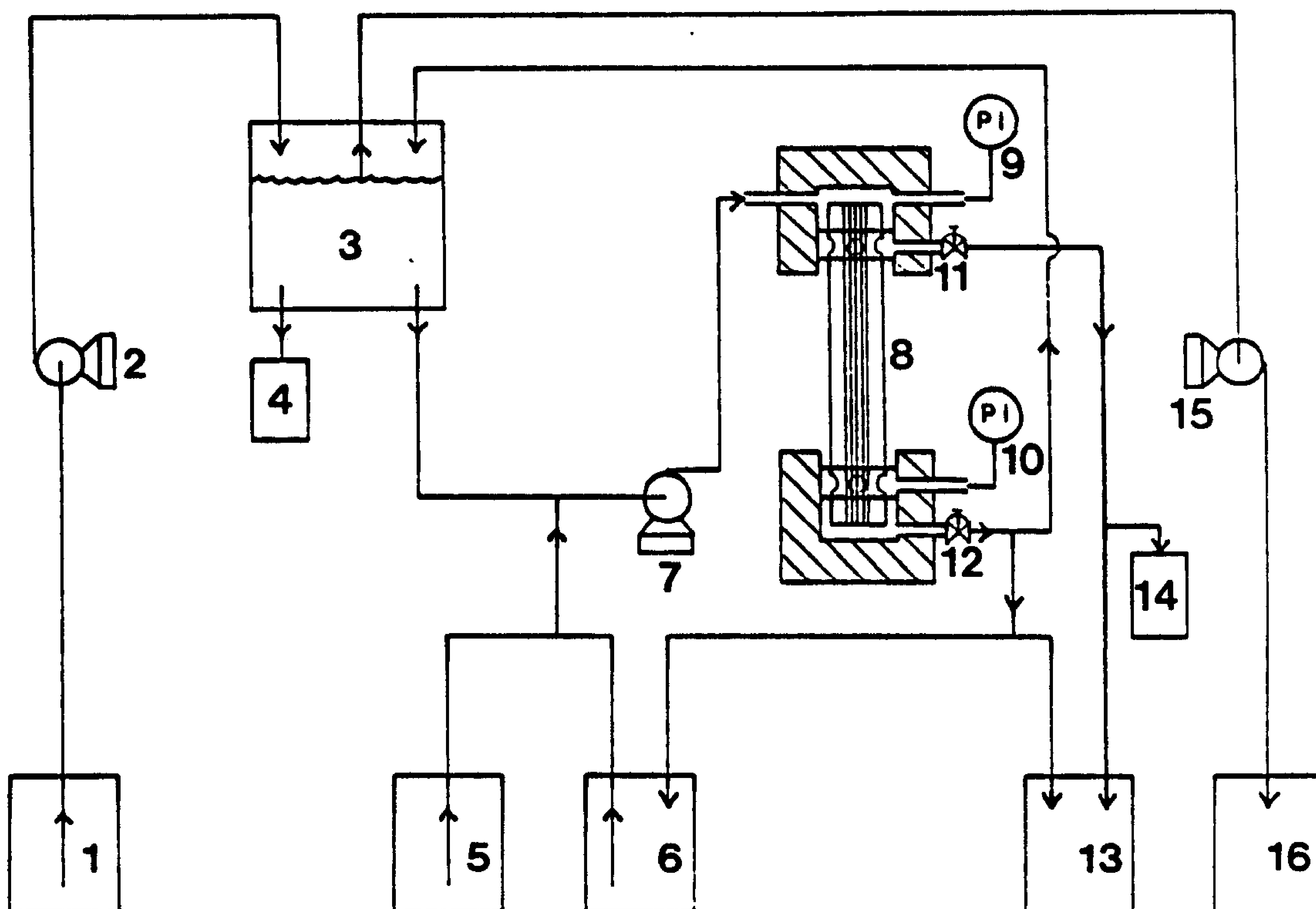
Following the manufacturer's recommendations, all culture streams that flowed through the H1P100-20 filter cartridge were pre-filtered to prevent blockage of the fibre lumens with particulate matter such as cell aggregations. During development, the prefilter was mounted inside the top end piece of the DH4 adaptor as supplied by Amicon Ltd. This arrangement gave rise to operational difficulties and in the final developed system it was replaced by a prefilter mounted in the culture stream between the circulation pump and the DH4 adaptor unit.

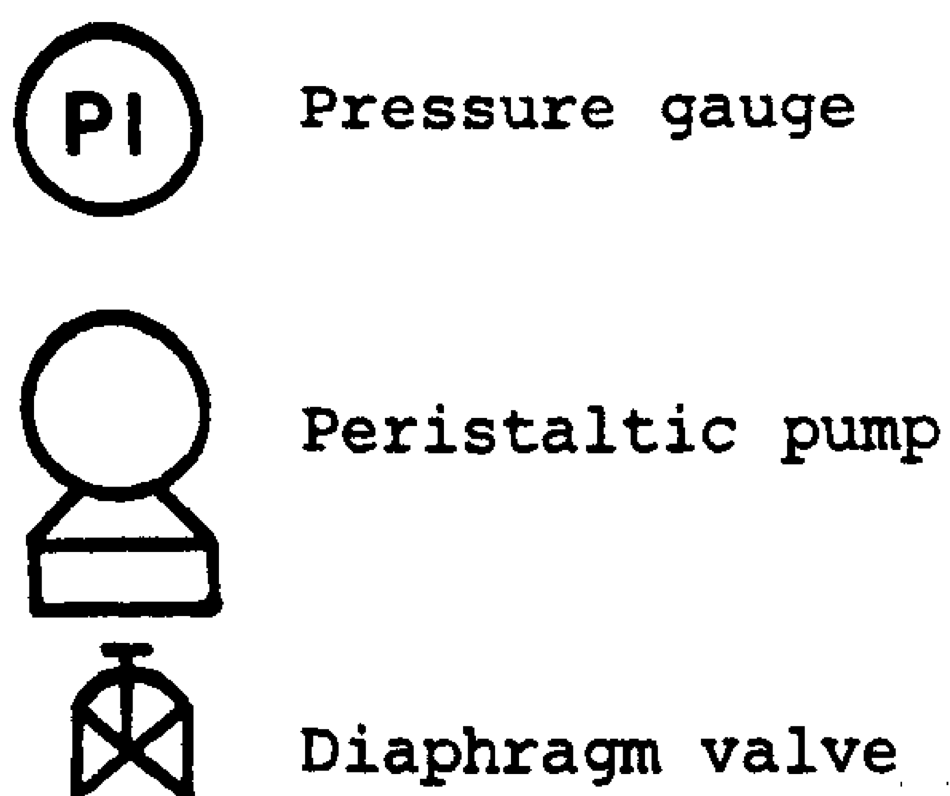
3.2 Developmental Fermenter Systems

3.2.1 First Stage Development

3.2.1.1 Main Features

A line diagram of the first stage development system is shown in Figure 3.3. Silicone tubing of 4.8 mm ID and 8.0 mm OD, was used throughout. The only pump in the recycle system was a 501 U/R twin-roller peristaltic pump with speed range 9 to 170 rpm (7 in Figure 3.3, Watson-Marlow, Falmouth, Cornwall). Diaphragm valves and pressure gauges were mounted on the DH4 end pieces so that the pressure at the culture inlet and filtrate outlet could be monitored and controlled (9,10,11,12). The filter unit (H1P100-20 joined to DH4) as set up for this system is shown in Figure 3.4. The flow rate of filtrate was controlled solely by balancing of the culture inlet and filtrate outlet pressures and no filtrate pump was used.





1. Growth medium reservoir
2. Growth medium pump
3. Fermentation vessel
4. Culture sampling point
5. Rinse water reservoir
6. Hypochlorite reservoir
7. Circulation pump
8. Hollow fibre filter unit
9. Culture inlet p ressure gauge
10. Filtrate pressure gauge
11. Filtrate flow control valve
12. Culture flow control valve
13. Filtrate/Rinse receiver
14. Filtrate sample point
15. Whole culture pump
16. Whole culture receiver

Figure 3.3 Line diagram of first stage development fermenter

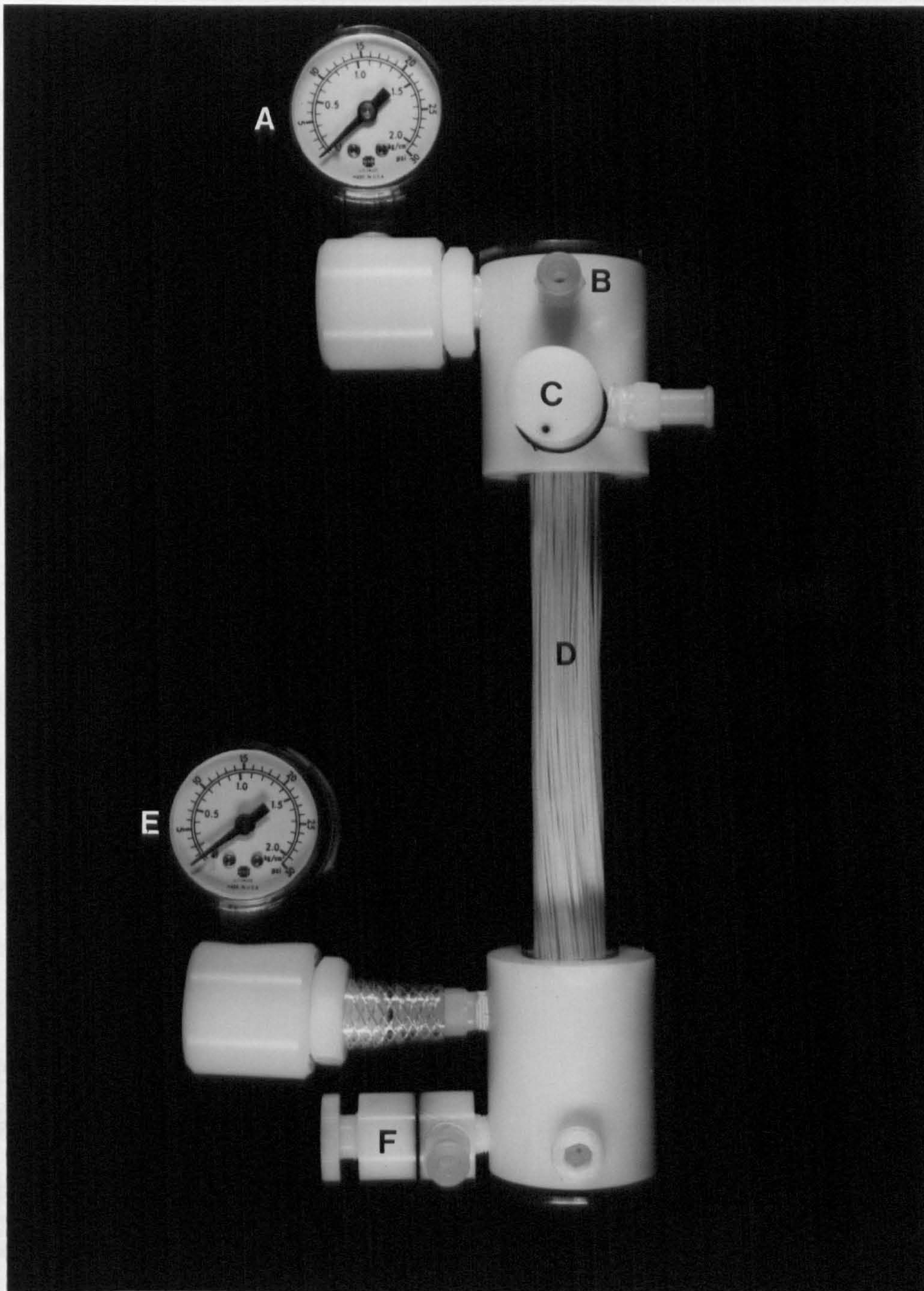


Figure 3.4 Filter unit configuration for first stage development

A: Culture inlet pressure gauge

B: Culture inlet

C: Filtrate outlet with back-pressure valve

D: Hollow fibre filter cartridge

E: Filtrate pressure gauge

F: Culture outlet with back-pressure valve

The filter unit was joined to the rest of the system by stainless steel connectors with screw collars in each line a short distance from the DH4 end units. The culture outflow and return lines terminated in stainless steel needle connectors for connection with the fermentation vessel by insertion through rubber septa held in ports in the bottom and top end plates respectively. The total volume of the cell recycle circuit was 125 mL giving a retention time within the circuit of 25s at a circulation rate of 0.3 L min^{-1}.

3.2.1.2 Sterilization and Operation

The hypochlorite and rinse reservoirs (20 L glass vessels) were filled with distilled water. The filled reservoirs, the rinse/filtrate receiver (20 L glass vessel) and all the lines were sterilized by autoclaving at 121°C for 30 min. When sterile, the system was joined to the filter unit and the needle connectors for culture outflow and return were aseptically joined to the fermentation vessel with the lines clipped off. Concentrated hypochlorite solution (agricultural grade, 40m L) was injected into the water in the hypochlorite reservoir and the diluted solution was circulated through the filter unit at 0.3 L min^{-1}. A stream of hypochlorite solution was drawn from the filtrate line to ensure sterility of the filtrate side of the unit. The circulation stream in the sterilization mode is shown in Figure 3.5. Hypochlorite solution was circulated in this way for 1 to 2 h and then the system was left overnight filled with the sterilizing solution.

The system was rinsed with autoclaved water pumped through at 0.3 L min^{-1}. The circulation stream in rinsing mode is shown in Figure 3.6. After being passed through the hollow fibre filter, the rinse water was discharged into a 20 L receiver. Water was drawn

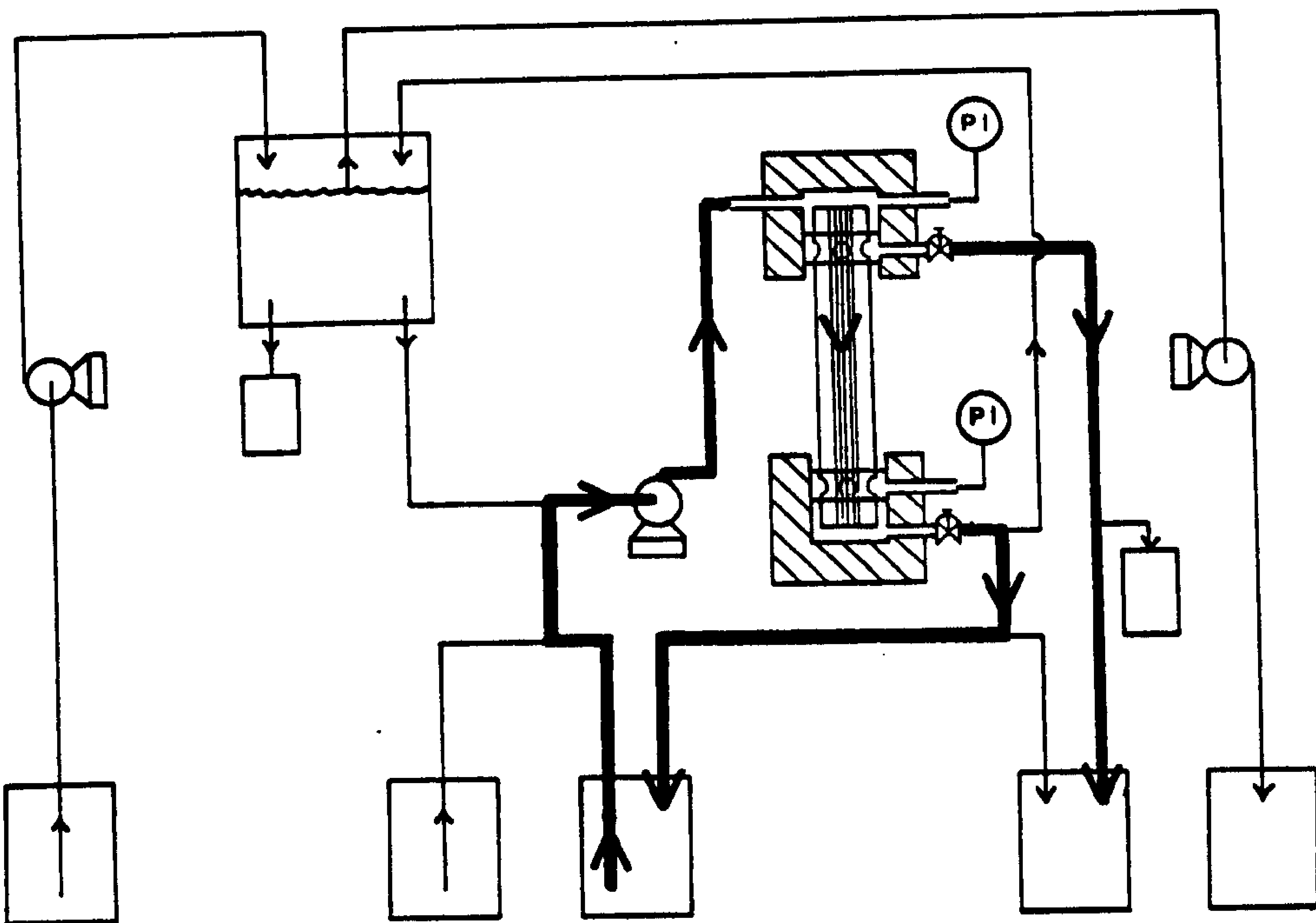


Figure 3.5 First stage development: Circulation in sterilization mode

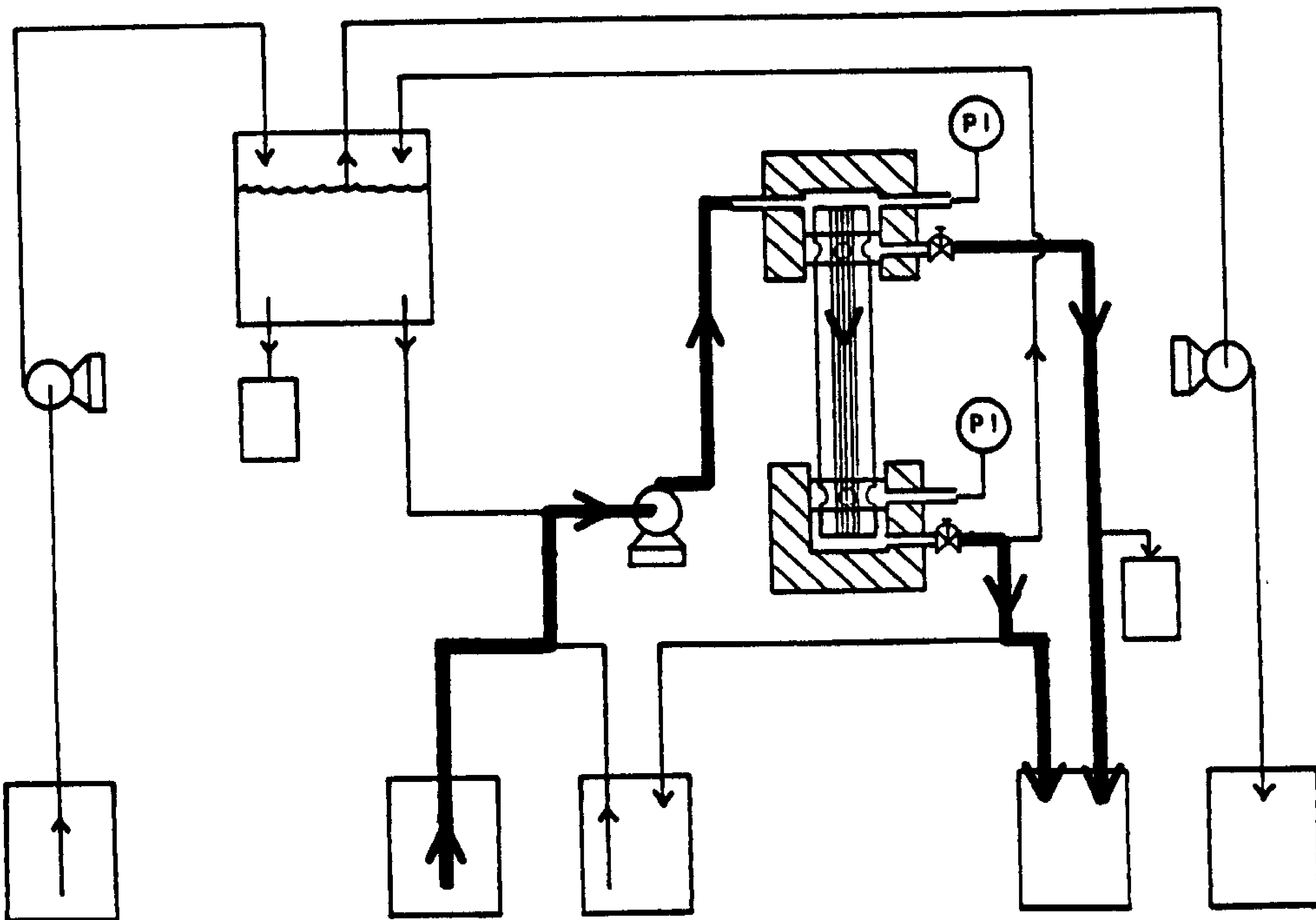


Figure 3.6 First stage development: Circulation in rinsing mode

through the filtrate line to remove hypochlorite from the filtrate side of the unit. This water was discharged into the same receiver. When 20 L rinsing water had been pumped through the filter, the water reservoir was aseptically exchanged for a full vessel which had been autoclaved at 121°C for 30 min. The receiver vessel was aseptically exchanged for a sterilized empty vessel and the rinsing was continued until the second 20 L volume had been pumped through.

When rinsing was complete, the sterilization system was isolated with screw clips and cell recycling was started. The circulation stream in cell recycle mode is shown in Figure 3.7. Culture was drawn from the bottom of the culture vessel and pumped through the filter unit at a rate of 0.3 L min^{-1}. The culture stream was controlled by a diaphragm valve at the culture outlet and the culture inlet pressure was monitored. The culture stream was fed back to the fermentation vessel entering via the top end plate. The stream of cell-free filtrate which was generated was controlled by a second diaphragm valve and the pressure in the filtrate side of the unit was monitored. The rate of filtrate flow was measured by directing the stream into a sampling line and measuring the volume of filtrate collected in sterile Universal bottles over three consecutive one minute periods. The rate of filtrate flow (and hence the recycle ratio) was controlled by altering the transmembrane pressure (Chapter 1, section 1.2.3). This latter was achieved by manipulation of the diaphragm valves at the culture and filtrate stream outlets from the filter unit. The filtrate stream was discharged into the rinse/filtrate receiver when the flow rate was not being measured.

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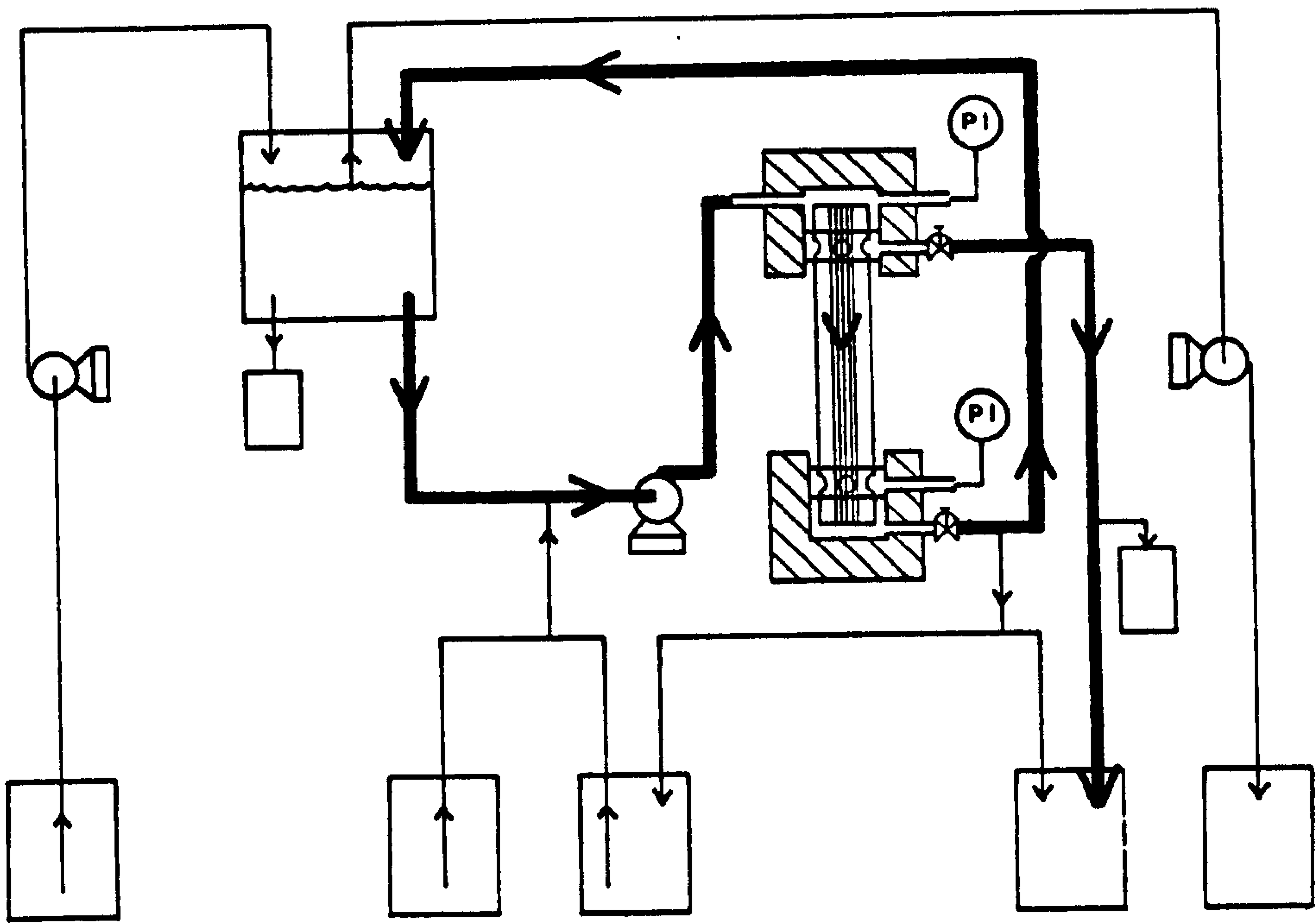


Figure 3.7 First stage development: Circulation in cell recycle mode

3.2.1.3 Comments on Performance

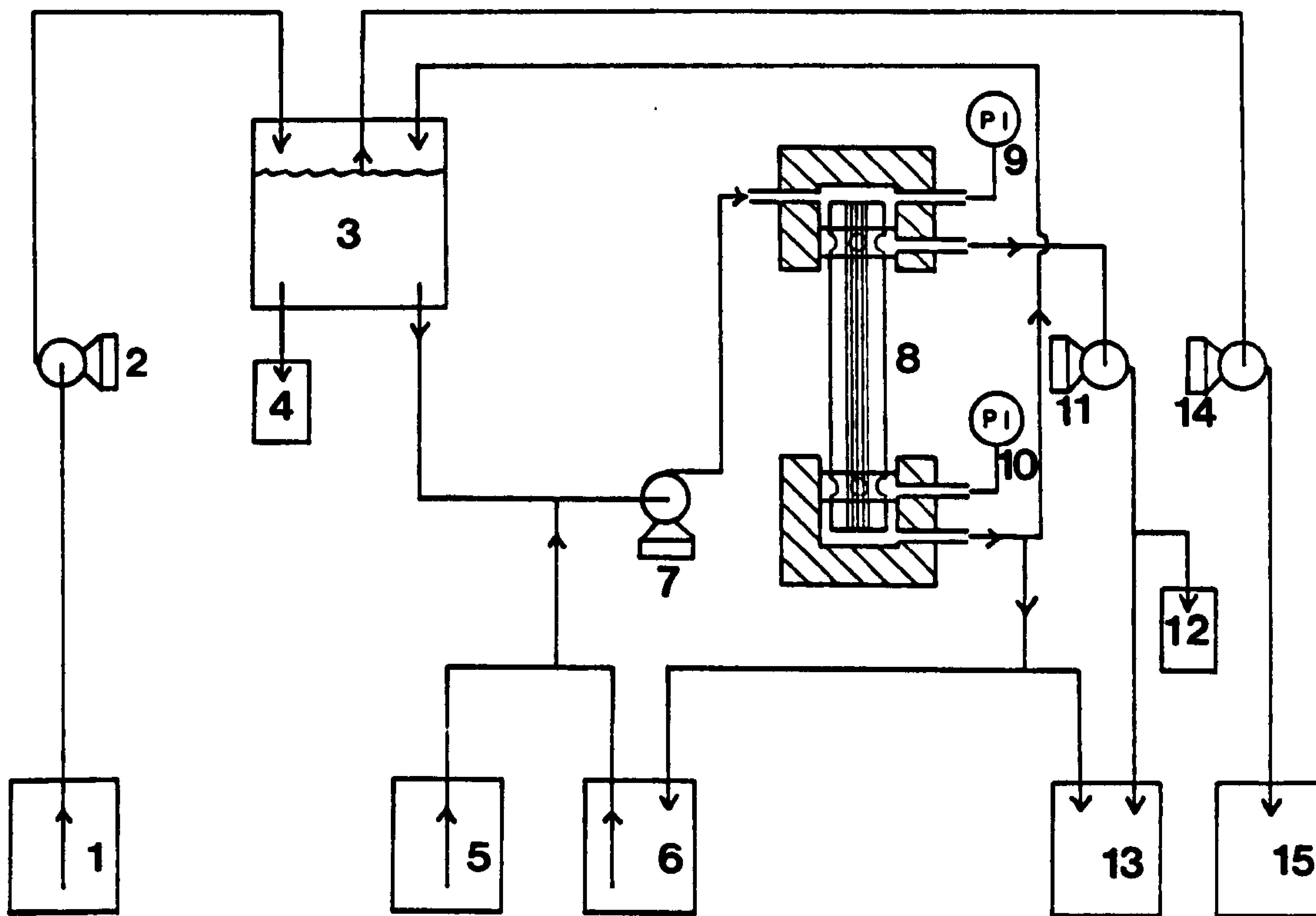
By reference to the objectives listed in Table 3.1, the first stage development proved to be inadequate in several important respects. The two main difficulties were in the control of filtrate flow rate and in culture pumping and pressure pulsing. The approach of controlling the rate of filtrate flow by simple manipulation of the transmembrane pressure was found to be ineffective in all but the very short term. A stable rate of filtrate flow could not be maintained overnight and often the flow ceased completely. The pumping of the circulating culture in this system presented several difficulties. Pump cavitation was severe due to the high pump head rotational speeds required to maintain the flow (approximately 120 rpm for 0.3 L min^{-1}). High overall pressure and pressure pulsing were produced. Pulsing from 0.5 to 0.8 bar occurred with each rotation of the pump and resulted in rapid wear of the pump tubing. The retention time of culture in the recycle circuit (25s) was unacceptably long.

Despite these difficulties, the first stage system contained features which were retained in subsequent developments. The use of a single pump for sterilizing, rinsing and cell recycling operations, with switching between these modes by changing of screw clip patterns, was found to offer great flexibility and potential for further development. The method of sampling and measuring the rate of filtrate flow was found to be practicable and was retained.

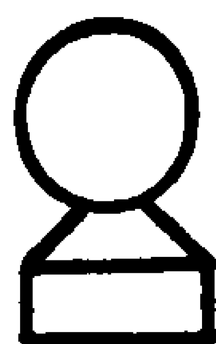
3.2.2 Second Stage Development

3.2.2.1 Main Features

The second stage system was developed to overcome the high speed pressure pulsing and instability of filtrate flow rate produced in the first stage development. A line diagram of the second stage development is shown in Figure 3.8.



Pressure gauge


Peristaltic pump

1. Growth medium reservoir
2. Growth medium pump
3. Fermentation vessel
4. Culture sampling point
5. Rinse water reservoir
6. Hypochlorite reservoir
7. Circulation pump
8. Hollow fibre filter unit
9. Culture inlet pressure gauge
10. Filtrate pressure gauge
11. Filtrate pump
12. Filtrate sample point
13. Filtrate/Rinse receiver
14. Whole culture pump
15. Whole culture receiver

Figure 3.8 Line diagram of second stage development fermenter

A large twin-roller peristaltic pump (7 in Figure 3.8) (601S; Watson-Marlow, Falmouth, Cornwall) was used to circulate culture and cleaning solutions through the hollow fibre unit (8). This pump allowed the use of larger bore tubing with thicker walls thereby producing longer tube life, higher flow rates, and lower pump speeds. 'Marprene' tubing of 12.5 mm ID and 19.5 mm OD (Watson-Marlow) was used to deliver 0.75 L min^{-1} at a pump speed of 25 rpm. The culture outflow line was modified to reduce pulsing and resistance to flow. Silicone tubing of 6 mm ID and 10 mm OD was used from the fermentation vessel to the pump and nylon reinforced pressure tubing of 10 mm ID and 16 mm OD from the pump to the hollow fibre filter unit. The screw connectors used in the first stage development were replaced with short lengths of stainless steel tubing of 8 mm ID and 10 mm OD which were pushed into the pressure tubing to make a connection. On the culture return line, the diaphragm valve was replaced by a simple tubing adaptor and the culture was carried back to the top end plate of the fermentation vessel by silicone tubing of 6 mm ID and 10 mm OD. The diaphragm valve was also removed from the filtrate line. The filter unit configuration for this system is shown in Figure 3.9.

In order to achieve stable and reproducible filtrate flow rates, a multi-roller peristaltic pump (11 in Figure 3.8) (Eyela; MBR Bioreactor Ltd., High Wycombe, Bucks.) was used to draw filtrate from the filter unit. The filtrate pump acted on silicone tubing of 1.6 mm ID and 4.8 mm OD. The volume of the cell recycle circuit in this system was 0.175 L giving a retention time of 10s at 0.75 L min^{-1} circulation.

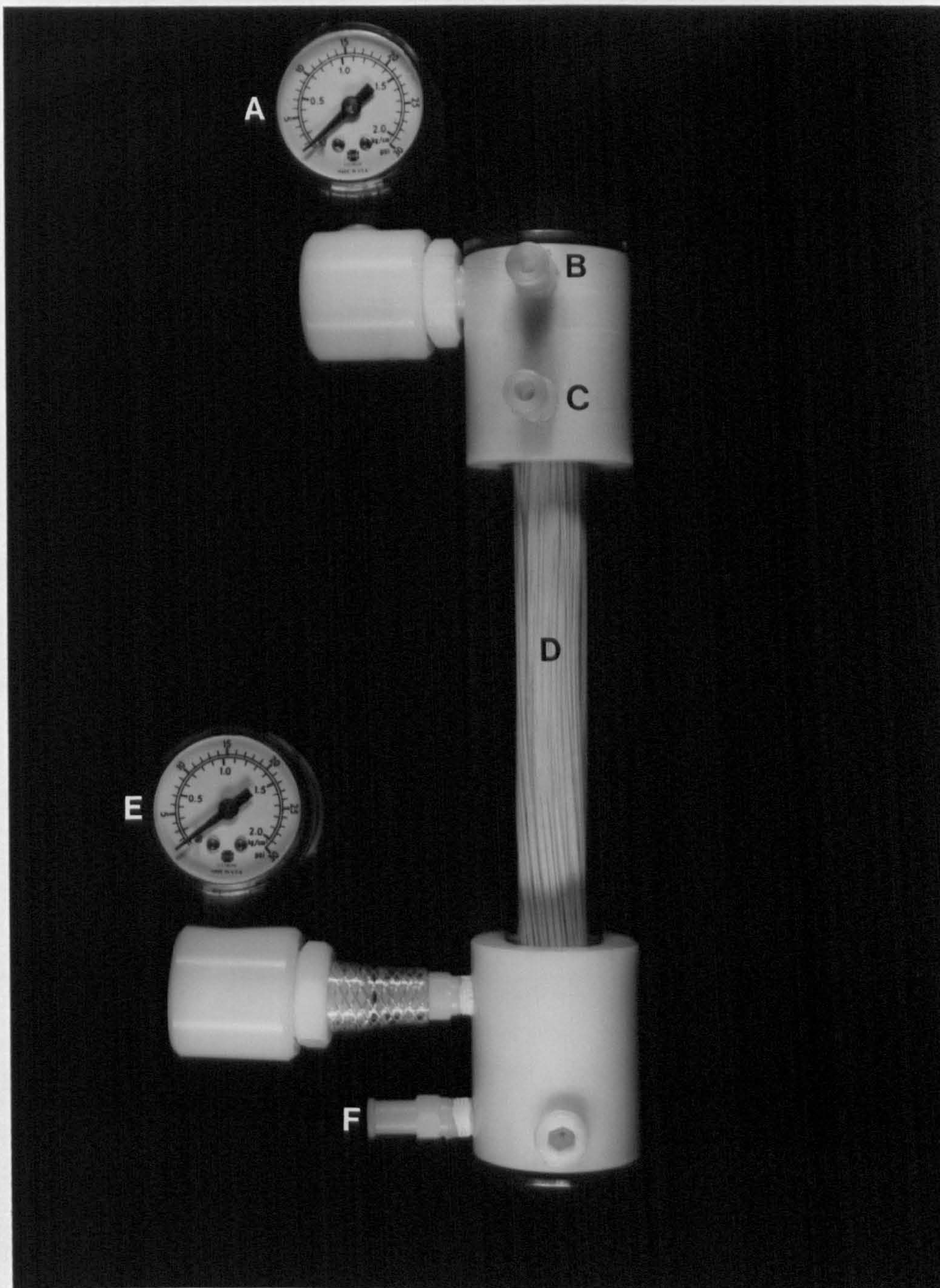


Figure 3.9 Filter unit configuration for second stage development

A: Culture inlet pressure gauge

B: Culture inlet

C: Filtrate outlet

D: Hollow fibre filter cartridge

E: Filtrate pressure gauge

F: Culture outlet

3.2.2.2 Sterilization and Operation

Sterilization and rinsing were performed in the same way as for the first stage development system except that the filtrate pump was used to draw hypochlorite solution and water through the filtrate side of the filter in the second stage development system. Circulation in the sterilization and rinsing modes is shown in Figures 3.10 and 3.11 respectively.

During the cell recycling mode, culture was pumped through the filter unit at 0.75 L min^{-1}. The pressures at the culture inlet and filtrate outlet were monitored but not controlled. Filtrate was drawn from the filter unit by the filtrate pump. The rate of filtrate flow was measured as in the first stage development (Section 3.2.1.2) and adjusted to give a particular recycle ratio by manipulation of the filtrate pump speed. The circulation in cell recycling mode is shown in Figure 3.12.

3.2.2.3 Comments on Performance

The second stage development proved to be a significant improvement on the first stage. Overall pressure and pressure pulsing were reduced (average pressure 0.25 bar with pulsing 0.15 to 0.35 bar) and stable, reproducible filtrate flow rates were achieved. However, in thus enabling cell recycling for longer periods, problems associated with longer term operation were revealed.

Some circulation pump cavitation was still present, caused by restriction of flow of culture from the fermentation vessel via the 3 mm ID needle connector. The stream of culture returning to the fermentation vessel via the top plate caused foaming at the culture level, a problem not encountered in the first stage development due to the lower circulation rate used.

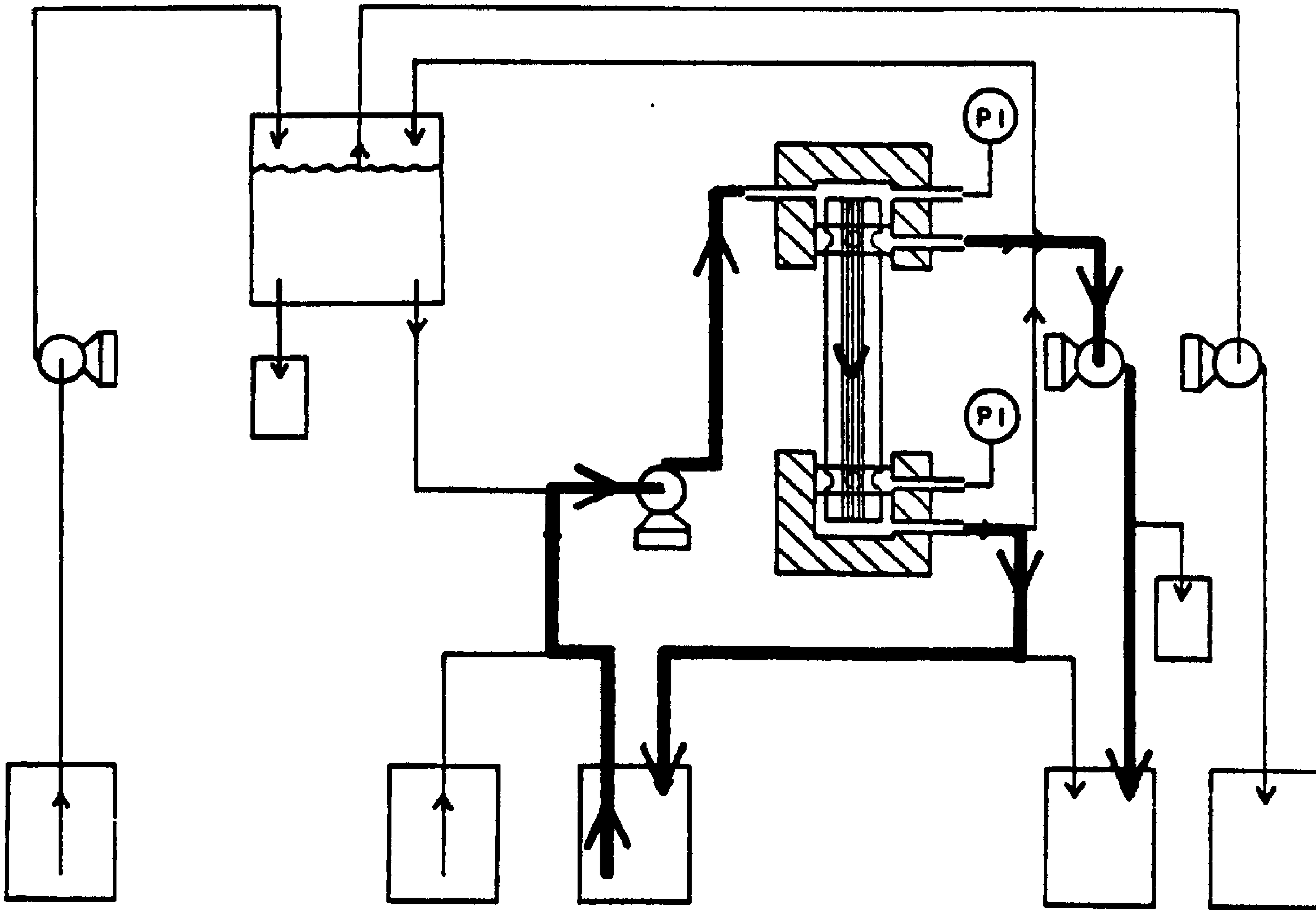


Figure 3.10 Second stage development: Circulation in sterilization mode

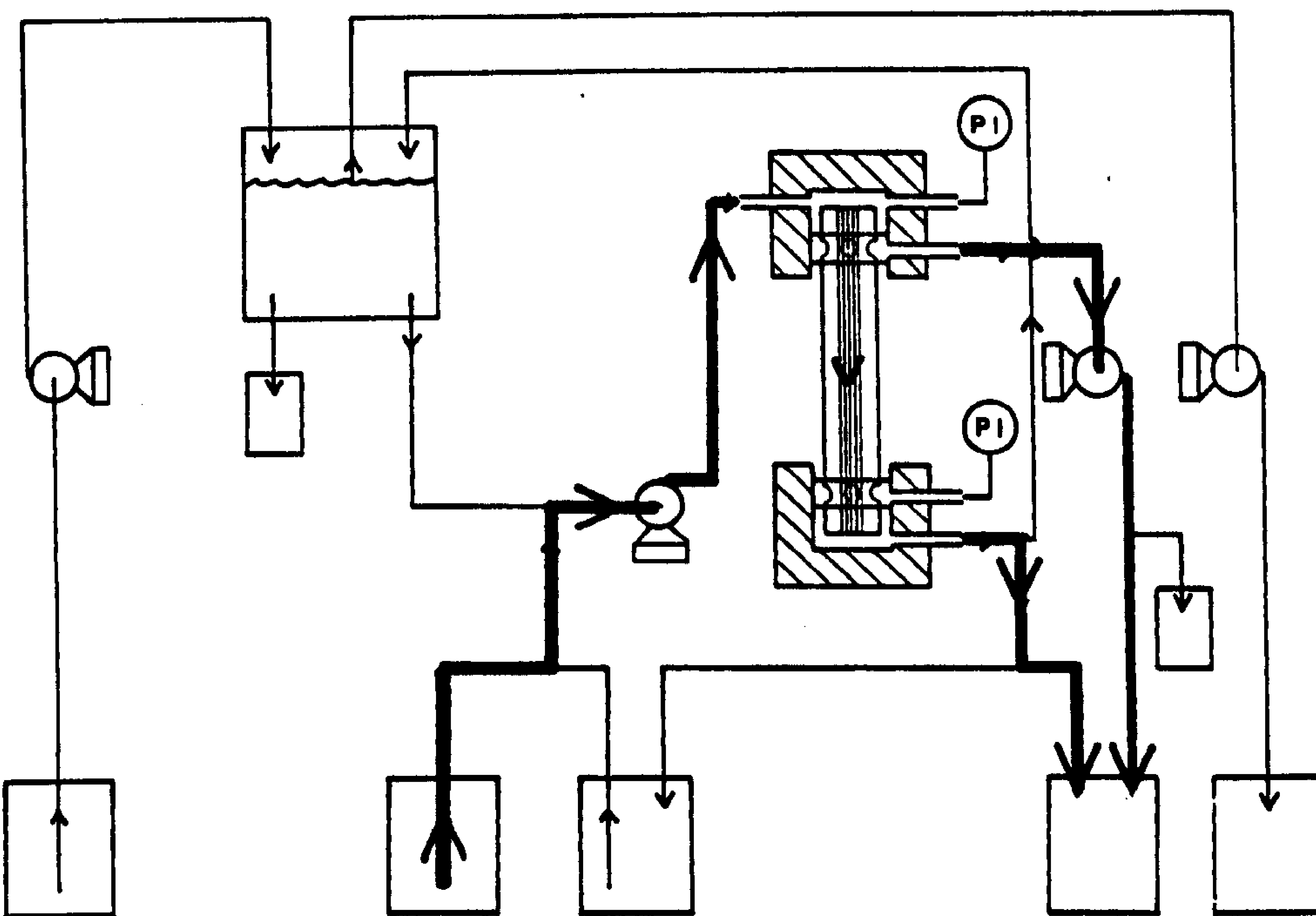


Figure 3.11 Second stage development: Circulation in rinsing mode

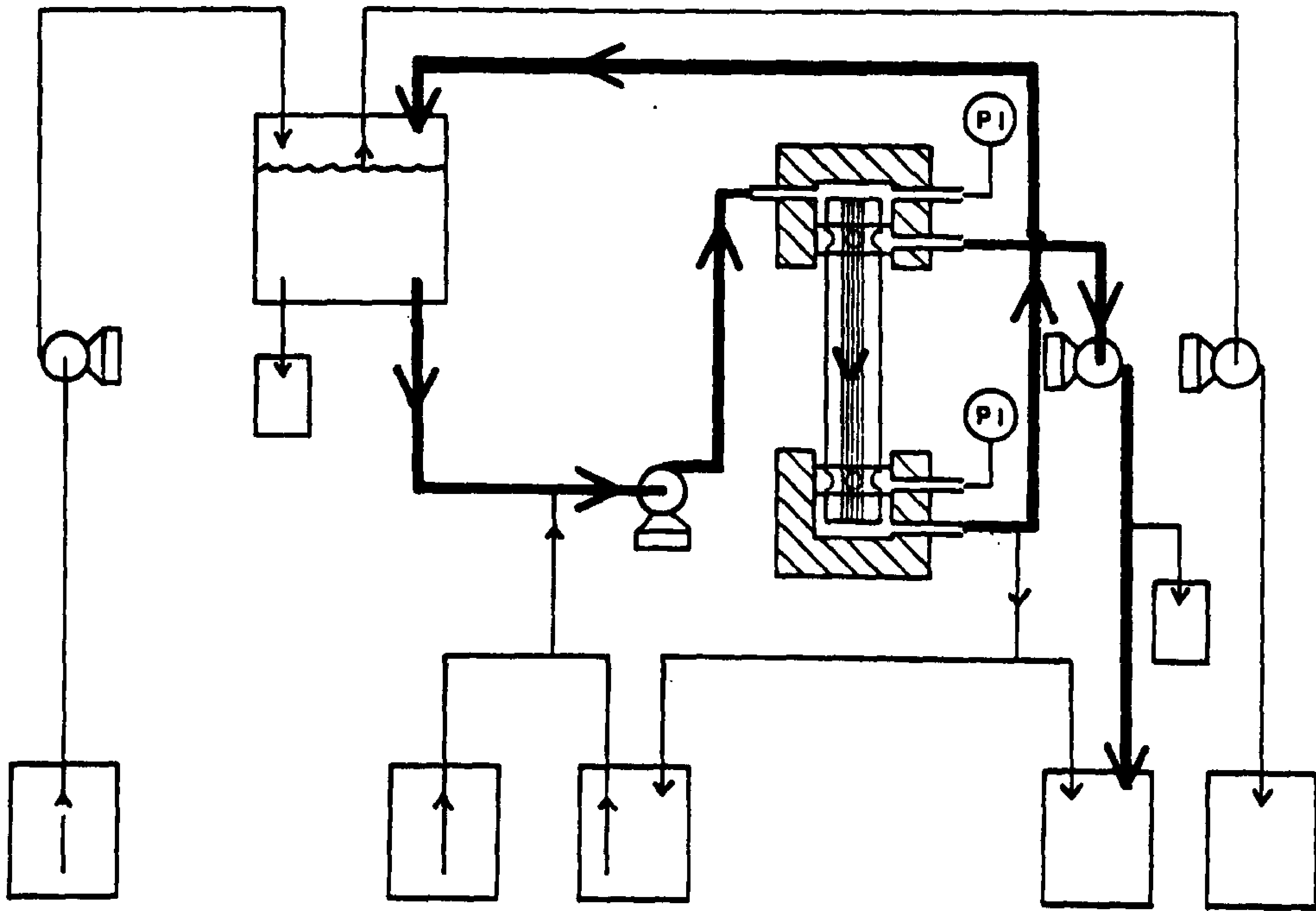


Figure 3.12 Second stage development: Circulation in cell recycle mode

As biomass was concentrated in the circulating stream, the prefilter in the top end DH4 unit became blocked. The culture inlet pressure increased towards the maximum permitted for the hollow fibre cartridge (1.725 bar) within 24 h circulation of biomass concentrations greater than approximately 10 g L^{-1}. When the prefilter was changed, normal inlet pressure (0.15 to 0.35 bar) was restored immediately but the new prefilter also became blocked within 24 h. The procedure necessary to change the prefilter was unsatisfactory. The circulation was stopped and the top end DH4 unit was pulled from the H1P100-200 cartridge to give access to the blocked prefilter disc. This procedure caused considerable spillage of culture and could not be performed aseptically. The prefilters were expensive and had to be discarded once blocked.

After 48 to 60 h recycling, microbial growth could be seen around the fibres in the filtrate side of the cartridge. This growth was removed by operating the system in the sterilization mode with the filtrate pump set to full speed to draw as much hypchlorite as possible through the filtrate side of the cartridge. However, when recycling was restarted after rinsing, growth reoccurred around the fibres. This suggested that the simple permeation of sterilizing solution through the fibres into the filtrate side of the cartridge did not produce adequate sterilization.

The system did not fail safe when the growth medium supply was interrupted. Circulation of culture continued and the filtrate pump continued to draw filtrate, resulting in intense concentration of the culture and a fall in the culture level, until the probes and heater elements in the fermentation vessel were exposed. The intensely concentrated culture caused severe blockage of the hollow fibre cartridge.

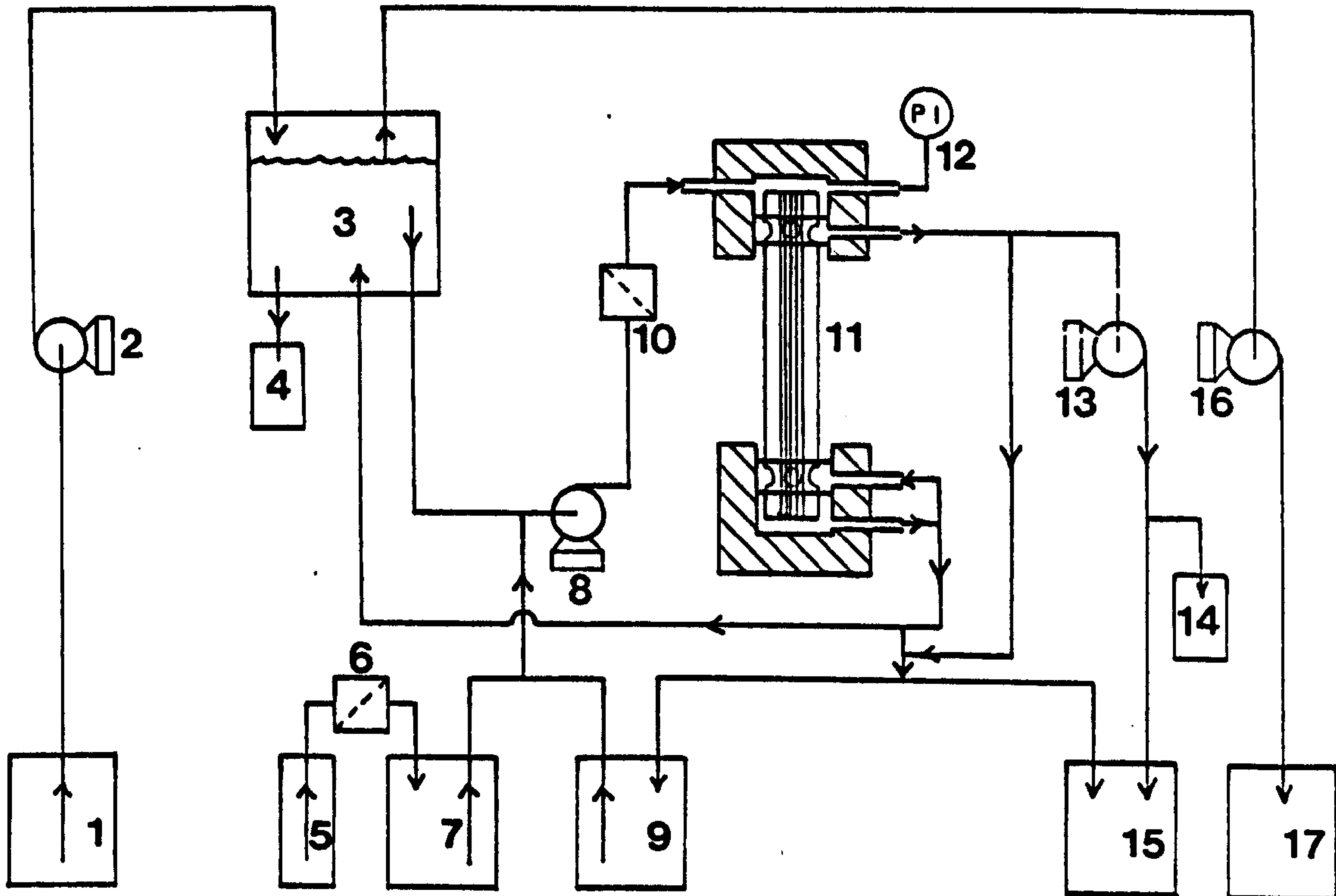
3.3 Final Developed System (R < 1.0)

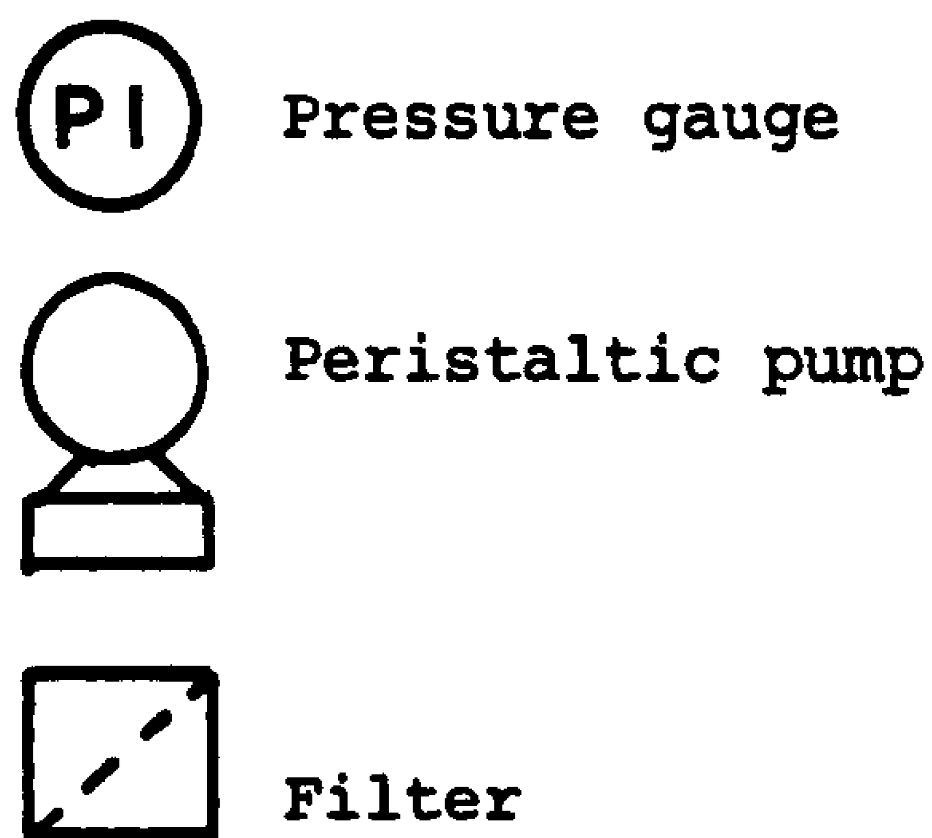
3.3.1 Main Features

The final partial cell recycle fermenter system was closely based on the second stage development and included improvements to the latter system in sterilization, rinsing, culture circulation and prefiltration. A line diagram of the final developed system is shown in Figure 3.13. A photograph of the system is shown in Figure 3.14.

An additional mode of circulation for sterilization and rinsing was developed to prevent microbial growth around the filter fibres. This mode, counter-current flow, involved redirecting the full flow passing down the lumen of the hollow fibres back up the outside of the fibres within the filtrate side of the cartridge. This circulation produced vigorous flushing of the filtrate side and is described in detail below (Section 3.3.2.1). In addition, an improvement was made to the supply of sterile water for rinsing. In the development stage systems, water was sterilized by autoclaving in 20 L volumes which were connected in turn to the rinsing line. In the final developed system, a stainless steel pressure vessel (5 in Figure 3.13, not shown in Figure 3.14) (Gelman Sciences Ltd., Cambridge) was used to pass water through a sterile filter (6) (Mini Capsule; Gelman) into a sterilized rinse reservoir. In this way a continuous supply of sterile rinsing water was provided.

The outflow of culture from the fermentation vessel was modified to reduce cavitation of the circulation pump and to allow the system to fail safe in case of interruption of the supply of growth medium. The needle connector on the culture outflow line was replaced by a 10 cm length of stainless steel tubing of 8 mm ID and 10 mm OD mounted in a silicone rubber bung in the bottom end plate of the fermentation vessel. The tube formed a submerged weir opening approximately 5 cm





1. Growth medium reservoir
2. Growth medium pump
3. Fermentation vessel
4. Culture sampling point
5. Pressure vessel - non sterile H_2O
6. Water filter
7. Rinse water reservoir
8. Circulation pump
9. Hypochlorite reservoir
10. Prefilter
11. Hollow fibre filter unit
12. Culture inlet pressure gauge
13. Filtrate pump
14. Filtrate sample point
15. Filtrate/Rinse receiver
16. Whole culture pump
17. Whole culture receiver

Figure 3.13 Line diagram of final developed system

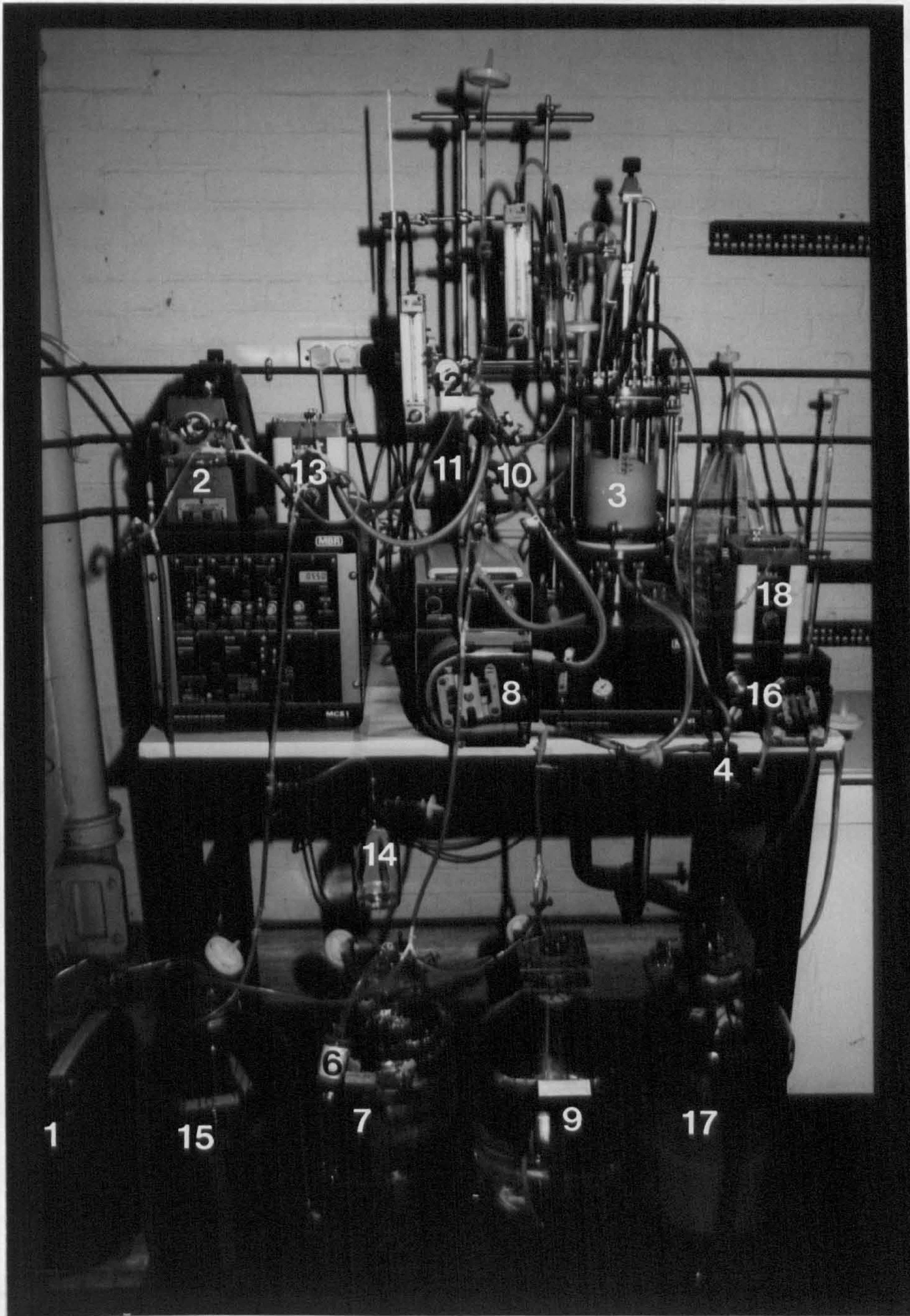


Figure 3.14 Photograph of final developed system

1: Medium reservoir
2: Medium pump
3: Fermentation vessel
4: Culture sampling point
5: Rinse water pressure vessel - not shown
6: Rinse water filter
7: Rinse water reservoir
8: Circulation pump
9: Hypochlorite reservoir
10: Culture prefilter
11: Hollow fibre filter
12: Inlet pressure gauge
13: Filtrate pump
14: Filtrate sampling point
15: Rinse/filtrate receiver
16: Culture overflow pump
17: Culture receiver
18: Alkali pump

below the culture level. This development greatly reduced the resistance to flow and allowed the cell recycle system to fail safe as culture could not be drawn below the level of the weir. Thus the potential fall in culture volume was limited, leaving the hollow fibre unit empty and the probes and heater elements covered. To reduce foaming, the culture stream was returned via a submerged needle convector in the bottom end plate of the fermentation vessel.

To avoid changing blocked prefilters in the top DH4 adaptor end piece, a sintered glass disc prefilter (10) was mounted in the culture supply line between the circulation pump and the hollow fibre unit. The sintered glass disc (Pyrex, 40 mm diameter, porosity 1; Scientific Furnishings Ltd., Chichester, Sussex) was enclosed in a rubber O-ring and mounted in a Swinnex filter disc holder (47 mm; Millipore (UK) Ltd., Harrow, Middx.) connected into the culture supply line. The sintered glass prefilter had the advantages of a larger area than the normal prefilter and changing of the prefilter could be performed rapidly and aseptically by unscrewing the filter holder to gain access to the disc. Used glass prefilters were cleaned for further use by soaking in 5% (v/v) Lipsol laboratory detergent.

The circulation pump was operated at 25 rpm delivering 0.75 L min^{-1} flow. The total volume of the cell recycle circuit was 175 mL (11% of the total fermenter volume) giving a retention time of 10 s.

3.3.2 Modes of Circulation

3.3.2.1 Sterilization and Rinsing

The sterilization and rinsing of the system was performed using two different patterns of circulation; permeate flow and countercurrent flow.

(a) Permeate Flow

The permeate flow modes of sterilization and rinsing were essentially those described for the second stage development (Section 3.2.2.2) and were characterised by the passage of solutions through the pores in the hollow fibre walls. Permeate flow sterilization mode was a closed loop circulation from which hypochlorite was drawn through the hollow fibre walls by the filtrate pump. Circulation in permeate flow sterilization mode is shown in Figure 3.15. This mode of circulation was used to ensure that the filter pores were clear and to sterilize the downstream parts of the filtrate line.

Permeate flow rinsing mode was a total-loss circulation with continuous filter sterilizing of water to fill the rinse reservoir. Water was drawn through the pores in the hollow fibre walls and rinsed the filtrate line. Circulation in permeate flow rinsing mode is shown in Figure 3.16.

(b) Counter-Current Flow

The counter-current flow modes of circulation were characterised by the redirection of flow which had been passed down the lumen of the hollow fibres back up the outside of the fibres via a by-pass loop attached to the filter unit; the unit as set up for the final developed system is shown in Figure 3.17. The flow passed out of the filter unit via the filtrate line and was redirected back to the sterilizing and rinsing system via a second bypass loop.

As with the permeate flow modes, the counter-current sterilization mode was a closed loop circulation and the counter-current rinsing mode was a total-loss circulation. The circulation for the latter modes are shown in Figures 3.18 and 3.19 respectively.

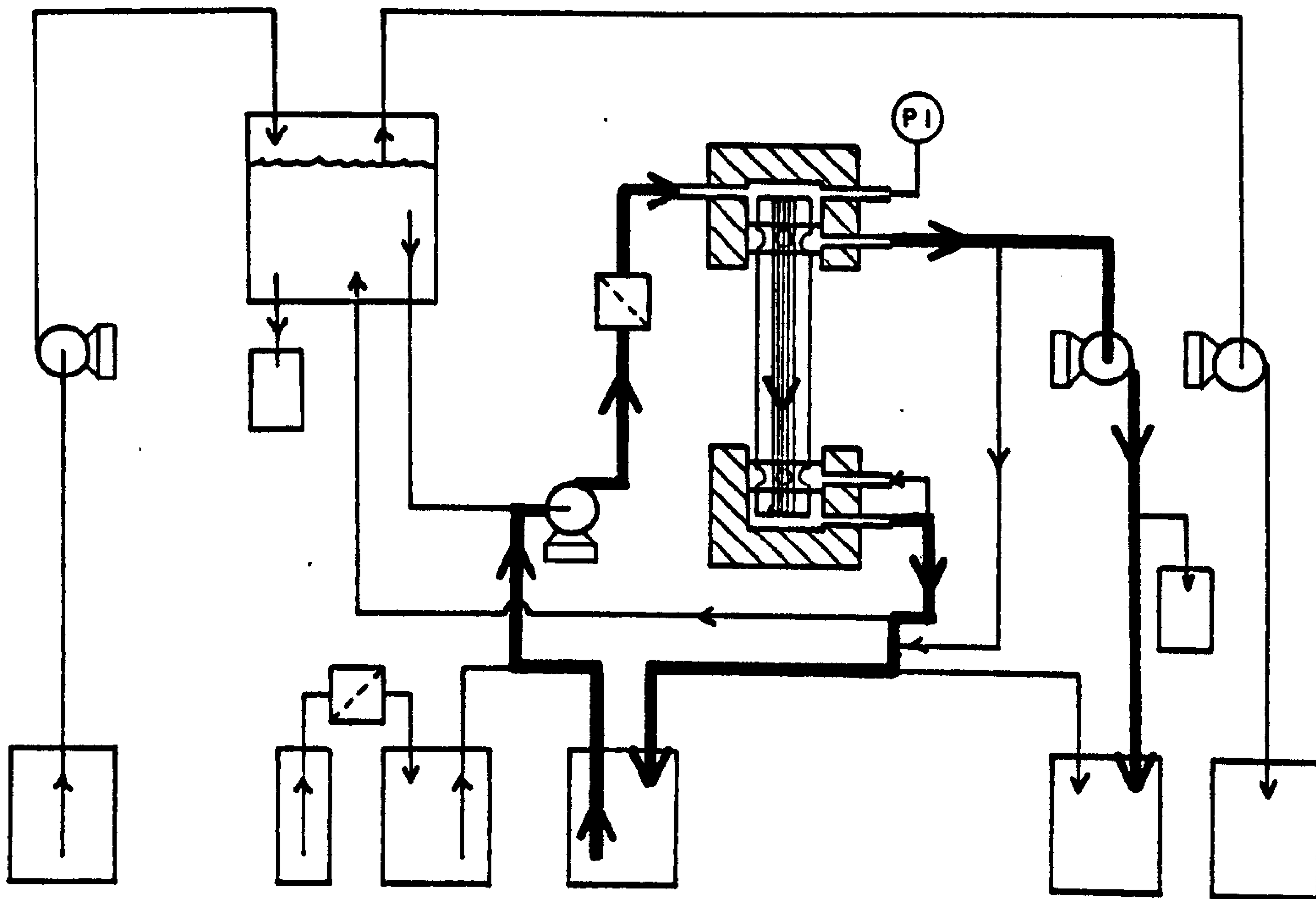


Figure 3.15 Final developed system: Circulation in permeate-flow sterilization mode

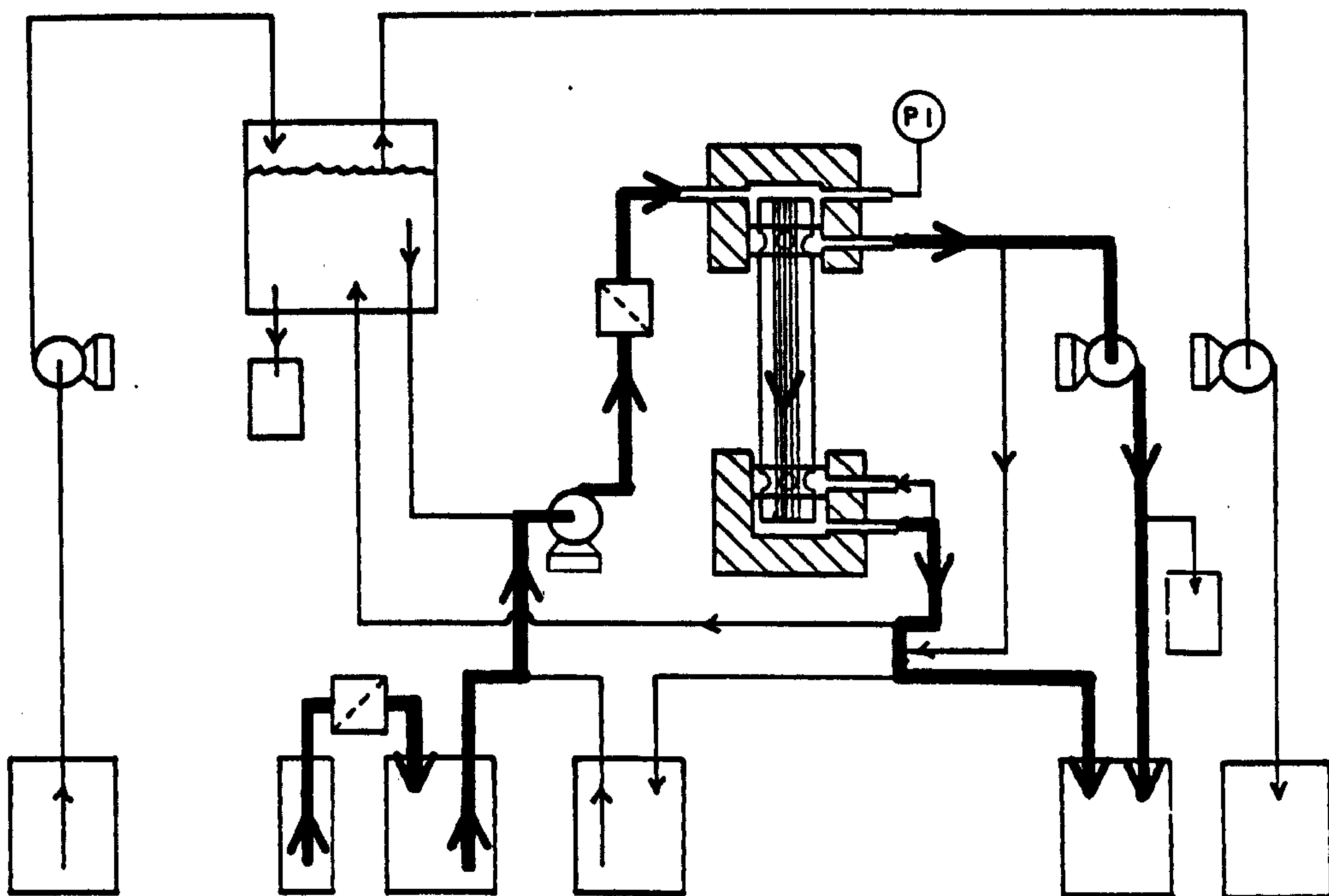


Figure 3.16 Final developed system: Circulation in permeate-flow rinsing mode

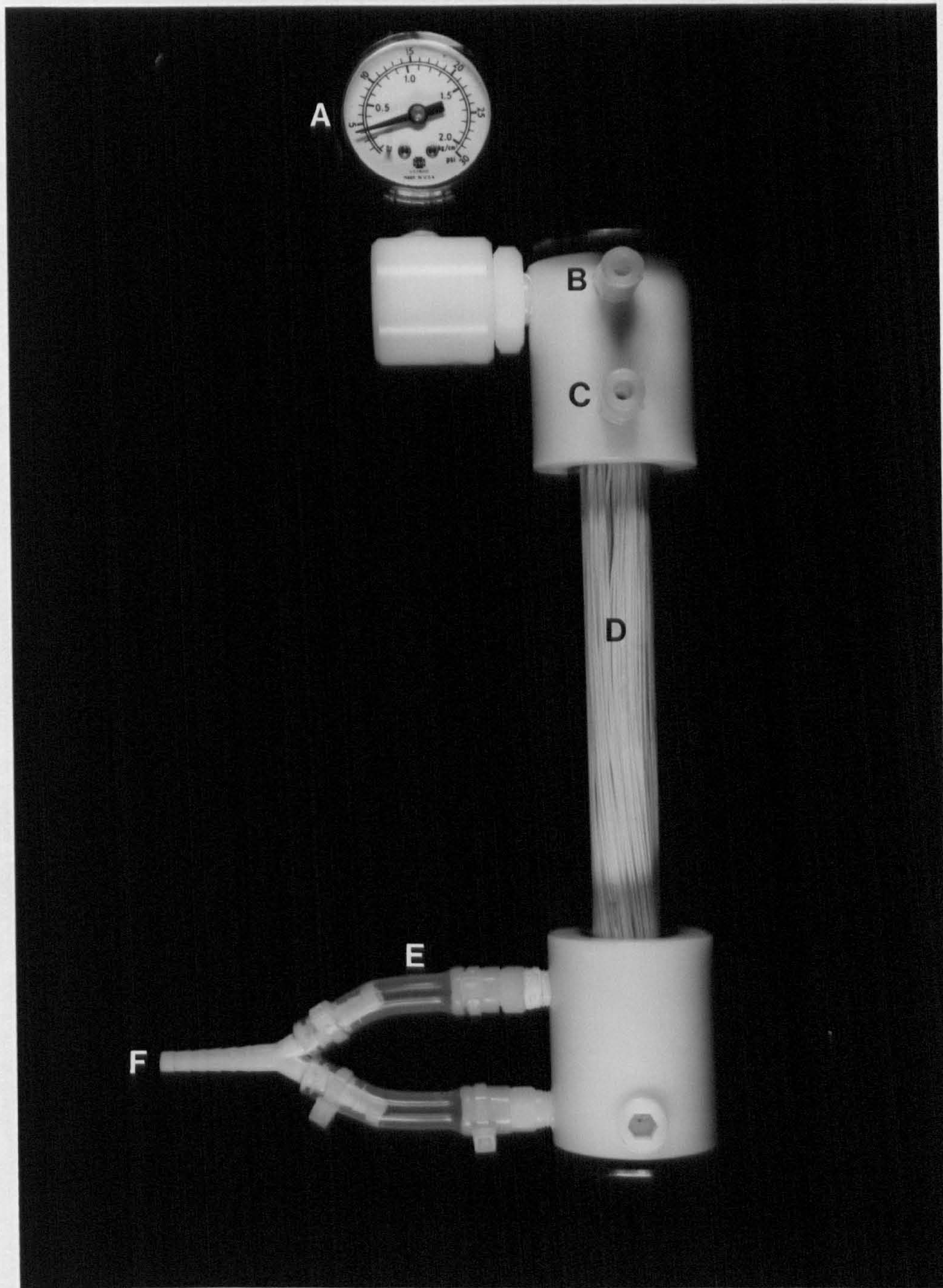


Figure 3.17 Filter unit configuration for final developed system

A: Culture inlet pressure gauge

B: Culture inlet

C: Filtrate outlet

D: Hollow fibre filter cartridge

E: Bypass loop for counter-current flow

F: Culture outlet

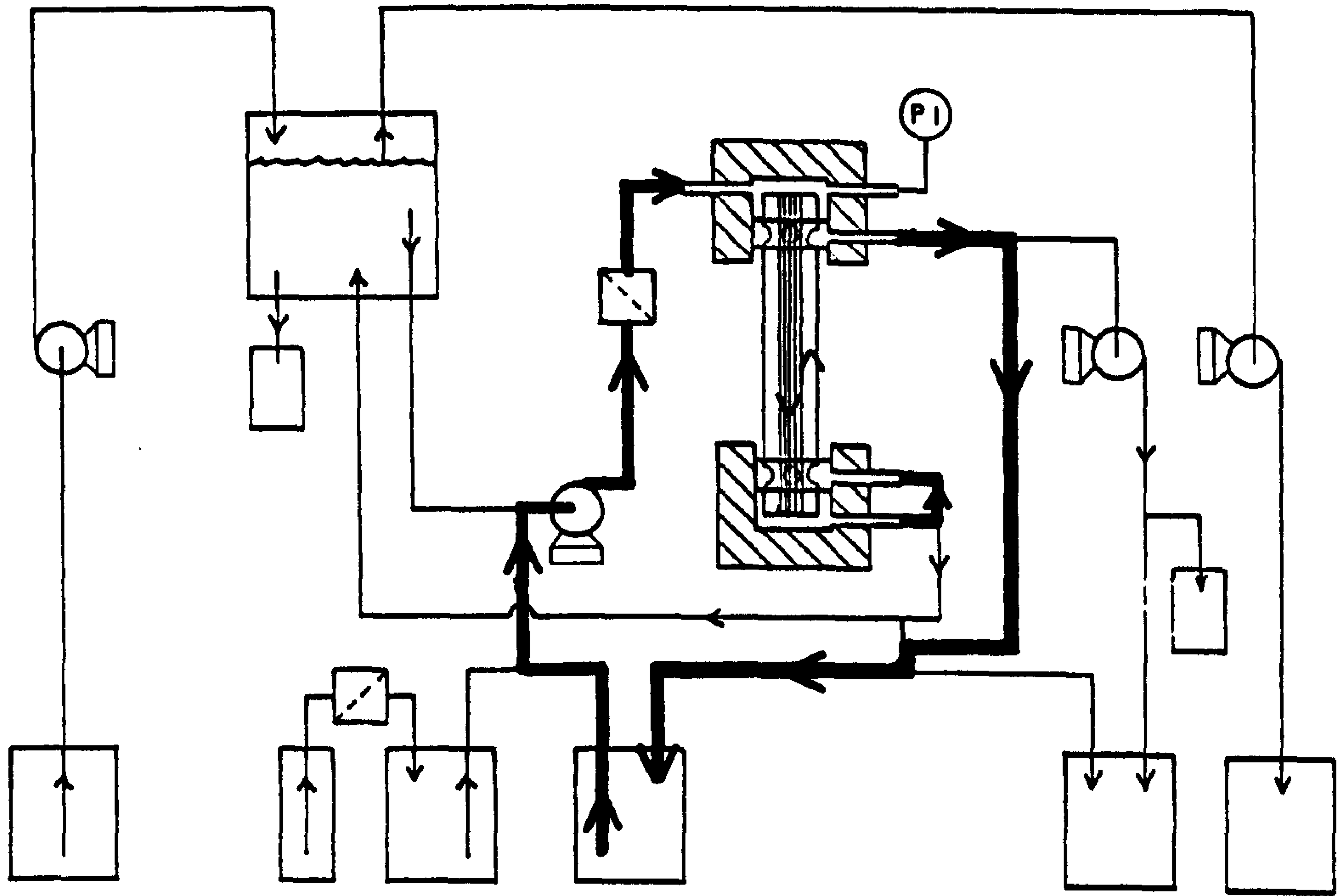


Figure 3.18 Final developed system: Circulation in counter-current sterilization mode

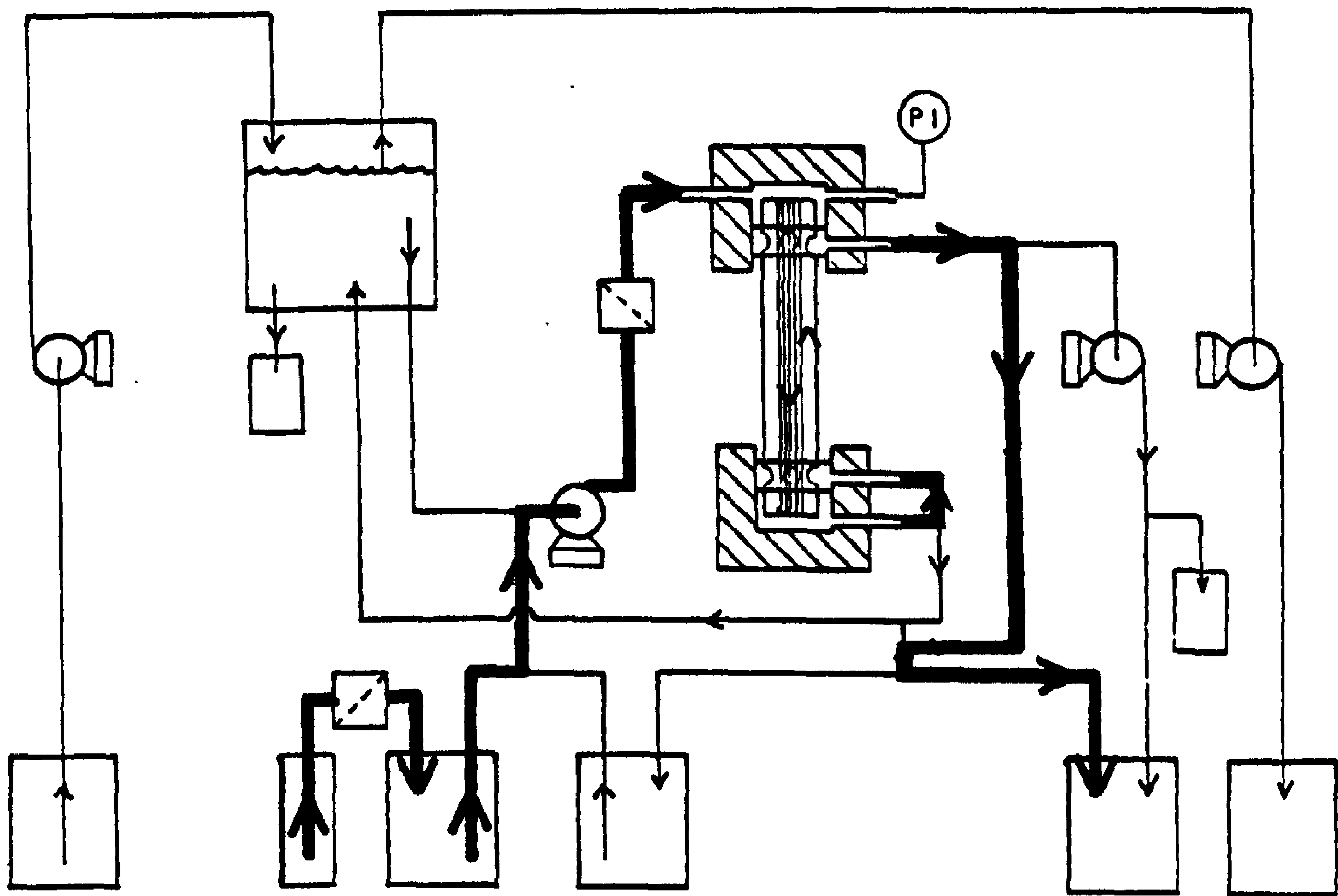


Figure 3.19 Final developed system: Circulation in counter-current rinsing mode

Pre-run sterilization and rinsing was performed as follows. All lines, reservoirs and receivers were sterilized by autoclaving and the diluted hypochlorite solution (200 ppm) made, as described in Section 3.2.1.2. The lines were connected to the filter unit and hypochlorite solution was circulated in permeate flow sterilization mode for 1h to clean the pores in the hollow fibres and to ensure sterility of the filtrate line. The circulation was then changed to counter-current flow sterilization and operated in this mode for 2 h. The system was rinsed with 20 L water in permeate flow mode to flush the hypochlorite from the filtrate line and rinsed with a further 40 L water in counter-current flow mode to completely flush the filter unit.

Circulation in permeate flow sterilization mode resulted in a fall in the level of hypochlorite solution in the reservoir due to the flow removed via the filtrate line. The hypochlorite reservoir could be emptied completely by running to waste (Figure 3.20) and refilled with fresh water from the rinse reservoir (Figure 3.21). Concentrated hypochlorite was then diluted in the water in the hypochlorite reservoir as described in Section 3.2.1.2.

3.3.2.2. Cell Recycling

Culture was drawn from the fermentation vessel via the submerged weir and passed through the sintered glass prefilter and into the hollow fibre filtration unit. The circulation rate was 0.75 L min^{-1} and the culture inlet pressure was monitored. The culture stream was returned to the fermentation vessel via a submerged needle connector in the bottom end plate of the vessel. The filtrate stream was drawn from the filter unit by the filtrate pump as described for the second system development (Section 3.2.2.2) and the rate of flow was measured as described for the first system development (Section

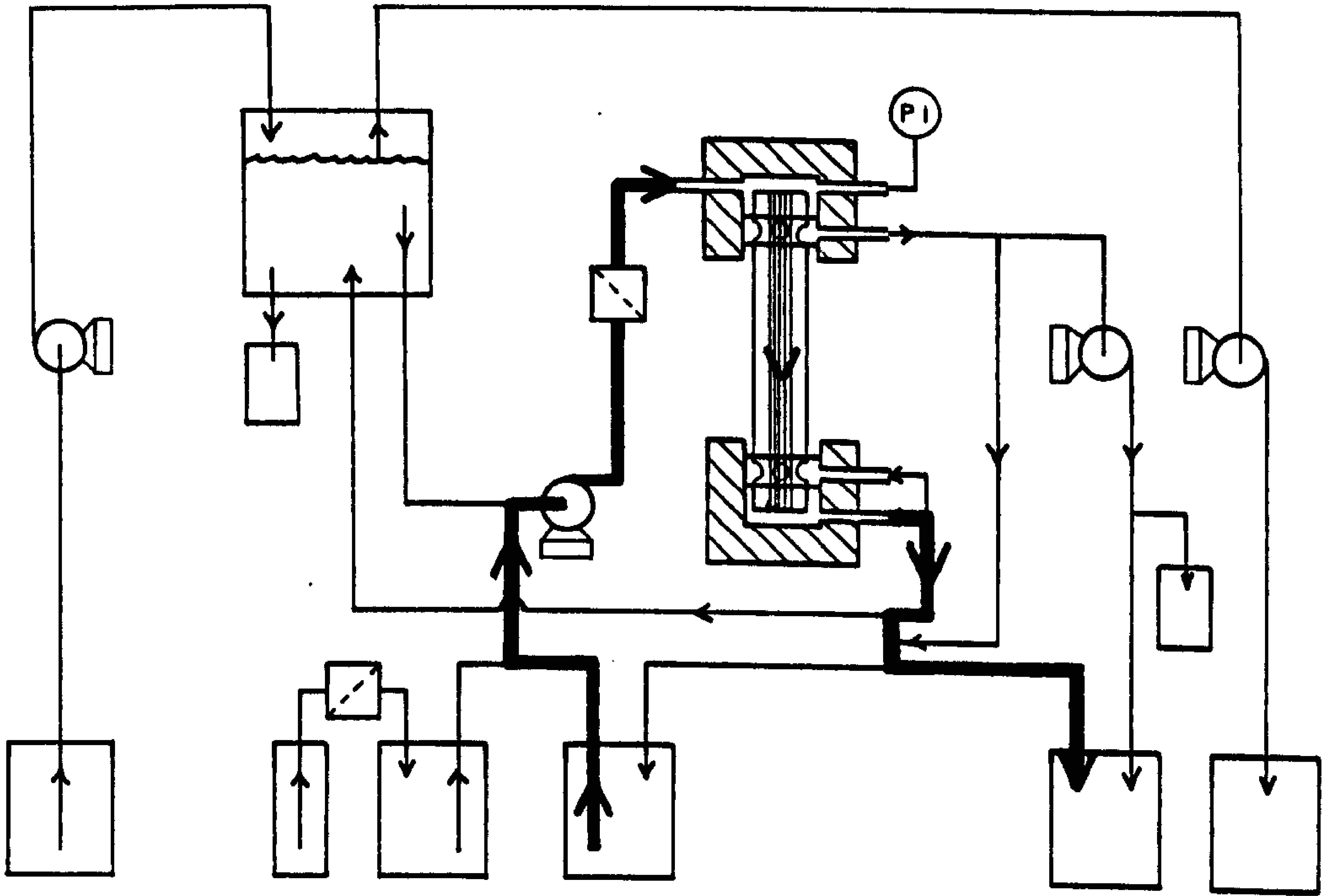


Figure 3.20 Final developed system: Hypochlorite reservoir emptying

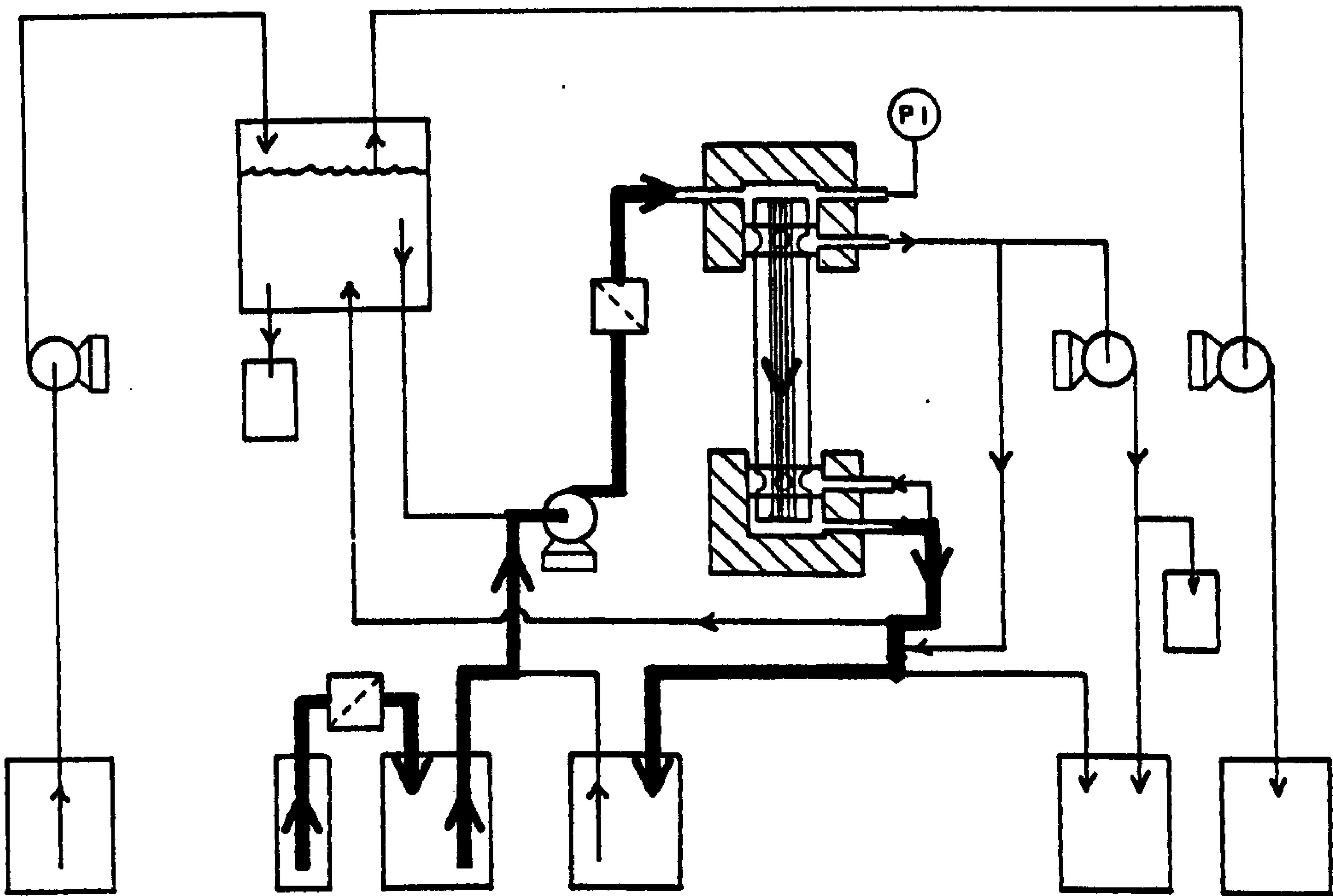


Figure 3.21 Final developed system: Hypochlorite reservoir filling

3.2.1.2). The rate was set to give a particular recycle ratio by manipulation of the filtrate pump speed. The circulation in cell recycling mode is shown in Figure 3.22 (filtrate to waste) and Figure 3.23 (filtrate sampling).

3.4 Total Cell Recycle System (R = 1.0)

The total cell recycle fermenter system was a development of the partial recycle system described in Section 3.3. The total recycle system operated at R = 1.0, i.e. the cell-free filtrate stream was the only liquid outflow of the fermenter system at a flow rate equal to the rate of growth medium supply.

Recycling at R = 1.0 would have been possible with the system described in Section 3.3 but exact matching of the filtrate to the medium supply rate would have been difficult to achieve by manual control of the filtrate pump. To overcome this difficulty, the filtrate pump was placed under the automatic control of the antifoam circuit of the bioreactor MCS1 control cabinet. A foam detection probe was mounted in the top plate of the fermentation vessel. The probe and the mains power supply of the filtrate pump were connected to the MCS1 cabinet. Culture was circulated through the hollow fibre filter unit and growth medium was fed at a set rate. The medium feed caused the culture level to rise until contact was made with the foam probe. This contact switched on the filtrate pump, which had been set at full speed, drawing filtrate from the circulating culture stream. As a result, the culture level fell below the foam probe and the filtrate pump was switched off. In this way total cell recycling with a constant culture volume was maintained automatically.

A line diagram of the circulation of the total cell recycle system is shown in Figure 3.24. Apart from the automatic control

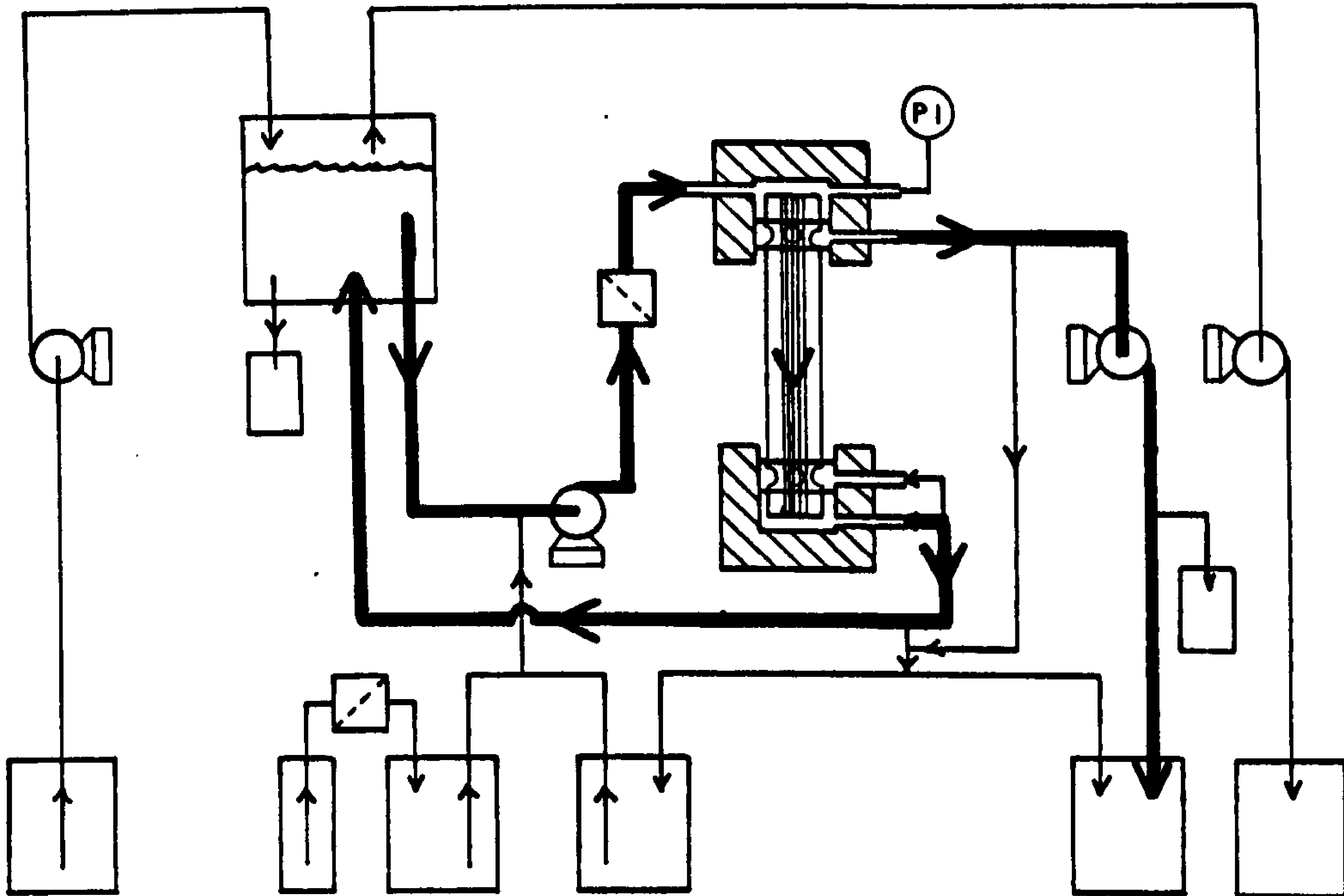


Figure 3.22 Final developed system: Circulation in cell recycle mode, filtrate to waste

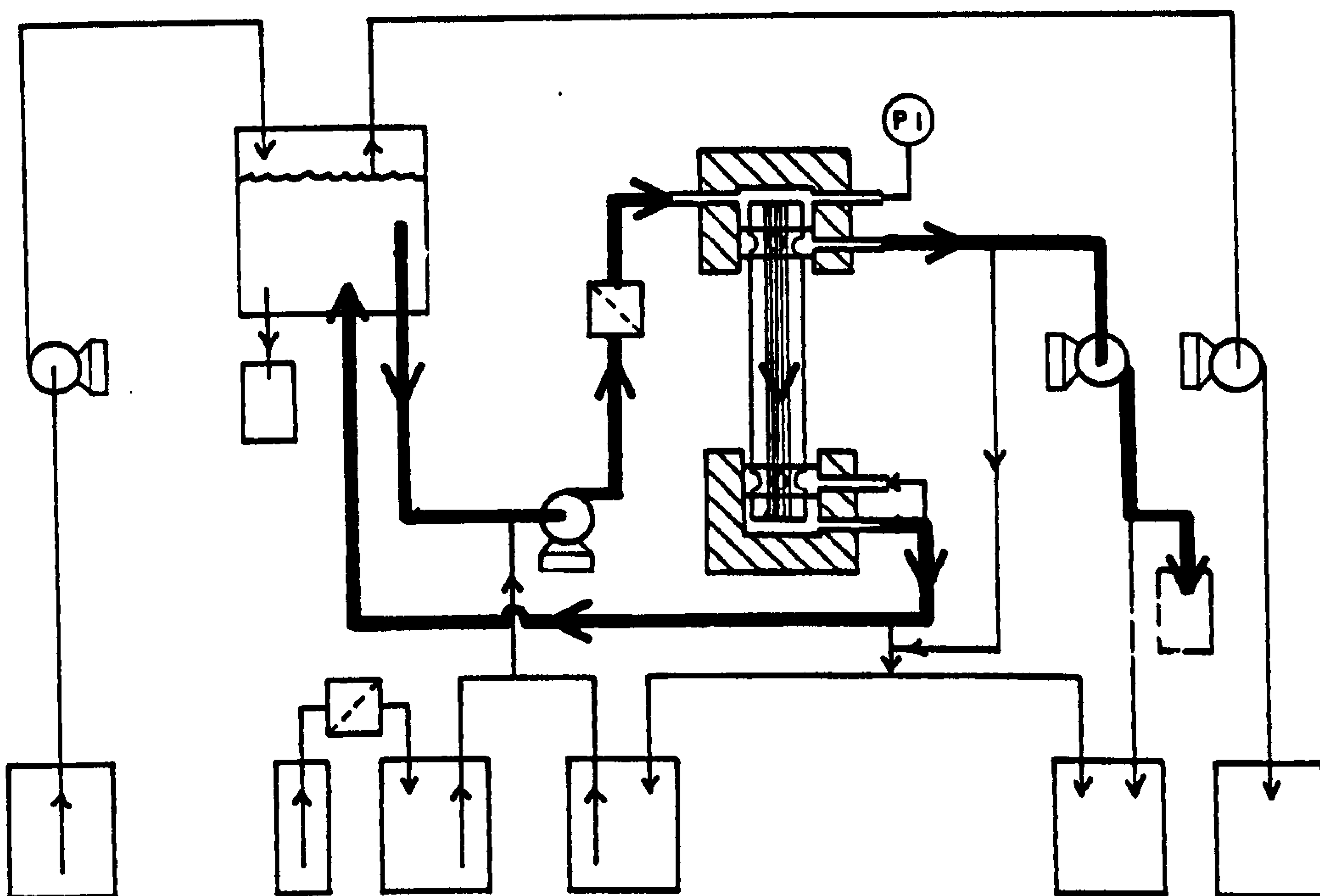
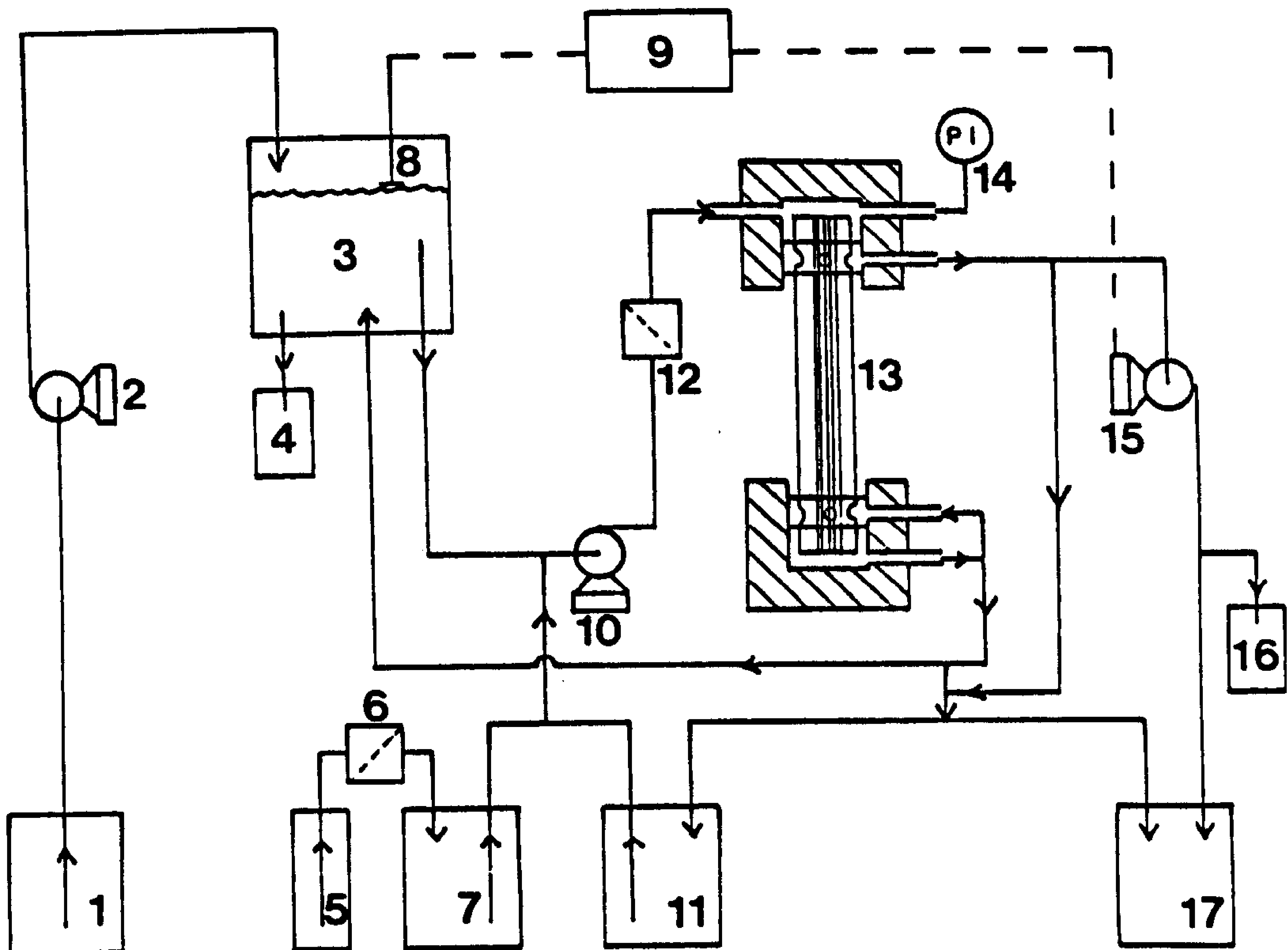
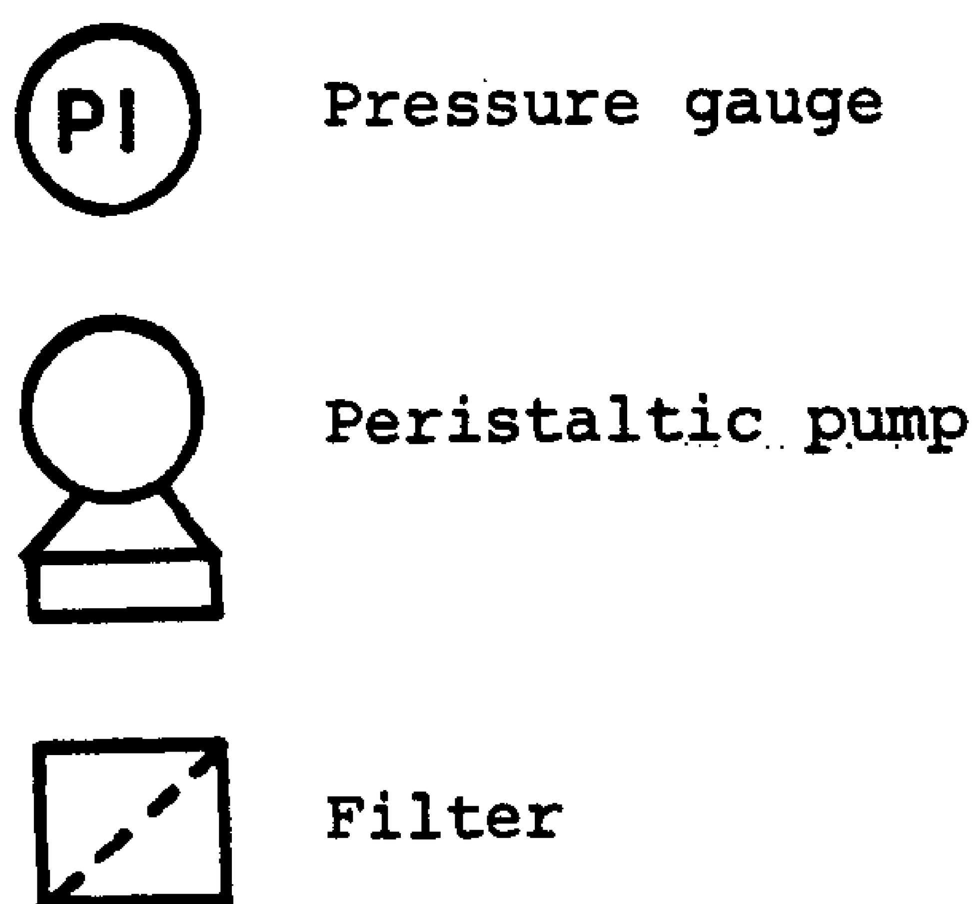


Figure 3.23 Final developed system: Circulation in cell recycle mode, filtrate sampling.





1. Growth medium reservoir
2. Growth medium pump
3. Fermentation vessel
4. Culture sampling point
5. Pressure vessel - non sterile H_2O
6. Water filter
7. Rinse water reservoir
8. Culture level sensor
9. Bioreactor control cabinet
10. Circulation pump
11. Hypochlorite reservoir
12. Prefilter
13. Hollow fibre filter unit
14. Culture inlet pressure gauge
15. Filtrate pump
16. Filtrate sample point
17. Filtrate/Rinse receiver

Figure 3.24 Line diagram of total cell recycle fermenter.

of the filtrate pump, all modes of circulation were as described for the final developed system (Section 3.3.2).

3.5 Experiments Performed

Experiments were performed at recycle ratio (R) values equal to 0.5, 0.8, 0.9 and 1.0, representing a five-fold range of concentrating effect exerted (equal to (1-R)) in partial cell recycle fermentation. The dilution rate in all experiments was 0.30 h^{-1}, close to the dilution rate giving maximum volumetric productivity in the chemostat (Chapter 5, Section 5.1.2). The cell recycle cultures were sampled at intervals of 12 to 36 h and the flow rates of growth medium and cell-free filtrate were checked 3 or 4 times daily. During each experiment, culture samples were collected on ice and analysed for biomass, lactic acid, glucose, ethanol, and acetate concentrations. During the R = 0.8 experiment, cell pellet samples were taken and stored at -20°C for subsequent analysis of lactate dehydrogenase specific activity and the cellular content of protein, polysaccharide, carbon, hydrogen and nitrogen. In addition, the culture viability was measured throughout the R = 0.8 experiment. A glucose pulse experiment was performed during the R = 0.9 steady state (after 311 h recycling). This latter experiment was performed as follows. An aliquot of culture (20 mL) was removed from the fermentation vessel and 20 mL sterile 50% (w/v) glucose monohydrate solution (50.5 mmol glucose) was injected into the culture via a hypodermic needle inserted through a rubber septum in the fermentation vessel top plate. The rate of acid production was monitored by measuring the volume of 2 M NaOH solution added from a 25 mL glass pipette over 2 min periods. The pipette was refilled from the NaOH reservoir every 8 min resulting in a 1 min gap in measurement with each refilling.

Culture samples were taken immediately before the glucose injection and 60 and 120 min post-injection. The samples were analysed for biomass dry weight, lactic acid, ethanol and acetate concentration.

CHAPTER 4

RESULTS: BATCH CULTURE

4.1 Strain Selection

The results of the shake flask comparison of the lactic acid productivity of *Lactobacillus delbreuckii* strains NCIB 8130, CN 960, 7473 and NRRL B-445 are shown in Table 4.1. The most productive strain was NRRL B-445 which exhibited the highest yield of lactic acid per g biomass. This latter strain was used in all subsequent fermentations.

4.2 pH-Controlled Batch Culture

The concentrations of biomass, lactic acid and glucose recorded throughout a pH-controlled batch culture are shown in Figure 4.1. Glucose was not detectable in the culture after 10 h growth and no further production of lactic acid was recorded after this latter point. After 12 h culturing, the yields of lactic acid from biomass and glucose were 6.13 gg^{-1} and 0.98 gg^{-1} respectively, the latter value being equal to 88% of the maximum theoretical yield, and the yield of biomass from glucose was 0.15 gg^{-1}. The natural logarithm of the biomass concentration is plotted against time in Figure 4.2. There was no observed lag period and logarithmic phase growth continued until 7 h culturing time had elapsed. The specific growth rate in logarithmic phase (μ_{max}) was 0.52 h^{-1}. Interestingly, deceleration in growth occurred between 7 and 10 h although glucose was still present in the culture over this period. It may be concluded that the depletion of glucose was not the cause of the onset of deceleration phase.

Strain	Biomass Concn. (gL^{-1})	Lactic Acid Concn. (gL^{-1})	Yield (glactic acid g^{-1} biomass)
NCIB 8130	0.10	0.21	2.10
CN 960	0.62	3.56	5.74
7473	0.82	4.86	5.96
NRRL B445	1.66	12.72	7.66

Table 4.1

Lactic Acid Production of four strains of *L. delbreuckii*

(24 h shake flask culture, MRS broth, 42°C)

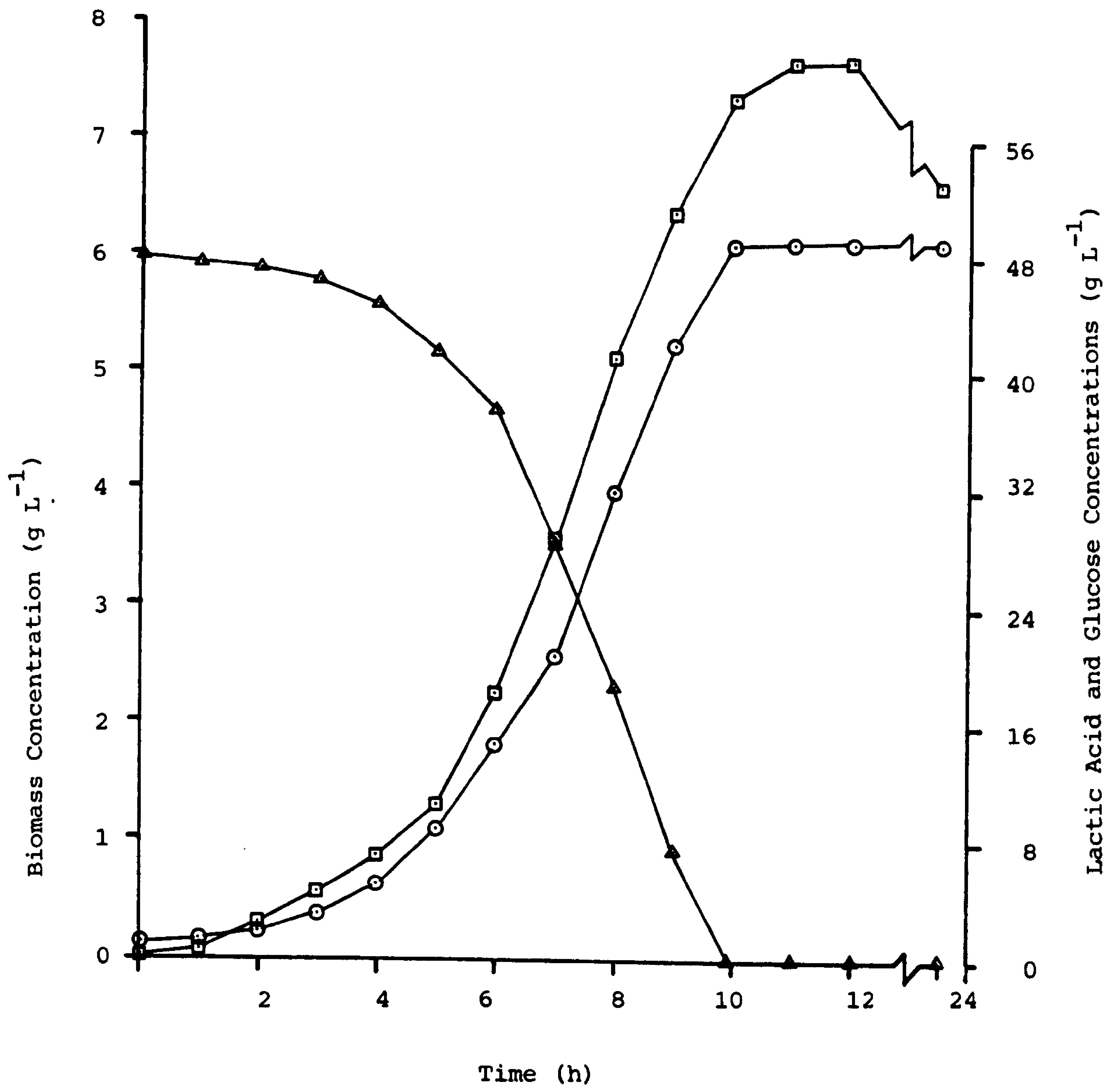


Figure 4.1 pH Controlled batch culture: biomass (⊡), lactic acid (⊙) and glucose (▲) concentrations Vs. time.

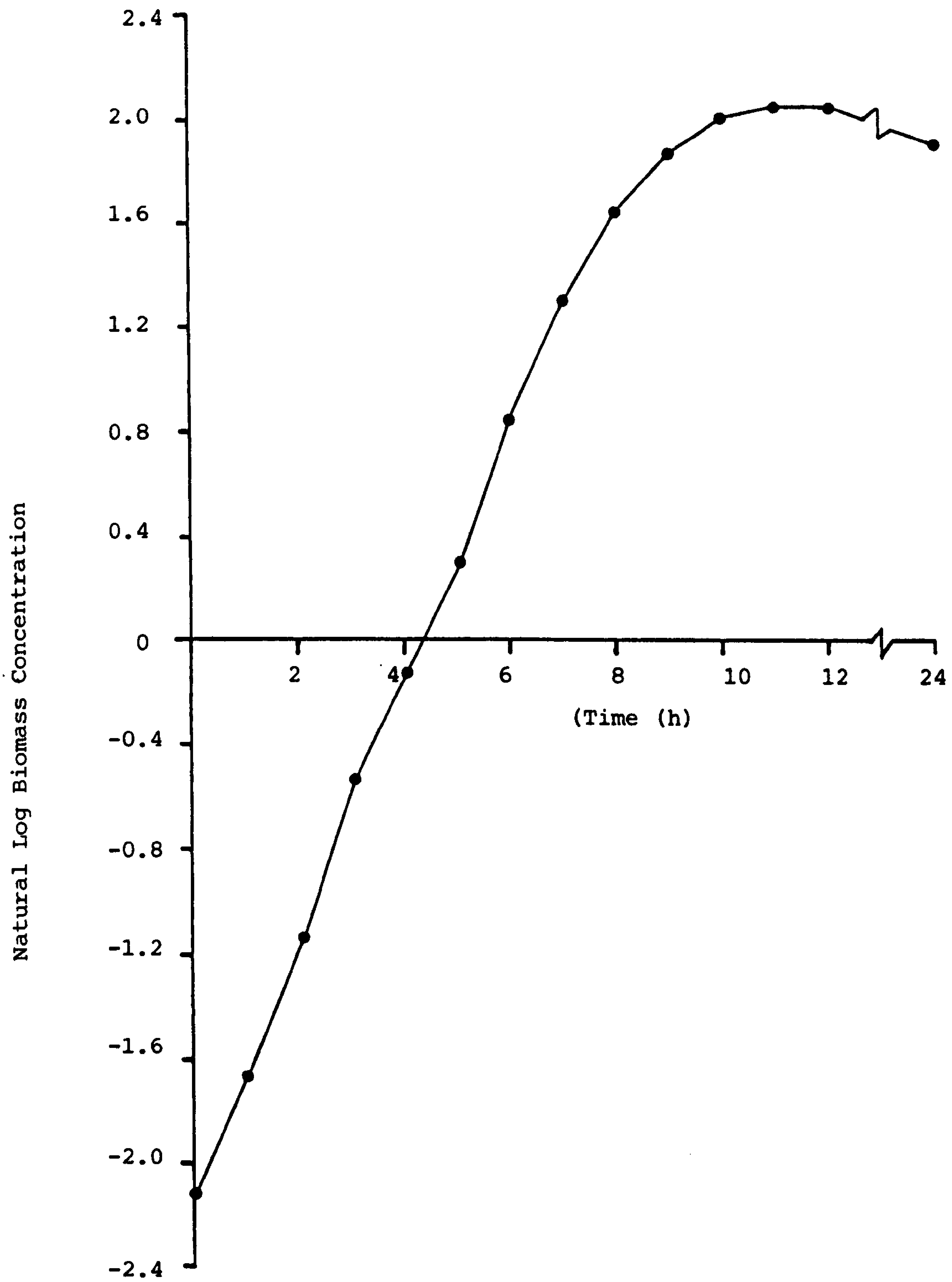


Figure 4.2 pH-Controlled batch culture: Natural logarithm biomass concentration Vs. time.

The specific growth rate (μ) was estimated at points throughout the period of culture by measuring the gradient of tangents taken from the curve in Figure 4.2. Similarly, tangents were taken from the curves of lactic acid and glucose concentration in Figure 4.1. The measured gradients of the latter tangents were taken to be estimates of the volumetric rate of lactic acid formation ($Q_{lactate}$) and the volumetric rate of glucose consumption ($Q_{glucose}$) respectively. The latter volumetric rates were divided by the biomass concentration recorded at each respective time point to provide estimates of the specific rate of lactic acid formation ($q_{lactate}$) and the specific rate of glucose consumption ($q_{glucose}$). Figure 4.3 shows μ, $q_{lactate}$ and $q_{glucose}$ plotted against culture time. It may be seen from Figure 4.3 that $q_{lactate}$ and $q_{glucose}$ were variable throughout the period of culture, maximum values for both parameters occurring in mid-logarithmic phase (after 5 h). The trends in $q_{lactate}$ and $q_{glucose}$ were similar throughout the fermentation but there was no similarity between the latter trends and that for μ until the onset of deceleration phase (after 7 h).

The similarity in the trends of $q_{lactate}$ and $q_{glucose}$ throughout the growth curve (Figure 4.3) suggests that a close relationship existed between the rates of glucose uptake and product formation throughout the batch culture. The evidence for such a relationship is strengthened by plotting $Q_{lactate}$ against $Q_{glucose}$ as in Figure 4.4. The plotted points in Figure 4.4 fall close to a straight line the slope of which, calculated by linear regression, represents the yield coefficient (1.05 gg^{-1}).

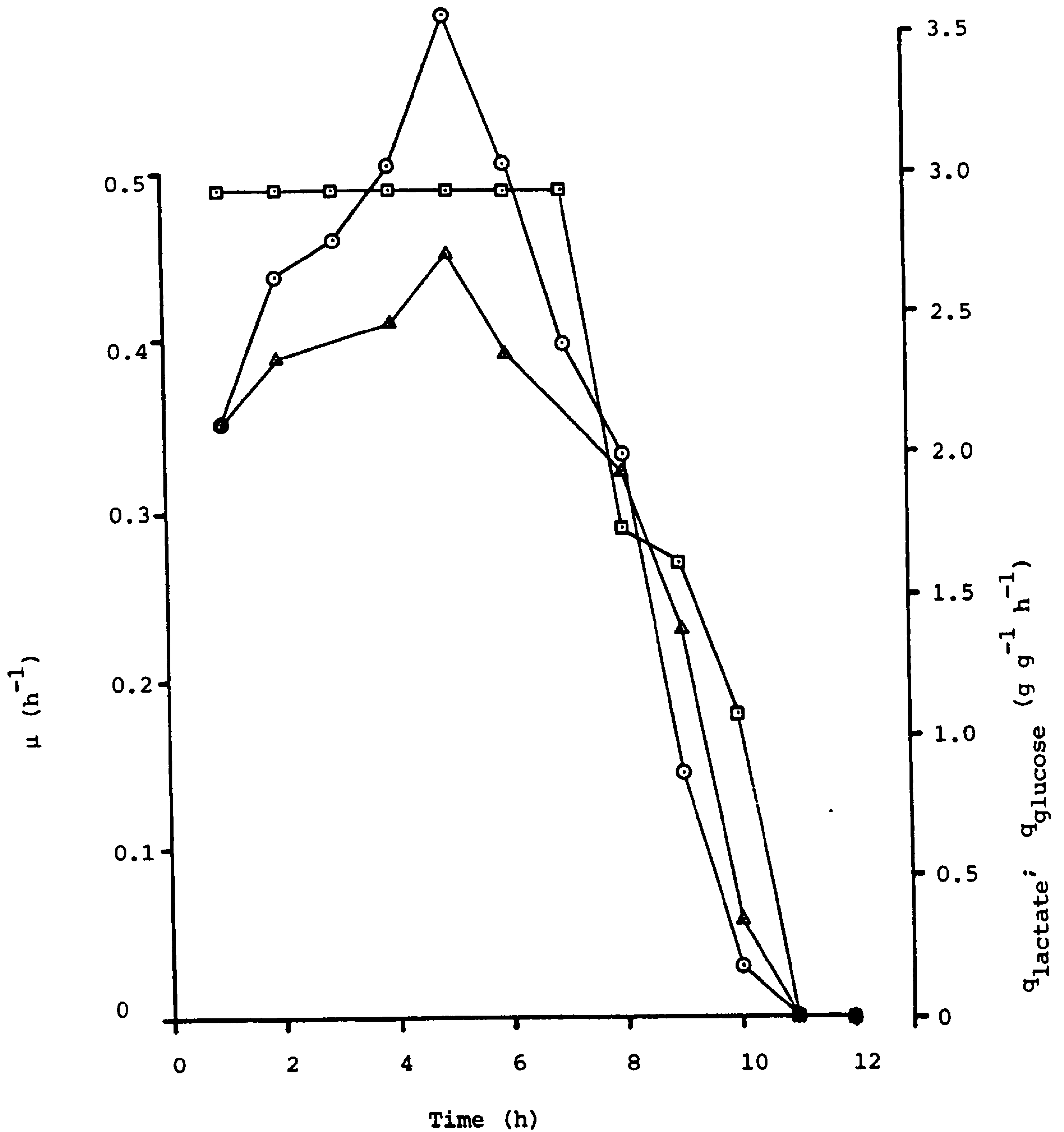


Figure 4.3 pH-Controlled batch culture: specific growth rate (μ, ⊡), specific rate of lactic acid formation ($q_{lactate}$, ⊙), and specific rate of glucose uptake ($q_{glucose}$, ▲) Vs. time.

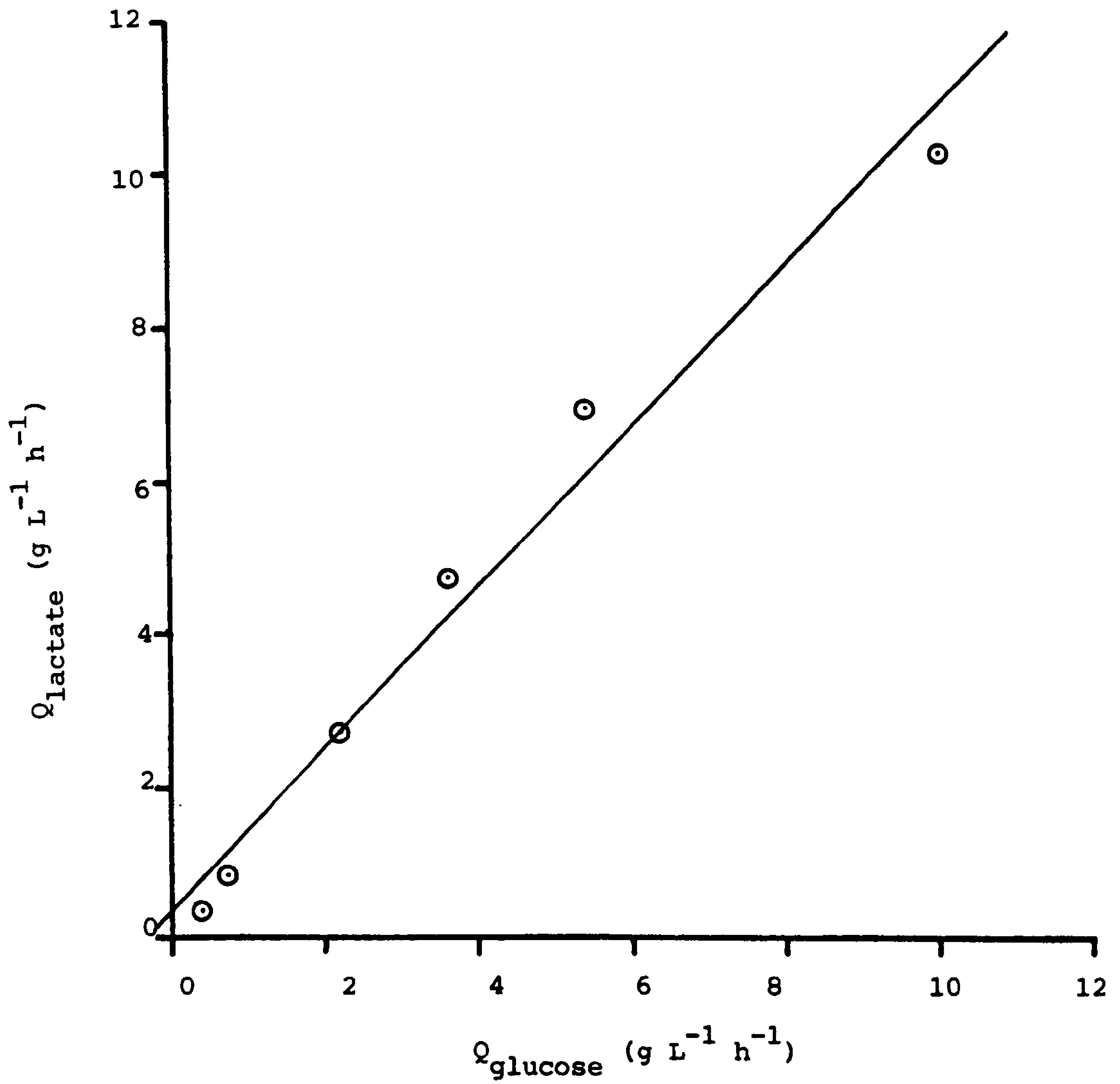


Figure 4.4 pH-Controlled batch culture: volumetric rate of lactic acid formation ($Q_{lactate}$) Vs. volumetric rate of glucose uptake ($Q_{glucose}$).

CHAPTER 5

RESULTS: CHEMOSTAT CULTURE

Some of the data presented in this Chapter have been previously published (Major & Bull, 1985).

5.1 Dilution Rate Profile

5.1.1 Biomass, Lactic Acid and Glucose Concentrations.

The steady state culture concentrations of biomass, lactic acid and residual glucose recorded at each dilution rate (D) are shown in Table 5.1. The concentrations of biomass and lactic acid were significantly lower at D = 0.50 h^{-1} than at D = 0.05 h^{-1} although some of the differences within this range were not significant (Student's t test, P = 0.05). These decreasing trends were contrasted with significant increases in supernatant glucose concentration with each step-wise increase in D. At D = 0.05 h^{-1} and D = 0.10 h^{-1}, no residual glucose was detectable in the culture by the qualitative or quantitative methods.

5.1.2 Specific Rates, Yields and Productivities

The specific rates of lactic acid formation ($q_{lactate}$) and glucose uptake ($q_{glucose}$) are plotted versus D in Figure 5.1. In each case the data fall close to a straight line with coefficients of correlation of 0.99. The gradients of the plots of $q_{lactate}$ and $q_{glucose}$ versus D represent respectively the yield of lactic acid from biomass (Yp/x) and the reciprocal of the yield of biomass from glucose (Yx/s)

Dilution Rate (h^{-1})	Biomass ($g\ L^{-1}$)	Lactic Acid ($g\ L^{-1}$)	Glucose ($g\ L^{-1}$)
0.05	5.40 ± 0.20	37.35 ± 1.25	< 0.004
0.10	5.68 ± 0.20	36.46 ± 0.24	< 0.004
0.15	5.42 ± 0.80	33.02 ± 1.05	4.07 ± 0.24
0.20	5.28 ± 1.00	31.65 ± 1.05	8.15 ± 0.48
0.30	4.04 ± 1.20	27.85 ± 0.87	12.39 ± 0.22
0.35	3.99 ± 0.80	25.50 ± 0.82	16.50 ± 0.47
0.40	3.48 ± 0.80	22.30 ± 0.59	19.90 ± 0.35
0.45	2.71 ± 1.20	17.34 ± 0.23	26.90 ± 0.22
0.50	1.82 ± 1.00	11.35 ± 1.05	34.07 ± 0.51

Table 5.1

Chemostat Culture: Steady State Biomass, Lactic Acid and Residual Glucose Concentrations (95% Confidence Limits Calculated by Student's t test)

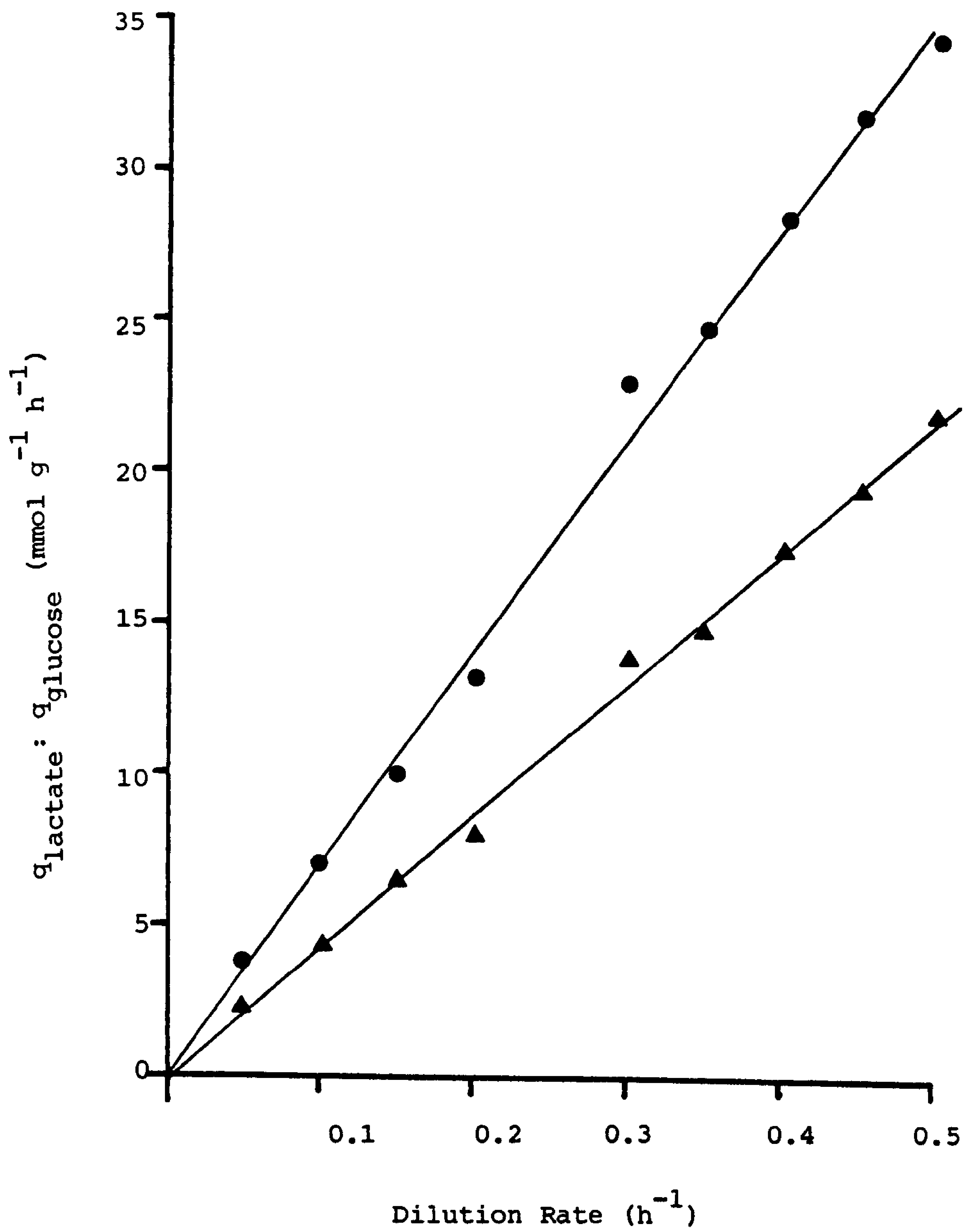


Figure 5.1 Chemostat Culture: specific rate of lactic acid formation ($q_{lactate}$, ●) and specific rate of glucose uptake ($q_{glucose}$, ▲) Vs. dilution rate.

(Pirt, 1975). It may be seen from Figure 5.1 that the yields remained constant throughout the range of D with values calculated by linear regression to be 70.6 mmol g^{-1} (6.35 gg^{-1}) for Yp/x and 21.6 $gmol^{-1}$ (0.12 gg^{-1}) for Yx/s. The intercept of $q_{glucose}$ on the D axis was very low (-0.03 mmol g^{-1} h^{-1} by linear regression) suggesting a negligible rate of glucose uptake for maintenance and other non-anabolic functions (Tempest & Neijssel, 1984). The relationship between the specific rates of glucose uptake and lactic acid formation may be seen by plotting $q_{lactate}$ versus $q_{glucose}$ as in Figure 5.2. The data fall close to a straight line (coefficient of correlation = 0.99) the gradient of which represents the molar yield of lactic acid from glucose (Yp/s). It may be seen from Figure 5.2 that Yp/s remained constant throughout the range of D with a value calculated by linear regression to be 1.48 mol mol^{-1}, (0.74 gg^{-1}) i.e. 74% of the theoretical maximum yield.

Figure 5.3 shows the volumetric productivities of biomass and lactic acid achieved as functions of D. The trends observed in volumetric productivity were the same for biomass and lactic acid, namely significant increases in productivity with each step-wise increase in D until maximum values were recorded at D = 0.35 h^{-1} and D = 0.40 h^{-1} respectively. The productivity of the culture declined at values of D greater than 0.40 h^{-1}. The maximum values of volumetric productivity were 1.40 g L^{-1} h^{-1} for biomass and 8.93 g L^{-1} h^{-1} for lactic acid.

5.1.3 Ethanol, Acetate and Molar Product Ratio

Table 5.2 shows the ethanol and acetate concentrations recorded in steady state cultures at six different values of D and the molar product ratio, lactic acid: ethanol plus acetate, recorded in each

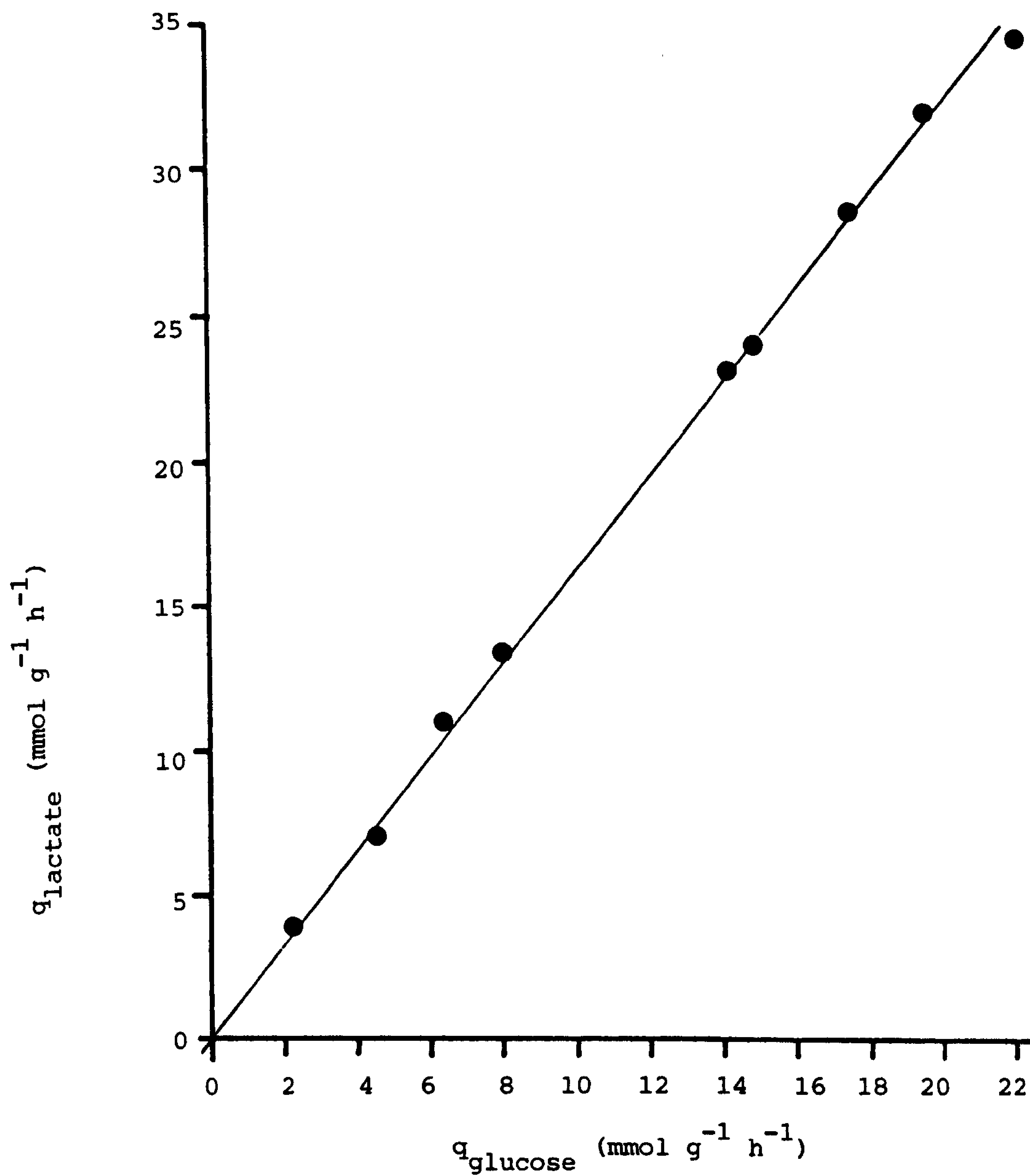


Figure 5.2 Chemostat culture: specific rate of lactic acid formation ($q_{lactate}$) Vs. specific rate of glucose uptake ($q_{glucose}$).

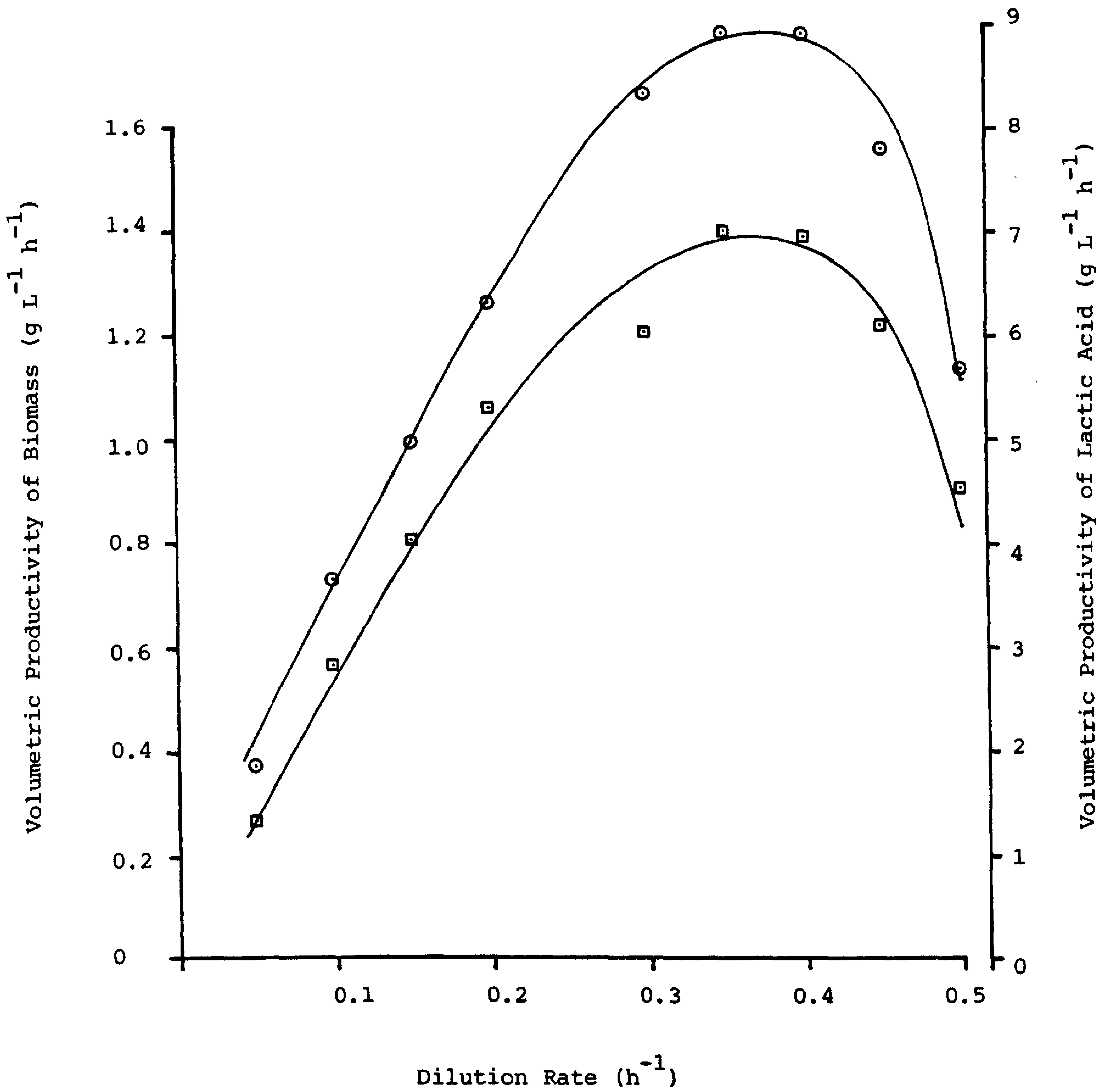


Figure 5.3 Chemostat culture: volumetric productivity of biomass (⊡) and lactic acid (⊙) Vs. dilution rate.

Dilution Rate (h^{-1})	Ethanol (mM)	Acetate (mM)	Molar Product Ratio
0.05	6.51	6.07	33:1
0.10	3.77	3.87	53:1
0.20	2.55	2.44	71:1
0.30	2.18	2.19	71:1
0.40	1.82	1.84	68:1
0.50	0.92	0.88	70:1

Table 5.2

Chemostat Culture: Steady State Ethanol and Acetate Concentrations and Molar Product Ratio, Lactate: Ethanol + Acetate

culture. Ethanol and acetate were produced in equimolar quantities (no significant difference by Student's t test; $P = 0.05$) and significant reductions in the concentration of each resulted from increases in D. The molar product ratio data show that ethanol and acetate were minor products in comparison with lactic acid at all dilution rates. However, a comparison of the molar product ratios for the glucose-excess ($D = 0.20$ to $0.50\ h^{-1}$) and the glucose-depleted ($D = 0.05$ and $0.10\ h^{-1}$) cultures shows a small significant shift towards the formation of ethanol and acetate in the latter cultures.

5.1.4 Lactate Dehydrogenase

A glucose-depleted ($D = 0.064\ h^{-1}$) and a glucose-excess ($D = 0.30\ h^{-1}$) culture were compared with reference to lactate dehydrogenase (LDH) specific activity, activation and inhibition, and kinetic parameters for pyruvate measured in cell-free extracts of biomass. In all the LDH assays performed, the background rate of NADH oxidation (rate in the absence of pyruvate as substrate) was 10 to 20% of the rate in the complete assay mixture (Chapter 2, Section 2.3.10). The LDH specific activity in the complete assay mixture was slightly less in the extract from the glucose-depleted culture than in that from the glucose-excess culture but this difference may not have been significant (significant at $P = 0.05$ but not at $P = 0.02$, Student's t test). In the extracts from both cultures, LDH specific activity was substantially reduced by the omission of Mn^{2+} or FDP from the assay mixture (Table 5.3).

The effect of phosphate ions at various concentrations on the LDH specific activity in cell-free extracts of the glucose-depleted and glucose-excess cultures is shown in Figure 5.4. Phosphate was

LDH Specific Activity (μmol NADH mg^{-1} protein min^{-1})

D (h^{-1})	Complete Mixture	Minus Mn^{2+}	Minus FDP
0.064	5.71	0.42	0.58
0.30	6.40	0.47	0.66

Table 5.3

Lactate dehydrogenase (LDH) Specific Activity in Cell-Free Extracts of Glucose-Depleted (D = 0.064 h^{-1}) and Glucose-Excess (D = 0.30 h^{-1}) Cultures in the Presence and Absence of Activators

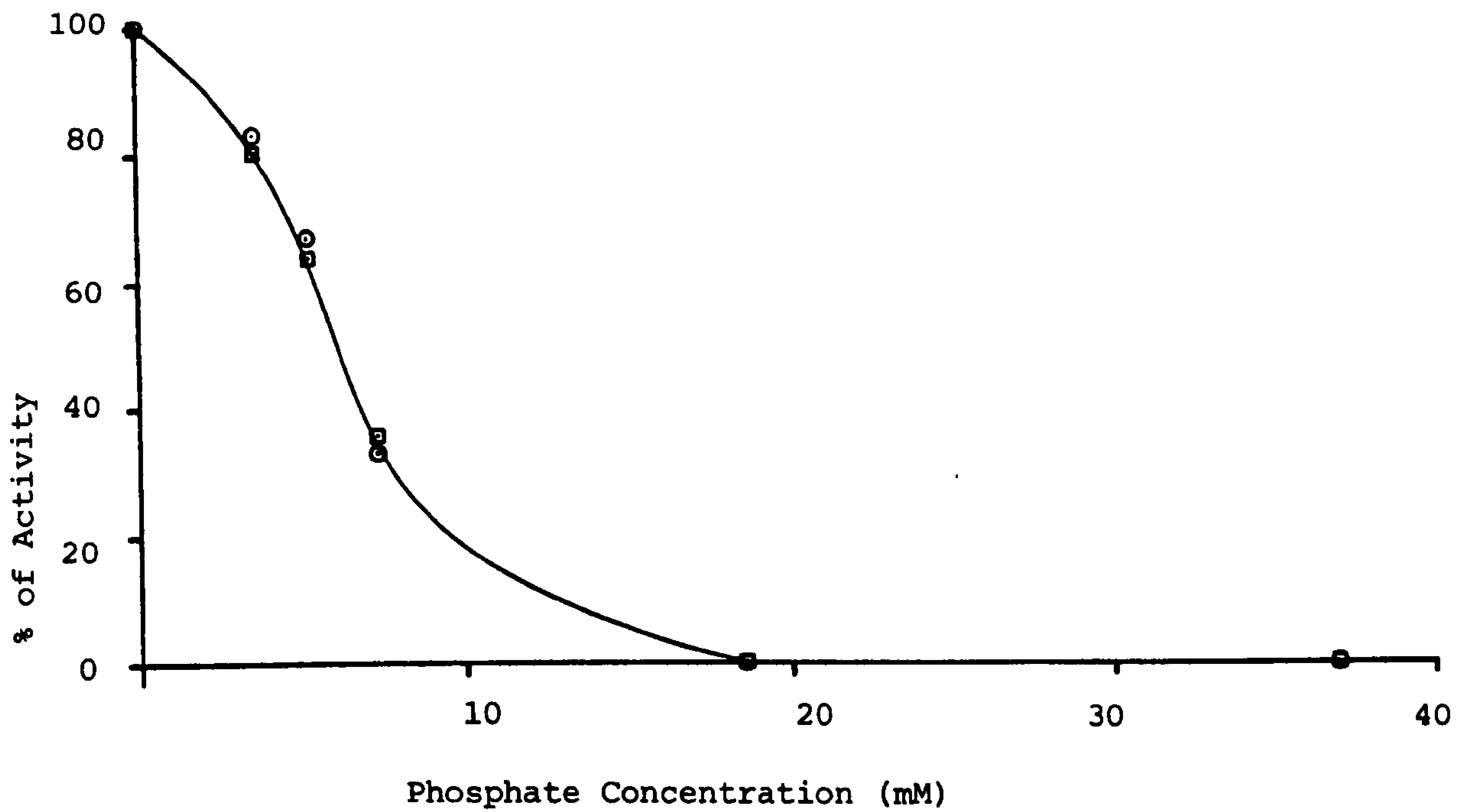


Figure 5.4 LDH specific activity in cell-free extracts of glucose depleted (D = 0.064 h^{-1}, ⊡) and glucose excess (D = 0.3 h^{-1}, ⊙) chemostat cultures in the presence of phosphate at various concentrations (as % of activity in absence of phosphate).

inhibitory to LDH activity in the extracts from both cultures giving sigmoidal responses to increasing concentrations of inhibitor and complete inhibition by 18.5 mM phosphate.

The effect on LDH specific activity of varying the pyruvate concentration in the assay mixture is shown as three Lineweaver-Burk plots in Figure 5.5. The latter Figure carries data from cell-free extracts of glucose-depleted (D = 0.064 h^{-1}) and glucose-excess (D = 0.30 h^{-1}) cultures with individual linear regression lines calculated from the data for each of the two extracts and a combined regression line calculated from all the data. The coefficients of correlation of the regression lines were 0.99 for each of the individual plots and 0.96 for the combined plot. These latter values suggest that, although the data obtained from both cultures closely fit different individual regression lines, the data may also be taken as fitting the single combined regression line with greater than 99% confidence (Student's t test on coefficient of correlation). It follows from the latter argument that there was no significant difference between the extracts from the two cultures in the response of LDH specific activity to varying pyruvate concentration. The kinetic constants of LDH for pyruvate. V_{max} and K_m, were calculated from the combined regression line to be 11.1 µmol NADH mg^{-1} min^{-1} and 9.3 mM respectively.

5.1.5 Cellular Composition

The total protein, polysaccharides, carbon, nitrogen and hydrogen content of chemostat-grown cells, expressed as a w/w percentage, is shown at various values of D in Table 5.4. The latter table illustrates a constancy of cellular composition; the variation in the cellular content of each chemical substance was less than 4% of dry weight over

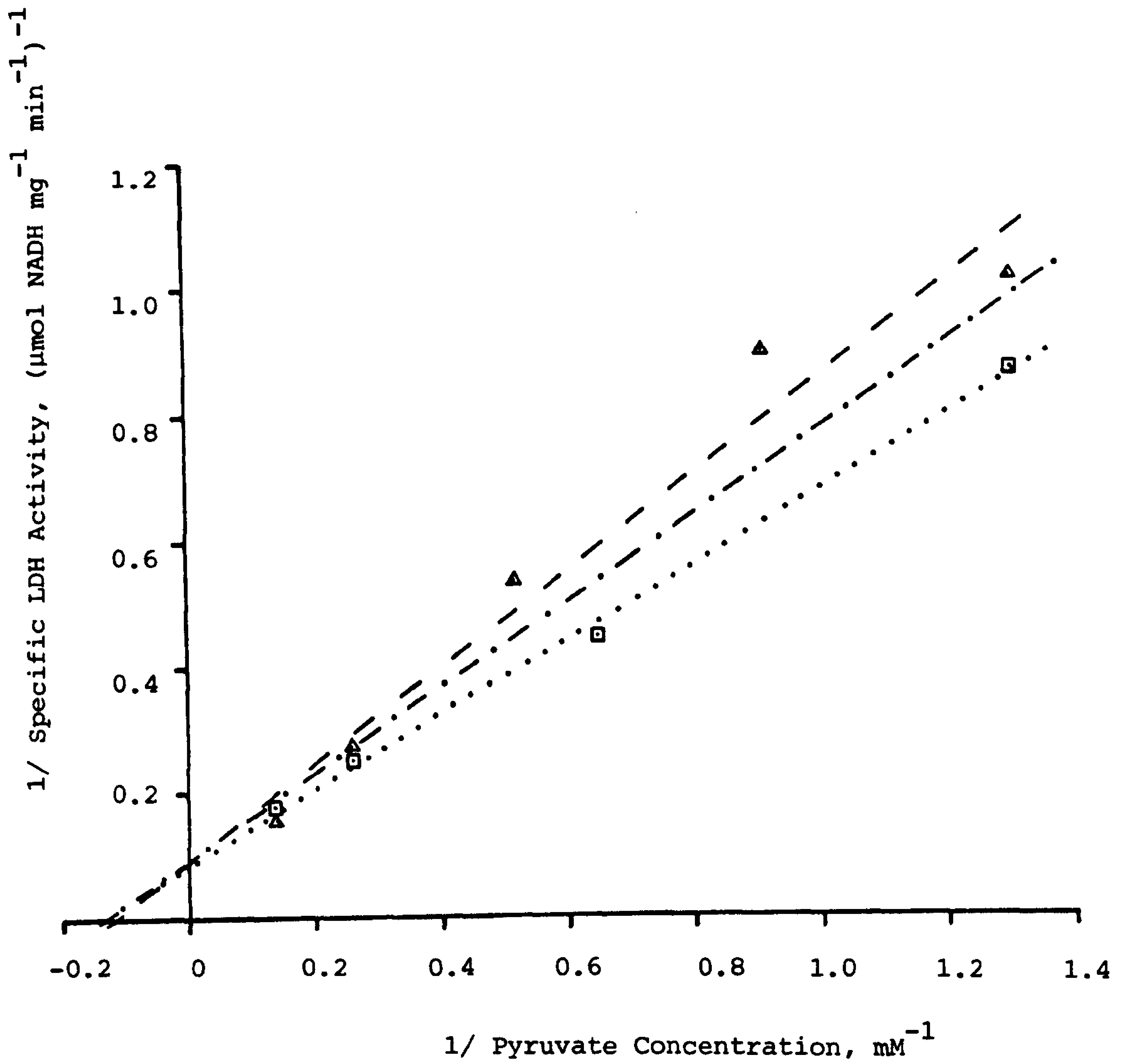


Figure 5.5 Lineweaver-Burk plots for LDH of cell-free extracts of glucose depleted (D = 0.064h^{-1}, ⊡) and glucose excess (D = 0.3 h^{-1}, ▲) chemostat cultures.

	Dilution Rate (h^{-1})			
	0.05	0.10	0.15	0.30
Protein	46.5	N.D.	N.D.	43.1
Polysaccharide	15.9	N.D.	N.D.	15.0
Carbon	43.6	41.6	41.6	41.3
Nitrogen	9.2	8.3	8.7	10.5
Hydrogen	7.0	6.9	6.6	6.5

Table 5.4

Chemical Composition of Chemostat-Grown Cells at Various Dilution Rates (% of Dry Weight, w/w)

N.D.: Not Determined

the range of D which included glucose-depleted (D = 0.05 and 0.10 h^{-1}) and glucose-excess (D = 0.15 and 0.30 h^{-1}) cultures.

5.2 Investigation of Growth Limitation

The steady-state concentrations of glucose, total carbon, total nitrogen, and total, organic, and inorganic phosphate recorded in glucose-excess culture supernatants are compared with those present in the feed medium in Table 5.5 (glucose, total carbon and total nitrogen from D = 0.40 h^{-1}; phosphates from D = 0.35 h^{-1}). The latter Table shows that each of the nutrients was present in considerable excess in the high D cultures. It is unlikely therefore that any of the nutrients listed in Table 5.5 was the growth limiting substrate in the latter cultures. This Section presents results obtained from experiments which attempted to identify the nature of the growth limitation in the higher D, glucose-excess, cultures.

5.2.1 Direct Injection of Nutrients

Figures 5.6 and 5.7 respectively show the response of culture absorbance (A_{640}) to the addition of solutions of complex amino acid sources (hydrolysates of casein) and defined mixtures of vitamins. The composition of the solutions is listed in Chapter 2 (Section 2.6.4.1, Tables 2.2 and 2.3). The liquid volumes injected were 100 mL of the casein hydrolysate solutions and 20 and 10 mL respectively of the three and ten vitamin mixture solutions.

The injection of the casein hydrolysate solutions immediately caused the pH of the culture to rise to 6.2 but the normal pH (6.0) was restored within 2 min in each case. The culture A_{640} fell sharply on addition of each of the solutions, the magnitude of the fall being greater than would have been caused by a simple dilution effect.

Nutrient	Concentration in Medium (mM)	Concentration in Supernatant (mM)
Glucose	278	111
Total Carbon	2620	2100
Total Nitrogen	238	185
Total PO_4	5.9	4.1
Organic PO_4	1.7	1.5
Inorganic PO_4	4.2	2.6

Table 5.5

Nutrient Concentrations in Growth Medium and Culture Supernatants (glucose, C and N from $D = 0.40\ h^{-1}$; phosphates from $D = 0.35\ h^{-1}$)

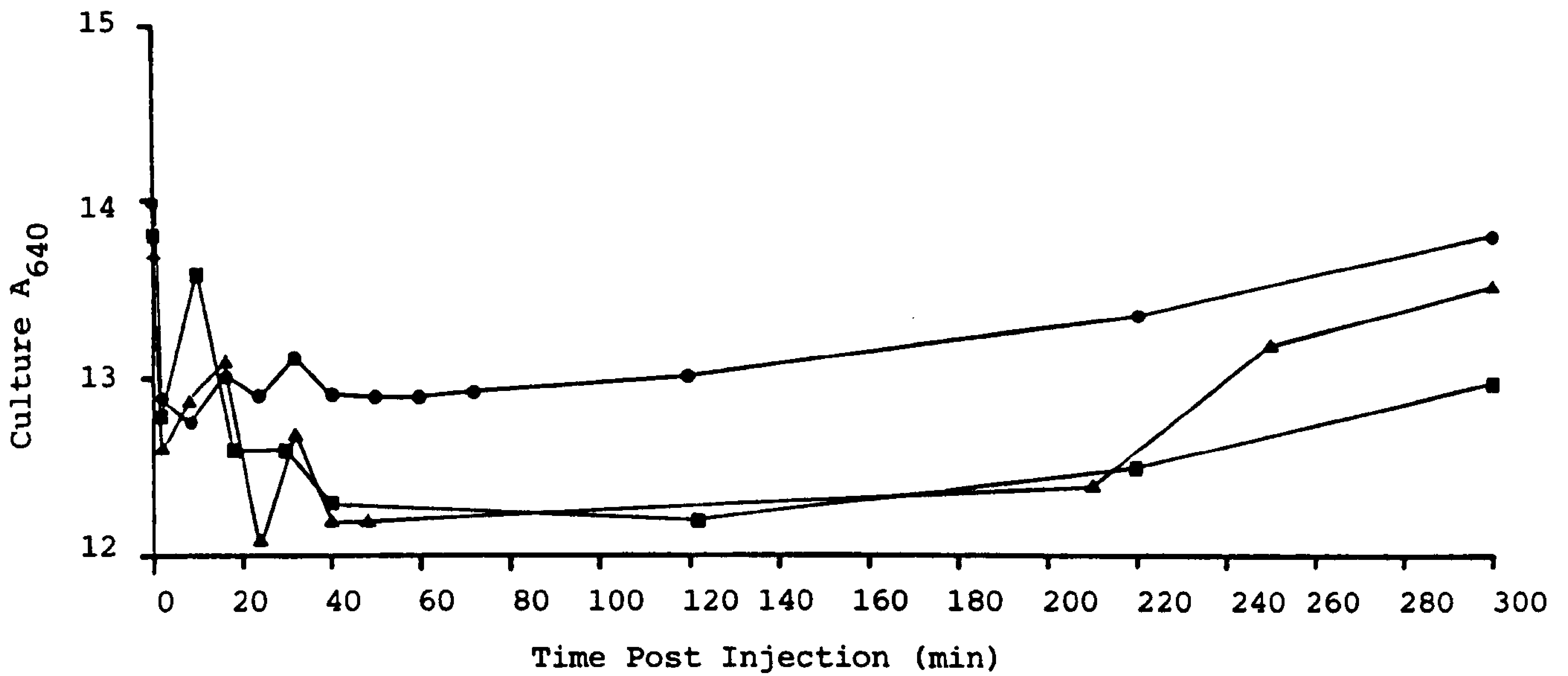


Figure 5.6 Culture A_{640} Vs. time post injection of acid hydrolysate of casein giving 10 gL^{-1} (▲) and 40 gL^{-1} ● in the fermenter and tryptic hydrolysate giving 10 gL^{-1} in the fermenter (■).

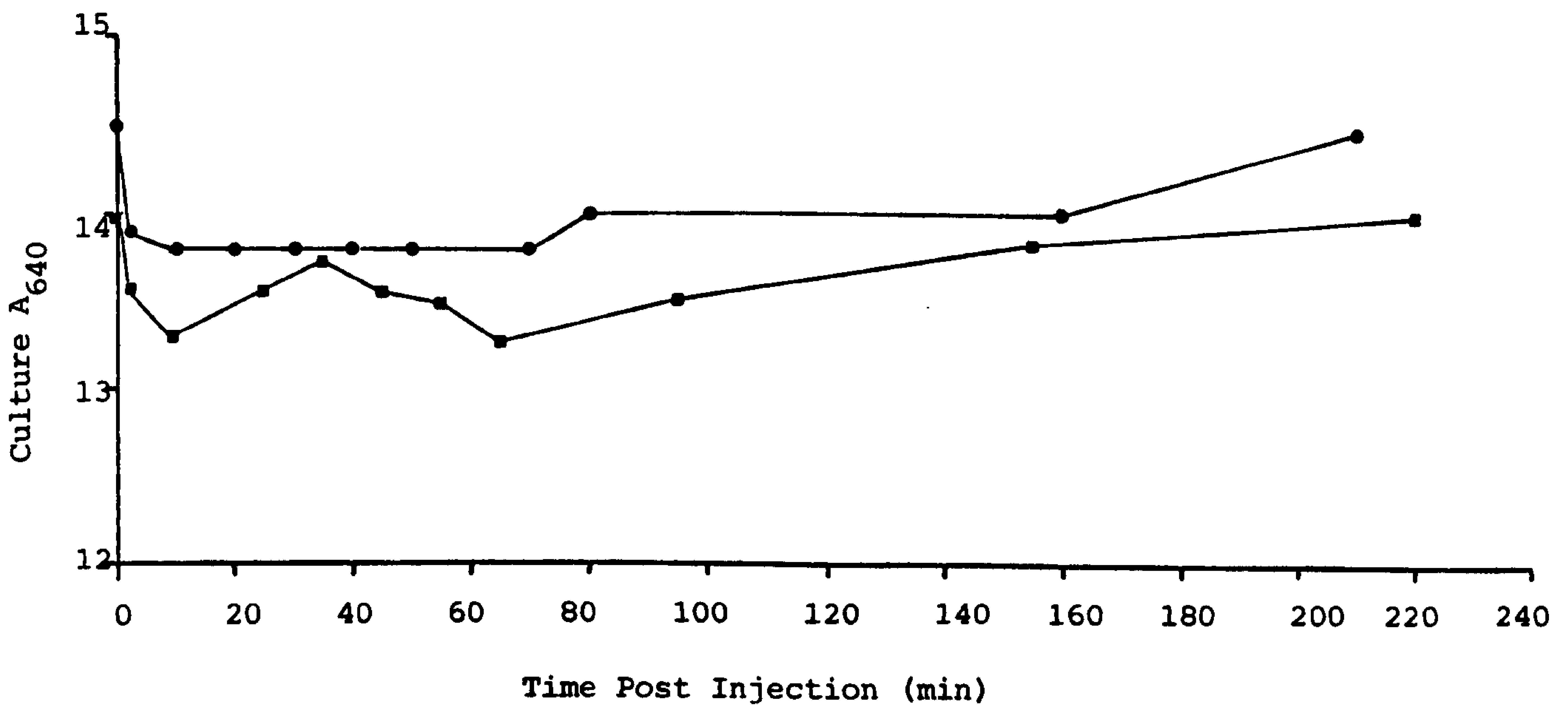


Figure 5.7 Culture A_{640} Vs. time post injection of 3 vitamin mixture (●) and 10 vitamin mixture (■); composition of mixtures listed in Chapter 2 (Table 2.3).

The initial fall in A_{640} recorded in the first 2 min following the injection of each of the casein hydrolysates was of roughly the same magnitude (1.0 to 1.1 A units) and thus seemed to be independent of the type or concentration of hydrolysate used. The initial fall in A_{640} following the injection of the vitamin solutions occurred over the first 8 min, the magnitude of the fall being equal (0.7 A units) in each case although the added liquid volumes were different.

In all experiments except the three vitamin addition, the culture A_{640} oscillated widely for a period of 40 to 60 min following the initial fall. In the casein addition experiments the degree of oscillation caused by the addition of the acid hydrolysates was greater than that caused by the tryptic hydrolysate. However, both concentrations of the acid hydrolysate produced roughly equal degrees of A_{640} oscillation in the cultures. Following the early oscillatory periods, the A_{640} of each culture gradually increased towards the pre-injection value. However, it should be noted that the larger intervals of culture sampling after the first 60 to 80 min may have overlooked further A_{640} oscillations occurring after the early period. In the vitamin injection experiments the A_{640} had risen to the pre-injection value after 0.70 to 0.73 residence tiems (210 to 220 min). In the casein addition experiments the A_{640} had not returned to the pre-injection values after 1 residence time (300 min) although the former value was re-established after 24 h in each case. This latter distinction between the vitamin and casein experiments may be related to the greater liquid volumes added in the latter experiments.

No stimulation of culture growth was achieved by any of the nutrient injections and thus, by the criteria of Mateles and Battat (1974), it was concluded that none of the nutrients added was growth-limiting in the glucose-excess cultures.

5.2.2 Growth Medium Supplementation

5.2.2.1 Yeast Extract

The steady state, D = 0.20 h^{-1}, culture concentrations of biomass and lactic acid recorded using a range of concentrations of yeast extract in the growth medium are shown in Figure 5.8. The base-state feeding of 30 g L^{-1} yeast extract (normal VR medium) was returned to three times during the course of the experiment with approximately 5% variation in the biomass concentration produced. Figure 5.8 shows that reducing the yeast extract feed concentration below 30 g L^{-1} resulted in approximately arithmetic decreases in the biomass and lactic acid concentrations produced. By contrast, increasing the yeast extract feed concentration above 30 g L^{-1} resulted in smaller, non-arithmetic, increases in biomass and product concentration.

The interpretation of Figure 5.8 is not straightforward. The observation that approximately arithmetic decreases in biomass and product concentration resulted from reducing the yeast extract concentration below 30 g L^{-1} suggests that some component of the extract may have been growth limiting at a feed concentration of 30 g L^{-1}, since sub-arithmetic decreases would have been expected had an excess existed in the base-state. That an increase in the concentrations of biomass and product, although small, resulted from the feeding of yeast extract at concentrations above 30 g L^{-1} supports the latter suggestion since no increase in biomass would have been expected had all components been in excess in the base-state. However, the consistently sub-arithmetic nature of the growth stimulation suggests that a different component (or components) of the extract, perhaps providing a lower yield of biomass, became limiting at feed concentrations greater than 30 g L^{-1}. In summary, it may be suggested that some

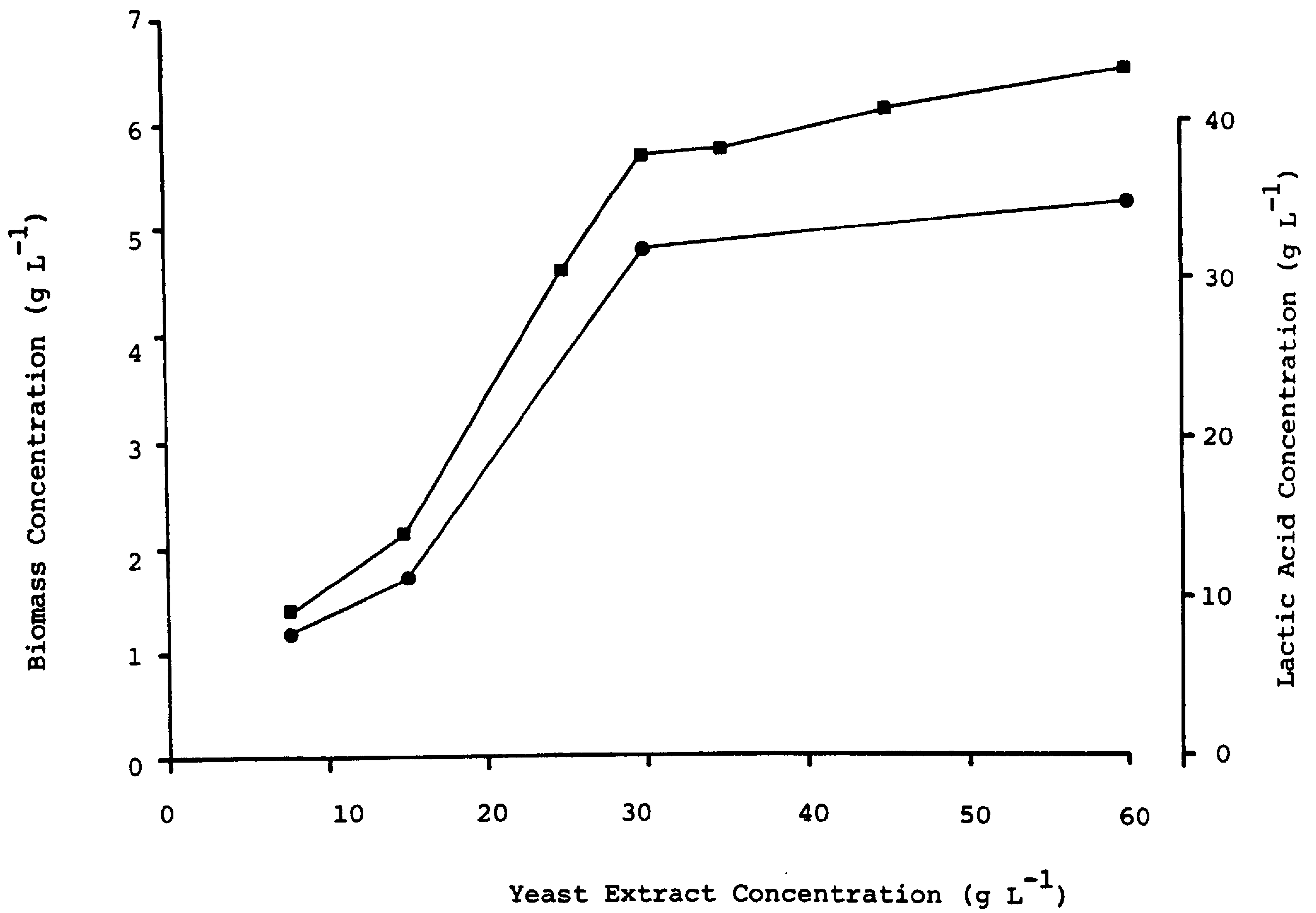


Figure 5.8 Steady state biomass (■) and lactic acid (●) concentrations Vs. yeast extract concentration in growth medium ($D = 0.2\ h^{-1}$).

component of yeast extract was the growth limiting substrate in the base-state, but this conclusion may be equivocal.

5.2.2.2 Other Nutrients

Table 5.6 presents the concentration of biomass and lactic acid observed in $D = 0.20\ h^{-1}$ cultures, at steady state, using unsupplemented VR growth medium and VR medium supplemented with a range of nutrients. The supplemented media are described in full in Chapter 2 (Section 2.6.4.2, Table 2.4). The base-state using unsupplemented VR medium was returned to three times during the course of the experiment with approximately 7% variation in the biomass concentration observed. Statistical analysis of the data presented in Table 5.6 reveals no significant difference between the biomass and lactic acid concentrations produced using the unsupplemented medium and those produced using any of the nutrient supplementations (Student's t test, $P = 0.05$). Stimulation of biomass concentration was observed on a single occasion using the medium supplemented with purines and pyrimidines (biomass = $6.03\ g\ L^{-1}$) but this stimulation was not found to be reproducible in subsequent chemostat cultures and thus the lower biomass concentration obtained is quoted in Table 5.6.

Table 5.6 shows that no reproducible stimulation of steady state biomass or lactic acid concentration was achieved by the supplementations made to VR medium. It may be concluded from the latter experiment that the growth of the $D = 0.20\ h^{-1}$, glucose-excess, chemostat culture was not limited by; amino acids present in tryptone, B vitamins, trace elements (Cu, Co, Ca, MoO_4, Zn, I, BO_3, Mn), or nucleic acid monomers.

Growth Medium	Biomass ($g\ L^{-1}$)	Lactic Acid ($g\ L^{-1}$)
Unsupplemented	5.20	31.80
+ Tryptone	4.70	30.82
+ Trace Elements	4.64	29.02
+ Riboflavin and Thiamine	4.79	33.48
+ Uridine and Thymidine	5.29	30.91
+ Purines and Pyrimidines	4.80	31.50
+ Manganese	4.70	31.90

Table 5.6

Steady State Biomass and Lactic Acid Concentrations Produced Using Unsupplemented and Supplemented Growth Media ($D = 0.20\ h^{-1}$)

CHAPTER 6

RESULTS: CELL RECYCLE FERMENTATION

6.1 Fermentation Time Profiles

In this Section, culture parameters are described as functions of time for four cell recycle fermentations. The results in Section 6.1.1 are from partial recycle experiments at recycle ratio (R) values equal to 0.9, 0.8 and 0.5 (6.1.1.1), and from a total recycle (R = 1.0) experiment (6.1.1.2). The results in Section 6.1.2 are from the R = 0.8 experiment.

6.1.1 Biomass, Lactic Acid and Product Balance

6.1.1.1 Partial Recycle

Profiles showing changes in the concentrations of biomass and lactic acid, and the balance of product formation (as molar product ratio; i.e. lactate: ethanol plus acetate), occurring with time, are presented for R = 0.9, 0.8 and 0.5 cultures in Figures 6.1, 6.2 and 6.3 respectively. The latter figures each illustrate a similar pattern of biomass accumulation. In each experiment three phases may be identified: firstly, a phase of rapid accumulation of biomass; secondly, a phase of much slower accumulation during which oscillations in biomass concentration were seen in the R = 0.9 and R = 0.8 experiments; and thirdly, the establishment of a steady state.

During the first phase of the R = 0.9 culture, (Figure 6.1) the biomass concentration increased by approximately four-fold over the first 25 h recycling. Lactic acid also accumulated in the R = 0.9 culture over the initial 25 h but the proportional increase in the concentration of the latter product (p) was not as great as that observed for the biomass concentration (x) representing a decrease

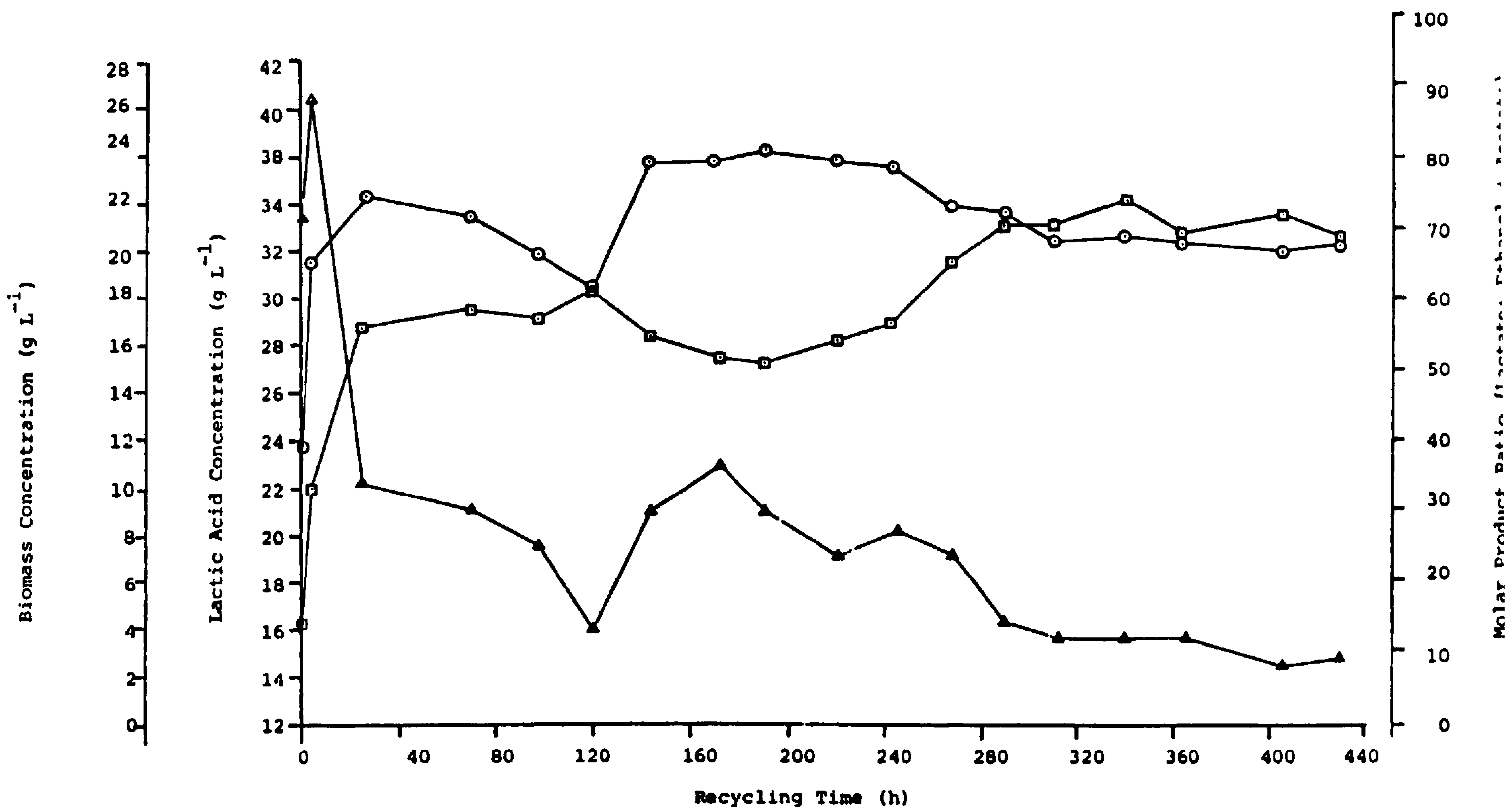


Figure 6.1 D = 0.3 h^{-1} R = 0.9. Biomass (▣) and lactic acid (◎) concentrations and molar product ratio (▲) Vs. recycling time.

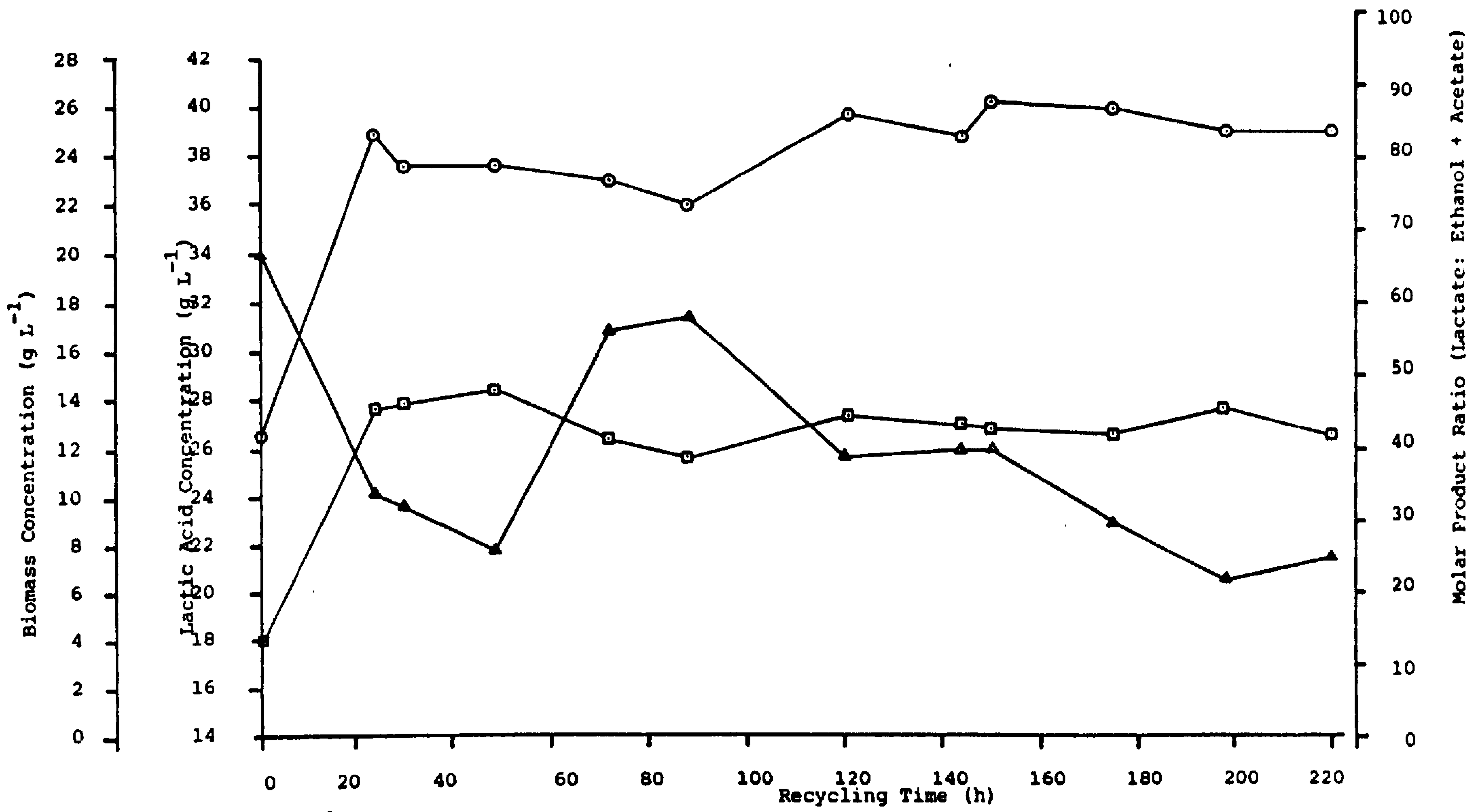


Figure 6.2 $D = 0.3\ h^{-1}$ $R = 0.8$. Biomass (□) and lactic acid (◎) concentrations, and molar product ratio (▲) Vs. recycling time.

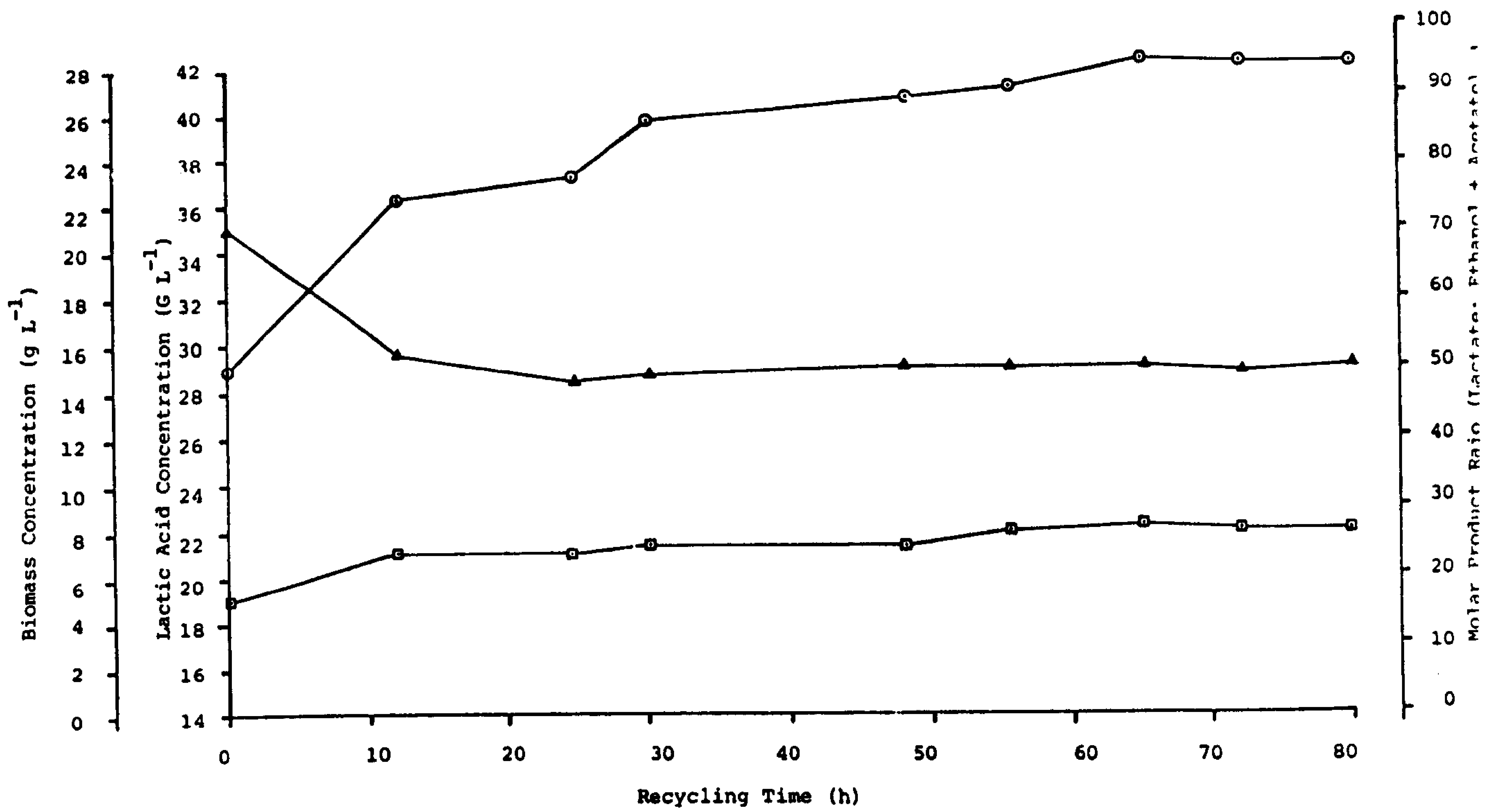


Figure 6.3 D = 0.3 h^{-1} R = 0.5 Biomass (□) and lactic acid (○) concentrations, and molar product raio (▲) Vs. recycling time.

in yield (Yp/x) from 6.0 g g^{-1} to 2.2 g g^{-1}. A similar response was observed during the first phase of the R = 0.8 culture (Figure 6.2) in which the concentrations of biomass and lactic acid increased (the former by approximately three-fold) over the first 24 h recycling. However as in the R = 0.9 experiment, Yp/x fell during this phase; from 6.6 g g^{-1} to 2.9 g g^{-1}. In the R = 0.5 experiment (Figure 6.3) the first phase was shorter, lasting only 12 h. The proportional increase in biomass concentration was less than two-fold and the associated fall in Yp/x was the least marked; from 6.4 g g^{-1} to 5.0 g g^{-1}.

In each experiment, the residual glucose initially present in the culture (glucose-excess chemostat, D = 0.30 h^{-1}) was essentially depleted during the first 4 to 6 h of recycling. Following the initial depletion, glucose was not detected throughout the duration of any of the experiments. The balance between the formation of lactic acid and the minor products, ethanol and acetate, as reflected by the molar product ratio, shifted during the first phase of rapid biomass accumulation. In the R = 0.9 experiment the molar product ratio increased from 70:1 to 90:1 during the first 4 h when the residual glucose was being utilized rapidly with the formation of lactic acid. However, between 4 h and 25 h in the same experiment, when the residual glucose was exhausted but biomass was still accumulating rapidly, the molar product ratio decreased from 90:1 to 33:1, favouring the formation of the minor products. Similarly, in the R = 0.8 and R = 0.5 experiments the balance of product formation shifted following the depletion of residual glucose, the molar product ratio decreasing from 70:1 to 35:1 and 55:1 respectively.

Following the initial phase of rapid accumulation, the rate of increase in biomass concentration declined in each experiment. During this second phase, marked oscillations in biomass concentration were

observed in the R = 0.9 and R = 0.8 experiments and changes in the major/minor product balance were seen which appeared to be linked to the pattern in biomass concentration. Whilst biomass was accumulating (between 25 h and 120 h, 191 h and 290 h in R = 0.9; between 24 h and 49 h, 98 and 121 h in R = 0.8) the molar product ratio decreased favouring the formation of the minor products, ethanol and acetate. Conversely, during periods of decline in biomass concentration (between 120 h and 191 h in R = 0.9; between 49 h and 98 h in R = 0.8) the formation of lactic acid was favoured and the molar product ratio increased. The oscillatory phase of the experiments was most pronounced in the R = 0.9 culture. The peak-to-trough variation in biomass concentration was the greatest (5.7 g L^{-1}) and the phase persisted for the longest time (265 h). Also, the link between the oscillations in biomass concentration and the balance of product formation was most clearly defined in the R = 0.9 culture in which the concentration of lactic acid oscillated in complete antiphase to the concentration of biomass. In the R = 0.5 experiment, very small oscillations in biomass concentration were seen following the initial rapid accumulation but the overall pattern was of a very gradual accumulation of biomass and lactic acid accompanied by a marginal reduction in molar product ratio.

Steady state (in terms of biomass and lactic acid concentration) was achieved within each culture. The time taken to establish steady state was not constant but varied in approximately constant proportion to the degree of recycle imposed, (1-R), as shown in Table 6.1. The steady states are described and analysed in Section 6.2.

Recycle Ratio (R)	Time to Steady State Hours	Hours x D	Hours x (1-R)
0.9	290	87	29
0.8	150	45	30
0.5	56	17	28

Table 6.1

Time taken to achieve steady state at different recycle ratios

($D = 0.3\ h^{-1}$)

6.1.1.2 Total Recycle

The R = 1.0 experiment lasted only 26 h during which the method of maintaining a constant culture volume with balanced flow rates of growth medium and cell-free filtrate, by use of a foam probe linked to the filtrate pump (Chapter 3, Section 3.4), was shown to be highly successful. The experiment was aborted because the accumulated biomass concentration proved to be too great for the filter units. Both the sintered glass prefilter and the hollow fibre cartridge blocked solidly causing a failure in the recycle loop line. Immediately prior to the loop failure the biomass concentration was 40.2 g L^{-1}, the lactic acid concentration was 38.5 g L^{-1} and the molar product ratio was 21:1.

6.1.2 Lactate Dehydrogenase Activity, Cellular Composition and Viability

The lactate dehydrogenase (LDH) specific activity and the total content of protein and polysaccharide measured in cells harvested throughout the R = 0.8 experiment are plotted against recycling time in Figure 6.4. The total content of carbon, hydrogen and nitrogen of similarly harvested cells are plotted against recycling time in Figure 6.5. The latter figures illustrate a constancy of chemical composition throughout the cell recycle fermentation. The mean cellular contents of protein and polysaccharide (as percentage of biomass dry weight) were 36.6% and 16.3% respectively. The mean cellular CHN composition was: Carbon, 44.3%; nitrogen, 6.9%; hydrogen, 10.5%. The latter figures also illustrated an approximately constant specific activity of LDH, a key enzyme in determining the metabolic fate of pyruvate and hence the balance of product formation, throughout the cell recycle culture. The mean specific activity was 6.3 µmol NADH mg^{-1} min^{-1}. Interestingly, both the cellular

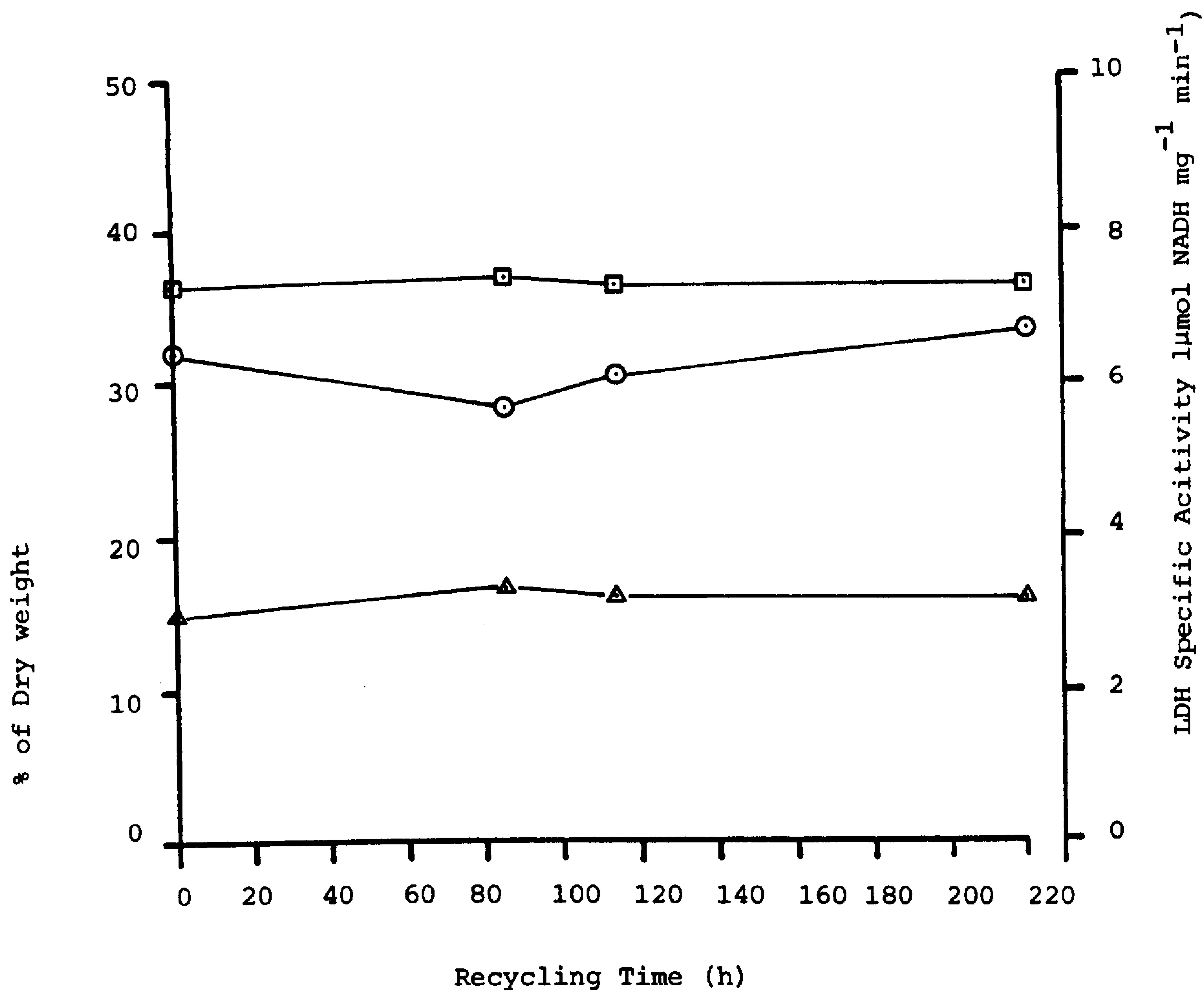


Figure 6.4 D = 0.3 h^{-1}, R = 0.8. LDH specific activity in cell-free extracts (⊙); total cellular protein (⊡) and polysaccharide (△) as % of dry weight Vs. recycling time.

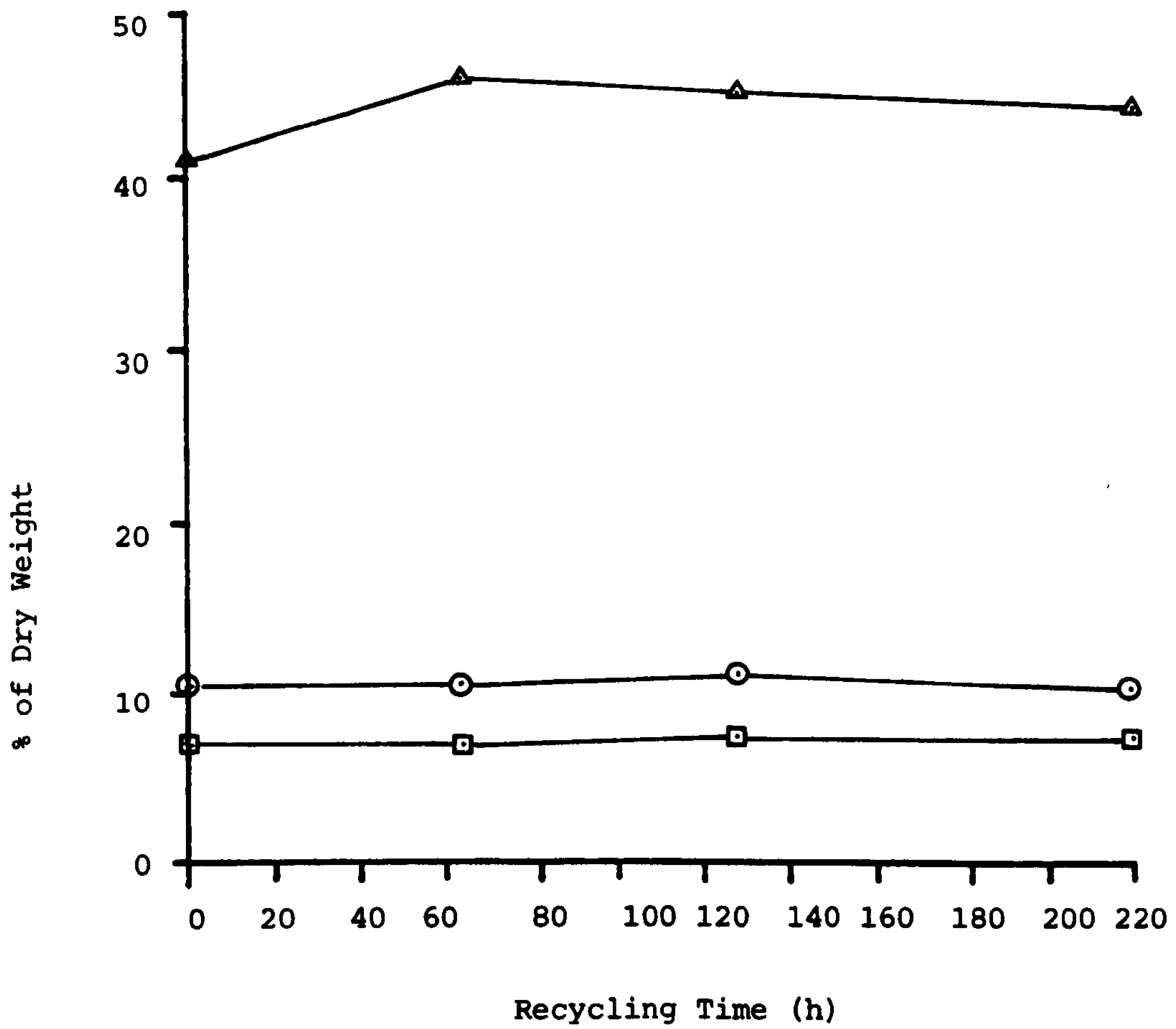


Figure 6.5 D = 0.3 h^{-1}, R = 0.8. Total cellular carbon (▲), nitrogen (⊡) and hydrogen (⊙) as a % of dry weight Vs. recycling time.

composition and LDH activity observations support the findings of the chemostat dilution rate profile which had illustrated such constancies throughout a range of dilution rate from 0.05 h^{-1} to 0.30 h^{-1} (Chapter 5, Sections 5.1.4 and 5.1.5).

The effect of cell recycling on the culture viability was assessed throughout the R = 0.8 experiment. The slide culture technique, used by Vick Roy *et al.* (1983) for viability measurements in cell recycle cultures, was found to be inappropriate for the organism grown in the present study. The latter approach relied on distinguishing between single cells (representing one non-viable unit) and microcolonies (representing one viable unit) by use of a microscope after incubation on solid medium. *Lactobacillus delbreuckii* was routinely to be found existing in chains of between two and twenty cells immediately on removal from the fermenter and single cells were rare. As a result it was impossible to make the viable/non-viable distinction on the basis of the presence/absence of chains of cells following incubation. The second approach taken was to compare colony counts on solid medium with total cell counts made with a counting chamber. It was assumed that each colony forming unit represented one chain of cells. Therefore, the colony counts were multiplied by the mean number of cells per chain in each sample to give the count of viable cells. The percentage of the total cell count which was viable throughout the R = 0.8 experiment is shown in Figure 6.6. This latter Figure suggests that cell recycling did not reduce the viability of the culture which remained between 66% and 76% viable (the D = 0.30 h^{-1} chemostat steady state culture was 69% viable).

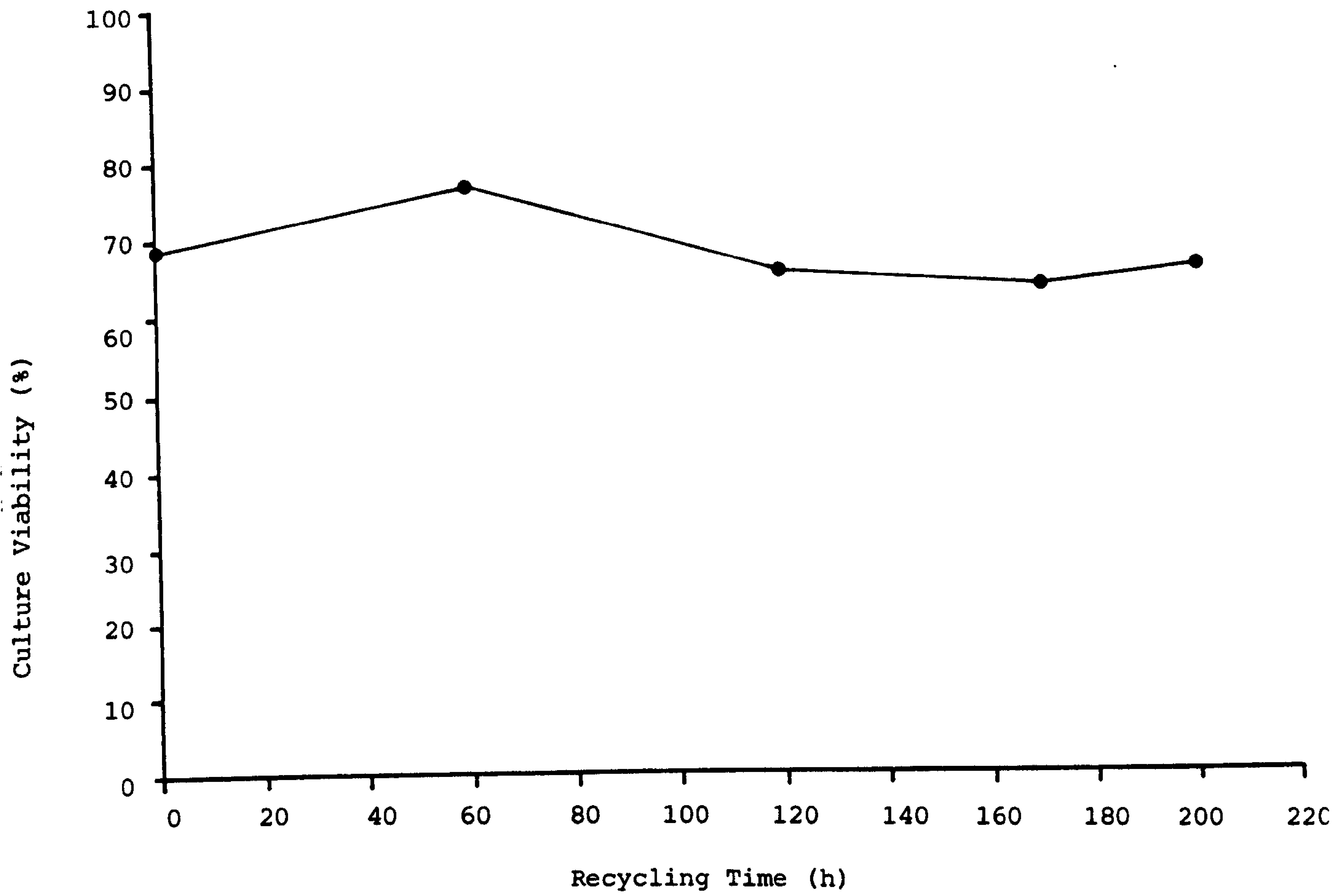


Figure 6.6. $D = 0.3\ h^{-1}$, $R = 0.8$. Culture viability Vs. recycling time.

6.2 Steady State Results

6.2.1 Biomass Production

The concentration of biomass achieved at steady state in each cell recycle fermentation was considerably greater than that at the same dilution rate in the chemostat. However, when account was taken of the physical concentration effect (dividing the biomass concentration by 1-R) it was found that the yield of biomass per mole of glucose consumed was reduced in the cell recycle fermenter, greater reductions being observed at higher recycle ratios. The steady state biomass concentrations and yields are shown in Table 6.2.

It was apparent that the enhancement of biomass concentration was not as great as would be predicted by the model of Bull & Young (1981) (Chapter 1, Section 1.2.1), which assumed the retention of a constant biomass yield in the cell recycle fermenter. An attempt was made to formulate a mathematical model which would more accurately describe the observed biomass production. The formulated model described the reduction in biomass yield in terms of a constant rate of consumption of glucose for non-anabolic functions. This latter model was derived as follows. The Bull & Young (1981) model for steady state biomass concentration ($\bar{x}$) is shown in equation 6.1:

$$\bar{x} = Y(s_o - \bar{s})/(1-R) \qquad \underline{\text{Equation 6.1}}$$

At steady state, s_o is very much greater than $\bar{s}$ and thus equation 6.1 approximates to:

$$\bar{x} = Ys_o/(1-R) \qquad \underline{\text{Equation 6.2}}$$

The reduction in growth yield caused by a constant specific maintenance rate of glucose consumption (q_m) was described by Tempest & Neijssel

Culture	Biomass Concentration, x (g L^{-1})	x/(1-R) (g L^{-1})	Growth Yield, [x/(1-R)]/glucose (g $mole^{-1}$)
Batch	7.70	N/A	27.72
Chemostat D = 0.3 h^{-1}	4.04	4.04	19.14
Recycle R = 0.5	8.15	4.08	16.11
R = 0.8	13.05	2.61	10.32
R = 0.9	20.92	2.09	8.26

Table 6.2

Biomass Concentrations and Growth Yields from Glucose in Batch (Stationary Phase), Chemostat and Cell Recycle Cultures (Steady state, D = 0.3 h^{-1})

(N/A: Not Applicable)

(1984) to be:

$$^{1}/_{Y} = ^{1}/_{Y^{max}} + q_m/\mu \qquad \text{Equation 6.3}$$

where Y^{max} is the maximum growth yield and μ is the specific growth rate. Substituting for Y in equation 6.2 with equation 6.3, and as $\mu = (1-R)D$ in cell recycle culture at steady state, we have:

$$\bar{x} = [s_o/(^{1}/_{Y^{max}} + q_m/(1-R)D)]/(1-R) \qquad \text{Equation 6.4}$$

In solving for $\bar{x}$, Y^{max} was defined as the mean growth yield observed in chemostat culture where the non-anabolic consumption of glucose was shown to be negligible (Chapter 5, Section 5.1); a value of 22.88 g mol^{-1} was obtained for Y^{max}. Equation 6.4 was solved for $\bar{x}$ over a range of R using different values of q_m. The value of q_m which best predicted the observed biomass concentration was derived by a least squares analysis of prediction and observation at R = 0.5, 0.8 and 0.9. The best-fitting value of q_m was 2.50 mmol glucose g^{-1} h^{-1} which produced an overall variation in $\bar{x}$ between prediction and observation of 1.0 g L^{-1} and a sum of squares variation of 4.19 g L^{-1}. Figure 6.7 compares the biomass concentrations at a range of R as predicted by the Bull & Young (1981) model (equation 6.2, Y = 22.88 g mol^{-1}) and the maintenance model presented here (equation 6.4, Y^{max} = 22.88 g mol^{-1}, q_m = 2.50 mmol g^{-1} h^{-1}), with those observed at R = 0.5, 0.8 and 0.9. The comparison shows that the Bull & Young (1981) model consistently overestimated the biomass concentration. By contrast, the maintenace model predicted the biomass concentration with reasonable accuracy. It was concluded that maintenance effects were significant in the cell recycle fermenter.

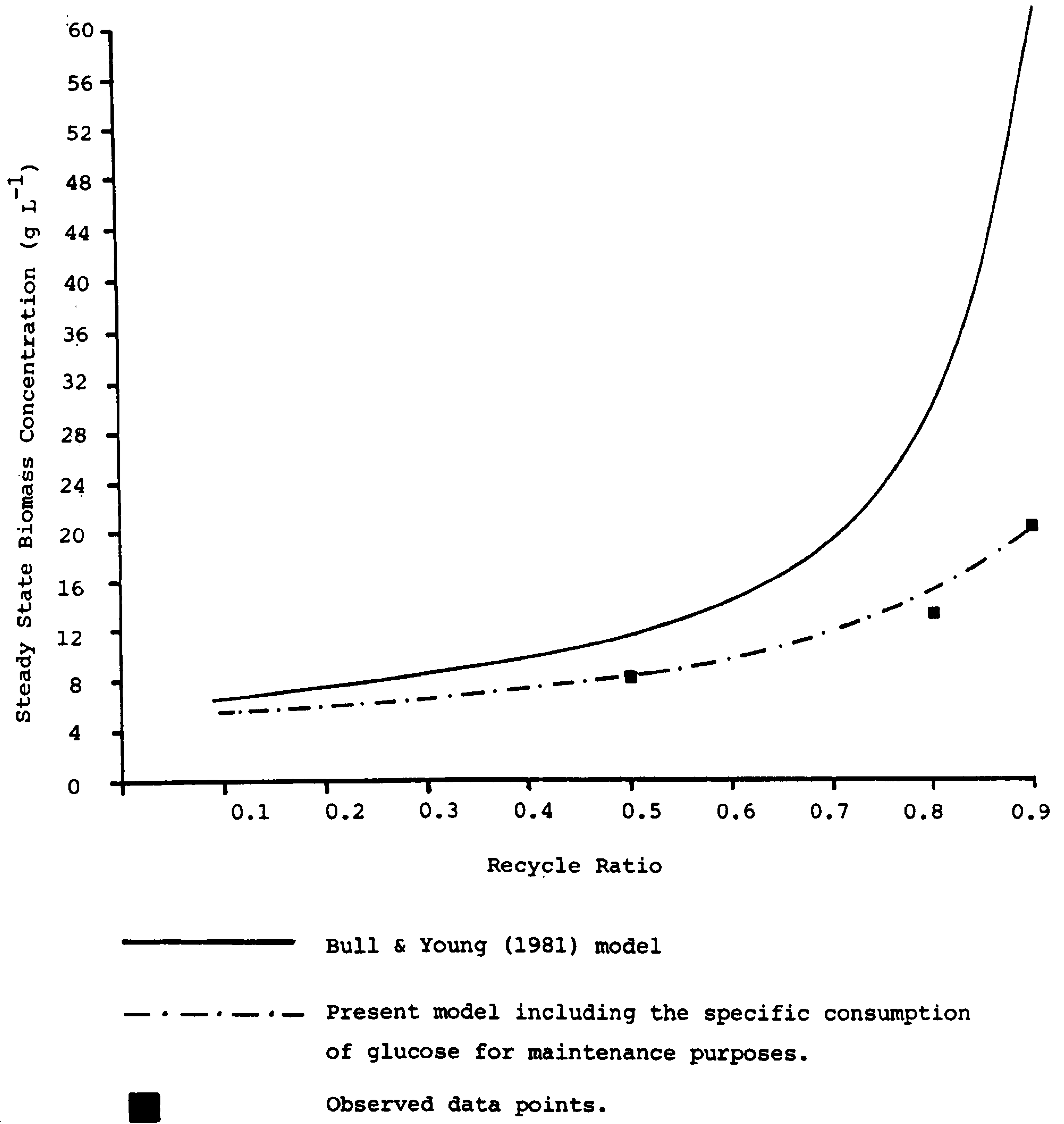


Figure 6.7 A comparison of prediction with observation for two models of biomass production in cell recycle fermentation

6.2.2 Product Formation

The concentrations of lactic acid, ethanol and acetate; the molar product ratio; the yield of lactic acid from biomass (Yp/x); and the volumetric productivity of lactic acid are shown in Table 6.3 for each cell recycle steady state and for the chemostat steady state at the same dilution rate ($D = 0.3\ h^{-1}$).
It may be seen from Table 6.3 that during each cell recycle fermentation the concentration of lactic acid was enhanced in comparison with the chemostat. However, the increases in the concentration of the latter product proportionally were not as great as those for biomass (Table 6.2) and thus Yp/x was reduced, greater reductions occurring at higher recycle ratios. In addition, a shift in the balance of product formation was seen which increased with recycle ratio. The steady state concentration of lactic acid declined and those of ethanol and acetate increased resulting in decreases in the molar product ratio (lactate: ethanol + acetate). The reduction in the concentration of lactic acid at $R = 0.8$ and 0.9 ensured that the latter cultures were less productive than the $R = 0.5$ culture but all the cell recycle fermentations were more productive than the chemostat. The $R = 0.5$ culture achieved a volumetric productivity 35.5% greater than the most productive chemostat state, due to the complete utilization of glucose. Despite the shift in product balance, lactic acid remained the major product; being formed at a molar concentration at least ten times greater than those of the minor products combined.

The nature of the observed shift in the product balance was elucidated by the glucose pulse experiment made during the $R = 0.9$ steady state (after 311 h recycling) in which 50 mmol glucose was injected directly into the culture. Table 6.4 shows the concentrations of biomass, lactic acid, ethanol and acetate, the yield of each

	Chemostat	Recycle Fermenter		
		Recycle Ratio		
		0.5	0.8	0.9
Lactic Acid Concn. (mM)	309	448	439	383
Ethanol Concn. (mM)	2.6	4.8	9.2	18.5
Acetate Concn. (mM)	1.4	4.5	8.0	17.4
Molar Product Ratio	70:1	48:1	25:1	10:1
$Y_{p/x}$ ($g\ g^{-1}$)	6.9	4.9	3.0	1.7
Volumetric Productivity ($g\ L^{-1}\ h^{-1}$)	8.3	12.1	11.9	10.3

Table 6.3

Steady State Product Formation in Chemostat and Recycle Fermenter ($D = 0.30\ h^{-1}$)

product from biomass, and the molar product ratio immediatley prior to glucose injection, 60 min and 120 min post-injection. Figure 6.8 shows the rate of total acid production over consecutive 2 min periods, estimated from measurements of alkali addition rate, following the glucose injection. Included in Figure 6.8 is an estimated response of acid production rate based on the selection of a higher acid-yielding organism growing at the maximum specific growth rate for the system, calculated using Equation 6.5:

$$r_t = r_o e^{\mu_{max} t} \qquad \text{Equation 6.5}$$

where t is the time post-injection, r_t is the acid production rate at time t, r_o is the pre-injection acid production rate, and μ_{max} is the maximum specific growth rate (0.52 h^{-1}). The calculated 'genotypic response' represents an estimate of the fastest increase in acid production rate which could result from mutant selection.

It may be seen from Table 6.4 that the biomass concentration decreased following the glucose pulse due to the diluting effect of the injection (1.1% fall in biomass, 1.2% v/v injection). The balance of product formation was temporarily altered by the glucose pulse. After 60 min, at the height of the burst of acid production (Figure 6.8), the concentration and yield of lactic acid had increased whilst those of the minor products had decreased with a resultant increase in the molar product ratio. The magnitude of the increase in the concentration of the major product (98 mmol) indicated that the glucose injected was converted almost quantitatively to lactic acid. After 120 min, the burst of acid production was over and the pre-injection balance was beginning to be re-established. The increase in acid production rate was almost instantaneous (Figure 6.8) and was clearly more rapid than the estimated fastest genotypic response. In summary, the

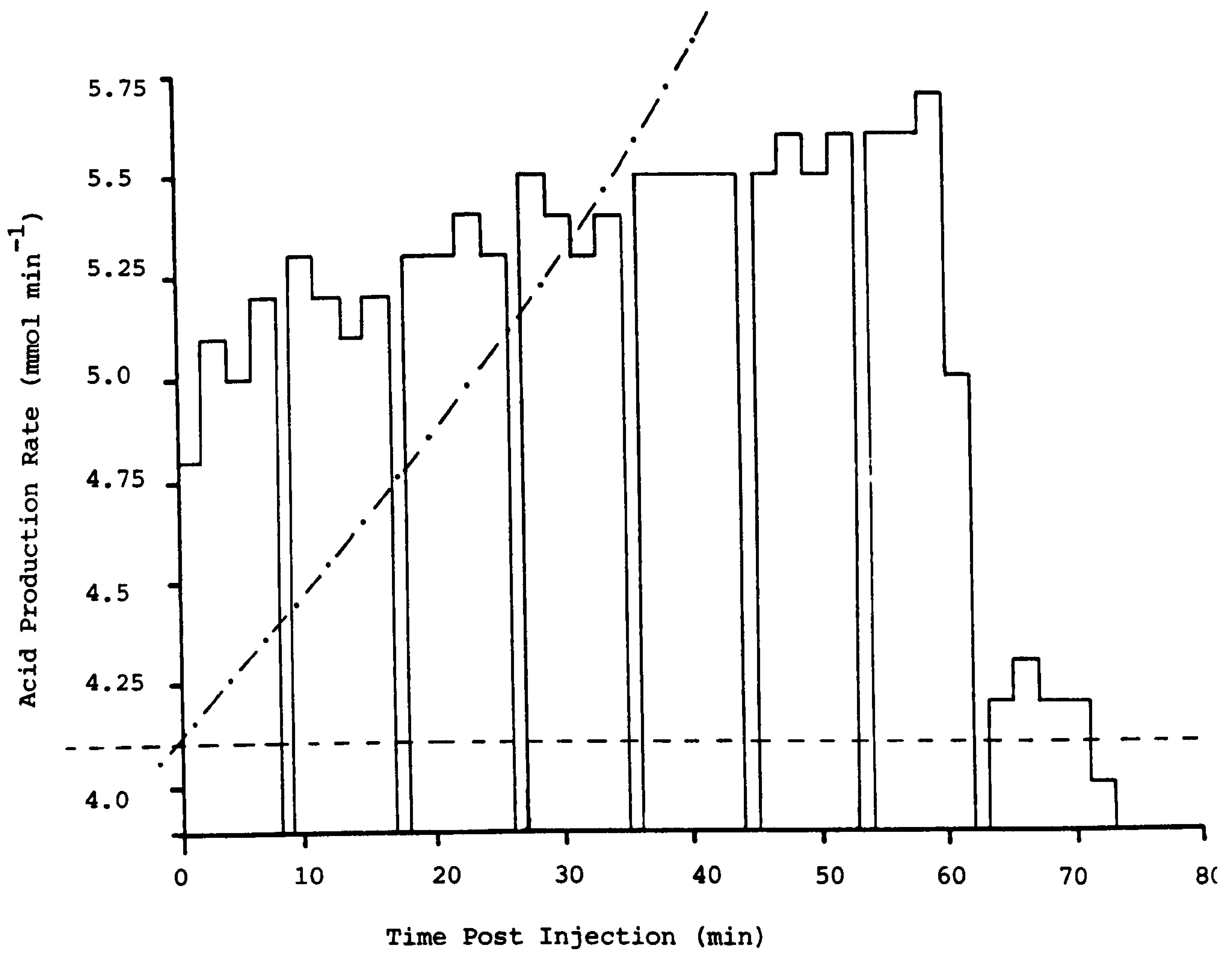


Figure 6.8 D = 0.3 h^{-1} R = 0.9. Total acid production rate following direct injection of glucose (50.5 mmol) into the culture.

	Time Post-Injection (min)		
	0	60	120
Biomass			
Concentration (g L^{-1})	20.93	20.71	20.63
Lactic Acid			
Concentration (mM)	382	478	422
Yield from Biomass (mmol g^{-1})	18.25	23.08	20.46
Ethanol			
Concentration (mM)	16.0	13.5	13.8
Yield from Biomass (mmol g^{-1})	0.76	0.65	0.70
Acetate			
Concentration (mM)	15.6	12.0	12.4
Yield from Biomass (mmol g^{-1})	0.75	0.58	0.60
Molar Product Ratio			
(Lactate: Ethanol + Acetate)	12:1	19:1	16:1

Table 6.4

Glucose Pulse Experiment Biomass and Product Concentrations Yields and Molar Product Ratio, Pre and Post Injection of Glucose (50 mmol), R = 0.9

results of the glucose pulse experiment suggest: (1) that the shift in product formation balance seen in the cell recycle fermenter was due to glucose limitation; (2) that the shift was phenotypic in nature because the rapidity of the acid production response suggested the presence of considerable excess lactic acid producing capacity within the culture which was subject to glucose limitation.

The specific rate of adenosine triphosphate (ATP) formation (q_{ATP}) was estimated from cell recycle culture product formation data assuming that 1 mole ATP was formed in the production of each mole of pyruvate plus 1 mole ATP in the conversionof pyruvate to acetate (De Vries *et al.*, 1970). The overall equation was:

$$q_{ATP} = \frac{[\text{lactate}] + 0.5\ [\text{ethanol}] + 1.5\ [\text{acetate}]}{[\text{biomass}]} \times \text{Dilution Rate}$$

Equation 6.6

In the same way, q_{ATP} values were calculated from chemostat data (Chapter 5, Tables 5.1 and 5.2). All the calculated q_{ATP} data are shown plotted against the specific rate of glucose uptake ($q_{glucose}$) in Figure 6.9 with a linear regression line constructed using the chemostat data only. The cell recycle data fall very close to the chemostat regression line suggesting that the yield of ATP per mole glucose was not enhanced in the cell recycle fermenter despite the observed shift in product balance. The q_{ATP} values plotted in Figure 6.9 are also shown in Table 6.5 with the specific growth rate (μ) and the yield of biomass per mole ATP formed ($Y_{ATP} = \mu/q_{ATP}$). It may be seen that in the chemostat Y_{ATP} remained broadly constant (mean 13.73 g mol^{-1}, standard deviation 0.77), but in the cell recycle fermenter Y_{ATP} decreased with increasing recycle ratio such that at R = 0.9, Y_{ATP} was 37.2% of the mean chemostat value. This latter

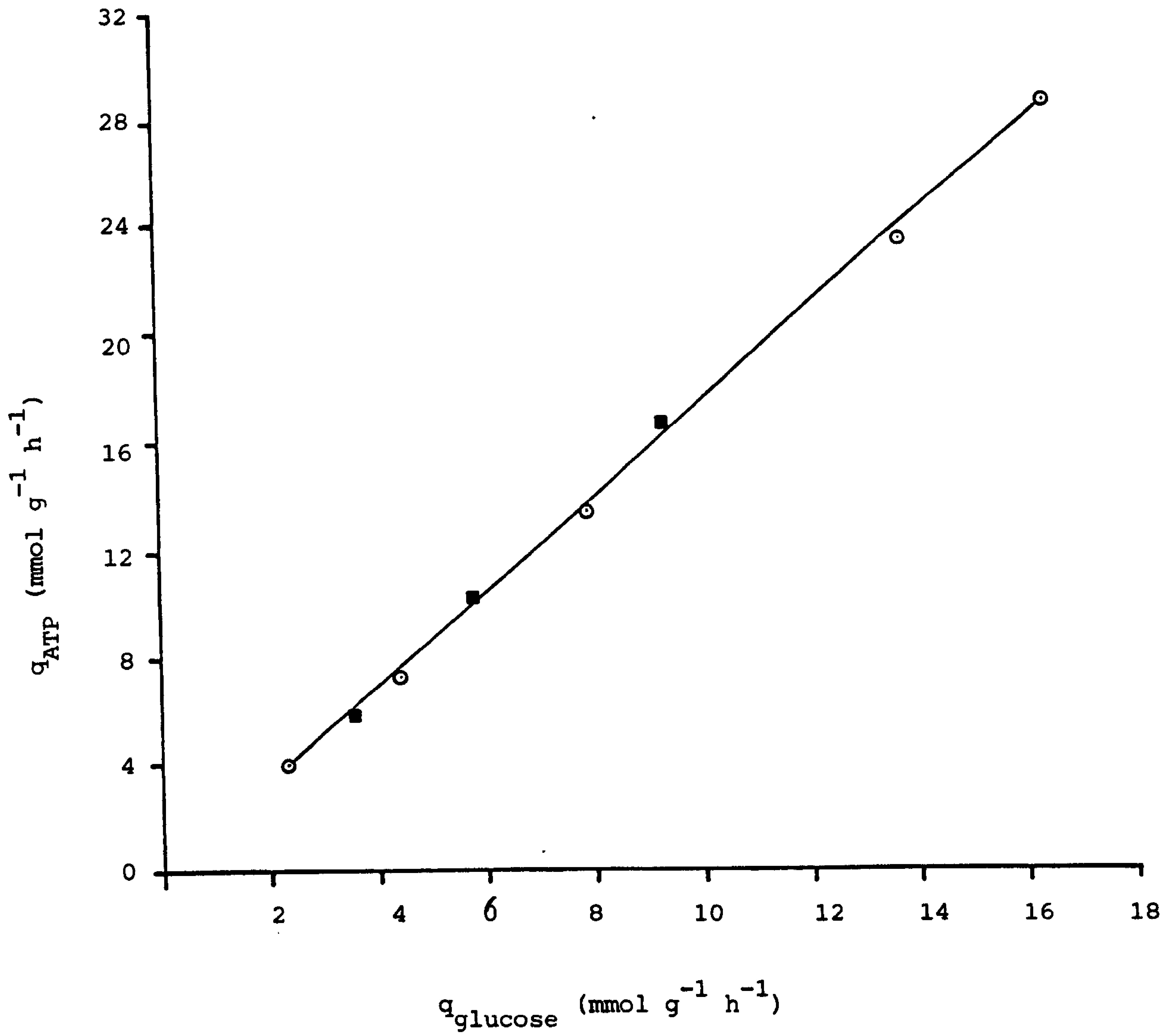


Figure 6.9 Specific rate of ATP formation (q_{ATP}, from product formation data) Vs. specific rate of glucose uptake ($q_{glucose}$) for chemostat (⊙) and cell recycle fermentation (■) data (regression line calculated from chemostat data only).

CHEMOSTAT

μ (h^{-1})	q_{ATP} (mmol g^{-1} h^{-1})	Y_{ATP} (g mol^{-1})
0.05	3.93	12.72
0.10	7.23	13.82
0.20	14.46	14.89
0.30	23.44	12.80
0.40	28.80	13.89
0.50	35.01	14.28

CELL RECYCLE

R	μ (h^{-1})	q_{ATP} (mmol g^{-1} h^{-1})	Y_{ATP} (g mol^{-1})
0.5	0.15	16.73	8.97
0.8	0.06	10.38	5.78
0.9	0.03	5.87	5.11

Table 6.5

Specific Growth Rate (μ), Specific Rate of ATP Formation (q_{ATP}) and Yield of Biomass from ATP (Y_{ATP}) for Chemostat and Cell Recycle Cultures

observation indicates that an analysis of product formation data supports the analysis of glucose uptake data in suggesting that glucose catabolism and cellular anabolism were less closely coupled in the cell recycle fermenter than in the chemostat.

CHAPTER 7

DISCUSSION

7.1 Conventional Fermentation

7.1.1 Batch Culture

The maximum specific growth rate (μ_{max}) and the yields of biomass and lactic acid from glucose (Yx/s and Yp/s) were consistent with previously reported data; μ_{max} was 0.52 h^{-1} and after 12 h culturing, Yx/s and Yp/s were 0.15 gg^{-1} and 0.98 gg^{-1} respectively. Using the same organism and growth conditions, Luedeking & Piret (1959a) reported a μ_{max} of 0.48 h^{-1} and Vick Roy *et al.* (1983) reported a μ_{max} of 0.65 h^{-1}, Yx/s of 0.16 gg^{-1} and Yp/s of 0.90 gg^{-1}. The specific rates of lactic acid formation ($q_{lactate}$) and glucose consumption ($q_{glucose}$) were variable throughout the batch fermentation with peak values occurring in the mid-exponential phase (Figure 4.3). By contrast, the specific growth rate (μ) was constant during the exponential phase (Figure 4.3) suggesting that μ_{max} was not determined by the rate of glucose consumption. This conclusion is supported by the observation that the onset of the deceleration phase occurred whilst 18.5 g L^{-1} glucose remained unused in the culture (Figure 4.1).

7.1.2 Chemostat Culture

The chemostat experiments yielded results which were broadly consistent with previous reports.

Maximum concentration of biomass and lactic acid were produced at low values of D (0.05 h^{-1}, 0.10 h^{-1}) and glucose consumption was complete only in these latter steady states. At D = 0.50 h^{-1}

34.07 g L^{-1} glucose (68% of the glucose in the feed stream) remained unused (Table 5.1). The presence of excess sugars in higher D cultures and their complete utilization at lower D has been reported by Keller & Gerhardt (1975) and Thomas, Ellwood & Longyear (1979). The former authors, reporting continuous cultures of *L. bulgaricus* on a whey feedstock, observed complete lactose utilization at D = 0.067 h^{-1} but 4.2% (w/v) residual lactose at D = 0.20 h^{-1}. Thomas *et al.* (1979) reported similar responses from two strains of *Streptococcus lactis* in a chemostat: for one strain, glucose utilization was complete at D = 0.48 h^{-1} and below but 5.11 mM residual glucose was present at D = 0.76 h^{-1}; for the other strain, glucose utilization was complete at D = 0.29 h^{-1} and below but 3.42 mM remained at D = 0.61 h^{-1}.

Linear correlations were observed for both $q_{lactate}$ and $q_{glucose}$ versus D (Figures 5.1). Similarly, linear correlation of $q_{lactate}$ versus $q_{glucose}$ was observed throughout the range of D (Figure 5.2). These responses indicate that Yp/x, Yx/s and Yp/s remained constant throughout the dilution rate range with values calculated by linear regression to be 6.35 gg^{-1}, 0.12 gg^{-1} and 0.81 gg^{-1} respectively. Yields which were broadly constant at different values of D have previously been reported by Hanson & Tsao (1972) (Yp/s 0.70 gg^{-1} to Yp/s 0.73 gg^{-1}). A similar Yp/s, 0.77 gg^{-1} (D = 0.175 h^{-1}), was reported by Luedeking & Piret (1959b) although a lower yield, 0.67 gg^{-1}, was reported at D = 0.48 h^{-1}.

Observations of <u>variable</u> yields as a function of D have been reported by De Vries *et al.* (1970) (*L. casei*) and Thomas *et al.* (1979) (*S. lactis* strain ML_3). In these latter reports Yp/s was minimal at low dilution rates; Yp/s = 0 at D = 0.14 h^{-1} and 0.125 h^{-1} (De Vries *et al.*, 1970), and Yp/s = 0.01 gg^{-1} at D = 0.11 h^{-1} (Thomas *et al.*, 1979).

These reductions in Yp/s were reflections of shifts in product profile from entirely homolactic at high D to essentially or entirely heterolactic at low D when the products were ethanol, acetate and formate. Thomas *et al.* (1979) reported that the decrease in Yp/s at low D was accompanied by an increase in Yx/s from 0.20 gg^{-1} at D = 0.76 h^{-1} to 0.24 gg^{-1} at D = 0.35 h^{-1} and below. Both De Vries *et al.* (1970) and Thomas *et al.* (1979) concluded that the observed changes in yields and product profile represented a channelling of pyruvate away from lactate dehydrogenase (LDH) towards the ATP-generating formation of acetate by acetate kinase involving pyruvate formate lyase (PFL) in the initial reaction. Thomas *et al.* (1979) suggested that pyruvate dehydrogenase may also have been involved in the initial reaction leading to acetate formation.

Interestingly, the changes in yields and product profile reported by Thomas *et al.* (1979) were strain-dependant. A second strain of *S. lactis* (ML_8) remained homolactic at all dilution rates and Yx/s remained constant. The observations in the present study are consistent with this latter response. A small shift in product profile was observed at low D; the molar product ratio, Lactate: ethanol plus acetate, decreased from about 70:1 at D = 0.20 h^{-1} (glucose-excess) to 33:1 at D = 0.05 (glucose-depleted) (Table 5.2). However, the small shift did not reduce Yp/s nor increase Yx/s (Figure 5.1 and 5.2).

De Vries *et al.* (1970) concluded that the observed changes in Yp/s and product profile on changing D were due to a reduction in the intracellular level of fructose-1, 6-diphosphate (FDP), a powerful activator of LDH, in glucose-limited *Lactobacilli*. The latter authors observed no difference in the specific activity of LDH in cells from glucose-limited and glucose-excess steady states. Thomas *et al.* (1978) suggested that the balance of product

formation by lactic streptococci was influenced by the intracellular FDP concentration and by a reduction in the specific activity of LDH in cells from low D, glucose-limited, steady states. Subsequently Thomas concluded that in lactic streptococci a complete diversion of products from lactate required changes in both the specific activity of enzymes and the intracellular concentration of effectors (Fordyce *et al.*, 1984).

In the present study LDH specific activity was slightly reduced in the glucose-depleted (D = 0.064 h^{-1}) cells but this reduction may not have been significant (Table 5.3), thus lending support to the observations of De Vries *et al.* (1970). No measurements of intracellular FDP concentration were made in the present study but the possibility that the level of the latter compound may have been responsible for control of the metabolism of pyruvate was highlighted since LDH activity was severely restricted in assay buffers ommitting FDP (Table 5.3). Interestingly, residual LDH activity was observed in the absence of FDP, a phenomenon also reported by Crow & Pritchard (1977). Manganese ions were required for complete LDH activity (Table 5.3) as also reported by De Vries *et al.* (1970) and Holland & Pritchard (1975). Phosphate ions inhibited LDH activity (Figure 5.4), an observation consistent with previous reports (Holland & Pritchard, 1975; Garvie, 1980).

Kinetic studies in which the concentration of pyruvate in the assay mixture was varied suggested that common V_{max} and K_m values could be ascribed to the LDH of cells from the glucose-depleted and glucose-excess steady states (Figure 5.5). The values of the latter parameters (11.1 µmol NADH mg^{-1} min^{-1} and 9.3 mM respectively) are consistent with previous reports (Garvie, 1980).

The plots of $q_{lactate}$ and $q_{glucose}$ vs D (Figure 5.1) both extrapolated very close to the origin, remaining linear at low values of D. Thus, analyses of product formation and glucose consumption both suggested that the demand for glycolytic activity to fulfil non-anabolic (e.g. maintenance) functions was negligible. De Vries *et al.* (1970) also reported a very low specific rate of maintenance metabolism (q_m; 1.52×10^{-3} mole ATP (g dry weight)$^{-1}$ h^{-1}) for anaerobic chemostat cultures of *L. casei* in a complex medium. Tempest & Neijssel (1984) have suggested that the method of estimation of q_m used in the present study (plotting $q_{glucose}$ vs D) is superior to the method used by De Vries *et al.* (1970) (plotting $^1/_Y$ vs $^1/_D$) since the latter approach places heavy emphasis on data obtained at low values of D in which inaccuracies of measurement may be more marked. Furthermore, plots of q vs D produce a more even distribution of data points, deviations from linearity are more recognizable, and q_m is estimated as an intercept point rather than as the gradient of a very shallow line as in double reciprocal plots. Pirt (1975) stated that the apparent growth yield (Yx/s) was related to the theoretical maximum growth yield (Y^{max}) as follows:

$$^1/_{Yx/s} = {^1/_{Y^{max}}} + q_m/D$$ Equation 7.1

In the present chemostat study, q_m has been shown to be very low and so the measured Yx/s may be taken to be an estimate of Y^{max}.

The biological interpretation of q_m and Y^{max} values requires circumspection. Changes in the chemical composition of microbial cells may obscure the true trend in growth yield (Tempest & Neijssel, 1984). In the present study, the cellular content of total protein, polysaccharide, carbon and nitrogen remained constant throughout a range of D from 0.05 h^{-1} to 0.30 h^{-1}. The mean polysaccharide content

(15.5% of dry weight) is consistent with a quoted value for the percentage of cell wall polysaccharides in bacteria (De Vries *et al.*, 1970) and suggests that no intracellular carbon storage occurred. The constant cellular nitrogen content (mean, 9.18% of dry weight) combined with a constant content of total protein (mean, 44.8% of dry weight) suggests that the total content of non-protein nitrogenous compounds (such as nucleic acids) also remained constant. The constancy of cellular chemical composition lends support to the suggestion that Yx/s in the chemostat may be taken as an estimate of Y^{max}.

In two glucose-excess steady states (D = 0.35 h^{-1} and 0.40 h^{-1}), total carbon, nitrogen and total organic and inorganic phosphate were also present in considerable excess in culture supernatants (Table 5.5). It is, therefore, unlikely that any of the latter nutrients was the growth-limiting substrate in the glucose-excess steady states. Experiments were performed to identify the growth limitation in the latter steady states. The results were largely negative and somewhat equivocal (Chapter 5, Section 5.2).

In the first set of experiments, nutrient solutions were injected directly into the culture in order to observe transient stimulation of growth, following the rationale of Mateles & Battat (1974). No stimulation of growth was observed following the injection of casein hydrolysates or defined mixtures of vitamins and culture A_{640} oscillated widely after each injection (Figures 5.6 and 5.7). By the criteria of Mateles & Battat (1974), it may be concluded that none of the amino acids and vitamins injected was growth-limiting. However, it may be that the growth-limiting substrate was indeed injected in one of the mixtures but that any stimulatory effect was masked by the oscillations in A_{640} caused by the traumatic injection of aliquots of concentrated nutrient solutions. If the growth-limiting

substrate had been one of the amino acids included in the casein hydrolysate injections, it is possible that growth stimulation may have been prevented by competition for the uptake of that particular amino acid by the others present in the injected solution (Payne, 1980). The experiments of Mateles & Battat (1974) were performed using cultures of a *Pseudomonas* organism, for more nutritionally versatile than a *Lactobacillus*, in a basal minimal salts medium. The injected solutions in the latter study were of single salts. It may be that this latter approach is useful only when applied to simple growth systems and is not applicable to cultures of fastidious organisms in complex media.

In the second set of experiments, the composition of the growth medium was varied and the biomass and lactic acid concentrations produced using the various media were compared under steady state conditions. Reducing the concentration of yeast extract in the medium to below the normal 30 g L^{-1} resulted in arithmetic decreases in biomass and lactic acid production. Increasing the yeast extract concentration above 30 g L^{-1} resulted in growth stimulation, although this was consistently sub-arithmetic in magnitude (Figure 5.7). These latter experiments suggest that some component of yeast extract was growth-limiting at the normal concentration of 30 g L^{-1}. Yeast extract is a highly complex mixture containing vitamins, peptides, nucleic acid derivatives, and trace elements. It may be that at feed concentrations greater than 30 g L^{-1} a different component of the growth medium became limiting, or that competition for uptake sites occurred between the various components of the yeast extract leading to sub-arithmetic growth stimulation.

Further experiments were performed in which various elements of yeast extract were separately added to the growth medium but none of

these nutrients gave a reproducible stimulation of biomass and lactic acid production (Table 5.6). These negative results suggest either: (a) that the growth-limiting substrate in the glucose-excess steady state was none of the nutrients added; (b) that the growth-limiting substrate was present in one of the supplementations but competition for uptake reduced the stimulatory effect or; (c) that a nutrient (or nutrients), existing at slightly supra-limiting levels within the unsupplemented culture, rapidly imposed a new growth limitation following the supplementation of the medium with the original growth-limiting substrate. Further experiments to clarify these latter points might include the supplementation of the growth medium with individual amino acids, vitamins, or trace elements. However, the nutritional requirements of lactobacilli are highly complex (Peters & Snell, 1954; Ramasamy & Natarajan, 1980) and thus such experiments would be complex and lengthy. The possibility of multiple limitationmentioned above (c) might prevent conclusive results being obtained.

The presence of excess lactose in chemostat cultures of *L. casei* at high values of D has been reported by Keller & Gerhardt (1975) who reasoned that since bacterial K_s values for carbohydrate substrates are of the order of milligrams per litre, the presence of excess lactose (at 4.2% w/v) demonstrated that the culture was not lactose-limited. Keller & Gerhardt (1975) concluded that inhibition by lactic acid was the growth-limiting factor. There are two reasons why such a conclusion may have been incorrect. Firstly, Keller & Gerhardt (1975) did not consider any substrate other than lactose as potentially growth-limiting. It may have been premature to conclude that growth limitation was non-nutritional on the basis of the consideration of a single substrate amongst a wide range of nuritional

requirements. Secondly, it may be that the inhibitory effect of lactic acid is not an important consideration in carbohydrate-excess cultures. The undissociated form of lactic acid has long been held to be growth inhibitory. However, few mechanisms for such limitation have been proposed. It has been shown that anaerobic cultures of lactic acid bacteria can generate ATP via the excretion of lactate in symport with protons. This endproduct efflux contributes to the electrochemical gradient of the proton motive force (pmf) and reduces the demand for ATP hydrolysis to maintain the pmf via the transmembrane ATPase (Otto *et al.*, 1980; TenBrink & Konings, 1980). A net benefit of 1ATP-equivalent per mol glucose consumed may be gained under optimum conditions (Konings & Veldkamp, 1983) leading to an increase in Yx/s under glucose limitation (TenBrink & Konings, 1980). The energy-yielding endproduct efflux system may be a possible site of action for lactate inhibition since it has been shown that as lactic acid accumulates within a culture, the lactate gradient across the cell membrane decreases and the stoichiometry of the lactate/proton efflux falls (Konings & Veldkamp, 1983). Thus, the net result of the accumulation of lactic acid within the culture is the loss of the extra ATP formation from endproduct efflux. However, it may be assumed that carbohydrate-excess anaerobic cultures are also energy (ATP)-excess (Tempest & Neijssel, 1984). Therefore, it may be suggested that lactate inhibition was not significant in the high D, glucose-excess, cultures since the resultant loss of the extra ATP formation by endproduct efflux would not be important to the ATP-excess organisms. The observation that dialysis applied to a batch culture of *L. delbreuckii* did not produce an increase in growth until glucose was depleted within the culture (Friedman & Gaden, 1970) supports such a hypothesis. The hypothesis that lactic

acid inhibition of growth was not significant in the glucose-excess cultures might be tested by applying dialysis to glucose-excess steady states, or by culturing *L. delbreuckii* in glucose-excess chemostat co-culture with a lactate-consuming organism such as *Pseudomonas stutzeri*. In a carbohydrate-limited chemostat, co-culture with the latter organism increased the molar growth yield of *S. cremoris* (Otto *et al.*, 1980b).

7.2 Cell Recycle Fermentation

During the cell recycle fermentation (CRF) experiments in the present study, two changes in microbial physiology occurred: (1) the profile of product formation shifted away from lactic acid towards ethanol and acetate; and (2) the growth yield from glucose was reduced.

7.2.1 Product Formation

A partial shift towards the formation of ethanol and acetate was observed at all values of recycle ratio (R) (Table 6.3). This phenomenon has not been previously reported in studies of CRF applied to lactic acid production. The magnitude of this shift increased with R and was reflected in decreases in the molar product ratio (i.e. lactate: ethanol + acetate) and the yield of lactic acid from biomass (Yp/x) in comparison with the conventional chemostat steady state at the same D (0.30 h^{-1}) (Table 6.3). The steady state concentration of lactic acid ($\bar{p}$) was enhanced under CRF compared with the chemostat at D = 0.30 h^{-1} without cell recycle due to the complete utilization of glucose. However, the shift in product profile ensured that the maximum $\bar{p}$ and volumetric productivity of lactic acid ($D\bar{p}$) (40.3 g L^{-1} and 12.10 g L^{-1} h^{-1} respectively) were

achieved using the minimum degree of recycle (R = 0.5). The latter $\bar{Dp}$ was 35.5% greater than the maximum achieved in the conventional chemostat. It should be emphasised that, despite the shift in product profile, lactic acid remained the major product and was produced to a molar concentration at least ten times those of the combined minor products at steady state.

It may be concluded that the shifts in product profile observed during CRF were extensions of the similar behaviour which was observed on switching to low D, glucose-depleted steady states in the conventional chemostat (Table 5.2). It may be that, as suggested in Section 7.1.2, these latter shifts are similar in nature to those which have been extensively reported for glucose-limited anaerobic cultures of lactic acid bacteria (De Vries *et al.*, 1970; Carlsson & Griffith, 1974; Thomas *et al.*, 1979; Fordyce *et al.*, 1984). The glucose pulse experiment made during the R = 0.9 steady state demonstrated that the shift in product profile under CRF was a response to glucose limitation. In this latter experiment glucose, directly injected into the culture, was quantitatively converted to lactic acid during a period of enhanced acid production which started immediately after the glucose injection and lasted for approximately 60 min (Figure 6.8). Within the period of enhanced acid production, the concentrations of ethanol and acetate in the culture decreased and the molar product ratio increased (Table 6.4). The rapidity of the onset of enhanced acid production was considerably greater than could have arisen by the genotypic selection of a more homofermentative organism and demonstrated that the shift in product profile towards the minor products was a phenotypic response to glucose limitation. A similar conclusion was reached by Thomas *et al.* (1979) following a switch in D of a *S. lactis* culture from that giving glucose limitation (and hetero-

lactic fermentation) to that giving a glucose excess. The glucose pulse experiment also demonstrated that the genotypic potential of the CRF culture for lactic acid production was under-exploited due to glucose limitation. Vick Roy *et al.* (1983) also reported the presence of excess lactic acid producing capacity in a CRF culture of *L. delbreuckii*. In the latter study the volumetric productivity of the culture doubled within 30 min., with no increase in cell mass, following an increase in D from 0.54 h^{-1} to 1.14 h^{-1}.

These shifts in product profile may result from a channelling of pyruvate away from lactate dehydrogenase towards other pathways and the ATP-yielding formation of acetate (De Vries *et al.*, 1970; Thomas *et al.*, 1979). Both the latter authors reported increases in growth yield (Yx/s) resulting from shifts in product profile. However, in the present study Yx/s declined under CRF (Table 6.2; Section 7.2.2). The specific rate of ATP formation (q_{ATP}) was calculated from chemostat and CRF product formation data. Plots of q_{ATP} versus $q_{glucose}$ revealed that the more extensive shift in product profile under CRF did not significantly increase the yield of ATP from glucose in comparison with the conventional chemostat (Figure 6.9). In summary, the shift in product profile under CRF was neither as extensive nor as energetically advantageous as those reported for *L. casei* (De Vries *et al.*, 1970) or *S. lactis* ML_3 Thomas *et al.*, 1979).

Fructose-1, 6-diphosphate (FDP) was required for maximum specific activity of LDH in cell free extracts of organisms from glucose-excess and glucose-depleted chemostat steady states (Table 5.3) suggesting that alteration in FDP-mediated activation of LDH was a possible mechanism for the partial shift in product profile observed in chemostat cultures (Section 7.1.2). This

suggestion may be extended to cover the CRF observations since LDH specific activity remained stable throughout the R = 0.8 experiment (Figure 6.4) although the molar product ratio declined from 70:1 to 25:1 over the same period. The observation that the shift in product profile was phenotypic in nature is consistent with the hypothesis that changes in the intracellular content of FDP were responsible for the shift away from lactic acid production.

Further experiments are required to clarify the nature and control mechanisms of the shift in product profile under CRF. The consistently equimolar production of ethanol and acetate suggests the formation of the latter from a common intermediate, possibly acetyl-CoA (Fordyce *et al.*, 1984). As outlined in Chapter 1 (Section 1.4.2), two alternative routes for the formation of acetyl-CoA have been demonstrated in anaerobic cultures of lactic acid bacteria, each of which yields a different C_1 by-product: pyruvate formate-lyase (PFL), which yields 1mol formate per mol pyruvate; and pyruvate dehydrogenase (PDH) which yields 1mol CO_2 per mol pyruvate. Analysis of the molar ratios of formation of formate and CO_2 to ethanol and acetate in CRF cultures would provide a clear indication of whether PFL, PDH, or both enzymes were involved in the conversion of pyruvate to ethanol and acetate (Thomas *et al.*, 1979). De Vries *et al.* (1970) concluded that only PFL was involved in the shift to heterolactic fermentation in *L. casei* whilst Thomas *et al.* (1979) and Fordyce *et al.* (1984) concluded that both PFL and PDH were involved in a similar product shift in *S. lactis* ML_3. Lloyd *et al.* (1980) and Hickey, Hillier & Jago (1983) reported the absence of PDH activity in cultures of *L. delbreuckii*. However, these two latter studies were of aerobic cultures, providing an interesting comparison with a study of PFL and PDH activity in anaerobic CRF cultures of *L. delbreuckii*.

A study of the control mechanisms involved in the shift of product profile would require the assay of intracellular metabolites. The present study has provided an indication that the intracellular concentration of FDP might be important in determining the metabolic fate of pyruvate in CRF cultures. Thus an analysis of FDP concentrations within cells from glucose-excess and glucose-depleted chemostat cultures and from CRF cultures at different values of R would be of great interest. In the study of Fordyce *et al.* (1984), *S. lactis* ML_3 exhibited a fall in intracellular FDP concentration from 25 to 10 mM on switching from homo- to heterofermentation whilst strain ML_8 (which demonstrated product profile shifts more comparable in scale with those in the present study) maintained FDP at higher levels. An analysis of the intracellular concentration of phosphoenolpyruvate (PEP) would be of interest since triose phosphates have been shown to be inhibitors of PFL (Mason *et al.*, 1981). An analysis of intracellular phosphate (P_i) concentrations in chemostat and CRF cultures would also be of interest since P_i was shown to be a powerful inhibitor of LDH from glucose-excess and glucose-depleted chemostat cultures (Figure 5.4).

As described in Chapter 1 (Section 1.4.2) the oxidation of lactate via lactate oxidase might also result in a shift in product profile towards acetate formation. However, such oxidation requires molecular oxygen (Kandler, 1983) and is thus most unlikely to have been active in the anaerobic CRF cultures.

7.2.2. Biomass Formation and Glucose Consumption

The steady state concentration of biomass ($\bar{x}$) produced within the recycle fermenter were considerably higher than in the conventional chemostat. However, when the physical concentrating effect was

considered (dividing $\bar{x}$ by $(1 - R)$), the concentration of biomass formed by anabolism alone was found to be reduced in the recycle fermenter. As a result, the growth yield of biomass from glucose (Yx/s) was reduced in the latter cultures, the reduction being greatest at the highest values of R (Table 6.2). The observed reductions in Yx/s ensured that predictions of $\bar{x}$ based on the mathematical treatment of Bull & Young (1981) (Chapter 1, Section 1.2.1) consistently overestimated the observed biomass data. A mathematical model was derived which predicted $\bar{x}$ values via a reduction in Yx/s defined by a constant specific rate of glucose consumption for non-anabolic (e.g. maintenance) functions (q_m) (Equation 6.4). It was argued within the present Chapter (Section 7.1.2) that the Yx/s observed in conventional chemostat culture provided a valid estimate of Y^{max} since maintenance consumption of glucose was negligible in the conventional fermenter. Using the latter Y^{max} value, Equation 6.4 was solved for $\bar{x}$ over a range of R using different values of q_m and a value of the latter was derived giving the least sum of squares variation between predicted and observed $\bar{x}$ at $R = 0.5$, 0.8 and 0.9. The best-fitting value of q_m was 2.50 mmol glucose g^{-1} h^{-1}. The present maintenance model fitted the observed CRF data more closely than did the model of Bull & Young (1981) (Figure 6.7).

Considerations of maintenance metabolism in CRF cultures have been addressed by Beyeler, Rogers & Fiechter (1984), Damiano *et al.* (1985) and Jobses, Hiemstra & Roels (1987). All the latter studies were of ethanol-producing fermentations and no such treatment of a lactic acid-producing fermentation has been reported. Beyeler *et al.* (1984) and Jobses *et al.* (1987) both reported that estimates of maintenance coefficient obtained via CRF were in good agreement with those obtained via conventional chemostat culture. In the

present study, however, the reduction in Yx/s by non-anabolic consumption of glucose appeared to be strictly a phenomenon of the recycle fermenter.

Pirt (1975) stated that reductions in Yx/s via maintenance activity were most significant at low specific growth rate (μ) since q_m formed a greater proportion of the total metabolic activity at low μ. Although μ was reduced in the CRF cultures (being equal to $[1 - R]D$ at steady state), it is unlikely that the Yx/s reduction was purely a low μ effect. At $R = 0.5$, μ was equal to $0.15\ h^{-1}$, a value well within the range studied in the chemostat, yet Yx/s was reduced to 84% of that observed in the conventional fermenter. Other factors which may have caused a reduction in Yx/s include; a reduction of glucose assimilation as polysaccharide within the biomass, or a loss of culture viability. Analysis of the $R = 0.8$ experiment suggests that neither of these latter factors was significant within the CRF. The chemical composition of the cells remained constant throughout the $R = 0.8$ experiment (Figures 6.4 and 6.5). The mean value for the polysaccharide content of CRF-grown cells (16.3% of dry weight) was similar to that of chemostat-grown cells (15.5% of dry weight) thus eliminating reduction in glucose assimilation as a possible source of Yx/s reduction. The constant cellular total carbon of CRF-grown cells further eliminates reduction in carbon assimilation from substrates other than glucose as a source of Yx/s reduction. The viability of the CRF culture remained close to the 69% viability observed in the $D = 0.30\ h^{-1}$ chemostat steady state (Figure 6.6) thus eliminating the loss of culture viability as a source of Yx/s reduction. The maintenance of culture viability whilst Yx/s decreased within a CRF fermentation has also been reported by Vick Roy *et al.* (1983). In the latter study

viability was maintained at greater than 95% although Yx/s was reduced to 0.09 gg^{-1} compared with 0.16 gg^{-1} in conventional batch culture. Vick Roy *et al.* (1983) did not compare CRF growth yields with chemostat data nor present a mathematical treatment describing the Yx/s reduction.

A possible explanation for the reduction in Yx/s within the CRF cultures may be that whilst the bacteria were not rendered "non-viable" as defined in the present study (i.e. unable to form visible colonies on solid medium), they may have been damaged in such a way as to increase the demand for glycolytic activity to fulfil maintenance functions. The physicochemical environment within the CRF culture may have affected the integrity of the bacterial cell membrane so as to increase the ionic permeability and the energetic demand to maintain gradients of physiologically important ions such as H^+ or K^+. The energetic consequences of increasing cell membrane permeability and reducing H^+ and K^+ gradients have been demonstrated by Neijssel (1977) and Hueting De Lange & Tempest (1979). These authors demonstrated that the addition of the protonophorous uncoupler 2,4-dinitrophenol to glucose-limited chemostat cultures of *Klebsiella aerogenes* increased the maintenance energy requirement at all growth rates; and that a linear relationship existed between the respiration rate and the electrochemical transmembrane K^+ gradient in glucose-limited chemostat cultures. It has been suggested that greater than 90% of the maintenance energy requirement of glucose-limited *K. aerogenes* cultures serves to support the transmembrane K^+ gradient (Tempest & Neijssel, 1984).

Holst *et al.* (1986) reported the absence of protein leakage from *S. lactis* cells subjected to total cell recycle via hollow

fibres and concluded that the microorganisms were not damaged. However, minute discontinuities in the cell membrane could increase the permeability to very small ionic species whilst still retaining cellular proteins. If the bacteria in the present study had been damaged within the CRF culture so that their cell membranes were more ion-permeable, such a change would constitute a heavy energetic burden under the stringent glucose limitation present in the CRF. However, such cells plated on to glucose-rich solid medium, as in the viability tests would grow as effectively as intact cells and thus appear completely viable since the energetic burden of glucose-limitation would be lifted on the plating medium.

Another possible explanation for the decreased Yx/s in the CRF cultures is an increased maintenance energy demand as a result of the initiation of stringent control. The stringent control response is a major readjustment of cellular activity which may occur in response to amino acid and energy source starvation and has been reviewed by Gallant (1979). These effects include: (a) a severe reduction in the rate of ribosomal and transfer ribonucleic acid accumulation; (b) modification in the rate of messenger RNA formation, certain gene transcriptions being enhanced, others being repressed; (c) increase in the rate of protein turnover; (d) reduced rates of synthesis of glycolytic intermediates, nucleotides, lipids, carbohydrates, polyamines, and peptidoglycans. The stringent response is triggered by the formation of guanosine 3'-diphosphate 5'-triphosphate (pppGpp) from ATP and guanosine triphosphate (GTP). The pppGpp is dephosphorylated to guanosine 3'diphosphate 5'diphosphate (ppGpp) (Arbige & Chesbro, 1982b) which has been shown to trigger the effects of the stringent response and is present at elevated levels in organisms under stringent control (Gallant, 1979). The general

benefit of the stringent control response is the maintenance of translational fidelity under starvation conditions, an objective which is achieved at the expense of metabolic energy (ATP, GTP) in the formation of regulatory nucleotides. The formation of ppGpp may thus be considered as a component of the maintenance energy requirement (Arbige & Chesbro, 1982b).

Chesbro, Evans & Eifert (1979) and Arbige & Chesbro (1982a,b) have observed the stringent control response in cultures of *Escherichia coli* and *Bacillus polymyxa* subjected to total cell recycle. The latter authors (Arbige & Chesbro, 1982b) reported enhanced intracellular concentrations of pppGpp and ppGpp and reduced total RNA content in recycled *B. polymyxa* organisms. The latter observations of the stringent response coincided with an increase in the maintenance requirement which was shown to be due to glucose limitation since a marked increase in the rate of biomass accumulation occurred once the glucose feed rate was increased.

An investigation of whether the stringent control response was initiated within the CRF culture of the present study would be particularly interesting because the stringent response has been shown to cause a reduction in the intracellular concentration of FDP in *E. coli* via the allosteric inhibition of glucosephosphate isomerase (Taguchi, Izui & Katsuki, 1978). Such an alteration in FDP levels could have profound effects on the metabolic fate of pyruvate since FDP was required for complete LDH activity in the present study (Section 7.1.2). Thus, the possibility is raised that both the decrease in Yx/s and the shift in product profile away from lactic acid formation were manifestations of the same stringent control response.

In summary, five possible explanations for the reduction in $Y_{x/s}$ in the CRF cultures have been proposed: (1) a low-μ effect not manifested at the generally higher μ studied in the chemostat; (2) a result of decreased glucose assimilation into biomass; (3) a reflection of a loss of viability within the CRF cultures; (4) sub-lethal damage to the recycled organisms imposing an increased energy burden (possibly via increased ion permeability); and (5) the initiation of the stringent control response. The first two possibilities have been excluded by the results of the present study. The third possibility might be excluded but the assessment of CRF culture viability by a different method, such as the measurement of ATP: ADP + AMP ratios (Walker *et al.*, 1986), would strengthen this conclusion. The fourth and fifth possibilities remain to be investigated further.

In investigating the fourth possibility, the effects of the physical and the physiological environments would have to be distinguished. In this connection it might be useful to compare a conventional chemostat culture with a CRF culture at R = 0. A CRF culture operating at R = 0 would be one in which culture fluid was circulated around the recycle loop with the filtrate pump switched off. Therefore, the concentration of biomass would not become enhanced and the effects of the physical environment could be examined alone. Such chemostat/CRF comparisons could be made under glucose-limited and glucose-excess conditions. If sub-lethal damage was imposing on energy burden on the recycled organisms then, by comparison with the chemostat, $Y_{x/s}$ in the CRF (R = 0) culture ought to be reduced under glucose limitation but not under glucose (i.e. energy) excess. In order to investigate the fifth possibility, that stringent control was initiated in the CRF cultures, the experiment proposed in Section 7.2.1 could be extended. This

proposed experiment involved the assay of intracellular FDP and triose phosphate concentrations in chemostat and CRF-grown organisms and might be extended to include the assay of intracellular ppGpp and RNA concentration. A study of the effect of ppGpp on FDP formation in CRF-grown cells might provide the link between Yx/s reductions and product shifts via the stringent control response.

7.3 Further Fermenter Development

The cell recycle fermenter developed during this study (Chapter 3, Section 3.3) was largely successful in operation. The chemical sterilization system offered great flexibility of operation and ensured that contamination of the filtrate stream occurred only after major failures of the hollow fibre filter. The use of a peristaltic pump to draw filtrate at predetermined rates was found to offer a fine degree of control over R and stable filtrate flow rates were easily established, measured and maintained. The total recycle fermenter system (Chapter 3, Section 3.4) was founded on the maintenance of balanced flow rates of growth medium and filtrate by linking the filtrate pump to a level sensor in the fermentation vessel via the antifoam circuit of the fermenter control unit.

However, the total recycle experiment emphasised certain deficiencies of the cell recycle fermenter which were also apparent in the partial recycle experiments. The choice of the Amicon H1P100-20 hollow fibre filter unit as the means of generating the cell-free filtrate stream could be improved upon in subsequent fermenter developments. This latter hollow fibre unit required the prefiltering of the biomass to avoid blockage of the narrow fibre lumens. The original mounting of the prefilter, within the top end unit of the filter housing, was found to be unsuitable since prefilter blockage

was frequent and changing the prefilter was difficult to achieve ascepticaly. An improvement was made by including a larger diameter sintered glass prefilter in the culture line and this modification allowed the long-term operation of the fermenter at $R < 1.0$. However, the sintered glass prefilter still required changing daily, involving a break in the culture line. During the $R = 1$ experiment the prefilter blocked solidly and then failed, allowing the blockage of the hollow fibre filter. Therefore, it would be highly desirable to develop the fermenter to eliminate the need for prefiltering. This objective might be achieved by using an Amicon hollow fibre filter unit with wider lumen fibres, such as the H1P100-43 unit.

The design of the Amicon filters is perhaps intrinsically unsuitable for long term CRF operation since difficulties were encountered with the leakage of bacteria into the filtrate stream. Two features of the design of the hollow fibre filter units were responsible for these failures: (1) Inadequate spatial separation of whole culture and filtrate streams, the bleeding of whole culture across the separating O-ring was observed after 200-300 h operation; (2) Delicate hollow fibre construction, two filter units had to be discarded following the rupture of fibres and the flooding of the filtrate stream with whole culture.

In summary, further development of the cell recycle fermenter should involve the selection of a filter system which; (a) requires no prefiltration, (b) has adequate spatial separation of whole culture and cell-free filtrate streams, and (c) has robust filter matrices. Attractive alternative filter systems include ceramic hollow fibres and flat sheet units without spacer screens, described in detail in Chapter 1 (Section 1.3.2).

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LACTIC ACID PRODUCTIVITY OF A CONTINUOUS CULTURE OF *LACTOBACILLUS DELBREUCKII*

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Summary

Maximum volumetric productivities of biomass ($1.40\ gl^{-1}h^{-1}$) and lactic acid ($8.93\ gl^{-1}h^{-1}$) for a continuous culture of *Lactobacillus delbreuckii* occurred between dilution rates $0.35h^{-1}$ and $0.40h^{-1}$. All major nutrients were in excess in these cultures. Glucose utilisation was complete at dilution rates of $0.1h^{-1}$ and lower. Product and biomass yields were constant in the dilution rate range studied ($0.05h^{-1}$ to $0.50h^{-1}$).

Introduction

The production of lactic acid by *Lactobacillus* species has recently been used as a model for novel bioreactor developments such as whole cell immobilization in alginate beads (Stenroos et al., 1982) immobilization in hollow fibres (Vick Roy et al., 1983a) and biomass recycle fermenters (Vick Roy et al., 1983b). In each of these studies the performance of the novel system has been compared with previously published reports of conventional continuous fermentation. However, in such reports the range of dilution rates studied has been small and incomplete and no systematic study of lactic acid productivity over a comprehensive range of dilution rates has been reported.

The objective of the present study was to provide a complete profile of substrate utilization, organism and product yields and volumetric productivities over a wide range of dilution rates in order to provide a baseline from which to compare the performance of chemostats with partial biomass recycle.

Materials and Methods

Organism and Growth Medium

The organism used was *Lactobacillus delbreuckii* NRRL-B445, a homofermentative lactic acid producer obtained from the Northern Regional Research Laboratory, Peoria, USA. The growth medium used was essentially that described by Vick Roy et al. (1983a,b) and contained (gl^{-1}): glucose monohydrate 50, yeast extract 30, sodium succinate hexahydrate 2, Na_2SO_4 2, $MgSO_4.7H_2O$ 0.3, KH_2PO_4 0.2, K_2HPO_4 0.2, $MnSO_4.4H_2O$ 0.03 and $FeSO_4.7H_2O$ 0.03. Silicone antifoam RD emulsion (Dow Corning Corp.) was included in the medium at a concentration of $0.5ml\ l^{-1}$. The pH of the medium was adjusted to 6.0 with 10M HCl.

Continuous culture

An MBR 'Mini' Bioreactor (Wellman Biotechnology, Warley, W. Midlands, England) was used. The working culture volume was 1.4l, the temperature was maintained at 42°C and the pH at 6.0 by the addition of 2M NaOH solution. The culture head space was filled with nitrogen gas.

The dilution rate (D) was varied in a stepwise manner from $0.05h^{-1}$ to $0.50h^{-1}$ and nine steady states were analysed. After changing the dilution rate the culture was left for between six and ten residence times to allow steady states to be established before triplicate samples were taken for analysis.

Analysis of Samples

Biomass dry weight was measured by drying aliquots of washed bacteria at 100°C overnight. Lactic acid concentrations in culture supernatants were determined by a lactate dehydrogenase/NAD reduction method (Sigma Chemical Company bulletin no. 826;UV). Glucose concentrations were determined by a glucose oxidase/peroxidase reduction method (Sigma Chemical Company bulletin no. 510).

Total and inorganic phosphate concentrations were determined in culture supernatants by the method described by Chen et al. (1956); organic phosphate was calculated by difference.

Total carbon and nitrogen concentrations in freeze dried culture supernatants were determined using a Carlo Erba simultaneous CHN analyser model 1106.

Results and Discussion

The maximum specific growth rate (μ_{max}) in pH-controlled batch cultures was $0.52h^{-1}$. Thus the range of dilution rates analysed in the present study was approximately $0.1\mu_{max}$ to μ_{max}.

The steady state biomass, lactic acid and glucose concentrations for each dilution rate are shown in Table 1.

Table 1 Steady state biomass, lactic acid and glucose concentrations.

Dilution Rate (h^{-1})	Biomass (gl^{-1})	Lactic Acid (gl^{-1})	Glucose (gl^{-1})
0.05	5.40±0.20	37.35±1.25	<0.004
0.10	5.68±0.20	36.46±0.24	<0.004
0.15	5.42±0.80	33.02±1.05	4.07±0.24
0.20	5.28±1.00	31.65±1.05	8.15±0.48
0.30	4.04±1.20	27.85±0.87	12.39±0.22
0.35	3.99±0.80	25.50±0.82	16.50±0.47
0.40	3.48±0.80	22.30±0.59	19.90±0.35
0.45	2.71±1.20	17.34±0.23	26.90±0.22
0.50	1.82±1.00	11.35±1.05	34.07±0.51

95% confidence limits calculated by Student's t test.

Biomass and lactic acid concentrations were significantly lower at D = 0.50h^{-1} than D = 0.05 h^{-1} although some of the differences in biomass concentrations within this range were not significant. These decreasing trends are contrasted with significant increases in supernatant glucose concentration at increased dilution rates.

Table 2 shows that this pattern of residual nutrient concentrations in higher dilution rate cultures is reflected by total carbon, total nitrogen and total inorganic and organic phosphate concentrations.

Table 2 Nutrient concentrations in growth medium and culture supernatants. Glucose, total carbon and total nitrogen from D = 0.40h^{-1}, phosphates from D = 0.35h^{-1}.

Nutrient	Concn. in Medium	Conc. in Supernatant
Glucose	278mM	111mM
Total Carbon	2.62M	2.10M
Total Nitrogen	238mM	185mM
Total PO_4	5.9mM	4.1mM
Organic PO_4	1.7mM	1.5mM
Inorganic PO_4	4.2mM	2.6mM

Thus it has been shown that the major nutrients are in excess at higher dilution rates. Furthermore, subsequent experiments (data not shown) have suggested that amino nitrogen may also be in excess in these cultures.

The presence of excess sugars in higher dilution rate cultures and their complete utilization at lower dilution rates has also been reported by Keller and Gerhardt (1975) for *L. bulgaricus* on a whey feedstock. These authors reported 4.2% residual lactose at D = 0.2h^{-1} but none at D = 0.067h^{-1} and lower dilution rates. The authors reasoned that a nutrient present in such large excess in culture supernatants was most unlikely to be the growth limiting substrate in the higher dilution rate cultures. By the same reasoning a similar conclusion may be reached here and extended beyond glucose to include total carbon, total nitrogen, phosphates and possibly amino nitrogen. Keller and Gerhardt (1975) suggested product concentration as the limiting factor in their cultures and the present results are not inconsistent with

this conclusion. However, the nutritional requirements of Lactobacilli are so complex (Peters and Snell, 1954; Ramasamy and Natarajan, 1980) that it is conceivable that some undefined vitamin or growth factor may be the growth limiting substrate in these cultures.

The yields of both lactic acid and biomass in terms of glucose consumed were found to be constant within the calculated confidence limits with mean values of $0.74gg^{-1}$ and $0.12gg^{-1}$ respectively. Previously reported product yields under similar conditions include $0.70gg^{-1}$ at $D = 0.25h^{-1}$ and $0.73gg^{-1}$ at $D = 0.13h^{-1}$ (Hanson and Tsao, 1972) and $0.77gg^{-1}$ at $0.175h^{-1}$ (Luedeking and Piret, 1959). However, Luedeking and Piret (1959) reported a lower yield of $0.67\ gg^{-1}$ at $D = 0.48h^{-1}$.

Thus product yields reported in this paper are in general agreement with published data.

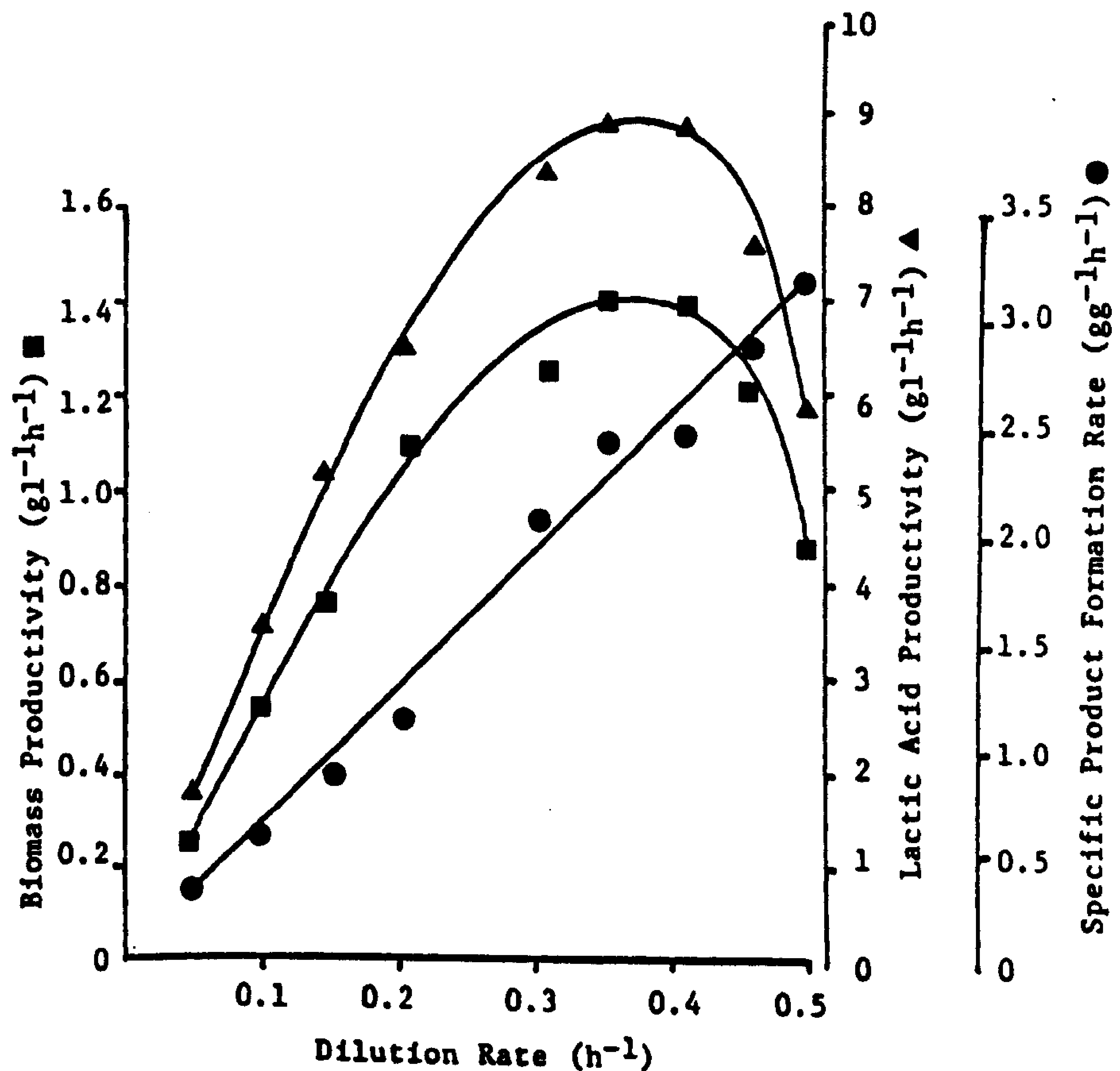

Figure 1 Biomass and Lactic Acid Volumetric Productivities and Specific Product Formation Rate vs. Dilution Rate.

Figure 1 shows the biomass ($D\bar{x}$) and lactic acid ($D\bar{p}$) volumetric productivities and the specific product formation rate (q_p) as a function of dilution rate. The trends observed in $D\bar{x}$ and $D\bar{p}$ are similar: significant increases resulting from each stepwise increase in dilution rate until maximum values of 1.40 $gl^{-1}h^{-1}$ for $D\bar{x}$ and 8.93 $gl^{-1}h^{-1}$ for $D\bar{p}$ are reached at between dilution rates $0.35h^{-1}$ and $0.40h^{-1}$. Calculation of $D\bar{p}$ from data reported by Leudeking and Piret (1959) shows reasonable agreement with the present trend with $D\bar{p}$ being equal to $6.6gl^{-1}h^{-1}$ at $D = 0.175h^{-1}$ and equal to $4.8gl^{-1}h^{-1}$ at $D = 0.48h^{-1}$.

The linear increase in q_p as a function of dilution rate shown in Figure 1 (Coefficient of Correlation = 0.99) is the expected response for the production of a primary metabolite and demonstrates that lactic acid production is growth linked.

This study has shown that there are two regions of dilution rate which are of particular interest concerning lactic acid production: between $D = 0.35h^{-1}$ and $0.40h^{-1}$ where the volumetric productivity of the system is at a peak, and below $D = 0.1h^{-1}$ where glucose utilisation is complete.

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