ASPECTS OF METABOLISM AND DEXTRANSUCRASE BIOSYNTHESIS IN *LEUCONOSTOC MESENTEROIDES*

A THESIS SUBMITTED TO THE UNIVERSITY OF MANCHESTER FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF TECHNOLOGY

ALEXANDER MCAVOY

DEPARTMENT OF CHEMICAL ENGINEERING

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ABSTRACT

ASPECTS OF METABOLISM AND DEXTRANSUCRASE BIOSYNTHESIS IN *LEUCONOSTOC MESENTEROIDES*

The bacterium *Leuconostoc mesenteroides* B512-F has been grown in fed-batch and continuous culture in order to study the characteristics of its growth and dextransucrase biosynthesis. Procedures for the analysis of medium components using HPLC were established and also used to develop a novel method for the enzyme assay. The accumulation of fructose in the medium during the metabolism of sucrose and its further conversion to mannitol in continuous culture were demonstrated.

A trace nutrient contained in the yeast extract was found to limit the rate of metabolism and production of dextransucrase at different values depending on the source of the yeast extract. The trace nutrient was shown to be folic acid. The nutrient limitation resulted in linear growth. A simple mechanistic description and kinetic theory was developed to describe the phenomenon.

D E C L A R A T I O N

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institution of learning.

Alexander McAvoy

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DEDICATION

I dedicate this work; in loving memory of my grandparents; to my parents; my wife, Karen, and all our family.

Sandy

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OD denotes Optical Density

HPLC denotes High Pressure Liquid Chromatography

NOMENCLATURE

k_s	Saturation coefficient for medium	$kg.m.^{-3}$
k_{si}	Saturation coefficient for cell contents	$kg.m.^{-3}$
m_t	Mass of trace material in batch	kg.
r_x	Growth rate	$kg.m.^{-3}h.^{-1}$
s	Substrate concentration in medium	$kg.m.^{-3}$
s_i	Substrate concentration inside cells	$kg.m.^{-3}$
s_{ic}	Critical substrate concentration inside cells	$kg.m.^{-3}$
t	Time	h
V	Volume of culture	m^3
v	Specific volume of liquid inside cells	$m^3.kg.^{-1}$
x	Cell concentration	$kg.m^{-3}$
x_c	Critical cell concentration when $s_i=s_{ic}$	$kg.m^{-3}$
μ, μ_m	Specific and maximum specific growth rate	h^{-1}
f_v	Feedrate	$m^3.h^{-1}$
m	Maintenance coefficient	$kg.kg.^{-1}.h.^{-1}$
Y	Yield coefficient	$kg.kg.^{-1}$
D	Dilution rate	h^{-1}
ρ	Density	$kg.l^{-1}$
c	Increment in culture volume per unit volumetric feed of substrate	$m^3.m^{-3}$
V_o	Initial volume of culture	m^3
x_o	Initial cell concentration	$kg.m^{-3}$

P	Pressure	atmospheres
T	Temperature	K
Vg	Gas volume	litres
R	Gas constant	$atm.deg.^{-1}mole^{-1}$
G_{mi}	Inlet molar gas flowrate	$moles.h^{-1}$
G_{mo}	Outlet molar gas flowrate	$moles.h^{-1}$
Q	Specific rate of gas consumption or production	$moles.kg.^{-1}h^{-1}$
M	Molar rate of gas consumption or production	$moles.h^{-1}$
V_f	Volume fraction of specified gas	
f	Feedrate	$m^3.h^{-1}$

Superscripts

o2	Oxygen
co2	Carbon dioxide
N	Nitrogen + Inert gases + Volatiles + Saturated water

Subscripts

f	Feed
w	Water
s	Substrate
i	In
o	Out

DSU	Dextransucrase unit	$0.52mg\ reducing\ sugar.h^{-1}$
RQ	Respiratory Quotient	

CHAPTER 1 INTRODUCTION

Dextran is produced commercially by the bacterium, Leuconostoc mesenteroides B512-F, in the presence of excess sucrose together with small amounts of other nutrients. The resulting broth has to undergo a number of hydrolysis and fractionation steps to achieve pharmaceutical grade products with average molecular weights of 70,000 and 40,000.

The extracellular enzyme, dextransucrase, produced by Leuconostoc mesenteroides B512-F, enzymatically catalyses the polymerisation of glucose from the sucrose, turning it into dextran with the release of fructose. An enzymatic route for the manufacture of dextran is thus possible. An enzymic process requires the culturing of the bacteria to produce the enzyme, dextransucrase. The final broth is processed to produce a cell free filtrate which can be concentrated by ultrafiltration. The concentrated enzyme solution is then used to produce dextran from sucrose. In such a process, the concentrations of the substrate and the active enzyme can be controlled and optimised.

Previous work (McAvoy, 1981) showed that a pH-linked mixed feed fed-batch system, using sucrose as the carbon source, is suitable for the operation of the dextransucrase manufacturing process. However, the details of this

fermentation process have not been explored and many aspects need examining. In particular a number of sugars, organic acids and alcohols are present in the medium and analysis of such components might be achieved using High Pressure Liquid Chromatography (HPLC). The use of HPLC in the development of an improved assay method for the dextransucrase is also a possibility.

Although some fed-batch runs have been carried out using sucrose as the carbon source, the effect of glucose needs exploring. The use of continuous culture techniques assists in such investigations but may also be advantageous for enzyme production. In addition, reported variations in the rate of growth associated with source and quantity of yeast extract could be due to variations in the level of some trace component such as folic acid. Linear growth, substrate uptake and dextransucrase production observed in the fed-batch process might be related to such nutrient limitations. A mechanism is needed to explain the linear growth phenomenon, from which a kinetic description can be developed.

Both fed-batch and continuous culture experiments have been carried out to explore some of the problems, identify the nutrient limitation and to develop a model for the linear growth.

CHAPTER 2 LITERATURE SURVEY

A detailed literature survey on Leuconostoc mesenteroides B512-F and dextransucrase has already been presented (McAvoy, 1981). However, a number of additional topics have been investigated in the present work. A literature survey has been completed on these additional areas.

2.1 FED-BATCH FERMENTATION

The term "fed-batch culture" was introduced in a review article by Burrows in the book entitled "The Yeasts" and became firmly established after its use by Yoshida et al in 1973. Fed-batch has been defined as a fermentation technique where one or more nutrients are fed to the bioreactor during cell growth and in which no broth is removed until the end of the fermentation. The main characteristic of the fed-batch fermentation is that the concentrations of the nutrients fed into the fermentation medium can be controlled by the changing of the feedrate (Yamane and Shimizu, 1984).

In fermentation technology there are two distinct methods of operation, batch fermentation and continuous fermentation. In the batch fermentation all the essential nutrients, possibly excepting oxygen, are added to the bioreactor, the medium is inoculated and some time later, is harvested for its product(s). During the fermentation cycle, cell, substrate and end-product concentrations

are changing continually, environmental factors such as pH and temperature will also change unless controlled. In the continuous fermentation, medium containing all the essential nutrients is fed continuously at a constant rate whilst broth is removed at an identical rate thus maintaining a constant bioreactor volume. In the continuous fermentation a steady-state is attained in which the cell, substrate, limited-substrate and end-product concentrations are constant. Fed-batch effectively exists between batch and continuous fermentation as a mode of operation (Yamane and Shimizu, 1984). Fed-batch is used for processes in which substrate inhibition, catabolite repression and the glucose effect are important factors.

The fed-batch fermentation has been in use since the early 1900s when it was discovered that in the production of yeast from malt wort, the concentration of wort in the medium had to be controlled carefully. Malt wort was fed at a rate which was lower than that which the yeast could use. This prevented excessive growth with accompanying anaerobic conditions and the formation of ethanol instead of yeast biomass. Following this example the fed-batch technique was tried successfully on a number of other fermentation processes. The number of applications and techniques tried are numerous and will not be detailed in this work unless relevant. Excellent papers by Whittaker (1980), Yamane and Shimizu (1984) and Minihane and Brown

(1986) review and list the applications to which the fed-batch technique has successfully been applied.

2.1.1 Classification of Fed-Batch Techniques

Yamane and Shimizu (1984) have classified fed-batch microbial processes according to the feeding mode; those without feedback control, and those with feedback control. These two feed modes are further divided with reference to the feedback parameter and the feed rate (Table 2.1).

The constant fed-batch culture is a batch culture to which the substrate is fed at a constant rate (Yoshida et al, 1973). Exponentially fed-batch culture is a batch culture to which substrate is fed at an exponentially increasing rate (Lim et al, 1977). Cyclic (Pirt, 1974) or repeated fed-batch cultures are those in which part of the fermenter contents are periodically withdrawn.

TABLE 2.1 Classification of the fed-batch technique in microbial processes (Yamane, 1978)

1. Without feedback control
   - 1.1 Intermittent addition
   - 1.2 Constant rate
   - 1.3 Exponentially increased rate
   - 1.4 Optimised
   - 1.5 Others

2. With feedback control

   2.1 Indirect feedback control

   2.2 Direct feedback control

   2a Constant-value control

   2b Optimal control

In direct feedback control, the concentration of the substrate in the broth directly controls the rate of addition of the substrate. The main drawback with this method is the lack of suitable, accurate, on-line sensors to monitor the concentration of the substrate in the broth (Minihane and Brown, 1986).

In indirect feedback control, other feedback parameters such as dissolved oxygen, pH, carbon dioxide evolution rate, oxygen uptake rate or respiratory quotient are used. For the indirect parameter to be of any use, the mathematical relationship between it and the substrate uptake must be accurately determined (Yamane and Shimizu, 1984; Minihane and Brown, 1986).

In fed-batch fermentations without feedback control, the substrate is fed to the fermenter according to some pre-determined profile or mathematical model of the process. In early fed-batch fermentations the feed was in the form of intermittent batches or at a constant rate. Advances in control equipment has enabled the investigation and use of exponentially increasing or optimal feedrates

(Martin and Felsenfeld, 1964; Yamane and Tsukano, 1977).

Recent advances in measurement and control techniques, and the advances and availability of computers and computer interface systems has resulted in more sophisticated control techniques in the optimisation of fed-batch fermentations. Several reviews of such applications have been published (Hatch, 1982; Parulekar and Lim, 1985; Rolf and Lim, 1982).

Yamane and Shimizu (1984) have completed an extensive survey of fed-batch processes. The fed-batch processes are classified and tabulated according to their feed substrate, feedback parameter and feeding mode.

2.1.2 Indirect Feedback Control Using pH

The use of the pH parameter for indirect feedback control is by no means novel. A linear relationship had been demonstrated between caustic addition and sucrose uptake by the cells (McAvoy, 1981). Various workers have used the technique for different fermentations (Solomons, 1969; Umbreit et al, 1964; Jarvis and Johnson, 1947; Nishio et al, 1977; Edwards et al, 1970).

Edwards et al (1970) fed a predetermined ratio of acetic acid and sodium acetate to a culture of Candida utilis ATCC 9226. Candida utilis is partially inhibited by sodium acetate concentrations above about 0.5% w/v. The

acetic acid/sodium acetate solution was fed by indirect feedback control using the pH. A ratio of acetic acid/ sodium acetate was found which resulted in a constant acetate concentration being maintained throughout the fermentation. The growth and cumulative feed profiles obtained from the fermentations, exhibited linear growth, rather than exponential growth. The reason(s) for this were not clear. However, it was proposed that limitation by ferrous iron was responsible, with reduced oxygen and nitrogen levels playing secondary roles.

Nishio et al (1977) fed a mixture of methanol-ammonia, in response to a direct signal of pH change, to cultures of the methanol-utilising bacteria, Pseudomonas AM-1 and Klebsiella species No 101. The concentration of methanol and production of biomass and vitamin B_{12} were monitored through the fermentations. During the fed-batch fermentations the residual methanol concentrations remained almost unchanged, ranging from 0.4 to 0.6% v/v in Pseudomonas, and 0.6 to 0.8% v/v in Klebsiella. Thus a fed-batch fermentation in which the methanol concentration was maintained below the limit at which its inhibitory effect on growth occurs, was established for Pseudomonas and Klebsiella. Both bacterial cultures exhibited exponential growth until oxygen became limiting.

Dumenil et al (1983) used the same fed-batch technique to study the growth and vitamin B_{12} production of a

facultative methylotrophic gram positive bacterium, Corynebacterium species XG. The methanol/ammonia mixture ratio was calculated to compensate methanol assimilation by the bacteria and pH variations. The exponential growth phase was maintained for 25 hours. The same workers (Dumenil et al, 1984) cultured the Corynebacterium species XG using a constant fed-batch technique developed by Yamane and Hirano (1977). In the fed-batch culture with a constant feedrate, the growth eventually became linear due to the limitation of the substrate supply.

2.1.3 Mathematical Models

There are a number of papers published on the modelling of the fed-batch fermentation (Yamane and Shimizu, 1984; Pirt, 1974; Dunn and Mar, 1975; Lim et al, 1977; Yamane and Hirano, 1977; Keller and Dunn, 1978).

Edwards et al (1970) examined what they termed "extended culture", a fed-batch fermentation in which the substrate concentration is kept constant by the feedrate of the substrate. An equation is presented which predicts exponential growth, at a specific growth rate, dependent upon the concentration of the substrate in the broth.

Dunn and Mar (1975) developed equations which describe "variable volume cultivations" and draw an analogy between the quasi-steady state in fed-batch fermentations (Pirt, 1974) and a dynamic steady-state in continuous

culture. This work only considered constant feedrate fed-batch fermentations indicating the decreasing specific growth rate as the substrate became limiting. However, it was suggested that for the purpose of obtaining a particular desired rate of change of the specific growth rate, the substrate feedrate could be changed progressively during the course of the fermentation.

Lim et al (1977) showed that, under certain conditions, an extended culture is an exponentially fed-batch culture and that an exponentially fed-batch culture can be maintained at a steady-state. Also, an exponentially fed-batch culture can be mimicked by the use of continuous culture at constant volume and constant flowrate.

Yamane and Hirano (1977) proposed a mathematical model which described a fed-batch fermentation with constant feedrate. In the model, the effect of dilution, by increasing volume of the broth, is evaluated separately from that of substrate feeding. The model predicts that when the maintenance energy of the micro-organisms is negligible, linear growth occurs, in which its specific growth rate is proportional to the mass feedrate of the substrate and the substrate concentration becomes lower than the saturation constant of the micro-organisms. A number of computer simulations are presented for sets of initial and operating conditions, and system parameters. An experimental study (Yamane and Hirano, 1977) was

undertaken to test the model. The model predicted cell concentrations in the exponential growth phase and linear phase and the glycerol concentration in the exponential phase. The glycerol concentration was too low to determine during the linear growth phase. The cell yield in the linear growth phase was the same as that in the exponential growth phase.

The previous papers all included a model of some form to describe the particular fed-batch technique being investigated. The papers by Keller and Dunn (1978), and Yamane and Shimizu (1984) present and discuss the general equations for fed-batch fermentations and give computer simulations for special cases. The following equations have been presented by the previous two papers but most of the equations have appeared before in the literature and some have been used to analyse experimental data.

The following assumptions have been made in deriving the equations.

1. A well-mixed variable volume, fed-batch fermenter.

2. The microbial growth is limited by the concentration of a growth-limiting substrate which is fed to the fermenter.

3. All other nutrients are in excess.

4. No culture broth is withdrawn from the fermenter.

5. If the growth-limiting substrate is an energy source, part of it will be used for maintenance of biomass.

6. Specific growth rate follows the Monod relationship.

Simple model for fed-batch fermentation.

Mass balance on the cells

$$\frac{d(Vx)}{dt} = \mu.V.x \qquad (1)$$

and for limiting substrate.

$$\frac{d(Vs)}{dt} = f_v.s_f - \frac{\mu.V.x}{Y} - m.V.x \qquad (2)$$

$$\frac{dV}{dt} = f_v + f_{acid} + f_{base} + f_{antifoam} - f_{vaporisation} \qquad (3)$$

since,

$$f_v \gg (f_{acid} + f_{base} + f_{antifoam} - f_{vaporisation})$$

then

$$\frac{dV}{dt} = f_v \qquad (4)$$

Substitution of equation (4) into equation (1) gives, for the biomass,

$$\frac{dx}{dt} = (\mu - \frac{f_v}{V}).x \qquad (5)$$

In these equations,

$$\mu = \frac{1}{Vx}.\frac{d(Vx)}{dt} \qquad (6)$$

The dilution rate can be represented (Pirt, 1974),

$$D = \frac{f_v}{V} \qquad (7)$$

Then, equations (5) and (2) become:

$$\frac{dx}{dt} = (\mu - D).x \qquad (8)$$

and

$$\frac{ds}{dt} = D.(s_f - s) - (\frac{\mu}{Y} + m)x \qquad (9)$$

Equation (2) describes the balance of the feed growth limiting substrate in the broth. The right hand side of equation (2) is comprised of the substrate input,

substrate consumption by cell growth and substrate consumption by cell maintenance, respectively. The increase in broth volume (equation 4) results from the volumetric feedrate of the substrate solution and from any other additions and subtractions. D in equation (7) is time variable and not constant, as V increases with time, whereas D of a continuous culture is constant. The only exception to this is in an exponentially fed-batch fermentation.

A number of 'special' cases are presented by Keller and Dunn (1978). The mass balance on substrate is given by,

$$\frac{d(Vs)}{dt} = s_f.f_v - \frac{\mu.x.V}{Y} \qquad (10)$$

This is the same as equation (2) without mVx.

Case A $\mu = \mu_m$

The maximum specific growth rate is maintained by keeping the substrate concentration high using the substrate feed solution. Then,

$$\frac{d(Vx)}{dt} = \mu_m.V.x \qquad (11)$$

Integrated,

$$x = \frac{x_o.V_o.}{V} e^{\mu_m.t} \qquad (12)$$

Case B μ = constant

The substrate concentration in the broth is kept constant.

Equation (11) can be integrated to give,

$$x = \frac{x_o.V_o}{V}.e^{\mu.t} \qquad (13)$$

From equation (10), for constant s,

$$f_v = \frac{\mu.V.x}{Y.(s_f-s)} \qquad (14)$$

Combining equations (13) and (14) gives the exponential feedrate which is required to keep s constant.

$$f_v = \frac{\mu.x_o.V_o}{Y.(s_f-s)} . e^{\mu.t} \qquad (15)$$

Case C $s \simeq o$

The substrate feedrate is so low that the substrate is consumed immediately, s will remain at a very low value.

From equation (10) with ds/dt = o and $s \simeq o$.

$$\mu.x.V = s_f \cdot f_v.Y \qquad (16)$$

This indicates that the growth rate is equal to that allowed by the substrate feed.

Substitution into equation (1) gives:

$$\frac{d(Vx)}{dt} = s_f.Y.f_v \qquad (17)$$

Then integration gives:

$$x = \frac{x_o.V_o}{V} + \frac{s_f.Y.f_v.t}{V} \qquad (18)$$

The biomass concentration becomes independent of the growth kinetics.

Yamane and Hirano (1977) have taken the modelling of the constant feedrate fed-batch culture a stage further demonstrating the effect of dilution on the behaviour of the system independent of substrate feeding. This is possible by considering the substrate content in the feed solution as an operating variable, defining the rate of volume increase as equation (19):

$$\frac{dV}{dt} = 10^{-3}.\left(\frac{f_w}{\rho_w} + c.\frac{f_s}{\rho_s}\right) \qquad (19)$$

A number of computer simulations demonstrated theoretically the effect of altering the concentration of the substrate in the feed solution. It is recommended to feed as concentrated a solution as possible. Yamane and Hirano (1977) were able to verify the equations of their model by growing fed-batch cultures of Enterobacter cloacae NCIB 8271 with constant feedrates of glycerol or its aqueous solution.

2.2 MANNITOL PRODUCTION

A literature survey of the carbohydrate metabolism of Leuconostoc mesenteroides (mainly glucose metabolism) is presented by McAvoy (1981).

The species Leuconostoc mesenteroides has been shown to ferment fructose and produce acid. The fermentation of mannitol to acid is variable and dependent on the strain (Bergey, 1974).

Blackwood and Blakley (1956) studied the carbohydrate metabolism of Leuconostoc mesenteroides PRL L33. Sixty-six carbohydrates were surveyed as potential substrates; 12 of the 43 sugars, none of the 10 sugar alcohols and 2 of the 13 sugar acids were utilised under aerobic or

anaerobic conditions. Analysis of the end-products of the anaerobic fermentation of 100 m.mol of D.-fructose gave 67 m.mol of carbon dioxide, 43.3 m.mol of ethanol, 15.7 m.mol of acetate, 63.5 m mol of lactate and 33 m.mol of mannitol. This represents a conversion of 3 moles fructose to 1 mole of mannitol in anaerobic fermentation. No other information on this topic could be found.

2.3 LINEAR GROWTH AND CONSERVATIVE SUBSTRATES

Linear growth caused by a constant feed of limiting substrate to a batch culture has been discussed in Section 2.1 and has been described in some detail by Sinclair and Kristiansen (1987) in the case of a constant supply of oxygen to a batch fermentation.

Linear growth has also been reported by a number of workers in connection with the utilisation of trace nutrient components. The rapid uptake of trace nutrients by relatively small quantities of cells in the early stages of the culture is a common observation. Streptococcus faecalis and Lactobacillus casei both assimilate and concentrate folic acid intracellularly (Wood and Hitchings, 1959; Henderson and Huennekens, 1974). In the study of the growth of Proteus vulgaris with limiting concentrations of nicotinic acid, linear growth was reported to occur after the disappearance of all detectable nicotinic acid from the medium. Horuchi (1959) reported the occurrence of linear growth at the

moment of complete removal of phosphate from the medium by Escherichia coli. Slezak and Sikyta (1957) showed that three different initial concentrations of phosphate in an Escherichia coli fermentation resulted in three different values of cell concentration at which linear growth was initiated and that the slopes of the linear growth phase were also different. Linear growth has been reported in studies of Streptococcus faecalis by Forrest and Walker (1965) and Anderson et al (1987). The linear growth phase was thought to be due to the limitation of some unknown substrate in the complex medium. Nyholm (1976) studied the uptake of phosphate by Chlorella pyrenoidosa and used the term 'conservative' substrate to describe substrates such as inorganic ions or vitamins that are not irreversibly consumed after uptake but are stored within the cells and may be used by successive generations.

2.3.1 Requirements and Uptake of Vitamins

Vitamins are growth factors, other than amino acids, essential for the normal growth and reproduction of cells. The vitamins are classed into two groups: the fat-soluble vitamins, A, D, E, K, ubiquinone, cholesterol and unsaturated fatty acids; and the water-soluble vitamins ascorbic acid, thiamine, riboflavin, pantothenic acid, pyridoxin, nicotinic acid, biotin, paraaminobenzoic acid, folic acid, cobalamin, mevalonic acid, choline and meso-inositol. Most of the water-soluble vitamins are components of coenzymes (Lehninger, 1977).

The term auxotroph is used to describe a micro-organism which requires a certain growth factor(s), such as a vitamin(s), to be supplied in the environment in order for growth to occur. Quantitative studies on vitamin requirements in terms of growth yield are few, and variations in requirements have been observed when organisms are grown in media of different pH value, temperature or chemical composition (Pirt, 1975; Rose, 1976).

Studies by Sauberlich and Baumann (1948), Whiteside-Carlson and Carlson (1949), Whiteside-Carlson and Rosano (1951) and Garvie (1967) demonstrate the high degree of variability of amino acid and vitamin requirements by strains belonging to the genus Leuconostoc. Due to the auxotrophic requirements of this genus, Leuconostoc strains have, in the main, been cultured in media containing natural product components such as yeast extract, liver extract, raw beet sugar, molasses and tomato juice which are complex in their chemical composition.

Folic acid, also known as pteroylglutamic acid, has been identified as a requirement for some strains of Leuconostoc (Whiteside-Carlson and Carlson, 1949, and Garvie, 1967) and Sauberlich and Baumann (1948) concluded that high amounts of folic acid stimulated maximum acid production by Leuconostoc citrovorum provided the culture

was incubated for 72 hours. Biochemically, folic acid functions in the transfer of certain one carbon groups and a symptom of folic acid deficiency is the impaired biosynthesis of purines and pyrimidine thymine (Lehninger, 1977). Dagley and Nicholson (1970) have published a summary of the structure of folic acid, its active forms and typical reactions.

Studies on folic acid uptake by Wood and Hitchings (1959), and Henderson and Huennekens (1974) on Streptococcus faecalis and Lactobacillus casei, respectively, demonstrated that both micro-organisms assimilate and concentrate folic acid intracellularly, that this process requires an energy source such as glucose and that it is dependent on cell concentration, pH and temperature. Linear growth observed in the culture of bacteria such as Leuconostoc species might thus arise directly from a mechanism associated with the uptake and conservation of folic acid by the cells.

2.4 HIGH PRESSURE LIQUID CHROMATOGRAPHY

From studies completed on the Leuconostoc mesenteroides B512-F fermentation, it appeared that the maximum production of the enzyme, dextransucrase, is dependent upon the initial sucrose concentration. However, it was also discovered that there was a large build-up of reducing sugar: 0·7 moles of reducing sugar (fructose equivalent) produced for each mole of sucrose fed to the

fermentation (McAvoy, 1981). Due to the nature of the fermentation a number of sugars and end-products may be present. Sucrose can be broken down to give the two reducing sugars, glucose and fructose. It is also the substrate for the enzyme, dextransucrase, forming dextran and fructose. However, at the controlled pH of 6·7, dextransucrase activity is minimal (Schneider et al, 1982). The bacterium, L. mesenteroides, has also been shown to metabolise fructose, producing the sugar alcohol, mannitol, as one of the end-products. In addition, lactic acid, acetic acid and carbon dioxide have all been shown to be end-products of the aerobic fermentation of L. mesenteroides (Johnson and McCleskey, 1957).

Due to the complex nature of the carbohydrate metabolism of L. mesenteroides and its possible links with dextransucrase production a quantitative study of the substrate and end-product patterns is required in order to formulate an accurate model of the fermentation.

HPLC was the chosen analysis method for the examination of the fermentation broth due to its speed of analysis and cost-effectiveness for a large number of samples, the ability to perform quantitative scans of mixtures of chemicals and its accuracy (Lenz and Zoll, 1982; Jackson, 1980).

High Performance Liquid Chromatography is an efficient

separation technique which utilises the equilibrium distribution of solute molecules between two immiscible phases; a mobile phase flowing through a stationary phase. Solute molecules preferentially distributed in the mobile phase will pass more rapidly through the system than those distributed in the stationary phase (Simpson, 1982). A suitable detector is required to detect and quantify the various chemical components as they elute from the column.

HPLC columns as described by Aitzetmuller (1978) were examined by Jackson (1980). These columns were silica packed and modified with a polyfunctional amine group. The polyfunctional amine group was dissolved in the eluent and was thus continually regenerating the stationary phase (silica) as it was pumped through the column.

Recent advances in column packing materials and methods have enabled the development of polystyrene resin columns. Such columns have the ability to separate carbohydrates or organic acids efficiently, utilising a slightly modified water-based eluent. Polystyrene resin columns, in comparison to silica-based columns are recommended for the separation of organic acids or carbohydrates because of their simplicity, good selectivity and stability through the entire pH range (Woo and Benson, 1983). Unfortunately little information is available on the separation of complex carbohydrate/organic acid mixtures

typical of the L. mesenteroides fermentation and to what degree of sensitivity.

2.5 EFFLUENT GAS ANALYSIS

In all the fermentations in which gas analysis was utilised, the aeration rate was 6 $1.min^{-1}$ and the level of dissolved oxygen was controlled by the rpm of the impeller. The rotameter and aeration rate were checked using a volumetric 'wet type' gas meter and found to be accurate. The air was taken from the mains compressed air line, the temperature of which was determined to be approximately 20 ^{o}C. Using equation (1) with P=1 atm, T=293·15 ^{o}K, R=0·08206 $atm.deg.^{-1}$ $mole^{-1}$ and n=1 mole.

$$V_g = nRT/P \qquad (1)$$

gives V_g = 24·056 litres (1 mole of ideal gas).

Aeration rate = 6 $1.min^{-1}$ = 360 $1.h.^{-1}$

The molar gas flowrate into the fermenter was then calculated to be:

$$G_{mi} = 360/24·056 \qquad (2)$$

$$= 14·965 \text{ moles air.h.}^{-1}$$

The water condensate present in the compressed air was removed by the Norgren combined air/water separator/ pressure reducer. However, the air was still saturated with water vapour. It was assumed that the air was 100 per cent saturated with water vapour, therefore, the water content of the air between 15 and 25 ^{o}C would be 0·017 - 0·032 g moles water/g mole dry air corresponding to 1·060 - 1·997% water - volume fraction (Foust et al, 1960). The water vapour content at 20 ^{o}C was determined to be 0·023 g moles water/g mole dry air which was equivalent to 1·436% water. The more accurate inlet molar dry gas flowrate:

$$14{\cdot}965 \times 0{\cdot}98564 = 14{\cdot}750 \text{ moles dry air.h.}^{-1}$$

was used in the following equations.

The oxygen and carbon dioxide analysers were calibrated and operated on a dry gas basis, the gas (sample or calibration) being dried by self-indicating silica gel prior to analysis.

The respiratory quotient (RQ) is the amount of carbon dioxide produced by a culture, divided by the amount of oxygen consumed by the culture, in a given period of time.

$$RQ = \frac{\text{Rate of } CO_2 \text{ production}}{\text{Rate of } O_2 \text{ consumption}} \qquad (3)$$

The RQ can be determined from the specific carbon dioxide produced, Q_{co2}, and specific oxygen consumed, Q_{o2}, or by the overall molar carbon dioxide produced, M_{co2}, and the overall molar oxygen consumed, M_{o2}. The RQ gives an indication of the type of metabolism that is operating; an RQ greater than 1 suggests that anaerobic type pathways are being utilised and CO_2 is being produced from substrates without using oxygen. An RQ less than 1 suggests that oxygen is being used to oxidise metabolic intermediates to end-products other than carbon dioxide.

The following equations can be used to calculate M_{o2} and M_{co2};

$$M_{o2} = G_{mi}.Vf_i^{o2} - G_{mo}.Vf_o^{o2} \qquad (4)$$

$$M_{co2} = G_{mo}.Vf_o^{co2} - G_{mi}.Vf_i^{co2} \qquad (5)$$

The division of the right hand side of equations (4) and (5) by the cell mass present (V.x) will give the specific uptake or production rates, Q_{o2} and Q_{co2}, respectively. If, from an organism's metabolic stoichiometry and pathways, the RQ is equivalent to 1 then it is reasonable to assume that:

$$G_{mi} = G_{mo} \qquad (6)$$

G_{mi} being determined from the inlet gas flowrate.

However, in the case of the microaerophillic bacterium Leuconostoc mesenteroides this is an unreasonable assumption since very little is known of its aerobic metabolism.

Therefore, in the case of Leuconostoc mesenteroides it can be assumed that:

$$G_{mi} \neq G_{mo} \tag{7}$$

In order to calculate Q_{co2} and Q_{o2}, G_{mo} must be measured or replaced in the equation. Measurement of G_{mo} to the required accuracy is very difficult and, therefore, the latter option was used.

The equations for the calculation of Q_{o2} and Q_{co2} were derived as follows:

$$G_{mi} = G_{mi}.Vf_i^{o2} + G_{mi}.Vf_i^{co2} + G_{mi}.Vf_i^{N} \tag{8}$$

$$G_{mo} = G_{mo}.Vf_o^{o2} + G_{mo}.Vf_o^{co2} + G_{mo}.Vf_o^{N} \tag{9}$$

where Vf^N = volume fraction (nitrogen + inert gases + volatiles + saturated water)

$$Vf^{o2} + Vf^{co2} + Vf^{N} = 1 \tag{10}$$

therefore,

$$Vf^{N} = 1 - Vf^{o2} - Vf^{co2} \quad (11)$$

Assume that,

$$G_{mi}.Vf_{i}^{N} = G_{mo}.Vf_{o}^{N} \quad (12)$$

Therefore,

$$G_{mo} = G_{mi}.Vf_{i}^{N}/Vf_{o}^{N} \quad (13)$$

Incorporating equation (11) into equation (13) gives:

$$G_{mo} = G_{mi}((1 - Vf_{i}^{o2} - Vf_{i}^{co2})/(1 - Vf_{o}^{o2} - Vf_{o}^{co2})) \quad (14)$$

Incorporating equation (14) into equations (4) and (5) gives:

$$M_{o2} = G_{mi}(Vf_{i}^{o2}-Vf_{o}^{o2}((1-Vf_{i}^{o2}-Vf_{i}^{co2})/(1-Vf_{o}^{o2}-Vf_{o}^{co2}))) \quad (15)$$

$$M_{co2}=G_{mi}(Vf_{o}^{co2}((1-Vf_{i}^{o2}-Vf_{i}^{co2})/(1-Vf_{o}^{o2}-Vf_{o}^{co2}))-Vf_{i}^{co2}) \quad (16)$$

The division of the right-hand side of equations (15) and (16) by the cell mass present (V.x) will give the specific uptake or production rates, Q_{o2} and Q_{co2}, respectively.

$$Q_{o2}=(G_{mi}/V.x)(Vf_i^{o2}-Vf_o^{o2}((1-Vf_i^{o2}-Vf_i^{co2})/(1-Vf_o^{o2}-Vf_o^{co2})))$$

(17)

$$Q_{co2}=(G_{mi}/V.x)(Vf_o^{co2}((1-Vf_i^{o2}-Vf_i^{co2})/(1-Vf_o^{o2}-Vf_o^{co2}))-Vf_i^{co2})$$

(18)

Equation (17) is the equation given by Reub et al (1982) for the calculation of the oxygen uptake rate in the units m.Moles oxygen./litre broth/h..

Equations (15), (16), (17) and (18) were used in the work presented on gas analysis in this thesis. Vf_i^{o2} and Vf_i^{co2} were assumed to be 0·2095 and 0·0004, respectively. This value of Vf_i^{co2} was chosen from the analysis of the inlet gas to the fermenter which gave results of 0·00037-0·00042.

3. EXPERIMENTAL EQUIPMENT

3.1 FERMENTER AND SERVICES

The fermentation equipment has been described previously (McAvoy, 1981). However, a number of modifications were carried out. Figure 3.1 illustrates a schematic flow diagram of the modified fermentation rig. All the modifications to the original experimental equipment are described in the following sections.

3.1.1 Steam Supply

An additional steam line was incorporated between the steam supply (X) and the air supply (Y) to enable 'in situ' steam sterilisation of the air filter. This additional line was taken from the steam supply after the gate valve (W) (ORANE 200) and prior to the air sparger and outlet/sample lines and was connected into the air supply after the rotameter and prior to the air filter through valve V(7). Two Saunders valves V(6) and V(7), with steam sterilisable rubber diaphragms, were fitted to enable the control and use of this additional line.

3.1.2 Water Supply

Cooling water was made available to the condenser that was added onto the effluent air line.

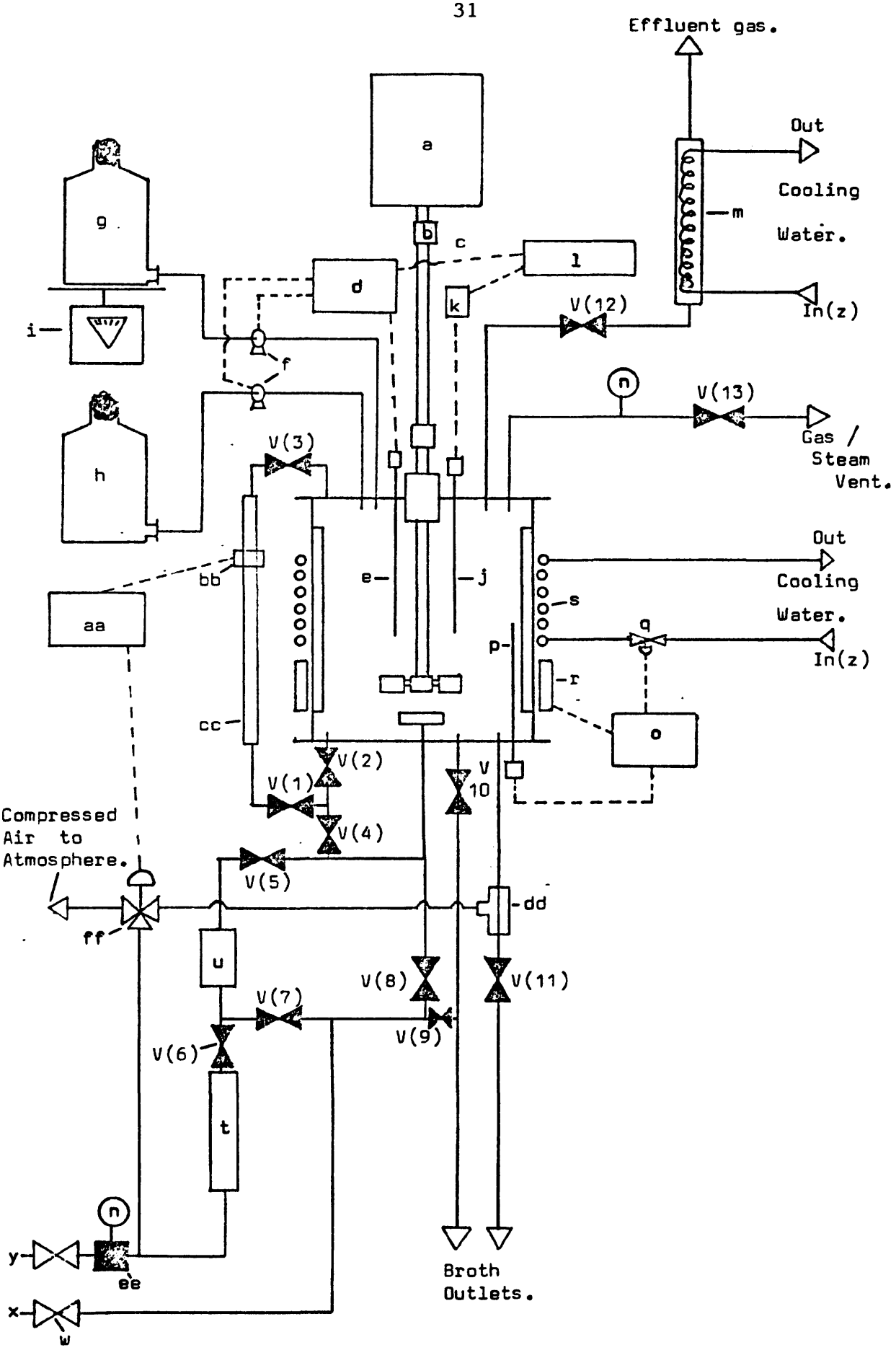


Figure 3.1
Schematic Flow Diagram of Experimental Equipment.

KEY TO SCHEMATIC DIAGRAM OF EXPERIMENTAL EQUIPMENT

a 0.25 HP electric motor

b Coupling

c Flexible drive shaft

d pH indicator/controller

e pH probe

f Peristaltic (Delta) pumps - 10 rpm max flow 240 $ml.min^{-1}$

g Substrate and/or alkali feed reservoir

h Acid reservoir

i Digital balance

j Dissolved oxygen probe

k 1 kohm potentiometer

l 0-10 mV 'Ether' recorder

m Condenser

n Pressure gauge 0-10 psig

o Temperature indicator/controller

p Temperature probe

q Solenoid valve

r Heating tape

s Cooling coils

t Rotameter 4-40 $l.min^{-1}$

u Steam sterilisable ('in-situ') air filter

V() Valve - Hoffman clip and identification number

w Gate valve

x Steam main

y Air main

z Water main

aa Fison's Fi-Monitor

bb Capacitance proximity switch

cc Glass tube

dd Pinch valve

ee Pressure reducer/condensate removal

ff Three-way solenoid valve

3.1.3 Air Supply

The two miniature in-line air filters (Microflow Ltd, Fleet, England) were replaced by a single filter supplied by Pall Process Filtration Ltd, Portsmouth. The new filter consisted of an air filter housing with condensate drain and a disposable filter cartridge. The housing was an Aluminium Junior housing model MDA 4463 G08Z3 and the filter an Emflon Junior filter cartridge model MCY 4463 FRP. The filter medium is hydrophobic, has an absolute rating of 0.2 microns and is constructed of polytetrafluoroethylene (PTFE) in a polypropylene assembly. It can be sterilised 'in situ' by direct steaming and had an expected continuous operational life of one year on a fermentation application.

3.1.4 Electrical Supply

No changes were made to the scheme previously described (McAvoy, 1981).

3.2 CONTROL SYSTEMS

The temperature control, pH control, dissolved oxygen and substrate feed systems have been described previously (McAvoy, 1981).

Due to the lack of available outlets/inlets on the fermenter top-plate, the automatic foam control system was replaced by the condenser for the effluent air. The fermenter was manually dosed with antifoam as required.

3.2.1 Continuous Culture

Initial work was carried out utilising an overflow weir consisting of a 0.5 inch diameter stainless steel tube inserted through a coupling in the fermenter baseplate and held in place by neoprene 'O' rings. The height of the tube into the fermenter determined the running volume of the system. The running volume had to be determined for each set of running parameters ie. air flow rate and stirrer speed. Considering that the broth was a bacterial culture, this method should have performed successfully. However, a number of problems were encountered:-

1. Frequent contamination by Mucor species which tended to grow on the exposed baffles and round the top of the fermenter. Mucor species are known to be aerial contaminants.

2. The experimental determination of accurate effluent broth flowrates proved to be difficult. For a known medium inlet flowrate of 0.6 $l.h.^{-1}$ (from a calibrated peristaltic pump) experimentally measured effluent broth flowrates ranged from 0.47 to 0.61 $l.h.^{-1}$.

Both of these problems were related to the use of an overflow weir. Due to the nature of the fermentation

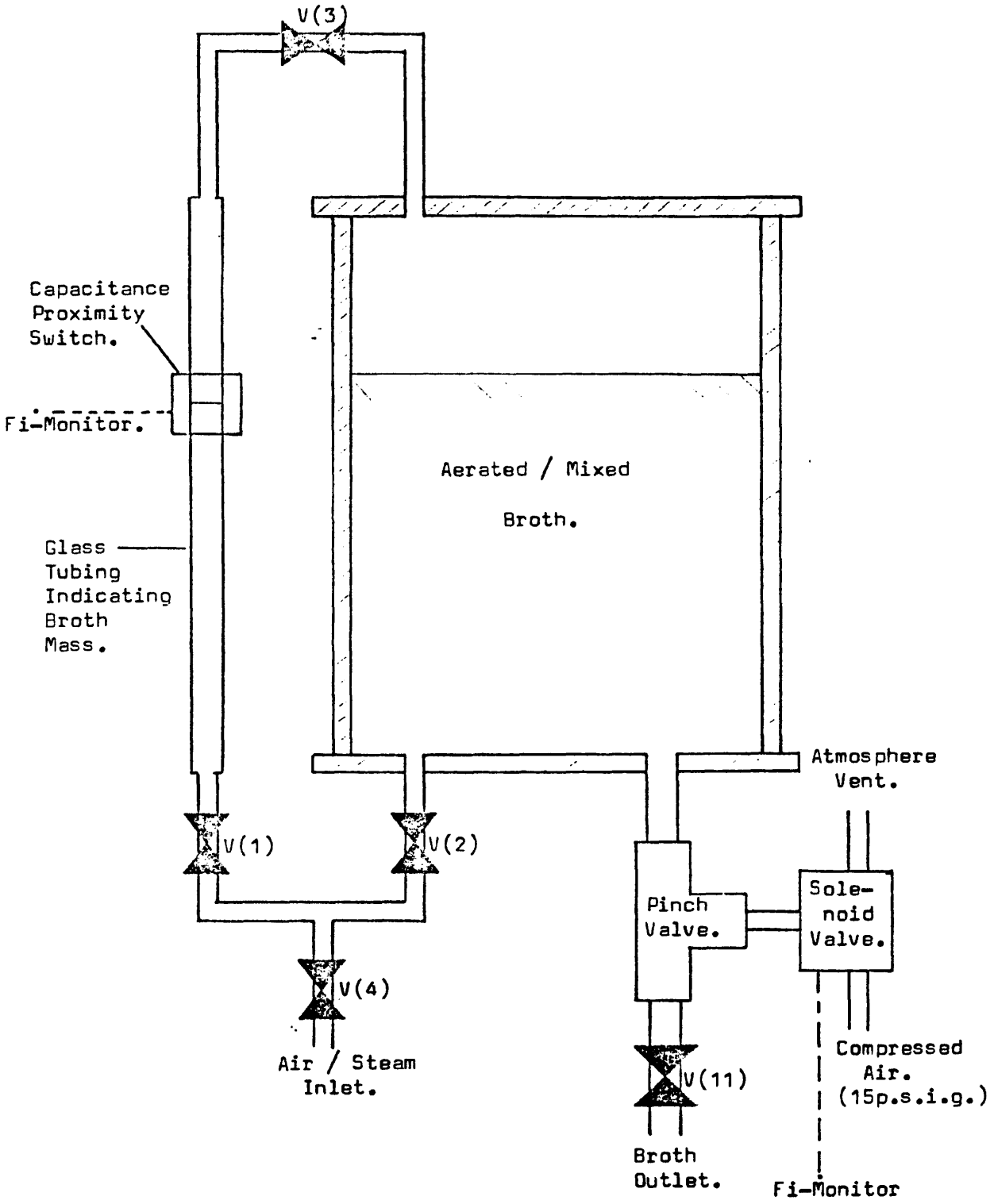


Figure 3.2

Schematic diagram of Pinch Valve System.

broth, foaming was persistent and variable. This excessive foaming was thought to cause the inaccuracies in the effluent broth flowrate.

The overflow weir was subsequently changed for an air operated pinch valve (Brown and Inkson, 1972) and a level detection system based on the work of Brown and Patel (1971) and shown in Figure 3.2. Details of the construction of the pinch valve are described in the paper by Brown and Inkson (1972). The valve is operated by compressed air at 15 psig collapsing a section of silicon tubing (6.4 mm internal diameter and 1.6 mm wall thickness) located in the valve housing. The valve is opened by exhausting the housing to atmosphere. A 240 V three-way solenoid valve (RS Components Ltd, Corby) determines whether the valve housing is pressurised by compressed air or vented to atmosphere.

Indication of the mass of the culture present in the fermenter was by an external loop made from a length of glass tubing (external diameter 7 mm, internal diameter 4 mm). The pressure exerted by the aerated culture was indicated by the level of the medium in the glass tube (Figure 3.2). The glass tubing of the loop was steam sterilised with the air filter, steam being passed through the air filter and then through the glass tubing. The glass tubing of the loop was filled with sterile medium at the start of the batch growth of the bacterium prior to continuous culture. Leuconostoc mesenteroides

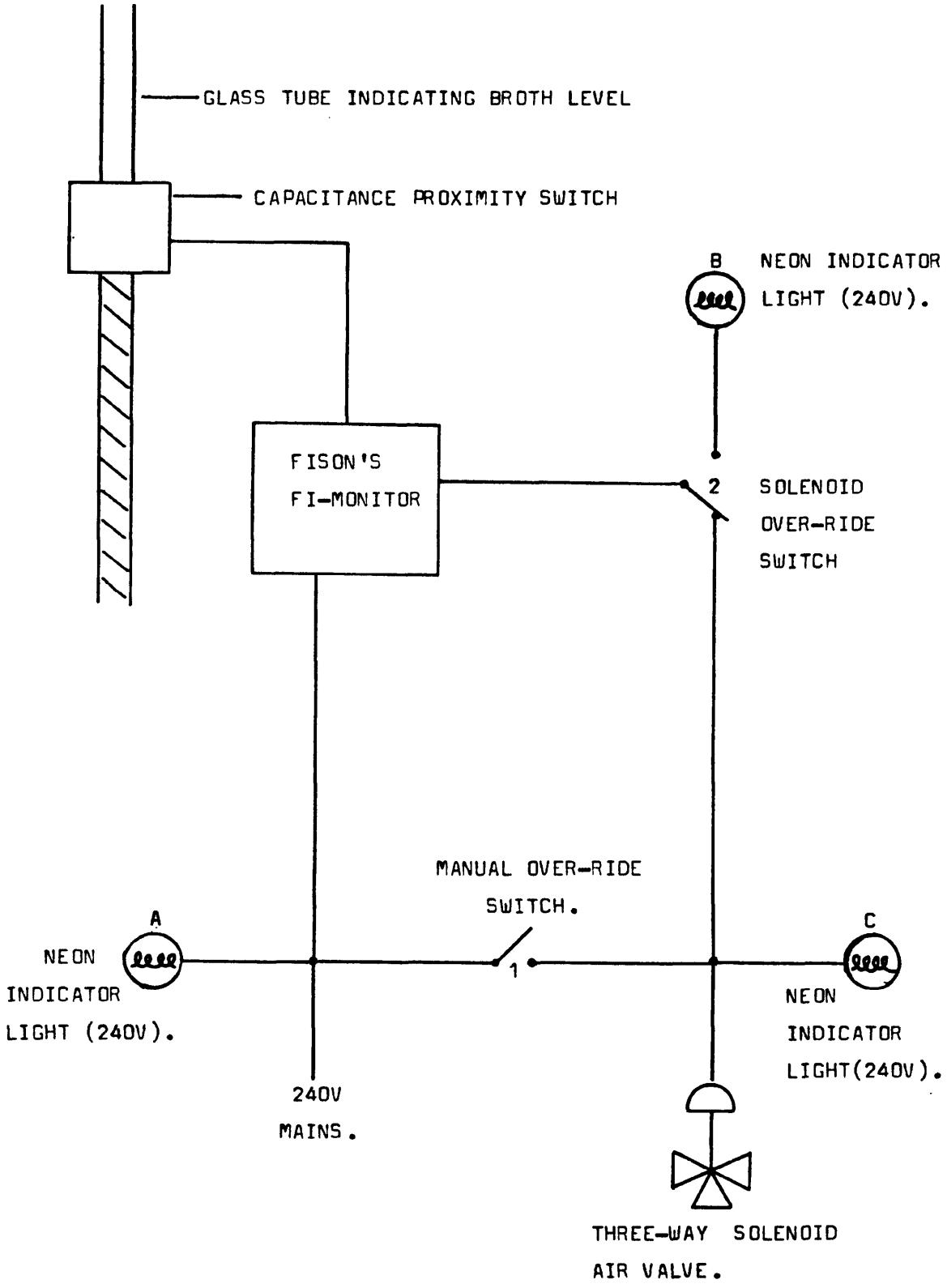


FIGURE 3.3

SIMPLE DIAGRAM OF PINCH VALVE CONTROL CIRCUIT.

B512-F is a non-motile bacterium. Therefore, it is unable to grow upwards through the medium in the glass tube. However, cells will settle under gravity to the lowest section of the loop. The loop was connected to the air sparger line of the fermenter to enable any cell debris collecting at the base of the loop to be blown back into the fermenter. Periodically, this was carried out by closing valves 3 and 1 (Figure 3.2) and opening valve 4. Valve 2 was already open when the level detection system was in use. This method maintained the air flowrate through the fermenter at the value set by the rotameter.

Changes of the medium level in the glass tubing were detected by a capacitance proximity switch as part of the Fi-monitor unit. The Fi-monitor has a sensitivity control which is adjusted according to the type of material to be detected. The Fi-monitor, in response to the capacitance proximity switch, switched a relay which controlled the power to the three-way solenoid valve. When the three-way solenoid valve was energised, it vented the pinch valve housing to atmosphere, allowing broth to flow from the fermenter. In the de-energised state, compressed air (15 psig) was applied to the pinch valve housing, effectively closing the valve.

Figure 3.3 illustrates the three-way solenoid valve control circuit. This simple circuit provided a number of advantageous facilities. The neon indicator A showed

that the Fi-monitor was switched on. Switch 1 provided a manual over-ride to bypass the Fi-monitor and energise the three-way solenoid. The neon indicator C indicated when the solenoid valve was energised. Switch 2 allowed the output from the Fi-monitor to be isolated from the three-way solenoid valve and directed to the neon indicator B. The neon indicator B was used to simulate the energising and de-energising of the three-way solenoid valve when the sensitivity and position of the capacitance proximity switch was being set.

A valve on the outlet line (Figure 3.1, valve 11) from the pinch valve allowed control of the broth flowrate from the fermenter. If this valve was fully open, then quantities of broth up to 50 mls in volume flowed from the fermenter in any on/off operation caused by the Fi-monitor. If, however, this valve was used to restrict the flow of broth from the fermenter then approximately 10 mls of broth could be allowed to flow in any on/off operation by the Fi-monitor (these values are quoted for a 3 litre fermentation at a dilution rate of 0.2 $h.^{-1}$).

The effluent broth was collected in a sterile 20 litre aspirator which was open to atmosphere via a miniature in-line air filter (Microflow Ltd, Fleet, England). For a known medium inlet flowrate of 0.6 $l.h.^{-1}$ (from a calibrated peristaltic pump) experimentally determined effluent broth flowrates ranged from 0.62 to 0.64 $l.h.^{-1}$.

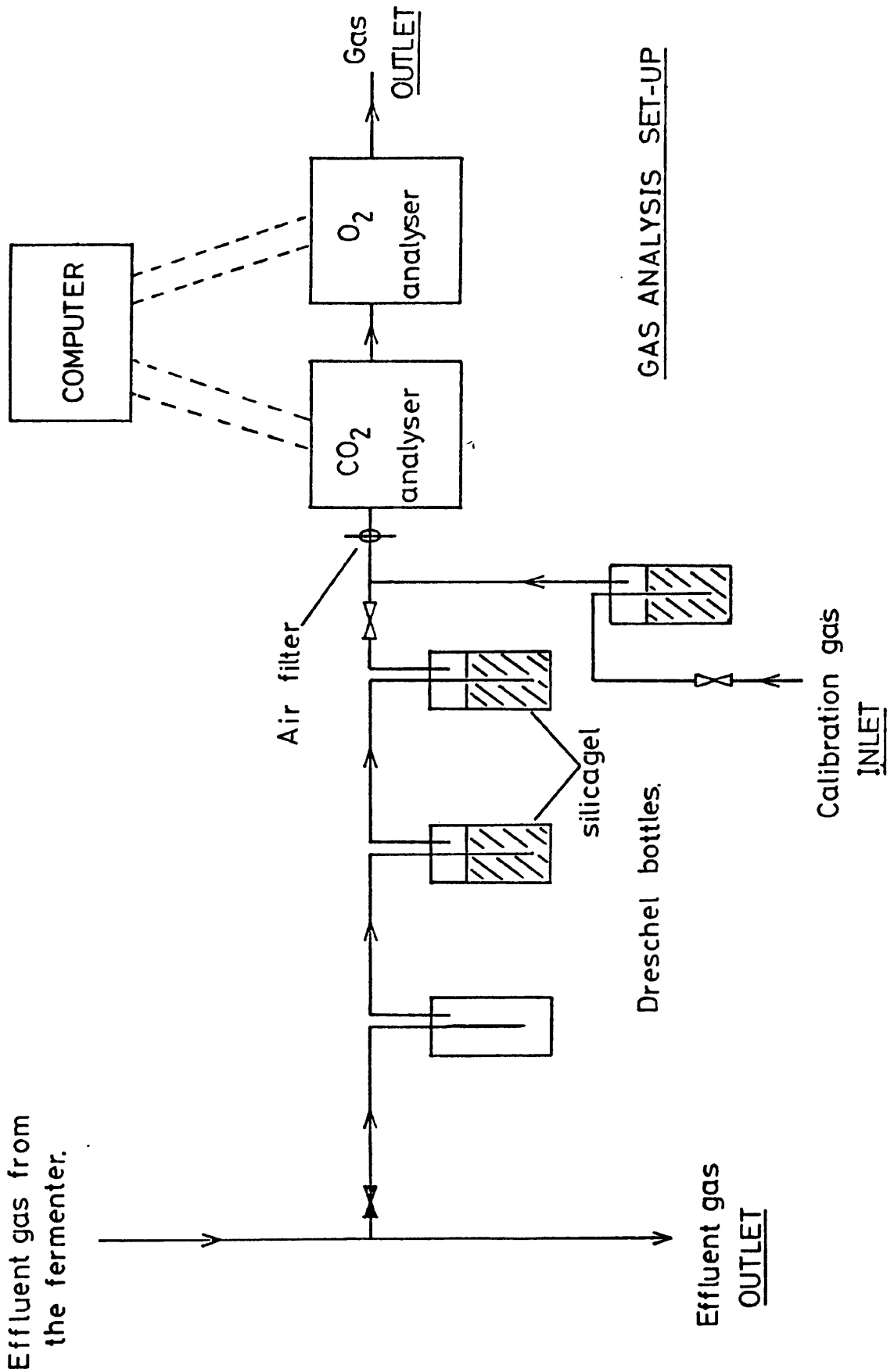

GAS ANALYSIS SET-UP
Gas OUTLET
COMPUTER
O_2 analyser
CO_2 analyser
Air filter
silicagel
Dreschel bottles.
Calibration gas INLET
Effluent gas from the fermenter.
Effluent gas OUTLET

Figure 3.4

3.3 GAS ANALYSIS EQUIPMENT

The on-line measurement and data logging of the composition of the effluent gas from a fermenter provides substantial information about the metabolism of the culture:-

1. Specific rates of oxygen uptake and carbon dioxide production.
2. Respiratory quotient (molar rate of CO_2 production/ molar rate of O_2 uptake).
3. Fast response, on-line parameters which are indicative of the rate of metabolism of the culture.

This information is important in the case of Leuconostoc mesenteroides B512-F because it is a microaerophillic bacterium with a complex carbohydrate metabolism when grown on sucrose.

Figure 3.4 illustrates a flow diagram of the gas analysis set-up. Effluent gas was drawn off the main effluent gas outlet line via 5 mm internal diameter silicone rubber tubing at a measured flowrate of 1 $l.min.^{-1}$ using the diaphragm pump of the CO_2 analyser. The effluent gas passed through a series of three 250 ml Dreschel bottles; the first was empty and acted as a liquid trap, the remaining two contained self-indicating silica gel which dried the gas. The self-indicating silica gel changed colour from blue to pink as it absorbed water. Pink indicated that the silica gel was exhausted. (The silica gel was

reactivated by placing it in a wide open-topped container and heating it to 90 ^{o}C for 24 hours in an oven). The dried effluent gas was then passed through a 0.3 micron air filter to remove any particulate matter before entering the analysis cells of the CO_2 and O_2 analysers. The gas was passed through the carbon dioxide analyser first since this instrument had a diaphragm pump and a rotameter with a needle valve control prior to the analysis cells. Gas was then passed to the oxygen analyser which had an outlet open to the atmosphere. Calibration gases were directed via a separate controlled inlet line to enable calibration of the instruments whilst the fermenter was running. Effluent gas was sampled at an identical flowrate to that used for the calibration gases (1 l.min.$^{-1}$). The outputs from both analysers were interfaced to an Electrolux computer control system (Henfrey Engineering, Epsom, England).

3.3.1 Carbon Dioxide Analysis

The concentration of carbon dioxide present in the effluent gas was determined using an Infra Red Gas Analyser (The Analytical Development Company Ltd, Hoddesdon, England) Type SS200. This unit has two analysis cells, one for each range, 0-2% and 0-10%. The range required is switch selectable, the position of the switch controlling internal solenoid valves which route the gas to the appropriate analysis cell. The second analysis cell is constantly flushed by CO_2-free air

provided by a flushing pump and a column of self-indicating soda lime located on the rear panel of the instrument. A zero option on the switch directs the flow of CO_2-free air to both analysis cells and facilitates quick and easy zeroing of the instrument. Once zeroed, predried and filtered gas of known composition (0.3% CO_2, 19.40% O_2, balance N_2; Special Gases Division, British Oxygen Corporation) was flushed through the respective cell. If the reading did not agree with the known CO_2 composition, a potentiometer on the front of the instrument was adjusted to give the correct value. Once the instrument had been calibrated it required very little adjustment. The zero was checked and reset, if necessary, twice a day, whereas, the calibration was checked weekly or prior to a fermentation. The self-indicating soda lime required frequent checking and renewal. The linearised output from the analyser was datalogged by a microcomputer.

3.3.2 Oxygen Analysis

The concentration of oxygen present in the effluent gas was determined using a Magnos 2 Paramagnetic Oxygen Analyser (Hartmann and Braun AG, Frankfurt, Germany) with zero suppression. The operational range of the instrument was 18.21 - 21 vol% oxygen. The majority of common atmospheric gases are diamagnetic, however, oxygen is strongly paramagnetic and it is this property which is exploited in its analysis (Ellis and Nunn, 1968).

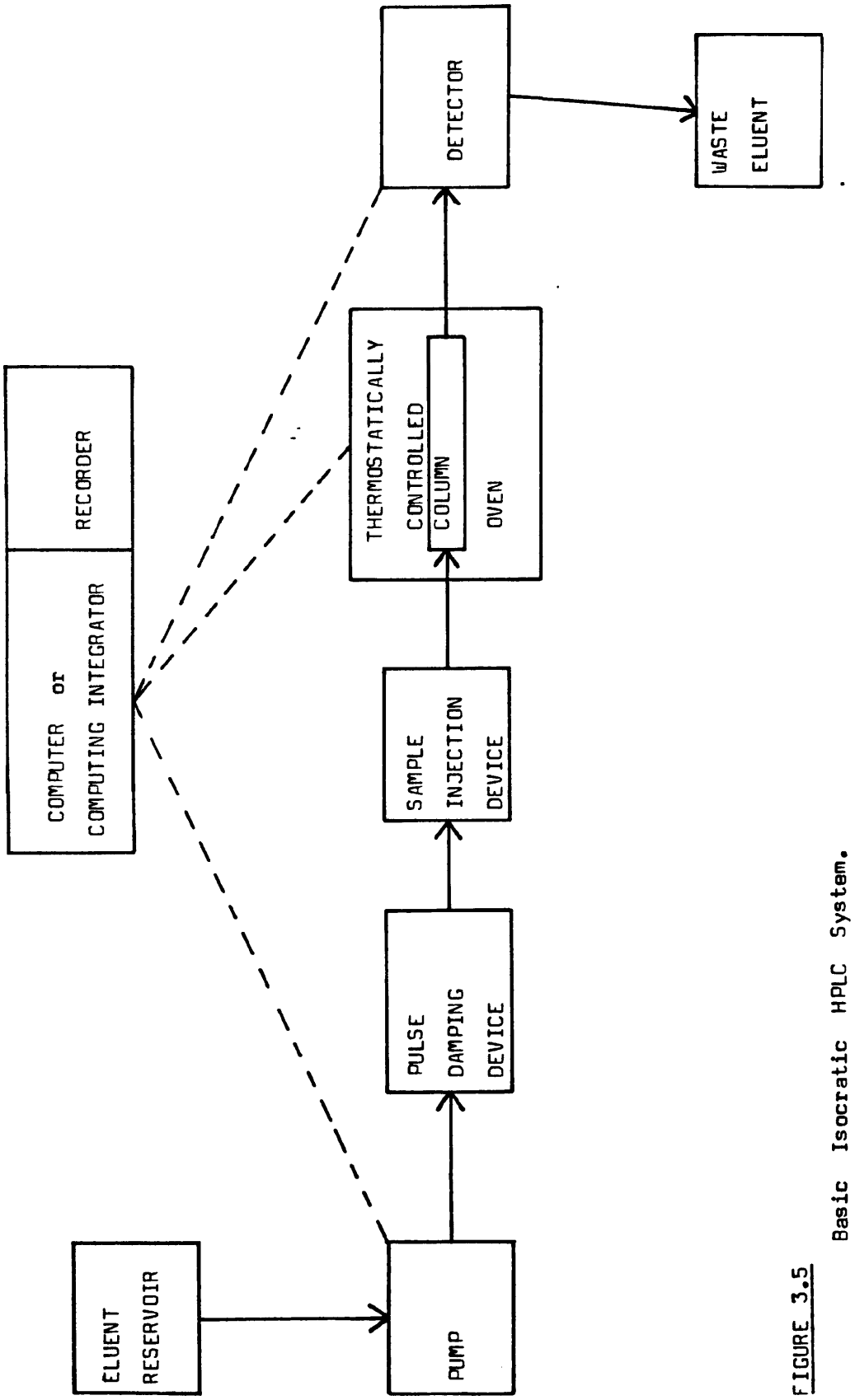


FIGURE 3.5 Basic Isocratic HPLC System.

Calibration gases of known composition (0.3% CO_2, 19.40% O_2, balance N_2; Special Gases Division, British Oxygen Corporation) were allowed to flow through the analysis cell to give the lower calibration value. Zero output from the instrument was taken as 18.21% O_2. Compressed air from the laboratory air mains was then passed through the analysis cell (oxygen content being taken as 20.95%) to give the upper calibration value. The maximum output from the instrument was taken as 21.00%. Calibration conversion of the output to the calibrated value and datalogging were undertaken by the microcomputer system.

3.4 BASIC HPLC SYSTEM

A basic isocratic HPLC system can be represented by the layout in Figure 3.5. The stationary phase is packed in a stainless steel column and located in a thermostatically controlled oven. The mobile phase is pumped at a known flowrate through the column from an eluent reservoir. The system attains a steady-state at which a known flowrate through the column will give a constant back pressure. A sample is introduced to the mobile phase at the column head by a suitable sample injection device. The sample migrates through the packed column where the various components of the sample are separated. An appropriate detector is situated on the effluent stream of the column and its output monitored and displayed by a computer or computing integrator. A number of different HPLC systems were used in this

section of the work. However, although the systems were different in makers' names, the methods of operation were essentially the same.

3.4.1 HPLC Columns Investigated

A number of HPLC columns were selected to investigate their suitability for use in the analysis of the *L. mesenteroides* fermentation broth.

3.4.1.1 Magnisphere Column

This column was packed with spherical silica of diameter 5 microns bearing a chemically bonded amine function (unknown composition). The amine function was marketed as HPLC Amine Modifier 1 by NATEC, Hamburg, GFR and was present in the eluent at a concentration of 0.01%. Use of this amine modifier is documented in a paper by Aitzetmuller, (1978). The mobile phase (eluent) was a predetermined ratio of acetonitrile:water.

3.4.1.2 LiChrosorb Column

This column was packed with irregular shaped silica of approximate size 5 microns with pores of 100 Ångstroms. As with the Magnisphere column, the silica bears a chemically bonded amine (Amine Modifier 1) function and the eluent was a predetermined ratio of acetonitrile: water.

3.4.1.3 Bio Rad HPX-87 Carbohydrate Column

This column was packed with Aminex HPX-87 resin; this is an 8% cross-linked polystyrene cation exchange resin of of 9 microns diameter in the calcium form. This column is optimized for the separation of mono- and di-saccharides typically found in foods and as biological metabolites. The recommended mobile phase is pre-filtered degassed water, although acetonitrile concentrations up to 10% can be used (Bio Rad Laboratories Ltd, Caxton Way, Watford).

3.4.1.4 Bio Rad HPX-87 Organic Acid Column

This column was packed with Aminex HPX-87 resin; this is an 8% crosslinked polystyrene cation exchange resin of 9 microns in the hydrogen form. The column is recommended for both the analysis of citric acid cycle acids in biological samples and the analysis of organic acids in beverages and foods. In general, organic acids elute from the column in order of increasing pKa when dilute sulphuric acid is used as the mobile phase. If analysis is by refractive index, it is possible to separate and detect a number of sugars on this column. The recommended mobile phase is acidified water (H_2SO_4) but this can be modified by the use of up to 10% acetonitrile.

4. EXPERIMENTAL METHODS

4.1 INTRODUCTION

The analytical methods, substrate feed system, start-up procedure, inoculum preparation and procedure for a batch or fed-batch fermentation have been previously described (McAvoy, 1981). However, in this work the ratio and substrate of the substrate feed system may differ from the earlier work but are clearly indicated.

A number of additional techniques have been employed and are described in the following sections.

4.2 STERILISATION PROCEDURE

The glass wall of the fermenter was enclosed by a securely joined cylinder of fine wire mesh prior to any sterilisation procedure, as a safety precaution against the bursting of the vessel. A short section of silicone tubing, which could be open or closed by a Hoffman clip, was attached to each inlet/outlet stainless steel pipe.

The sterilisation procedure was carried out in two stages. Firstly, the sterilisation of the air filter and the glass tube of the level detection system. Secondly, the sterilisation of the fermentation vessel, pinch valve, inlet/outlets and the condenser. Initially, all the inlet/outlets of the fermenter were opened. Using the following terminologies, the sterilisation operations can be described.

Valve (number); + open; - closed; +/- partially open

4.2.1 Stage 1

The sterilisation of the air filter and the glass tubing of the level detection system. Valve conditions,

V	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
	+	+/-	+	+	+	-	+	-	-	+/-	+/-	+	-

(see figure 3.1)

Steam was passed through the air filter to the valve network of the glass tubing of the level detection system. The air filter condensate run-off valve was partially opened to allow the escape of condensate. The air filter only allowed the passage of 'dry' steam, water droplets were collected as steam condensate.

The first sterilisation stage lasted for 24 hours. All waste steam from this stage was passed out via the fermenter, pinch valve and condenser. After 24 hours the steam flow was lowered and the valve conditions altered to:-

V	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
	+	+/-	+	+	+	+	+	-	-	+/-	+/-	+	-

The air flow was adjusted to 10 $l.min^{-1}$, the steam was closed off and the valve conditions altered to:-

V	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
	+	+/-	+	+	+	+	-	-	-	+/-	+/-	+	-

All steamed areas of the vessel were dried with sterile air at 10 $1.min^{-1}$. This air flowrate was maintained until the next stage in the sterilisation process was undertaken.

4.2.2 Stage 2

The sterilisation of the fermentation vessel, pinch valve, inlet/outlets and the condenser.

Initially, all the inlet/outlets were open. Most areas to be sterilised in this stage had already been subjected to the waste steam from Stage 1 for 24 hours.

Continuing from Stage 1, the air flowrate was lowered to 5 $1.min.^{-1}$ (air escaping via the air filter condensate run-off) and the valve conditions altered to:-

V	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
	-	+/-	-	+/-	-	+	-	+	-	+/-	+/-	-	+/-

The main steam valve was opened, directing steam to the fermenter. When a steady stream of steam was observed to come from the open ports, the Hoffman clips on the short lengths of silicone tubing were partially closed thus steadily increasing the pressure within the fermenter. Valve V(12) was then partially opened to

allow steam to escape from the fermenter via the condenser. The pressure in the fermenter was accurately controlled by the adjustment of the Hoffman clips on the inlet/outlet ports. A good flow of steam was observed to be flowing through the condenser. Stage 2 sterilisation was conducted at 5-6 psig for 2 hours. After 2 hours the steam flow was lowered until the pressure in the fermenter was 1 psig. Valve V(5) was opened (+) and sterile air was allowed to flow to the fermenter. The air filter condensate run-off valve and the main steam valve were closed. The valve conditions were:-

V	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
	-	+/-	-	+/-	+	+	-	-	-	+/-	+/-	+/-	+/-

All steamed areas of the fermenter were dried with sterile air at 10 $1.min.^{-1}$. After drying the valve conditions were altered to:-

V	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
	-	-	-	-	+	+	-	-	-	+/-	-	+/-	-

The aforementioned air flowrate and a positive pressure of 2 psig were maintained until the fermenter was required for the aseptic transfer of sterile medium.

4.2.3 Miscellaneous Sterilisation

When the silicone tubing from the feed bottles was aseptically connected to the fermenter, the stainless steel pipe endings and the silicone tube endings were liberally sprayed with an ethanol/water mixture (70:30). The dissolved oxygen and pH probes were immersed in the alcohol mixture for 30 minutes and then rinsed with fresh alcohol mixture prior to aseptically placing them in the fermenter.

The sterilisation of the outlet sample line, acid (HCl), sucrose and sodium hydroxide for the feed were as described previously (McAvoy, 1981).

If the effluent broth from the fermenter was not required for further work, it was sterilised at 15 psig for a specified time determined by the volume. After each fermentation, the fermenter, probes and all lines were free steamed to cleanse all surfaces of nutrients and bacteria. After free steaming the fermenter was dried with sterile air. This procedure was used to prevent the growth and build-up of any contaminating organisms in the fermenter during periods of non-operation.

4.3 GLUCOSE ANALYSIS

The glucose concentration present in the fermentation broths was determined using the reducing sugar method described previously (McAvoy, 1981). However, in this case the fructose standard was replaced by a glucose standard of known concentration (2 $g.l^{-1}$).

4.4 CONTINUOUS CULTURE

For continuous culture a batch fermentation was initiated using a medium (Appendix 1.1) which was similar to the basic feed medium. The procedure was the same as that described for a batch fermentation. As the culture attained the stationary growth phase, the feed pump for the continuous culture medium was switched on. The dilution rate (h^{-1}) was calculated by measuring the flow-rate ($l.h.^{-1}$) from the overflow tube or pinch valve.

$$\text{Dilution Rate } (h^{-1}) = \frac{\text{Flowrate } (l.h^{-1})}{\text{Volume } (l)}$$

Six residence times (equivalent to 18 litres of medium for a 3 litre fermentation) were allowed to pass to ensure that the culture was at steady-state.

$$1 \text{ Residence Time } (h) = \frac{1}{\text{Dilution Rate } (h^{-1})}$$

4.5 SAMPLING

4.5.1 Batch or Fed-Batch Fermentation

Samples were taken from the fermenter via a steam sterilised silicone tube, the flow being controlled by a Hoffman clip. The total volume of the sample removed from the fermenter was 50 ml. The sample taken was in the form of three volumes:

1. The first 20 ml was taken and discarded. This was the dead volume associated with the outlet port and not representative of the fermentation broth.

2. A 20 ml sample was taken for analysis from V10 (fig.3.1).

3. 10 ml of broth was lost on sterilisation of the outlet after sampling.

Care was exercised in ensuring that the sampling apparatus was cool prior to the sample being taken. This was to prevent the denaturation of the enzyme or the destruction of the cells by heat.

4.5.2 Continuous Culture

Samples were collected from the overflow tube or pinch valve after the statutory six residence times. With the pinch valve system a sample of the culture in the fermenter could be taken by operating the manual override switch (Figure 3.3, switch 1).

All samples taken from the continuous culture fermentations were examined for contamination. Sodium Azide ($Na\ N_3$) to a concentration of 0.03% w/v was added to each sample taken to halt growth, then the sample was placed in a 25 ml sterile sample bottle and stored at 4 ^{o}C until required. Samples for HPLC analysis were prefiltered through 0.2 micron filters to remove bacteria and the filtrate stored at 4 ^{o}C until required for analysis.

4.6 HPLC

Work was undertaken to examine the separation of a synthetic fermentation broth utilising the packed columns modified with polyfunctional amine groups and the more recent polystyrene resin packed columns. These columns have already been described in 3.4.1. Details of HPLC running conditions and materials are presented with the results. Samples for HPLC analysis were filtered through a 0.2 micron filter prior to injection onto the column.

4.6.1 Dextransucrase Assay

The Reducing Sugar assay for dextransucrase is not specific, any contaminating enzymes such as levansucrase or invertase will produce reducing sugars from sucrose. and cause error in the calculated enzyme activity. To examine for the presence of contaminating enzymes and check the reducing sugar assay, an HPLC assay was developed. Since the HPLC assay examines both enzyme substrate and end-products it can be considered enzyme specific. An example of each method, using an identical broth sample, is presented in Section 4.6.2 and 4.6.3, and discussed in Section 6.2.

Initially the enzyme activity of a typical sample of centrifuged L. mesenteroides B512-F broth was determined by the reducing sugar method. The enzyme activity was 260 DSU/ml (Section 4.6.2).

1 DSU converts 1 mg of sucrose to dextran and fructose in 1 hour at pH 5.2 and 25 ^{o}C. Therefore, for a reaction time of approximately 2 hours, 1 ml (260 DSU) of dextransucrase solution was added to 25 ml of a 2% sucrose substrate solution (500 mg sucrose). The sucrose substrate was prepared in the following manner:

2% w/v sucrose was added to a 0.1 M acetate buffer, pH 5.2 containing 0.005% w/v calcium chloride.

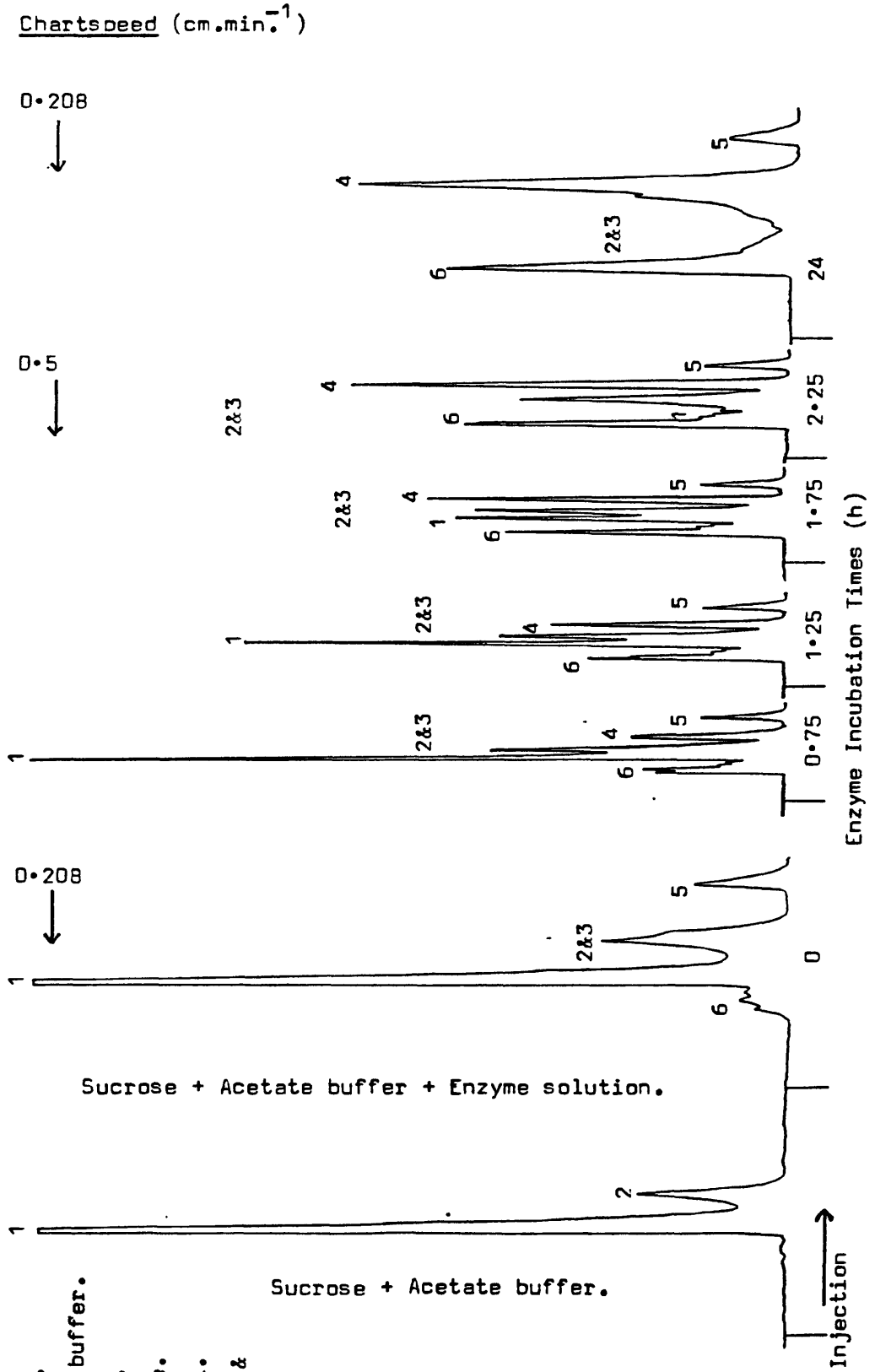


Figure 4.1 HPLC Chart Read-out For Enzyme Assay.

The calculated volume (1 ml) of dextransucrase solution was added to the sucrose substrate solution and incubated in a water bath at 25 ^{o}C. A 2 ml sample was taken at time zero and at 30 minute intervals from 0.75 h. All the samples were immediately boiled at 100 ^{o}C for 5 minutes in boiling tubes to denature the enzyme and stop the reaction. The boiled samples were then cooled, placed in labelled, sterile sample bottles and stored at 4 ^{o}C until required for HPLC analysis.

A Bio-Rad HPX-87 carbohydrate column was used to separate the relevant sugars. The HPLC system conditions were:

Eluent	distilled, degassed water
Flowrate	0.75 ml.min.$^{-1}$
Temperature	85 ^{o}C
Chartspeed	0.208 or 0.5 cm.min.$^{-1}$ (as indicated)
Refractometer	x 4 attenuation

Each sample was analysed for sucrose, fructose, glucose and dextran. Using the peak areas of the sugars in the sample, it was possible to calculate the concentrations of the sugars present in the sample (Section 4.6.3). The HPLC chart readouts (Figure 4.1) give a graphic display of the reaction progress and a quantitative display of the substrate and end-products of the reaction.

4.6.2 Enzyme Assay By Reducing Sugar Method

The conventional assay for dextransucrase involves a simple reducing sugar analysis. A more reliable procedure was developed based on the use of HPLC measurements of sucrose and frutose (Section 4.6.3).

A 1:10 dilution of centrifuged L. mesenteroides B512-F broth was prepared for assay. The centrifuged broth was the 11th hour sample from Run 35 discussed in Section 6.6.1 (Appendices 6.1 to 6.4) which was stored at 4 ^{0}C prior to assay.

ENZYME INCUBATION TIME (MINS)	OPTICAL DENSITY (530 nm)
0	0.07
5	0.15
10	0.23
15	0.30
20	0.39
Fructose Standard (2.0 $g.l.^{-1}$)	0.70

Correlation = 0.999 Slope = 0.0158

$$\therefore \text{Enzyme Activity} = \frac{0.0158 \times 1154 \times 10}{0.70}$$

$$= 260.47 \text{ DSU.ml.}^{-1}$$

A detailed description of the Reducing Sugar Method enzyme assay has been reported elsewhere (McAvoy, 1981).

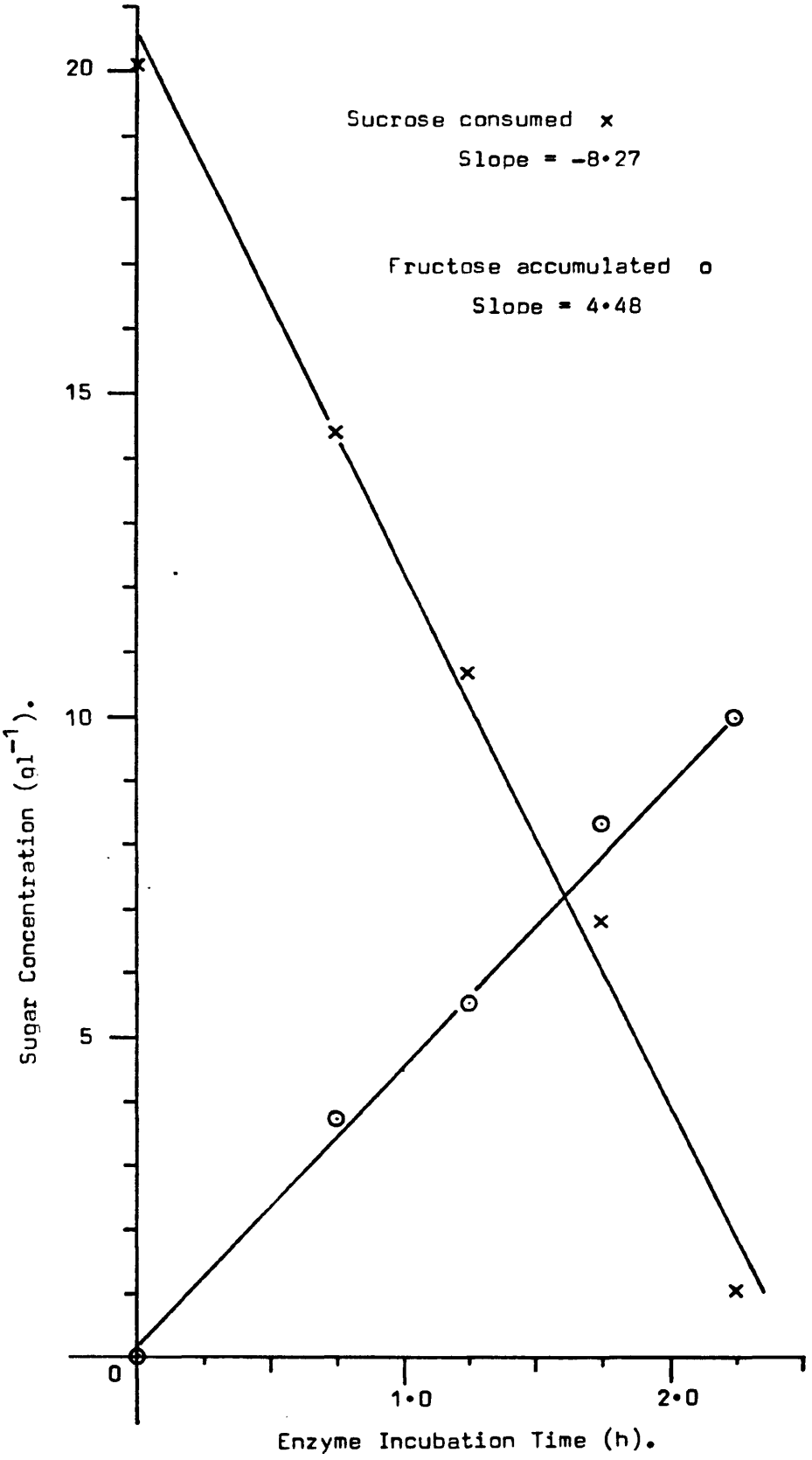


Figure 4.2 Sucrose consumption and Fructose accumulation vs Time (h).

4.6.3 Enzyme Assay By HPLC

The following are the sucrose and fructose concentrations determined from the HPLC traces (Figure 4.1). The sample assayed was the same as that reported in Section 4.6.2.

TIME (h)	SUCROSE CONC^{N} ($g.l.^{-1}$)	FRUCTOSE CONC^{N} ($g.l.^{-1}$)
0	20.10	0
0.75	14.42	3.74
1.25	10.70	5.52
1.75	6.82	8.32
2.25	1.04	9.98

The above results are plotted graphically (Figure 4.2).

Sucrose correlation 0.996 slope = - 8.27

Fructose correlation 0.998 slope = 4.48

Rate of sucrose consumption = 8.27 $g.l.^{-1}$ $h.^{-1}$

Rate of fructose accumulation = 4.48 $g.l.^{-1}$ $h.^{-1}$

The enzyme assay procedure will be discussed further in Section 6.2 of the discussion.

CHAPTER 5 - EXPERIMENTAL RESULTS

5.1 INVESTIGATION OF HPLC COLUMNS

The analysis of fermentation filtrates and other solutions for components such as sugars and organic acids is most conveniently carried out using high pressure liquid chromatography. It was necessary to assess a number of different columns for this purpose and these have already been detailed in Section 3.4.1. The test traces are shown in Figures 6.1 to 6.7. The test sample composition, column conditions, eluent composition and sample component retention times were as indicated at the bottom of each figure or to the right of each trace.

5.2 FERMENTATION RESULTS

A number of shake flask experiments and forty-one experimental runs are described in this work. The results from the experiments are presented graphically in the discussion chapter in chronological order. Some degree of grouping is possible.

5.2.1 Glucose Mixed-Feed Fed-Batch Fermentations (Runs 1-6)

Six fermentations were undertaken to demonstrate the use of the mixed-feed fed-batch technique to maintain the glucose within a narrow concentration range. The basic medium and feeds for Runs 1-6 are detailed in Appendices 1.2 and 1.3 respectively. The initial fermentation conditions for these experiments are listed in Table 5.1.

TABLE 5.1 GLUCOSE FED-BATCH INITIAL FERMENTATION CONDITIONS (RUNS 1-6)

Fermentation Run Number	1	2	3	4	5	6
Initial Glucose Concentration ($g.l.^{-1}$)	30	5	10	10	10	10
Initial Yeatex Concentration ($g.l.^{-1}$)	40	40	40	50	50	10
Initial Fermentation Volume (l.)	4	4	4	4	4	3.5
Inoculum Volume Added (ml.)	300	300	300	300	300	300
Inoculum Biomass Added (g.dry wt.)	0.63	0.63	0.52	0.42	0.52	0.35
Temperature	23 ^{o}C					
Dissolved Oxygen (% saturation)	Manually controlled at 40-60% by impeller rpm and air flowrate					
Initial Air Flowrate	$8\ l.min.^{-1}$					
Initial Stirrer Speed	400 rpm					
pH	6.7					

Run 1: Determination of the NaOH-to-glucose ratio. Raw data are listed in Appendices 1.4.1.1 - 1.4.1.6 and are plotted out in Figures 6.8 - 6.10.

Runs 2-5: Testing of the NaOH-to-glucose ratios. Raw data are listed in Appendices 1.4.2.1 -1.4.5.1 and are plotted out in Figures 6.11 - 6.18.

Run 6: A low 'Yeatex' concentration fermentation eventually fed with a glucose/NH_4OH solution. Raw data are listed in Appendices 1.4.6.1 and 1.4.6.2 and are plotted out in Figures 6.19 and 6.20.

5.2.2 Continuous Culture Experiments Using Sucrose as the Carbon Source (Runs 7-32)

Twenty-six continuous culture runs were undertaken to investigate the relationships between sucrose concentration, enzyme production and end-product concentrations. The media for Runs 7-15 and 16-32 are detailed in Appendices 2 and 3 respectively. Initial fermentation conditions for Runs 7-15 and 16-32 are detailed in Tables 5.2 and 5.3 respectively.

The results of Runs 7-15 are listed in Tables 6.2 and 6.3 and are plotted in Figures 6.21 and 6.22. The results of Runs 16-32 are listed in Tables 6.4 and 6.5.

TABLE 5.2 FERMENTATION CONDITIONS FOR CONTINUOUS CULTURE (RUNS 7-15)

Fermentation Run Number	7	8	9	10	11	12	13	14	15
Sucrose Concentration ($g.l.^{-1}$)	20	20	25	30	30	35	40	45	50
Fermentation Volume (l.)	3	3	3	3	3	3	3	3	3
Effluent Broth Outlet flowrate ($l.h.^{-1}$)	0.522	0.582	0.525	0.540	0.603	0.612	0.576	0.471	0.537
Temperature	25 ^{o}C								
Dissolved Oxygen (%saturation)	0	Manually controlled at 40-60% by impeller rpm							
Air Flowrate	6 $l.min^{-1}$ (2 VVM)								
pH	6.7 (using 2M NaOH)								
Stirrer Speed	0	Variable rpm. Manually adjusted to maintain required dO_2%.							

BASIC FERMENTATION MEDIUM FOR RUNS 7-15 PRESENTED IN APPENDIX 2.

TABLE 5.3.A FERMENTATION CONDITIONS FOR CONTINUOUS CULTURE (RUNS 16-32)

Fermentation Run Number	16	17	18	19	20	21	22	23	24
Sucrose Concentration ($g.l.^{-1}$)	60	60	50	40	40	40	40	40	40
Fermentation Volume (l.)	3	3	3	3	3	3	3	3	3
Effluent Broth Outlet Flowrate ($l.h.^{-1}$)	0.576	0.558	0.531	0.564	0.585	0.630	0.564	0.594	0.567
Temperature	25 ^{o}c								
Dissolved Oxygen (%saturation)	0	Manually controlled at 40-60% by impeller rpm							
Air Flowrate	6 $l.min.^{-1}$ (2 VVM)								
pH	6.7 (using 2M NaOH)								
Stirrer Speed	Variable rpm. Manually adjusted to maintain required dO_2%								

FERMENTATION MEDIA FOR CONTINUOUS CULTURE RUNS 16-32 PRESENTED IN APPENDIX 3.i.

TABLE 5.3.B FERMENTATION CONDITIONS FOR CONTINUOUS CULTURE (RUNS 16-32)

Fermentation Run Number	25	26	27	28	29	30	31	32
Sucrose ($g.l.^{-1}$) Concentration	40	40	40	40	80	40	60	40
Fermentation (l.) Volume	3	3	3	3	3	3	3	3
Effluent Broth Outlet Flowrate ($l.h.^{-1}$)	0.630	0.642	0.630	0.621	0.621	0.621	0.621	0.639
Temperature	25 ^{o}C							
Dissolved Oxygen (%saturation)	Manually controlled at 40-60% by impeller rpm							
Air Flowrate	6 $l.min.^{-1}$ (2 VVM)							
pH	6.7 (using 2M NaOH)							
Stirrer Speed	Variable rpm. Manually adjusted to maintain required dO_2%							

FERMENTATION MEDIA FOR CONTINUOUS CULTURE RUNS 16-32 PRESENTED IN APPENDIX 3.1..

5.2.3 Shake Flask Experiments to Test Media Sterilisation and Possible Alternative Nitrogen Sources

A series of shake flask experiments were undertaken to investigate the effect of sterilisation on the STEP III and YMB media. Media composition, preparation and experimental procedure are detailed in Appendices 4.1 - 4.3; results are listed in Table 6.6.

Three possible alternative nitrogen sources were tested for their suitability for supporting L. mesenteroides growth. Media composition, preparation and experimental procedure are detailed in Appendix 4.4; results are listed in Table 6.7.

5.2.4 Sucrose Mixed-Feed Fed-Batch Fermentations to Investigate the Effect of Different Types of Yeast Extracts (Runs 33-37)

Media composition for Runs 35-37 are presented in Appendix 5.5. Initial fermentation conditions for Runs 35-37 are detailed in Table 5.4.

Runs 33 and 34: Typical sucrose fed-batch fermentation using 'Bovril Yeatex' as the nitrogen source. Medium composition and preparation are detailed in Appendix 5.1, feeds are detailed in Appendix 5.2. Initial fermentation conditions are detailed in Table 5.5.

Raw and derived data are listed in Appendices 5.3 and 5.4 and are plotted out in Figures 6.23 - 6.28.

TABLE 5.4 SUCROSE FED-BATCH/BATCH INITIAL FERMENTATION CONDITIONS (RUNS 35-41)

Fermentation Run Number	35	36	37	38	39 (batch)	40	41
Initial Sucrose ($g.l.^{-1}$) Concentration	6	6	6	6	100	6	6
Initial Fermentation Volume (l.) (inclusive of inoculum)	3	2.5	2.5	2.5	3	3	2.5
Inoculum Volume (ml)	200	200	200	200	200	200	200
Inoculum Biomass Added (g)	0.64	0.64	0.64	0.64	0.64	0.64	0.64
Temperature (^{o}C)	23-26.5	23-32.5	23	23	23	23	23
Dissolved Oxygen (%saturation)	Manually controlled at 40-50% by impeller rpm						
Air Flowrate ($l.min.^{-1}$)	6						
Initial Stirrer Speed (rpm)	400						
pH	6.7						

TABLE 5.5 INITIAL CONDITIONS OF SUCROSE FED-BATCH FERMENTATION (RUNS 33 AND 34)

Fermentation Run Number	33	34
Initial Sucrose Concentration ($g.l.^{-1}$)	6	6
Initial Fermentation Volume (l.)	3	3
Inoculum Volume Added (ml)	150	210
Inoculum Biomass Added (g.dry wt.)	0.32	0.41
Temperature (^{o}C)	23	23
Dissolved Oxygen (%saturation)	20-30%	40-50%
Initial Air Flowrate ($l.min.^{-1}$)	6	6
Initial stirrer Speed	50 rpm	400 rpm
pH	6.7	6.7

SEE APPENDIX 5 FOR MEDIUM COMPOSITION AND PREPARATION AND FEED COMPOSITION AND PREPARATION

Run 35; Sucrose fed-batch fermentation using 'Ohly' yeast extract.

Raw and derived data are listed in Appendices 6.1 - 6.7. These results are plotted out in Figures 6.29 - 6.33.

Run 36: Sucrose fed-batch fermentation using 'Ohly' yeast extract with phosphate feed and pulsed addition of yeast extract.

Details of the phosphate feed and addition of the yeast extract are presented in Table 5.6. Raw and derived data are listed in Appendices 7.1 - 7.7. These results are plotted out in Figures 6.34 - 6.38.

TABLE 5.6 FERMENTATION ADDITIONS TO RUN 36

Key	Time (h)	Composition	Volume (ml)
A	11.00 (feed on)	50 g K_2HPO_4	250
B	13.00	26.5 g Yeast Extract (Gist-Brocades)	100
C	13.25	Phosphate feed finished Feedrate 111 $ml.h.^{-1}$	

Run 37: Sucrose fed-batch fermentation with a 'Gist-Brocades' yeast extract feed.

Details of the yeast extract feed and feedrate are presented in Table 5.7. Raw and derived data are listed in Appendices 8.1 - 8.7. These results are plotted out in Figures 6.39 - 6.43.

TABLE 5.7 FERMENTATION ADDITIONS TO RUN 37

Key	Time (h)	Composition	Feedrate ($ml.h.^{-1}$)
A	08.00 (feed on)	185 $g.l.^{-1}$ Yeast Extract (Gist-Brocades)	32.5
B	11.00	Increase Yeast Extract feedrate	80.0
C	16.00	Yeast Extract feed exhausted	
		0.498 l. of Yeast Extract solution fed to the fermenter	

5.2.5 Sucrose Mixed Feed Fed-Batch Fermentations with 'Pulsing of Nutrients to Identify an Unknown Limiting Substrate (Runs 38-41)

Media composition for Runs 38-41 are presented in Appendix 5.5. Initial fermentation conditions are detailed in Table 5.5.

Run 38: Pulsing of alternative nitrogen sources. Details of the fermentation additions are presented in Table 5.8. Raw and derived data are listed in Appendices 9.1 - 9.7. These results are plotted out in Figures 6.44 - 6.47.

TABLE 5.8 FERMENTATION ADDITIONS TO RUN 38

Key	Time (h)	Composition	Volume (ml)
A	08.75	9 g Sucrose	50
B	09.50	2.5 g Inositol	50
C	10.50	12.5 g Peptone P (Oxoid L49)	50
D	12.00	12.5 g Tryptone T (Oxoid L43)	50
E	13.00	12.5 g Casein Hydrolysate (oxoid L41)	50
F	14.00	12.5 g Yeatex (Bovril)	50
G	16.50	12.5 g Yeast Extract (Gist-Brocades)	50

Run 39: Pulsing of vitamin mixture.

Details of the vitamin mixture composition and time of addition are presented in Table 5.9. Raw and derived data are listed in Appendices 10.1 - 10.6. These results are plotted out in Figures 6.48 - 6.52.

TABLE 5.9 FERMENTATION ADDITIONS TO RUN 39

Key	Time (h)	Composition	Volume (ml)
A	10.00	0.2225 g Vitamin B_1	
		0.0462 g Folic Acid	
		0.0526 g Riboflavin	
		0.0732 g Pantothenic Acid	
		0.0809 g Nicotinic Acid	
		0.0253 g Biotin	
			50

Run 40: Sequential pulsing of individual vitamins. Details of the vitamin additions are presented in Table 5.10. Raw and derived data are listed in Appendices 11.1 - 11.7. These results are plotted out in Figures 6.53 - 6.56.

TABLE 5.10 FERMENTATION ADDITIONS TO RUN 40

Key	Time (h)	Composition	Volume (ml)
A	10.00	0.2090 g Vitamin B_1	10
B	11.00	0.0620 g Folic Acid	10
C	12.00	0.0494 g Riboflavin	10
D	13.50	0.1000 g Pantothenic Acid	10
E	14.00	0.1175 g Nicotinic Acid	10
F	14.75	0.0275 g Biotin	10

Run 41: Pulsing of folic acid.

Details of the fermentation additions are presented in Table 5.11. Raw and derived data are listed in Appendices 12.1 - 12.7. These results are plotted out in Figures 6.57 - 6.60.

TABLE 5.11 FERMENTATION ADDITIONS TO RUN 41

Key	Time (h)	Composition	Volume (ml)
A	0.825	0.1026 g Folic Acid	10
B	11.50	0.1811 g Folic Acid	10
C	12.00	27 g Tryptone T (Oxoid L43)	-
D	13.00	10 g K_2HPO_4	-
E	13.50	Medium - STEP III (see Appendix 4.1 used Gist-Brocades yeast extract)	100

CHAPTER 6 DISCUSSION

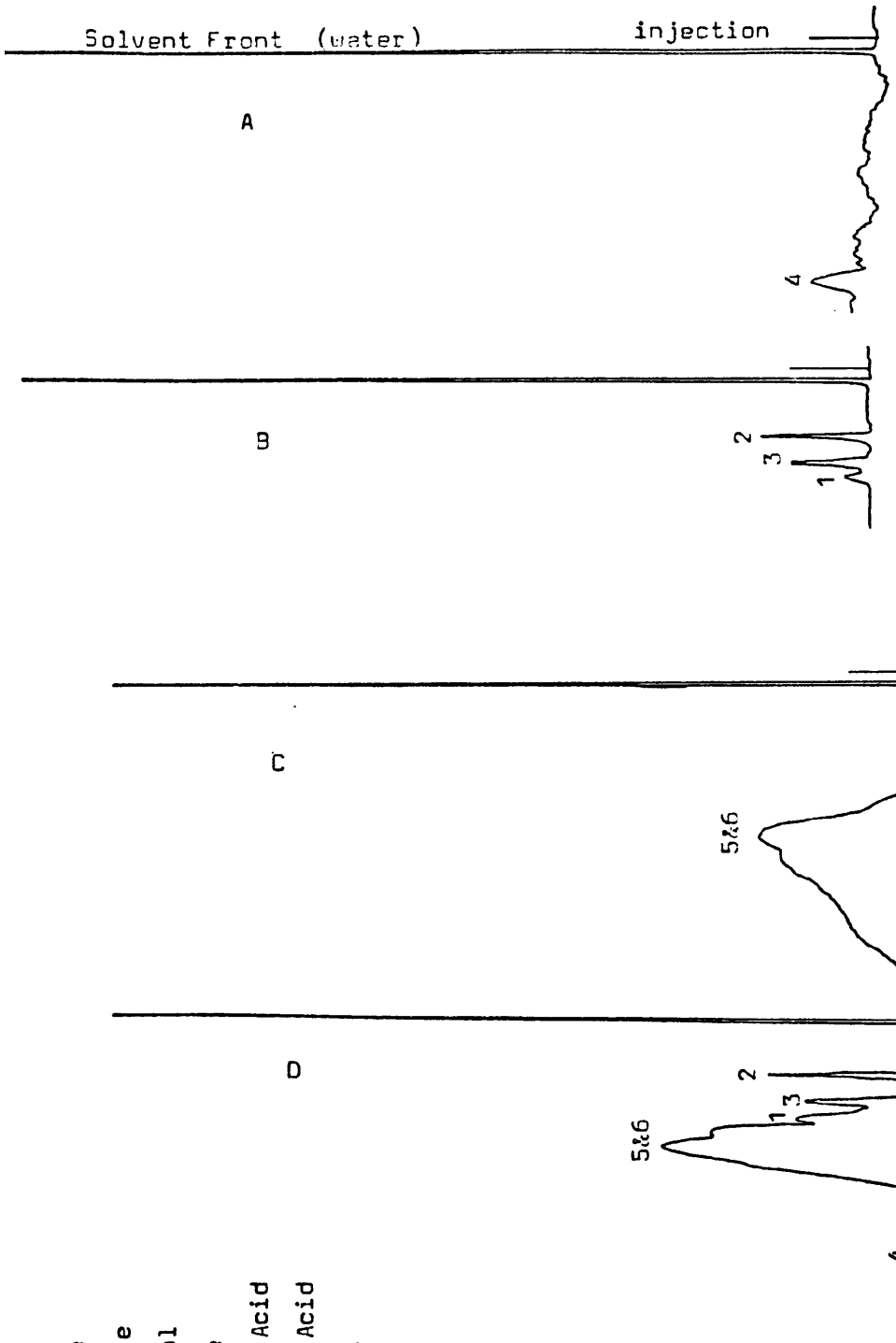
A number of sugars, organic acids and other carbon compounds are involved in the culture of Leuconostoc mesenteroides and suitable HPLC systems were needed to evaluate their compositions in the culture media. A study of the growth on a medium containing only glucose was undertaken in order to confirm the need for sucrose in the production of dextransucrase. The possibility of enzyme biosynthesis in continuous culture was of considerable interest, but its investigation was interrupted by trace component limitations. These limitations were studied in fed-batch cultures, the component tentatively identified and a mechanism is proposed for an associated linear growth phenomenon.

6.1 HIGH PRESSURE LIQUID CHROMATOGRAPHY

As indicated in Section 2.4 of the literature survey, it was necessary to identify a combination of columns which would separate and quantify a typical spectrum of known end-products from the culture of L. mesenteroides.

6.1.1 Magnisphere Column

The charts in Figure 6.1 A and B demonstrate that the Magnisphere packing was able to separate the four sugars glucose, fructose, mannitol and sucrose. However, it proved difficult to determine concentrations less than 0.1% w/v. Ethanol eluted too close to the solvent peak to enable the determination of the peak area and thus the concentration. Figure 6.1 C and D



1. Glucose
2. Fructose
3. Mannitol
4. Sucrose
5. Lactic Acid
6. Acetic Acid
7. Ethanol

Figure 6.1

Column MAGNISPHERE 5u Temp. 25°C Flow 2ml.min^{-1} Pressure 2,200p.s.i.

Eluent Acetonitrile : water (85 : 15, + 0·01% Amine Modifier.)

Detector WATERS REFRACTOMETER R401

show that acetic and lactic acid did not elute from the column as good peaks. Their presence in a fermentation sample in any reasonable concentration thus caused inaccuracies in the calculation of the sugar concentrations.

6.1.2 LiChrosorb Si 100 Column

The LiChrosorb Si 100 packing gave good peaks for the four sugars but their separation was very limited (Figures 6.2.E and 6.3.A). Although both acetic and lactic acid produced better peaks (Figures 6.2.C, D, F, G, and 6.3.B) than those of the Magnisphere packing, they still interfered greatly with the sugars (Figures 6.2.H and 6.3.C). However, if either or both acids were present in a fermentation sample in any reasonable concentration they caused inaccuracies in the calculated concentrations of the sugars present. The calibration and calculation of sugar concentrations to an accuracy of at least 0.05% was possible if background interference was at a minimum.

6.1.3 Bio Rad HPX-87 Carbohydrate Column

The Bio Rad HPX-87 carbohydrate column gave good separation of the four sugars and ethanol (Figures 6.4.D, E, F, G and 6.5.D and E). The conditions specified for Figure 6.5.E were found to be optimal. Concentrations of 0.166% gave very distinct peaks (Figures 6.4.B and C) and the calculation of concentrations as low as 0.01% w/v

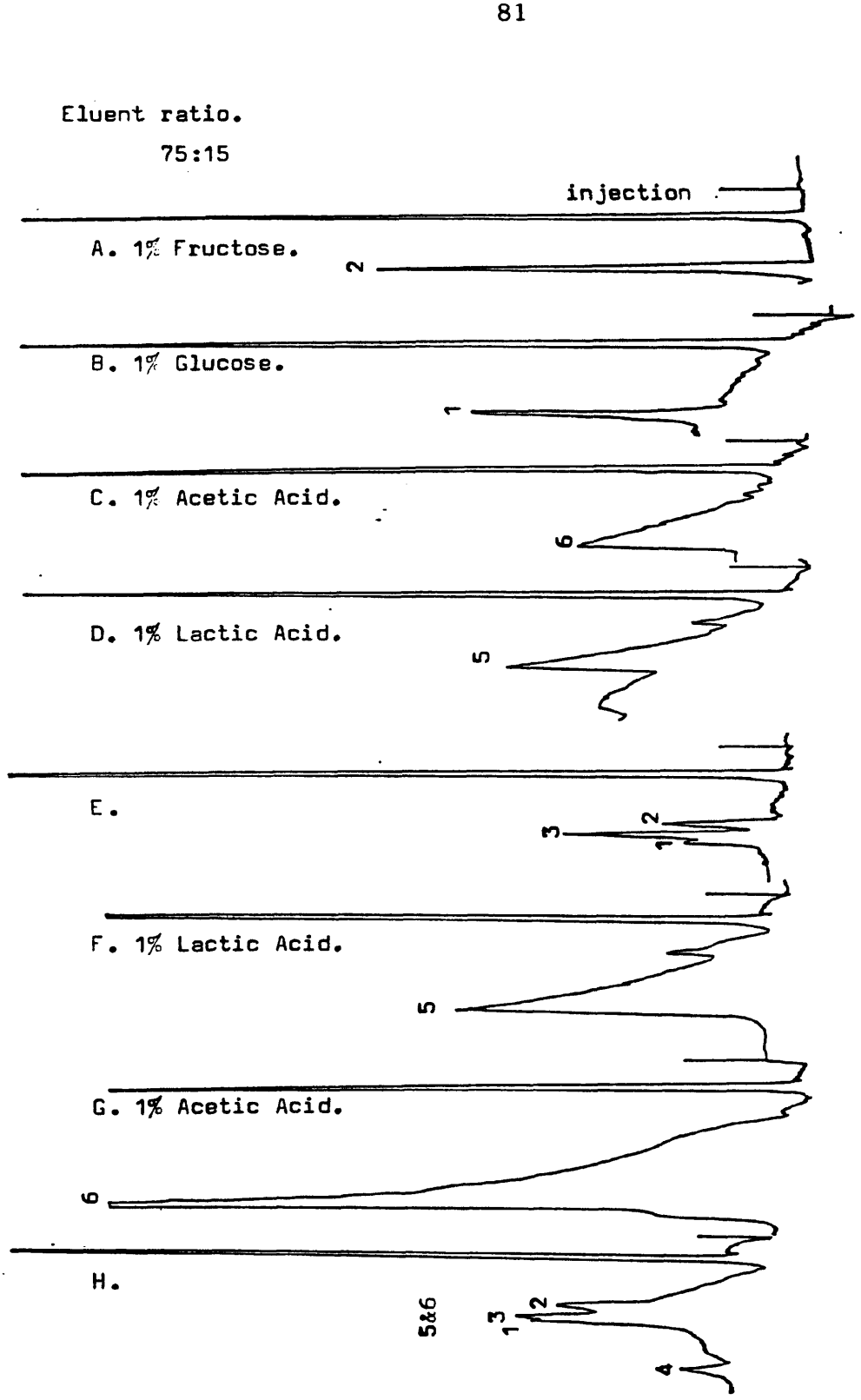

Eluent ratio.
75:15
injection
A. 1% Fructose.
2
B. 1% Glucose.
1
C. 1% Acetic Acid.
6
D. 1% Lactic Acid.
5
E.
3
2
1
F. 1% Lactic Acid.
5
G. 1% Acetic Acid.
6
H.
5&6
3
1
2
4
1. Glucose
2. Fructose
3. Mannitol
4. Sucrose
5. Lactic Acid
6. Acetic Acid
Column LiChrosorb Si 100 5um Temp. 25°C Flow 1ml.min⁻¹ Pressure 1,500p.s.i.
Eluent Acetonitrile : water (+ 0·01% Amine Modifier)
Ratio as specified.
Detector WATERS REFRACTOMETER R401.

Figure 6.2

1. Glucose
2. Fructose
3. Mannitol
4. Sucrose
5. Lactic Acid
6. Acetic Acid

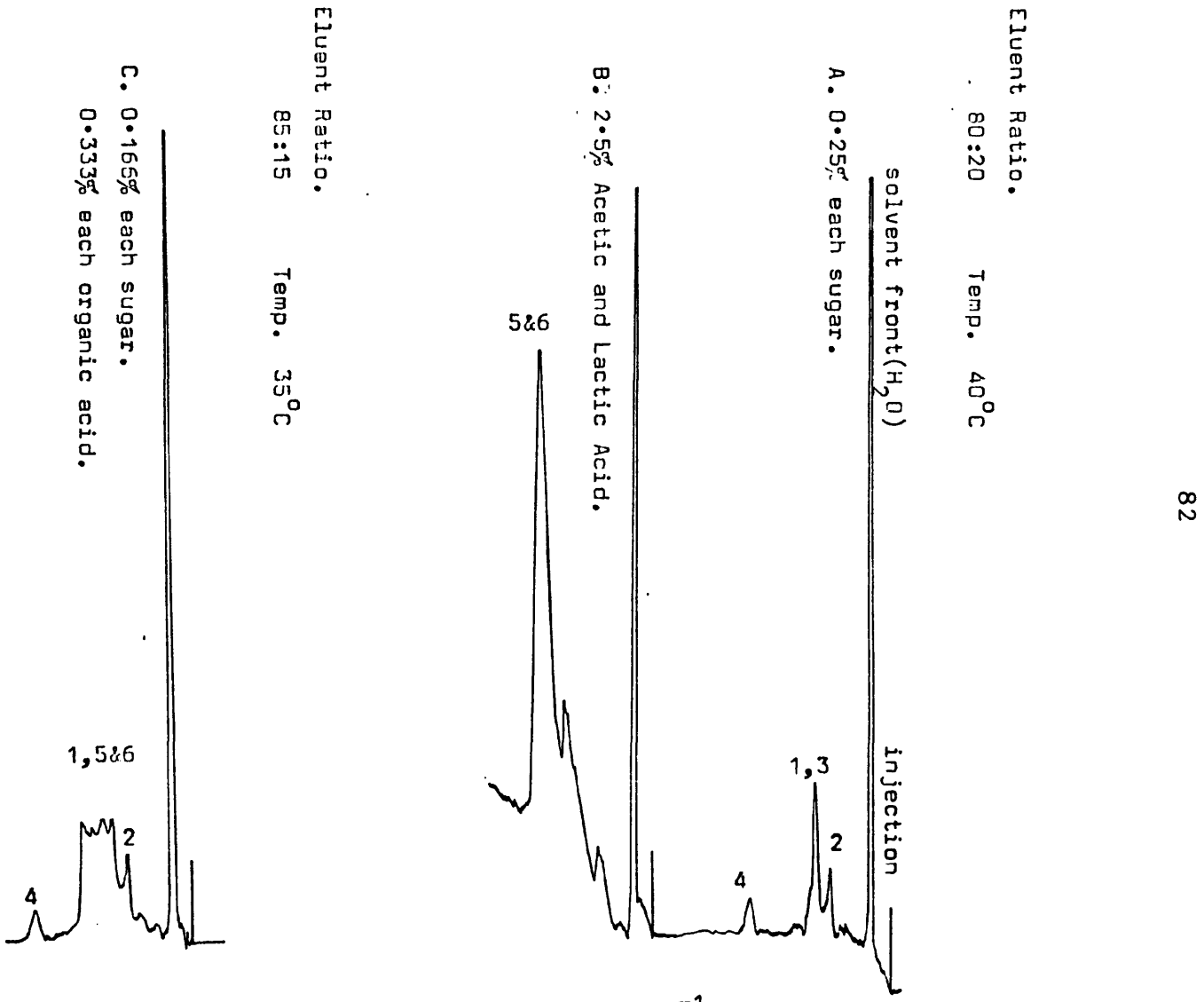


Figure 6.3

Column LiChrosorb Si 100 5um. Flow 1·5ml.min^{-1}

Eluent Acetnitrile : water(+0·01% Amine Modifier.) Detector WATERS REFRACTOMETER R401
Ratio as specified. x4 attenuation.

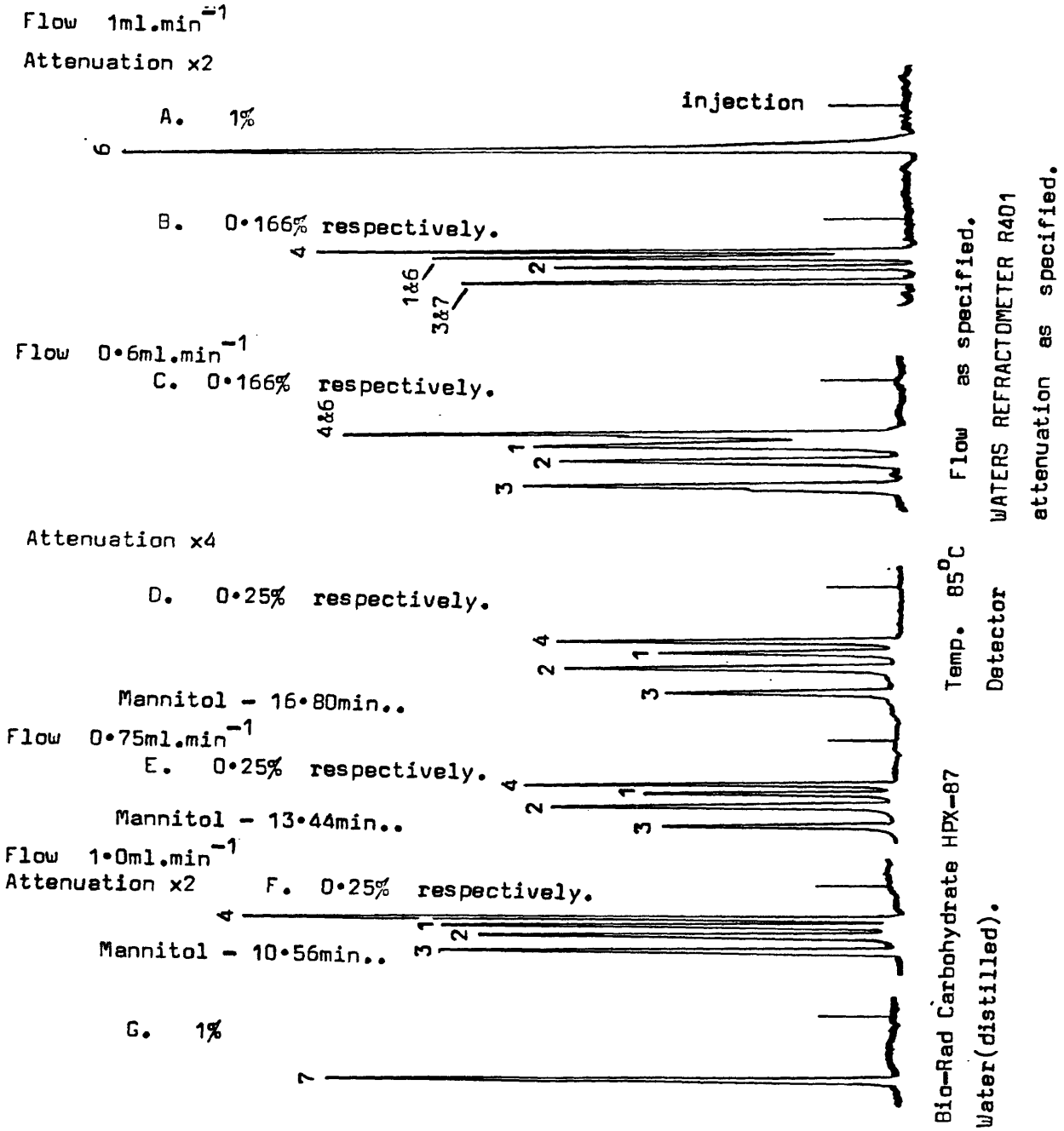

Flow 1ml.min⁻¹
Attenuation x2
A. 1%
injection
6
B. 0·166% respectively.
4
2
1&6
3&7
Flow 0·6ml.min⁻¹
C. 0·166% respectively.
4&6
1
2
3
Attenuation x4
D. 0·25% respectively.
2 4
1
3
Mannitol - 16·80min..
Flow 0·75ml.min⁻¹
E. 0·25% respectively.
4
2
1
3
Mannitol - 13·44min..
Flow 1·0ml.min⁻¹
Attenuation x2
F. 0·25% respectively.
4
1
2
3
Mannitol - 10·56min..
G. 1%
7
Flow as specified.
Temp. 85°C
Detector WATERS REFRACTOMETER R401
attenuation as specified.
Column Bio-Rad Carbohydrate HPX-87
Eluent Water(distilled).
1. Glucose
2. Fructose
3. Mannitol
4. Sucrose
5. Lactic Acid
6. Acetic Acid
7. Ethanol
Concentrations as indicated.

Figure 6.4

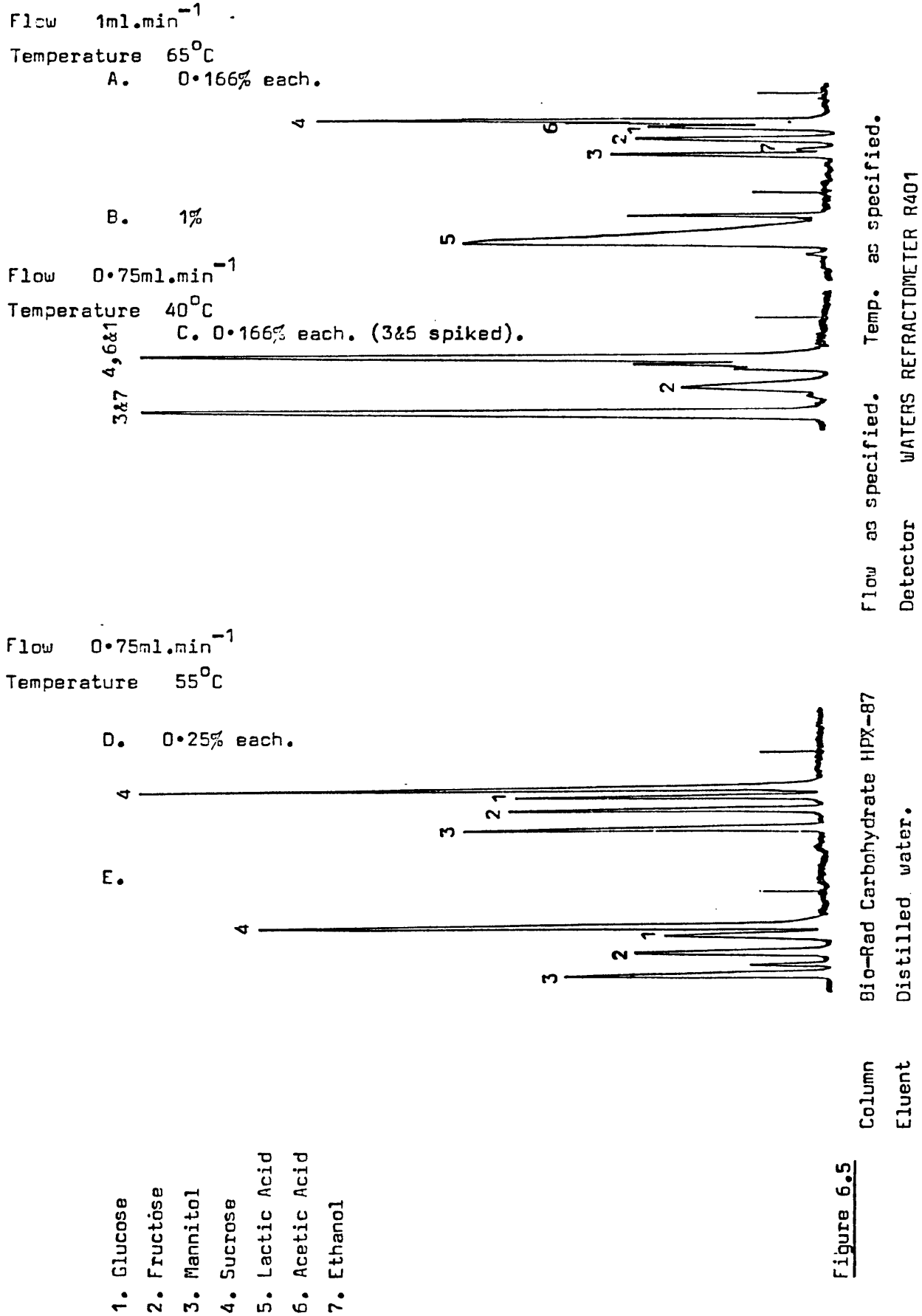

Flow 1ml.min^{-1}
Temperature 65°C
A. 0·166% each.
B. 1%
Flow 0·75ml.min^{-1}
Temperature 40°C
C. 0·166% each. (3&6 spiked).
Flow 0·75ml.min^{-1}
Temperature 55°C
D. 0·25% each.
E.
1. Glucose
2. Fructose
3. Mannitol
4. Sucrose
5. Lactic Acid
6. Acetic Acid
7. Ethanol
Column Bio-Rad Carbohydrate HPX-87
Eluent Distilled water.
Flow as specified. Temp. as specified.
Detector WATERS REFRACTOMETER R401

Figure 6.5

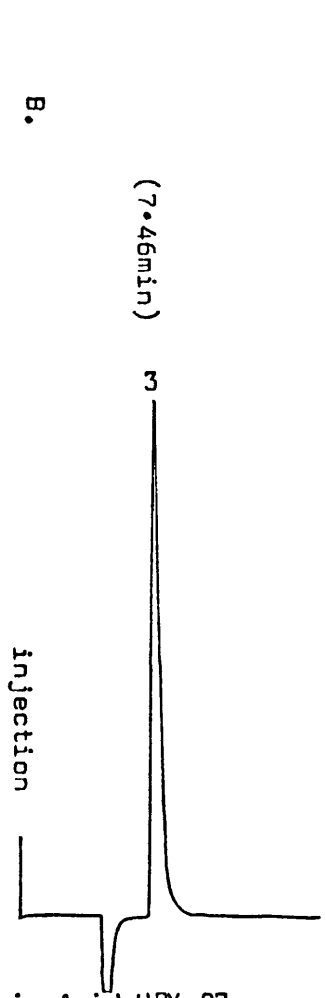
was possible. Ethanol required special conditions, a column temperature of 55 ^{o}C and a flowrate of 0.75 ml.min^{-1} (Figure 6.5.E) to separate it from mannitol. Acetic acid gave a well-defined peak but eluted with either the glucose or sucrose peak depending on the column conditions (Figures 6.4.A, B, C, and 6.5.A and C). Lactic acid eluted as two peaks (Figure 6.5.B) which interfered with the sucrose, glucose, fructose and ethanol peaks. Conditions which would enable the separation of lactic and acetic acids from the sugars and ethanol could not be found. Also, if lactic and acetic acids were present in any significant quantities they caused inaccuracies in the calculation of the concentrations of the sugars present.

6.1.4 Bio Rad HPX-87 Organic Acid Column

With the exception of fructose and mannitol, the Bio Rad HPX-87 organic acid column was able to separate sucrose, glucose, ethanol, lactic and acetic acids producing good, well-defined peaks (Figure 6.6.A). Fructose and mannitol had similar retention times (Figure 6.6.A and B) but individually they eluted as good peaks.

A sample from a fermentation broth was analysed by both the Bio Rad HPX-87 carbohydrate and organic acid columns under the indicated conditions. The results are presented in Figure 6.7.

	g/l
1. Glucose	0•5
2. Fructose	2•5
3. Mannitol	-
4. Sucrose	2•5
5. Lactic Acid	3•75
6. Acetic Acid	2•5
7. Ethanol	0•5



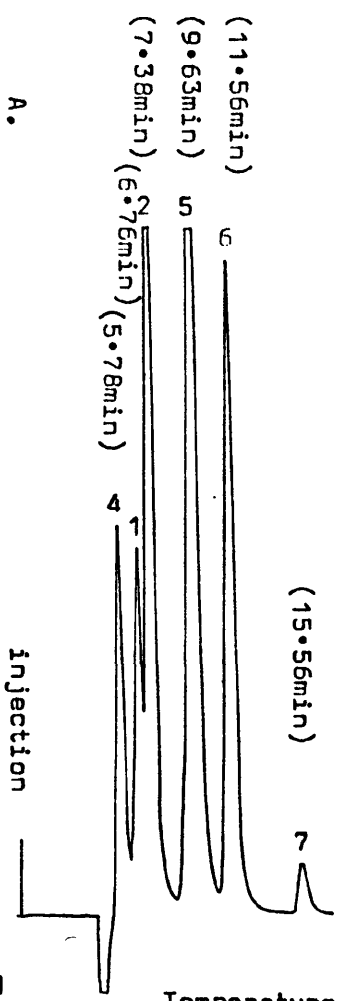
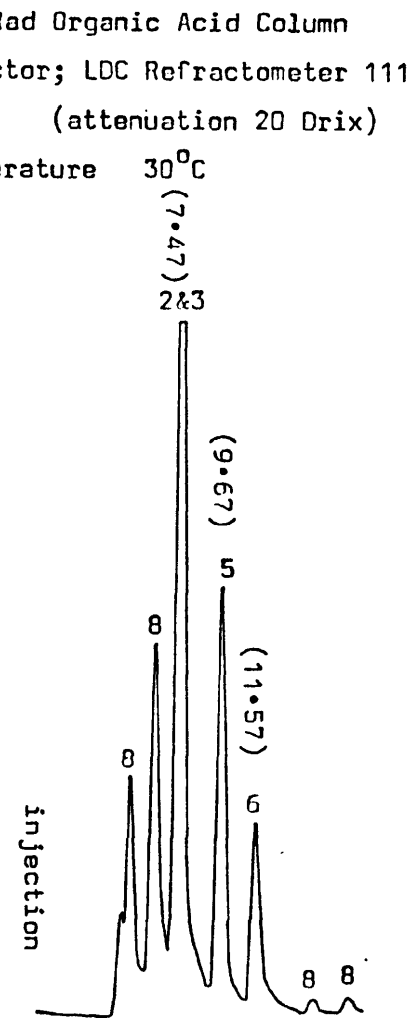


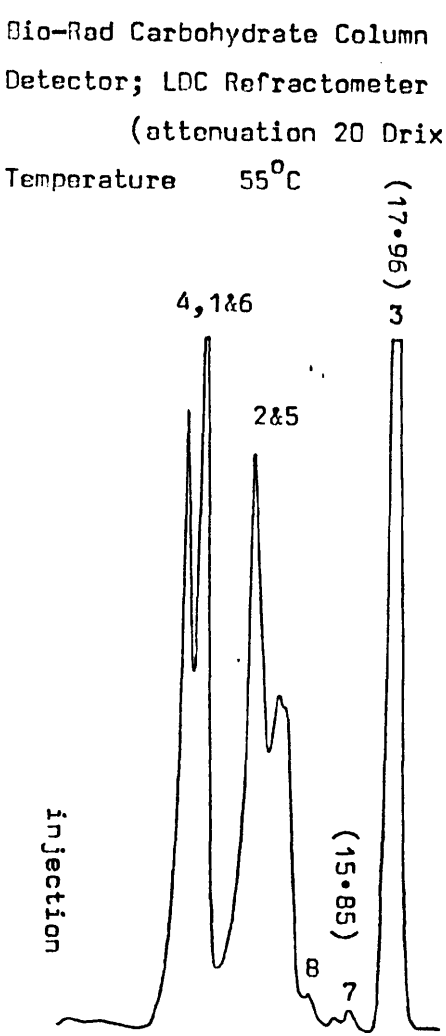
Figure 6.6

Column Bio-Rad Organic Acid HPX-87 Flow 0•8ml.min^{-1} Temperature 25^{o}C

Eluent 0•01468M sulphuric acid. Detector LDC REFRACTOMETER 111 (attenuation 20 Drix).

1. Glucose
2. Fructose
3. Mannitol
4. Sucrose
5. Lactic Acid
6. Acetic Acid
7. Ethanol
8. Unknown





Figures in the brackets are retention times in minutes.

Figure 6.7
Example of fermentation broth analysed by both Bio-Rad Carbohydrate and Organic Acid Columns.

6.1.5 Summary

Both the Magnisphere and LiChrosorb packings in conjunction with the Amine Modifier 1 separated the four relevant sugars adequately. However, neither packing was compatible with the organic acids which eluted as broad, ill-defined peaks at elution times which interfered with those of the sugars. These columns under the aforementioned conditions were not suitable for the analysis of the Leuconostoc mesenteroides broth.

The Bio Rad HPX-87 carbohydrate column gave excellent separation of all four sugars and ethanol (Figure 6.5.E). Sensitivity was better than that of the amine-modified columns reported by Aitzetmuller (1978) and Jackson (1980). Analysis times were also reduced by a factor of 3; from over 30 minutes for the amine-modified columns to just over 10 minutes for the Bio Rad HPX-87 carbohydrate column. Both lactic and acetic acids gave good peaks on this column. Unfortunately, they eluted at similar times to some of the sugars. The Bio Rad HPX-87 carbohydrate column could, therefore, only be used to analyse for mannitol and ethanol in the broth typical of the Leuconostoc mesenteroides fermentation. This column is excellent for the identification and quantification of low sugar concentrations in fermentations in which there is no organic acid end-products.

The Bio Rad HPX-87 organic acid column was the only column that was investigated which would separate the relevant sugars from the organic acids. However, both fructose and mannitol have similar elution times and could not be distinguished and quantified if present together in a sample. A combination of analyses from both the Bio Rad HPX-87 carbohydrate and organic acid columns enabled the identification and quantification of the sugars, ethanol and organic acids present in a Leuconostoc mesenteroides fermentation (Figure 6.7). The Bio Rad HPX-87 carbohydrate column enabled the analysis of mannitol and fructose. A further advantage of these columns was that their eluent was water or water-based, in comparison to the costly HPLC grade acetonitrile required by the amine-modified silica columns.

Using the Bio Rad columns, an HPLC trace of a fermentation medium prior to inoculation showed an initial unknown peak (assumed to be proteins and high molecular weight material) and then a peak for the carbohydrate source used in the medium. Examination of Figure 6.7 and Appendices 2.2.1 and 2.2.2 illustrates that there were other unknown fermentation end-products at low concentrations present in the broth. In this work it was assumed that other interfering carbohydrate and organic compounds were at much lower concentrations than those to be examined. No attempt was made to identify the compounds responsible for the unknown peaks.

6.2 DEXTRANSUCRASE ASSAY USING HPLC

The work of Lawford, Kligerman and Williams (1979) suggested that the presence of enzymes such as invertase and levansucrase in the fermentation broth of L. mesenteroides might interfere with the dextransucrase assay. The possibility of the presence of such contaminating enzymes had not been considered by previous workers. Since HPLC will characterise each individual end-product in the enzymatic reaction it was possible to check for the presence of these contaminating enzymes. Both invertase and levansucrase produce one mole of glucose from one mole of sucrose substrate. A glucose peak would, therefore, become evident in any HPLC examination of a dextransucrase reaction.

A centrifuged L. mesenteroides B512-F fermentation broth of known dextransucrase activity, calculated by the reducing sugar method (McAvoy, 1981), was analysed by HPLC to characterise the end-products.

The HPLC chart readout in Figure 4.1 clearly illustrates the reaction progress. As the incubation time increased, sucrose was consumed and dextran and fructose accumulated. No other end-products can be seen to elute from the column. Glucose and mannitol were present in the reaction mixture as carry-over from the fermentation broth and their concentrations do not change throughout the reaction. These results demonstrate that dextransucrase appears to

be the only detectable enzyme present in the broth. The chart readout for the 24 h incubation time shows clearly that the reaction has gone to completion. From the calculations (Section 4.6.3 and Figure 4.2) of the rates of sucrose consumption and fructose accumulation it can be concluded that active dextransucrase was present. If the dextransucrase activity was calculated by the Reducing Sugar Method (Section 4.6.2), then the rate of sucrose consumption would be approximately 10 $g.l.^{-1}$ $h.^{-1}$ with a rate of fructose accumulation of 5.2 $g.l.^{-1}$ $h.^{-1}$. The HPLC experimental results gave the rate of sucrose consumption to be 8.27 $g.l.^{-1}$ $h.^{-1}$ and the rate of fructose accumulation to be 4.48 $g.l.^{-1}$ $h.^{-1}$. Both sets of experimental values gave good linear plots (Figure 4 . 2) with correlations of greater than 0.99. Although the HPLC analysis was set up to calculate accurately the fructose concentration and thus the rate of fructose accumulation, the rate of sucrose consumption agrees well with that of the rate of fructose accumulation. Fructose accumulation of 4.48 $g.l.^{-1}$ $h.^{-1}$ would give a sucrose consumption of 8.62 $g.l.^{-1}$ $h.^{-1}$ in comparison to the experimentally derived value of 8.27 $g.l.^{-1}$ $h.^{-1}$. A fructose accumulation rate of 4.48 $g.l.^{-1}$ $h.^{-1}$ represents an initial enzyme activity of 224 DSU which is 13.85% lower than the 260 DSU calculated to have been added. This loss may have been due to the concentrated form of the enzyme.

On examination of the HPLC chart readouts (Figure 4.1) of this experiment the individual sugar peaks do not exhibit as good a resolution as work presented earlier on a similar analysis (Figures 6.4 and 6.5). This lack of resolution was due entirely to the use of a Waters Refractometer R403 and the lack of availability of a more suitable R401. The R403 has a larger refractometer cell volume and thus causes a degree of mixing in the eluent stream which in turn gives broader based peaks and lower resolution.

Both the HPLC assay and the Reducing Sugar assay have their advantages and disadvantages. The HPLC assay is enzyme specific, minimising the inaccuracies in the assay due to other enzymes or chemicals. However, HPLC equipment is expensive, time consuming to set up and the analysis for each individual assay takes at least 120 minutes. This length of time assumes an enzyme incubation time of 20 minutes and at least five samples being analysed for fructose and sucrose concentration. (Refer to Section 4.6.2).

The Reducing Sugar assay is not enzyme specific and is susceptible to interference from contaminating enzymes such as invertase and levansucrase. The main advantages of the Reducing Sugar assay are the relatively shorter assay time of 40 minutes and the cheaper and simpler equipment and chemicals.

6.3 GLUCOSE/NaOH FED-BATCH FERMENTATIONS

Glucose can be aerobically metabolised by Leuconostoc mesenteroides to produce carbon dioxide, acetic and lactic acid and possibly ethanol (Bergey, 1974). The glucose concentration in the fermentation broth can be easily determined using the Reducing Sugar Method (McAvoy, 1981). This overcomes one of the main drawbacks of the earlier work (McAvoy, 1981) using the pH-linked substrate feed system in which it was not easy to determine the background sucrose concentration to ascertain whether it was being maintained within the desired concentration range (2-10 $g.l.^{-1}$).

A batch run fermentation (Run 1) was completed to determine the ratio of NaOH to glucose consumed. Five fed-batch fermentations were run to examine experimentally the pH-linked substrate feed system using glucose as the carbohydrate source.

6.3.1 Run 1 Determination of NaOH to Glucose Ratio

A batch fermentation was completed using an initial glucose concentration of approximately 30 $g.l.^{-1}$ as the carbon source. The molar glucose consumption and molar NaOH consumption to maintain the pH at 6.7 were determined (Appendices 1.4.1.1, 1.4.1.3, 1.4.1.4, and 1.4.1.5).

An approximate determination from Figure 6.8 (Appendix 1.4.1.6) gave a ratio of 1.82 moles of NaOH to 1 mole of glucose and this ratio was used to test the glucose/NaOH

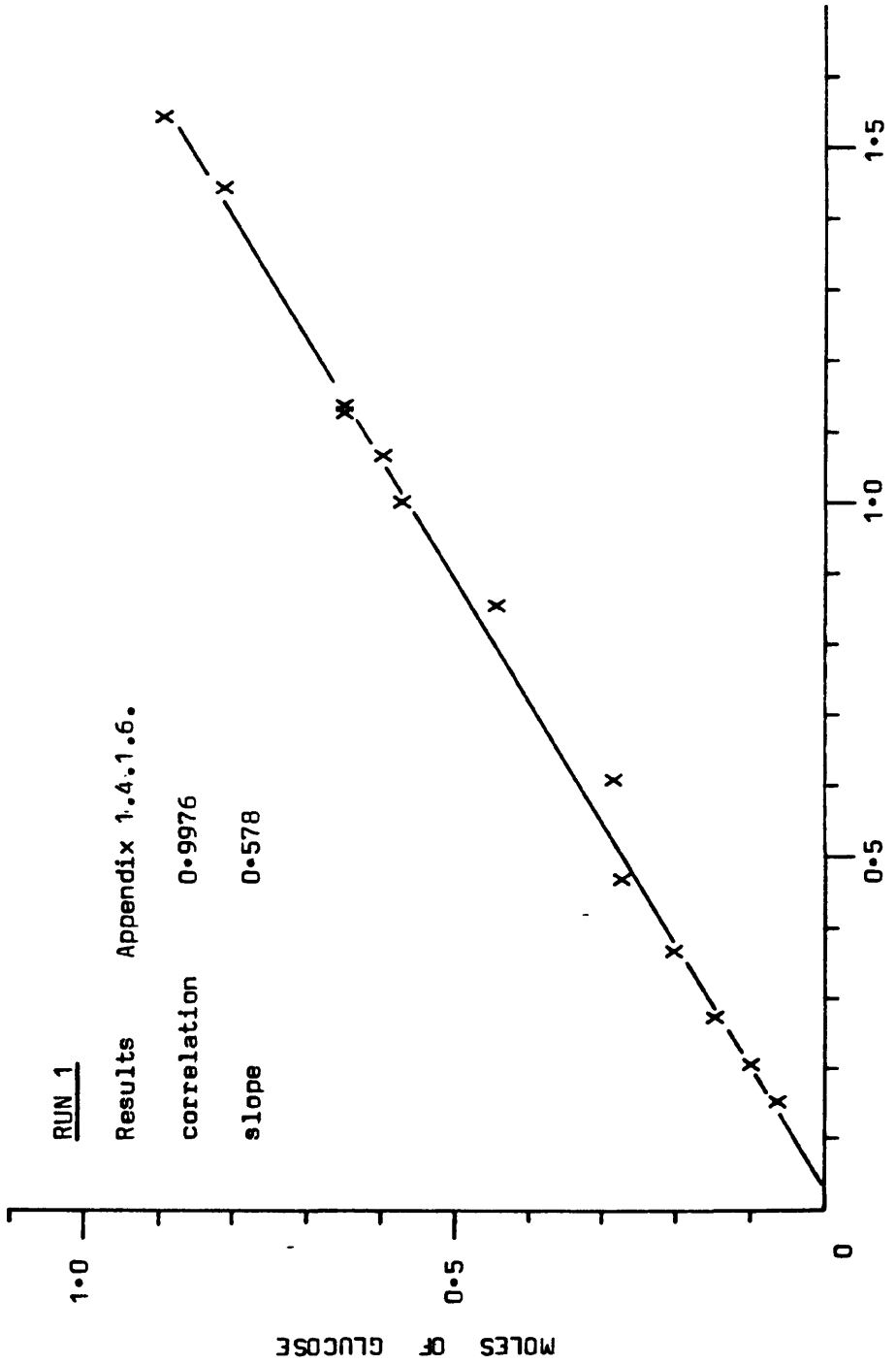


Figure 6.8 Run 1; Moles of NaOH vs Moles of Sucrose.

fed-batch fermentation in Run 2. However, linear regression applied to the data in Appendix 1.4.1.6 at a later date gave a ratio of 1.73 moles of NaOH to 1 mole of glucose with a linear correlation coefficient of 1.00.

A maximum optical density of 0.6 was attained in 12 h (Figure 6.9) and at 8 h the rates of bacterial growth, glucose consumption and NaOH requirement decreased markedly. Although the glucose concentration at 8 h was 8.1 $g.l.^{-1}$, such an abrupt reduction in growth suggested a carbon limitation. At 10.5 h, 40 g of glucose was added to the fermenter changing the glucose concentration at that time from 4.9 $g.l.^{-1}$ to 7.0 $g.l.^{-1}$. The NaOH requirement increased almost immediately together with the glucose consumption, but with no significant bacterial growth. At 12.5 h, as the glucose concentration dropped to 3.9 $g.l.^{-1}$, the NaOH requirement, again, decreased dramatically. It appeared from this batch experiment that glucose could be rapidly metabolised at concentrations greater than 4.0 $g.l.^{-1}$ but that microbial growth was still limited by some other component. A maximum specific growth rate was estimated from the linear portion of Figure 6.10 (Appendix 1.4.1.2) to be 0.39 $h.^{-1}$.

6.3.2 Run 2

The ratio of 1.82 moles of NaOH to 1 mole of glucose was used for this pH-linked fed-batch fermentation. The glucose/NaOH solution was prepared as described in

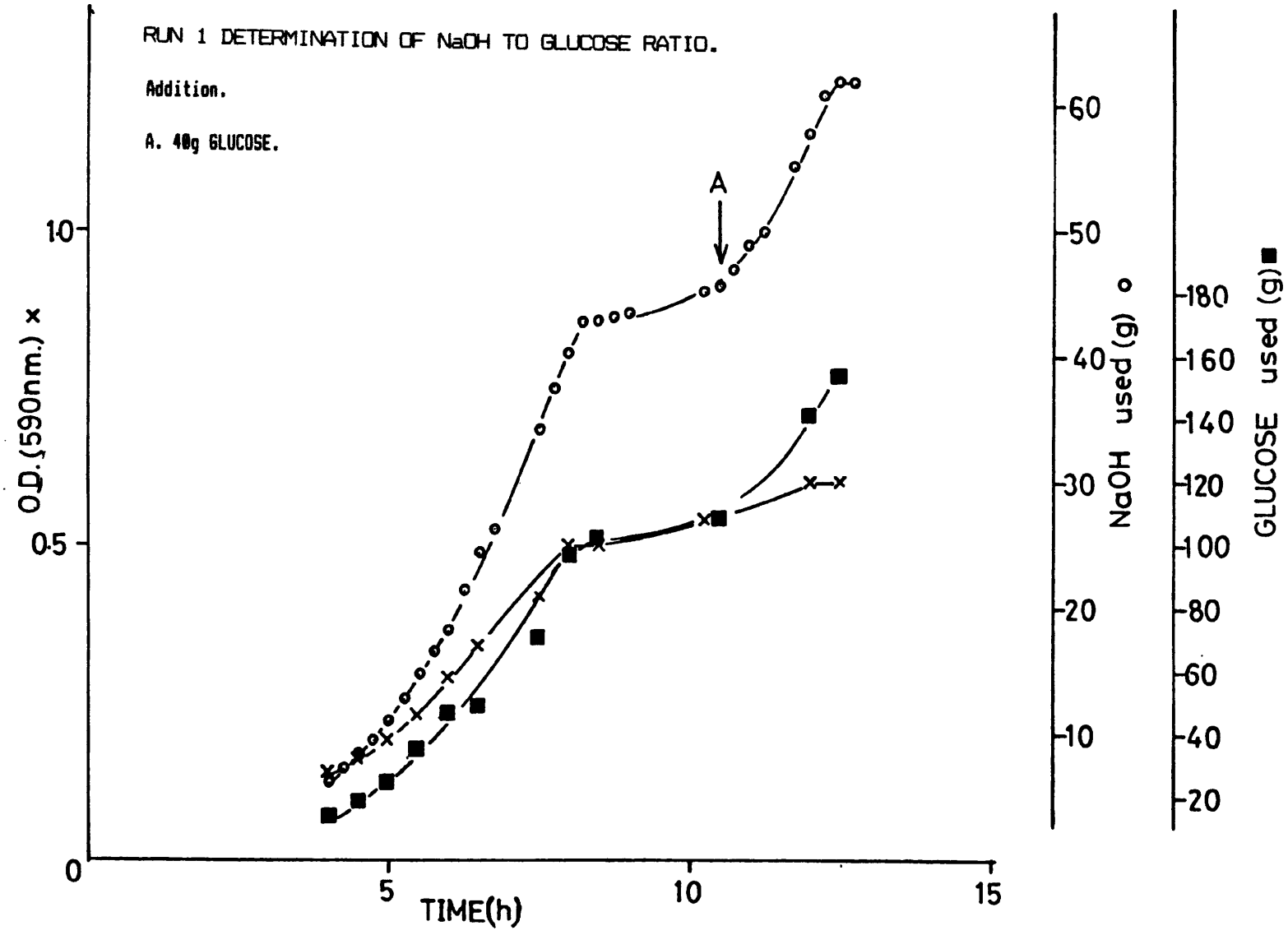


Figure 6.9 Run 1; O.D.,NaOH Used and Glucose Used vs Time.

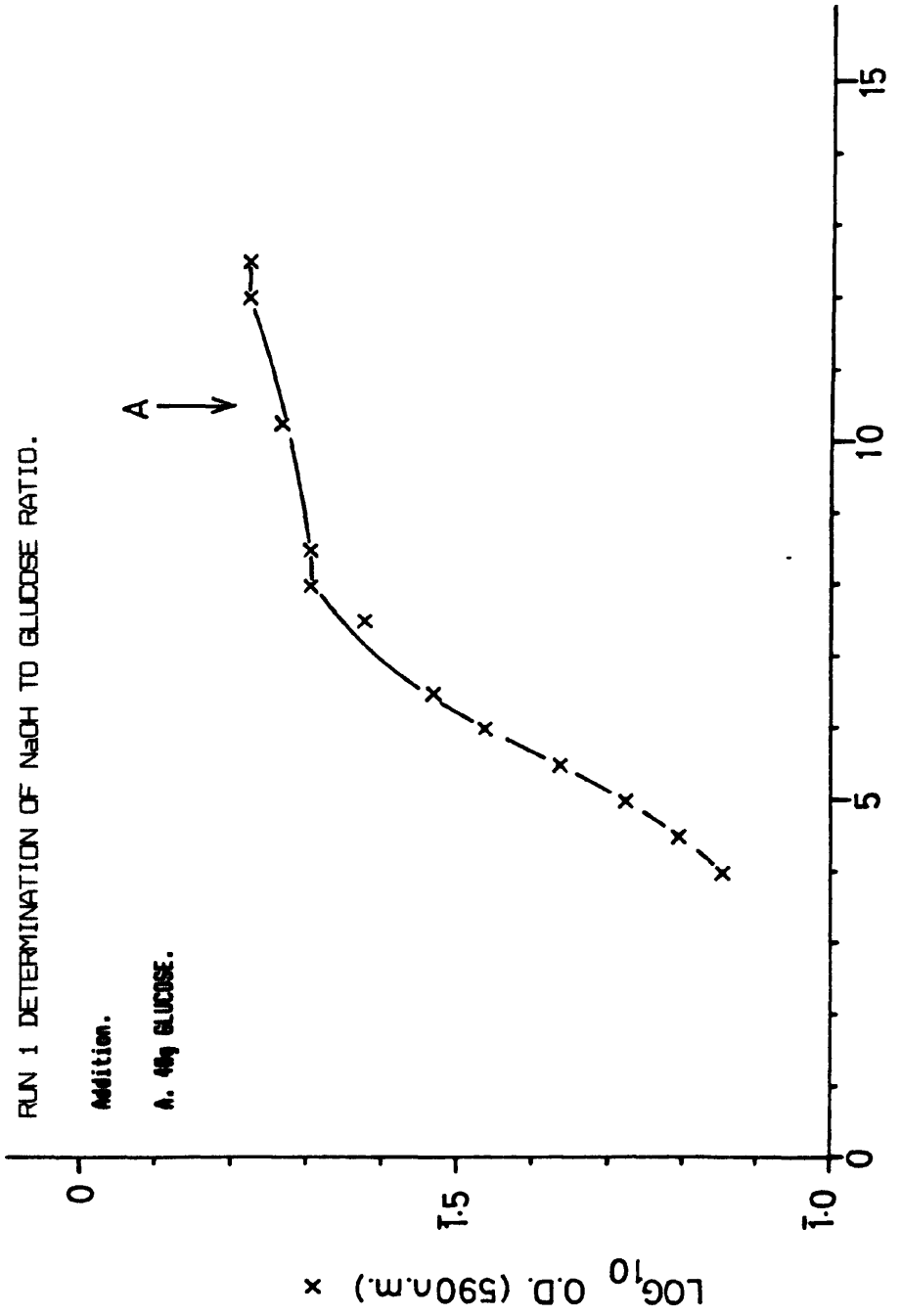


Figure 6.10 Run 1; Log O.D. vs Time.

Appendix 1.3.2. As the two cool solutions were mixed, a degree of caramelisation occurred. This yellowing of the glucose/NaOH solution increased with time.

The results of this experiment are plotted in Figures 6.11 and 6.12. Examination of Figure 6.11 (Appendix 1.4.2.1) illustrates the decreasing glucose concentration indicating that the NaOH/glucose ratio of 1.82 was too high. At 5 h, when the glucose concentration was 5.6 $g.l.^{-1}$, the rate of bacterial growth and NaOH requirement decreased markedly. At 6.75 h the glucose concentration was 4.7 $g.l.^{-1}$, the rate of bacterial growth and NaOH requirement were low. 80 g of Yeatex in 400 ml of water was added at 6.75 h (Point A, Figure 6.11). The slightly acidic nature of this addition triggered the pH system, adding 1.95 g glucose with the NaOH. The glucose concentration increased from 4.7 $g.l.^{-1}$ to 6.1 $g.l.^{-1}$ but no marked increase in the rate of bacterial growth, glucose consumption or NaOH requirement was observed.

At 11 h, 24 g of glucose was added to the broth increasing the glucose concentration from 5.4 $g.l.^{-1}$ to 11.8 $g.l.^{-1}$. The rate of bacterial growth, glucose consumption and NaOH requirement increased markedly. No growth rates were calculated due to a lack of suitable data (Figure 6.12, Appendix 1.4.2.2).

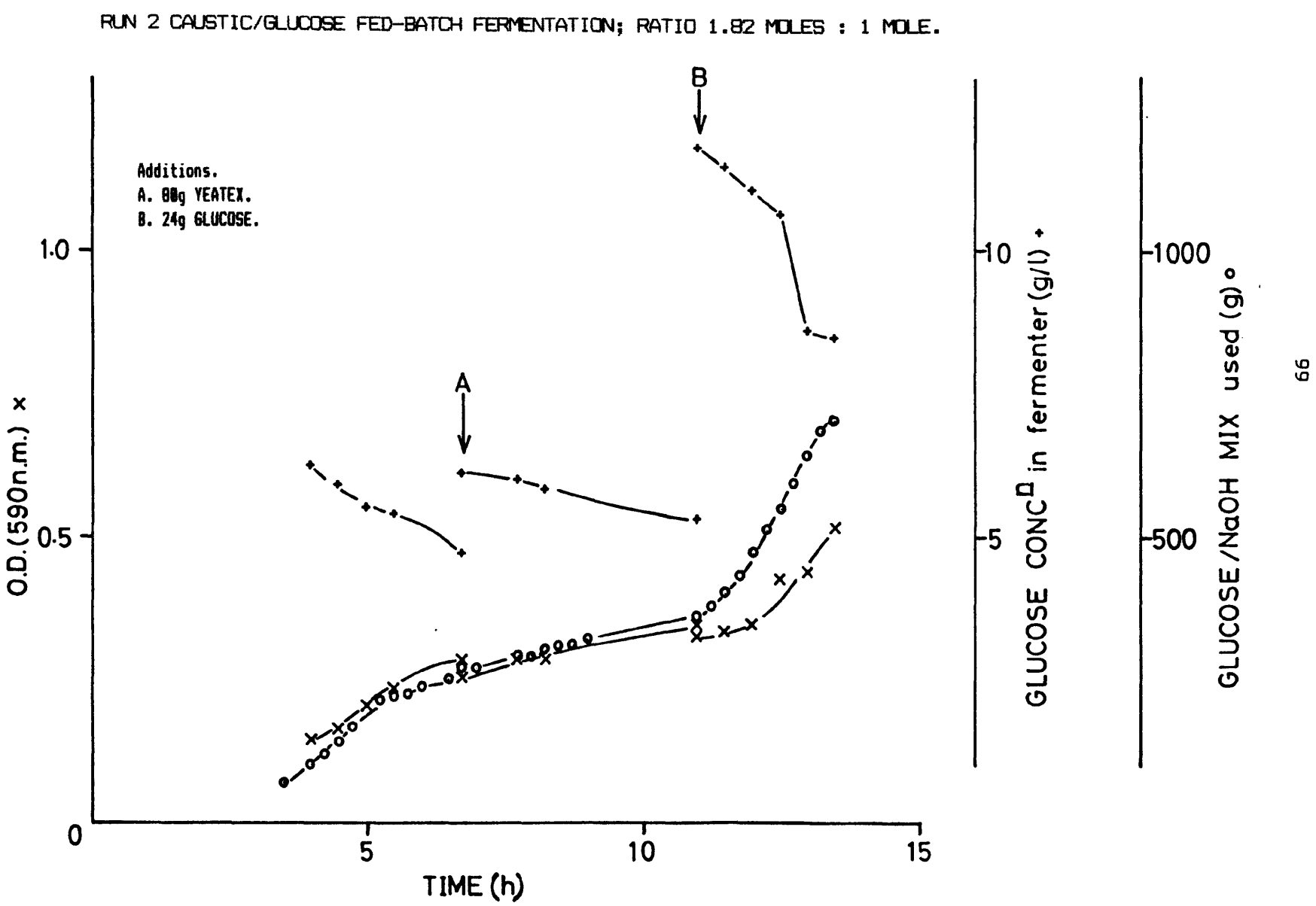


Figure 6.11 Run 2; O.D.,Glucose Conc^{n} and Glucose/NaOH Used vs Time.

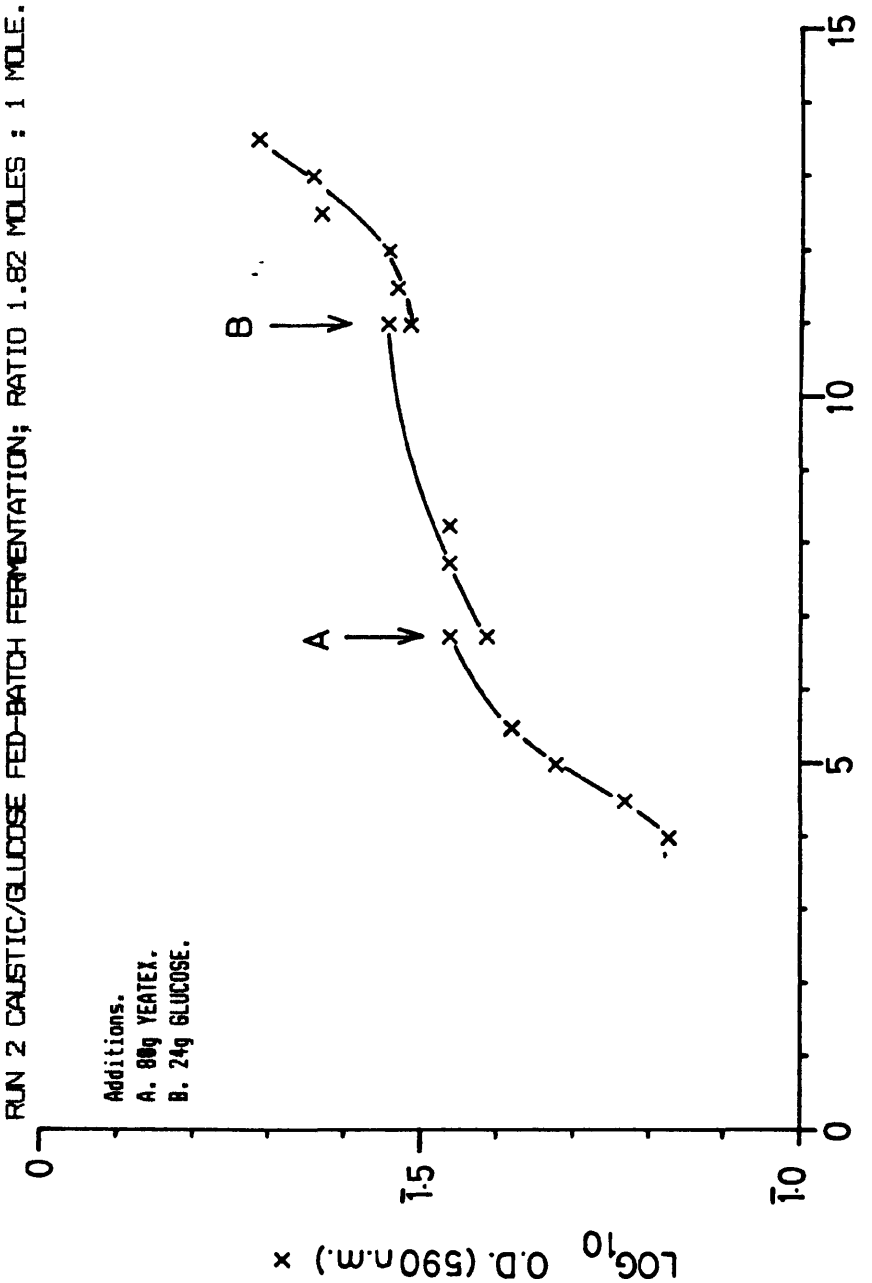


Figure 6.12 Run 2; Log O.D. vs Time.

The limiting substrate at 6.75 h appears to have been glucose since the addition of 24 g of glucose at 11 h resulted in increased bacterial growth, glucose consumption and NaOH requirement. The addition of glucose to Run 2 at 11 h resulted in a marked increase in bacterial growth (Figure 6.11) in contrast to Run 1 (Figure 6.10) in which the addition of 40 g of glucose at 10.5 h resulted in little cell growth. However, the cell concentration in Run 1 (Figure 6.10) had already reached an OD of approximately 0.5 before the addition of the glucose supplement whereas in Run 2 (Figure 6.11) the OD was approximately 0.3 before the addition of the glucose supplement. In Run 1 growth inhibiting end-products, such as organic acids, may have reached a concentration at which they adversely affected the bacterial growth. However, in Run 2 this point may not have been reached at the time of the addition of the additional glucose.

6.3.3 Run 3

The molar ratio of NaOH to glucose was reduced to 1.48 in Run 3 (Appendix 1.3.3). This ratio successfully maintained the glucose concentration between 10 and 17.3 $g.l.^{-1}$ over 12.5 h. The ratio of NaOH/glucose was now too low since the glucose concentration began to increase in a linear fashion from 10 $g.l.^{-1}$ at 4 h to 17.3 $g.l.^{-1}$ at 12.5 h (Figure 6.13 and Appendix 1.4.3.1). The rate of increase of the glucose concentration was calculated to be 0.80 g $glucose.l.^{-1}broth.h.^{-1}$ with a linear correlation coefficient of 0.98.

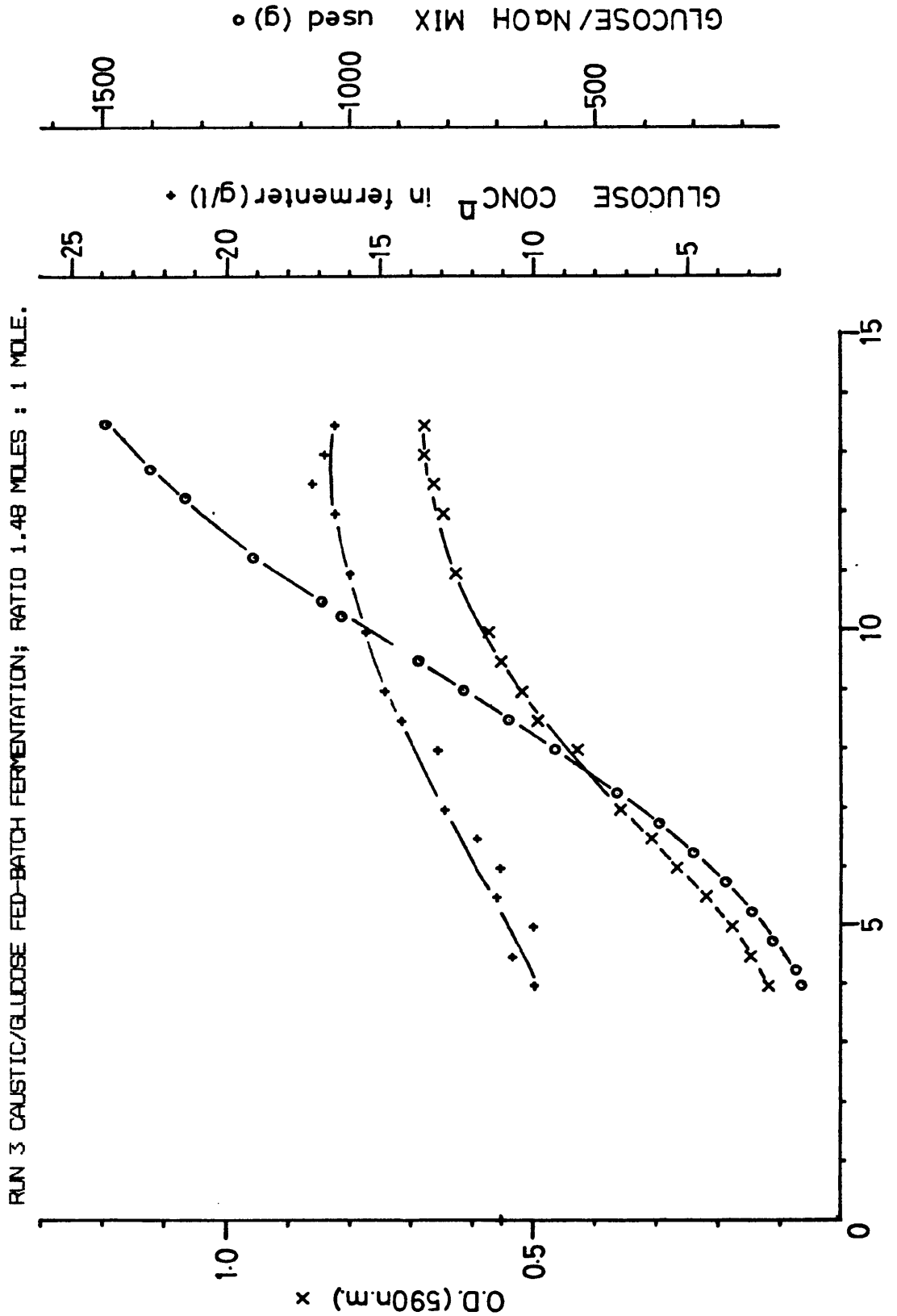


Figure 6.13 Run 3; O.D.,Glucose Conc^{n} and Glucose/NaOH Used vs Time.

A maximum OD of 0.68 was attained at 13 h and a maximum growth rate was calculated from the linear portion in Figure 6.14 to be 0.406 $h.^{-1}$ in close agreement with 0.39 $h.^{-1}$ for Run 1 (Figure 6.14 and Appendix 1.4.3.2). The maximum rate of addition of the glucose/NaOH solution of 185.5 $g.h.^{-1}$ corresponding to 37.1 g glucose $h.^{-1}$ occurred between 7 and 11 hours. The linear correlation coefficient for this constant feedrate was calculated to be 1.0.

6.3.4 Run 4

This fed-batch fermentation was a repeat of Run 3. However, when the cell growth entered the stationary phase, 2 molar ammonium hydroxide was used to control the pH and feed nitrogen (Appendix 1.3.4). 60 g of glucose was also added to the broth.

As in Run 3, the glucose/NaOH solution was successful in feeding glucose to the fermenter. The glucose concentration started to increase in a linear fashion from 15.5 $g.l.^{-1}$ at 5 h to 20 $g.l.^{-1}$ at 10.75 h (Figure 6.15 and Appendix 1.4.4.1). The rate of increase of glucose concentration was calculated to be 0.73 g.glucose l^{-1} broth.$h.^{-1}$ with a linear correlation coefficient of 0.91.

The maximum specific growth rate was calculated to be 0.33 $h.^{-1}$ (Figure 6.16 and Appendix 1.4.4.2). The maximum rate of addition of glucose/NaOH of 165.7 $g.h.^{-1}$

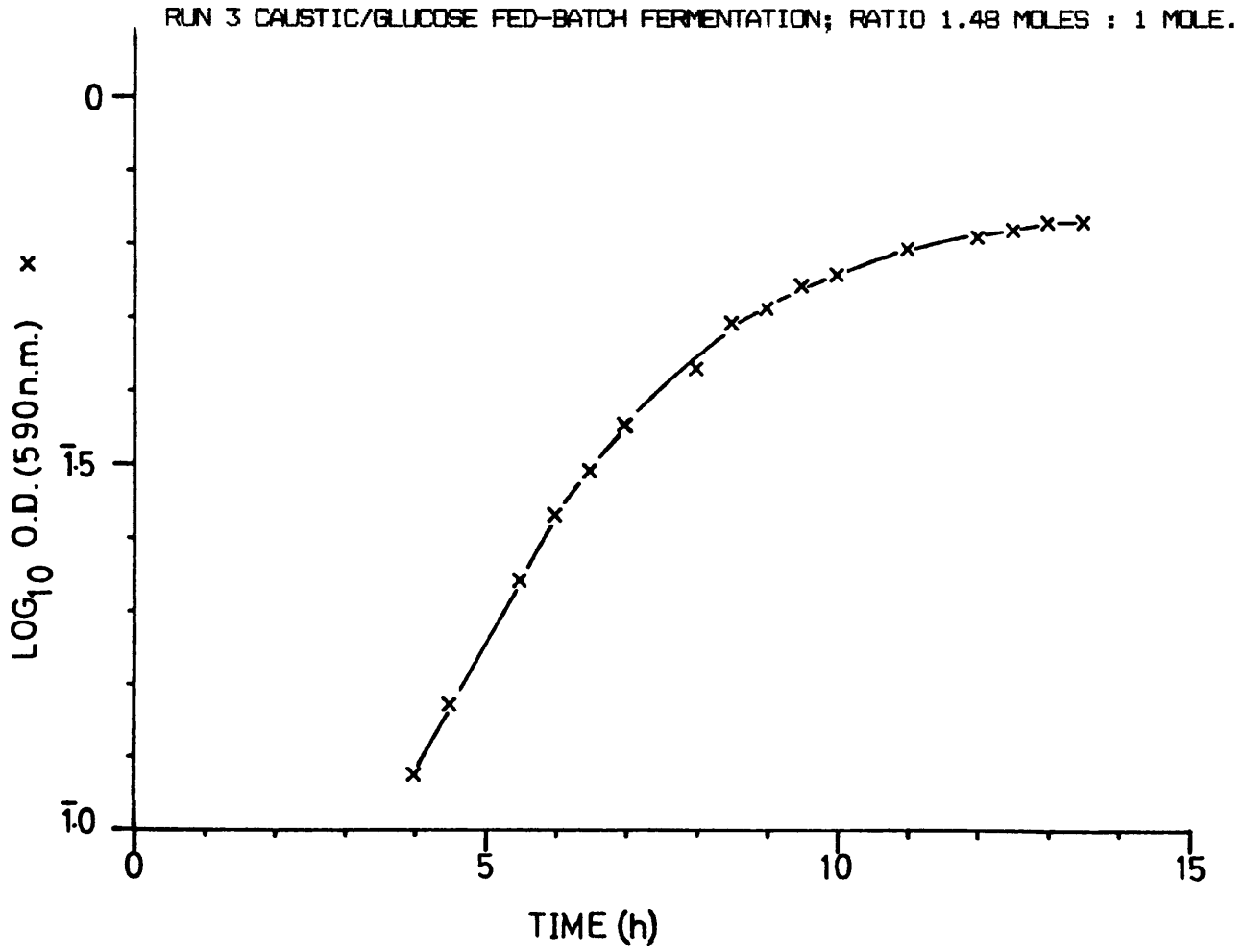


Figure 6.14 Run 3; Log O.D. vs Time.

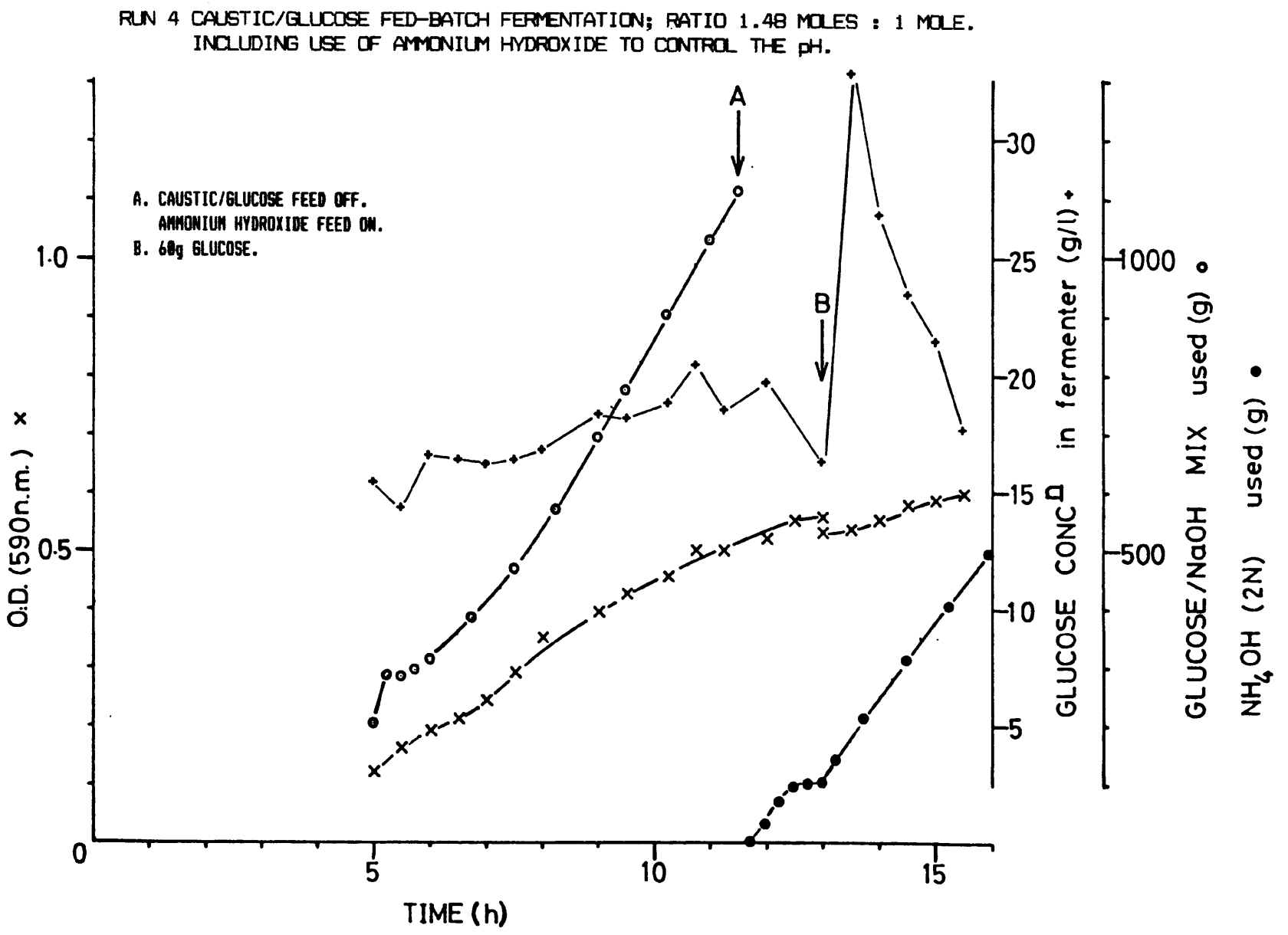


Figure 6.15 Run 4; O.D., Glucose Conc^{n}, Glucose/NaOH Used and Ammonium Hydroxide Used vs Time.

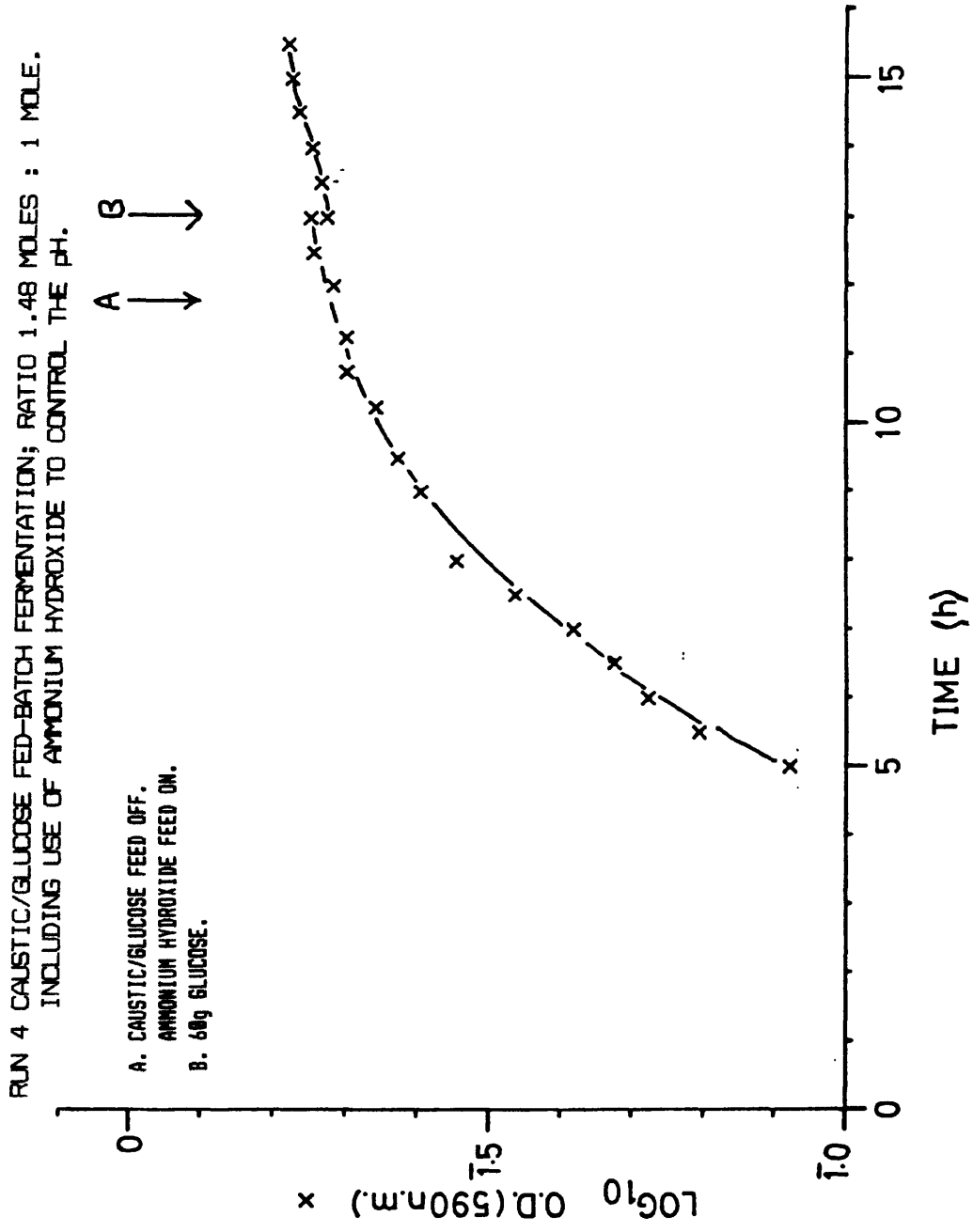


Figure 6.16 Run 4; Log O.D. vs Time.

corresponding to 33.1 g glucose.h.$^{-1}$ occurred between 8 and 11.75 hours. The linear correlation coefficient for this maximum constant feedrate was calculated to be 1.0.

At 11.75 h the glucose/NaOH feed solution was replaced with 2 molar ammonium hydroxide solution to control the pH. Consumption of ammonium hydroxide halted after 12.5 h with a corresponding halt in cell growth. The glucose concentration at 13 h was high at 16.3 g.l.$^{-1}$.

On the addition of 60 g of glucose at 13 h, the glucose concentration increased to 32.9 g.l.$^{-1}$. There was an almost immediate demand for 2 molar ammonium hydroxide and cell growth was observed to occur (Figure 6.15). The feedrate of 2 molar ammonium hydroxide from 13-16 h was constant at 129.3 g.h.$^{-1}$ with a linear correlation coefficient of 1.0. The optical density increased from 0.54 to 0.60 over the same period.

Although growth was entering the stationary phase, 2 molar ammonium hydroxide was being consumed at the constant rate of 129.3 g solution.h.$^{-1}$ thus suggesting a constant rate of production of organic acids.

6.3.5 Run 5

Run 5 was a repeat of Run 3 and the initial stages of Run 4. The glucose/NaOH solution ratio was 1.48 moles of NaOH to 1 mole of glucose (Appendix 1.4.5).

The glucose concentration started to increase in a linear fashion from 13 $g.l.^{-1}$ at 4 h to 17.5 $g.l.^{-1}$ at 10.25 h (Figure 6.17 and Appendix 1.4.5.1). The rate of increase of glucose concentration was calculated to be 0.74 g glucose $l.^{-1}$ broth $h.^{-1}$ with a linear correlation coefficient of 0.98. This again confirmed that the ratio of NaOH to glucose was on the low side of the stoichiometric ratio. The maximum $\overset{\text{specific}}{\wedge}$ growth rate was calculated to be 0.440 $h.^{-1}$ (Figure 6.18 and Appendix 1.4.5.2). The optical density was 0.55 at 10.25 h and still rising, but was clearly entering the stationary phase (Figures 6.17 and 6.18). The maximum rate of addition of glucose/NaOH solution of 193.5 $g.h.^{-1}$ corresponding to 38.6 g glucose.$h.^{-1}$ occurred between 6 and 10.25 hours. The linear correlation coefficient for the maximum constant feedrate was calculated to be 1.00.

6.3.6 Run 6

The initial stage of Run 6 was identical to Runs 3, 4 and 5 (Appendix 1.4.6).

The rate of consumption of glucose/NaOH was poor, although bacterial cell growth measured as optical density appeared to be reasonable (Figure 6.19). The maximum specific growth rate of 0.38 $h.^{-1}$ occurred between 2.5 h and 5 h, much earlier than in Runs 1-5 (Figure 6.20 and Appendix 1.4.6.2). A constant feedrate of 25.56 g solution.$h.^{-1}$ corresponding to 5.11 glucose.$h.^{-1}$ was

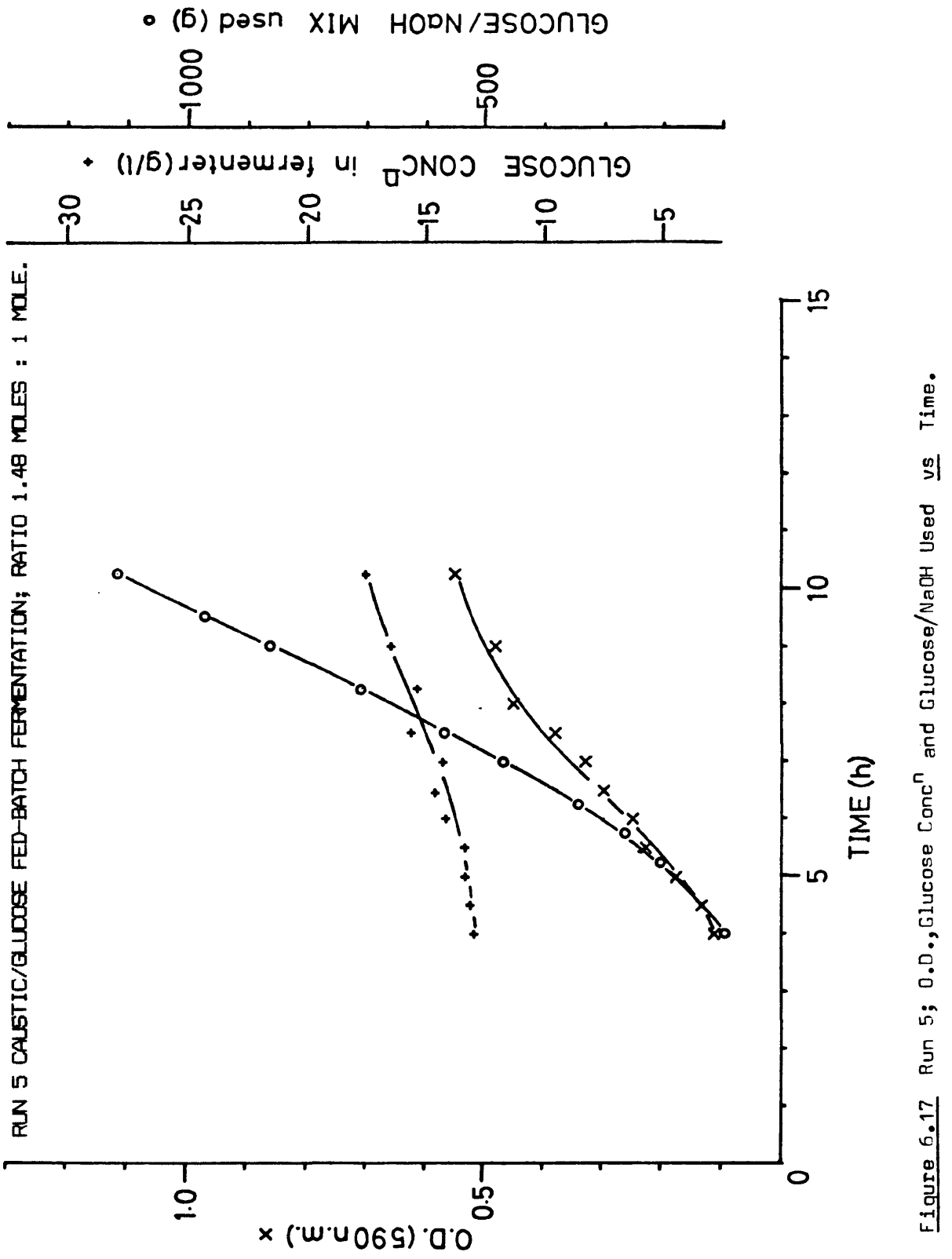


Figure 6.17 Run 5; O.D., Glucose Conc^{n} and Glucose/NaOH Used vs Time.

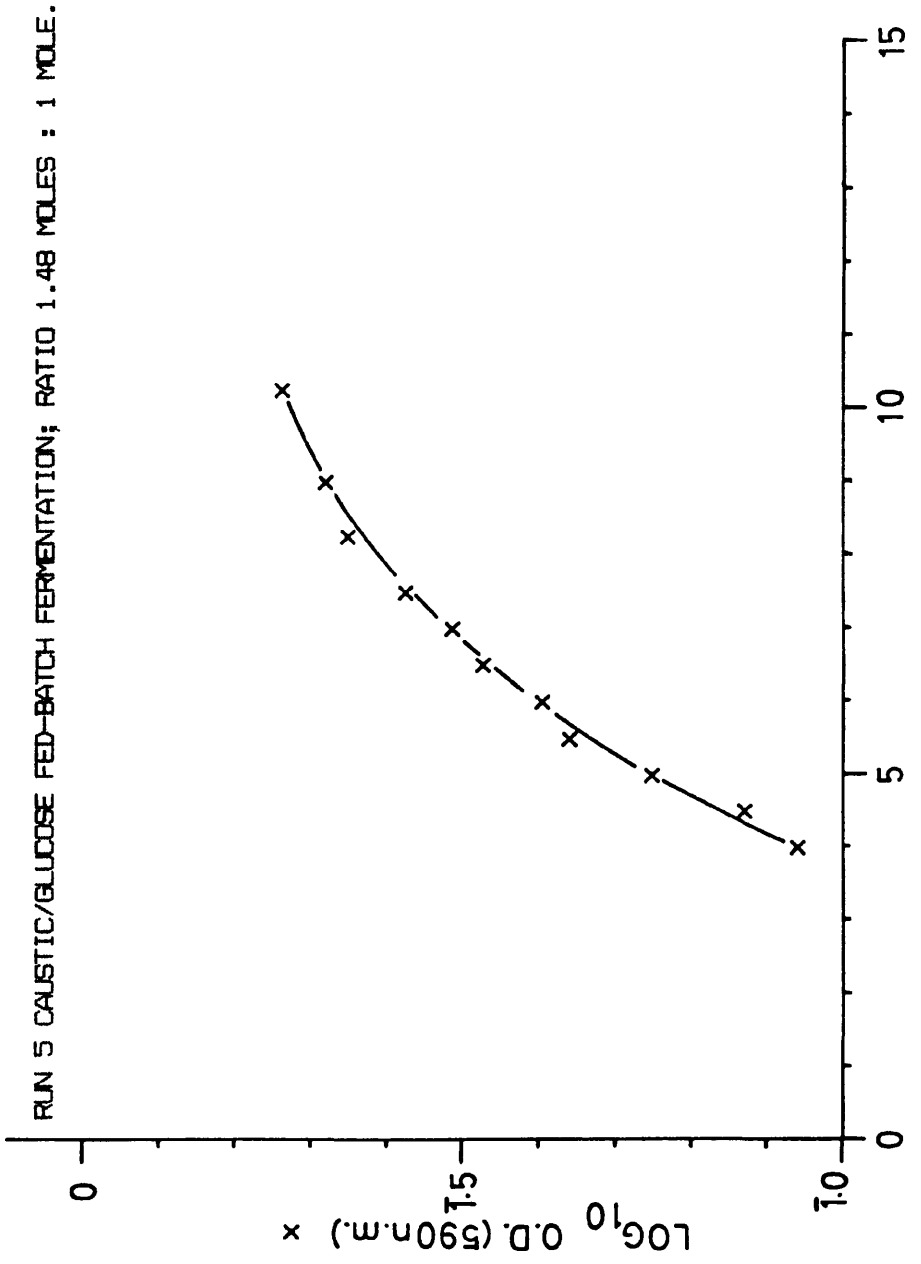


Figure 6.18 Run 5; Log O.D. vs Time.

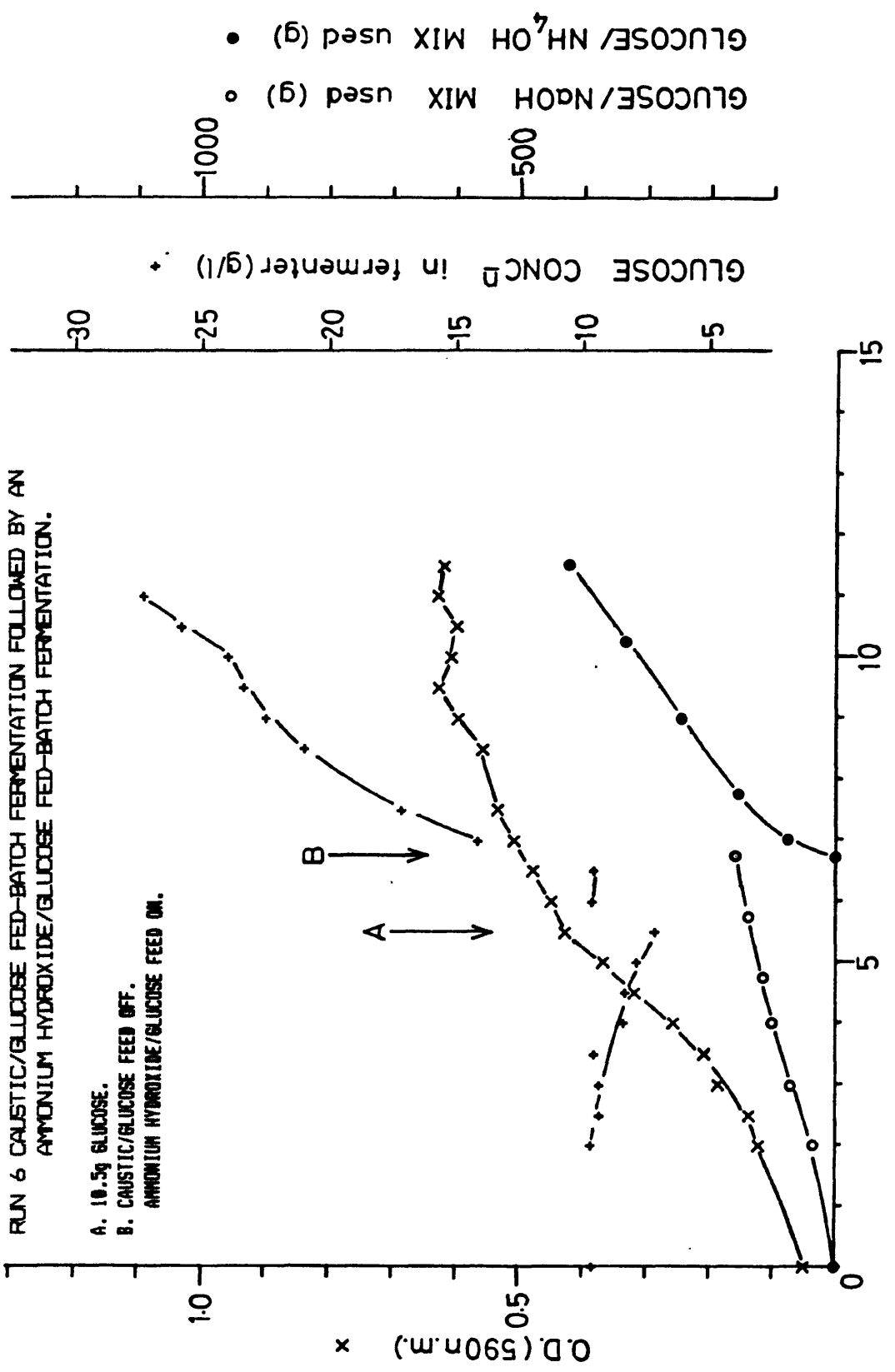


Figure 6.19 Run 6; O.D., Glucose Conc^{n}, Glucose/NaOH Used and Glucose/Ammonium Hydroxide Used vs Time.

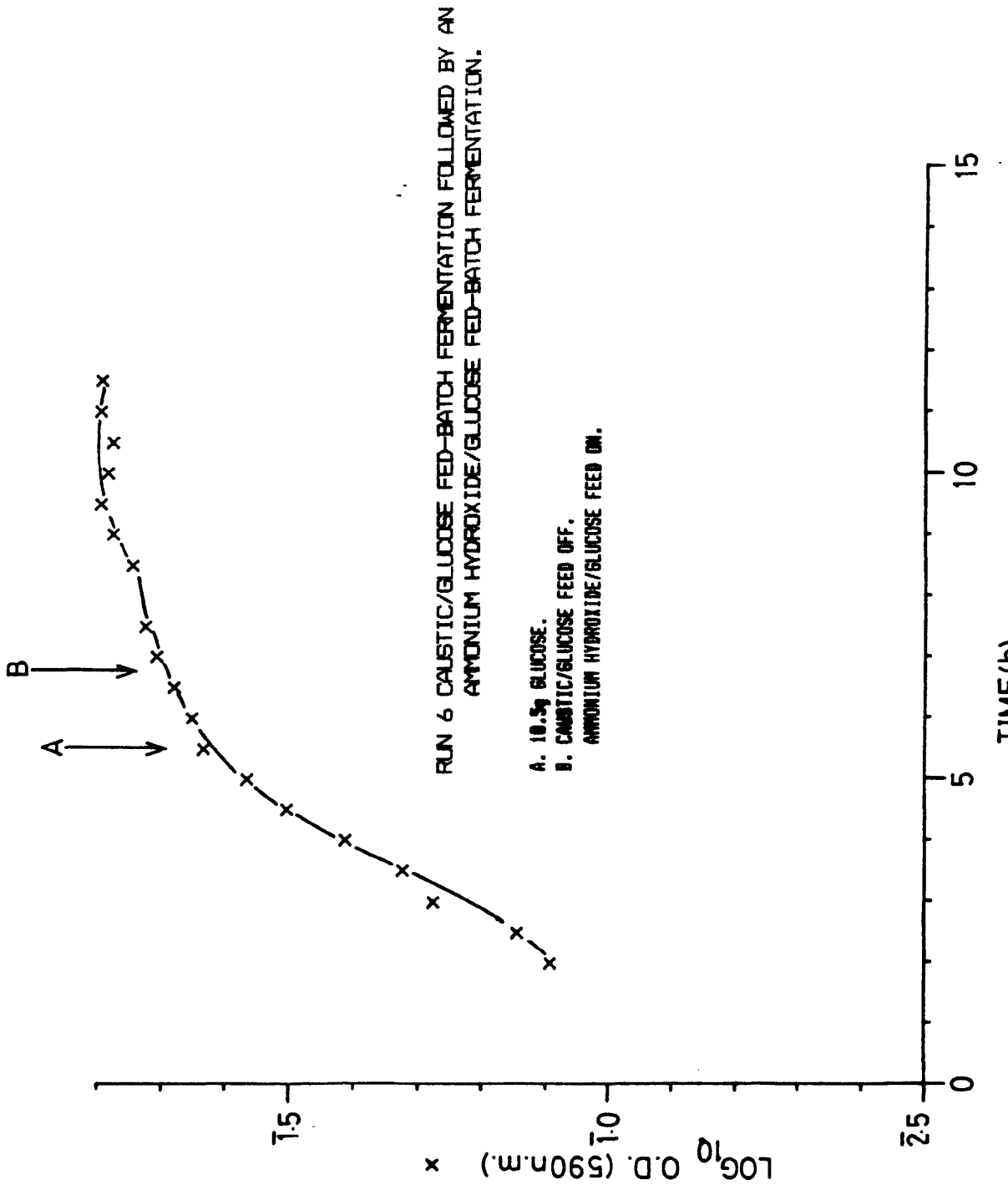


Figure 6.20 Run 6; Log O.D. vs Time.

calculated between 2 and 6.5 h. Unlike Runs 3, 4 and 5, the glucose concentration decreased from 9.7 $g.l.^{-1}$ at 2 h to 7.2 $g.l.^{-1}$ at 5.5 h (Figure 6.19). The addition of 10.5 g of glucose at 5.5 h (Point A, Figure 6.19) did not alter the rates of growth or metabolism.

At 6.75 h the glucose/NaOH solution was exchanged for a glucose/NH_4OH solution with the ratio, 2.25 moles of NH_4OH to 1 mole of glucose (Point B, Figure 6.19). The ratio of NH_4OH to glucose was low and the glucose concentration in the broth increased from 9.6 $g.l.^{-1}$ at 6.5 h to 33.8 $g.l.^{-1}$ at 11.5 h. The consumption of glucose/NH_4OH was constant from 7.5 h to 11.5 h at a rate of 71.53 g solution.$h.^{-1}$ corresponding to 14.9 g glucose.$h.^{-1}$. The excess glucose was accumulating in the fermentation broth.

6.3.7 Summary

A linear relationship exists between the glucose consumed by Leuconostoc mesenteroides and the NaOH required to maintain the pH at 6.7. A ratio of 1.73 moles of NaOH to 1 mole of glucose was calculated from the data in Run 1.

Fermentations using a pH-linked, mixed feed, fed-batch technique demonstrated the feasibility of using this method to control the range of glucose concentration throughout the fermentation. In Run 2 a ratio of 1.82 moles of NaOH to 1 mole of glucose is shown to be high since the initial glucose concentration decreased (Figure

6.11). In Runs 3, 4 and 5 a ratio of 1.48 moles of NaOH to 1 mole of glucose is shown to be low since the initial glucose concentration increased. The rate of increase of glucose concentration for Runs 3-5 was 0.73-0.80 $g.l.^{-1}h.^{-1}$ with linear correlation coefficients better than 0.9 (Table 6.1). The linearity of the increase of the glucose concentration also indicates that it is the NaOH/glucose concentration which is low at 1.48 moles of NaOH to 1 mole of glucose. No attempts were made to optimise the ratio of NaOH to glucose due to the halting of the bacterial metabolism when the glucose concentration dropped below $8.g.l.^{-1}$ (Run 2, Figure 6.11; Run 4, Figure 6.15) on the assumption that it was better to err on the low side of the ratio.

Ammonium hydroxide was used to control the pH in the fermentation (Run 5, Figure 6.17) and a mixture of NH_4OH/ glucose was proposed as a method of feeding nitrogen, carbon and controlling the pH. However, growth problems with Run 6 prevented confirmation of this method.

Bacterial biomass was determined by the optical density of the culture. No optical density versus cell dry weight calibration graph was determined for a glucose substrate culture. Due to the difference of the cell size between bacteria grown on sucrose and on glucose, it is not possible to use the OD versus cell dry weight calibration graph (sucrose substrate culture) to determine the cell

TABLE 6.1

RUN NUMBER	3	4	5
Maximum Specific Growth Rate ($h.^{-1}$)	0.401	0.331	0.440
@ Time (h)	4-6	5-8	4-5.5
Linear Correlation Coefficient (In OD)	1.00	0.99	0.99
Maximum Glucose Feedrate ($g.h.^{-1}$)	37.1	33.1	38.6
@ Time (h)	7-11	8-11.75	6-10.25
Linear Correlation Coefficient	1.00	1.00	1.00
Rate of Glucose Concentration Increase ($g.l.^{-1}$broth$.h.^{-1}$)	0.80	0.73	0.74
Linear Correlation Coefficient	0.98	0.91	0.98

dry weight of a glucose-substrate culture (Brooker, 1979). Maximum ODs at 1:25 dilution and 590 nm varied between 0.60 and 0.68 when no problems were encountered during the fermentation

A maximum specific growth rate of 0.39 $h.^{-1}$ was determined from Run 1, the batch fermentation. Maximum specific growth rates of 0.33-0.44 $h.^{-1}$ were determined from the fed-batch fermentations.

In Runs 3, 4 and 5 the feed system operated with no problems. No exponential phase is apparent on examination of the NaOH/glucose feed profiles for Runs 3, 4, and 5. This is unusual since the feed profile should mimic that of an exponential bacterial growth profile. The bacterial growth profiles do not show any clear exponential phase but this is thought to be due to a short exponential phase and a lack of data points. The NaOH/glucose feed profiles have a distinct linear period during which the glucose feedrate is at a maximum for the respective fermentation (Figures 6.13, 6.15 and 6.17). Table 6.1 lists the calculated maximum specific growth rates and maximum glucose feedrates over their respective time periods. It is clear that the maximum glucose feedrate occurs immediately after the period determined as the exponential phase of growth. The linear correlation coefficients for each set of data used to calculate both the growth rate and glucose

feedrate are excellent at 0.99-1.00. Fed-batch fermentations, Runs 3-5, were identical in their set-up and materials. However, there is a noticeable difference between the maximum specific growth rate and glucose feedrate for each fermentation (Table 6.1). There is agreement between the maximum specific growth rate and maximum glucose feedrate for each fermentation, in that a higher specific growth rate has a higher glucose feedrate. These results suggest that each fermentation has a very short exponential phase during which a substrate, other than carbon, becomes limiting. This limitation forces the bacterial culture to grow and metabolise linearly rather than exponentially and this is clearly shown in the carbon/alkali and alkali feeds.

All the fermentations were assayed for the presence of dextransucrase. No dextransucrase activity was detected in any of the glucose fermentations. This is in agreement with previous work (Neely and Nott, 1962) which indicates that sucrose is an inducer required for the production of dextransucrase. However, it must be noted that none of these fermentations were glucose-limited for any period.

In a number of the fermentations (Runs 1, 2 and 4), the cell growth and rate of metabolism decreased dramatically at glucose concentrations less than 8 $g.l.^{-1}$ (Figures 6.10 and 6.11). In the case of Run 4 at 16 $g.l.^{-1}$

(Figure 6.15), however, this occurred towards the end of the fermentation (13 h) and may have been influenced by other factors. The reason(s) for this is not known since the saturation constant, K_s for glucose is generally about 10^{-5}M (Pirt, 1975). Shehata and Marr (1971) quote glucose K_s values of 0.18 g.l.$^{-1}$ and 0.068 mg.l.$^{-1}$ for E. coli. The Reducing Sugar Method is not specific for any one sugar and will give a positive result for any free aldehyde or an alpha hydroxy ketone (Allinger, et al, 1976). Therefore, it is possible to have a background reducing sugar concentration not due to glucose. However, the initial glucose concentration of each fermentation agreed well with each expected glucose concentration. No high background reducing sugar concentrations were detected in similar sucrose (non-reducing) fed-batch fermentations (McAvoy, 1981). This suggests that a background reducing sugar value cannot be attributed to the other medium components such as Yeatex or phosphate. The glucose concentration at which cell growth and metabolism halted was constant throughout the fermentation suggesting that a background reducing sugar value was not due to any end-product since this would be increasing in concentration. It, therefore, appears that Leuconostoc mesenteroides B512-F has a high K_s value for glucose when grown under the specified cultural conditions. However, an analysis of the broth using a more specific analytical technique such as high pressure liquid chromatography would resolve this problem.

Bacterial growth and metabolism were very poor in Run 6. The addition of glucose did not alleviate this problem. The main difference between this Run and Runs 1-5 was the low Yeatex concentration, 10 $g.l.^{-1}$ in contrast to 40 and 50 $g.l.^{-1}$. The rates of glucose/NaOH and NH_4OH/glucose feeds were low and linear suggesting a nutrient limitation. The results of this fermentation suggest that Leuconostoc mesenteroides requires a high concentration of yeast extract to maintain a good rate of growth and metabolism. The feed of NH_4OH/glucose did not alleviate the limitation, indicating that the limiting substrate was not glucose or inorganic nitrogen. Also, these results indicate that this fermentation is not suitable to test the feasibility of an NH_4OH/glucose feed system since the bacteria require a high yeast extract concentration for good growth and metabolism.

6.4 CONTINUOUS CULTURE

A number of continuous culture fermentations were undertaken to examine the effect of the sucrose concentration on dextransucrase production and to derive some kinetic data. A continuous culture base medium was selected which was essentially the same as the fed-batch medium with the yeast extract concentration halved to 20 $g.l.^{-1}$. Details of the continuous culture media can be found in Appendices 2.1 and 3.1. Details of the batch culture medium and the method used to initiate the continuous culture can be found in Appendix 1.1.

Maximum specific growth rates of approximately 0.26-0.44 $h.^{-1}$ were determined from the glucose fed-batch fermentations (Section 6.3.7) and previous sucrose fed-batch fermentations (McAvoy, 1981). An approximate dilution rate of 0.2 $h.^{-1}$ was thus chosen for these continuous culture experiments although an actual dilution rate was determined and recorded for each steady-state. A steady-state was assumed to exist after six residence times. A residence time being defined as 1/dilution rate ($h.^{-1}$). Continuous culture fermentation conditions can be found in Table 5.2; Runs 7-15, and Table 5.3; Runs 16-32.

The continuous culture results are reported as two distinct sections Runs 7-15 (Tables 6.2 and 6.3) and Runs 16-32 (Tables 6.4 and 6.5). As will be discussed in the next two sections, Runs 7-15 behaved in relatively

predictable ways whereas Runs 16-32 were erratic.

6.4.1 Continuous Culture Runs 7-15

As the inlet feed sucrose concentration was increased the dextransucrase activity increased. These results clearly demonstrate the importance of the sucrose feedrate on dextransucrase production. The bacterial biomass concentration, measured as optical density, was in the range 0.22-0.31 (Table 6.2 and Figure 6.21). The enzyme yield on sucrose was calculated and found to be at a maximum value at a sucrose feed concentration of 30 $g.l.^{-1}$.

The primary end-products of the bacterial metabolism, that is, ethanol, lactic and acetic acid, were all approximately constant for Runs 7-15. Run 7 was dissolved oxygen limited, and therefore, the concentration of acetic acid was depressed and ethanol elevated. For Runs 8-15 the lactic acid concentration was in the range 3.8-4.6 $g.l.^{-1}$ and the acetic acid concentration in the range 1.2-1.7 $g.l.^{-1}$ (Table 6.3). Ethanol concentrations of 0-1.0 $g.l.^{-1}$ were detected but these results must be viewed with care considering the volatile nature of ethanol and the high aeration rate of the fermentation. No sucrose was detected in the effluent broth from any of these fermentations. The fructose concentration appeared to be increasing slowly as the sucrose feed concentration was increased and a marked increase was obvious when the sucrose concentration in the feed was 50 $g.l.^{-1}$. The

TABLE 6.2 THE RESULTS OF CONTINUOUS CULTURE RUNS 7-15

FERMENTATION RUN NO	FEED SUCROSE CONCENTRATION ($g.l.^{-1}$)	SUBSTRATE FLOW ($l.h.^{-1}$)	DILUTION RATE ($h.^{-1}$)	OD (590 nm) 1:25 dilution	CELL DRY WEIGHT ($g.l.^{-1}$)	ENZYME ACTIVITY ($DSU.ml^{-1}$)	ENZYME YIELD ON SUCROSE (DSU/g sucrose)
7	20	0.522	0.174	0.23	1.65	11.07	554
8	20	0.582	0.194	0.26	1.80	6.41	321
9	25	0.525	0.175	0.24	1.75	6.41	256
10	30	0.540	0.180	0.31	2.25	4.81	160
11	30	0.603	0.201	0.27	1.95	46.16	1,539
12	35	0.612	0.204	0.28	2.05	50.11	1,432
13	40	0.576	0.192	0.26	1.90	56.55	1,414
14	45	0.471	0.157	0.22	1.60	66.05	1,479
15	50	0.535	0.179	0.24	1.75	68.33	1,367

TABLE 6.3 THE HPLC ANALYSIS OF THE CONTINUOUS CULTURE EFFLUENT BROTH, RUNS 7-15

FERMENTATION RUN NO	FEED SUCROSE CONCENTRATION ($g.l.^{-1}$)	EFFLUENT BROTH END-PRODUCT CONCENTRATION ($g.l.^{-1}$)					
		SUCROSE	FRUCTOSE	MANNITOL	LACTIC ACID	ACETIC ACID	ETHANOL
7	20	0	0.11	0	4.16	0.54	2.89
8	20	0	0.19	0	4.09	1.32	0
9	25	0	0.14	0	4.15	1.32	0
10	30	0	0.29	0.11	4.07	1.38	0.41
11	30	0	0.17	2.91	3.82	1.25	0.94
12	35	0	0.28	10.33	4.30	1.27	1.00
13	40	0	0.26	16.06	4.19	1.15	1.02
14	45	0	0.40	17.94	4.10	1.23	0.91
15	50	0	2.53	21.07	4.58	1.68	0.89

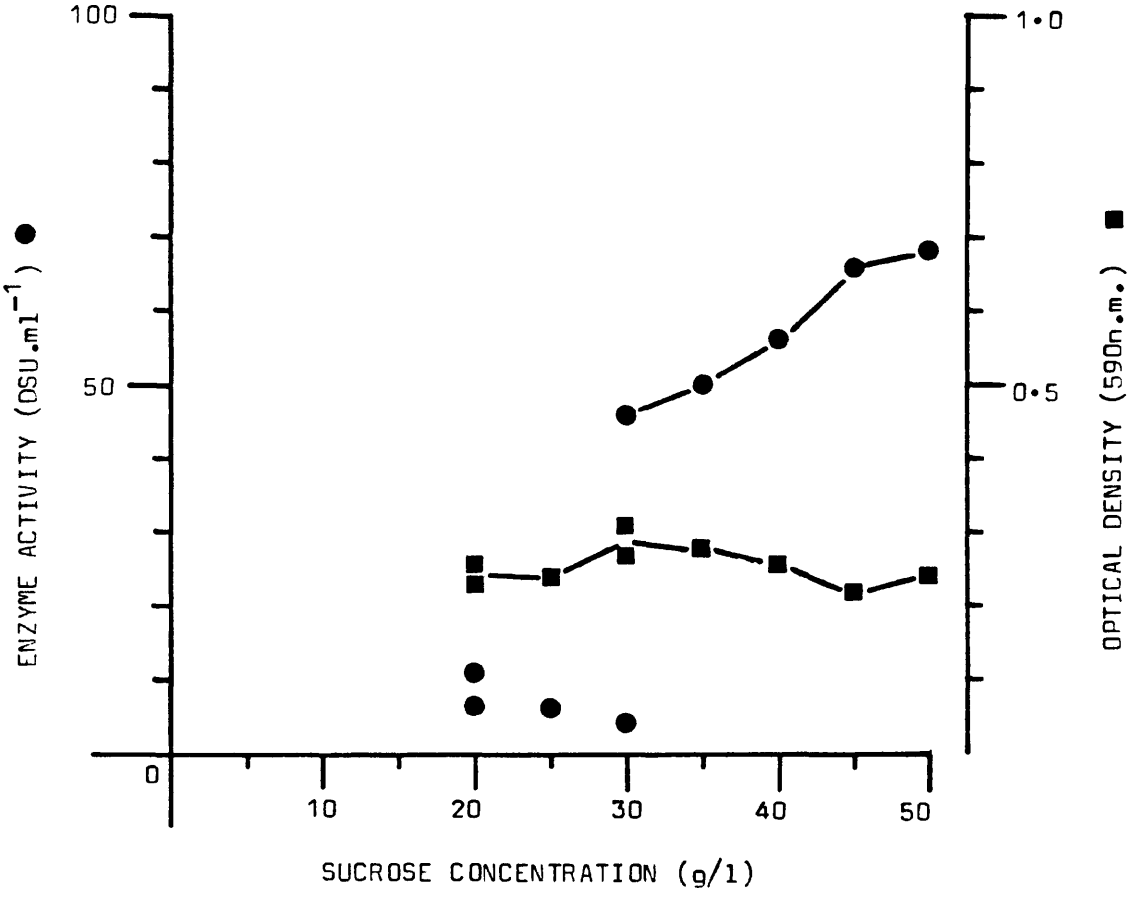


Figure 6.21 Enzyme Activity and O.D.(590 n.m.) vs Sucrose Concentration in Feed.

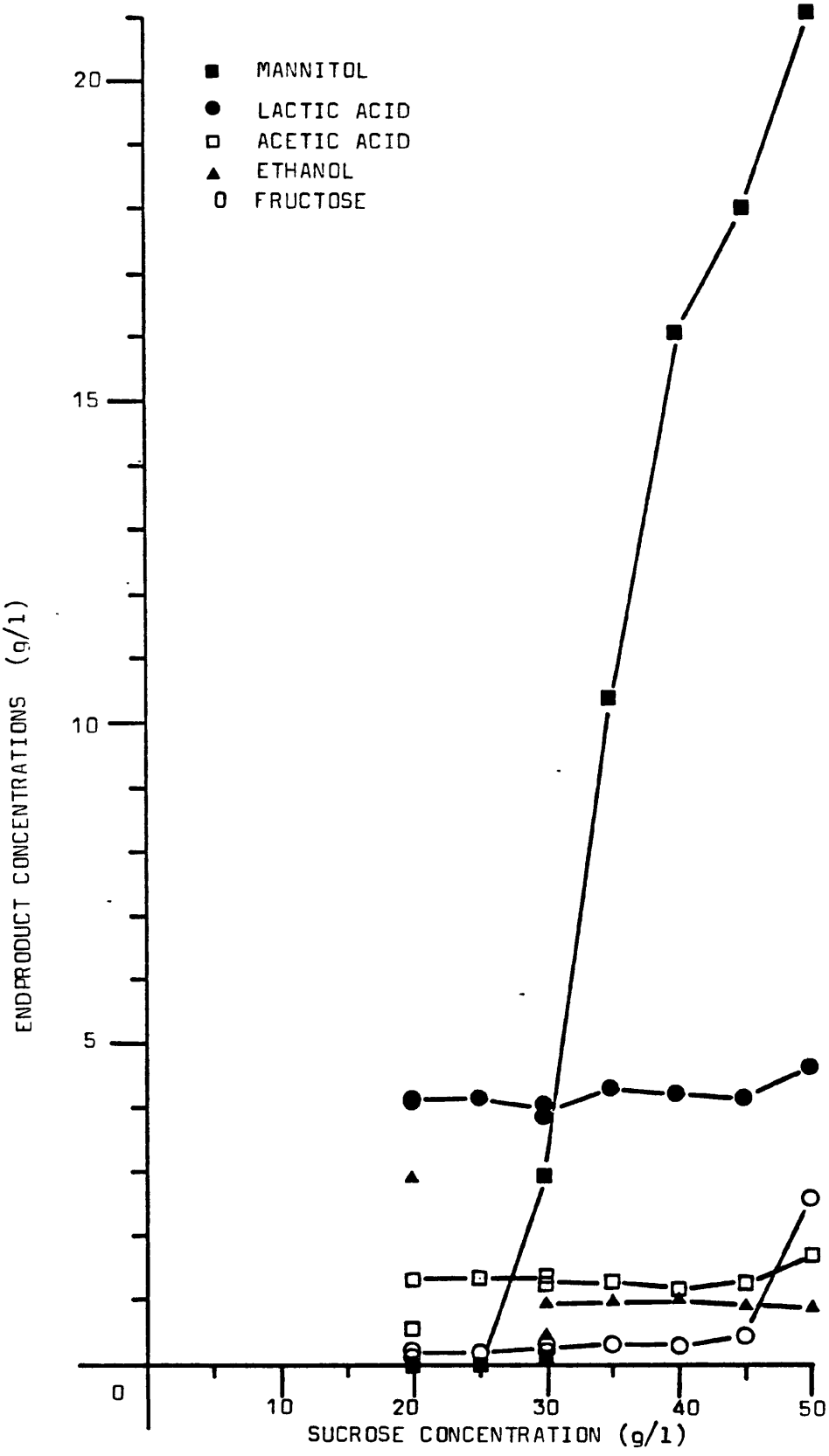


Figure 6.22 Endproduct concentrations vs Sucrose concentration in Feed.

mannitol concentration appeared and began to increase rapidly when the sucrose feed concentration was increased from 30 to 50 $g.l.^{-1}$. Figure 6.22 graphically illustrates the results from Table 6.3

The level of bacterial biomass remained relatively constant for Runs 7-15 whilst the sucrose feed concentration had increased two-fold. The concentrations of lactic and acetic acid also remained relatively constant. These factors indicated that the bacteria were being limited by a substrate other than sucrose.

The lack of consistency of biomass and end-product concentrations from run to run suggests that the concentration of the unknown limiting substrate is variable. This is clear from the results of Runs 10 and 11 which also indicate the importance of the limiting substrate concentration with respect to enzyme production.

Although the cells were obviously limited by an unknown substrate, they did have the ability to convert the excess sucrose in the feed to other metabolites than the primary end-products. Figure 6.22 clearly illustrates that the cells' metabolism was able to convert excess carbon in the form of sucrose to fructose and ultimately into mannitol. Therefore, the determination of a biomass yield on sucrose must not be treated too seriously.

6.4.2 Continuous Culture Runs 16-32

In Runs 7-15 the sucrose feed concentration had only been increased to 50 $g.l.^{-1}$ with corresponding increases in enzyme activity and mannitol concentrations (Figures 6.2 1 and 6.2 2). On attempting to run the fermentation at the higher sucrose concentration of 60 $g.l.^{-1}$ the culture washed out. This fermentation was repeated and a similar result was obtained (Runs 16 and 17, Table 6.4).

Run 18 was a repeat of Run 15. However, the results of Run 18 (Table 6.4 and 6.5) differed from those of Run 15. Run 18 gave a lower OD, lower enzyme activity and the end-product concentrations were markedly different.

Runs 19-28 were direct repeats of Run 13. As can be seen from the results in Tables 6.4 and 6.5 no consistent steady-state could be attained. The raw materials and fermentation conditions were identical for each run and it was suspected that variations in the concentration of the unknown limiting substrate of Runs 7-15 was somehow causing the very erratic results found in Runs 16-32.

The batch cultures, used to initiate the continuous culture Runs 16-32, also exhibited signs of being variable and inconsistent.

It was evident that the organism could be grown in continuous culture and produce enzyme at commercially

TABLE 6.4 THE RESULTS OF CONTINUOUS CULTURE RUNS 16-32

FERMENTATION RUN NO	FEED SUCROSE CONCENTRATION ($g.l.^{-1}$)	DILUTION RATE ($h.^{-1}$)	OD (590 nm) 1:25 dilution	CELL DRY WEIGHT ($g.l.^{-1}$)	ENZYME ACTIVITY ($DSU.ml^{-1}$)
16	60	0.192	W A S H O U T		
17	60	0.186	W A S H O U T		
18	50	0.177	0.19	1.4	8.8
19	40	0.188	W A S H O U T		
20	40	0.195	0.43	3.1	28.9
21	40	0.210	W A S H O U T		
22	40	0.188	0.36	2.6	12.7
23	40	0.198	0.39	2.8	18.5
24	40	0.189	0.43	3.1	19.1
25	40	0.210	0.22	1.6	0
26	40	0.214	0.38	2.75	0
27	40	0.210	0.35	2.6	0
28	40	0.207	0.26	1.85	0
29	80	0.207	0.23	1.65	0
30	40	0.207	0.085	0.625	0
31	60	0.207	W A S H O U T		
32	40	0.213	0.29	2.1	0

TABLE 6.5 THE HPLC ANALYSIS OF THE CONTINUOUS CULTURE EFFLUENT BROTH, RUNS 18-32

FERMENTATION RUN NO	FEED SUCROSE CONCENTRATION ($g.l.^{-1}$)	EFFLUENT BROTH END-PRODUCT CONCENTRATION ($g.l.^{-1}$)						
		SUCROSE	FRUCTOSE	GLUCOSE	MANNITOL	LACTIC ACID	ACETIC ACID	ETHANOL
18	50	0	0	0	12.46	12.70	5.47	1.68
20	40	0	1.75	3.40	14.27	9.26	2.38	1.78
22	40	0	0	0	1.50	9.74	5.11	0
23	40	0	1.13	0	16.26	13.19	7.08	2.35
24	40	0	0.17	0	8.17	17.24	8.16	1.21
25	40	0	11.37	2.16	6.77	10.85	5.85	0
26	40	7.16	0	0	7.38	17.33	9.42	0
27	40	6.47	0	0	7.04	16.92	8.69	0
28	40	0	1.07	0	13.41	14.34	7.85	0
29	80	0	19.38	10.77	12.50	8.51	4.49	0
30	40	0	10.92	8.52	8.01	6.18	3.08	0
32	40	0	0	0	10.84	15.92	8.21	0.44

viable concentrations. In addition, under the unknown nutrient limitation, L. mesenteroides was able to convert fructose into mannitol. Many questions remain to be answered with regard to the continuous culture system. However, due to the inconsistent steady-state found in the continuous culture Runs 16-32 it became necessary to examine in some detail the nutrient requirements of this organism.

6.5 SHAKE FLASK EXPERIMENTS ON NUTRIENT REQUIREMENTS

The results of the continuous culture and glucose fed-batch work had suggested that a variable nutrient limitation problem existed with the type of media used. Shake flask experiments were set up to discover;

1. Whether or not the sterilisation cycle was causing the denaturation of an essential nutrient or the synthesis of some growth inhibitor.

2. Whether or not other nitrogen sources with vitamin supplements could support L. mesenteroides B512-F growth.

6.5.1 Sterilisation Effects

Two types of medium, Yeast Molasses Broth (YMB) and Step III, were prepared (Appendices 4.1, 4.2 and 4.3) and sterilised for varying time periods over the range 0-60 mins. The flasks were inoculated and grown under the conditions specified.

Table 6.6 lists the results of the shake flask sterilisation experiments. No major differences in bacterial growth could be detected from any of the shake flasks. Yeast Molasses Broth medium gave an OD of 0.04 at 12 hours in static or shaken flasks. Step III medium gave ODs in the range 0.26-0.32 at 12 hours in static or shaken flasks. The unsterilised shaken Step III shake flask did appear to be marginally better than the others.

TABLE 6.6 TEST OF AUTOCLAVED SHAKE FLASK MEDIA

Sterilisation Period at 10 psi (minutes)	OD (590 nm) after 12 hours growth			
	STATIC		SHAKEN	
	YMB	STEP III	YMB	STEP III
Unsterilised	0.04	0.26	0.03	0.32
5	0.04	0.28	0.03	0.28
10	0.04	0.29	0.04	0.29
15	0.04	0.29	0.03	0.27
20	0.04	0.29	0.03	0.28
60	0.04	0.28	0.03	0.29

Growth temperature 30 ^{o}C Orbital shaker 180 rpm - shaken cultures only

Medium composition and preparation Appendices 4.1 and 4.2

Experimental method Appendix 4.3

The information gained from such experiments is very limited and must be viewed with care. However, it was concluded that the sterilisation cycle does not markedly effect the growth potential of L. mesenteroides B512-F on these media.

6.5.2 Alternative Nitrogen Sources

The medium used in the continuous culture and fed-batch work consisted of glucose or sucrose as the carbon source, an inorganic phosphate, a trace element supplement and a complex nitrogen source in the form of yeast extract.

It was considered possible that the limiting substrate causing the erratic continuous culture results was a chemical component of the complex nitrogen source. An attempt was, therefore, made to find another more defined and consistent nitrogen source. The alternative nitrogen sources were supplemented with known essential vitamins for the growth of Leuconostoc mesenteroides (Bergey, 1974). The alternative nitrogen sources were chosen from "The Oxoid Manual", 5th edition, 1982. The nitrogen sources were, Casein hydrolysate (Oxoid L41), Peptone (Oxoid L49) and Tryptone T (Oxoid L43). Fisons Ltd (Private communication) reported that they had successfully used Casein hydrolysate as the nitrogen source for the Whole Cell Method of dextran production.

The amino acid content for each of these nitrogen sources (The Oxoid Manual, 1982) was used to calculate an equivalent to the amount of yeast extract (Appendix 4.4).

The results in Table 6.7 show that only the shake flasks using yeast extract as the nitrogen source exhibited any reasonable growth. The ODs of 0.31 and 0.30 were similar to those attained in Table 6.6.

TABLE 6.7 ALTERNATIVE NITROGEN SOURCES PLUS VITAMIN SUPPLEMENTS

Nitrogen Source	OD (590 nm) after 12 hours growth	
+ Vitamins		
Yeast Extract (Bovril Yeatex)	0.31	0.30
Casein Hydrolysate (Oxoid L41)	0.02	0.02
Peptone P (Oxoid L49)	0.02	0.01
Tryptone T (Oxoid L43)	0.02	0.08

Growth temperature 30 °C

Orbital shaker 180 rpm - shaken cultures only

Medium composition and preparation Appendices 4.4 and 4.2

Experimental method Appendix 4.3

This work demonstrated the importance of using yeast extract as a nitrogen source and suggested the possibility that the inconsistencies and erratic fermentation results previously obtained might be due to variations in the quantity of some unknown component in the yeast extract.

6.6 SUCROSE MIXED FEED FED-BATCH FERMENTATIONS

A. Preliminary Experiments

Due to the erratic results of the continuous culture Runs 16-32, a re-examination of the L. mesenteroides B512-F fermentation was undertaken using the sucrose mixed feed fed-batch technique. Similar erratic results were obtained ranging from no growth to those of Runs 33 and 34 which were more successful. Runs 33 and 34 differed only in the nature of their feed; Run 33 was a sucrose/NaOH mix whereas Run 34 was a sucrose/KOH mix. Appendix 5.1 details the medium composition and preparation. Table 5.4 lists the initial fermentation conditions of Runs 33 and 34. The results of Run 33 are detailed in Appendices 5.3.1 - 5.3.4 and Figures 6.23 - 6.25. Figure 6.23 presents the fermentation results on a concentration basis whereas Figure 6.24 presents the same fermentation results on a mass basis thus checking for any misleading effects due to changing volume and allowing realistic comparison between runs.

The maximum OD of 0.56 was attained at 15 h (Figure 6.23) and a maximum specific growth rate for the exponential phase was calculated to be 0.67 $h.^{-1}$ (Figure 6.25). A maximum enzyme activity of 47 $DSU.ml^{-1}$ was achieved at 11 h (Figure 6.23). From 8-14 h the sucrose/NaOH feedrate was 0.089 $l.h.^{-1}$ with a linear correlation coefficient of 1.00. No

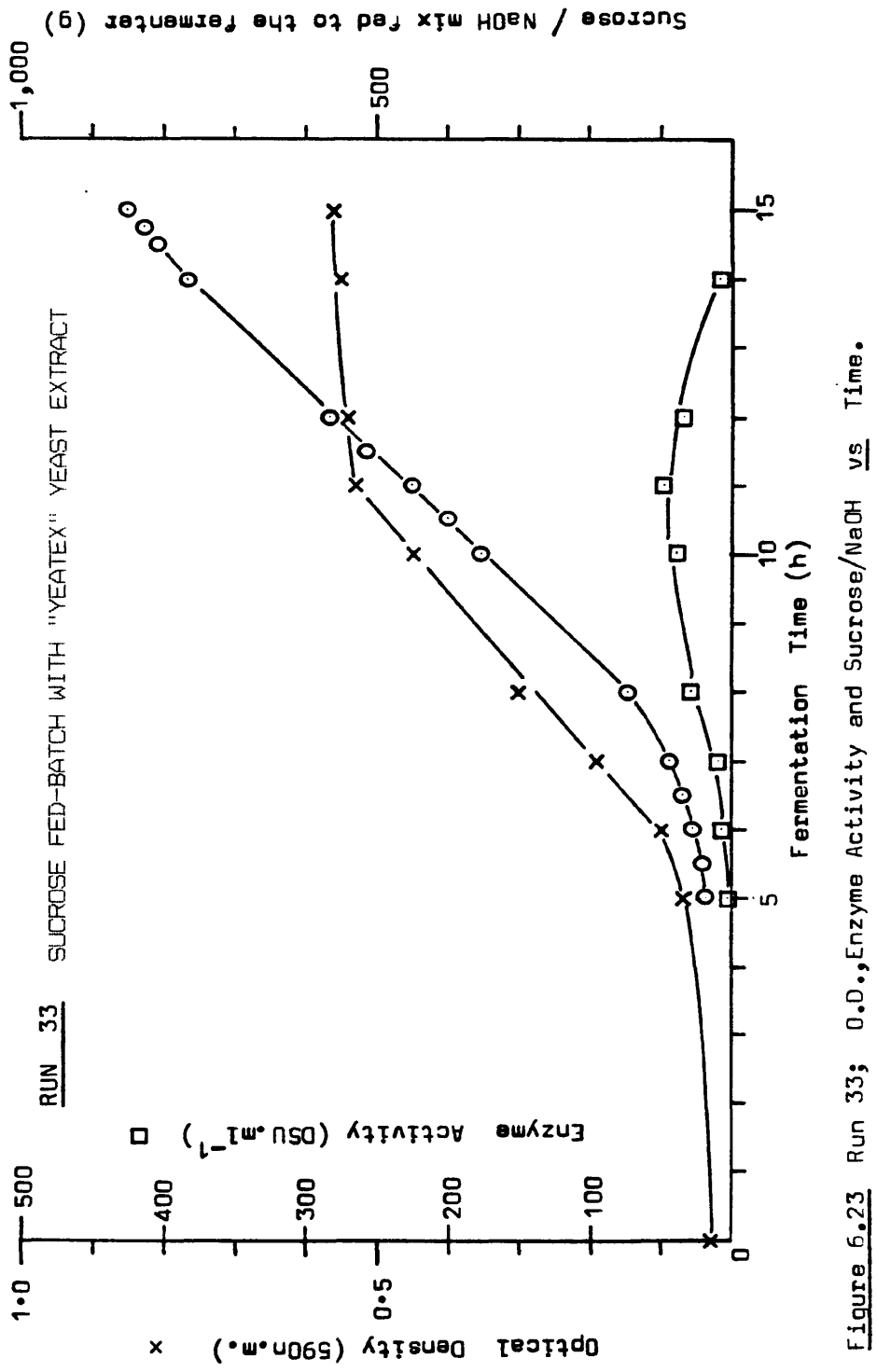


Figure 6.23 Run 33; O.D.,Enzyme Activity and Sucrose/NaOH vs Time.

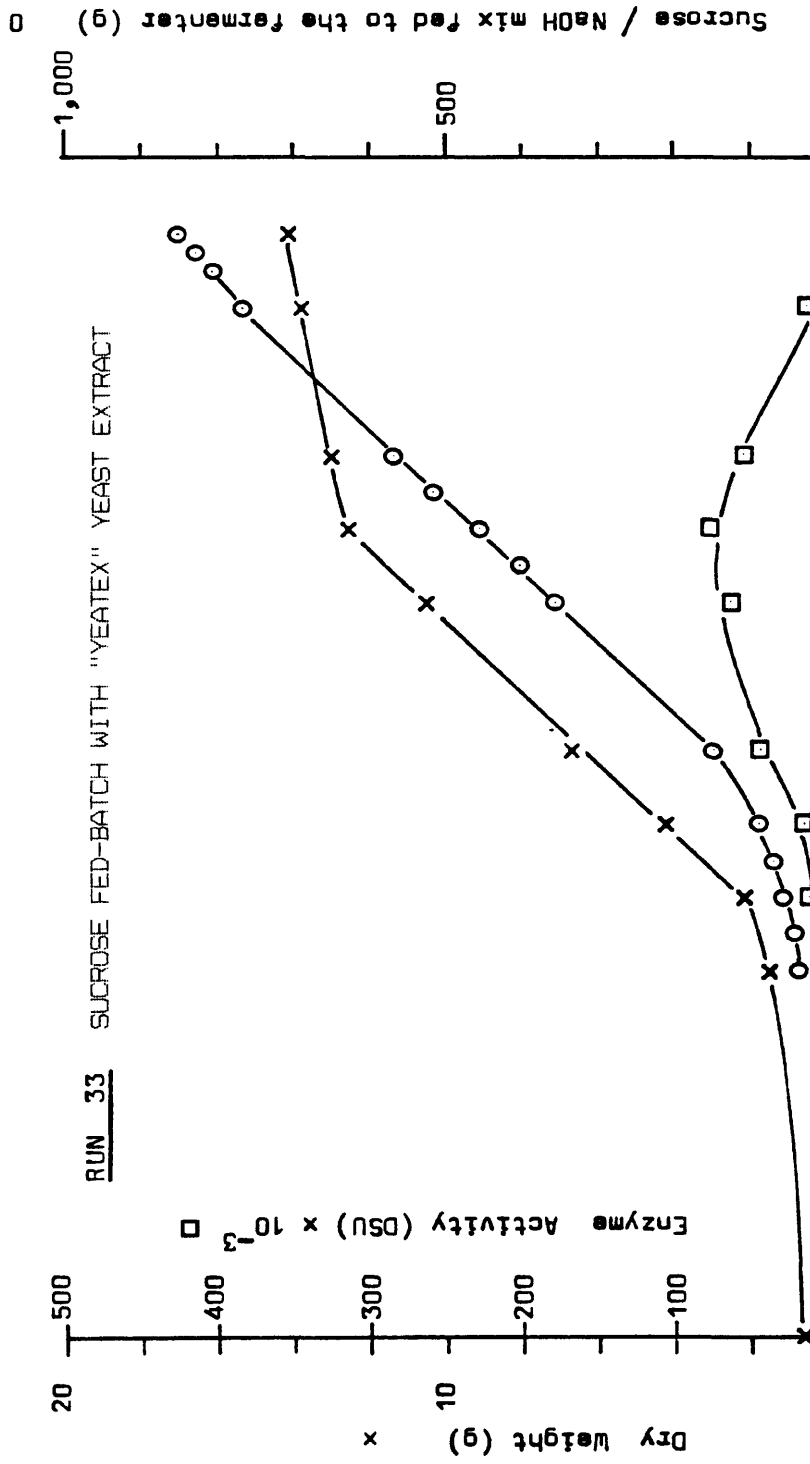


Figure 6.24 Run 33; Dry Weight, Enzyme Activity and Sucrose/NaOH Used vs Time.

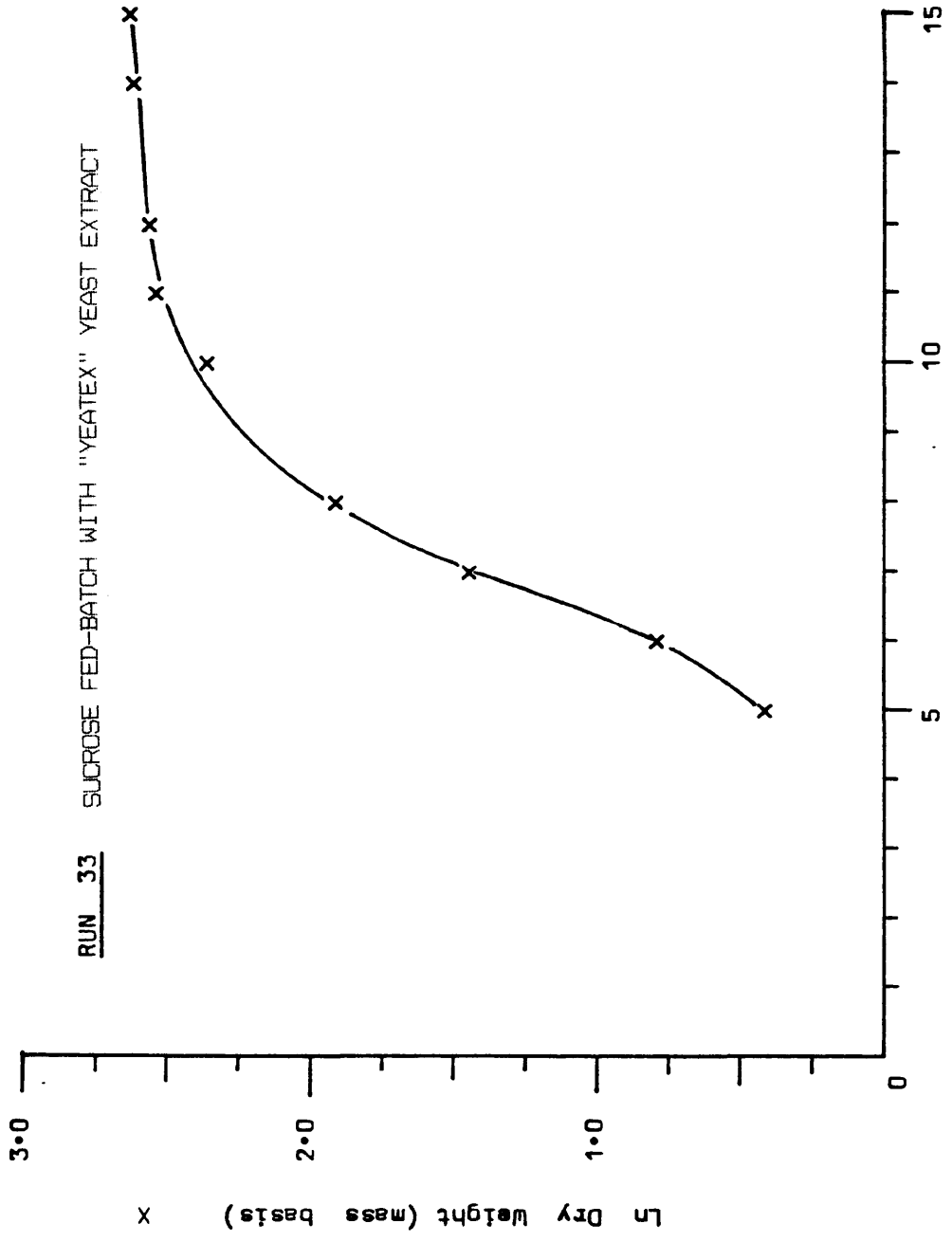


Figure 6.25 Run 33; Ln Dry Weight vs Time.

unusual effects were observed due to volume variation (comparison of Figures 6.23 and 6.24).

The results of Run 34 are detailed in Appendices 5.4.1 and 5.4.4 and Figures 6.26 - 6.28. Figure 6.26 presents the fermentation results on a concentration basis whereas Figure 6.27 presents the same results on a mass basis.

The maximum OD of 0.70 was attained at 13.5 h (Figure 6.26) and a maximum specific growth rate was calculated to be 0.38 $h.^{-1}$ (Figure 6.28). A maximum enzyme activity of 152 $DSU.ml^{-1}$ was achieved at 11.5 h (Figure 6.26). From 6.5 - 14.5 h the sucrose/KOH feedrate was 0.104 $l.h.^{-1}$ with a linear correlation coefficient of 1.00. Figure 6.27 shows a sharp increase in the enzyme activity between 7 and 8.5 h which is not evident from Figure 6.26. This clearly demonstrates the rapid production of the enzyme in a narrow time interval during which the bacterial culture is subjected to ideal enzyme producing conditions.

Although Runs 33 and 34 were the best of a number of sucrose fed-batch fermentations the ODs and enzyme yields were poor and variable. In previous work using this technique (McAvoy, 1981), ODs of between 1.22 - 1.38 and enzyme yields of 220-280 $DSU.ml^{-1}$ were attainable in 12-15 h with no obvious

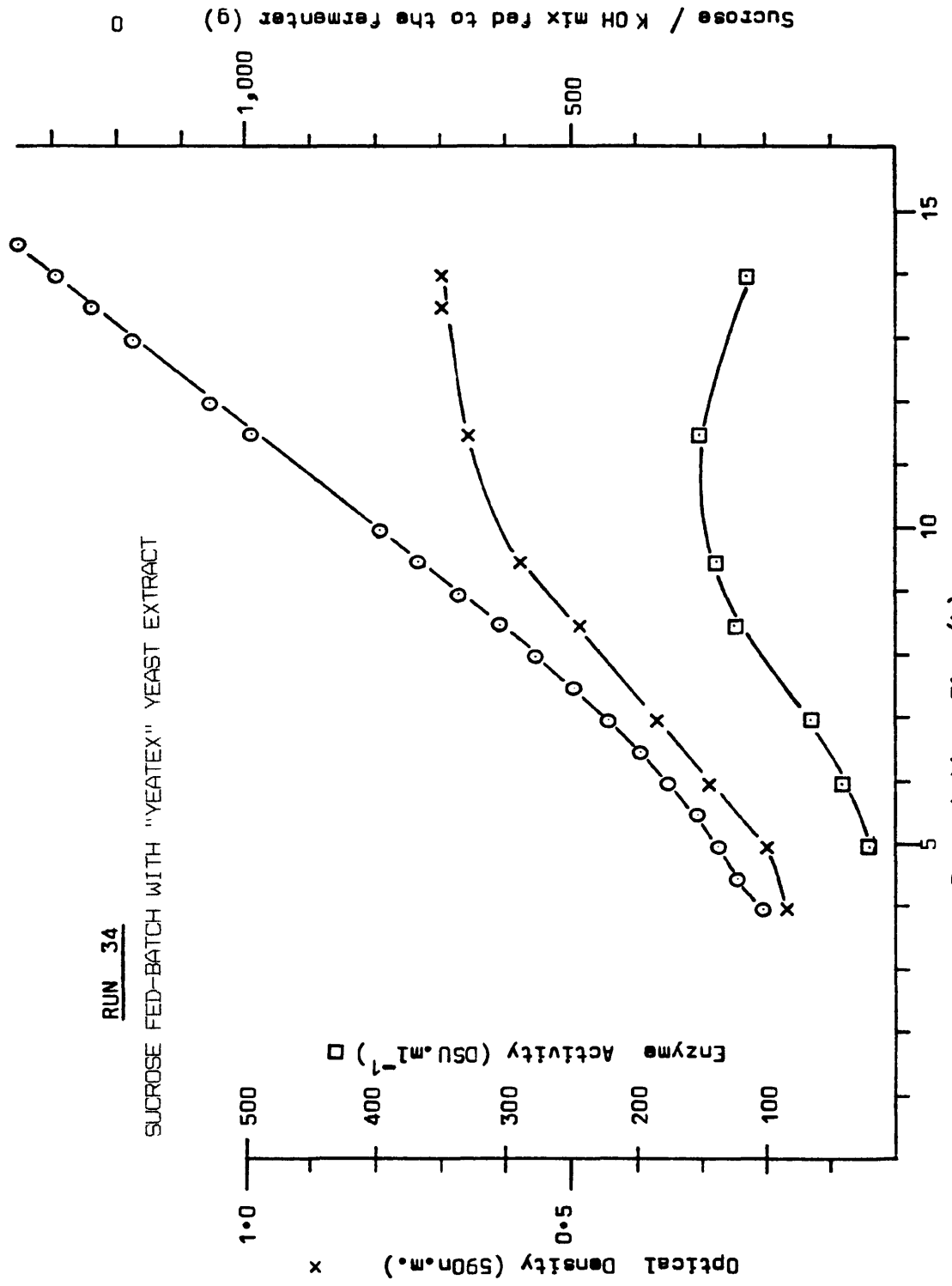


Figure 6.26 Run 34; O.D.,Enzyme Activity and Sucrose/KOH Used vs Time.

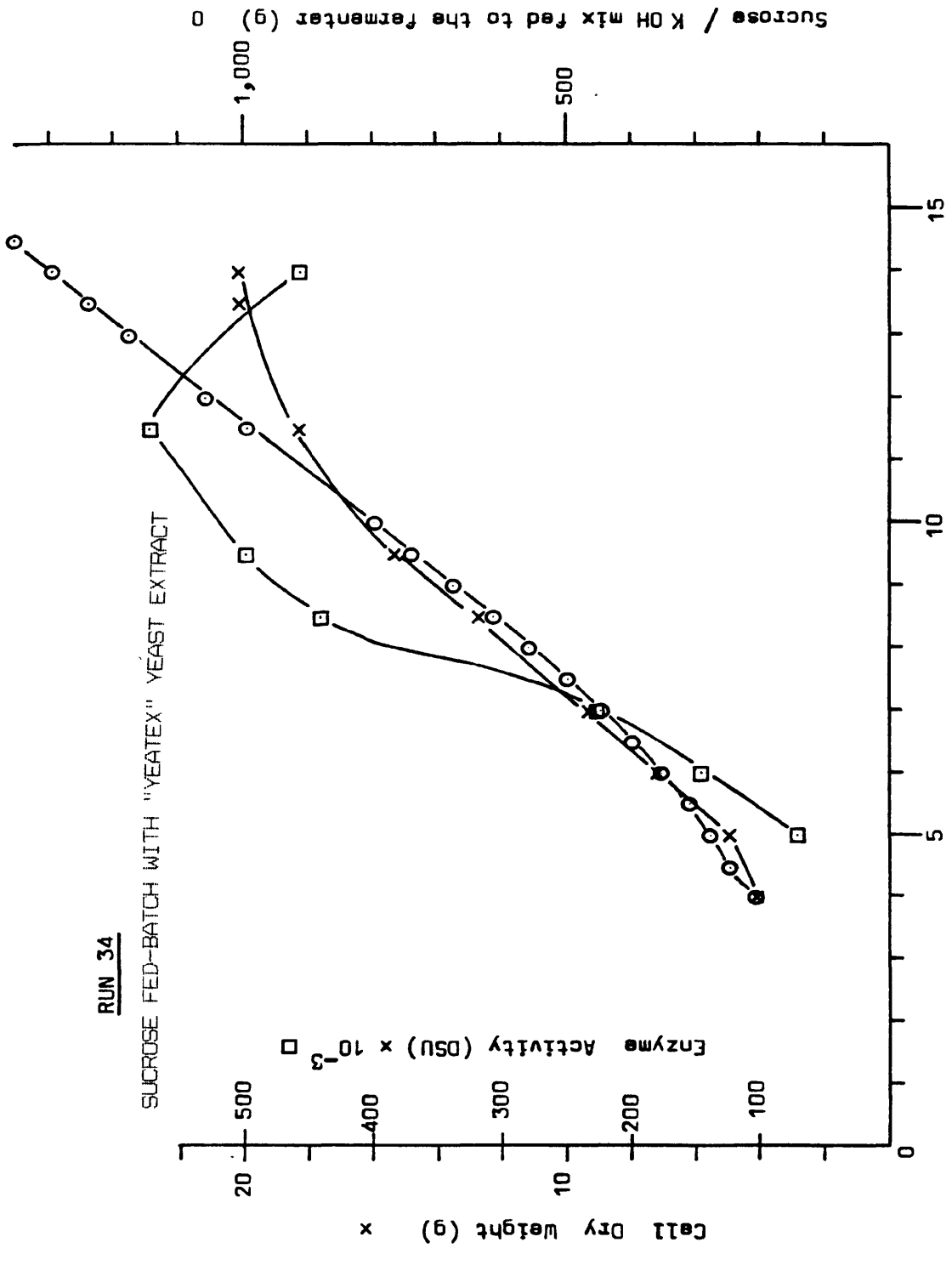


Figure 6.27 Run 34; Dry Weight, Enzyme Activity and Sucrose/KOH Used vs Time.

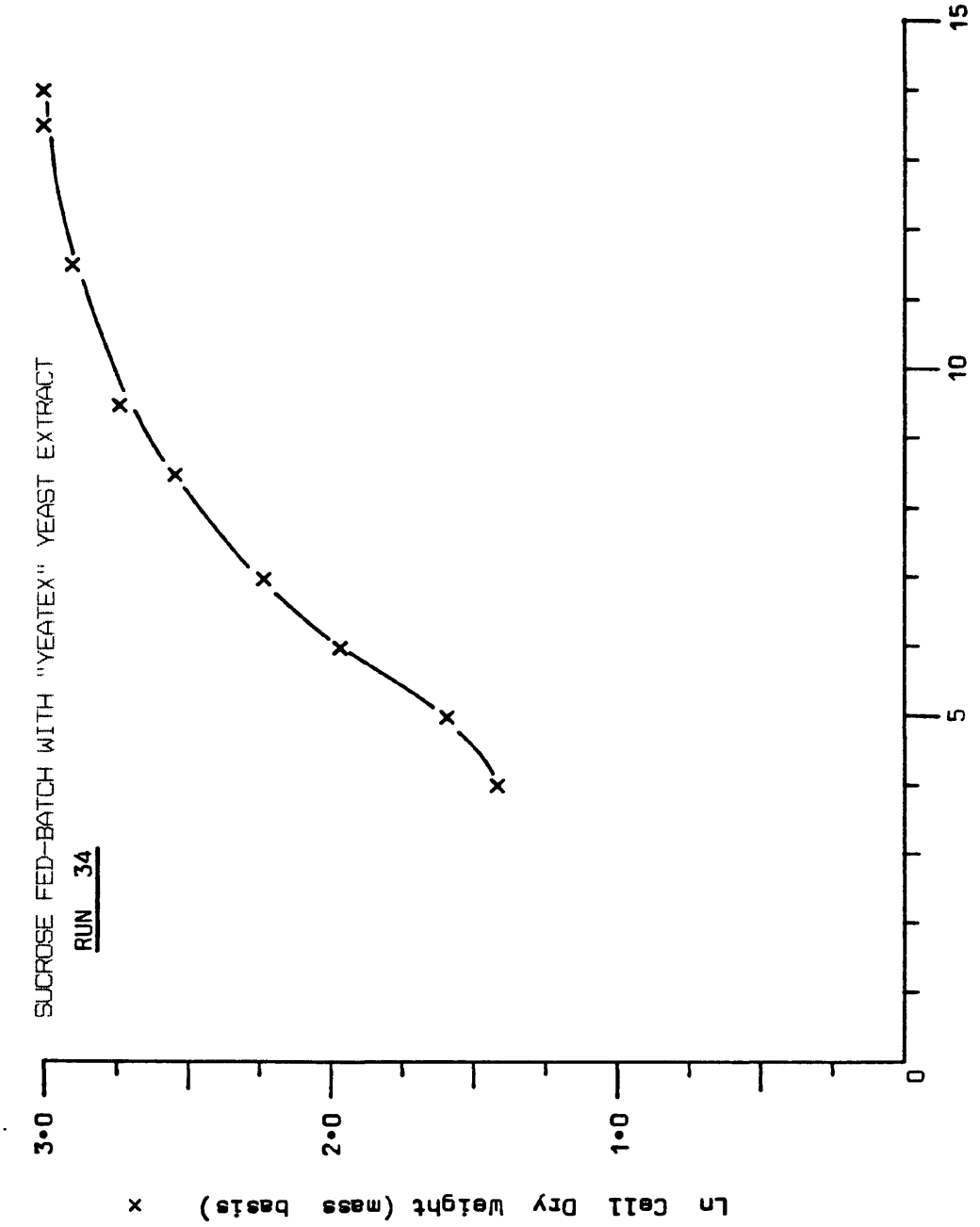


Figure 6.28 Run 34; Ln Dry Weight vs Time.

growth problems. As with the continuous culture Runs 16-32 there appeared to be a variable growth limitation.

B. Studies with Different Yeast Extracts

Preliminary shake flask work had failed to discover an alternative nitrogen source to yeast extract (see Section 6.5) and therefore, a closer examination of the type of yeast extract was made.

Bovril "Yeatex" yeast extract is prepared from spent brewery yeast which is collected from a number of breweries. The yeast is hydrolysed and then either concentrated to a paste or spray dried to a powder. It is reasonable to assume that the quality of the yeast extract varies since the yeast itself must vary from brewery to brewery.

A number of yeast extract manufacturers produce their own yeast under known conditions resulting in a consistent product. This yeast is hydrolysed and concentrated to yeast extract paste. It might, therefore, be expected that the quality of these yeast extract pastes will be more consistent than that of Bovril. Two types of "continental" yeast extracts were acquired, one from Ohly and one from Gist-Brocades.

Ohly yeast extract was used in Runs 35 and 36, Gist-Brocades yeast extract was used in Runs 37-41.

6.6.1 Fed-Batch with Ohly Yeast Extract (Run 35)

Run 35 conditions were a repeat of Runs 33 and 34 but using Ohly yeast extract in place of Bovril "Yeatex". The results of this fermentation were similar to those obtained in previous work (McAvoy, 1981) demonstrating that the erratic results obtained in the continuous culture Runs 16-32 and sucrose fed-batch Runs 33 and 34 were due to the variability of some essential nutrient in the yeast extract.

A maximum specific growth rate of 0.39 $h.^{-1}$ was calculated between 7-9.25 h (Appendix 6.1 and 6.7; Figures 6.29 and 6.31). The maximum enzyme activity of 265 $DSU.ml^{-1}$ was reached at 11 h and then began to decrease due to the adverse pH of 6.7 (Figure 6.29). The optimum pH for enzyme production is 6.7 whereas maximum enzyme stability is obtained at 5.2. Figure 6.30 (Appendix 6.5) illustrates the fermentation results on a mass basis to compensate for the increasing volume during the fermentation (Appendix 6.4). No unusual effects were observed due to volume variation (comparison of figures 6.29 and 6.30). Between 10.25-14 h the sucrose/NaOH feedrate was calculated to be 0.199 $1.h.^{-1}$ corresponding to 85.4 g $sucrose.h.^{-1}$ with a linear correlation coefficient of 1.00.

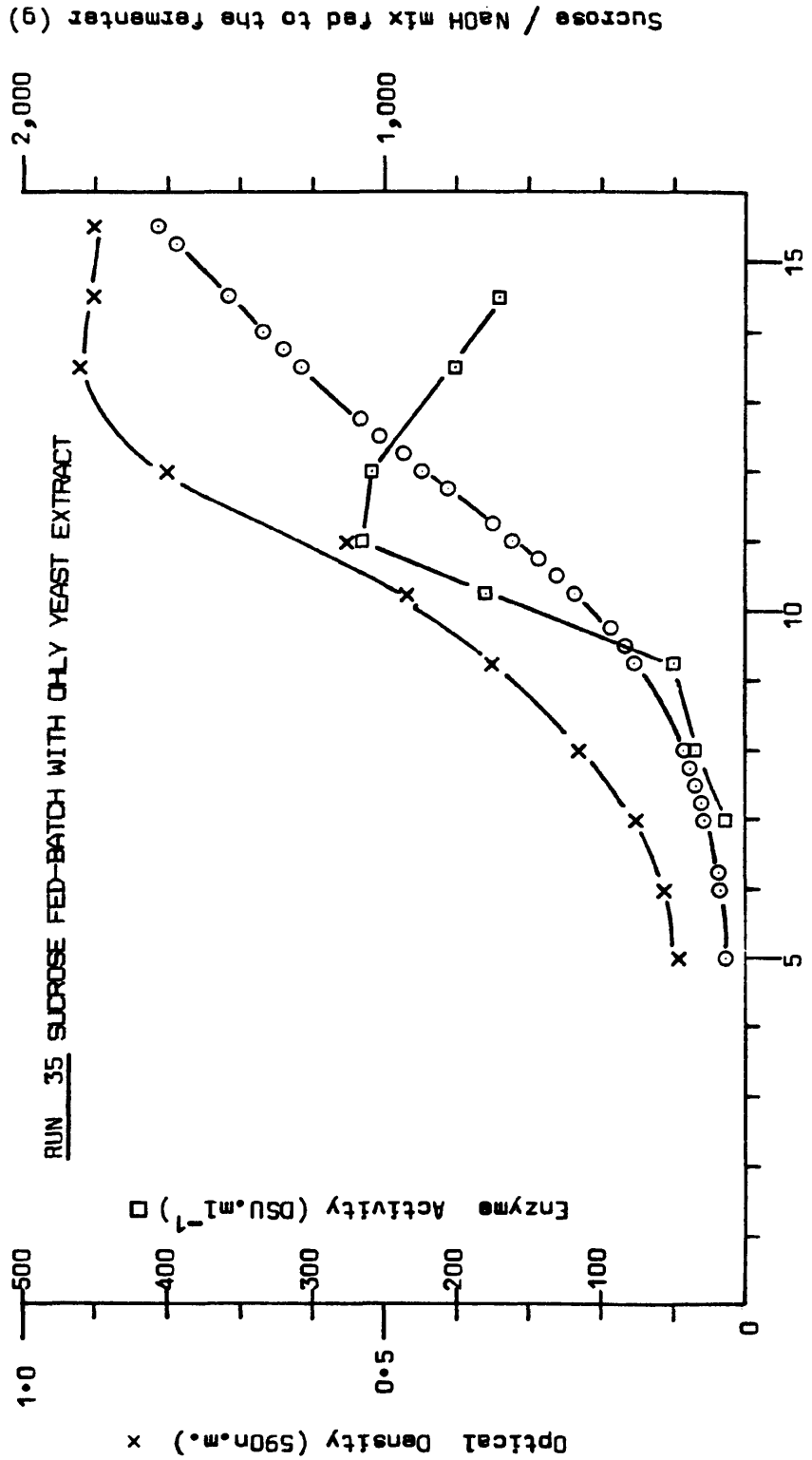


Figure 6.29 Run 35; O.D.,Enzyme Activity and Sucrose/NaOH Used vs Time.

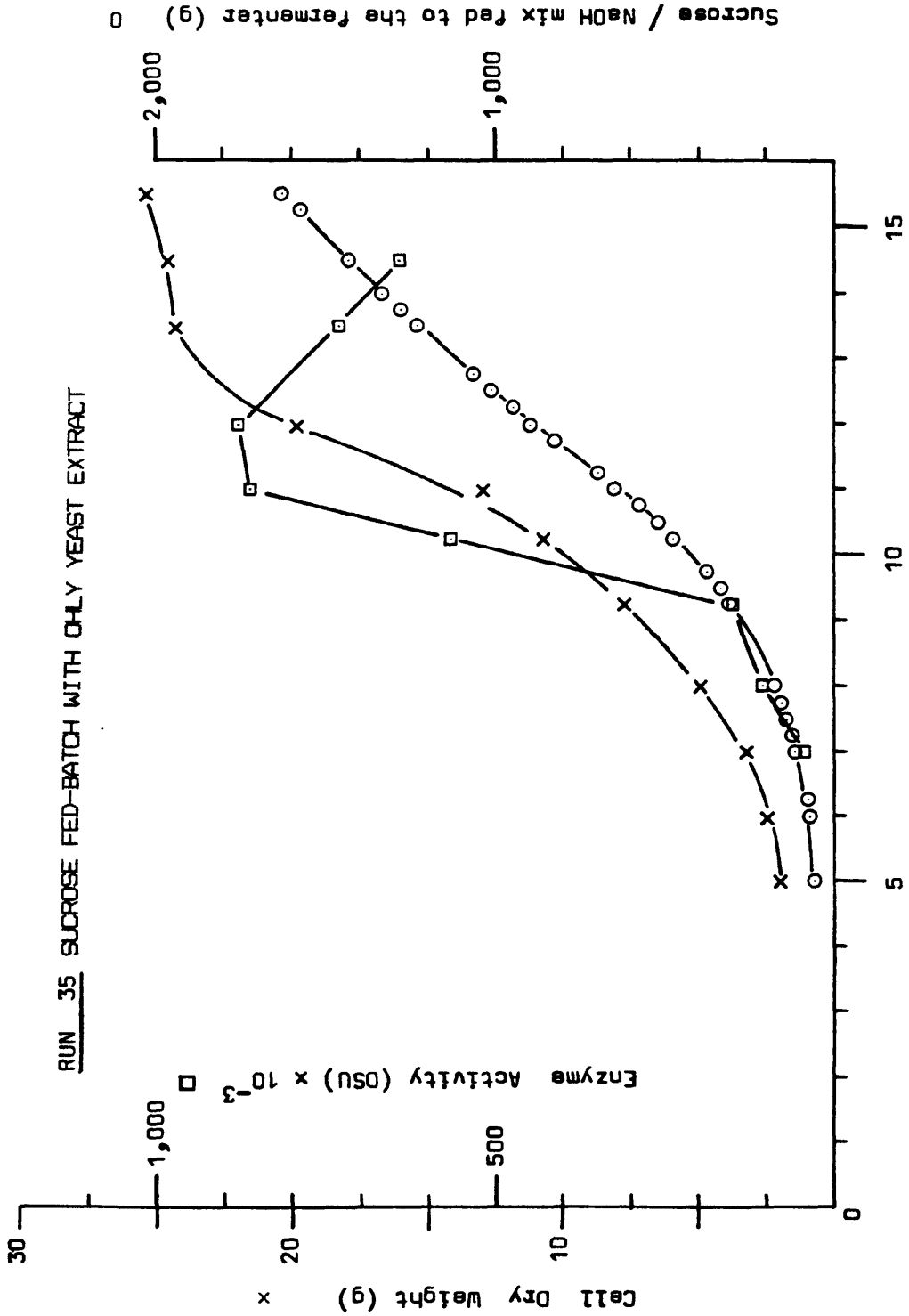


Figure 6.30 Run 35; Dry Weight, Enzyme Activity and Sucrose/NaOH Used vs Time.

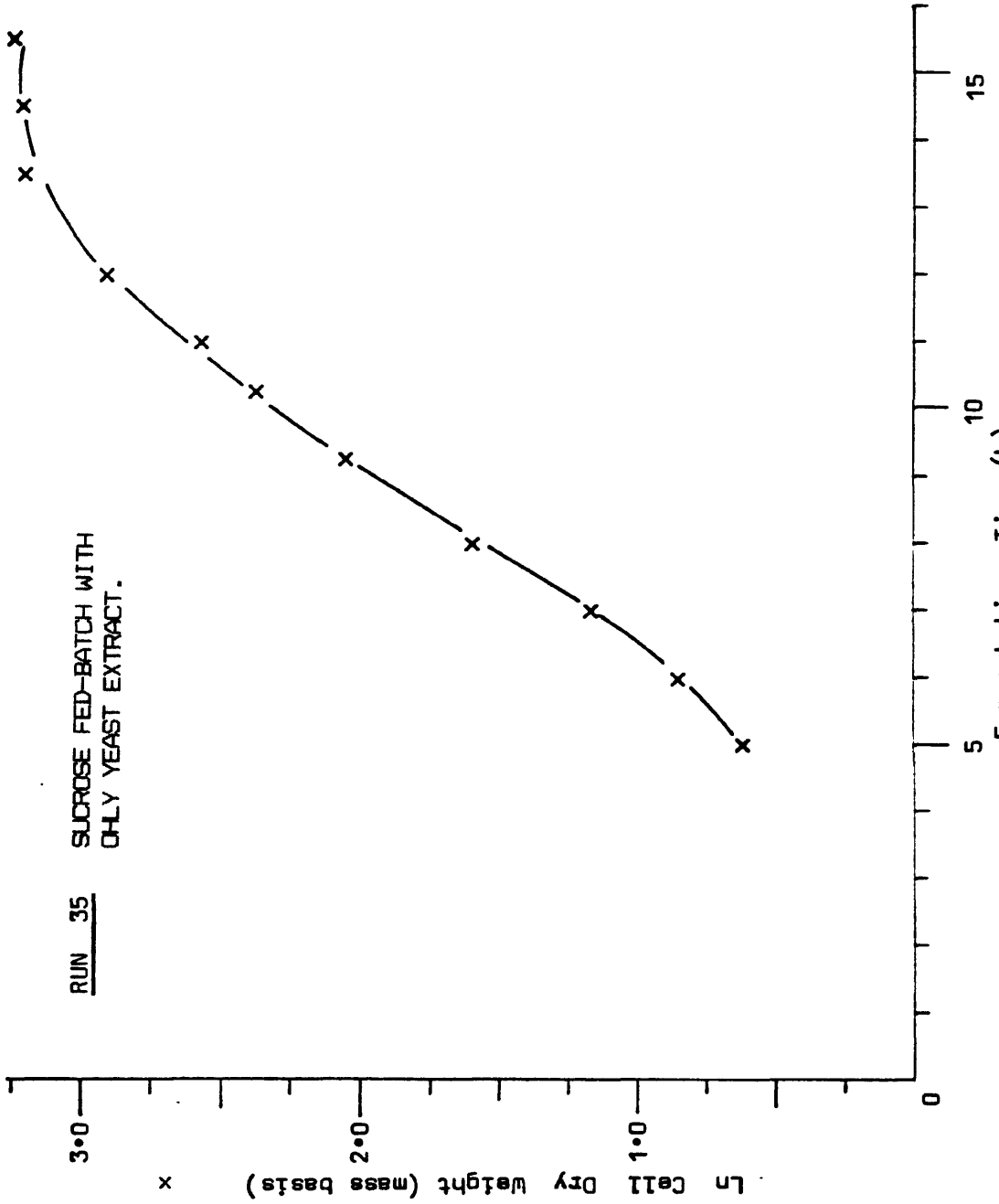


Figure 6.31 Run 35; Ln Dry Weight vs Time.

Figure 6.32 (Appendix 6.2) illustrates the oxygen and carbon dioxide concentrations detected in the effluent gas. This data was used to calculate the molar carbon dioxide production and oxygen consumption rates (Appendix 6.6 and Figure 6.33). Both peak at approximately 11-11.5 h with rates of 151.5 m.Moles O_2 $h.^{-1}$ and 168.0 m. Moles CO_2 $h.^{-1}$. These peaks correspond with the peak in the enzyme activity and occur when the OD is relatively low at 0.55. These results suggested that the fermentation was subjected to a nutrient limitation at 11-11.5 h. The occurrences of this limitation is, however, not obvious from the OD data.

The results of the HPLC analysis of the broth (Table 6.8) clearly demonstrate the ability of the mixed feed fed-batch technique to feed sucrose to the fermentation. These results also show the presence and build-up of the glucose, fructose and mannitol concentrations through the fermentation and that the bacteria were not subjected to a carbon limitation.

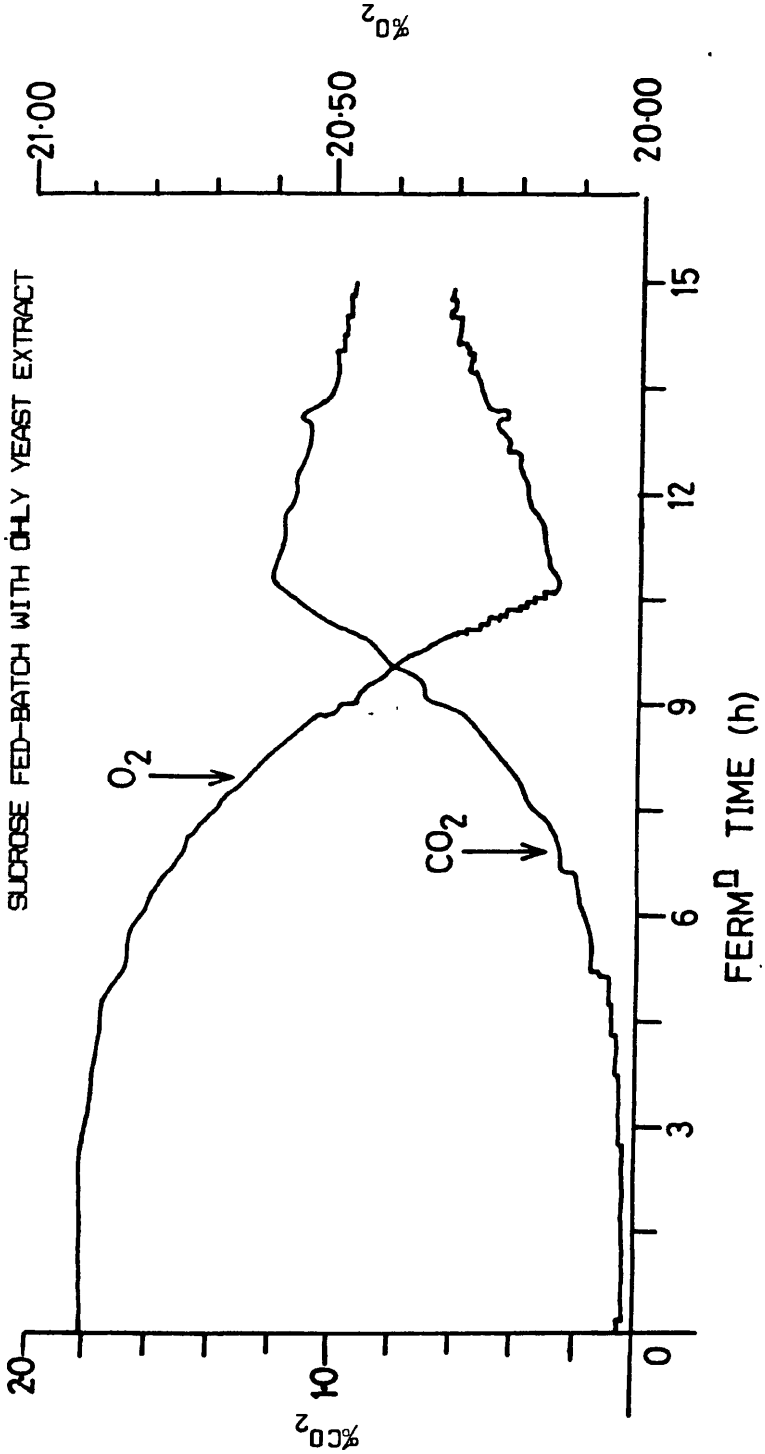


Figure 6.32 Run 35; Effluent Gas Composition vs Time.

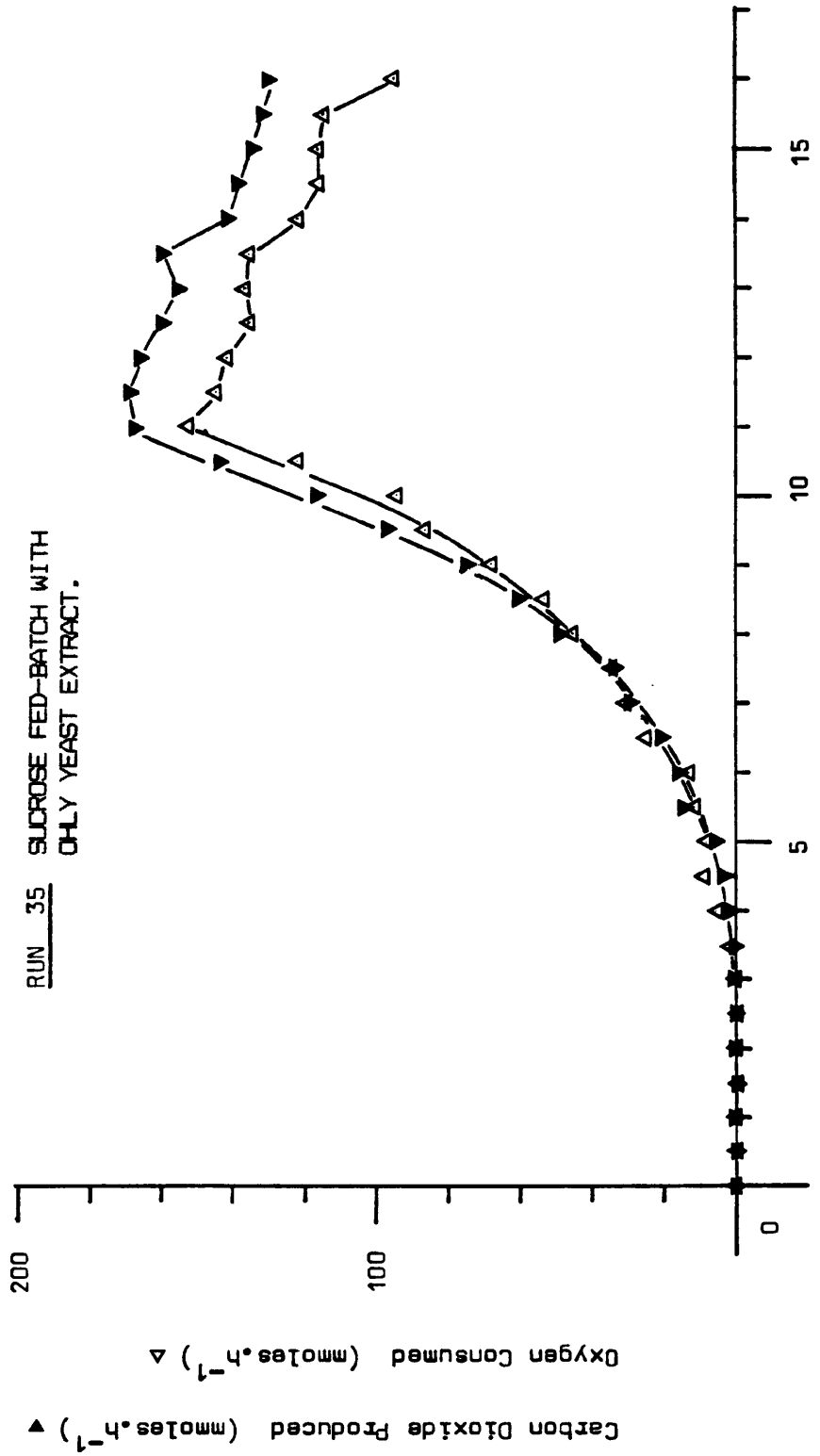


Figure 6.33 Run 35; Rate of CO2 Evolution and O2 Consumption vs Time.

TABLE 6.8

RUN 35 HPLC ANALYSIS OF SUGARS

	CONCENTRATIONS ($g.l.^{-1}$)			
TIME (h)	SUCROSE	GLUCOSE	FRUCTOSE	MANNITOL
5	4.9	0.5	0.8	2.3
6	4.6	0.6	1.1	2.4
7	4.6	0.7	1.6	2.4
8	3.4	1.4	2.8	3.5
9.25	7.3	1.5	5.1	3.4
10.25	7.5	2.5	7.2	4.6
11	4.2	3.6	8.8	6.1
12	12.4	6.0	11.9	7.8
13.5	14.7	8.1	15.3	8.7
14.5	0	10.9	19.9	17.0
15.5	0	14.7	26.9	20.6

6.6.2 Fed-Batch with Continuous Phosphate Addition (Run 36)

In Run 35 there appeared to be a nutrient limitation which affected the bacterial metabolism at 11-11.5 h. There was no carbon shortage indicated by HPLC (Table 6.8) so it was postulated that phosphate might help the carbon uptake. Run 36 was a repeat of Run 35, however, when the onset of the limitation was detected by the gas analysis equipment (Figure 6.34) two actions were undertaken; a solution of K_2HPO_4 was fed continuously (Point A Figures 6.34 - 6.38) and 26.5 g of Ohly yeast extract was added to the fermenter (Point B Figures 6.34 -6.38). Point C Figures 6.34 to 6.38 indicates the end of the phosphate feed (see Table 5.6). The phosphate feed did not alleviate the limitation, however, the addition of Ohly yeast extract did. This can be clearly observed in the gas analysis data (Appendix 7.2) shown in Figure 6.34, molar CO_2 production and O_2 consumption (Appendix 7.6) in 6.35, temperature and feed profiles shown in Figures 6.36 and 6.37. No dextransucrase activity was detected in this fermentation due probably to the elevated fermentation temperatures of 29-32.5 ^{o}C. A fault in the temperature control system prevented the cooling of the broth.

A maximum OD of 0.96 was attained at 15 h (Appendix 7.1) and can be seen in Figure 6.36. Due to the increasing fermentation temperature, a maximum specific growth rate was not calculated from the cell concentration data

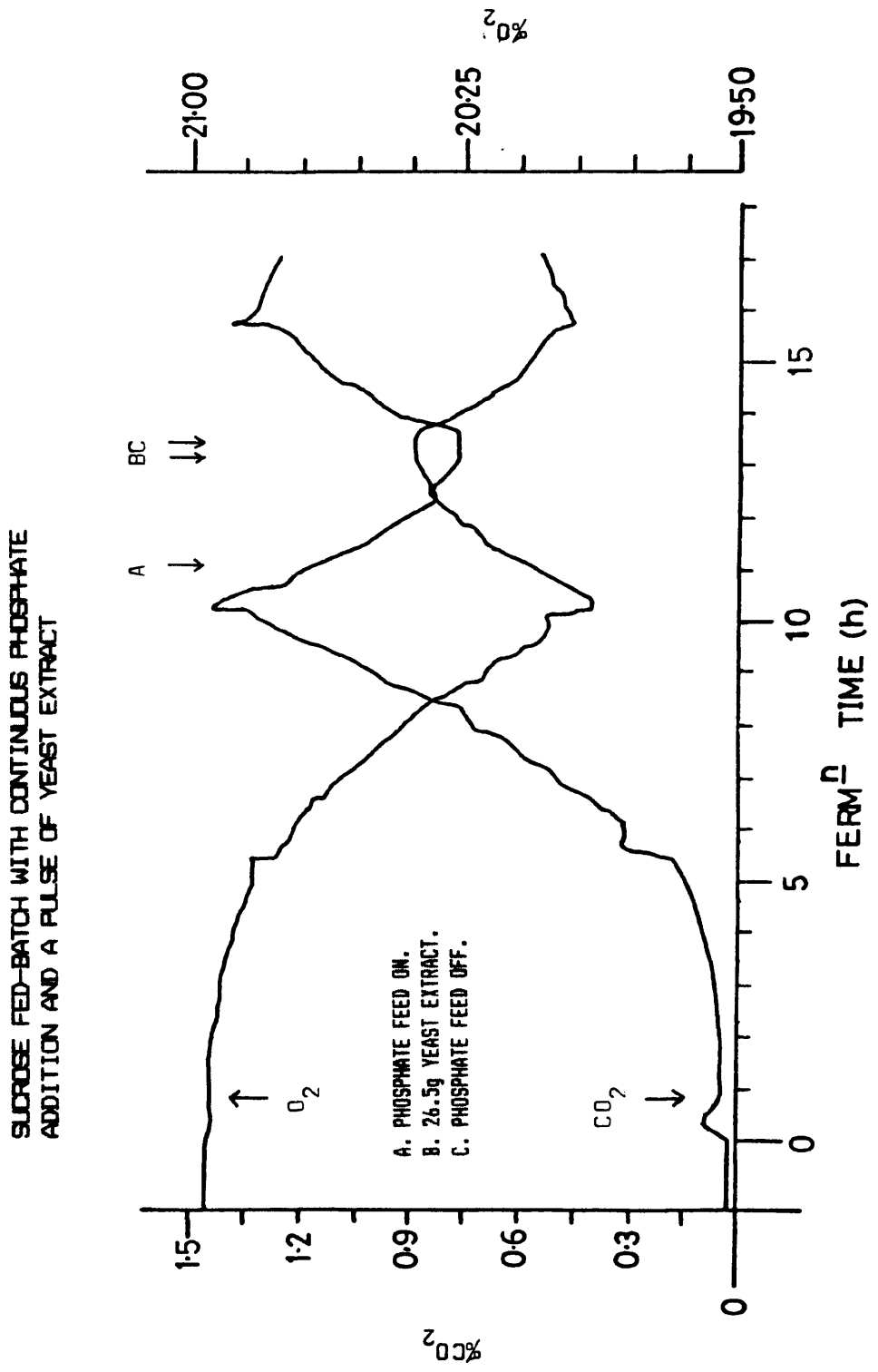


Figure 6.34 Run 36; Effluent Gas Composition vs Time.

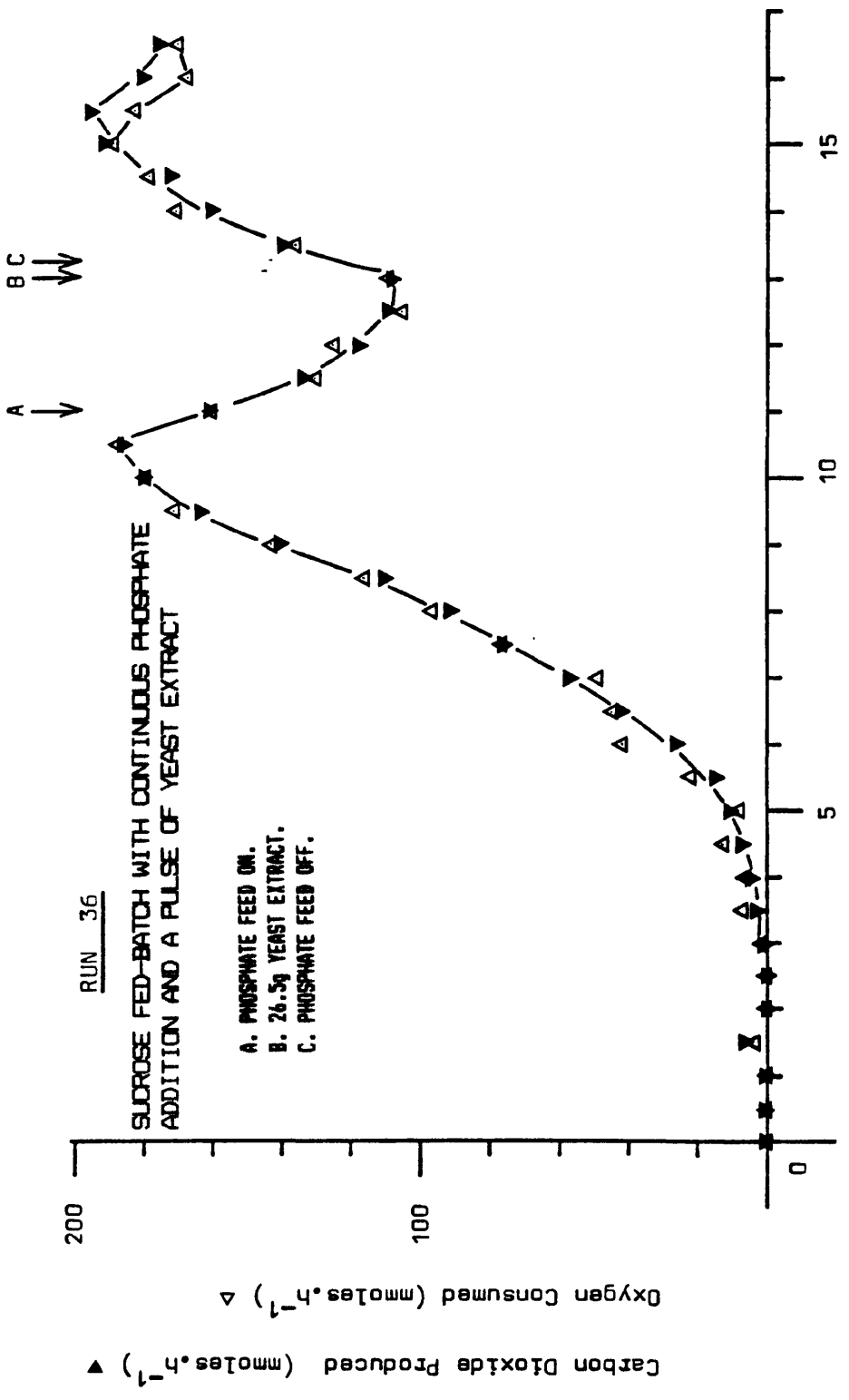


Figure 6.35 Run 36; Rate of CO2 Evolution and O2 Consumption vs Time.

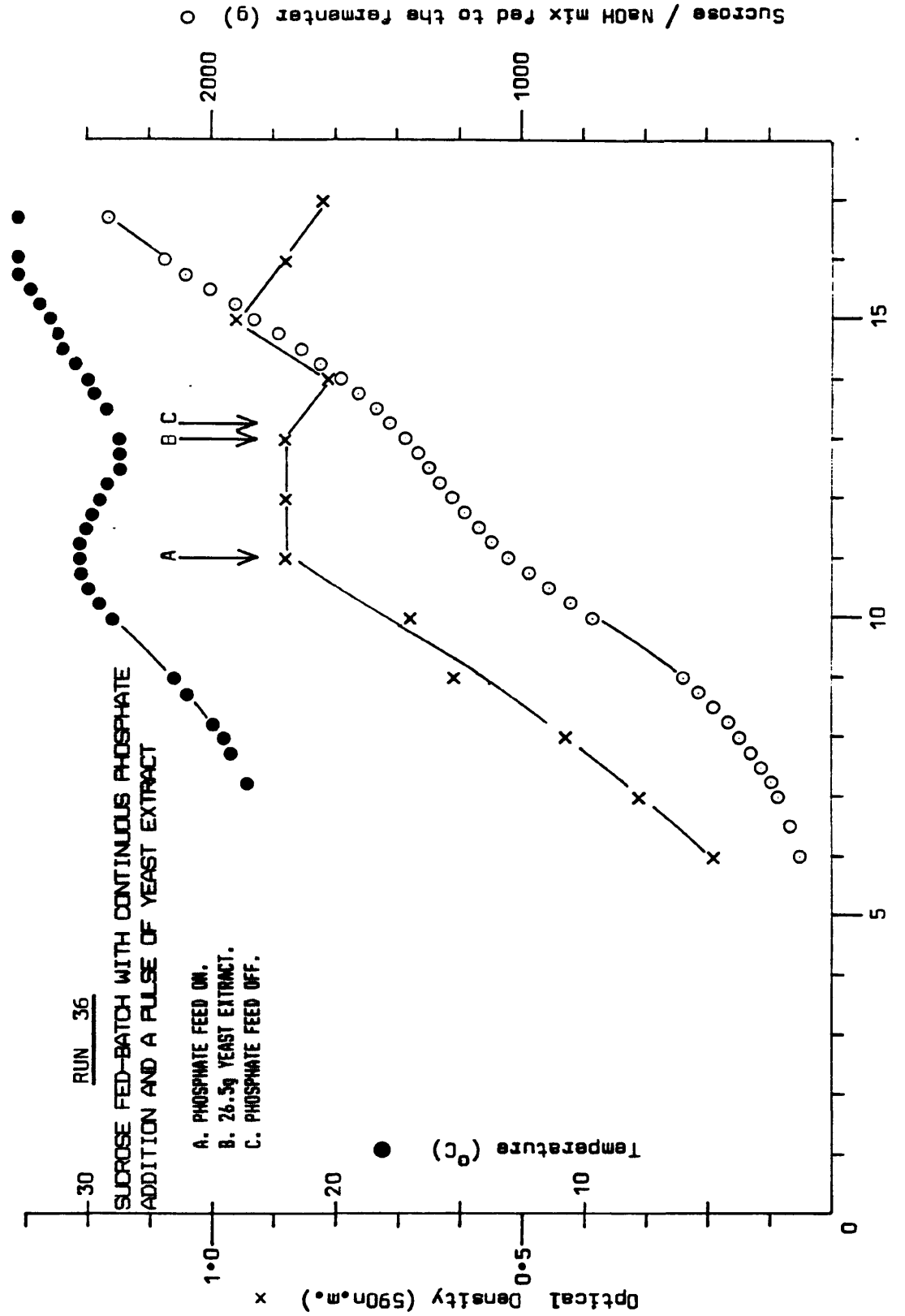


Figure 6.36 Run 36; O.D., Temperature and Sucrose/NaOH Used vs Time.

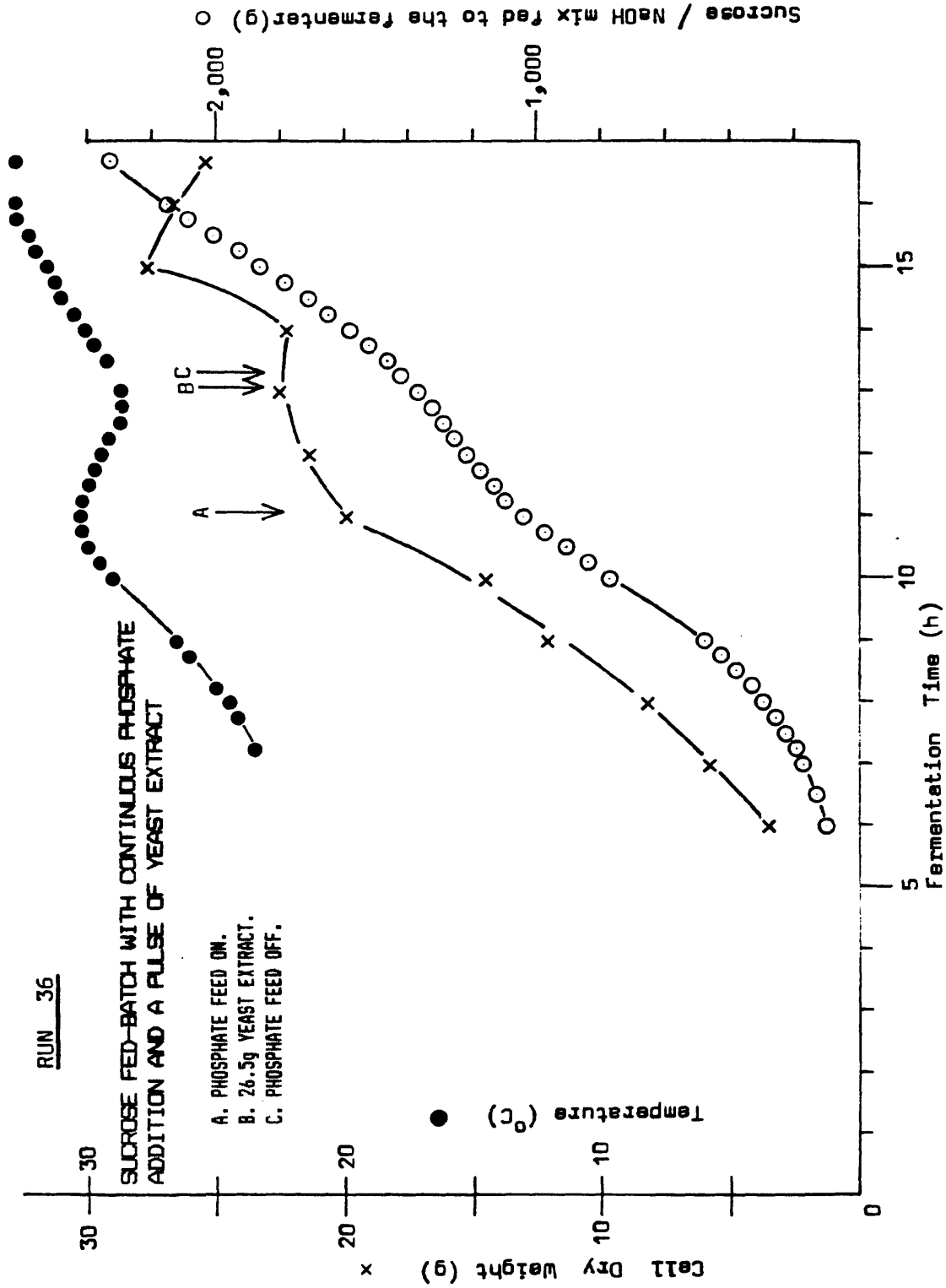


Figure 6.37 Run 36; Dry Weight, Temperature and Sucrose/NaOH Used vs Time.

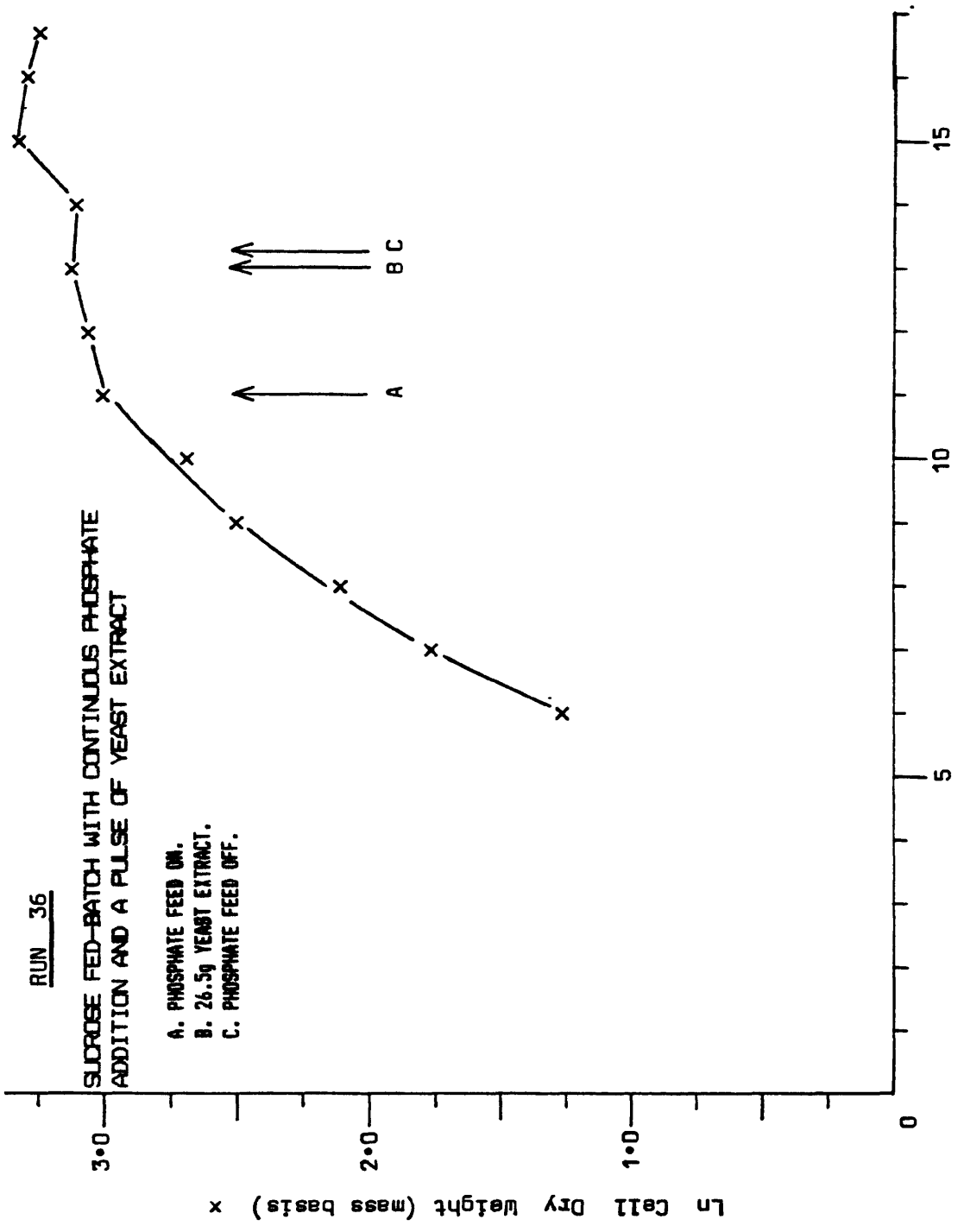


Figure 6.38 Run 36; Ln Dry Weight vs Time.

(Appendix 7.7) seen plotted in Figure 6.38. Figure 6.37 (Appendix 7.5) illustrates the fermentation results on a mass basis to compensate for the increasing volume (Appendix 7.4) during the fermentation. Figure 6.37 shows clearly the increase in the cell dry weight (g) on addition of the Ohly yeast extract (Point B).

Two distinct linear periods of sucrose/NaOH feed can be determined from Figure 6.36; 8.5-10.5 h with a feedrate of 0.232 $1.h.^{-1}$ corresponding to 99.5 g. sucrose. $h.^{-1}$ and 14-16.7 h with a feedrate of 0.241 $1.h.^{-1}$ corresponding to 103.4 g. sucrose. $h.^{-1}$. Both linear periods had correlation coefficients of 1.00. From 10.5-14 h there exists a transient which is due to a combination of the nutrient limitation and the lack of temperature control.

Figure 6.35 (Appendix 7.6) illustrates the molar carbon dioxide and oxygen consumption rates. The graph clearly demonstrates the ability of the Ohly yeast extract to restore the rate of the cell metabolism. Both CO_2 production and O_2 consumption peaked at 10.25 h with rates of 199 m Moles O_2 $h.^{-1}$ and 203 m Moles CO_2 $h.^{-1}$. The unknown nutrient limitation reduced these values to 109 m Moles O_2 $h.^{-1}$ and 108 m Moles CO_2 $h.^{-1}$ at 13 h. The addition of 26.5 g of Ohly yeast extract resulted in almost complete restoration of the respiration rates to 188 m Moles O_2 $h.^{-1}$ and 191 m Moles CO_2 $h.^{-1}$ at 15-15.25 h. Figure 6.35 also clearly demonstrates that the respiratory quotient (RQ) of the growing bacteria is very

TABLE 6.9

RUN 36 HPLC ANALYSIS OF SUGARS

	CONCENTRATIONS ($g.l.^{-1}$)				
TIME (h)	SUCROSE	GLUCOSE	FRUCTOSE	MANNITOL	ETHANOL
6	9.2	1.4	2.4	3.4	0
7	8.8	2.2	4.0	4.7	0.21
8	14.3	2.2	6.7	5.0	0
9	14.6	4.4	10.1	11.6	0
10	14.2	5.7	16.2	18.1	0
11	33.4	3.2	20.0	17.6	0
12	44.8	1.6	26.4	19.1	0
13	43.2	0	26.0	18.2	0
14	47.0	0	27.8	19.0	0
15	51.2	0	29.2	19.0	0
16	65.8	0	33.1	20.3	0

close to 1.

Table 6.9 lists the results of the HPLC analysis of the broth. From 6-10 h the sucrose concentration varied between 8.8-14.6 $g.l.^{-1}$ after which it increased markedly to 65.8 $g.l.^{-1}$ at 16 h. This increase was due to the change in the stoichiometry when growth ceased at 11 h. The sucrose/NaOH feed was then supplying excess sucrose. The glucose, fructose and mannitol concentrations increased steadily up to approximately 11 hours, after which fructose and mannitol remained constant until the addition of yeast extract when they continued to rise. However, glucose did not follow this trend and by 13 h no glucose could be detected in the broth.

6.6.3 Continuous Feeds of Yeast Extract (Run 37)

Runs 35 and 36 had shown that some component of the yeast extract was bringing about a nutrient limitation early in the fermentation, and at approximately the same time the enzyme production peaked. It was, therefore, considered possible to control the cell growth and metabolism by feeding the yeast extract. For Run 37 Gist-Brocades yeast extract was used in the starting medium at an initial concentration of 10 $g.l.^{-1}$. When the onset of the limitation was detected by gas analysis equipment (Point A Figure 6.39) at 8 h a solution of Gist-Brocades (185 $g.l.^{-1}$) yeast extract (see Table 5.7) was fed continuously to the fermenter at a rate of 32.3 $ml.h.^{-1}$. After 3 h (Point B figures 6.39-6.43) the feedrate of the yeast extract solution was increased by

approximately 2.5 times to 80 ml.h.$^{-1}$ (see Table 5.7. Point C, Figures 6.39 to 6.43 signify yeast extract feed end. At approximately 4.5 h (Figures 6.39 and 6.42) a decrease in the CO_2 production and O_2 consumption can be seen. This was due to a fault in the sucrose/NaOH feed, starving the fermentation of NaOH and consequently sucrose. On correcting the fault at 5 h the bacterial metabolism can be seen to respond almost immediately.

Figures 6.40 and 6.41 illustrate the linearity of the cell growth and sucrose/NaOH feed and Figure 6.42 the linearity of the molar CO_2 production and O_2 consumption during the two periods of yeast extract feed. On increasing the yeast extract feed by 2.5 times the linear rates of the above parameters also increased.

At the lower yeast extract feedrate, sucrose/NaOH was being fed to the fermenter at 0.076 1.h.$^{-1}$ with a linear correlation coefficient of 1.00. Cell growth (9-12 h) was also linear at a rate of 0.90 g.l.$^{-1}$ with a correlation coefficient of 0.97. During this lower yeast extract feedrate, dextransucrase activity peaked at 373 DSU.ml.$^{-1}$ and then began to decrease due probably to the adverse pH (Figures 6.43 and 6.40).

At the higher yeast extract feedrate, sucrose/NaOH was being fed to the fermenter at 0.161 1.h.$^{-1}$ with a linear correlation coefficient of 1.00. This sucrose/NaOH feedrate is 2.3 times that calculated for the lower

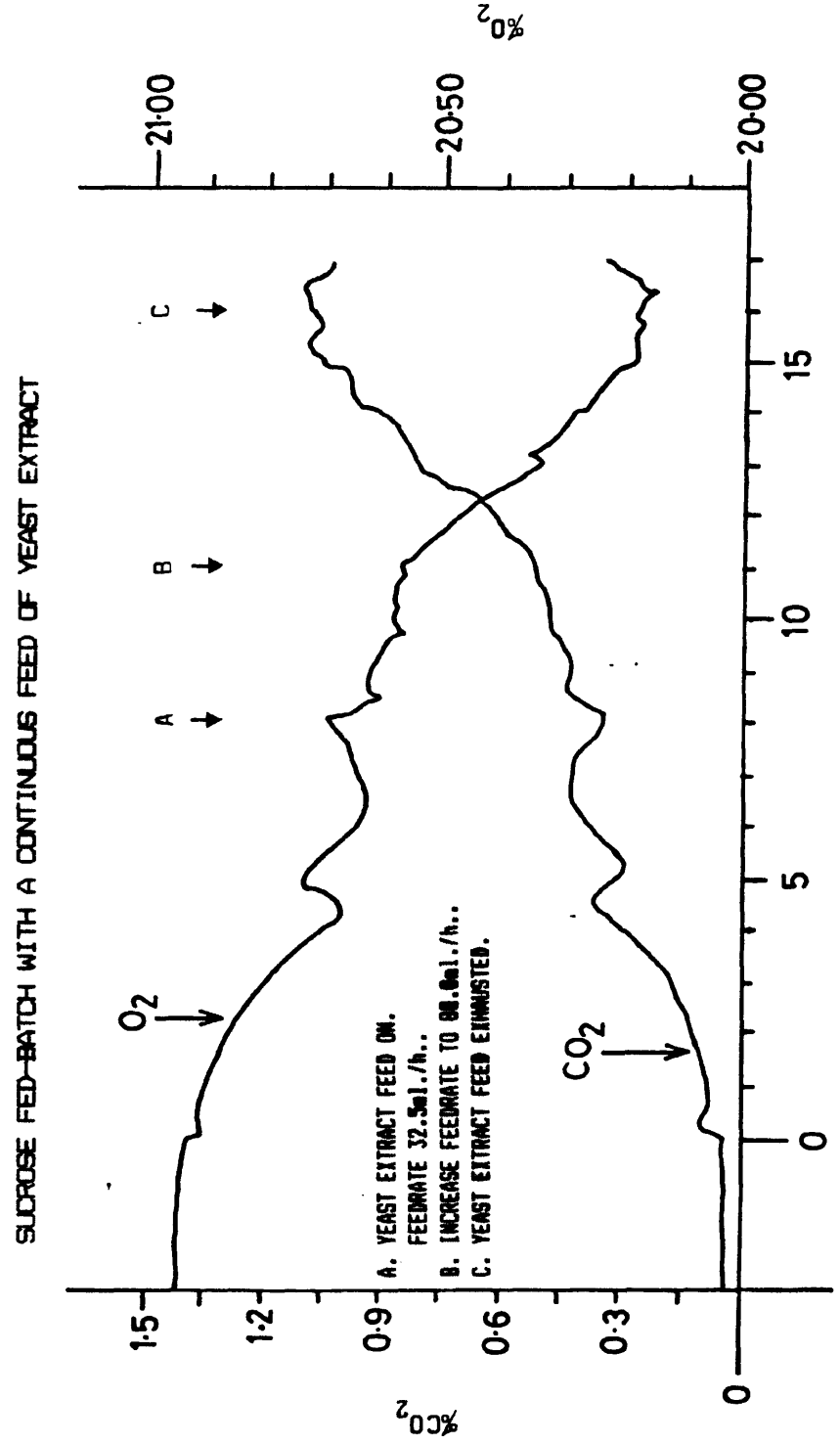


Figure 6.39 Run 37; Effluent Gas Composition vs Time.

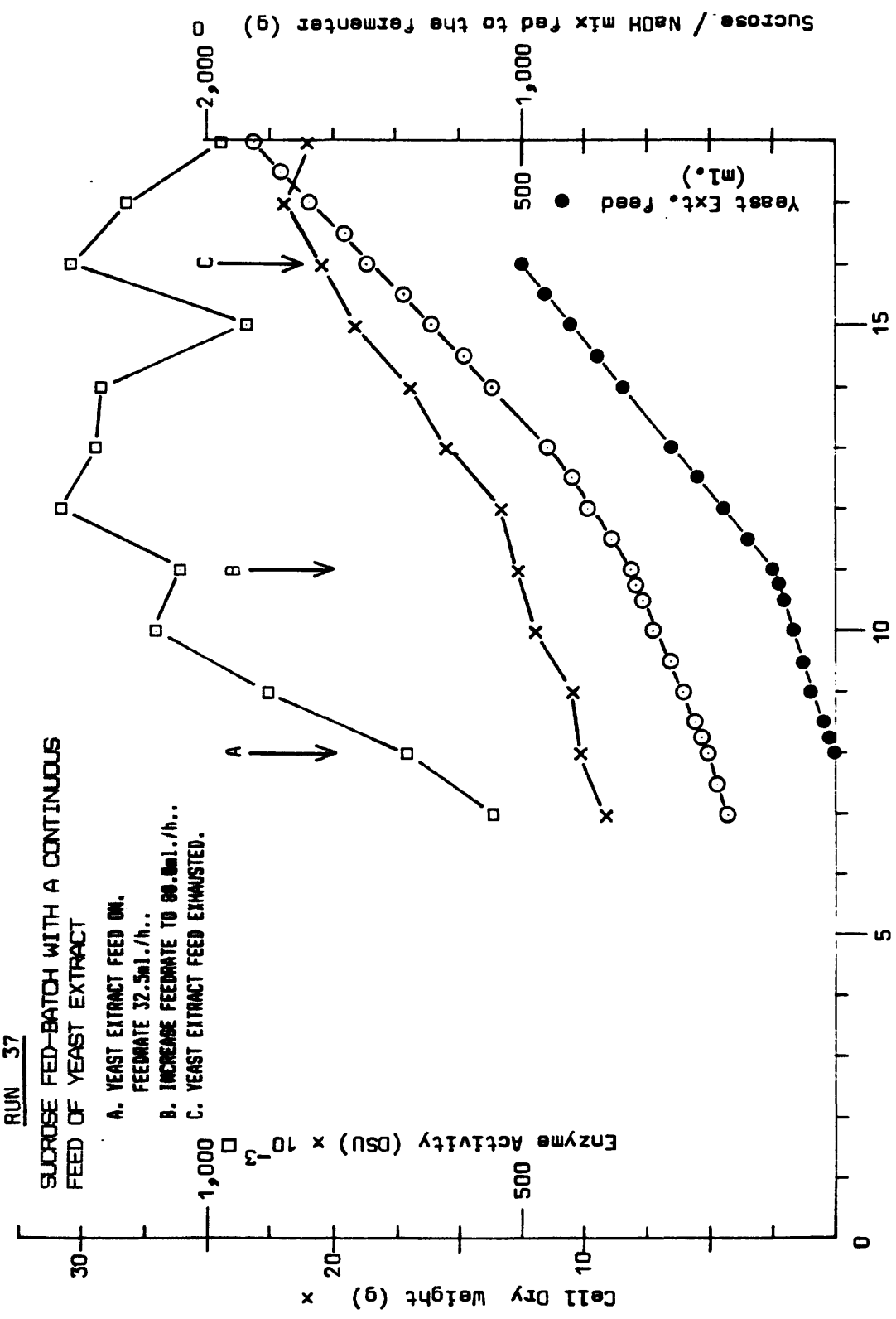


Figure 6.40 Run 37; Dry Weight, Enzyme Activity, Yeast Extract Fed and Sucrose/NaOH Used vs Time.

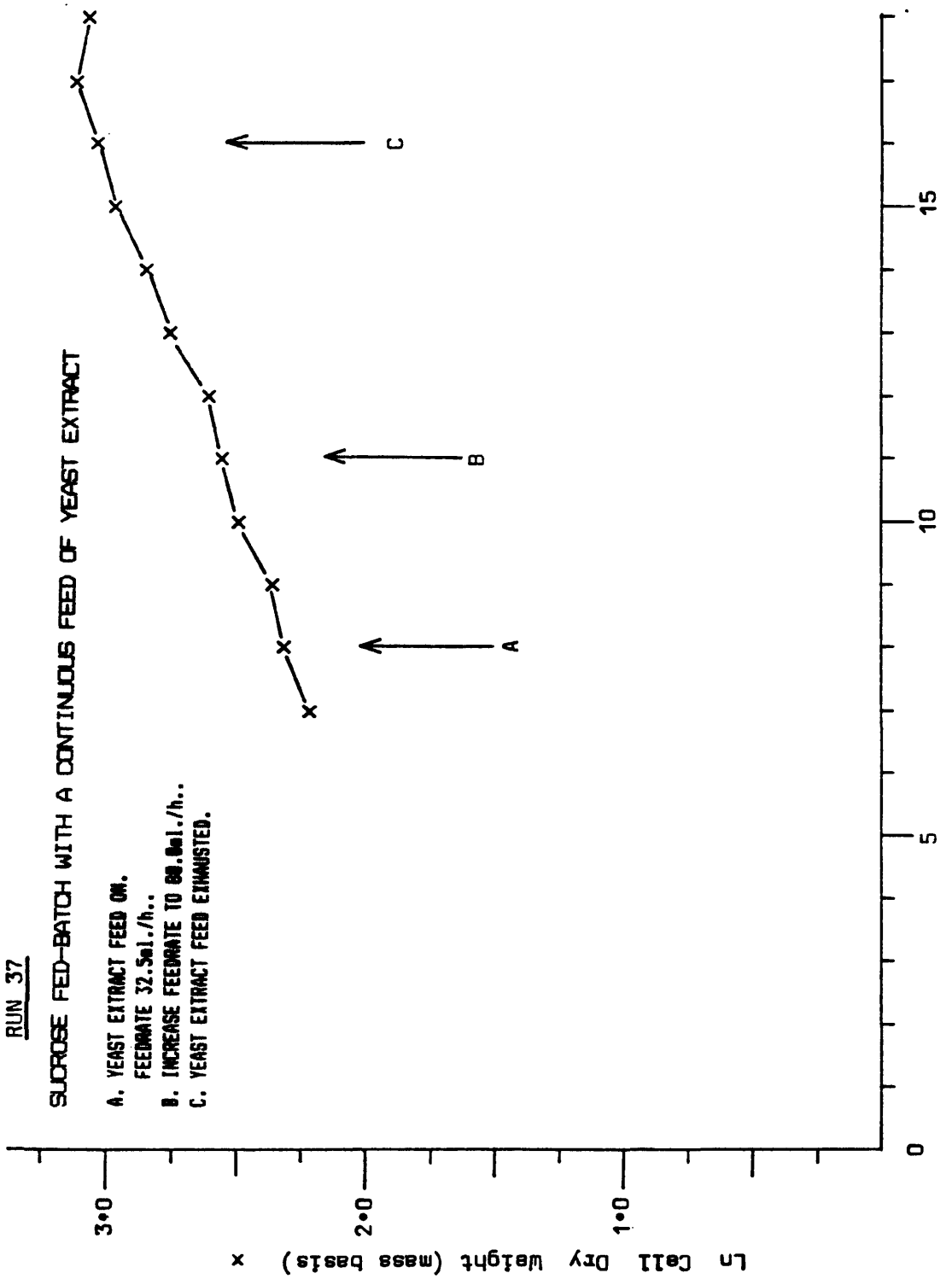


Figure 6.41 Run 37; Ln Dry Weight vs Time.

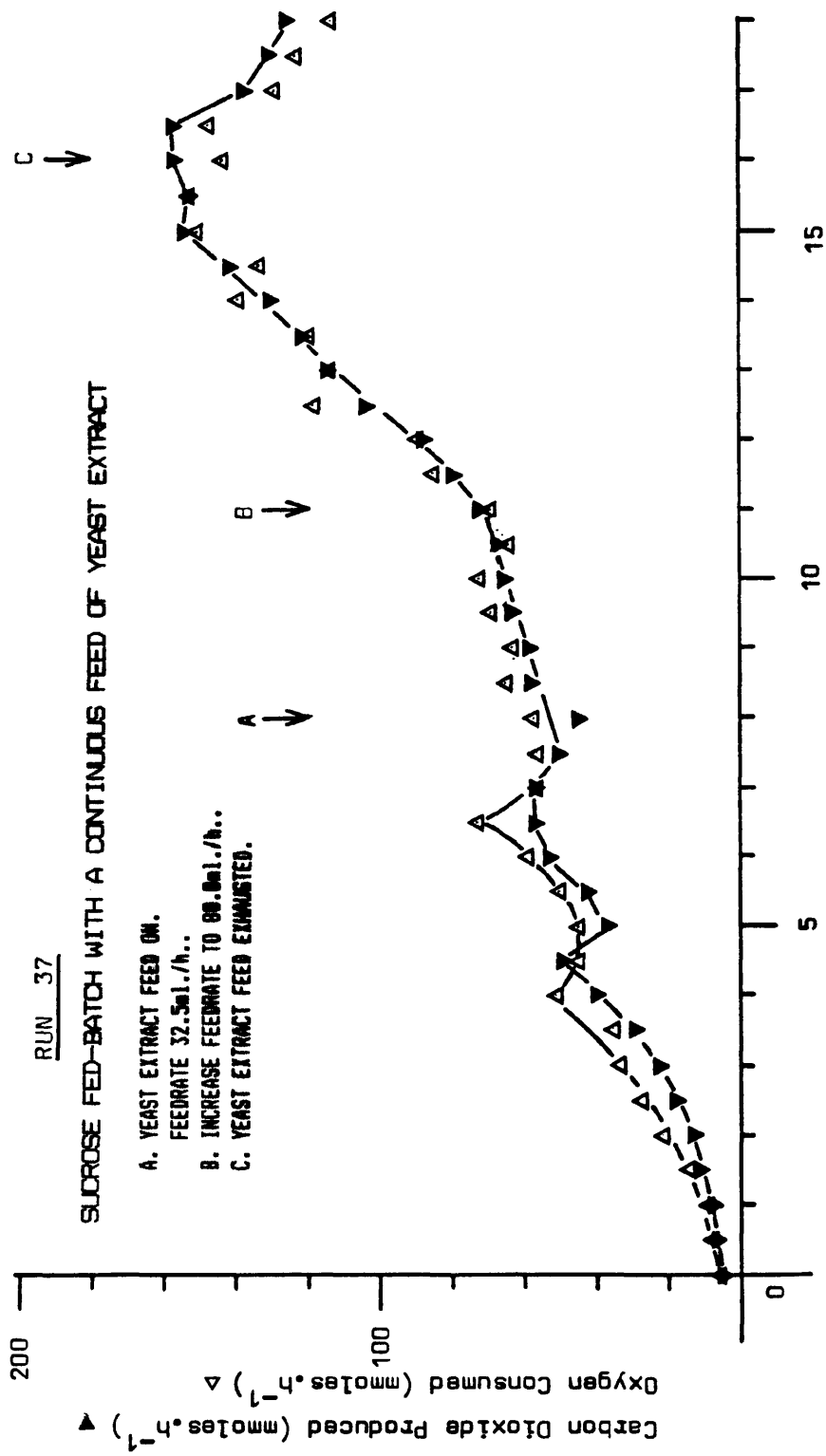


Figure 6.42 Run 37; Rate of CO2 Evolution and O2 Consumption vs Time.

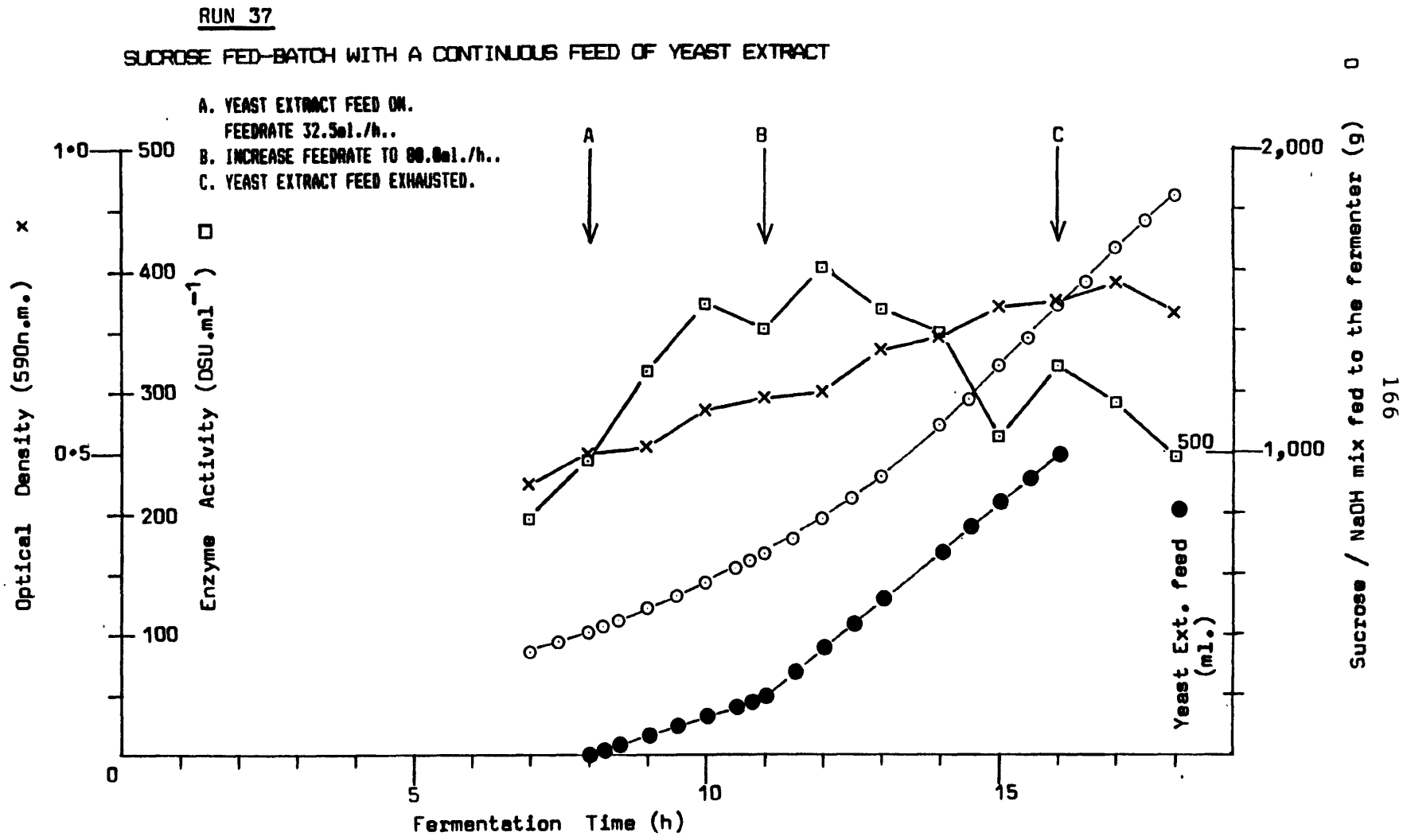


Figure 6.43 Run 37; O.D.,Enzyme Activity,Yeast Extract Feed and Sucrose/NaOH Used vs Time.

yeast extract feedrate. Cell growth (12-17 h) was also linear at a rate of 1.73 $g.h.^{-1}$ with a linear correlation coefficient of 1.00. The dextransucrase activity peaked at 403 $DSU.ml.^{-1}$ (Figures 6.43 and 6.40).

The dextransucrase activity in this run was higher than previously achieved in sucrose fed-batch fermentations without yeast extract feed. The total yeast extract used in this fermentation was equivalent to 46.8 $g.l.^{-1}$ initial volume. However, the yeast extract feed could have been halted any time after 12 h (corresponding to 23.14 $g.l.^{-1}$ initial volume) since the maximum enzyme yield had been attained by that time (Figure 6.40).

The control of the cell metabolism is illustrated by Figure 6.42, the molar gas consumption and production rates. Both molar CO_2 production and O_2 consumption rates reached their highest values of 158 m $Moles.h.^{-1}$ at 16.25 h.

Table 6.10 lists the HPLC analysis results of the broth. The sucrose concentration was zero between 9 and 12 h; glucose, fructose and mannitol increased steadily in the broth until 14 h. The sucrose concentration increased in the broth from 13 to 17 h. Thus, the ratio of sucrose/ NaOH required to maintain a constant mass of sucrose in the fermenter had changed. The fermentation was not carbon-limited since there were high concentrations of both glucose and fructose in the broth throughout the fermentation.

TABLE 6.10

RUN 37 HPLC ANALYSIS OF SUGARS

	CONCENTRATIONS ($g.l.^{-1}$)				
TIME (h)	SUCROSE	GLUCOSE	FRUCTOSE	MANNITOL	ETHANOL
7	0	3.5	10.2	8.4	0
8	1.9	4.6	14.4	10.0	0
9	0	4.6	16.1	10.6	0
10	0	5.7	21.8	13.4	0
11	0	6.0	27.9	16.4	0
12	0	2.5	23.7	20.1	0
13	2.7	9.9	28.9	18.9	0
14	1.6	7.9	33.9	20.9	0
15	9.5	7.4	35.2	20.8	0
16	27.8	8.5	34.0	20.8	0
17	46.7	10.8	34.8	25.0	0

6.6.4 Fed-Batch with Nitrogen Source Additions (Run 38)

Earlier shake flask work failed to discover an alternative nitrogen source to the yeast extract, even when they included a vitamin supplement. The absence of the essential nutrient apparently present in the Ohly and Gist-Brocades yeast extracts, may have been the reason for no growth on those alternative nitrogen sources. The various nitrogen sources (see Table 5.8) tested in the shake flask work were, therefore, added to a sucrose fed-batch fermentation which had initially been grown on 7.5 $g.l.^{-1}$ of Gist-Brocades yeast extract and was then exhibiting the signs of the nutrient limitation as determined by the gas analysis (see Figure 6.44). The effluent gas was monitored to detect any response in the cell metabolism to the addition of any of the alternative nitrogen sources. Table 5.8 lists the type, quantity and time at which each alternative nitrogen source was added.

The results of the gas analysis clearly show that only the addition of yeast extracts (Figures 6.44 and 6.45, Points F and G) alleviated the nutrient limitation. None of the alternative nitrogen source additions resulted in any marked response in the bacterial metabolism as monitored by the gas analysis. The additions of the yeast extracts were made in the latter stages of the fermentations. Figures 6.46 and 6.47 show that the addition of Bovril yeast extract (Point F) did increase

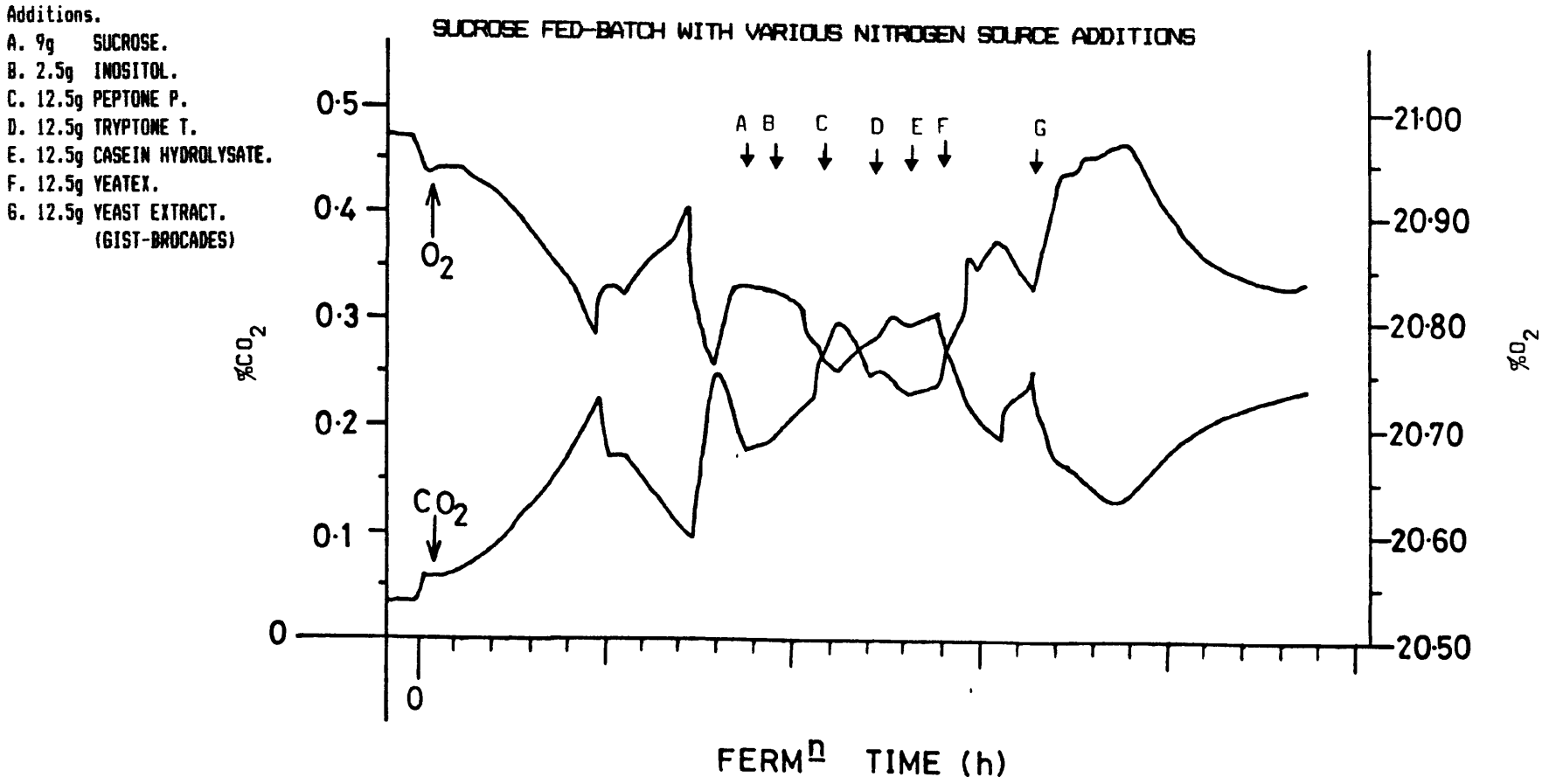


Figure 6.44 Run 38; Effluent Gas Composition vs Time.

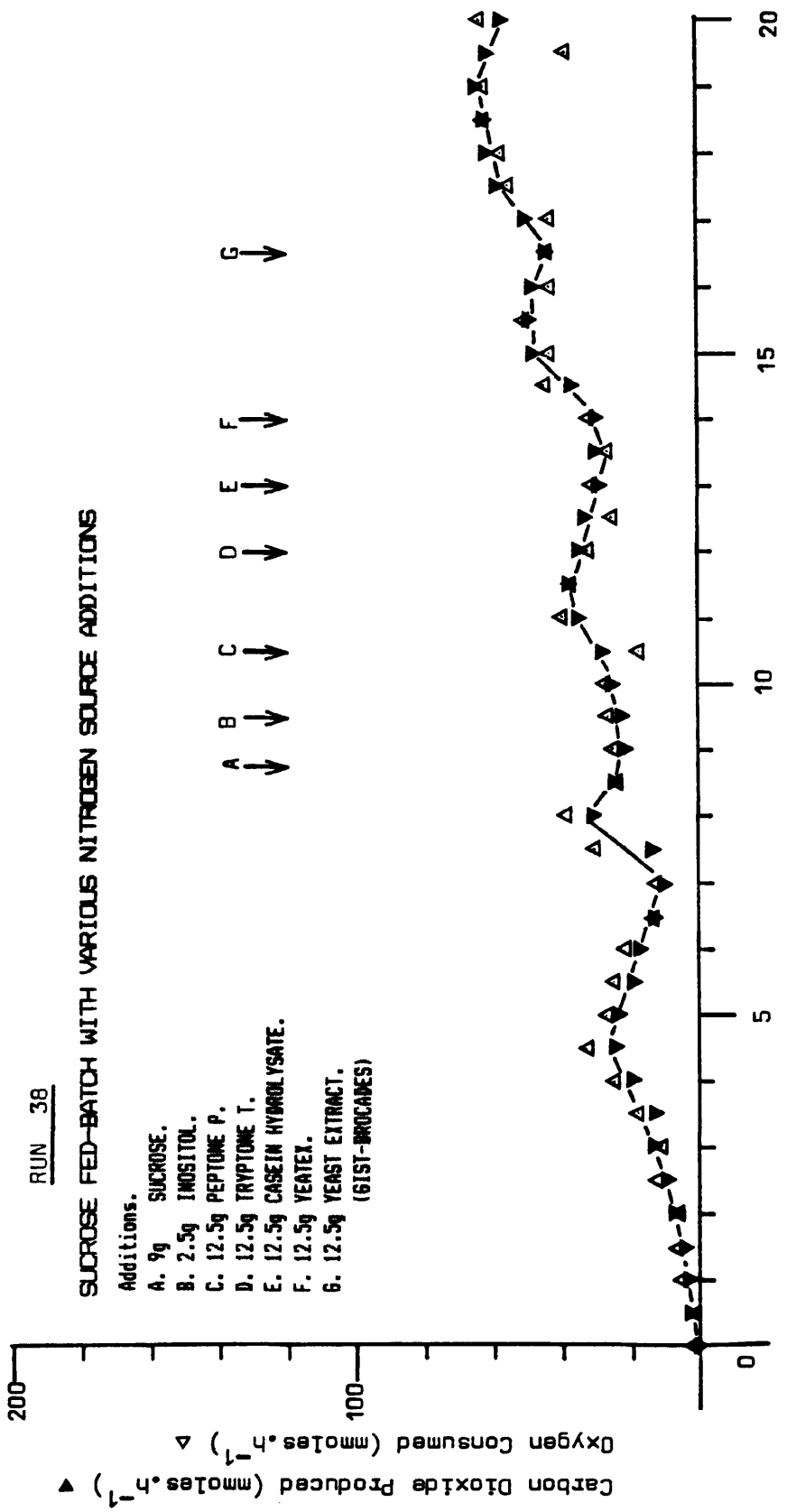


Figure 6.45 Run 38; Rate of CO2 Evolution and O2 Consumption vs Time.

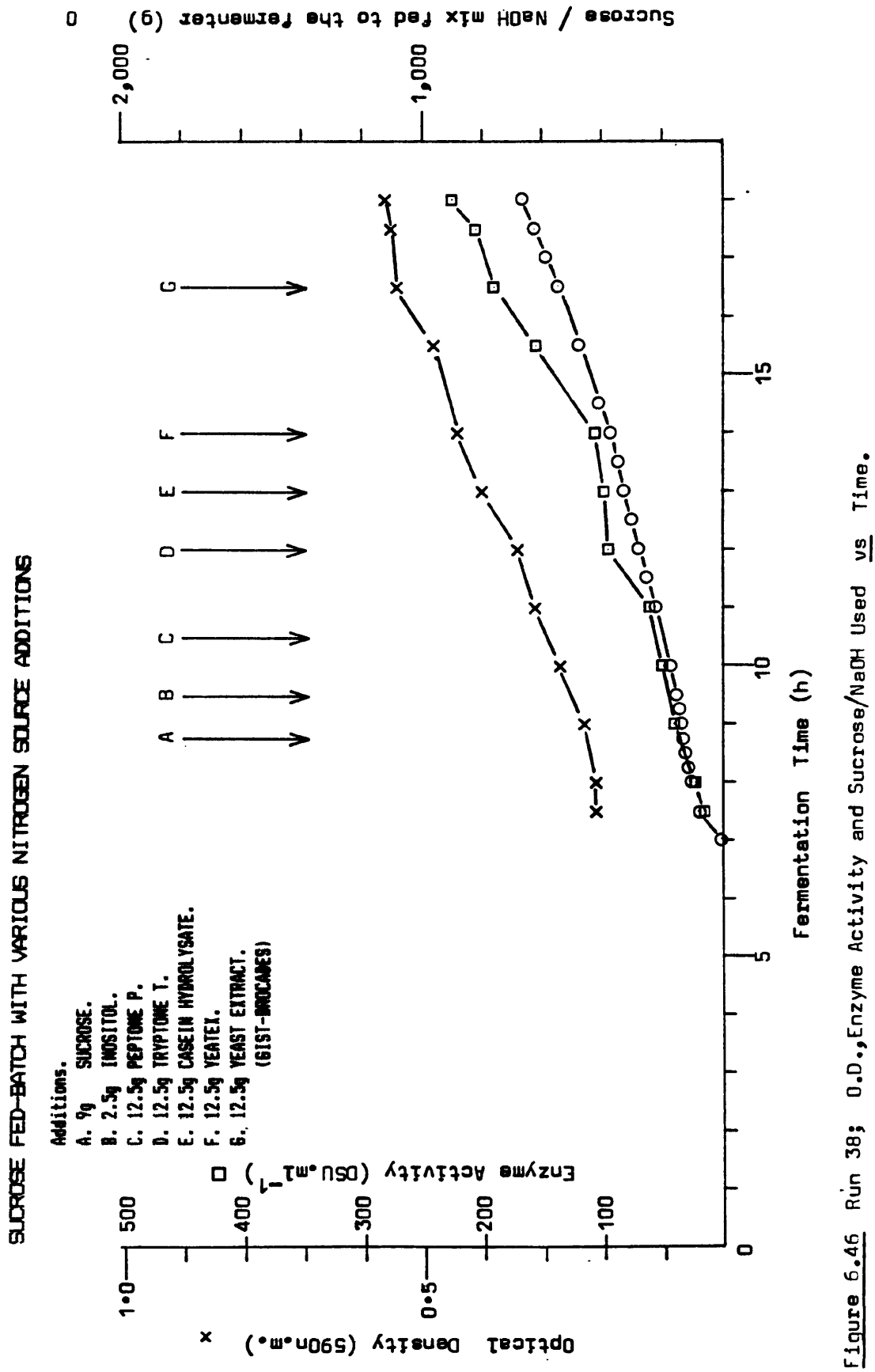


Figure 6.46 Run 38; O.D.,Enzyme Activity and Sucrose/NaOH Used vs Time.

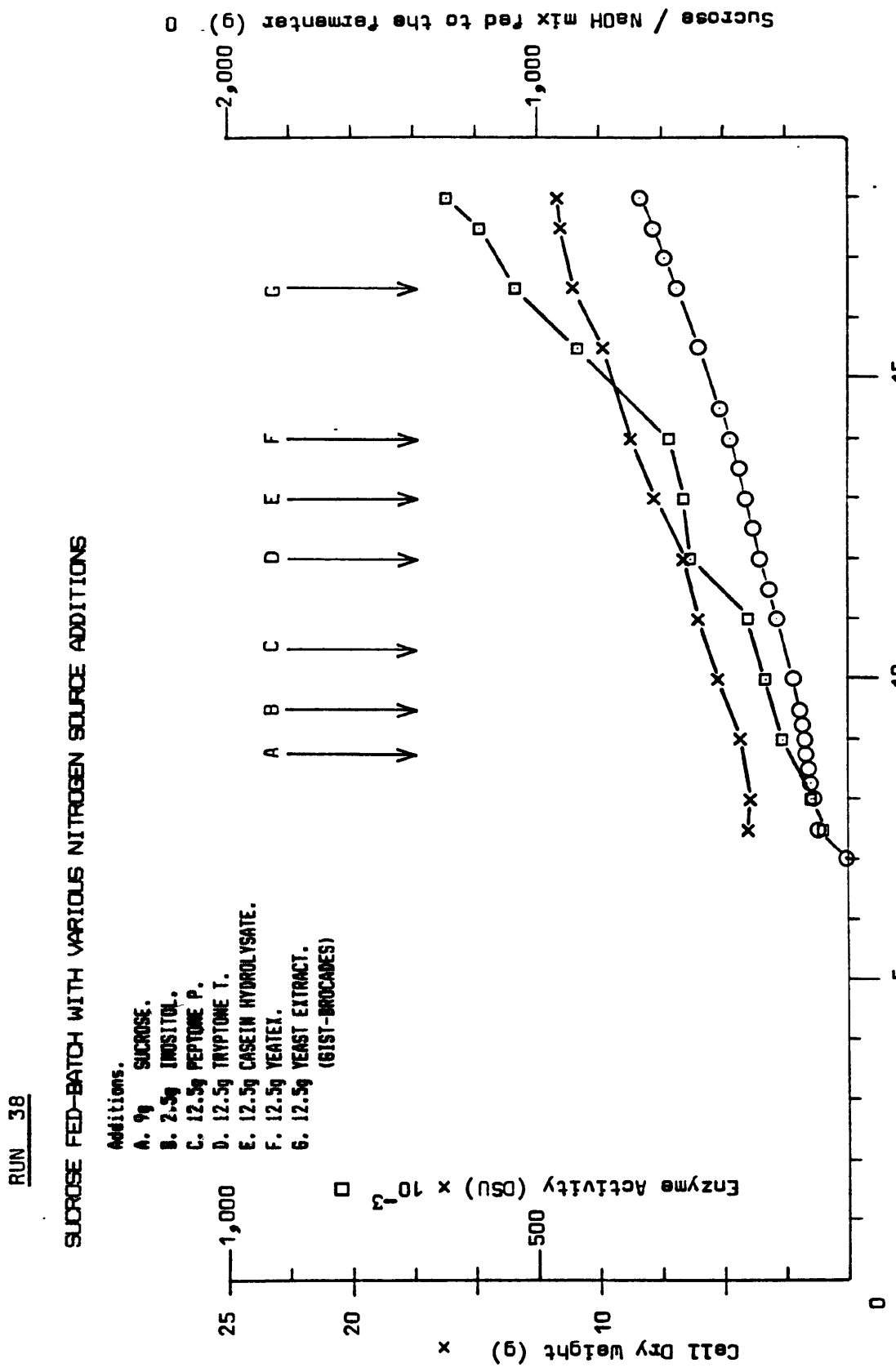


Figure 6.47 Run 38; Dry Weight, Enzyme Activity and Sucrose/NaOH Used vs Time.

the rate of production of the enzyme significantly. The enzyme activity increased from 105 DSU.ml.$^{-1}$ to a maximum of 225 DSU.ml.$^{-1}$ after the addition of the yeast extract at 14 h.

The cell growth from 9 to 17.5 h was linear (Figure 6.47) at a rate of 0.87 g.h.$^{-1}$ with a correlation coefficient of 1.00. The sucrose/NaOH feed had two linear periods, 9.5-14 h and 14.5-18 h with feedrates of 0.042 1.h.$^{-1}$ and 0.063 1.h.$^{-1}$, respectively. Both linear periods have correlation coefficients of 1.00. The increase in the sucrose/NaOH feedrate coincided and was the result of the addition of the yeast extract.

The molar rate of CO_2 production and O_2 consumption are graphically represented in Figure 6.45. The two yeast extract additions, F and G, each resulted in an increase in the cell metabolism. The increases were not great but this may have been due to the size of the addition of only 12.5 g of yeast extract at any one time. Larger additions of yeast extract would probably have resulted in more marked increases in the fermentation respiration rates. Figures 6.44 and 6.45 clearly show that the yeast extract is the only substrate that was examined which produced an increase in respiration, presumably because it contained the limiting nutrient.

Table 6.11 lists the HPLC analysis results of the broth.

TABLE 6.11

RUN 38 HPLC ANALYSIS OF SUGARS

	CONCENTRATIONS ($g.l.^{-1}$)				
TIME (h)	SUCROSE	GLUCOSE	FRUCTOSE	MANNITOL	ETHANOL
7.5	0	1.4	1.3	3.9	0
8	2.9	0	0	6.2	0
9	0	4.1	3.3	5.2	0
10	0	2.4	6.8	6.1	0
11	0	1.9	6.0	9.0	0
12	0	0	4.1	14.7	0
13	0	0.4	1.4	8.6	0
14	0	0.6	1.4	7.9	0
15.5	0	0.8	1.6	9.8	0
16.5	0	2.1	2.3	11.9	0
17.5	0	2.0	2.1	11.5	0
18	0	1.3	2.6	11.7	0
31.0	0	8.5	6.3	19.9	0

The sucrose concentration was zero throughout the fermentation. Glucose and fructose concentrations were low and mannitol, as in previous fermentations, increased in concentration throughout the fermentation. The cell growth and metabolism were proceeding at a sub-optimal rate due to the nutrient limitation, this is evident from Figures 6.45, 6.46 and 6.47. The nutrient limitation at approximately 8 h halted the increase in the bacterial metabolism and thus changed the ratio of sucrose/NaOH that was necessary to maintain a constant mass of sucrose in the fermenter. As in Run 37, 9-12 h, a higher ratio of sucrose/NaOH would have been required to maintain a constant mass of sucrose. However, in contrast to Run 37, sucrose did not accumulate at the end of the fermentation. This was probably due to the late batch additions of yeast extracts. The fermentation was not carbon-limited since there were low concentrations of glucose and fructose in the broth throughout the fermentation.

6.6.5 Addition of Vitamin Mixture (Run 39)

The strains of the genus Leuconostoc are known auxotrophs for a wide range of amino acids and vitamins. Yeast extract is known to be an excellent source of amino acids and vitamins. The alternative nitrogen sources tested in the shake flask work and Run 38 are known to be good sources of amino acids but poor sources of vitamins. A sucrose batch (no sucrose feed, only pH

control) was thus run to examine the response of the cell growth and metabolism to the presence of a number of vitamins (Table 5.9) known to be essential to some strains of Leuconostoc. Literature from Bovril and Gist-Brocades indicates the presence of all these vitamins in their respective yeast extracts. Dextransucrase was not assayed during this fermentation.

Figures 6.48 and 6.49 show the positive response by the bacterial metabolism to the vitamin mixture added after 10.5 h. This result indicates that the nutrient limitation encountered in this work is due to a vitamin or combination of vitamins contained within the mixture. The OD profile of cell growth (Figure 6.50) is unusual and shows no clear exponential phase. Figure 6.51 being on a mass basis gives a better representation of the cell growth. The cell growth does not have an exponential phase (Figure 6.52) and appears to be linear. The rate of cell growth from 6-9 h has been calculated at 2.8 $g.h.^{-1}$ with a linear correlation coefficient of 1.00.

Between 7 and 12.5 h the 2M NaOH feedrate, to control the pH at 6.7, was 0.117 $l.h.^{-1}$ with a linear correlation coefficient of 1.00.

The molar rate of CO_2 production and O_2 consumption (Figure 6.49) illustrates the lack of an exponential phase with CO_2 production and O_2 consumption increasing

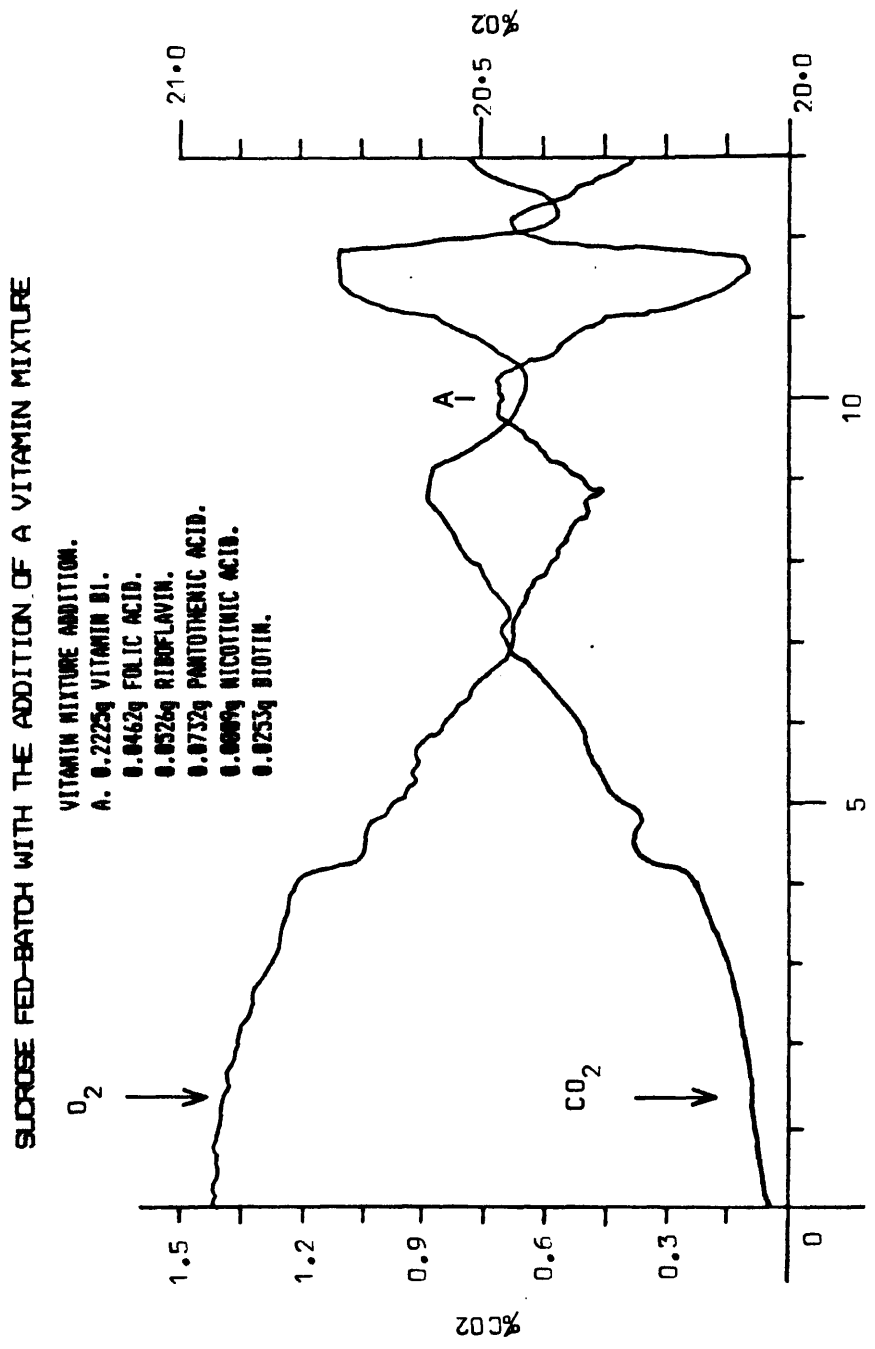


Figure 6.48 Run 39; Effluent Gas Composition vs Time.

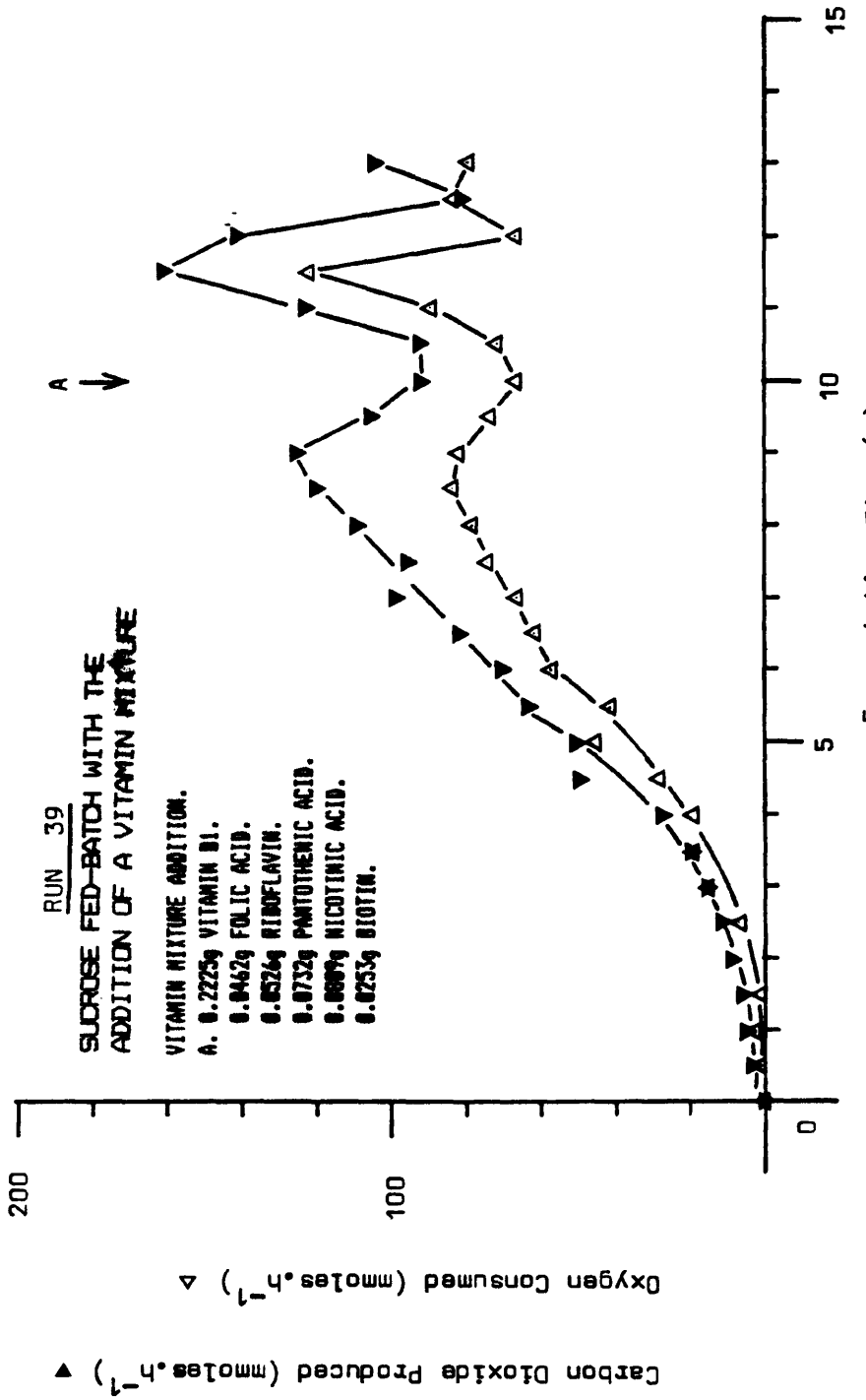


Figure 6.49 Run 39; Rate of CO2 Evolution and O2 Consumption vs Time.

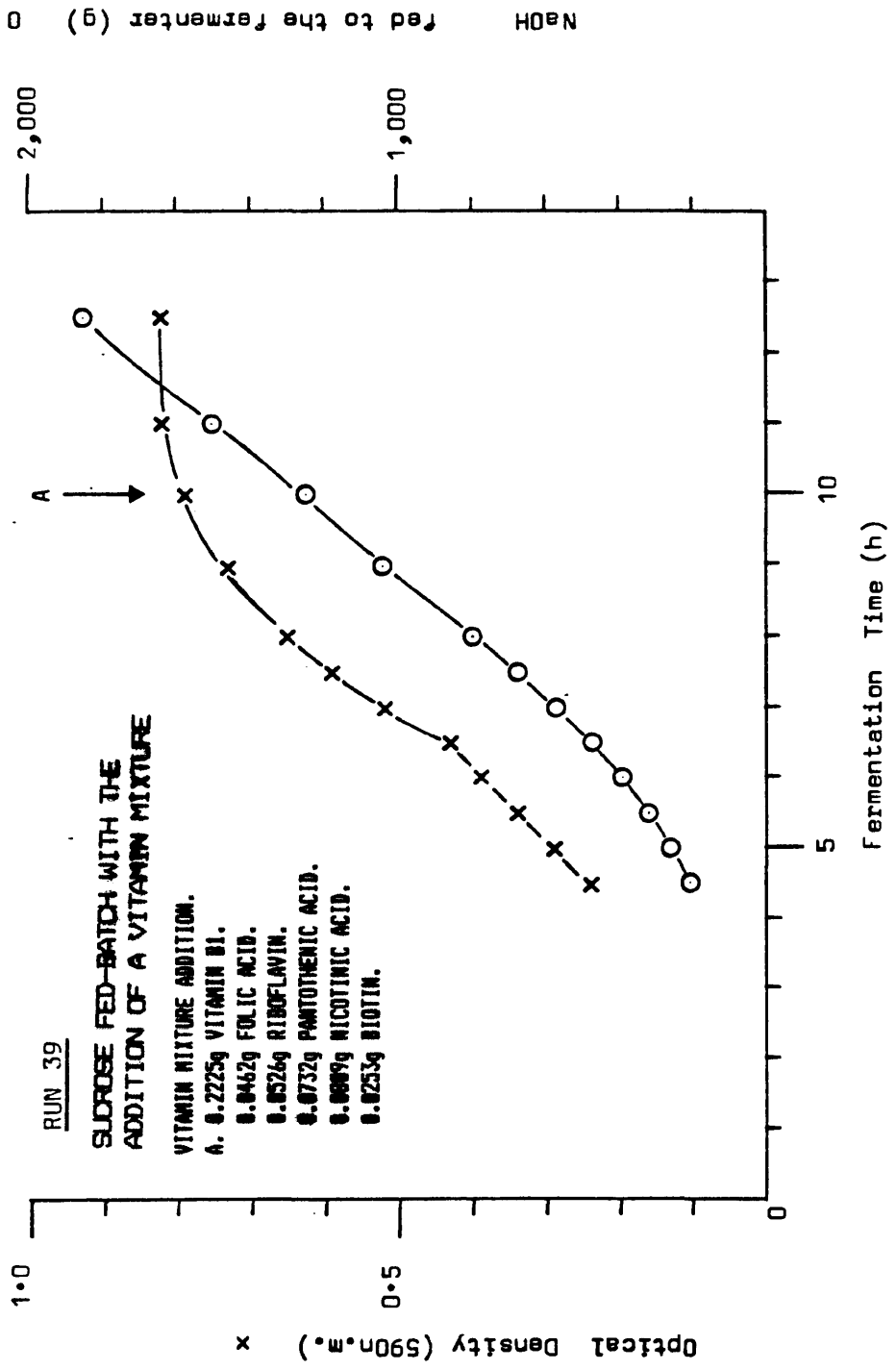


Figure 6.50 Run 39; O.D. and NaOH Used vs Time.

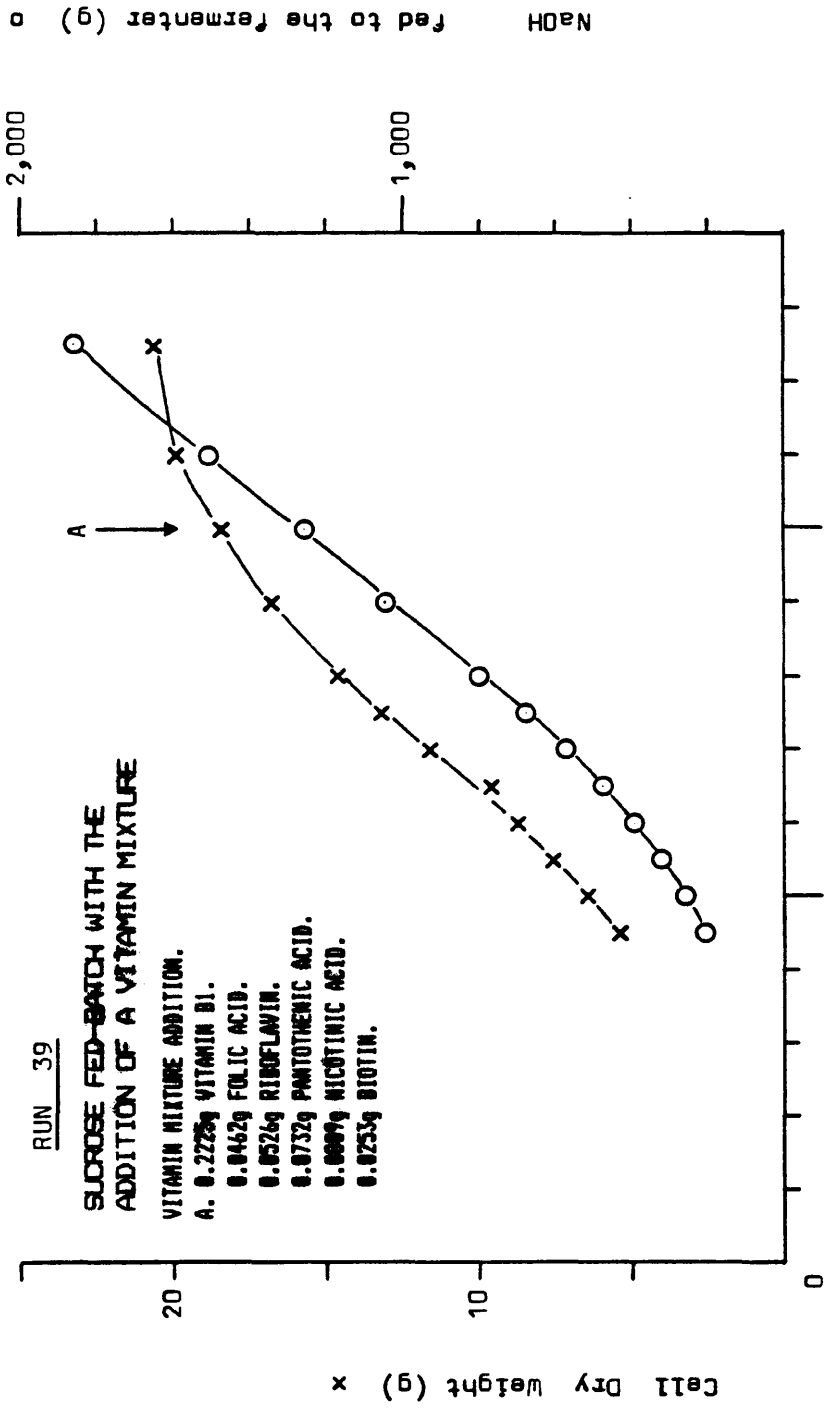


Figure 6.51 Run 39; Dry Weight and NaOH Used vs Time.

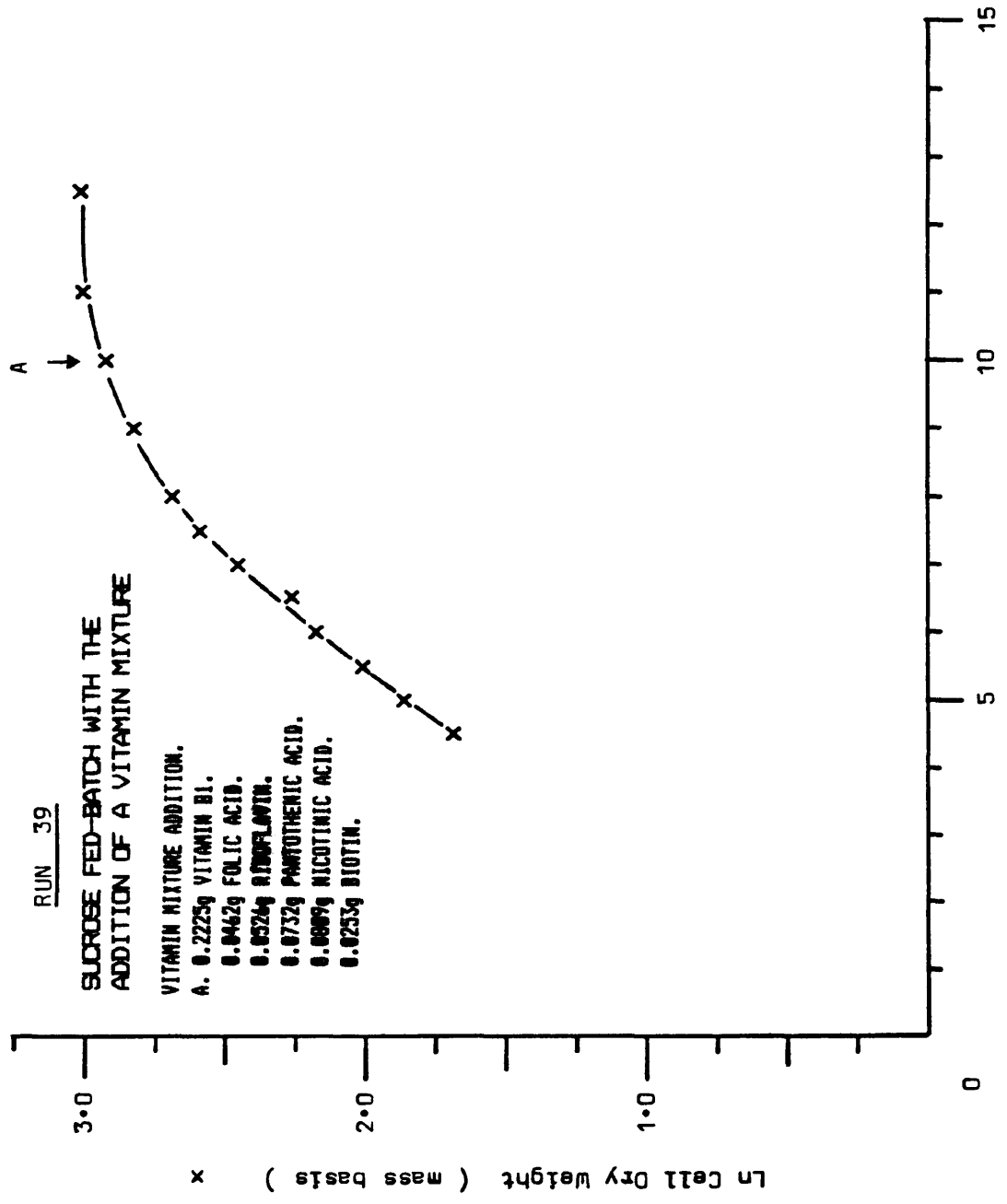


Figure 6.52 Run 39; Ln Dry Weight vs Time.

linearly from approximately 5 to 9 h. At 9 h the nutrient limitation caused the respiration rate to decrease from 83 m Moles O_2.h.$^{-1}$ to 66 m Moles O_2.h.$^{-1}$ and 124 m Moles CO_2.h.$^{-1}$ to 91 m Moles CO_2.h.$^{-1}$. At this point the vitamin mixture was added to the broth; CO_2 production increased from 91 m Moles.h.$^{-1}$ to 158 m Moles.h.$^{-1}$ and O_2 consumption from 66 m Moles.h.$^{-1}$ to 121 m Moles.h.$^{-1}$. This was a batch fermentation and it is thought that the subsequent decrease in the respiration rate was due to the exhaustion of the sucrose substrate. Unfortunately no HPLC analysis of the broth was undertaken for this run.

6.6.6 Separate Vitamin Additions (Run 40)

A sucrose mixed feed fed-batch fermentation was run to determine which vitamin or combination of vitamins were responsible for the initiation of growth and metabolism in Run 39. As soon as the limitation was evident from the gas analysis data, the vitamins (Table 5.10) were added sequentially to the fermenter. A positive response was achieved on the addition of folic acid (Point B, Figure 6.53) at 11 h. Figures 6.53 and 6.54 clearly illustrate the increase in the rate of the bacterial metabolism as determined by the cell respiration rate. From these results it was concluded that the nutrient limitation was due to the folic acid or a combination of the folic acid and vitamin B_{12}.

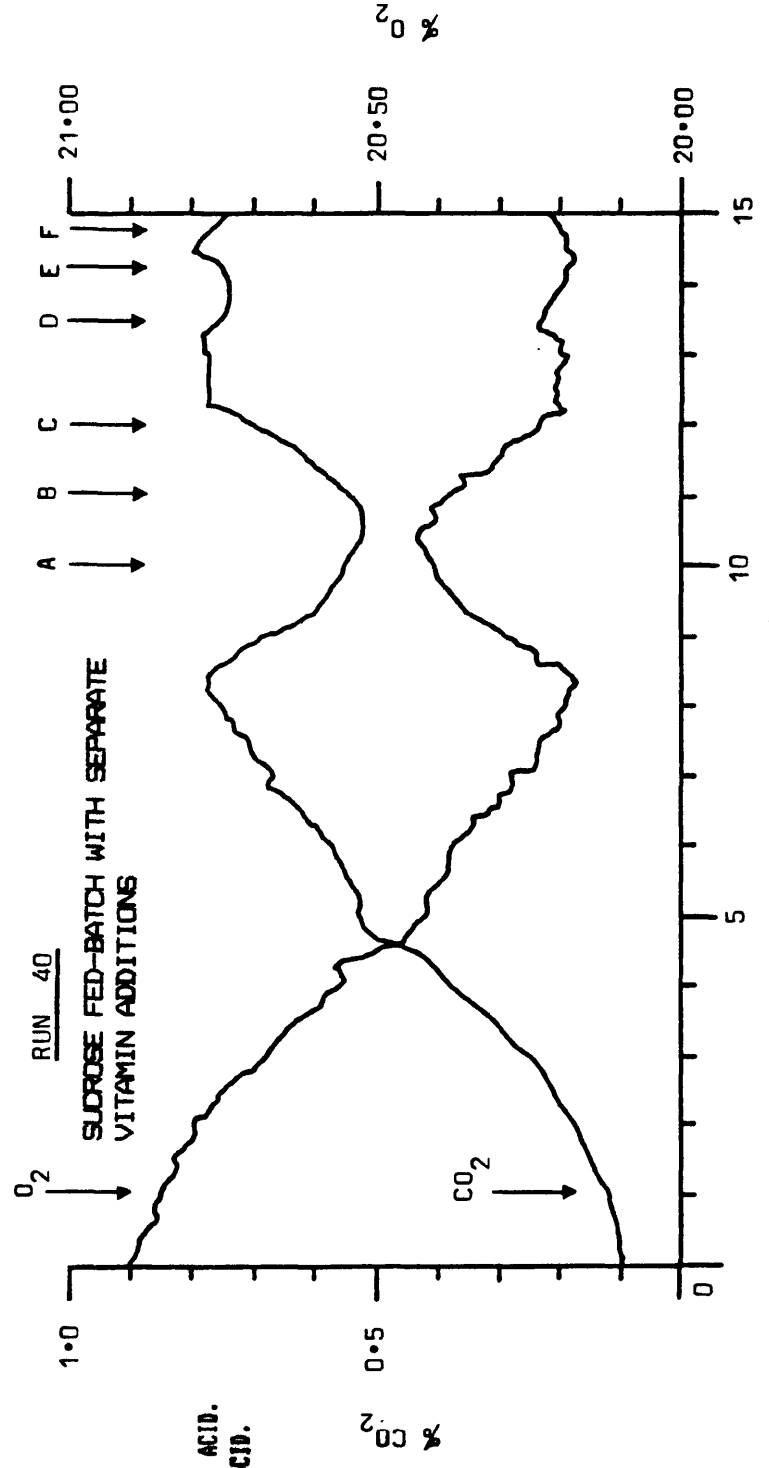


Figure 6.53 Run 40; Effluent Gas Composition vs Time.

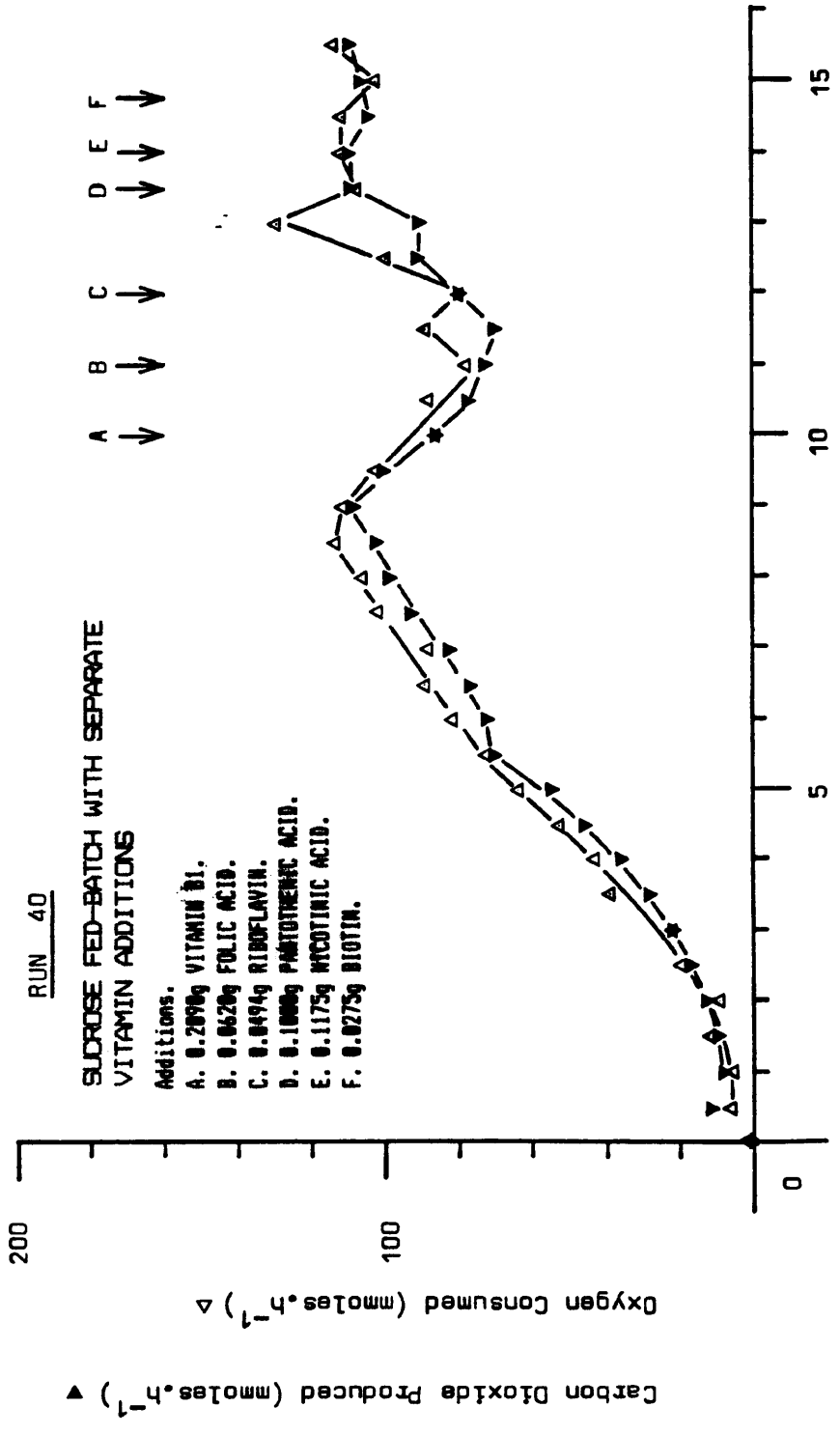


Figure 6.54 Run 40; Rate of CO2 Evolution and O2 Consumption vs Time.

From Figures 6.55 and 6.56 the cell growth appears to be linear from approximately 5.5 to 10 h. The rate of cell growth from 5.5 to 10 h has been calculated at 2.02 $g.h.^{-1}$ with a correlation coefficient of 1.00. A maximum OD of 0.96 was attained in 15 h.

As in Run 36, two distinct linear periods of sucrose/NaOH feed can be determined from Figure 6.55; 7.5-9 h with a feedrate of 0.132 $l.h.^{-1}$ corresponding to 53.7 g sucrose. $h.^{-1}$ and 12-15.5 h with a feedrate of 0.117 $l.h.^{-1}$ corresponding to 47.6 g sucrose.$h.^{-1}$. Both linear periods had correlation coefficients of 1.00.

The molar rate of CO_2 production and O_2 consumption (Figure 6.54) illustrate the lack of an exponential phase with CO_2 production and O_2 consumption increasing linearly between approximately 5 and 9 h. The respiration rate profiles of Runs 39 and 40 are very similar (Figures 6.49 and 6.54). At 9 h the nutrient limitation caused the respiration rate to decrease from 111 m Moles $O_2.h.^{-1}$ to 78 m Moles $O_2.h.^{-1}$ and 108 m Moles $CO_2.h.^{-1}$ to 70 m Moles $CO_2.h.^{-1}$. The addition of 0.062 g of folic acid increased the rate of O_2 consumption to 130 m Moles.$h.^{-1}$ and CO_2 production to 109 m Moles.$h.^{-1}$.

Table 6.12 lists the results of the HPLC analysis of the broth. Between 7 and 14 h the sucrose concentration varied within the range 11.3-15.2 $g.l.^{-1}$ with an average

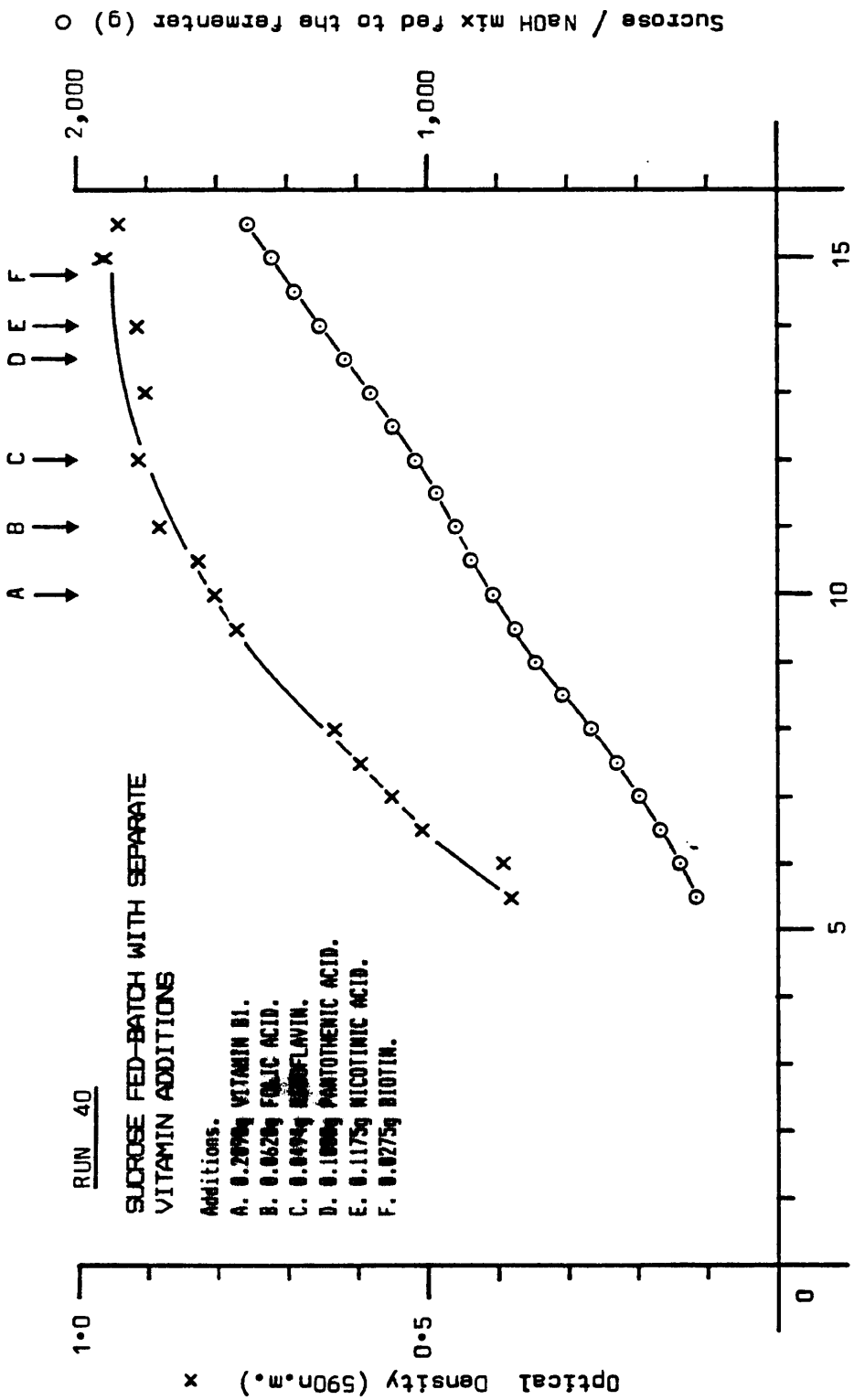


Figure 6.55 Run 40; O.D. and Sucrose/NaOH Used vs Time.

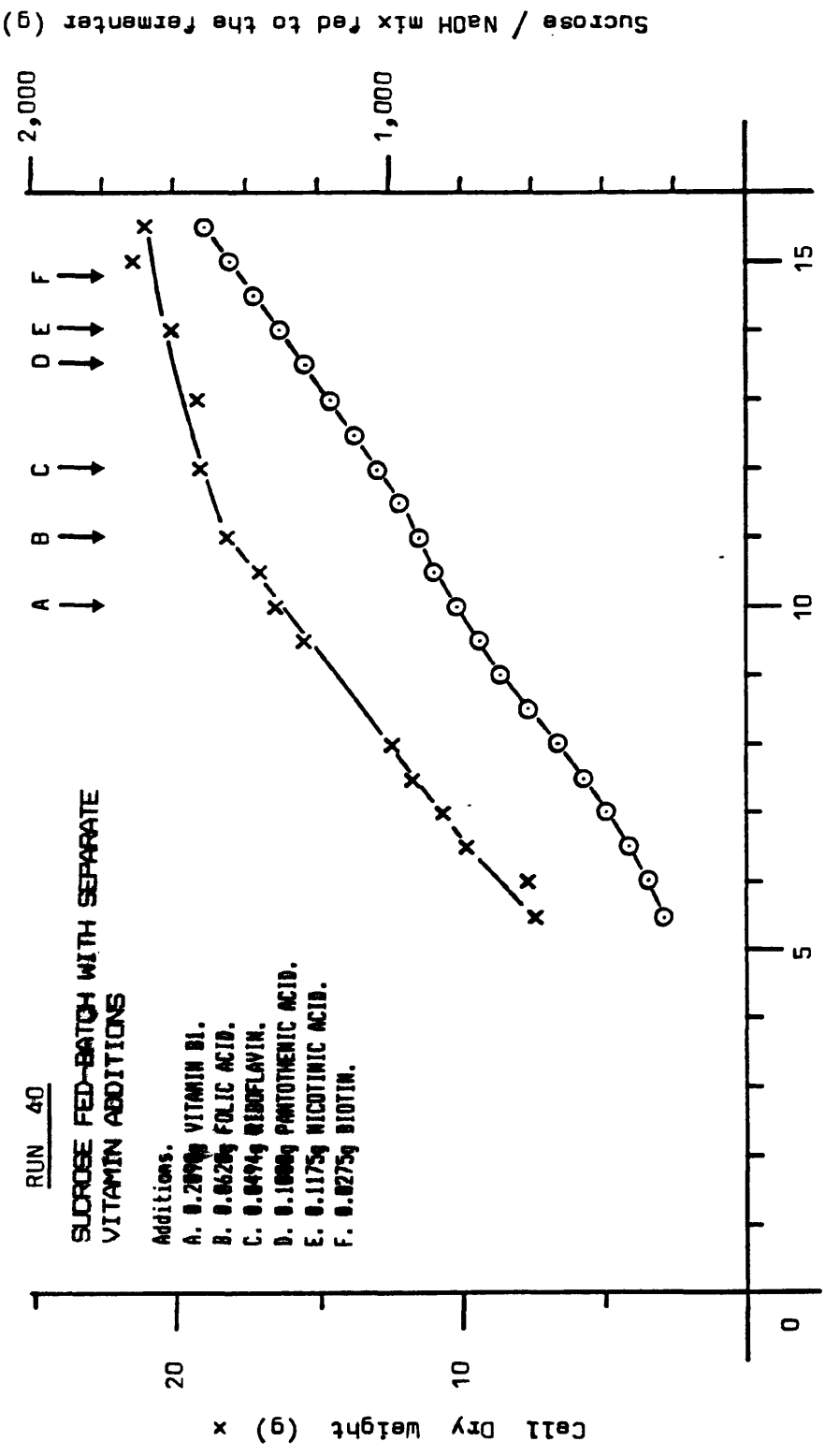


Figure 6.56 Run 40; Dry Weight and Sucrose/NaOH Used vs Time.

TABLE 6.12 RUN 40 HPLC ANALYSIS OF SUGARS AND ORGANIC ACIDS

FERMENTATION TIME	5.5	6	7	8	9.5	10	11	12	13	14	15
SUCROSE	7.1	9.3	13.4	15.2	-	11.3	13.9	13.9	13.7	12.6	-
GLUCOSE	0.2	0.6	1.9	2.5	3.2	3.0	4.0	4.0	5.2	5.8	6.7
FRUCTOSE	1.2	1.4	2.3	2.9	2.9	3.4	3.0	2.7	3.6	3.9	3.5
MANNITOL	11.2	11.9	18.0	19.4	25.8	31.1	39.3	29.1	37.2	40.5	43.9
ETHANOL	0	0	0	0	0	0	0	0	0	0	0
LACTIC ACID	-	-	24.0	27.6	34.9	41.5	43.9	-	-	-	-
ACETIC ACID	-	-	7.9	9.2	13.4	15.1	16.9	-	-	-	-

- indicates no data.

Values are concentration ($g.l.^{-1}$)

of 13.4 $g.l.^{-1}$ indicating that the sucrose/NaOH ratio was approximately correct for this fermentation. Glucose and fructose were present throughout the fermentation at concentrations below 7 $g.l.^{-1}$. Mannitol increased through the fermentation and at 15 h the concentration was 44 $g.l.^{-1}$.

Lactic and acetic acids increased in concentration throughout the fermentation achieving levels of 43.9 $g.l.^{-1}$ and 16.9 $g.l.^{-1}$, respectively, at 11 h. At such high concentrations it is not unreasonable to assume that the presence of the organic acids will become inhibiting to growth and enzyme production.

6.6.7 Additions of Folic Acid (Run 41)

Run 40 had shown that the nutrient limitation was due to folic acid and vitamin B_1. Run 41 was undertaken to demonstrate that the addition of folic acid was responsible for the increase in the cell respiration rate. Figures 6.57 and 6.58 illustrate the response of the cell respiration rate to the addition of 0.1026 g of folic acid at 8.25 h. From this result it was concluded that the nutrient limitation was due to folic acid.

At roughly 10-12 h the cell growth, respiration and enzyme production came to a halt; the culture having effectively reached the stationary phase. A number of additional nutrients (Table 5.11) were added to the

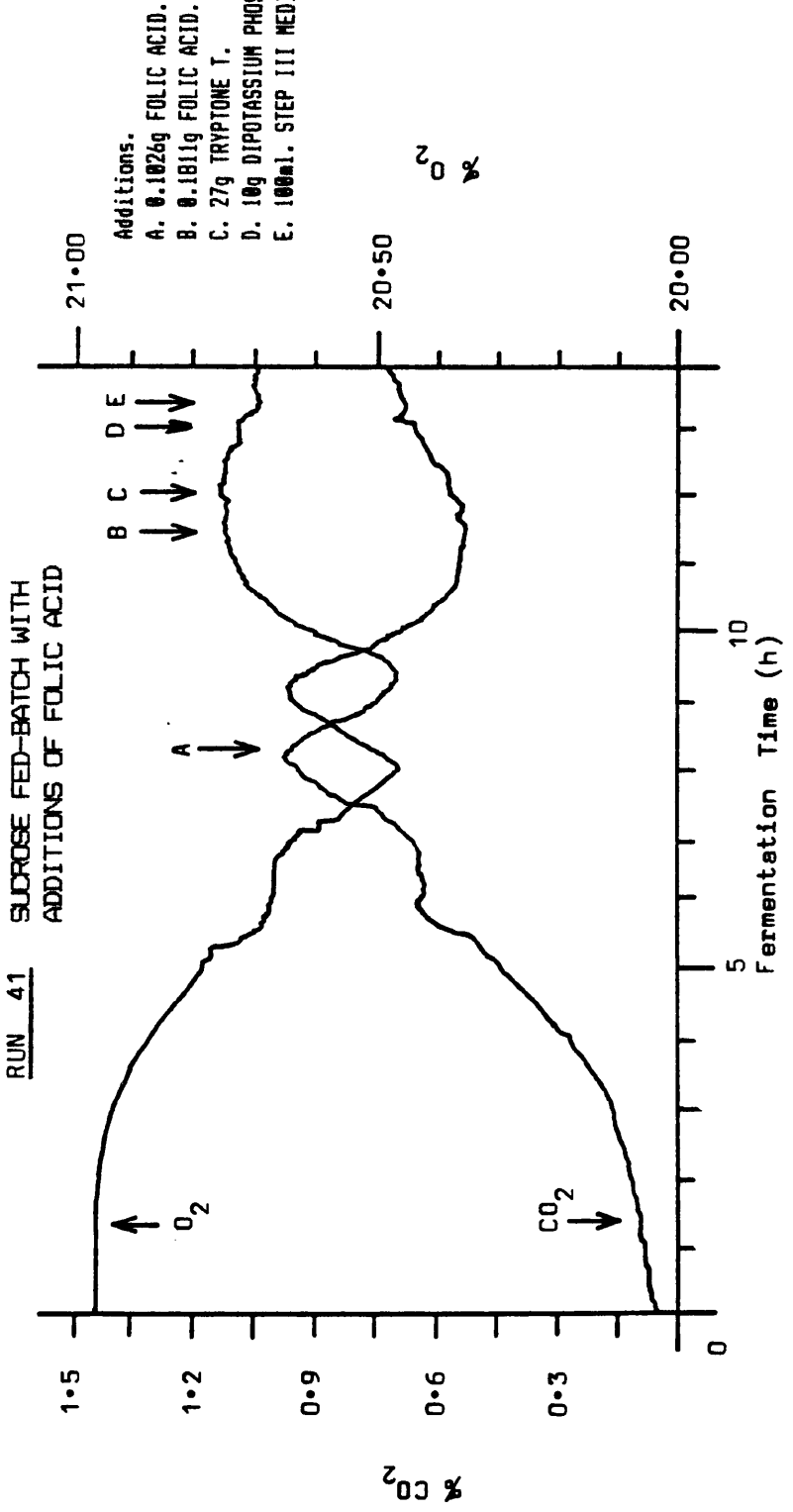


Figure 6.57 Run 41; Effluent Gas Analysis vs Time.

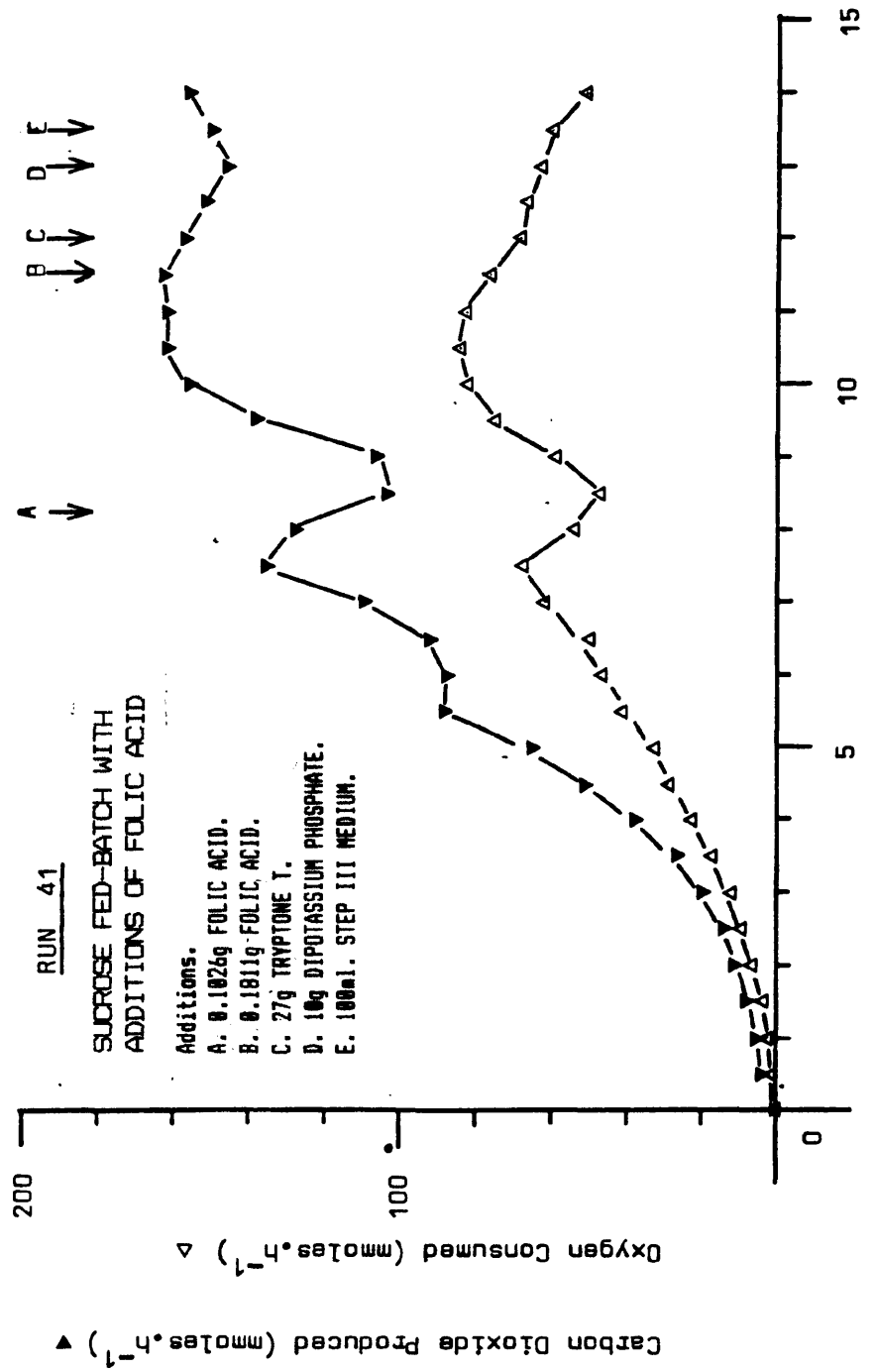


Figure 6.58 Run 41; Rate of CO2 Evolution and O2 Consumption vs Time.

fermentation to investigate the possible occurrences of another nutrient limitation. No response was evident to any of these additions. The final addition (Point F) at 13.5 h was equivalent to the complete growth medium. This did not cause a measurable change in the oxygen uptake but this may have been due to the presence of high concentrations of metabolic end-products which are inhibitory to the cell metabolism and growth. The existence of potentially inhibitory materials was shown by the HPLC analysis of the broth (Table 6.13).

The cell growth showed two periods of linear growth, 6-8 h and 10-14 h (Figure 6.59). The rate of cell growth from 6-8 h has been calculated at 2.8 $g.h.^{-1}$ with a linear correlation coefficient of 1.00 and for 10-14 h at 0.9 $g.h.^{-1}$ with a linear correlation coefficient of 0.99. A maximum OD of 0.84 was attained in 14 h.

The enzyme activity peaked at 395 $DSU.ml.^{-1}$ in 12 h. This high enzyme yield was only matched by that of Run 37 when Gist-Brocades yeast was fed to the fermenter.

As with the cell growth, the sucrose/NaOH had two linear periods (Figures 6.59 and 6.60) at 6.5-8 h and 9-13 h. The sucrose/NaOH feedrate at 6.5-8 h was calculated to be 0.133 $l.h.^{-1}$ corresponding to 54.1 g $sucrose.h.^{-1}$ and at 9-13 h was 0.165 $l.h.^{-1}$ corresponding to 66.8 g sucrose. $h.^{-1}$. Both sets of data had linear correlation

TABLE 6.13 RUN 41 HPLC ANALYSIS OF SUGARS AND ORGANIC ACIDS

FERMENTATION TIME	5	6	7	8	9	10	11	12	13	14
SUCROSE	2.8	0.9	+	+	+	1.2	2.6	3.9	6.2	5.3
GLUCOSE	+	+	0.3	1.1	2.0	2.6	5.8	5.0	7.8	7.2
FRUCTOSE	0.5	0.4	0.5	1.3	1.1	1.1	2.8	1.9	3.1	4.7
MANNITOL	13.3	17.6	18.6	22.3	25.6	26.2	36.2	37.5	39.4	43.1
LACTIC ACID	14.0	20.7	24.0	28.3	29.5	30.7	41.9	47.2	51.1	55.9
ACETIC ACID	5.3	8.8	8.6	10.1	10.4	12.0	16.5	19.4	19.4	22.5

+ indicates present in very low concentrations.

Values are concentration ($g.l.^{-1}$)

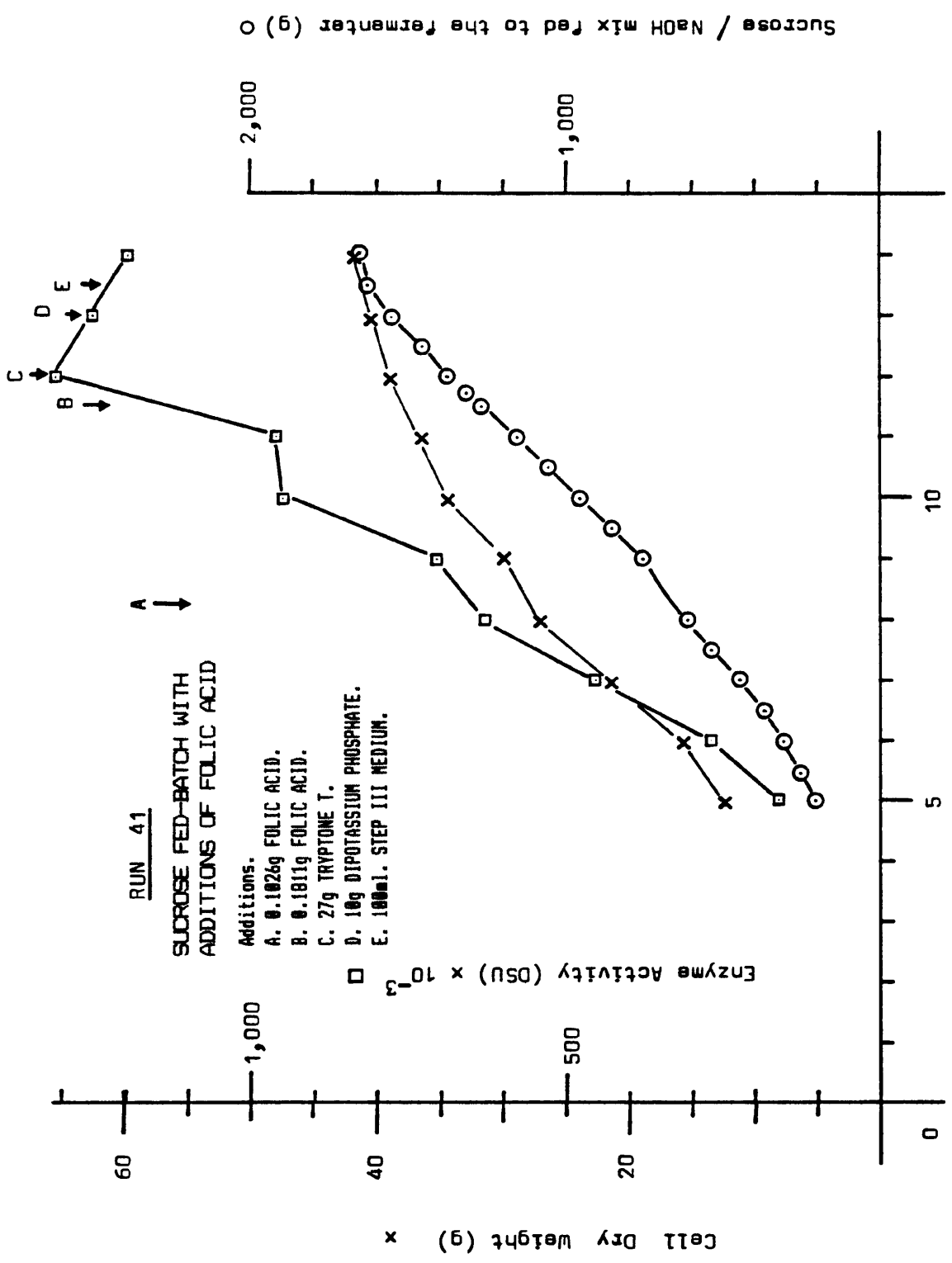


Figure 6.59 Run 41; Dry weight, Enzyme Activity and Sucrose/NaOH Used vs Time.

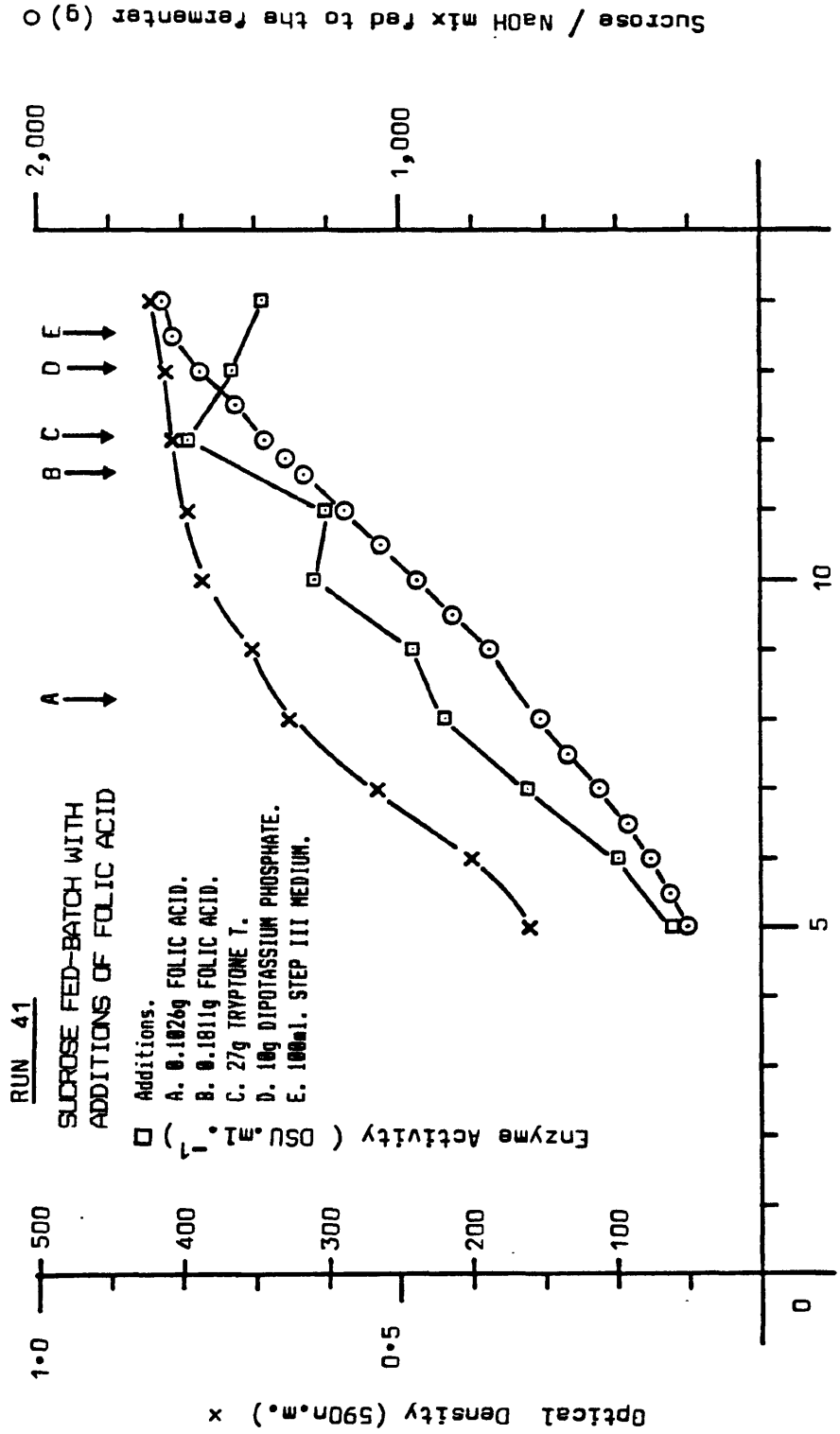


Figure 6.60 Run 41; O.D.,Enzyme Activity and Sucrose/NaOH vs Time.

coefficients of 1.00.

As in previous runs, the molar rates of CO_2 production and O_2 consumption (Figure 6.58) illustrate the lack of an exponential phase. Only the O_2 consumption had a linear period, CO_2 production increased until 5 h then halted for approximately 1.5 hours before increasing again. At 7.5-8 h the nutrient limitation caused the respiration rate to decrease from 67 m Moles $O_2.h.^{-1}$ to 46 m Moles $O_2.h.^{-1}$ and 138 m Moles $CO_2.h.^{-1}$ to 97 m Moles $CO_2.h.^{-1}$. The addition of 0.1026 g folic acid increased the rate of O_2 consumption to 83 m Moles $O_2.h.^{-1}$ and CO_2 production to 163 m Moles $CO_2.h.^{-1}$.

Table 6.13 tabulates the results of the HPLC analysis of the fermentation broth. Sucrose, glucose and fructose were present throughout the fermentation with sucrose at a very low concentration from 6-9 h. As in previous fermentations, the mannitol concentration increased throughout the fermentation to 43.1 $g.l.^{-1}$ at 14 h.

Lactic and acetic acids increased in concentration throughout the fermentation, achieving levels of 55.9 $g.l.^{-1}$ and 22.5 $g.l.^{-1}$, respectively, at 14 h. At 11 h the mannitol, lactic and acetic acid concentrations were very similar to those of Run 40. This comparison has been made on a concentration basis and not a mass basis. However, both fermentations had identical initial

volumes and similar volumes at 11 h. As in Run 40 it is not unreasonable to assume that such high concentrations of organic acids will become inhibitory to growth and enzyme production.

6.7 LINEAR GROWTH AND CONSERVATIVE NUTRIENTS

The overriding factor in all of this work was the presence of an unknown limiting nutrient. Earlier work (McAvoy, 1981) on the sucrose mixed feed fed-batch fermentation did not clearly show the presence of a nutrient limitation although profiles of the sucrose/NaOH feed were linear and not exponential as expected. With respect to the available data, this linearity was initially thought to be due to some aspect of the feed system. Optical density data and thus cell dry weights derived from OD measurements are thought to be unreliable since it has been clearly demonstrated that sucrose concentration has a dramatic effect on the cell wall of Leuconostoc mesenteroides; an increase in sucrose concentration present in the medium resulting in an increase in surface coat dimensions (Brooker, 1976 and 1977). The addition of oxygen and carbon dioxide gas analysers to the fermenter enabled the monitoring of the cells' respiration, giving a more reliable indicator as to how the cells were growing.

Sucrose Mixed Feed Fed-batch (SMFF) with Ohly yeast extract (Run 35) demonstrated that the yeast extract was very important to the bacterial growth, metabolism and enzyme production. The gas analysis data exhibited a sharp halt which occurred at approximately the same time as the halt in the enzyme production. No sharp halt to growth was evident from the OD data and, HPLC data

indicated there was no carbon limitation (Table 6.8).

Run 36, an SMFF with Ohly yeast extract demonstrated that the sharp halt in the bacterial respiration was not due to a phosphate limitation but to something in the Ohly yeast extract. The addition of a pulse of Ohly yeast extract resulted in increased bacterial respiration and bacterial heat evolution.

Continuous feeds of Gist-Brocades yeast extract (Run 37) confirmed the importance of the yeast extract in controlling the bacterial growth, respiration and enzyme production. Feeding of Gist-Brocades yeast extract achieved the highest yields of dextransucrase and for relatively lower quantities of yeast extract.

Pulsed additions of other nitrogen sources to a SMFF (Run 38) showed that only the yeast extract additions markedly affected the bacterial respiration, growth and enzyme production.

The pulsed addition of a vitamin mixture (Run 39) to a SMFF which had demonstrated the sharp halt in bacterial respiration and which subsequently recovered, indicated that the limiting component observed in the yeast extract was a vitamin. The addition of each vitamin to a SMFF fermentation (Run 40) resulted in a response to folic acid. The addition of folic acid to a SMFF fermentation

and the resultant response demonstrated and confirmed that folic acid was the limiting substrate in the yeast extract.

Initial exponential growth followed by linear growth has been shown to occur when a batch process is fed with a limiting substrate at a constant rate. Examples of linear growth produced by feeding glycerol at a constant rate have been reported for Enterobacter cloacae by Yamane and Hirano (1977) and for Klebsiella pnuemoniae by Esener et al (1981). Continuous feed of Gist-Brocades yeast extract (Run 37) to the Leuconostoc mesenteroides culture produced similar results.

Linear growth rates and feed rates have also been observed for SMFF fermentations in which the identified nutrient limitation was demonstrated to be a batch component, folic acid (Runs 39, 40 and 41). As presented in Section 2 of the literature survey a number of workers have reported clear examples of linear growth in connection with the utilisation of trace nutrient components. Nyholm (1976) studied the uptake of phosphate by Chlorella pyrenoidosa, recognised that the early scavenged uptake of the trace nutrient could be followed by its distribution to successive generations and so described such components as 'conservative' nutrients. This rapid uptake of the trace nutrients by relatively small quantities of cells in the early stages of the

culture, out of proportion to the requirements indicated by the large values of the yield coefficients, is a common observation.

Wood and Hitching (1959) demonstrated that Streptococcus faecalis assimilates and stores folic acid even in the presence of a concentration of the vitamin greater than the physiological range. The amount assimilated was dependent on the number of cells. Anderson et al (1987) examining the optimum growth conditions for the induction of tyrosine carboxylase observed exponential growth followed by linear growth which they suggested was due to the limitation of some medium component.

The following is a proposed kinetic formulation to describe linear growth linked to a conservative trace nutrient uptake such as folic acid by Leuconostoc mesenteroides.

A mass balance on the cell growth gives:

$$\frac{dx}{dt} = r_x = \mu x \qquad (1)$$

Michaelis-Menten kinetics for the effect of substrate concentration in the medium states that:

$$\mu = \mu_m \frac{s}{k_s + s} \qquad (2)$$

At high s, $\mu = \mu_m$ and Equation (1) gives:

$$\frac{dx}{dt} = \mu_m x \qquad (3)$$

This results in the familiar exponential growth curve.

At $k_s >> s$, assuming that s in the denominator is zero:

$$\mu = \frac{\mu_m s}{k_s} \qquad (4)$$

and:

$$\frac{dx}{dt} = \frac{\mu_m . s . x}{k_s} \qquad (5)$$

Linear growth requires that dx/dt = constant and this would mean that sx = constant. However, previous work indicates that folic acid as a conservative substrate is consumed almost completely from the medium before the linear growth phase starts and does not decline in proportion to the increase in the cell concentration.

It is proposed here that the specific growth is controlled by the concentration of the folic acid, s_i, inside the cells but still according to Equation (2):

$$\mu = \mu_m \frac{si}{k_{si} + s_i} \qquad (6)$$

If it is assumed that the fixed mass of folic acid (m_t) is scavenged completely by the cells and that it is incorporated into the cellular structure in such small quantities that it can be considered to remain constant, then the internal concentration at all times will be:

$$s_i = \frac{m_t}{xVv} \qquad (7)$$

q

At $s_i \gg k_{si}$, $\mu = \mu_m$,

At low s_i:

$$\mu = \mu_m \quad \frac{m_t}{k_{si}} \cdot \frac{}{xVv} \qquad (8)$$

Then from Equation (1):

$$\frac{dx}{dt} = \mu_m \quad \frac{}{k_{si}} \cdot \frac{m_t = \text{constant}}{Vv} \qquad (9)$$

The implications of this analysis are that from Equation (7), the value of x at which $s_i = s_{ic}$ (when μ changes from μ_m) is proportional to m_t and from Equation (9) the slope of the linear growth is also proportional to m_t. The assumptions are that the given mass of trace nutrient

is rapidly consumed by the cells, is not effected measurably by conversion during metabolism and that the specific growth rate is controlled by the concentration of the trace nutrient inside the cells. This simple analysis has been successfully used to describe the effect of different trace nutrient concentrations on the growth pattern of Bacillus subtilis (Brown et al, 1988) included in Appendix 13.

In the Leuconostoc mesenteroides fermentation the kinetic analysis shows that the mass of trace nutrient in the initial medium determines both the cell concentration at which the linear growth starts and also the slope of the linear growth and sucrose/NaOH feed phases. Experimentally this is clearly demonstrated if Runs 33,34, 35, 40 and 41 are compared (see Table 6.14). Assuming the linear rate of sucrose/NaOH feed is proportional to the linear growth rate and, therefore, a measure of the initial concentration of the folic acid as the conservative limiting nutrient (m_t).

In this work, Yeatex was determined to be a 'poor' yeast extract for bacterial growth and enzyme production (Table 6.14, Runs 33, and 34). Ohly and Gist-Brocades yeast extracts were determined to be 'good' yeast extracts for bacterial growth and enzyme production. (Table 6.14, Runs 35, 40 and 41). From Table 6.14 it can be seen that the 'good' yeast extracts have higher

TABLE 6.14

RUN NUMBER	33	34	35	40	41
Yeast Extract Type	Yeatex	Yeatex	Ohly	Gist-Brocades	Gist-Brocades
Concentration ($g.l.^{-1}$)	40	40	40	40	40
Specific Feedrate of Sucrose/NaOH Solution ($l.l.^{-1}$.medium.$h.^{-1}$)	0.030	0.035	0.066	0.053	0.053
Correlation Coefficient of Feedrate	1.00	1.00	1.00	1.00	1.00

specific sucrose/NaOH feedrates than the bad yeast extract. These results indicate that the 'good' yeast extracts have a higher/more available concentration of folic acid than the bad yeast extract and are in agreement with those predicted by the kinetic analysis. It is probable that the erratic results observed during the continuous culture experiments and attempts at fed-batch experiments prior to Run 33 were due to low and variable values of folic acid present in the Bovril Yeatex extract available at that time.

CHAPTER 7 CONCLUSIONS

1. LiChrosorb and Magnisphere packed columns using an Amine Modified eluent will separate glucose, fructose sucrose and mannitol enabling the determination of their concentrations.

2. Lactic and acetic acids elute from the LiChrosorb and Magnisphere columns as broad, ill-defined peaks which interfere with the peaks of the four sugars. this prevents the use of these columns in investigating samples containing both the sugars and any one of the organic acids.

3. The Bio Rad HPX-87 carbohydrate column can be used to analyse and quantify fructose, mannitol and ethanol. Lactic and acetic acids give good peaks using this column, however, their elution times interfere with those of sucrose and glucose.

4. The Bio Rad HPX-87 organic acid column will separate and quantify the organic acids from the relevant sugars. However, fructose and mannitol have similar elution times and cannot be quantified.

5. The combination of the Bio Rad HPX-87 carbohydrate and organic acid HPLC columns enable the qualitative and quantitative determination of the broad spectrum

of end-products found in the L. mesenteroides B512-F sucrose fermentation.

6. Dextransucrase activity can be assayed by monitoring the rate of sucrose consumption and fructose accumulation using the Bio Rad HPX-87 carbohydrate column. This assay is more specific than the Reducing Sugar Assay which can be erroneous due to the presence of contaminating enzymes.

7. The L. mesenteroides B512-F broth containing the dextransucrase does not contain any detectable levels of contaminating enzymes which hydrolyse the sucrose molecule.

8. The glucose mixed feed fed-batch technique was successful in feeding glucose to the fermentation and maintaining the glucose concentration within a narrow range. A ratio of 1.73 moles of NaOH to 1 mole of glucose was calculated from a glucose batch fermentation. Experimentally, a ratio of 1.82 moles of NaOH to 1 mole of glucose was found to be too high and 1.48 moles of NaOH to 1 mole of glucose to be low. At 1.48 moles of NaOH to 1 mole of glucose the glucose concentration was increasing in a linear fashion at the approximate rate of 0.76 g glucose $l.^{-1}$ broth. $h.^{-1}$ for a 3 litre initial volume fermentation using 40 $g.l.^{-1}$ Yeatex.

9. Glucose fed-batch fermentations did not produce any dextransucrase, confirming the requirement of sucrose as an inducer.

10. The source of yeast extract predetermines the rate of cell growth and enzyme production of L. mesenteroides.

11. The 'continental' yeast extracts were found to be more suitable than Bovril "Yeatex" for L. mesenteroides B512-F cell growth, enzyme and organic acid production.

12. Folic acid was shown to be a limiting factor in the continental yeast extracts clearly demonstrated by off-gas composition changes.

13. Continuous feeds of continental yeast extracts resulted in linear growth and resulted in the highest enzyme yield of 403 DSU.ml.$^{-1}$.

14. Lactic and acetic acid concentrations were found to be high at the end of the fermentation (10-15 hours), typically 55.9 g.l.$^{-1}$ and 22.5 g.l.$^{-1}$ respectively.

15. Linear growth and linear sucrose/NaOH feeds are believed to be caused by the limitation of metabolism by low concentrations of the conservative trace nutrient folic acid.

16. It is proposed that folic acid is scavenged from the medium early in the fermentation and considered to be a 'conservative' nutrient.

17. A simple mechanistic analysis of the linear growth arising from conservative trace nutrient limitation is proposed. The mass of the conservative trace nutrient determines the rates of the linear growth, linear feed of sucrose/NaOH and linear rate of dextransucrase production.

CHAPTER 8 FURTHER WORK

1. Further investigations of the fed-batch process with different initial concentrations of yeast extract and folic acid and different feed rates of the same materials should be made to maximise the production of dextransucrase.

2. Studies should be made of the scavenging uptake of folic acid and to determine the yield coefficient, to further illucidate the mechanism of conservative trace nutrient limitation.

3. Continuous culture experiments should be undertaken to further assess the effects of different nutrient limitations, especially sucrose, yeast extract and vitamins, and the dilution rate on growth and enzyme production.

4. Attempts should be made to analyse the stoichiometry of the metabolism using respiration data and organic chemicals concentrations from HPLC measurements.

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APPENDICES

APPENDIX 1.1

The batch culture used to initiate a continuous culture was grown on the following medium.

Component	Concentration ($g.l.^{-1}$)
Sucrose	25
Yeatex	20
K_2HPO_4	10
Antifoam (PPG 2000)	0.5 $ml.l.^{-1}$
R* salts	5 $ml.l.^{-1}$

R* salts:-

$MnSO_4$ H_2O	2.0
$MgSO_4$ $7H_2O$	40.0
NaCl	2.0
$FeSO_4$ $7H_2O$	2.0

Initially, the medium was prepared and then sterilised in a five litre aspirator at 10 p.s.i. for 20 minutes. However, later work involved sterilisation of the medium in three separate amounts.

1. Sucrose, R* salts and antifoam.
2. Yeast extract (Yeatex).
3. K_2HPO_4.

The individual media were sterilised at 10 p.s.i. for 20 minutes and then mixed in the fermenter when cool. Mass and volume measurements were calculated to give the correct final concentrations.

APPENDIX 1.2

Basic medium for Runs 1, 2, 3, 4, 5 and 6.

1.2.1 Composition

Nutrient	Concentration ($g.l.^{-1}$)
Glucose	variable (as indicated)
Yeast extract (Yeatex PASTE)	variable (as indicated)
Dipotassium hydrogen orthophosphate (K_2HPO_4)	20
R* salts	5 $ml.l.^{-1}$
Antifoam (PPG 2000) (Polypropylene Glycol 2000)	0.5 $ml.l.^{-1}$

R* salts:-

$MgSO_4$ $7H_2O$	40
$MnSO_4$ H_2O	2
NaCl	2
$FeSO_4$ $7H_2O$	2

1.2.2 Preparation

The glucose, yeast extract, phosphate, R* salts and antifoam were dissolved in distilled water to the correct concentration, placed in a 5 litre aspirator and sterilised at 10 p.s.i. for 30 minutes. After sterilisation and cooling, the medium was transferred aseptically to the sterile fermenter.

APPENDIX 1.3

FEEDS

1.3.1 Run 1

1.5 litres of 2M NaOH was sterilised in a conical flask for 30 minutes at 10 p.s.i. and then transferred aseptically to a sterile 2 litre aspirator.

For calculations:-

1,500 ml. of 2M NaOH = 1,567g (in feed system)

1 ml. = 1.0448g weight

1.3.2 Run 2

a. 90g NaOH dissolved in 400 ml. of distilled water.

b. 222g glucose disssolved in 600 ml. of distilled water.

Each one of the above solutions was sterilised in a conical flask at 8 p.s.i. for 20 minutes then allowed to cool. Once cooled the solutions were ready for aseptic transfer and mixing in a sterile 2 litre aspirator (feed reservoir) prior to inoculation of the fermenter.

For calculations:-

1g mass of feed = 0.89494 ml.

1 ml. of feed = 1.11739g mass

1.3.3 Run 3

a. 90g NaOH dissolved in 400 ml. of distilled water.

b. 273g glucose dissolved in 600 ml. of distilled water.

Sterilisation procedure as in Run 2.

For calculations:-

1g mass of feed = 0.89072 ml.

1 ml. of feed = 1.12269g mass

1.3.4 Run 4

Glucose/NaOH feed as previously described for Run 3. Also, 1.0 litre of 2M Ammonium Hydroxide (NH_4OH). 35g of Ammonia (2 moles of NH_3) aseptically made up to 1.0 litre in a sterile 2 litre aspirator with sterile distilled water.

Mass of feed = 980g

1.3.5 Run 5

Glucose/NaOH feed as previously described for Run 3.

1.3.6 Run 6

Two feed solutions were prepared, a glucose/NaOH mix and a glucose/NH_4OH mix.

a. 45g NaOH dissolved in 200 ml. of distilled water.

b. 136.5g glucose dissolved in 300 ml. of distilled water.

Sterilisation procedure as in Run 2.

1 ml. of feed = 1.11739g mass of feed

c. 273g glucose dissolved in 500 ml. of distilled water.

Solution (c) was sterilised in a 2 litre aspirator at 8 p.s.i. for 20 minutes and allowed to cool before use.

d. 38.25g of NH_3(124.2 ml. of NH_3 solution) mixed with 500 ml. of sterile distilled water.

Mass of feed = 1.281g

APPENDIX 1.4

1.4.1.1

RUN 1 Fermentation Results

Time (h)	O.D. (590n.m)	Mass of 2M NaOH Fed to the Fermenter (g)	Glucose Concentration (gl^{-1})
0	-	0	32.2
4	0.14	77	28.7
4.5	0.16	103	27.7
5	0.19	138	26.1
5.5	0.23	184	23.6
6	0.29	234	20.6
6.5	0.34	305	20.1
7	-	-	-
7.5	0.42	430	13.3
8	0.50	504	8.1
8.5	0.50	535	7.0
9	-	543	-
9.5	-	-	-
10.25	0.54	565	4.9
10.50	-	570	4.9
A. 10.5	addition of 40g glucose		
11	-	609	-
12	0.6	722	7.0
12.5	0.6	773	3.9

1.4.1.2

RUN 1 Bacterial Growth

Time (h)	O.D. (590n.m)	Log_{10} O.D.	In O.D.
4	0.14	-1.146	-1.966
4.5	0.16	-1.204	-1.833
5	0.19	-1.279	-1.661
5.5	0.23	-1.362	-1.470
6	0.29	-1.462	-1.238
6.5	0.34	-1.531	-1.079
7.5	0.42	-1.623	-0.868
8	0.50	-1.699	-0.693
8.5	0.50	-1.699	-0.693
10.25	0.54	-1.732	-0.616
12	0.6	-1.778	-0.511
12.5	0.6	-1.778	-0.511

$$\mu \text{ max} = \frac{-1.079 + 1.661}{1.5}$$

$$= 0.388 \ h^{-1}$$

1.4.1.3

RUN 1 Volume Variation

Time(h)	Mass of 2M NaOH Fed to the Fermenter (g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	-	4,300	-	4,300
4	77	74	4,374	50	4,324
4.5	103	99	4,349	100	4,299
5	138	132	4,332	150	4,282
5.5	184	176	4,326	200	4,276
6	234	224	4,324	250	4,274
6.5	305	292	4,342	300	4,292
7.5	430	412	4,412	350	4,362
8	504	482	4,432	400	4,382
8.5	535	512	4,412	450	4,362
9	543	520	4,370	-	4,370
10.25	565	541	4,391	500	4,341
A. 10.5	100 ml of glucose solution		4,441	-	4,441
10.5	570	546	4,446	-	4,446
11	609	583	4,483	-	4,483
12	722	691	4,591	550	4,541
12.5	773	740	4,590	600	4,540

1.4.1.4

RUN 1 NaOH Fed to the Fermenter

Time(h)	Mass of 2M NaOH Fed to the Fermenter (g)	Mass of NaOH Fed to the Fermenter (g)	Mass of NaOH Fed to the Fermenter (M)
4	77	6.19	0.155
4.5	103	8.22	0.206
5	138	11.01	0.275
5.5	184	14.68	0.367
6	234	18.72	0.468
6.5	305	24.40	0.610
7.5	430	34.36	0.859
8	504	40.29	1.007
8.5	535	42.82	1.071
9	543	43.46	1.087
10.25	565	45.21	1.130
10.5	570	45.57	1.139
11	609	48.72	1.218
12	722	57.73	1.443
12.5	773	61.83	1.546

1.4.1.5

RUN 1 Glucose Consumed

Time(h)	Volume of Broth Prior to Sample Taken (ml)	Glucose Concentration (gl^{-1})	Mass of Glucose Present (g)	Mass of Glucose Consumed (g)	Moles of Glucose Consumed (M)
0	4,300	32.2	138.5	0	0
4	4,374	28.7	125.5	13.0	0.072
4.5	4,349	27.7	120.5	18.0	0.100
5	4,332	26.1	113.1	25.4	0.141
5.5	4,326	23.6	102.1	36.4	0.202
6	4,324	20.6	89.1	49.4	0.274
6.5	4,342	20.1	87.3	51.2	0.284
7.5	4,412	13.3	58.7	79.8	0.443
8	4,432	8.1	35.9	102.6	0.570
8.5	4,412	7.0	30.9	107.6	0.598
10.25	4,391	4.9	21.5	117.0	0.650
10.5	4,446	4.9	21.8	116.7	0.648
A. Added 40g of glucose					
12	4,591	7.0	32.1	146.4	0.813
12.5	4,590	3.9	17.9	160.6	0.892

1.4.1.6

RUN 1 Moles of Glucose and NaOH Fed to The Fermenter

Time (h)	Moles of NaOH Fed to the Fermenter (M)	Moles of Glucose Consumed (M)
0	0	0
4	0.155	0.072
4.5	0.206	0.100
5	0.275	0.141
5.5	0.367	0.202
6	0.468	0.274
6.5	0.610	0.284
7.5	0.859	0.443
8	1.007	0.570
8.5	1.071	0.598
10.25	1.130	0.650
10.50	1.139	0.648
12	1.443	0.813
12.5	1.546	0.892

Moles NaOH -vs- Moles Glucose

r = 0.99

a = 0.018

b = 0.578

RUN 2 Fermentation Results

Time (h)	O.D. (590n.m)	Mass of Glucose/ NaOH Mix Fed to the Fermenter(g)	(ml.)	Glucose Concentration (gl^{-1})
3.5	-	76	(85)	-
4	0.15	108	(121)	6.3
4.5	0.17	148	(165)	6.0
5	0.21	201	(225)	5.6
5.5	0.24	225	(251)	5.4
6	-	241	(269)	-
6.5	-	259	(289)	-
6.75	0.29	264	(295)	4.7
A. 6.75	Added 80g YEATEX + 400 ml H_2O 1.95g glucose added from feed system to correct deviation due to yeast extract acidity			
6.75	0.26	275	(307)	6.1
7	-	275	(307)	-
7.75	0.29	293	(327)	6.0
8	-	300	(335)	-
8.25	0.29	305	(341)	5.9
8.5	-	312	(349)	-
9	-	326	(364)	-
11	0.35	367	(410)	5.4
B. 11	Add 24g of glucose			
11	0.33	367	(410)	11.8
11.5	0.34	407	(455)	11.5
12	0.35	478	(534)	11.1
12.5	0.43	551	(616)	10.6
13	0.44	646	(722)	8.6
13.5	0.52	703	(786)	8.5

1.4.2.2

RUN 2 Bacterial Growth

Time (h)	O.D. (590n.m)	$\log_{10}$O.D.	ln O.D.
4	0.15	-1.176	-1.897
4.5	0.17	-1.230	-1.772
5	0.21	-1.322	-1.561
5.5	0.24	-1.380	-1.427
6.75	0.29	-1.462	-1.238
A. addition 80g YEATEX + 400 ml H_2O + 1.95g glucose			
6.75	0.26	-1.415	-1.347
7.75	0.29	-1.462	-1.238
8.25	0.29	-1.462	-1.238
11	0.35	-1.544	-1.050
B. addition 24g of glucose			
11	0.33	-1.519	-1.109
11.5	0.34	-1.532	-1.079
12	0.35	-1.544	-1.050
12.5	0.43	-1.634	-0.844
13	0.44	-1.644	-0.821
13.5	0.52	-1.716	-0.654

RUN 3 Fermentation Results

Time (h)	O.D. (590n.m)	Mass of Glucose/ NaOH Mix Fed to the Fermenter(g)	Glucose Concentration (gl^{-1})
0	-	0	11.1
4	0.12	78	10.0
4.5	0.15	119	10.7
5	0.18	159	10.0
5.5	0.22	207	11.2
6	0.27	271	11.1
6.5	0.31	338	11.9
7	0.36	411	13.0
8	0.43	580	13.2
8.5	0.50	675	14.1
9	0.52	768	14.9
9.5	0.56	862	-
10	0.58	956	15.6
10.5	-	1,053	-
11	0.63	1,148	16.0
12	0.65	1,302	16.5
12.5	0.67	1,367	17.3
13	0.68	1,435	16.9
13.5	0.68	1,494	16.5

Feedrate 7 - 11h $r = 1.00$

$a = -897.74$

$b = 185.51\ g.h^{-1}$

1g mass of feed = 0.89072 mls volume

$\therefore$ Feedrate 7 - 11h $= 185.51\ g.h^{-1}$

$= 37.10\ g\ glucose.h^{-1}$

1.4.3.2

RUN 3 Bacterial Growth

Time (h)	O.D. (590n.m)	$\log_{10}$O.D.	ln O.D.
4	0.12	-1.079	-2.120
4.5	0.15	-1.176	-1.897
5.5	0.22	-1.342	-1.514
6	0.27	-1.431	-1.309
6.5	0.31	-1.491	-1.171
7	0.36	-1.556	-1.022
8	0.43	-1.634	-0.844
8.5	0.50	-1.699	-0.699
9	0.52	-1.716	-0.654
9.5	0.56	-1.748	-0.580
10	0.58	-1.763	-0.545
11	0.63	-1.799	-0.462
12	0.65	-1.813	-0.431
12.5	0.67	-1.826	-0.400
13	0.68	-1.833	-0.386
13.5	0.68	-1.833	-0.386

4 - 6h $r = 1.00$

$a = -3.715$

$b = 0.401$

μ max $= 0.401 h^{-1}$

1.4.4.1

RUN 4 Fermentation Results

Time (h)	O.D.(590n.m)	Mass of Glucose/ NaOH Mix Fed to the Fermenter(g)	Glucose Concentration (gl^{-1})
0	-	0	10.2
5	0.12	205	15.5
5.5	0.16	287	14.4
6	0.19	314	16.7
6.5	0.21	357	16.4
7	0.24	412	16.3
7.5	0.29	470	16.4
8	0.35	532	16.9
9	0.4	695	18.4
9.5	0.43	778	18.2
10.25	0.46	901	19.0
10.75	0.5	990	20.5
11.25	0.5	1,061	18.6
11.75	-	1,157	-
A. 11.75	Glucose/NaOH mix replaced with 2M NH_4OH		
11.75	-	0	-
12	0.52	34	19.8
12.5	0.56	100	-
13	0.56	106	16.33
B. 13	60g of glucose was added to the fermenter. Volume 150 ml.		
13	0.54	-	-
13.5	0.54	176	32.9
14	0.56	245	26.8
14.5	0.58	314	23.5
15	0.59	377	21.5
15.5	0.60	438	17.7
16	-	500	-

1.4.4.1 (CONT'D)

Feedrate 8 - 11.75h $r = 1.00$

$a = -795.5$

$b = 165.71\ g.h^{-1}$

$\therefore$ Feedrate $= 165.71\ g.h^{-1}$

$= 33.14\ g\ glucose.h^{-1}$

Feedrate of 2M NH_4OH 13 - 16h

$r = 1.00$

$a = -1564.88$

$b = 129.26$

$\therefore$ Feedrate $= 129.26\ g.h^{-1}$

$= 131.90\ mls.h^{-1}$

1.4.4.2

RUN 4 Bacterial Growth

Time (h)	O.D. (590n.m)	$\log_{10}$O.D.	In O.D.
5	0.12	-1.079	-2.120
5.5	0.16	-1.204	-1.833
6	0.19	-1.279	-1.661
6.5	0.21	-1.322	-1.561
7	0.24	-1.380	-1.427
7.5	0.29	-1.462	-1.238
8	0.35	-1.544	-1.080
9	0.40	-1.597	-0.916
9.5	0.43	-1.633	-0.844
10.25	0.46	-1.663	-0.777
10.75	0.50	-1.699	-0.693
11.25	0.50	-1.699	-0.693
12	0.52	-1.716	-0.654
12.5	0.56	-1.748	-0.580
13	0.56	-1.748	-0.580

B. Addition. 60g glucose dissolved in water. Volume 150 ml.

13	0.54	-1.732	-0.616
13.5	0.54	-1.732	-0.616
14	0.56	-1.748	-0.580
14.5	0.58	-1.763	-0.580
15	0.59	-1.771	-0.528
15.5	0.60	-1.778	-0.511

μ max 5 - 8h $r = 0.99$

$a = -3.71$

$b = 0.331$

μ max $= 0.331 h^{-1}$

RUN 5 Fermentation Results

Time (h)	O.D.(590n.m)	Mass of Glucose/ NaOH Fed to the Fermenter (g)	Glucose Concentration (gl^{-1})
0	-	0	11.6
4	0.12	98	13.0
4.5	0.14	132	13.0
5	0.18	178	13.4
5.5	0.23	31	13.4
6	0.25	299	14.2
6.5	0.30	377	14.6
7	0.33	467	14.3
7.5	0.38	568	15.6
8.25	0.45	710	15.3
9	0.48	860	16.5
10.25	0.55	1,115	17.5

Feedrate 6 - 10.25h $r = 1.00$

$a = -878.3$

$b = 193.46\ g.h^{-1}$

$\therefore$ Feedrate $= 193.46$ g solution.h^{-1}

$= 38.6$ g glucose.h^{-1}

1.4.5.2

RUN 5 Bacterial Growth

Time (h)	O.D.(590n.m)	$\log_{10}$O.D.	ln O.D.
4	0.12	-1.079	-2.120
4.5	0.14	-1.146	-1.966
5	0.18	-1.255	-1.715
5.5	0.23	-1.362	-1.470
6	0.25	-1.398	-1.386
6.5	0.30	-1.477	-1.204
7	0.33	-1.519	-1.109
7.5	0.38	-1.580	-0.968
8.25	0.45	-1.653	-0.799
9	0.48	-1.681	-0.734
10.25	0.55	-1.740	-0.598

μ max 4 - 5.5h r = 0.99
a = -3.91
b = 0.44

μ max = $0.440h^{-1}$

RUN 6 Fermentation Results

Time (h)	O.D.(590n.m)	Mass of Feed Fed to the Fermenter(g)	Glucose Concentration (gl^{-1})
0	0.05	0	9.6
2	0.13	39	9.7
2.5	0.14	53	9.5
3	0.19	76	9.4
3.5	0.21	88	9.6
4	0.26	103	8.5
4.5	0.32	114	8.4
5	0.37	122	8.0
5.5	0.43	133	7.2

5.5 10.5g of glucose added to the fermenter. Volume 30 ml.

6	0.45	147	9.7
6.5	0.48	156	9.6

a. 6.75 Glucose/NaOH mix replaced with Glucose/NH_4OH mix

6.75	-	0	-
7	0.51	77	14.2
7.5	0.54	133	17.2
8.5	0.56	214	21.0
9	0.60	247	22.5
9.5	0.63	282	23.4
10	0.61	318	24.0
10.5	0.60	353	25.8
11	0.63	386	27.3
11.5	0.62	423	33.8

Glucose/NaOH
Feedrate 2 - 6.5h $r = 0.99$
$a = -5.6$
$b = 25.56$

Glucose/NaOH Feedrate = 25.56 g solution.h^{-1}

= 5.11 g glucose.h^{-1}

Glucose/NH_4OH Feedrate

r = 1.00

a = -398.6

b = 71.53

Glucose/NH_4OH Feedrate = 71.53 g solution.h^{-1}

= 14.9 g glucose.h^{-1}

1.4.6.2

RUN 6 Bacterial Growth

Time (h)	O.D.(590n.m)	$\log_{10}$O.D.	ln O.D.
0	0.05	-2.699	-2.996
2	0.13	-1.114	-2.040
2.5	0.14	-1.146	-1.966
3	0.19	-1.279	-1.661
3.5	0.21	-1.322	-1.561
4	0.26	-1.415	-1.347
4.5	0.32	-1.505	-1.139
5	0.37	-1.568	-0.994
5.5	0.43	-1.634	-0.844
6	0.45	-1.653	-0.799
6.5	0.48	-1.681	-0.734
7	0.51	-1.708	-0.673
7.5	0.54	-1.728	-0.616
8.5	0.56	-1.748	-0.580
9	0.60	-1.778	-0.511
9.5	0.63	-1.799	-0.462
10	0.61	-1.785	-0.494
10.5	0.60	-1.778	-0.511
11	0.63	-1.799	-0.462
11.5	0.62	-1.792	-0.478

μ max 2.5 - 5h $r = 0.99$

$a = -2.87$

$b = 0.38$

μ max $= 0.38h^{-1}$

APPENDIX 2

Basic medium for continuous culture Runs 7 to 15.

2.1.1 Composition

Nutrient	Concentration ($g.l.^{-1}$)
Sucrose	variable (as indicated)
Yeast extract (Yeatex)	20
Dipotassium hydrogen orthophosphate (K_2HPO_4)	20
R* salts	5 $ml.l^{-1}$
Antifoam - PPG 2000 (Polyproplylene Glycol 2000)	0.5 $ml.l^{-1}$

R* salts:-

$MgSO_4$ $7H_2O$	40
$MnSO_4$ H_2O	2
NaCl	2
$FeSO_4$ $7H_2O$	2

2.1.2 Preparation

The sucrose, yeast extract, R* salts, antifoam and phosphate were dissolved in distilled water in the order indicated. The concentrated solution was then placed in a 20 litre aspirator and distilled water added to give the correct concentrations. The medium was then sterilised at 12-13 p.s.i. for 1 hour. Sterilisation pressure was increased because of a Bacillus contaminating 20 litre batches of medium sterilised at 10 p.s.i. for 1 hour. A fine precipitate was present in all the media prepared.

Appendix 2.2.

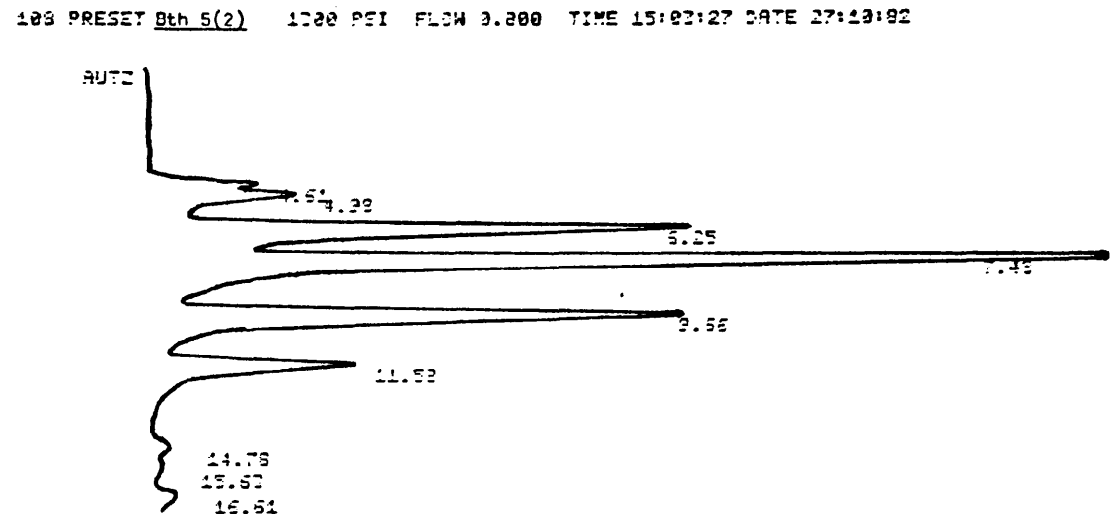
2.2.1. Bio-Rad HPX-87H Organic Acid Column.

Fermentation (continuous culture) Run 13.

Initial Sucrose Concentration 40 gl^{-1}

Dilution Rate 0·192 h^{-1}

1:5 dilution of centrifuged fermentation broth.



RUN TIME 17.9
SOLVN : 0.01468N H2SO4
DET 1 : RI AT 20 DRIX
COL : HPX 97H AMINEX ION EXC
OPERATOR : A MCAVOY
NAME : CONT.CULT.FERM.BROTHS
VOL : 20UL LOOP

INT1

EXTERNAL STANDARD METHOD

TIME	AREA		G/L	NAME
4.61	52234	P		
4.99	120182	P		
6.25	458555	P		
7.48	966305	P		FRUCTOSE / MANNITOL
9.66	469948	P	.33799902	LACTIC ACID
11.58	198018		.22935172	ACETIC ACID
14.78	13382	P		
15.63	4364		.00445234	ETHANOL
16.61	7504			
	2294092			TOTAL

CHART No RC-3038

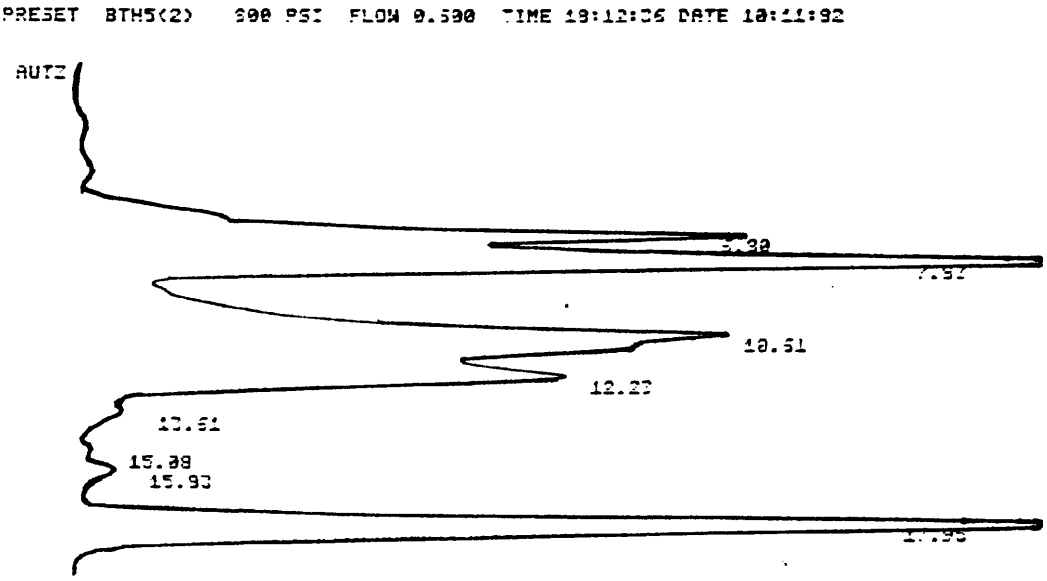
2.2.2. Bio-Rad HPX-87 Carbohydrate Column.

Fermentation (continuous culture) Run 13.

Initial Sucrose Concentration 40 gl^{-1}

Dilution Rate 0·192 h^{-1}

1:5 dilution of centrifuged fermentation broth.



RUN TIME 19.9
SOLVN : WATER
DET 1 : RI AT 20 DRIX
COL : CARBO. HPX-87 AMINEX
OPERATOR : A.MCAVOY
NAME : CONT.CULT.FERM.BROTHS
CONC : VARIABLE
VOL : 20 UL

INT1

EXTERNAL STANDARD METHOD

TIME	AREA		G/L	NAME
6.80	690684	P		
7.82	1227853	P		
10.61	1331902	P		
12.23	656983	P		
13.61	27769			
15.93	5325	P		
15.93	25243		.20491541	ETHANOL
17.95	1127771		3.21189202	MANNITOL
	5153530			TOTAL

ART No RC-3038

APPENDIX 3.1 Media for Continuous Culture Runs 16-32

Fermentation Run Number	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Sucrose (gl^{-1})	60	60	50	40	40	40	40	40	40	40	40	40	40	80	40	60	40
Yeast Extract (Bovril Yeatex) (gl^{-1})	20	20	20	20	20	20	20	20	20	20	20	20	20	20	5	0	20
K_2HPO_4 (gl^{-1})	20	20	20	20	20	20	20	20	20	5	2.5	10	20	5	20	1	20
R* salts ($ml.l^{-1}$)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	-
NH_4Cl (gl^{-1})	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.3	-
R** salts ($ml.l^{-1}$)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
Citric Acid (gl^{-1})	-	-	-	-	-	-	-	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antifoam - PPG 2000 ($ml.l^{-1}$)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Medium Preparation - see Appendix 3.4

R* salts - Appendix 3.2

R** salts - Appendix 3.3

APPENDIX 3.2

R* salts

$MgSO_4\ 7H_2O$	49.0 gl^{-1}
$MnSO_4\ H_2O$	2.0 gl^{-1}
NaCl	2.0 gl^{-1}
$FeSO_4\ 7H_2O$	2.0 gl^{-1}

A fresh solution of the above composition was prepared in distilled water for each medium.

APPENDIX 3.3

R** salts

$MgSO_4\ 7H_2O$	100.0 gl^{-1}
$CaCl\ 2H_2O$	15.0 gl^{-1}
$FeSO_4\ 7H_2O$	3.0 gl^{-1}
$MnSO_4\ 7H_2O$	2.0 gl^{-1}
$ZnSO_4\ 7H_2O$	0.5 gl^{-1}
$CuSO_4\ 5H_2O$	0.1 gl^{-1}
$CoCl_2\ 6H_2O$	0.1 gl^{-1}

A fresh solution of the above was prepared in distilled water when required for use.

APPENDIX 3.4

Medium Preparation

Continuous Culture Runs 16-19 and 21

The sucrose, yeast extract, R* salts, antifoam and phosphate were dissolved in distilled water and added to a 20 litre aspirator in the order indicated. Distilled water (roughly 14 litres) was then added to the aspirator to bring the final volume up to 20 litres. The medium was then sterilised at 12-13 p.s.i. for 1 hour.

A precipitate was always present in the final sterilised media but appeared to be heavier than those of Runs 7-15. The reason for this change was unknown since there had been no alteration to the media composition or preparation. The precipitate was suspected to be due to the high phosphate concentration of the medium.

Continuous Culture Runs 20 and 22-32

Each 20 litres of medium was prepared in two separate 10 litre volumes.

1. Sucrose, trace elements and antifoam.
2. Yeast extract (Bovril Yeatex) and phosphate.

When citric acid was used it was split between the two solutions. Each 10 litre volume was therefore a double concentrate of the indicated solution (except citric acid). The 10 litre volumes were then sterilised at 12-13 p.s.i. for 45 minutes. When cooled, the solution containing the Yeatex and phosphate was added to the solution containing the sucrose and trace elements and the resulting solution mixed.

APPENDIX 4

Media for the test of the effect of sterilisation times on growth.

4.1 Composition

Yeast, Molasses Broth (Y.M.B.)

Sucrose	40 gl^{-1}
Molasses (sugar beet)	20 gl^{-1}
K_2HPO_4	0.4 gl^{-1}
Yeast Extract (Bovril Yeatex)	5 gl^{-1}

Step III

Sucrose	40 gl^{-1}
Yeast Extract (Bovril Yeatex)	40 gl^{-1}
K_2HPO_4	20 gl^{-1}
R* salts	5 $ml.l^{-1}$

R* salts:-	gl^{-1}
$MgSO_4$ $4H_2O$	40
$MNSO_4$ H_2O	2
NaCl	2
$FeSO_4$ $7H_2O$	2

4.2 Preparation

The sucrose, yeast extract, R* salts and phosphate were dissolved in distilled water in the order indicated. 100 ml. volumes were then distributed to pre-labelled 500 ml. conical flasks which were then sterilised for the indicated time period at 10 p.s.i. The unsterilised flasks were stored in a refridgerator at 4°C until required for use. All flasks were duplicated.

4.3 Experimental Method

MacCartney bottles containing 10 ml. of Y.M.B. were loop inoculated from a Leuconostoc mesenteroides B512-F slope and incubated at 30°C for 24 hours. Each 500 ml. conical flask containing 100 ml. of Y.M.B. was inoculated with a 10 ml. Y.M.B. of Leuconostoc mesenteroides B512-F and incubated under the desired conditions, i.e. static or stationary, at 30°C. After 12 hours the optical densities (O.D.) at 590(n.m) of 1:25 dilutions of the cultures were determined. The STEP III media were then inoculated with 10 ml. of culture from the 20 minute sterilisation time Y.M.B. flasks and incubated under the desired conditions, i.e. static or stationary, at 30°C. After 12 hours the optical densities (O.D.) at 590(n.m) of 1:25 dilutions of the cultures were determined. All flasks were duplicated.

4.4 Alternative Nitrogen Sources

Composition

Basal Media	(gl^{-1})
Nitrogen Source	variable (as indicated)
Sucrose	40
K_2HPO_4	20
R* salts (as Appendix 3.2)	5 $ml.l^{-1}$
Thiamine	0.4 $mg.l^{-1}$
Biotin	0.04 $mg.l^{-1}$
Nicotinic Acid	16.0 $mg.l^{-1}$
Pantothenic Acid	2.0 $mg.l^{-1}$

Nitrogen Sources	(gl^{-1})
1. Bovril Yeatex Yeast Extract	40
2. Casein Hydrolysate Oxoid L41	30
3. Peptone P Oxoid L49	51.43
4. Tryptone T Oxoid L43	38.92

The preparation and experimental methods were as that in Appendix 4.2. All media was sterilised at 10 p.s.i. for 20 minutes. The flasks were inoculated from the duplicate 20 minute sterilisation time Y.M.B. flask.

APPENDIX 5

Basic medium for Runs 33 and 34.

5.1.1 Composition

Nutrient	Concentration (gl^{-1})
Sucrose	6
Yeast Extract (Yeatex Paste)	40
Dipotassium Hydrogen Orthophosphate (K_2HPO_4)	20
R* salts (as Appendix 1.2.1)	5 $ml.l^{-1}$

5.1.2 Preparation

In Run 33 the sucrose, yeast extract, R* salts and phosphate were dissolved in distilled water to the correct concentration, placed in a 5 litre aspirator and sterilised at 10 p.s.i. for 30 minutes.

In Run 34 the yeast extraact, R* salts and phosphate were dissolved in distilled water to 2.5 litres and the pH then adjusted to 5.0. The sucrose was dissolved in distilled water, the final volume being 500 ml. Each solution was sterilised at 10 p.s.i. for 20 minutes and mixed in a sterile fermenter when cool.

5.2 Feeds

5.2.1 Run 33

a. 90g NaOH dissolved in 400 ml. of distilled water.
b. 600g sucrose dissolved in 600 ml. of distilled water.
Each one of the above solutions was sterilised in a conical flask at 8 p.s.i. for 20 minutes then allowed to cool. When cool, the solutions were ready for aseptic transfer and mixing in a sterile 2 litre aspirator (feed reservoir) prior to inoculation of the fermenter.
For calculations:-

1 ml. of feed = 1.175 g mass

5.2.2 Run 34

a. 126g KOH dissolved in 400 ml. of distilled water.

b. 600g sucrose dissolved in 600 ml. of distilled water.

Volume = 1,420 ml.

Each of the above solutions was sterilised in a conical flask at 8 p.s.i. for 20 minutes then allowed to cool. When cool, the solutions were ready for aseptic transfer and mixing in a sterile 2 litre aspirator (feed reservoir) prior to inoculation of the fermenter.

For calculations:-

1 ml. of feed = 1.175 g mass

5.3.1

RUN 33 Fermentation Results

Time (h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter (g)
0	0.03	-	0
5	0.07	3.0	34
5.5	-	-	40
6	0.10	6.7	52
6.5	-	-	69
7	0.19	9.2	88
8	0.30	27.7	147
10	0.45	38.5	353
10.5	-	-	400
11	0.53	46.5	454
11.5	-	-	516
12	0.54	33.0	567
14	0.55	7.0	767
14.5	-	-	806
14.75	-	-	827
15	0.56	-	850

5.3.2

RUN 33 Volume Variation

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0	3,150	50	3,100
5	34	29	3,129	100	3,079
5.5	40	34	3,084	-	3,084
6	52	44	3,094	150	3,044
6.5	69	59	3,059	-	3,059
7	88	75	3,075	200	3,025
8	147	125	3,075	250	3,025
10	353	300	3,200	300	3,150
10.5	400	340	3,190	-	3,190
11	451	384	3,234	350	3,184
11.5	516	439	3,239	-	3,239
12	567	483	3,283	400	3,233
14	767	653	3,403	450	3,353
14.5	806	686	3,386	-	3,386
14.75	827	704	3,404	-	3,404
15	850	723	3,423	500	3,373

5.3.3

RUN 33 Fermentation Results on a Mass Basis

Time(h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. (gl^{-1})	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Cell Dry Wt. (g)	Enzyme Activity $(DSU)x10^{-2}$
0	3,150	0.19	-	0.60	-
5	3,129	0.48	3.0	1.50	9.4
6	3,094	0.70	6.7	2.17	20.7
7	3,075	1.37	9.2	4.21	28.3
8	3,075	2.19	27.7	6.73	85.2
10	3,200	3.30	38.5	10.56	123.2
11	3,234	3.89	46.5	12.58	150.4
12	3,283	3.96	33.0	13.00	108.3
14	3,403	4.04	7.0	13.75	23.8
15	3,423	4.11	-	14.07	-

Cell dry weight was determined from O.D.(590n.m) vs. Dry Weight graph.

The above results do not include cells and enzyme lost due to sampling.

5.3.4

RUN 33 Natural Log of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Weight (g)	Ln Mass of Cell Dry Weight (g)
0	0.60	0.51
5	1.50	0.41
6	2.17	0.77
7	4.21	1.44
8	6.73	1.91
10	10.56	2.36
11	12.58	2.53
12	13.00	2.56
14	13.75	2.62
15	14.07	2.64

5.4.1

RUN 34 Fermentation Results

Time (h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Sucrose/ KOH Mix Fed to the Fermenter (g)
4	0.17	-	205
4.5	-	-	243
5	0.20	20.7	274
5.5	-	-	308
6	0.29	42.5	351
6.5	-	-	396
7	0.37	65.9	444
7.5	-	-	498
8	-	-	556
8.5	0.49	124.6	612
9	-	-	673
9.5	0.58	138.5	737
10	-	-	797
11.5	0.66	152.3	992
12	-	-	1,057
13	-	-	1,175
13.5	0.70	-	1,238
14	0.70	116.2	1,293
14.5	-	-	1,351

5.4.2

RUN 34 Volume Variation

Time(h)	Mass of Sucrose/KOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0	3,210	0	3,210
4	205	174	3,384	50	3,334
4.5	243	207	3,367	-	3,367
5	274	233	3,393	100	3,343
5.5	308	262	3,372	-	3,372
6	351	299	3,409	150	3,359
6.5	396	337	3,397	-	3,397
7	444	378	3,438	200	3,388
7.5	498	424	3,434	-	3,434
8	556	473	3,483	-	3,483
8.5	612	521	3,531	250	3,481
9	673	573	3,533	-	3,533
9.5	737	627	3,587	300	3,537
10	797	678	3,588	-	3,588
11.5	992	844	3,754	350	3,704
12	1,057	900	3,760	-	3,760
13	1,175	1,000	3,860	-	3,860
13.5	1,238	1,054	3,914	400	3,864
14	1,293	1,100	3,910	450	3,860
14.5	1,351	1,150	3,910	-	3,910

5.4.3

RUN 34 Fermentation Results on a Mass Basis

Time(h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. (gl^{-1})	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Cell Dry Wt. (g)	Enzyme Activity $(DSU)x10^{-3}$
4	3,384	1.22	-	4.13	-
5	3,393	1.44	20.7	4.89	70.2
6	3,409	2.11	42.5	7,19	144.9
7	3,438	2.70	65.9	9.28	226.6
8.5	3,531	3.59	124.6	12.68	440.0
9.5	3,587	4.26	138.5	15.28	496.8
11.5	3,754	4.85	152.3	18.21	571.7
13.5	3,914	5.15	-	20.16	-
14	3,910	5.15	116.2	20.14	454.3

5.4.4

RUN 34 Natural Log of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Weight (g)	Ln Mass of Cell Dry Weight (g)
4	4.13	1.42
5	4.89	1.59
6	7.19	1.97
7	9.28	2.23
8.5	12.68	2.54
9.5	15.28	2.73
11.5	18.21	2.90
13.5	20.16	3.00
14	20.14	3.00

APPENDIX 5.5

Medium and feed composition for Runs 36-41

Run Number	35	36	37	38	39	40	41
Sucrose ($g.l.^{-1}$)	6	6	6	6	100	6	6
K_2HPO_4 ($g.l.^{-1}$)	20	20	20	20	20	20	20
Ohly U.E. ($g.l.^{-1}$)	40	40	-	-	-	-	-
Gist-Brocades Y.E. ($g.l.^{-1}$)	-	-	10	7.5	40	40	40
R* salts ($ml.l^{-1}$)	5	5	5	5	5	5	5
FEED							
NaOH	90	90	90	90	2M	90	90
Sucrose	600	570	570	570	-	570	570

Medium preparation as Appendix 5.1.2.

Sucrose/NaOH feed preparation as Appendix 5.2.

6.1

RUN 35 Fermentation Results

Time (h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter (g)
0	-	-	0
5	0.09	-	55
6	0.11	-	66
6.25	-	-	76
7	0.15	13.5	109
7.25	-	-	120
7.50	-	-	137
7.75	-	-	151
8	0.23	34.0	171
9.25	0.35	48.3	302
9.50	-	-	331
9.75	-	-	372
10.25	0.47	179.0	472
10.50	-	-	518
10.75	-	-	573
11	0.55	264.8	643
11.25	-	-	696
11.75	-	-	821
12	0.80	257.4	896
12.25	-	-	946
12.50	-	-	1,010
12.75	-	-	1,061
13.50	0.92	200.2	1,227
13.75	-	-	1,279
14	-	-	1,334
14.50	0.90	170.0	1,428
15.25	-	-	1,575
15.50	0.90	-	1,622

6.2

RUN 35 Effluent Gas Results

Aeration rate = 6 $l.min.^{-1}$

Time (h)	Oxygen %	Carbon Dioxide %
0	20.95	0.040
0.5	20.95	0.040
1	20.95	0.040
1.5	20.95	0.040
2	20.95	0.040
2.5	20.95	0.040
3	20.95	0.040
3.5	20.94	0.041
4	20.92	0.048
4.5	20.90	0.059
5	20.90	0.074
5.5	20.87	0.136
6	20.86	0.148
6.5	20.79	0.175
7	20.75	0.231
7.5	20.72	0.273
8	20.64	0.369
8.5	20.58	0.444
9	20.48	0.542
9.25	20.44	0.574
9.5	20.35	0.693
10	20.28	0.825
10.25	20.19	0.866
10.5	20.10	1.01
11	19.90	1.17
11.5	19.94	1.18
12	19.96	1.16
12.5	20.00	1.12
13	20.00	1.09
13.5	20.00	1.12
14	20.10	0.996
14.5	20.14	0.976
15	20.14	0.948
15.5	20.15	0.928

6.3

RUN 35 Volume Variation

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0	3,000	0	3,000
5	55	47	3,047	50	2,997
6	66	56	3,006	100	2,956
6.25	76	65	2,965	-	2,965
7	109	93	2,993	150	2,943
7.25	120	102	2,952	-	2,952
7.5	137	117	2,967	-	2,967
7.75	151	129	2,979	-	2,979
8	171	146	2,996	200	2,946
9.25	302	257	3,057	250	3,007
9.50	3.31	282	3,032	-	3,032
9.75	372	317	3,067	-	3,067
10.25	472	402	3,152	300	3,102
10.50	518	441	3,141	-	3,141
10.75	573	488	3,188	-	3,188
11	643	547	3,247	350	3,197
11.25	696	592	3,242	-	3,242
11.75	821	699	3,349	-	3,349
12	896	763	3,413	400	3,363
12.25	946	805	3,405	-	3,405
12.50	1,010	860	3,460	-	3,460
12.75	1,061	903	3,503	-	3,503
13.50	1,227	1,044	3,644	450	3,594
13.75	1,279	1,089	3,639	-	3,639
14	1,334	1,135	3,685	-	3,685
14.50	1,428	1,215	3,765	500	3,715
15.25	1,575	1,340	3,840	-	3,840
15.50	1,622	1,380	3,880	550	3,830

1 ml volume of feed = 1.175g mass

RUN 35 Fermentation Results on a Mass Basis

Time(h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. (gl^{-1})	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Cell Dry Wt. (g)	Enzyme Activity $(DSU)x10^{-3}$
5	3,047	0.64	-	1.95	-
6	3,006	0.79	-	2.37	-
7	2,993	1.08	13.5	3.23	40.4
8	2,996	1.66	34.0	4.97	101.9
9.25	3,057	2.53	48.3	7.73	147.7
10.25	3,152	3.40	179.0	10.72	564.2
11	3,247	3.98	264.8	12.92	859.8
12	3,413	5.80	257.4	19.80	878.5
13.5	3,644	6.67	200.2	24.31	729.5
14.5	3,765	6.52	170.0	24.55	640.1
15.5	3,880	6.52	-	25.30	-

Cell dry weights derived from O.D.(590n.m) vs. Dry Wt. (gl^{-1}) graph.

Feedrate 10.25 -14h $r = 1.00$

$a = -1,642.9$

$b = 199.2\ ml.h^{-1}$

$\therefore F = 0.199\ l.h^{-1}$

6.5

RUN 35 Molar Oxygen Consumption and Carbon Dioxide Production Rates

Time (h)	Mo_2(m.moles.h^{-1})	Mco_2(m.moles.h^{-1})	RQ.
0	0	0	-
0.5	0	0	-
1	0	0	-
1.5	0	0	-
2	0	0	-
2.5	0	0	-
3	0	0	-
3.5	1.8	0.1	0.08
4	5.3	1.2	0.22
4.5	8.6	2.8	0.33
5	8.0	5.0	0.63
5.5	11.2	14.2	1.27
6	12.6	15.9	1.27
6.5	24.5	19.9	0.81
7	29.8	28.0	0.94
7.5	33.7	34.3	1.02
8	44.9	48.4	1.08
8.5	53.1	59.5	1.12
9	67.9	73.9	1.09
9.25	74.1	78.6	1.06
9.5	86.3	96.2	1.12
10	94.2	115.7	1.23
10.25	109.3	121.6	1.11
10.5	120.6	143.0	1.19
11	151.5	166.4	1.10
11.5	143.8	168.0	1.17
12	140.8	165.1	1.17
12.5	134.9	159.2	1.18
13	136.1	154.7	1.14
13.5	134.9	159.2	1.18
14	121.1	140.9	1.16
14.5	114.5	138.0	1.21
15	115.5	133.8	1.16
15.5	114.4	130.8	1.14
16	94.2	130.4	1.39

RUN 35 Natural Log Graph of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Wt. (g)	Ln Mass of Cell Dry Wt. (g)
5	1.95	0.67
6	2.37	0.86
7	3.23	1.17
8	4.97	1.60
9.25	7.73	2.05
10.25	10.72	2.37
11	12.92	2.56
12	19.80	2.99
13.5	24.31	3.19
14.5	24.55	3.20
15.5	25.30	3.23

$$7 - 9.25h \quad \mu \text{ max} = \frac{2.05 - 1.17}{2.25}$$

$$= 0.39h^{-1}$$

7.1

RUN 36 Fermentation Results

Time(h)	O.D.(590n.m)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter(g)	Temperature (^{o}C)
0	-	0	23
6	0.19	100	-
6.5	-	131	-
7	0.31	171	-
7.25	-	196	23.5
7.50	-	226	-
7.75	-	258	24.25
8	0.43	294	24.5
8.25	-	333	25.0
8.5	-	380	-
8.75	-	424	26.0
9	0.61	478	26.5
10	0.68	773	29.0
10.25	-	841	29.5
10.5	-	908	30.0
10.75	-	977	30.25
11 A.	Phosphate (50g K_2HPO_4) feed on.		Volume 250 ml.
11	0.88	1,041	30.25
11.25	-	1,097	30.25
11.5	-	1,134	30.0
11.75	-	1,180	29.75
12	0.88	1,221	29.50
12.25	-	1,260	29.25
12.5	-	1,292	28.75
12.75	-	1,327	28.75
13 B.	Addition of 26.5g Yeast Extract (Gist-Brocades). Volume 100 ml.		
13	0.88	1,367	28.75

7.1 (CONT'D)

Time(h)	O.D.(590n.m)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter(g)	Temperature (°C)
13.25	-	1,421	-
C. All of phosphate added.			
13.5	-	1,465	29.25
13.75	-	1,525	29.75
14	0.81	1,582	30
14.25	-	1,648	30.5
14.5	-	1,709	31.0
14.75	-	1,783	31.25
15	0.96	1,861	31.5
15.25	-	1,925	32.0
15.5	-	2,005	32.25
15.75	-	2,084	32.5
16	0.88	2,152	32.5
16.7	0.82	2,330	32.5

7.2

RUN 36 Effluent Gas Results

Aeration rate = 6 $l.min.^{-1}$

Time (h)	Oxygen %	Carbon Dioxide %
0	20.95	0.040
0.25	20.95	0.040
0.5	20.95	0.040
0.75	20.95	0.040
1	20.95	0.040
1.25	20.95	0.040
1.5	20.92	0.082
1.75	20.92	0.061
2	20.95	0.046
2.25	20.95	0.041
2.5	20.95	0.041
2.75	20.94	0.042
3	20.94	0.045
3.25	20.92	0.049
3.5	20.91	0.054
3.75	20.92	0.060
4	20.91	0.068
4.25	20.90	0.077
4.5	20.87	0.087
4.75	20.86	0.097
5	20.89	0.113
5.25	20.81	0.127
5.5	20.81	0.145
5.75	20.78	0.164
6	20.69	0.216
6.25	20.66	0.312
6.5	20.65	0.327
6.75	20.60	0.373
7	20.55	0.427
7.25	20.45	0.498
7.5	20.43	0.559
7.75	20.36	0.607

7.2 (CONT'D)

Time (h)	Oxygen %	Carbon Dioxide %
8	20.30	0.660
8.25	20.24	0.732
8.5	20.17	0.785
8.75	20.07	0.893
9	19.98	0.993
9.25	19.92	1.05
9.5	19.80	1.15
9.75	19.76	1.25
10	19.72	1.33
10.25	19.59	1.42
10.5	19.68	1.30
10.75	19.77	1.20
11	19.88	1.10
11.25	19.97	1.01
11.5	20.06	0.949
11.75	20.11	0.895
12	20.11	0.834
12.25	20.19	0.814
12.5	20.23	0.779
12.75	20.22	0.764
13	20.21	0.773
13.25	20.10	0.874
13.5	20.02	0.991
13.75	19.93	1.03
14	19.81	1.13
14.25	19.81	1.17
14.5	19.75	1.21
14.75	19.72	1.25
15	19.67	1.34
15.25	19.67	1.32
15.5	19.71	1.30
15.75	19.75	1.28
16	19.80	1.26
16.25	19.88	1.24
16.5	19.79	1.23

RUN 36 Volume Variation

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0	2,500	0	2,500
6	100	85	2,585	50	2,535
6.5	131	111	2,561	-	2,561
7	171	146	2,596	100	2,546
7.25	196	167	2,567	-	2,567
7.5	226	192	2,592	-	2,592
7.75	258	220	2,620	-	2,620
8	294	250	2,650	150	2,600
8.25	333	283	2,633	-	2,633
8.5	380	323	2,673	-	2,673
8.75	424	361	2,711	-	2,711
9	478	407	2,757	200	2,707
10	773	658	2,958	250	2,908
10.25	841	716	2,966	-	2,966
10.5	908	773	3,023	-	3,023
10.75	977	831	3,081	-	3,081
11 A.	Phosphate (K_2HPO_4) feed on. Feedrate 111.1 $ml.h^{-1}$.				
11	1,041	886 (0)	3,136	300	3,086
11.25	1,097	934 (28)	3,162	-	3,162
11.5	1,134	965 (56)	3,221	-	3,221
11.75	1,180	1,004 (83)	3,288	-	3,288
12	1,221	1,039 (111)	3,351	350	3,301
12.25	1,260	1,072 (139)	3,362	-	3,362
12.5	1,292	1,100 (167)	3,418	-	3,418
12.75	1,327	1,129 (194)	3,475	-	3,475
13	1,367	1,163 (222)	3,537	400	3,487
13 B.	Addition of 26.5g Yeast Extract (Gist-Brocades). Volume 100 ml.				

7.3 (CONT'D)

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
13.25	1,421	1,209 (250)	3,661	-	3,661
13.25	C. All of phosphate added.				
13.5	1,465	1,247	3,699	-	3,699
13.75	1,525	1,298	3,750	-	3,750
14	1,582	1,346	3,798	450	3,748
14.25	1,648	1,403	3,805	-	3,805
14.5	1,709	1,454	3,856	-	3,856
14.75	1,783	1,517	3,919	-	3,919
15	1,861	1,584	3,986	500	3,936
15.25	1,925	1,638	3,990	-	3,990
15.5	2,005	1,706	4,058	-	4,058
15.75	2,084	1,774	4,126	-	4,126
16	2,152	1,831	4,183	550	4,133
16.7	2,330	1,983	4,285	600	4,235

The figures in brackets represent volume addition due to feed of phosphate.

7.4

RUN 36 Fermentation Results on a Mass Basis

Time (h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. (gl^{-1})	Mass of Cell Dry Wt. (g)
6	2,585	1.37	3.54
7	2,596	2.24	5.82
8	2,650	3.11	8.25
9	2,757	4.42	12.18
10	2,958	4.93	14.57
11 A. Phosphate feed on.			
11	3,136	6.38	20.00
12	3,351	6.38	21.37
13 B. Addition of 26.5g Yeast Extract (Gist Brocades).			
13	3,537	-	22.57
13.25 C. Phosphate feed finished.			
14	3,798	5.87	22.29
15	3,986	6.96	27.74
16	4,183	6.38	26.69
16.7	4,285	5.94	25.46

Feedrate 8.5 - 10.5h $r = 1.00$

$a = -1,666.3$

$b = 232.2\ ml.h^{-1}$

$\therefore F = 0.232\ l.h.^{-1}$

Feedrate 14 - 16.7h $r = 1.00$

$a = -2,032.1$

$b = 241.0\ ml.h.^{-1}$

$\therefore F = 0.241\ l.h.^{-1}$

7.5

RUN 36 Molar Oxygen Consumption and Carbon Dioxide Production Rates

Time (h)	Mo_2(m.moles.h^{-1})	Mco_2(m.moles.h^{-1})	RQ.
0	0	0	-
0.25	0	0	-
0.5	0	0	-
0.75	0	0	-
1	0	0	-
1.25	0	0	-
1.5	4.0	6.2	-
1.75	4.8	3.1	-
2	0	0.9	-
2.25	0	0	-
2.5	0	0	-
2.75	1.8	0.3	0.17
3	1.7	0.7	0.44
3.25	5.2	1.3	0.25
3.5	6.9	2.1	0.30
3.75	4.8	2.9	0.61
4	6.4	4.1	0.65
4.25	7.9	5.4	0.69
4.5	13.1	6.9	0.53
4.75	14.5	8.4	0.58
5	8.3	10.7	1.29
5.25	7.8	12.8	1.64
5.5	22.0	15.4	0.70
5.75	26.8	18.2	0.68
6	41.5	25.9	0.62
6.25	43.4	40.0	0.92
6.5	44.6	42.2	0.95
6.75	52.2	49.0	0.94
7	59.4	56.9	0.96
7.25	75.2	67.4	0.90
7.5	76.6	76.4	1.0
7.75	87.7	83.4	0.95
8	96.8	91.2	0.94

7.5 (CONT'D)

Time (h)	Mo_2(m.moles.h^{-1})	Mco_2(m.moles.h^{-1})	RQ.
8.25	105.2	101.8	0.97
8.5	116.1	109.6	0.94
8.75	130.5	125.5	0.96
9	143.4	140.2	0.98
9.25	152.3	148.6	0.98
9.5	170.7	163.2	0.96
9.75	174.4	178.1	1.02
10	178.8	190.2	1.06
10.75	199.4	203.1	1.02
10.5	187.2	185.4	0.99
10.75	174.4	170.6	0.98
11	157.8	155.9	0.99
11.25	144.6	142.7	0.99
11.5	130.3	133.8	1.03
11.75	123.0	125.8	1.02
12	125.3	116.8	0.93
12.25	111.3	113.9	1.02
12.5	105.2	108.8	1.03
12.75	107.6	106.5	0.99
13	109.1	107.8	0.99
13.25	125.7	122.7	0.98
13.5	136.1	140.0	1.03
13.75	151.2	145.6	0.96
14	169.6	160.3	0.95
14.25	168.1	166.2	0.99
14.5	177.7	172.1	0.97
14.75	181.7	178.0	0.98
15	187.6	191.3	1.02
15.25	188.3	188.3	1.00
15.5	181.7	185.4	1.02
15.75	175.1	182.5	1.04
16	166.6	179.7	1.08
16.25	161.8	176.8	1.09
16.5	169.6	175.2	1.03

7.6

RUN 36 Natural Log Graph of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Wt. (g)	Ln Mass of Cell Dry Wt. (g)
6	3.54	1.26
7	5.82	1.76
8	8.25	2.11
9	12.18	2.50
10	14.57	2.68
11	20.00	3.00
12	21.37	3.06
13	22.57	3.12
14	22.29	3.10
15	27.74	3.32
16	26.69	3.28
16.7	25.46	3.24

8.1

RUN 37 Fermentation Results

Time(h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter(g)
0	-	-	0
7	0.45	195.7	340
7.5	-	-	376
8	0.50	244.0	404
8 A.	$185g.l^{-1}$ Yeast Extract (Gist Brocades) feed on. Flowrate 32.5 $ml.h^{-1}$.		
8.25	-	-	423
8.50	-	-	443
9	0.51	317.0	481
9.5	-	-	524
10	0.57	372.5	570
10.5	-	-	617
10.75	-	-	642
11	0.59	350.8	666
11 B.	Yeast Extract (Gist Brocades) feedrate increased to 80.0 $ml.h^{-1}$.		
11.5	-	-	717
12	0.6	403.2	779
12.5	-	-	846
13	0.67	366.7	920
14	0.69	346.2	1,090
14.5	-	-	1,175
15	0.74	261.5	1,285
15.5	-	-	1,376
16	0.75	321.1	1,495
16 C.	Yeast Extract feed exhausted.		

8.1 (CONT'D)

Time(h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter(g)
16.5	-	-	1,560
17	0.78	289.9	1,677
17.5	-	-	1,761
18	0.73	245.4	1,846

8.2

RUN 37 Effluent Gas Results

Aeration rate = 6 $l.min.^{-1}$

Time (h)	Oxygen %	Carbon Dioxide %
0	20.91	0.077
0.25	20.90	0.094
0.5	20.90	0.082
0.75	20.89	0.084
1	20.89	0.090
1.25	20.89	0.101
1.5	20.86	0.110
1.75	20.82	0.119
2	20.82	0.130
2.25	20.79	0.143
2.5	20.78	0.159
2.75	20.77	0.173
3	20.74	0.187
3.25	20.74	0.208
3.5	20.72	0.233
3.75	20.62	0.274
4	20.62	0.314
4.25	20.60	0.349
4.5	20.64	0.369
4.75	20.68	0.327
5	20.66	0.287
5.25	20.64	0.287
5.5	20.62	0.325
5.75	20.58	0.367
6	20.56	0.396
6.25	20.52	0.416
6.5	20.48	0.424
6.75	20.55	0.424
7	20.57	0.417
7.25	20.55	0.399
7.5	20.58	0.380
7.75	20.61	0.352

8.2 (CONT'D)

Time (h)	Oxygen %	Carbon Dioxide %
8	20.58	0.339
8.25	20.56	0.366
8.5	20.52	0.437
8.75	20.51	0.436
9	20.53	0.432
9.25	20.51	0.442
9.5	20.49	0.465
9.75	20.45	0.481
10	20.47	0.481
10.25	20.49	0.487
10.5	20.51	0.496
10.75	20.45	0.521
11	20.48	0.525
11.25	20.43	0.540
11.5	20.38	0.576
11.75	20.36	0.606
12	20.35	0.631
12.25	20.27	0.664
12.5	20.17	0.745
12.75	20.14	0.798
13	20.18	0.820
13.25	20.12	0.837
13.5	20.14	0.861
13.75	20.11	0.888
14	20.02	0.922
14.25	20.05	0.990
14.5	20.04	0.994
14.75	20.00	1.01
15	19.93	1.08
15.25	19.92	1.10
15.5	19.92	1.07
15.75	19.93	1.08
16	19.96	1.10
16.25	19.88	1.11
16.5	19.94	1.10

8.2 (CONT'D)

Time (h)	Oxygen %	Carbon Dioxide %
16.75	20.01	1.04
17	20.07	0.965
17.25	20.09	0.942
17.5	20.11	0.918
17.75	20.11	0.889
18	20.17	0.885

8.3

RUN 37 Volume Variation

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)		Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0		2,500	-	2,500
7	340	289		2,789	50	2,739
7.5	376	320		2,770	-	2,770
8	404	344		2,794	100	2,744
8 A. Yeast Extract (Gist-Brocades) feed on. Flowrate 32.5 ml.h						
8.25	423	360	(8)	2,768	-	2,768
8.5	443	377	(16)	2,793	-	2,793
9	481	409	(33)	2,842	150	2,792
9.5	521	443	(49)	2,842	-	2,842
10	570	485	(65)	2,900	200	2,850
10.5	617	525	(81)	2,906	-	2,906
10.75	642	546	(89)	2,935	-	2,935
11	666	567	(98)	2,965	250	2,915
11 B. Yeast Extract Feedrate increased to 80.0 $ml.h^{-1}$						
11.5	717	610	(138)	2,958	-	2,958
12	779	663	(178)	3,051	300	3,001
12.5	846	720	(218)	3,098	-	3,098
13	920	783	(258)	3,201	350	3,151
14	1,090	928	(338)	3,376	400	3,326
14.5	1,175	1,000	(378)	3,438	-	3,438
15	1,285	1,094	(418)	3,572	450	3,522
15.5	1,376	1,171	(458)	3,639	-	3,639
16	1,495	1,272	(498)	3,780	500	3,730
16 C. Yeast Extract feed reservoir exhausted.						

8.3 (CONT'D)

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
16.5	1,560	1,328	3,786	-	3,786
17	1,677	1,427	3,885	550	3,835
17.5	1,761	1,499	3,907	-	3,907
18	1,846	1,571	3,979	600	3,929

8.4

RUN 37 Fermentation Results on a Mass Basis

Time(h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. (gl^{-1})	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Cell Dry Wt. (g)	Enzyme Activity $(DSU)x10^{-3}$
7	2,789	3.26	195.7	9.09	545.8
8	2,794	3.62	244.0	10.11	681.7
8 A. Yeast Extract (Gist-Brocades) feed on. Flowrate 32.5 ml.h					
9	2,842	3.69	317.0	10.49	900.9
10	2,900	4.13	372.5	11.98	1,080.3
11	2,965	4.27	350.8	12.66	1,040.1
11 B. Yeast Extract feedrate increased to 80.0 $ml.h^{-1}$					
12	3,051	4.35	403.2	13.27	1,230.2
13	3,201	4.85	366.7	15.52	1,173.8
14	3,376	5.00	346.2	16.88	1,168.8
15	3,572	5.36	261.5	19.15	934.1
16	3,780	5.43	321.1	20.53	1,213.8
16 C. Yeast Extract feed exhausted.					
17	3,885	5.65	289.9	21.95	1,126.3
18	3,979	5.29	245.4	21.05	976.4

Feedrate 8.25 - 11h $r = 1.00$

$a = -267.0$

$b = 75.5\ ml.h.^{-1}$

$\therefore F = 0.076\ l.h.^{-1}$

Feedrate 13 - 18h $r = 1.00$

$a = -1,320.3$

$b = 161.0\ ml.h.^{-1}$

$\therefore F = 0.161\ l.h.^{-1}$

8.4 (CONT'D)

Cell Growth	9 - 12h	r	= 0.97
		a	= 2.63
		b	= 0.90 $g.h.^{-1}$
	12 - 17h	r	= 1.00
		a	= -7.26
		b	= 1.73 $g.h.^{-1}$

8.5

RUN 37 Molar Oxygen Consumption and Carbon Dioxide Production Rates

Time (h)	M_{O_2} (m.moles.h^{-1})	M_{CO_2} (m.moles.h^{-1})	RQ.
0	6.0	5.5	0.91
0.25	7.2	8.0	1.10
0.5	7.7	6.2	0.81
0.75	9.5	6.5	0.69
1	9.2	7.4	0.80
1.25	8.8	9.0	1.02
1.5	14.1	10.3	0.73
1.75	21.2	11.6	0.55
2	20.7	13.3	0.64
2.25	25.8	15.2	0.59
2.5	27.1	17.5	0.65
2.75	28.4	19.6	0.69
3	33.4	21.7	0.65
3.25	32.6	24.8	0.76
3.5	35.4	28.5	0.80
3.75	52.4	34.5	0.66
4	50.8	40.4	0.79
4.25	53.2	45.6	0.86
4.5	45.0	48.5	1.08
4.75	39.2	42.3	1.08
5	44.4	36.4	0.82
5.25	48.2	36.4	0.76
5.5	50.4	42.0	0.83
5.75	56.2	48.2	0.86
6	58.8	52.5	0.89
6.25	65.5	55.4	0.85
6.5	72.6	56.6	0.78
6.75	59.6	56.6	0.95
7	56.2	55.6	0.99
7.25	60.6	52.9	0.87
7.5	55.7	50.1	0.90
7.75	51.2	46.0	0.90
8	57.3	44.1	0.77

8.5 (CONT'D)

Time (h)	M_{O_2} (m.moles.h^{-1})	M_{CO_2} (m.moles.h^{-1})	RQ.
8.25	60.0	48.0	0.80
8.5	64.7	58.5	0.91
8.75	66.6	58.4	0.88
9	63.0	57.8	0.92
9.25	66.4	59.3	0.89
9.5	69.2	62.7	0.91
9.75	76.0	65.0	0.86
10	72.3	65.0	0.90
10.25	68.4	65.9	0.96
10.5	64.3	67.3	1.05
10.75	74.5	70.9	0.95
11	68.8	71.6	1.04
11.25	77.5	73.7	0.95
11.5	85.4	79.0	0.93
11.75	87.9	83.5	0.95
12	88.8	87.2	0.98
12.25	102.4	92.0	0.90
12.5	117.9	103.9	0.88
12.75	121.4	111.7	0.92
13	113.2	115.1	1.02
13.25	112.6	117.6	1.04
13.5	119.1	121.1	1.02
13.75	123.6	125.1	1.01
14	139.0	130.0	0.94
14.25	130.9	140.2	1.07
14.5	132.6	140.8	1.06
14.75	139.4	143.1	1.03
15	149.7	153.4	1.02
15.25	150.8	156.4	1.04
15.5	151.9	151.9	1.00
15.75	149.7	153.4	1.02
16	143.4	156.5	1.09
16.25	157.8	157.8	1.00
16.5	147.1	156.5	1.06
16.75	136.4	147.6	1.08

8.5 (CONT'D)

Time (h)	M_{O_2} (m.moles.h^{-1})	M_{CO_2} (m.moles.h^{-1})	RQ.
17	128.1	136.5	1.07
17.25	125.3	133.1	1.06
17.5	122.5	129.6	1.06
17.75	123.6	125.2	1.01
18	112.6	124.7	1.11

8.6

RUN 37 Natural Log Graph of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Wt. (g)	Ln Mass of Cell Dry Wt. (g)
7	9.09	2.21
8	10.11	2.31
9	10.49	2.35
10	11.98	2.48
11	12.66	2.54
12	13.27	2.59
13	15.52	2.74
14	16.88	2.83
15	19.15	2.95
16	20.53	3.02
17	21.95	3.09
18	21.05	3.05

9.1

RUN 38 Fermentation Results

Time(h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter(g)
pH system out of operation until 7.0th hour			
7	-	-	0
7.5	0.21	15.0	89
8	0.21	22.5	105
8.25	-	-	117
8.5	-	-	121
8.75	-	-	128
8.75 A. Added 50 ml. of sucrose solution (9g)			
9	0.23	40.0	134
9.25	-	-	142
9.5	-	-	154
9.5 B. Added 50 ml. of inositol solution (2.5g)			
10	0.27	50.0	173
10.5 C. Added 50 ml. of Peptone P (Oxoid L49) (12.5g)			
11	0.31	60.0	223
11.5	-	-	248
12	0.34	94.9	279
12 D. Added 50 ml. of Tryptone T (Oxoid L43) (12.5g)			
12.5	-	-	300
13	0.40	97.4	323
13 E. Added 50 ml. of Casein Hydrolysate (Oxoid L41) (12.5g)			

9.1 (CONT'D)

Time(h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter(g)
13.5	-	-	346
14	0.44	104.9	370
14 F.	Added 50 ml. of Yeatex (Bovril) (12.5g)		
14.5	-	-	406
15.5	0.48	154.4	474
16.5	0.54	189.8	543
16.5 G.	Added 50 ml. of Yeast Extract (Gist-Brocades) (12.5g)		
17	-	-	584
17.5	0.55	205.0	626
18	0.56	225.2	664
31	0.76	177.2	1,596

9.2

RUN 38 Effluent Gas Results

Aeration rate = 6 $l.min.^{-1}$ (initially 2.4 V.V.M.)

Time (h)	Oxygen %	Carbon Dioxide %
0	20.94	0.043
0.25	20.94	0.061
0.5	20.94	0.058
0.75	20.92	0.060
1	20.95	0.063
1.25	20.91	0.067
1.5	20.91	0.073
1.75	20.91	0.080
2	20.90	0.086
2.25	20.91	0.095
2.5	20.90	0.104
2.75	20.87	0.114
3	20.89	0.125
3.25	20.87	0.133
3.5	20.83	0.145
3.75	20.83	0.156
4	20.79	0.169
4.25	20.83	0.183
4.5	20.74	0.200
4.75	20.75	0.218
5	20.77	0.196
5.25	20.78	0.170
5.5	20.79	0.172
5.75	20.81	0.166
6	20.81	0.155
6.25	20.83	0.142
6.5	20.86	0.131
6.75	20.86	0.121
7	20.86	0.110
7.25	20.87	0.101
7.5	20.87	0.130
7.75	20.77	0.189

9.2 (CONT'D)

Time (h)	Oxygen %	Carbon Dioxide %
8	20.70	0.246
8.25	20.70	0.241
8.5	20.74	0.205
8.75	20.79	0.185
9	20.79	0.185
9.25	20.78	0.183
9.5	20.78	0.186
9.75	20.78	0.193
10	20.77	0.207
10.25	20.75	0.214
10.5	20.82	0.223
10.75	20.73	0.245
11	20.69	0.270
11.25	20.70	0.286
11.5	20.70	0.298
11.75	20.73	0.287
12	20.73	0.271
12.25	20.70	0.251
12.5	20.77	0.255
12.75	20.72	0.247
13	20.74	0.239
13.25	20.73	0.231
13.5	20.77	0.239
13.75	20.77	0.237
14	20.74	0.237
14.25	20.74	0.256
14.5	20.66	0.286
14.75	20.69	0.307
15	20.65	0.361
15.25	20.61	0.356
15.5	20.61	0.369
15.75	20.64	0.375
16	20.65	0.366
16.25	20.66	0.356
16.5	20.65	0.340

9.2 (CONT'D)

Time (h)	Oxygen %	Carbon Dioxide %
16.75	20.60	0.345
17	20.65	0.384
17.25	20.61	0.408
17.5	20.57	0.439
17.75	20.56	0.447
18	20.56	0.452
18.25	20.56	0.456
18.5	20.53	0.462
18.75	20.56	0.469
19	20.53	0.469
31	20.68	0.290

9.3

RUN 38 Volume Variation

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0	2,500	-	2,500
7	0	0	2,500	-	2,500
7.5	89	76	2,576	50	2,526
8	105	89	2,539	100	2,489
8.25	117	100	2,500	-	2,500
8.5	121	103	2,503	-	2,503
8.75	128	109	2,509	-	2,509
8.75 A. Added 50 ml. of sucrose solution (9g)					2,559
9	134	114	2,564	150	2,514
9.25	142	121	2,521	-	2,521
9.5	154	131	2,531	-	2,531
9.5 B. Added 50 ml. of inositol solution (12.5g)					2,581
10	173	147	2,597	200	2,547
10.5 C. Added 50 ml. of Peptone P, Oxoid L49 (12.5g)					2,597
11	223	190	2,640	250	2,590
11.5	248	211	2,611	-	2,611
12	279	237	2,637	300	2,587
12 D. Added 50 ml. of Tryptone T, Oxoid L43 (12.5g)					2,637
12.5	300	255	2,655	-	2,655
13	323	275	2,675	350	2,625
13 E. Added 50 ml. of Casein Hydrolysate, Oxoid L41 (12.5g)					2,675

9.3 (CONT'D)

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
13.5	346	294	2,694	-	2,694
14	370	315	2,715	400	2,665
14 F. Added 50 ml. of Yeatex (Bovril) (12.5g)					2,715
14.5	406	346	2,746	-	2,746
15.5	474	403	2,803	450	2,753
16.5	543	462	2,812	500	2,762
16.5 G. Added 50 ml. of Yeast Extract (Gist-Brocades) (12.5g)					2,812
17	584	497	2,847	-	2,847
17.5	626	533	2,883	550	2,833
18	664	565	2,865	600	2,815
31	1,596	1,358	3,608	650	3,558

RUN 38 Fermentation Results on a Mass Basis

Time(h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. (gl^{-1})	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Cell Dry Wt. (g)	Enzyme Activity $(DSU)x10^{-3}$
7.5	2,576	1.51	15.0	3.90	38.6
8	2,539	1.51	22.5	3.83	57.1
8.75 A. Added 50 ml. of sucrose solution (9g)					
9	2,564	1.66	40.0	4.26	102.6
9.5 B. Added 50 ml. of inositol solution (12.5g)					
10	2,597	1.95	50.0	5.07	129.9
10.5 C. Added 50 ml. of Peptone P, Oxoid L49 (12.5g)					
11	2,640	2.24	60.0	5.92	158.4
12	2,637	2.46	94.9	6.48	250.3
12 D. Added 50 ml. of Tryptone T, Oxoid L43 (12.5g)					
13	2,675	2.89	97.4	7.74	260.5
13 E. Added 50 ml. of Casein Hydrolysate, Oxoid L41 (12.5g)					
14	2,715	3.18	104.9	8.65	284.8
14 F. Added 50 ml. of Yeatex (Bovril) (12.5g)					
15.5	2,803	3.47	154.4	9.74	432.8
16.5	2,812	3.91	189.8	11.00	533.7
16.5 G. Added 50 ml. of Yeast Extract (Gist-Brocades) (12.5g)					

9.4 (CONT'D)

Time(h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. (gl^{-1})	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Cell Dry Wt. (g)	Enzyme Activity $(DSU)x10^{-3}$
17.5	2,883	3.98	205.0	11.48	591.0
18	2,865	4.06	225.2	11.62	645.2
31	3,608	5.51	177.2	19.88	639.3

Feedrate 9.5 - 14h $r = 1.00$

$a = -265.7$

$b = 41.6\ ml.h.^{-1}$

$\therefore F = 0.042\ l.h.^{-1}$

Feedrate 14.5 - 18h $r = 1.00$

$a = -568.9$

$b = 62.8\ ml.h.^{-1}$

$\therefore F = 0.063\ l.h.^{-1}$

RUN 38 Molar Oxygen Consumption and Carbon Dioxide Production Rates

Time (h)	Mo_2(m.moles.h^{-1})	Mco_2(m.moles.h^{-1})	RQ.
0	1.7	0.4	0.25
0.25	1.0	3.1	2.97
0.5	1.2	2.7	2.29
0.75	4.8	2.9	0.61
1	0	3.4	
1.25	6.4	4.0	0.62
1.5	6.2	4.9	0.79
1.75	7.8	5.9	0.76
2	5.7	6.8	1.20
2.25	7.2	8.1	1.13
2.5	12.4	9.4	0.76
2.75	8.3	10.9	1.32
3	11.6	12.5	1.08
3.25	18.7	13.7	0.73
3.5	18.3	15.5	0.85
3.75	17.8	17.1	0.96
4	24.7	19.0	0.77
4.25	16.8	21.1	1.26
4.5	32.8	23.5	0.72
4.75	30.3	26.2	0.87
5	27.4	23.0	0.84
5.25	26.6	19.1	0.72
5.5	24.6	19.4	0.79
5.75	21.1	18.5	0.88
6	21.6	16.9	0.78
6.25	18.4	15.0	0.82
6.5	13.2	13.4	1.01
6.75	13.6	11.9	0.88
7	12.2	10.3	0.85
7.25	12.5	9.0	0.72
7.5	30.0	13.3	0.44
7.75	40.8	21.9	0.54
8	38.6	30.4	0.79

9.5 (CONT'D)

Time (h)	Mo_2(m.moles.h^{-1})	Mco_2(m.moles.h^{-1})	RQ.
8.25	31.3	29.6	0.95
8.5	23.4	24.3	1.04
8.75	18.6	21.4	1.15
9	24.2	21.4	0.88
9.25	23.4	31.4	1.34
9.5	26.0	21.5	0.83
9.75	25.7	22.6	0.88
10	27.1	24.6	0.91
10.25	30.5	25.7	0.84
10.5	17.1	27.0	1.58
10.75	33.0	30.2	0.92
11	39.5	33.9	0.86
11.25	37.0	36.3	0.98
11.5	36.6	38.1	1.04
11.75	31.4	36.5	1.16
12	32.0	34.1	1.06
12.25	38.4	31.1	0.81
12.5	25.2	31.7	1.26
12.75	34.8	30.5	0.88
13	31.4	29.4	0.93
13.25	33.6	28.2	0.84
13.5	25.8	29.4	1.14
13.75	25.9	29.1	1.12
14	31.5	29.1	0.92
14.25	30.7	31.9	1.04
14.5	44.5	36.3	0.82
14.75	38.1	39.4	1.03
15	43.4	47.4	1.09
15.25	51.1	46.6	0.91
15.5	50.6	48.5	0.96
15.75	44.8	49.4	1.10
16	43.3	48.1	1.11
16.25	41.8	46.6	1.12
16.5	44.3	44.3	1.00
16.75	53.4	45.0	0.84

9.5 (CONT'D)

Time (h)	Mo_2(m.moles.h^{-1})	Mco_2(m.moles.h^{-1})	RQ.
17	42.6	50.8	1.19
17.25	49.1	54.3	1.11
17.5	55.3	58.9	1.06
17.75	56.9	60.1	1.06
18	56.7	60.8	1.07
18.25	56.5	61.4	1.09
18.5	61.9	62.3	1.01
18.75	56.0	63.3	1.13
19	61.6	63.3	1.03
19.5	38.0	61.3	1.62
20	63.3	56.9	0.90
20.5	47.6	53.0	1.11
21	52.1	49.7	0.95
21.5	45.3	47.4	1.05
22	41.9	46.0	1.10
22.5	44.0	45.1	1.03
23	38.8	43.8	1.13
23.5	42.5	43.8	1.03
24	49.9	43.9	0.88
24.5	36.8	44.1	1.20
25	38.7	44.0	1.13
25.5	46.2	44.1	0.96
26	37.0	43.4	1.17
26.5	39.1	42.6	1.09
27	37.2	42.8	1.15
27.5	35.6	41.6	1.17
28	37.5	41.5	1.10
28.5	37.8	40.6	1.07
29	37.8	40.4	1.07
29.5	30.7	39.3	1.28
30	32.9	37.9	1.15
30.5	31.1	37.5	1.20
31	40.6	36.9	0.91
31.5	36.5	38.5	1.06

9.5 (CONT'D)

Time (h)	M_{O_2} (m.moles.h^{-1})	M_{CO_2} (m.moles.h^{-1})	RQ.
32	30.5	32.8	1.07
32.5	24.4	27.7	1.14
33	17.9	23.9	1.33

9.6

RUN 38 Natural Log Graph of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Wt. (g)	Ln Mass of Cell Dry Wt. (g)
7.5	3.90	1.36
8	3.83	1.34
9	4.26	1.45
10	5.07	1.62
11	5.92	1.78
12	6.48	1.87
13	7.74	2.05
14	8.65	2.16
15.5	9.74	2.28
16.5	11.00	2.40
17.5	11.48	2.44
18	11.62	2.45

10.1

RUN 39 Fermentation Results

Time (h)	O.D. (590n.m)	2M NaOH Fed to the Fermenter (g)
4.5	0.24	102
5	0.29	129
5.5	0.34	159
6	0.39	196
6.5	0.43	238
7	0.52	287
7.5	0.59	338
8	0.65	399
9	0.73	521
10	0.79	626

10 A. Addition of vitamin mixture.

11	0.82	752
12.5	0.82	928

10.2

RUN 39 Effluent Gas Results

Aeration rate = 6 $l.min.^{-1}$

Time (h)	Oxygen %	Carbon Dioxide %
0	20.95	0.037
0.5	20.94	0.058
1	20.93	0.069
1.5	20.94	0.080
2	20.94	0.095
2.5	20.90	0.116
3	20.85	0.141
3.5	20.82	0.176
4	20.81	0.222
4.5	20.73	0.374
5	20.64	0.381
5.5	20.64	0.465
6	20.55	0.505
6.5	20.51	0.588
7	20.46	0.704
7.5	20.42	0.686
8	20.38	0.768
8.5	20.34	0.844
9	20.34	0.877
9.5	20.41	0.749
10	20.47	0.656
10.5	20.44	0.660
11	20.30	0.861
11.5	20.08	1.110
12	20.40	0.982
12.5	20.39	0.590
13	20.28	0.745

10.3

RUN 39 Volume Variation

Time(h)	Mass of 2M NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0	3,000	-	3,000
4.5	102	102	3,102	50	3,052
5	129	129	3,079	100	3,029
5.5	159	159	3,059	150	3,009
6	196	196	3,096	200	3,046
6.5	238	238	3,088	250	3,038
7	287	287	3,087	300	3,037
7.5	338	338	3,088	350	3,038
8	399	399	3,099	400	3,049
9	521	521	3,171	450	3,121
10	626	626	3,226	500	3,176
10 A.	Addition of vitamin mixture (50 ml.)				3,226
11	752	752	3,352	550	3,302
12.5	928	928	3,478	600	3,428

RUN 39 Fermentation Results on a Mass Basis

Time (h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. (gl^{-1})	Mass of Cell Dry Wt. (g)
4.5	3,102	1.73	5.37
5	3,079	2.10	6.45
5.5	3,059	2.46	7.52
6	3,096	2.82	8.74
6.5	3,088	3.11	9.61
7	3,087	3.77	11.62
7.5	3,088	4.27	13.20
8	3,099	4.71	14.59
9	3,171	5.29	16.77
10	3,226	5.73	18.47
10 A.	Addition of vitamins.		
11	3,352	5.94	19.91
12.5	3,478	5.94	20.66

Feedrate 7 - 12.5h $r = 1.00$

$a = -535.5$

$b = 116.9\ ml.h.^{-1}$

$\therefore F = 0.117\ l.h.^{-1}$

Cell Growth 6 - 9h $r = 0.99$

$a = -8.12$

$b = 2.8\ g.h.^{-1}$

10.5

RUN 39 Molar Oxygen Consumption and Carbon Dioxide Production Rates

Time (h)	M_{O_2} (m.moles.h^{-1})	M_{CO_2} (m.moles.h^{-1})	RQ.
0	0	0	-
0.5	1.2	2.7	2.29
1	2.6	4.3	1.65
1.5	0.3	5.9	19.56
2	-	8.1	-
2.5	6.4	11.2	1.76
3	14.7	14.9	1.01
3.5	18.9	20.1	1.06
4	19.0	26.9	1.41
4.5	28.0	49.3	1.76
5	44.5	50.3	1.13
5.5	41.3	62.8	1.52
6	56.5	68.7	1.21
6.5	60.8	81.0	1.33
7	65.6	98.2	1.50
7.5	73.8	95.4	1.29
8	78.1	107.6	1.38
8.5	82.6	118.9	1.44
9	81.3	123.8	1.52
9.5	73.2	104.8	1.43
10	65.6	91.0	1.39
10.5	71.0	91.6	1.29
11	89.4	121.4	1.36
11.5	120.8	158.2	1.31
12	66.1	139.7	2.11
12.5	83.0	81.1	0.98
13	78.9	104.2	1.32

RUN 39 Natural Log Graph of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Wt. (g)	Ln Mass of Cell Dry Wt. (g)
4.5	5.37	1.68
5	6.45	1.86
5.5	7.52	2.02
6	8.74	2.17
6.5	9.61	2.26
7	11.62	2.45
7.5	13.20	2.58
8	14.59	2.68
9	16.77	2.82
10	18.47	2.92
10 A. Addition of vitamins.		
11	19.92	2.99
12.5	20.66	3.03

RUN 40 Fermentation Results

Time (h)	O.D.(590n.m)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter (g)
0	-	0
5.5	0.38	228
6	0.39	275
6.5	0.505	333
7	0.55	390
7.5	0.595	458
8	0.63	532
8.5	-	608
9	-	691
9.5	0.77	750
10	0.80	810
10 A.	Addition of Vitamin B_1 (0.2090g)	
10.5	0.825	868
11	0.88	918
11 B.	Addition of Folic Acid (0.0620g)	
11.5	-	971
12	0.91	1,032
12 C.	Addition of Riboflavin (0.0494g)	
12.5	-	1,097
13	0.90	1,158
13.5	-	1,227
13.5 D.	Addition of Pantothenic Acid (0.1000g)	
14	0.915	1,302

11.1 (CONT'D)

Time (h)	O.D. (590n.m)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter (g)
14	E. Addition of Nicotinic Acid (0.1175g)	
14.5	-	1,378
14.75	F. Addition of Biotin (0.0275g)	
15	0.96	1,442
15.5	0.94	1,508

11.2

RUN 40 Effluent Gas Results

Aeration rate = 6 $l.min.^{-1}$

Time (h)	Oxygen %	Carbon Dioxide %
0	20.94	0.039
0.5	20.90	0.121
1	20.91	0.094
1.5	20.87	0.109
2	20.88	0.130
2.5	20.82	0.157
3	20.80	0.189
3.5	20.70	0.228
4	20.67	0.285
4.5	20.60	0.351
5	20.53	0.410
5.5	20.46	0.511
6	20.41	0.529
6.5	20.36	0.564
7	20.36	0.602
7.5	20.27	0.672
8	20.24	0.710
8.5	20.20	0.735
9	20.20	0.775
9.5	20.26	0.720
10	20.37	0.617
10.5	20.37	0.566
11	20.43	0.533
11.5	20.37	0.518
12	20.41	0.581
12.5	20.29	0.655
13	20.19	0.769
13.5	20.22	0.780
14	20.20	0.779
14.5	20.21	0.745
15	20.25	0.759
15.5	20.19	0.771

11.3

RUN 40 Volume Variation

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0	2,500	-	2,500
5.5	228	194	2,694	50	2,644
6	275	234	2,684	50	2,634
6.5	333	283	2,683	50	2,633
7	390	332	2,682	50	2,632
7.5	458	390	2,690	50	2,640
8	532	453	2,703	50	2,653
8.5	608	517	2,717	-	2,717
9	691	588	2,788	-	2,788
9.5	750	638	2,838	50	2,788
10	810	689	2,839	50	2,789
10.5	868	739	2,839	50	2,789
11	918	781	2,831	50	2,781
11.5	971	826	2,826	-	2,826
12	1,032	878	2,878	50	2,828
12.5	1,097	934	2,884	-	2,884
13	1,158	986	2,939	50	2,889
13.5	1,227	1,044	2,947	-	2,947
14	1,302	1,108	3,011	50	2,961
14.5	1,378	1,173	3,026	-	3,026
15	1,442	1,227	3,080	50	3,030
15.5	1,508	1,283	3,086	50	3,036

RUN 40 Fermentation Results on a Mass Basis

Time (h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. ($g.l.^{-1}$)	Mass of Cell Dry Wt. (g)
5.5	2,694	2.75	7.41
6	2,684	2.85	7.65
6.5	2,683	3.65	9.79
7	2,682	3.95	10.59
7.5	2,690	4.35	11.70
8	2,703	4.60	12.43
9.5	2,838	5.60	15.89
10	2,839	5.80	16.47
10.5	2,839	6.00	17.03
11	2,831	6.40	18.12
12	2,878	6.60	18.99
13	2,939	6.50	19.10
14	3,011	6.65	20.02
15	3,080	6.95	21.41
15.5	3,086	6.80	20.98

Feedrate 7.5 - 9h $r = 1.00$

$a = -598.7$

$b = 131.6\ ml.h.^{-1}$

$\therefore F = 0.132\ l.h.^{-1}$

12 - 15.5h $r = 1.00$

$a = -533.2$

$b = 117.3\ ml.h.^{-1}$

$\therefore F = 0.117\ l.h.^{-1}$

Cell Growth 5.5 - 10h $r = 1.00$

$a = -3.56$

$b = 2.02\ g.h.^{-1}$

11.5

RUN 40 Molar Oxygen Consumption and Carbon Dioxide Production Rates

Time (h)	Mo_2(m.moles.h^{-1})	Mco_2(m.moles.h^{-1})	RQ.
0	1.9	0.1	-
0.5	6.2	12.0	1.9
1	5.4	8.0	1.5
1.5	12.2	10.2	0.8
2	9.5	13.3	1.4
2.5	19.7	17.3	0.9
3	22.2	22.0	1.0
3.5	39.3	27.7	0.7
4	42.6	36.1	0.8
4.5	53.1	45.8	0.9
5	63.9	54.5	0.9
5.5	73	69.5	1.0
6	81.6	72.1	0.9
6.5	89.5	77.2	0.9
7	88.1	82.9	0.9
7.5	102.1	93.2	0.9
8	106.2	98.8	0.9
8.5	112.7	102.4	0.9
9	111.2	108.4	1.0
9.5	102.2	100.3	1.0
10	85.7	85.1	1.0
10.5	87.6	77.5	0.9
11	77.7	72.7	0.9
11.5	89.4	70.4	0.8
12	79.6	79.8	1.0
12.5	99.1	90.7	0.9
13	128.6	90.5	0.7
13.5	107.3	109.2	1.0
14	111.0	109.0	1.0
14.5	110.5	103.9	0.9
15	102.5	106.1	1.0
15.5	113.2	107.8	1.0

11.6

RUN 40 Natural Log Graph of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Wt. (g)	Ln Mass of Cell Dry Wt. (g)
0	-	-
5.5	7.41	2.00
6	7.65	2.03
6.5	9.79	2.28
7	10.59	2.36
7.5	11.70	2.46
8	12.43	2.52
9.5	15.89	2.77
10	16.47	2.80
10.5	17.03	2.83
11	18.12	2.90
12	18.99	2.94
13	19.10	2.95
14	20.02	3.00
15	21.41	3.06
15.5	20.98	3.04

12.1

RUN 41 Fermentation Results

Time(h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml.^{-1}$)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter (g)
0	-	-	0
5	0.32	60.7	205
5.5	-	-	250
6	0.40	98.7	305
6.5	-	-	371
7	0.53	162.0	452
7.5	-	-	539
8	0.65	217.6	613
8.25 A.	Addition of 0.1026g Folic Acid		
9	0.7	237.9	754
9.5	-	-	850
10	0.77	308.8	949
10.5	-	-	1,048
11	0.79	299.7	1,151
11.5	-	-	1,258
11.5 B.	Addition of 0.1811g Folic Acid		
11.75	-	-	1,310
12	0.81	394.7	1,369
12 C.	Addition of 27g Tryptone T. (Oxoid L43)		
12.5	-	-	1,445
13	0.82	364.7	1,547
13 D.	Addition of 10g K_2HPO_4		
13.5	-	-	1,620

12.1 (CONT'D)

Time(h)	O.D.(590n.m)	Enzyme Activity ($DSU.ml.^{-1}$)	Mass of Sucrose/ NaOH Mix Fed to the Fermenter (g)
13.5	E.	Addition of 100 ml. of Complete Medium	
14	0.84	344.7	1,650

RUN 41 Effluent Gas Results

Aeration rate = 6 $l.min.^{-1}$ = 2.4 V.V.M.

Time (h)	Oxygen %	Carbon Dioxide %
0	20.95	0.04
0.25	20.94	0.045
0.5	20.94	0.064
0.75	20.94	0.073
1	20.93	0.079
1.25	20.92	0.087
1.5	20.92	0.094
1.75	20.91	0.102
2	20.90	0.114
2.25	20.89	0.124
2.5	20.88	0.137
2.75	20.87	0.152
3	20.86	0.169
3.25	20.84	0.191
3.5	20.82	0.218
3.75	20.81	0.252
4	20.78	0.291
4.25	20.76	0.336
4.5	20.73	0.380
4.75	20.72	0.426
5	20.69	0.472
5.25	20.64	0.509
5.5	20.61	0.627
5.75	20.59	0.641
6	20.58	0.629
6.25	20.59	0.643
6.5	20.56	0.655
6.75	20.51	0.706
7	20.47	0.773
7.25	20.43	0.888
7.5	20.40	0.947
7.75	20.44	0.971

12.2 (CONT'D)

Time (h)	Oxygen %	Carbon Dioxide %
8	20.49	0.891
8.25	20.54	0.797
8.5	20.56	0.730
8.75	20.54	0.700
9	20.49	0.751
9.25	20.42	0.874
9.5	20.36	0.966
9.75	20.32	1.04
10	20.30	1.08
10.25	20.29	1.10
10.5	20.28	1.12
10.75	20.29	1.13
11	20.29	1.12
11.25	20.31	1.14
11.5	20.32	1.13
11.75	20.35	1.10
12	20.37	1.09
12.25	20.38	1.06
12.5	20.39	1.05
12.75	20.40	1.04
13	20.42	1.01
13.25	20.43	1.02
13.5	20.43	1.04
13.75	20.43	1.04
14	20.47	1.08

12.3

RUN 41 Volume Variation

Time(h)	Mass of Sucrose/NaOH Fed to the Fermenter(g)	Volume of Feed Added (ml)	Volume of Broth Prior to Sample Taken (ml)	Total Samples Taken (ml)	Broth Volume (ml)
0	0	0	2,500	0	2,500
5	205	168	2,688	50	2,618
5.5	250	205	2,655	-	2,655
6	305	251	2,701	50	2,651
6.5	371	305	2,705	-	2,705
7	452	371	2,771	50	2,721
7.5	539	442	2,792	-	2,792
8	613	503	2,853	50	2,803
8.25	646	531	2,831	-	2,831
9	754	619	2,919	50	2,869
9.5	850	698	2,948	-	2,948
10	949	779	3,029	50	2,979
10.5	1,048	861	3,060	-	3,060
11	1,151	945	3,145	50	3,095
11.5	1,258	1,033	3,183	50	3,183
11.75	1,310	1,075	3,225	-	3,225
12	1,369	1,124	3,274	50	3,224
12.5	1,445	1,186	3,286	-	3,286
13	1,547	1,270	3,370	50	3,320
13.5	1,620	1,330	3,380	-	3,380
14	1,650	1,355	3,405	50	3,355

RUN 41 Fermentation Results on a Mass Basis

Time(h)	Volume of Broth Prior to Sample Taken (ml)	Cell Dry Wt. ($g.l^{-1}$)	Enzyme Activity ($DSU.ml^{-1}$)	Mass of Cell Dry Wt. (g)	Enzyme Activity $(DSU)x10^{-3}$
0	2,500	0.29	0	0.73	0
5	2,668	2.30	60.7	6.14	162.1
6	2,701	2.88	98.7	7.77	269.6
7	2,771	3.83	162.0	10.60	453.8
8	2,853	4.70	217.6	13.41	629.0
9	2,919	5.10	237.9	14.89	705.3
10	3,029	5.65	308.8	17.11	947.1
11	3,145	5.75	299.7	18.08	958.1
12	3,274	5.90	394.7	19.32	1,307.1
13	3,370	5.95	364.7	20.05	1,248.7
14	3,405	6.10	344.7	20.77	1,191.9

Feedrate 6.5 - 8h $r = 1.00$

$a = -559.0$

$b = 133.0\ ml.h.^{-1}$

$\therefore F = 0.133\ l.h.^{-1}$

9 - 13h $r = 1.00$

$a = -863.7$

$b = 164.6\ ml.h.^{-1}$

$\therefore F = 0.165\ l.h.^{-1}$

Cell growth 6 - 8h $r = 1.00$

$a = -9.15$

$b = 2.82\ g.h.^{-1}$

10 - 14h $r = 0.99$

$a = 7.92$

$b = 0.93\ g.h.^{-1}$

12.5

RUN 41 Molar Oxygen Consumption and Carbon Dioxide Production Rates

Time(h)	Mo_2(m.moles.h^{-1})	Mco_2(m.moles.h^{-1})	RQ.
0	0	0	-
0.25	1.7	0.7	0.41
0.5	0.9	3.5	3.89
0.75	0.6	4.9	8.17
1	2.2	5.7	2.59
1.25	3.8	6.9	1.82
1.5	3.5	8.0	2.29
1.75	5.0	9.1	1.82
2	6.4	10.9	1.70
2.25	7.9	12.4	1.57
2.5	9.3	14.3	1.54
2.75	10.5	16.5	1.57
3	11.7	19.0	1.62
3.25	14.6	22.2	1.52
3.5	17.3	26.2	1.51
3.75	17.8	31.2	1.75
4	21.9	37.0	1.69
4.25	23.9	43.6	1.82
4.5	27.7	50.1	1.81
4.75	27.8	56.9	2.05
5	31.6	63.7	2.02
5.25	39.5	69.2	1.75
5.5	40.5	86.7	2.14
5.75	43.7	88.7	2.03
6	46.0	86.9	1.89
6.25	43.6	89.0	2.04
6.5	48.8	90.8	1.86
6.75	56.1	98.3	1.75
7	61.0	108.2	1.77
7.25	64.0	125.3	1.96
7.5	67.3	134.1	1.99
7.75	58.9	137.8	2.34

12.5 (CONT'D)

Time(h)	M_{O_2}(m.moles.h^{-1})	M_{CO_2}(m.moles.h^{-1})	RQ.
8	52.7	125.9	2.39
8.25	47.0	111.9	2.38
8.5	45.9	101.9	2.22
8.75	50.7	97.4	1.92
9	58.1	105.0	1.81
9.25	66.4	123.2	1.86
9.5	74.0	136.9	1.85
9.75	78.6	147.9	1.88
10	80.8	153.8	1.90
10.25	81.9	156.8	1.91
10.5	83.0	159.8	1.93
10.75	80.8	161.3	2.00
11	81.2	159.8	1.97
11.25	76.7	162.8	2.12
11.5	75.2	161.4	2.15
11.75	70.8	156.9	2.22
12	67.4	155.5	2.31
12.25	66.7	151.0	2.26
12.5	65.2	149.5	2.29
12.75	63.7	148.0	2.32
13	61.2	143.6	2.35
13.25	58.9	145.1	2.46
13.5	58.1	148.1	2.55
13.75	58.1	148.1	2.55
14	49.1	154.2	3.14

12.6

RUN 41 Natural Log Graph of Cell Dry Weight Mass

Time (h)	Mass of Cell Dry Wt. (g)	Ln Mass of Cell Dry Wt. (g)
0	0.73	-0.31
5	6.14	1.81
6	7.77	2.05
7	10.60	2.36
8	13.41	2.60
9	14.89	2.70
10	17.11	2.84
11	18.08	2.89
12	19.32	2.96
13	20.05	3.00
14	20.77	3.03

APPENDIX 13

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LINEAR GROWTH IN BATCH CULTURE CAUSED BY CONSERVATIVE TRACE NUTRIENT LIMITATION

D E BROWN+ and R N GADDUM
Biotechnology Centre, Cranfield Institute of Technology,
Cranfield, Bedford MK43 OAL
and
A McAVOY*
Department of Chemical Engineering, UMIST, Manchester M60 1QD

SUMMARY

Examples of linear growth are reviewed and some are identified with conservative trace nutrient limitation. A kinetic description of the limitation mechanism is proposed and shown to describe the limited batch culture of Bacillus subtilis.

INTRODUCTION

Conventional analysis of the growth kinetics of micro-organisms in batch culture, as pioneered by Monod, predicts an exponential increase in the cell population in the presence of unlimited supplies of nutrients. This analysis has been verified on countless occasions, so that it has been accepted as the basis of most microbial kinetic growth models. Consequently, there is a danger that workers might be tempted to force an exponential fit when perhaps the data do not warrant it.

Linear growth in batch culture caused by the constant rate of supply of oxygen to the system is well understood and described in some detail by Sinclair and Kristiansen (1987). Initial exponential growth is followed by linear growth started at cell concentrations that are proportional to the aeration rates and proceeded at linear growth rates that are also proportional to the aeration rates. Analogous effects occur when a batch process is fed with the limiting substrate at a constant rate. Examples of linear growth produced by feeding glycerol at a constant rate have been reported for Enterobacter cloacae by Yamane and Hirano (1977) and for Klebsiella pnuemoniae by Esener et al (1981).

A number of workers have reported clear examples of linear growth in connection with the utilisation of trace nutrient components such as

+ To whom all correspondence should be addressed.
* Present address: Sturge Biochemicals Ltd, Dennison Road, Selby YO8 8EF.

nicotinic acid by Proteus vulgaris (Jackson and Copping (1952)), phosphate by Escherichia coli (Slezak and Sikyta (1957), and Horiuchi (1959)) and some unknown factor, possibly a component of yeast extract, by Streptococcus faecalis (Anderson et al (1987)). Reduction in the growth rate, arising from trace element limitation, has also been discussed by Pirt (1975).

The rapid uptake of the trace nutrients by relatively small quantities of cells in the early stages of the culture, out of proportion to the requirements indicated by large values of yield coefficients, is a common observation. This scavenging process was reported for nicotinic acid by Proteus vulgaris (Jackson and Copping (1952)) and the moment of complete removal of phosphate from the medium by Escherichia coli (Horiuchi (1959)) correlated with the start of the linear growth phase. Slezak and Sikyta (1957) showed that three different initial concentrations of phosphate resulted in three different values of cell concentration at which linear growth was initiated and that the slopes of the linear growth phases were also different. Nyholm (1976) studied the uptake of phosphate by Chlorella pyrenoidosa, recognised that the early scavenged uptake of the trace nutrient could be followed by it's distribution to successive generations and so described such components as 'conservative' nutrients.

Although linear growth linked to conservative trace nutrient uptake has been observed by several workers no satisfactory kinetic formulation has yet been proposed.

THEORETICAL

A mass balance on the cell growth gives:

$$\frac{dx}{dt} = r_x = \mu x \qquad (1)$$

Michaelis-Menten kinetics for the effect of substrate concentration in the medium states that:

$$\mu = \mu_m \frac{s}{k_s + s} \qquad (2)$$

From this analysis, at high s, then $\mu = \mu_m$ and Equation 1 gives $dx/dt = \mu_m x$ and the familiar exponential growth results. At low s, assuming that s in the denominator is zero, gives $\mu = \mu_m s/k_s$

and:

$$\frac{dx}{dt} = \frac{\mu_m . s . x}{k_s} \qquad (3)$$

Linear growth requires that dx/dt=constant and this would mean that sx=constant. However, the evidence suggests that a conservative substrate is consumed almost completely from the medium before the linear growth phase starts and does not decline in proportion to the increase in cell concentration.

It is suggested here that the specific growth rate is controlled by the concentration of the trace nutrient inside the cells but still in a similar manner to Equation (2):

$$\mu = \mu_m \frac{s_i}{k_{si} + s_i} \qquad (4)$$

If it is assumed that the fixed mass of trace nutrient (m) is scavenged completely by the cells and that it is incorporated into the cellular structure in such small quantities that it can be considered to remain constant, then the internal concentration at all times will be:

$$s_i = \frac{m}{xVv} \qquad (5)$$

Thus, at high values of s_i, $\mu = \mu_m$. However, at low values of s_i the specific growth rate is:

$$\mu = \frac{\mu_m}{k_{si}} \cdot \frac{m}{xVv} \qquad (6)$$

Then from Equation (1):

$$\frac{dx}{dt} = \frac{\mu_m}{k_{si}} \cdot \frac{m}{Vv} = \text{constant} \qquad (7)$$

The implications of this analysis are that from Equation (5), the value of x at which $s_i = s_{ic}$ (when μ changes from μ_m) is proportional to m and from Equation (7) the slope of the linear growth is also proportional to m.

EXPERIMENTAL

In order to test the assumptions developed above for growth under conservative trace nutrient limitation, batch growth studies were carried out on Bacillus subtilis B20 at two different initial concentrations of yeast extract, which was observed to contain some unknown trace nutrient essential for growth (Gaddum 1988).

The organism was grown in a standard LKB laboratory fermenter, with 3 dm^3 culture medium in a 5 dm^3 vessel. The medium consisted of 10% w/v glucose together with either 1.5% or 3.0% w/v Lab M yeast extract. The starting pH was 6.4 and controlled with sodium hydroxide to 6.0 if necessary. A silicone antifoam (ASAAF, ABM Chemicals) was available on demand. The fermentations were run at 35°C, with an air flow rate of 3 dm^3 per minute and the stirrer speed varied in a range up to 8 $rev.s^{-1}$, maintaining dissolved oxygen tension greater than 50% sat. A 3% v/v inoculum, grown in a shake-flask medium containing 5% w/v yeast extract and 2% w/v malt extract, was used to start each run.

The dry weight measurement was determined by spinning a 10 cm^3 sample at 133 $rev.s^{-1}$ for 10 minutes at 4°C. The pellet was re-suspended in 10 cm^3 of distilled water and re-centrifuged. The second pellet was re-suspended in 2 cm^3 of distilled water and dried to constant weight at 105°C in a foil boat.

The results of the growth experiments, as dry weight versus time are shown in Figure 1. In order to super-impose the initial stages of growth, the data for the 3% w/v yeast extract were moved backwards in time by 1.5 hours. The ratio of the yeast extract quantities is m_1/m_2 = 1.5/3.0 = 0.50. Values of the slopes of the linear growth periods are 0.28 and 0.62 $kg.m^{-3}.h^{-1}$ giving a ratio of 0.45. The cell concentration at which exponential growth ceased, was 0.98 $kg.m^{-3}$ for 1.5% w/v yeast extract and 1.80 $kg.m^{-3}$ for 3.0% w/v yeast extract, giving a value of 0.54 for the x_c ratio.

The ratios of both the slopes and the intercepts are thus in close agreement with those of the trace component compositions as described by the simple kinetic model. These results are further supported by the data of Slezak and Sikyta (1967). Three values of inorganic phosphate were used of 4.3, 7.1 and 13.7 $\mu gP.cm^{-3}$ giving a ratio of 1:1.65:3.19. The ratios of x_c were determined to be 1:1.63:2.94 in close agreement with the phosphate ratios. However, the ratios of the slopes were approximately 1:1.42:1.75 which shows a gradual deviation from the model at high cell concentrations.

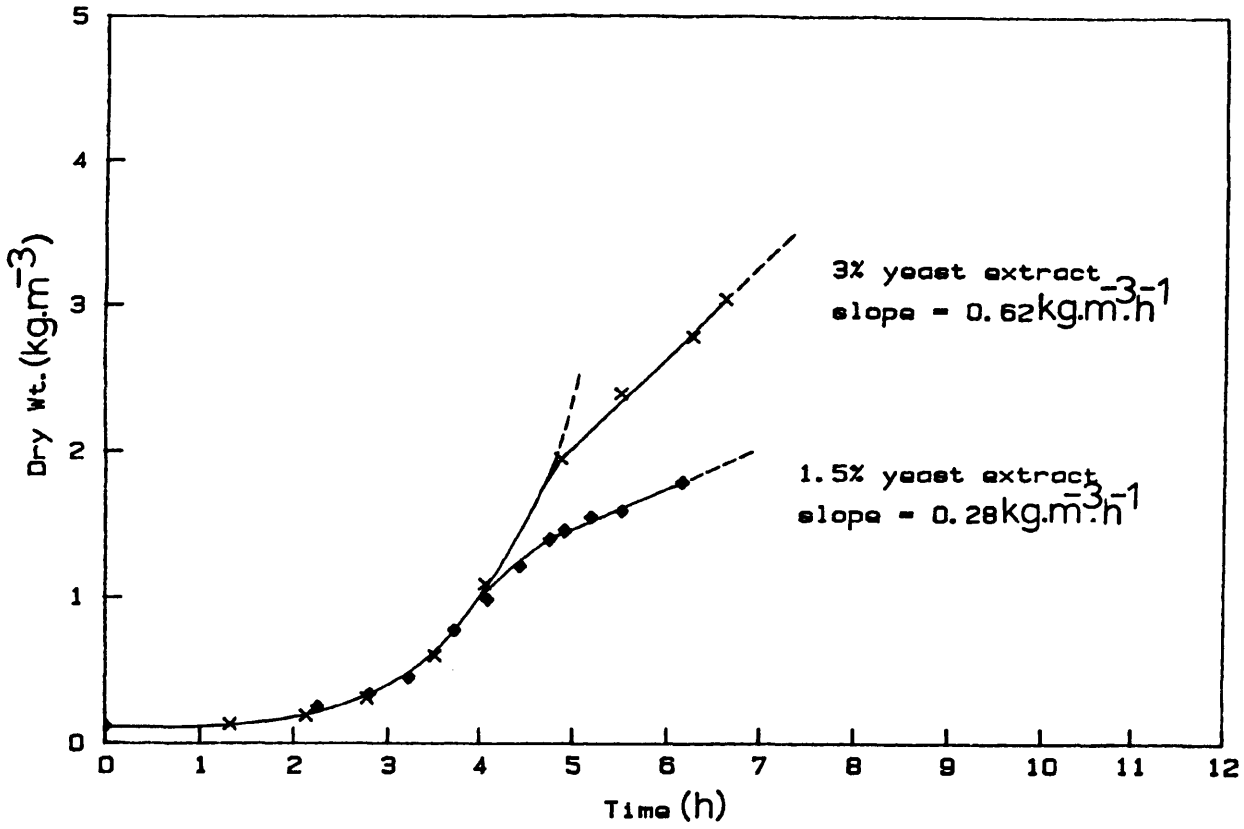

Figure 1. Effect of yeast extract concentration on the batch culture of Bacillus subtilis.

CONCLUSIONS

A simple mechanistic analysis of the linear growth reported by several workers, arising from conservative trace nutrient limitation, has been proposed. The assumptions are that the given mass of trace material is rapidly consumed by the cells, is not effected measurably by conversion during metabolism and that the specific growth rate is controlled by the concentration of the trace material inside the cells. The kinetic analysis shows that the mass of trace material in the initial medium determines both the cell concentration at which linear growth starts and also the slope of the linear growth phase. Experimental values determined from batch growth of Bacillus subtilis on different concentrations of yeast extract showed close agreement with the kinetic analysis. The broad implication is that many fermentations based on the need for complex medium components may suffer from conservative trace nutrient limitation. A linear growth rate, linear rates of substrate uptake or product formation together with a constant outlet gas composition, might be indications of this phenomenon.

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NOMENCLATURE

k_s	Saturation coefficient for medium	$kg.m^{-3}$
k_{si}	Saturation coefficient for cell contents	$kg.m^{-3}$
m	Mass of trace material in batch	kg
r_x	Growth rate	$kg.m^{-3}.h^{-1}$
s	Substrate concentration in medium	$kg.m^{-3}$
s_i	Substrate concentration inside cells	$kg.m^{-3}$
s_{ic}	Critical substrate concentration inside cells	$kg.m^{-3}$
t	time	h
V	Volume of culture	m^3
v	Specific volume of liquid inside cells	$m^3.kg^{-1}$
x	Cell concentration	$kg.m^{-3}$
x_c	Critical cell concentration when $s_i=s_{ci}$	$kg.m^{-3}$
μ, μ_m	Specific and maximum specific growth rate	h^{-1}

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