PRODUCTION OF 2,3-BUTANEDIOL FROM RENNET WHEY PERMEATE BY *KLEBSIELLA PNEUMONIAE* IMMOBILIZED IN ALGINATE GEL

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ABSTRACT

The successful immobilization of cells of Klebsiella pneumoniae(NCIB 8017) in sodium alginate gels was demonstrated. A cell to alginate ratio of 1ml original cell culture to 1.25ml sodium alginate solution(20g/l) was found to be optimum for butanediol production from rennet whey permeate. Preliminary batch fermentation studies revealed that immobilized cells incubated in a non-agitated mode produced a higher concentration of 2,3-butanediol than those in an agitated mode. Smaller beads(1.8mm diameter) produced higher quantities of 2,3-butanediol than larger beads(5.5mm diameter), while bead storage at 4°C in either 0.1M Tris-HCl buffer or the gelating agent proved satisfactory although some activation was required to realise the full butanediol producing potential of the beads. Supplementation of 3g/l calcium chloride to the whey permeate was non-inhibitory to butanediol production and led to enhanced calcium alginate bead stability. Acclimatization of cells in high lactose concentration prior to cell immobilization did not result in enhanced butanediol production or lactose utilization. Product and substrate inhibition effects were not detected. In batch fermentation, a butanediol productivity of 0.11g/l.h was obtained. In continuous fermentation in a CSTR, the productivity was increased to 0.74g/l.h. Using packed columns operated in the vertical mode, similar productivities to those using the CSTR was attained. However, the columns suffered from an accumulation of carbon dioxide bubbles. This problem was overcome by placing a stainless steel mesh inside the column, and operating at an angle of 10° to the horizontal. Under these conditions, a butanediol productivity of 2.40g/l.h was achieved, representing an improvement over values reported in the literature.

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CHAPTER 1

INTRODUCTION

Whey is a by-product of cheese and casein manufacture. It contains approximately 5% lactose, 0.9% nitrogenous materials and small amounts of vitamins and minerals (Sandhu and Waraich, 1983). There are many types of whey produced by various manufacturing processes. In New Zealand, the estimated volume of whey produced and the volume processed for the 1982-1983 dairying season is presented in Table 1.1(Hobman, 1982). Whey permeate is the material remaining after ultrafiltration of whey to recover soluble proteins. The chemical co…pusition of whey permeate is shown in Table 1.2. The abundance of this dairy effluent, coupled with its high biochemical oxygen demand(ca. 33,000mg/1) means that it cannot be discharged directly into natural water systems, and thus pose a waste disposal problem to the dairy industry. In New Zealand, it is common practice to spray irrigate pasture with this material, which in turn helps to maintain soil and pasture fertility. In recent years, the dairy industry has recognised whey as a by-product rather than a waste product(Maddox and Archer, 1984). Processes such as the extraction of protein using ultrafiltration have become quite common in dairy factories. More recently, the realization that whey and its permeates are potentially useful substrates for microbial fermentation has generated interest in many temperate countries. A list of some microbial products that can be obtained via fermentation using various microorganisms is shown in Table 1.3.

The ever-increasing international demand for crude oil has resulted in fear and uncertainty as to the future supply of this dwindling natural resource . Thus, the idea of producing chemical feedstocks and liquid fuels, such as 2,3-butanediol, from naturally renewable resources such as whey, becomes an intriguing possibility for the future. The importance of 2,3-butanediol lies in its chemical properties. Its dehydration yields the industrial

Table 1.1 An estimate of the volume of whey produced and the volume processed for the 1982-1983 dairying season in New Zealand(Hobman, 1982)

	Base Product	
	Cheese	Casein
Quantity(tonnes)	120,000	57,000
Volume of whey produced (m^3)	912,000	1,480,000
Volume of whey used(m^3) for:		
Whey cheese	2,600	-
Lactose	373,000	-
Whey powder	100,000	108,000
Whey protein (extraction only)	-	293,000
Whey protein extraction plus lactose utilization	60,000	371,000
Miscellaneous	500	-
Total	536,100	772,000
Percentage of whey partially or totally processed	59	52
Percentage of whey not processed	41	48

Table 1.2 Chemical composition of whey permeates (Matthews, 1978)

	Sulphuric casein whey permeate	Lactic casein whey permeate	Rennet whey permeate
Total solids %	5.69	5.93	5.40
Ash %	0.78	0.71	0.44
Lactose %	4.26	4.21	4.77
Na g/kg	0.6	0.48	0.36
Ca g/kg	1.17	1.36	0.36
K g/kg	1.45	1.53	1.41
Cl g/kg	0.09	0.98	1.06
PO_4 g/kg	1.92	2.0	0.68
SO_4 g/kg	1.51	-	-
Lactate g/kg	-	0.64	-

Table 1.3 Some products obtained from whey by microbial fermentation

Organism	Product	Reference
Yeast	ethanol	Maddox & Archer (1984)
Yeast	ethanol (whey wine)	Kosikowski (1979)
Kluyveromyces ragi	ethanol	King & Zall (1983)
Kluyveromyces fragilis	single cell protein	Sandhu & Waraich (1983) Kosikowski(1979)
Wingea robertsii	single cell protein	Sandhu & Waraich (1983)
Fusarium moniliforme	gibberellic acid	Gohlwar *et al* (1984)
Clostridium acetobutylicum	butanol	Maddox & Archer (1984)
Methanogens	methane	-do-
Asperigillus niger	citric acid	-do-
Klebsiella pneumoniae	2,3-butanediol	Lee & Maddox (1984)

solvent methyl ethyl ketone. Further dehydration produces 1,3-butadiene which is the monomer of synthetic rubber. Dimerization of butadiene by the Diels-Alder reaction gives styrene, an important aromatic intermediate in the lucrative polymer industry.

The advantages of fermentation systems based on immobilized cell technology have resulted in enhanced productivities over those observed in traditional batch systems. The objective of the present work was to investigate the feasibility of producing 2,3-butanediol from rennet whey permeate based on the principles of continuous fermentation and immobilized cell technology.

CHAPTER 2

LITERATURE REVIEW

2.1 Immobilized Cell Technology

Interest in the utilisation of immobilized cells has grown remarkedly during the last decade. This is evident by the increasing number of publications in this field. Thus, whereas in 1973 only seven papers appeared on immobilized cells(as compared with 200 on immobilized enzymes), four years later more than 50 dealt with this new technology (Dunnill, 1980). The term "cell immobilization" implies the prevention of free movement of a material of one phase within another and is therefore a physical confinement or localization of a microorganism that permits the economical reuse of the microorganism.

2.1.1 Reasons for cell immobilization

Cells are immobilized for a variety reasons of varying complexity. Some of the reasons are listed below (Abbot, 1977) :-

(a) to present a single enzyme produced by and contained within the cells in a convenient form for commercial use.

(b) to make available for use a sequence of enzymes produced by the cell.

(c) to provide a highly concentrated source of viable cells for use in the biotransformation of substances/substrates, or as a large inoculum for conventional fermentation technology.

(d) to bring together biochemical systems that could not normally exist together.

(e) to allow the medium-term storage of the cells for commercial use without further growth.

(f) to preserve cell lines stored only with difficulty by conventional means.

2.1.2 Advantages of cell immobilization

Immobilized microorganisms may display properties quite different from those of freely suspended cells. The specific advantages are dependent on the immobilization method employed, and on the characteristics of the process used for the comparison(Abbot, 1977). The most obvious benefit of cell immobilization is the capability of recycling or reusing the microbial cells. Reuse of the cells provides a means for making batch processes continuous and for maintaining a high cell population so as to achieve fast reaction rates. In continuous processes, immobilized cells are less subject to the effects of inhibitory compounds or nutrient depletion. The introduction of a toxic compound or depletion of a nutrient causes a washout of cells from conventional continuous fermentation. However, microorganisms can be retained in an immobilized-cell reactor until a favourable environment is restored. Furthermore, a continuous immobilized-cell reactor can be operated at high dilution rates exceeding the maximum specific growth rate of the organism concerned. Another advantage is that higher volumetric productivities may be possible because of the greater cell densities which can be incorporated into an immobilized-cell reactor(Bucke, 1983).

When microorganisms are attached to a relatively large solid support, fluid viscosity is lower than when comparable numbers of cells are freely suspended. This lower viscosity tends to contribute to better mixing and mass transfer properties in the reactor. Two other factors may also enhance mass transfer in immobilized cell systems. Thus, Hattori(1972) suggested that the solid supports employed in the immobilization process, when placed in a dilute medium, may concentrate nutrients at the liquid-solid interface. The entrapped microorganisms are therefore exposed to a higher nutrient concentration than that which exists in the bulk solution. The second factor by which immobilization improves mass transfer is through an increase in the apparent density of the microorganism(Martin et al, 1976). The density of freely suspended cells is very similar to the density of the surrounding medium. As a

result, the differential velocity between the cells and suspending liquid medium is relatively small and because diffusional distances are large, the low differential velocities result in slow nutrient diffusion rates. On the other hand, the effective density of immobilized cells is essentially the same as the density of the support matrix. Thus, when a dense matrix is employed, the resultant increase in differential velocities will lead to enhanced nutrient diffusion rates. Lastly, cell immobilization avoids the use of costly enzyme immobilization procedures and allows multistep enzymatic reactions otherwise not possible with enzyme immobilization.

2.1.3 Disadvantages of cell immobilization

One of the primary disadvantages of cell immobilization is the loss of some desirable catalytic activity. Such losses may be due to enzyme inactivation during the immobilization process or to diffusional barriers that hinder substrate and product diffusion to and from the cell matrix. Such a diffusional barrier is the NERNST Layer which is the unstirred liquid layer surrounding the immobilized particle. Further, underlying cells, located deeper in the support matrix, often do not gain access to nutrients and thus act as no more than a support layer to superficial cells(Abbot, 1977). The adsorption of reaction products on the support matrix may result in reduction of productivities if the entrapped microorganism is sensitive to product inhibition. The support matrix may itself be toxic to the cells;for example Starostina et al(1983) found that cells of E. coli, when immobilized in polyacrylamide gel, were severely inhibited with regard to cell division and nucleic acid production, and the osmotic stability of the cell membrane was diminished.

2.1.4 Immobilization methods

Mosbach(1983) and Bucke(1983) classified cell immobilization procedures into four principal approaches. These are:- covalent attachment(binding), entrapment (including microencapsulation), adsorption(including bioadsorption to a biospecific ligand such as concanavalin

A), and cross-linking leading to insoluble aggregates. Of the various procedures, entrapment within different polymeric gels is by far the most widely applied method.

2.1.4.1 Covalent attachment

This approach has been widely and sucessfully used for enzyme immobilization. However, the attachment of whole cells to surfaces using binding agents of a generally toxic nature has not been very successful (Mattiasson, 1983). If viable cells are immobilized in this manner, any newly formed cells could not be bound and substantial cell leakage would result. Most studies have therefore employed non-viable cells and the reported productivities of the systems are relatively poor. Examples of cell immobilization employing this procedure include production of uronic acid(Jack and Zajic, 1971) and gluconic acid(Nelson, 1976) . Kennedy(1979) has proposed an interesting approach using hydrous titanium(IV) and zirconium(IV) as support materials. He postulated that partial covalent bonds were formed between cell components, such as protein and carbohydrates, and the support materials. Other polymer supports and covalent coupling agents which have been used include derivatized porous glass with glutaraldehyde, carboxymethyl-cellulose with carbodiimide, and hydroxyalkyl methacrylate gel with glutaraldehyde (Blanch, 1984). Cell loadings were, however, low.

2.1.4.2 Adsorption

This method involves the formation of ionic and hydrogen bonds between the cell surface and the support, and which are primarily determined by the charge distribution of carboxy and amino groups on the cell wall. Consequently the properties of the support will determine the resulting behaviour of the system(Dunnill, 1980, Mosbach, 1983; Blanch, 1984). For example, cell loading will be determined by the surface area of the support material, its charge and the available pore size. As adsorption is a reversible process, cell leakage is a characteristic feature. Table 2.1 lists some of the variety of supports

Table 2.1 Cell immobilization by adsorption (Blanch, 1984)

Product	Organism	Support Material
Succinic acid	*Escherichia coli*	Ion exchange resin
Acetic acid	*Acetobacter*	Ceramic support
Lactic acid	*Lactobacillus* and yeast	Gelatin
Ethanol	*Saccharomyces cerevisiae*	Anion exchanger
Ethanol	*Saccharomyces carlsbergenesis*	PVC and porous glass
Ethanol	*Zymomonas mobilis*	Glass fibre pads
Phenol degradation	*Pseudomonas* sp	Anthracite

and organisms which have been investigated to date.

2.1.4.3 Cross-linking leading to insoluble aggregates

Perhaps the best example of this method is that cited by Bungard et al(1979) in which bacterial cells containing glucose isomerase were immobilized simply by heating the flocculated cell mass, or by cross-linking with bifunctional reagents such as glutaraldehyde (Hemmingsen, 1979). One disadvantage of this method is that some cells are sensitive to cross-linking agents such as glutaraldehyde and cell lysis may occur as a result.

2.1.4.4 Entrapment

Immobilization of living cells by entrapment was first reported by Mosbach(1966) who used lichen cells to catalyse the enzymic sequence of;

$$\text{depside ester} \xrightarrow{\text{esterase}} \text{orsellinic acid} \xrightarrow{\text{decarboxylase}} \text{orcinol}$$

Later, Mosbach and Larsson(1970) demonstrated that immobilized cells could grow while present in polyacrylamide supports. Since then, extensive research has been reported in the literature relating to the use of different support materials for the entrapment of whole cells. Entrapment is now the most commonly used method of cell immobilization. Although a variety of support materials have been employed, they can be classified into natural and synthetic polymers. The use of synthetic polymers often results in cell damage because of the harsh polymerization conditions involved. Some of the synthetic polymers which have been used in the past include polyacrylamide, polyvinylchloride and polyurethane. Polyacrylamide gel was the first gel used to entrap living microbial cells(Chibata and Tosa, 1977). Although polyacrylamide gel was extensively used in the past, its toxic nature towards entrapped cells resulted in an investigation by Starostina et al(1983) who reported that cell division of entrapped E. coli was blocked. The cells were elongated and the synthesis of nucleic acid

was inhibited. A decrease in cell osmotic stability was also observed. In contrast to polyacrylamide gel, photocrosslinkable resin, a synthetic polymer which has been recently developed,(Sonomoto et al, 1982) is suitable for immobilized living cell systems mainly because the immobilization can be performed under mild conditions(Chibata et al, 1983). Similarly the use of polyurethane has been quite extensive due to the use of a prepolymer to avoid the exposure of the cells to harsh reaction conditions(Mattiasson, 1983). The advantage of polyurethane lies in its fairly open structure and its flexibility to be produced in any desired size or shape for different kinds of bioreactor.

Amongst the natural polymers, K-carrageenan and calcium alginate gels are by far the most widely investigated support materials for cell immobilization by entrapment. The popularity of these two matrices is due to their mild conditions towards cells during the immobilization procedure. Further, the method is simple and does not require any specific environmental conditions. Wada et al(1979) were the first to use K-carrageenan and this support material has since been intensively investigated. In several cases polyacrylamide entrapment has been replaced by K-carrageenan for use in industrial processes(Chibata, 1979).

Like K-carrageenan, alginate(alginic acid) is an algal polysaccharide consisting of copolymer of D-mannuronic and L-glucuronic acids with the detailed structure varying with the source. The most important property of alginate for cell immobilization is its gel strength. Gels are formed with divalent cations which cross-link glucuronic acid units of different molecules. Thus, alginic acids with a high glucuronic acid content are generally preferred. For example, alginic acids derived from Laminaria species generally have more glucuronic groups as compared to those derived from Marocystis or Ascophyllum species, which have a high proportion of mannuronic groups. Since the early work by Hackel et al(1975), calcium alginate gel has been used

for the immobilization of cells and enzymes. Kierstan and Bucke(1977) found that alginate gel provided a suitable matrix for the immobilization by entrapment of whole microbial cells, subcellular organelles and isolated enzymes. Their studies also revealed that immobilized cells retained a very high effectiveness, and the procedure was mild and not detrimental to cell activity. Thus, immobilized Saccharomyces cerevisiae cells were found to retain their ability to produce ethanol for several months when supplied only with glucose or sucrose. The mechanical stability of alginate gel beads was studied by Cheetham(1979) who demonstrated the feasibility of using calcium alginate beads on a large scale in packed bed reactors, where there were only low intrinsic pressure drops. No channelling, abrasion of pellets or compression were observed.

Although calcium alginate has been the preferred matrix in many investigations, it is not without its disadvantages. The calcium may be removed by chelating agents such as phosphate and EDTA or displaced by other cations such as Mg^{2+}, K^{+} and Na^{+}. Strontium and barium alginates are more stable to chelating agents and have been used successfully to entrap bacterial chromatophores (Paul and Vignais, 1980).

Other natural polymers which have been used to entrap whole cells include agar, collagen and gelatin. However, these polymers have lost favour among many workers mainly because of the high cell leakage and practical problems often encountered upon scaling-up operations.

A recent development of entrapment is to immobilize cells between membranes. The advantage of this new technique is that various geometries are possible such as flat sheets, speres, hollow fibres and spiral wound fibres. This method is also a gentle approach to immobilization and has been extensively used for plant

and mammalian cell immobilization(Brodelius and Mosbach, 1982; Mattiasson, 1983), and also for bacterial cells in hollow fibre geometry(Vick Roy et al, 1983). Some examples of biochemicals produced by cells immobilized via entrapment are listed in the literature reviewed by Blanch(1984), Chibata(1983), Abbot(1977), Mattiasson(1983), Mosbach(1983) and Dunnill(1980). However, as calcium alginate is the support matrix used in the present investigation, examples of biochemicals produced by various microorganisms entrapped in this polymer are listed in Table 2.2

2.1.5 Factors influencing product formation using alginate immobilized cells

The factors which contribute to enhance product formation may also be detrimental when they occur in excess. Thus, most researchers strive for the optimum combination of factors or parameters which when operated simultaneously would bring about a sustained and high level of product formation. Some of the factors which contribute to enhanced metabolite production include cell density, bead size and the concentrations of calcium chloride and sodium alginate used in the immobilization procedure.

With respect to cell density, most reports, especially in alcohol research, have revealed that an increase in cell density results in a corresponding increase in ethanol production. Thus, Williams and Munecke(1981) reported that when the concentration of entrapped cells of Saccharomyces cerevisiae was increased, ethanol production also increased but the specific bead productivity was higher at lower cell loading. The authors attributed this to the limitations of substrate and product diffusion at high cell loads. Similar patterns of increased cell density were also reported by Marwaha and Kennedy(1984). Suihko and Poutanen(1984) also demonstrated that high volumetric rates of ethanol production were possible with high cell density, but the

Table 2.2 Some biochemicals produced by microorganisms entrapped in calcium alginate gel

Organism	Substrate	Product/ reaction	Reference
Saccharomyces cerevisiae	glucose	ethanol	Cho et al(1982)
-do-	-do-	-do-	Cho & Choi(1981)
-do-	-do-	-do-	Williams & Munecke(1981)
-do-	-do-	-do-	Mcghee et al(1982)
-do-	-do-	-do-	Lee et al(1983)
-do-	-do-	-do-	Doran & Bailey(1984)
-do-	-do-	-do-	Holsberg & Margalith(1981)
-do-	D- xylulose	-do-	Suiko & Poutanen(1984)

Table 2.2(Cont.)

Organism	Substrate	Product/ reaction	Reference
Saccharomyces cerevisiae and -glucosidase	cellobiose	ethanol	Hagerdal(1984)
Saccharomyces cerevisiae (Brewer's yeast)	wort	-do-	White & Portno(1978)
Kluyveromyces fragilis	whey	-do-	Linko et al(1981)
-do-	Jerusalem artichoke tubers	-do-	Margaritis & Bajpai(1981)
Kluyveromyces ragi	whey	-do-	King & Zall(1983)

Table 2.2(Cont.)

Organism	Substrate	Product/ reaction	Reference
Kluyveromyces marxianus	whey	ethanol	Marwaha & Kennedy(1984)
Zymomonas mobilis	glucose	-do-	Margaritis et al(1981)
-do-	-do-	-do-	Klein & Kressdorf(1983)
Clostridium butyricum	-do-	iso-propanol butanol	Krouwel et al(1980)
Clostridium acetobutylicum	-do-	acetone butanol	Haggstrom(1980)
-do-	-do-	acetone, acetic acid, butanol and butyric acid	Haggstrom & Molin(1980)

Table 2.2(Cont.)

Organism	Substrate	Product/ reaction	Reference
Clostridium beijerinckii	glucose	iso-propanol, butanol and ethanol	Krouwel *et al*(1983)
Aspergillus niger	sucrose	citric acid	Vaija *et al*(1982)
Aspergillus niger	glucose	-do-	Eikmeier & Rehm(1984)
Lactobacillus delbrueckii	-do-	lactic acid	Stenroos *et al*(1982)
Lactobacillus vaccinostercus	xylose	-do-	Tipayang & Rozaki(1982)
Aspergillus niger	glucose	gluconic acid	Linko(1981)

Table 2.2(Cont.)

Organism	Substrate	Product/ reaction	Reference
Trigonopsis variabilis	amino acid	α-keto acid	Brodelius *et al*(1980)
Streptomyces species	mannitol	tylosin and nikomycin	Veelken & Pape(1982)
Penicillium chrysogenum	glucose	penicillin G	Morikawa *et al*(1979)
Propionibacterium species	-do-	vitamin B_{12}	Yongsmith & Chutma(1983)
Arthobacter simplex	hydrocortisone	Δ^1-dehydrogenation	Kloosterman IV & Lily(1985)
Curvularia lunata	cortisol	steroid Δ_1 - dehydrogenation	Ohlson *et al*(1980)

Table 2.2(Cont.)

Organism	Substrate	Product/ reaction	Reference
Rhizopus nigricans	progesterone	11-α-hydroxylation	Maddox et al(1981)
Pseudomonas species	phenol	phenol degradation	Betmman & Rehm(1984)
Pseudomonas denitrificans	water	water denitrification	Nilsson et al(1980)
Anabaena species	photoproduction	ammonia	Kerby et al(1983)
-do-	-do-	-do-	Musgrave et al(1982)

high productivity could not be sustained for long periods under anaerobic conditions due to cell death. However, other workers, notably Stenroos et al(1982), demonstrated that varying the cell mass of Lactobacillus delbrueckii during immobilization had little effect on subsequent lactic acid production. These authors postulated that the relative ease of cell growth within the gel matrix had contributed to a stable cell density. Decreased metabolite production as a result of increased cell loading was also reported by Hiementra et al(1983) who observed that a corresponding decrease in oxygen diffusion resulted in lower rates of methanol oxidation by entrapped Hansenula polymorphia cells. The limitation of oxygen transfer was also noted by Adlercreutz and Mattiasson (1984) who recorded a substantial decrease in dihydroxy-acetone production by immobilized cells of Gluconobacter oxydans. Another report of an increase in cell density was noted by Brodelius et al(1980) who revealed that a large increase in cell mass resulted in only a small increase in α-ketoacid production. The authors attributed this to mass transfer limitations.

Unlike cell density, increasing the calcium chloride concentration, and thus the amount of cross-linking within the gel, is often detrimental to the metabolism of the entrapped microorganism although mechanical stability of the alginate pellets is often enhanced. Thus, Holsberg et al(1981) found that ethanolic fermentation ability by entrapped Saccharomyces cerevisiae was inhibited at calcium chloride concentrations above 0.05M. This pattern was also reported by Marwaha and Kennedy (1984) who demonstrated that beads formed at calcium chloride concentrations greater than 0.1M led to poor ethanol yields. However, a majority of workers have reported that an optimum calcium chloride concentration exists and this concentration varies with the microorganism to be entrapped. For example, Tipayang and Rozaki(1982) found that a concentration of 20-30g/l was the optimum concentration for immobilizing cells of Lactobillus vaccinostercus, while Banerjee et al(1984) reported that

maximum enzymatic activity of Saccharomyces cerevisiae cells was obtained when a 10g/l solution of calcium chloride was used in the immobilizing procedure. Other workers such as Margaritis et al(1981) found that the presence of calcium ions in the substrate was necessary for both bead hardening and metabolism of Zymomonas mobilis.

The effect of the sodium alginate concentration on the metabolic behaviour of entrapped microorganisms and the physical properties of the alginate beads has been investigated by several workers, notably Kierstan and Bucke(1977) and Cheetham et al(1979). The former group found that an increase in sodium alginate concentration led to an increase in the retention efficiency of high molecular weight compounds while the gel matrix did not appear to form a barrier to diffusion. The latter group however, disclosed that a low alginate concentration resulted in beads of poor mechanical strength but with good substrate diffusion properties. Alginate beads were not formed if a concentration of only 1g/l was employed. A later investigation by Ohlson et al(1979) revealed that an alginate concentration of 20-30g/l was optimal for high steroid $\triangle^1$-dehydrogenase activity of immobilized cells of Arthrobacter simplex. These authors also noted that concentrations higher than 30g/l led to high viscosity, and they encountered practical difficulties in forming the alginate pellets. Other workers have also reported critical alginate concentrations for maximum product formation. Thus, Yongsmith and Chutma(1983) revealed that maximum vitamin B_{12} production from entrapped Propionibacterium sp was obtained using an alginate concentration of 12g/l. Banerjee et al(1984) reported that entrapped cells of Saccharomyces anamensis exhibited highest enzymatic activity at an alginate concentration of 20g/l. Jain and Ghose(1984) indicated that 40g/l of alginate gave the best stability and enzymatic activity of immobilized cells of Pichia etchelsii. However, many reports reveal a broad optimum alginate concentration. Tramper et al(1983) found there was no significant

difference in the oxidizing activity of entrapped Gluconobacter oxydans when 15 or 20g/l of sodium alginate were used. Marwaha and Keenedy(1984) revealed that the concentration range 10-20g/l of sodium alginate did not affect ethanol production by immobilized cells of Kluyveromyces marxianus. However, concentrations higher than 25g/l decreased ethanol yields, but there was no cell leakage which was characteristic of lower concentrations. It appears therefore that the optimum sodium alginate concentration employed in the cell immobilization is dependent on the species of microorganism.

Another factor which influences product formation by entrapped cells is that of bead size. The majority of reports state that an increase in bead size leads to a corresponding decrease in product formation and enzymatic activity. This has been attributed to limitations to substrate and product diffusion due to greater distances for the substrate end product to diffuse into and out of the gel matrix(Ohlson et al, 1979; Margaritis et al, 1981; Tramper et al, 1983; Veelken and Pape, 1982; Yongsmith and Chutma, 1983). Klein et al(1980) noted that an increase in bead size led to an increase in the critical compression ratio and also an increase in abrasion rates of the gel pellet during stirred tank reactor experiments. Thus, the parameter of bead size seems to have an inverse relationship with the metabolic behaviour of entrapped microorganisms.

Since one of the claimed advantages of immobilized cells is their reuse, the problem of storage has been investigated by several workers, most of whom reported no loss of activity provided the beads were stored in buffer or in the gelating solution at $4^{o}C$ or lower(Ohlson et al, 1979; Nilsson et al, 1980; Paul and Vignais, 1980; Banerjee et al, 1984; Jain and Ghose, 1984).

The problem of excessive carbon dioxide evolution using immobilized cells in packed columns has been encountered by many investigators. As a result of excessive carbon dioxide accumulation, the back pressure increases within the column resulting in bead compaction(Cho and Choi, 1981). These authors suggested the use of a

fluidized bed to avoid the problem, whilst Margaritis et al(1981) found that the inclusion of a steel mesh into the column enhanced carbon dioxide removal from the column.

Reports in the literature regarding the effect of temperature on the performance of immobilized cells are rather conflicting. Thus, Nilsson et al(1980) revealed that there was no difference in the optimum temperature of entrapped and free cells of Pseudomonas dentrificans. Similarly, Stenroos et al(1982) found that lactic acid production by free and immobilized cells of Lactobacillus delbrueckii had the same temperature range. In contrast to these findings, Williams and Munecke(1981), using Saccharomyces cerevisiae, showed that the optimum for free cells was 37°C whilst that of immobilized cells was 30°C. However, Banerjee et al(1984) found that immobilized Saccharomyces cerevisiae had a higher optimum temperature(37°C) than that of free cells (30°C). Unlike temperature, most investigators have reported no significant difference inpH optimum between free and immobilized cells(Nilsson et al, 1980; King and Zall, 1983).

2.1.6 Methods used in alginate bead stabilization

The approaches employed by investigators to maintain calcium alginate integrity as the result of the adverse effects of phosphate ions can be broadly divided into four types. These are; the addition of calcium chloride to the substrate itself(so as to precipitate out the phosphate ions), partial drying of the alginate pellets, increasing the alginate concentration, and surface coating of the alginate beads with cross-linking agents.

2.1.6.1 Addition of calcium chloride to the substrate

The earliest report of this method was made by Klein et al(1979) who demonstrated that the incorporation of calcium ions into the substrate could enhance alginate bead stability considerably. A year later, Nilsson et al(1980) also revealed that the presence of calcium chloride(5mM) in the substrate also enhanced

bead stabilization. Krouwel et al(1983) did not detect any inhibitory effect of calcium ions(5g/1) in glucose fermentation with free cells of Clostridium beyerinckii A recent investigation by Schoutens et al(1985) using Clostridium beyerinckii revealed that a calcium chloride concentration of 5g/1 was adequate in maintaining bead integrity in whey permeate, as well as being noninhibitory noninhibitory to butanol production.

2.1.6.2 Partial drying of alginate beads

This procedure was reported by Klein et al(1980) who demonstrated that alginate beads dried by hot air currents had the highest mechanical strength and operational stability. This finding was later confirmed by Krouwel et al(1982).

2.1.6.3 Surface coating with crosslinking agents

This approach was adopted by many investigators from the early research done with immobilized enzymes. Cheetham et al(1979) reported that although the addition of 1g/1 of glutaraldehyde enhanced bead stability, it also decreased immobilized yeast activity. However, Haggstrom(1980) did not notice any inhibitory effect of glutaraldehyde(1g/1) on the activity of entrapped Clostridium acetobutylicum. This result was also reported by Linko et al(1980) who used 2.5% glutaraldehyde on entrapped Saccharomyces cerevisiae cells. Other investigators who used different cross-linking agents such as polyethyleneimine(Veliky, 1981), sodium metaperiodate and carbodiimide(Birnbaum, 1981), and partially quaternized polyethyleneimine(Tanaka et al, 1984) also found that these compounds were ideal for hardening alginate beads, but the cell activity varied with the concentrations, duration and species of organism entrapped within the gel matrix.

2.1.6.4 Other miscellaneous methods of bead stabilization

Paul and Vignais(1980) investigated the use of other cations such as barium and strontium. They

reported that barium alginate gave the best stability. The highest metabolic activity from entrapped bacterial chromatophores was also obtained from this gel pellet. These authors also noted that the barium alginate pellets were smaller than those of strontium and calcium alginate. Another approach which has been practised is to increase the sodium alginate concentration at the expense of product formation. Thus, Williams and Munecke(1981) found that although bead stability was enhanced with an increase in the sodium alginate concentration, ethanol production decreased correspondingly.

2.1.7 Industrial applications of immobilized cells

Perhaps the best known industrial immobilized cell process is that of the Tanabe Seiyaku company which employs E. coli entrapped in polyacrylamide gel for the stereoselective conversion of fumaric acid to L-aspartic acid.The half-life of the immobilized cell catalyst is 120 days and the cost of production of aspartic acid by this method has been estimated to be 40% less than that using a conventional process employing freely suspended cells(Dunnill, 1980). Recent developments in Japan have indicated that continuous alcohol production using alginate-immobilized yeast has reached the pilot plant stage, and a project undertaken by the Research Association for Petroleum Alternatives Development(RAPD) has been initiated to commercialise this process(Nagashima et al, 1983). Some of the industrial applications of immobilized cells in industry are summarized in Table 2.3.

2.2 Microbial Production of 2,3-Butanediol

2.2.1 Biochemistry

2,3-Butanediol can be produced by fermentation of sugars. The biochemical reactions leading to the formation of 2,3-butanediol from a hexose are depicted in Fig 2.1. Juni(1952) was the first to report that Klebsiella pneumoniae forms acetoin(3-hydroxybutanone) from pyruvate by the action of acetolactate synthetase and acetolactate decarboxylase. Eight years later,

Table 2.3 Industrial processes employing immobilized cells(Bucke, 1983)

Microorganism	Support Material	Application	Operating since
Escherichia coli	polyacrylamide	production of aspartic acid	1973
Brevibacterium ammoniagenes	polyacrylamide	production of L-malic acid	1974
Bacillus coagulans	cell paste cross-linked with glutaraldehyde	source of glucose isomerase	1979
Arthrobacter sp	polyelectrolyte	source of glucose isomerase	1979
Saccharomyces cerevisiae	calcium alginate	ethanol production	pilot plant 1983

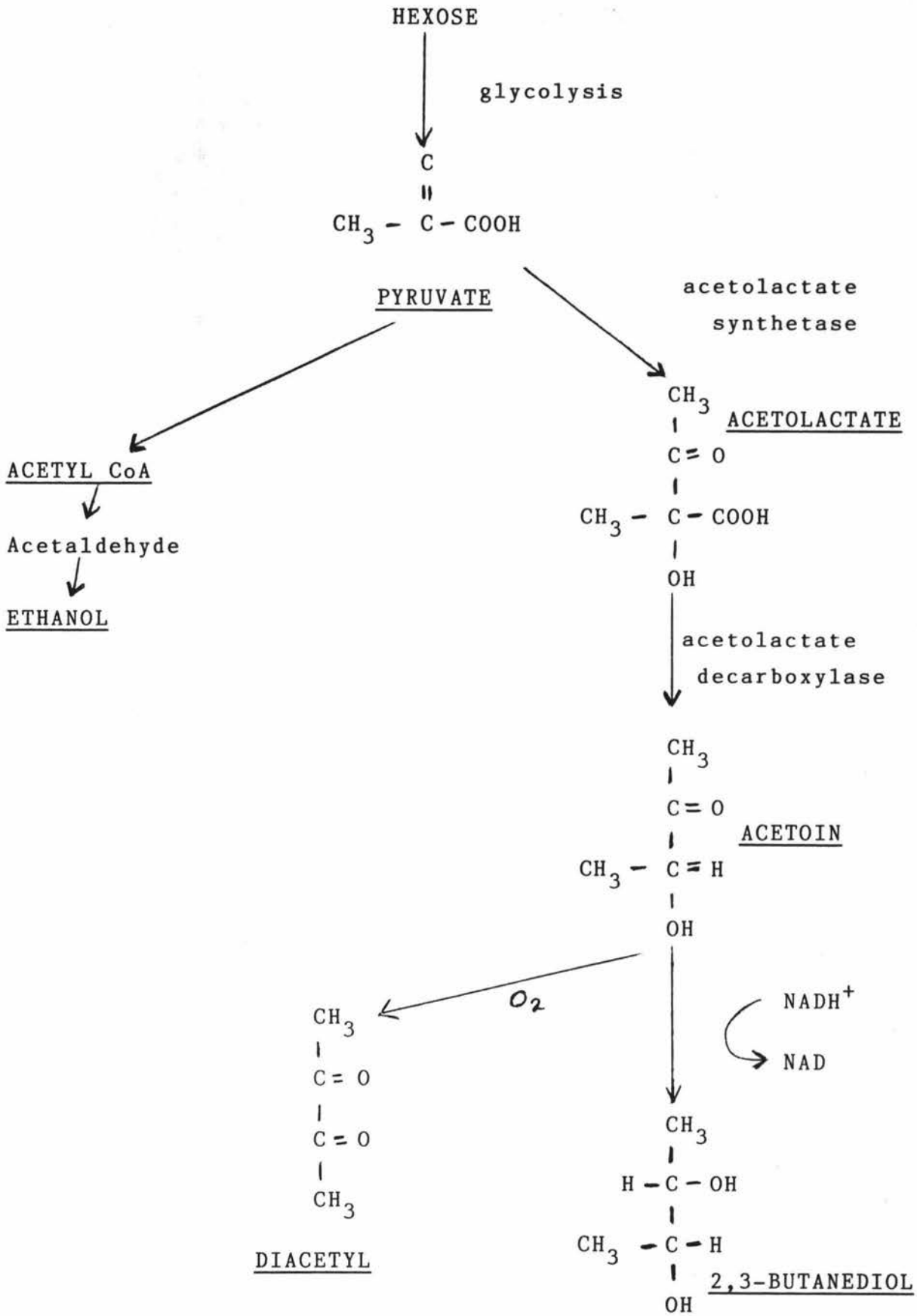
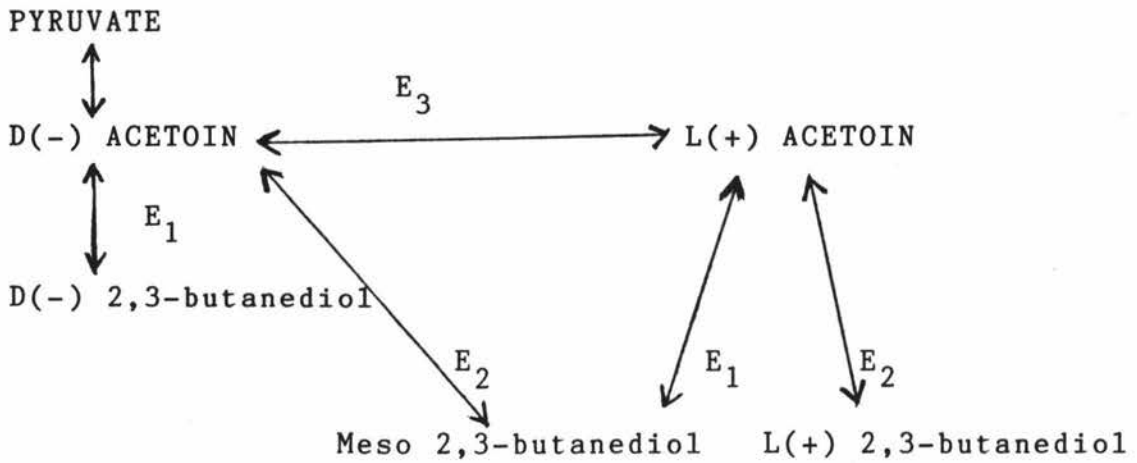


Fig 2.1 The biochemical pathway leading to the formation of 2,3-butanediol from a hexose(Taylor and Juni, 1960; Voloch et al, 1983)

Taylor and Juni(1960) pioneered the first model(Fig. 2.2) elucidating the formation of 2,3-butanediol stereoisomers from glucose. Their model postulated that as a consequence of glycolysis, acetoin is formed from pyruvate by the action of two enzymes; (i) acetolactate synthetase which catalyses the condensation of two pyruvate molecules with a single decarboxylation to yield acetolactate, and (ii) acetolactate decarboxylase which catalyses the decarboxylation of acetolactate to acetoin. The acetolactate decarboxylase is specific for the dextrorotatory isomer of acetolactate and the reaction product is D(-) acetoin. The acetoin so produced can either be oxidized to diacetyl(by oxygen present in the medium) or reduced enzymatically to 2,3-butanediol using $NADH^+$ as cofactor. Ethanol is also an end-product. The model proposed by Taylor and Juni(1960) was based on the observed optical rotation of acetoin produced from pyruvate, the composition of the 2,3-butanediol stereoisomers formed during the fermentation and the rate of oxidation of the 2,3-butanediol stereoisomers. These authors proposed the existence of three enzymes, acetoin racemase, L(+) 2,3-butanediol dehydrogenase and D(-) 2,3-butanediol dehydrogenase. The dehydrogenases were assumed to be nonspecific with respect to acetoin stereoisomers in that they would accept either isomer as substrate. The authors, however, were unable to demonstrate the presence of acetoin racemase.

A later study by Voloch et al(1983) suggested that the conversion of acetoin to 2,3-butanediol by Klebsiella pneumoniae involved the presence of acetoin racemase and two acetoin reductases, one specific for D(-) acetoin and the other for L(+) acetoin. Their model, shown in Fig. 2.3, postulated that the enzyme E_1 catalyses the reduction of D(-) acetoin to meso 2,3-butanediol but does not utilise L(+) acetoin. A systematic name for E_1 was thought to be meso 2,3-butanediol NAD^+ oxidoreductase D(-) acetoin-forming. The enzyme E_2 was envisaged to catalyse the reduction of L(+) acetoin and a possible systematic name for E_2 could be L(+) 2,3-butanediol:

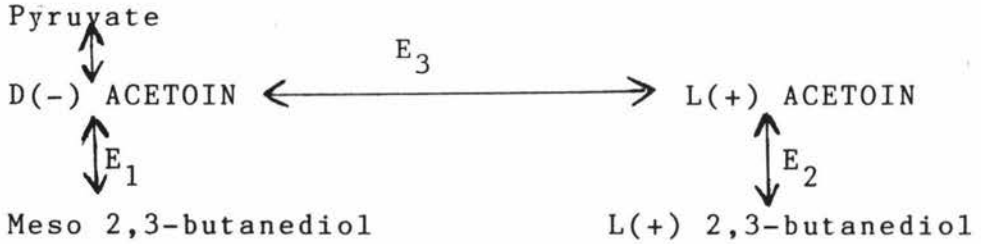


E_1 = D-dehydrogenase

E_2 = L-dehydrogenase

E_3 = Racemase

Fig 2.2 Mechanism for the formation of 2,3-butanediol stereoisomers by bacteria as proposed by Taylor and Juni(1960) for Klebsiella pneumoniae



E_1 = D(-) Acetoin reductase

E_2 = L(+) Acetoin reductase

E_3 = Acetoin racemase

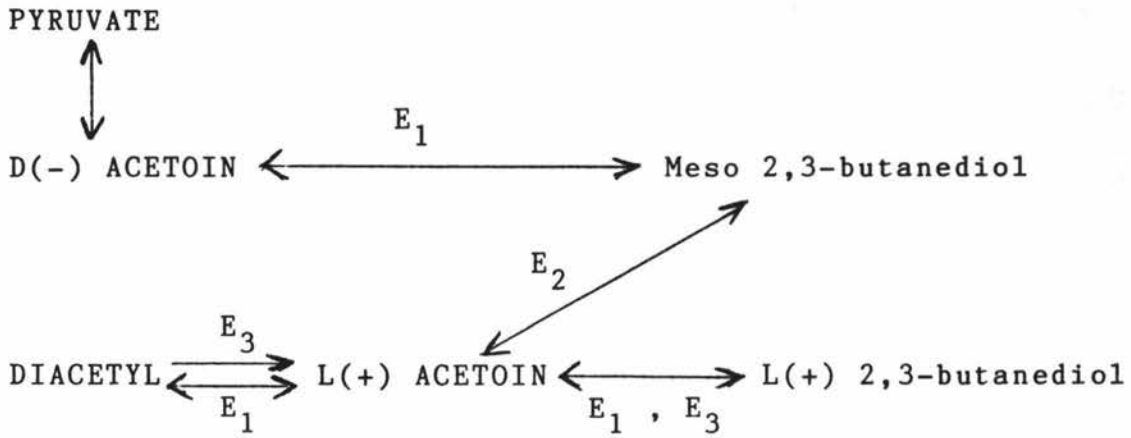
Fig 2.3 Proposed mechanism for the formation of 2,3-butanediol stereoisomers by Klebsiella pneumoniae(Voloch et al, 1983)

NAD^+ oxidoreductase L(+) acetoin-forming. The basic difference between this model from that of Taylor and Juni lies in the stereospecificity of the acetoin reductase. However neither models allows for the conversion of L(+) acetoin to D(-) 2,3-butanediol or conversion of D(-) acetoin to L(+) 2,3-butanediol.

More recently, Ui et al(1985) proposed another model (Fig. 2.4) to explain the formation of 2,3-butanediol isomers. This model assumes the participation of three different 2,3-butanediol dehydrogenases and is different from that offered by Voloch et al(1983) in that the earlier group assumed the presence of an acetoin racemase for the L(-) 2,3-butanediol isomer formation. The present model assumes the successive formation of meso 2,3-butanediol, L(+) acetoin and L(+) 2,3-butanediol, all being catalyzed by three dehydrogenases(E_1, E_2 and E_3) without the participation of an acetoin racmase. These authors noted that the racemase activity observed by Voloch and coworkers might be ascribed to the catalytic activity for interconversion between D(-) acetoin and L(-) acetoin mediated through meso-2,3-butanediol as an intermediate.

2.2.2 Production of 2,3-butanediol by *Klebsiella pneumoniae*

One of the earliest references to the natural occurance of 2,3-butanediol was that of Hemminger(1882), who isolated what was then thought to be isobutylene glycol from wine. Later Harden and Walpole(1906) proved that 2,3-butanediol could be produced by bacteria in appreciable quantities. The culture colonies used by these authors could now be classified as *Aerobacter aerogenes*. It was left to Perlman(1944) who revealed that *Aerobacter aerogenes* was able to ferment wood hydrolyzates into 2,3-butanediol. This author recorded a combined acetoin and 2,3-butanediol yield in excess of 30%. A year later, Ward et al(1945) also reported that it was possible to produce 2,3-butanediol from hydrolysed



E_1 = Meso - 2,3-butanediol dehydrogenase (D(-) Acetoin forming)

E_2 = Meso - 2,3-butanediol dehydrogenase (L(+) Acetoin forming)

E_3 = L(+) 2,3-butanediol dehydrogenase (L(+) Acetoin forming)

Fig 2.4 Mechanism for the formation of 2,3-butanediol stereoisomers by Klebsiella pneumoniae as proposed by Ui et al(1985)

starch mashes by the same organism. The solvent concentration obtained was 42.3g/l representing a yield of 0.49g/g of starch utilised. In an attempt to determine the optimal conditions for the 2,3-butanediol fermentation, Freeman(1947) reported that when Aerobacter aerogenes(B-199) was cultured in a sucrose medium under aerated conditions at a temperature of 35^{o}C and initial pH of 5.8, the organism was able to utilize all the sucrose giving a solvent concentration of 43.4g/l which represented a yield of 0.39g/g of sucrose utilized. This author also noted that increasing the initial sucrose concentration resulted in an increase in the lag phase and that the overall fermentation time also increased. A similar study was made by Ledingham and Neish(1954) who showed that aerating the medium caused a suppression of ethanol and lactic acid production whilst 2,3-butanediol formation increased. However an excess of aeration resulted in an increase in acetoin at the expense of 2,3-butanediol production.

In one of the earliest studies on continuous fermentation using Aerobacter aerogenes, Pirt and Gallow (1958) recorded a maximum volumetric butanediol productivity of 3.3g/l.h which was three times higher than that ever recorded in a batch fermentation. The dilution rate employed was 0.11h^{-1} and sucrose was the carbon source. These authors also showed that a further increase in dilution rate led to lower rates of 2,3-butanediol production, while an increase in oxygen flow rate favoured biomass formation. Traces of ethanol, lactic and formic acids were formed in partially aerobic fermenting conditions. However, when the system was anaerobic, the investigators noted that there was an increase in production of formic and lactic acids which subsequently caused a decrease in the percentage conversion of substrate to 2,3-butanediol. The optimal pH and temperature ranges in the continuous mode were 5.6 - 6.0 and 35 - 37^{o}C, respectively. Later investigations by Long and Patrick (1961) indicated that citrus press-liquor and molasses were excellent substrates for 2,3-butanediol production.

A butanediol concentration of 48.3g/l, representing a yield of 0.312g of butanediol per g sugar utilized was attained. These studies also revealed that supplements of yeast extract and urea were essential for maximum solvent concentration whilst supplementation with calcium carbonate and potassium dihydrogen phosphate depressed the yield. Aeration and agitation of the fermentation medium were beneficial. Although the authors practised prior adaptation of culture, the procedure had little effect on total butanediol production.

As a result of the fuel crisis in the 1970's, research into the use of agricultural residues to produce liquid fuel was intensified. Initial interest was mainly from North America and one of the earliest preliminary investigations was conducted by Veeraraghaven et al(1979) who reported the production of 2,3-butanediol from xylose using a bacterium code-named AU-1-d3 which was isolated from wood piles. These investigators recorded a butanediol concentration 35g/l and noted that the optimal conditions for fermentation were 32^{o}C, pH 5.5 and an oxygenation rate of 0.3mg/L/min. Product formation by this bacterium was found to be non-growth-associated.

From 1979 to the present, interest in the utilization of lignocellulosic residues and other agricultural wastes as substrates for the microbial production of 2,3-butanediol by Klebsiella pneumoniae had continued to flourish. This led to several publications by the Canadian Forintek Corporation. Thus, Yu and Saddler (1982a), investigated the utilization of the sugars present in wood hemicellulose. The sugars evaluated were arabinose, xylose, galactose, glucose, mannose and cellobiose, and the strain employed was ATCC 8724. Their studies revealed that the production of 2,3-butanediol occurred during late exponential phase, concomitant with a culture pH drop, and continued into the stationary phase of growth. The production of 2,3-butanediol from all sugars was optimum under anaerobic conditions with the exception of xylose, which required a finite air supply for maximum solvent yield. The solvent concentration recorded for

this sugar was 2.7g/l representing a yield of 0.27g/g of xylose utilized. The concentrations of 2,3-butanediol for the other tested sugars ranged from 2.7 - 3.5g/l while the yields did not exceed 0.35g/g sugar utilized. In a later study, Yu and Saddler(1982b) demonstrated that sugar concentrations in excess of 20g/l could not be completely utilized even when a higher inoculum size was used. Supplementation of the culture medium with end-products at concentrations above those normally attained in the fermentation failed to reveal any product inhibition. However the inclusion of 0.5% acetic acid to the culture medium greatly enhanced both sugar utilization and butanediol production. The authors attributed this to the "inducer effect" of acetic acid on the three enzymes involved in the formation of 2,3-butanediol from pyruvate. A further investigation by Yu and Saddler (1982c) revealed that when Klebsiella pneumoniae(ATCC 8724) was grown on acid-hydrolysed wood hemicellulose, the organism could produce a butaneiol concentration of 6.02g/l representing a yield of 0.5g/g of monosaccharide utilised. The authors attributed their high yield to the presence of uronic acids in the wood samples which the organism could utilize. However, it was also noted that fermentation efficiency decreased when higher levels of sugar were used and this was due to the presence of furfural and hydroxymethyl furfural which acted as inhibitors. The main sugar component of the acid hydrolyzate was xylose. In a further investigation to their previous work, Yu and Saddler(1983) utilized a fed-batch fermentation mode to enhance the production of 2,3-butanediol by Klebsiella pneumoniae(ATCC 8724). The organism was cultured under finite air conditions and in the presence of added acetic acid(1g/l). Under these conditions, 50g/l of glucose and xylose were converted to 25 and 27g/l respectively, of 2,3-butanediol. The corresponding solvent yields were 0.5 and 0.54g/g sugar utilized. The efficiency of bioconversion was found to decrease with increasing sugar concentrations. The authors explained this effect as being due to the build up of

gaseous end-products and gas pressure in the vessel which in turn inhibited further cell growth, sugar utilization and butanediol production. Another reason given was the decrease in water activity caused by the increased sugar concentration. These studies also showed that high butanediol concentrations were obtained for cultures grown at an initial sugar concentration of 150g/l provided that the inoculum was first acclimatized to high sugar level.

Although the Forintek group has dominated research in the 2,3-butanediol fermentation from lignocellulosic residues, there is also interest in the use of other easily available agricultural wastes as substrates for the microbial production of this solvent. Thus, Lee and Maddox(1984) reported the first published work on the production of 2,3-butanediol from whey permeate using Klebsiella pneumoniae. The authors screened four bacterial strains and found that Klebsiella pneumoniae(NCIB 8017) was far superior to Bacillus polymyxa and two strains of Enterobacter cloacae. A butanediol concentration of 7.5g/l representing a yield of 0.46g/g of lactose utilized was obtained after 96h fermentation. The authors also revealed that enzymatically hydrolysed whey permeate gave a higher butanediol concentration than normal whey permeate, which appears to suggest that lactose utilization might be a limiting step in the fermentation process.

2.2.3 Production of 2,3-butanediol by Bacillus polymyxa

Bacillus polymyxa is a more versatile organism than Klebsiella pneumoniae in that it is diastatic. However, its solvent yields appear to be lower than those produced by Klebsiella pneumoniae. In 1926, Donker published the first important contribution to the Bacillus polymyxa fermentation. Later, Scheffer(1928) also reported that Bacillus polymyxa could produced 2,3-butanediol. The major attribute of Bacillus polymyxa is its ability to produce the pure levo-isomer of 2,3-butanediol(an important constituent in antifreeze manufacture as

distinct from the meso form which is produced by Klebsiella pneumoniae(Long and Patrick, 1963). Long and Patrick(1965) revealed that Bacillus polymyxa(NRRL B510) was able to produce 2,3-butanediol from citrus wastes supplemented with urea. The butanediol concentration and yield were however much lower than those produced by Aerobacter aerogenes from the same substrate. These authors also noted that the addition of KH_2PO_4 or $CaCO_3$ as buffers resulted in decreased production of the solvent.

The versatility of Bacillus polymyxa was further demonstrated when Speckman and Collins(1982) reported that the organism was able to ferment lactose in cheese whey resulting in maximum solvent production of 3.65g/l representing a yield of 0.16g/g of lactose utilized. These values were obtained after a 168h fermentation. In a follow-up investigation, Shazer and Speckman(1984) demonstrated that when the fermentation was conducted in a continuous mode with biomass recycle, butanediol productivity was 43 times higher than that achieved in batch fermentation. The volumetric rate of production recorded was 1.04g/l.h whilst that of a batch mode was 0.024g/l.h. The optimal dilution rate employed was $1.04h^{-1}$. These authors noted that acetate concentrations greater than 5mM were detrimental to the growth of the organism but a concentration of 75mM seemed to increase productivity by at least 17-fold.

The ability of Bacillus polymyxa to ferment whey lactose was also studied by Lee and Maddox(1984) who conducted a screening trial and found that Bacillus polymyxa(NCIB 8526) gave a poorer butanediol yield and concentration than Klebsiella pneumoniae(NCIB 8017), but performed better than Enterobacter cloacae. In the same year, the Canadian investigators Laube et al(1984a) demonstrated that strain NRCC 9033 produced the best solvent concentration from xylose and other components of hemicellulose. A butanediol concentration of 4.3g/l representing a yield of 0.18g/g xylose utilized was obtained. The authors observed that the addition of 10g/l of yeast extract significantly improved butanediol

production. In a further investigation, Laube et al (1984b) revealed that the beneficial effect of increased yeast extract concentration on butanediol production was in fact due to the presence of manganese, magnesium and iron cations in the nutrient supplement. An addition of 1.7μM of manganese ion resulted in maximum stimulatory effect on butanediol production.

In a different investigation, the same Canadian group(Gro.leau et al, 1985) showed that nitrogen sparging of the medium resulted in best 2,3-butanediol production at a low yeast extract concentration, but aeration produced best solvent production when high yeast extract levels were used. Twice as much butanediol and five times as much ethanol accumulated with nitrogen sparging. The authors concluded that nitrogen sparging was the best approach towards enhanced butanediol production by Bacillus polymyxa(NRCC 9033).

2.2.4 2,3-butanediol production by other organisms

The concentration and yield of 2,3-butanediol produced by organisms other than Bacillus polymyxa and Klebsiella pneumoniae are rather poor. This might be due to the lesser interest and lack of available information, particularly on the fermentation conditions. Nevertheless, those organisms shown in Table 2.4 may possess genetic and biochemical characteristics which the geneticist can utilize in the future for genetic manipulation.

2.2.5 Production of 2,3-butanediol by immobilized cells

There are only two publications to date which relate to the production of 2,3-butanediol by immobilized cells. The first was by Chua et al(1980) who used K-carrageenan to entrap cells of Enterobacter aerogenes (IAM 1133). The authors obtained 11g/l of the solvent from a glucose medium in a batch mode. The batch volumetric productivity was 0.5g/l.h. However, when the system was studied using a packed column in a continuous mode, the volumetric rate of 2,3-butanediol recorded was 1.32g/l.h at a dilution rate of $0.4h^{-1}$. Repeated runs

Table 2.4 Organisms which produce minor amounts of 2,3-butanediol

Organism	Substrate	Reference
Streptococcus diacetilactis	untreated cheese whey	Speckman & Collins (1982)
Streptococcus faecalis	-do-	-do-
Torulopsis colliculosa	-do-	-do-
Bacillus subtilis	glucose	Ui *et al* (1983)
Bacillus cereus	-do-	-do-
Bacillus coagulans	-do-	-do-
Serratia marcescens	-do-	-do-
Enterobacter cloacae	-do-	-do-
Proteus vulgaris	-do-	-do-
Staphylococcus aureus	-do-	-do-
Pseudomonas trifolii	-do-	-do-
Vibrio metschnikovii	-do-	-do-
Pseudomonas hydrophilla	blackstrap cane molasses	Long & Patrick (1963)

Table 2.4(Cont.)

Organism	Substrate	Reference
Enterobacter cloacae	whey permeate	Lee & Maddox (1984)
Aeromonas hydrophilla	soluble starch	Willets (1984)

resulted in no loss of activity of the immobilized cells. The importance of low cell loading was emphasized by these workers as the cells occupied about 30% of the gel matrix. Thus a high cell loading would result in gel disintegration due to rapid cell growth upon activation. A more recent report(Anonymous, 1984) has described the use of immobilized cells of Klebsiella pneumoniae in both batch and continuous production of 2,3-butanediol. Unfortunately, no information was provided regarding the substrate or the productivities obtained.

2.2.6 Factors affecting the 2,3-butanediol fermentation

In thorough reviews of the 2,3-butanediol fermentation, Long and Patrick(1963) and Jansen and Tsao (1983) reported that the factors which influence the 2,3-butanediol fermentation include; type of substrate, organism employed, culture acclimatization, temperature, pH, aeration and agitation(oxygen uptake rate), nutrient supplementation, sugar concentration and water activity.

2.2.6.1 Type of substrate and organism employed

Current interest in the 2,3-butanediol fermentation is centred on the two bacterial species of Bacillus polymyxa and Klebsiella pneumoniae. The choice of organism will of course depend on the ability to metabolise the sugar substrate and to produced butanediol in high concentrations and yield. Bacillus polymyxa, being diastatic, would be the preferred organism using a starch substrate but not necessarily for simpler sugars. Of the few systematic comparisons reported, Lee and Maddox(1984) demonstrated the superiority of Klebsiella pneumoniae over Bacillus polymyxa when using rennet whey permeate as substrate, and Long and Patrick(1963) observed similar results using citrus wastes.

2.2.6.2 Acclimatization of cultures

This practice is often adopted when the organism is sensitive to substrate inhibition as a result of the presence of high concentrations of sugar. The

procedure is also frequently followed to ameliorate any adverse effect of a new substrate. However, acclimatization of culture does not always lead to higher butanediol production. Thus, Long and Patrick(1961, 1963 and 1965) found that prior adaptation of Bacillus polymyxa and Aerobacter aerogenes to citrus molasses did not have any significant effect on final butanediol yields. A similar result was reported by Speckman and Collins(1982) while investigating the production of 2,3-butanediol from cheese whey by Bacillus polymyxa. However, Yu and Saddler (1983) reported that prior acclimatization of Klebsiella pneumoniae cultures resulted in higher butanediol production from glucose and xylose.

2.2.6.3 Temperature

Pirt and Gallow(1958), while investigating the continuous fermentation of sucrose by Aerobacter aerogenes, recorded a maximum sucrose uptake rate at 37°C. In the same experiment, maximum butanediol production occurred between 35 - 37°C. Later investigations by Long and Patrick(1963) revealed that when the fermentation temperature was increased within certain limits, the rate of fermentation also increased but the increase in rate was accompanied by a corresponding decrease in the total butanediol yield. Maximum butanediol yields however could be attained within the optimum temperature range for growth of the particular species used in the fermentation. Unfortunately, no other systematic study on the optimum temperature appears to have been reported and most investigators perform the fermentation between 30 - 37°C.

2.2.6.4 pH

Neish and Ledingham(1949) reported that the yield of butanediol from the anaerobic fermentation of glucose by Klebsiella pneumoniae reached a maximum in the pH range of 5.2 - 5.6 and decreased to near zero when the pH was above 7. Later investigations by Pirt and Gallow(1958) revealed that for partially aerobic

growth, the optimum pH for butanediol production from sucrose occurred between pH 5.0 and 6.0 and production decreased sharply above pH 6.5. Long and Patrick(1961) reported that when citrus molasses were fermented by Aerobacter aerogenes, reduced butanediol production was observed when the pH of the substrate dropped from 7.2 to 5.2. The optimum pH range for maximum butanediol production was noted to be between 6.0 and 6.2. The authors emphasized that pH control of the substrate at this optimum range was very important. However, Lee(1984) using Klebsiella pneumoniae observed that rennet whey permeate possess its own intrinsic buffering characteristics which render pH control unnecessary, and that maximum butanediol production occurred at pH 5.0. Jansen(1982) using the same organism, reported that when xylose was the carbon source, the maximum butanediol yield occurred between pH 5.0 and 6.0 and declined sharply when the pH was above 6.5. In a later report, Jansen et al(1984a) attributed this decrease in butanediol yield to the decreasing activity of the butanediol-producing enzymes. This later investigation also revealed that at pH values below 4.2, the organism did not grow. The relationship of pH to butanediol production, therefore, appears to involve the interplay of a number of factors such as the nature of the substrate, the type of buffer used, as well as the fermentation conditions.

2.2.6.5 Aeration and agitation(oxygen transfer rate)

Although 2,3-butanediol is a product of anaerobic metabolism, aeration has been shown to increase its production by Klebsiella pneumoniae(Ledingham and Neish, 1954). This stimulatory effect of aeration was originally explained by McCall and Georgi(1954) as being due to the removal of carbon dioxide from the medium. However Pirt(1957) and Pirt and Gallow(1958), demonstrated that aeration increases butanediol production by increasing the cell concentration. Their studies also showed that

the rate of glucose uptake by the organism was dependent on the amount of available oxygen in the system. Under anaerobic conditions, cell synthesis and carbon dioxide production were at the minimum level and most of the glucose was converted to ethanol, formic acid, 2,3-butanediol, acetoin and lactic acid. Small amounts of oxygen were noted to suppress the formation of ethanol and formic acid but still permit the production of 2,3-butanediol. However, a large supply of oxygen was found to favour biomass production at the expense of metabolite formation. A later study by Harrison et al (1969), using Klebsiella pneumoniae, revealed that the transition from an aerobic to an anaerobic type of metabolism took place at a low dissolved oxygen tension (DOT), and that there was a critical DOT range between 2 and 10mm Hg. DOT values above the critical range resulted in a constant oxygen uptake rate and metabolism tends to be aerobic.

As a result of these studies, Wimpenny and Necklen(1971) classified the metabolic phases of Klebsiella aerogenes into four types on the basis of redox potential measurements; Phase 1, Anaerobiosis, at less than 0mV; Phase 2, Limited Aeration, at about +100 mV, where cytochrome levels are maximal; Phase 3, Aerobiosis, between +200 and 300mV; Phase 4, in which extreme aeration occurred at values greater than 300mV. Recent studies by Jansen et al(1984a, b) showed that during oxygen limited growth, Klebsiella oxytoca metabolized xylose by fermentation into 2,3-butanediol and also converted the sugar to carbon dioxide and cell mass by direct respiratory oxidation. They also reported that the production of 2,3-butanediol was partially growth-associated and product inhibition occurred at concentrations greater than 65g/l. These authors singled out the oxygen transfer coefficient as the most important variable affecting 2,3-butanediol production.

A study by Sablayrolles and Goma(1984) showed that the growth of Aerobacter aerogenes(NRRL B199) was inhibited in oxygen-limited conditions, while an excess of oxygen was toxic to metabolite formation. The optimum oxygen transfer coefficient determined by these authors was between 50 - 100h^{-1}. More recently, Lee (1984) reported that increased aeration and agitation rates did not improve butanediol production from whey permeate.

It appears that the poor attempts to quantitate the oxygen requirements of Klebsiella pneumoniae are due to inaccuracy of both equipment and methods employed. However, the majority of investigators agree that maximum 2,3-butanediol production occurs when the fermentation medium is anaerobic. The problem is to find a compromise between maximum butanediol production and maximum biomass concentration since the production of 2,3-butanediol is partially growth associated.

2.2.6.6 Nutrient supplementation

With respect to Klebsiella pneumoniae, Yu and Saddler(1982a) revealed that medium supplementation with vitamins was unnecessary and that ammonium salts could provide the sole source of nitrogen for growth. However, the addition of acetic acid was found to enhance butanediol yields. With regard to whey permeate, Lee(1984) has shown that nutrient supplementation does not result in greater butanediol production. It appears therefore that whey permeate possesses its own inherent nutrient and buffering capacity for butanediol production by Klebsiella pneumoniae.

2.2.6.7 Sugar concentration

Fulmer et al(1933) demonstrated that high initial sucrose concentrations affected both product yields and reaction rates. Butanediol yield and production rates were both maximum at an initial sucrose concentration of 80g/l. At higher sugar concentrations, the yield and rate of butanediol production decreased significantly.

Long and Patrick(1963) suggested that the optimum initial sugar concentration often depended on the particular raw material used as the carbon source. Thus, as the sugar concentration in the raw material was increased, the level of any accompanying toxic material also increased resulting in poor substrate utilization.

Ohlson and Johnson(1948) reported that glucose at 100g/l in a chemically defined medium was rapidly consumed by Klebsiella pneumoniae. However, when an acid-hydrolysed wheat mash medium was employed, the butanediol yield fell and carbohydrate utilization became incomplete at sugar concentrations above 90g/l. To overcome this problem and to increase the amount of sugar utilized the authors used a "slow feed" bioreactor in which a concentrated glucose solution was added to the fermentor slowly. In this way, glucose could be utilized at a total concentration of 265g/l. A later investigation by Jansen et al(1984a) showed that the apparent substrate inhibition of Klebsiella pneumoniae grown in xylose concentrations above 20g/l may be explained by a decrease in water activity.

2.2.6.8 Water activity

Jansen and Tsao(1983) commented that species of Klebsiella are not as osmotolerant as many other bacterial species. Their study, shown in Fig 2.5, revealed that the growth rate of Klebsiella pneumoniae was very dependent on water activity. At a water activity of 0.985, the growth rate was only 50% of the maximum while at water activities below 0.975, growth rates became less than 10% of the maximum. These authors postulated that the decrease in growth rate with decreasing water activities could explain why high initial sugar concentrations are not suitable for the butanediol process.

2.2.7 Product recovery

Some physical properties of 2,3-butanediol are shown in Table 2.5. Due to its high boiling point and affinity towards water, the extraction of 2,3-butanediol

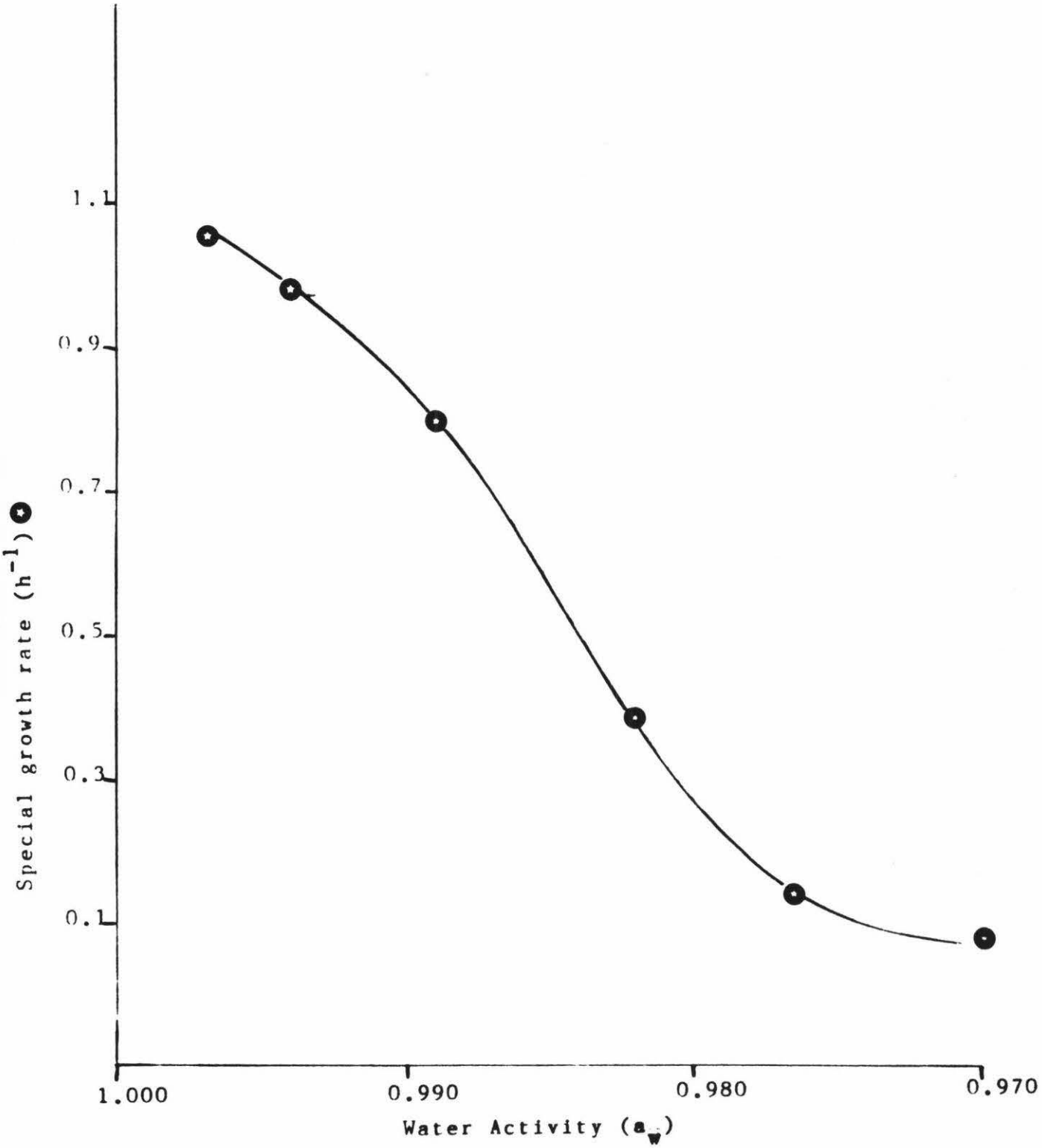


Fig2.5 Specific growth rate of Klebsiella pneumoniae versus water activity (Jansen et al, 1983)

Table 2.5 Physical properties of the stereoisomeric 2,3-butanediol(Underkofler and Hickey , 1954)

Constant	Meso - 2,3-butanediol	D(-) 2,3-butanediol	Racemic - 2,3-butanediol
Melting point, ^{o}C	34.4	19.0	7.6
Boiling point, ^{o}C at 745mm	181 - 182	179 - 180	177
Specific rotation at $26^{o}C$(D line)	0.00^{o}	-13.34^{o}	0.00^{o}
Density, g/ml at $25^{o}C$	0.9939	0.9869	-
Refractive index at $25^{o}C$	1.4366	1.4308	-
Surface tension, dynes per cm at $25^{o}C$	-	30.61	-

Table 2.5(Cont.)

Constant	Meso - 2,3-butanediol	D(-) 2,3-butanediol	Racemic - 2,3-butanediol
Viscosity, centipoises at 35°C	65.6	21.8	-
Specific viscosity at 30°C	15.72	5.34	-
Melting point of di-p-nitrobenzoate, °C	193	143	128
Specific rotation of di-p-nitrobenzoate in chloroform at 25°C	-	51.0°	-

from a fermenting broth poses problems which have not been satisfactorily addressed. In the past, fractional distillation was described as a means of product recovery(Prescott and Dunn, 1959), but its economic feasibility on a commercial scale is unknown. Speckman and Collins(1982) noted that butanediol recovery was not a problem when a Sephadex G-10 column was used. These authors suggested that if further research were conducted on product recovery by this system, a more economical method could be developed in the foreseeable future. The rapid development of various membrane and chromatographic separation techniques may also provide a suitable product recovery technique.

2.3 Conclusion

Unlike the fermentation processes for ethanol or butanol production, the butanediol process has never been operated on a commercial scale. However, with the increasing prices of chemical feedstocks, and the ease with which butanediol can be converted to butadiene, research into the fermentation process is accelerating. Much is now known about the process, but the newer fermentation technologies which have been developed in recent years remain to be applied

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microbiological media

Nutrient agar was obtained from Oxoid Ltd., London, United Kingdom.

3.1.2 Rennet whey permeate

This substrate was obtained from the New Zealand Dairy Research Institute, Palmerston North, prepared as described by Matthews et al(1978).

3.1.3 Chemicals

The chemicals used were all of analytical grade. Calcium acetate, sodium alginate(ex Laminaria hyperborea), calcium chloride hexahydrate, strontium chloride, barium chloride, concentrated hydrochloric acid, ethanol and dipotassium hydrogen phosphate were all obtained from BDH Chemical Company Ltd., Palmerston North, New Zealand. 2,3-butanediol, tris(hydroxymethyl) methylamine and lactose were supplied by Sigma Chemical Company, U.S.A., while yeast extract and Bacto peptone were obtained from Gibco Ltd., Wisconsin, U.S.A., and Difco Laboratories, Detroit, Michigan, U.S.A., respectively.

3.1.4 Organism

Klebsiella pneumoniaeNCIB 8017 was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, United Kingdom, and was maintained on nutrient agar slopes. The organism was subcultured bimonthly, by inoculating into a fresh slope and incubating at 30^{o}C for 1 day, followed by storage at 4^{o}C.

3.2 Methods

3.2.1 Media preparation

All media used in cell cultivation and fermentation experiments were adjusted to pH 7 by the addition of 4M sodium hydroxide prior to sterilization. In addition, the rennet whey permeate used in the continuous fermentation studies was supplemented with 3g/l $CaCl_2.6H_2O$.

3.2.2 Sterilization of media, chemicals, reagents and apparatus

Chemical solutions, media and rennet whey permeate(2l volume or less) were sterilized in an autoclave at 121°C for 15min, with the exception of sodium alginate solutions which were autoclaved for 10min at 121°C. Larger volumes of rennet whey permeate in carboy container were autoclaved for 25min at 121°C. Gas filters, centrifuge tubes, pipettes and miscellaneous glassware were sterilized in a hot-air oven at 160°C for 24h.

3.2.3 Cleaning of glassware

All glassware was washed in hot pyroneg(R) solution(Diversey Wallace Company, Auckland, New Zealand), rinsed in tap water followed by distilled water, and then hot-air dried.

3.2.4 Cell cultivation

A medium containing lactose(50g/l), Bacto peptone (5g/l), yeast extract(5g/l) and dipotassium hydrogen phosphate(2g/l) was used to culture cells of *Klebsiella pneumoniae* for immobilization. Inoculation was by direct transfer using a loop from a nutrient agar slope into 100ml of medium contained in a 250ml conical flask. Incubation was at 30°C on a Lab-line junior orbit shaker (Lab-line Instruments Inc., Illinois, U.S.A) at 150rpm for 18h.

3.2.5 Cell harvest

Cells(18h old) were harvested aseptically by centrifugation.

3.2.6 Cell immobilization procedure

Harvested cells were mixed with sterile sodium alginate solution(20g/l) in a ratio of 1ml original culture to 1.25ml of sodium alginate solution. The suspension was thoroughly mixed with a sterile glass rod and extruded with the aid of a peristaltic pump, through a 1ml pipette, into a gently stirred 0.1M $CaCl_2.6H_2O$ solution in 0.1M Tris-HCl buffer pH 7(for barium alginate and strontium alginate beads, $BaCl_2$ and $SrCl_2$ were used, respectively). The sperical beads so formed were 3mm$\pm$ 0.5mm in diameter. the alginate beads were allowed to cure at room temperature for 1 - 2h after which they were washed successively with the buffer and distilled water. For experiments in batch fermentation and packed columns, the beads, were prepared in a beaker and then transfered aseptically to the experimental equipment. For continuous fermentation experiments in a continuous stirred tank reactor(CSTR), the beads were prepared in the reactor.

3.2.7 Analytical methods

3.2.7.1 pH measurement

pH measurements of samples were performed using a Metrohm model E520 pH meter.

3.2.7.2 Sugar analysis

Quantitative analysis of whey lactose was carried out using a Waters Associates model ALC/GPC 244 high performance liquid chromatograph(Waters Associates Inc., Milford, Massachusetts, U.S.A). A sugar-Pak 1 column(6.5mm ID x 300mm, Waters Associates) was used. The detector was a model R 401 differential refractometer (Waters Associates). The solvent employed was calcium acetate(20mg/l) and the flow rate was 0.5ml/min. The

column temperature was 90°C. Samples, diluted as appropriate, were membrane filtered prior to injection of 50μ1. Lactose was quantitated by measurement of peak heights and comparison with a standard curve.

3.2.7.3 Ethanol, acetoin and 2,3-butanediol analysis

A gas liquid chromatograph model GC 8A(Shimadzu Corporation, Kyoto, Japan) was used with a flame ionization detector. The column was 5%FFAP on chromosorb^{R} WAW(Smith Biolab, Wellington, New Zealand). The chromatograph was operated at an initial column temperature of 100° which was increased to 240°C at a rate of 32°C per min. A nitrogen carrier gas flowrate of 30ml/min was employed and the volume of sample injected was 2μ ml. Quantitation was performed using an internal standard of isobutanol and comparison with standard mixtures.

3.2.8 Batch fermentation

Most batch experiments were performed using barium alginate beads. The beads, corresponding to 20ml of original culture, were suspended in 100ml of whey permeate contained in 250ml conical flasks. The flasks were incubated at 30°C in a static mode but with occassional manual shaking. However, in some experiments, flasks were agitated on a Lab-line junior orbit shaker(Lab-line Instruments Inc., Illinois, U.S.A) at 150rpm.

3.2.9 Continuous fermentation

Two types of continuous fermentation system were employed. These were a continuous stirred tank reactor (CSTR) and packed columns. The whey permeate used in both systems was supplemented with 3g/l of calcium chloride hexahydrate to enhance calcium alginate bead stability. The substrates were continuously surface-flushed aseptically with oxygen free nitrogen prior to entry into the reactor. Both systems were incubated at 30°C.

3.2.9.1 Continuous stirred tank reactor

The experimental system is depicted in Fig 3.1. A New Brunswick multigen 2.01 fermentor vessel with a working volume of 1.501 was employed. The bead volume used was 1.301 which was the maximum volume of alginate pellets the reactor could accomodate while retaining adequate mixing. The bioreactor was magnetically stirred at 150rpm. The loss of beads from the reactor was prevented by a wire mesh placed in front of the overflow. The substrate was fed into the bioreactor using a peristaltic pump. The system was cultured in a batch mode for 40h prior to initiation of medium flow.

3.2.9.2 Packed column

Three glass columns of identical diameter (24mm) but with lengths of 216mm, 432mm and 648mm(volumes 100, 200 and 300ml) were employed. The two shorter columns were operated in a vertical mode with upward flow of medium. The longest column was fitted with a cylindrical steel mesh(1mm mesh diameter) on the inside and operated at 10^{o} to the hori zontal with upward flow of medium(Margaritis et al, 1981). The experimental layout of the packed column is depicted in Fig 3.2 and Plates 3.1 and 3.2. Beads of known volume were introduced into the columns via a glass funnel. The void volume of each column was calculated by initially filling the packed column with sterile substrate and then draining off the medium from the column and measuring the volume of medium. The bead plus void volumes of each column gives the total packed volume of the respective columns. As with CSTR, the packed columns were incubated in a batch mode for 40h prior to initiation of the medium flow.

3.2.10 Calculations of dilution rate, retention time and butanediol productivity in CSTR and packed column experiments

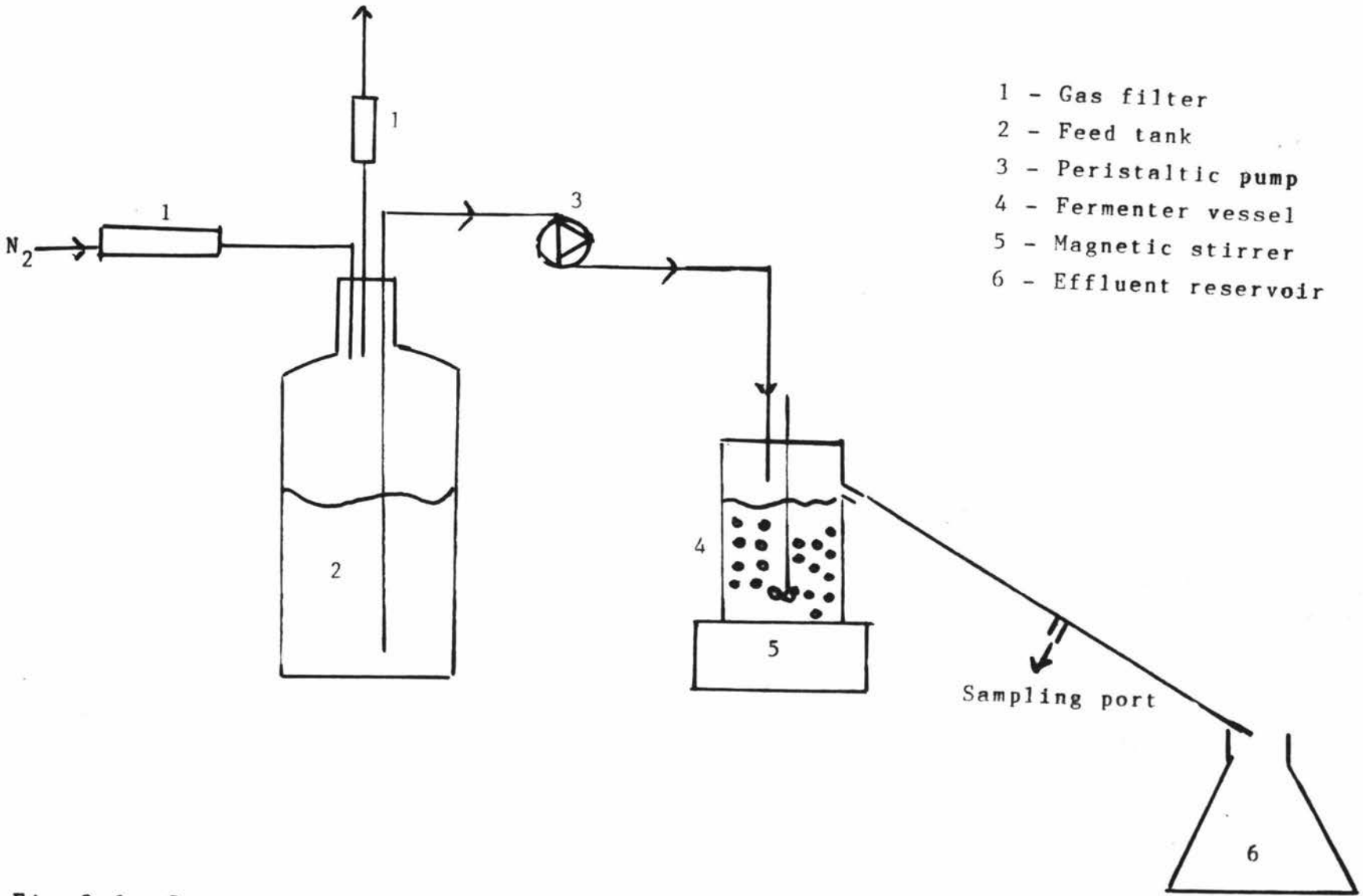


Fig 3.1 Continuous fermentation in a continuous stirred tank reactor

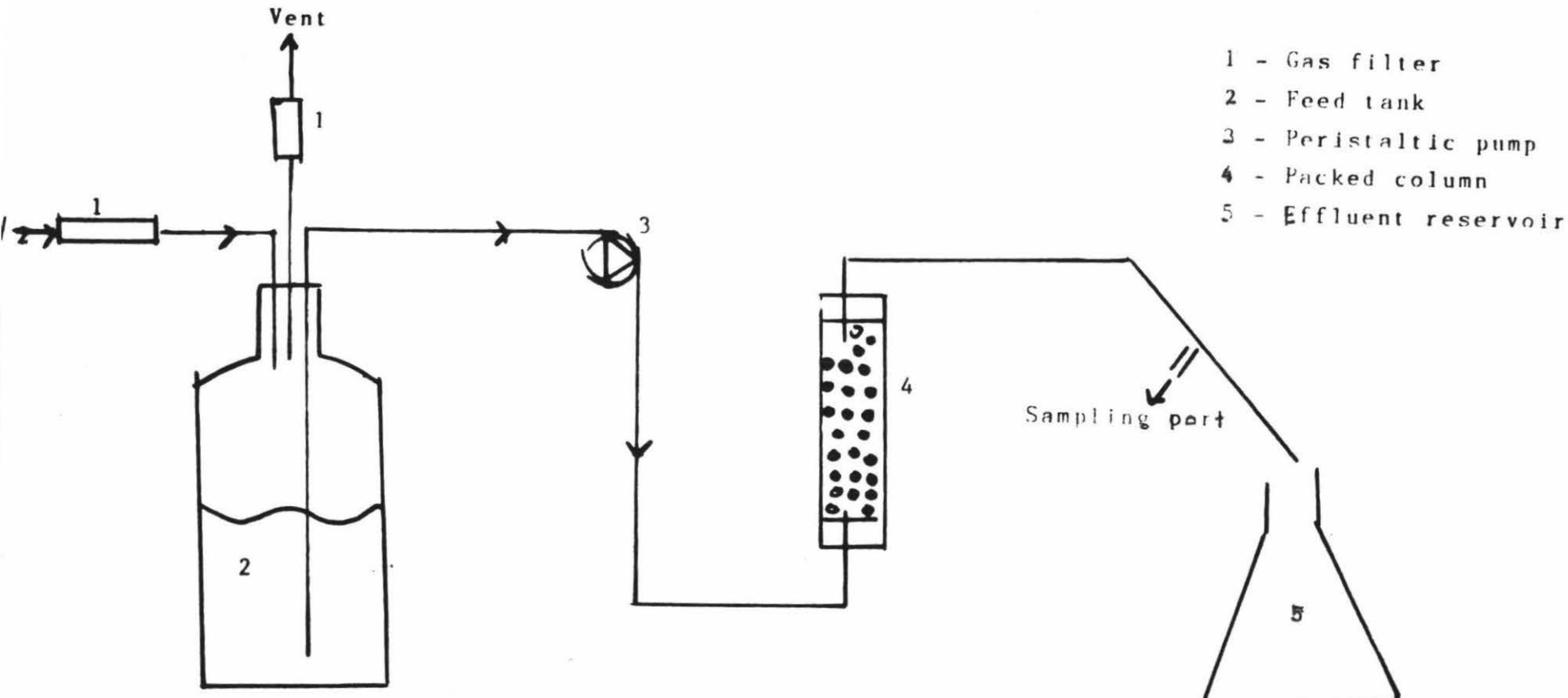


Fig 3.2 Continuous fermentation in a packed column reactor

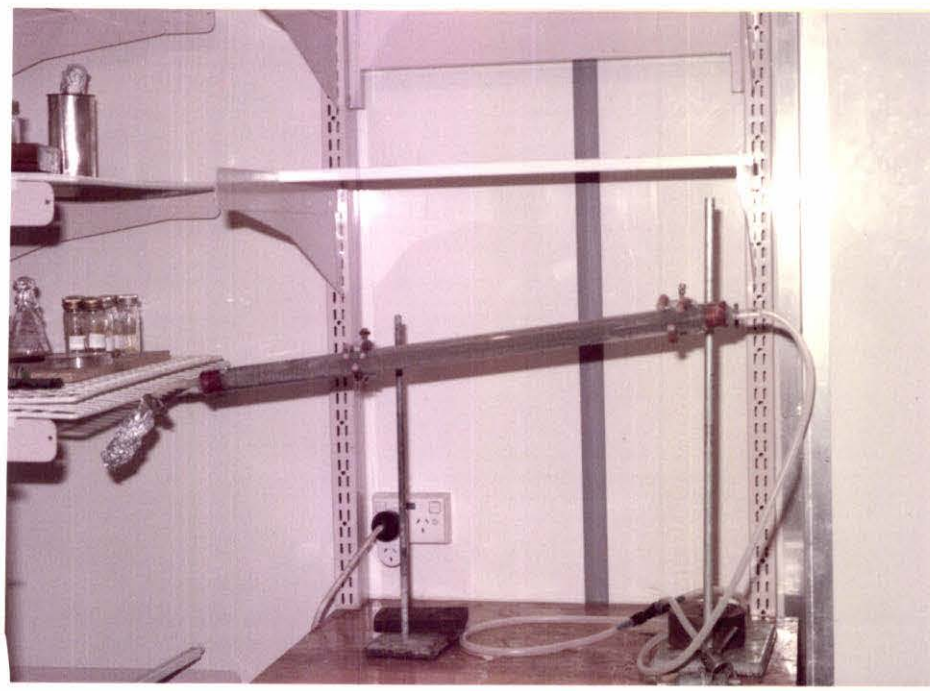


Plate 3.1 Meshed column inclined at 10^{o} to the horizontal

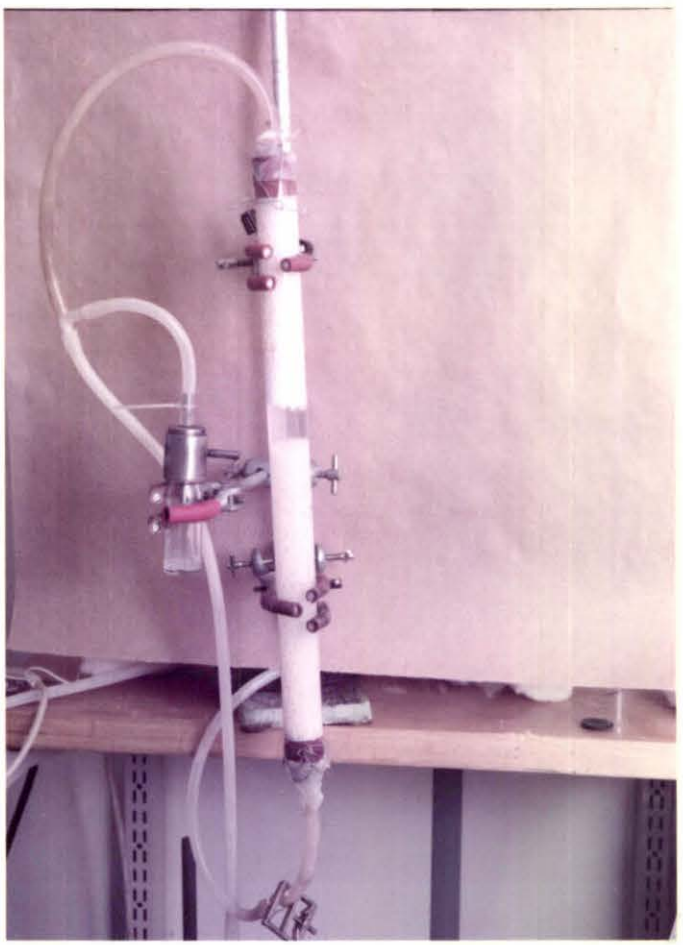


Plate 3.2 Short column in a vertical mode, without a steel mesh

3.2.10.1 CSTR

The calculations of dilution rate, retention time and butanediol productivity(volumetric productivity) were based on continuous fermentation principles(Pirt, 1975). The following formulae were employed in the calculations:-

$$D = F/V$$

where D = dilution rate h^{-1}

F = substrate flow rate 1/h

V = working volume(1.5l)

τ = residence time h = 1/D

Butanediol productivity g/l.h = D x butanediol concentration g/l at steady state.

3.2.10.2 Packed column

The determination of butanediol productivity, dilution rate and residence time was based on the method of Luong and Tseng(1984). The following formulae were used;

Butanediol productivity = F.P/V g/l.h

where F = feed flow rate 1/h

P = 2,3-butanediol concentration in the effluent stream g/l at steady state

V = total volume of the column 1

Retention time, τ = V/F h

Dilution Rate D = $1/\tau$ = F/V h^{-1}

CHAPTER 4

RESULTS

4.1 Batch fermentation

Batch fermentation studies were conducted in 100ml whey permeate contained in 250ml-shakeflasks using 27ml of barium alginate beads representing the cells obtained from 20ml of original culture. The purpose of these preliminary batch fermentation experiments was to gather relevant data such as the effects of cell immobilization, agitation(aeration), storage and increased lactose concentration on butanediol production. These data could then be used in designing the continuous fermentation studies.

4.1.1 Effects of cell immobilization on 2,3-butanediol production

The purpose of this experiment was to determine whether cell immobilization of Klebsiella pneumoniaeNCIB 8017 was detrimental to butanediol production. Cells from 20ml of original culture were either immobilized in barium alginate or used as free cells. Both the immobilized and free cells were suspended in 100ml of whey permeate and incubated at 30^{o}C in a static mode with periodic manual shaking.

The results of this experiment are depicted in Fig 4.1 and 4.2 for immobilized and free cells, respectively. These data reveal that immobilized cells produced a higher solvent concentration than that of free cells. A maximum butanediol concentration of 9.6g/l was achieved by immobilized cells after 120h of incubation compared to 6.5g/l attained by free cells over the same period. These product concentrations represent yields of 0.28 and 0.24 g/g lactose utilized for immobilized and free cells, respectively. The data also reveal that lactose utilization by immobilized cells was more complete than that of free cells. The corresponding ethanol and acetoin concentra-

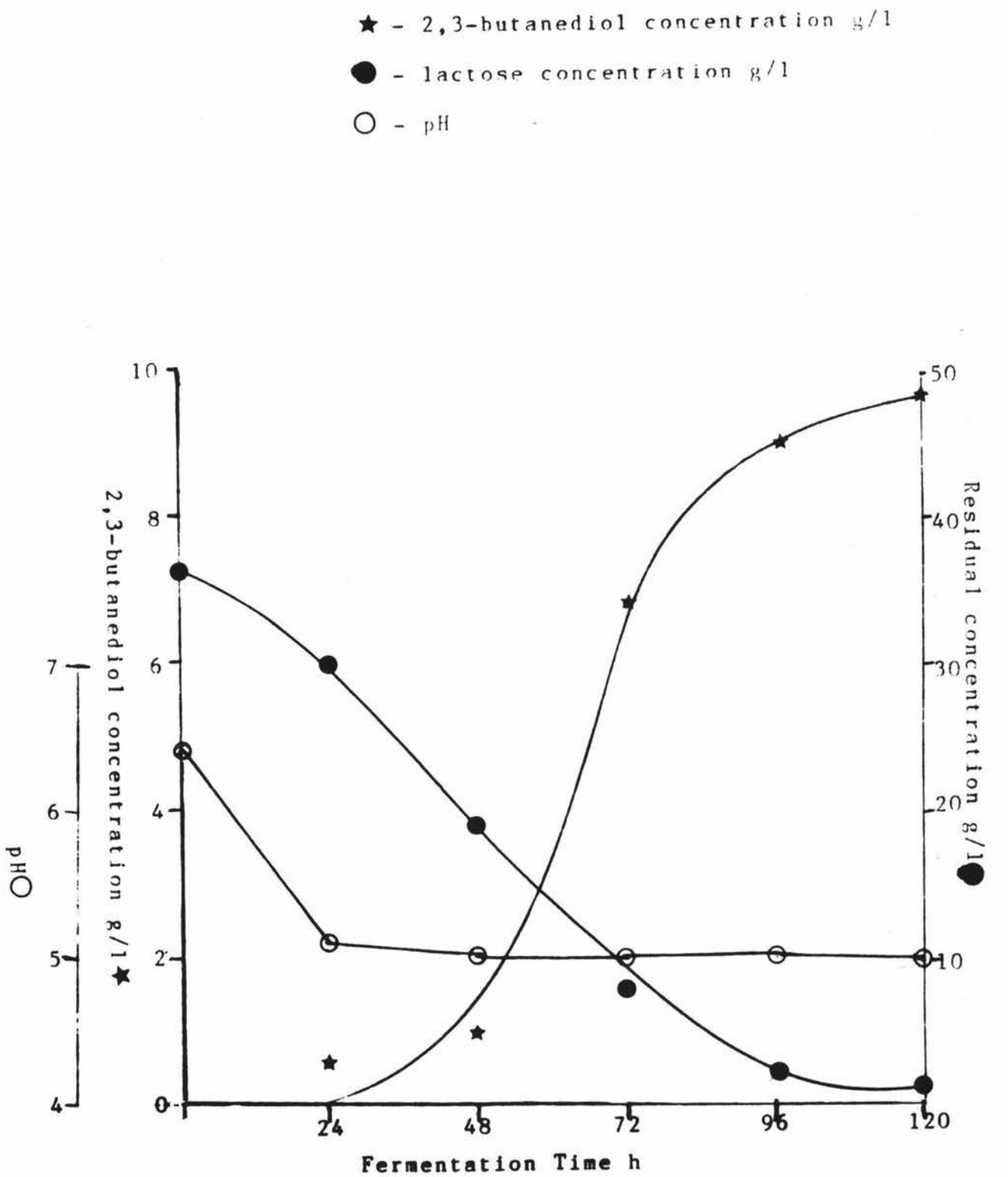


Fig 4,1 2,3-butanediol production by *Klebsiella pneumoniae* immobilized in barium alginate beads

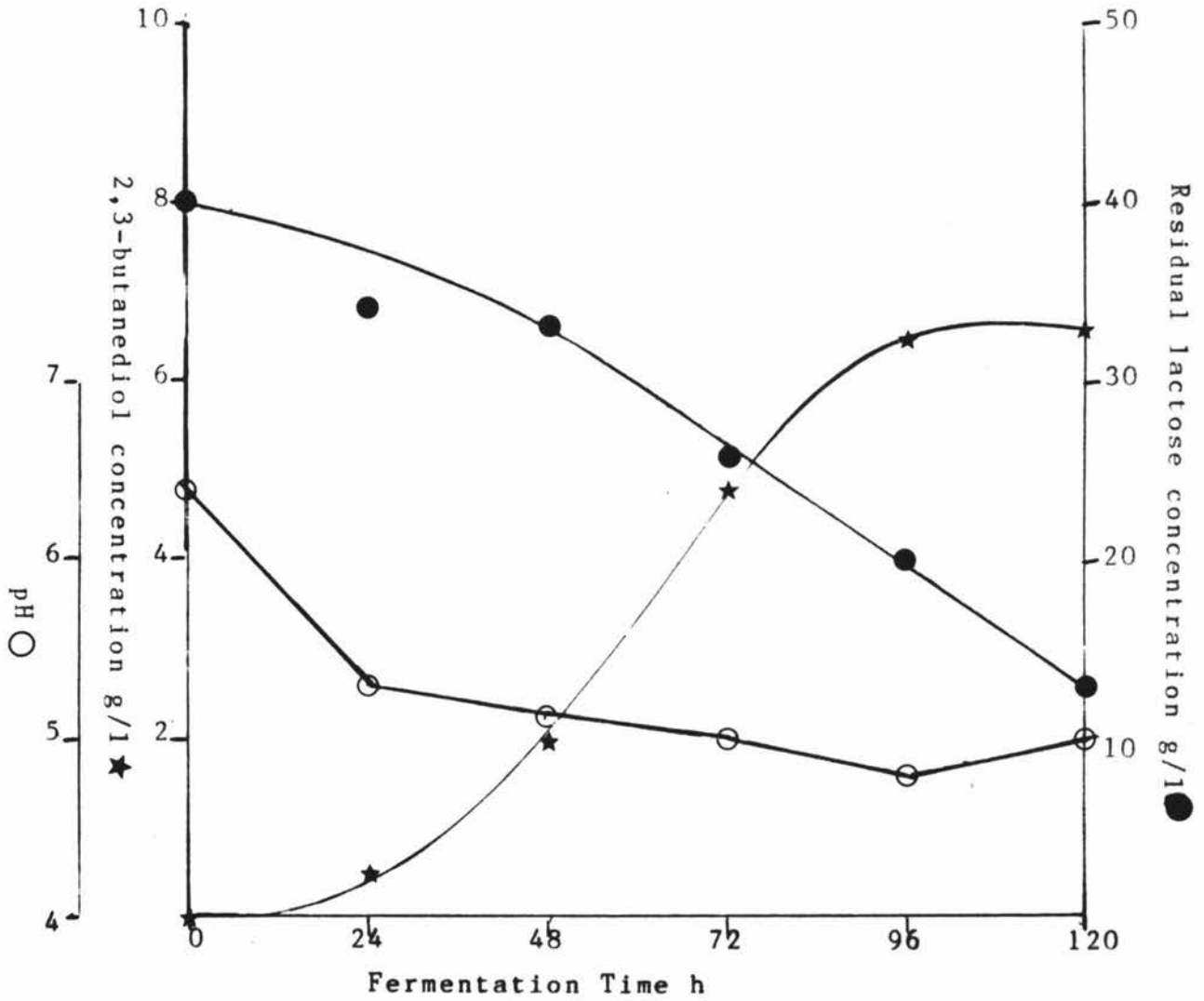


Fig 4.2 2,3-butanediol production by free cells of **Klebsiella pneumoniae**

tions(Appendix Table 1A) show that immobilized and free cells produced approximately the same ethanol concentration; however, free cells produced higher amount of acetoin than immobilized cells. The pH value during butanediol production was in the range of 4.8 - 5.2. The increased solvent production achieved by immobilized cells may be due to the more anaerobic microenvironment experienced by the entrapped cells. Thus cell immobilization of Klebsiella pneumoniae appears to be beneficial with respect to butanediol production.

4.1.2 Effect of agitation on butanediol production by free and immobilized cells

An experiment was carried out to determine the effect of agitation(i.e aeration) on butanediol production by free and immobilized cells. The experiment was performed as described in section 4.1.1 and in addition, duplicate flasks were incubated with continuous agitation as decribed in section 3.2.8. The results of this experiment are shown in Table 4.1. The most noticeable feature about these results is that both free and immobilized cells produced higher butanediol concentrations when incubated in a non-agitated mode, and the solvent concentration recorded by immobilized cells was approximately twice that attained by free cells. The probable reason for the higher solvent production in a non-agitated mode is the more anaerobic conditions experienced under these conditions. The concentrations of ethanol for both free and immobilized cells were also higher in a non-agitated mode(Appendix Table 2A). The acetoin concentrations did not exceed 1g/l in either mode for both free and immobilized cells.

The results of this experiment have therefore demonstrated that butanediol production by either free or immobilized cells was far superior when no agitation was provided. Thus, all further batch experimentation in shakeflasks was conducted in a static mode.

Table 4.1 Maximum butanediol production and yields by free and immobilized cells under agitated and non-agitated conditions

	With agitation 2,3-butanediol		No agitated 2,3-butanediol	
	g/l	g/g lactose utilised	g/l	g/g lactose utilised
Free Cells	2.7	0.27	4.1	0.4
Immobilized Cells	6.5	0.3	8.7	0.5

4.1.3 Effect of cell numbers on butanediol production by immobilized cells

In this experiment, a constant ratio of 1.0ml original cell culture to 1.25ml sodium alginate solution was maintained. The cell number and the respective sodium alginate volumes employed are shown in Table 4.2. The beads thus formed were placed in 100ml of whey permeate. The aim of this experiment was to determine the cell number which would give the highest butanediol concentration. The cell immobilization procedure was as previously described in sections 3.2.6 and 3.2.8. The results of this experiment, shown in Fig 4.3, reveal that the cell number for maximum butanediol production was obtained from 20ml of original culture. Further increase in cell number resulted in a sharp decrease in butanediol concentration, although the lactose consumption increased. The reason for this result is not completely clear. One possible explanation is that if butanediol production is partly growth-associated and partly non-growth associated, then too low initial cell numbers would result in growth rather than butanediol production, while too high initial numbers lead to too little growth due to competition for nutrients. The maximum butanediol concentration recorded was 14.4g/l representing a yield of 0.53g/g lactose utilized. The ethanol and acetoin concentrations attained by the various cell loadings are shown in Appendix Table 3A which reveals that ethanol concentrations did not exceed 4g/l whilst acetoin concentrations were rather low. On the basis of the data obtained in this experiment, the cell numbers used in all further experiments was the cells from 20ml original culture/100ml of whey permeate. The cell: alginate ratio was maintained at 1.0 : 1.25.

4.1.4 Comparison of barium, calcium and strontium ions on alginate bead stability and butanediol production

Barium, calcium and strontium chloride solutions at 0.1M concentration in 0.1M Tris-HCl buffer(pH 7.0) were employed as gelating agents during cell immobilization.

Table 4.2 Cell number and amount of sodium alginate solution used to test the effect of cell numbers on butanediol production by immobilized cells

Vol. of original cell culture (ml)	Vol. of sodium alginate (ml)
1.0	1.25
5.0	6.25
20	25
40	50
60	75
80	100

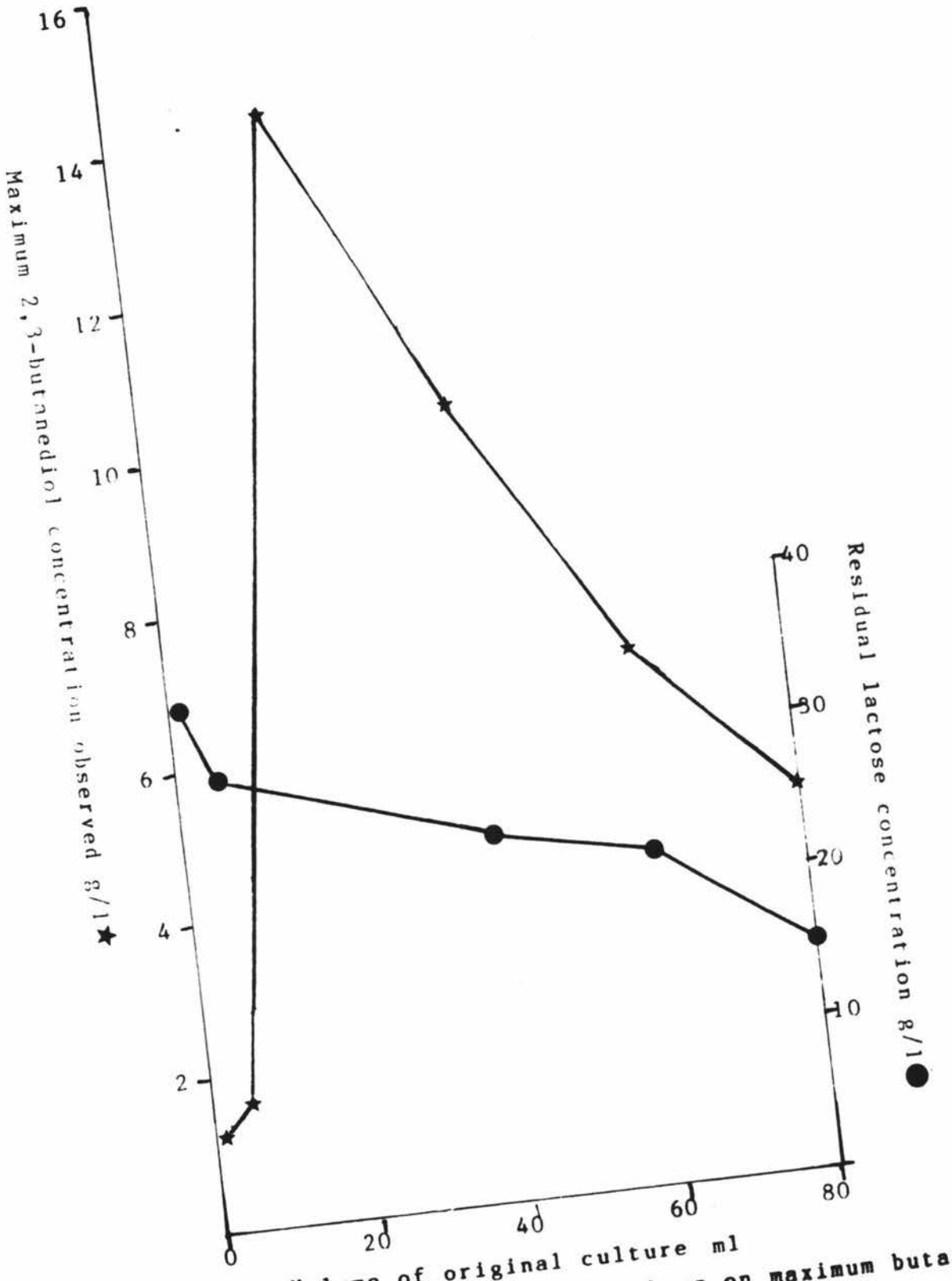


Fig 4.3 Effect of cell numbers on maximum butanediol production

The three types of alginate beads so obtained were all made using the same equipment. 27ml of respective alginate beads were suspended in 100ml whey permeate contained in 250ml shakeflasks. The flasks were incubated at 30°C in a static mode with periodic manual shaking. The purpose of this experiment was to assess the mechanical stability and butanediol-producing ability of entrapped cells when immobilized in barium, calcium and strontium alginate gels. The results, shown in Figs 4.4 - 4.6, reveal that the barium alginate beads produced the highest butanediol concentration of 21.8g/l after 120h incubation, while the calcium and strontium alginate beads gave lower concentrations of 14.2 and 14.7g/l respectively. Lactose utilization by all the three types of alginate beads was complete after 144h of fermentation. This coincided with a sharp decrease in butanediol concentration for all three bead types suggesting that the organism was consuming the butanediol as an alternative carbon source. Ethanol and acetoin concentrations(Appendix Table 4A) recorded by all three alginate beads did not exceed 5g/l and 1g/l, respectively. Visual examination of the beads revealed that calcium alginate pellets were the least stable of the three bead types whilst strontium alginate pellets appeared to be the most stable. Barium alginate pellets were fragile and had the smallest bead diameter(2mm) amongst the three bead types. This smaller bead size would mean shorter diffusion distances between nutrients present in the bulk solution and the entrapped cells. This could possibly explain the better butanediol production by barium alginate beads compared with those of calcium and strontium alginate. Because of the favourable results obtained using barium alginate beads, barium chloride was selected as the gelating agent for most of the subsequent batch fermentation experiments.

4.1.5 Supplementation of barium chloride or calcium chloride to whey permeate and their effects on butanediol production by entrapped cells

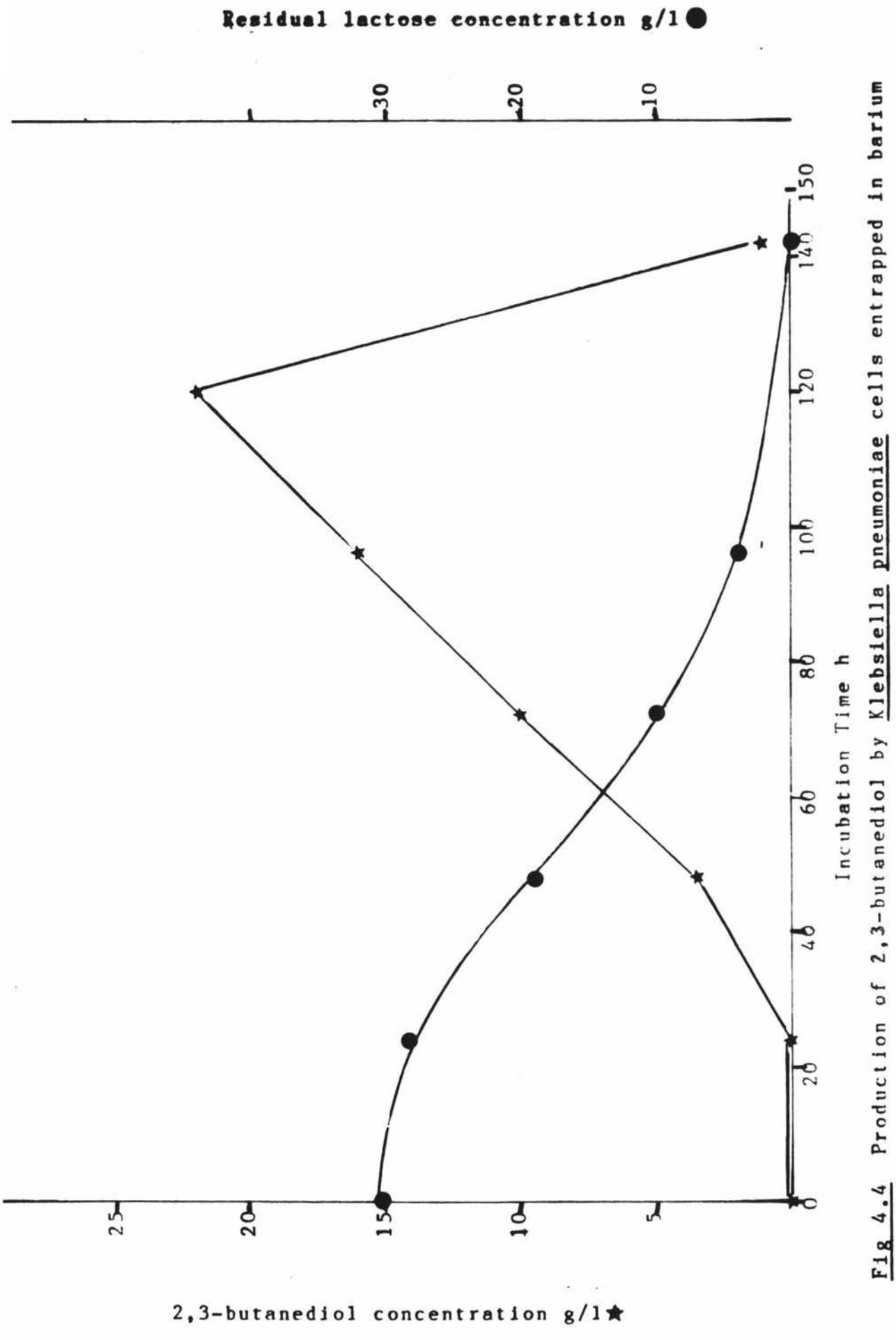


Fig 4.4 Production of 2,3-butanediol by Klebsiella pneumoniae cells entrapped in barium alginate gels

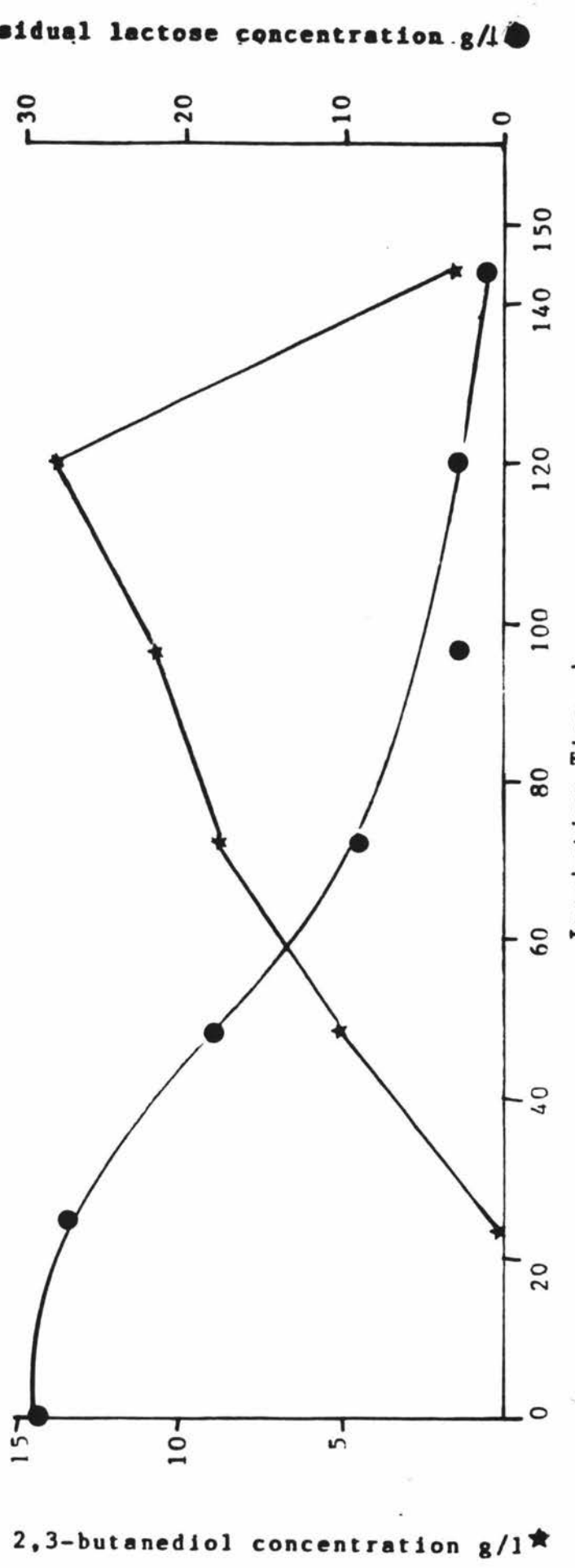


Fig 4.5 Production of 2,3-butanediol by Klebsiella pneumoniae cells entrapped in calcium alginate gels

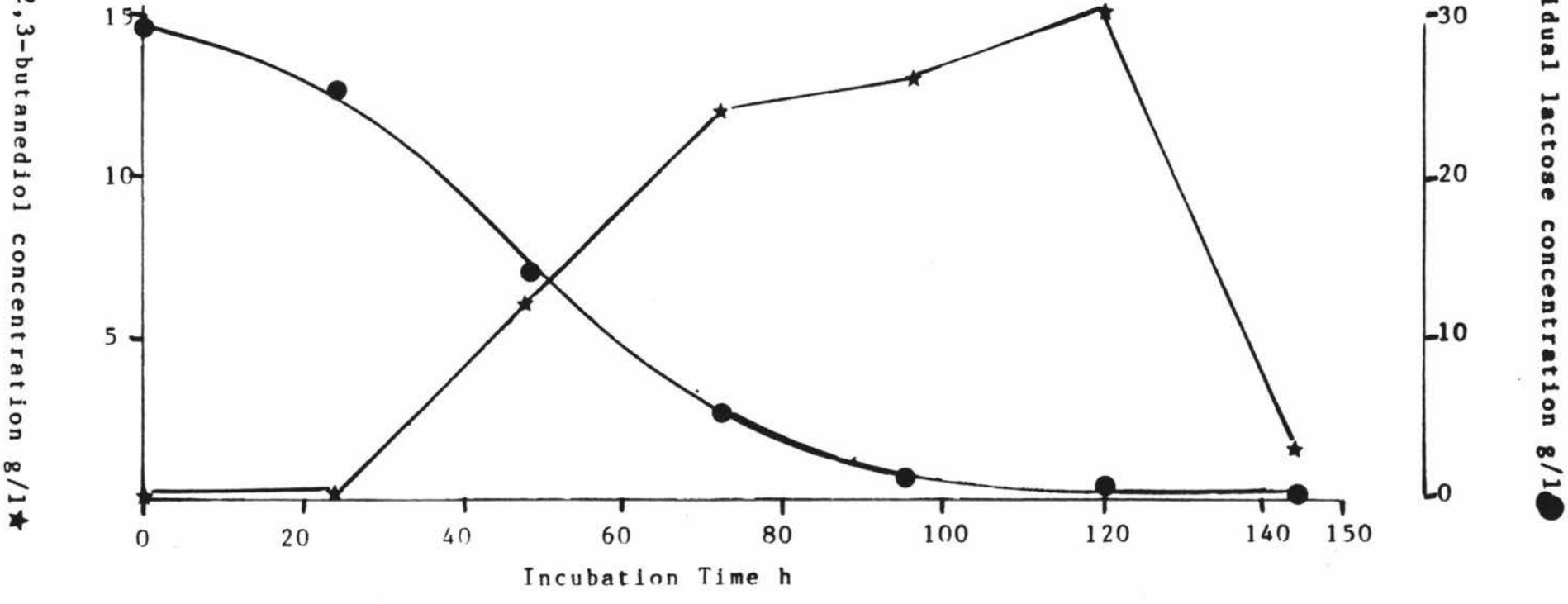


Fig 4.6 Production of 2,3-butanediol by Klebsiella pneumoniae in strontium alginate gels

Having obtained preliminary information regarding the mechanical stability and butanediol-producing capability of barium, calcium and strontium alginate beads, further experiments were performed with the aim of enhancing bead stability and hence the smooth operation of later continuous fermentation experiments. Barium and calcium alginate pellets containing entrapped cells were prepared as previously stated(sections 3.2.6 and 3.2.8). Equal volumes of the two bead types were inoculated into whey permeate containing barium or calcium chloride as appropriate, at concentrations ranging from 1 - 5g/l. The results of this experiment are shown in Table 4.3. The data in this Table reveal that as the concentration of barium chloride increases, the maximum butanediol production decreases correspondingly, implying an inhibitory effect of barium ions on butanediol production. In contrast, the presence of calcium ions did not appear to have such an inhibitory effect. As there was little significant difference in terms of butanediol production between calcium chloride concentrations of 2 and 3g/l, the latter concentration was chosen to supplement whey permeate for continuous fermentation experiments because of the excellent stability of the beads at this calcium ion concentration.

4.1.6 Effect of bead size on butanediol production

The aim of this experiment was to determine the optimum bead diameter for maximum butanediol production. The practical difficulties of preparing large quantities of beads were also investigated, particularly with respect to later experimentation in a continuous mode. In this experiment, a constant ratio of 20ml original cell culture to 25ml of sodium alginate solution was maintained during cell immobilization. Barium alginate bead sizes ranging from 1.8mm to 5.5mm diameter were made manually with the aid of sterile disposable syringes, 1ml and 10ml pipettes. The results of this study, shown in Table 4.4, reveal that butanediol production decreased with increasing bead diameter. This could possibly be due to larger diffusion

Table 4.3 Effect of barium or calcium chloride supplements to whey permeate on butaendiol production by entrapped cells

Concentration of $BaCl_2$ or $CaCl_2$ (g/l)	Calcium Chloride Maxiumm butanediol concentration(g/l)	Barium Chloride Maximum butanediol concentration(g/l)
1	13.1	10.5
2	12.0	8.5
3	12.3	3.5
4	9.0	3.5
5	10.0	3.0

Table 4.4 Effect of bead diameter on butanediol production by barium alginate immobilized cells

Bead diameter(mm)	Maximum production of 2,3-butanediol(g/l)
1.8	11.3
2.4	10.7
3.0	9.4
4.5	8.6
5.5	8.2

distances associated with larger bead sizes. Thus, larger beads would result in mass transfer limitations and the nutrients would need a longer time to reach the entrapped cells. The overall butanediol productivities for all bead sizes tested were approximately 0.09g/l.h while the yields were approximately 0.3g/g lactose utilized. Ethanol production, like butanediol, decreased with increasing bead sizes but acetoin did not appear to follow a similar pattern(Appendix Table 5A). Thus, although the data in this experiment indicate that smaller alginate bead sizes should be used in further experiments, the practical difficulties of making small beads via disposable syringes added constraints to the choice of an optimum bead size.

Thus, since 3.0mm diameter beads could be easily made(using a 1ml pipette), and butanediol production using this size was comparatively higher than that of larger beads, this bead size was selected and employed in all further batch and continuous experiments.

4.1.7 Effect of bead storage and bead recycle on butanediol production

The main aim of this experiment was to test the effects of long term bead storage on subsequent butanediol production. The effect of bead recycle was also studied to test for longevity of activity.

Separate lots of barium alginate beads(27ml per lot) containing entrapped Klebsiella pneumoniae cells were stored for up to four weeks in 8.5g/l barium chloride or 0.1M Tris-HCl buffer(pH 7.5) at both 4°C and 30°C. After the appropriate storage period the beads were removed from their storage environment, washed with sterile distilled water and inoculated into 250ml conical flask containing 100ml of unsupplemented whey permeate. The flasks were then incubated at 30°C in a static mode. After 72h incubation, the butanediol concentration was recorded, and the beads were recycled into fresh whey permeate. After a furhter 72h incubation, the butanediol production was again recorded, and the beads were transfered

to fresh whey permeate for a third and final incubation (second recycle). The results of this experiment, shown in Fig 4.7 and Tables 4.5 - 4.7, indicate two main trends. First, increased storage time led to decreased butanediol production during the first incubation period after retrieval from storage(Fig 4.7). Beads stored at 30°C were the most seriously affected. Second, although activity was lost during storage, it was subsequently revived on successive incubation in whey permeate(Tables 4.5 - 4.7). The cause of the loss in activity is not clear, but possible reasons could include some cell death or a temporary inactivation of the enzyme system necessary for butanediol production. However since whey permeate is known to be a satisfactory growth medium for Klebsiella pneumoniae(Lee, 1984), it is not surprising that any surviving cells could be subsequently reactivated. The corresponding ethanol and acetoin concentrations recorded (Appendix Table 6A) showed a similar pattern to that of butanediol production. The results obtained from this experiment suggest that long-term continuous fermentation of whey permeate should be feasible.

4.1.8 Effect of lactose concentration during culturing of cells on subsequent butanediol production and lactose utilization by immobilized cells

This experiment was designed to determine whether prior acclimatization of cells in a high initial lactose concentration would result in higher butaendiol production from whey permeate supplemented with additional lactose. The possible adverse effects of substrate and product inhibition were also assessed. Cells of Klebsiella pneumoniae were cultivated in a semi-synthetic medium as described previously in section 3.2.4. However, in this experiemnt, two levels of lactose were employed. These were 50 and 100g/1. After cell immobilization the whey permeate used for incubation was supplemented with 45g/1 lactose. Unsupplemented whey permeate was also used as a control. The results of this experiment are summarized in Tables 4.8 and 4.9. The data in these

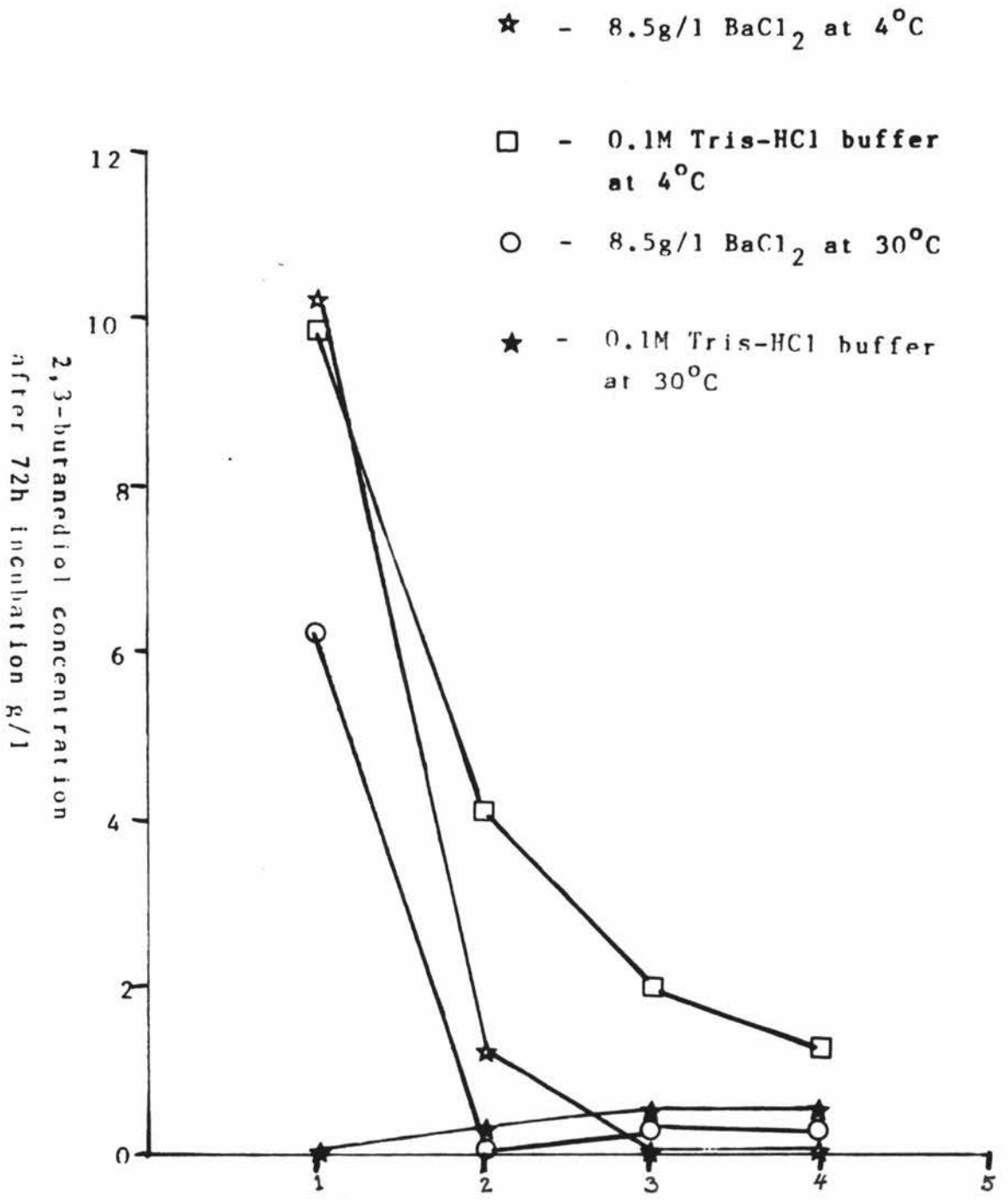


Fig 4.7 Effect of storage time and conditions on 2,3-butanediol production(1st incubation)

Table 4.5 Effect of 2 weeks storage and bead recycle on butanediol production

Storage conditions	2,3-butanediol concentration after 72h incubation(g/l)		
	Incubation No:		
	1	2	3
8.5g/l $BaCl_2$ at 4°C	1.2	7.9	13.1
8.5g/l $BaCl_2$ at 30°C	0	DNA	DNA
0.1M Tris-HCl buffer (pH 7.5) at 4°C	4.1	7.0	14.1
0.1M Tris-HCl buffer (pH 7.5) at 30°C	0	DNA	DNA

*DNA - Data Not Available

Table 4.6 Effect of 3 weeks storage and bead recycle on butanediol production

Storage conditions	2,3-butanediol concentration after 72h incubation(g/l)		
	Incubation No:		
	1	2	3
8.5g/1 $BaCl_2$ at 4°C	0	6.9	11.2
8.5g/1 $BaCl_2$ at 30°C	0	DNA	DNA
0.1M Tris-HCl buffer (pH 7.5) at 4°C	2.0	12.4	11.9
0.1M Tris-HCl buffer (pH 7.5) at 30°C	0	DNA	DNA

*DNA - Data Not Available

Table 4.7 Effect of 4 weeks storage and bead recycle on butanediol production

Storage conditions	2,3-butanediol concentration after 72h incubation(g/l)		
	Incubation No:		
	1	2	3
8.5g/l $BaCl_2$ at 4°C	0.7	10.8	11.2
8.5g/l $BaCl_2$ at 30°C	0	DNA	DNA
0.1M Tris-HCl buffer (pH 7.5) at 4°C	1.3	5.1	11.5
0.1M Tris-HCl buffer (pH 7.5) at 30°C	0	DNA	DNA

*DNA - Data Not Available

Table 4.8 Effect of lactose concentration during growth on subsequent 2,3-butanediol production by immobilized cells incubated in unsupplemented whey permeate

Lactose concentration during cell growth(g/l)	Fermentation parameters of immobilized cells				
	Maximum 2,3-butanediol conc.(g/l)	Initial lactose conc.(g/l)	Final lactose conc.(g/l)	Yield g/g lactose utilized	Overall productivity (g/l.h)
50	7.1	21.9	1.4	0.36	0.064
100	6.9	27.0	1.5	0.30	0.071

Table 4.9 Effect of lactose concentration during growth on subsequent 2,3-butanediol production by immobilized cells incubated in lactose-supplemented whey permeate

Lactose concentration during cell growth(g/l)	Fermentation parameters of immobilized cells				
	Maximum 2,3-butanediol conc.(g/l)	Initial lactose conc.(g/l)	Final lactose conc.(g/l)	Yield g/g lactose utilized	Overall productivity (g/l.h)
50	24.7	68.1	6.2	0.4	0.11
100	24.5	66.7	5.8	0.4	0.11

Tables indicate that increased lactose concentration during cell growth had no significant effect on subsequent butanediol production or lactose utilization when the cells were immobilized and incubated in whey permeate. A similar pattern was observed when the permeate was supplement with additional lactose. Comparing the performances of immobilized cells in ordinary permeate and lactose supplemented permeate, butanediol production and lactose utilization were higher in the latter, and the productivities were increased. There was also a slight increase in solvent yield. The data in the two Tables also suggest that substrate inhibition effects are not present using unsupplemented whey permeate. In addition, using unsupplemented whey permeate, product inhibition does not seem to be a problem.

4.1.9 Discussion

The batch fermentation studies generated information which was useful in the planning and operation of continuous fermentation investigations. The highlight of these studies was that cell immobilization of Klebsiella pneumoniae in alginate gels is not detrimental to butanediol production, but is infact, beneficial. The butanediol concentration recorded were much higher than with free cells. The immobilization procedure adopted was found to be mild and non-toxic to the entrapped microorganism. The optimum cell number was found to be 20ml original cell culture per 100ml of whey permeate. Agitation of suspended alginate beads resulted in poor butanediol production while static incubation gave higher solvent concentrations. Although barium alginate beads were employed throughtout the batch studies, and were more stable and superior to calcium alginate, barium ions which were included in whey permeate resulted in severe inhibition of butanediol production. However, this pattern was not observed with calcium alginate beads. The stability of the latter beads was enhanced when a calcium chloride concentration of 3g/l was added to the permeate. These beads were then chosen for continuous

fermentation studies. With regards to bead size, on the basis of ease of preparation, and satisfactory butanediol production, a 3mm bead diameter was noted to be the ideal choice. The effects of bead storage and bead recycle revealed that long term bead storage was detrimental to butanediol production irrespective of storage conditions. In particular, storage of beads at 30°C was found to severely affect butanediol production. However, storage of beads at 4°C in either 0.1M Tris-HCl buffer or 8.5 g/l $BaCl_2$ was satisfactory but required repeated reviving with whey permeate to attain maximum butanediol levels. Prior acclimatization of cells in high lactose concentrations did not have any significant effect on subsequent butanediol production by immobilized cells incubated in either ordinary or lactose supplemented whey permeate. Substrate and product inhibitions were not detected but entrapped cells were observed to consume butanediol when the lactose in the whey permeate was exhausted. The overall butanediol productivity of immobilized cells in batch fermentation was relatively poor(0.11g/l.h). Hence the need to conduct continuous fermentation experiments with a view to improving productivity.

4.2 Continuous fermentation

The results of the batch fermentation studies had shown that overall butanediol productivity was rather poor. Thus, continuous fermentation experiments were performed with a view to improve productivity. Two types of bioreactors were evaluated. These were a continuous stirred tank reactor(CSTR) and packed columns. In contrast to batch fermentation, calcium alginate beads were employed. To ensure mechanical stability of these beads, the whey permeate was supplemented with 3g/l calcium chloride. The function of this was to counteract the phosphate ions present in the permeate, thus partially removing the adverse effect of phosphate interaction with the gel matrix. The substrate was kept anaerobic by surface-flushing aseptically with oxygen-free

nitrogen gas. The cell immobilization procedure was as described in section 3.2.6.

In all experiments, a particular dilution rate was employed until steady-state was attained. Only then was the D value changed to attain a new steady-state.

4.2.1 Continuous stirred tank reactor

In an attempt to enhance productivity, a fermentor vessel with a working volume of 1.5l was filled with 1.3l of calcium alginate beads. This bead volume was the maximum the vessel could accomodate while retaining good mixing. The beads and whey permeate were incubated for 40 - 45h at 30°C prior to initiation of the medium feed. The stirrer speed was adjusted to 150rpm to ensure satisfactory uniform mixing. Five continuous fermentation runs were conducted. However, due to severe contamination of the substrate in the initial three experiments, the results of only two favourable runs are shown. The results of the first experiment, shown in Fig 4.8, reveal that upon batching the system for 45h, there was a rapid decline in lactose concentration indicating that lactose was rapidly consumed. The butanediol concentration attained was, however, rather low(1.2g/l). Upon feed initiation at a dilution rate of $0.06h^{-1}$, there was a rapid increase in residual lactose concentration while the increase in butanediol production was rather gradual. A steady state was reached after 90h of fermentation. The butanediol productivity at this dilution rate was 0.12g/l.h. An increase of the dilution rate to $0.14h^{-1}$ resulted in only a marginal increase in productivity. Steady state was achieved after 130h of fermentation. A further increase in dilution rate resulted in a sharp decrease in butanediol concentration and productivity with a corresponding increase in residual lactose concentration. When the dilution rate was now decreased to $0.06h^{-1}$, the butanediol concentration and productivity did not recover to their original values. In contrast to these results the data in Fig 4.9 were more encouraging. A butanediol productivity of 0.39g/l.h was attained at a D value of $0.074h^{-1}$ after 90h of fermentation.

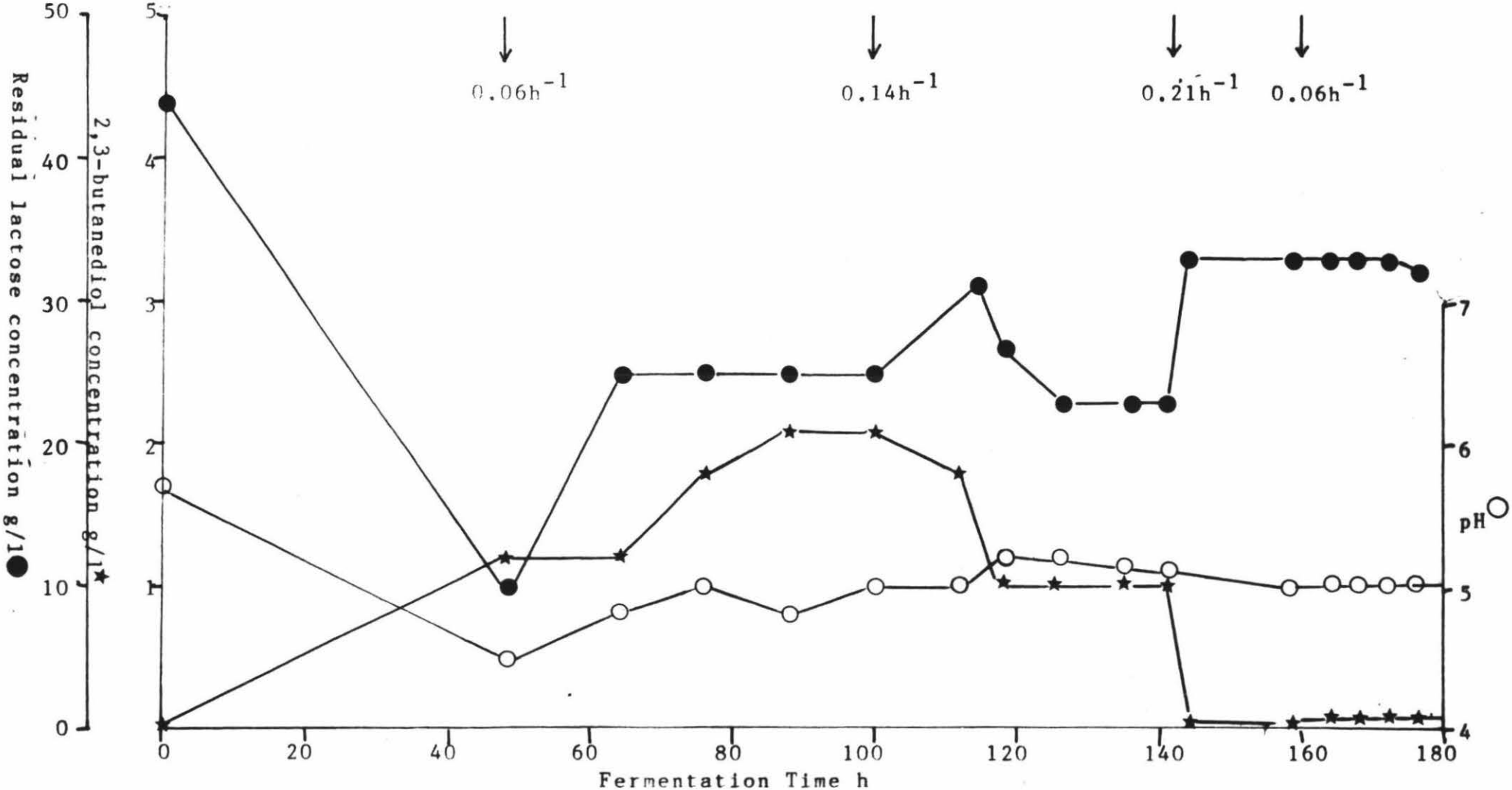


Fig 4.8 Production of 2,3-butanediol and lactose utilization in a CSTR

Bead volume = 1.31, working volume = 1.51 (1st Run)

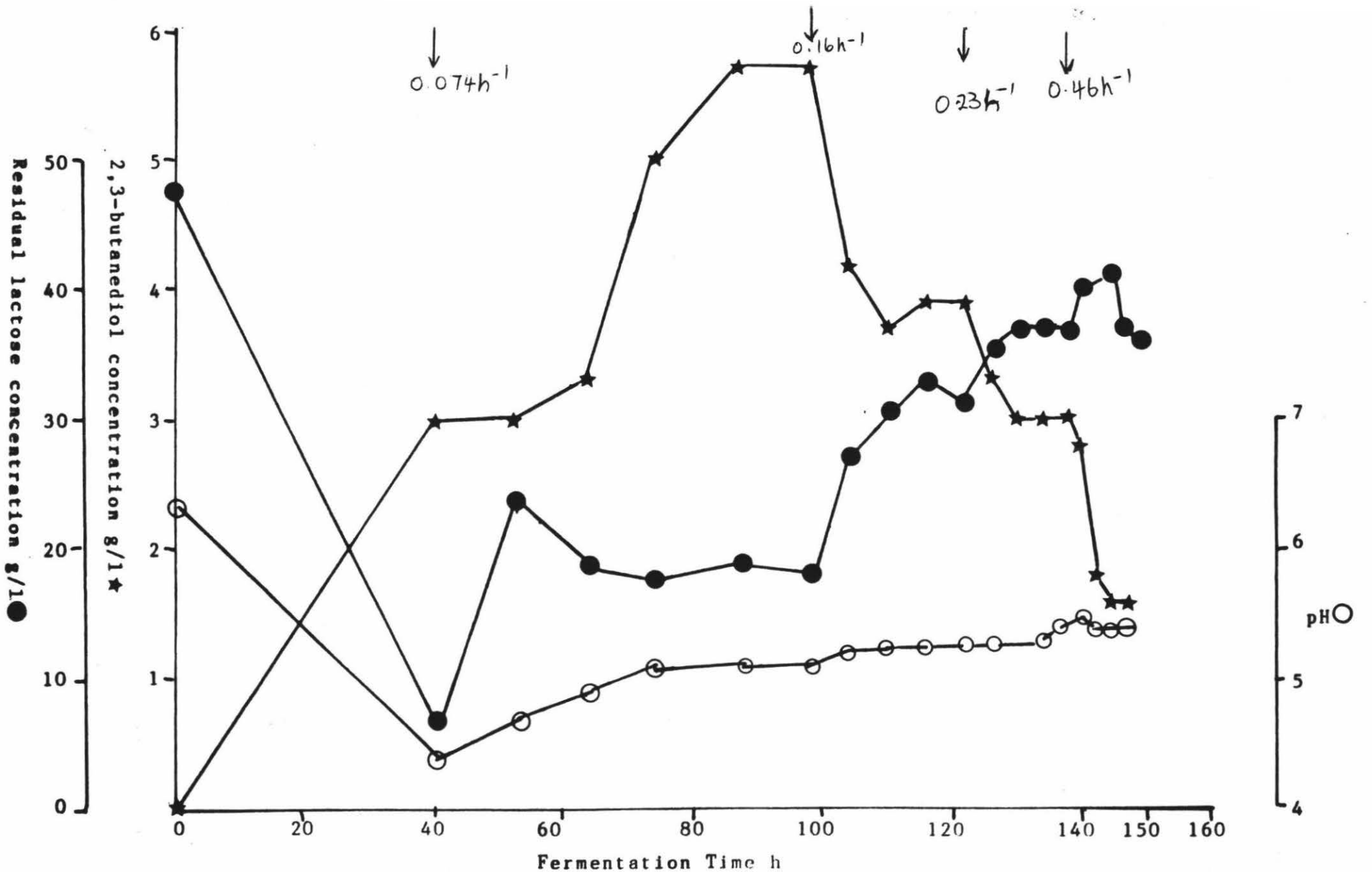


Fig 4.9 Production of 2,3-butanediol and lactose utilization in a CSTR

Bead volume = 1.31, working volume = 1.51 (2nd Run)

Further increases in dilution rate resulted in similar increases in butanediol productivity. For example, at a D value of $0.16h^{-1}$, the productivity recorded was 0.68g/l.h. However, above this D value further increases resulted in no corresponding increases in butanediol productivity. Another feature of this second experiment was that at no time did the butanediol concentration or productivity decrease to zero. Lactose utilization for both runs was not complete at any D value. However, the maximum productivity achieved was seven times that recorded in batch fermentation. The results of these two fermentation runs are summarized in Fig 4.10. It appears therefore that for this particular CSTR system dilution rates greater than $0.2h^{-1}$ do not generate any further increase in butanediol productivity suggesting that the entrapped cells have reached their maximum metabolic capability. A major disadvantage of the CSTR is the abrasive action of the agitating system which causes bead flaking and eventual bead disintegration.

4.2.2 Packed columns

Three glass columns with volumes of 100, 200 and 300ml, with identical diameters of 24mm, were employed in this study. The substrate was fed into the lower end of each column. The two shorter columns were operated in a vertical mode while the longest column was inclined at 10^{o} to the horizontal and fitted internally with a stainless steel mesh. Calcium alginate beads were employed and were prepared as described in section 3.2.6. The aim of the packed column experiments was to evaluate the performance of entrapped cells when these cells were exposed to a substrate having a nutrient gradient.

4.2.2.1 100ml column

The results of this experiment, shown in Figs 4.11 and Fig 4.12, reveal that the maximum butanediol productivity attained was 0.9g/l.h at a D value of $0.56h^{-1}$. This value was slightly greater than that obtained in the CSTR. However, a further increase

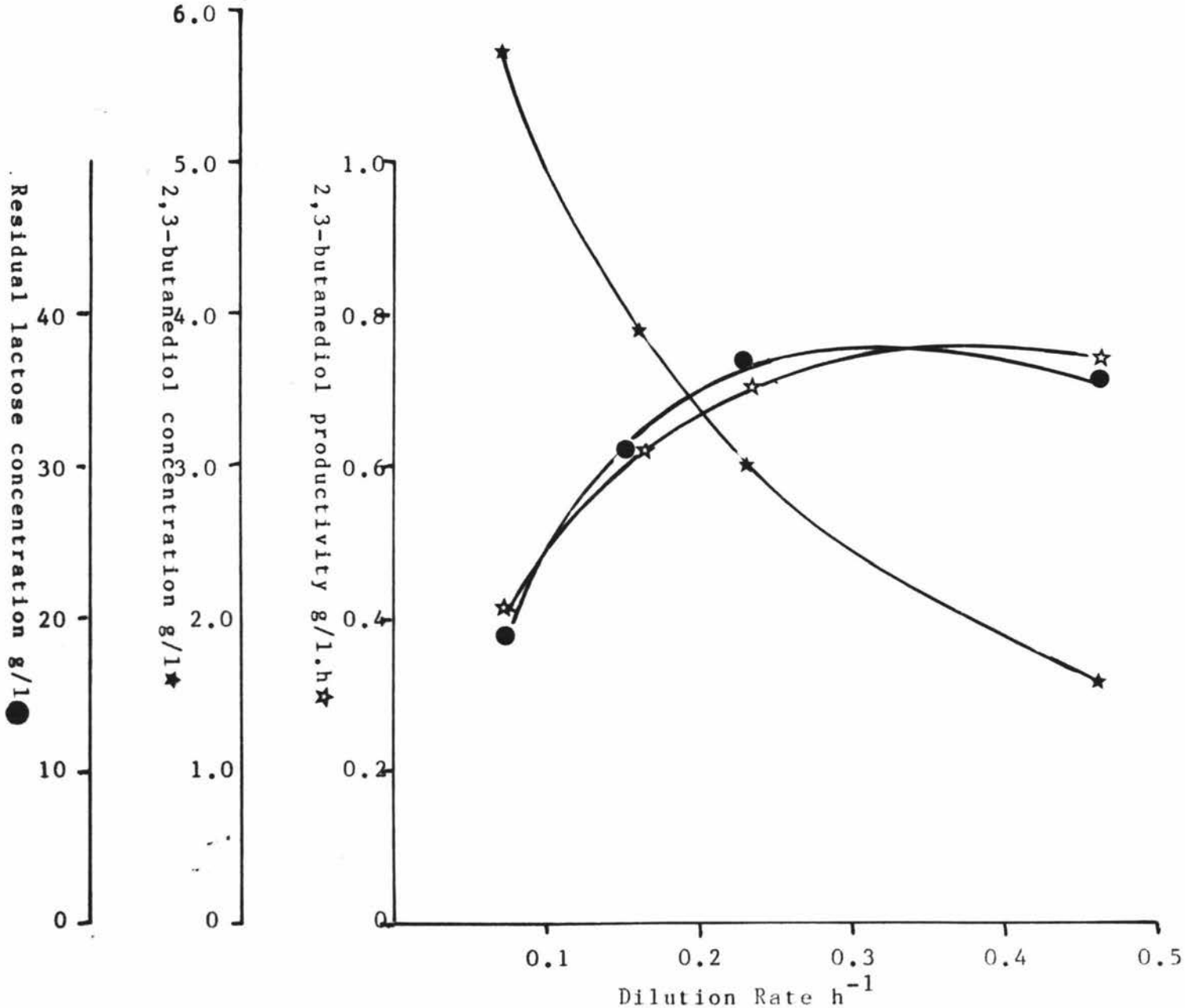


Fig 4.10 Effect of dilution rate on butanediol productivity, concentration and lactose utilization in a CSTR

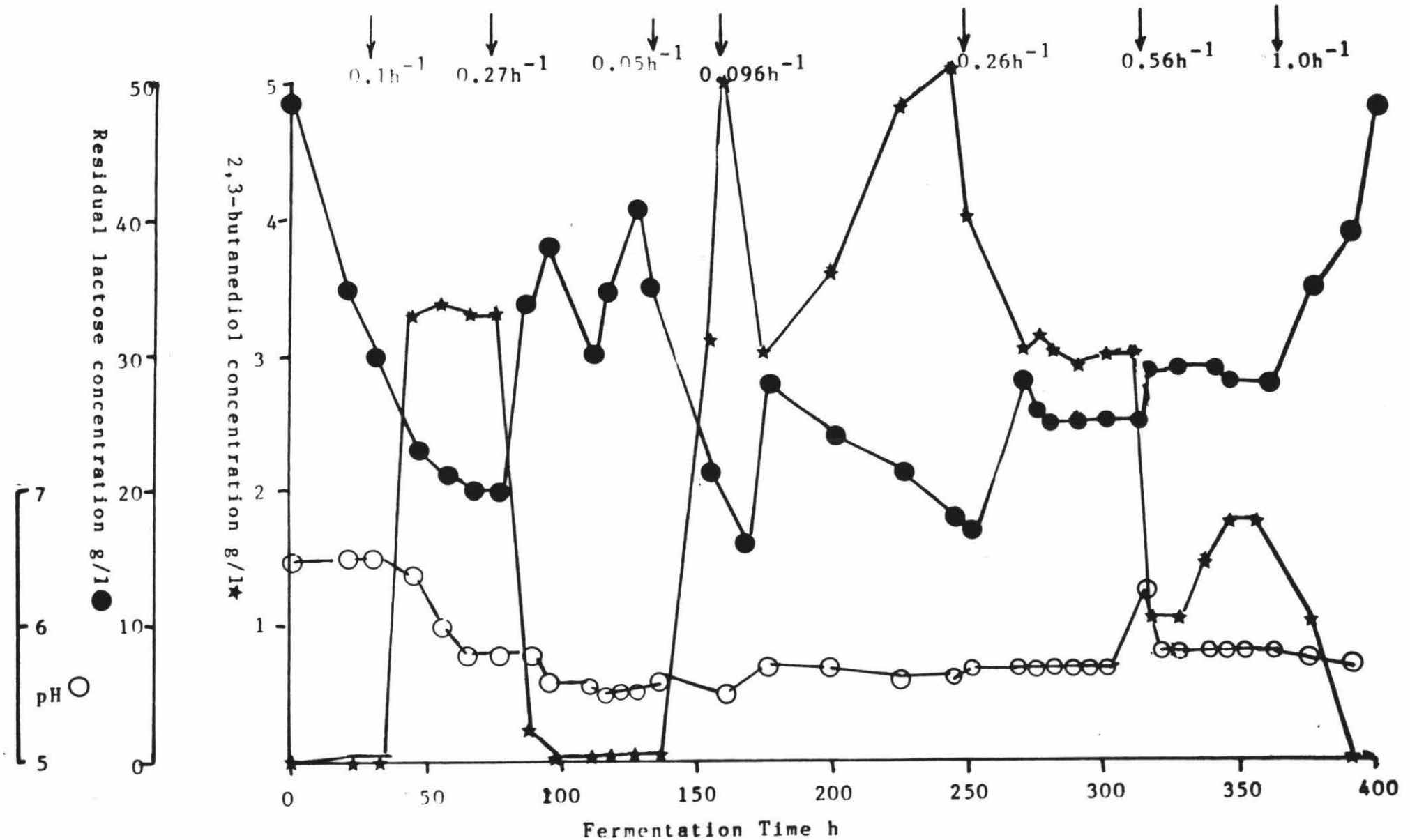


Fig 4.11 Production of 2,3-butanediol and lactose utilization in a 100ml column
Bead volume = 67.0ml, void volume = 33.0ml

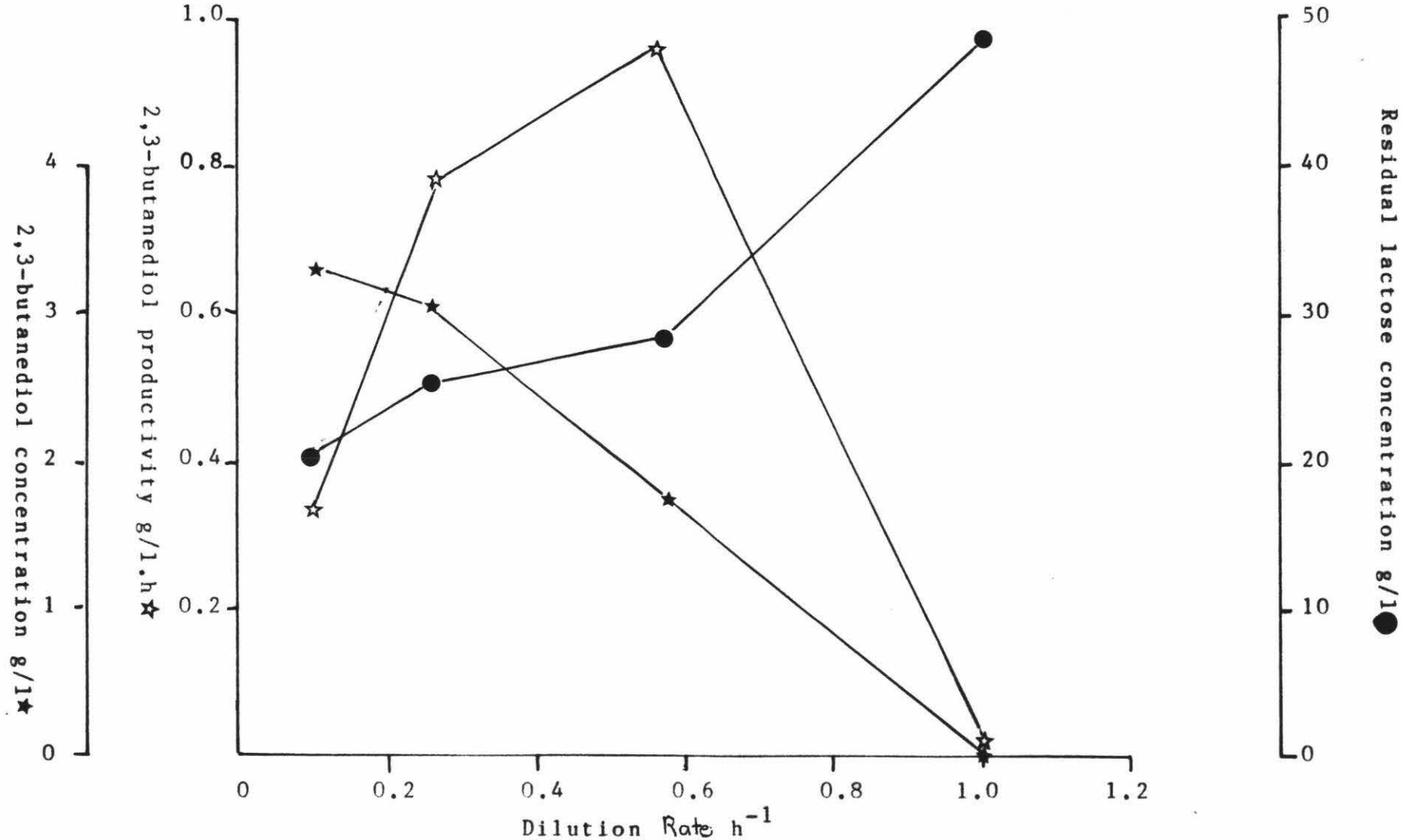


Fig 4.12 Effect of dilution rate on butanediol productivity, concentration and lactose utilization in a 100ml column

in dilution rate resulted in a decrease in butanediol concentration and productivity with a corresponding increase in residual lactose concentration. This decline in butanediol productivity can be explained by the adverse effect of carbon dioxide accumulation within the column(Plate 4.1) which in turn caused bead compaction (Plate 4.2) and subsequent feed channelling along the sides of the glass walls, preventing nutrients gaining access to entrapped cells.

4.2.2.2 200ml column

Since the butanediol productivity obtained from the 100ml column was only slightly greater than that obtained using the CSTR, the column was lengthened with a view to increase the butanediol productivity. Three fermentation runs were performed and the results are shown in Figs 4.13 - 4.15 and summarized in Fig 4.16. In the first run(Fig 4.13), medium feed was initiated at a dilution rate of $0.07h^{-1}$ and steady state was achieved after 125h of fermentation giving a productivity of 0.42 g/l.h. The dilution rate was then doubled and the new steady state was reached after 185h of fermentation. The productivity at this steady state was 0.61g/l.h. The D value was again increased($0.27h^{-1}$) and steady state was attained after 260h of fermentation giving a butanediol productivity of 0.14g/l.h. A further increase in D value led to a sharp decline in butanediol productivity accompanied by a corresponding increase in residual lactose concentration. This sharp decrease was again due to the adverse effects of carbon dioxide accumulation in the system previously encountered with the 100ml column. In the second run, (Fig 4.14) the initial dilution rate was $0.25h^{-1}$ in an attempt to attain maximum productivity in the shortest possible fermentation time thereby avoiding the rapid build up of carbon dioxide in the column. This approach was however, found to be detrimental to the smooth operation of the packed column. A steady state was achieved at this D value after 110h of fermentation giving a productivity of

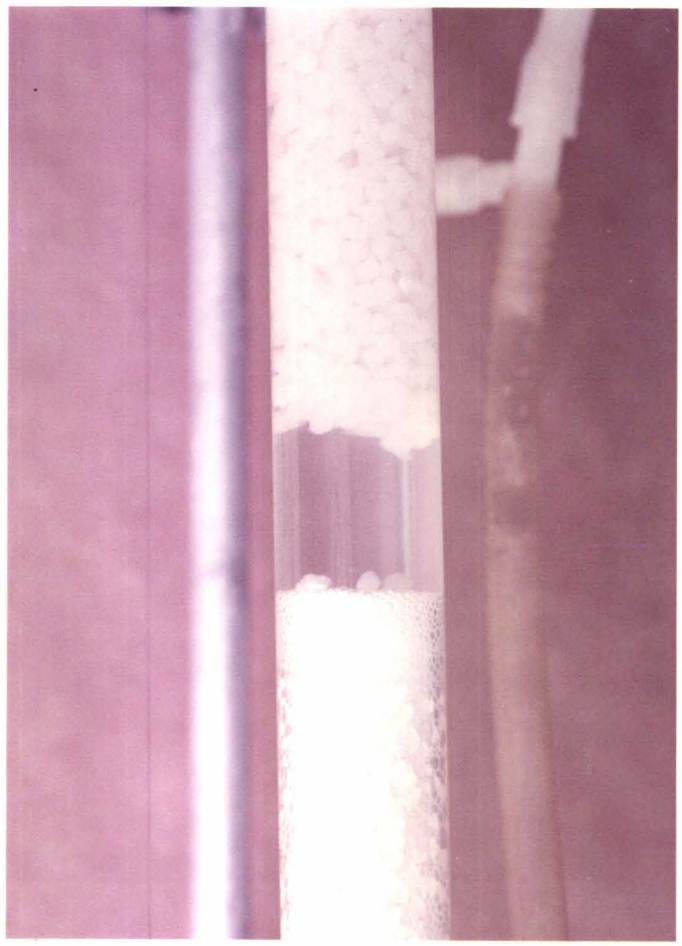


Plate 4.1 Adverse effect of CO_2 build up in the column

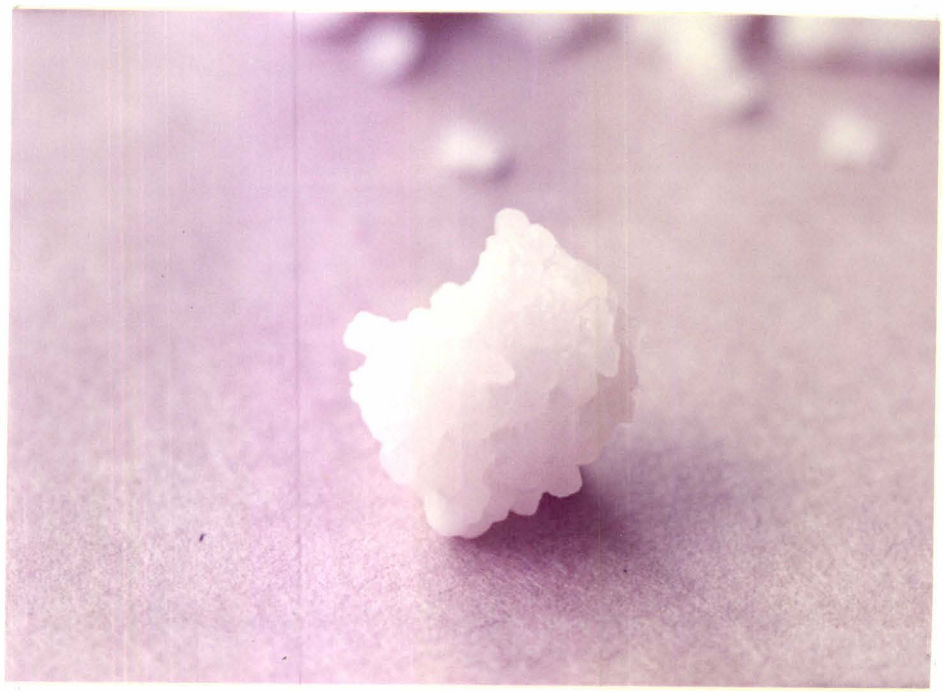


Plate 4.2 Bead compaction as a result of CO_2 build up in column

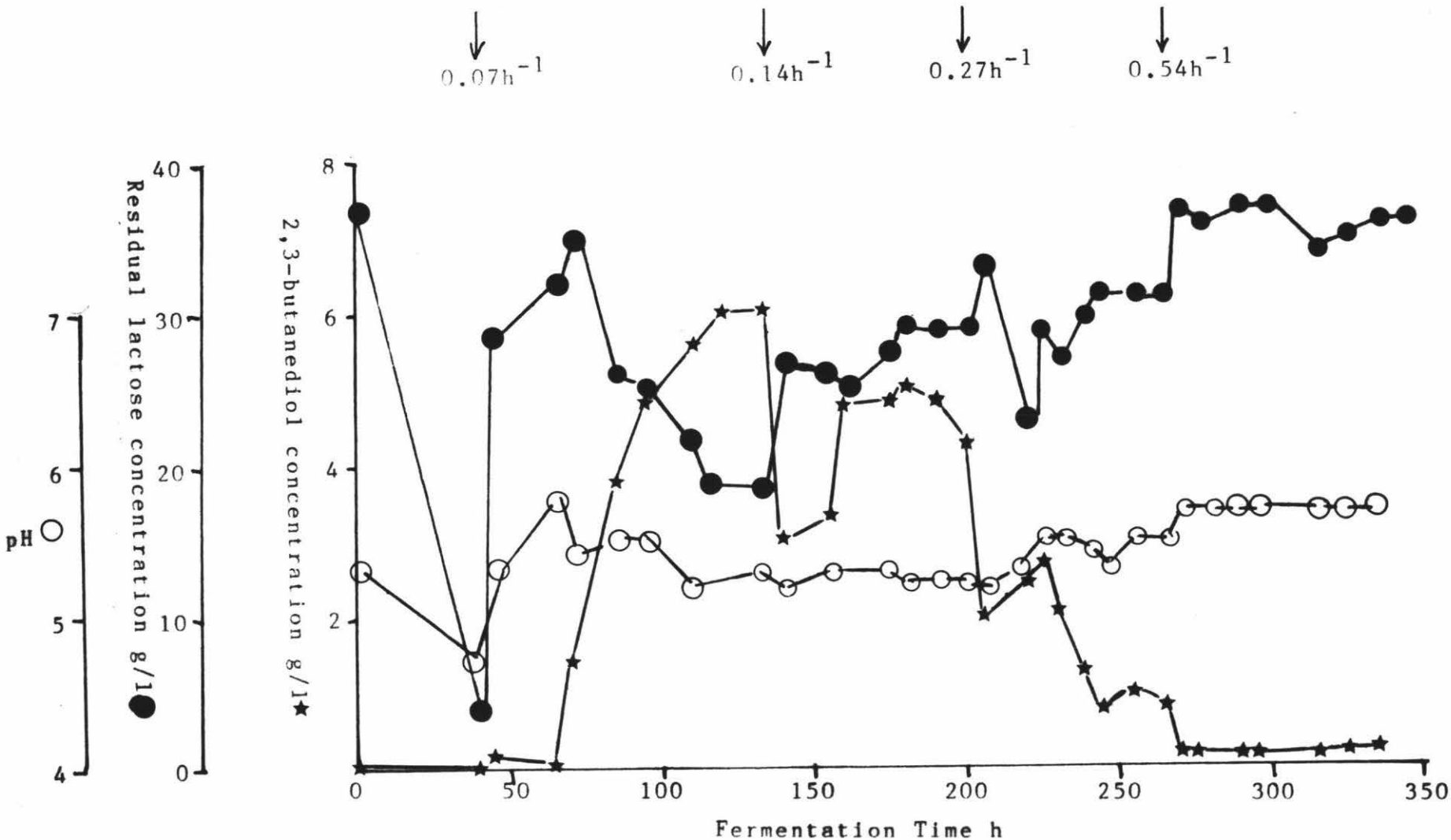


Fig 4.13 2,3-butanediol production and lactose utilization in a 200ml packed column
Bead volume = 130ml, void volume = 70ml (1st Run)

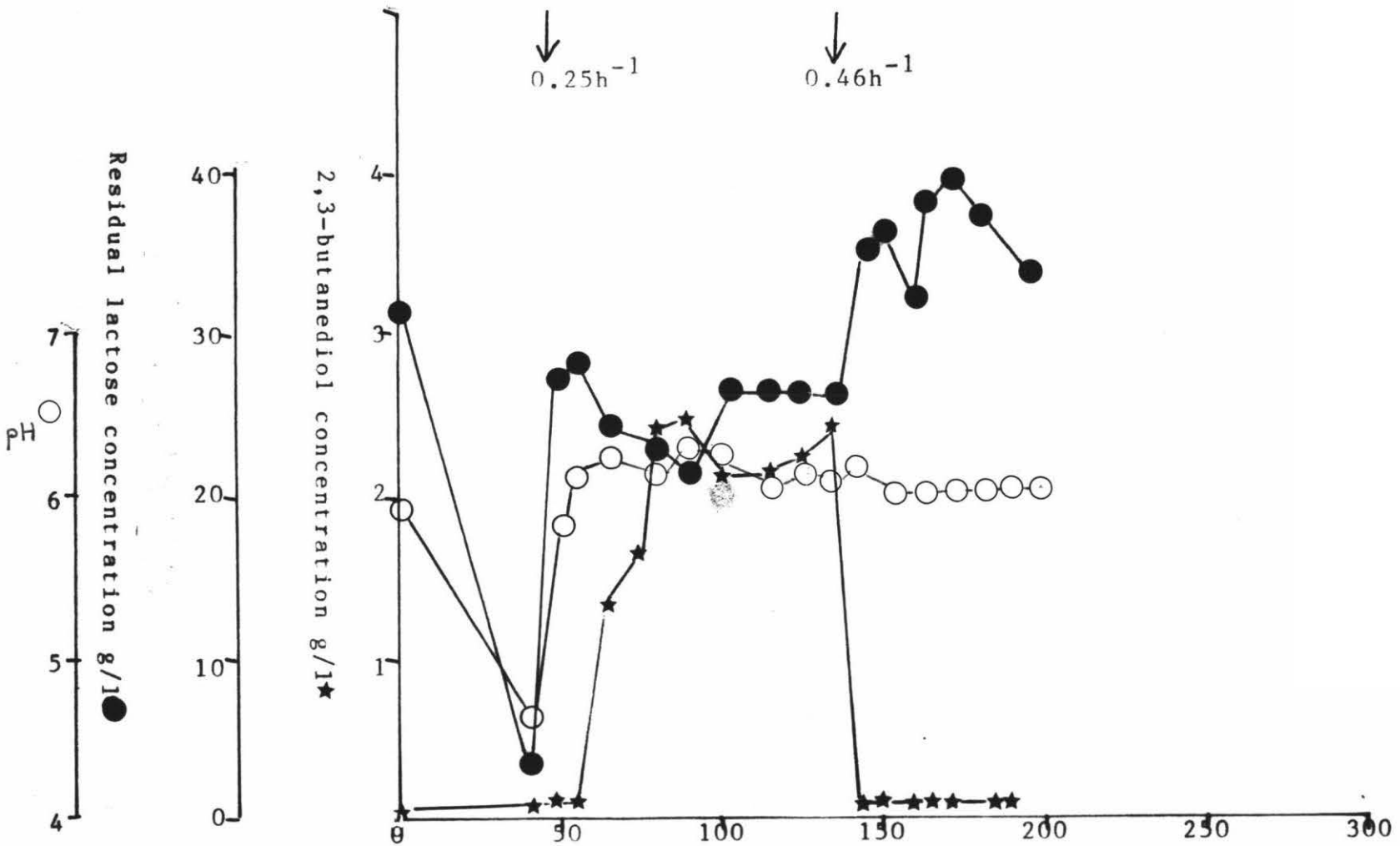


Fig 4.14 2,3-butanediol production and lactose utilization in a 200ml column
Bead volume = 165ml, void volume = 70ml (2^{nd} Run)

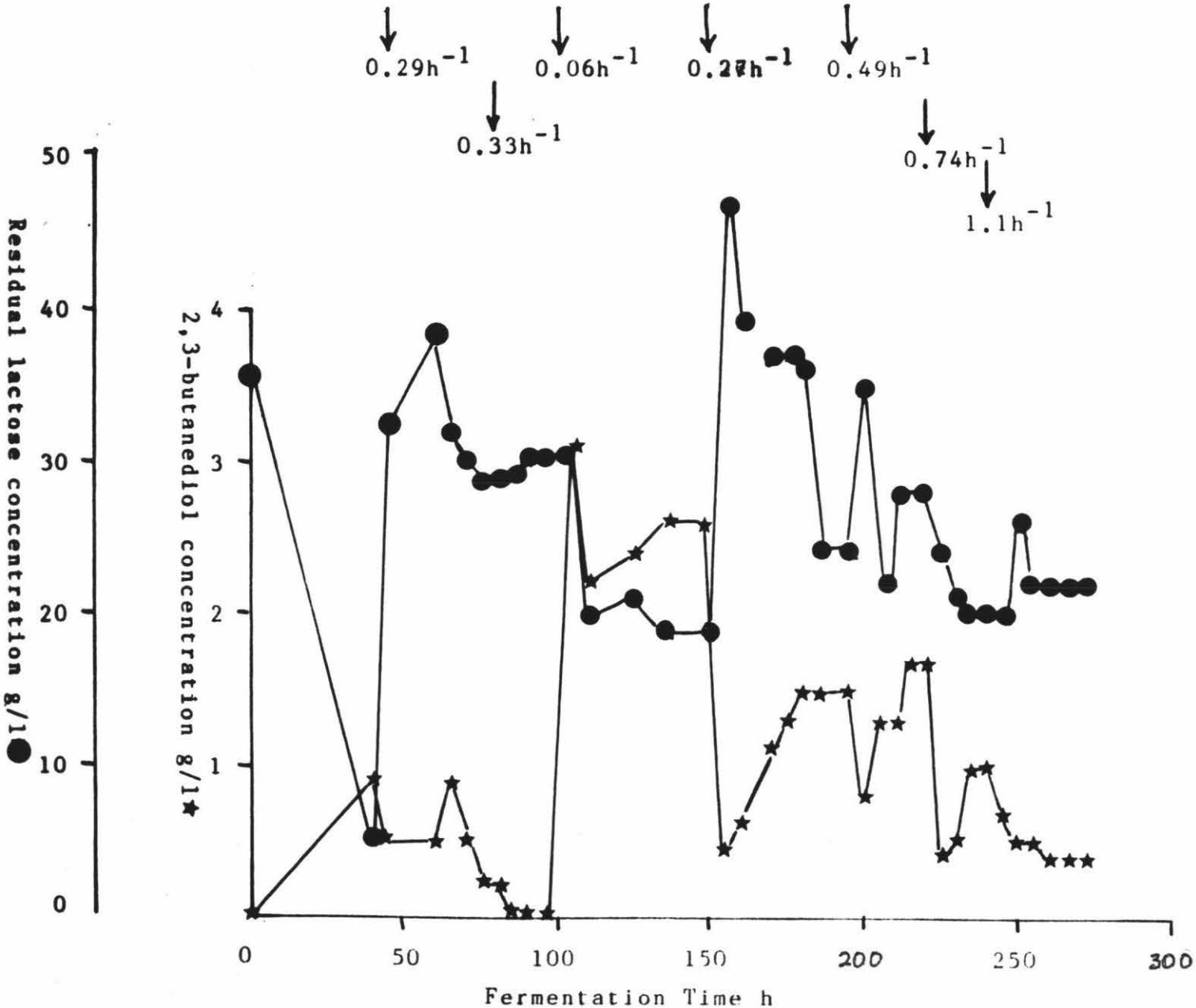


Fig 4.15 2,3-butanediol production and lactose utilization in a 200ml packed column
Bead volume = 200ml, voud volume = 70ml (Final Run)

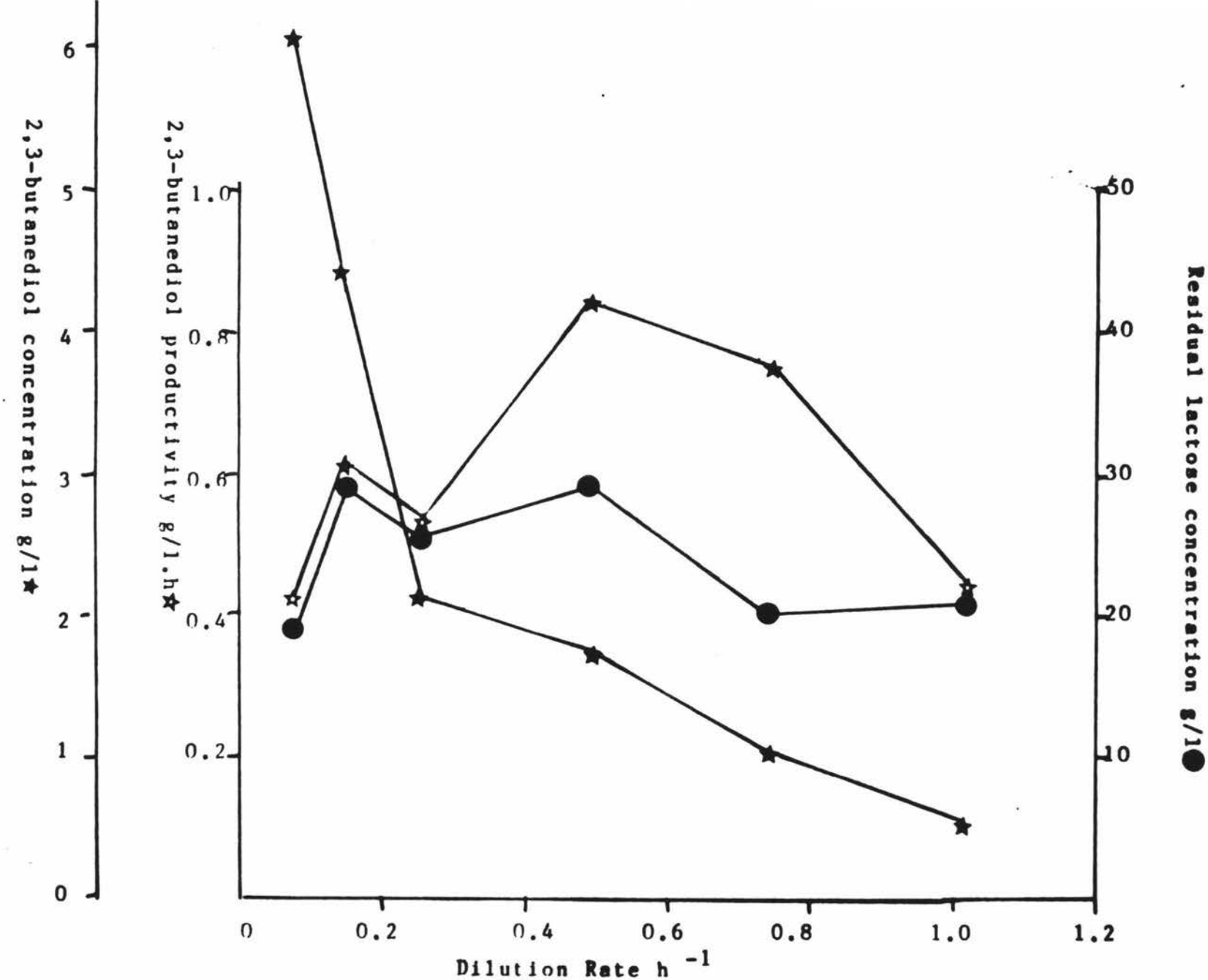


Fig 4.16 Effect of dilution rate on butanediol productivity, concentration and lactose utilization in a 200ml column

0.53g/1.h. A further increase in D value resulted in a sharp decrease in butanediol productivity. This was again due to the rapid evolution of carbon dioxide in the column. In the final run(Fig 4.15), the initial dilution rate was adjusted to $0.29h^{-1}$. The purpose of this was to confirm whether high initial D value was detrimental to butanediol production. The results reveal that a butanediol productivity of only 0.08g/1.h was achieved at steady state after 80h of fermentation. A further increase in D value($0.33h^{-1}$) resulted in no butanediol production. When the D value was subsequently reduced to $0.06h^{-1}$, butanediol production was reactivated and steady state was attained after 140h of fermentation giving a productivity of 0.16g/1.h. The D value was then increased to $0.27h^{-1}$ so as to verify the initial D value data. A steady state was reached after 215h of fermentation. The corresponding productivity was 0.40g/1.h. This improved productivity following a low D value appears to suggest that a low initial D value is essential for the start up of a packed column experiment. An increase in D value from $0.27h^{-1}$ to $0.49h^{-1}$ resulted in a further increase in butanediol productivity to 0.84 g/1.h. Further increase in D values of $0.74h^{-1}$ and $1.1h^{-1}$ gave lower productivities of 0.74 and 0.44g/1.h respectively. The overall results of the three fermentation runs, summarized in Fig 4.16, reveal that the maximum butanediol productivity of 0.84g/1.h was attained at a D value of $0.49h^{-1}$. Further increase in D values did not result in corresponding increases in productivities or lactose utilization. The productivities obtained using the 200ml column were not significantly greater than those obtained using the CSTR or the 100ml column. The adverse effect of carbon dioxide build up in the column was again the main cause of discontinuous column operation.

4.2.2.3 300ml column

In an attempt to overcome the problem of gas build-up in the two previous columns, and to further increase the butanediol productivity, the 300ml column

was operated as described in section 3.2.9.2. Two runs using this meshed column were performed and the results are shown in Figs 4.17 - 4.19. During the first run (Fig 4.17), substrate was fed into the column at a dilution rate of $0.19h^{-1}$ and a steady state was achieved after 135h of fermentation. The butanediol productivity at this stage was 1.48g/1.h. When the dilution rate was increased, the new steady state was achieved after 190h of fermentation with a butanediol productivity of 1.76 g/1.h. The dilution rate was then increased to $0.77h^{-1}$ and the butanediol productivity attained was 2.4g/1.h. The dilution rate was then lowered with the aim of confirming the initial productivity value of 1.48g/1.h. However, at this new D value($0.11h^{-1}$) a productivity of only 0.83g/1.h was obtained. This poorer value could be due to the fact that the entrapped cells had aged. However, the main point to note from the run was that the presence of the steel mesh facilitated the removal of carbon dioxide from the column(Plate 4.3), thereby preventing bead compaction and hence enhancing nutrient accessibility to the entrapped cells.

Another feature of the run was that the butanediol productivities obtained were much higher than those achieved using the CSTR or the two shorter columns.

In the second and final run(Fig 4.18), substrate was initially fed into the column at a higher dilution rate($0.29h^{-1}$) than that which occurred in the previous run. The purpose was to determine whether maximum butanediol productivity could be attained within a shorter fermentation time. The results, shown in Fig 4.18, reveal that the initial higher dilution rate resulted in very poor butanediol productivity and concentration. This appears to suggest that during the initial start-up of the column the D value should not be too high. This was confined when the dilution rate was decreased to $0.04h^{-1}$, where the butanediol production was activated. The butanediol productivity attained at this new D value ($0.04h^{-1}$) was 0.23g/1.h. Subsequent increases in dilution

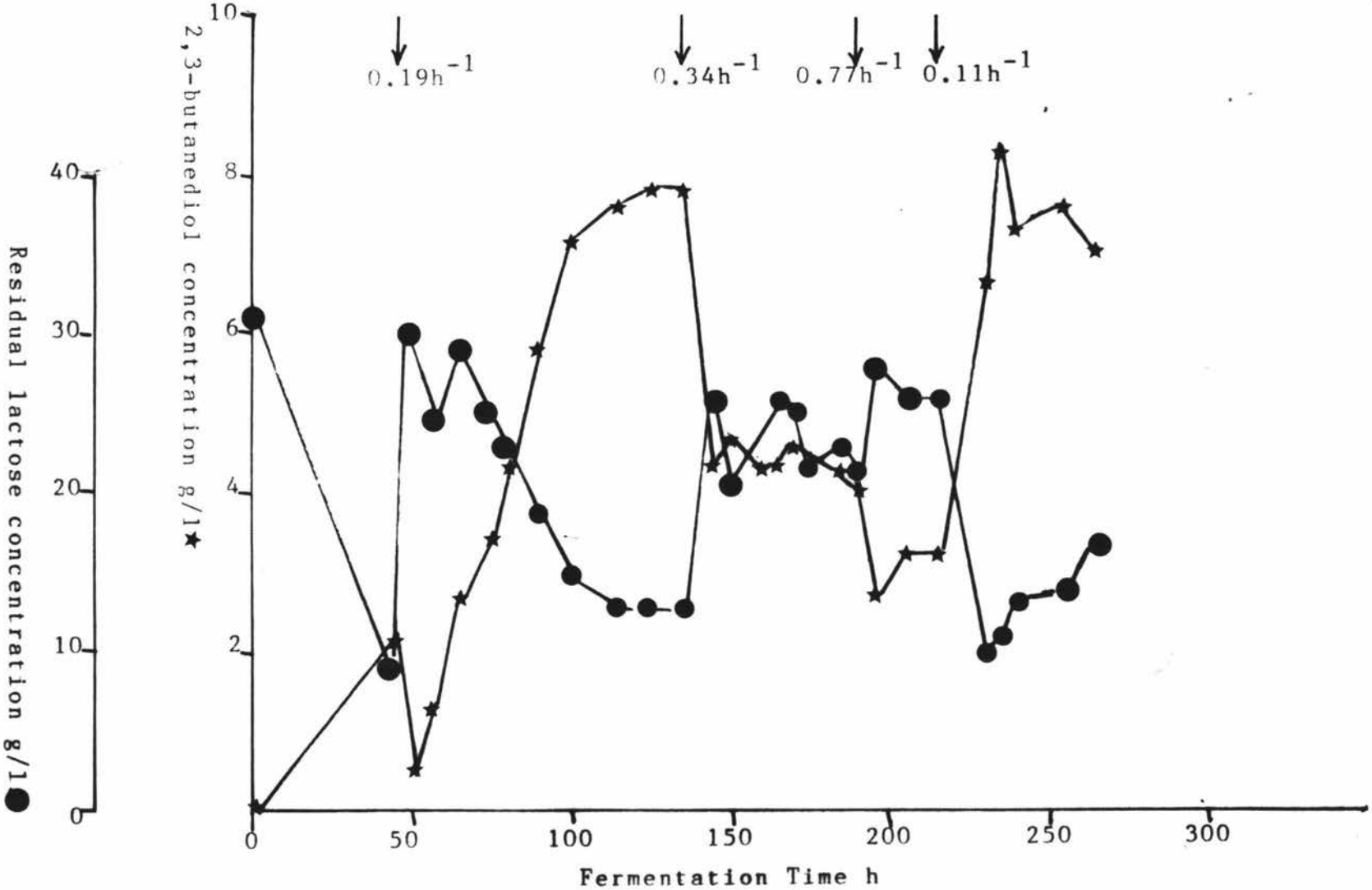


Fig 4.17 2,3-butanediol production and lactose utilization in a 300ml meshed column

Bead volume = 150ml
Void volume = 155ml
} 305ml = Total packed volume (1st Run)

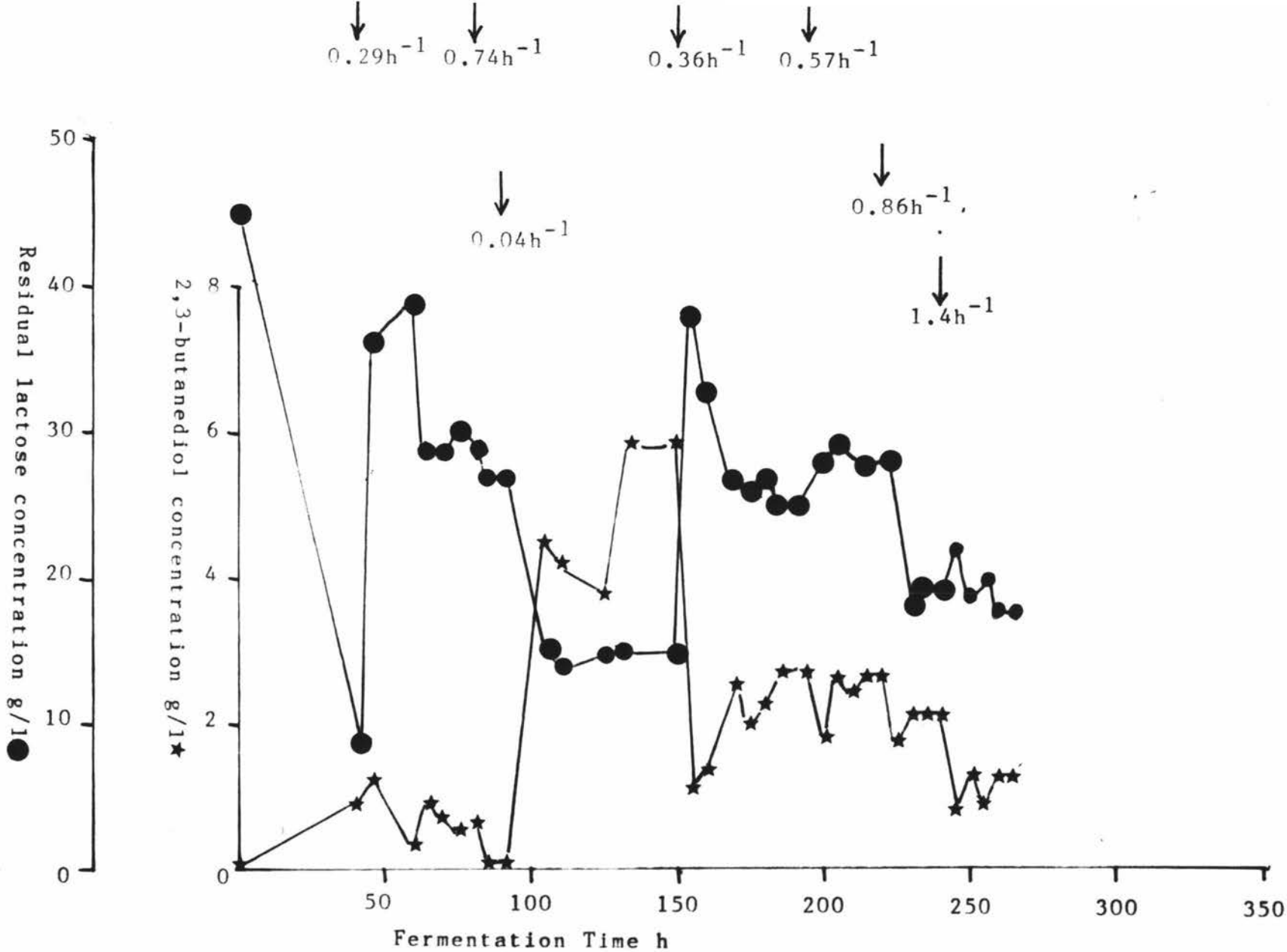


Fig 4.18 2,3-butanediol production and lactose utilization in a meshed column

Bead volume = 200
Void volume = 148
} 348ml Total packed column volume (Final Run)

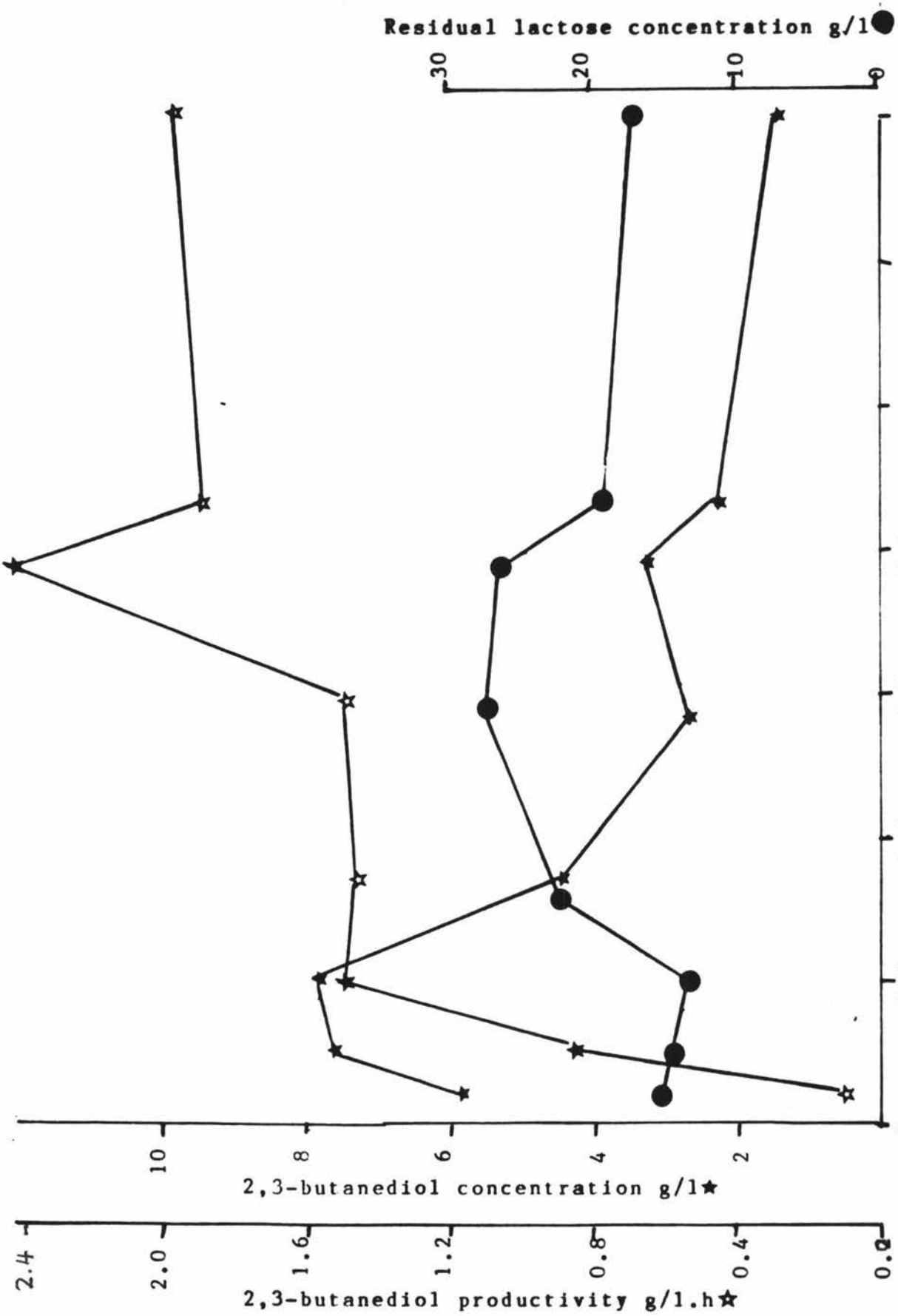


Fig 4.19 Effects of dilution rates on butanediol productivity, concentration and lactose utilization in a 300ml meshed column

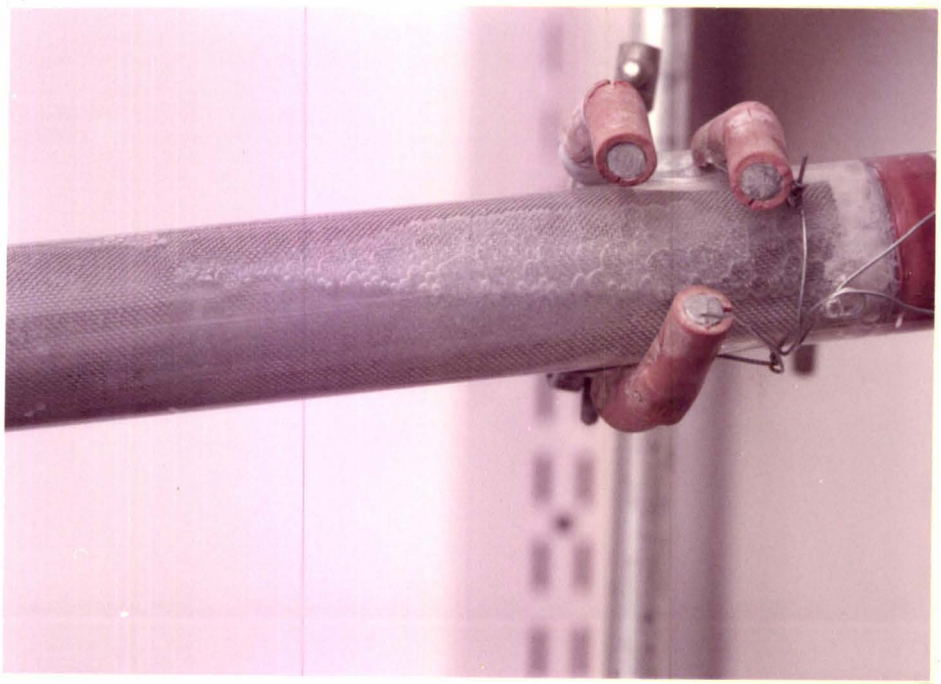


Plate 4.3 Transport of CO_2 bubbles out of column

rates to $0.36h^{-1}$, $0.57h^{-1}$, $0.86h^{-1}$ and $1.40h^{-1}$ resulted in butanediol productivities of 1.0, 1.48, 1.89 and 1.96g/l.h respectively. It appears, therefore, that dilution rates greater than $0.86h^{-1}$ do not have any significant effect in further increasing the butanediol productivity. The results of these two runs, summarized in Fig 4.19, highlight two main features. Maximum butanediol productivity of 2.4g/l.h was obtained at a D value of $0.77h^{-1}$. Further increases in dilution rate did not result in significant increases in the productivities. A low dilution rate for starting the continuous run was noted to be essential for the attainment of maximum butanediol productivity.

With respect to the stability of the column, the second run was operated for six weeks at a dilution rate of $0.14h^{-1}$ and the butanediol producing activity of the entrapped cells remained stable and high. A constant butanediol productivity of 0.95g/l.h was attained at this dilution rate.

4.3 Discussion

The results of the continuous fermentation experiments demonstrated that the butanediol productivities obtained were much higher than those achieved in batch fermentation studies(Table 4.10). In particular, the productivity using the meshed column was 24 times higher than that obtained in a batch fermentation. The productivity of the CSTR was poor compared to that of the meshed column. A possible reason for this could be the absence of a nutrient gradient in the CSTR, but which occurred in the packed column. Thus, the existence of a nutrient gradient appears to favour the attainment of high butanediol productivities. The inclusion of a steel mesh into the glass column solved the problem of adverse carbon dioxide build-up and this technique appears to show promise for the future.

Table 4.10 Summary of maximum butanediol productivities achieved by immobilized cells in batch and continuous fermentation

Bioreactor	2,3-butanediol productivity (g/l.h)
Shakeflask (Batch)	0.11
100ml column	0.96
200ml column	0.84
300ml column (with mesh)	2.40
CSTR	0.74

CHAPTER 5

FINAL DISCUSSION AND CONCLUSION

The results of the batch fermentation studies revealed that cell immobilization of Klebsiella pneumoniae NCIB 8017 in alginate gels was beneficial towards to production of 2,3-butanediol from whey permeate. The butanediol concentration produced by immobilized cells was higher than that produced by free cells reported earlier by Lee(1984) and Lee and Maddox(1984). With respect to the effect of agitation, the results confirm the reports by Jansen et al(1984a) and Sablayrolles and Goma(1984) in that butanediol production occurs during oxygen limited conditions. The results also agree with those of Lee (1984) who had earlier demonstrated that aeration did not increase butanediol production. The ability of barium alginate gels to withstand the chelating actions of phosphate ions in the whey permeate confirmed the findings of Paul and Vignais(1980) using bacteria chromatophores. The method of supplementing whey permeate with calcium chloride to enhance calcium alginate bead stability, was in agreement with the method used by Schoutens et al (1985) for butanol production by Clostridium beyerinckii. With regard to bead size and bead storage, the results confirm the studies by Ohlson et al(1979), Banerjee et al (1984) and Jain and Ghose(1984) albeit using different organisms. Smaller beads were shown to perform better than larger beads presumably because of fewer mass transfer limitations. Storage of beads at $4^{o}C$ in either 0.1M Tris-HCl buffer or 8.5g/l $BaCl_2$ was reasonably satisfactory, although the beads required activating. Prior acclimatization of Klebsiella pneumoniae in a medium of high lactose concentration did not result in higher butanediol production by immobilized cells from either ordinary whey permeate or lactose-suplemented whey permeate. These results appear to contradict those of Yu and Saddler(1983), who used Klebsiella pneumoniae in a xylose-based synthetic medium, and obtained improved

butanediol production after acclimatization. The reason for the difference is not clear, but it may reflect the fact that in the present study, the cells were physically separated from the bulk medium.

The overall butanediol productivity using a batch fermentation process(0.11g/1.h) was poor in comparison to that obtained in continuous fermentation experiments. Using the CSTR, a butanediol productivity of about seven times of a batch fermentation was observed. Although this value was higher, the continuous operation of the CSTR posed a serious problem because of the abrasive actions of the agitation system on alginate beads which resulted in gradual bead flaking and eventual bead disintegration. Consequently, this bioreactor was not suitable for the continuous production of 2,3-butanediol. The butanediol productivities obtained using the 100ml and 200ml columns were not significantly greater than that of the CSTR. In addition, due to the severe problem of carbon dioxide build-up and subsequent bead compaction, the bioreactors could not be operated in excess of 250h and are therefore unsuitable in terms of long term butanediol production. The bioreactor of choice was the meshed column. This column was not only stable over a six week period, but achieved a butanediol productivity of 2.4g/1.h. This value exceeds that reported by Shazer and Collins(1984) who used Bacillus polymyxa in a continuous mode with biomass recycle and obtained a butanediol productivity of 1.04g/1.h. The butanediol productivity achieved using the meshed column was about 24 times that obtained in a batch fermentation and is higher than any value recorded in the literature. Therefore, the use of this bioreactor for the commercial production of 2,-3-butanediol production from rennet whey permeate seems promising.

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A P P E N D I X

Appendix Table 1A Production of ethanol and acetoin by immobilized and free cells of Klebsiella pneumoniae(NCIB 6017)

Incubation Time (h)	Free Cells			Immobilized Cells		
	Ethanol(g/l)	Acetoin(g/l)	pH	Ethanol(g/l)	Acetoin(g/l)	pH
0	-	-	6.5	-	-	6.5
24	0.8	-	5.3	0.8	0.2	5.2
48	1.9	0.4	5.2	2.8	0.5	5.0
72	3.6	0.6	5.0	4.6	0.2	4.9
96	6.2	0.8	4.9	6.4	0.4	4.9
120	4.5	0.8	5.0	5.4	0.2	5.0

Appendix Table 2A Ethanol and acetoin concentration at time of maximum butanediol production by free and immobilized cells of *Klebsiella pneumoniae* (NCIB 6017) under agitating and non agitating conditions

	With Agitation		No Agitation	
	Ethanol (g/l)	Acetoin (g/l)	Ethanol (g/l)	Acetoin (g/l)
Free Cells	0.6	0.06	2.0	0.42
Immobilized Cells	0.6	0.4	1.3	0.3

Appendix Table 3A Ethanol and acetoin concentrations at time of maximum butanediol production by 1, 5, 20, 40, 60 and 80ml cells

Cell density (ml)	Ethanol (g/l)	Acetoin (g/l)
1.0	2.9	0.4
5.0	3.4	0.3
20.0	3.2	0.09
40.0	4.0	-
60.0	2.4	-
80.0	1.4	-

Appendix Table 4A Ethanol and acetoin concentrations at time of maximum butanediol production obtained by barium, calcium alginate beads

Alginate bead	Ethanol (g/l)	Acetoin (g/l)
Barium	2.3	0.4
Calcium	4.6	0.4
Strontium	2.8	0.3

Appendix Table 5A Ethanol and acetoin concentrations produced by various bead sizes at time of maximum butanediol production

Bead size(mm)	Ethanol(g/l)	Acetoin(g/l)
1.8	6.2	0.8
2.4	5.3	1.1
3.0	5.4	0.2
4.5	4.9	1.1
5.5	4.2	1.1

Appendix Table 6A Concentrations of ethanol and acetoin after 72h incubation under different storage conditions

Storage Time (wk)	8.5g/1 $BaCl_2$ at 4°C					
	Incubation No:					
	1		2		3	
	Etoh g/1	Acetoin g/1	Etoh g/1	Acetoin g/1	Etoh g/1	Acetoin g/1
1	3.9	0.4	5.9	0.5	3.5	0.3
2	4.0	0.1	5.4	0.3	7.5	0.2
3	2.5	-	7.8	0.2	6.2	0.2
4	3.0	-	6.4	0.2	4.5	0.4

Appendix Table 6A(Cont.)

Storage Time (wk)	$BaCl_2$ at 30°C					
	Incubation No:					
	1		2		3	
	Etoh g/l	Acetoin g/l	Etoh g/l	Acetoin g/l	Etoh g/l	Acetoin g/l
1	1.7	0.3	3.8	0.4	4.9	0.4
2	DNA	DNA	DNA	DNA	DNA	DNA
3	DNA	DNA	DNA	DNA	DNA	DNA
4	DNA	DNA	DNA	DNA	DNA	DNA

* DNA - Data not available

Appendix Table 6A(Cont.)

Storage Time (wk)	0.1M Tris-Hcl at 4°C					
	Incubation No:					
	1		2		3	
	Etoh g/l	Acetoin g/l	Etoh g/l	Acetoin g/l	Etoh g/l	Acetoin g/l
1	3.8	0.3	4.7	0.2	5.9	0.2
2	3.1	-	6.6	0.4	6.7	0.1
3	2.3	0.3	8.1	0.2	4.6	0.6
4	2.9	0.1	6.1	0.4	2.6	0.2

Appendix Table 6A(Cont.)

Storage Time (wk)	0.1M Tris-Hcl at 30°C					
	Incubation No:					
	1		2		3	
	Etoh g/l	Acetoin g/l	Etoh g/l	Acetoin g/l	Etoh g/l	Acetoin g/l
1	0.7	-	1.7	0.2	3.9	0.5
2	DNA	DNA	DNA	DNA	DNA	DNA
3	DNA	DNA	DNA	DNA	DNA	DNA
4	DNA	DNA	DNA	DNA	DNA	DNA

*DNA - Data not available

Appendix Table 7A Ethanol concentrations at time of maximum butanediol production recorded by free and immobilized cells precultured in 50 and 100g/l lactose.

Treatment	Ethanol Concentration g/l	
	Untreated whey permeate	Treated whey permeate
Free cells pre cultured in 50g/l lactose	4.7	3.2
Free cells pre cultured in 100g/l lactose	4.4	2.2
Immobilized cells pre cultured in 50g/l lactose	2.5	5.3
Immobilized cells pre cultured in 100g/l lactose	2.7	4.3