THE FERMENTATION OF CHEESE WHEY BY *Lactobacillus helveticus*

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DECLARATION

This thesis has not been nor is currently being submitted for the award of any other degree or similar qualification.

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The Fermentation Of Cheese Whey by Lactobacillus helveticus

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ABSTRACT

The lactic acid fermentation of cheese whey permeate by *Lactobacillus helveticus* was studied. Precipitate formation during autoclaving of whey permeate was examined. Precipitation was found to be pH and temperature dependent. Qualitative analysis suggested that the precipitate was a calcium-phosphate complex. Solubilisation was achieved both by acidification and use of the sequestering agent EDTA. Optimisation of *L. helveticus* growth in whey permeate was carried out using factorial design, as opposed to a traditional univariate approach. Using this technique, the variation of specific growth rate with pH, temperature and stirrer speed was assessed.

Cell growth and lactic acid formation in whey permeate containing various supplements, were investigated. Yeast extract was the most effective nitrogen/growth factor supplement. Maximum lactic acid production was achieved in permeate containing yeast extract (0.75% w/v) , Tween 80 (0.1% v/v) and sodium acetate (0.05% w/v). Optimisation of lactic acid production in supplemented whey permeate was performed using factorial design. Optimum conditions for both acid formation and cell growth were pH 5.9, temperature 42°C and stirrer speed 200 rpm.

Fourier transform infrared spectroscopy was applied to the on line and off line quantitative analysis of lactose and lactic acid during the fermentation process. This technique enabled substrate and product levels to be assessed quickly and simply, with no sample pre-treatment. Continuous culture of *L. helveticus* in MRS medium and supplemented whey permeate was carried out. Substrate conversion and lactic acid productivity decreased with increasing dilution rate. Maximum productivity corresponded to a dilution rate of 0.3 h^{-1}, whereas minimum residual substrate occured at a dilution rate of 0.1 h^{-1}. Translation of the fermentation process from bench scale (1l) to pilot scale (16l) appeared to be successful. Completion times, productivity and lactose utilisation compared favourably with bench scale results.

NOMENCLATURE

General Abbreviations Used

BOD	Biological oxygen demand
CaH	Caesin hydrolysate
COD	Chemical oxygen demand
CSL	Corn steep liquor
F1	Fermenter 1
F2	Fermenter 2
FIA	Flow injection analysis
FTIR	Fourier transform infrared
GC	Gas chromatography
GS	Glucose synthetic
HPLC	High performance liquid chromatography
IR	Infrared
LaH	Lactalbumin hydrolysate
LS	Lactose synthetic
MRS	de Man, Rogosa, Sharpe
MW	Molecular weight
OD	Optical density
ODi	Optical density before autoclaving
ODa	Optical density after autoclaving
PDPW	Partially deproteinised whey
PEP	Bacteriological peptone
PER	Protein equivalence ratio
pHi	pH before autoclaving
pHa	pH after autoclaving
UF	Ultrafiltration
WP	Whey permeate

WPC	Whey protein concentrate
YE	Yeast extract

Mathematical Abbreviations

ANOVA	Analysis of variance
b	Coefficient of regression equation
df	Degrees of freedom
F ratio	Fisher variance ratio
LOF	Lack of fit
MS	Mean squares
MS_T	Total mean squares
PE	Pure error
REG	Regression
RES	Residual
P	pH factor
p	pH effect
SE	Standard error
S	Stirrer speed factor
s	Stirrer speed effect
SS	Sum of squares
SS_T	Total sum of squares
T	Temperature factor
t	Temperature effect
μ	Specific growth rate constant
μ_1	Specific growth rate for fermenter 1
μ_2	Specific growth rate for fermenter 2
μ_{av}	Average specific growth rate
x	Any factor
-	Lower level of factor
+	Upper level of factor

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CHAPTER 1. INTRODUCTION

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1. INTRODUCTION

1.1 CHEESE / WHEY PRODUCTION

Whey is the major by-product of cheese making, with approximately 5-10 lb of whey generated per lb of cheese produced[1]. World wide cheese manufacture has been rising steadily and in 1981 reached 11 million tons. The total amount of liquid whey generated in this activity was near 104 million tons, constituting 83% of the volume of milk used[2]. This represents 67 million tons of lactose and 600,000 tons of proteins[3]. Fig. 1.1.1. shows the increase in whey production for the US from 1973 to 1985. By 1988, whey production in the US was estimated to be 26 million tons[4].

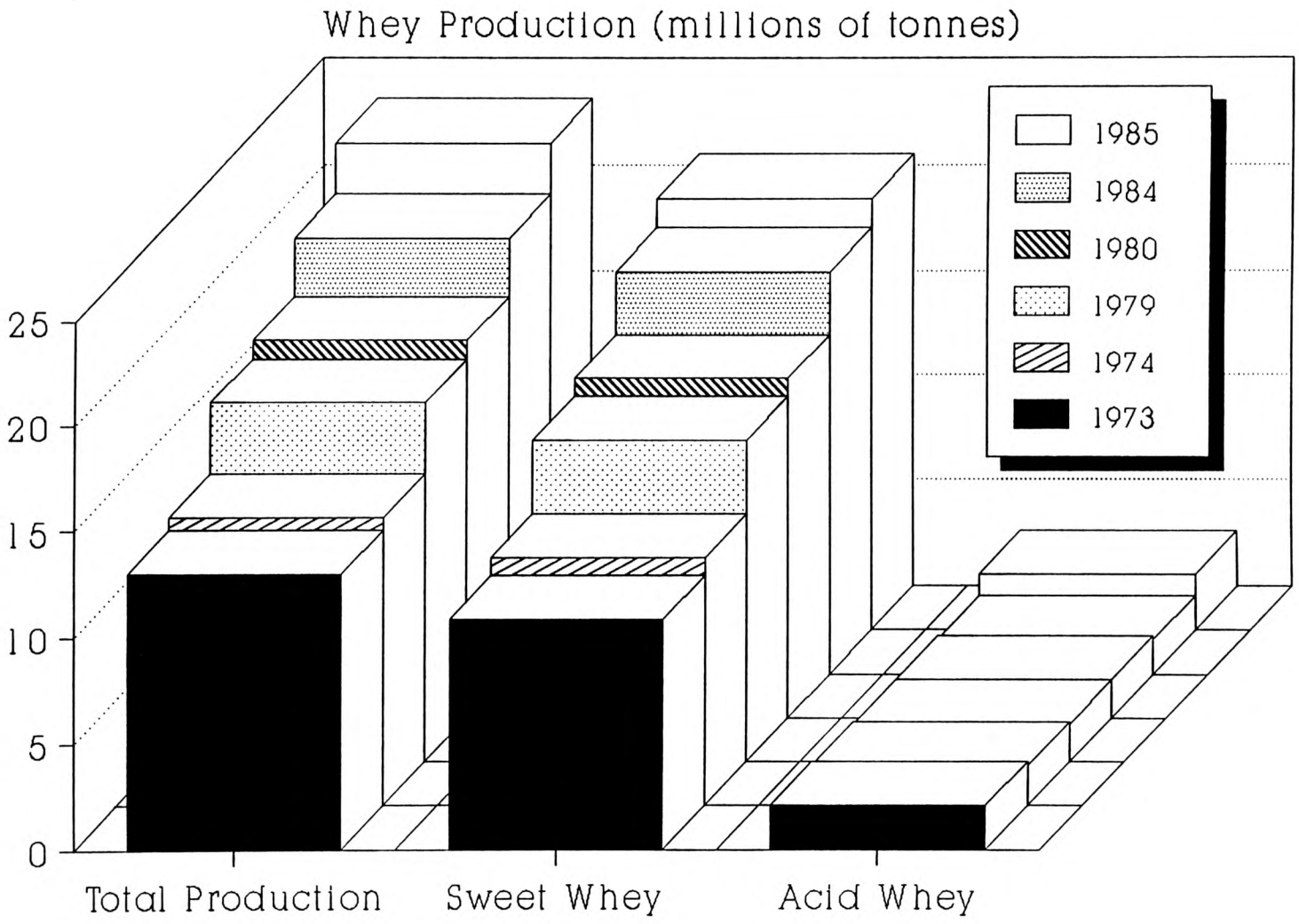
In general, the stages of cheese making are[5]:

1. milk pre-treatment
2. coagulation
3. separation of solid curd from fluid whey
4. forming of curd
5. cheese ripening

This is illustrated in Fig. 1.1.2. Milk with a relatively low bacterial count is most suitable for cheese production. A high count can lead to problems associated with off flavour development and excessive gas production. Several milk enzymes can affect cheese quality. Lactoperoxidase for example, inhibits some lactose fermenting lactic acid bacteria important in cheese manufacture. Pasteurization can reduce the microbial and enzymic activity of milk. Cottage and cream cheeses usually employ pasteurized milk. Homogenization of milk, which reduces the size of fat globules, is used in the manufacture of cream cheeses, some soft cheeses and cheese spreads.

Milk coagulation is generally achieved by the combined processes of pH reduction and milk clotting enzymes like rennet. Acidification is initiated by the use of lactic acid bacte-

FIG 1.1.1. Estimated U.S. Fluid Whey Production 1973-1985[6]



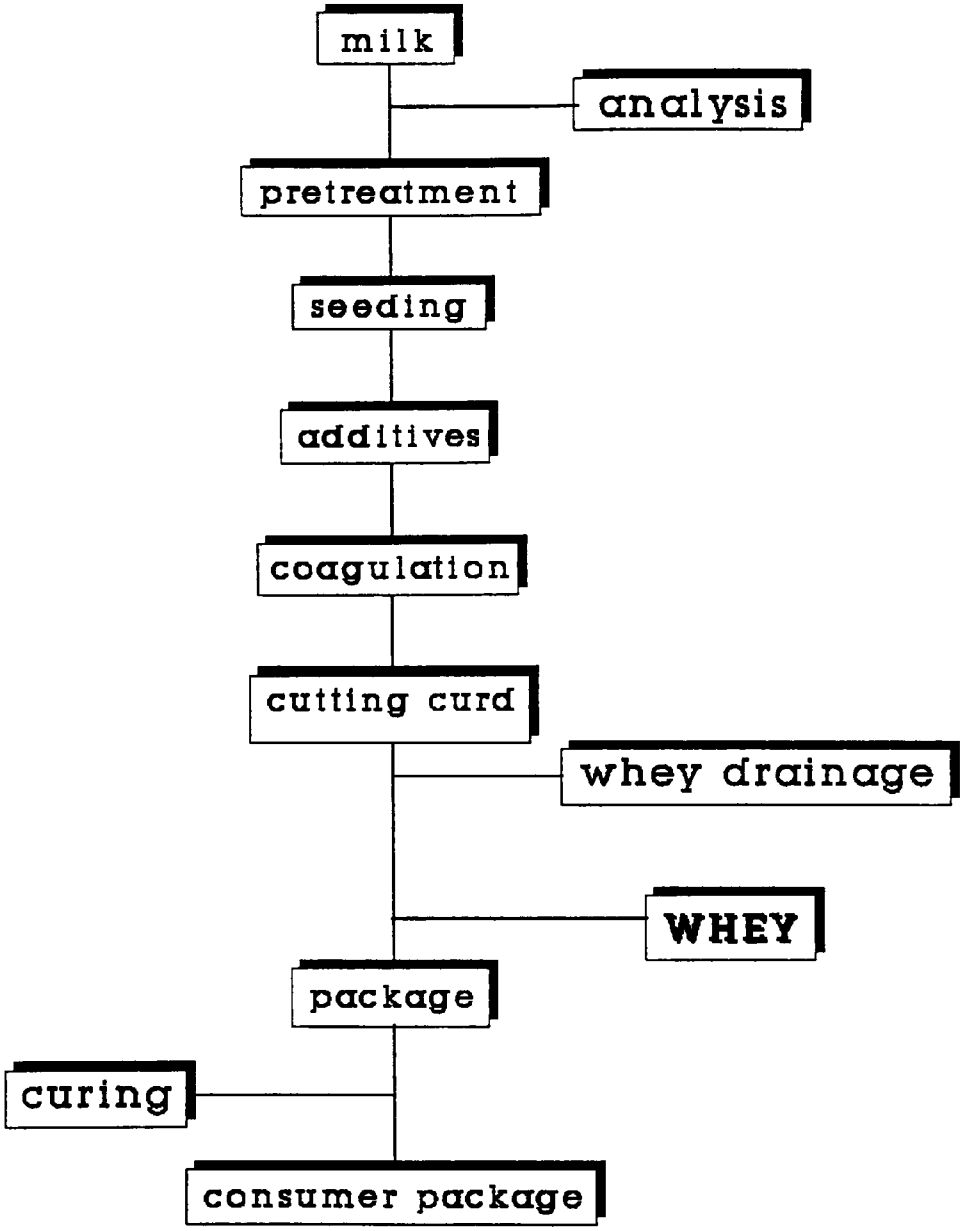


FIG 1.1.2. Flow Diagram for Cheese Production

ria, such as *Lactobacillus helveticus*, as starter cultures. Acid production reduces casein solubility as the pH approaches 4.6, the isoelectric point of casein, and accelerates enzymic cheese coagulation. In addition, acidification suppresses development of an undesirable microflora, for example *Staphylococcus aureus*, and contributes to the elasticity, texture and flavour of cheeses.

Following coagulation, the hot curd is separated from the liquid whey and allowed to coalesce. This process of whey syneresis (extraction) is accelerated by cutting of the curd, pH reduction, increasing temperatures and several physical separation techniques. The overall texture and taste of cheeses depend on differences in their physical composition (water, fat and protein content), the composition of the starter culture used to seed the milk and the ecology of the secondary microflora that grow in or on the cheese as it matures[7].

1.2 WHEY COMPOSITION

The composition of whey depends on its origin (ewe, goat or cow) and the cheese making technique employed[8] (Table 1.2.1.). For example, coagulation with rennet as in Cheddar production, yields sweet whey (pH 4.5-6.7) with high lipid content. Coagulation by lactic acid fermentation yields acid whey (pH 3.9-4.5), containing smaller quantities of proteins and lactose. Lactose constitutes 75% of the solids in whey. It is a disaccharide composed of glucose and galactose moieties, and exists in two anomeric forms, α and β. Since it is water soluble, almost 90% of milk lactose is present in whey, existing primarily in the α form. 14% of whey solids are proteins and these amount to 20% of total milk proteins. In addition, whey contains a number of vitamins and trace elements (Tables 1.2.2. and 1.2.3.).

1.3 WHEY: DISPOSAL versus UTILISATION

1.3.1 Background

The disposal of whey has been an inherent part of cheese making for centuries. However, it was not until the advent of factory production that the effluent was seen as a

Table 1.2.1. Composition of different types of liquid whey (g/l)[8]

		COW		EWE	GOAT
Curd type	*Rennet*	*Mixed*	*Lactic*	*Rennet*	*Lactic*
Lactose	51.8	50.8	45.3	51.0	39.2
Lipids	5.06	3.38	0.85	6.46	0 .40
Ash	5.252	5.888	7.333	5.654	8.361
Total Nitrogen	1.4	1.5	1.2	2.9	1.5
Non-protein Nitrogen	0.4	0.4	0.5	0.8	0.7
Ammonium Nitrogen	4×10^{-2}	0.1	0.1	0.1	0.2
Urea Nitrogen	0.1	0.1	0.1	0.1	0.1
Lactic acid	0.3	2.2	7.6	1.8	8.7
Citric acid	1.3	1.1	0.3	1.0	0.2
Phosphorus	0.41	0.47	0.65	0.55	0.70
Calcium	0.47	0.63	1.25	0.49	1.35
Potassium	1.46	1.49	1.49	1.28	1.81
Sodium	0.51	0.54	0.53	0.62	0.43
Chlorides (as NaCl)	2.20	2.21	2.09	2.37	3.29
TOTAL dry matter	70.702	70.808	69.293	75.124	66.941

Table 1.2.2. Average content of the main trace elements in whey and whey permeate (mg / 100 g dry matter)[8]

Element	Whey	Permeate
Mg	0.01 - 0.04	0.5 - 0.8
Cu	0.5 - 5	1 - 3
Fe	1 - 7	3 - 11
Zn	5 - 9	30 - 33

Table 1.2.3. Average vitamin content of whey and whey permeate (mg / 100 g dry matter)[8]

Vitamin	Whey	Permeate
Vitamin A	100	80
Pantothenate	30 - 70	50 - 60
Cobalamin	0.01 - 0.05	0.02 - 0.05
Thiamin	4 - 6	5 - 6
Pyridoxin	6 - 10	5 - 10
Riboflavin	7 - 30	15 - 20
Biotin	0.2 - 0.3	0.1 - 0.3
Vitamin C	30 - 50	20 - 40

ous pollutant[3]. Previously, whey had been regarded as a waste product and was discharged into rivers or used as animal feed. The huge volume of liquid whey presently generated by dairy operations creates severe pollution problems because of its high biological oxygen demand (BOD), averaging 45 g/l and high chemical oxygen demand (COD), typically 65 g/l. This is due mainly to the lactose present at concentrations between 4.5% and 5.0% w/v[2].

Cheese whey, containing an excellent source of nutrients, is abundantly available at low or no cost. If not utilised, it must be treated to destroy these nutrients, which is both costly and unacceptable in today's "Green" climate. Concern about environmental pollution, an appreciation of the unique functional and nutritional properties of whey, and the need to maximise returns on raw materials, has recently encouraged much research into the modification and uses of whey. At present, approximately 50% of whey produced by the dairy industry is utilised (Table 1.3.1, Figs 1.3.1 and 1.3.2).

Table 1.3.1. Whey utilisation in Europe and the USA[6,9] . Figures are in millions of tons

	USA	EUROPE
Whey production (fluid basis)	23.09	NA
Whey production (solids basis)	1.50	2.00
Dry solids utilised	0.70	1.00
% utilised (solids basis)	47	50

1.3.2 Whey Utilisation

Whey processing and utilisation is one of the most important growth and development areas in the field of dairy science and technology. There are numerous outlets for whey, described by Moulin & Galzy[8] as a "potential substrate for biotechnology," and its pro-

FIG 1.3.1. Whey Utilisation in New Zealand 1985/1986[10]

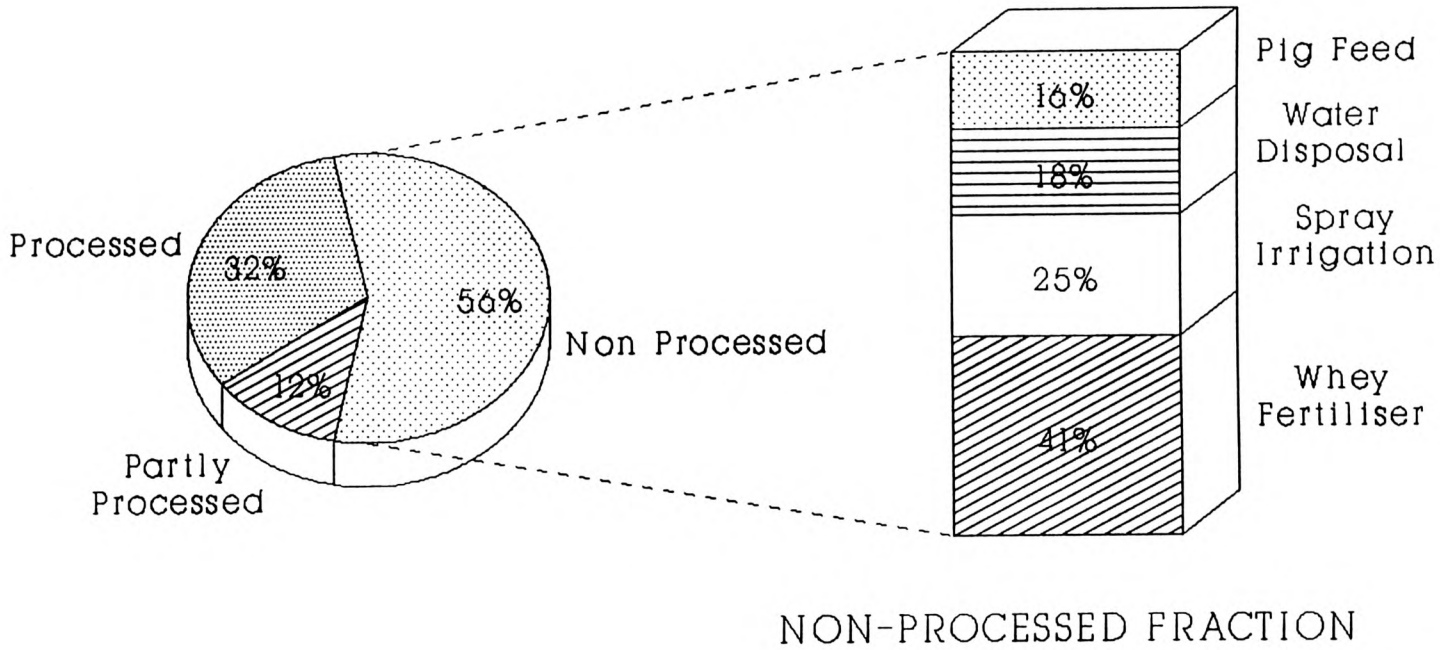
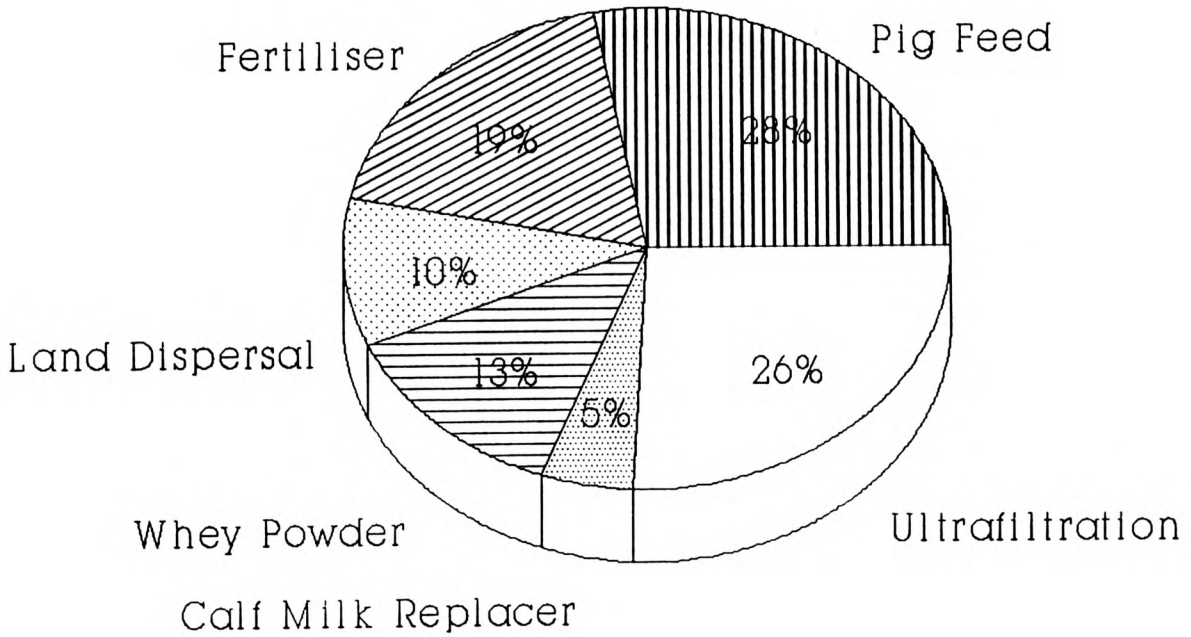


FIG 1.3.2. Disposal and Utilisation of Cheese Whey in Australia 1985[10]



ducts. These range from fuels, fertilisers and animal feeds to various branches of the food industry[11,12].

The use of whey and whey products for pig feed has been reported from countries such as Denmark, Argentina, Poland and Australia. However, due to the constant increase in output from cheese plants, redistribution of large volumes of this byproduct to farmers has proved difficult. A large proportion of the liquid whey generated in Australia and New Zealand is used in spray irrigation and as a fertiliser. In New Zealand, researchers have found that the application of whey to grasslands at an annual rate of 40,000 l/ hectare contains sufficient plant-available nutrients to effectively replace conventional fertilisers[11,12]. This form of whey utilisation is clearly a viable economic proposition for both dairy farmers and dairy companies.

1.3.3. Whey as a Fermentation Substrate

Fermentation is used widely in the manufacture of dairy products. Globally, the quantity of milk fermented to cheese annually exceeds beer production by a factor greater than two[10]. Use of whey as a substrate for fermentations has appealed to workers faced with the problem of upgrading this raw material into useful products which can be manufactured and marketed profitably. Because it is a highly nutritious liquid, whey represents an excellent substrate for controlled microbial growth and metabolism. Numerous fermentations employ whey as a low cost carbon source. Processes include the production of ethanol, food acids, single cell proteins (SCP), gums, methane, antibiotics, amino acids, vitamins, enzymes and other biochemicals[1,11]. The Kraft company in the US for instance, have established a fuel ethanol unit employing whey permeate as a substrate. Elsewhere in the US, an animal feed made from whey ammonium lactate and lactosylurea has been produced. This "Lactowhey" has been used as a cattle feed supplement since 1978. Research groups in France and the US have described procedures for manufacturing biogas from whey. Anaerobic digestion for methane production is attractive since the product does not have to be marketed and the COD of the effluent is markedly

reduced. In the French study, the COD decreased by 90%, 0.59 m^3 of biogas being produced per Kg of COD removed[11].

1.3.4. Whey: Transport and Handling

Since the start of industrialised cheese and caesin production, whey handling has posed problems in terms of storage and transport. Expansion and centralisation of these dairy operations has made the problem more pronounced[13]. In order to minimise costs, concentration processes for whey were developed. The manufacture of whey powder from permeate and whey concentrates, although expensive, is greatly used since whey powder is easy to store and distribute. According to a US dairy expert, roughly 61% of whey produced in the US is dried[11]. With regard to animal feed, powdered whey is used extensively in hog rations and also in milk replacers for unweaned calfs. In Australia, approximately 5% of whey is used as calf milk replacer[10]. Studies on the incorporation of whey powder into human foods have shown that sweet whey can be added advantageously to items such as frozen desserts, cheese products, dried soups etc[14]. The utilisation of natural and modified whey as the base of drinks is common place. Subsequent enrichment with the required taste, aroma and flavour additives creates a whole range of beverages. For example, in Poland, light alcoholic beverages (similar to chainpagne) are manufactured[15].

1.3.5. Whey Proteins

Over the past 25 years, there has been an increasing awareness that the proteins in whey (Table 1.3.5.) are nutritionally valuable. This period has seen the rapid growth of food technology, the emergence of the use of functional proteins in processed foods and the economic recovery of proteins from whey. Three protein concentrating processes, ultrafiltration (UF), adsorption and heat precipitation are in common commercial use. The whey proteins are noted for their high nutritional quality and their high degree of functionality. These functional properties include solubility over a wide pH range, forming stable foams and heat induced gels, and emulsifying significant amounts of lipid[16].

Table 1.3.5. Whey protein characteristics[16]

Protein	% of whey protein	Approximate weight contribution (g/l)	Molecular weight
Lactoglobulin	55 - 65	3.3	18 400
Lactalbumin	15 - 25	1.2	14 200
Proteose-peptone	10 - 20	0.2	4 000 - 80 000
Immune globulins	10 - 15	0.5	80 000 - 900 000
Bovine serum albumin	5 - 6	0.3	66 300
Beta-caesins	1 - 2	< 0.1	24 000
Minor proteins	< 0.5	< 0.05	30 000 - 100 000

	Isoelectric point	Stability to heat
Lactoglobulin	5.4 - 5.5	heat labile
Lactalbumin	4.4 - 4.5	slightly heat labile
Proteose-peptone	5.1 - 6.0	heat stable
Immune globulins	5.5 - 8.3	very heat labile
Bovine serum albumin	5.1	heat labile
Beta-caseins	4.7	-
Minor proteins	-	-

The supplementation of staple foods with whey protein can be used to improve the protein quality of an inferior diet. Soluble whey proteins are of superior nutritional quality with only egg protein having a greater protein efficiency ratio (PER)[14]. In Mexico tortillas have been supplemented with whey protein concentrate (WPC), resulting in a doubling of their PER value[3].

With such an array of uses for whey, it is surprising that in some locations it is still viewed as a waste product. In this time of increased ecological and environmental awareness, the pressure to maximise returns on our resources will force industries to regard materials such as whey in a different light. No doubt in the years to come many more innovative applications of whey will emerge.

1.4 LACTIC ACID: PRODUCTION and USES

1.4.1 Synthesis and Applications

The souring of milk has been observed for centuries but it was only in 1780 that Scheele discovered the causative agent, lactic acid[5]. The first commercial production of lactic acid by a microbiological process was in 1881, as a means of obtaining its calcium salt[17]. The venture was unsuccessful in its attempt to market calcium lactate as a substitute for cream of tartar in baking powder. Today about half of the world demand is produced by fermentation[18]. The remainder is generated synthetically. In 1989, total lactic acid production worldwide was estimated to be 30,000 tons[19]. Ryder[20] offered a subjective assessment of economic, market and technological factors associated with the manufacture of some fermentation products. He suggested that the overall economic potential of food acids, such as lactic acid, is unknown. However with increasing petrochemical feedstock prices, synthesis of lactate by fermentation is becoming more cost effective.

Lactic acid (2-hydroxypropionic acid, $C_3H_6O_3$) exists in two optically active forms. Sarcolactic acid, the only lactic acid "natural" to humans and mammals is the L(+) isomer. The D(-) form of lactic acid is the other optical isomer. Lactic acid is a commodity biochemical sold in three major grades: technical, food, pharmaceutical. The grades are listed in order of increasing purity. Manufacturing costs are mainly attributable to product recovery and purification[21]. The market for lactic acid is competitive and highly sensitive to production costs[22]. It is employed as a food acidulant (50% of the market) and serves as a food preservative[18]. Crude grades are used for the deliming of hides in the leather industry and it is used in the textile industry for fabric treatment. Its ability to form polymeric polylactic acids finds application in production of various resins[5]. Manufacture of stearoyl-2-lactylate accounts for 20% of lactic acid production. It is also used in metal pickling, pharmaceuticals and as a starting material in specialised chemical processes.

1.4.2 Microrganisms, Raw Materials and Fermentation Process

Two kinds of lactic acid bacteria are recognised, **heterofermentative** and **homofermentative**[17]. Homofermentative organisms such as *L. helveticus* catabolize glucose via the Embden-Meyerhof pathway. Pure or almost pure lactic acid is formed. (Appendix A). Heterofermentative organisms such as *L. brevis* produce a range of byproducts and are not suitable for commercial purposes.

One of the first investigations of lactic acid fermentation was carried out by Rogers[23] in 1928. He looked at the influence of aeration, pH and product accumulation on bacterial growth. A symposium on the lactic acid bacteria in 1952 dealt with the classification, nutritional requirements, use in microbiological assays and industrial significance of these organisms[24]. Luedeking[25] undertook an in-depth study of the kinetics of batch lactic acid fermentation at controlled pH. From this work, an expression relating bacterial growth and cell density with product formation was established. A wide range of articles describe the use of lactic acid bacteria in whey fermentation. Campbell[26] as early as 1953,

outlined a commercial operation in Canada for calcium lactate and lactic acid manufacture. Several papers detail the manufacture of ammonium lactate, a ruminant feed supplement.[27,28].

The recent use of *L. helveticus* in preference to other bacterial species was argued by Roy et al[29]. He indicated that this particular species produced higher concentrations of acid and that the product obtained was racemic. Strains such as *L. delbruckii* and *L. bulgaricus* produce only the D(-) isomer, which is not metabolised by man.

Optimum conditions for whey fermentation by *L. helveticus* in batch culture were determined to be pH 5.5, temperature 42°C and stirrer speed 200 rpm[29]. An investigation into growth and product formation kinetics of *L. helveticus* showed that choice of temperature, medium and pH influenced the mechanism of acid formation[30]. Utilisation of *L. helveticus* in continuous culture revealed an increase in lactic acid production of 1.5g l^{-1} h^{-1} over batch cultures[31]. Recent studies have shown that immobilisation of *L.helveticus* cells in calcium alginate beads reduced fermentation time by up to 60%[32,33].

Industrially, L(+) lactic acid is produced by *L. helveticus* and related homofermentative strains. Media such as semi-refined corn sugar (dextrose), molasses, sulphite waste water and whey are utilised. Molasses or malt are added to provide the necessary accessory nutrients[5]. Since these organisms are microaerophilic, complete exclusion of oxygen from bioreactors is not essential. The fermentation rate depends primarily on the pH, temperature, concentration of nitrogenous nutrients and the lactic acid concentration. Normally, pH is controlled in the region 5-6.5, temperature is maintained between 45°C-60°C and fermentation time is 3 days. Yields of 90-95% are typically obtained[5,18,34].

1.5. FACTORIAL DESIGNS

Historically, factor analysis was developed early this century to assist in formulating mathematical models of human ability as assessed by psychological tests[35]. Winkler[35] proposed that computer accessibility has widened the sphere of practical application of this statistical technique. Indeed, the advent of high speed digital computers has been the driving force behind the rapidly expanding field of chemometrics[36]. Traditionally optimisation of laboratory procedures has relied on the one variable at-a-time (**univariate**) approach[37]. Chatfield[38] stated that the most serious defect of univariate experiments is that interaction between factors (e.g pH, temperature) cannot be detected.

Factorial designs are a popular class of experimental designs which enable the influence of individual factors (main effects) to be estimated simultaneously, together with any interactions which may exist[39]. In addition they provide more efficient screening of process variables. Comprehensive accounts of the design, execution and analysis of factorial experiments are found in a number of texts[39,40,41].

A **factor** is any feature of the experimental conditions which is of interest to the investigator[38]. There are two types. A **quantitative** factor refers to anything that may be controlled such as the level of a variable (pH, temperature etc)[37]. A **qualitative** factor is one whose values cannot be arranged in order of magnitude. The presence or absence of a catalyst, or the type of catalyst employed are examples of the latter. One of the major advantages of factorial designs is that they can be used to reveal the existence of factor interaction when it is present in a system[40].

Important descriptors of such designs are the number of factors involved and the number of **levels** in each factor. For instance, if a factorial design has 3 factors (x_1, x_2, x_3) at two levels (low and high), it is said to be a 2 x 2 x 2 or 2^3 factorial[40]. It is useful to represent the lower level of a quantitative variable by a - and its higher level by a +. For qualitative factors, + and - represent the presence or absence of the factor respectively. A

display of the experimental details is termed a design matrix, shown here for a 2^3 design[36].

$$\begin{bmatrix} x_{11} & x_{21} & x_{31} \\ x_{12} & x_{21} & x_{31} \\ x_{11} & x_{22} & x_{31} \\ x_{12} & x_{22} & x_{31} \\ x_{11} & x_{21} & x_{32} \\ x_{12} & x_{21} & x_{32} \\ x_{11} & x_{22} & x_{32} \\ x_{12} & x_{22} & x_{32} \end{bmatrix} = \begin{bmatrix} - & - & - \\ + & - & - \\ - & + & - \\ + & + & - \\ - & - & + \\ + & - & + \\ - & + & + \\ + & + & + \end{bmatrix}$$

Fig 1.5.1. shows a geometric view of the design.

It is often convenient to present the results of a factorial experiment as a **response surface**, a graph of a system response (such as growth rate), plotted against one or more system factors e.g temperature, stirrer speed etc[40]. If only one factor is studied, the response may be plotted against the level of the factor. If the factor is examined at 2 levels then it may only be modelled as a straight line. However if the design allows 3 or more levels of the factor to be selected, then the curvature in the response may be estimated[37]. In 2 factor systems, a response surface can be represented by a true surface, either flat or curved, existing in three dimensional experimental space (2 factor dimensions and 1 response dimension)[40]. With more than 2 factors tested, a multi-dimensional surface needs to be visualised.

Moresi[42] applied factor analysis to the fermentation of whey by *Kluveromyces fragilis* to screen variables most relevant to biomass yield. Oxygenation of the medium was found to have a marked influence on cell growth. Factorial design has also been used to optimise COD reduction in whey fermentation by *K. fragilis*[43]. In a recent paper by Kemp[44], response surface optimisation was employed in a study of *Lactobacillus plantarum* batch

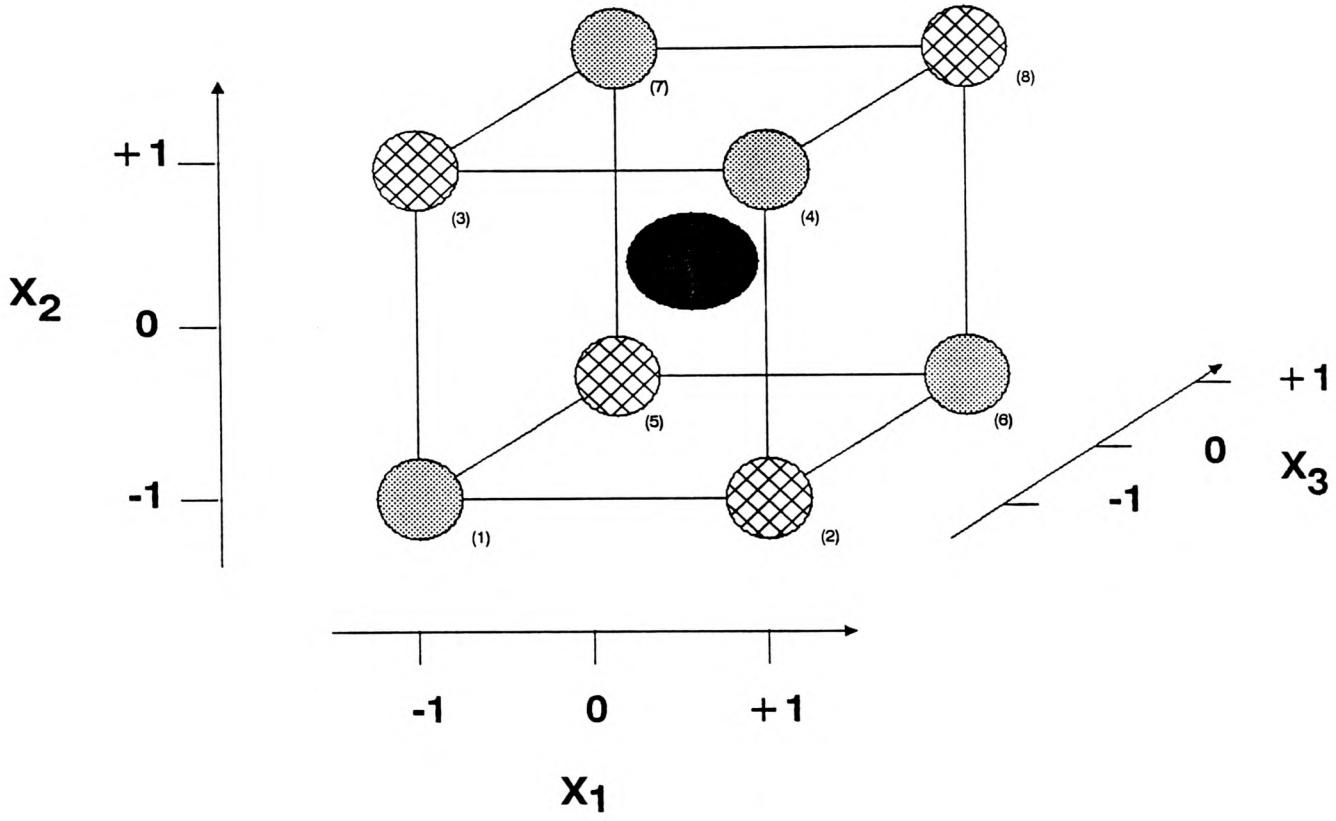


Fig 1.5.1. Generalised 2^3 factorial design. The dotted and striped regions constitute the two half fractions of the design. The shaded central region indicates the centre point conditions for the design matrix. X_1, X_2 and X_3 represent the three factors .

culture. Optimal conditions for biomass production in de Man, Rogosa, Sharpe (MRS) medium[45] were determined.

1.5.1. Fractional Factorial Designs

A disadvantage of a full 2^k factorial design is that the number of runs increases geometrically as k is increased[41]. For example, 2 level factorial designs with k = 2,3,4,5,6 & 7 require 4, 8, 16, 32, 64 & 128 runs to estimate all parameters in a full factorial model[39]. At some point higher order interactions tend to become negligible and can be disregarded. In addition, when a large number of variables are incorporated into a design, frequently some have no distinguishable effects at all. Thus there tends to be "redundancy" in a 2^k design for large values of k. Fractional factorial designs exploit this redundancy[41]. They enable efficient screening of the effects of many factors by using a small number of experimental runs. Fig 1.5.1. shows the two half fractions of a 2^3 factorial design.

1.6. FTIR SPECTROSCOPY and QUANTITATIVE ANALYSIS

1.6.1. An Historical Perspective

Infrared (IR) spectroscopy first came to prominence during the 1940's. Much of the early development work was carried out at large chemical companies such as Dow, Shell and American Cynamid. The need to perform quality control (QC) of synthetic rubbers and petroleum fractions provided the necessary impetus[46,47]. In the UK, the technique was developed in order to analyse Luftwaffe fuel during World War II. This early work was carried out in the Thompson School at Oxford University and in Southerland's group at Cambridge University. In the 1950's however, GC (gas chromatography) replaced IR in many quantitative applications[48]. The relegation of IR to a "qualitative only" technique was attributable to two main drawbacks:

1. the technique was "noise limited"
2. data extraction from the spectrum was crude. The only available form of the spectrum was a chart recording.

In the 1970's evolution of FTIR (Fourier Transform infrared) spectroscopy, where the computer became an integral part of the instrumental system, signalled a major turning point in spectroscopy. The coupling of data processing with IR spectroscopy opened up new avenues for QC and quantitative applications. Continued improvements in FTIR spectrometer design during the 1980's in conjunction with advances in computer technology has lead to further improvements in the field.

1.6.2. Advantages of FTIR Spectroscopy

The principles of FTIR and traditional dispersive IR spectroscopy is discussed in a number of texts[49,50,51,52]. The following is a brief description of each technique:

Dispersive IR Spectroscopy

A simplified diagram of a typical double beam dispersive instrument is shown in Fig 1.6.1. The source (a heated filament) emits a continuum of wavelengths that pass through a prism or grating which disperses the continuum into radiation of different wavelengths. Rotation of the prism or grating enables the different wavelengths to pass through through the focusing slits, producing light of a single wavelength (mononchromatic). This light is split into two beams of equal intensity at a beam splitter. The beams are guided and focussed by silvered mirrors before passing through the sample and reference cells. The two beams then strike an IR detector which compares their intensities. The detector output is converted into an electrical signal, amplified and sent to a recorder that traces the IR spectrum. The rotation of the grating or prism is synchronised with the wavelength (λ) and wavenumber ($1/\lambda$) scales on the recorder to provide a sample spectrum that is a plot of light transmittance versus wavelength (or wavenumber).

FTIR Spectroscopy

The basic optical arrangement of a FT instrument is shown in Fig 1.6.2. It derives from the classical attempt by Michelson in the 19th century to measure the 'ether wind', by

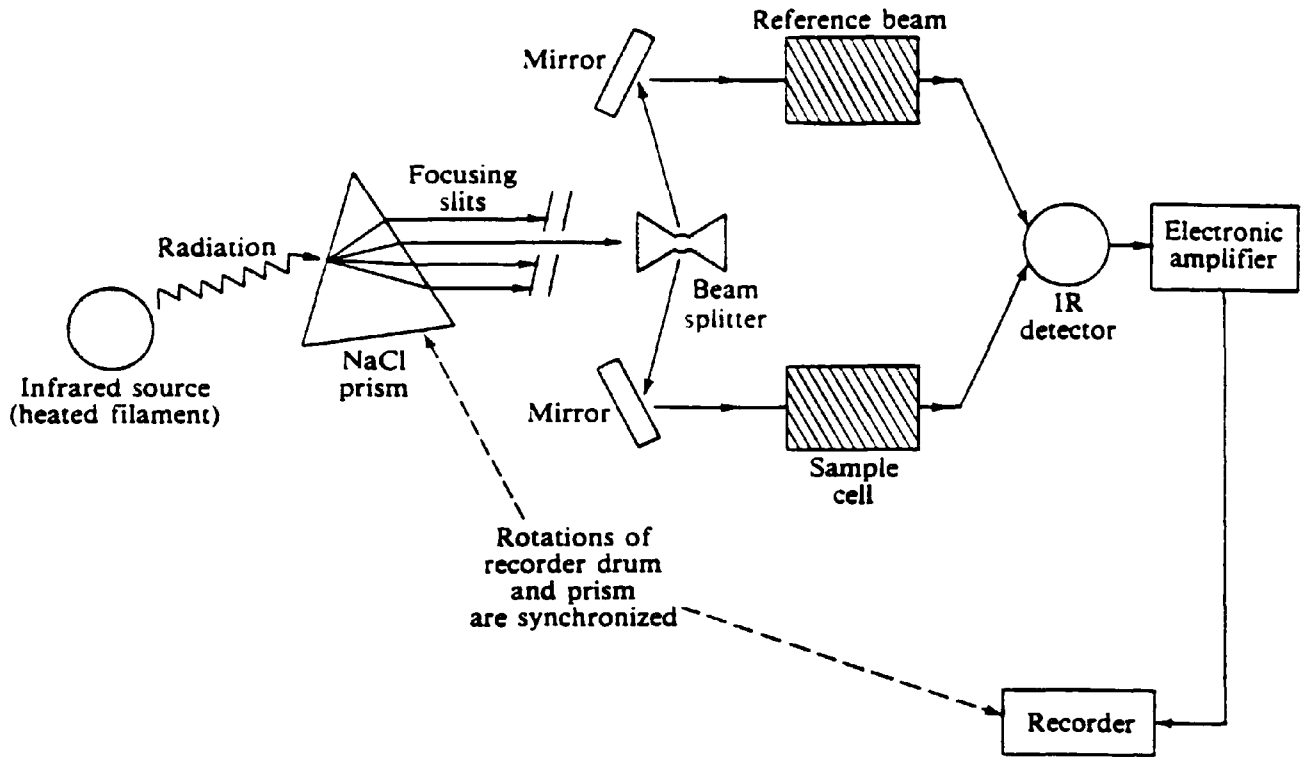


Fig 1.6.1. Simplified view of a double-beam dispersive IR spectrophotometer[50]

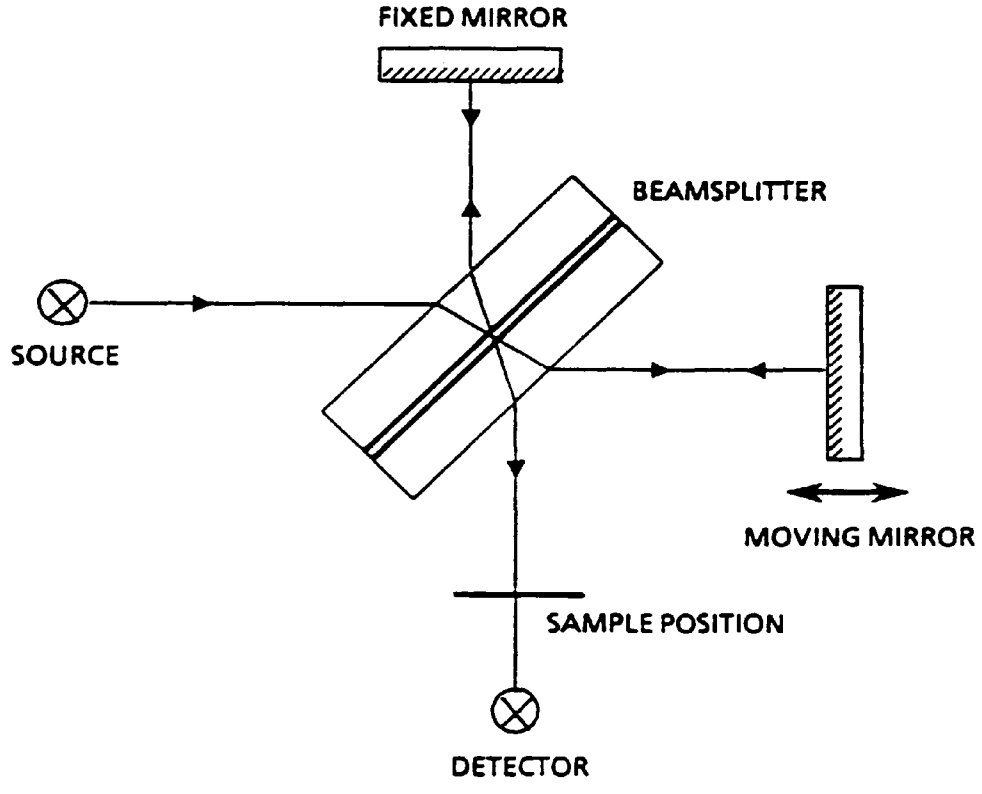


Fig 1.6.2. The interferometer unit of a Fourier transform spectrometer[52]

determining the velocity of light in two perpendicular directions[50]. Radiation is directed from the source to the beam splitter (e.g. KBr coated with germanium) which transmits about 50% of the incident beam and reflects the remainder. Thus half the radiation goes to a fixed mirror and half to a mirror that can be moved to introduce a varying difference in pathlength. When the beams are recombined at the beam splitter, an interference pattern is obtained as the path difference is changed. If the recombined beam is directed through a sample before reaching the detector, sample absorptions will appear as gaps in the frequency distribution. Conversion of this interference pattern (interferogram) into a normal absorption spectrum is achieved by the mathematical process of Fourier Transformation, which is carried out by computer.

The FTIR spectrometer has several advantages over classical dispersive instruments. Of these, the Multiplex (or Fellgett) Advantage and the Jacquinot (or Throughput) Advantage are possibly the most important.

Multiplex Advantage: In conventional dispersive instruments, each element or observation point of the spectrum is recorded *consecutively*. In an interferometer, all the points are measured *simultaneously*.

Jacquinot Advantage: In a dispersive instrument radiation from the source is invariably brought to focus on a slit. In an interferometer, the absence of slits leads to a higher energy throughput at a given resolution.

The greater energy throughput of FTIR gives signal-to-noise ratios several orders of magnitude better than dispersive instruments. This has permitted the introduction of sampling methods based on reflection techniques, such as Attenuated Total Reflection (ATR)- see section 1.6.4. Previously such techniques were considered to have insufficient energy throughput for routine use. Additionally it has also enabled techniques such as microscopy and GC to be used in tandem with FTIR (hyphenated techniques). The

use of rapid scanning interferometers (in association with the Multiplex Advantage) has enabled the whole spectrum range to be scanned in a couple of seconds. Thus in the time taken for a single scan of a dispersive instrument, the FTIR can co-add (average) many scans to further improve the signal-to-noise ratio.

1.6.3. Quantitative Analysis: Background

The fundamental premise is that as the concentration of a chemical species increases, a corresponding increase is observed in spectral response. The relationship between concentration and spectral response is described by the Beer-Lambert Law. For the simple case of a single compound at a single frequency:

$$A = abc$$

where :

A = absorbance at a given frequency (also known as optical density (OD))

a = absorptivity of the compound at that frequency

b = pathlength of the sample

c = concentration of the sample.

Since few systems of practical application contain only one component, the Beer-Lambert Law must be modified to accommodate many components[53]. In complex systems the situation is further complicated by apparent departures from linear Beer-Lambert behaviour. These deviations arise through chemical/physical effects e.g. dissociation, solvation and H-bonding, and instrumental conditions such as stray radiation and reflection losses[54]. Consequently a number of mathematical approaches have been devised to accommodate multicomponent systems and possible apparent deviations from Beer's Law. Four of the most common are classical least squares (CLS), inverse least squares (ILS), partial least squares (PLS) and principal components regression (PCR)[55]. It should be noted that the least squares method was first developed in 1959 by Bauman[56]. However

these increasingly sophisticated quantitative routines have only emerged in recent years as a result of improvements in FTIR computer systems.

1.6.4. Quantitative Analysis of Aqueous Solutions

Historically quantification of aqueous samples using IR spectroscopy has been difficult because water is a strong absorber in the mid-infrared (MIR) region. Since only a small amount of energy passes through an aqueous solution, conventional transmission cells have short pathlengths (approximately 0.015mm). The narrow cells are difficult to use because of problems in filling and cleaning.

A new technique for analysing aqueous solutions became commercially available during the early 1980's. The cylindrical internal reflectance (CIR) cell was developed by Paul Wilks[60]. The version used in this work is known as the CIRCLE® (cylindrical internal reflectance cell for liquid evaluation) cell (Spectra-Tech Europe LTD, Warrington, UK). This sampling accessory overcomes many of the difficulties associated with a fixed, short pathlength transmission cell. The CIRCLE® cell is suitable for the analysis of opaque and viscous materials, virtually all liquids including strong absorbers such as water. Details of the CIRCLE® cell can be found in a number of papers[58,59,60,61,62].

Quantitative FTIR spectroscopy has been used in a wide range of applications: in the field of clinical chemistry, the determination of glucose in whole blood[63], the quantification of human plasma protein mixtures[64] and the spectral analysis of human blood plasma[65]. Fuller et al[66] successfully employed the technique in the analysis of commercial detergents. Analysis of polychlorinated biphenyls (PCB's) in transformer oils[67] and the on line monitoring of a polymer process stream[68] have been reported. Further examples include analysis of fructose, glucose and sucrose in fermentation broths[69] the study of ethanol production from whey[70] and the on line analysis of lactic acid and lactose during the fermentation of whey[71].

CHAPTER 2. EXPERIMENTAL WORK

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2. EXPERIMENTAL WORK

2.1 MEDIUM PREPARATION

2.1.1 Formation of Partially Deproteinised Whey (PDPW)

Materials and Equipment

Whole sweet whey was obtained from Castle Dairies, Pontygwindy Industrial Estate, Caerphilly. This was the sole source of whey for all experimental work. 2M NaOH and 2M H_2SO_4 (BDH Ltd, Poole, Dorset). 1l Buchner flask, Whatman No 541 and Whatman No 42 filter papers.

Experimental Procedure

Details are provided in Fig 2.1.1.

2.1.2. Preparation of Whey Permeate (WP)

Materials and Equipment

Whole sweet whey. Amicon DC10L ultrafiltration system and an Amicon H5P10-43 hollow fibre cartridge, with 10,000 molecular weight (MW) cut-off (Amicon, Stonehouse, Gloucester).

Experimental Procedure

Ultrafiltration (UF) is defined as a "physiochemical fractionation and concentration technique in which a pressurised solution flows over a porous membrane"[73]. Fig 2.1.2 shows details of the UF unit set in concentration mode. Fluid (whey) is drawn from the reservoir through the course prefilter (removes colloidal particles, thereby minimising membrane fouling), pumped through the lumen of the hollow fibres and returned to the reservoir. Species of MW lower than the membrane cut-off (10,000) pass through the fibre walls and emerge as permeate (ultrafiltrate), WP in this case. Retained species in the process solution are progressively concentrated in the reservoir. Operation continues until the desired concentration is achieved. This yielded whey protein concentrate

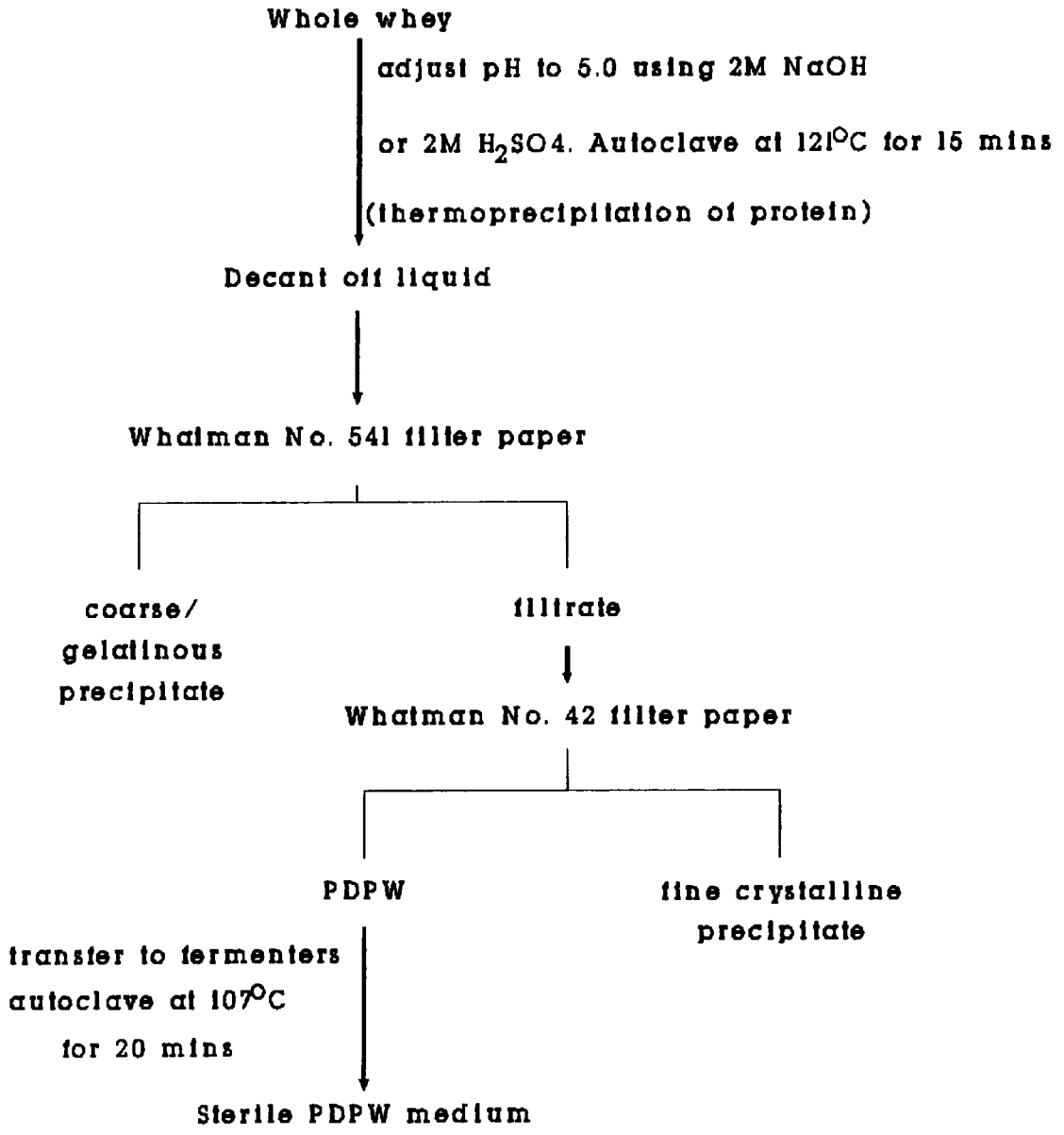


FIG 2.1.1. Production of PDPW

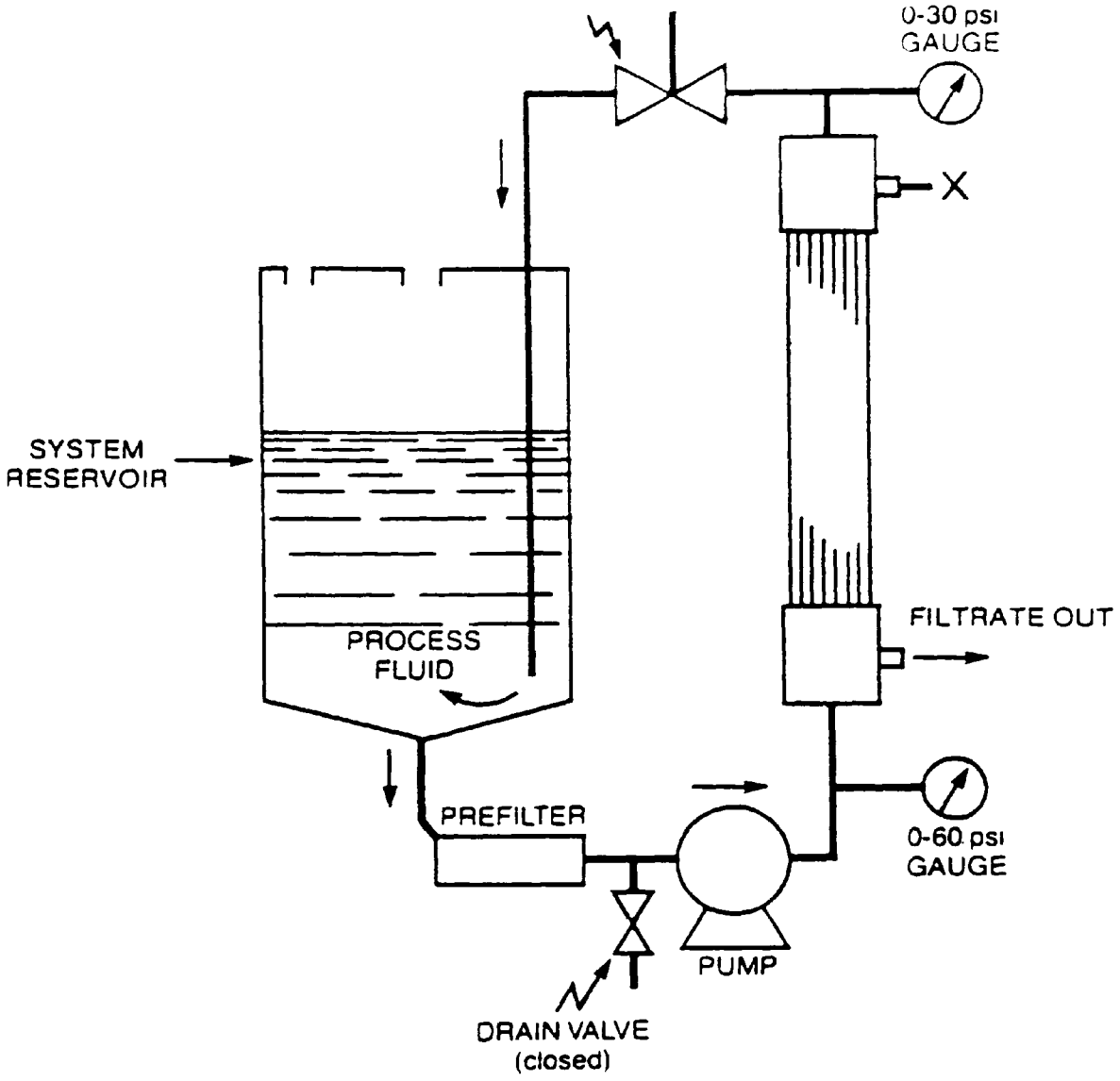


Fig 2.1.2. Ultrafiltration apparatus - concentration mode[74]

Arrows indicate direction of fluid flow

(WPC). Pressure for UF is generated by the force required to pump the liquid through the fibres and by a valve in the return line. A pressure limit switch controls maximum inlet pressure to prevent rupture of the fibres[74]. Upon completion of the operation, both the WP and WPC were stored at -18°C until required.

2.1.3. Determination of Protein Content in Whey Protein Concentrate

Protein content of the WPC was assessed spectrophotometrically using the Biuret method[75]. Comprehensive practical details of this test are given by Harris[76].

2.2. INVESTIGATION of PRECIPITATE FORMATION

2.2.1. Effect of Yeast Extract (YE) on Precipitate Formation

Materials and Equipment

Gallenkamp pH stick (Fisons Scientific, Loughborough, Leiecestershire), WP and YE (technical grade, Difco Laboratories, Detroit, Michigan, USA). This particular brand/grade of YE was used for all subsequent studies.

Experimental Procedure

YE was dissolved in WP to give a range of concentrations 0.5% to 2.0% w/v. pH_i, the pH of each solution prior to autoclaving, was measured. NOTE: The autoclave was run for 15 mins at a temperature of 115°C (10 lb/in^2 pressure) for **all** precipitate experiments. The optical density at 620 nm following autoclaving, (OD_a), was recorded (Beckman DU-5 UV/vis spectrophotometer, Beckman Instruments, Fullerton, California, USA). NOTE: this wavelength was employed for **all studies**

2.2.2. Influence of Temperature on Precipitate Formation

Materials and Equipment

WP, a hotplate and a thermometer (range 0-150°C).

Experimental Procedure

The temperature of 250ml of WP was raised from 15°C to boiling point. OD was monitored.

2.2.3. Impact of pH on Precipitate Formation in WP

Materials and Equipment

Two lots of WP were tested. The first whey batch was received and processed in March 1989, the second in September 1989. 2M H_2SO_4 and 2M NaOH.

Experimental Procedure

80 ml of WP was poured into a number of 100ml bottles and the pH adjusted to give a range of 4.1 to 6.0. The OD of each solution before autoclaving (OD_i) was measured. The solutions were then heat sterilised (autoclaved). The pH after autoclaving (pH_a) and the corresponding OD_a values were noted.

2.2.4. Effect of Acid Addition on Precipitate Solubility

Materials and Equipment

WP, YE, 2M HCl and 2M NaOH.

Experimental Procedure

WP-YE (0.5% w/v), pH_i 5.59, was autoclaved. On cooling, acid was added dropwise to the medium. pH and OD were measured following each addition. Changes in OD on readjustment of pH to the original value of 5.59 using NaOH, were similarly monitored.

2.2.5. Influence of pH on Precipitation in Supplemented WP

Materials and Equipment

WP, YE, $CaCl_2$, 2M NaOH and 2M H_2SO_4.

Experimental Procedure

The following media were used:

1. WP
2. WP-$CaCl_2$ (0.1% w/v)
3. WP-YE (0.5% w/v)

Solutions of pH_i 4.0 to 7.5 were made up for each medium, and OD_i values recorded. After autoclaving, pH_a and OD_a were noted.

2.2.6. Effect of pH Adjustment on Solubility of Precipitate

Materials and Equipment

1M HCl and 1M NaOH

Experimental Procedure

Acid was added to WP-YE (0.5% w/v) solution, pH_i 7.00, which had been autoclaved. Changes in OD were recorded as pH decreased. The pH of the solution was readjusted to its original value of 7.00 using NaOH. The medium was autoclaved again and the subsequent OD_a and pH_a values noted.

2.2.7. Influence of EDTA on Precipitation

Materials and Equipment

0.5M EDTA solution and 1M NaOH

Experimental Procedure

Ethylenediamine tetraacetic acid (EDTA) was added dropwise to WP (pH_i 6.49) previously autoclaved during experiment 2.2.5., until a minimum OD was attained. The pH was readjusted to 6.5 with alkali and the medium autoclaved once more. pH_a and OD_a were measured.

2.2.8. Analysis of WP Precipitate for Calcium and Phosphate

Materials and Equipment

Dilute ethanoic acid and ammonium ethandioate. Conc. HNO_3, ammonium molybdate, dilute HCl, aqueous ammonia, $MgCl_2$ and NH_4Cl. Perkin Elmer 1760X FTIR spectrometer (Perkin Elmer, Norwalk, Conn, USA).

Experimental Procedure

WP precipitate obtained during experiment 2.2.5. was filtered off from three bottles, combined, and evaporated to dryness. It was then tested for calcium and phosphate.

Calcium, Ca^{2+}

Approximately 0.1g of precipitate was dissolved in dilute ethanoic acid. Ammonium ethandioate was added. A white precipitate indicates the presence of Ca^{2+}

Phosphate, PO_4^{3-}

(i) To the solid, 5 drops of conc. HNO_3 and 2 drops of ammonium molybdate were added. A bright yellow precipitate indicates PO_4^{3-}.

(ii) The precipitate was dissolved in dilute HCl. Solid $MgCl_2$ and NH_4Cl were added, and the tube shaken. Aqueous ammonia was slowly poured down the side of the tube. A white ring at the junction of the 2 layers shows that phosphate is present.

(iii) An FTIR spectrum of the precipitate (KBr disc, 1 part sample : 9 parts KBr) was recorded. The spectrum was examined for absorption features characteristic of phosphate.

2.3 OPTIMISATION of STARTER CULTURE PERFORMANCE

Organism and Inoculum

Lactobacillus helveticus 8652 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen. Stock cultures were maintained on agar slopes at $4^{o}C$.

Modified MRS medium[45] was used. This contained: 15 g agar, 10 g peptone, 10 g beef extract, 5 g yeast extract, 20 g lactose, 1 ml Tween 80, 2 g K_2HPO_4, 5 g sodium acetate.$3H_2O$, 2 g triammonium citrate, 0.2 g $MgSO_4.7H_2O$ and 0.2 g $MnSO_4.4H_20$ in 1 l deionized water. The pH of the medium was adjusted to 6.1.

Starter Culture

Active (starter) cultures for seeding the fermenters were prepared by loop inoculating 100ml of MRS lactose (pH initially 6.2) from an MRS agar slope. The culture was propagated at 42°C without agitation. This procedure was adopted for all subsequent experiments, unless otherwise stated.

Culture Medium

WP, containing no supplements.

Equipment

All fermentations were carried out in 1 l working volume fermenters (LH Fermentation 500 series, LH Fermentation, Reading, Berkshire) under conditions of controlled pH, temperature and stirrer speed. pH was maintained through automatic addition of 2M NaOH.

Analysis

Bacterial growth was determined by measuring OD (620nm) using a Beckman DU-5 UV/vis spectrophotometer. OD readings were converted to dry weight using a calibration curve (see section 2.9). Each run was carried out in duplicate. Unless otherwise stated, this procedure was adopted for all subsequent experiments.

NOTE: ASEPTIC TECHNIQUE WAS USED THROUGHOUT

Experimental Procedure

Mid exponential phase inoculum: 40ml inocula (OD = 0.571) were transferred to the fermenter vessels. For late exponential and stationary phase starter cultures, 25ml

(OD = 0.809) and 15ml (OD = 1.372) inocula were transferred to the fermenters. Assuming OD is proportional to biomass, the **number** of cells transferred was approximately the same. Growth of *L. helveticus* was compared in each case.

2.4. OPTIMISATION of L. helveticus GROWTH USING a 2^{3-1} FRACTIONAL FACTORIAL DESIGN.

Starter Culture

Starter cultures (MRS lactose medium) were propagated for 12 hours at 42°C without agitation. 3 % v/v inocula were transferred to the fermenters.

Culture Medium

WP was supplemented with 0.5% w/v YE. The fermenters, containing WP-YE medium, were autoclaved at 115°C (10lb/in^2) for 15 minutes.

Experimental Method.

The influence of the 3 factors pH (P), temperature (T) and stirrer speed (S) on *L. helveticus* growth was assessed in a 2^{3-1} fractional factorial design at two levels. The levels of the factors used were:

P	- = 5.5	0 = 5.9	+ = 6.3
T	- = 38°C	0 = 42°C	+ = 46°C
S	- = 150rpm	0 = 200rpm	+ = 250rpm.

Four experimental runs, together with 2 centre point runs were carried out. Growth of *L. helveticus* was monitored, and the specific growth rate, μ, calculated for each run. (For a plot of $\log_e$ cell dry weight vesus time, μ corresponds to the slope of the line).

2.5. OPTIMISATION of L. helveticus GROWTH in WP USING a 2^3 FULL FACTORIAL DESIGN

Starter Culture

As 2.4.

Culture Medium.

WP, supplement free, heat sterilised at 115°C (10lb/in^2) for 15 minutes.

Experimental Method

The effect of the 3 factors pH (**P**), temperature (**T**) and stirrer speed (**S**) on *L. helveticus* growth was examined using a 2^3 full factorial design at two levels. The factor levels employed were:

P	- = 5.1	0 = 5.5	+ = 5.9
T	- = 38°C	0 = 42°C	+ = 46°C
S	- = 150rpm	0 = 200rpm	+ = 250rpm.

The main effects *p,t* and *s*, the two factor interactions *pt*, *ps* and *st*, and the three factor interaction *pts*, were assessed in 8 experimental runs. The variance of the system was estimated by carrying out 3 centre point runs.

2.6. SUPPLEMENTATION of WP

The effect of various supplements on growth and lactic acid production by *L. helveticus* was investigated.

2.6.1. Nitrogen/Growth Factor Supplements

Materials

Peptonized milk (PM), skim milk (SM) powder, lactalbumin hydrolysate (LAH), casein hydrolysate (CAH) (Oxoid, Basingstoke, Hampshire), bacteriological peptone (PEP) (lab M, Bury, Lancs) and YE (Difco).

Starter Culture

100 ml of MRS lactose medium (loop inoculated) was propagated at 42°C for 12 hours without agitation. 3% v/v inocula were used to seed the fermenters.

Analysis

L. helveticus growth was determined by measuring OD (620nm) and converting to dry weight. Lactic acid and lactose were measured off line by FTIR spectroscopy (see section 2.10.3). This procedure was adopted for all supplement studies.

Fermentation Media

WP, WP-PM, WP-LAH, WP-CAH, WP-SM, WP-PEP, WP-YE. Each supplement was added to give a final concentration of 0.5% w/v.

Experimental Procedure

Growth and final lactic acid concentration were assessed for each medium.

2.6.2. The Effect of Yeast Extract Concentration on Growth and Acid Production

Fermentation media

WP plus 0.00, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50% w/v YE respectively.

Experimental Procedure

Lactic acid production / lactose utilisation and cell growth were studied.

2.6.3. The Influence of Additional Supplements on Growth and Acid Production

Materials

$MgSO_4.7H_2O$, $MnSO_4.4H_2O$, Tween 80, K_2HPO_4 and Na acetate. $3H_2O$ (BDH, Poole, Dorset)

Fermentation media

1. WP-YE (0.75% w/v)
2. WP-YE plus $MgSO_4.7H_2O$ (0.02% w/v) and $MnSO_4.4H_2O$ (0.02% w/v)
3. WP-YE plus Tween 80 (0.1% v/v)

4. WP-YE plus K_2HPO_4 (0.05% w/v)
5. WP-YE plus Na acetate (0.05% w/v).

Experimental Procedure

Lactic acid formation and growth of *L. helveticus* were compared in the various media.

2.6.4. Supplemented WP versus MRS and LS Media

Growth and and lactic acid production in supplemented whey was compared with MRS and Lactose Synthetic (LS) media.

Fermentation Media

1. WP_o (whey permeate optimum) medium. This contained 0.75% w/v YE, 0.1% v/v Tween 80 and 0.05% w/v Na acetate.

2. MRS lactose medium. Composition as described previously, except that the lactose concentration was increased to 4.2% w/v.

3. LS Medium. This is based on the Glucose Synthetic (GS) medium developed by Friedman & Gaden[77]. This consisted of: 50 g lactose, 30 g YE, 0.6 g $MgSO_4$, 0.03 g $FeSO_4$, 0.03g $MnSO_4$, 1.0 g Na-acetate, 0.5 g KH_2PO_4 and 0.5 g K_2HPO_4 in 1 l deionised water.

Experimental Procedure

Following inoculation of the fermenters, lactate formation and cell growth were assessed for each medium.

2.6.5. The Effect of Starter Culture Medium on Lactic Acid Production

Starter Media

WP_o, MRS lactose medium (containing 3% w/v lactose) and skim milk. The pH of the MRS and LS media was adjusted to 6.1 and both were autoclaved at 121°C (15lb/in^2) for

15 mins. The pH of the WP_0 medium was adjusted aseptically to 6.1 **after** autoclaving, using sterile 2M NaOH, in order to prevent precipitation.

Starter Cultures

100 ml of each medium was loop inoculated from an MRS agar slope. The cultures were propagated for 13 hours at 42°C.

Fermentation Medium

WP_0 medium

Experimental Procedure

3% v/v inocula from each starter medium were used to seed the fermenters. Subsequent cell growth (OD, converted to dry weight) and acid formation (off line FTIR analysis) were examined.

2.7. CHEMICAL OXYGEN DEMAND ANALYSIS

Starter Culture

100 ml of MRS lactose, loop inoculated and propagated at 42°C for 12 hours.

Fermentation Medium

WP_0 medium

Experimental Procedure

Two fermenters were filled with 850 ml of WP_0 medium and autoclaved (121°C/ 15lb/in^2, 15 mins). Stirrer speed was set to 200 rpm, pH and temperature were maintained at 5.9 and 42°C respectively. 50 ml samples were removed from each fermenter prior to inoculation and at the end of the fermentation (T = 16.5 hours). The samples were centrifuged at 4°C (Chilspin 2 centrifuge, 4,000 rpm/2790 xg, 10 mins). COD analysis was carried out on the supernatant according to the Yorkshire Water Authorities Method of Analysis[79]:

5.0 ml of COD reagent (May & Baker, Dagenham) and 0.25 ml of mercuric sulphate (BDH, Poole) was added to 2.5 ml of sample. The solution was then heated in sealed tubes at 150°C for 2 hours. On cooling, the solution was transferred to a conical flask. The sample was titrated with ferrous ammonium sulphate (0.026 mol/l) (BDH, Poole) using ferroine (1,10 - phenanthroline ferrous sulphate complex) indicator (BDH, Poole). An orange/brown colour is obtained at the end-point.

2.8. CALIBRATION CURVE

A calibration curve relating OD at 620nm to dry weight of cells was constructed. It should be emphasised that strictly speaking, a calibration curve should be produced for each medium used. This, however, would inevitably prove extremely time consuming. Therefore, it has been assumed that this curve provides a reasonable approximation for all media.

Starter Culture

100 ml of MRS lactose was loop inoculated from an agar slope. The starter culture was propagated at 42°C, without agitation for 12 hours. A 3% v/v inoculum was transferred to the fermenter.

Fermentation Medium

WP containing 0.5% w/v YE and 0.1% v/v Tween 80. pH and temperature were controlled at 5.9 and 42°C respectively and the stirrer speed set at 200 rpm.

Experimental

33 universal bottles (with caps) were labelled and dried for 25 mins in a microwave oven (Toshiba Deltawave III), on the auto-defrost setting. The bottles were then transferred to a desiccator. On cooling, the bottles were weighed on a four figure balance (Ohaus GA110). They were placed in the microwave for a further 10 mins, before returning to the desiccator. 800 ml of the fermenter culture was removed after 12 hours - the cells were in stationary phase. The cells were harvested by centrifugation (MSE Hi-spin 21

Centrifuge, 10 000 rpm/18 000xg, 15 mins). They were washed three times with saline (0.9% w/v NaCl), before final resuspension in fresh saline. A number of dilutions of this 'stock' cell suspension were made to give calibration samples with OD's ranging from 0 - 2 (the OD of each suspension was measured at least 3 times, and the average calculated). Three 10ml portions of each suspension were transferred to the pre-weighed bottles using a Gilson pipette. The bottles were placed in a conventional oven at 105°C overnight. Complete drying was ensured by microwaving the bottles for 10 mins, before transfer to the desiccator. On cooling, the bottles were re-weighed. A calibration graph was then constructed.

Note: a modified approach to this technique was attempted. Trial cell suspensions were placed in the microwave oven to dry for 20 mins. However, despite using the time and power setting (auto-defrost) recommended by McNeil[80] and Bu'Lock[81], the method was not suitable. Vigorous boiling resulted in loss of sample. Thus, a conventional oven was employed to carry out initial drying.

2.9. OPTIMISATION of LACTIC ACID PRODUCTION by L. helveticus in WP_O MEDIUM USING a 2^3 FULL FACTORIAL DESIGN

Starter Culture

As section 2.8. above

Culture Medium

WP_O medium

Analysis

Lactose and lactic acid were measured off line by FTIR spectroscopy. Samples were withdrawn from the fermenters after 13.5 hours, cooled using ice and centrifuged. Analysis (in duplicate) was carried out on the supernatant.

Experimental Method

The effect of the 3 factors pH (**P**), temperature (**T**) and stirrer speed (**S**) on acid formation by *L. helveticus* was examined using a 2^3 full factorial design at two levels. The factor levels employed were:

P	- = 5.5	0 = 5.9	+ = 6.3
T	- = 38°C	0 = 42°C	+ = 46°C
S	- = 150rpm	0 = 200rpm	+ = 250rpm.

The main effects *p*, *t* and *s*, the two factor interactions *pt*, *ps* and *st*, and the three factor interaction *pts*, were assessed in 8 experimental runs. The variance of the system was estimated by carrying out 4 centre point runs.

2.10. QUANTITATIVE ANALYSIS by FTIR SPECTROSCOPY

Materials and Equipment

Lithium lactate (AnalaR) and lactose (BDH, Poole, Dorset). Perkin Elmer 1760X FTIR spectrometer and the QUANT software package (Perkin Elmer, Norwalk, Conn., USA). A macro CIRCLE® cell (Spectra-Tech Europe LTD, Warrington, Cheshire).

2.10.1. Quantitative Analysis: Method Development

The development of a quantitative method involves the following steps:

1. Understand the system/sample
2. Determine the best method of sampling
3. Prepare a set of standards of known composition
4. Acquire spectral data on the standard set
5. Reduce the spectral range to those areas which show activity for the components to be analysed
6. Prepare validation samples and evaluate the method
7. Analyse the sample

Here we are dealing with a complex two-component system (lactic acid and lactose). There is considerable spectral overlap between the two components (Figs 2.10.1 & 2.10.2.). The situation is further complicated by solvation/H-bonding between the components and water, as well as interaction between the components themselves. The CIRCLE® cell, an FTIR accessory designed for liquid analysis, was used for sampling.

Table 2.10.1 Composition of standard solutions.

Lactose % w/v	**Lactic acid** % w/v	**Lactose** % w/v	**Lactic acid** % w/v
0.250	4.557	2.602	0.150
2.256	1.593	3.054	2.625
3.663	3.758	5.103	1.239
4.883	2.191	0.337	0.418
1.583	1.783	1.114	3.024
0.746	1.109	0.505	0.219
1.881	0.932	0.088	0.064
4.210	0.424		

The number of standards required must be equal to or greater than the number of components to be analysed. In this instance, the minimum is two. However 15 standard solutions were employed (Table 2.10.1.). Use of more than the minimum number of reference solutions is known as **overdetermination**. The pH of the standards was adjusted to 5.9 and the solutions warmed to a temperature of 42°C before scanning. Thus the standards resembled the actual fermentation samples as closely as possible. Selection of calibration samples is crucial to the success of the quantitative analysis. In choosing the composition of the standards the following points should be considered:

Fig 2.10.1 Spectra of lactose (4.875% w/v) and lactic acid (4.848% w/v) super-imposed

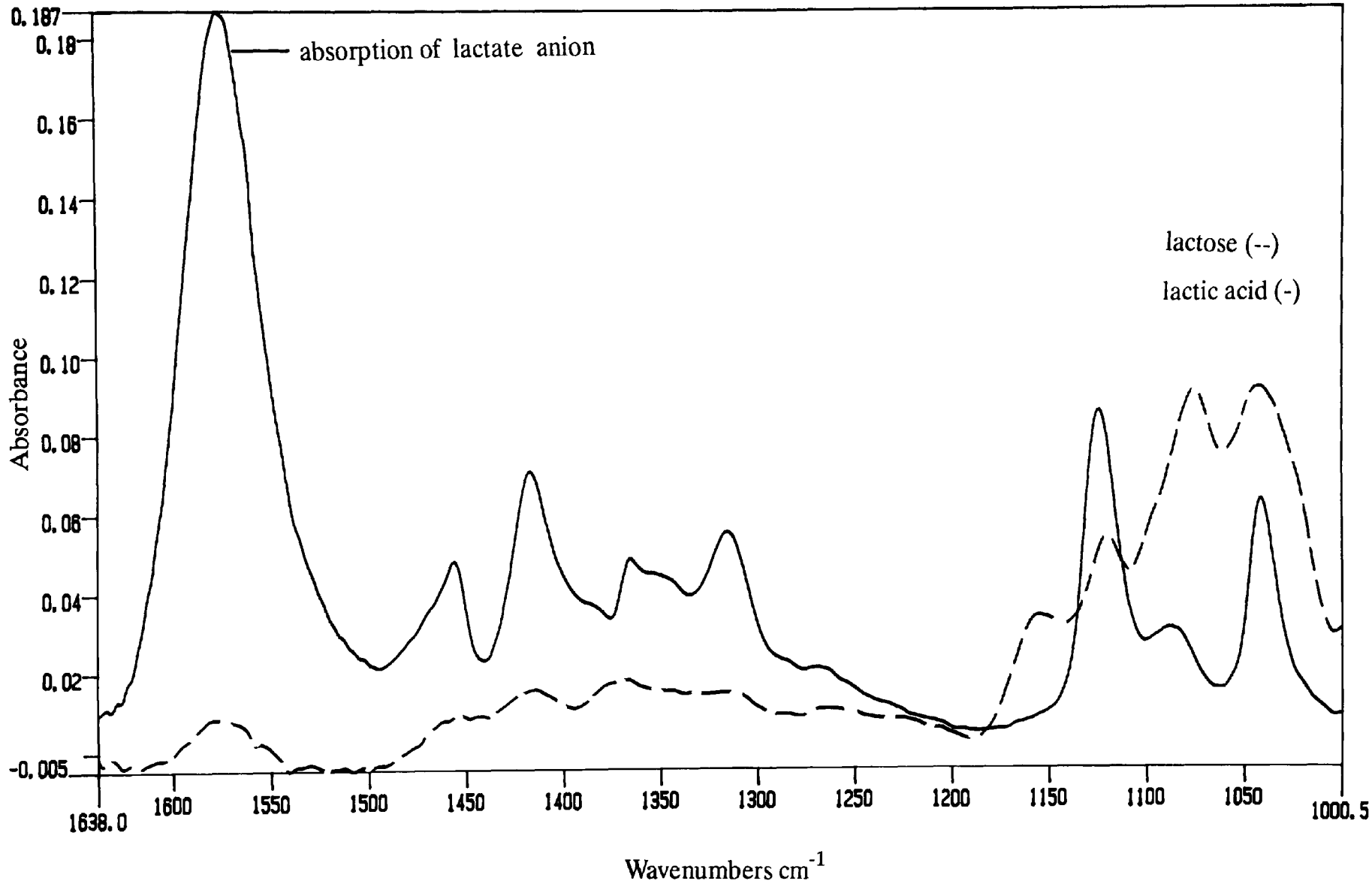
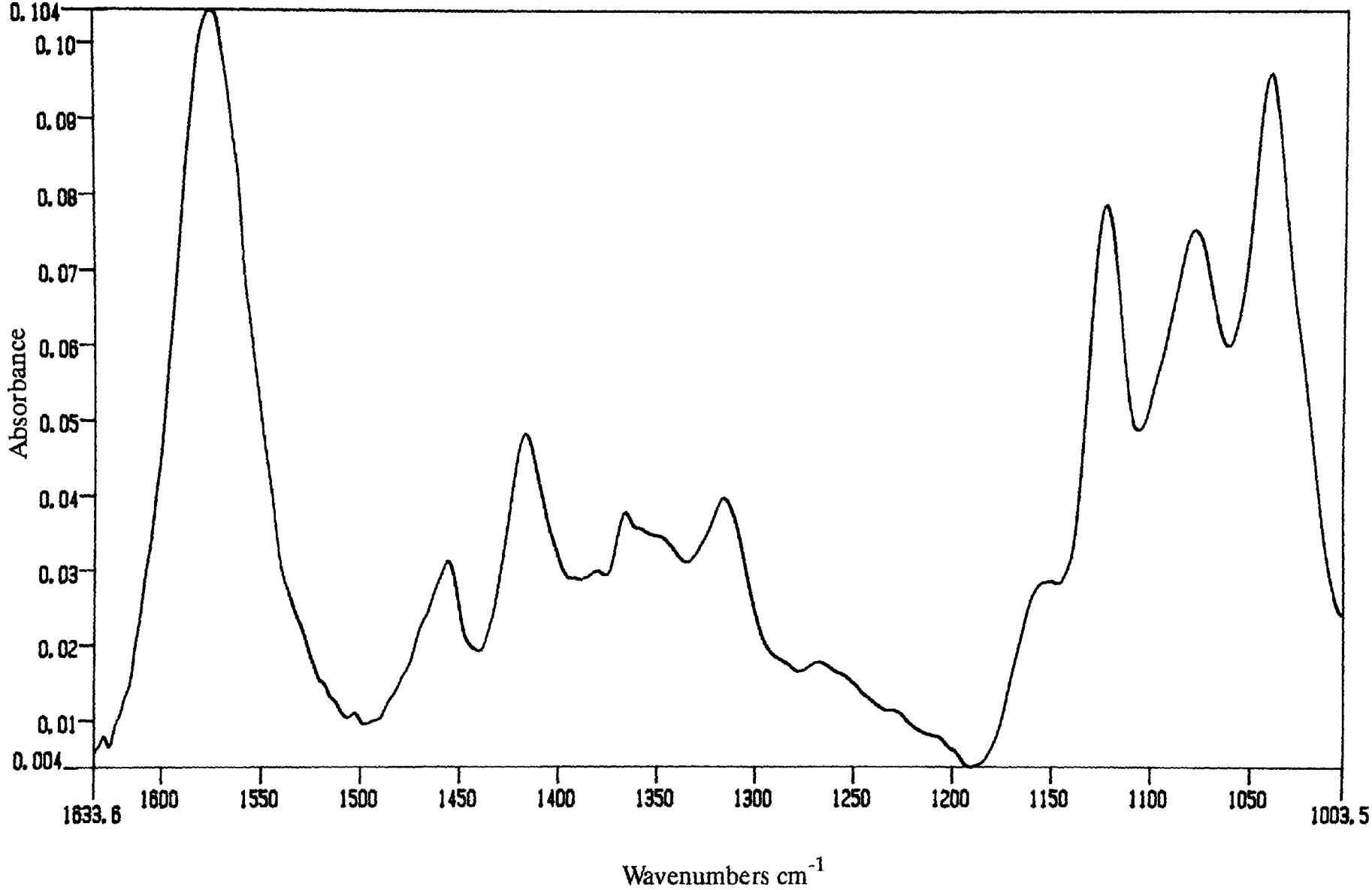


Fig 2.10.2 Spectrum of a lactose (3.054% w/v) / lactic acid (2.625% w/v) solution



1. The calibration set should encompass the expected range in concentration of the sample components. For example, the concentration of lactose in the whey medium was found, using an enzyme kit (Boehringer Mannheim), to be approximately 4.5% w/v. Thus, the concentrations of lactose and lactic acid in the standards were varied over the range 0-5% w/v.

2. Each standard solution should be a unique mixture. Redundant (equivalent or duplicate) standards prepared by diluting a stock solution, provide no independent spectral information and undermine the quality of the calibration. This is the so-called '*stock solution syndrome*'.

3. The sum of the component concentrations in the standards should not add to a constant. This is known as the '*100% syndrome*'. This causes problems during the mathematical operations of multicomponent analysis and may generate erroneous results.

4. Calibration samples should be selected to ensure that the range of variation in component concentrations is large relative to the precision to which calibration samples can be made.

Mixtures were used in order to allow for possible molecular interactions. The standards were stable for several weeks at a temperature of 4°C. Five validation samples were also prepared. These are solutions of known composition prepared in the same manner as the standards, but **not** included in the calibration set. They were used to test and optimise the quantitative method devised. This entailed scanning the validation samples and comparing calculated component concentrations with the actual concentrations. Optimum results were achieved using the region 1600 - 1007cm^{-1}.

Analysis of an 'unknown' using QUANT involves finding the linear combination of standard spectra, which, according to a least squares criterion, gives the best fit to the

sample spectrum. The calculated concentration of each component is found by adding together the contribution for each component from all the standards in the best fit combination[81].

2.10.2. On line Fermentation Analysis

The fermenter was connected to a macro CIRCLE®cell via PTFE (polytetrafluroethene) tubing (Fig 2.10.3.). The fermenter, containing the whey medium, and the CIRCLE® cell, were sterilised by autoclaving at 121°C (15lb/in^2) for 15 min. (Alternatively, the cell was flushed with 70% ethanol and rinsed with sterile distilled water). Medium was continuously circulated through the cell via a peristaltic pump (Watson Marlow 501U, Watson Marlow, Falmouth, Cornwall) (Fig 2.10.4.). Mid IR spectra (32 scans, range 1600 - 1007 cm^{-1}, 4 cm^{-1} resolution) were recorded automatically by means of a short program written using OBEY, a Perkin Elmer macro language (Fig 2.10.5.). Quantitative analysis was carried out on the spectral data obtained using QUANT.

2.10.3. Off line analysis

25 ml samples were withdrawn from the fermenter, cooled using ice and centrifuged at 5°C (Chilspin 2 centrifuge, 4000 rpm/2790xg, 10 mins). Analysis was carried out on the supernatant using the CIRCLE® cell in the 'open boat' mode.

2.10.4. Enzymatic Off line Analysis

D,L- lactic acid and lactose were measured off line using enzyme kits (Catalogue numbers 139 084 & 986 119, Boehringer Mannheim, Germany). Samples (5ml) were removed from the fermenter, quenched using ice and centrifuged at 5°C (Chilspin 2 centrifuge, 4000 rpm/2790xg, 10 mins). The supernatant was then tested.

2.10.5 Software Comparison

The performance of two software packages was assessed. QUANT uses least squares curve fitting as a means of multicomponent analysis. This program is supplied by Perkin Elmer. PLSCAL (Sprouse Scientific Systems ,California, USA) implements a partial

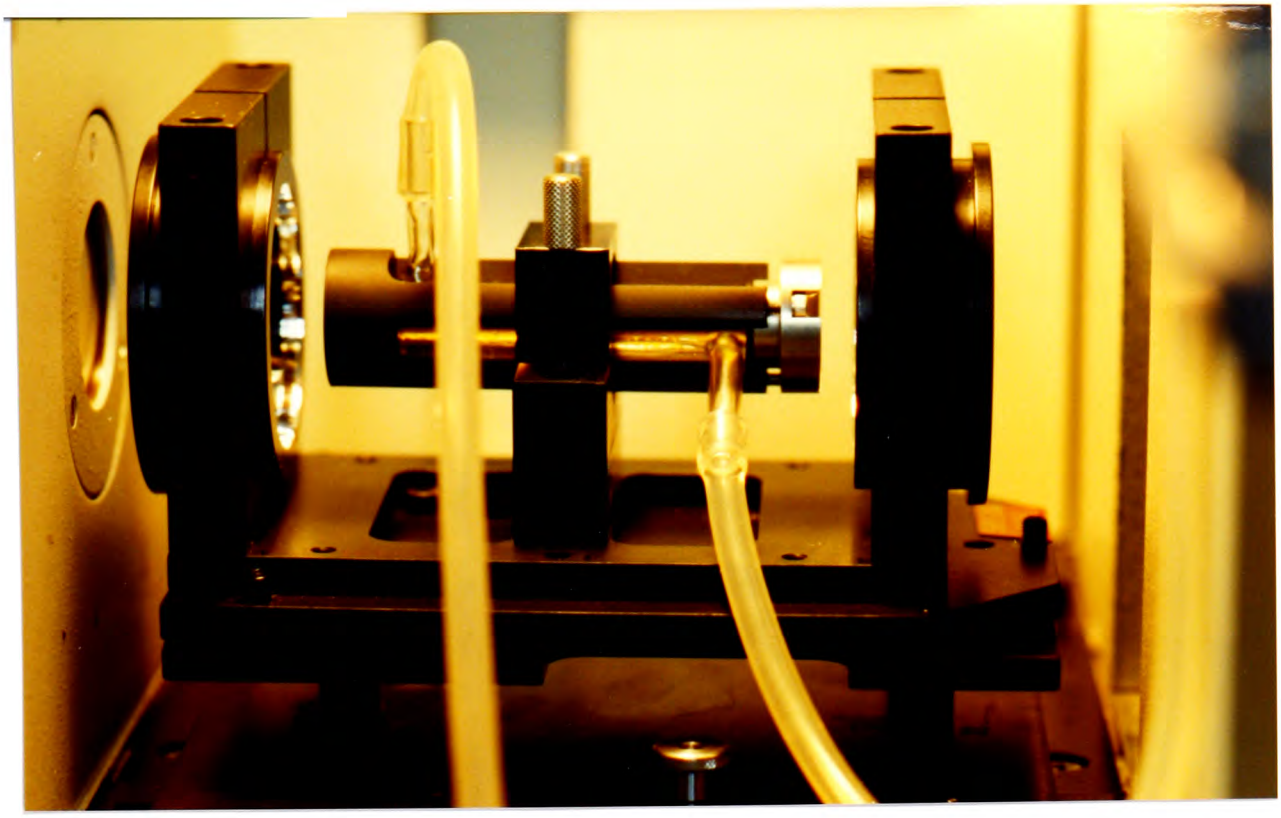


Fig 2.10.3. CIRCLE® cell positioned in the sample compartment of the FTIR spectrometer. Note the PTFE tubing via which the fermentation broth was circulated

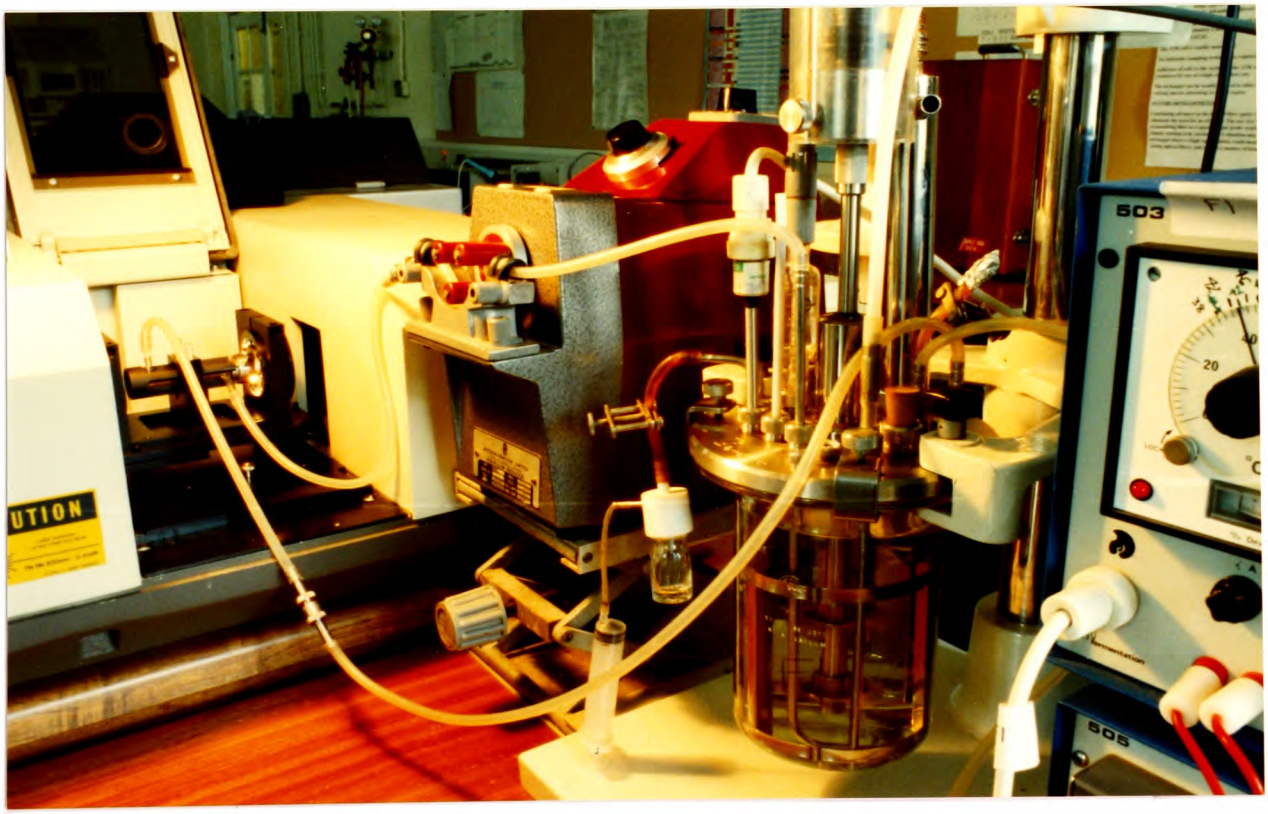


Fig 2.10.4. Fermenter connected on line to the FTIR spectrometer. Fermentation broth was continuously circulated through the CIRCLE® cell by the peristaltic pump.

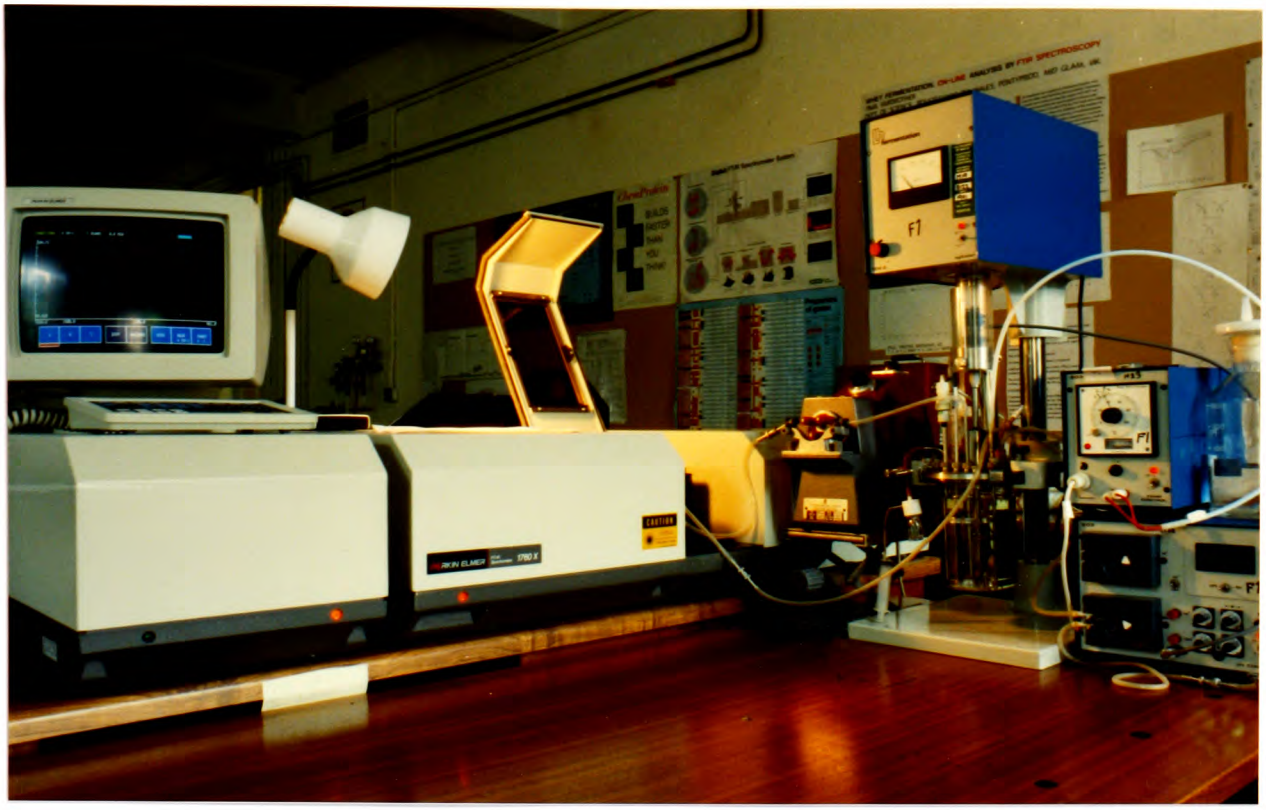


Fig 2.10.5 FTIR spectrometer and fermenter (LH Fermentation 500 series) configured for on line analysis

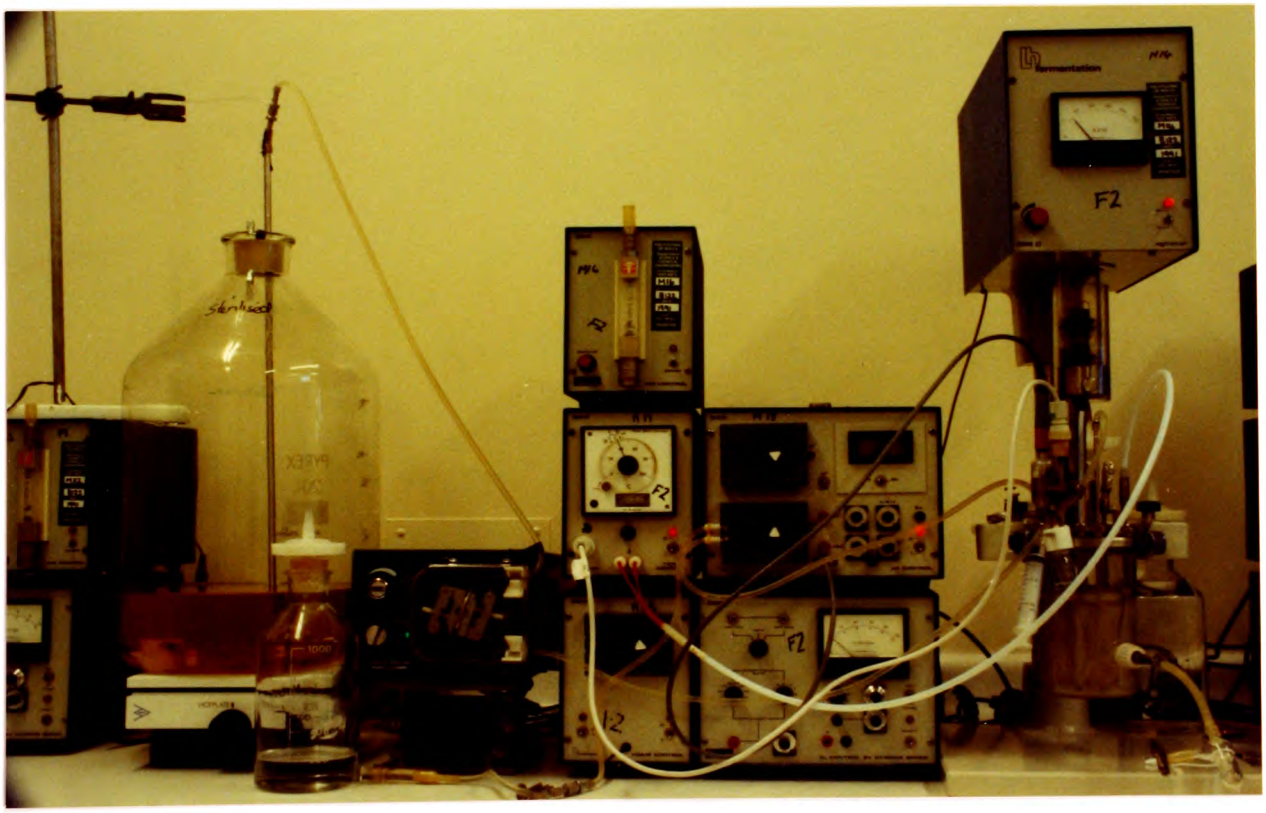


Fig 2.11.1 LH Fermentation 500 series fermenter adapted for continuous culture of *L. helveticus*

least squares approach to quantitative analysis. QUANT runs on an Epson personal computer (PC) (Epson UK LTD, Hemel Hempstead, Herts) dedicated to the FTIR spectrometer. PLSCAL was installed on an adjacent Viglen PC (Viglen LTD, Alperton, Middlesex). Both are standard IBM AT 'clones'.

The 15 standard spectra, together with the 5 validation spectra, were exported from the Epson to the Viglen PC. The spectra were converted from Perkin Elmer to Sprouse format, and copied into the PLSCAL program. Thus, the calibration spectra, quantitative method and 'unknowns' (validation samples) on both machines were the same. The accuracy and time taken for sample analysis by the two programs were assessed.

2.11. CONTINUOUS CULTURE of L. helveticus

2.11.1. Preliminary Study

Equipment

LH Fermentation 500 series fermenter adapted for continuous culture (Fig 2.12.1.). Fresh medium was supplied from a feed vessel at a pre-determined rate by a peristaltic pump (Watson-Marlow 501U). The volume of medium in the fermenter vessel was controlled by a weir-type overflow. Spent medium passed into a collecting vessel.

Starter Culture

100 ml of MRS lactose was loop inoculated and incubated at 42°C for 12 hours.

Fermentation Medium

MRS lactose

Experimental Procedure

Following autoclaving (121°C-15lb/in^2, 15 mins), the temperature and stirrer speed of the fermenter were maintained at 42°C and 250 rpm respectively. The pH controller was set at 5.9. A 3% v/v inoculum was used to seed the fermenter. When the OD of the culture reached approximately 0.7 (corresponding to mid-exponential growth phase), the

peristaltic pump was engaged. The dilution rate (D) was set at 0.15 h^{-1}. A minimum of three retention (residence) times (R) elapsed before sampling. This ensured that steady state was achieved. 5 ml of culture was removed hourly for 4 hours and analysis for lactose and lactic acid carried out using enzyme kits (Boehringer Mannheim), as described in section 2.10.4. This procedure was repeated for D = 0.30 h^{-1}.

2.11.2. Continuous Culture of L. helveticus in WP_O Medium

The above operation was repeated with minor adjustments. WP_O was used as the fermentation medium instead of MRS. D rates of 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8 h^{-1} were employed. For each value of D, 30 ml samples were removed hourly for 5 hours. Cell growth (OD readings converted to dry weight) was assessed. Substrate and product levels were measured off line by FTIR spectroscopy (see section 2.10.3.).

2.12. FERMENTATION SCALE UP

Translation of the fermentation process from a 1 l to a 16 l working volume fermenter was assessed.

Equipment

LH 2000 series fermenter (LH Fermentation, Reading, Berkshire) with modular analogue controller (Figs 2.11.1 & 2.11.2.).

Starter Medium

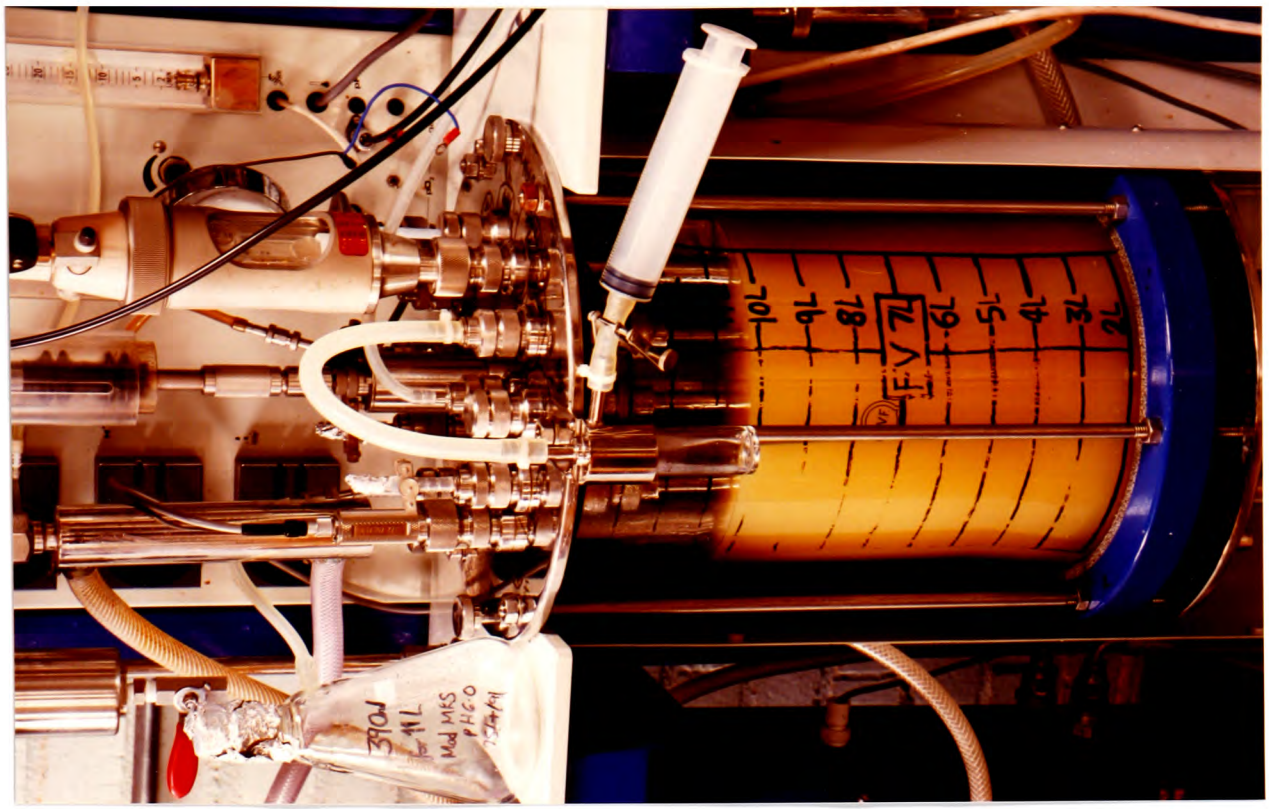
MRS lactose

Fermentation medium

WP_O medium

Experimental Procedure

A two-stage starter culture was used. 150 ml of MRS medium was loop inoculated from an MRS agar slope. The culture was grown for 10 hours, without agitation, at 42°C. 10 ml was then transferred by sterile syringe to a modified Buchnner flask containing 390



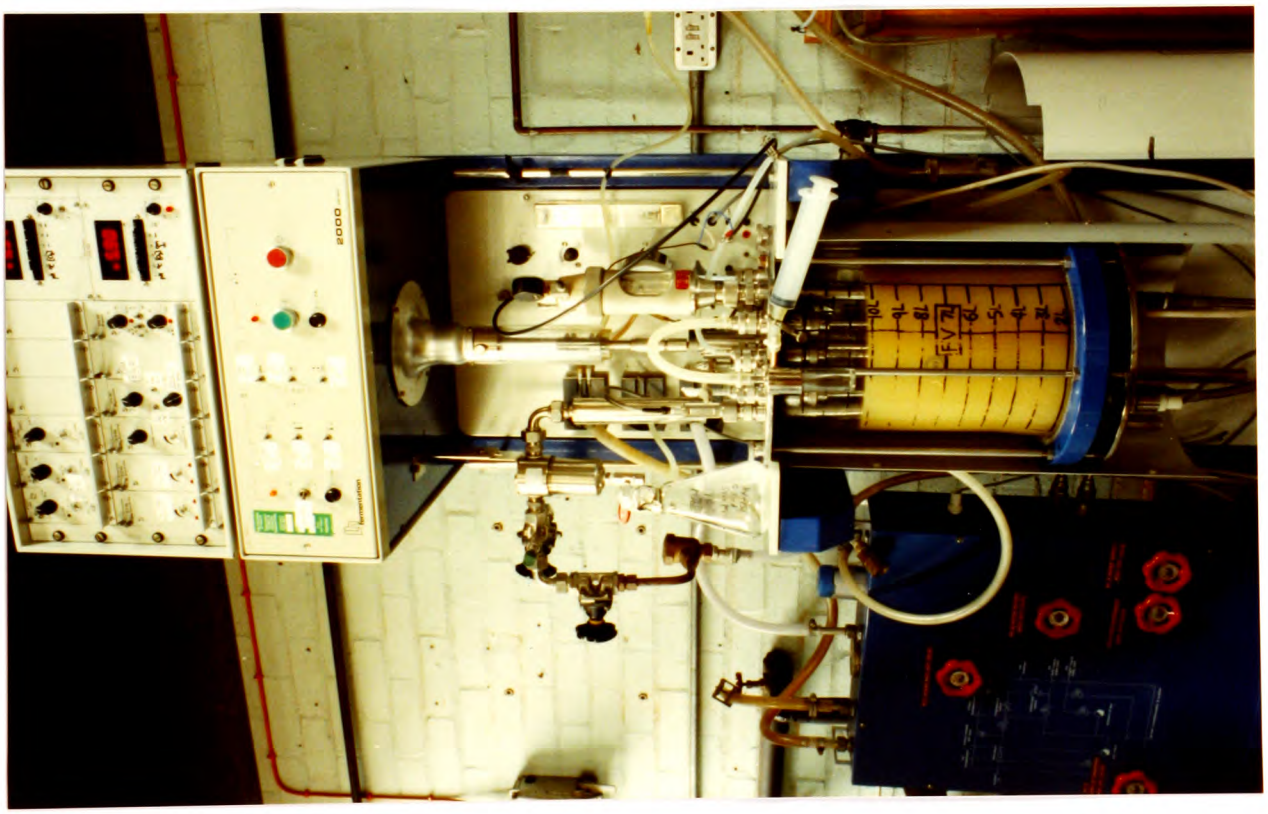


Fig 2.12.1. (left) LH Fermentation 2000 series pilot plant fermenter. The steam generator used for in situ sterilisation is located to the left of the fermenter vessel.

Fig 2.12.2. (right) Close-up of the fermenter vessel, containing a 16 hour old culture of *L. helveticus* grown in WP_o medium.

ml of MRS medium. The Buchner flask had been stoppered and a short length of PTFE tubing, complete with 'steri' connector, attached to the side-arm. This culture was propagated for 12 hours. The contents were transferred to the fermenter via the Buchner flasks's side-arm. (Note: the fermenter, containing 12 l of WP_o medium, had been sterilised *in situ* using a steam generator). Temperature was controlled at 42°C and pH maintained at 5.9 by automatic addition of 6M NaOH. Stirrer speed was set at 200 rpm. Thus the inoculum size/state and fermentation conditions employed were comparable with the bench scale (1 l) set up.

Growth of *L. helveticus* (OD converted to dry weight) and lactic acid synthesis / lactose depletion (off line FTIR analysis) were monitored.

CHAPTER 3. RESULTS & DISCUSSION

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3. RESULTS & DISCUSSION

3.1 WHEY PRE-TREATMENT

Ideally, whey generated during cheese production would be pumped into fermentation vessels, steam sterilised *in situ*, and fermented. Filter sterilisation would prove time consuming and lead to considerable down times between runs. Use of ethylene oxide would be impractical. Unfortunately, attempts to heat sterilise the whey lead to precipitate formation.

Pasteurisation, sterilisation and other heat treatments cause variable amounts of whey protein modification including denaturation, protein-protein interaction and protein-lactose interaction[82]. Between 4°C and 60°C reversible physiochemical changes such as hydrophobic association and partial unfolding occur. In the range 60°C-100°C, protein denaturation induces mainly irreversible physiochemical changes[83]. Unfolding of the globular whey proteins leads to exposure of sulphydryl (-SH) groups, allowing coagulation to occur, especially in the presence of calcium[73]. Between 100°C and 150°C, irreversible chemical changes e.g. Maillard reaction and cys-breakdown takes place[83].

The degree of denaturation depends on the protein components, total protein and solids concentration, time of exposure, temperature, pH and ionic strength. Of the whey proteins, imunoglobulins are most sensitive to temperature, whereas the proteose-peptone fraction is stable over a wide pH range[73] (Table 1.3.5.). Precipitation would pose problems during the fermentation process. The solid formed may interfere with bacterial growth. Monitoring of the reaction would prove difficult. Use of on-line FTIR spectroscopy would be adversely affected, due to protein deposition on the CIRCLE® cell crystal. The lifetime and efficiency of HPLC columns and enzyme electrodes would be reduced due to fouling. Product separation and purification would in turn become more costly.

In an effort to overcome these problems the procedure outlined in Fig 2.1.1. was employed. This process was developed during previous studies of ethanol production from whey using the yeast *Candida pseudotropicalis*. The PDPW generated was subsequently used as the fermentation medium for *L. helveticus*. Unfortunately, precipitation remained a major problem. This was due to the protein remaining in PDPW following thermoprecipitation. In the case of acid whey for example, a minimum holding time of 10 minutes at 90°C only precipitates approximately 75% of the protein present[73].

In an attempt to overcome these hurdles and maximise use of this raw material, UF was employed to remove the whey proteins. Although an expensive technique, UF generates an extra product, WPC, which has good market potential. Whey permeate was generated at the rate of 5-6 l/h. It was stored at -18°C until needed, together with the WPC. Protein content of the WPC was estimated to be 174 mg cm^{-3} using the Biuret method .

3.2. PRECIPITATE FORMATION

After autoclaving WP and WP-YE, a fine white precipitate was frequently observed in the fermenters. (The precipitate was subsequently analysed qualitatively). In one set of experiments, the pH of WP-YE medium was adjusted to 5.5 in the first fermenter and 5.9 in the second. The fermenters were allowed to equilibrate overnight at 42°C. By the following morning, the OD had risen from 0.020 to 0.65 in fermenter 1 and from 0.025 to 0.88 in fermenter 2. The results in Fig 3.2.1. reinforced the idea that pH and/or the concentration of yeast extract, [YE], were significant. As [YE] (and consequently initial pH) increased, the amount of precipitate generated during autoclaving rose.

3.2.1. Influence of Temperature on Precipitation

From Table 3.2.1., no solid was generated as the temperature of WP (pH_i 4.20) was increased to 100°C. This suggested that initial pH was too low, temperature alone was not sufficient to induce precipitation, or that YE is necessary for precipitation to occur.

FIG 3.2.1. Influence of Yeast Extract Concentration/ Initial pH on Precipitate Formation

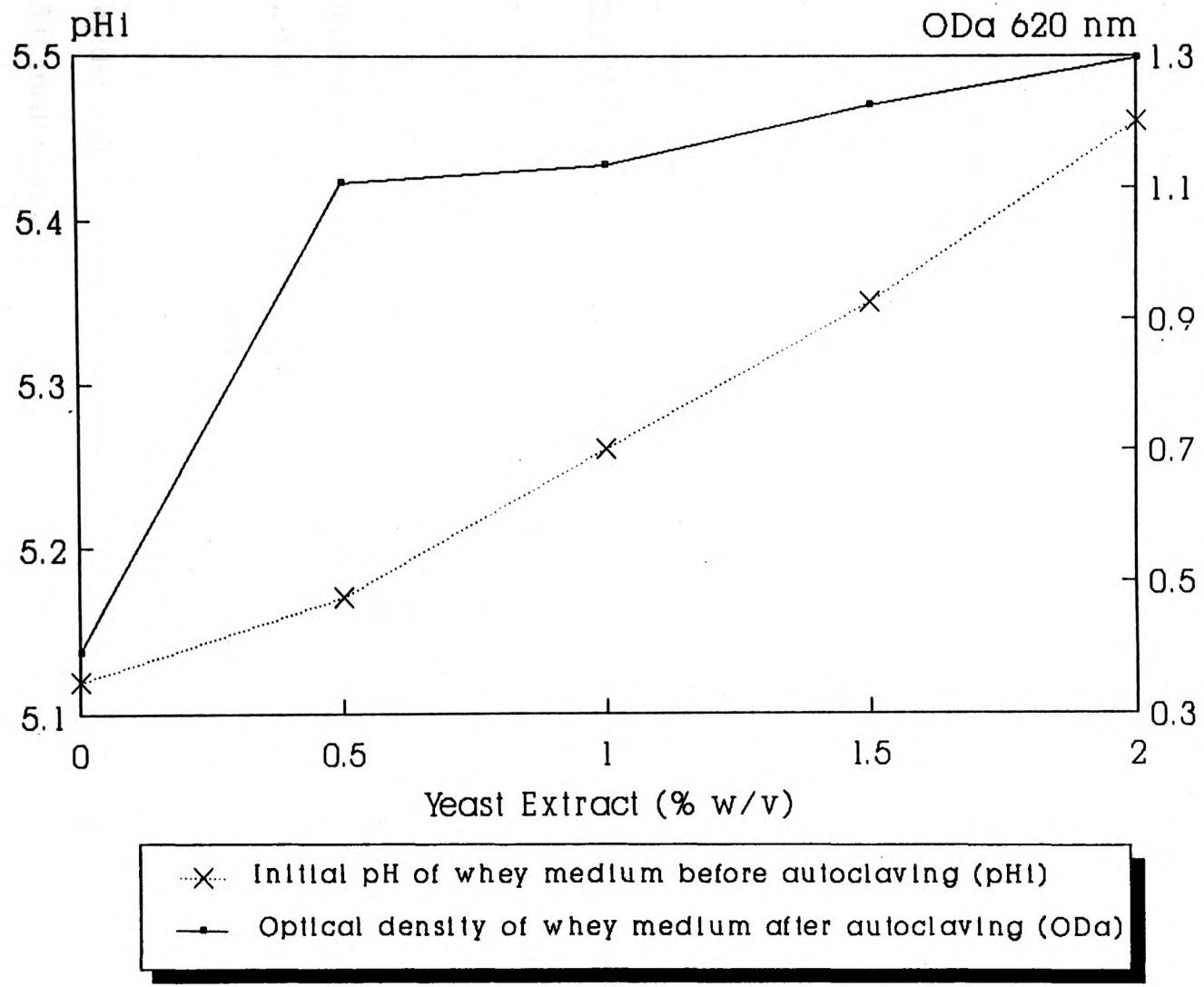


Table 3.2.1. Effect of heating on precipitate formation in WP

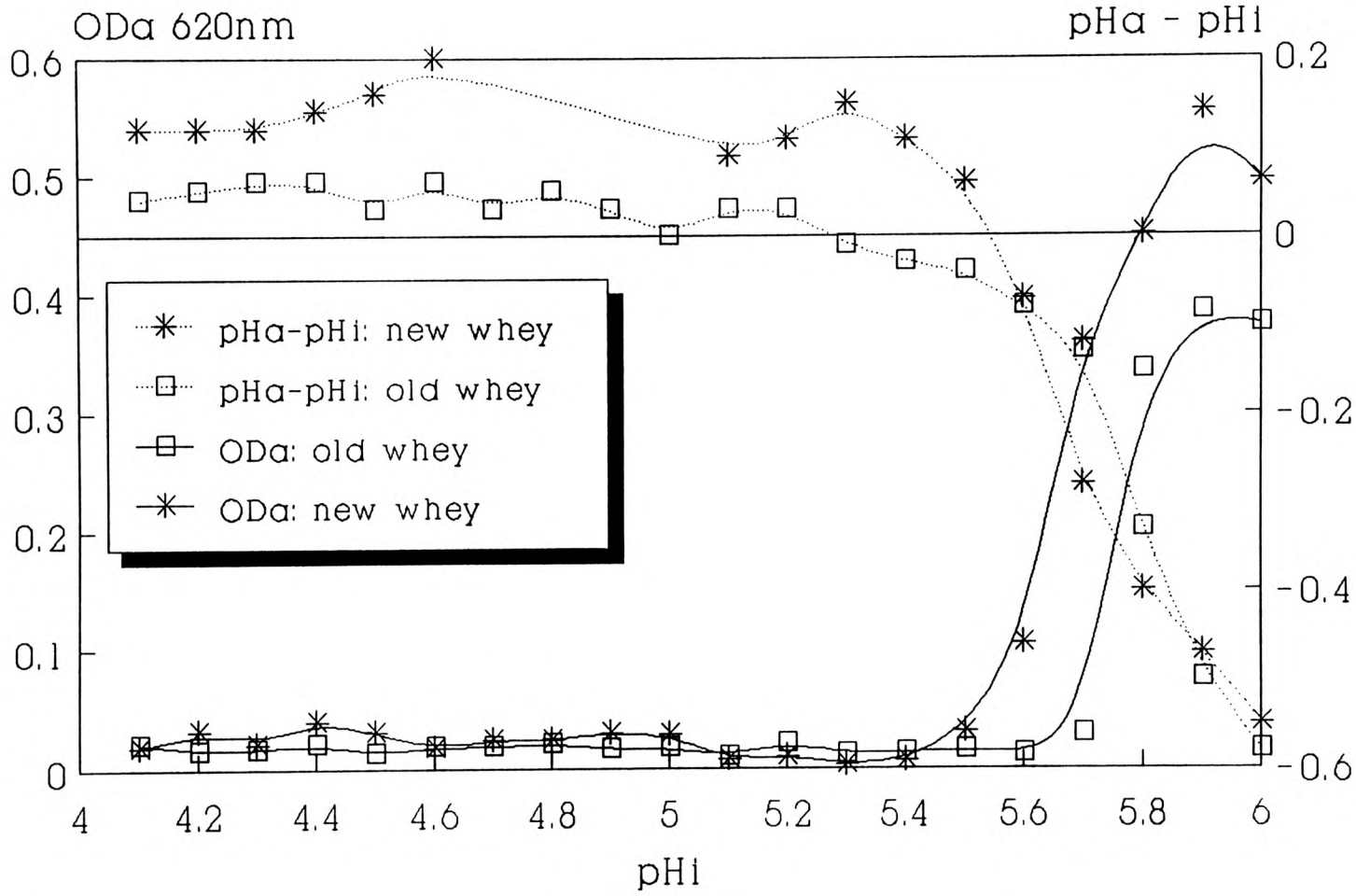
Temperature (°C)	ODa (620 nm)
15	0.012
25	0.015
30	0.017
35	0.016
40	0.017
55	0.018
65	0.021
70	0.021
85	0.021
90	0.020
100	0.023

3.2.2. Relationship Between pH and Generation of Precipitate

Fig 3.2.2. demonstrates that precipitation is pH dependent. Solid formation commenced at around pH 5.5 for the new batch of WP (September 1989) and pH 5.6 for the previous batch (March '89). Solubilisation of the precipitate was found to be induced by acid (Fig 3.2.3.). Subsequent readjustment of medium pH to its initial value of 5.5 using 2M NaOH had no effect. This inferred that generation of solid is a function of temperature and pH.

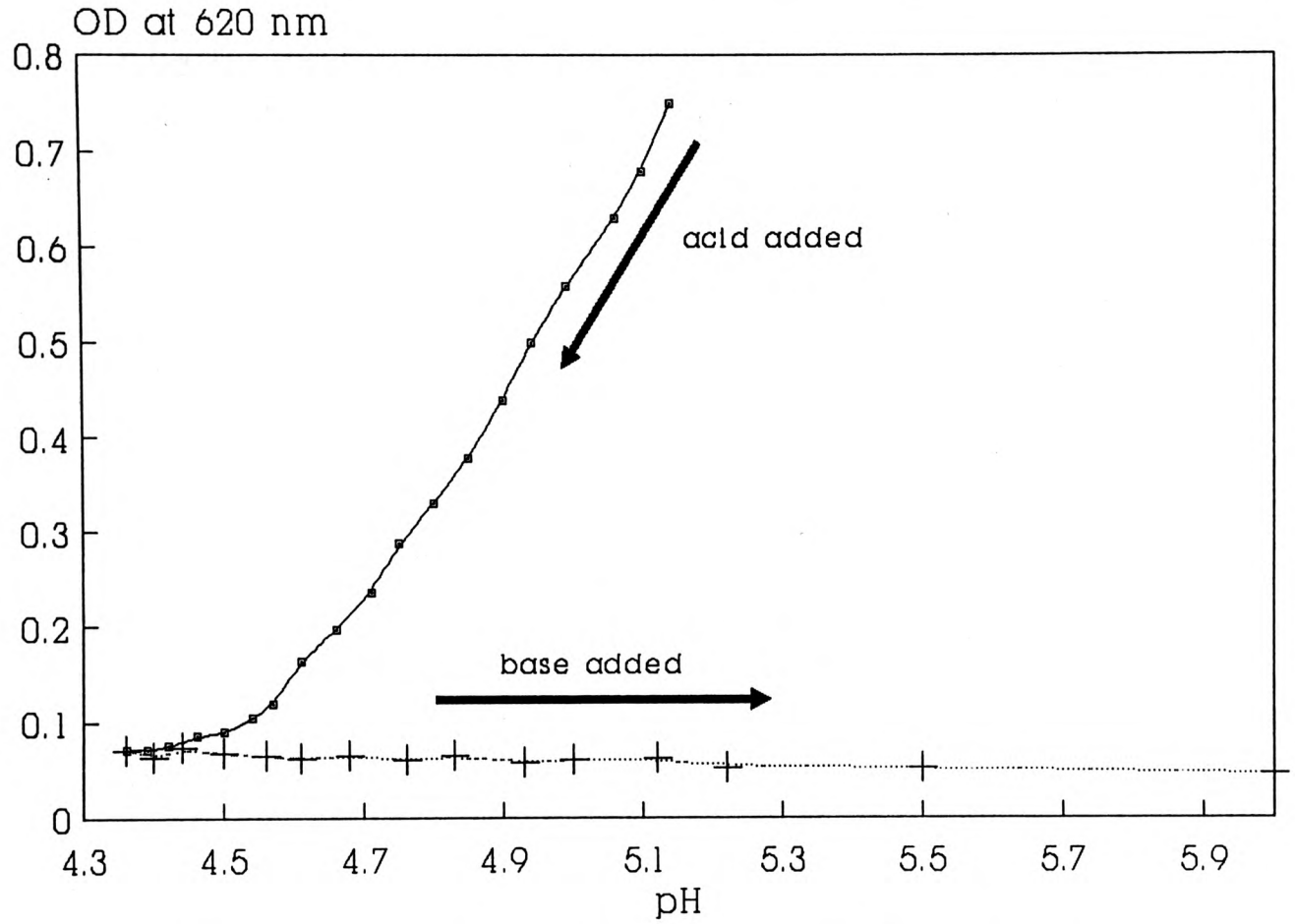
Further evidence of the influence of pH_i on precipitation is presented in Fig 3.2.4. At pH_i 4.0-5.0, small increases in pH_a and OD_a were observed. As initial pH was increased, precipitate formation became more apparent. Maximum OD_a values corresponded to pHi of 7.0. pHa showed a corresponding decrease over the range pHi 5.5-7.5 relative to

FIG 3.2.2. Influence of Initial pH on Precipitate Formation in Whey Permeate During Autoclaving



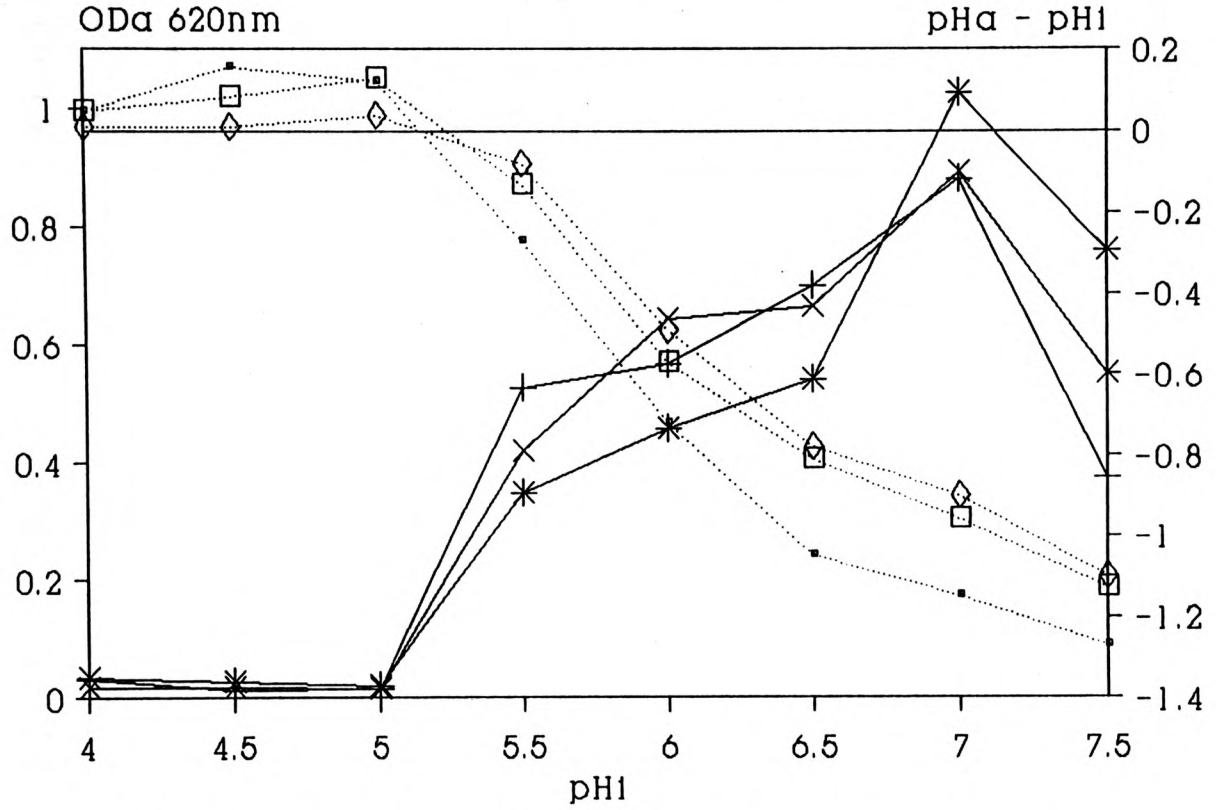
pHi & ODi = pH and OD before autoclaving
pHa & ODa = pH and OD after autoclaving

FIG 3.2.3. Effect of pH Adjustment on Precipitate Solubility



2M HCl was added dropwise to autoclaved whey permeate -yeast extract (0.5% w/v) medium until a minimum optical density was attained. The pH was then readjusted with 2M NaOH and the changes in optical density recorded.

FIG 3.2.4. Influence of Initial pH on Precipitation in Supplemented Whey Permeate During Autoclaving



Whey permeate : optical density after autoclaving (ODa)

Whey permeate-$CaCl_2$: optical density after autoclaving

Whey permeate-YE : optical density after autoclaving

Whey permeate : pH after autoclaving minus initial pH (pHa-pHi)

Whey permeate-$CaCl_2$: pH after autoclaving minus initial pH

Whey permeate-YE : pH after autoclaving minus initial pH

pHi. Little difference was observed in the properties of WP and WP-YE . This contrasts with the data presented in Fig 3.2.1. Here, OD increased 3-fold on autoclaving WP supplemented with 0.5% YE. Clearly, this can now be explained in terms of the rise in pHi on addition of YE, as opposed to YE induced precipitation. Addition of acid was again found to solubilise the solid produced:

Using WP-YE, pHi = 6.54 ODi = 0.094

pHa = 5.57 ODa = 0.846

On adding 1M HCl:

pH	5.01	4.67	4.48	4.35	4.29
OD	0.395	0.218	0.145	0.116	0.115

A minimum OD of 0.115 corresponded to pH =4.29. (In Fig 3.2.3., maximum solubility (lowest OD) occurred at pH =4.36). On readjusting to pH_i = 6.54 with base, no significant change in OD (0.115 to 0.132) was observed. The medium was autoclaved once more. Again, precipitation resulted, pHa = 5.55 and ODa = 1.257.

3.2.3. Influence of EDTA on Precipitate Solubility

Using WP, pHi = 6.49 ODi = 0.064

pHa = 5.68 ODa = 0.666

Following addition of the sequestering agent EDTA, a fall in OD from 0.730 to 0.097 was noted. Readjustment to pHi = 6.48 with alkali had no effect (OD decreased from 0.097 to 0.088). Subsequent autoclaving lead to only a marginal increase in OD from 0.088 to 0.103

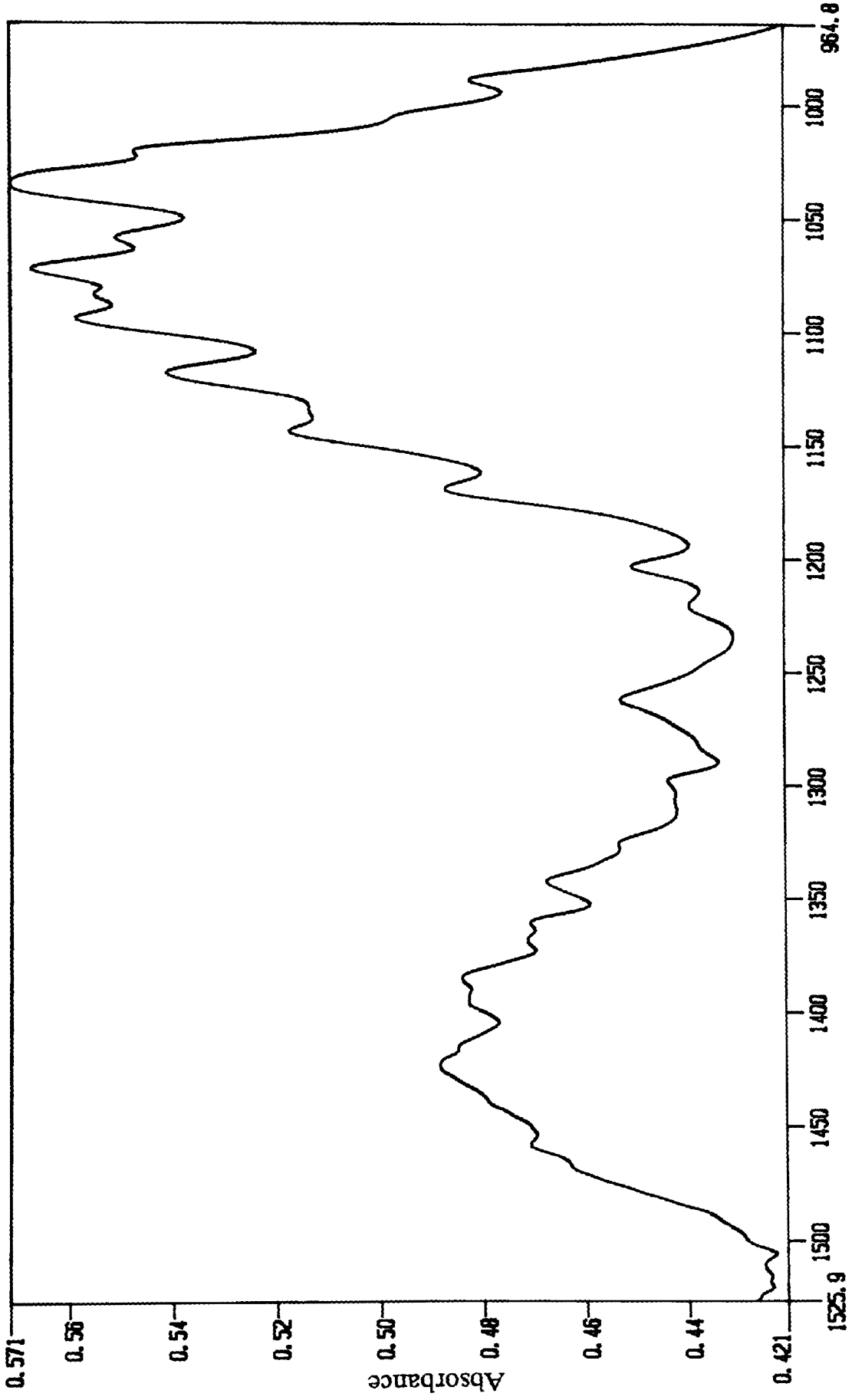


Fig 3.2.5. Spectrum of whey permeate precipitate (run as a KBr disc)

Analysis of the precipitate gave positive results for Ca^{2+} and phosphate. Additionally, qualitative analysis of the solid by FTIR spectroscopy (Fig 3.2.5.), revealed absorption characteristics (1200 cm^{-1} - 1000 cm^{-1}) consistent with the presence of phosphate.

It is evident that precipitation is influenced by pH and temperature and may be due to formation of Ca^{2+} complexes. In the past $CaCl_2$ has been used in the precipitation of heat denatured WP, complete precipitation occurring at pH 5.5-5.8[84]. (Recall Fig 3.2.2. - precipitation was seen to commence at pH 5.5 and pH 5.6 respectively). Ca^{2+} is frequently the cation that cross links macromolecules, or induces large conformational changes in them. Ca^{2+} is usually six co-ordinate and can bind up to 6 different protein functional groups at the same time. With small ligands, the cation is often octa co-ordinate[85]. The total phosphate in whey and WP is derived from phospholipid and free phosphate[86]. Polypeptides and peptides in whey are mainly proteolytic products of casein. They are mostly phosphorylated and do not occur in a compact globular structure[83]. Free phosphate, lipids and proteins thus contain potential binding sites for Ca^{2+}.

Sharpe[87] stated that the quantity of complex formed is pH dependent, since most ligands are bases in the Bronsted sense, i.e. H^+ acceptors. (N.B. at pH values below their isoelectric point, whey proteins have a net positive charge and behave as cations. At higher values of pH, the proteins are anionic with a resultant negative charge[73]). It should also be noted that solubility of calcium phosphate decreases on heating[89]. Resolution of precipitate by acid is probably due to competition between H^+ and Ca^{2+} for available binding sites, leading to disaggregation of Ca^{2+} complexes. As pH rose from 5.5-7.0, [H^+] decreased. This facilitated reassociation of Ca^{2+}, leading to higher ODa values. The corresponding drop in pHa could have been due to removal of negatively charged phospolipids, peptides and phosphate, now complexed with Ca^{2+}, from solution. At pH 7.0, OH^- competes for Ca^{2+} binding, leading to reduced precipitate levels. An analogous situation is seen in the effect of pH on the thermal denaturation of the calcium metalloprotein alpha-lactalbumin (α-la)[88].

Formation of such colloidal protein-mineral-lipid particles is one of the major contributors to membrane fouling in UF of whey. This problem has been similarly alleviated by acidification of whey prior to UF, to solubilise colloidal material[82].

EDTA is a hexadentate sequestering agent with a high affinity for Ca^{2+}. Addition of EDTA to WP effectively removed Ca^{2+} (and other free metal ions) from the medium. Thus no precipitate appeared on re-autoclaving. In a study of calcium-caesin co-precipitates, three classes of precipitate were described. Solubility experiments revealed that pH adjustment of low calcium coprecipitates was sufficient to solubilise the solid. For medium and high calcium coprecipitates, a sequestering agent such as EDTA was required for maximum solubility[84]. Complexing agents are also occasionally added to whey before UF in order to remove Ca^{2+}, thereby improving flux rates.

A method for whey clarification, based on thermal aggregation of lipoproteins through ionic calcium bindings, has recently been proposed by Maubois [90]. He applied this pretreatment in order to improve flux rates during UF of whey and to increase WPC purity. Clarification reduces Ca^{2+} content of whey through complex formation with lipoprotein, probably as phosphate salts. It also reduces phosphate and nitrogen.

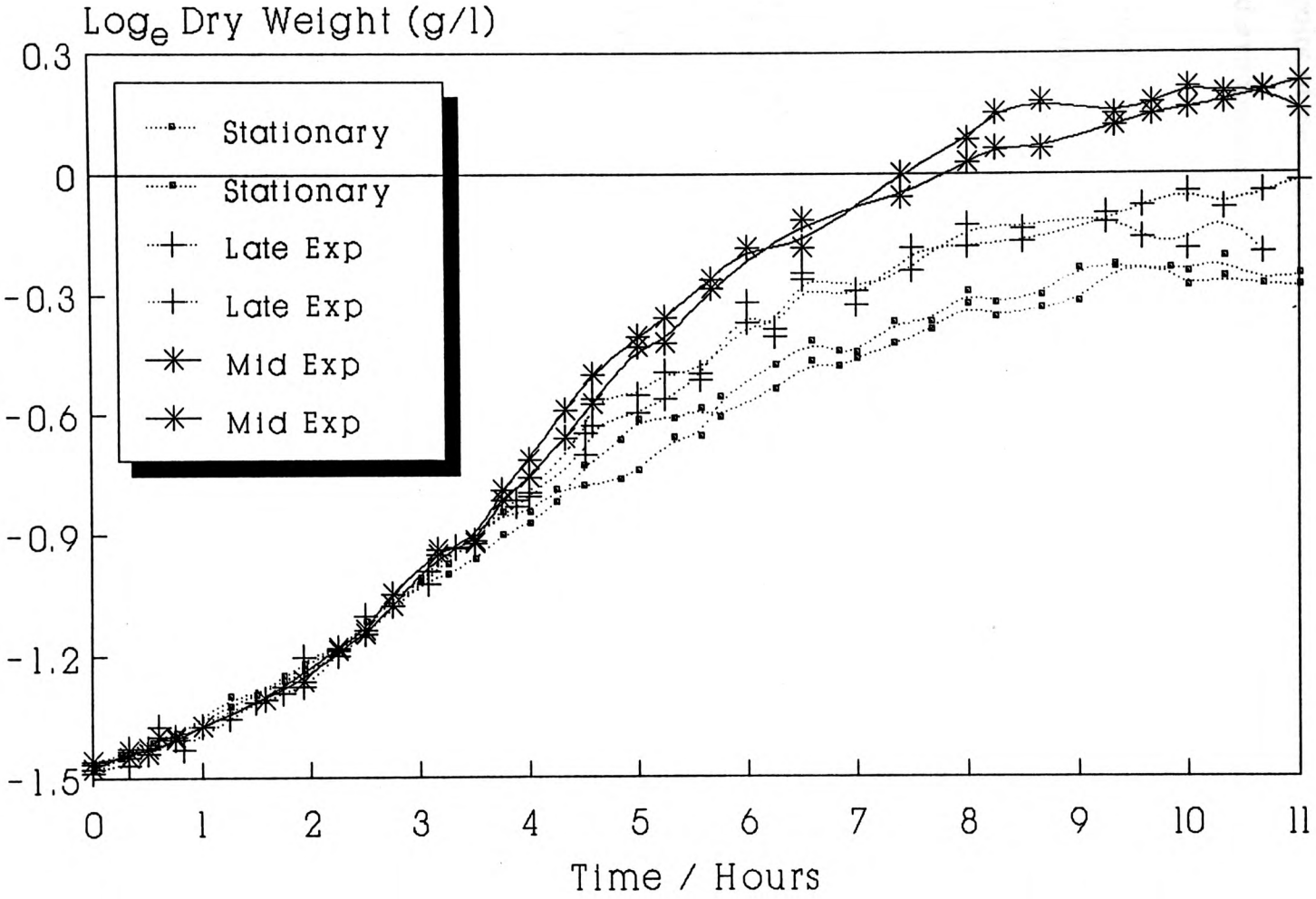
Based on these findings, when autoclaving or simply heating WP based media:

1. $4.0 < pHi < 5.2$ to avoid precipitate formation
2. should precipitate appear, it can be solubilised by acid or addition of EDTA

3.3. STARTER CULTURE DEVELOPMENT

Fig 3.3.1. shows that a higher final cell concentration was attained in WP using a mid-exponential phase inoculum. During this stage of growth, the inoculating culture is as active as possible and characterised by a maximal division or doubling rate. As we move to late exponential and stationary phases, the age of the culture increases. Substrate/nutrient limitations and product (lactic acid) inhibition/toxicity become progressively more

FIG 3.3.1. Influence of Growth State (Age) of Inoculum on Batch Growth of *L. helveticus* in WP



important. (Note: the undissociated, electroneutral form of lactic acid, rather than lactate, is believed to be the species which inhibits the fermentation[91]). The number of viable cells per unit volume of inoculum is reduced with respect to a mid exponential culture. This was manifested in lower final cell concentrations in the fermenter. From an economic point of view, given the high capital and operating costs of fermentation equipment, a mid-exponential inoculum would minimise fermentation times.

A second important variable is the **size** of the transferred inoculum. Reddy et al[92] investigated the influence of inoculum size during whey fermentation. No difference was observed in the fermentation rate when 2.5, 5.0 and 10.0% v/v inocula were employed. During the course of this work, a 3.5% v/v inoculum was generally used. Choice of starter culture medium is also of key importance. This is discussed in section 3.6.5.

3.4. OPTIMISATION of L. helveticus GROWTH: 2^{3-1} FRACTIONAL FACTORIAL DESIGN

The results obtained (Table 3.4.1.) suggest that optimum conditions for growth of *L .helveticus* in WP-YE (0.5% w/v) are:

pH 5.9 temperature 42°C stirrer speed 200 rpm

This coincided with the centre point conditions used. Similarly, Roy et al[29] found that maximum lactic acid and biomass production by *L. helveticus* strain *milano*, occurred at pH 5.9, 42°C and 200 rpm in WP-CSL (corn steep liquor) medium.

Table 3.4.1. Treatment combinations and responses obtained for the 2^{3-1} design. μ_{av} is the mean specific growth rate for fermenters 1 and 2.

P	S	T	μ_1 (h^{-1})	μ_2 (h^{-1})	μ_{av} (h^{-1})
0	0	0	0.663	0.715	0.689
0	0	0	0.635	0.676	0.656
-	-	+	0.622	0.492	0.557
+	-	-	0.564	0.530	0.547
+	+	+	0.540	0.560	0.550
-	+	-	0.595	0.577	0.586

This is illustrated geometrically in Fig 3.4.1.

The effect on μ of moving from the lower (-) level of pH (pH = 5.5) to the upper (+) pH level (pH = 6.3), the p main effect, is given by: the value of μ_{av} at the higher level of pH minus μ_{av} at the lower pH level, divided by four

i.e. $$p = (0.547 + 0.550) - (0.586 + 0.557)/4$$
$$= -0.012$$

Similarly, the temperature and stirrer speed main effects, t and s respectively, are:

$$t = 0.008$$
$$s = -0.007$$

The influence on μ of moving from low to high factor levels is shown in Fig 3.4.2. The small main effects (p, t and s) observed are attributable to the fact that the runs carried out "circled" the optimum conditions. Thus no real improvements in response (μ) were achieved on varying the level of each factor. For instance on moving from 38°C to 46°C, we passed through the optimum temperature of 42°C.

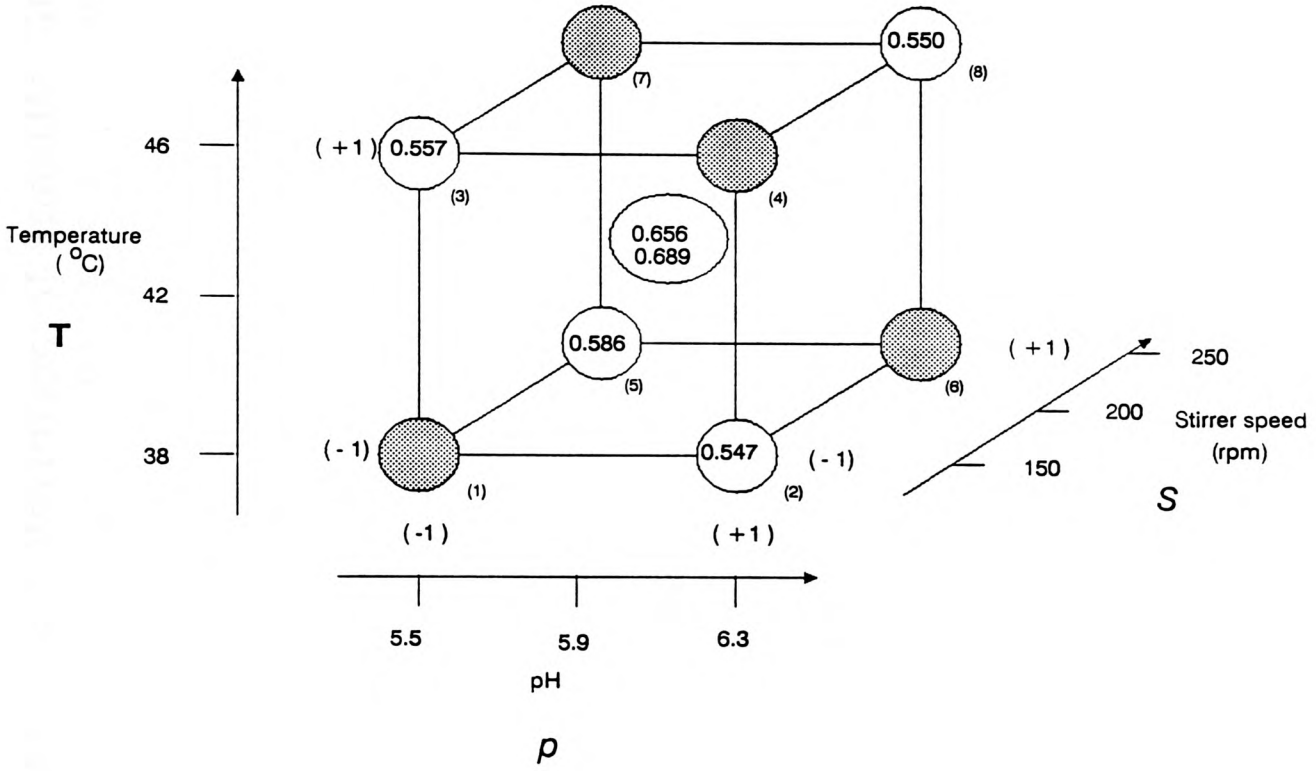
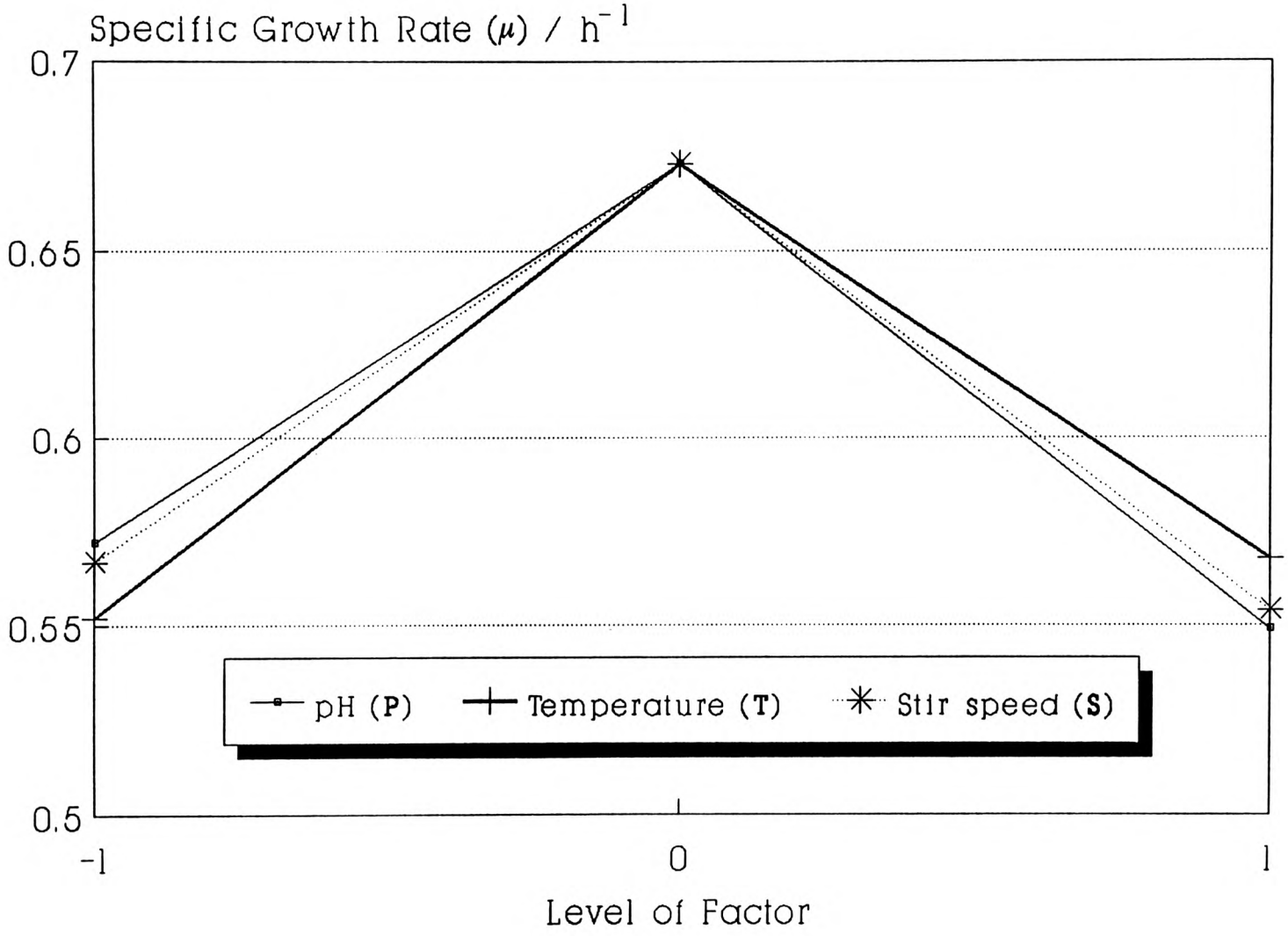


Fig 3.4.1. Growth optimisation. Specific growth rates (μ) calculated for the half fraction carried out are indicated. μ values for the centre point conditions are situated at the cube centre.

FIG 3.4.2. Variation of Specific Growth Rate With Factor (P, S & T) Levels



A fractional factorial design, as opposed to a full 2^3 design, was employed in order to provide flexibility. If the growth rates had indicated that optimum conditions lay at the periphery or outside the experimental region defined by the existing factor levels, then a new matrix could have been generated. Consider for instance, if μ at pH 5.5, temp 38°C and stirrer speed 150 rpm (- - -) had been significantly higher than in the other runs. We could have "moved towards" this elevated response value and created a new design to investigate this region. In this case, the optimum lay within the area defined by the design matrix. The other half fraction could then have been performed, if desired, to give a full 2^3 factorial design.

3.5. GROWTH OPTIMISATION USING A 2^3 FACTORIAL DESIGN

See Appendix B for details of ANOVA (analysis of variance).

Table 3.5.1. 2^3 design matrix and responses (μ) obtained.

RUN	P	S	T	μ_1 (h^{-1})	μ_2 (h^{-1})	μ_{av} (h^{-1})
1	-	-	-	0.613	0.654	0.634
2	+	-	-	0.697	0.718	0.708
3	-	+	-	0.738	0.673	0.706
4	+	+	-	0.803	0.801	0.802
5	-	-	+	0.743	0.717	0.730
6	+	-	+	0.652	0.623	0.638
7	-	+	+	0.800	0.814	0.807
8	+	+	+	0.862	0.896	0.879
9	0	0	0	0.909	0.861	0.885
10	0	0	0	0.850	0.846	0.848
11	0	0	0	0.833	0.914	0.874

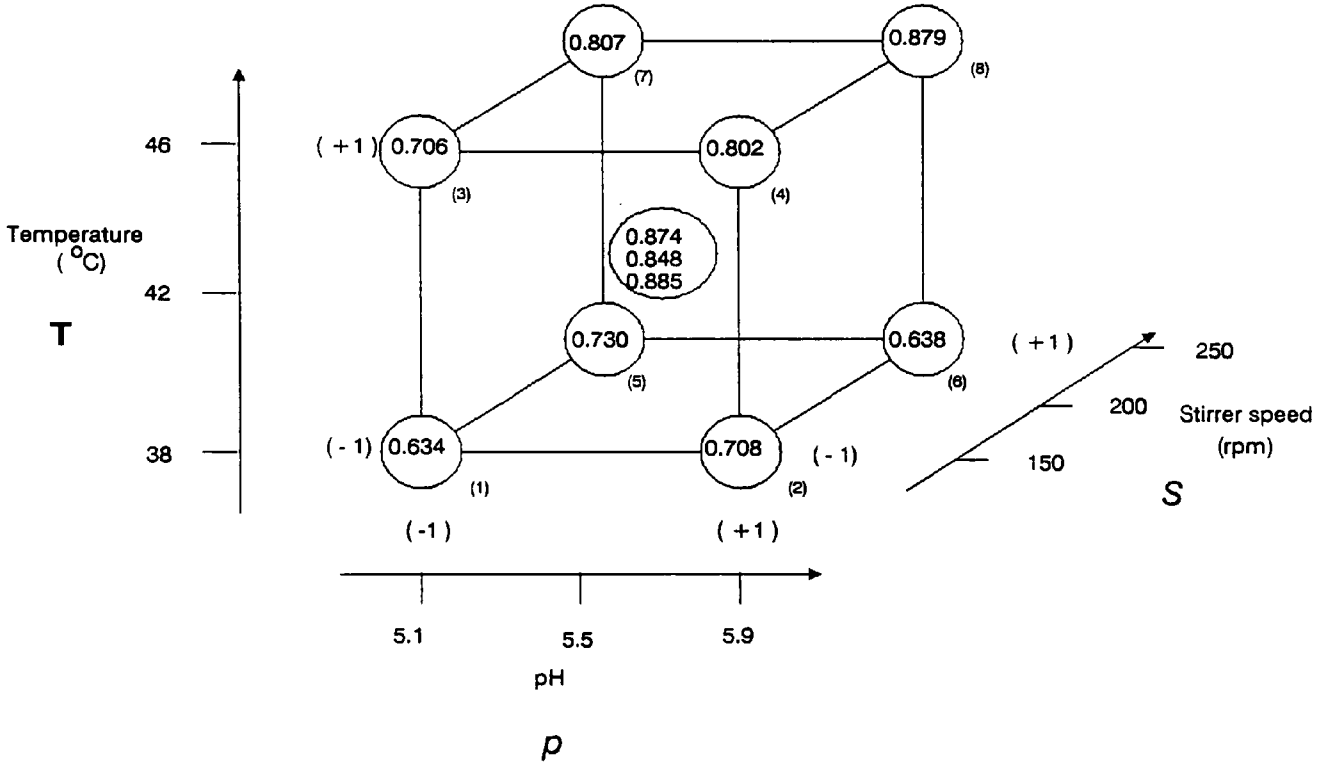


Fig 3.5.1. 2^3 factorial design. Specific growth rates (μ) for runs 1 to 8 are shown. μ values for the centre point conditions (runs 9 to 11) are located at the cube centre.

The results obtained for the design matrix employed are shown in Table 3.5.1. above and depicted graphically in Fig 3.5.1.

Using a statistical program developed by Dr Edward Morgan[93], the following parameters were calculated:

Main Effects	Two Factor Interactions
$p = 0.019$	$pt = 0.023$
$t = 0.061$	$ps = -0.024$
$s = 0.025$	$ts = 0.019$

Three factor interaction

$pts = 0.018.$

Based on the experimental data obtained, a polynomial expression relating response, in this case μ, to the process variables can be generated. The generalised form is:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_{12} + b_{13}x_{13} + b_{23}x_{23} + b_{123}x_{123}$$

where b_0 is the intercept, b_1 is the slope in the direction of factor x_1, $b_{12}x_{12}$ is a 2 factor interaction term etc. Using the results obtained, the equation takes the form:

$$y = 0.7737 + 0.019p + 0.061t + 0.025s + 0.023pt - 0.024ps + 0.019ts + 0.018pts$$

NOTE: strictly, this only applies to the coded factor levels, + or -. Based on the regression equation formed, estimated values of μ were produced and compared with the true values (Table 3.5.2.).

Table 3.5.2. Observed responses, predicted values and the residuals obtained from the regression equation.

Run	Observed μ_{av}	Estimated μ_{av}	Residual
1	0.634	0.670	-0.036
2	0.708	0.744	-0.036
3	0.706	0.742	-0.036
4	0.802	0.838	-0.036
5	0.730	0.766	-0.036
6	0.638	0.674	-0.036
7	0.807	0.843	-0.036
8	0.879	0.843	-0.036
9	0.874	0.774	0.100
10	0.848	0.774	0.074
11	0.885	0.774	0.111

Response surfaces showing the variation of μ with factor levels were generated using this equation (Figs 3.5.2., 3.5.3. & 3.5.4.). ANOVA was also carried out to assess the effectiveness of the model (Tables 3.5.3. & 3.5.4).

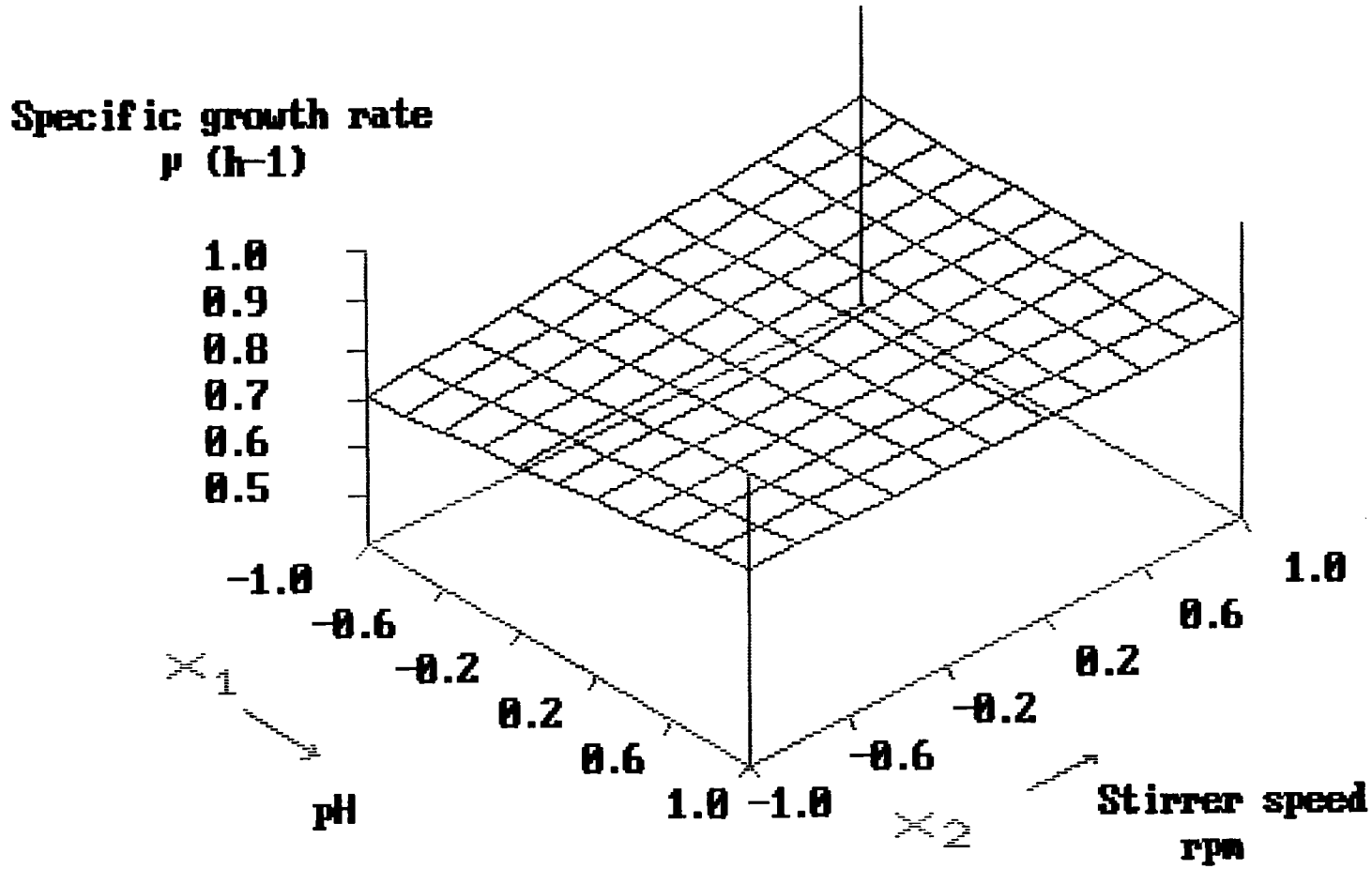


Fig 3.5.2. Response surface showing the variation of μ with pH & stirrer speed

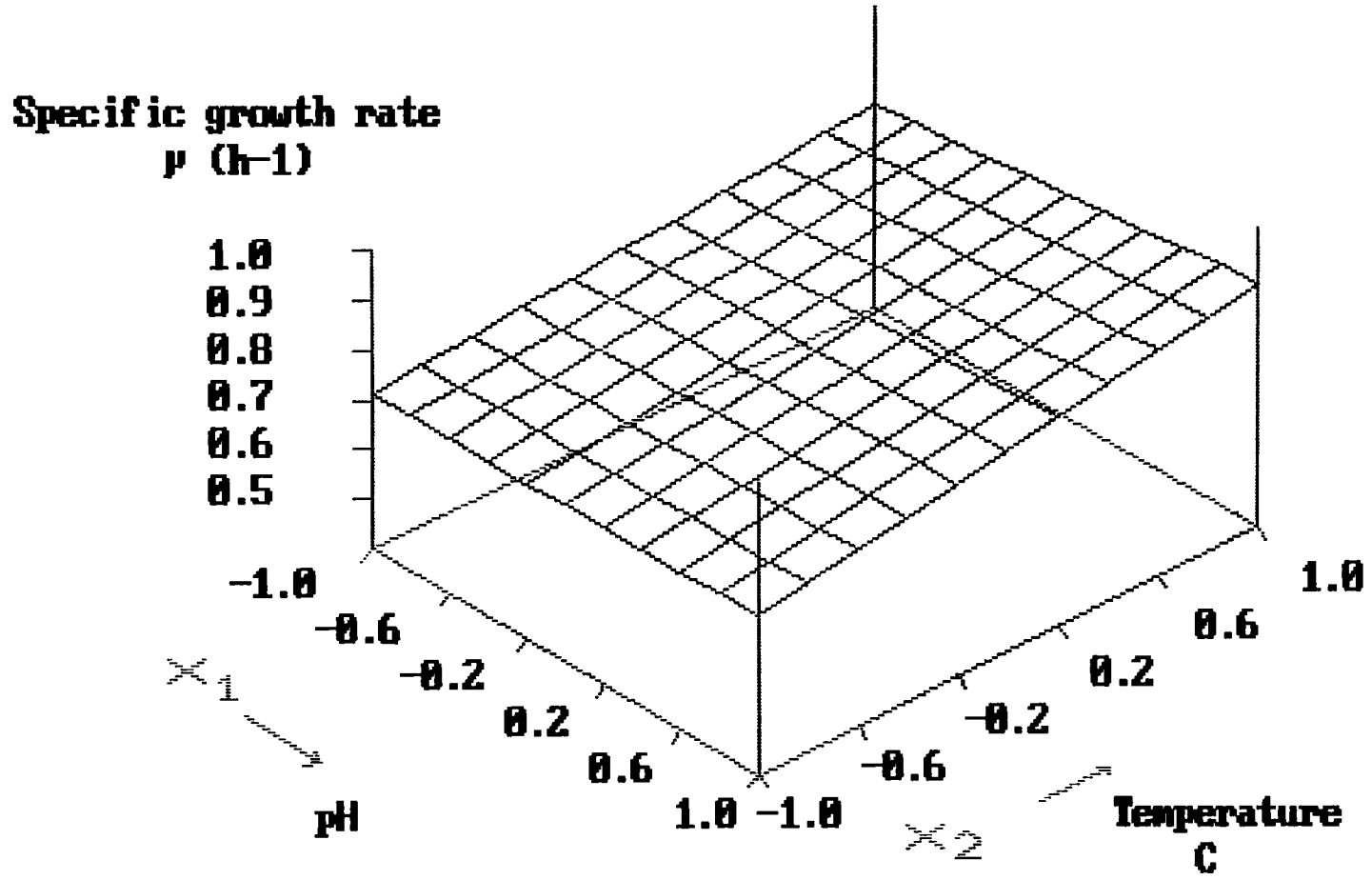


Fig 3.5.3. Response surface showing the variation of μ with pH & temperature

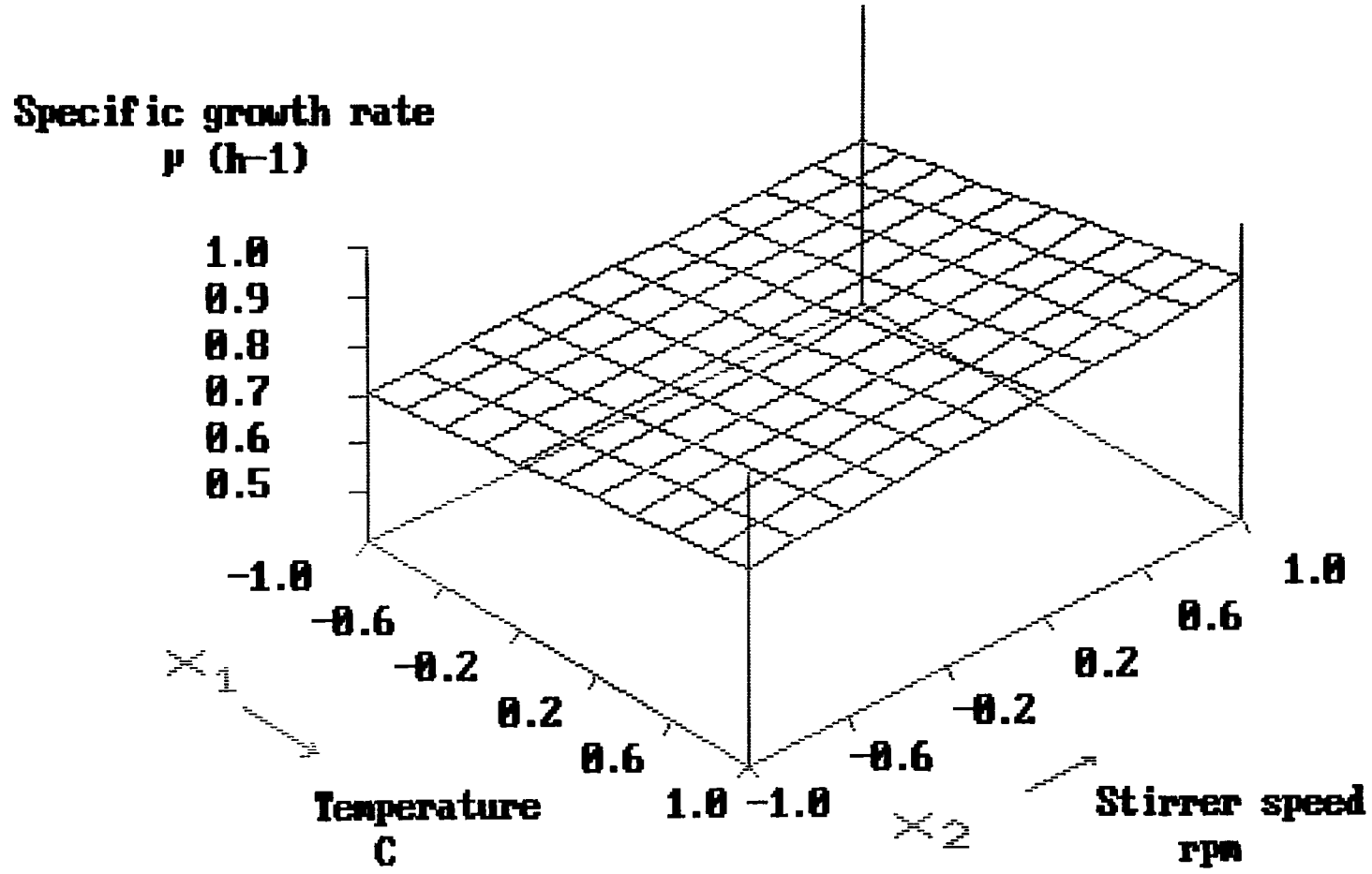


Fig 3.5.4. Response surface showing the variation of μ with temperature & stirrer speed

Table 3.5.3. ANOVA for 2^3 design.

Source of error	Sum of squares	df	Mean squares	Variance (F) ratio
Total	0.089	10		
Regression	0.051	7	0.007	0.579
Residual	0.038	3	0.013	
Lack of fit	0.010	1	0.010	0.731
Pure error	0.028	2	0.014	

Table 3.5.4. Estimation of the significance of the regression coefficients using a t-test

Coefficient	b_0	b_1	b_2	b_3
Value	0.774	0.019	0.061	0.025
t-value	22.752	0.470	1.517	0.639
% prob < > 0	>99.5	<90	<90	<90
Coefficient	b_{12}	b_{13}	b_{23}	b_{123}
Value	0.023	-0.024	0.019	0.018
t-value	0.583	0.596	0.476	0.445
% prob < >0	<90	<90	<90	<90

The residuals (difference between calculated and observed values of μ) for runs 1-8 were constant. This indicates that a poor fit between the model and data was achieved. Usually, the residuals follow a normal distribution. From the ANOVA (Table 3.5.3.), SS_{REG} as a percentage of the SS_T, was 57.4%. Just over half of the variance of the system was accounted for by the regression equation. The variance ratio of the MS_{REG} to the MS_{RES}, is less than the value of 8.89 derived from a one-tailed F distribution for 7 and 3 df (degrees of freedom), at a probability of 95%. This implies that the model equation is not significant and does not describe the system particularly well. SS_{PE} is determined from the centre point data. For LOF and PE, the F ratio of 0.731 was significantly lower than the value of 18.31 obtained for 1 and 2 df at a probability of 95%. Since $F_{calculated} < F_{table}$, variance due to PE is significantly greater than that due to LOF. The weakness of the model is attributable to the high PE, due to the considerable variation in μ observed for the centre point experiments.

The regression coefficients were then individually tested to see if they differed significantly from zero. Ratios of the b coefficients to their standard errors were formed to give t-values. These were compared with the corresponding value of 2.353 obtained from a t-distribution for 3 df, at a probability level of 95% (Table 3.5.4.). For b_0 (the offset or intercept parameter), $t_{calc} > t_{table}$. Thus at the 95% level of confidence, b_0 is non-zero. This is simply a function of the μ values observed. For the main effects and interaction coefficients, $t_{calc} < t_{table}$, indicating that deviation from zero was not significant. This again shows inadequate fit between the regression equation and the experimental results. However, from the t-values generated, it appears that the most important factor is temperature.

A stationary point with coordinates -0.7784 **P**, 0.9828 **T** and -0.6711 **S** was generated using the regression equation. This corresponds to a maximum response for the system. At the stationary point, μ was calculated to be 0.7876, compared with a mean of 0.86 observed for the 3 centre point runs.

It is evident that the regression equation does not fit the data particularly well. This may be due to:

(i) The complex nature of the whey medium. As such, the system may be susceptible to greater inherent variation than defined (synthetic) media, such as MRS and lactose synthetic (LS).

(ii) The growth state of the starter culture will influence the course of *L .helveticus* growth. Attempts were made to use 'standardised' mid exponential phase inocula. Inevitably however, variation between starter cultures would affect bacterial growth.

(iii) Half of the experimental data was effectively discarded since μ_{av} values were used to create the regression equation. Attempts to utilise both F1 (fermenter 1) and F2 (fermenter 2) growth rates resulted in a matrix too large to be manipulated using the computer program.

(iv) An improved model may have been obtained if rates of acid production and lactose depletion had been utilised instead of μ. Possible sources of error in OD values include: deviation from the Beer-Lambert Law at high values, differences in mixing and therefore homogeneity of the samples and contribution of any precipitate present to OD readings.

In order to characterise the optimum conditions more effectively, additional centre point runs could have been carried out in an attempt to minimise variation in the system. Although the regression equation did not describe the system particularly well, factorial design enabled the most effective use of the data produced. If a univariate approach had been adopted, the experimental domain would have been scanned less efficiently and the variation observed in the system would not have been detected. Interaction

between factors could not have been assessed and a "pseudo optimum" may have been attained.

The optimum conditions obtained, pH 5.5, temperature 42°C and stirrer speed 200 rpm support the findings of Roy et al[29]. In the first batch of whey used by Roy, optimum conditions for lactic acid production and cell growth were pH 5.9, 42°C and 200 rpm. For a second batch, optimum conditions identical to ours were attained. Roy explained that different absolute values of μ under optimum conditions of temperature and pH were probably due to variation in composition between whey batches. He proposed that this difference probably lay in the low molecular weight components (peptides, free amino acids etc.) of the cheese whey. These results are further reinforced by the findings of Aeschlimann[31]. He stated that maximum lactate and biomass productivity were realised at pH 5.5 and temperature 42°C, using *L. helveticus* in continuous culture. In addition, Bergey[95] suggests that the optimal pH for lactobacilli is 5.5-5.8 and 40-42°C represents the optimum temperature for *L .helveticus.*

3.6. MEDIUM SUPPLEMENTATION

Lactobacilli are known to require various amino acids and B complex vitamins for growth and have been described as "metabolic cripples"[96]. Riboflavin, niacin calcium pantothenate and pyridoxal or pyridoxamine are known to be essential for growth of *L. helveticus* [24]. Early fermentations therefore used supplemented whole whey / WP. Campbell[26] for example in 1953, used whey supplemented with 0.65% w/v corn steep liquor (CSL) for the industrial production of calcium lactate. Reddy et al[92] added 0.1% CSL or 0.2% YE in the formation of ammonium lactate (a ruminant feed supplement). It has been observed that in media containing free amino acids, the essential vitamins and other key nutrients, partial hydrolysates of proteins frequently increase the the rate of growth and are sometimes even crucial for growth of certain lactic acid bacteria. The identity and and mechanism of action of these stimulatory peptides is often unknown[24].

FIG 3.6.4. Effect of Yeast Extract Concentration on the Growth of *L. helveticus* in WP

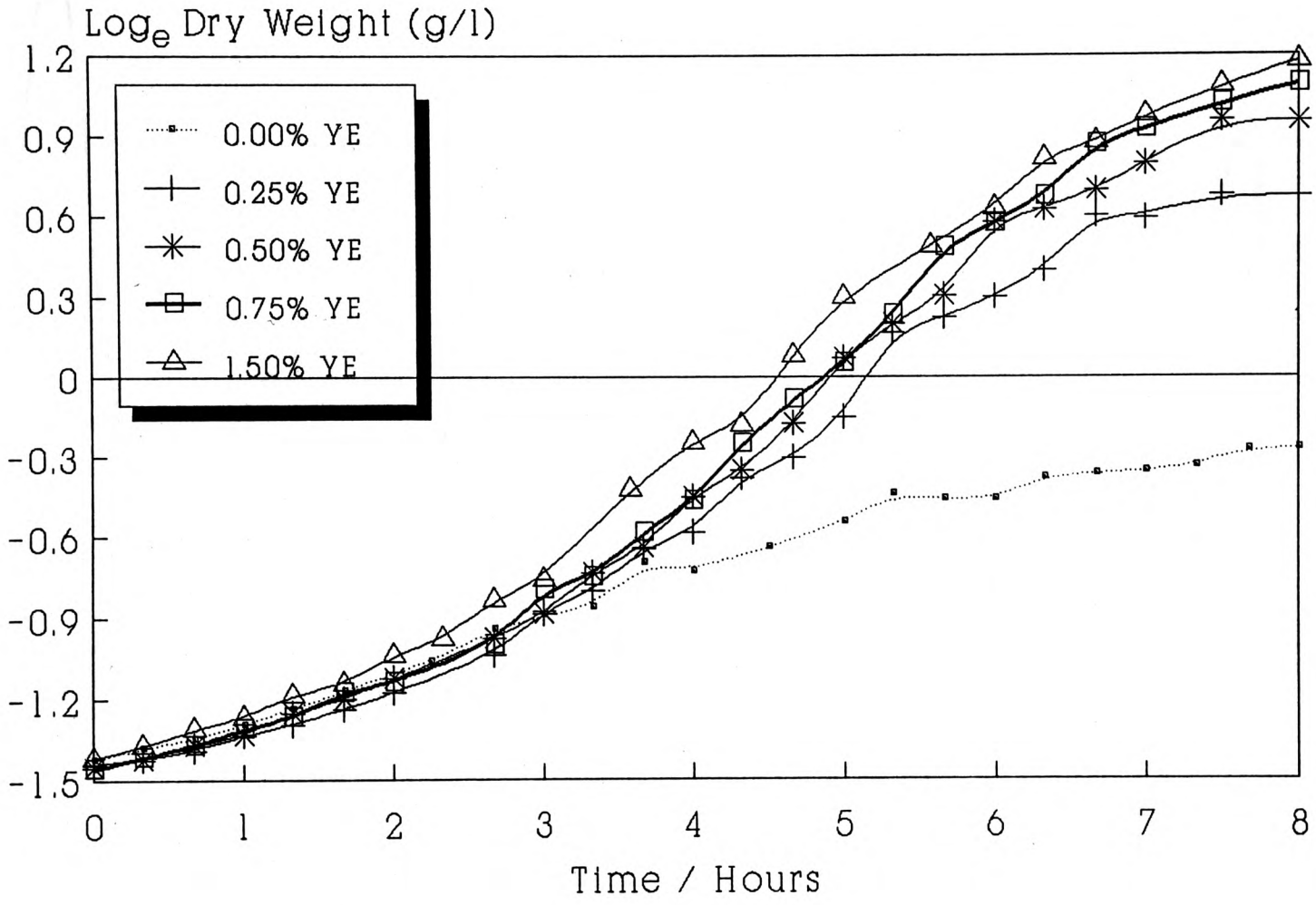


Fig 3.6.2. Spectra showing the effect of various growth supplements on lactic acid production

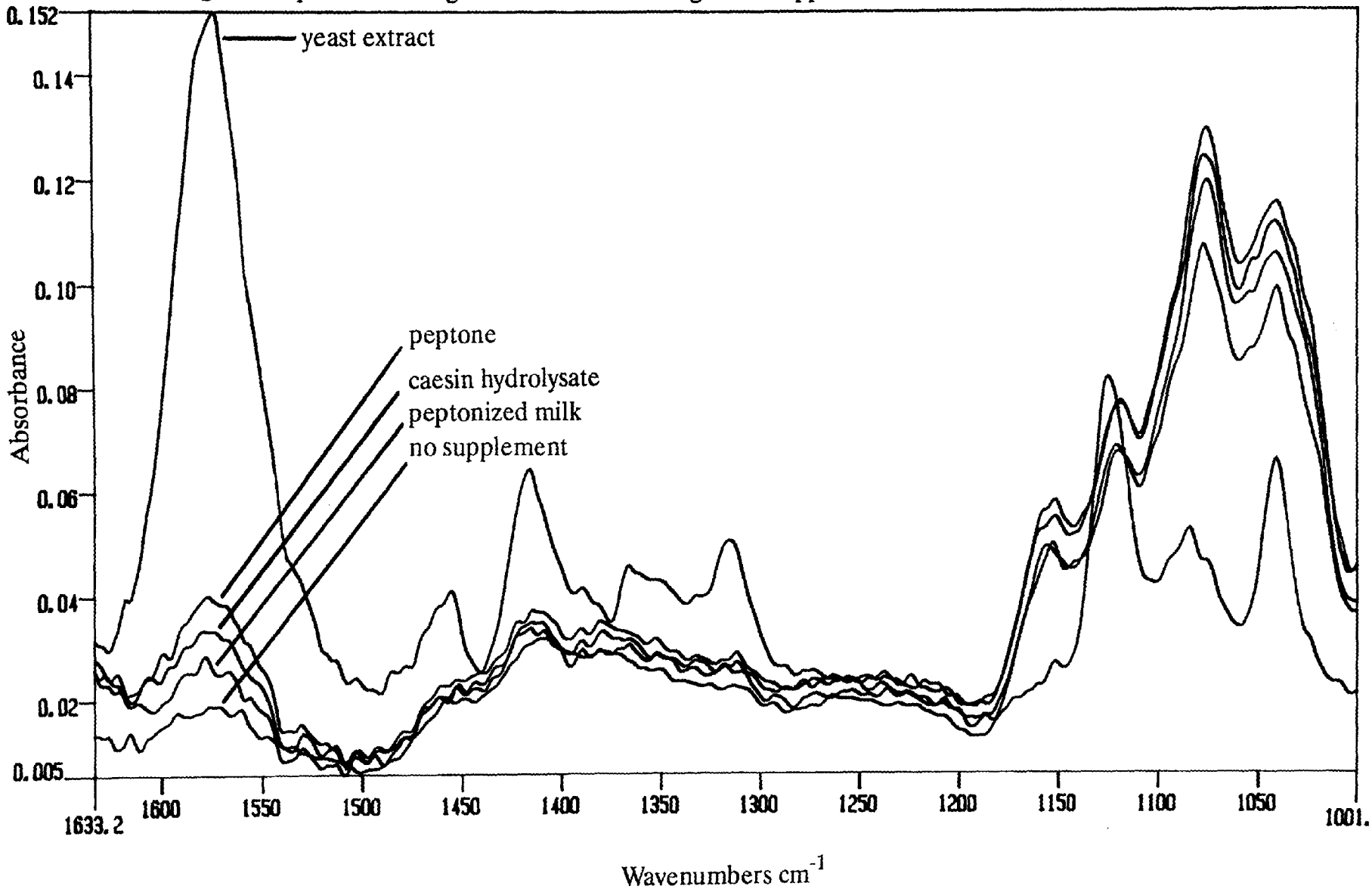
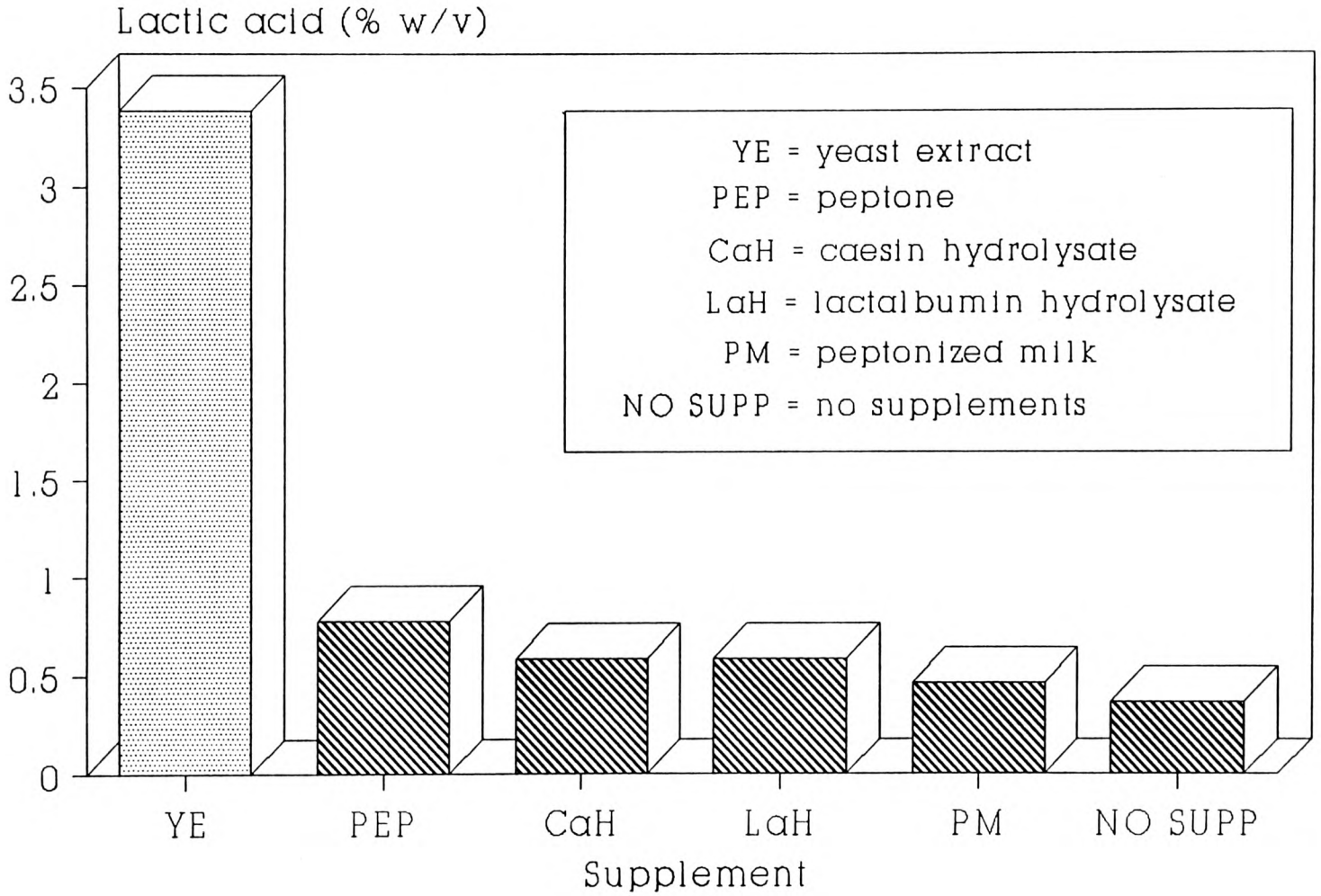


FIG 3.6.3. Effect of Growth Supplements on Lactic Acid Production by *L. helveticus* in WP



3.6.1. Effect of Various Nitrogen Sources on Growth & Acid Formation

The influence of 5 nitrogen/growth factor supplements on growth and lactic acid production was investigated. These were:

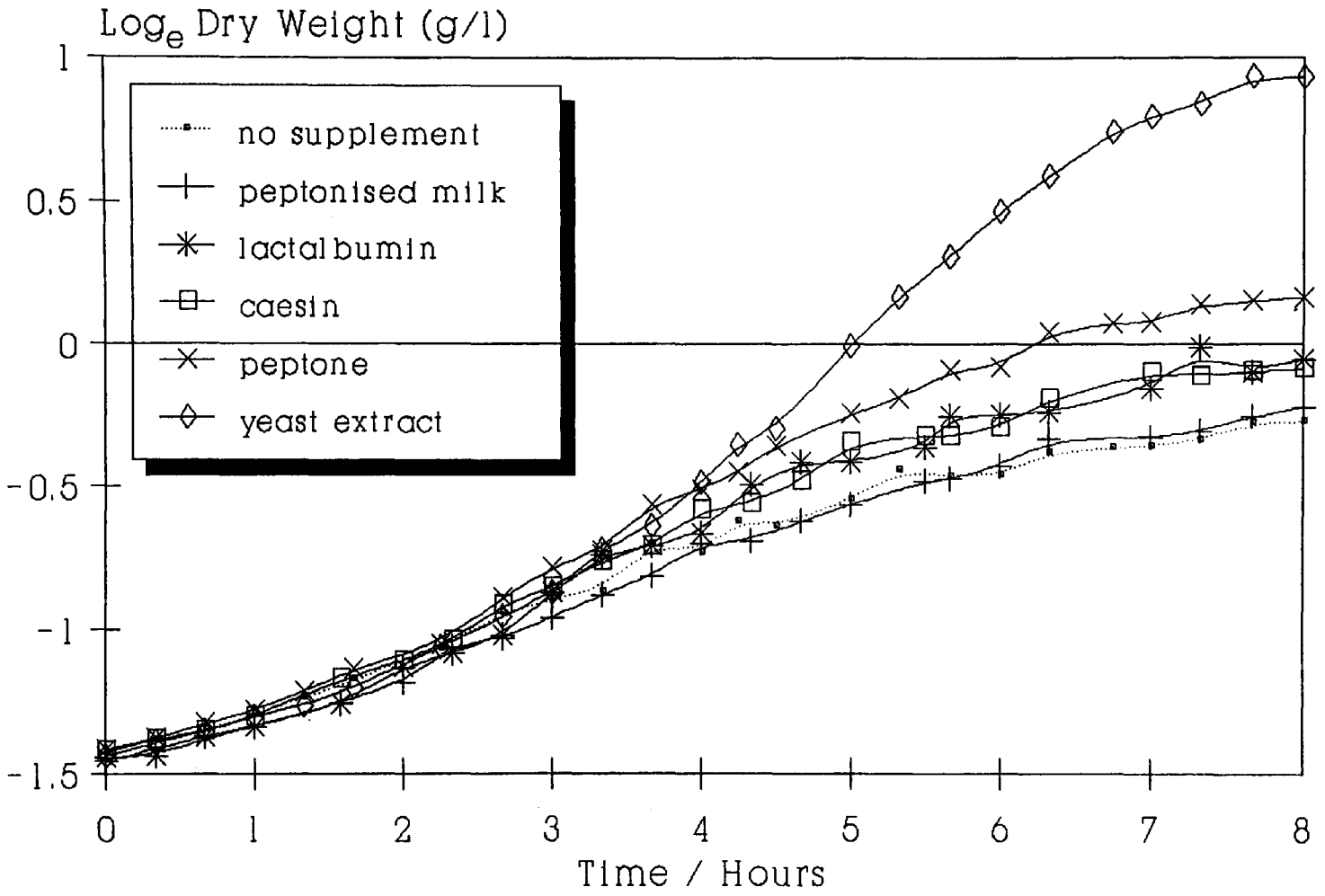
1. Lactalbumin hydrolysate (LaH) - a pancreatic digest of this protein containing high levels of essential amino acids
2. Casein hydrolysate (CaH) - an acid hydrolysed digest, high in amino acids but low in vitamins
3. Peptonized milk (PM) - a pancreatic digest which is a more readily available N source than milk or milk powder
4. Bacteriological peptone (PEP) - acts as a readily available N source, rich in peptides and amino acids
5. Yeast extract (YE) - rich source of B complex vitamins, peptides and amino acids

The effect of these supplements on growth and acid production is shown in Figs 3.6.1, 3.6.2 and 3.6.3. YE is clearly the most effective supplement, generating an 8-fold increase in final lactic acid concentration and a 3-fold increase in biomass. The order of efficacy is :

$$YE > PEP > CaH = LaH > PM$$

Vahvaselka & Linko[97] found that 0.5% w/v YE gave a 3-fold increase in acid production over unsupplemented milk ultrafiltrate. Additionally, in contrast to this study, they also noted that CaH and whey protein hydrolysate had a marked influence on acid production by *L. helveticus*. This was attributed to the provision of short peptides and amino acids. Roy et al[29] concluded that YE was a more effective N/growth factor source than CSL and tryptone ultrafiltrate. Addition of 1.5% w/v YE doubled lactic acid productivity to 2.7 $g\ l^{-1}\ h^{-1}$. They suggested that the active compounds responsible for stimulating growth and acid synthesis were $< 50\ 000$ MW.

FIG 3.6.1 The Effect of Various Nitrogen /Growth Factor Supplements on the Growth of *L. helveticus* in WP



concentration of supplements = 0.5% w/v

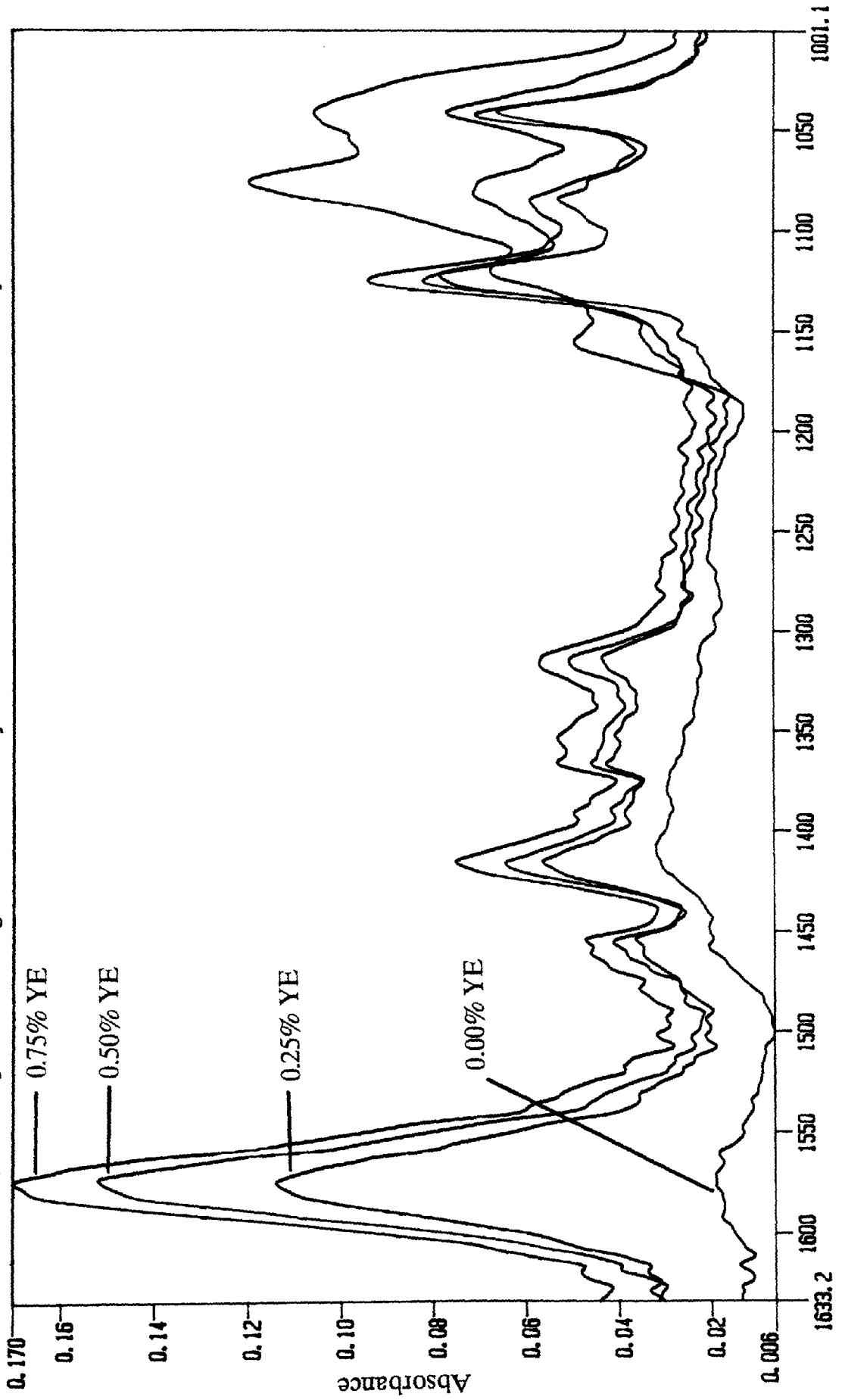
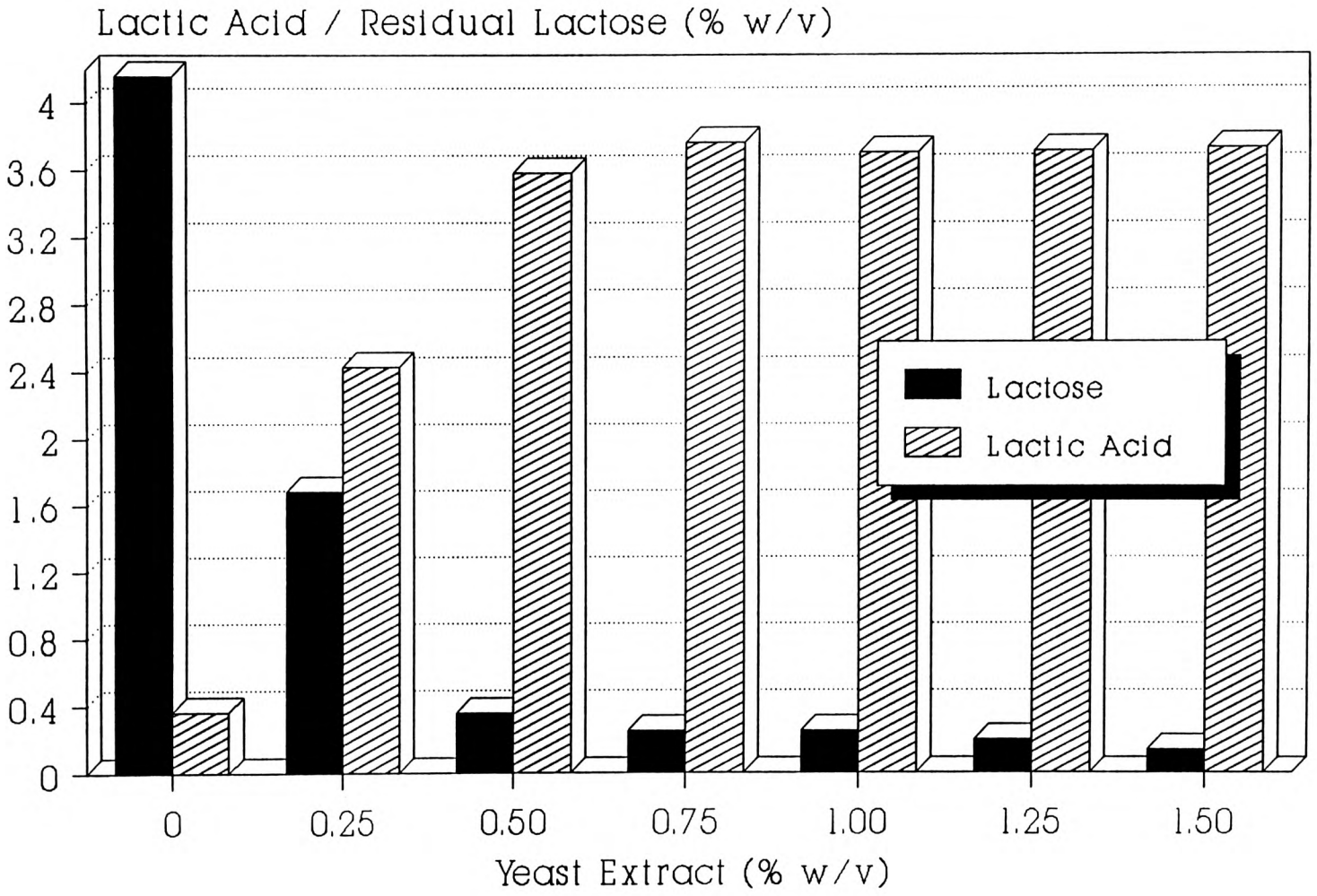


Fig 3.6.5. Spectra showing the influence of yeast extract concentration on lactic acid production

FIG 3.6.6. Effect of Yeast Extract Concentration on Lactic Acid Production/ Lactose Utilisation in WP



For this particular batch/source of WP, it appears that B complex vitamins may be of key importance. The relative ineffectiveness of PEP and the hydrolysates implied that the WP already contained the necessary essential amino acids and peptides. Subsequent addition of YE, a potent source of B complex vitamins, lead to a marked improvement in acid and biomass production over PEP. It should be stressed that discrepancies between these results and the findings of other workers can be related to differences in composition and pre-treatment of the whey used.

3.6.2. Effect of Yeast Extract Concentration on Lactate Production

The influence of YE concentration on lactic acid production and growth of *L. helveticus* is presented in Figs 3.6.4., 3.6.5. & 3.6.6. The optimum level of YE appeared to be 0.75% w/v. Moving from 0.25% to 0.50% w/v YE lead to a 48% increase in final acid concentration. A further 5% improvement was attained on increasing the level of YE from 0.50% to 0.75% w/v. At a concentration of 0.75% w/v YE, 87% of the available lactose was utilised, of which 92% was converted to lactate. No improvement in acid production was achieved on increasing the level of YE still further. However, a marginal decrease in residual lactose was observed with YE concentrations of 1.25% and 1.50% w/v. Since cell concentrations were slightly higher under these conditions, the extra lactose may have been used to satisfy the maintainance requirements of the larger bacterial populations. The optimum YE concentration of 0.75% is lower than than that previously proposed by Aeschliman[98] (1% w/v) and Roy et al[29] (1.5% w/v). Again, these differences can be related to variations in whey composition.

3.6.3. Influence of Additional Supplements

In addition to B complex vitamins and various amino acids, lactic acid bacteria are also known to need K^+, Mn^{2+}, Mg^{2+} and phosphate[24]. In the development of MRS medium, Man et al[45] noted that reducing the concentration of these ions and the level of acetate, resulted in diminished growth of lactobacilli. Indeed, sodium acetate is known to stimulate the growth of most lactic acid bacteria[24]. It should be emphasised that in addition to its stimulatory role, acetate can function as a buffer and can help suppress

FIG 3.6.7. Effect of Tween 80, $MgCl_2$ and $MnCl_2$ on the Growth of *L. helveticus* in WP Supplemented with 0.75% w/v YE

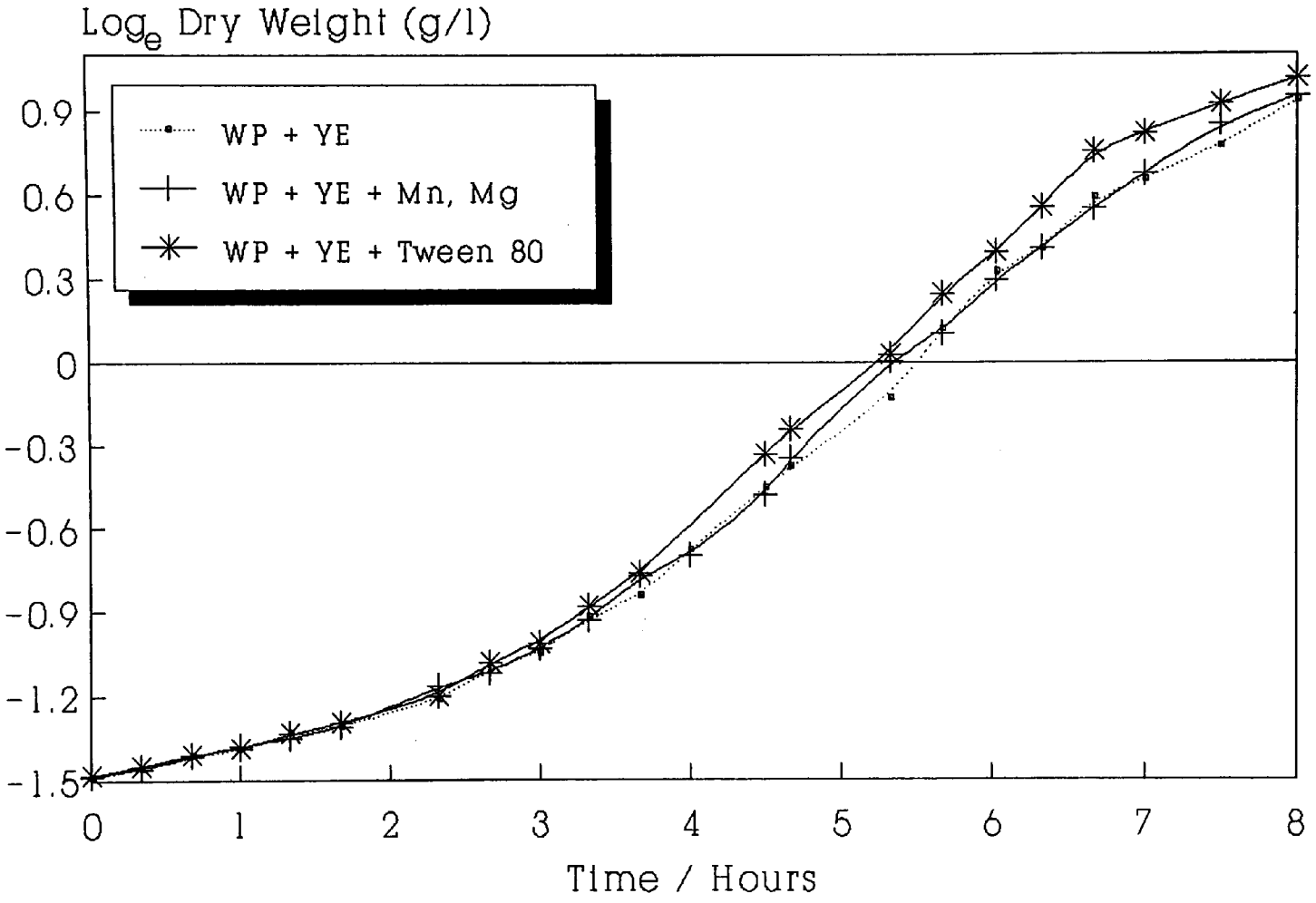


FIG 3.6.8. Effect of Na Acetate and K_2HPO_4 on the Growth of *L. helveticus* in WP Supplemented with 0.75% w/v YE

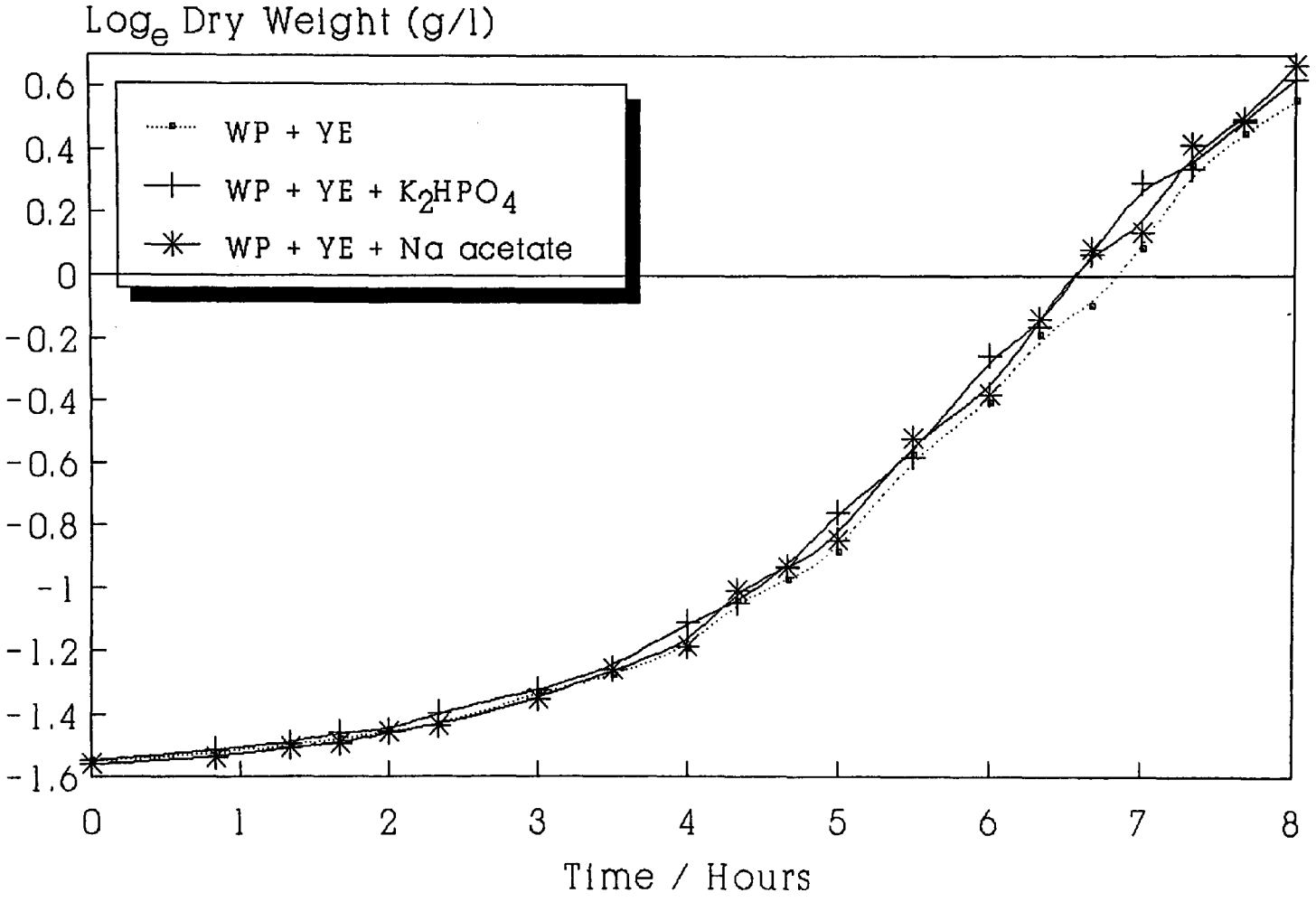
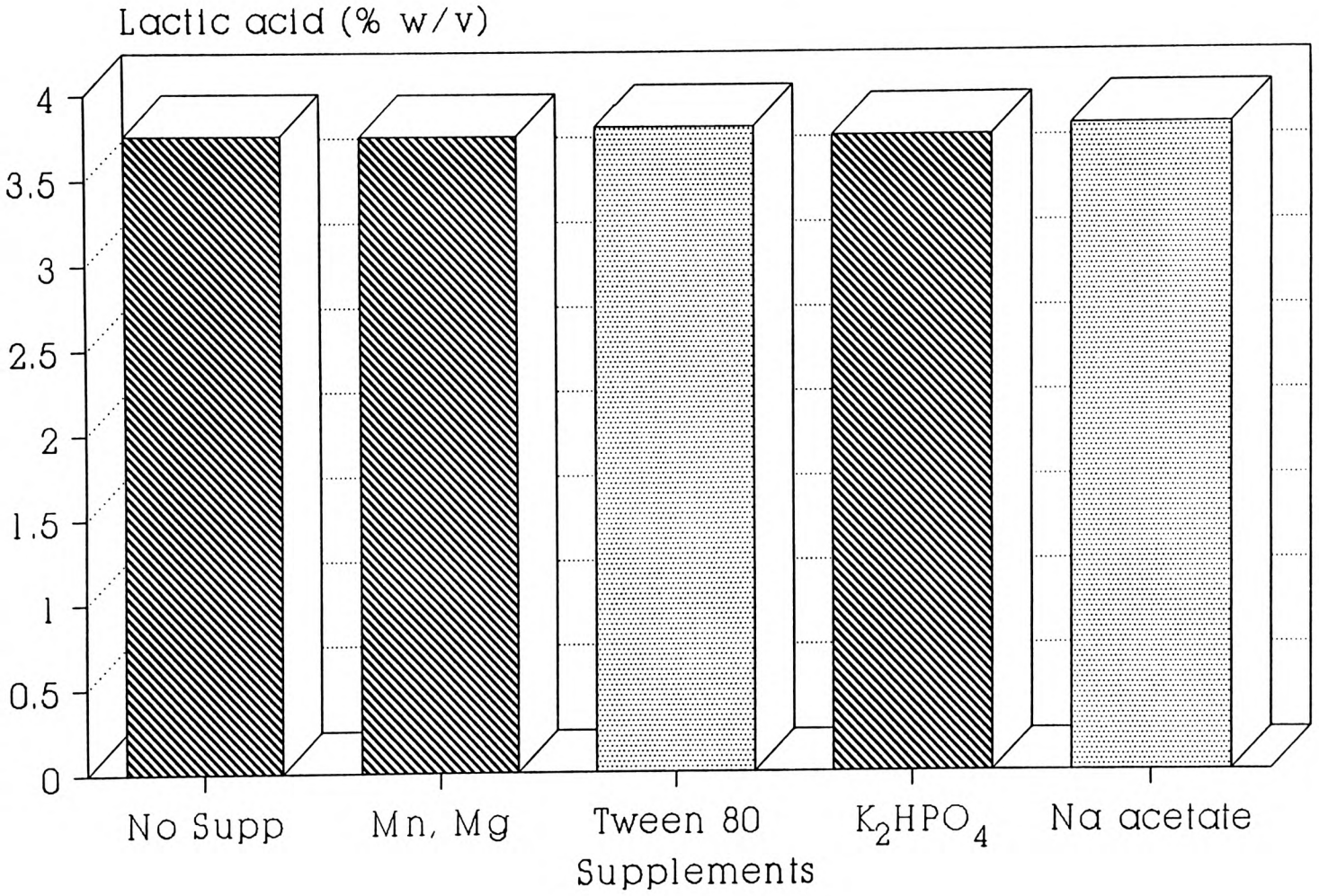


FIG 3.6.9. Effect of Additional Growth Supplements on Lactic Acid Production by *L. helveticus* in WP + 0.75% w/v YE



growth of the main competitor groups: streptococci, pediococci and leuconostocs. However since it is present in MRS medium at a concentration of only 0.05% w/v, its buffering capacity is minimal. Man et al[45] also noted diminished growth of several strains of lactobacilli on decreasing the concentration of Tween 80 (polymethylene (20) sorbitan mono-oleate), a non-ionic surfactant. Tween 80 acts to lower interfacial tension around bacteria, facilitating entry of compounds into the cell[99].

The effect of these supplements on cell growth and acid synthesis in WP containing 0.75% w/v YE is shown in Figs 3.6.7, 3.6.8. and 3.6.9. Minor increases in biomass and final lactic acid concentration (1%) were observed for sodium acetate and Tween 80. No improvements were seen for Mn^{2+}, Mg^{2+} and $K^{+}{}_2HPO_4^{2-}$. This suggested that sufficient quantities of these ions were already present in YE supplemented WP. Based on these findings, the optimum medium for *L. helveticus* in terms of lactic acid production and cell growth is:

WP, YE (0.75% w/v) , Tween 80 (0.1% v/v) and Na acetate (0.05% w/v).

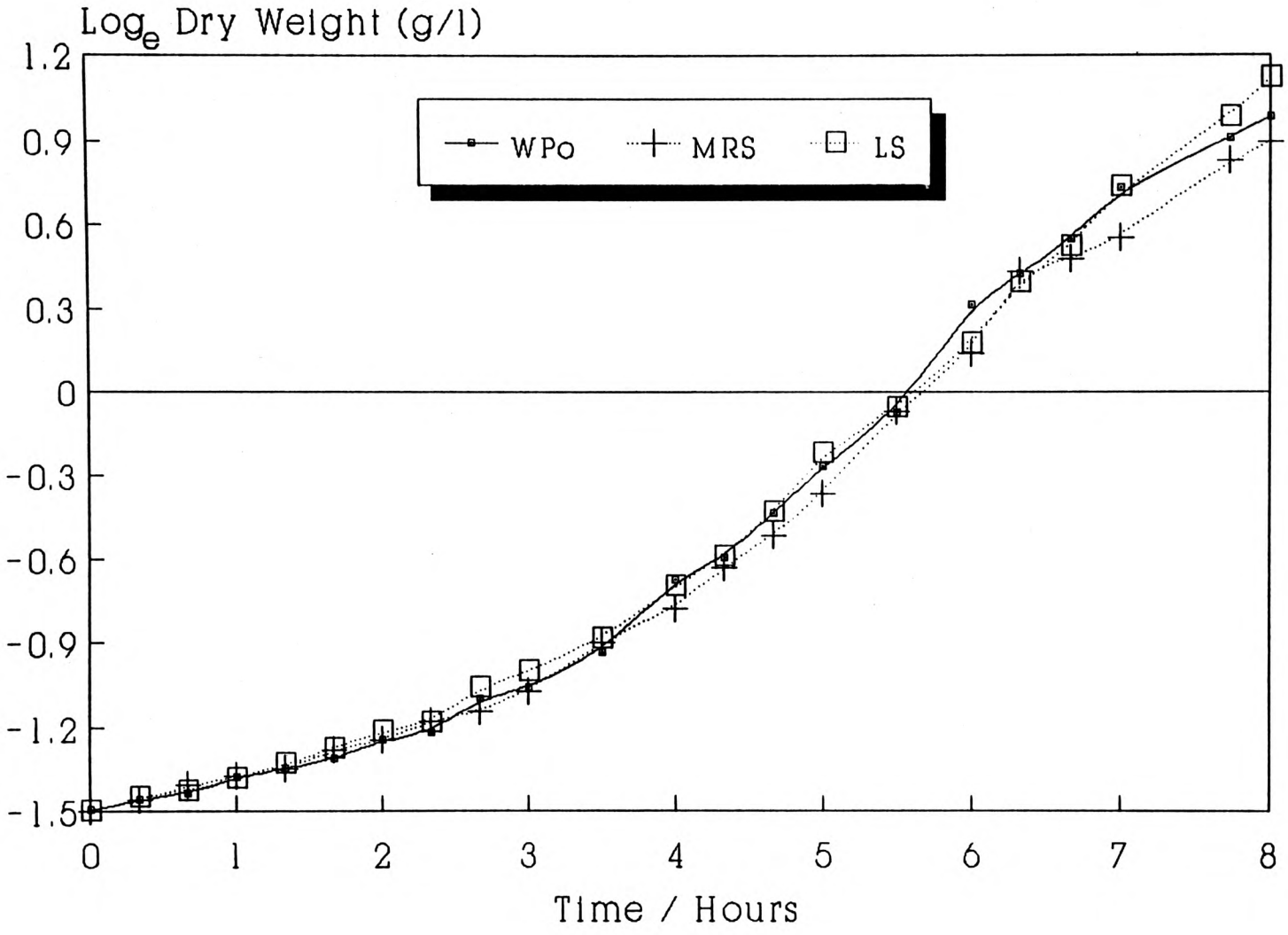
This shall be referred to as WP_o ($WP_{optimum}$) medium.

3.6.4. WP_o versus LS & MRS Media

Table 3.6.4. Lactic acid production by *L. helveticus* in WP_o, LS and MRS media

Medium	Lactic acid (% w/v)	Maximum cell dry weight (g/l)
WP_o	3.224	2.37
LS	2.979	2.82
MRS	2.889	2.65

FIG 3.6.10. Growth of *L. helveticus* in MRS, LS and WPo Media



The performance of WP_o medium compared with LS[77] and MRS media was assessed, Fig 3.6.10. and Table 3.6.4. (above).

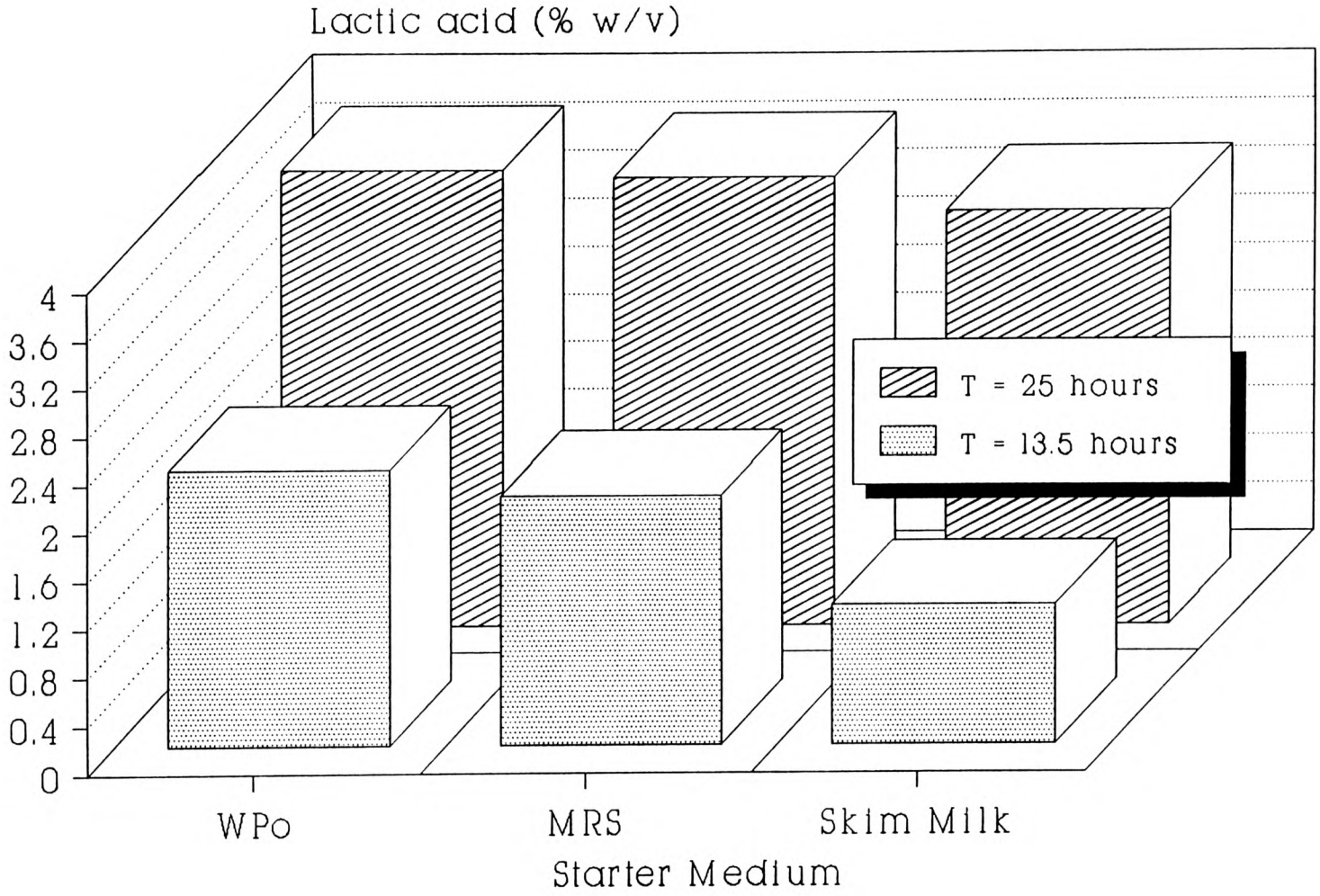
The synthetic media proved to be superior in terms of cell growth. However, final acid concentration in WP_o was 7.5% and 10% higher than in LS and MRS media respectively. Also, substrate conversion (85%) was 5% higher in WP_o compared with LS and MRS. In all three cases, lactose utilisation was approximately 89%. This is in agreement with Roy et al[29] who determined that YE supplemented WP gave the highest productivity of lactic acid (2.7 g l^{-1} h^{-1}) compared with LS and GS media. It appears therefore that WP_o is the most effective medium for lactic acid production by *L. helveticus*.

3.6.5. Comparison of Starter Culture Media

From Fig 3.6.11. it is evident that WP_o is more effective than MRS medium in terms of lactic acid production. The concentrations of acid at 13.5 and 25 hours respectively, indicated that the rate of synthesis and final yield of lactate was slightly higher for inocula propagated in WP_o rather than MRS medium. For the fermenters seeded with cells derived from the SM starter, the acid production rate was 50% lower compared with MRS and WP_o, although the final lactate concentration (3.448% w/v) was only 8% less. This reduced rate of acid synthesis can be linked to the poor growth of *L. helveticus* in the SM medium, giving rise to smaller (in terms of cell concentration) and less active inocula.

Vahvaselka and Linko[97] found that the rate of lactic acid production for inocula propagated in SM was twice that for a whey inoculum. Roy et al[29] determined that lactate formation and maximum productivity were 32.5g/l, 20.2 g/l and 1.43 g l h^{-1}/ 0.75 g l h^{-1} for inocula propagated in SM and WP-CSL media respectively. The apparent contradictions between our findings and the data of these workers is again related to whey composition. Vahvaselka and Linko used unsupplemented WP, whilst Roy et al employed WP containing 0.5% w/v CSL. It was shown earlier that WP on its own supports poor growth of

FIG 3.6.11 The Effect of Starter Culture Medium on Lactic Acid Production by *L. helveticus* in WPo Medium



L. helveticus. Improvements can be achieved by addition of various N/growth factor sources such as PEP and YE. Indeed Vahvaselka and Roy both concluded in the same studies that YE is the most effective growth supplement, yet neither group compared YE supplemented WP with SM as a starter medium!

Returning to our own data, given the inherent variability in whey composition, coupled with continued efforts to improve genetic stability of starter culture strains, use of MRS as the preferred starter medium would seem prudent. This would hopefully enable consistently performing ('standardised') inocula to be produced.

3.7. CHEMICAL OXYGEN DEMAND ANALYSIS

The COD of the WP_o medium was 32.4 g/l. Upon completion of the fermentation process, the COD of the solution remaining (allowing for cell and lactate removal) was 2.4 g/l. This represents a 92% reduction in the COD level. Clearly, lactic acid fermentation is an effective means of decreasing the polluting power of this dairy by-product. Similar reductions (45 g/l to 2.3 g/l) have been reported for production of methane from whey by anaerobic digestion[100].

3.8. OPTIMISATION of LACTIC ACID PRODUCTION

Results obtained for the 2^3 design matrix employed are shown graphically in Fig 3.8.1. The following parameters were calculated:

Main Effects	Two Factor Interactions
p =-0.093	pt = 0.033
t = 0.220	ps =-0.034
s = 0.095	st = 0.111

Three factor interaction

pts = 0.023.

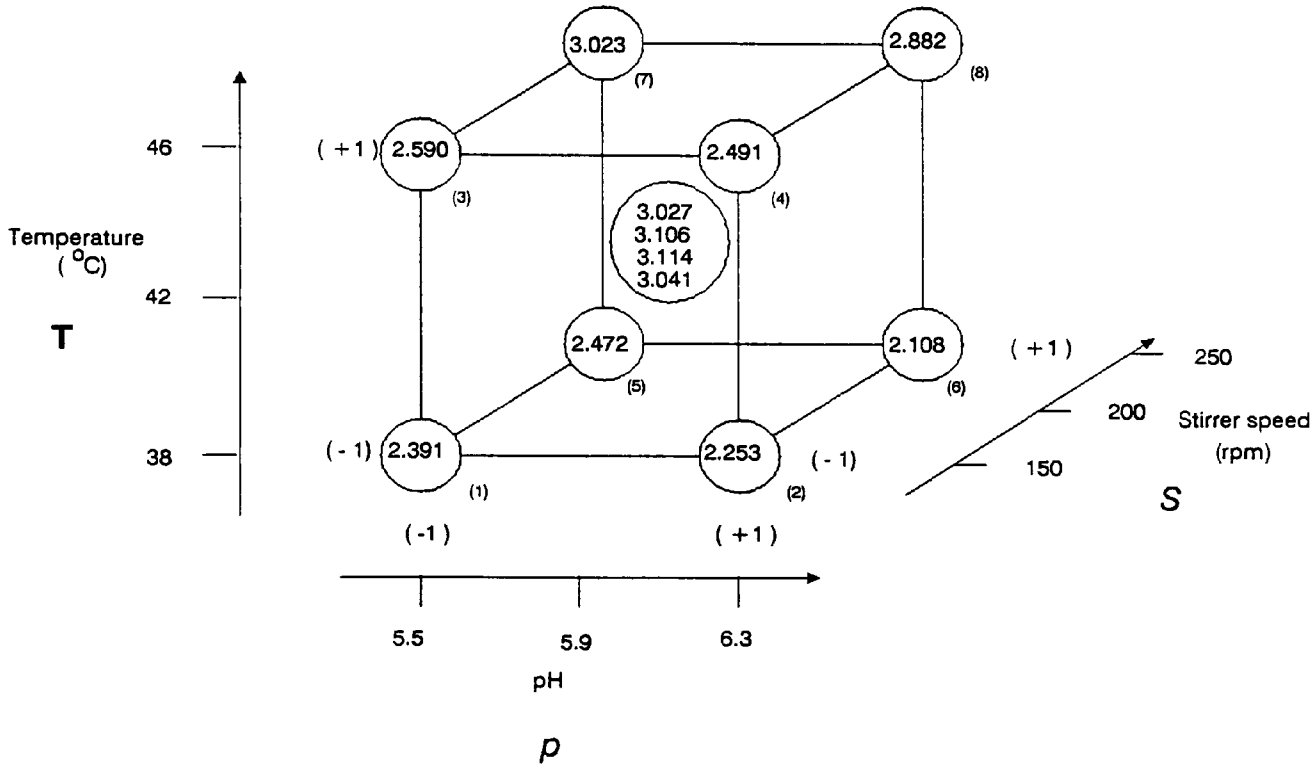


Fig 3.8.1. 2^3 factorial design. The concentration of lactic acid (% w/v) produced for runs 1 to 8 are positioned at the cube corners. Lactic acid production for the centre point conditions (runs 9 to 12) are situated at the cube centre.

Based on the experimental data obtained, the following polynomial expression relating response (lactic acid concentration) to the process variables was generated:

$$y = 2.708 - 0.093p + 0.220t + 0.095s + 0.033pt - 0.034ps + 0.111ts + 0.023pts$$

NOTE: strictly, this only applies to the coded factor levels, + or -. Based on the regression equation formed, estimated values of lactate concentration were derived and compared with the true values (Table 3.8.1.).

Table 3.8.1. Observed responses, predicted values and the residuals obtained from the regression equation.

Run	Observed μ_{av}	Estimated μ_{av}	Residual
1	2.391	2.573	-0.182
2	2.253	2.435	-0.182
3	2.472	2.654	-0.182
4	2.108	2.290	-0.182
5	2.590	2.772	-0.182
6	2.491	2.673	-0.182
7	3.023	3.205	-0.182
8	2.882	3.064	-0.182
9	3.114	2.708	0.406
10	3.041	2.708	0.333
11	3.106	2.708	0.398
11	3.027	2.708	0.319

Response surfaces showing the variation of lactic acid production with factor levels were generated using this equation (Figs 3.8.2, 3.8.3 & 3.8.4.). ANOVA was also carried out

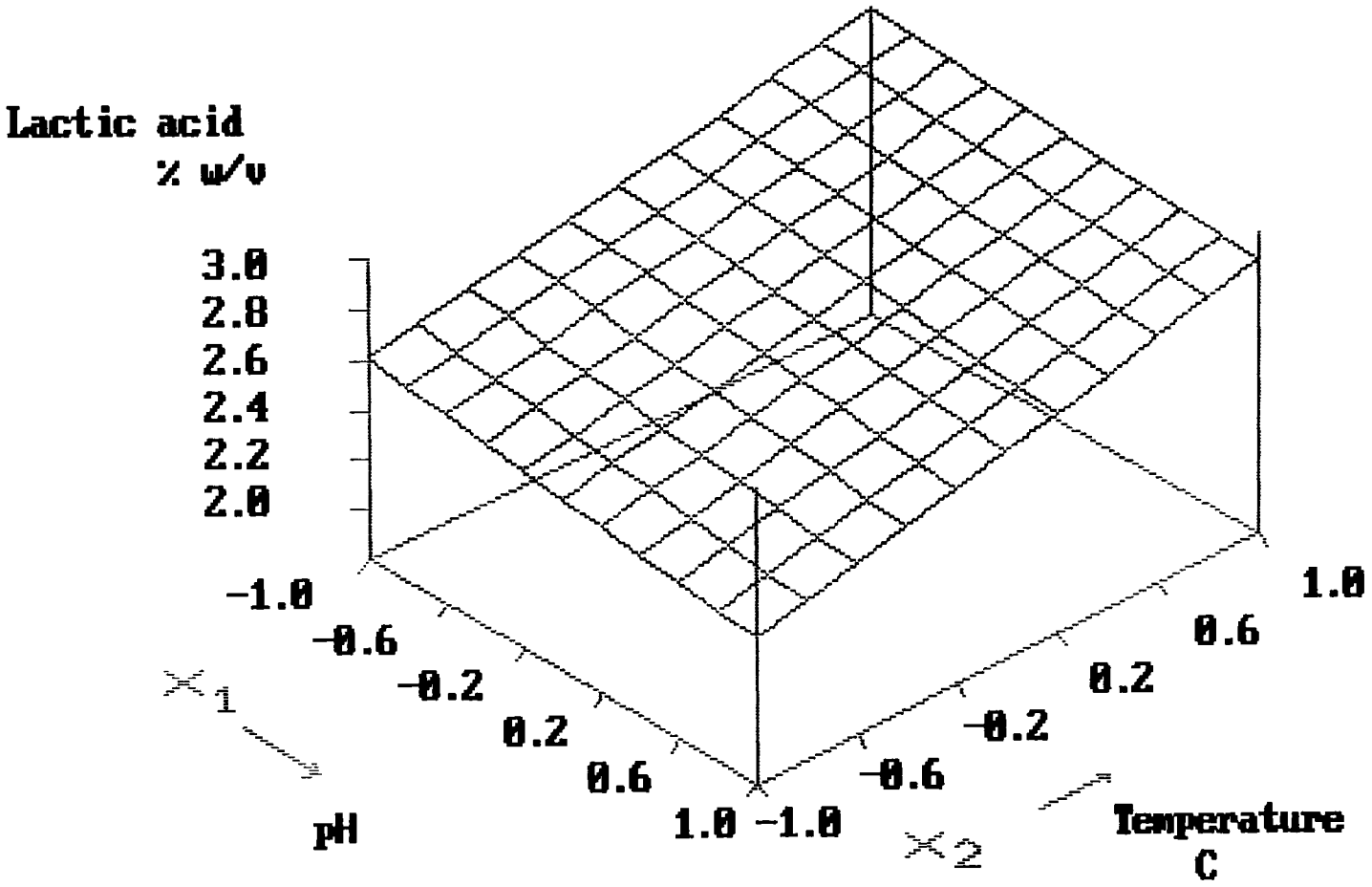


Fig 3.8.2. Response surface showing the variation of lactic acid concentration with pH & temperature

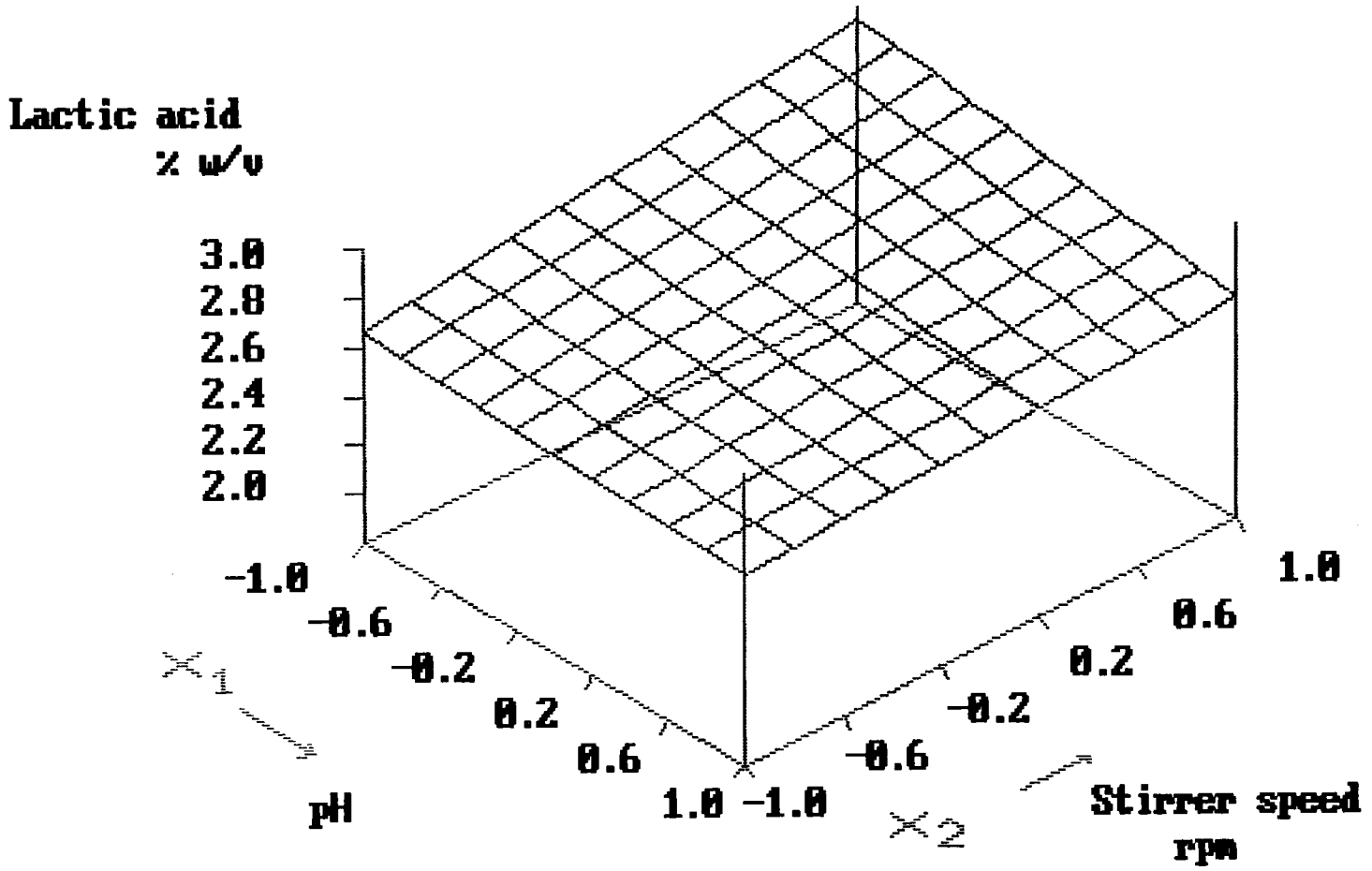


Fig 3.8.3. Response surface showing the variation of lactic acid concentration with pH & stirrer speed

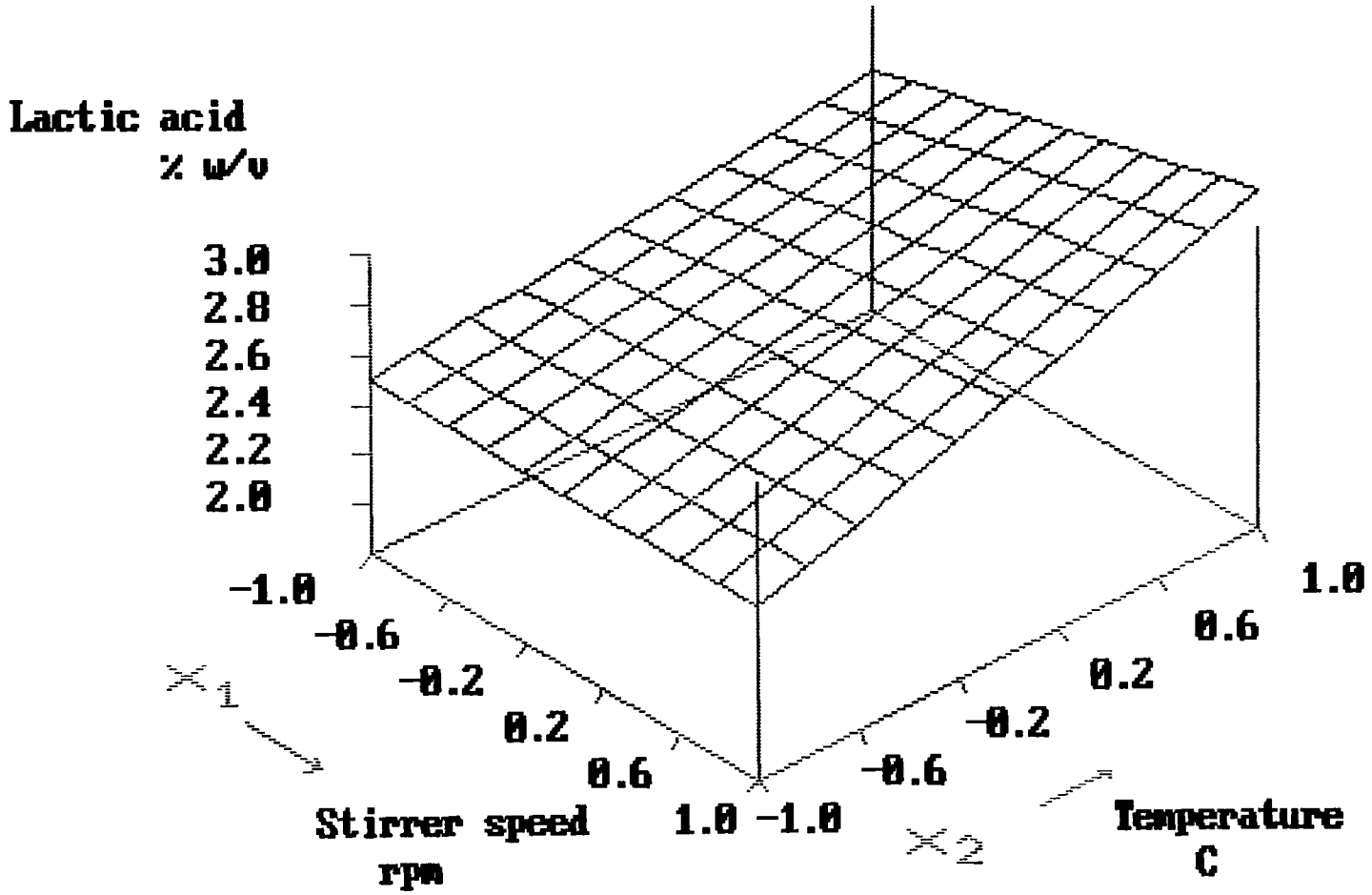


Fig 3.8.4. Response surface showing the variation of lactic acid concentration with temperature & stirrer speed

to assess the effectiveness of the model (Tables 3.8.2 & 3.8.3.).

Table 3.8.2. ANOVA for 2^3 design.

Source of error	**Sum of squares**	**df**	**Mean squares**	**Variance (F) ratio**
Total	1.449	11		
Regression	0.649	7	0.093	0.464
Residual	0.800	4	0.200	
Lack of fit	0.264	1	0.265	1.483
Pure error	0.535	3	0.178	

Table 3.8.3. Estimation of the significance of the regression coefficients using a t-test

Coefficient	**b_0**	**b_1**	**b_2**	**b_3**
Value	2.708	-0.093	0.095	0.220
t-value	20.975	0.587	0.602	1.393
% prob < > 0	> 99.5	< 90	< 90	< 90

Coefficient	**b_{12}**	**b_{13}**	**b_{23}**	**b_{123}**
Value	-0.034	0.033	0.111	0.023
t-value	0.212	0.207	0.702	0.145
% prob < > 0	< 90	< 90	< 90	< 90

Based on the ANOVA and the testing of the regression coefficients, a poor fit between model and data was attained. Only 45% of the system variance was accounted for by the regression equation. Additionally, the coefficients of the equation did not differ significantly from zero. However, as with the optimisation of growth, from the t-values generated temperature again appeared to be the most important factor.

Here, the weakness is attributable to LOF, unlike the previous growth model, which related μ to pH, temperature and stirrer speed, where high PE undermined the efficacy of the model. A possible explanation is the large fluctuations in laboratory temperature. This can be related to the nature of the building and the laboratory's heating/air conditioning system, which were beyond our control. Clearly, this introduced an extra source of variation, which could not be accounted for by the model.

Again, despite the model's weakness, use of factorial design meant that the experimental domain was scanned efficiently, with a minimum number of experiments since all variables were changed simultaneously and studied at multiple levels. Optimum conditions for lactic acid production were:

pH 5.9 temperature 42°C stirrer speed 200 rpm

This coincided with centre point conditions employed. This is in agreement with the results obtained during optimisation of *L. helveticus* growth in WP-YE (section 3.4.) and reinforces the findings of Roy et al[29].

3.9. MEASUREMENT and CONTROL

In order to exploit the full biosynthetic potential of microorganisms, optimal conditions must be maintained during cell growth and product formation. Control and optimisation of fermentation processes is based on an ability to accurately measure parameters such as substrate and product levels, and cell mass. Mathematical models generated using such data form the basis of systems for **real-time** process control. Substrate and product

concentrations can be monitored directly and indirectly. In addition, measurements can be conducted **off line**, where a sample is aseptically removed for analysis, or **on line**, where the sensor is connected to the fermenter or in contact with the process stream[18]. The two approaches are compared in table 3.9.1.

3.9.1. On Line versus Off Line Analysis

Table 3.9.1. Advantages and disadvantages of on line and off line analysis[101]

	ON LINE	OFF LINE
FOR	measures *in situ*	easy to make many different measurements
	real time	use of many different analytical principles
AGAINST	restricted number of suitable techniques	delayed measurement
	sterility problems	changes may occur during sample transport
	need for autoclavable equipment	difficulties in taking a representative sample
	growth of cells onto sensor may be a problem	growth of cells onto sampling device may cause difficulties

In recent years there has been a move towards development of on line techniques for measurement of reaction moieties in the bioconversion/fermentation. Indeed at a seminar entitled "Analytical Instrumentation: Future Trends" at the 1991 Pittsburgh Conference, one clear trend was identified. "**The analysis is moving closer to the sampling point, approaching the ultimate goal of real time analysis and continuous control of the environment and process**"[102]. Further to this, at a recent conference on "Measurement, Modelling and Control in Biotechnology", it was stressed that fast on-line and *in situ* measurement techniques are badly needed for optimal control of processes[103]. On line techniques already applied to the lactic acid fermentation of whey include HPLC[104], flow injection analysis (FIA)[105,106], enzyme electrodes[107] and fibre-optic biosensors[108].

3.9.2 On Line Quantitative Analysis by FTIR Spectroscopy

Advantages

1. Speed. The time required for scanning and quantitative analysis is approximately 3 minutes. This is dependant on variables such as the number of scans taken and the resolution selected. This compares with combined sampling and analysis times of 10 mins for FIA, 20 mins for HPLC and 1 min for the enzyme electrode. It should be stressed however that the electrode detects L-lactate only.

2. Simplicity Although care must be taken in method development, once the method has been established, analysis of samples is straightforward. No sample pretreatment is necessary, the fermentation culture is simply circulated through the CIRCLE® cell. FIA requires sample filtration and dilution prior to mixing in the reaction chamber. HPLC depends on cross flow ultrafiltration of the fermentation broth before analysis. An elaborate sampling procedure is also employed in conjunction with the enzyme electrode.

3. Versatility. FTIR spectroscopy can be readily applied to other reactions involving species absorbing in the IR region. FIA is dependant on the availability of suitable enzyme systems. HPLC can only be used if a column is available for the required separ-

ation/detection. Enzyme electrodes are in the early stages of development. At present applications are limited and there is no combined biosensor for analysis of both lactose and lactic acid.

4. Durability. The ZnSe crystal fitted in the CIRCLE® cell is durable and relatively inert. It is therefore suitable for use with a wide range of samples such as biological fluids, aqueous solutions and organic solvents. In addition the cell can be used at temperatures in excess of 100°C. Biosensors (immobilised enzymes/electrodes) tend to be stable for only a few days and have relatively narrow temperature and pH operating ranges because of enzyme inhibition.

5. Visualisation. Changes in substrate and product levels, manifested in peak changes in the spectral region of interest, are displayed on the FTIR computer monitor - see Fig 3.9.8. Thus the operator can relate the concentration data presented to spectral changes in the reaction broth. Data is not, for example, simply presented as a series of figures on an LCD display .

It should be emphasised that the flexible design of the CIRCLE® cell means that it can be used for off line and on line analyses[71]. In the "open boat" configuration (for off line analysis), the sample is poured into the cell, the measurement made and the cell washed with a suitable solvent. For automated sampling or reaction studies, such as fermentation monitoring (on line analysis), a flow cell is used - see Fig 2.6.2. earlier.

Disadvantages

1. Interference. Unexpected components in the sample with overlapping spectral features can introduce errors in the analysis. Care should therefore be taken to include all potential components in the standards. If an impurity is detected, the results from these solutions should be checked by independant analytical methods. Note: on comparing spectra recorded during whey fermentation with those of the standards, no additional spectral features were observed. This suggested that interference was not a problem. On

a positive note, the appearance of unusual absorption peaks in a sample may warn of possible microbial/chemical contamination.

2. Adsorption. Protein adsorption onto the surface of the ZnSe crystal in the CIRCLE® cell has been noted during studies of blood and blood plasma[53,63,67]. This can reduce the energy throughput of the cell and lead to inaccuracies during quantitative analysis. The deposited material can be removed by polishing the crystal with CeO_2 powder.

3. Air bubbles. The presence of air bubbles in the cell leads to non-reproducible contact with the crystal. As a consequence, errors will arise in the quantification of components. It is therefore important to eliminate any air bubbles that appear. This can often be achieved by inverting the cell.

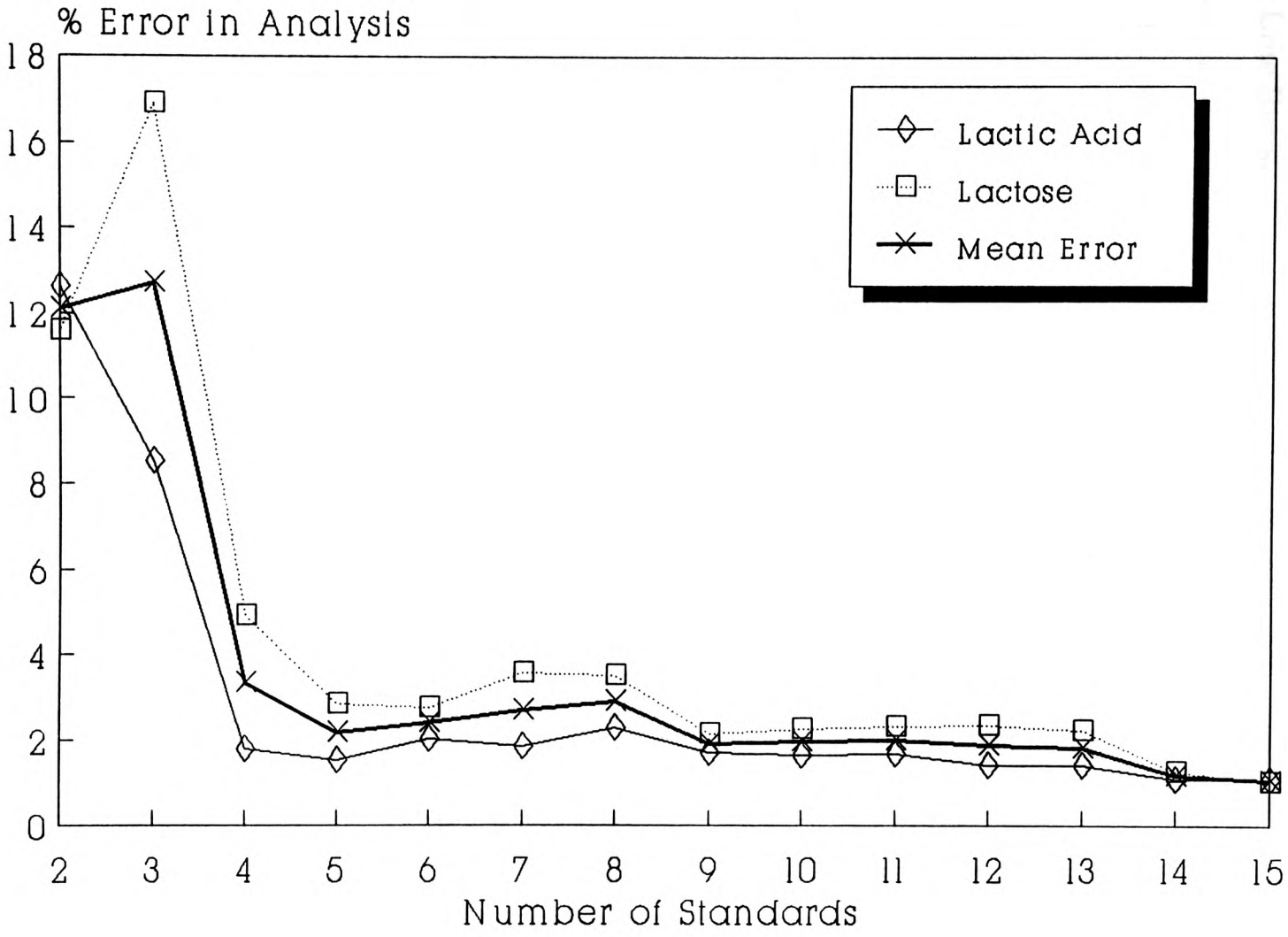
4. Sample homogeneity. Samples for analysis should be representative, well mixed and as homogeneous as possible. Inhomogeneiety leads to distortions in relative band intensities and causes serious errors in analysis[67].

5. Cost. The technique is not cheap. The complete package for carrying out quantitative analysis: FTIR spectrometer, CIRCLE® cell and appropriate software, costs in the region of £20 000.

3.9.3. Future Developments

Continuing advances in the field of fibre optics could eliminate the need for a CIRCLE® cell. The use of a MIR transmitting fibre as a spectroscopic probe will enable remote sensing to be carried out. A situation may then be envisaged where a single spectrophotometer could monitor (using optical fibres) and control a number of fermenters. Low-cost, near-IR probes capable of operating over more than 1 Km, are already being used industrially for remote reaction monitoring[109]. New sampling systems such as the

FIG 3.9.1 The Effect of Number of Standards Used on Quantitative Accuracy



Specac 'Fibreprobe' are now available. The use of chalcogenide fibres extend the operating range of this system to 900 cm^{-1}. Such probes have already been used for *in situ* monitoring of resin curing reactions[110].

3.9.4. On Line Runs

The influence of the number of standards used on quantitative accuracy is shown in Fig 3.9.1. **Overdetermination** (see section 2.10.1.) increases precision by averaging the the effects of errors in sample preparation. In addition, it serves to minimise irrelevant spectral features such as noise. With 15 standards, mean errors in the measurement of lactic acid and lactose were 0.9% and 1.1% respectively (Table 3.9.2.).

Table 3.9.2. Method validation. Comparison of actual concentrations of lactose and lactic acid with calculated values.

Lactose (% w/v)			Lactic acid (% w/v)		
Actual	**Calculated**	**Error %**	**Actual**	**Calculated**	**Error %**
5.524	5.557	0.6	3.889	3.884	0.1
1.236	1.227	0.7	3.347	3.331	0.5
3.954	3.977	0.6	0.851	0.878	3.2
0.581	0.600	3.3	2.858	2.872	0.5
3.019	3.007	0.4	2.638	2.636	0.1
	Mean error	1.1		**Mean error**	0.9

In this study, lactose and lactate were detected with reasonable accuracy (10% error) at a level of 0.08% w/v. Braue & Pannella[57] have suggested that the reliable sensitivity limit in the analysis of aqueous solutions is 0.05% w/v. Miller et al[111] have claimed that detection limits of 0.02% w/v are possible, thereby supporting the specified limits of the CIRCLE® cell.

Fig 3.9.2. Spectra recorded on line at T = 0 (--) & T = 47 (-) hours during the fermentation of supplemented whey permeate by *L. helveticus*. (Run 1)

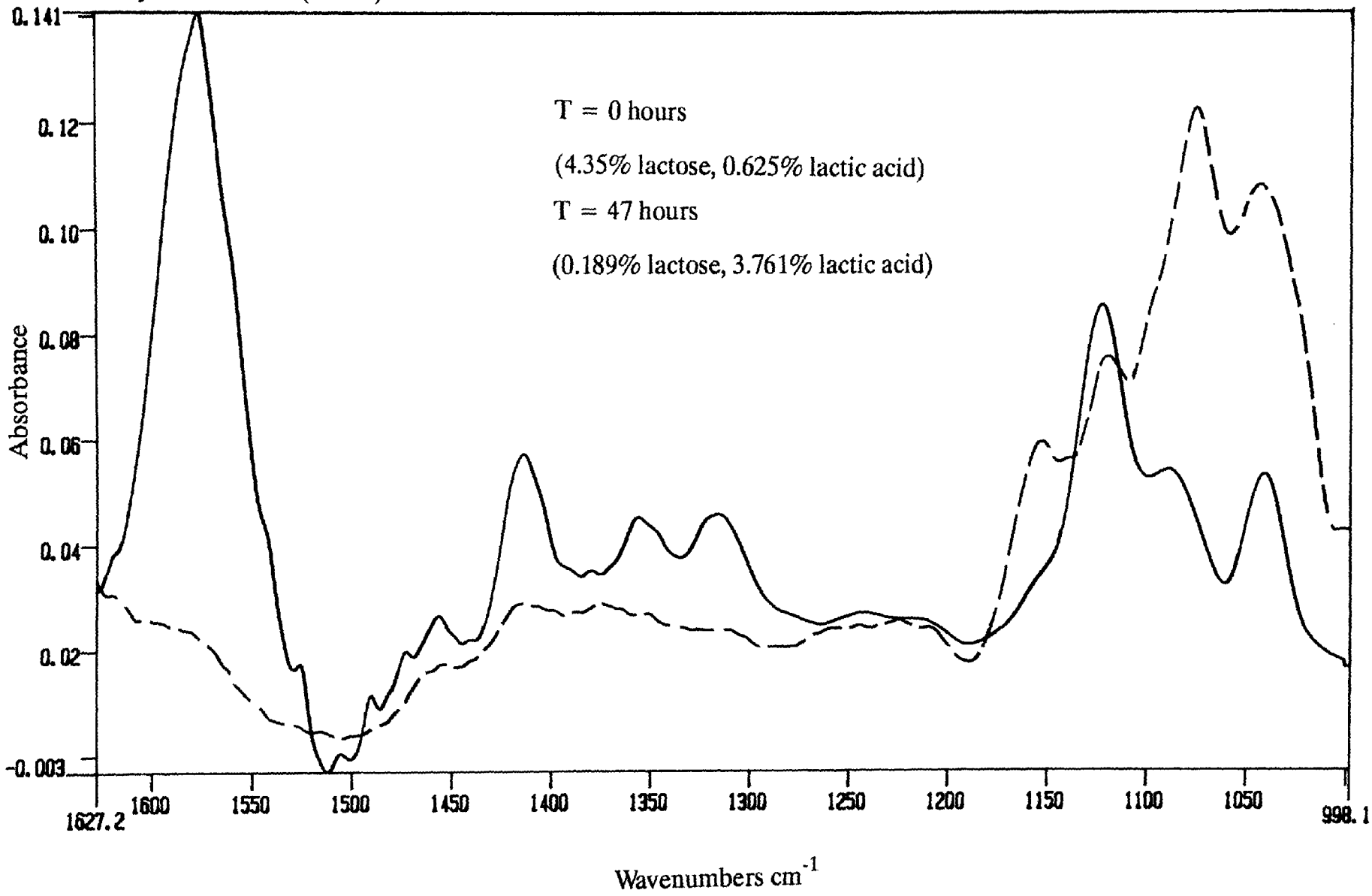
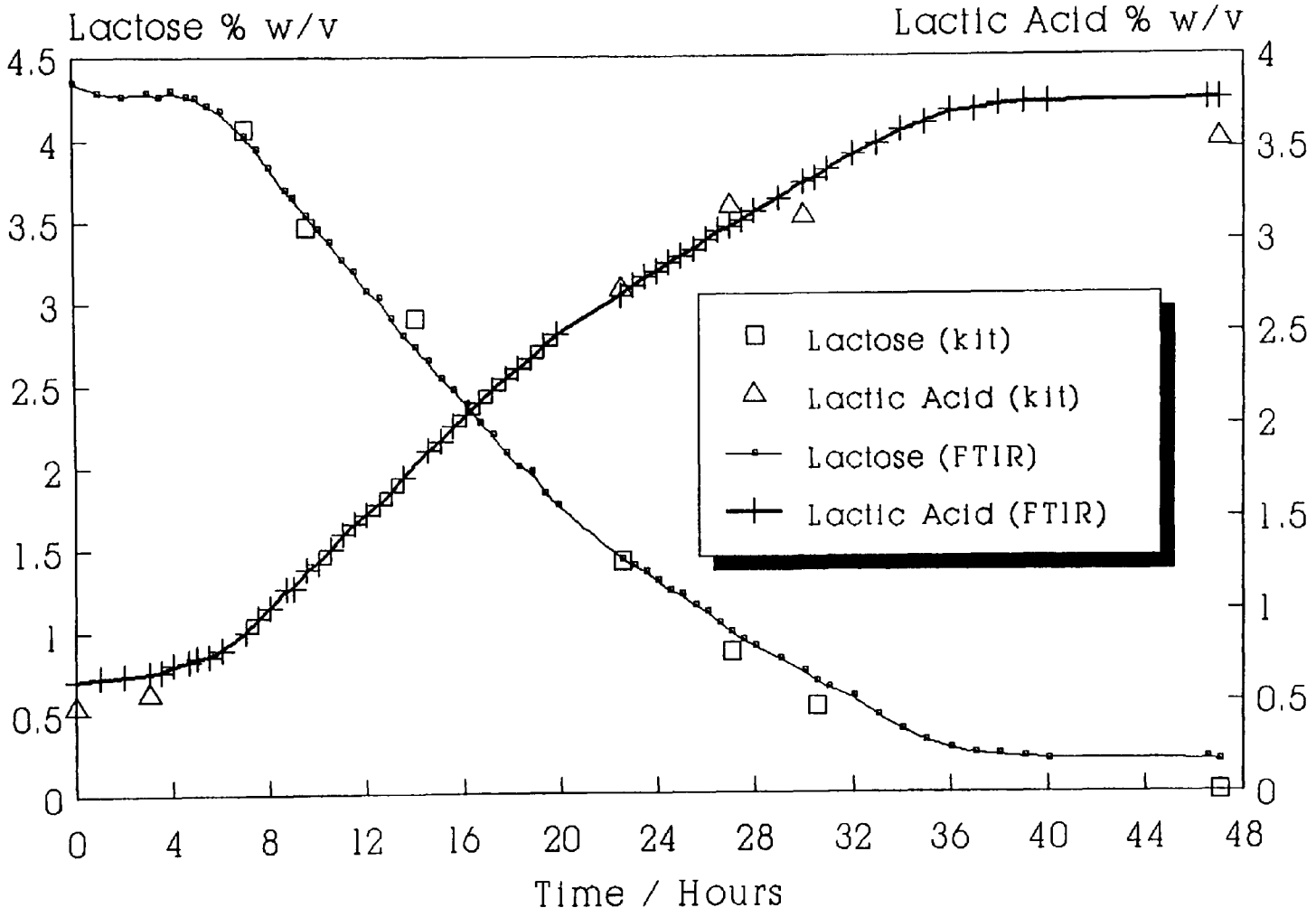
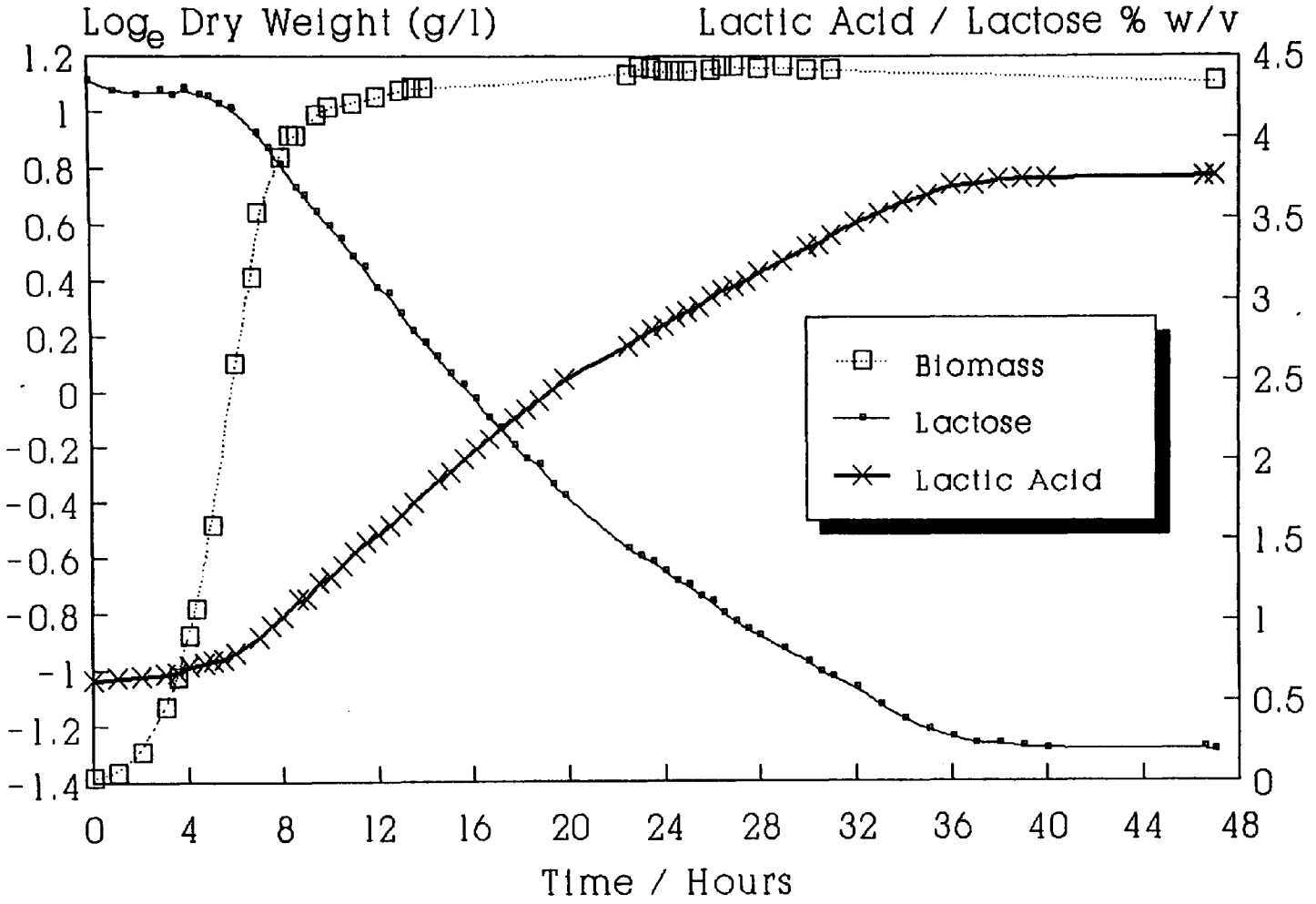


FIG 3.9.3. Whey Fermentation. On-line Analysis of Lactose & Lactate by FTIR. Off-line Results (Enzyme Kit) also Shown



Run 1

FIG 3.9.4. Relationship Between Growth, Lactic Acid Production and Lactose Depletion by *L. helveticus*



Run 1

Fig 3.9.5. Spectra recorded on line at T = 0 (--) & T = 21 (-) hours during the fermentation of WP_0 by *L. helveticus*. (Run 2)

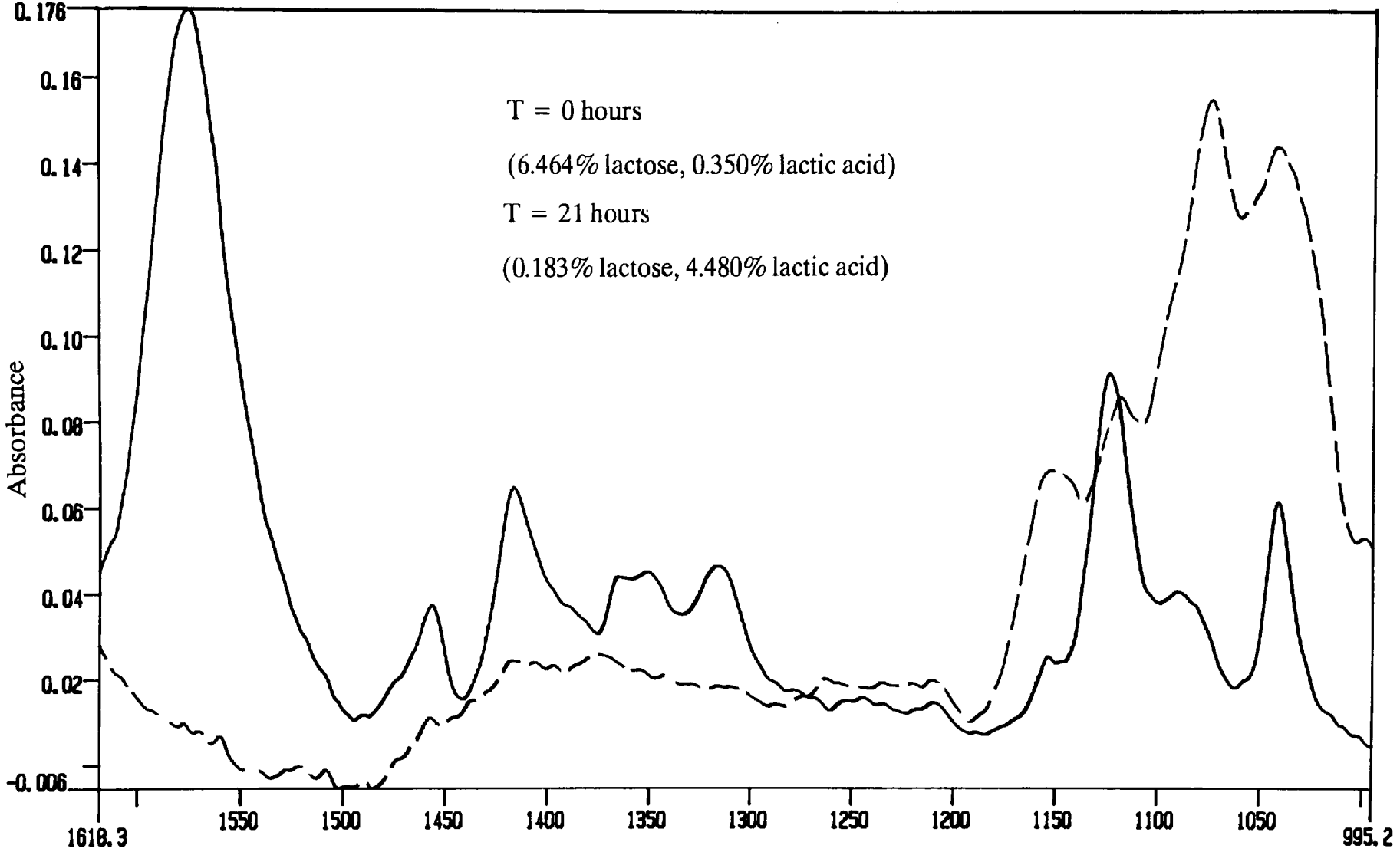
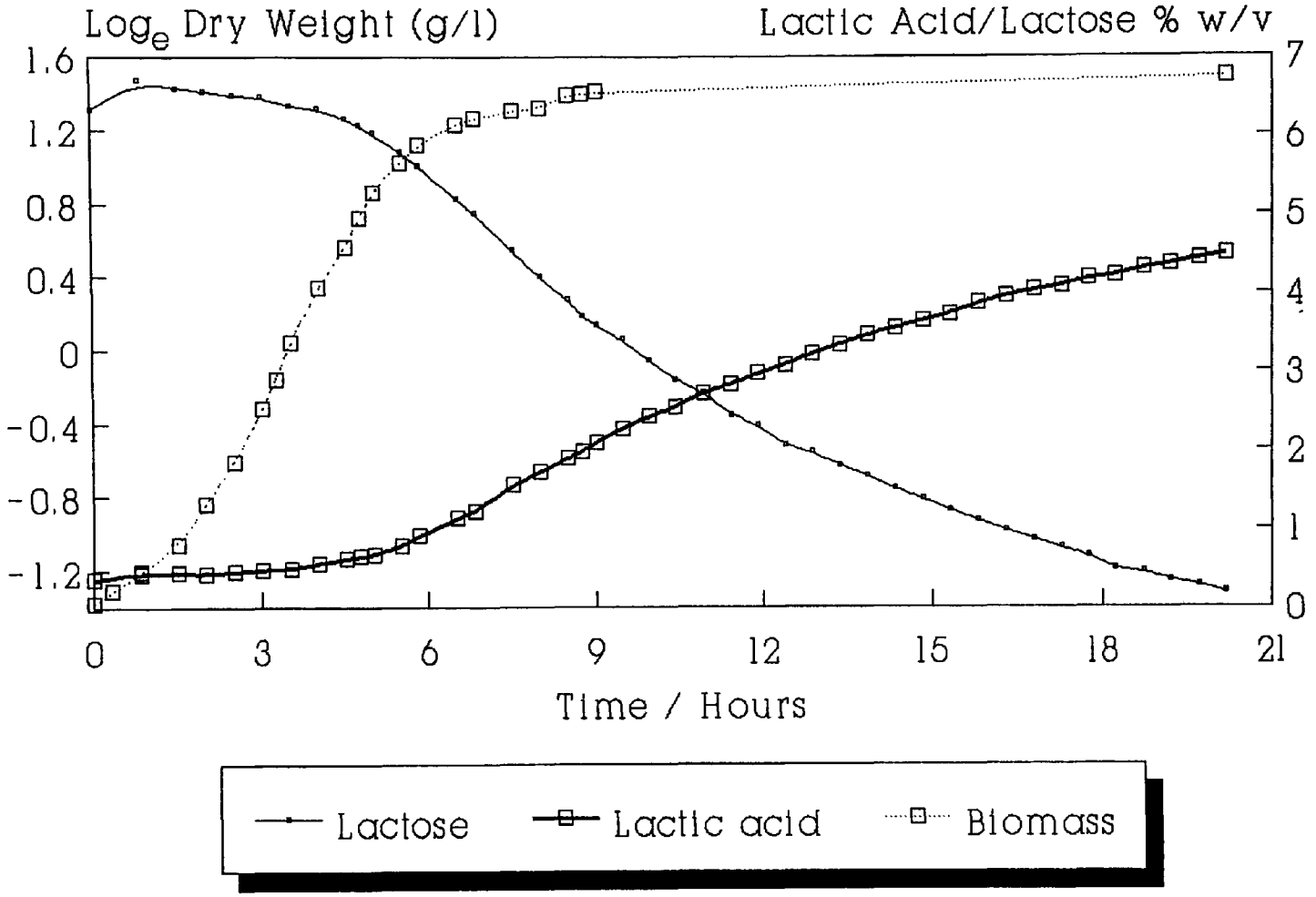


FIG 3.9.6. *L. helveticus* Growth and Lactic Acid Production / Lactose Utilisation in WPo Medium



Run 2

Fig 3.9.7. Spectral changes recorded on line during the fermentation of WP_o medium by *L. helveticus.* (Run 3). Note the diminution of lactose bands at 1156 and 1100-1000 cm^{-1} and the growth of lactate bands at 1570, 1440-1300, 1125 & 1040 cm^{-1}

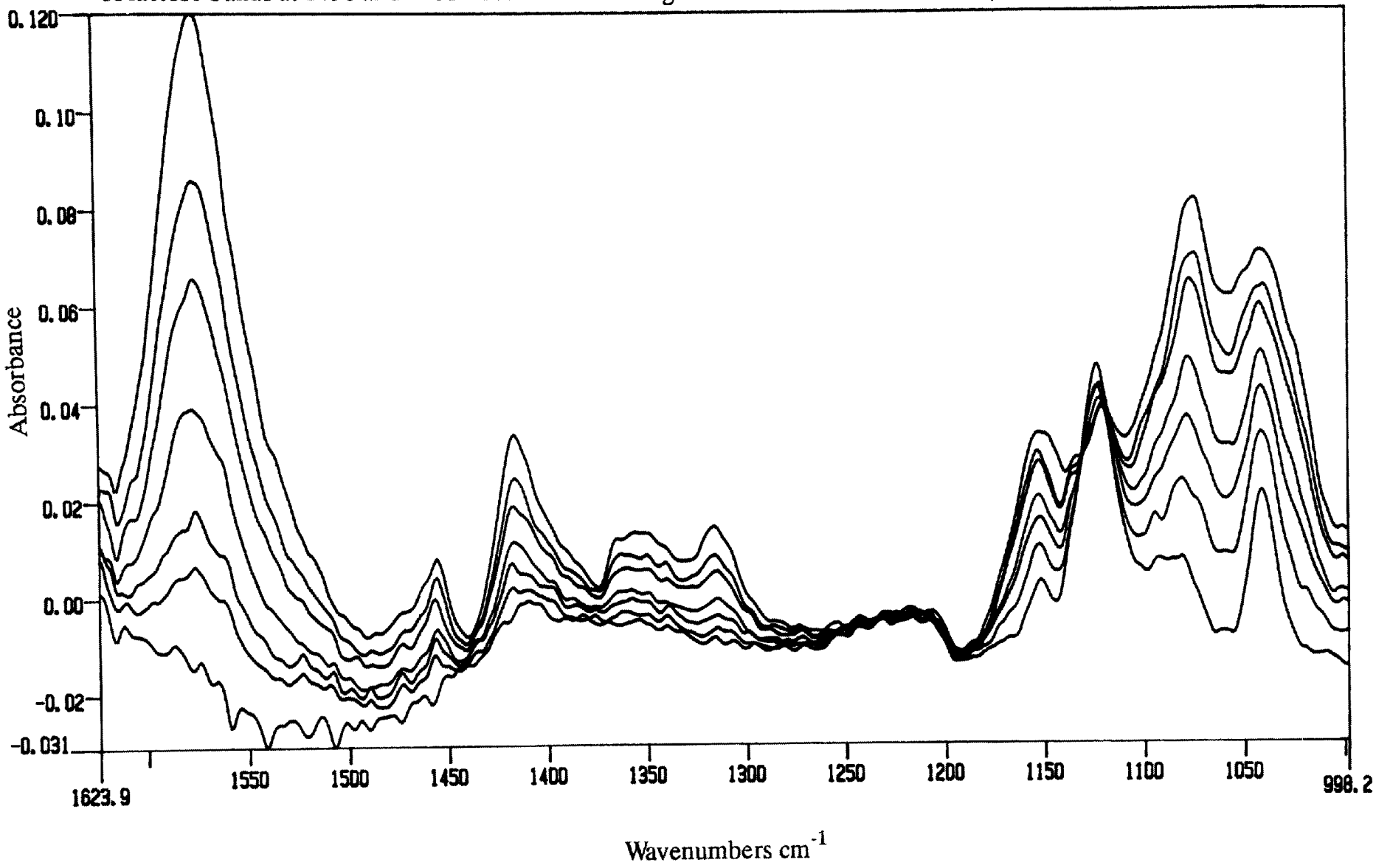
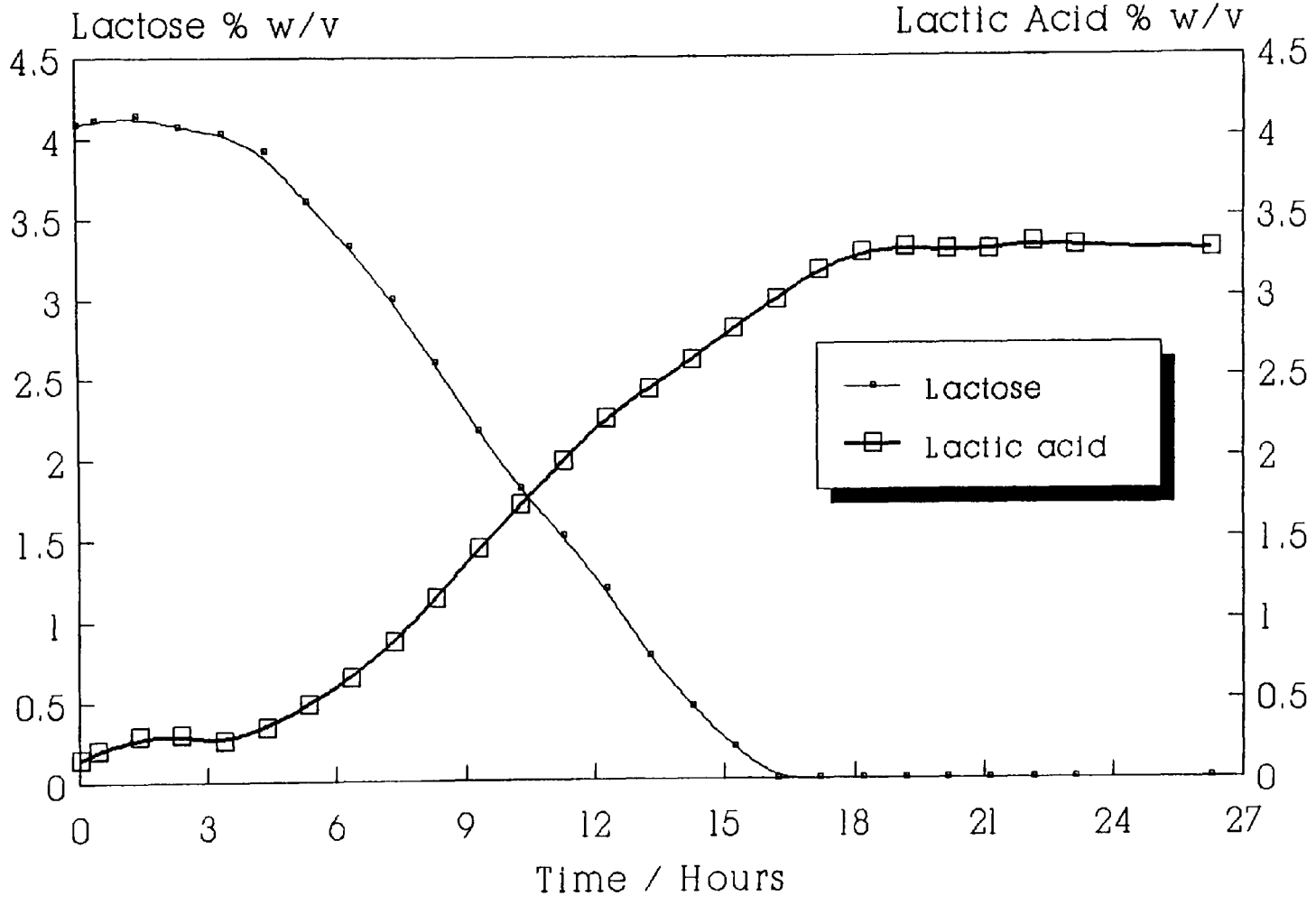


FIG 3.9.8. On-line Analysis of Lactose Depletion & Lactic Acid Production by *L. helveticus* in WPo Medium



Run 3

Fig 3.9.2. shows the changes in the spectrum during the course of the fermentation. Note the emergence of the peak at 1575 cm^{-1}, and to a lesser extent the peaks in the region 1450 - 1300 cm^{-1}, due to the lactate anion. Also, note the differences in the region 1200 - 1007 cm^{-1} due to lactose depletion and lactate formation. Changes in substrate and product levels are illustrated in Fig 3.9.3. Good agreement (correlation coefficient = 0.997) is observed between on line data and results obtained by off line analysis with enzyme kits. Fig 3.9.4. shows the relationship between bacterial growth, lactose depletion and acid production. Figs 3.9.5, 3.9.6. and 3.9.7., 3.9.8. show results for subsequent fermentations monitored on line. Table 3.9.3. summaries the parameters of interest.

Table 3.9.3. Fermentation data for on line runs

	Run 1	**Run 2**	**Run 3**
Completion Time (hours)	37	20.5	18
Productivity ($g\ l^{-1}\ h^{-1}$)	1.04	2.22	1.72
Substrate utilisation (%)	96	97	100
Substrate conversion (%)	84	71	82

There is a marked difference in the time required for complete fermentation between run1 and runs 2 and 3. This can be related to differences in media composition and inocula. For run1, the WP contained only 0.5% w/v YE and the inocula used to seed the fermenter was about 19.5 hours old (in stationary phase). Runs 2 & 3 were carried out after optimisation of starter performance and medium supplementation. WP_o medium was used (0.75% w/v YE) in conjunction with mid-exponential phase starters. Clearly this had a major impact on lactate productivity and reduced fermentation times by almost 50%. The lower substrate conversion during run 2 may have been linked to the unusually high lactose content (6.7% w/v) of that particular batch of whey.

Reproducibility of analysis by FTIR spectroscopy was assessed during run 3. Seven consecutive measurements were made on the whey medium prior to inoculation. Standard deviations of 0.016 and 0.017 % w/v were calculated for lactose and lactic acid respectively. This suggested that reproducibility of this technique was good.

In run 1, 21% of the lactic acid was formed during the exponential growth phase, compared with 79% during stationary phase. For run 2, the corresponding figures are 19% and 81% respectively. Growth and product synthesis are only partly linked (mixed growth associated). This is in agreement with the findings of Roy et al[30]. They observed that under optimal conditions for lactic acid production, the contribution of the non-growth associated product formation mechanism was superior to that of the growth linked mechanism. Similarly, in the fermentation of milk permeate by *L. helveticus*, Vahvaselka & Linko[97] noted that 70% of lactate was formed after the exponential growth phase. Keller & Gerhardt[27] in their study of *L. helveticus* in continuous culture, found that 40% of lactic acid was formed due to growth and 60% due to maintainance metabolism under optimal conditions.

3.9.5. Software Comparison

The performance of the QUANT and PLSCAL quantitative analysis programs is summarised below (Table 3.9.4.). In this particular case, QUANT has a slight edge in terms of quantitative accuracy, but is considerably slower in terms of calculation speed. However, the difference in calculation times may be attributable to differences in computer hardware as opposed to software related. PLSCAL has an additional advantage, in that the number of calibration standards which can be used is effectively only limited by memory size. QUANT can only utilise a maximum of 15 standards. Thus in systems containing 5, 6, 7 or more components, PLSCAL would probably be more suitable. Thus for this particular application, there is a possible trade-off between the slightly higher accuracy of QUANT and the speed of PLSCAL.

It should be noted that for this test, we have assumed that file transfer from the Epson PC/QUANT to the Viglen PC/PLSCAL and subsequent file conversion was 100% successful. Obviously, errors in this process would undermine the validity of the comparison.

Table 3.9.4. Software assessment. Comparison of actual concentrations of lactic acid and lactose with calculated concentrations.

QUANT					
Lactose (% w/v)			Lactic acid (% w/v)		
actual	calculated	error (%)	actual	calculated	error (%)
0.571	0.553	3.2	0.848	0.850	0.2
1.225	1.219	0.5	3.316	3.305	0.3
1.976	2.034	2.9	2.888	2.925	1.3
3.832	3.911	2.1	0.854	0.902	5.6
5.592	5.632	0.7	3.867	3.826	1.1
	Mean error	1.9		**Mean error**	1.7
PLSCAL					
Lactose (% w/v)			Lactic acid (% w/v)		
actual	calculated	error (%)	actual	calculated	error (%)
0.571	0.566	0.9	0.848	0.863	1.8
1.225	1.245	1.6	3.316	3.318	0.1
1.976	2.049	3.7	2.888	2.908	0.7
3.832	4.127	7.7	0.854	0.766	10.3
5.592	5.682	1.6	3.867	3.801	1.7
	Mean error	3.1		**Mean error**	2.9

Time taken for each analysis: QUANT 20-25 seconds

PLSCAL 1-2 seconds

This example serves to highlight the considerable interest in exchange of spectral data between instruments/computer systems within a laboratory and between institutions. In the last few years the field of communication in spectroscopy was opened by the development of an exchange format for infrared data. The JCAMP.DX (Joint Committee on Atomic and Molecular Physical Data. Data Exchange) protocol enables for example a spectrum collected on a Perkin Elmer FTIR spectrometer to be transferred to a different instrument, such as a Mattson FTIR, in another location. This format is currently being adopted for NMR, mass spectroscopy and chromatography[112].

3.10. CONTINUOUS CULTURE OF L. helveticus

3.10.1. Background Theory

Fermentations may be carried out as batch, fed-batch or continuous processes, or variations of these procedures. Batch culture may be viewed as a **closed system** (except for aeration) containing a limited amount of medium[18]. The inoculated culture passes through a number of phases: lag, exponential, stationary and death respectively. In fed-batch operations, substrate is added in increments throughout the process. Batch or fed-batch culture systems are used in the majority of industrial fermentation processes and are particularly suitable for the formation of mixed-growth and non-growth associated products. The major drawback of batch fermentations for the synthesis of growth-associated products, is that efficient product formation only occurs during a fraction of the fermentation cycle (Fig 3.10.1.).

Continuous culture may be regarded as an **open system** in which fresh medium is continuously added to the bioreactor while an equal volume of fermented medium, containing cells and products, is simultaneously removed. A **steady state** can be maintained such that the concentration of cells, the specific growth rate and culture environment

(e.g. nutrient and product concentrations) are constant. This contrasts with growth in batch systems, where culture conditions such as concentration of dissolved oxygen, nutrient and product/by-product levels, viscosity, change in complicated interacting ways[116]. As a result, continuous culture offers the opportunity to investigate the response of the microorganisms to their environment and for the extended production of cell mass or other products under optimal conditions. Continuous systems can consequently be more efficient in terms of fermenter productivity for certain applications, since non-productive lag or stationary phases (for growth-associated products) can be avoided and down times reduced[18].

Several types of continuous culture systems exist such as the turbidostat and plugflow reactor, but the most common is the **chemostat.** In the chemostat, the medium is designed so that all but a single essential nutrient are available in excess. The single growth limiting nutrient (carbon source, hydrogen donor, nitrogen source etc) controls the size of the steady state cell population[96]. In a homogenously-mixed, single stage continuous reactor, substrate addition to the reactor is balanced by displacement of culture from the vessel at an equal volumetric rate. Under such conditions a steady state is achieved where biomass formation is balanced by cell loss from the culture. In this situation the specific growth rate is controlled by the dilution rate:

$$\mu = D$$

The dilution rate, D (h^{-1}), represents the rate of flow of medium, F ($m^3 h^{-1}$), per unit volume of the culture, V (m^3).

$$D = F/V$$

When $D < 0.9\mu_{max}$ (maximum specific growth rate), the rate of cell production in the reactor is balanced by the rate of cell output - a steady state is established. When D approaches or exceeds μ_{max}, loss of cells in the effluent exceeds the rate of production and **washout** occurs.

The growth rate can be expressed as a function of the limiting substrate using the Monod model[117]:

$$\mu = \frac{\mu_{max} s}{Ks + s}$$

where s = residual concentration of the limiting nutrient in the growth chamber

Ks = the saturation constant and is equivalent to the substrate concentration at which μ is half the maximal rate ($\mu = 0.5 \mu_{max}$)

This model describes the growth rate as a function only of the limiting nutrient concentration and assumes no dependence on the concentration of other nutrients or other environmental factors.

At steady state, with $\mu = D$:

$$D = \frac{\mu_{max} s}{Ks + s}$$

The steady state concentration of the limiting nutrient in the chemostat is determined by the dilution rate according to the equation:

$$s = \frac{Ks\ D}{\mu_{max} - D}$$

The biomass concentration in the chemostat at steady state is given by

$$x = Y(So - s)$$

where x = cell concentration

Y = the yield coefficient for the limiting substrate (grams of biomass per gram of substrate consumed)

So = original concentration of limiting nutrient in the feed vessel

Fig 3.10.1. Batch-type fermentation cycle for a growth associated product[18]

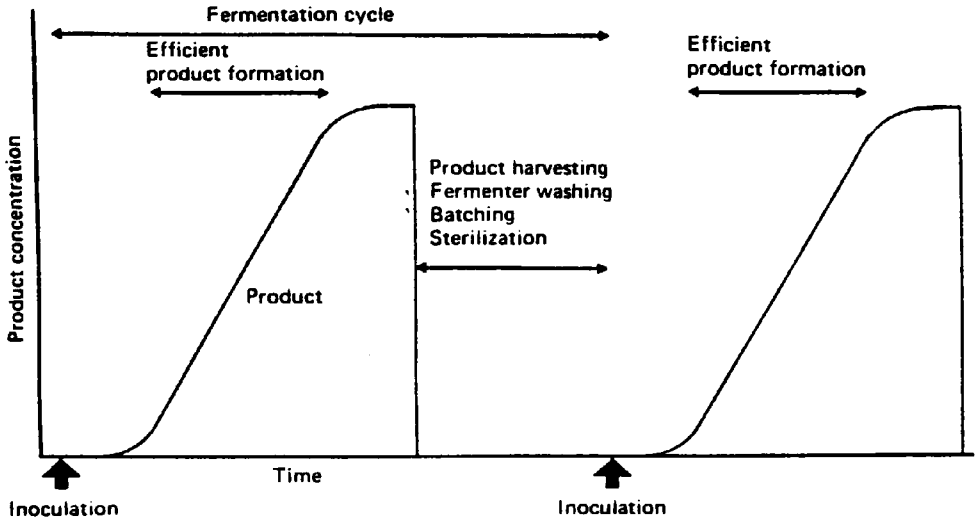
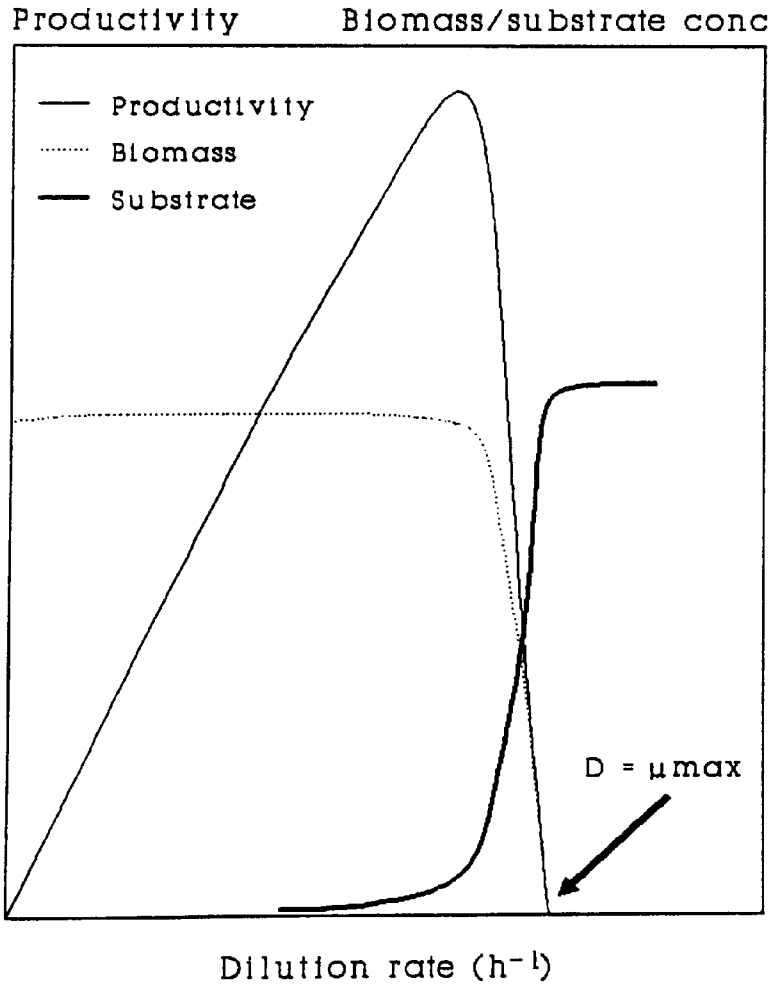


FIG 3.10.2. Effect of D on Steady State Biomass & Substrate Concentrations & Biomass Productivity



When using this equation it is assumed that:

(i) cell yield is independent of the growth or dilution rate

(ii) the value of the cell concentration is independent of of all nutrients except for the limiting one

Substituting for s:

$$x = Y\left[So - \frac{KsD}{\mu_{max} - D}\right]$$

Using these equations the theoretical behaviour of a chemostat may be depicted as in Fig 3.10.2.

3.10.2. Discussion of Results

The results of a preliminary study using MRS medium are presented in Fig 3.10.3. A doubling of the dilution rate caused substrate utilisation to fall from 93% to 60% and subsequent conversion of lactose into lactic acid to drop 9% to 81%. This indicated that the optimum value of D in terms of minimal residual lactose and maximum acid concentration lay below 0.3 h^{-1}.

The influence of dilution rate on growth and lactic acid formation in WPo medium is shown in Figs 3.10.4. and 3.10.5. Fig 3.10.6. depicts the variation in substrate utilisation/conversion and productivity with dilution rate.

The high residual lactose present over the whole range of dilution rates investigated suggests that lactose was not the limiting substrate. Typical Ks values for various carbon sources are in the range 1-10 mg/l. Cells will grow at rates close to μ_{max} if the limiting carbon source is greater than 10Ks or 10-100 mg/l[18]. The production of ammonium lactate from whey using *L. bulgaricus* has been studied by Stieber & Gerhardt[28,118]. In a single-stage continuous culture, Ks was calculated to be 70 mg lactose/l[118]. A value of

FIG 3.10.3. Effect of Dilution Rate on Lactic Acid Production by *L. helveticus* in MRS Medium: A Preliminary Study

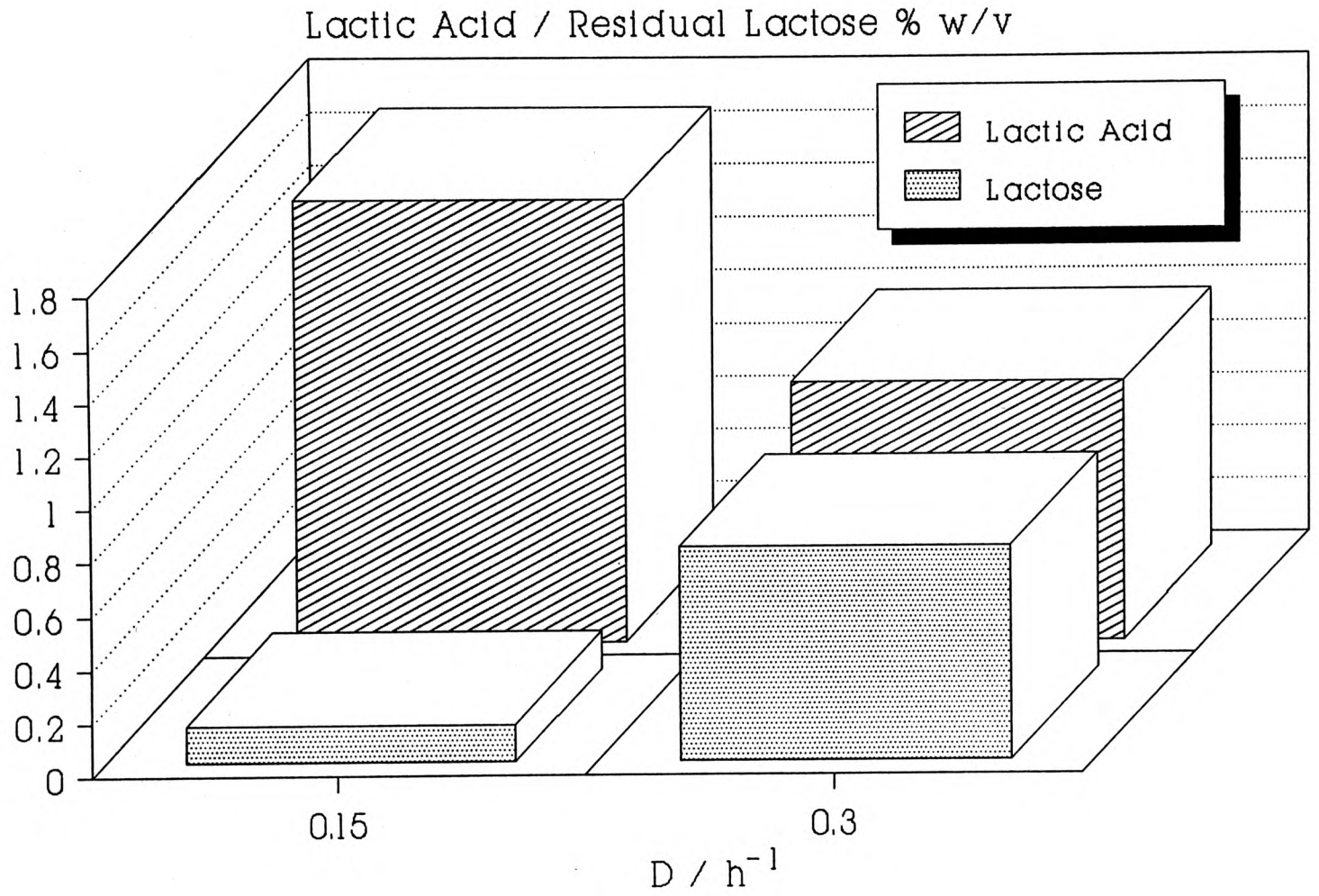


FIG 3.10.4. Effect of Dilution Rate on *L. helveticus* Growth & Lactose Depletion / Lactic Acid Production in WPo Medium

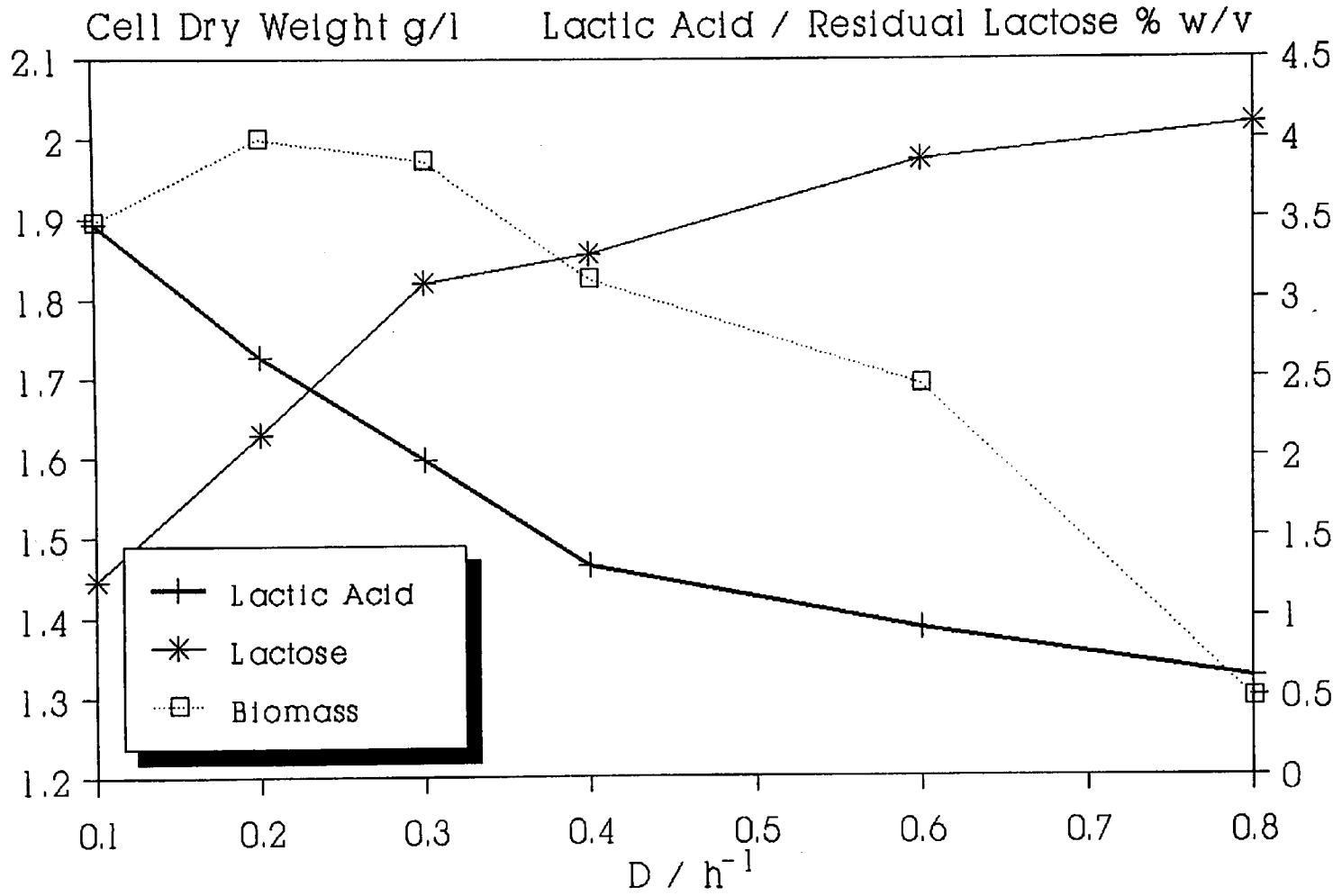
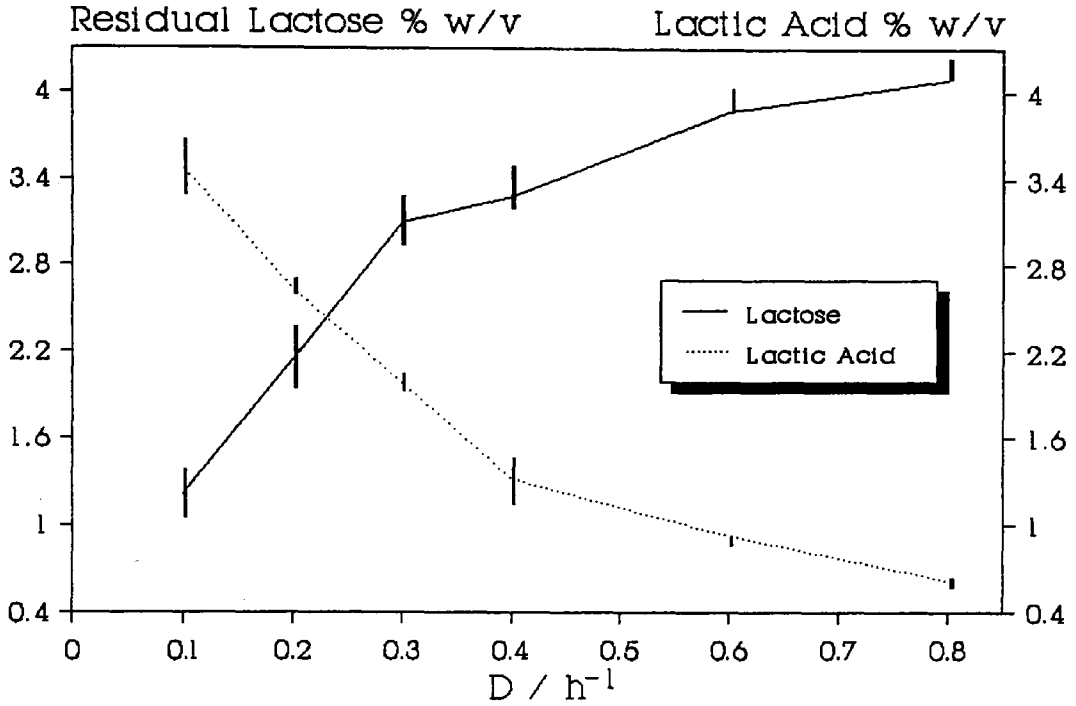


FIG 3.10.5. Range Bars For The Measurement Of Lactic Acid, Lactose & Biomass During The Course Of The Continuous Culture



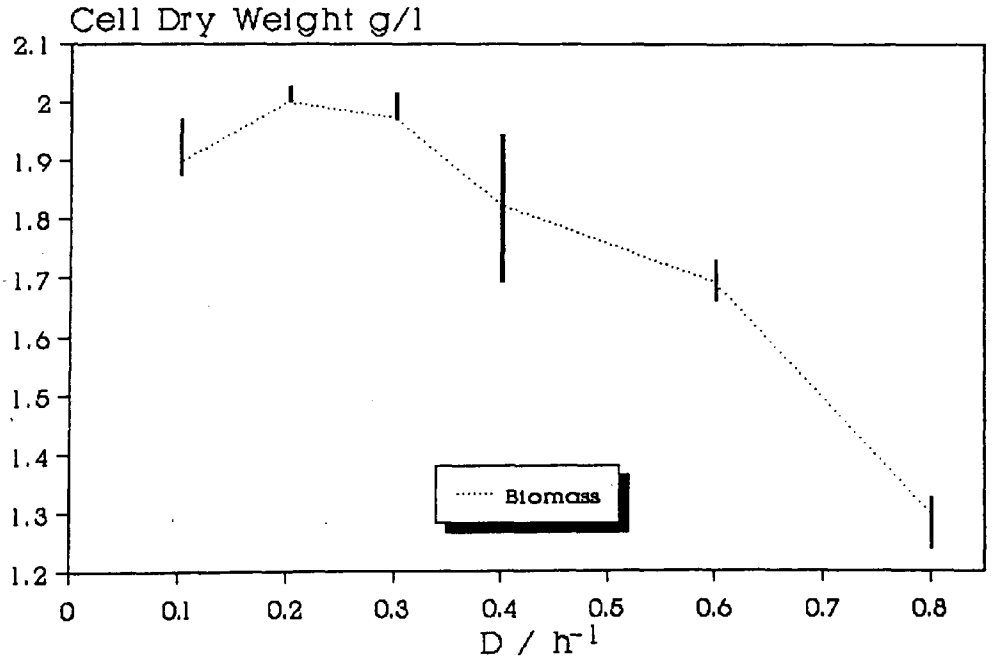
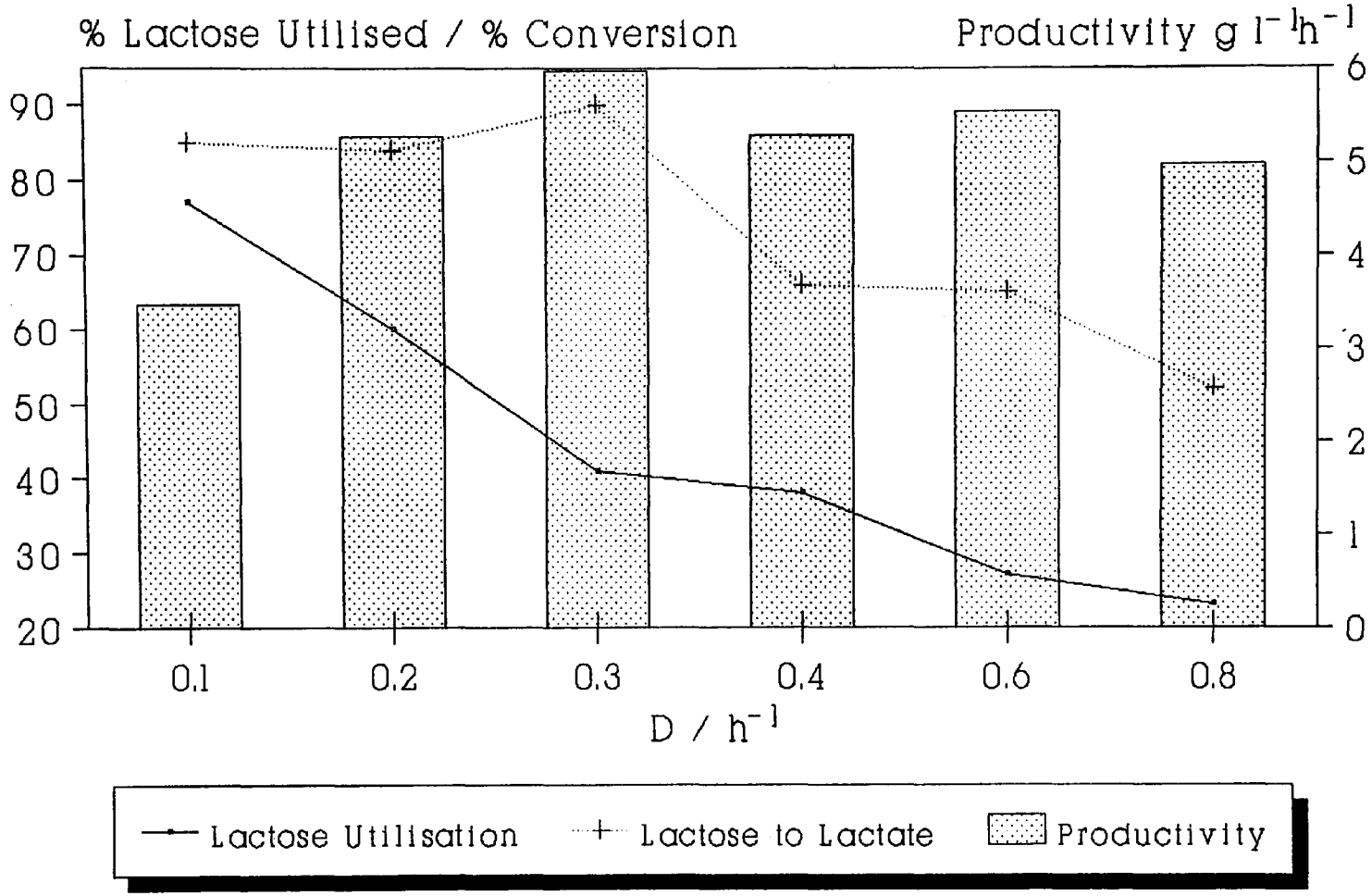


FIG 3.10.6. Effect of D on Productivity & Substrate Conversion / Utilisation by *L. helveticus* in WPo Medium

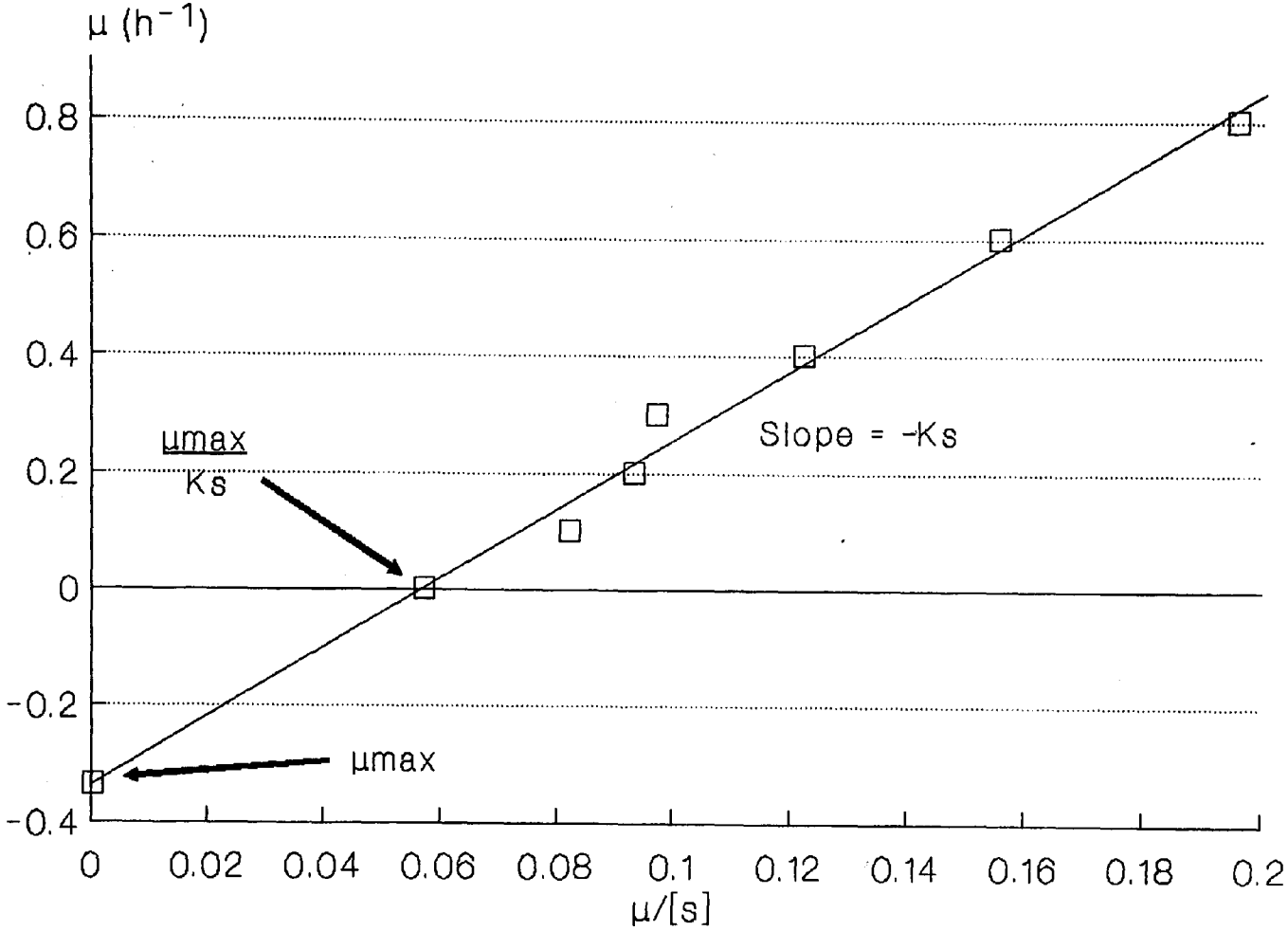


0.4 mg/l was proposed for *L. bulgaricus* grown in a dialysis continuous culture[28]. Using our data, an Eadie[119]-Hofstee[120] plot was produced (μ versus μ/[s]) in order to determine Ks for *L. helveticus* (Fig 3.10.7.). Using linear regression (method of least squares) Ks = -59 g/l (-5.9% w/v) lactose and μ_{max} = -0.335 h^{-1}. Clearly these figures are not realistic. This supports the idea that lactose was not limiting. As a consequence, since it is not certain that the carbon source or another single nutrient was limiting, the Monod equation is not strictly applicable.

The high residual lactose present at low dilution rate may have been due to product inhibition. Ohleyer[121] reported severe lactate inhibition for *L. delbrueckii* at around 4% w/v on lactose-yeast extract medium. Stieber & Gerhardt and Keller & Gerhardt[27] proposed that in the production of ammonium lactate by *L. bulgaricus*, the fermentation process was inhibited by increasing product concentration and not substrate exhaustion. However in our study, the relatively low concentration of 3.5% w/v lactic acid generated at D = 0.1 h^{-1}, implies that product inhibition may have been only a contributory factor. In comparison, lactic acid levels in excess of 4% w/v were observed for batch runs, with considerably lower residual lactose.

Possible limitation in growth factors may also have had an effect. In a preliminary study of lactic acid production from whey using *L. helveticus*, Aeschlimann & von Stockar[31] concluded that the high residual lactose observed was attributable to growth factor limitation. In that particular experiment, the whey permeate was supplemented with only 0.4% w/v YE. A subsequent investigation of the effect of YE levels on lactate production revealed that at D = 0.2 h^{-1}, 2% w/v YE was required to achieve maximum biomass concentration and 2.5% w/v was necessary to attain maximum lactic acid concentration[98]. A recent study by Roy[113] used whey permeate containing 1.5% w/v YE in the continuous culture of *L. helveticus*. Thus it is possible that the optimum YE level of 0.75% w/v determined in batch culture (section 3.6.2.) may have been inadequate for continuous culture of *L. helveticus*.

FIG 3.10.7. Determination of Ks & μmax Using The Eadie-Hofstee Plot



Ks = substrate concentration at which growth rate is μmax/2

s = steady state residual substrate concentration

The situation is further complicated since the whey used was subject to inherent variation in composition from batch to batch. Given the complex nutritional requirements of lactobacilli and the undefined nature of the whey medium, it is difficult to identify the limiting medium component(s). Furthermore, because the cell demands change as the growth rate changes, the actual limiting nutrient may vary with the growth rate.

From Figs 3.10.4 & 3.10.5., the amount of lactic acid produced diminished with increasing dilution rate, whilst residual lactose levels rose. The maximum concentration of acid/mimimum residual lactose occured at D = 0.1 h^{-1}. A corresponding decrease in substrate conversion (lactose to lactate) was observed as the dilution rate was raised (Fig 3.10.6.). Since maximum productivity corresponded to D = 0.3 h^{-1}, inevitably there would be a trade-off between high product concentration/low substrate concentration, which is desirable in an effluent treatment process, and maximum productivity.

This departure from the expected behaviour illustrated in Fig 3.10.2. can be related to the fact that lactic acid synthesis is only partially growth-linked (mixed-growth associated) - see section 3.9.4. Thus at low dilution rates (low growth rates) most of the lactic acid formed is due to maintainance or endogenous metabolism (substrate is used for non-anabolic functions). With increasing growth rate, the proportion of lactate formed due to bacterial growth rises. Keller & Gerhardt[27] in their study of whey ammonium lactate formation, found that at D = 0.2 h^{-1}, residual lactose = 4.2% w/v and lactic acid = 1.6% w/v. They calculated that 70% of the lactate formed was attributable to bacterial growth and 30% was due to maintainance metabolism. At D = 0.067 h^{-1}, residual lactose was reduced to 0.7% w/v. Maintainance metabolism accounted for 60% of the lactic acid formed, the remainder being due to bacterial growth. In our earlier study of *L. helveticus* in batch culture (section 3.9.4.), 80% of lactic acid was generated during the stationary phase (maintainance metabolism). Major & Bull[122] noted an increase in maintainance requirements for *L. delbrueckii* in a cell recycle fermenter compared with a normal chemostat system. They concluded that increased maintainance requirements

are advantageous for the production of lactic acid or any other primary product of energy producing metabolism. Oner et al[123] stated that when product formation is the desired goal, maintainance requirements should be as high as possible and growth yields small. They indicated that product yield and specific rate of product formation are enhanced with increasing values of the maintainance coefficient.

The changes in biomass are difficult to explain, particularly since the limiting nutrient(s) is unknown. The cell concentration would have been expected to remain relatively steady until D approached 0.9 μ_{max} (approximately 0.75 h^{-1} based on batch data). If the high residual substrate/ low product levels observed at D = 0.1 h^{-1} were due, at least in part, to nutrient limitation, it is possible that levels of this nutrient (or nutrients) were inadequate for the demands of the bacterial population as the growth rate rose. Consequently, the drop in biomass observed at D = 0.4 and 0.6 h^{-1} may also have been a result of limitation in growth factors.

In order to improve the efficiency of the operation, one possible option would be to use a two stage chemostat. The first stage would be geared towards growth of *L. helveticus* cells and thus a relatively high dilution rate (0.6 $h^{-1}$to 0.7 h^{-1}) would be employed. The second stage, with D = 0.1 h^{-1}, would be optimised for lactic acid production. Use of multi-stage processes however tend to be inherently more expensive and complicated than a single stage operation. Keller & Gerhardt[27] proposed that whey medium with initial substrate concentrations in excess of 5% w/v, as in this study, would benefit from staging.

3.11. SCALE UP

Development of microbiological processes is often carried out in three scales or stages:

1. bench scale
2. pilot plant
3. plant

Typically, strain selection, medium development and initial optimisation of operational parameters is carried out at bench level. Further improvements/refinements are made at the pilot scale before bringing the process to economic fruition on a plant scale[114].

The transfer of the fermentation process to production scale is referred to as **scale-up.** A complex of techniques and methodologies are employed in the translation process. The behaviour of a population of microorganisms should be independent of scale provided all aspects of the environment are also scale invariant and the size of the initial population is adjusted for scale. Movement from one scale to another would then be relatively straightforward if precisely the same conditions could be provided on both scales. On the other hand, if the response of the organisms to all the important variables e.g. pH, temperature, agitation speed, heat and mass transfer etc were known, accurate scale up would be possible[124].

In practice, reliable translation (either up or down) between scales is usually difficult due to the different ways in which process variables are affected by the size of the unit. As a consequence the problem resolves itself into one of identifying the key parameters involved in a process and selecting the one to which the process is most sensitive. This parameter can then be made the "criterion of scale-up" and systems designed to maintain this parameter at the same value at all scales of operation[125]. Parameters of particular importance are: gas-to-liquid mass transfer, liquid-to-cell mass transfer, fluid shear, macro-mixing and heat evolution. These parameters exert their effect on the fermentation by controlling the value of state variables such as temperature, pH, concentration of nutrients, dissolved oxygen and their homogeneity throughout the culture. In this respect culture rheology, the flow regime (fully turbulent or not) and the geometrical similarity of the systems involved need to be checked and compared.

Geometric similarity, from a practical viewpoint, is important in that it simplifies the prediction of fermenter performance on the larger scale. For geometric similarity the fol-

lowing ratios should be the same for both vessels:
vessel diameter/impeller diameter, liquid height/impeller diameter,
impeller height from base of vessel/impeller diameter, baffle width/vessel diameter[126]

Rheological behaviour of the culture is important as it influences mixing and mass and heat transfer. The fermentation broth can be described as Newtonian (viscosity independent of shear rate) or non-Newtonian (viscosity dependent on shear rate). Cultures of filamentous algae for instance exhibit non-Newtonian characteristics and increasing stirrer speed can reduce the apparent viscosity of the culture[127].

Maintainance of dynamic similarity is also important. Two possible strategies are[126]:
Scale-up based on ensuring that the Reynolds number (Re), which describes the degree of turbulence induced by agitation, is the same in both vessel sizes. This approach is suited to anaerobic processes.
For aerobic processes, since we are dealing with a two-phase system (liquid/gas bubbles) a better scale-up criterion is the Weber number, We, (inertia forces/rising bubble forces).Various other approaches can be adopted in translating between two scales, including:

1. constant volumetric O_2 transfer rate
2. Constant impeller tip speed
3. Equal mixing times
4. Constant volumetric power input
5. Similar momemtum factors

For reasons of simplicity, scale up of the fermentation process from bench (1 l) to pilot plant (16 l) was initiated using constant tip speed. As mentioned in section 3.8., the optimum conditions for lactate production (pH 5.9, temperature 42°C and stirrer speed 200 rpm) determined at bench scale, were implemented at pilot scale. Additionally, a 2 stage

Fig 3.11.1 Spectral changes recorded on line during the pilot scale fermentation of WP_o medium by *L. helveticus*.

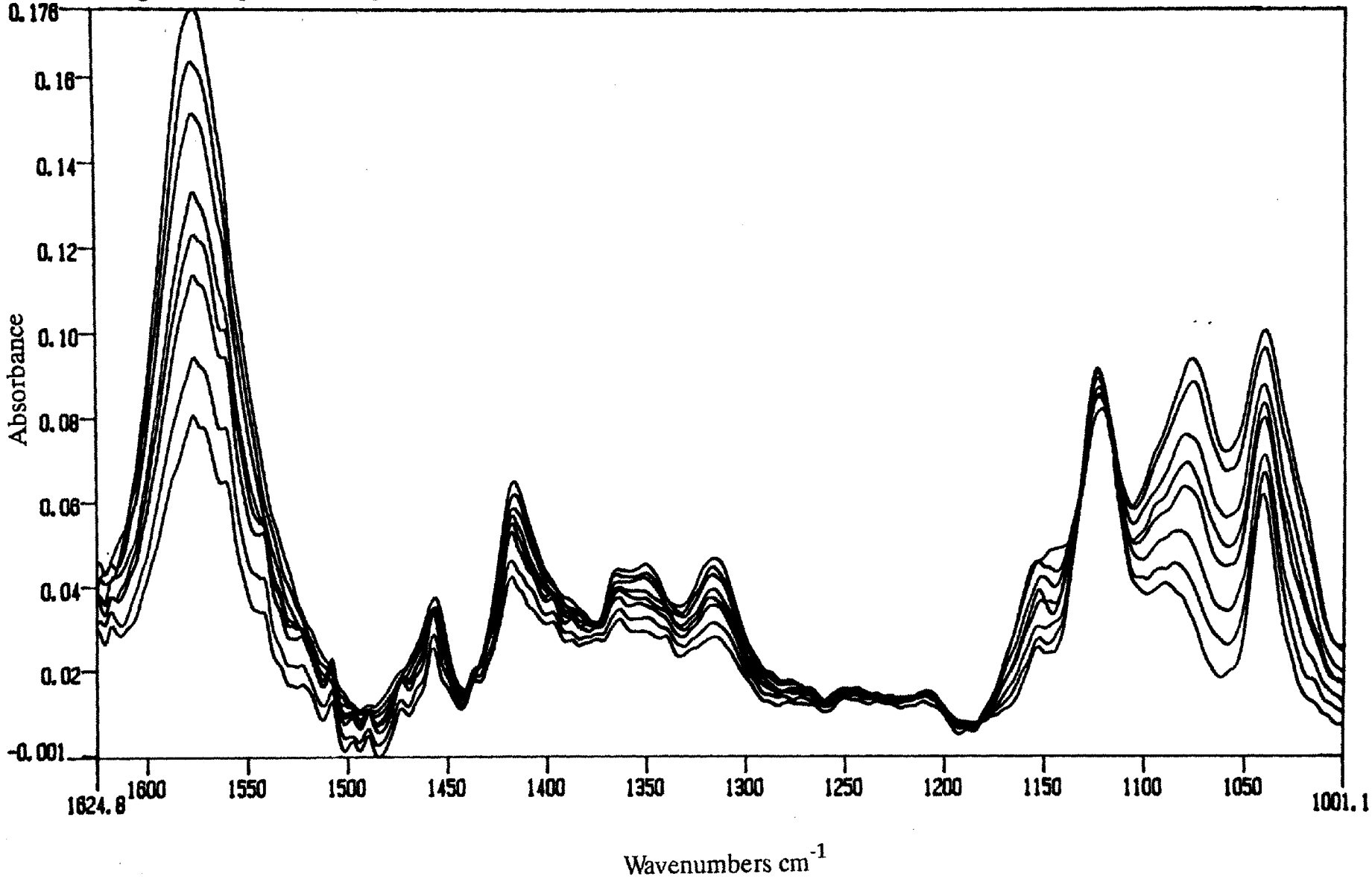
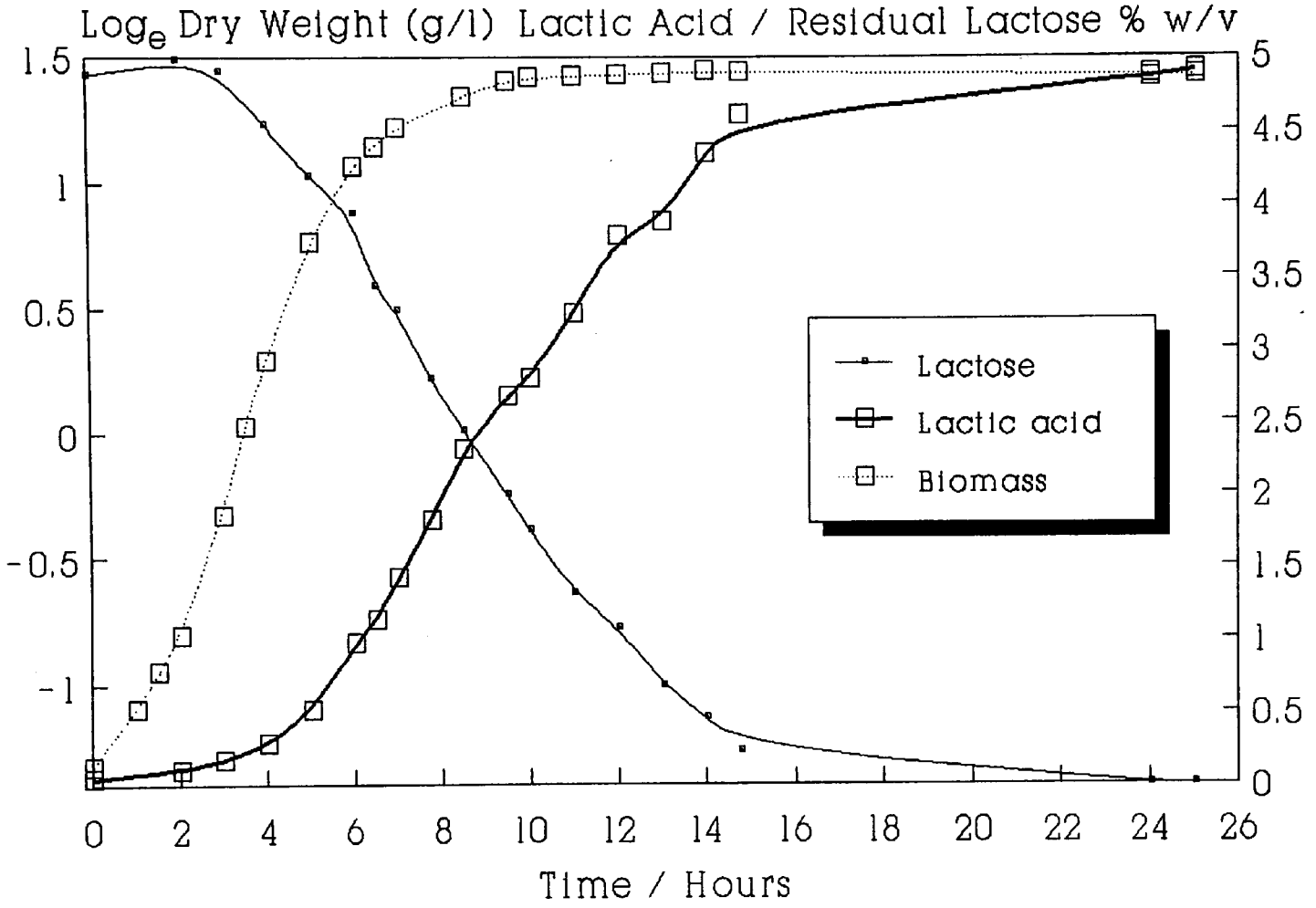


FIG 3.11.2. Scale Up: *L. helveticus* Growth & Lactose Depletion / Lactic Acid Production in a Pilot Plant Fermenter



starter culture was used in conjunction with the 16 l fermenter. This ensured that that the inocula used to seed the fermenters at both bench and pilot scales were comparable.

Figs 3.11.1 & 3.11.2 show the time course of the fermentation in the pilot plant fermenter. Relevent parameters for the fermentation are:

completion time:	approx 18 hours
productivity:	2.13 $g\ l^{-1}\ h^{-1}$
substrate utilisation:	100%
substrate conversion:	96%

The translation from bench to pilot scale appeared to be successful. The completion time, productivity and substrate utilisation are comparable with the results for on line runs 2 & 3 (bench scale) - Table 3.9.3. and batch fermentations monitored off line. Again, most of the lactate was formed during the stationary phase, with only 15% generated during exponential growth. However there is a clear difference in substrate conversion. On average, between 90% and 95% of lactose was converted to lactate during bench scale runs monitored **off line**. A figure of 96% was recorded for the pilot scale runs, which were also monitored **off line**. However, substrate conversion was only 84%, 71% and 82% for **on line** runs 1, 2 and 3 respectively. This may have been due to increased agitation/aeration as the fermentation broth was continuously circulated through the CIRCLE® cell by the peristaltic pump. (N.B. *L .helveticus* is microaerophilic). If this was the case, the effect would perhaps become less pronounced with increasing fermentation vessel size.

Note: during the operation of the pilot fermenter, off line as opposed to on line analysis was carried out due to logistical problems. For operational reasons, the FTIR could not

be moved to the Bioengineering Laboratory where the fermenter was sited, and the fermenter could not be transported to the Instrument Laboratory .

3.12. FTIR SPECTROSCOPY & PROCESS CONTROL : A FINAL REFLECTION

The basis of understanding and controlling a biological process is the data obtained from the sensors and instrumentation employed. Data generated from monitoring variables such as substrate and product levels can be used in process modelling and development of a fermentation strategy, as well as for control purposes[128].

Computer control can be divided into two kinds: direct digital control (DDC) and set point control. Each control loop is composed of[129]:

the process (fermentation)

a sensor

a controller (e.g. a computer)

an actuator or final control element e.g. a pump or valve

The algorithms executed by the controller or control system can range from simple on/off control to intricate strategies which take into account interactions among many of the variables[130]. The trend towards more automated process control creates a demand for methods which can quantitatively measure biological molecules on-line[18]. With regard to monitoring techniques, consideration should be given to response time, sensitivity, accuracy and stability of the instrument[131].

The advantages and disadvantages of on-line analysis by FTIR spectroscopy were discussed in section 3.9.2. However, in this present context, the analysis time of 3 minutes is clearly unsatisfactory for the monitoring of reactions involving bacteria such as *Escherichia coli* and *Beneckea natriegens*. In such cases, during circulation of the fermenter contents, data acquisition and quantitative analysis, the medium composition will change significantly. Inevitably there would be a discrepancy between the calculated levels of

substrate and product and the true values at any given time.

Use of an infrared probe (section 3.9.3.) would eliminate the need to transfer medium from the fermenter to the CIRCLE® cell, thereby avoiding possible changes in sample composition during circulation. In addition, the latest generation of FTIR spectrometers such as the Nicolet 60 SXR (Nicolet Analytical Instruments, Madison, Wisconsin, USA) and the Bio-Rad 896 (Bio-Rad, Hemel Hampstead, Herts, UK), are capable of recording up to 50 scans per second. These instruments could carry out the same lactic acid/lactose analysis in 10 seconds or less. Such improvements would serve to widen the sphere of practical application of this technique.

4. CONCLUSIONS & PROPOSALS FOR FUTURE WORK

CONCLUSIONS

In the heat sterilisation of whey permeate, initial pH was found to have a major influence on precipitate formation. Solubilisation of this calcium-phosphate precipitate was achieved by acidification and use of the sequestering agent EDTA. Adjustment of medium pH to between 4.0 and 5.2 prior to autoclaving effectively prevented precipitation occuring.

In the study of whey permeate supplementation, yeast extract was found to have a marked effect on *L. helveticus* growth and lactic acid production. The optimum concentration of this nitrogen/growth factor source was 0.75% w/v. Of the other supplements tested, addition of Tween 80 and sodium acetate resulted in a slight improvement in acid production. WP containing these three supplements (known as WP_o medium) was found to be superior in terms of acid formation than synthetic MRS and LS media. The COD of WPo medium was reduced by over 90% during the course of the fermentation. Thus the polluting power of this dairy by-product was significantly reduced.

Application of chemometric techniques, more specifically factorial designs, to the optimisation of cell growth and acid formation was partially successful. Although the models developed did not describe the system particularly well, factorial design enabled the most effective use of the data generated. The recent expansion in the field of chemometrics will undoubtedly lead to the increased use of such statistical approaches to a wide range of problems.

FTIR spectroscopy was shown to be an effective method for on line and off line analysis of lactic acid and lactose. Sample analysis was fast, simple and almost completely automated. Process kinetics could be studied by following the spectral changes occuring

during the course of the fermentation. Since a computer is an integral part of the instrument, an FTIR spectrometer could provide the basis of a system for real time measurement and control of the fermentation process. This technique could prove to be a viable alternative to flow injection analysis, which currently appears to be the preferred method for on line fermentation monitoring.

In the continuous culture of *L. helveticus*, increases in dilution rate lead to reductions in substrate conversion and lactic acid productivity. Inevitably a balance would need to be struck between maximum productivity ($D = 0.3\ h^{-1}$) and minimum residual lactose ($D = 0.1h^{-1}$). Staging of the process may prove to be a feasible option. Translation of the fermentation process from laboratory to pilot scale appeared to be successful. Completion times, cell growth, lactose utilisation and productivity all compared favourably with bench scale results.

FUTURE WORK

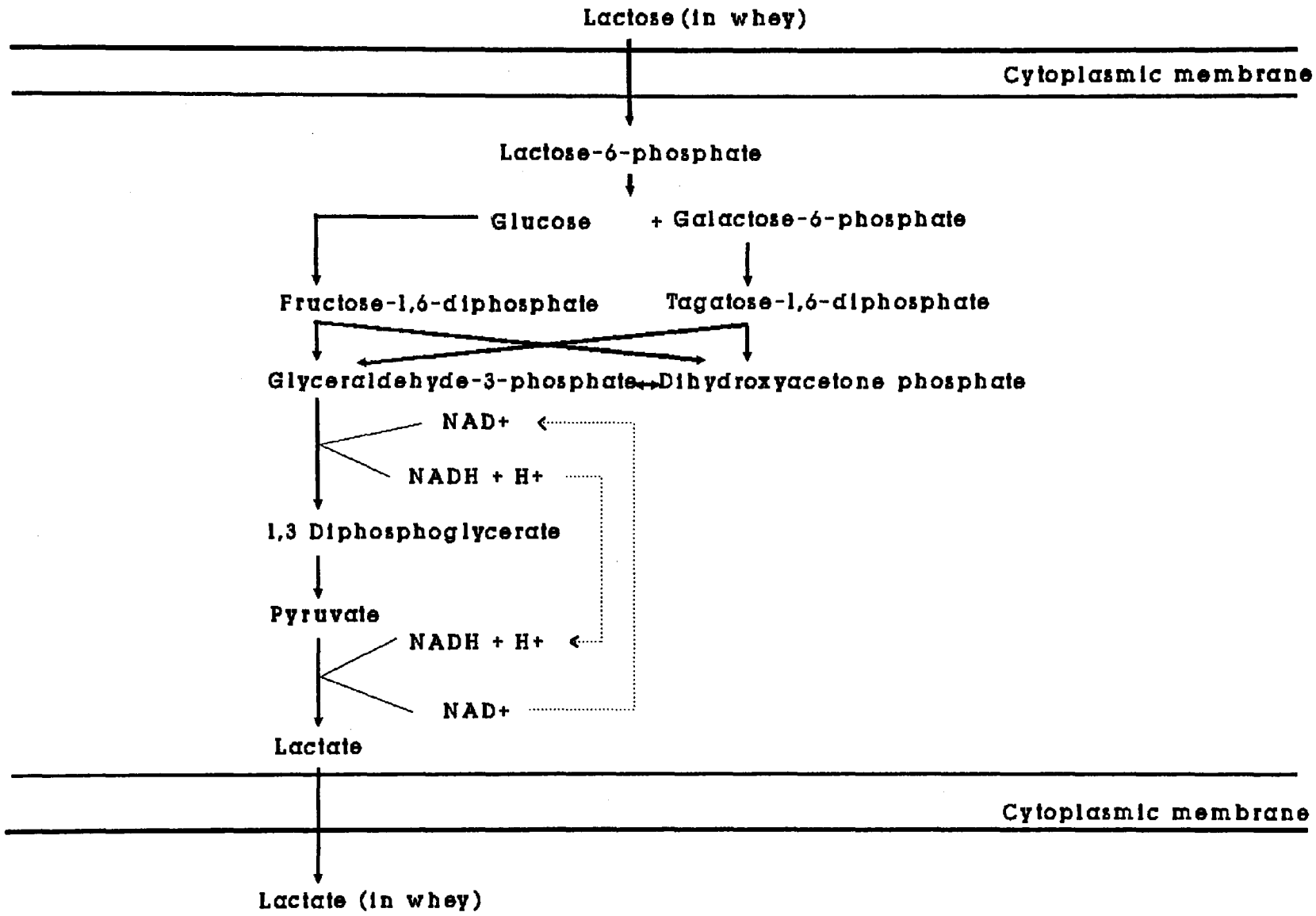
The need to monitor and control increasingly sophisticated biotechnological processes has been emphasised in recent years. With FTIR spectroscopy, we have a method for on line (and off line) measurement of lactose and lactic acid. Two associated areas require development. Firstly, the interfacing of the instrument to the fermenter's temperature, stirrer and pH units. Secondly, formation of an effective mathematical model to describe the fermentation. From this, a system for the automated measurement and control of the lactic acid fermentation could be devised. It should be emphasised that this approach could be adopted for a whole range of systems, not necessarily microbiological, involving species absorbing in the IR region.

A feasibility study on the use of optical fibres as spectroscopic probes would be desirable. Such probes, which have become commercially available over the last couple of years, could offer a realistic alternative to the CIRCLE® cell in certain applications, including fermentation monitoring.

Further investigation into the application of chemometric techniques, such as factorial design, to the fermentation of whey permeate. Modifications in the experimental design used in the optimisation procedures may lead to more reliable models for the system. Such multivariate approaches may lead to improvements in production or process economy on an industrial scale.

5. APPENDICES

APPENDIX A: The Metabolic Interconversions of Lactic Acid Fermentation.[115]



APPENDIX B

A Brief Explanation of ANOVA

ANOVA (analysis of variance) was used to estimate the efficiency of the model developed. A measure of the total variance of the data is obtained by determining the variance for n results. This is done by calculating the deviations from the overall average and squaring them. This provides a value known as the **total sum of squares (SS_T)**. This has ***n* degrees of freedom (df)** associated with it, where *n* is the total number of experiments. Dividing by *n*-1 gives an overall measure of the variance of the data. This is known as the **total mean squares (MS_T)**[37]

$$SS_T = SS_{REG} + SS_{RES}$$

SS_{REG} is the **sum of squares due to regression** i.e. sum of squares due to the factors (coefficients) as they appear in the model. The **residual sum of squares (SS_{RES})**, sometimes called the sum of squares about the regression, can be partitioned into[40]:

a **sum of squares due to lack of fit (SS_{LOF})**

a **sum of squares due purely to experimental error (SS_{PE})**

$$SS_{RES} = SS_{LOF} + SS_{PE}$$

Each sum of squares divided by its associated degrees of freedom gives an estimated variance (MS_{RES}, MS_{LOF} and MS_{PE})[37].

A measure of the adequacy of the model is assessed using an F-test.

$$\text{Fisher Variance (F) Ratio} = \frac{\text{variance of regression}}{\text{variance of residuals}} = \frac{MS_{REG}}{MS_{RES}} \qquad (1)$$

If $F_{calc} < F_{table}$, the regression equation is not significant.

The significance of the LOF is gauged by:

$$F = \frac{MS_{LOF}}{MS_{PE}}$$

If $F_{calc} > F_{table}$, the variance of LOF is significantly greater than the variance due to PE. Thus the model exhibits significant lack of fit. Conversely, if $F_{calc} < F_{table}$ then the model is adequate. This latter assessment of variance is only important if the regression equation proves to be acceptable based on (1).

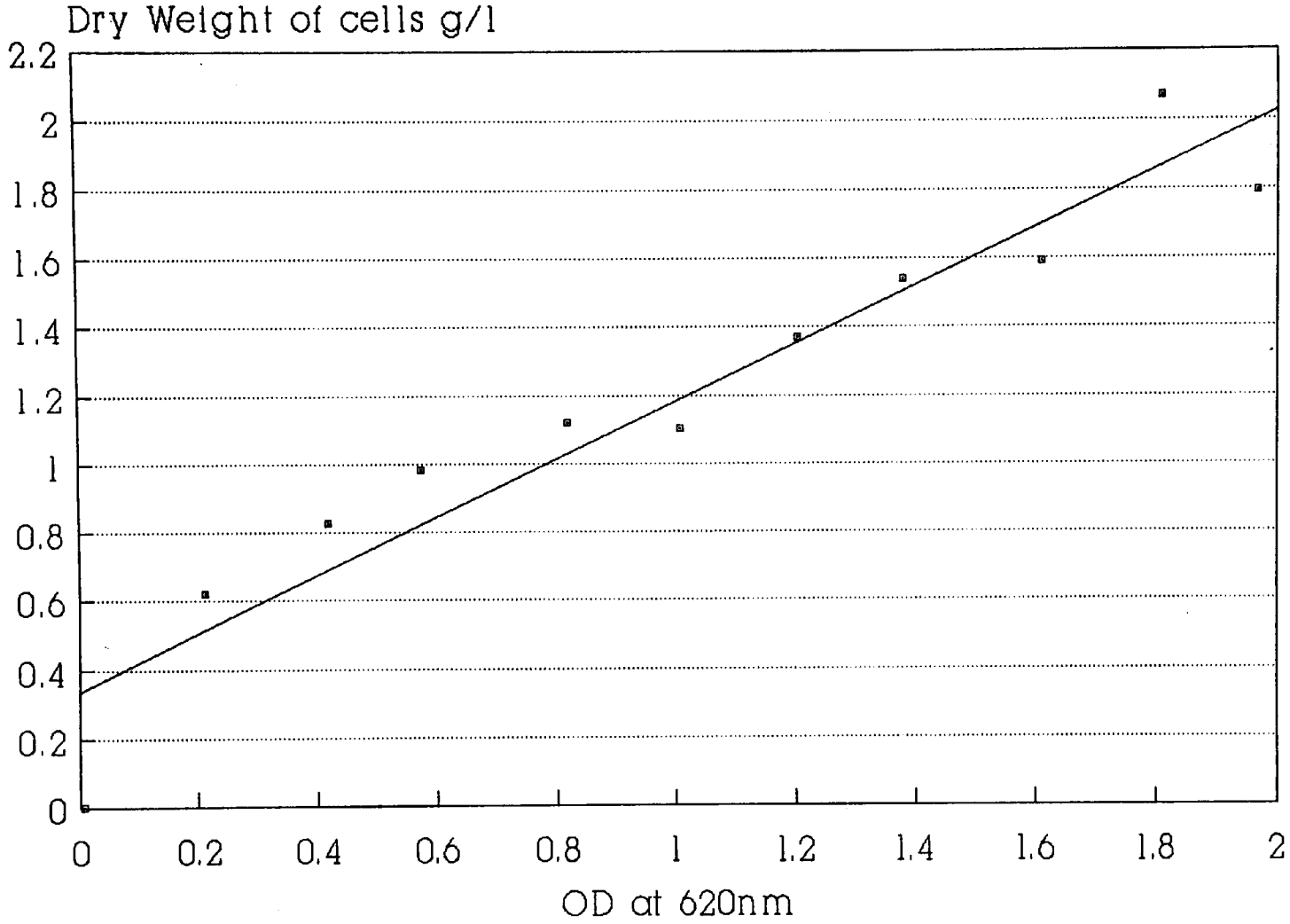
A t-test is used to assess the significance of the *b* terms (coefficients of the model equation). The t value of a given coefficient is given by:

$$\frac{b}{\text{SE of } b \text{ coefficient}}$$

SE is standard error

If $t_{calc} > t_{table}$, then *b* is non zero. If the reverse is true, $t_{calc} < t_{table}$, the minimum confidence interval is less than the critical confidence interval, and there is no reason to believe *b* is significantly different from zero.

APPENDIX C: Calibration Graph. Plot of Cell Dry Weight v Optical Density



APPENDIX D

'Raw' Data For The Continuous Culture of L. helveticus in WPo Medium

D = 0.1 h^{-1}			D = 0.2 h^{-1}		
Biomass (g/l)	Lactic Acid (% w/v)	Lactose (% w/v)	Biomass (g/l)	Lactic Acid (% w/v)	Lactose (% w/v)
1.88	3.458	1.239	2.01	2.654	2.111
1.87	3.539	1.141	2.00	2.583	2.354
1.95	3.233	1.417	2.00	2.673	2.171
1.87	3.475	1.218	2.00	2.633	1.941
1.90	3.634	1.095	2.00	2.640	2.139

D = 0.3 h^{-1}			D = 0.4 h^{-1}		
Biomass (g/l)	Lactic Acid (% w/v)	Lactose (% w/v)	Biomass (g/l)	Lactic Acid (% w/v)	Lactose (% w/v)
1.98	1.997	3.237	1.76	1.226	3.318
1.97	2.058	3.013	1.86	1.341	3.254
1.99	1.927	3.178	1.68	1.146	3.427
1.99	1.898	2.929	1.93	1.417	3.233
2.00	2.053	3.131	1.88	1.484	3.161

D = 0.6 h^{-1}			D = 0.8 h^{-1}		
Biomass (g/l)	Lactic Acid (% w/v)	Lactose (% w/v)	Biomass (g/l)	Lactic Acid (% w/v)	Lactose (% w/v)
1.73	0.926	3.891	1.31	0.658	4.159
1.71	0.897	3.888	1.25	0.592	4.028
1.67	0.943	3.842	1.31	0.618	4.091
1.66	0.942	3.791	1.33	0.617	4.095
1.70	0.906	3.941	1.30	0.624	4.087

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