STUDIES OF β - (1,3)(1,4) - GLUCANASE PRODUCTION BY A RECOMBINANT YEAST STRAIN IMMOBILISED IN CALCIUM ALGINATE GEL

A Thesis

presented to

Dublin City University,

For the

Degree of Master of Science

by

Gearóid Cahill, B Sc
under the supervision of Mr Padraig Walsh
and Dr Thecla Ryan

School of Biological Sciences
Dublin City University
Dublin 9

August 1990

Padraig Walsh (Internal examiner)

Donald E. Brown (External examiner)

I hereby declare that the research described within this thesis is based entirely upon my own work

Gearóid Cahill

Gearóid Cahill

Date 17 Aug 1990

Studies of β - (1,3)(1,4) - glucanase production by a recombinant yeast strain immobilised in calcium alginate gel

Gearóid Cahill, B Sc

ABSTRACT

Production of the recombinant enzyme β - (1,3)(1,4) - glucanase in *Saccharomyces cerevisiae* (DBY746), containing the plasmid pJG317 was studied for both free cells and cells immobilised in calcium alginate During prolonged growth, β-glucanase production decreased exponentially in the case of free cells No decrease in enzyme production was observed for immobilised cells

Plasmid stability of both free and immobilised cells was studied The percentage of plasmid-containing cells in suspension culture decreased to 24% in 96 hours The plasmid stability of cells throughout the immobilisation matrix was measured It was found that cells in the centre of the gel beads retained the recombinant plasmid to a greater degree than free cells, or cells at the surface of the matrix

Continuous stable production of β-glucanase by free cells in non-selective medium was not possible Enzyme production by the immobilised yeast strain, however, was stable for 10 days, with no sign of decrease The enzyme productivity for immobilised cells in a continuous reactor (using non-selective medium), was 48 U/ml/h, compared to 3 6 U/ml/h for free cells in batch culture using non-selective medium

ACKNOWLEDGEMENTS

I wish to thank sincerely my supervisors Mr Padraig Walsh and Dr Thecla Ryan, for their excellent guidance and assistance A special thanks to Padraig, for his practical help and suggestions, both in the laboratory and with the preparation of this thesis

Many thanks to Margaret Duffy and Sharon Hunter for their help in the Genetics Laboratory

I would also like to thank Deirdre O'Grady for her assistance in developing image analysis techniques for studying bead cross-sections

Finally, I would like to thank my parents, for their continued support and encouragement during this research

To Angela and Rebecca

TABLE OF CONTENTS

1 INTRODUCTION

1 1 HETEROLOGOUS PROTEIN PRODUCTION BY MICROORGANISMS

There is increasing interest in recombinant protein production by micro-organisms The potential markets for such proteins are vast These include the chemical and pharmaceutical industries, the food industry, the area of wastewater treatment and other applications such as analytical, biomedical and diagnostically related areas (Nicaud, 1986) There is therefore much research channelled into expanding the host range and expression vectors for recombinant protein production

The most characterised host system is *Escherichia coli* and many recombinant proteins are expressed at high levels and secreted to varying degrees by this organism There are, however, limitations to the applications of using recombinant *Escherichia coli* including

1) The possibility of endotoxins contaminating the final product
2) Secretion of proteins from a host system is most desirable to enable continuous purification to be performed easily *Escherichia coli* is not generally suitable for efficient secretion of recombinant proteins (Nicaud, 1986)
3) Many mammalian proteins, once synthesised, must be folded correctly and may require post-translational modifications to activate the protein Such modifications are not readily possible using this procaryotic organism

Much research is ongoing with Bacillus species as they are known to naturally secrete many enzymes (Pugsley, 1985) Being gram positive, there is only one cell membrane and therefore secretion of recombinant proteins is easier than in gram negative organisms There are however, problems with using Bacillus species They naturally secrete many proteases (Ulmanen, 1985) and also plasmids in Bacillus are generally unstable (Nicaud, 1986)

Interest in yeast as a producer of recombinant proteins is increasing for a number of reasons Yeasts are classified as generally regarded as safe (GRAS) which renders them suitable for healthcare product synthesis (Martin, 1989) The technology for growing yeast to high cell densities on a large scale is well developed Yeast can generally be propagated using inexpensive media Also yeast being a eucaryotic organism, shares many of the features of post-translational modification and protein processing of mammalian cells

1 1 1 Three dimensional folding

Most mammalian proteins produced in yeast have some degree of biological activity, implying correct three-dimensional folding, e g Hepatitis B surface antigen (McAleer, 1984) In the case of some intracellularly produced proteins however, random disulphide bond formation has been observed It is possible that by directing proteins of interest through the yeast secretory pathway where they undergo particular modifications, they are more likely to be correctly folded

1 1 2 Post-translational modifications

Many mammalian proteins require post-translational modification for full biological activity Such events include acetylation, phosphorylation and glycosylation Since most of these events are unique to eucaryotes, yeast is potentially an ideal host, and indeed yeast has been shown to be capable of carrying out many of these modifications For example, yeast has the pathways for N-linked and O-linked glycosylation Core glycosylation is the same as in other eucaryotes but the side chains can differ substantially To further complicate matters, it has been found that mammalian glycosylation can be species specific and, in some cases even cell specific

Despite these complications, yeast can modify many mammalian proteins correctly (Fujimana, 1986, Sambucetti, 1986) In cases where total activation is not possible in yeast, partially activated proteins could be produced and secreted These proteins could than be fully activated by the *in vitro* addition of the appropriate side groups

1 1 3 Secretion

Overproduction of heterologous proteins within yeast cells generally results in the appearance of insoluble protein in inclusion bodies (Van Brunt, 1986) The proteins can become entrapped within the cell wall Protein recovery is possible by mechanically breaking the cells to release

the product or by osmotic shock of spheroplasts (Derynck, 1983) This however, adds an extra step to the purification process and also adds multiple *contaminating* proteins to the protein solution Furthermore, the recombinant product, upon recovery, often has to be reactivated Secretion is very desirable as it eliminates the need for cell breakage and as yeast naturally secretes few proteins, purification of the desired recombinant product is simplified

In order for a protein to be directed into the secretory pathway of yeast, it must have a specific peptide sequence (referred to as the signal peptide) covalently attached to its left-hand end Thus, by fusing the gene of interest to a yeast signal peptide sequence, it is possible to target a particular protein into the medium More recently, it has been found that many heterologous proteins can be secreted in yeast using their native secretion signals (Kingsman, 1987) Unfortunately, not all of the recombinant protein produced in this manner ends up in the medium Some of it is found in an insoluble form in inclusion bodies within the cell (Mellor, 1983) Super-secreting mutants have been found that can secrete up to 80% of synthesised recombinant protein (Smith, 1985)

In studies of recombinant prochymosin production by *Saccharomyces cerevisiae* , it was found that, unless the protein passed through the secretion pathway, the protein could not be primed to its active form (Smith, 1985) This illustrated the importance of eucaryotic protein processing and also that secretion is an integral part of post-translational modification

1 1 4 Heterologous protein stability

Homologous proteins generally are resistant to the protease action of their host apart from the natural turnover rate of proteins within a cell Heterologous proteins, however, because of their foreign amino acid sequence, may be prone to rapid digestion Proteins which are to be secreted may avoid digestion in the cellular compartments of the host like the Golgi apparatus and the endoplasmic reticulum Digestion may also be avoided if the foreign proteins are glycosylated (Luzikov, 1988)

Proteases that normally exist in the endoplasmic reticulum and Golgi apparatus as activating enzymes to native proteins, may actually digest the foreign protein because of its amino acid sequence (Bitter, 1984) It may be possible to modify heterologous proteins to restructure the regions susceptible to digestion in the host without affecting biological activity

1 2 β-GLUCANASES

Glucanases are found naturally in cereals such as barley and oats (Jackson, 1986), and are produced by many bacillus and fungal species for example, *Bacillus subtilis*, *Aspergillus niger* These enzymes are of commercial importance in the brewing industry The substrate for these enzymes is β - glucan, a major component of the endosperm walls of barley and many other cereals (Anderson, 1976)

1 2 1 The role of glucanases in malting and germination

During germination or malting of barley, β - glucanase is secreted from the aleurone layer and scutellum into the endosperm (Stuart, 1986) In barley, 75% of the endosperm

wall is β - glucan (Fincher, 1975) β - Glucanase action on the cell walls allows access of other hydrolytic enzymes to the starch and protein reserves found in the endosperm

1 2 2 The role of glucanases in mashing and fermentation

The action of β - glucanases in the mashing process helps other hydrolytic enzymes to access their substrate, and is very important for fast and thorough extraction There is, however, substantial loss of β-glucanase activity during the kilning of the malt and in the mashing steps prior to fermentation (Jackson, 1986) Insufficient enzyme action results in the appearance of substantial amounts of high molecular weight β - glucans in the wort Extraction is also less efficient (Bathgate, 1975) Unhydrolysed β - glucans can cause difficulties in filtration of the wort and beer (Scott, 1972, Leedham, 1975) The use of adjuncts in brewing can also add to the shortage of β - glucanase during mashing, because they add to the amounts of substrate that have to be hydrolysed without supplying additional enzymes (Jackson, 1986)

The enzyme found naturally in barley is β-(1,3) (1,4)-glucanase This enzyme is produced by fungal and Bacillus species Preparations of these enzymes can be added at the mashing, fermentation or conditioning stages of brewing (Bamforth, 1982, Leedham, 1975) β - Glucanase from these species is more resistant to higher temperatures than the barley enzyme, which is desirable in terms of getting prolonged enzyme activity during the mashing process Cloning of barley β - glucanase (Jackson, 1986),

and Bacillus β - glucanase (Cantwell, 1985) genes into haploid yeast strains have been reported Ideally, genes should be inserted into diploid brewing strains where expression and secretion of β - glucanase could occur during fermentation, hydrolysing high molecular weight β - glucans, thus making downstream processing easier and eliminating haze formation due to the presence of β - glucan

1 3 IMMOBILISATION - PRINCIPLES AND APPLICATIONS

The use of immobilised microbes has been exploited by mankind for at least 150 years in the case of vinegar production (Kierstan, 1985) Immobilised micro-organisms have also been used to process waste streams In these two examples the immobilised cells are in the form of a biofilm This is only one form of immobilisation

Immobilisation in the most general terms can refer to complete restriction of movement of cells or enzyme molecules eg adsorption of cells to polymeric supports (Jirku, 1980(a)), or to the retention of the biocatalyst in a reaction vessel using an ultrafiltration or microfiltration recycle system There are many examples and applications of immobilised cell and enzyme systems The following, however, will deal exclusively with cellular immobilisation

1 3 1 Advantages of cell immobilisation

Immobilised cells can be used to varying degrees of reaction complexity The lowest level consists of a catalytic step involving a single enzyme In this case the

immobilised cells act as a source of the enzyme of interest The medium normally contains only the substrate of interest This approach avoids the expensive isolation of the enzyme to immobilise it on a support The next level of complexity involves using an entire metabolic pathway of a given organism to yield the product of interest The next highest degree of reaction complexity is total cell maintenance to obtain the desired product The highest degree of complexity involves cell growth and reproduction where product formation is growth associated (Bailey, 1986)

Cell density in immobilised reactors can be many times that of a free cell system (Tampion, 1987) This allows increased volumetric productivity Because the cells are immobilised, cell washout is not possible In a continuous system, a dilution rate greater than the maximum specific growth rate of the organism is therefore possible, thus increasing the reactor productivity Separation of cells from the product stream in an immobilised system is easier than in a free cell system as only cells that are leaked from the immobilisation matrix need to be removed

1 3 2 Methods of immobilisation

There are three basic approaches to cell immobilisation, entrapment, adsorption and covalent cross-linking Each has its own advantages and limitations and no method in particular suits all immobilisation systems However entrapment is the most widely used technique (Kolot, 1988)

1 3 2 1 Entrapment

Immobilisation of cells is achieved by enclosing the cells in a lattice of macromolecules This type of immobilisation does not require any special properties of the cells being entrapped The range of macromolecular gels that may be used for this technique is quite diverse, and these gels can be grouped in the manner in which they solidify Agarose, egg white and agar all solidify with a decrease in temperature Epoxy resins and polyacrylamide solidify by polymerisation Sodium alginate solidifies to a gel in the presence of di- or multivalent cations (Tampion, 1987)

A suspension of cells to be immobilised is added to the immobilisation matrix whilst it is in a liquid state After mixing, the slurry is subjected to gelling conditions and the matrix hardens Droplets of the slurry may be produced before gelling to form beads or the gel may be hardened as a slab and diced after gelling

Encapsulation is a variation on this idea, where beads of calcium alginate containing cells are formed These are then treated with a solution of polylysine Polylysine forms a permanent polysalt membrane on the outside of the beads by displacing the surface layer of calcium ions Finally the calcium alginate is dissolved using a buffered citrate solution (Lim, 1988) The resulting cells are mobile within the capsules Pore size can be controlled by varying the concentration of polylysine and the chain length of the polymer

1 3 2 2 Adsorption

Adsorption to many surfaces occurs naturally for a variety of micro-organisms eg *Saccharomyces carlsbergensis* to wood, brick and PVC (Navarro, 1976), *Candida tropicalis* to ceramics (Marcipar, 1979) Some micro-organisms form an anchorage disc to hold the cell to the support Others are held by their surface charges The strength of these charges varies greatly from microbe to microbe and is dependent on the nature of the support

1 3 2 3 Covalent cross-linking

This technique is more commonly used for immobilising enzymes although cells may be immobilised by the same method ie by the reaction between an amino acid residue on a protein with an active group on the support material (Lamptey, 1987) Unlike adsorption, this method requires a cross-linking agent to covalently bind the cell to the support (Kolot, 1988)

A commonly used cross-linking agent is gluteraldehyde, and there are reports of the use of isocyanate, carbodiimine and amino-silane (Tampion, 1987) Supports can be organic eg carrageenan, agar, cellulose and polystyrenes or inorganic eg silica, diatomaceous earths, glass and ceramics (Tampion, 1987)

A major advantage of this technique is that there are practically no diffusional resistances to the transport of substrates to the immobilised cells, unlike the entrapment methods However the coupling agents may be toxic to the cells and may reduce their activity and viability Cell loadings using this method are lower than those using entrapment (Tampion, 1987)

Activation of the support surface is necessary in order to covalently link the cells Attached cell numbers per unit surface area are drastically reduced if the surface is not initially activated (Navarro, 1977) There are reports of attachment of *Saccharomyces cerevisiae*, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* to silica beads using either glutaraldehyde or carbodiimide as coupling agents (Navarro, 1977, Nelson, 1976, Jack, 1977)

Another example of covalent immobilisation is based on the use of metal hydroxide precipitates, where the carboxyl or amino groups on the cell surface bind to the metal hydroxide (Barber, 1975) When titanium or zirconium chloride salts are added to a suspension of cells in water a gel / precipitate forms entrapping the cells Acetic acid production has been reported using this method (Kennedy, 1980)

1 4 PHYSIOLOGY OF IMMOBILISED MICRO-ORGANISMS

Immobilisation of cells is a relatively new field of study Apart from the use of different methods of immobilisation, and different applications, the behaviour and physiology of cells in immobilised systems is gaining much attention The behaviour of immobilised cells has been found in many cases to differ from that of free non-immobilised cells

1 4 1 Physical environment of entrapped cells

Supply of nutrients to cells in a fermentation is central to the success and efficiency of the process This

task becomes more difficult as cell numbers increase One advantage of entrapped systems is that higher cell densities are possible Therefore the supply of all nutrients to immobilised cells is of great importance, and diffusion of substrate and product through the immobilisation matrix needs much attention

In general, oxygen diffusion in matrices decreases with increasing cell concentration, bead size and gel concentration (Gosmann, 1988) The diffusivity of oxygen in unoccupied agar has been measured to be 70% of that in distilled water (Sato, 1983) This diffusion problem is more apparent with immobilised cells of high respiratory activity A heterogeneous distribution of cell growth usually develops within beads that are inoculated with a high cell loading Abundant growth occurs in the outer regions of the matrix where nutrients, especially oxygen, are more readily available More uniform growth may be observed if the beads are inoculated at a low level, with the appearance of microcolonies throughout the whole of the immobilisation matrix (Gosmann, 1988)

The specific oxygen uptake rate (OUR) in immobilised systems is usually slightly lower than that of free cells at low cell concentrations in the matrix, and as cell numbers increase the specific OUR decreases due to diffusional limitations, with increased oxygen consumption per bead The specific OUR is also influenced by gel concentration but to a lesser degree than by cell densities (Gosmann, 1986) The specific OUR in the case of beads with

a gradient of growth is higher than that of beads with the same number of cells evenly distributed throughout the matrix (Gosmann, 1988) Where the cells are evenly distributed in the gel oxygen must penetrate further into the bead to supply the same number of cells as in the case of a bead with a heterogeneous distribution of growth Typical penetration depths for oxygen in matrices of high cell density are 50 - 200 μm (Chang, 1988)

Diffusion of glucose in cell-free 2% calcium alginate gel is similar to diffusion in water (Tanaka, 1984) However, for larger molecules the rate of diffusion is reduced As with oxygen, the demand for a carbon source per unit volume of gel is much greater than the demand per unit volume of free cell suspension because of the greater cell densities in immobilised systems The supply of these nutrients is aided by mixing as well as diffusion in the free cell case With immobilised cells, however, supply of nutrients depends solely on diffusion into the matrix (Sato, 1983)

As with oxygen, the rate of diffusion of glucose through gels decreases with increasing gel concentration (Nguyen, 1986), and in the case of xylose decreases with increasing biomass present in the gel (Sakaki, 1988) The presence of proteins in the fermentation medium may reduce further the rate of diffusion of glucose into the immobilisation matrix (Nguyen, 1986)

Greater cell densities can be achieved using immobilised systems compared to free cell systems, although cell growth

and metabolic activity may be reduced in the centre of the matrix Most of the growth therefore occurs at the outer regions of the beads, leaving most of the biocatalyst volume functioning sub-optimally, if at all While conditions therefore in the bulk media may be optimal in terms of nutrients and oxygen supply, the nutrient supply to immobilised cells may be decreased and may influence the metabolism of the immobilised organism

Natural macromolecules are more commonly used as entrapment matrices eg alginate and carrageenan Cells being confined by these molecules are usually exposed to a lower water activity than free cells Reduced water activity can be accomplished in solution using dextran and polyethylene glycol (Mattiasson, 1982) This stress can bring about a reaction in cells to counteract this phenomenon Glycerol production by *Saccharomyces cerevisiae* increases substantially when grown in an environment of reduced water activity (Edgley, 1978), whereas non-halophilic bacteria experience an increase in their amino acid pool in reduced water activity conditions (Measures, 1975) With changed pools of internal metabolites, alternative metabolic pathways may be switched on

In an environment of low water activity, reduced growth rate has been observed with an increase in maintenance metabolism (Esener, 1981), as well as changes in product formation (Troller, 1981) Alginate added to media at levels as low as 0 5% can also reduce the water activity 50% increases in ethanol productivity have been reported

for yeast grown under these conditions (Hahn-Hagerdal, 1982)

1 4 2 Cellular changes due to immobilisation

There have been many reports on differences in the metabolism and morphology of cells once they are immobilised Optimum conditions for growth may vary from those of free cells (Williams, 1981) Product yields may be altered (Tyagi, 1984), as well as morphology (Jirku, 1980(b)) Navarro (1977) has reported higher specific product formation rates Some changes in metabolism may be due to nutrient limitations as a result of immobilisation but this cannot explain all the changes observed

For bacteria, the intracellular levels of RNA vary almost linearly with growth rate (Harder, 1982) In a study of immobilised *Zymomonas mobilis*, Monbouquette (1988) found that cells in the outer section of alginate beads had higher levels of RNA than cells in the inner core RNA levels are known to be a function of growth rate for *Zymomonas mobilis* (Jobses, 1985) In an immobilised system therefore, a heterogeneous cell population exists, as environmental conditions vary throughout the immobilisation matrix

Doran and Bailey (1986) found substantial changes in the metabolism of *Saccharomyces cerevisiae* when this organism was covalently attached to the surface of gelatin particles This work showed that the observed changes in the behaviour of immobilised organisms are not solely due to nutrient availability Doran and Bailey found that the

specific growth rate and cellular yield coefficient decreased on immobilisation Coupled with this however was an increase in glucose consumption and in specific ethanol productivity It was found that much of the glucose was abnormally channelled into intracellular polysaccharide stores and structural components The DNA content of the immobilised cells was found to be almost four times that of free cells and yet cellular RNA levels are less than a quarter of those for free cells Perhaps the decreased RNA levels are as a result of decreased growth rate as reported by Monbouquette (1988) previously Doran and Bailey (1986) suggest that the budding of the yeast is inhibited in some way by immobilisation Whereas budding may be restricted, DNA replication may proceed, as well as structural component synthesis They suggest this as a possible reason for improved ethanol productivity as there are reports that polyploid yeast strains have improved fermentative capabilities compared to normal diploid yeast (Kosikov, 1975)

In further studies on covalently coupled yeast Doran and Bailey (1987) found that cellular NADH levels varied during starvation conditions for free and immobilised yeast Measuring NADH in cells gives an indication of the activity of the glycolytic cycle With free cells under starvation conditions the levels of NADH remain constant, indicating little or no carbohydrate metabolism Free yeast cells use their carbohydrate stores very sparingly under starvation conditions (Sols, 1971) Upon feeding with

glucose and using potassium cyanide to inhibit respiration, Doran and Bailey (1988) showed the levels of NADH oscillate in the cell indicating operation of the glycolytic cycle With starved immobilised cells there are oscillations in the levels of NADH observed before glucose feeding This is contrary to the findings for free cells These results imply that the immobilised yeast cells are metabolising their carbohydrate reserves at an appreciable rate The carbohydrate reserves are higher for yeast attached to gelatin compared to free cells (Doran and Bailey, 1986)

Immobilisation, whether by entrapment or direct coupling, can lead to many changes in the behaviour of immobilised micro-organisms An immobilised cell system is a heterogeneous system as there are two phases, a solid gel matrix and culture medium Cell distribution within the gel matrix is also heterogeneous The cell population, in terms of morphology, age and metabolic activity, may also vary depending on nutrient supply within the matrix The immobilisation matrix itself may affect the behaviour of the cells in metabolism and in cell division/budding Division or budding may also be influenced by the fact that the immobilised cells are in close contact with each other as opposed to the relative cell to cell proximity in a free cell system

1 5 PLASMID STABILITY OF RECOMBINANT MICRO-ORGANISMS

In order for recombinant organisms to be of use in the production of novel products in appreciable quantities, they must be able to retain the ability to produce these

substances for a prolonged period of time With transformed cells this means that they must retain the inserted plasmids throughout the course of the fermentation Plasmid instability arises from two distinct types of phenotype loss

1 Complete plasmid loss due to poor segregation of the plasmids from parent to daughter cells
2 Structural instability of the recombinant plasmid

1 5 1 Segregational plasmid instability

Segregational instability can occur with low copy number plasmids where there is no effective partitioning function on the plasmid In general, plasmid stability increases with increasing plasmid copy number (PCN) (Caunt, 1988, Zabriskie, 1986), as the chance of random equal distribution of the plasmid from parent to daughter cells at division is increased It must be noted, however, that there is a threshold copy number level beyond which increased plasmid stability is not observed It has also been shown that yeast can lose high copy number plasmids (Futcher, 1984)

1 5 2 Structural plasmid instability

Structural instability may occur in recombinant plasmids due to insertions, resulting in either the loss or rearrangement of DNA sequences Generally, this type of instability is more likely in plasmids with large inserts (Caunt, 1988) Structural instability is dependent on the individual plasmid make-up and no real hard and fast rules apply Structural instability can result in cells which have retained the selection marker (eg antibiotic

resistance) but have lost the ability to synthesise the product of interest (Wei, 1989)

1 5 3 Selection pressure

Recombinant plasmids in yeast and bacteria are unstable to varying degrees, unless a selective pressure is applied to the growing organisms to force them to retain the plasmids With bacteria, antibiotics are commonly used where the host is sensitive to the drug and the plasmid encodes resistance to the antibiotic, as well as containing the gene which encodes the product of interest (Reinikainen, 1989) In the case of yeast, nutritional selection is more commonly used, where the host is auxotrophic for a particular amino acid due to a mutation in one of the genes involved in the synthesis of that amino acid The plasmid contains genes encoding the missing enzyme and the product of interest The selective medium lacks the amino acid concerned (Impoolsup, 1989)

Selective media generally will not stop the occurrence of segregational and structural plasmid loss but will inhibit the growth of plasmid-free cells in the media With nutritional selection it may be possible for plasmid-free cells to replicate under selection conditions because of pools of the missing precursor or the amino acid in the cell cytoplasm (Futcher, 1984)

It should be borne in mind that selective media may not yield simply plasmid-free or plasmid-containing cells, but a heterogeneous population of plasmid-containing cells as

well as plasmid-free cells (Dennis, 1985) Plasmid-containing cells may vary in their plasmid and gene product content If the selection pressure is extreme (eg high antibiotic concentration) then even low copy number plasmid-containing cells will be inhibited, thus forcing a population of high PCN Using ampicillin as a selection agent, PCN heterogeneity was observed in cultures of recombinant *Escherichia coli* (Dennis, 1985)

Growth medium incorporating either nutritional or antibiotic selection usually maintains high plasmid stability On a practical note, the high cost of these media, however, makes it uneconomical for large scale processes Hence there is much interest in ways of maintaining plasmid stability using non-selective complex media

1 5 4 Plasmid copy number

Plasmid copy number is a measure of the gene dosage for a recombinant protein in a cell Specific product formation is known to be directly related to PCN, where the more copies of the gene present the greater the synthesis rate of the protein (Uhlin, 1977, Seo, 1985, Engberg, 1975)

High levels of recombinant protein production puts the host cell under stress Greater amounts of energy, intracellular enzymes and precursors are channelled into producing a protein which is of no benefit to the organism As recombinant protein production increases due to increased PCN, the maximum specific growth rate of the host usually decreases (Parker, 1987) The demands of highly amplified plasmids on a recombinant cell are overwhelming

and can lead to reduced cell viability (Uhlin, 1979) It is evident that PCN, plasmid stability and growth rates of the plasmid-containing and plasmid-free cells are all closely related Changes in one of these parameters can affect both of the others

Klemperer (1978), found that cell yields from a given carbon-source were reduced as PCN increased There is therefore a trade-off between low PCN (low stability, low expression and higher growth rate) and high PCN (higher stability, higher expression, reduced growth rate and reduced viability of the organism)

The stability of 2 μm based plasmids generally increases as PCN increases However, a corresponding decrease in the specific growth rate of the yeast was reported (Caunt, 1988) The converse holds for autonomously replicating sequence (ARS) plasmids

The optimum PCN need not necessarily be the maximum PCN as high levels of plasmid can burden recombinant cells Plasmid-free cells may have a growth rate advantage over plasmid-containing cells (Zabriskie, 1986) It has been suggested that recombinant protein production could be optimised by selecting an optimal PCN which could be dictated by the dilution rate of a continuous system (Seo, 1985) In the case of recombinant bacteria, selection of optimal PCN was also suggested by varying antibiotic concentrations in the production media In the case of *Escherichia coli*, it was reported that as PCN increased the minimum inhibitory concentration of antibiotic increased also (Dennis, 1985)

1 5 5 Growth rate

The growth rate of a recombinant micro-organism has been found to influence plasmid loss rates There are conflicting reports in the literature on the effect of growth rate on plasmid stability Plasmid stability of yeasts in selective media decreases with decreasing growth rate (Parker, 1987) As growth rate increased the specific production of the organism decreased This correlates with the findings of Caunt (1988), mentioned previously ie PCN (and gene expression) decrease as the growth rate is increased In agreement with these findings, the plasmid stability of a recombinant yeast in a defined non-selective medium was found to decrease with decreasing growth rate (Kleinman, 1986) Similar findings were reported for bacteria (Wouters, 1980, Noack, 1981, Aiba, 1984 and Siegel, 1985)

In the case of yeast, at lower growth rates the daughters are 'born' smaller and have longer cycle times than the mother cells (Lord, 1980) The fact that the buds are smaller implies that the amount of cytoplasm transferred from mother to daughter is less, hence random equal partition of the plasmid is less likely At lower dilution (growth) rates, the cells are in a nutrient deficient environment, which may favour the growth of cells which can assimilate nutrients and grow faster, perhaps favouring the plasmid-free cells

Contrary to the findings above, plasmid stability of *Saccharomyces cerevisiae* was found to decrease with

increasing growth rate in complex non-selective media (Impoolsup 1989) It is thought, that at higher growth rates the time for DNA replication and correction of errors is reduced, leading to increased plasmid loss at higher growth rates

In batch studies with yeast (Hjortso, 1985) and bacteria (Pinches, 1985) it was found that plasmid stability decreased substantially in the post-exponential phase of growth The reasons for this are unclear Possibly the reduced growth rate or reduced nutrient levels in the media were responsible Watson (1986), found that plasmid loss per generation in *Bacillus subtilis* in non-selective medium decreased with increasing dilution rate However when these results were expressed as plasmid loss versus fermentation time, similar curves were obtained for all growth rates indicating that the plasmid loss rate appears to be independent of growth rate

The growth rate of an organism affects its metabolism and behaviour in many different ways In trying to understand growth rate effects on overall plasmid stability it must be remembered that growth rate changes affect PCN, cell yield, and expression levels, all of which can have an effect on plasmid stability

1 5 6 Oxygen effects

Changes in the oxygen supply to micro-organisms can have profound effects on the growth rate and metabolic activity of the organism In continuous culture studies using *Saccharomyces cerevisiae* , plasmid stability was studied in

complex media sparged with air, oxygen or nitrogen gases (Lee, 1987) The plasmid contained the gene for killer toxin production (and the corresponding immunity gene) Plasmid stability was best with air sparging, and in all cases plasmid stability increased with increasing dilution rate Expression of the toxin was best however in an anaerobic environment (N_2 sparging), and increased with increasing dilution rate From these results, air sparging of the culture appears to decrease plasmid expression and increase plasmid stability

The study of dissolved oxygen (dO_2) shocks on plasmid stability of *Saccharomyces cerevisiae* shows that where a sudden decrease in dO_2 occurs (and thereafter remains below 10% of saturation), plasmid stability decreased at a faster rate than if a dO_2 shock had not been introduced (Caunt, 1989) The decrease in plasmid stability after dO_2 shock was more pronounced at lower dilution rates In anaerobic conditions the energy yield is much less from carbohydrate sources, and it is thought that the sudden scarcity of energy in these experiments rendered plasmid maintenance even more unfavourable Anaerobic growth of yeast in non-selective conditions showed slightly disimproved plasmid stability in continuous culture compared to aerobic culture (Lee, 1987)

Other dO_2 shock experiments using *Escherichia coli* in which the dO_2 was allowed drop to 5% of maximum saturation and then increased to 100% saturation immediately, showed similar detrimental effects on plasmid stability (Hopkins, 1987) In batch culture (with uninterrupted aeration), in

the presence or absence of selection (ampicillin), plasmid loss was not substantial However when the batch culture was subjected to dO_2 shock in exponential phase of growth, the plasmid stability deteriorated rapidly The percentage of plasmid-containing cells decreased to 1% even in the presence of ampicillin

These negative responses to dO_2 changes in terms of plasmid stability are very important when large scale processes are considered Imperfect mixing is more likely to occur in large fermenters, producing pockets of media where the dO_2 may be lower than the average bulk concentration

1 5 7 Nutrition

Initial investigations into the nutritional requirements of plasmid-containing and plasmid-free cells indicate that there are differences in the requirements of each (Klemperer, 1979) In batch culture, *Escherichia coli* containing the R plasmid RP1 had a greater requirement for Mg^{2+}, K^{+}, and PO_4^{3-} than the host strain At low concentrations of phosphate, the growth rate of plasmid-free cells was greater than that of plasmid-containing cells The growth rate of both was similar in a simple salts medium which presumably was not phosphate limited

Knowledge of nutritional requirements of plasmid-free and plasmid- containing cells can be used to increase plasmid stability in non-selective media Caunt (1988), suggested finding an essential element of growth that is assimilated at the same rate by recombinant and host cells

If this nutrient is limited, in a continuous system, then the plasmid-free cells should have no growth advantage over plasmid-containing cells This approach will not overcome plasmid instability completely but may eliminate one of the reasons for plasmid-free cell dominance in non-selective conditions Under nitrogen limitation, improved plasmid stability of *Pseudomonas putida* has been achieved (Ensley, 1986)

1 5 8 Improving plasmid maintenance

Recombinant plasmids are generally unstable in their host cells unless an external selection pressure is applied As this is not feasable in general on large scale, alternative methods of increasing plasmid stability have been studied including, integration of the plasmid into chromosome, the use of centromeric plasmids, the use of the toxin gene as a dominant selection system (the presence of essential functions on the plasmid), separation of the cell growth and protein production stages of a process and also immobilisation of the recombinant cells

1 5 8 1 Integration into chromosome

Yeast integrating plasmids are non-autonomously replicating plasmids which integrate into the yeast chromosome by homologous recombination (Parent, 1985) Inserting heterologous DNA is possible via these plasmids (Hinnen, 1978)

1 5 8 2 Centromeric plasmids

Plasmids of this type contain yeast chromosome centromeres They behave therefore like miniature

chromosomes and are very stable As with integrating plasmids, the copy number and expression levels are very low (Parent, 1985)

1 5 8 3 Natural immunity

Using yeast strains which are sensitive to killer toxins, it is possible to use plasmids encoding the toxin and immunity genes without the need for further selection (autoselection) The immunity function is encoded in a sequence which overlaps the toxin gene (Bussey, 1988) The plasmid-containing cells produce the killer toxin and are immune to it, while the plasmid-free cells are sensitive to the toxin and are killed (Caunt, 1988) Killer toxins are very effective at low concentrations and are not thought to confer any adverse effects on the product

1 5 8 4 Separation of growth and production

Using an inducable promoter it is possible to suppress expression of recombinant plasmid genes during growth of the organism in order to build up large amounts of recombinant biomass Plasmid loss should be minimal if expression is not ongoing, as the plasmid makes little demand on the host (Noack et al 1981) The promoter can then be activated (or derepressed) to allow production of the recombinant protein In this way plasmid loss is insignificant during biomass build-up Activation occurs when large quantities of plasmid-containing biomass have been produced

1 5 8 5 Immobilisation of recombinant cells

With further understanding of the behaviour of immobilised cells and an increased awareness of the advantages of using immobilised systems, studies of recombinant protein production by immobilised transformed cells have been reported Inloes (1983), reported a 100-fold increase in productivity of β-lactamase using an immobilised recombinant *Escherichia coli* strain compared to free cell production Improved productivity was due to the increased cell density achieved in the reactor, by immobilising the cells in hollow fibre membranes Specific product formation (protein/cell) however was only 10% of that achieved by a free cell system Similar findings are reported for catechol 2,3 - dioxygenase production by immobilised *Escherichia coli* cells (Dhulster, 1984)

Plasmid stability of a recombinant *Escherichia coli* strain was found to improve substantially upon immobilisation in carrageenan gel (De Taxis du Poet, 1986) Increased volumetric productivity of the recombinant protein was achieved due to increased cell density in the reactor compared to a free cell reactor When cells released from the gel were grown in a free cell culture the plasmid loss rate was found to be very similar to that of free cells The microenvironment within the gel may be responsible for improved plasmid maintenance

Berry (1988), found that full retention of plasmids was possible, if the immobilisation matrix was inoculated at very high cell densities (10^{10}cells g^{-1}gel) for *Escherichia coli* in carrageenan) If the inoculation was low ($5x10^{3}$ cells g^{-1}gel), plasmid stability decreased to a lower level

than for higher inoculation rates and then remained constant In all cases, plasmid stability of the immobilised organism was better than the free cell systems using non-selective media

The reasons for improved plasmid stability of immobilised recombinant organisms are still unclear and there is ongoing research in the area As discussed in Section 1 4 already, the growth environment in an immobilised system can be radically different from that of free cell growth This altered environment, as well as direct effects of the immobilisation matrix on the cells and increased cell - cell contact may influence the ability of a cell to retain its recombinant plasmids

2 MATERIALS AND METHODS

2 1 ORGANISM

A *Saccharomyces cerevisiae* haploid strain DBY746 (α *his*3-Δ1 *leu*2-3 *leu*2-112 *ura*3-52 *trp*1-289$_a$) transformed with the plasmid pJG317 was utilised in all experiments pJG317 (a derivative of pAAH5) encodes the endo β-(1,3)(1,4)-glucanase gene from *Bacillus subtilis*, under the control of the yeast ADH1 promoter For convenience, the organism will be referred to simply as pJG317 throughout this work

Commercially available dried baker's yeast was also used in some of the immobilisation studies

2 2 MEDIA

All media was sterilised by autoclaving at 121° C (15 psi) for 15 minutes Two different media were used throughout the experimental work, selective and non-selective media

2 2 1 Selective medium

Selective medium (minimal medium) contained the following components per litre,

Yeast nitrogen base (without amino acids)		6 7g
Glucose		20 0g
Uracil		20 0mg
Succinic acid	Used for Buffering unless otherwise stated	10 0g
Sodium hydroxide		6 0g

Both glucose and uracil were added separately to media after autoclaving from sterilised stock solutions of 40% (w/v) and 0 25% (w/v) respectively 20 0 g/L Oxoid technical agar no 3 was added to the above for solid medium preparation

2 2 2 Non-selective medium

Non-selective medium (YEPD) contained the following components per litre,

Yeast extract (Oxoid)	10 0g
Bacteriological peptone (Oxoid)	20 0g
Glucose	20 0g

When this medium was used for immobilised cell growth it was supplemented with 0 015% $CaCl_2$ to help maintain the structural integrity of the beads In both yeast extract and peptone, there are significant amounts of phosphates which may disrupt the gel 20 0 g/L Oxoid technical agar no 3 was added to the above for solid medium preparation

2 3 CULTIVATION CONDITIONS

The organism was maintained on minimal medium agar plates grown for 72 hours at 30°C and then stored for a maximum of two weeks at 4°C before transferring to fresh selective medium

2 3 1 Inoculation preparation

In all experiments the yeast strain was grown up for 48 hours at 30°C in buffered selective medium The inoculum was grown up in 400 ml of medium in a 1 litre unbaffled Erylenmeyer flask on an orbital shaker at 150 rpm Cell counts were carried out on the culture and the required volume added to flasks for free cell culture

In the case of immobilised cell fermentations the inoculum culture was centrifuged at 5000 rpm for 20 minutes at 4°C The pellet was resuspended in 20 ml 0 01% peptone

and the cell concentration measured using a haemocytometer The required volume of concentrated cell suspension was then added to pre-sterilised alginate and mixed prior to bead formation

2 3 2 Cell immobilisation

Cells were immobilised in either 2% or 3% calcium alginate gel A solution of sodium alginate was made up at a concentration of 2 0 g or 3 0 g per 70 ml depending on which concentration was required The solution was boiled to help dissolution, and then autoclaved at 121°C for 15 minutes When cooled, a calculated amount of inoculum yeast suspension was added to the alginate solution to yield the desired inoculum density and the volume was brought up to 100 ml using sterile distilled water

The solution / suspension was mixed, drawn up into a sterile syringe and the desired alginate volume expelled dropwise into a sterile solution of 0 2 M $CaCl_2$ from a height of approximately 20 cm from the surface of the $CaCl_2$ solution The beads were allowed to harden for at least one hour in this solution at room temperature The beads were then washed in sterile 0 01% peptone and inoculated into growth media All preparation work was carried out in a laminar flow cabinet

Using a hypodermic needle (gauge 21), beads of 3 mm diameter were obtained Replacing the needle with a short piece of sterile tubing (outside diameter 4 mm), yielded beads of 5 mm bead diameter To obtain very small beads, a hypodermic needle was used in a stream of sterile air

Droplets of 1 mm diameter were whipped off the needle and into the $CaCl_2$ solution Greater variability in the size of these beads was observed, compared with the methods for making the larger beads

It was not possible to make beads larger than 5 mm in diameter In order to study cell development in larger beads, cylinders of alginate, 18mm in diameter and 25 mm in length were produced (see Figure 2 1) Dialysis tubing with a molecular weight cut-off of 10,000 was cut into lengths of approximately 30 cm and boiled in 10 mM EDTA for ten minutes to regulate pore size Then the tubing was washed in distilled water and placed in a small bottle of distilled water for autoclaving at 121° C for 20 minutes The alginate slurry was prepared as outlined above The sterilized dialysis tubing was clipped at one end and then filled with the alginate - cell suspension The tubing was then clipped at the top and suspended vertically in a sterilized graduated cylinder containing sterilized 0 2 M calcium chloride and allowed to harden over night Once hardened the cylinder of calcium alginate was removed from the tubing and sliced aseptically into 25mm long cylinders and added to flasks of medium All manipulations were carried out in a laminar flow cabinet

In all immobilisation experiments, the volume of gel beads added to shake flasks or used in continuous reactors was 10% of the liquid volume

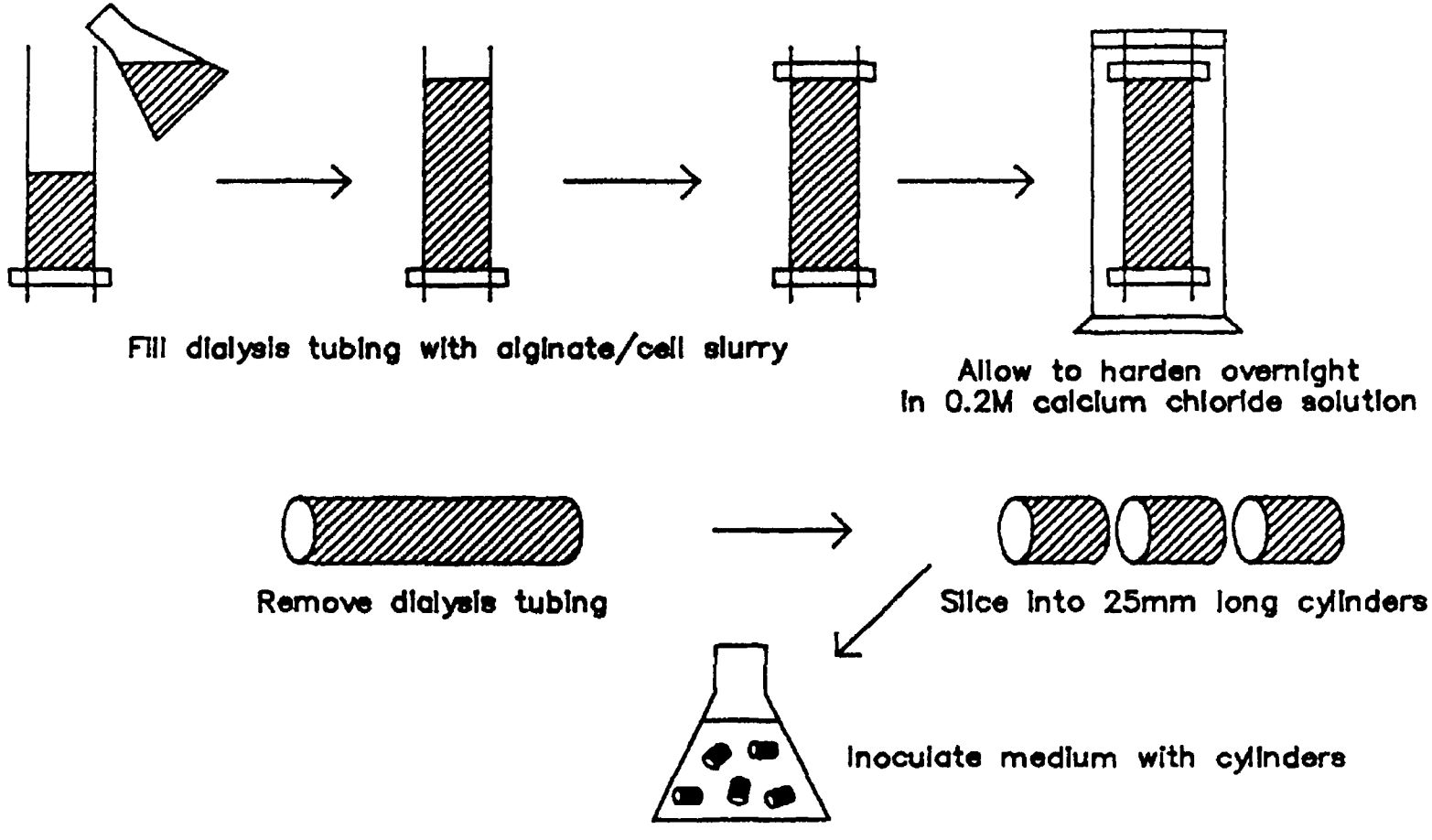

Figure 2.1 Schematic representation of 18mm diameter alginate cylinder preparation

2 3 3 Shake flask cultivation

Unless otherwise stated, all shake flask cultures were carried out in 400 ml of medium, whether selective or non-selective, in 1 litre unbaffled Erylenmeyer flasks at 30°C, on an orbital shaker operating at 150 rpm

2 3 4 Repeated batch cultivation

2 3 4 1 Free cell repeated batch

In the repeated batch system used in this experiment, a fresh flask of YEPD (pre-shaken at 30°C) was inoculated to give a cell concentration of 10^4 ml^{-1} This flask was then incubated at 150 rpm and 30°C for 24 hours, then a fresh pre-warmed, pre-aerated flask was inoculated from this flask to give an initial cell concentration of 10^4 ml^{-1} This flask was then incubated as above for 24 hours and so on Samples were taken at the end of each batch for enzyme measurement and plasmid stability measurement

2 3 4 2 Immobilised cell repeated batch

Initially beads were prepared and added to a fresh flask of pre-warmed, pre-aerated YEPD medium and incubated at 150 rpm at 30°C for 24 hours The beads were removed from the medium and washed twice in sterile 0 01% peptone Then all the beads were added to a fresh flask of YEPD and so on Samples of beads and medium were taken at the end of each batch for enzyme and plasmid stability measurement

2 3 5 Continuous fermentation

Continuous fermentations were carried out in a 2 L Life Sciences bioreactor Operating parameters were as follows,

Temperature	30°C
pH controlled at	5 00
Agitator speed	400 rpm

Liquid volume	1 3 L or 0 5 L
Aeration	1 v/v/min
pH controlled with	1 M H_2SO_4 / 2 M NaOH

Dilution rates are specified for each individual experiment In all fermenter runs silicone anti-foam (7000 K) was added to medium at a dosage rate of 0 1ml per litre prior to autoclaving

The fermentation vessel and medium were autoclaved at 121°C for 30 minutes Reservoirs of medium were autoclaved in batches of 8 litres for 30 minutes Air supplied to the fermenter was filtered through a 0 45 μm Sartorius PTFE air filter

Temperature was monitored using a resistance thermometer Temperature was controlled to ± 0 1°C using a water jacket

Using a pH controller with an Ingold sterilisable pH probe, the pH was controlled to ± 0 02 pH units

The vessel was agitated by two sets of four-blade turbine impellers of diameter 45 mm and spaced 55 mm apart The vessel contained two baffles of length 120 mm and width 20 mm

2 3 6 Continuous fermentation at high dilution rates

With immobilised cells, it was necessary to operate at high dilution rates to eliminate enzyme production by free cells which had leaked from the gel particles In order to do this, a conical flask with a working volume of 185 ml was used in place of the Life Sciences reactor The glass vessel was immersed in a water bath at 30°C, and mixing was

accomplished by aeration through a ring sparger at 10 8 v/v/min The pH of this reactor could not be controlled, but remained between pH 4 5 and 5 5 The inlet air to this reactor was filtered using a 0 45 μm Sartorius PTFE air filter The vessel plus the medium that it contained was autoclaved at 121°C for 30 minutes

2 4 SAMPLING AND MEASUREMENT OF PARAMETERS

During all fermentation runs, unless otherwise stated, samples of medium plus cells (free and/or immobilised) were removed at various times for cell enumeration and medium analysis For immobilised cell shake flask cultures, flasks were well mixed and samples poured into a sample bottle allowing beads to flow into the sample bottle The risk of contamination was reduced using this method, as opposed to removing beads with a sterilised spatula

Free cell counts were performed on samples, which were then centrifuged at 4500rpm for 15 minutes, and stored at 4°C for further analysis

Gel beads were removed from samples and dissolved to allow cell enumeration to be performed (see 2 4 1 below) Free cell counts and further analysis were carried out as for suspended cell samples

2 4 1 Cell enumeration

Total cell counts were performed on all samples using a Neubauer haemocytometer All counts were performed in duplicate

For immobilised cell samples, beads were washed in distilled water and drained on tissue paper Between five

and ten 3 mm beads were weighed accurately and then dissolved in 5 ml of a 10% sodium citrate solution at room temperature Approximately the same weight of 5mm and 1mm beads was dissolved per 5 ml of citrate When fully dissolved, samples were suitably diluted and counted using a Neubauer haemocytometer Free cell numbers are expressed per ml of medium, whereas immobilised cell numbers are expressed per gram of alginate gel

2 4 2 Measurement of cell growth at the surface and core of alginate beads

A method was developed to estimate cell numbers in the centre of the bead and at the outer surface Using two razor blades, separated by a feeler gauge of 70 μm thickness, cross-sections of beads were made through the centre of the beads For analysing 3 mm diameter beads, micro-pipette tips were modified to give an opening of 2 mm diameter These tips were used to excise the outer section of the bead cross-section from the rest of the sample (see Figure 2 2)

In the case of 5 mm diameter beads, it was possible to divide bead cross-sections into three distinct areas, outer, middle and inner sections using modified micro-pipette tips with openings of 4 mm and 3 mm in diameter (See Figure 2 2)

This was carried out on five beads from each sample and the cores and outer sections were weighed accurately and dissolved separately These were then counted as described in Section 2 4 1 previously

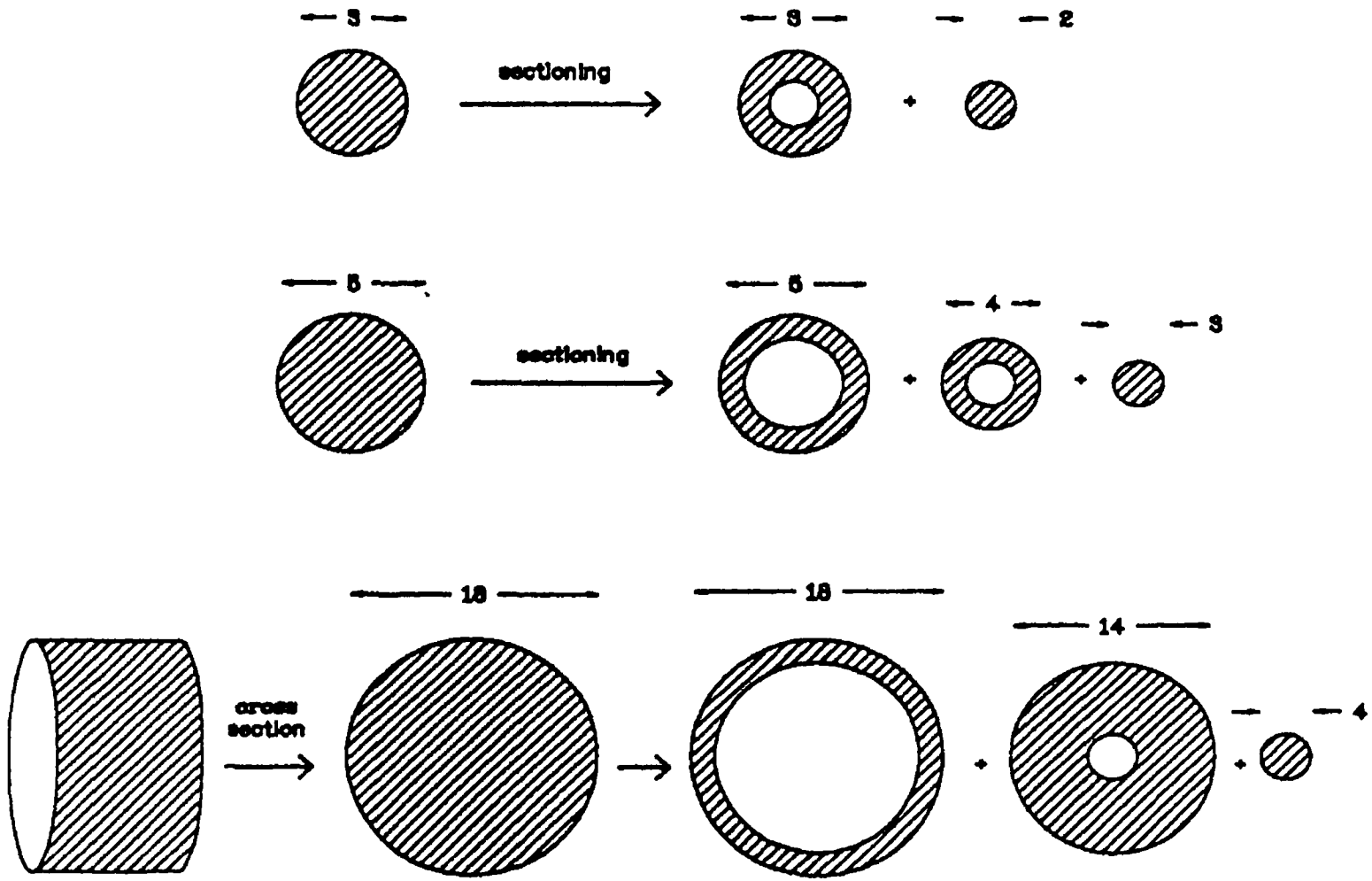

Figure 2.2 Schematic representation of bead–sectioning techniques
(All dimensions shown are in millimetres)

In the case of 18 mm alginate cylinders, a 2 mm thick cross section was sliced equidistant from both of the ends (See Figure 2 2) Using sterile cork borers the outer 2 mm layer of the gel disc was removed The inner core, 4 mm in diameter, was removed also These sections were weighed and dissolved separately in 10% sodium citrate Cell concentration was measured as outlined in Section 2 4 1

2 4 3 Cell viability

Cell viability was measured using a crystal violet stain The composition of the stain per 100 ml was as follows,

Crystal Violet	1 0g
Tri-sodium citrate di-hydrate	2 0g

The stain was filtered through Whatman no 1 filter paper before use 0 2 ml of stain was added to 1 ml of sample Cell counts were carried out after 10 minutes using a haemocytometer Dark blue cells were counted as non-viable

Cross-sections of beads were also stained using a crystal violet stain Tri-sodium citrate dissolves calcium alginate gel and therefore was replaced with sodium hydroxide (same concentration)

2 4 4 Glucose analysis

Glucose analysis was carried out on all samples using the dinitrosalicyclic acid (DNS) method of Miller (1959) Analysis was carried out on suitably diluted samples of cell-free medium

2 4 5 Estimation of reducing and non-reducing sugars

Sucrose concentrations were estimated by hydrolysing the sucrose present in samples, to glucose and fructose Then these reducing sugar concentrations were measured A commercial preparation of invertase was used, diluted 1/100 in phosphate buffer pH 7 0 0 5 ml of this concentrated enzyme solution was added to 2 ml of sample and incubated at 30°C for one hour to ensure maximal conversion Reducing sugar analysis was then carried out using the DNS method, taking into account the sample dilution due to enzyme addition

This measurement represents total reducing and non-reducing sugar concentrations Reducing sugar concentrations were measured as in Section 2 4 4 on untreated samples Sucrose concentration was estimated as the difference between the two measurements

2 4 6 Estimation of invertase activity in medium

This assay was used to measure the amount of invertase activity in sample supernatants The results are only used to demonstrate the presence of the enzyme in the medium The assay conditions were as follows

Substrate	10% sucrose
Buffer	Acetate at pH 4 5
Reaction temperature	30°C
Reaction time	10 min

0 5 ml of sample was added to 1 ml of pre-warmed substrate and allowed to react for ten minutes The reaction was stopped with 5 ml of 1M NaOH 1 ml of this mixture was added to 1 ml of distilled water and 2 ml of DNS reagent and assayed as previously described for

glucose Blanks were prepared for each sample by adding 5 ml of 1M NaOH to the substrate before sample addition The NaOH serves to stop enzyme action and also, in this case, to dilute the reducing sugars before analysis

2 4 7 β-Glucanase assay

This assay measures the reducing sugar levels released from β-glucan by the enzyme per unit time Since the levels of reducing sugars in fermentation medium are initially high and thereafter vary with time, it is necessary to remove this background sugar Dialysis removes the sugars effectively without loss of enzyme

Dialysis tubing with a molecular weight cut-off of 10,000 was boiled in 10 mM EDTA for ten minutes to regulate the pore size After washing in distilled water, 5 ml samples were placed in the tubing and sealed with dialysis tubing clips To ensure minimal volume increase during dialysis, the samples were pressurised into the smallest amount of tubing This served to prevent excess buffer inflow into the tubing which would have caused dilution of the sample

Samples were dialysed overnight at 4°C, against phosphate buffered saline (PBS), with three changes of buffer at least three hours apart The composition of PBS per litre was as follows,

KH_2PO_4	1 09 g
Na_2HPO_4	1 705g
NaCl	9 0 g

The enzyme substrate was made up fresh before each assay 1% β-glucan was dissolved in 0 1 M phosphate buffer

at pH 7 0 by boiling The solution was cooled and aliquoted in 1 ml amounts to the reaction test tubes using a Gilson 1 ml micro-pipette, for accurate dispensing without solution droplets adhering to the dispenser These tubes were allowed to attemperate for 10 minutes in a 50°C waterbath 1 ml of sample was added to 1 ml of pre-warmed substrate and allowed to react for precisely 10 minutes The reaction was stopped with 2 ml of DNS reagent Sample blanks consisted of 1 ml of 1% β-glucan, 2 ml of DNS reagent and finally 1 ml of sample added and incubated for 10 minutes at 50°C The presence of the DNS reagent inhibited enzyme action, thus the blanks were a measure of the residual sugar in the samples after dialysis

After incubation, samples were boiled for 10 minutes, cooled rapidly in a tap water bath and after addition of 10 ml of distilled water to each tube and mixing, the sample's absorbance at 540 nm was measured and compared to a glucose standard curve Blank values were subtracted from the sample values Units of β-glucanase activity are expressed as μg of glucose equivalents, released per minute per ml of sample

2 4 8 Plasmid stability measurement

2 4 8 1 Free cells

Samples were suitably diluted into the range of 2 - 5 x 10^3 cells ml^{-1} , based on total cell counts and 0 1 ml was spread on YEPD plates in duplicate After incubation at 30°C for 48 hours, 100 or 200 colonies were randomly replica plated onto selective media and onto YEPD (In the

experiment concerned, the number of replicas chosen will be mentioned) Colonies that did not grow on the selective medium but did grow on YEPD were counted as plasmid-free cells, whereas colonies that grew on both were counted as plasmid-containing cells

2 4 8 2 Immobilised cells

Firstly immobilised samples were dissolved using sterile 1% sodium citrate Then after suitable dilution the samples were spread onto YEPD plates and treated similarly to the free cell samples (Section 2 4 8 1)

2 4 9 Measurement of plasmid stability of cells at the surface and core of alginate beads

Two methods of measuring plasmid stability of different sectors of alginate beads were developed

Firstly, using the same method as described in Section 2 4 2, (except under aseptic conditions), samples of inner core and outer sections of beads were dissolved in sterile 1% tri-sodium citrate Samples were plated out on YEPD plates and plasmid stability measured as described in Section 2 4 8

The second method was based on the selective dissolution of alginate beads in sterile 1% tri-sodium citrate Approximately five beads were added to 5 ml of sterile 1% citrate and vortexed for 10 seconds, allowed to stand for 5 seconds and this procedure was then repeated two more times The citrate solution was drained from the beads into a sterile test tube and the rest of the beads was dissolved in fresh sterile citrate solution Samples of each were

then plated out for plasmid stability testing as described in Section 2 4 8

2 4 10 Image analysis of bead cross-sections

An AMS image analyser attached to a Leitz microscope was used to quantify the heterogeneous growth pattern exhibited by yeast in alginate beads The image analyser consisted of a video camera attached to a microscope, a high speed image processing unit and an 8-bit micro-computer

A given sample when viewed by the monochrome video camera is developed into an image of black, white and 255 shades of grey Depending on the application, the threshold level of 'greyness' can be varied Any grey areas equalling or darker than the threshold level was registered by the analyser All bead cross-sections were stained using crystal violet for one hour before analysis

The sensitivity of the analyser was set so that biomass levels at the start of fermentations registered a value close to zero As biomass concentrations increased with time, there was a corresponding increase in the analyser reading For a set of samples from a batch fermentation the sensitivity remained unchanged

Two different approaches to growth quantification were used in studying cell distribution in calcium alginate beads during growth in batch fermentations Micro-colony size was estimated in different areas of bead cross-sections for low cell densities and also gel occupancy was measured across bead sections for higher cell densities within the matrix

2 4 10 1 Image analysis at low cell densities

At low cell densities (approximately 0 2g dry weight per litre of gel), micro-colonies form as growth proceeds Therefore a circular reading frame a little larger than the largest micro-colonies observed was used (see Figure 2 3) The 3 mm bead was visually divided into three areas,

- The outer 0 25 mm layer
- A layer inside the above of 0 25 mm thickness
- The inner core of 2 mm diameter

Samples were positioned so that a single micro-colony appeared in the reading frame at a time This was readily possible because of the random low number of single colonies within the matrix Measurements were taken of a number of micro-colonies in each section at various sample times

2 4 10 2 Image analysis at high cell densities

At high inoculation concentrations (approximately 20 g dry weight per litre of gel), single discrete micro-colonies grew as before but, because of the initial high cell density, these micro-colonies quickly came in contact with each other Sizing of single colonies was not possible so an average measurement of a larger area was taken The reading frame chosen was rectangular (10 x 150 pixels) A thin rectangular frame was chosen to enable a lot of readings to be taken across the bead sections whilst still measuring an area sufficiently large to average out fluctuations (see Figure 2 4) Initial measurements were taken with the reading frame aligned along the bead

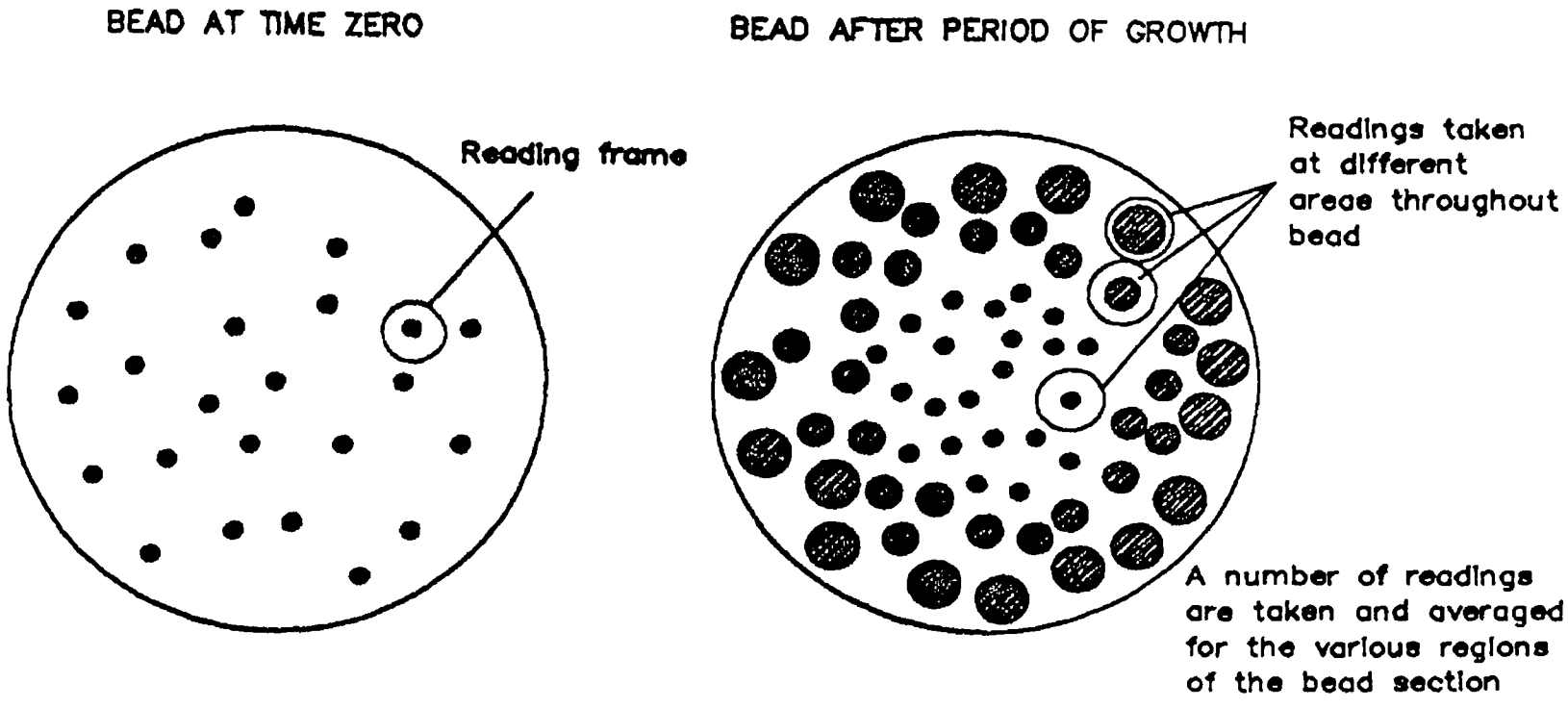

Figure 2.3 Image analysis of bead cross-sections using a circular reading frame (low initial cell loadings)

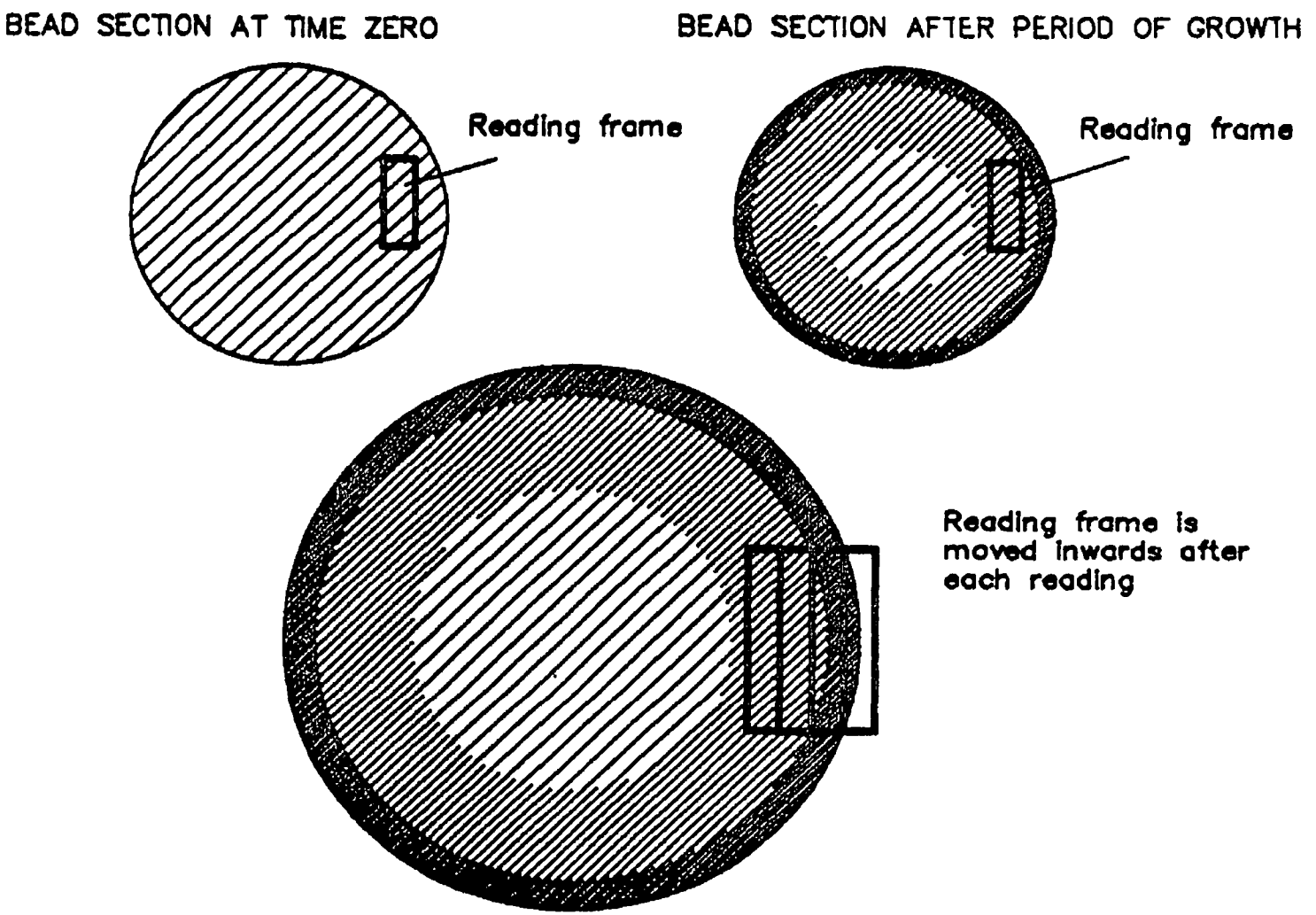

Figure 2.4 Image analysis of bead cross-sections using a rectangular reading frame (high initial cell loadings)

surface The reading frame was then moved inwards the width of the frame and the next measurement taken Continuing in this manner measurements were taken into the bead interior

3 RESULTS AND DISCUSSION

3 1 PRELIMINARY RESULTS

3 1 1 Background sugar levels in β-glucanase assay

The enzyme endo β-(1,3)(1,4)-glucanase hydrolyses β-glucan releasing dextrins with reducing ends In this work, the DNS method (Miller, 1959) was used to measure these reducing ends Measurement of the enzyme's activity is only accurate and reproducible if either the background amounts of reducing sugars remain constant or are eliminated In fermentations using reducing sugars as a carbon source, this background is initially high and then decreases with time as it is utilised there after

Initial work on the assay involved dilution of fermentation samples to reduce this background level However, in doing so, the enzyme activity was diluted below measurable amounts Whereas enzyme detection at the end of batch fermentation was possible with most of the reducing sugars utilised, this gave very little information on where in the batch growth curve most enzyme production occurred Therefore, it was decided to use sucrose, a non-reducing carbohydrate source in place of glucose

3 1 2 Sucrose as a carbon source to eliminate assay background sugar

Growth of pJG317 in minimal medium with sucrose was found to be similar to growth in minimal medium plus glucose (See Figure 3 1)

It was expected that the background reducing sugar levels would remain insignificant during fermentations

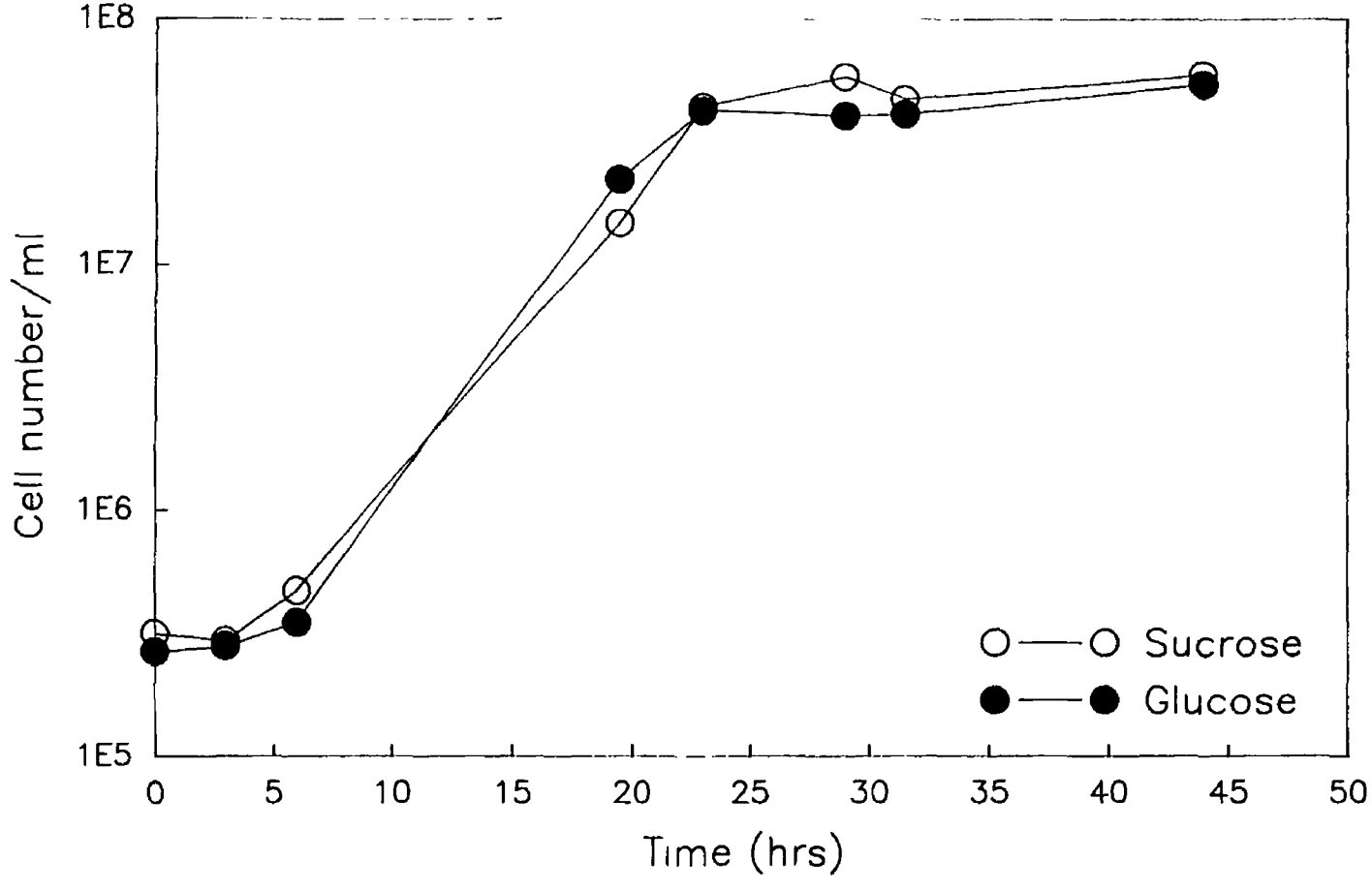

Figure 3 1 Growth of pJG317 cells on minimal medium with sucrose and glucose as carbon sources

using sucrose as the sole carbon source However significant amounts of reducing sugars were detected as the fermentation progressed and because of this, β-glucanase analysis was not possible Yeast invertase activity was found in the fermentation medium This enzyme hydrolyses sucrose to fructose and glucose which are both reducing sugars Figures 3 2 and 3 3 show typical batch growth curves of the clone pJG317 (m/m) and the parent strain DBY746 (YEPD) in media containing sucrose in place of glucose These figures illustrate reducing and non-reducing sugar levels as well as invertase activity in the culture medium The use of alternative non-reducing carbohydrate sources was not investigated

3 1 3 Development of assay conditions

A method was developed for removing the background reducing sugar levels, without affecting the enzyme activity of the sample

Dialysis of samples was found to remove the sugars effectively without loss of enzyme Table 3 1 lists activities of a commercial preparation of β - (1,3)(1,4) - glucanase (Novo Cereflo) when dialysed overnight at 4°C Enzyme preparations containing 10 g/L glucose were assayed after dialysis to check the ability of the method to render sugar containing samples assayable for β-glucanase

The commercial preparation was diluted 1/8000 to give activities in the range of the assay Separate dilutions were necessary in PBS and PBS plus 10 g/L glucose, thus yielding discrepancies in the activity All samples are in

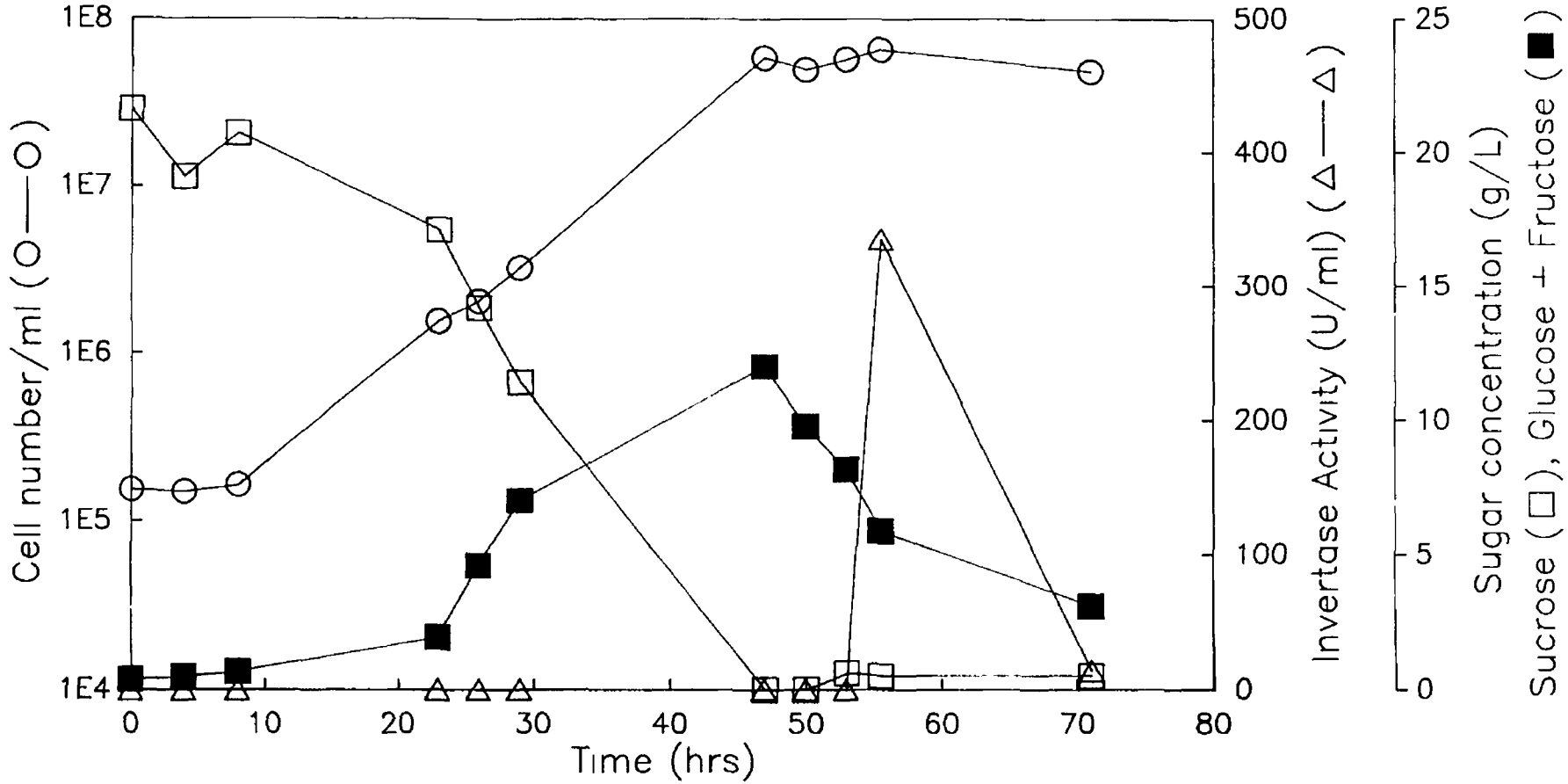

Figure 3 2 Growth of pJG317 cells in minimal medium containing sucrose as a carbon source, with measurement of invertase activity sucrose, glucose and fructose concentrations

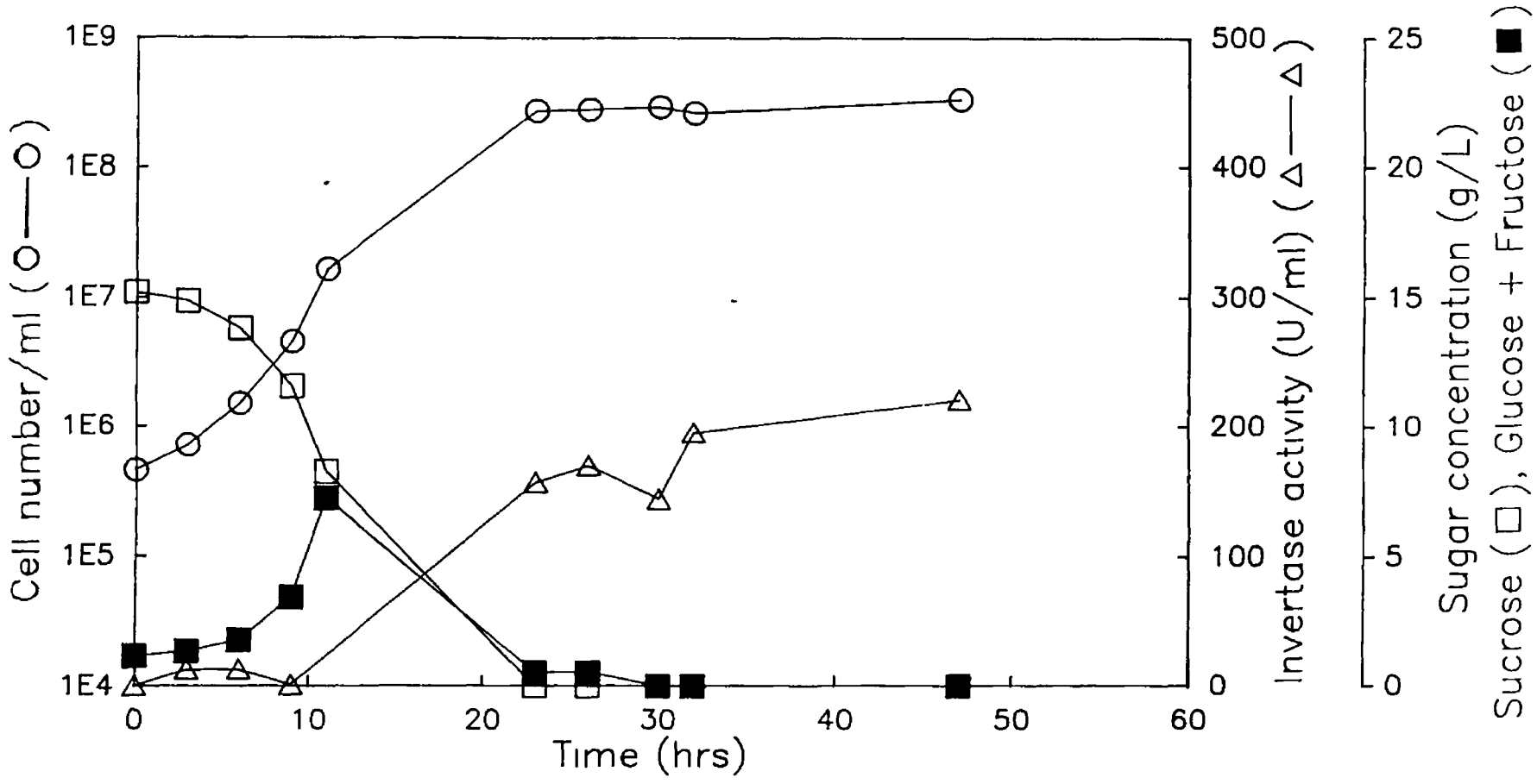

Figure 3 3 Growth of pJG317 cells in YEPD containing sucrose as a carbon source with measurement of invertase activity, sucrose, glucose and fructose concentrations

TABLE 3 1 ENZYME ACTIVITIES OF DIALYSED SAMPLES

Sample	Activity (U/ml)
Initial enzyme concentration	70 6
Sample dialysed overnight in PBS	78 25
Sample containing 10 g/L glucose dialysed overnight in PBS	82 4

the same range, whether dialysed or not Results in Table 3 1 are an average of duplicates

Maximum enzyme activity is achieved at temperatures between 50 and 60°C (Novo literature) However at 60°C, there is a drop in enzyme activity of 35% after 45 minutes, whereas at 50°C there is no loss in activity with the same incubation time Because of improved enzyme stability at the lower temperature of 50°C without reduction in activity a temperature of 50°C was chosen for the β-glucanase assay

Using a Novo preparation of β-glucanase, the activity of various dilutions was measured over a range of pH values The results of this experiment are presented in Table 3 2

The results are presented in absorbance units, as

TABLE 3 2 β-GLUCANASE ASSAY ABSORBANCE RESULTS AT VARIOUS ASSAY pH VALUES

	Absorbance at 540 nm					
Sample Dilution	pH 5 0	pH 5 5	pH 6 0	pH 6 5	pH 7 0	pH 7 5
1/8000	0 107	0 274	0 375	0 447	0 466	0 382
1/9000	0 102	0 246	0 331	0 442	0 443	0 360
1/10000	0 092	0 206	0 298	0 400	0 405	0 369
1/11000	0 101	0 206	0 283	0 371	0 399	0 319
1/12000	0 092	0 179	0 267	0.347	0.357	0 281
1/15000	0 106	0 158	0 227	0 302	0 306	0 235

opposed to enzyme activity units, as the values are only required for comparison purposes Sample blanks are not necessary here, as there is no sugar background in the commercial enzyme preparation or buffers used

In order to ensure that these findings apply to the enzyme secreted by pJG317, the activity of the secreted and intracellular enzyme was measured at various pH values The results are presented in Table 3 3 In this case results are presented in enzyme activity units per ml as the background levels of reducing sugars in the secreted and intracellular samples differed

There appears to be a slight difference in the pH optimum of the secreted and intracellular samples The protein processing involved in secretion may be responsible for the shift in pH optimum Since this work is concerned only with secretion of the enzyme, the pH chosen for enzyme activity analysis is pH 7 0

A study of the effect of substrate concentration on enzyme activity in the assay was carried out, in order to find the minimum concentration of substrate to use, which

TABLE 3 3 EFFECT OF pH ON THE ACTIVITY OF INTRACELLULAR AND EXTRACELLULAR β-GLUCANASE

	Enzyme Activity (U/ml)				
Sample	pH 4 0	pH 5 0	pH 6 0	pH 7 0	pH 8 0
Intracellular enzyme	0	42	80	77	36
Secreted enzyme	3	47	82	85	55

TABLE 3 4 EFFECT OF β-GLUCAN CONCENTRATION ON THE ENZYME ACTIVITY DETECTED FOR VARIOUS DILUTIONS OF COMMERCIAL ENZYME

Enzyme Dilution	Enzyme Activity (U/ml) β-glucan concentrations 0 1%	0 5%	1 0%	2 0%
1/8000	15	55	95	105
1/9000	11	41	89	103
1/10000	11	40	85	92
1/11000	10	38	83	87
1/12000	9	36	71	75
1/15000	8	28	57	56

still yielded accurate results Table 3 4 lists enzyme activities at pH 7 0 with various β-glucan concentrations

The sensitivity of the assay at 0 1% and 0 5% β-glucan concentrations is reduced, compared to the results for 1% and 2% β-glucan The difference between the results for 1% and 2% substrate is not as pronounced as that between 0 5% and 1% β-glucan Based on these results and on the expense of using 2% β-glucan routinely during assays, a 1% β-glucan solution was used in all β-glucanase activity assays

3 1 4 Free cell enzyme production in selective medium

Enzyme production by pJG317 was monitored in a suspended cell (free cell) shake flask culture using a selective medium, to investigate where in the batch growth cycle, enzyme production occurs The results are presented in Figure 3 4

It is clear from these results that enzyme production is growth associated This is important to bear in mind when

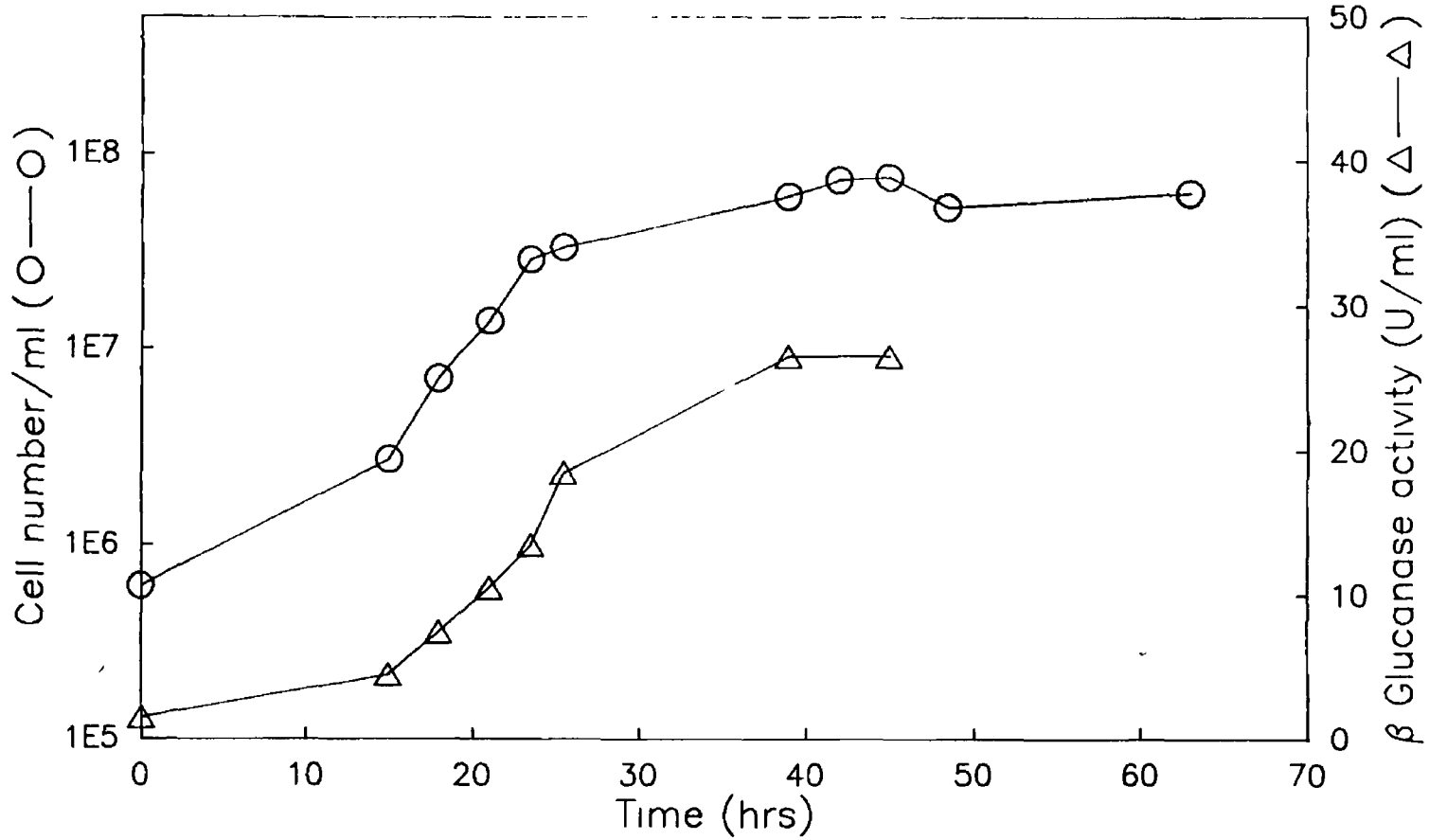

Figure 3 4 Cell growth and β glucanase activity vs time
– pJG317 in mininal medium – shake flask

an immobilised system is being developed In such a system growth must be encouraged in order to maintain enzyme production Immobilisation of micro-organisms has often served to contain high densities of biomass for bioconversions and other reactions where the cells are metabolically active but do not replicate (Durand, 1978) From the results of Figure 3 4, it is clear that growth and division of cells are necessary for enzyme synthesis The immobilisation matrix must be capable of retaining large numbers of cells initially in order to maximise productivity and also to allow growth and replication of the immobilised cells, insuring continued production of enzyme

3 1 5 Immobilisation considerations

The presence of many salts in the components of growth medium can have deleterious effects on the structural integrity of calcium alginate (Kolot, 1988) In particular, phosphates and chelating salts (eg EDTA) can readily dissolve calcium alginate There are significant amounts of phosphate in yeast extract, peptone and yeast nitrogen base Their presence in the growth medium may soften the beads as the fermentation proceeds To counteract this, all media used for immobilised cell growth is supplemented with calcium chloride

3 1 6 Calcium chloride and media precipitation

In a production medium used generally for yeast growth (not used in this work) containing various salts and yeast extract, it was found that addition of an excessive amount

of $CaCl_2$ led to precipitation in the medium This presumably was the precipitation of calcium salts This is undesirable as it may limit certain nutrients and cause variation in medium quality Various concentrations of $CaCl_2$ were added to this medium to measure the maximum level of $CaCl_2$ that could be added without causing precipitates to form The results are presented in Table 3 5 below

When 0 015% (w/v) $CaCl_2$ was added to YEPD and m/m, no precipitation was observed Previous researchers have reported the use of $CaCl_2$ in the growth medium of cells immobilised in calcium alginate (Adlercreutz, 1982, Lovitt, 1986) In all experiments involving growth in calcium alginate beads, the growth medium was supplemented with 0 015% (w/v) $CaCl_2$ unless otherwise stated

3 2 BATCH FERMENTATION RESULTS

Immobilised cell systems have many advantages over conventional free-cell reactor systems including increased biomass density and reduction of biomass loss in continuous reactors (Bailey and Ollis, 1986) Immobilised systems

TABLE 3 5 EFFECT OF CALCIUM CHLORIDE CONCENTRATION ON PRECIPITATION OF MEDIUM

$CaCl_2$ concentration %(w/v)	mM	Precipitation at pH 5 0
0 0015	0 1	-
0 015	1 0	-
0 0735	5 0	++
0 15	10 0	+++

usually infer systems of a continuous or re-usable nature, like repeated batch or continuously fed fermentations For experimental purposes, however, batch experiments are very useful In this work, batch fermentations were conducted using immobilised cells and these results were compared with free cell batch growth Whereas immobilised cell fermentation systems may not be batch processes in practice, a lot of information can be obtained by studying the behaviour of immobilised cells in batch culture The volume of gel beads used in the batch fermentations conducted was 10% of the medium volume

3 2 1 Steady state immobilised cell numbers in batch growth

There have been many reports of growth and division of micro-organisms entrapped in natural gel polymers such as carrageenan and alginate (Dhulster, 1984, Gosmann, 1986, Godia, 1987)

Initial studies of growth of pJG317 in an immobilised state in selective medium showed that there is a limit on the cell concentration obtainable within the gel This maximum cell concentration is achieved irrespective of the initial cell loading As with free cells the batch growth of cells in calcium alginate exhibits a lag, exponential and stationary phase When cells are immobilised using crosslinking agents growth does not usually occur (Laretta Garde, 1981) The results of two growth curves are illustrated in Figure 3 5 Even with a ten-fold increase in initial cell numbers, the cell counts in both cases reach

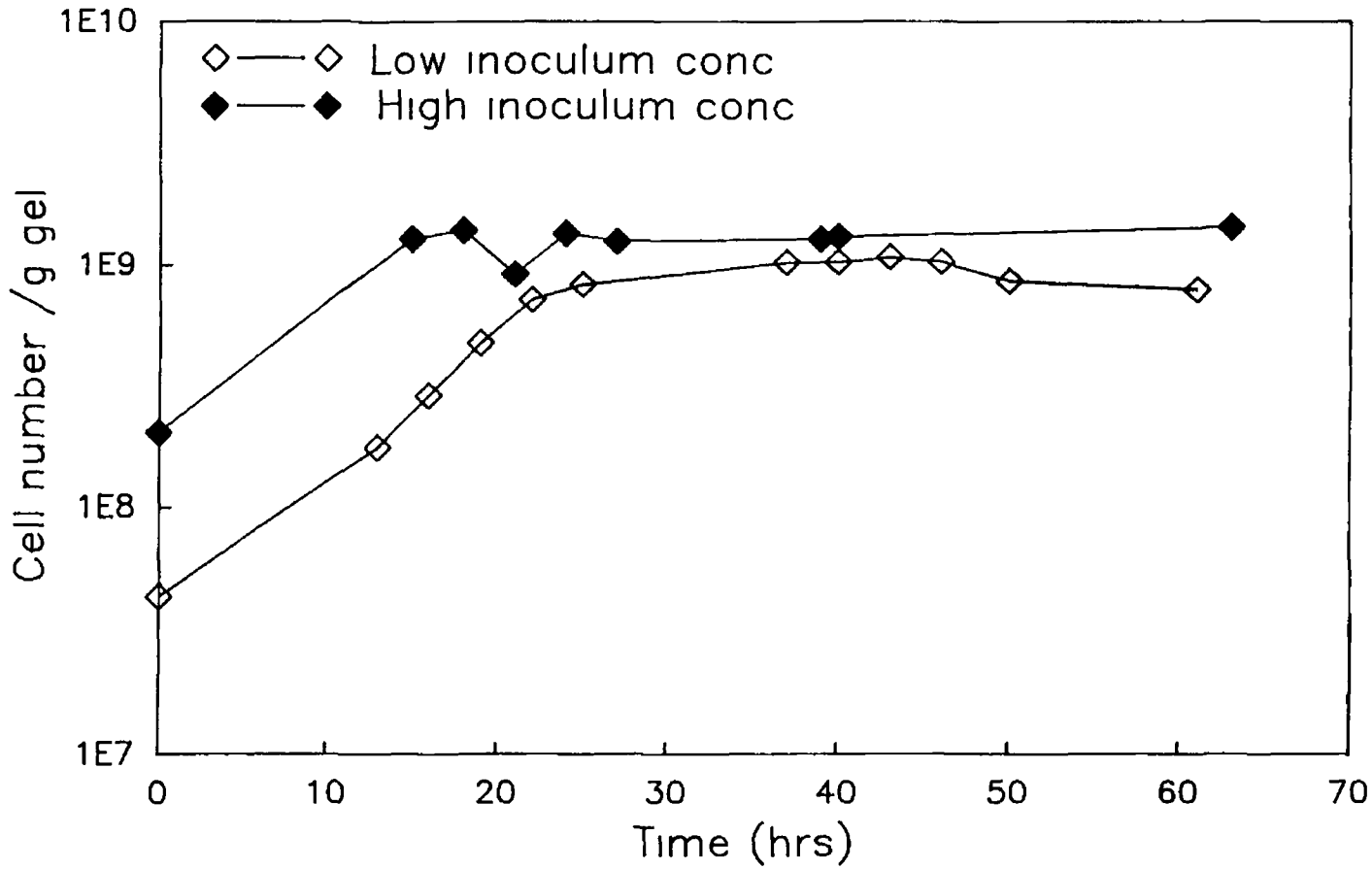

Figure 3 5 Immobilised cell concentration during batch growth of pJG317 in 3mm alginate beads at different inoculation concentrations

1 x 10^9 cells g^{-1}gel and remain constant These results compare well with those of Godia (1987), who found that yeast cell numbers reached 10^9 g^{-1}gel from inoculation concentrations ranging from 10^5 $g^{-1}$to 10 $^9g^{-1}$ Cell numbers reported did not exceed the order of 10^9 cells g^{-1}gel

3 2 2 Cell leakage from calcium alginate

Even though cell numbers do not exceed the order of 10^9 g^{-1} gel it should be noted that the cell biomass does not completely occupy the total gel volume The reported cell occupancy for *Escherichia coli* in carrageenan in the outer 50 μm dense layer of the immobilisation matrix is 50%, with percentage occupancies in the bead core as low as 10% (Marin-Iniesta, 1988) The more likely reason for the levelling off of the immobilised biomass concentration is the restriction of active growth and division to the outer surface of the beads With prolonged growth, cell numbers increase and disrupt the alginate matrix at the surface, causing it to swell as the cells grow in micro-colonies This increase in biomass at the surface causes matrix disruption and release of cells into the medium Further growth of cells in the matrix leads to release of more cells into the medium rather than further increase in cell density within the matrix Wada (1980) found that leakage of yeast cells did not occur until the average cell concentration in the matrix exceeded 10^9 cells /g of carrageenan beads Below this concentration no cell leakage was observed

In batch studies of immobilised yeast, cell leakage was

observed to a considerable degree In an experiment using two different sizes of alginate beads (1 mm and 5 mm diameter) it was found that greater cell leakage occurred in the case of the smaller beads (See Figure 3 6) This may be explained by the fact that the smaller beads have a larger surface area (per unit volume), and cell leakage relates to the area available for leakage

In batch studies, even if cell leakage is very small, the released cells remain in the vessel and replicate, unlike the situation of a continuous reactor, where free cells are washed out When an increase in free cell numbers is observed in an immobilised batch fermentation it is important to remember that the increase is due to cell leakage from the matrix and growth of free cells in the medium In a batch system, it is therefore difficult to accurately quantify or characterise cell leakage

3 2 3 Growth and gradients in alginate beads

Knowing that yeast growth and replication occurs in calcium alginate (See Section 3 2 1), it is of interest to compare the rate of cell replication of immobilised cells to that of free cells Figure 3 7 shows a typical batch growth curve for both free and immobilised pJG317 grown in m/m Although free cell numbers are expressed per ml and immobilised cell numbers per gram of alginate, the growth curves are similar showing growth rates of both to be of the same order This is also reported for bacteria immobilised in carrageenan (Dhulster, 1984, Berry, 1988) The growth rate of yeast appears therefore to be unaffected

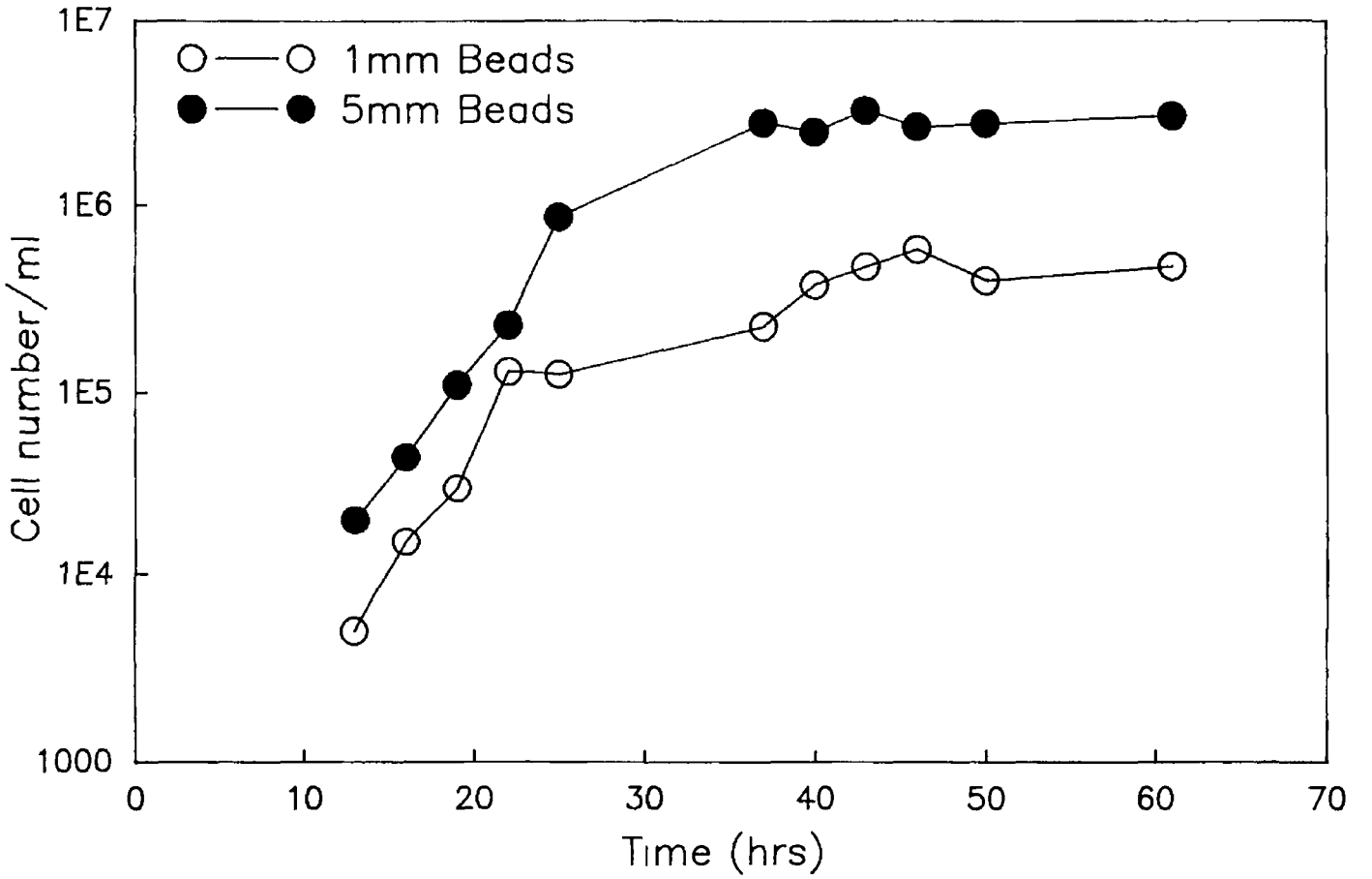

Figure 3 6 Plot of appearance of free cells during batch culture of pJG317 using 1mm and 5mm alginate beads

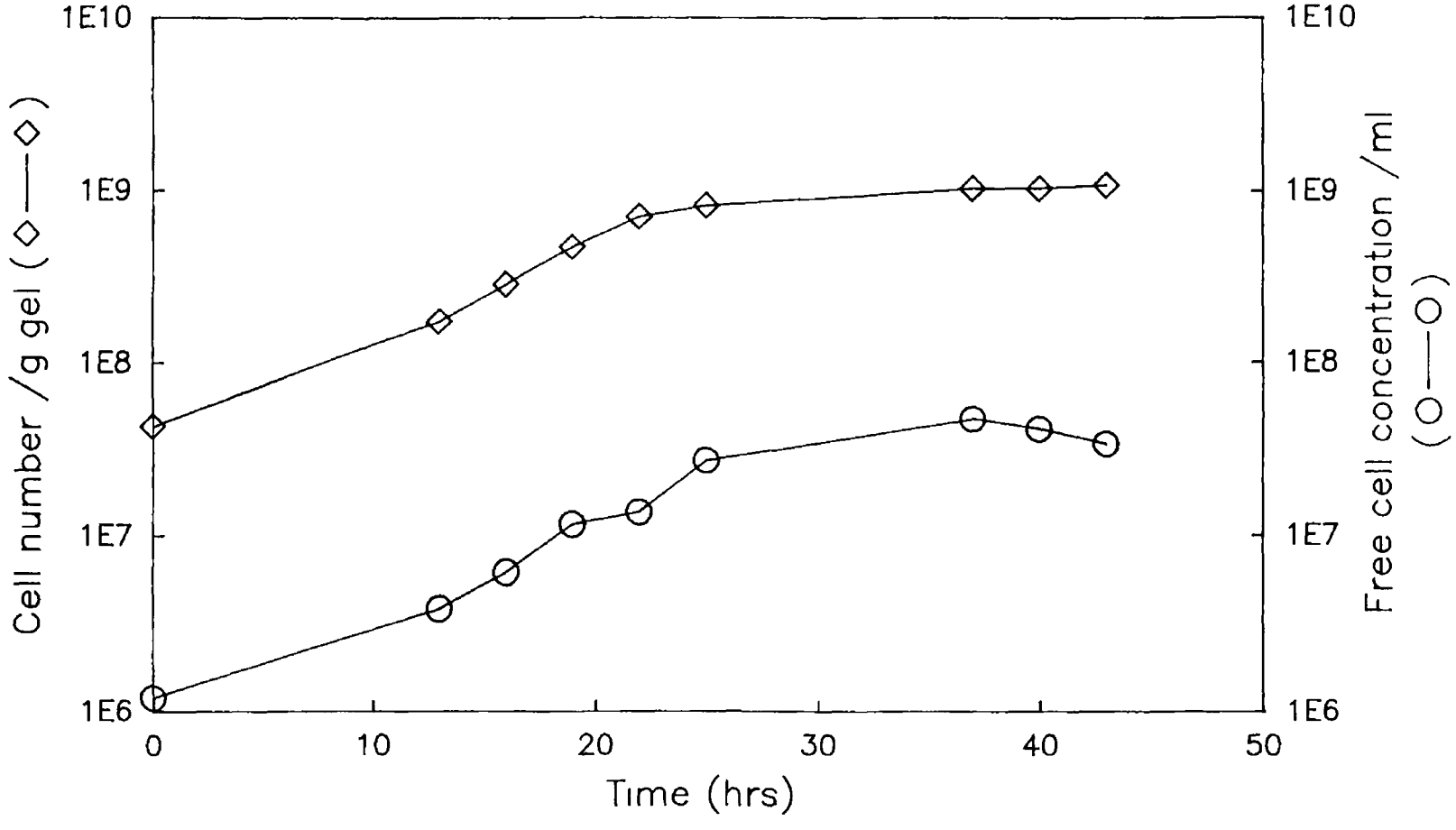

Figure 3 7 Growth of free and immobilised pJG317 cells in batch culture – minimal medium in shake flasks

by immobilisation, with the average growth rate of immobilised yeast in beads equalling that of free cells in suspension

It is important to note that Figure 3 7 represents the *average* concentration of cells in the beads In the case of immobilised cell systems there are three additional resistances to mass transfer i e resistance in the liquid film surrounding the matrix, at the liquid - matrix interface and resistance within the bead particle (Radovich, 1985) More detailed studies of cell growth within the matrix are therefore necessary and in particular the distribution of cell growth within the matrix

3 2 4 Image analysis of bead cross-sections

In looking closer at the growth patterns of yeast within the beads, it was observed that gradients of growth occur in the matrix, with most of the growth at the outer bead surface This phenomenon has been reported for various entrapped organisms, bacteria (Monbouquette, 1988), bacteria and yeast (Gosmann, 1986), fungi (Eikmeier, 1984, El-Sayed, 1986) Growth of cells in the core of the beads is restricted due to nutrient and oxygen limitations The rate of diffusion of nutrients through calcium alginate decreases with increasing cell populations within the matrix (Pu, 1988) The rate of consumption of these nutrients by cells at the bead surface exceeds their rate of diffusion into the gel As cell numbers increase, growth of cells in the bead interior is inhibited, as found by Monbouquette (1988)

Image analysis of beads with an initial cell density of 20 g/L dry weight of baker's yeast illustrates the biomass concentration gradients throughout the bead cross sections (See Methods Section 2 4 10 2) The results are illustrated in Figure 3 8 and show cell density gradients of bead cross-sections at various times during batch growth

The dense layer of cells at the bead surface at 15 hours is approximately 120 μm deep, indicating rapid growth of cells in this layer Cell growth, although slower inside this layer, reaches maximal growth by 22 hours The dense layer thickness at this time is about 350 μm Dense cell layers for immobilised bacteria have been estimated at 50 μm by Marin-Iniesta (1988), and between 50 and 150 μm by Berry (1988)

Initially it would seem from the results of Figure 3 8, that a thin layer of dense growth develops and deepens as growth proceeds It is clear that towards the end of batch growth, the surface of the bead shows signs of structural deterioration, with cell numbers at the surface decreasing, presumably due to cell leakage and break-up of the matrix surface

At lower cell loadings (2g dry weight of baker's yeast per litre of alginate), micro-colonies formed evenly throughout the matrix Berry (1988) found, in the case of *Escherichia coli* at an inoculation concentration of less than 6×10^6 g^{-1} carrageenan, that micro-colonies were observed which were evenly distributed throughout the matrix With higher initial cell loadings most growth was

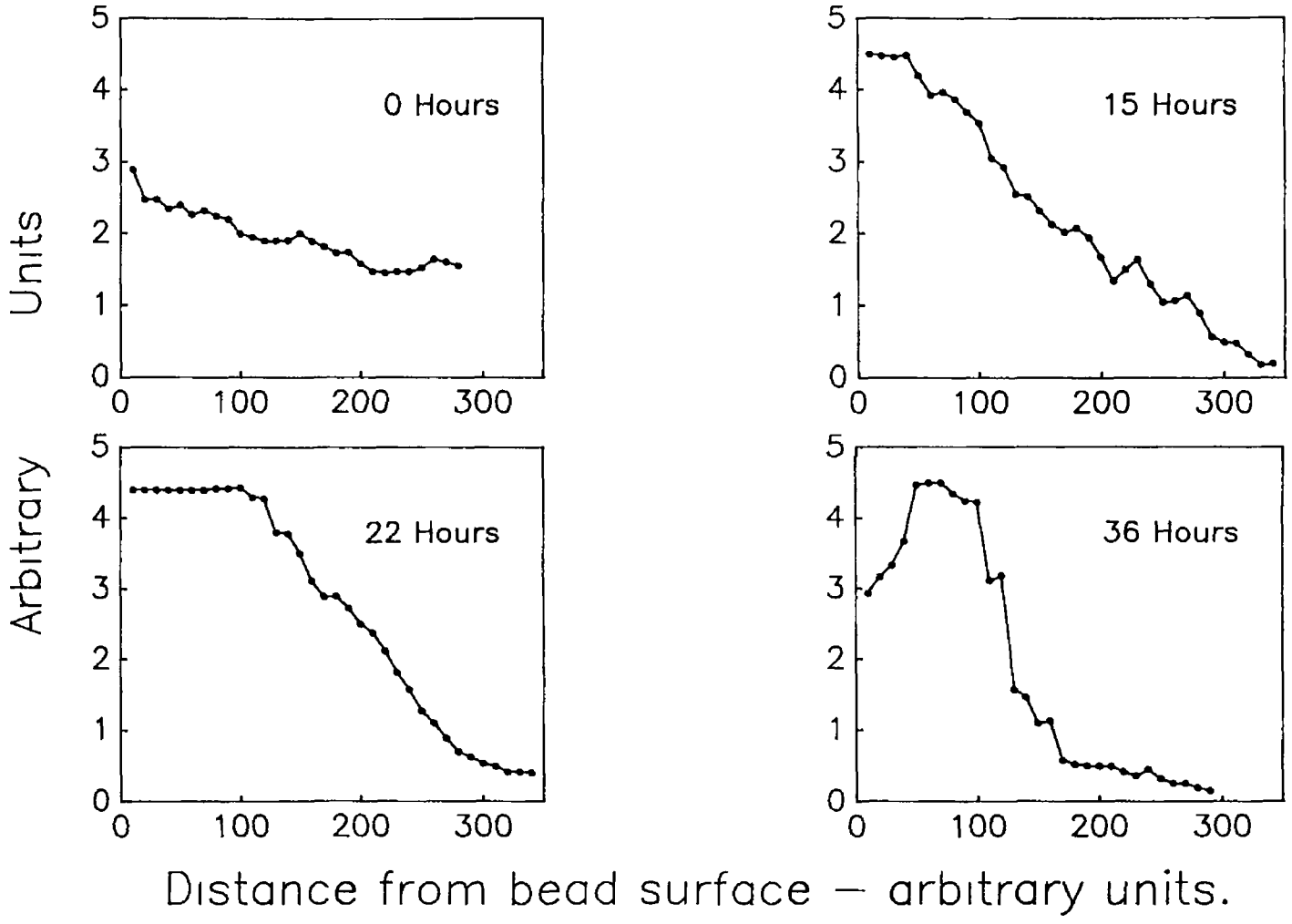

Figure 3 8 Image analysis of bead cross — sections at different times during batch growth of baker's yeast Inoculum cell density was 20 g/L dry weight

restricted to the outer 50 - 150 μm of the bead

Image analysis of 3 mm bead cross-sections (see methods Section 2 4 10 1) during batch growth show in Figure 3 9 that as with the higher cell loading case (Figure 3 8) cell growth is greater towards the bead surface and retarded in the inner sections of the beads Micro-colonies were evenly distributed throughout the matrix but colony size increased towards the bead surface These results compare well to the findings of Marin-Iniesta (1988) for *Escherichia coli* entrapped in carrageenan

At low cell loading concentrations (approx 2 g dry weight per litre of gel) cell growth is uniform initially, until a certain cell density is reached, then gradients of cell growth occur At high cell densities, cell growth is restricted to the outer layer of alginate (Berry, 1988) This helps to explain why similar growth rates were observed for free and immobilised cells Immobilised cells can grow at the same rate as free cells, but this only applies to cells at the bead exterior If all immobilised cells are considered, there is a gradient of growth rates from a maximum at the bead surface to a low growth rate at the bead core

3 2 5 Enumeration of cells in bead cross-sections

In this section, the heterogeneous distribution of cell growth in the matrix was estimated by measuring cell numbers in various sections of the matrix during batch growth

Cell numbers per gram of matrix were measured for

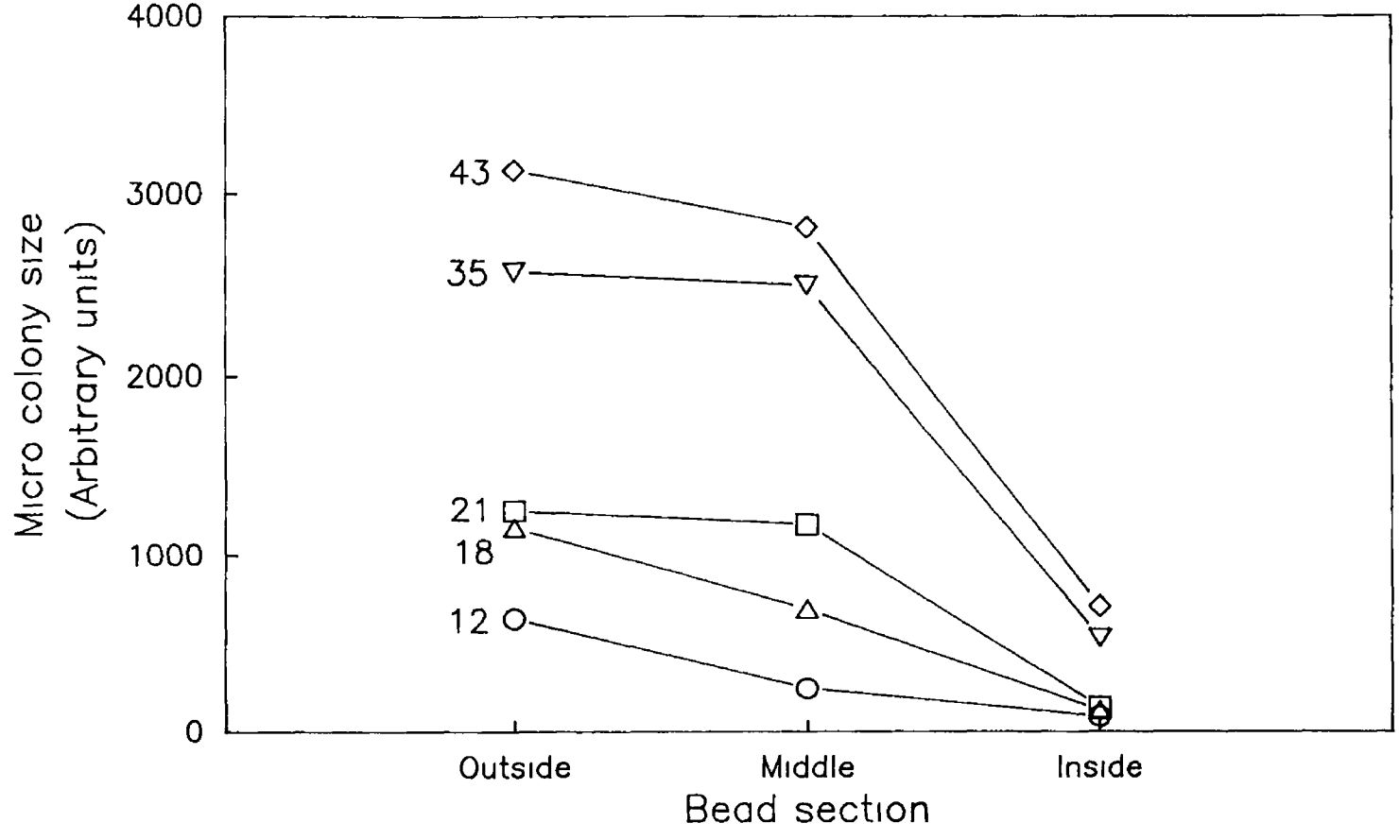

Figure 3 9 Plot of micro–colony size in sections of 5mm alginate beads during batch growth (Numbers on graph refer to hours of batch growth)

various sections of the beads, representing the outer surface layer, the very core of the bead and the middle section between the two Results of these observed gradients are presented in Figure 3 10

Initially, cell distribution is practically uniform and with incubation in growth medium, cell numbers increase to a greater degree in the outer sections of the beads Cell growth and replication takes place in the core of the 5 mm beads but at a reduced rate compared to the outer section of the beads

These results are similar to the image analysis results presented in Figure 3 8, although it is not possible to directly measure the biomass concentration at every point in the bead cross section Cell numbers in the outer section based on these results are 4 3 times that of the bead core at the end of batch growth Cell numbers estimated for the outer dense layer of growth are probably underestimated because the samples were 500 μm thick whereas the dense layer of growth is estimated to be limited to a region of thickness 200 - 350 μm based on the results presented in Figure 3 8

3 2 5 Growth and enzyme production of free cells at various pH values in selective medium

In batch studies using buffered minimal medium at various pH values, growth and enzyme production were monitored The medium contained 0 2 M buffers to maintain constant pH The pH of all flasks remained within 0 2 of a pH unit of the initial value Table 3 6 lists the

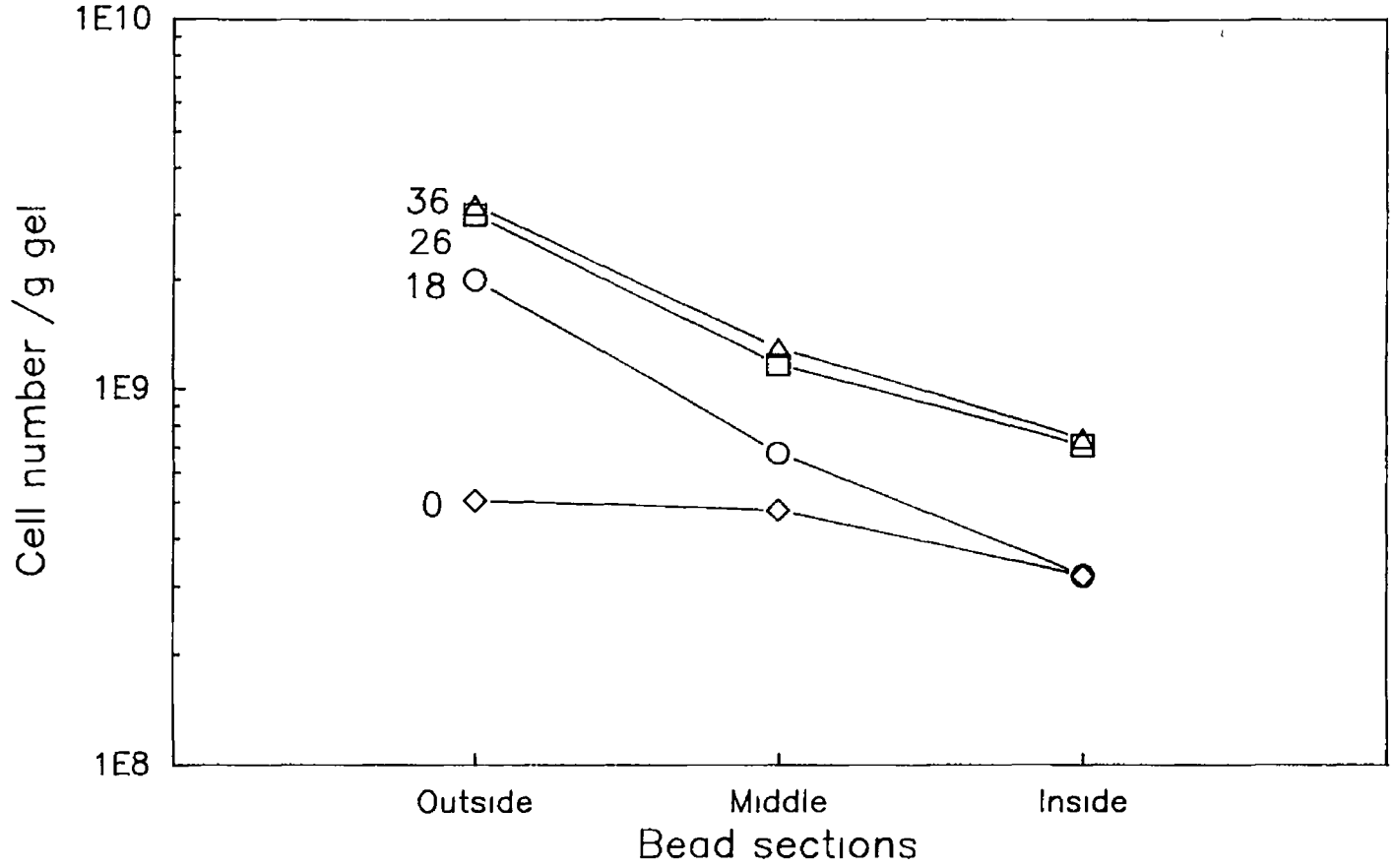

Figure 3 10 Plot of cell concentration of bead sections of 5mm alginate beads during batch growth of baker's yeast (Numbers on graph refer to hours of batch growth)

TABLE 3 6 PREPARATION OF BUFFERS FOR GROWTH MEDIA

pH	Amount added per 400 ml of medium (g)			
	Succinic Acid	NaOH	Na_2HPO_4	NaH_2PO_4
4	4 72	0 64	-	-
5	4 72	1 708	-	-
6	4 72	2 784	-	-
7	-	-	6 928	3 744

quantities of the various buffering salts used (Gomori, 1955)

When the buffered medium was made up and autoclaved, the actual pH was somewhat different than expected The pH values were, however, in the desired range and remained constant throughout the experiments

Figure 3 11 shows a typical set of results for batch growth and enzyme production at various pH's Best growth was obtained at pH 4 0 and pH 4 6 However, best enzyme production occurred at pH 4 6 The enzyme appears to be unstable at pH 3 7 and to a lesser degree at pH 4 6, whereas at pH 5 6 and 6 8, it appears stable, even when incubated and shaken at 30°C The reason for higher yields of enzyme at pH 4 6 rather than 3 7 (despite similar patterns of growth), may relate to the stability of the enzyme in the medium once secreted It may well be that enzyme production at both these pH values is similar, but that the enzyme is less likely to be inactivated at the higher pH The enzyme activity optimum is at pH 7 0 (see Section 3 1 3) so it may have a reduced stability at lower pH values

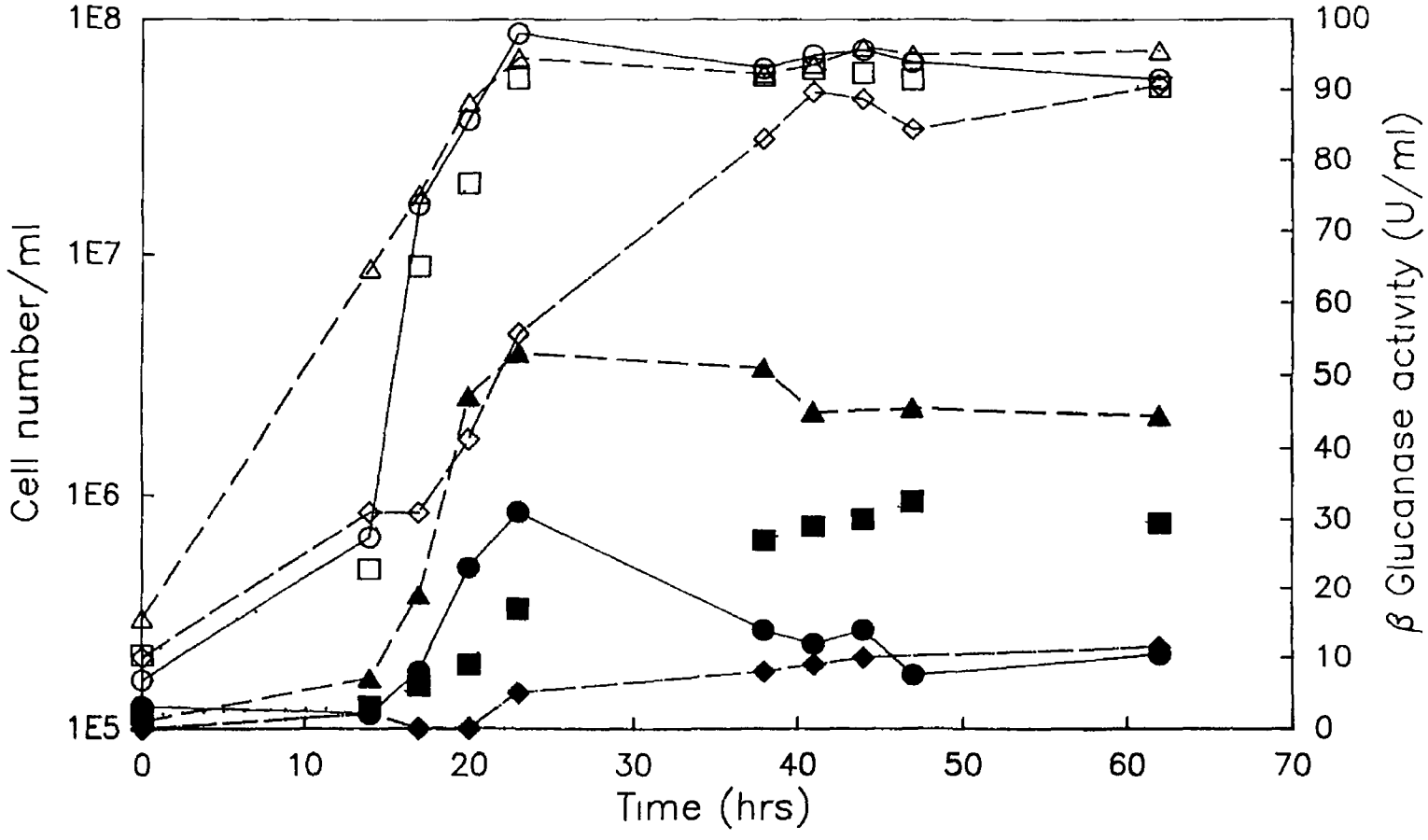

Figure 3 11 Cell growth and β glucanase production in batch culture of pJG317 at various pH values.
○——○ pH 3 7, △– –△ pH 4 6, □ □ pH 5 6, ◇——◇ pH 6 8
Closed symbols – enzyme activity, open symbols – cell concentration

3 2 7 Growth and enzyme production of immobilised cells at various pH values in selective medium

The conditions for optimum growth and enzyme production with free cells may differ from the optimum conditions required for cells in an immobilised state Therefore, it is necessary to find the optimal pH for growth and production for pJG317 using immobilised cells As with free cells, the studies were conducted with buffered media in shake flasks The medium was supplemented with 0 015% (w/v) $CaCl_2$ Phosphate buffering was replaced with Tris/HCl to avoid bead dissolution When $CaCl_2$ was added to this medium, precipitation readily occurred Therefore at pH 7 0, $CaCl_2$ was omitted from the medium In 400 ml of m/m, 4 846g of Tris-(hydroxymethyl)-aminomethane (Tris) plus 176 8 ml of 0 2 M HCl were added to buffer the medium at pH 7 0 Cell growth and enzyme production are illustrated in Figure 3 12

Growth and enzyme production are very similar to the free cell system of Figure 3 11 Immobilised cells produce the enzyme during the exponential phase just as in the free cell case No growth was observed at pH 7 0 This may be because of the change in buffer to Tris/HCl

Cell growth within the gel was quite similar for media pH values 3 8, 4 6 and 5 5, while no growth was observed at pH 7 0 Enzyme production at pH 4 6 and 5 5 was similar unlike in the free cell experiments where enzyme production was higher at pH 5 0 (see Figure 3 11) Based on these findings all shake flasks and fermenters were operated at

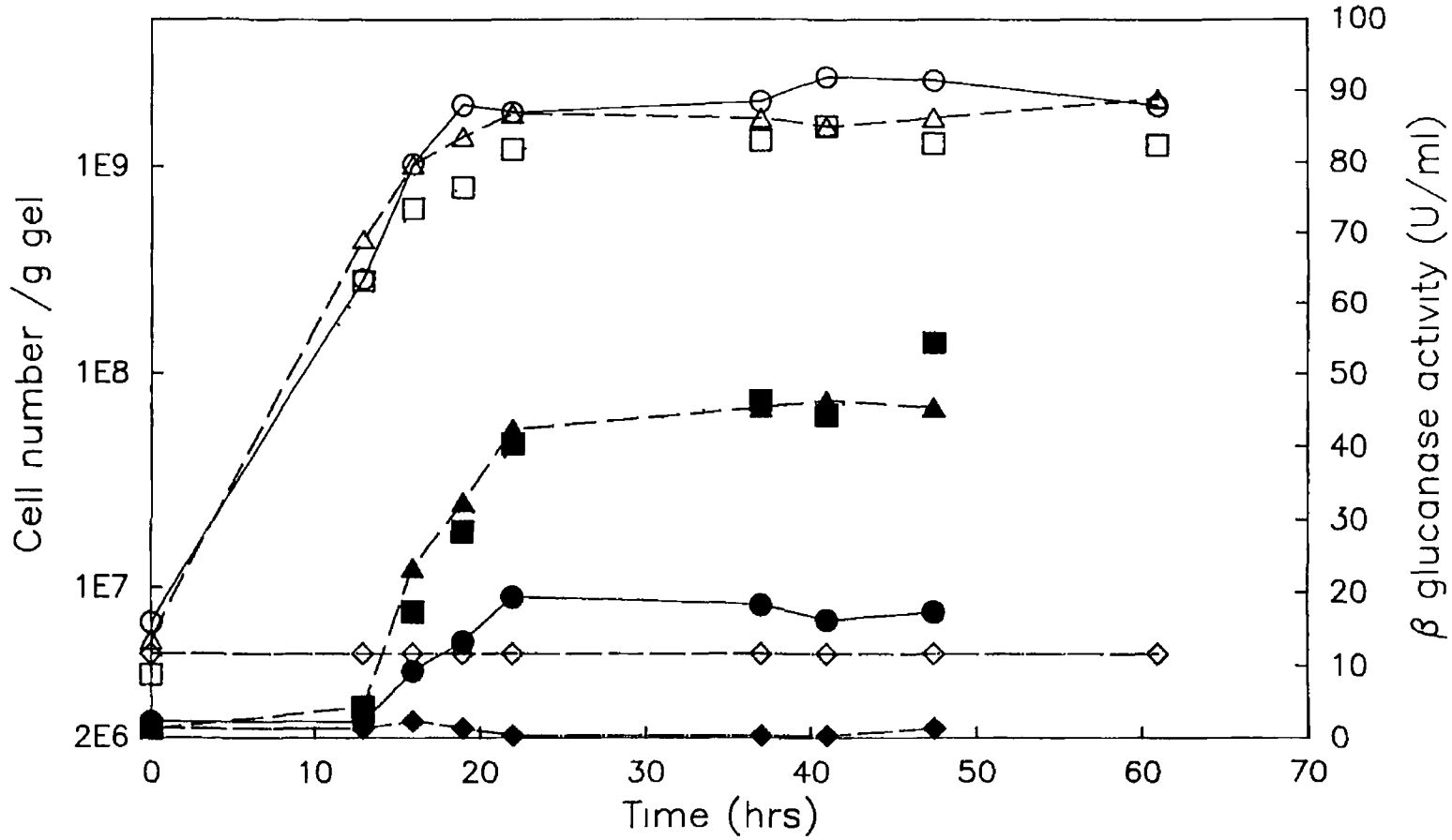

Figure 3 12 Cell growth and β glucanase production by immobilised pJG317 at various pH values

●—● pH 3 8, ▲--▲ pH4 6, ■ ■ pH 5 5, ◆--◆ pH 7 0

Closed symbols – enzyme activity, open symbols – cell concentration

pH 5 0 where maximal enzyme production occurs

Specific enzyme productivities were calculated for free and immobilised cells at pH 4 6 These are illustrated in Figure 3 13 Enzyme production per cell is presented during the exponential phase of growth where enzyme production occurs In the case of free cells the specific productivity is calculated by dividing the enzyme activity per ml by the cell number per ml at that time With the immobilised cells the enzyme activity per ml is divided by the cell number per gram of alginate and the result multiplied by 10 because the bead volume is only 10% of the medium volume The results for the immobilised cells are overestimated as they do not include enzyme production by cells that have been released into the medium Even with this overestimation, the enzyme production per cell in the free cell case is higher than that for the immobilised cells This would imply that the immobilised cells (or at least a fraction of them), are producing enzyme sub-optimally This result may suggest nutrient limitation of the immobilised cells since they cannot produce as much enzyme per cell as the free cells

3 2 8 Enzyme production using different size beads in selective medium

Figure 3 14 represents growth and enzyme production for immobilised cells using various bead sizes i e 5 mm, 3 mm and 1 mm diameter The growth pattern in all three systems is similar, as is enzyme production Immobilised cell numbers are presented only as cells per gram of alginate

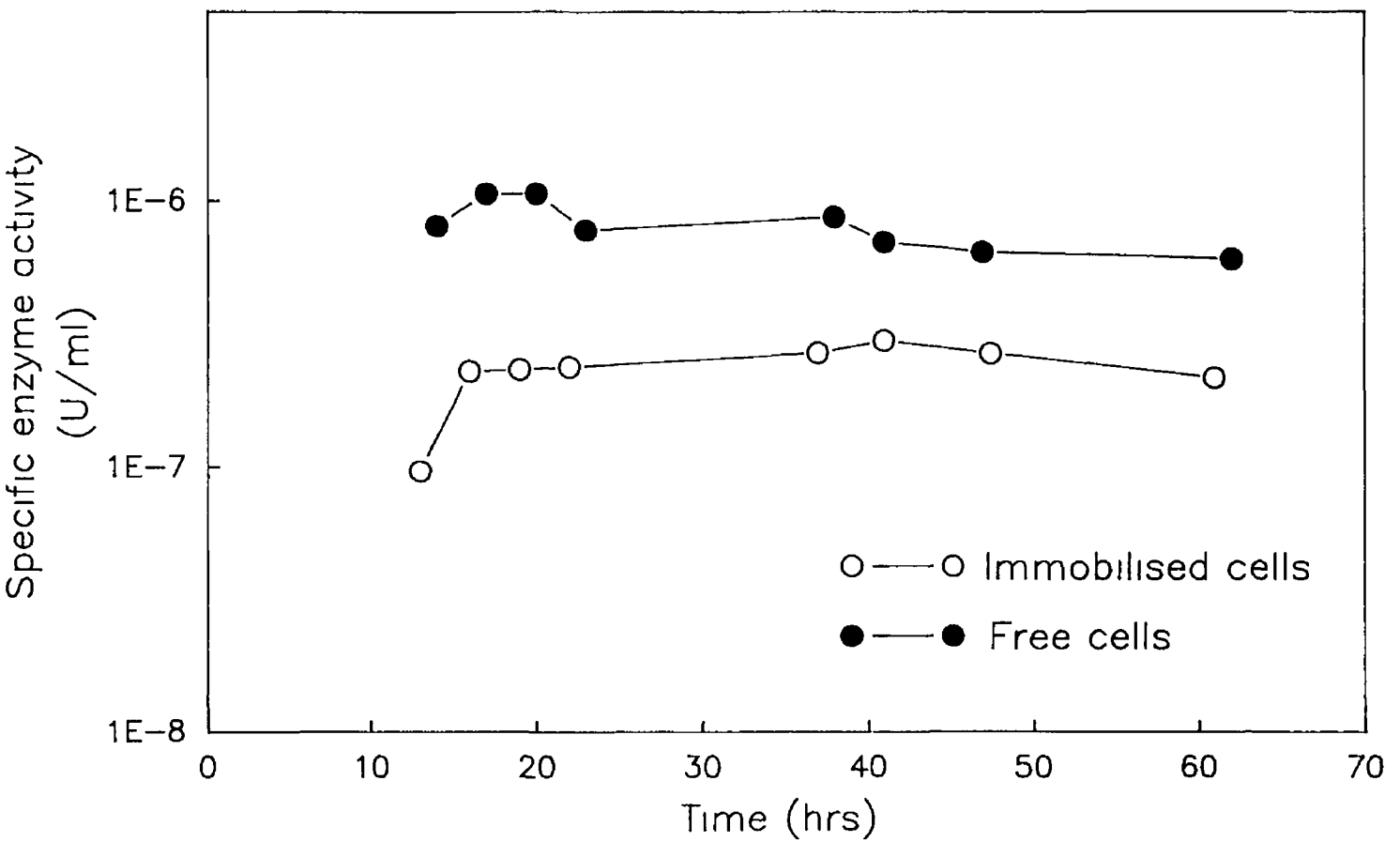

Figure 3 13 Plot of specific enzyme activities for free and immobilised pJG317 cells in batch culture

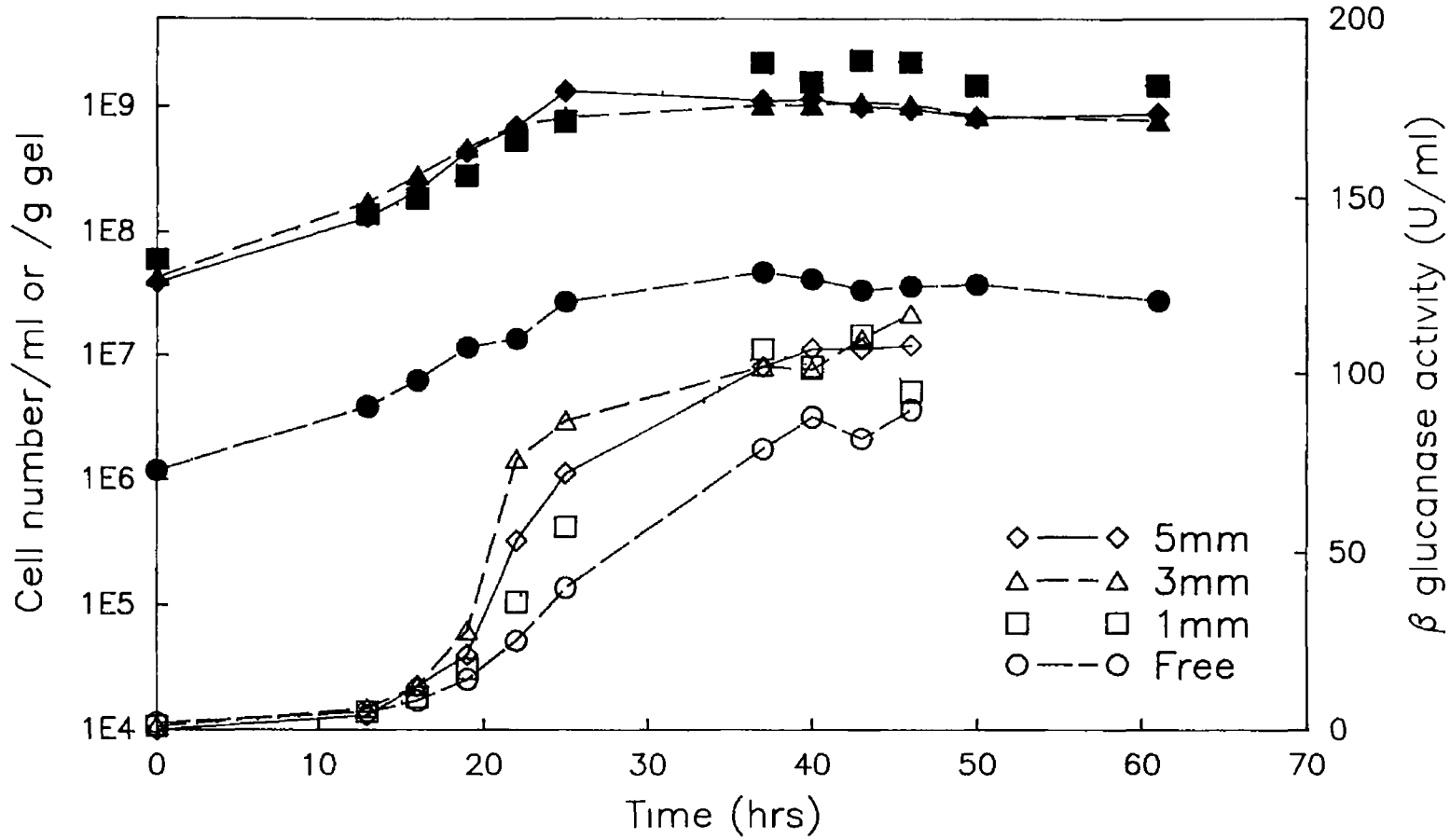

Figure 3 14 Cell growth and enzyme production by free and immobilised pJG317 cells Various bead sizes employed
Closed symbols – cell numbers, open symbols – enzyme activity

Free cell numbers initially were zero and then increased to 10^5 ml^{-1} for the larger beads and 10^6 ml^{-1} for the 1 mm beads Free cell enzyme production is also illustrated Improved production appears to occur in the immobilised systems However if specific production per cell is calculated, the free cell production per cell is highest Specific enzyme productivity results are presented in Figure 3 15

As indicated by the specific productivity results of Figure 3 13, enzyme production per immobilised cell is less than free cell specific production As gradients of growth are known to occur in the immobilisation matrix, it is therefore assumed that the enzyme production rate of cells in the inner core of the matrix is less than that of cells at the surface Even if the specific productivity of cells at the surface of the matrix equals that of free cells, the overall average productivity per cell is less than that for free cells, because of the lower enzyme production rate of cells at the bead centre

From the cell number data in Figure 3 14 it is important to note that the highest overall cell density was achieved in the case of the smallest beads (1 mm) This does not imply that higher cell densities were obtained at the bead surface of the smaller beads compared to the larger beads however The surface area per unit volume for the smaller beads is greater than that of the large beads, therefore there is a greater 'outer layer' volume for cells to occupy in the case of the smaller beads This means that for

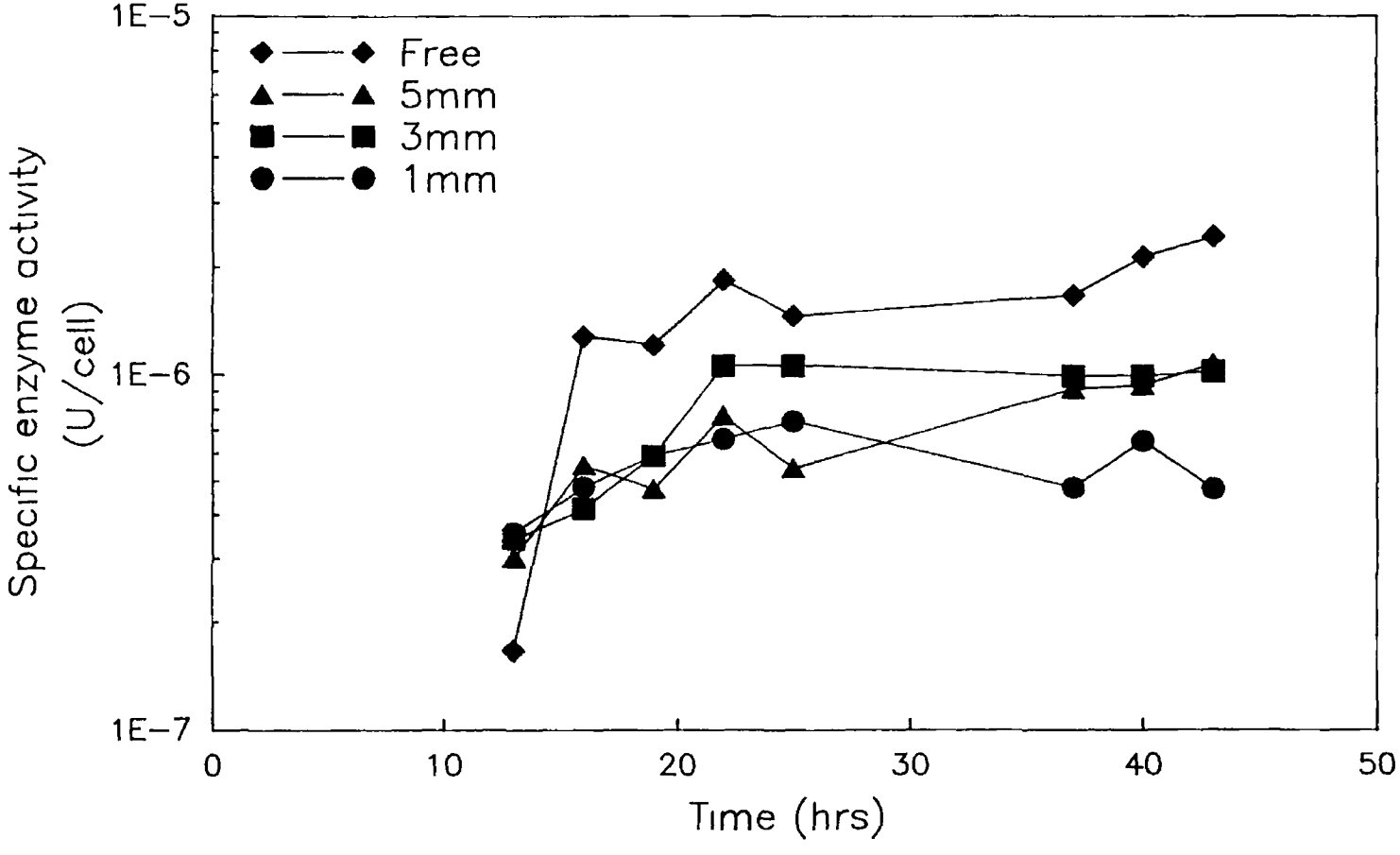

Figure 3 15 Plot of specific enzyme activities during batch growth of free and immobilised pJG317 cells Various bead sizes employed

smaller beads, a higher percentage of cells are growing maximally or almost maximally because more cells in the bead are proximal to the bead liquid interface

3 2 9 Enzyme production in selective and non-selective medium

In simple batch experiments cell growth and enzyme production were measured for cells in suspension Final enzyme concentrations in selective and non-selective media were 71 4 U ml^{-1} and 80 0 U ml^{-1} respectively These results are presented in Figure 3 16

In a batch system therefore it is evident that better enzyme production is possible using non-selective (rich) medium despite the fact that the organism may be unstable in this medium Cell numbers in YEPD typically increase to ten-fold the numbers attainable in m/m. This increase in cell number therefore must compensate for plasmid loss in batch culture

Specific enzyme production per cell however, is higher in the case of cells grown in selective medium, as opposed to non-selective medium (See Figure 3 17) This would indicate that the plasmid stability of pJG317 in YEPD decreases during batch growth which is understandable due to the absence of selection pressure Similar yeast plasmid instabilities were reported in rich non-selective medium by Impoolsup (1989)

In the batch system described above, final enzyme concentrations were higher for a rich non-selective medium than for a selective minimal medium This may not be the

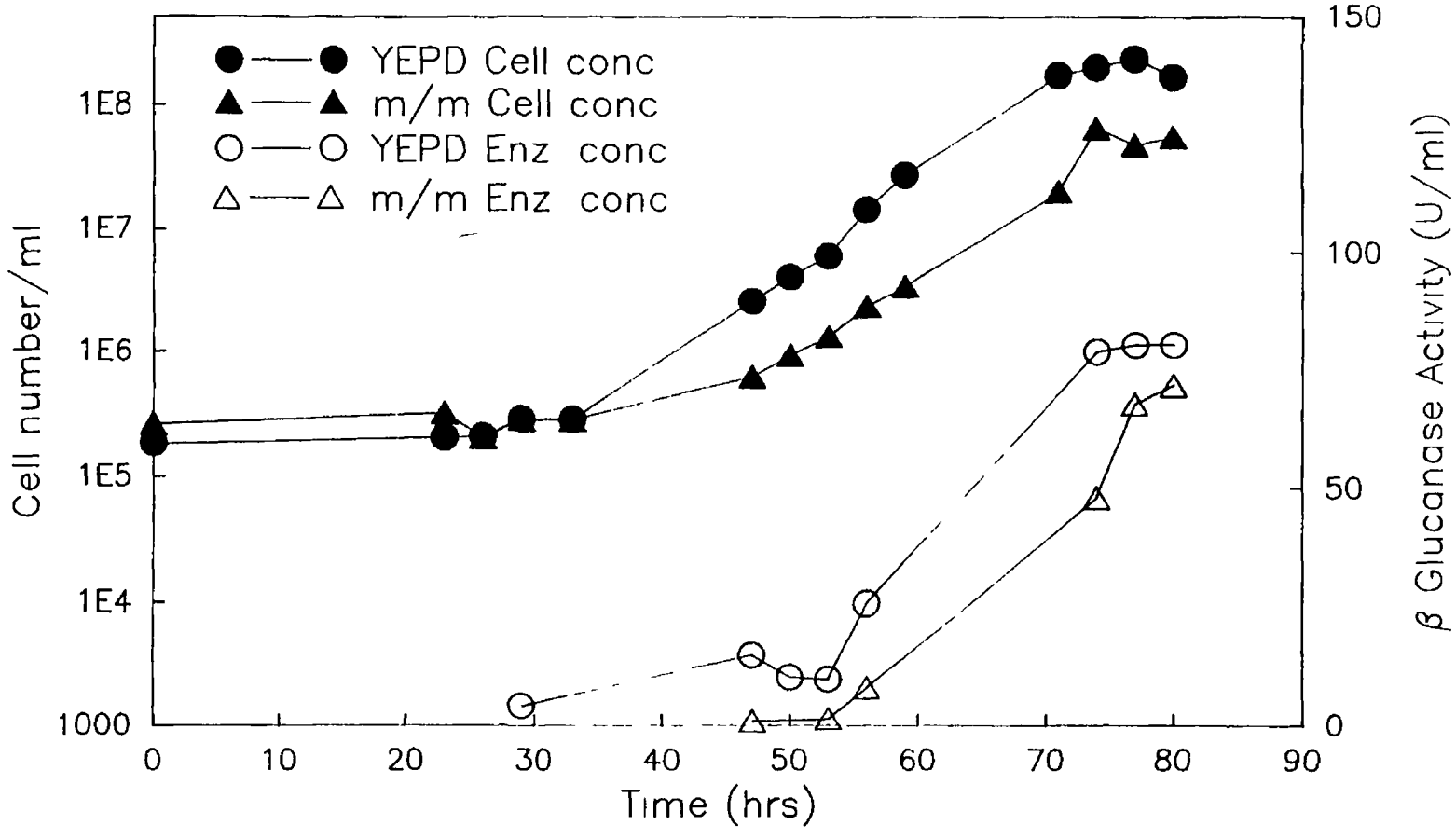

Figure 3 16 Free cell growth and enzyme production in selective and non-selective medium (Selective medium – m/m, non-selective medium – YEPD)

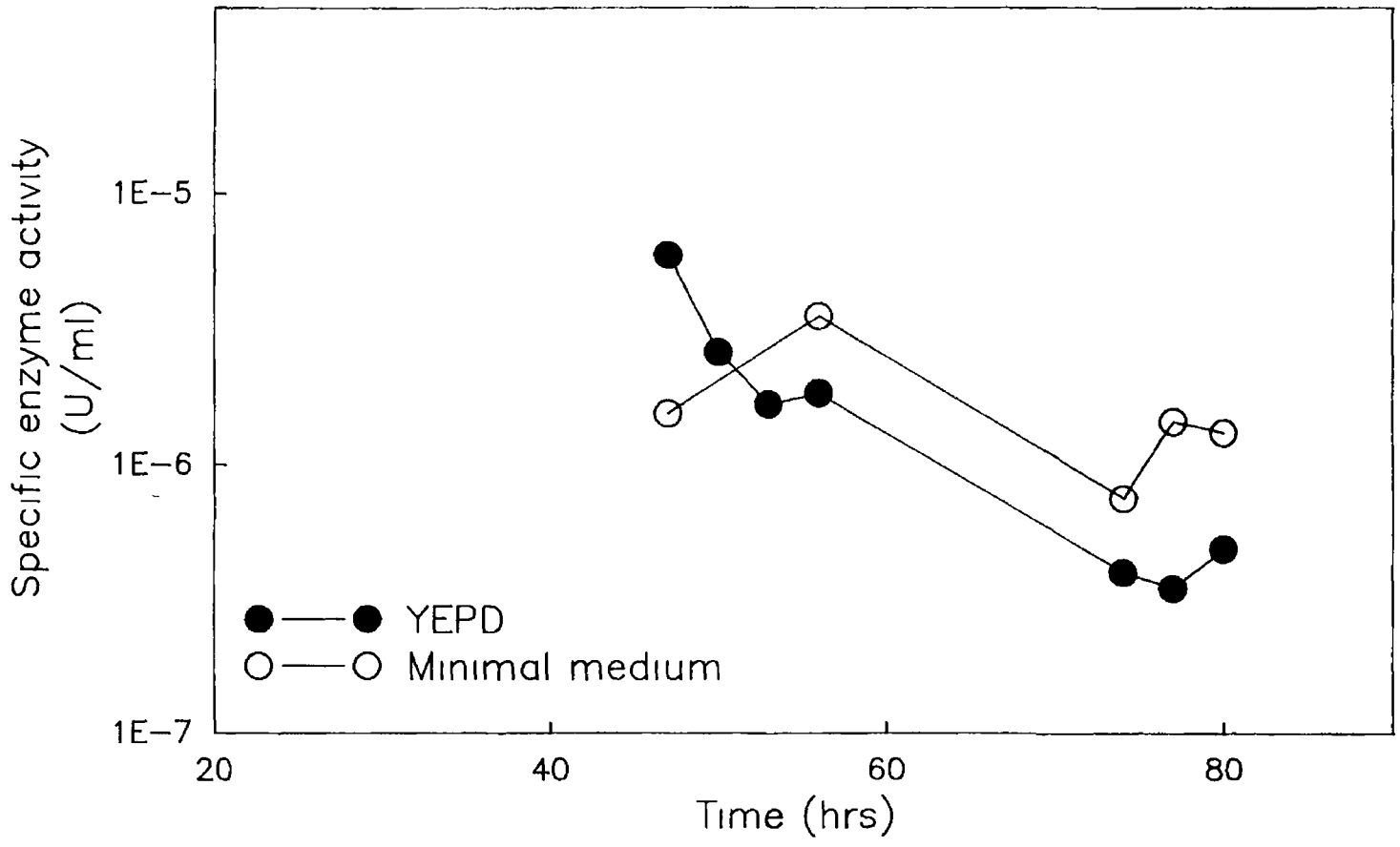

Figure 3 17 Plot of specific enzyme production during batch growth of pJG317 cells in YEPD and minimal medium

case, however, in a continuous reactor, where long term stability of the organism is essential in order to maintain steady enzyme concentrations in the product stream In developing a continuous reactor system for enzyme production using a recombinant strain, it is important to quantify plasmid loss rates for free and immobilised cells in non-selective medium

In order for a continuous reactor system to be successful the cells must remain in an actively growing state to ensure continuous enzyme production This is possible for free cells in a chemostat system, but with immobilised cells, it has been shown already that cell numbers reach a threshold level in the beads, with further growth causing continuous release of cells into the medium Therefore, it is not known if this compartmentalised nature of growth in the bead will maintain continuous enzyme production

One of the advantages of immobilised cell systems is their application to continuous systems or their re-use in repeated batch systems as mentioned earlier It is essential, therefore, to characterise the behaviour of immobilised recombinant cells in continuous and repeated batch experiments

3 3 REPEATED BATCH AND CONTINUOUS CULTURE RESULTS

3 3 1 Plasmid stability of free and immobilised cells in non-selective medium

As discussed in Section 1 5, the plasmid stability of recombinant cells decreases if they are grown in the

absence of a selection pressure In order to insure good plasmid maintenance, a selection pressure is usually required The use of selective medium on a large scale may not be economically viable however, so it is therefore important to characterise and quantify plasmid loss by recombinant cells in non-selective medium

3 3 1 1 Free cell plasmid loss in chemostat

A chemostat was set up in the 2 L Life Sciences Bioreactor, with a working volume of 1 3 L, to measure plasmid loss by pJG317 in non-selective medium (YEPD) The pH was not controlled but remained between 4 8 and 5 2 Plasmid stability was measured by replicating 100 colonies per sample Initially, a batch culture was set up and after 24 hours, a continuous feed was started at a dilution rate of 0 106 h^{-1} A logarithmic plot of plasmid stability versus generation number is presented in Figure 3 18 Generation number was calculated based on cell numbers for initial batch growth and based on the dilution rate for the continuous fermentation results Cell numbers remained relatively constant during the continuous run Plasmid loss in non-selective medium is significant with a reduction in the percentage of cells retaining the plasmid from 68% to 24% in 96 hours (approximately 18 generations)

In the case of batch fermentations, the number of yeast cell doublings is limited by the initial supply of nutrients Therefore the amount of plasmid loss during batch growth may not be too dramatic Earlier results indicate better enzyme production in YEPD compared to m/m

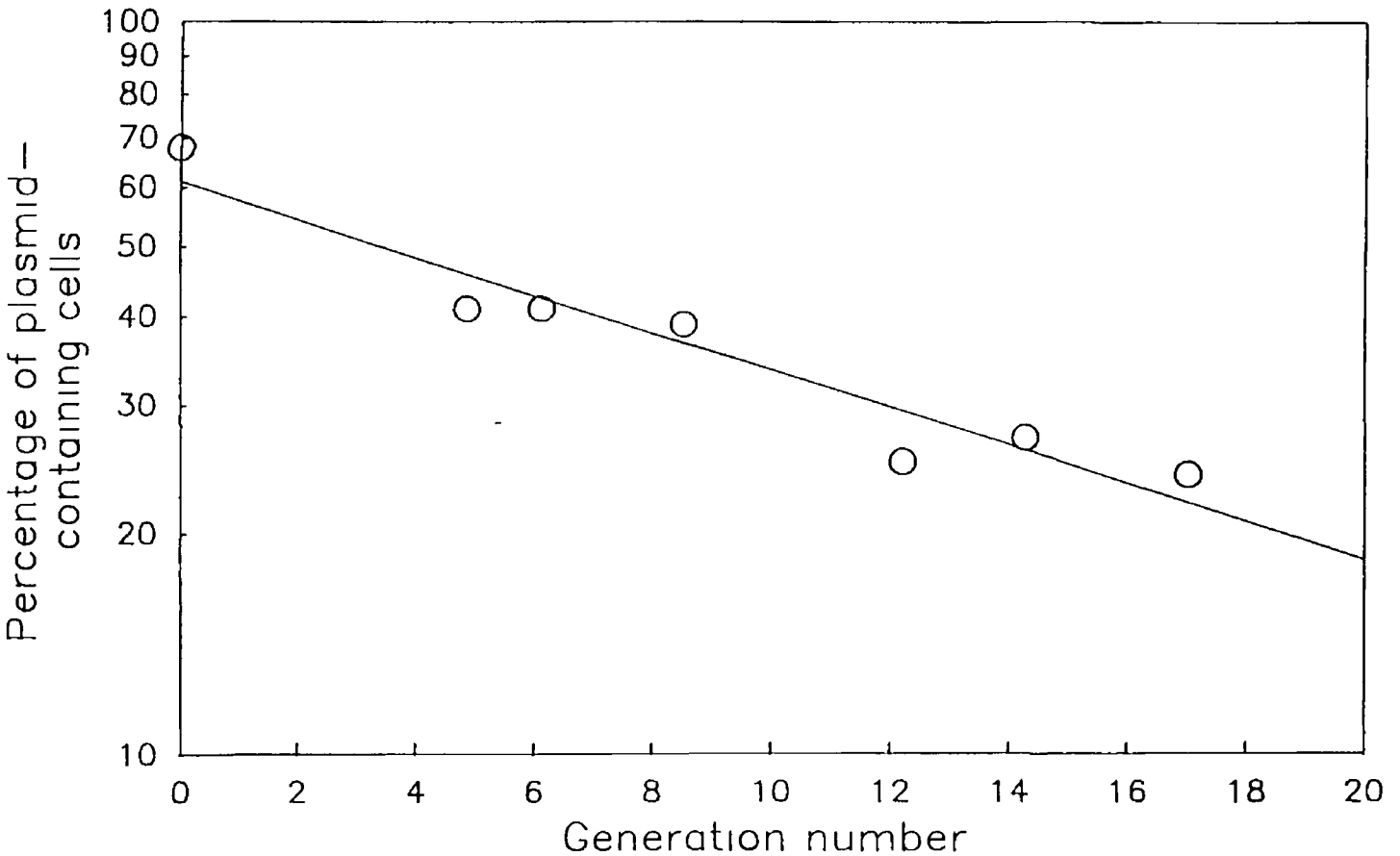

Figure 3 18 Percentage of plasmid-containing pJG317 cells vs generation number in a chemostat with a dilution rate D = 0 106 hr^{-1}

because of increased cell numbers despite assumed plasmid loss

In continuous culture of free cells in YEPD it appears unlikely that the reactor will produce enzyme without decline based on the plasmid stability results of Figure 3 18

3 3 1 2 Free cell plasmid stability and enzyme production in repeated transfer fermentations using non-selective medium

Knowing now that the plasmid PJG317 is unstable in the yeast strain DBY746, it is important to monitor enzyme production in tandem with plasmid stability A drop in plasmid stability may not directly infer a drop in expression of the enzyme Expression can relate to many parameters as discussed previously in Section 1 5

In order to study enzyme production and plasmid stability over a prolonged period of growth, a repeated transfer experiment was set up as described in Section 2 3 4 1 Plasmid stability was measured by replicating 200 colonies onto YEPD and m/m

The results of this experiment are presented in Figure 3 19 Enzyme concentrations, as well as the number of plasmid containing cells at the end of each batch are presented There is a direct relationship between the numbers of plasmid containing cells and the final enzyme concentration The total cell numbers at the end of each batch were similar and so enzyme production is directly proportional to the plasmid stability of the population This reconfirms the idea that production of β-glucanase

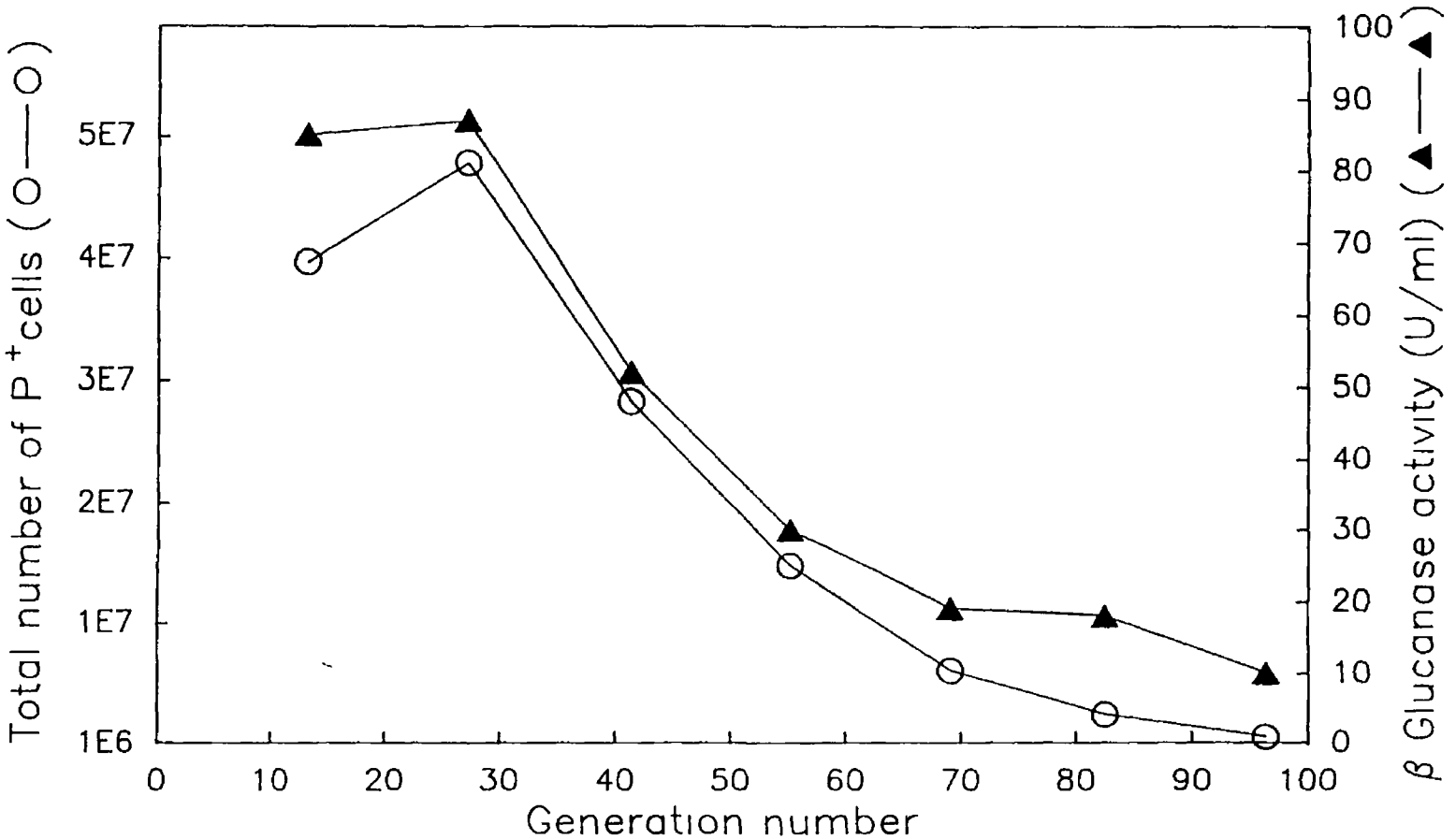

Figure 3 19 Plot of number of plasmid—containing cells and enzyme activity during growth of pJG317 cells in a free cell repeated batch experiment using non—selective medium

under non-selective conditions is not feasible in a continuous free cell system

3 3 1 3 Immobilised cell plasmid stability and enzyme production in repeated transfer fermentations using non-selective medium

A repeated batch system was used to study plasmid loss and enzyme production in the case of immobilised cells Plasmid stability was measured by replicating 100 colonies onto selective and non-selective medium The results of this experiment may not be directly comparable to the free cell equivalent for a number of reasons

All alginate beads were transferred to a fresh flask at the end of each batch Each day therefore, a flask was inoculated with a large cell number The number of generations that the immobilised cells can go through before the glucose substrate is utilised, in any one batch culture, is less than the the number of generations in the free cell repeated transfer experiment

This problem with the immobilised system is also compounded by the appearance of released cells in each batch Free cell numbers were substantial at the end of each batch run, increasing from 10^7 ml^{-1}after 24 hours to 10^8 ml^{-1}after 71 hours Before the beads were transferred to a new flask they were washed twice in sterile 0 01% peptone solution to wash off the free cells These released cells replicate in the medium, therefore limiting even more the number of generations that the immobilised cells can go through before the substrate is utilised Therefore in the free cell system, the cells analysed at

any time have gone through a greater number of generations than the immobilised cells

As described in Section 3 2 4, there is a gradient of growth from the inside to the outside of the beads Generation number calculations therefore, cannot describe the total cell population, as there is a gradient of generation number from high at the bead surface to low at the bead core Plasmid stability and enzyme production are therefore represented against time rather than generation number

Cell number and enzyme activity results are presented in Figures 3 20 and 3 21 for 5 mm beads of 2% and 3% alginate respectively Enzyme production does not appear to decrease with time As mentioned previously the results cannot be directly compared to a free cell system, but the result is promising in terms of continuous enzyme production using immobilised pJG317

Free cell numbers in this experiment increased over 70 hours and level out at 10^8 cells ml^{-1} Enzyme concentration at the end of each batch run appears to follow a similar trend The beads seem to act as a source of free cells to inoculate the medium of each batch

Plasmid stability was measured for the released cells and the cells within the bead It was decided to look at plasmid stability in the beads more closely than previously reported in the literature Previous reports on plasmid stability of immobilised cells compared free-cell plasmid stability to the average plasmid stability of cells in the

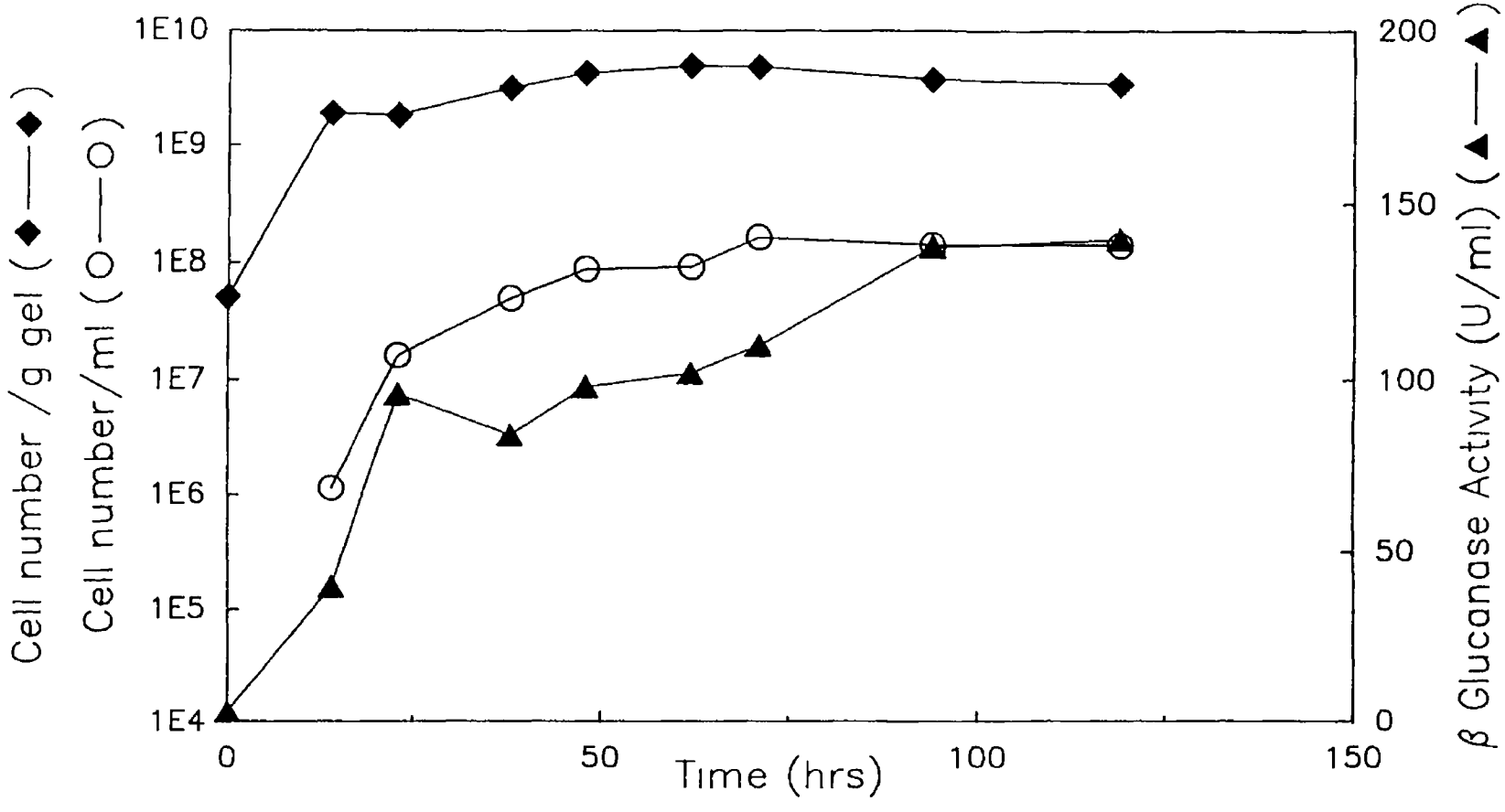

Figure 3 20 Plot of enzyme activity, immobilised and free cell numbers during growth of pJG317 cells in a repeated batch experiment (2% alginate beads)

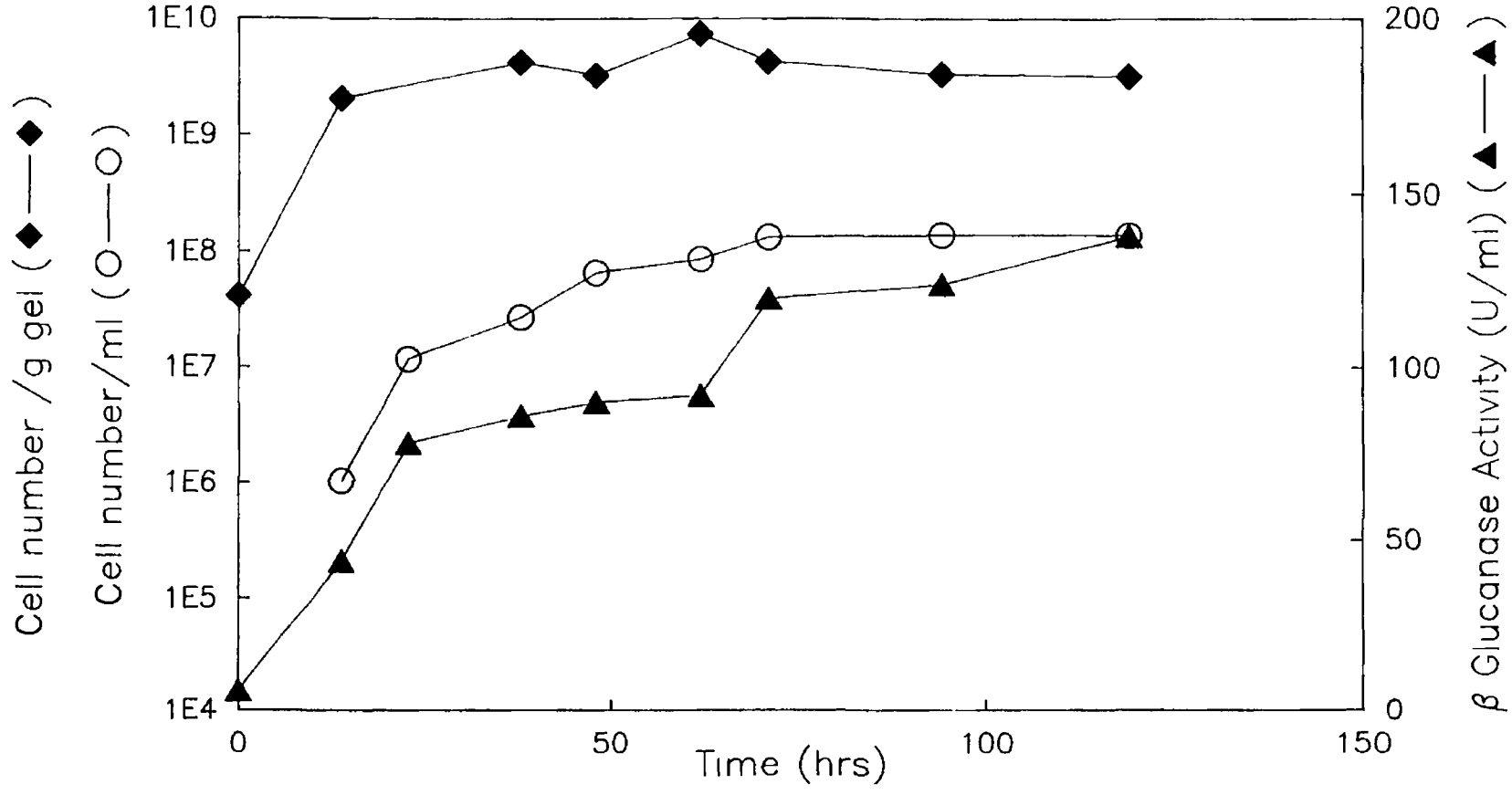

Figure 3 21 Plot of enzyme activity, immobilised and free cell cell numbers during growth of pJG317 cells in a repeated batch experiment (3% alginate beads)

immobilisation matrix (Oriel, 1988, Sayadi, 1989, Marin-Iniesta, 1988, Nasri, 1987, Berry, 1988) In order to gain a better insight into plasmid stability of cells within the matrix, measurements were made for cells in the outer sections of beads and in the bead core Two methods were utilised as described in Section 2 4 9 The results for these methods are presented in Figures 3 22(a), 3 22(b), 3 22(c), and 3 22(d)

As there is a large amount of scatter in the data, linear regression was performed The cells at the centre of the beads seem to retain the plasmid more stably than cells elsewhere in the matrix The result would perhaps be clearer if more than 100 replicas were chosen for plasmid stability measurement

The method of selective dissolution is less accurate than the dissection method (See Section 2 4 7) This is because during fermentation the beads soften due to the action of certain components in the medium, and also due to vessel agitation and growth of yeast throughout the matrix As they soften, more cells are released on the first dissolving step The technique is therefore not reproducible from sample to sample With later studies, the dissection method only is used for plasmid stability measurement of bead sections

All free cells in the medium are directly leaked from the bead surface or are the daughters of same The plasmid stability of the immobilised cells is higher than in a free cell system After each transfer therefore, cells are at

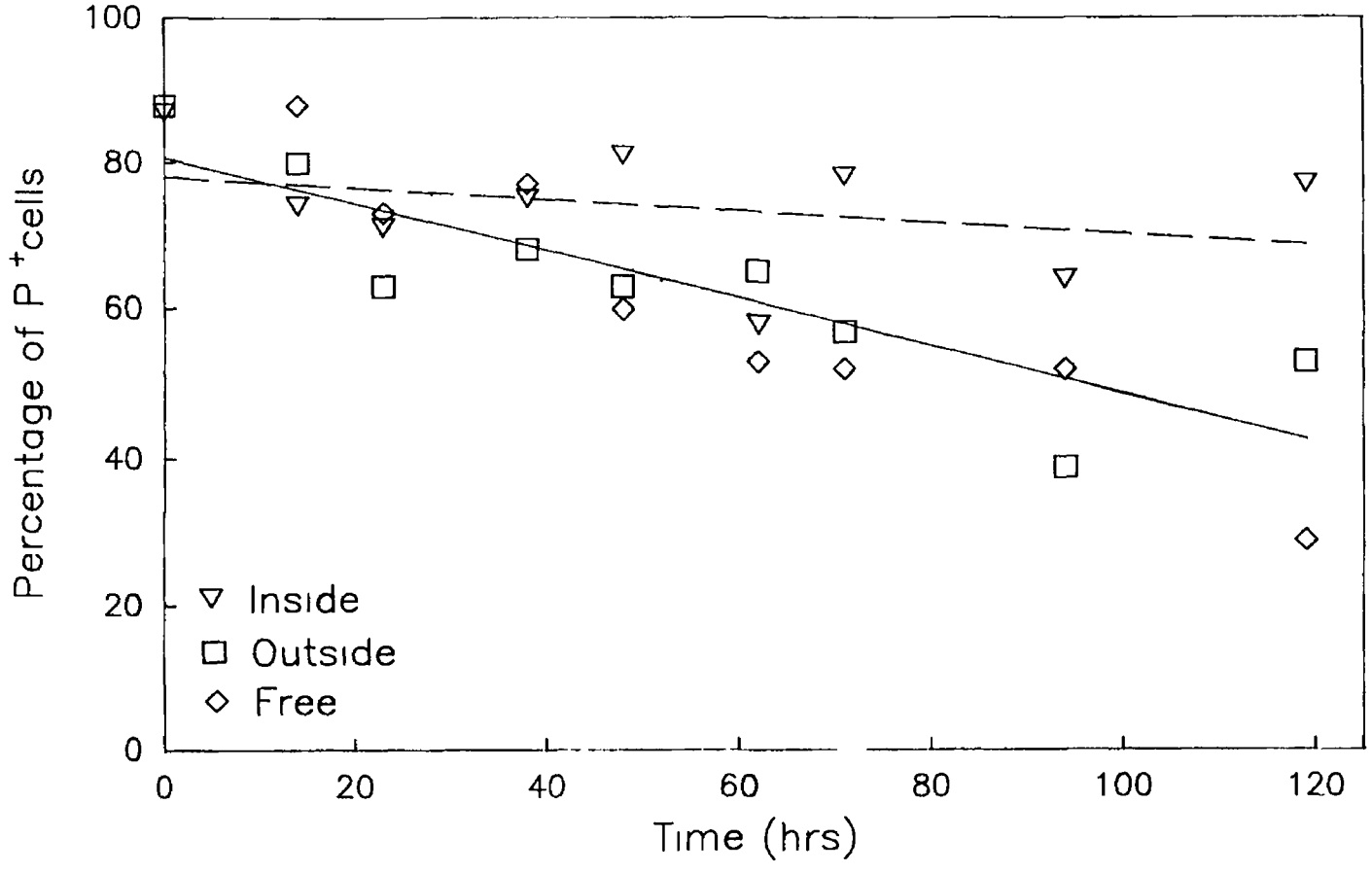

Figure 3 22 (a) Plasmid stability of bead sections in a repeated batch immobilised system 2% Alginate Dissection method 100 replicas

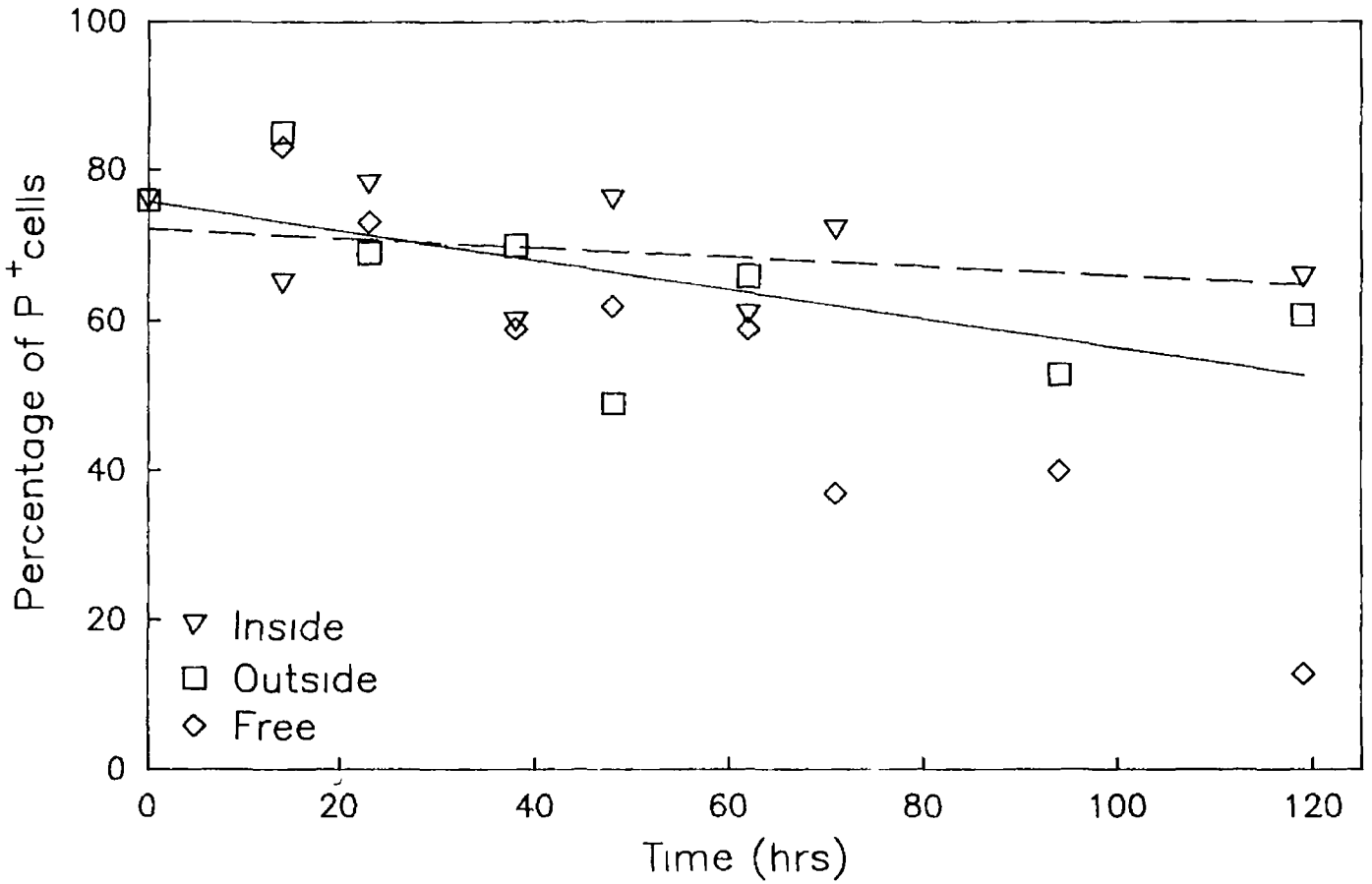

Figure 3 22 (b) Plasmid stability of bead sections in a repeated batch immobilised system 3% Alginate Dissection method 100 replicas

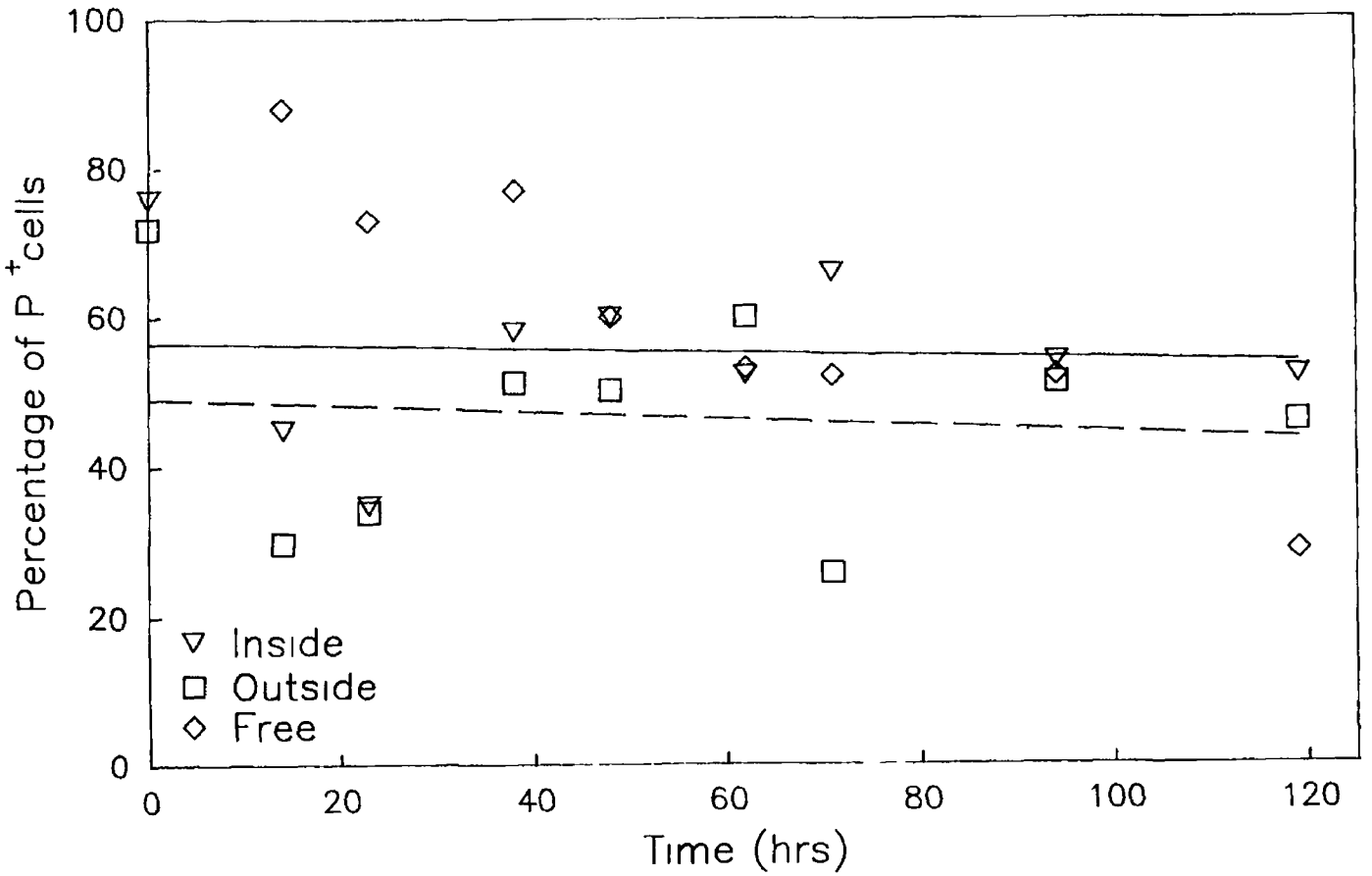

Figure 3 22 (c) Plasmid stability of bead sections in a repeated batch immobilised system 2% Alginate Dissolving method 100 replicas

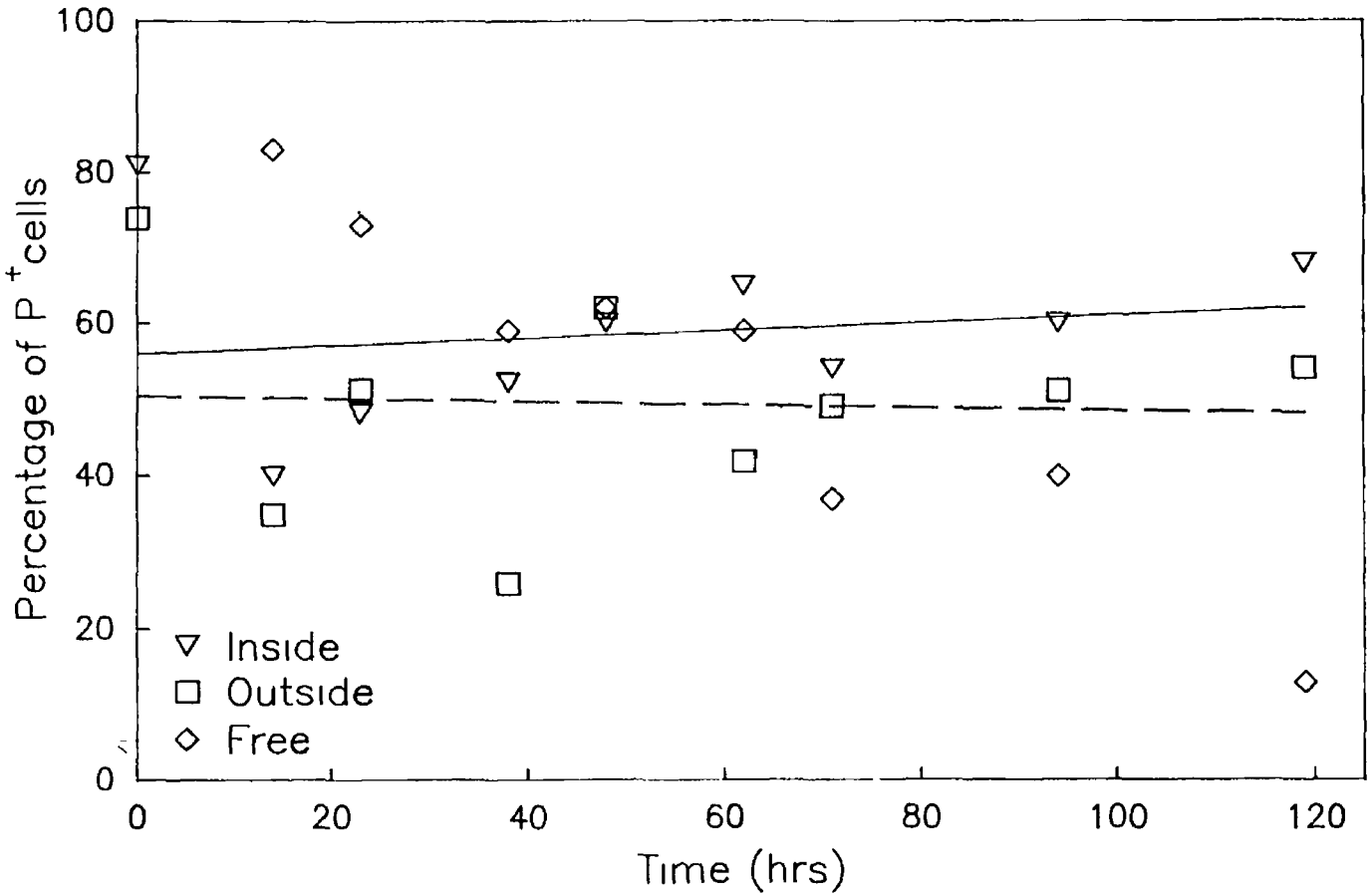

Figure 3 22 (d) Plasmid stability of bead sections in a repeated batch immobilised system 3% Alginate Dissolving method 100 replicas

the matrix surface grow and divide, releasing cells into the medium A higher proportion of these cells are plasmid-containing cells compared to free cells after a similar period of growth Cell leakage occurs to a substantial degree when immobilised cells are grown using a rich medium In batch and repeated batch studies, the leaked cells replicate in the medium and there is a free and immobilised fermentation occurring at the same time which hinders characterisation of the immobilised system It was hoped therefore, to gain a better insight into the behaviour of immobilised recombinant cells using continuous reactors under non-selective conditions

3 3 2 Immobilised cell continuous reactor experiments in non-selective medium

3 3 2 1 Immobilised CSTR at a dilution rate of 0 36 and 0 6 h^{-1}

In studying the growth and enzyme production characteristics of immobilised cells, only a certain amount of knowledge can be gained from batch experiments, because of the appearance of free cells, as discussed in Section 3 2 2 Therefore, in continuous reactor studies using non-selective medium, a more accurate picture of plasmid stability and cell leakage is possible From a batch experiment in the 2L fermenter a specific growth rate of 0 25 h^{-1} was calculated from an optical density trace at 600 nm (results not included) Based on this, a continuous reactor was set up in the 2L fermenter with a working volume of 500 ml and a dilution rate of 0 36 h^{-1} , to minimise free cell numbers in the reactor

The fermenter was inoculated with 50 ml of 2% alginate beads at a cell density of 2 x 10^8 cells g^{-1} The bead diameter was 3 mm and plasmid stability was measured by replicating 100 colonies of samples onto selective and non-selective media Continuous feed commenced at 28 5 hours, and from this time on, the glucose concentration remained below 1 5 g/L Figure 3 23 represents both free and immobilised cell counts and enzyme activity Enzyme production for 116 hours is relatively constant and shows little sign of decline This result shows continuous enzyme production by an unstable recombinant micro-organism in non-selective medium This was not possible using pJG317 in free cell culture (See Figure 3 19)

Plasmid stability of bead cross-sections shows improved plasmid maintenance in the centre of the matrix as opposed to the bead surface The results fluctuate initially but the underlying trend shows an improved plasmid stability in the bead centre (see Figure 3 24) Released cells have the lowest plasmid stability Free cell numbers increased to the order of 10^8 /ml in 44 hours The dilution rate was 1 5 times the specific growth rate calculated from batch fermentation data The low plasmid stability of the released cells may be because of free cell replication in the fermenter due to the low dilution rate which may lead to increased plasmid loss

A similar continuous fermentation run was set up at a dilution rate of 0 6 h^{-1} to try and reduce the number of free cells present This dilution rate was 2 4 times the

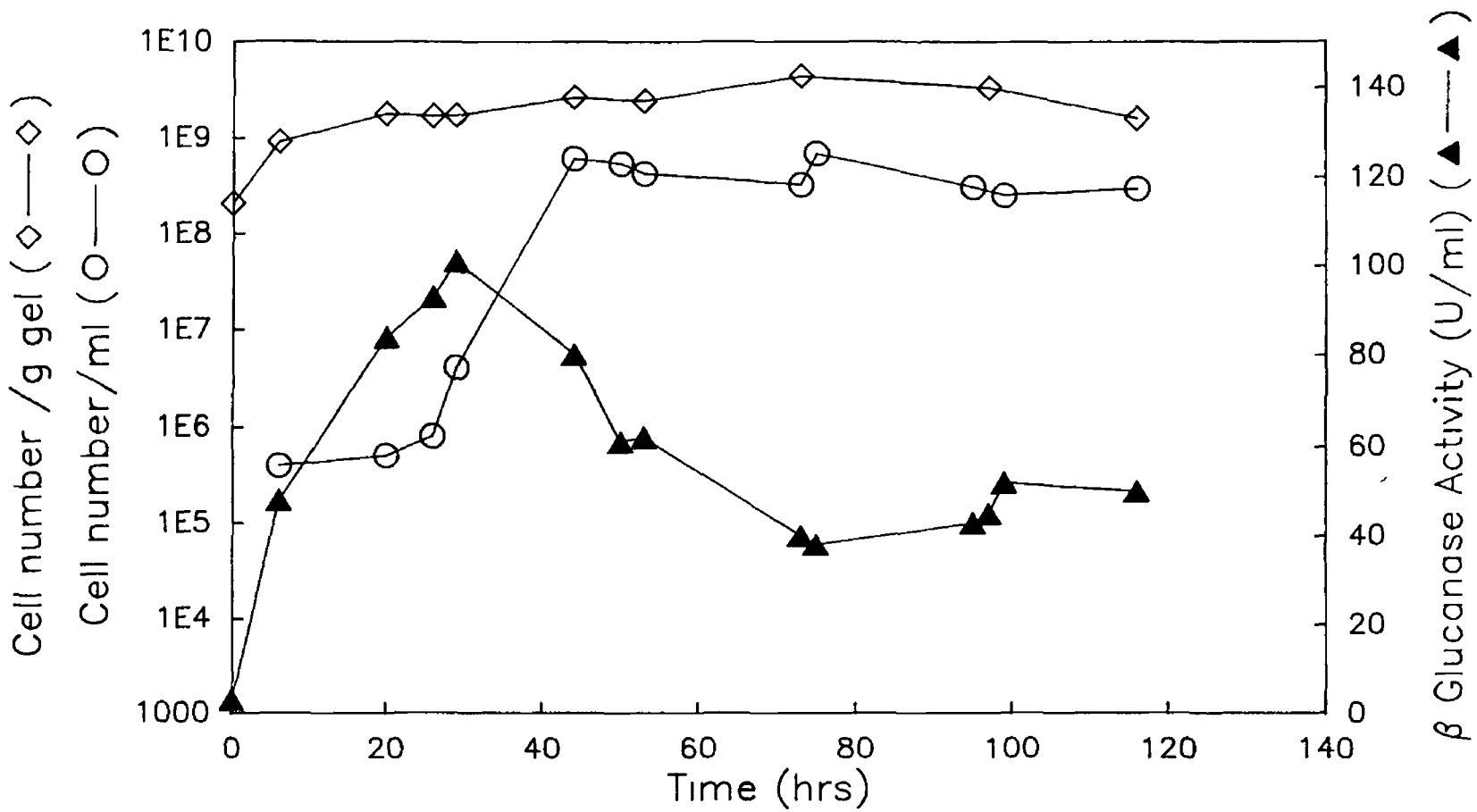

Figure 3 23 Plot of free and immobilised cell numbers and enzyme concentration for a continuous immobilised reactor D = 0 36 hr^{-1}

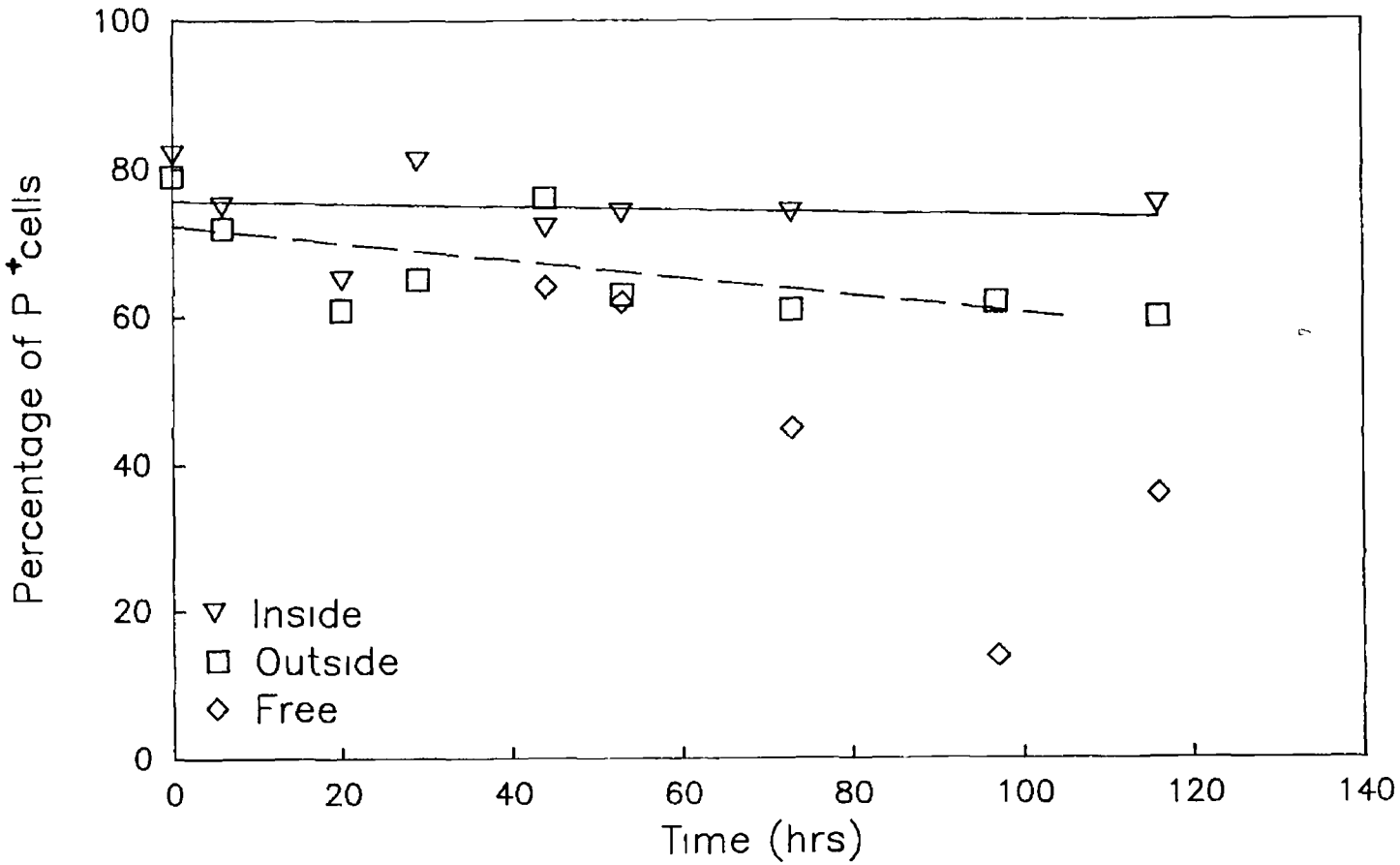

Figure 3 24 Plasmid stability of bead sections in a continuous immobilised cell reactor D = 0 36 hr $^{-1}$.

specific growth rate calculated from batch data This reactor ran for 197 hours At the end of the run the feed was stopped and the fermentation was allowed to continue in a batch manner until all the glucose present was depleted During continuous feeding the glucose concentration remained below 5 g/L The glucose concentration in the feed medium was 20 g/L This final batch produced almost the same concentration of enzyme as the first batch, before the onset of continuous feed It should be noted, however, that at the onset of this batch growth phase, there was approximately 5 g/L glucose present and an enzyme concentration of 30 U ml^{-1}

Enzyme production during continuous feeding was relatively constant and showed little sign of decrease with time Enzyme production and cell numbers are illustrated in Figure 3 25

Plasmid stability of bead sections and released cells are presented in Figure 3 26 From these continuous experiments, it seems that the plasmid maintenance of recombinant yeast is improved when the cells are immobilised Cells in the centre of the beads appear to retain the plasmid more effectively than the faster growing cells at the bead surface This improved plasmid stability may be because of the changed micro-environment of the matrix or perhaps because of the different growth rates from the bead surface to the centre of the beads

All plasmid stability results of bead sections so far have indicated that the centre of the beads contain cells

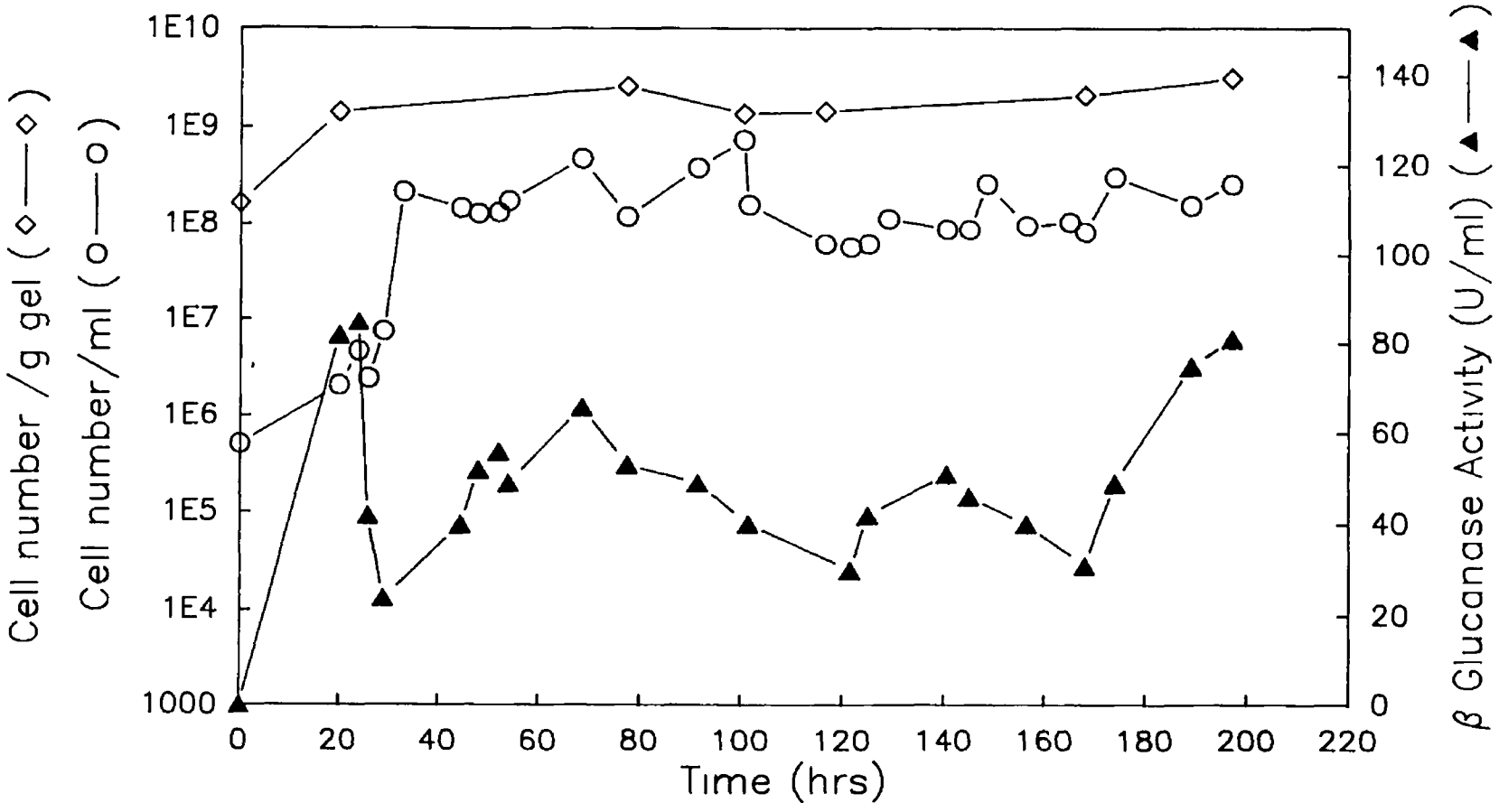

Figure 3 25 Plot of free and immobilised cell numbers and enzyme concentration for a continuous immobilised reactor. D = 0 6 hr^{-1}

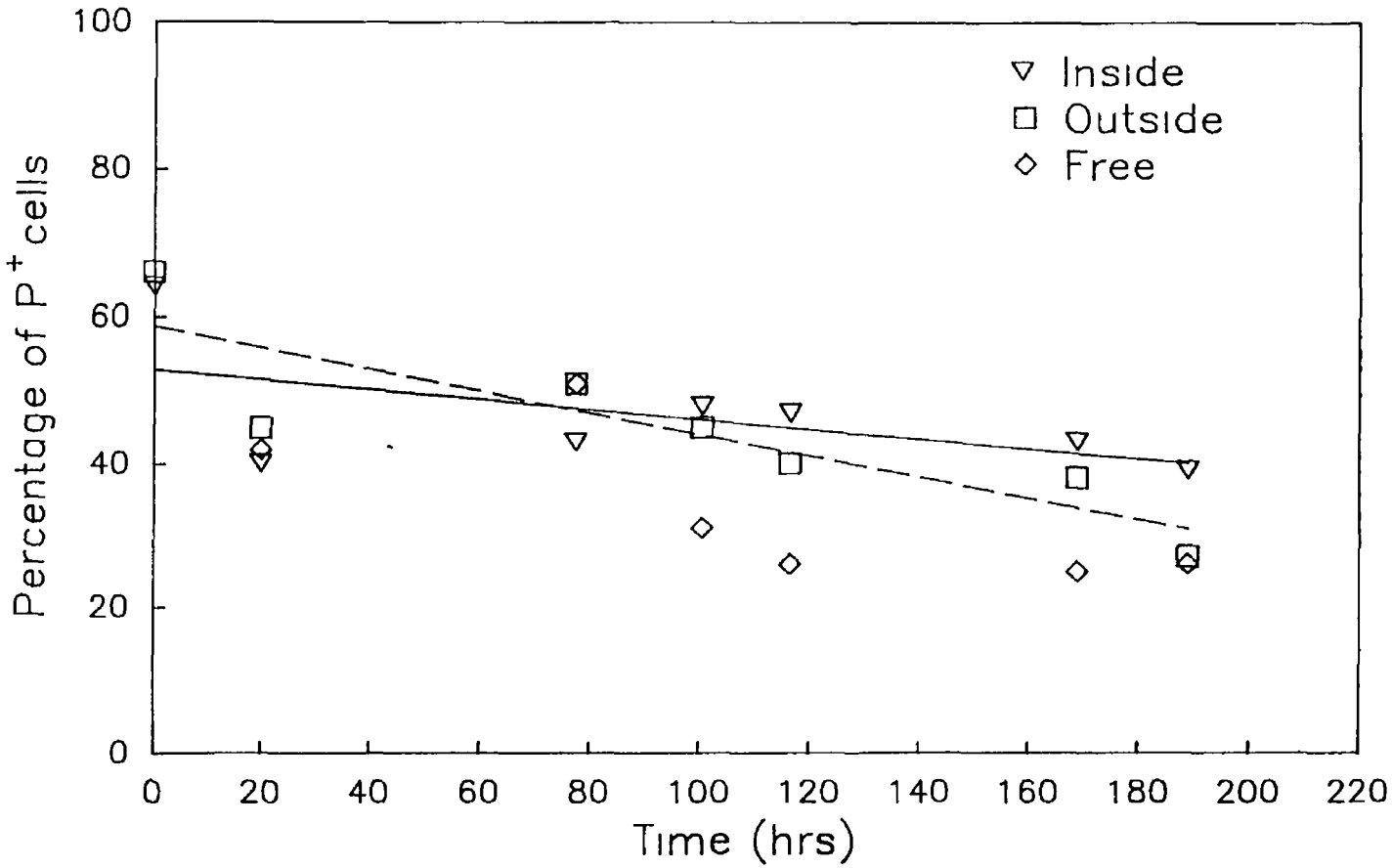

Figure 3 26 Plasmid stability of bead sections in a continuous immobilised cell reactor D = 0 6 hr^{-1}

of higher plasmid stability than the outer surface of the beads or the free cell population Whereas it is not known whether the gel environment has an effect on plasmid stability the beads certainly act as a reservoir of plasmid-containing cells

During this experiment, average bead weight was monitored to try and account for shedding of cells and alginate from the beads during long-term fermentations It was found that there was a dramatic decrease in bead weight and overall size with prolonged fermentation Figure 3 27 shows the average bead weight for 3 mm beads in a continuous fermentation at a dilution rate of 0 6 h^{-1} in a stirred vessel As a comparison the average weight of 5 mm beads of 2% and 3% alginate in repeated batch shake flasks is illustrated

Initially bead weight increases The alginate beads swell in the medium This was noted in all immobilised cell experiments It is not clear whether this is because of cell growth, media effects on the alginate or water uptake by the matrix After this expanding phase a decrease in weight is observed The decrease in the stirred vessel (400 rpm) is quite dramatic with a reduction to 27% of initial bead weight Even where agitation is mild i e in shake flasks, there is a decrease in bead weight

The bead weight loss in the stirred reactor is due to shearing by the impeller and because of disruption of the gel surface due to abundant cell growth The loss in the shake flasks is due to cell growth and also attrition due

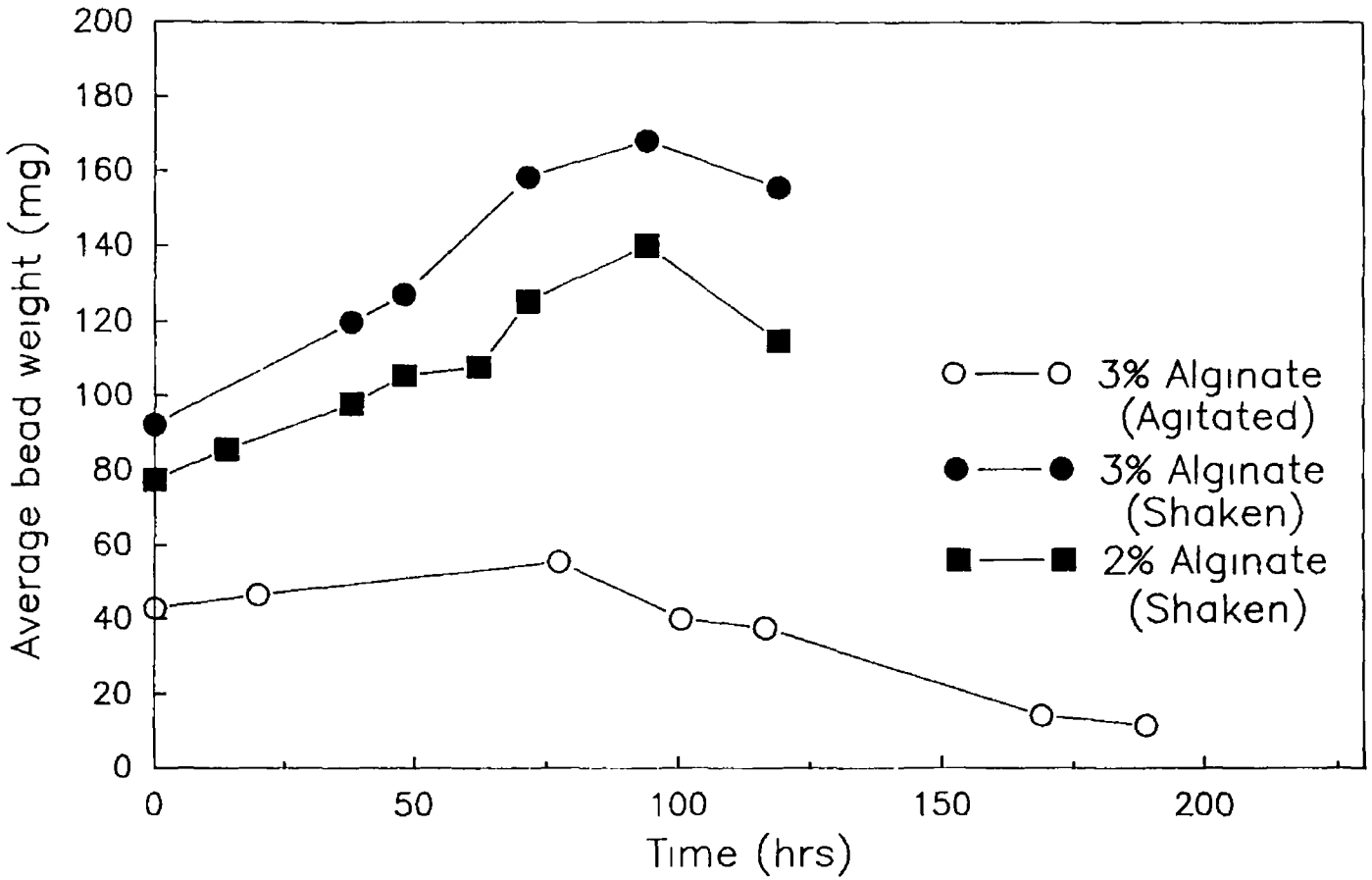

Figure 3 27 Average bead weight of an alginate bead vs time during fermentation of mechanically agitated and shaken immobilised cultures

to bead-bead and bead-vessel contact

With this continuous run there is a substantial presence of free cells and as the fermentation proceeds the relative amount of immobilised cells to free cells decreases Based on bead weight data (Figure 3 27) and free and immobilised cell numbers for this continuous run, the percentage of total cells in the reactor which are immobilised is plotted in Figure 3 28 Immobilised cell numbers drop to as low as 13% of total cell numbers This emphasises the need to run the immobilised reactor in such a way as to eliminate the presence of free cells to a considerable degree

It is still unclear as to whether the immobilised cells have the greater contribution to the stable undiminishing enzyme production or whether this is attributable to the released cells, which may have been altered by the micro-environment of the matrix

3 3 2 2 Washout chemostat to measure μ_{max}

It is desirable to run a continuous immobilised reactor system without the presence of free cells or with low levels of free cells In such a system, cell leakage can be quantified and a more accurate picture of plasmid stability in the matrix can be established A continuous reactor method was used to calculate μ_{max} for DBY746 containing pJG317 (Pirt, 1975) (Calculations in Appendix A) Using this value for μ_{max} , it was proposed to run a continuous immobilised reactor at a dilution rate of 1 5 μ_{max}

The aeration rate of this washout chemostat was 2 v/v/min Continuous feed of YEPD commenced at 22 5 hours

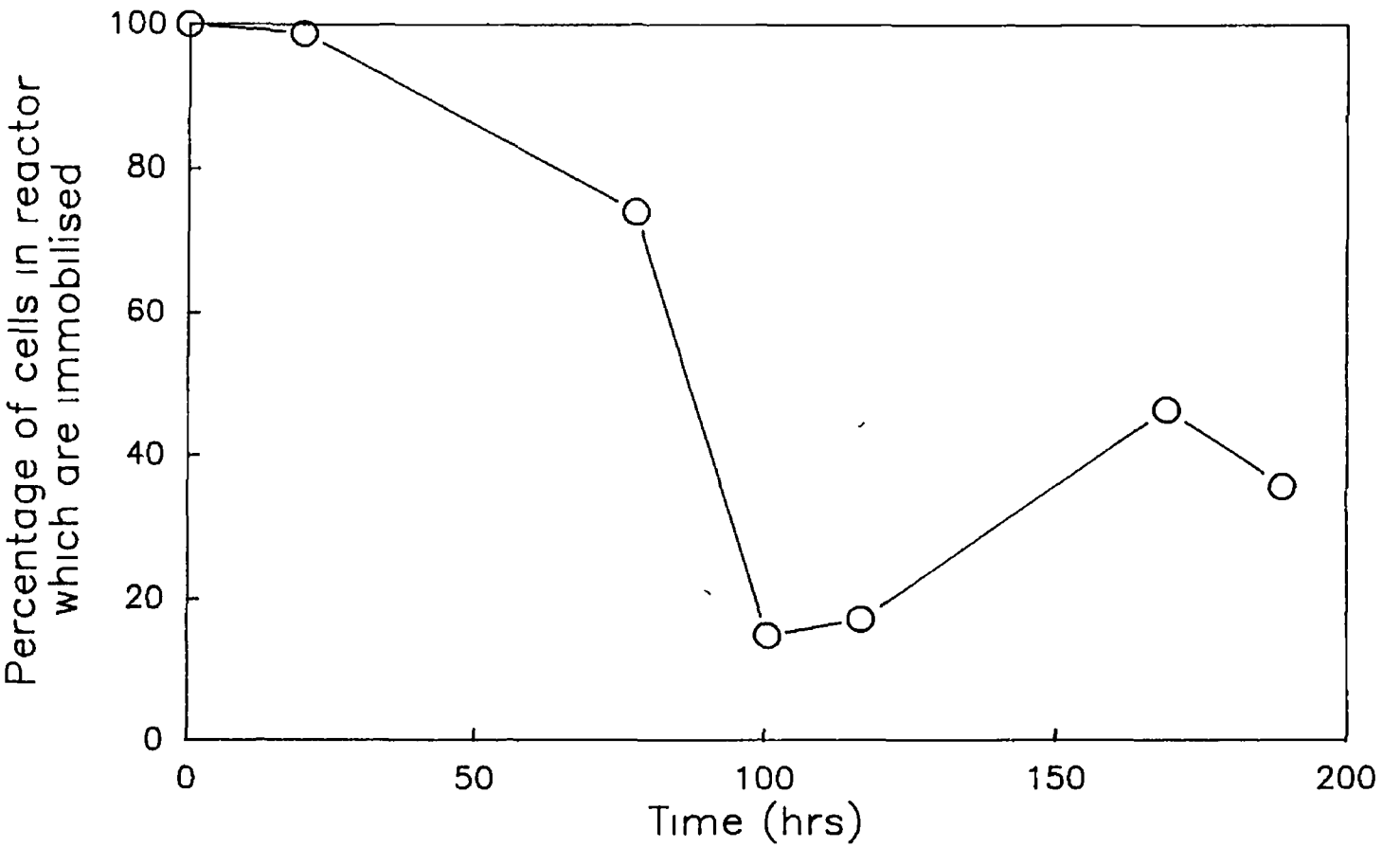

Figure 3 28 Estimated percentage of total cells in a continuous reactor which is immobilised (D = 0 6 hr $^{-1}$)

and the dilution rate was increased in steps Figure 3 29 shows a logarithmic plot of cell number versus time for the reactor After each change in dilution rate, the system was allowed to equilibrate When cell numbers levelled off, the dilution rate was increased until a dilution rate was reached where cell numbers did not remain constant but decreased with time The calculations for μ_{max} are presented in Appendix A The maximum specific growth rate for free cells in YEPD using this method was estimated to be 0 8 h^{-1}

3 3 2 3 Immobilised reactor at D = 1 5 μ_{max} in non-selective medium

Knowing the maximum specific growth rate for free yeast cells, a continuous immobilised cell reactor was set up to run at a dilution rate of 1 5 μ_{max} (D = 1 2 h^{-1}) using 3 mm beads of 3% alginate If the reactor volume was maintained at 500 ml, as with previous continuous experiments (See Section 3 3 2 1), the volume of medium required *daily* would be 14 4 L with a dilution rate of 1 2 h^{-1} The reactor volume was reduced to 185 ml due to the impracticalities of operating at high dilution rates and high volumes (see Section 3 2 7) The volume of alginate beads in the reactor was 20 ml, and because of this low volume, only released cell samples were taken during fermentation An extra volume of beads was made up initially and a sample analysed for cell numbers and plasmid stability Plasmid stability was measured by replicating 200 colonies onto selective and non-selective

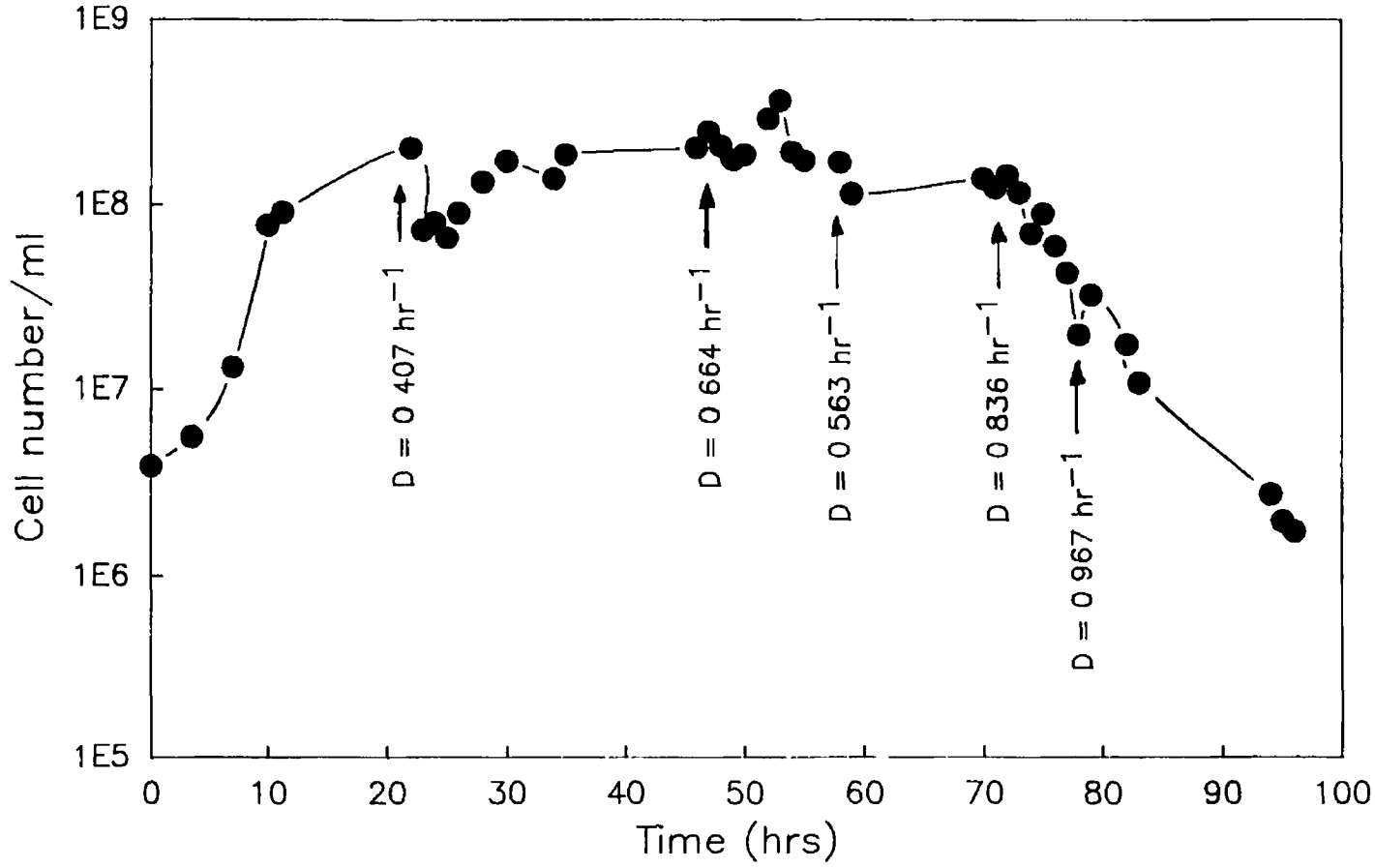

Figure 3 29 Washout chemostat experiment to measure μ max of pJG317 cells using non–selective medium

medium At the end of the run, immobilised cell plasmid stability and cell numbers were measured The pH was not controlled but remained between 4 5 and 5 5

Two similar fermentations were carried out under these conditions, and the results are presented in Figures 3 30 and 3 31 The first system ran for 104 hours and the second for 241 hours In both these systems, enzyme production remained constant and did not diminish with time The plasmid stability of the released cells was approximately constant at 30% The plasmid stability of the immobilised cells at the end of the fermentation was higher for both runs than that of the released cells In the first run, the final plasmid stability of the free (released) and immobilised cells was 32 5% and 41 3% respectively In the second run, the final stabilities were 27% for free cells and 50% for the immobilised cells The results for the immobilised cells are an average of the bead in total From previous results (See Section 3 3 1 3), it may be reasonably assumed that the plasmid stability in the centre of these beads is higher than these overall values

The reduction in bead weight in these reactors was 23% for the shorter run and 19% for the longer run This is a considerable decrease in bead loss compared to the stirred tank run in which bead weight was reduced to a quarter of their original value, presumably due to increased agitation It is unlikely that the bead structure would have remained intact for 240 hours had the fermentation been carried out in a stirred tank reactor

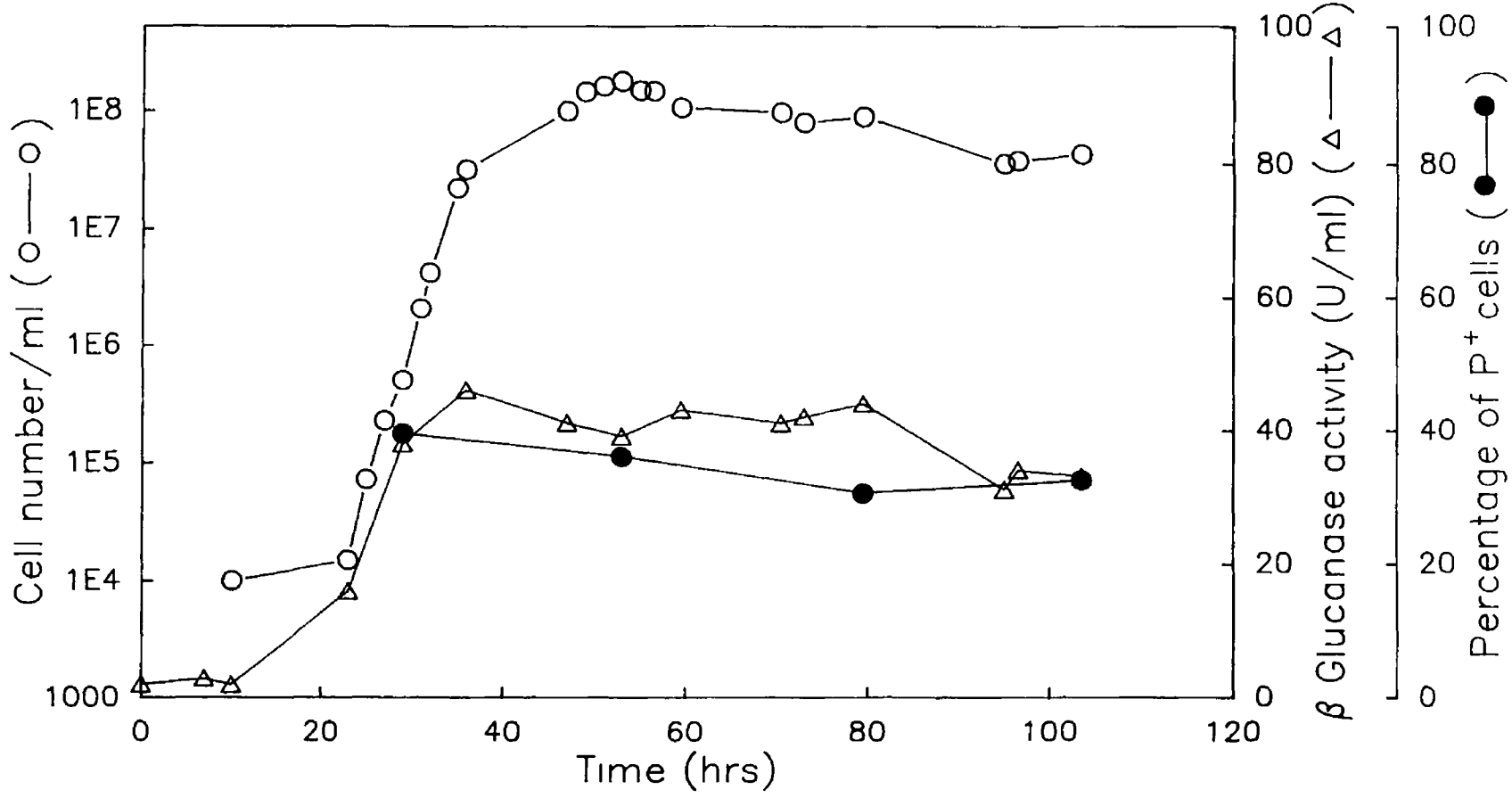

Figure 3 30 Plot of free cell concentration, enzyme activity and released cell plasmid stability for a continuous reactor (D = 1 2 hr $^{-1}$)

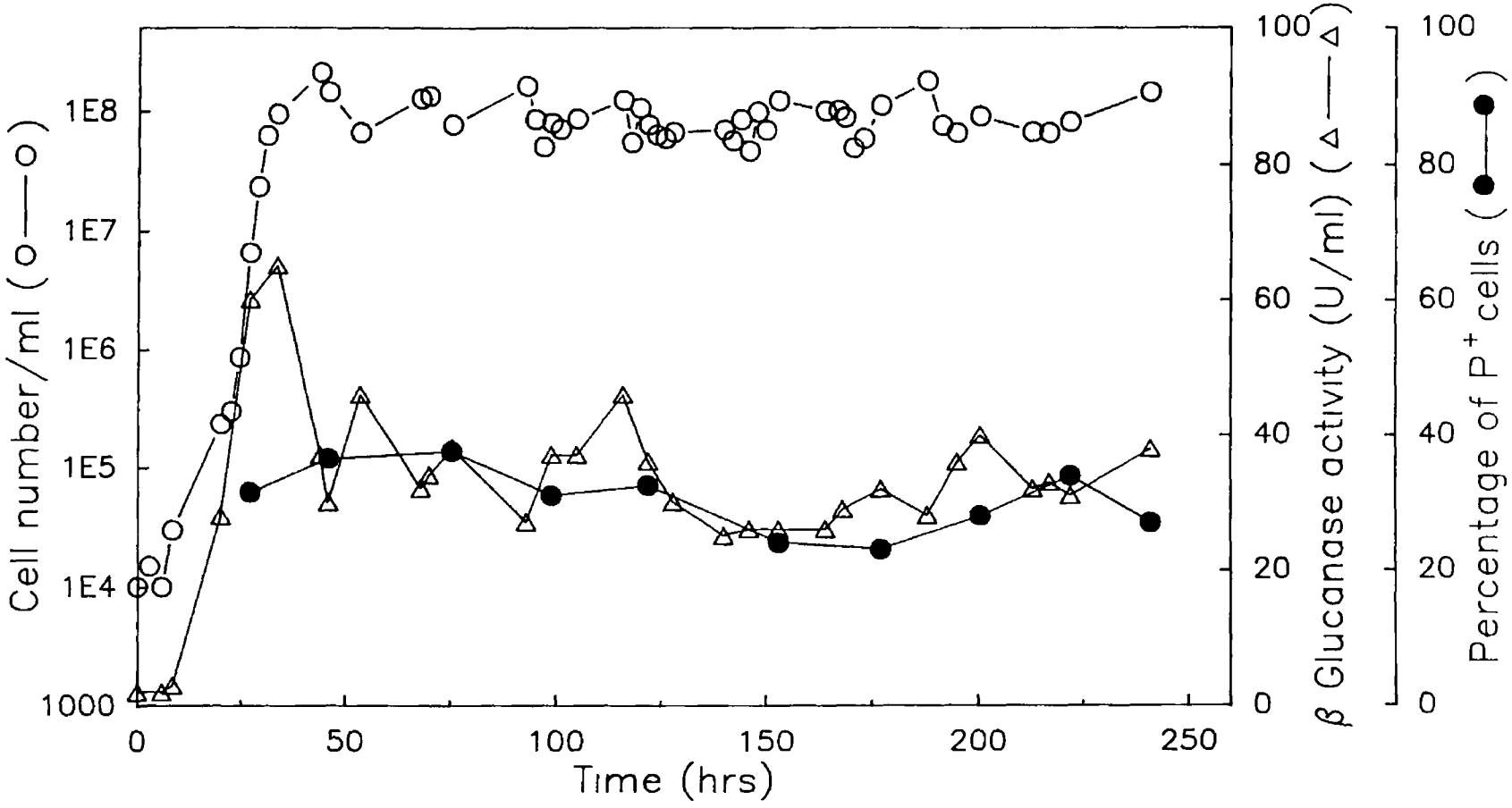

Figure 3 31 Plot of free cell concentration, enzyme activity and released cell plasmid stability for a continuous reactor (D = 1 2 hr $^{-1}$)

The free cell numbers increased over a period of 40 hours to the order of 10^8 ml^{-1} This was a most surprising result as the dilution rate should not allow free cell replication in the reactor The rate of release of cells from the matrix into the media was very high and cannot be accounted for purely as a result of cell leakage from the surface area of 20 ml of 3 mm beads Park (1985), found that yeast growth rates increased when aerated with 95% air and 5% CO_2 There are also reports that immobilised yeast cells exhibited faster growth rates than free cells in some cases (Wei, 1981) This was thought to be due to a higher percentage of CO_2 present in the matrix because of diffusional resistances In the case of immobilised bacteria, Mori (1989), found that the growth rate of cells released from an immobilisation matrix exceeded the normal maximum specific growth rate of free cells for a period of time after release from the matrix surface

It does appear from these results that the growth rate of the released cells is increased somewhat The rate of release of cells from the matrix into the medium in these fermentations is phenomenal Say for instance that the outer dense layer of cells in alginate beads contains an estimated 10^{10} cells per gramme This is a reasonable assumption as measured cell numbers in Figure 3 10 reach 3×10^9 g^{-1} These measurements are an average of the outer 500 μm layer of beads From Section 3 2 4 the approximate depth of dense cell growth is between 200 and 350 μm Therefore, cell numbers in the outer 200 μm layer of cells

have been underestimated Cells can only leak from the outermost surface of the beads If we assume here that cells can leak from the outer 150 μm layer of the matrix, then the rate of free cell loss from the beads is about 40% of this total layer per hour, assuming that no free cell replication occurs in the vessel at a dilution rate of 1 2 h^{-1} It is likely, from these results, that the maximum specific growth rate of the released cells is somewhat greater than that of free cells

3 3 3 Growth patterns and plasmid stability of immobilised yeast

3 3 3 1 Plasmid stability of immobilised pJG317 cells

The results up until this point for plasmid stability of bead sections have fluctuated somewhat To overcome this it was decided to immobilise the strain in very large beads (see Methods Section 2 3 2) Plasmid stability was measured by replicating 200 colonies per sample onto selective and non-selective medium

The results of this sectional analysis of larger beads are presented in Figure 3 32 Here it may be clearly seen that the outer section of the beads contains the cells of lowest plasmid stability, whereas the centre of the bead contains a greater proportion of plasmid-containing cells The outer sections of the bead samples were 2 mm deep When thinner slices were removed from the outer surface (approximately 1 mm thickness) at 165 hours, the plasmid stability measured was lower than a 2mm thick outer section at the same time (37% as opposed to 50%) This would imply

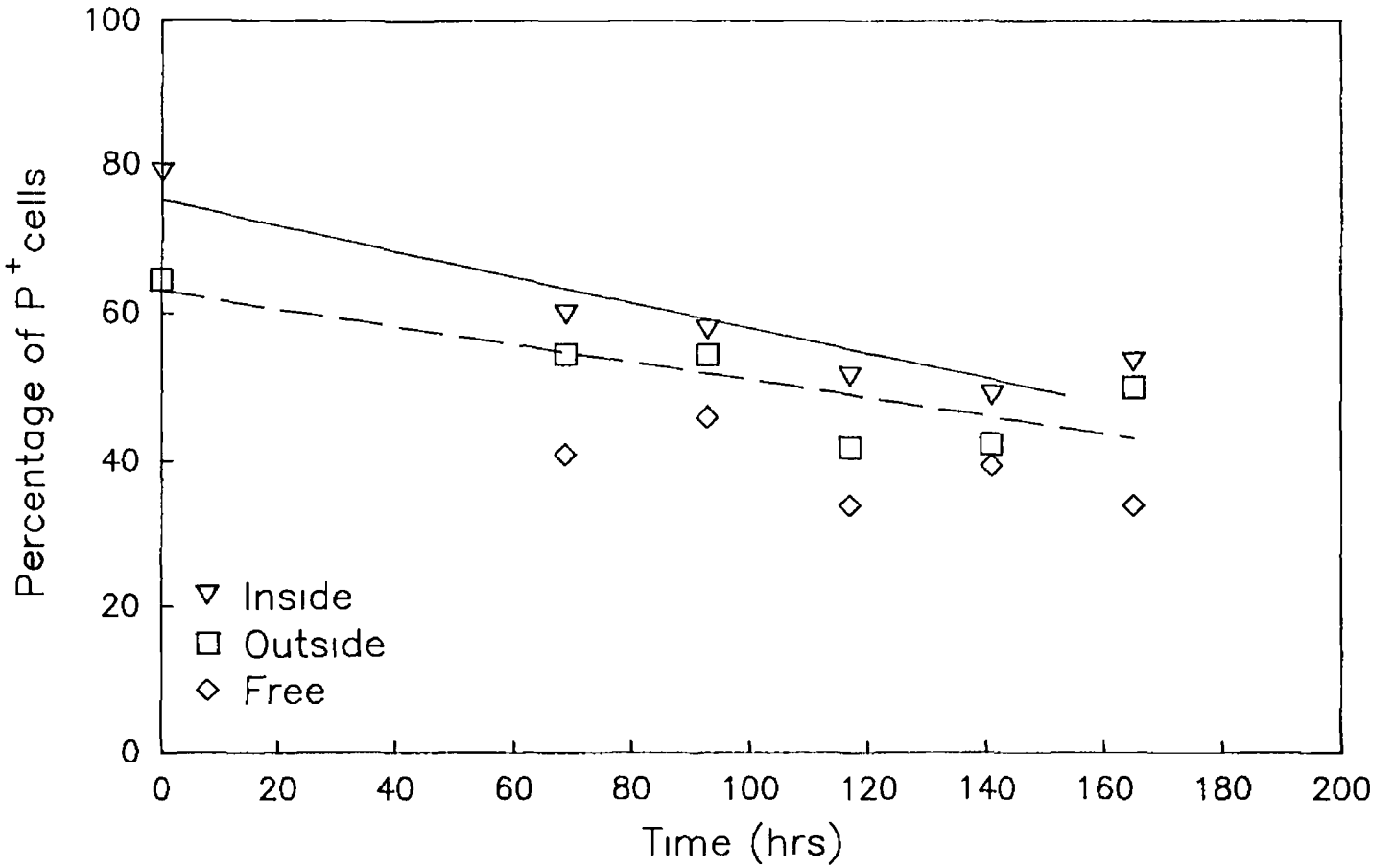

Figure 3 32 Plasmid stability of bead sections in a repeated batch immobilised system (3% alginate, 18mm diameter cylinders, 200 replicas)

that, if extremely thin sections of the outer surface could be sliced off, the plasmid stability measured may tend towards that of the released cells The overall trend of plasmid stability in the matrix appears to be inversely related to the cell density distribution within the beads Where cell numbers and cell growth are maximal, plasmid stability is low and vice-versa This would suggest that it is the compartmentalised nature of the growth within the immobilisation matrix that accounts for the increased plasmid stability in the matrix

The centre of the gel matrix, therefore, acts as a reservoir of cells of high plasmid stability This has been suggested previously for recombinant *Escherichia coli* immobilised in carrageenan (Nasri, 1987, Marin-Iniesta, 1988 and Berry, 1988) Cells growing on the outside of the matrix produce enzyme as they grow and in the process of division, lose the plasmid at a certain rate After a certain number of generations the cells are shed from the matrix surface, due to the disruption of the gel caused by cell growth, agitation and softening due to medium components As these cells are leaked from the alginate surface, they make way for diffusion of nutrients to cells which, until then, were unable to grow at an appreciable rate due to nutrient depletion Thus, at a microscopic level, the bead surface is receding, exposing fresh cells of higher plasmid stability to nutrients

Nasri (1987), estimated that *Escherichia coli* could only replicate approximately 10 to 16 times before being

released from the surface of carrageenan beads Growth of *Escherichia coli* in gel beads is restricted to the outer 50 μm layer (Nasri, 1987, Marin-Iniesta, 1988) Therefore, plasmid loss in an immobilised system, under non-selective conditions, is only possible during a limited number of generations before the cells are leaked from the matrix and washed out of the reactor

3 3 3 2 Growth and viability in large beads

The growth pattern of yeast in the 18 mm beads described above is presented in Figure 3 33 Most of the growth occurs in the outer section of the beads Initially growth occurs in all regions of the bead to varying degrees When exterior cell numbers exceed 10^9 g^{-1} , growth in the middle and core regions ceases presumably due to nutrient or oxygen depletion Berry (1988), previously reported that gradients of growth do not occur within the matrix until the immobilised cell numbers have reached a critical value (6×10^6 cells g^{-1}gel) Once this value has been exceeded, growth of cells in the interior of the matrix is inhibited

Although cell numbers in the outer section also level off, growth continues in this region resulting in continuous release of cells into the medium as discussed earlier in Section 3 2 2

Despite the fact that growth is retarded in the centre of the bead, the viability of the cells was 90% after 165 hours of repeated batch growth, while the outer section of the beads had a cell viability of 98% This implies that

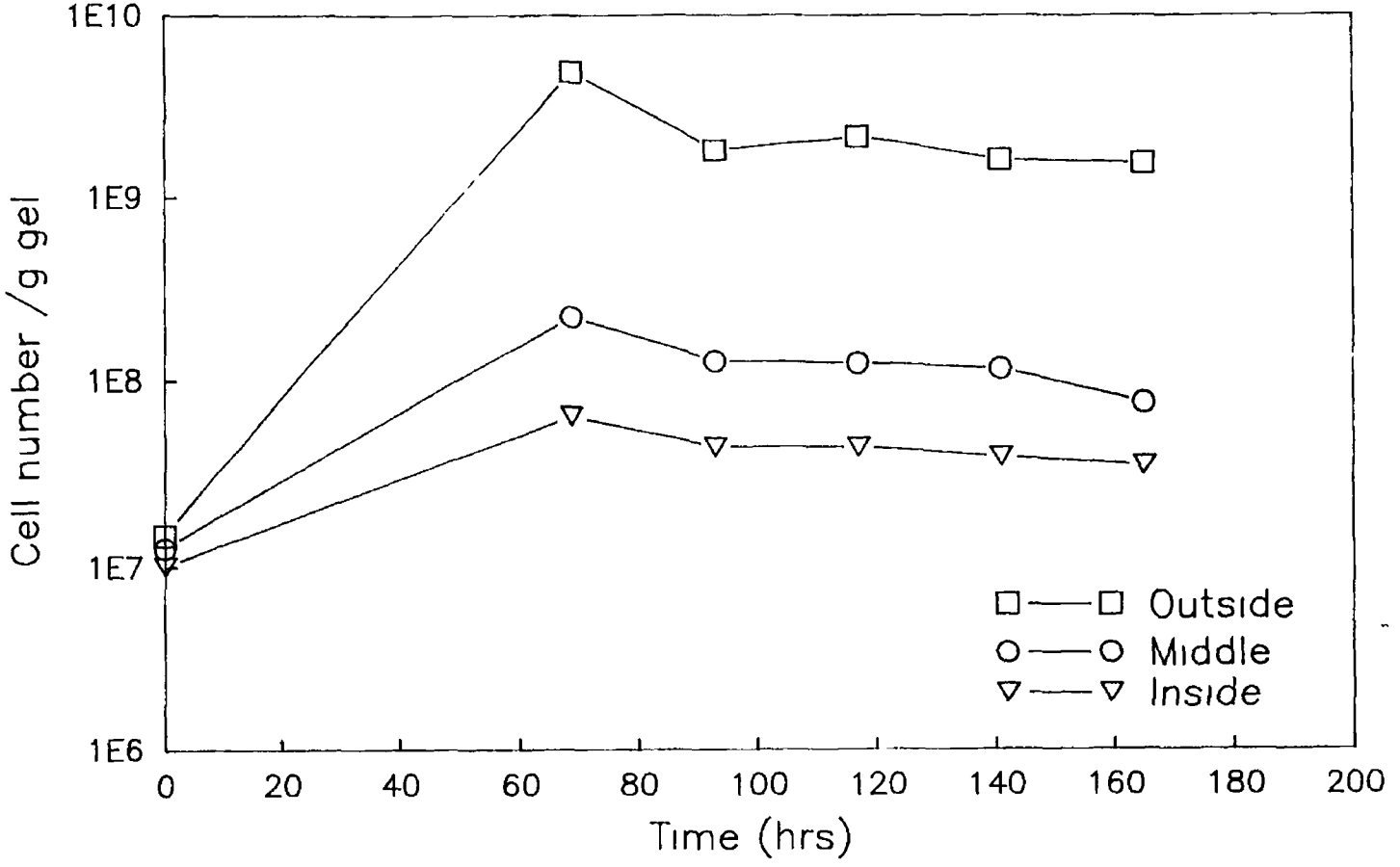

Figure 3 33 Cell concentration in bead sections during repeated batch growth of pJG317 (3% alginate, 18mm diameter cylinders)

the centre of very large beads contains viable cells and that development of necrotic centres does not occur with this yeast strain

From results in Section 3 3 2 3, the beads appear to act as reservoirs of plasmid-containing cells For compartmentalisation of cell growth within the entrapment matrix to be of use in continuous systems, it is essential that the cells in all parts of the matrix remain viable so that they can actively grow and replicate when exposed to more favourable conditions This ability of cells to remain viable even in the centre of large beads has been demonstrated above for periods up to 165 hours

3 3 3 3 Plasmid stability at different growth rates

Knowing that a gradient of cell growth occurs throughout the alginate bead (Section 3 2 3) and also that there is a gradient of plasmid stability throughout the immobilisation matrix (Sections 3 3 1 3, 3 3 2 and 3 3 3 1), it is of interest to see if the growth rate of the yeast strain affects the rate of plasmid loss in non-selective medium This is of importance as there is a gradient of growth rates from the bead surface to the core of the immobilisation matrix In a repeated batch experiment with free cells, two flasks were initially grown under similar conditions with daily changes of non-selective medium Plasmid stability was measured daily At 48 hours one flask was incubated at 25°C without shaking to decrease the growth rate, while the other was maintained at 30°C and shaken at 150 rpm The trends of plasmid loss are

illustrated in Figure 3 34

Initially, the rate of plasmid loss is similar in both cases and then, with a decrease in growth rate, the rate of plasmid loss decreased This means that, at lower growth rates the rate of plasmid loss per generation for yeast cells in suspension, is reduced compared to free cells at a higher growth rate This is in agreement with the findings of Impoolsup (1989), who conducted experiments in rich non-selective medium similar to YEPD Other researchers have reported results that conflict with these findings (Kleinman, 1986, DiBiasio, 1986) These reports, however, concerned work with minimal medium with and without selection pressure It is possible that the rate of plasmid loss at different growth rates depends on the nutritional status of the yeast cells

Although these results apply to free cells growing in YEPD, it may well be the case that immobilised cell plasmid stability improves at lower growth rates also If this is the case, then reduced generation number and improved plasmid maintenance (due to a reduced growth rate) may contribute to the higher plasmid stability observed in the centre of the alginate beads

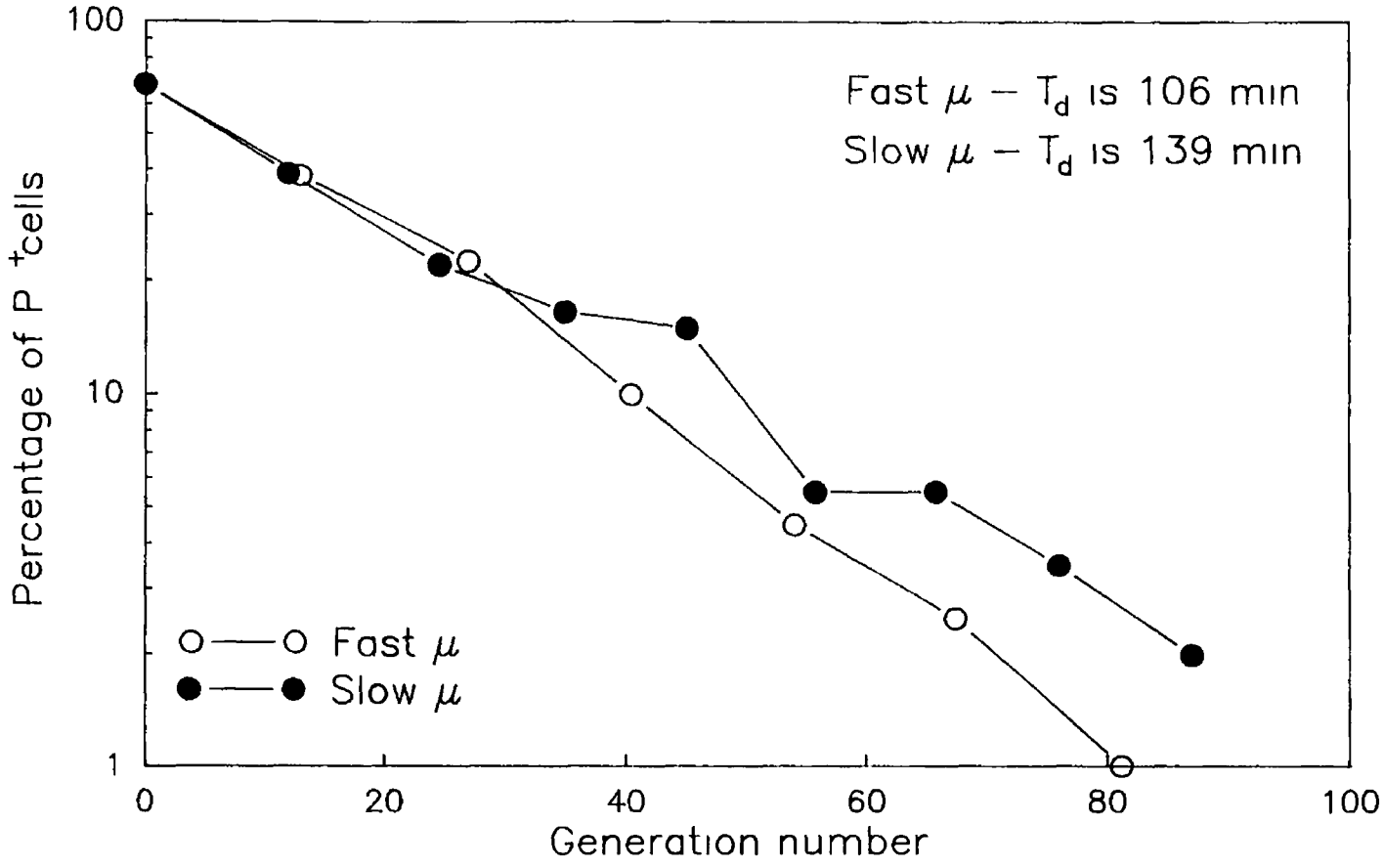

Figure 3 34 Plot of plasmid stability vs generation number for pJG317 cells grown in a repeated batch manner at two different growth rates

4 CONCLUSIONS

The yeast strain DBY746 containing the plasmid pJG317 produces the enzyme β - (1,3)(1,4) - glucanase Enzyme production only occurs during cell growth and division In free cell culture, enzyme is only produced during the exponential growth phase Growth of the clone occurs throughout the calcium alginate immobilisation matrix and enzyme production is comparable to that of free cells under selective conditions

The plasmid is not stably maintained in the yeast strain under non-selective conditions In free cell culture, plasmid-free cells quickly appear and dominate the fermentation

With immobilised cells, however, the decrease in the fraction of plasmid-containing cells was not as dramatic as in the free cell case Immobilisation has been reported to improve the plasmid stability of recombinant organisms (Berry, 1988, Marin-Iniesta, 1988, Sayadi, 1989) The reasons for this are not fully understood Improved plasmid maintenance was observed for immobilised recombinant yeast in this work also

It is thought that the physical environment of the matrix affects the cells in a manner that leads to improved plasmid maintenance Sayadi (1989), found that for *Escherichia coli* , plasmid copy number increased upon immobilisation This may however be related to a decreased growth rate within the matrix, which was reported by the author to cause an increase in plasmid copy number

It has also been suggested that the compartmentalised nature of growth within the beads restricts competition between plasmid-bearing and plasmid-free cells Most growth occurs in a very thin layer at the outer surface of the gel (Gosmann, 1988) This phenomenon limits the number of generations that cells can undergo in this layer before they are released from the bead surface In continuous reactors, these cells are then washed out of the reaction vessel Therefore competition between plasmid-bearing and plasmid-free cells occurs only during a limited number of generations before these cells are removed from the reactor In the case of immobilised recombinant *Escherichia coli* competition may last only for 3 to 4 generations (Berry, 1988) This is not the case for a free cell continuous reactor where competition between plasmid-bearing and plasmid-free cells occurs throughout the entire fermentation

Continued cell growth in an immobilised cell system leads to continuous cell leakage from the surface of the gel, rather than an increase in biomass within the gel This is clear from the results of continuous fermentations presented here De Taxis Du Poet (1986), suggests that the bead surface is receding on a microscopic scale due to disruption of the gel caused by abundant growth and enlargement of micro-colonies at the bead surface The release of these cells from the matrix increases the accessibility of oxygen and nutrients to other micro-colonies within the bead Disruption and breakdown of the

bead surface is evident in the image analysis results presented in Figure 3 9

Marin-Iniesta (1988), found that the plasmid stability of immobilised cells remained higher than cells leaked from the gel surface These released cells had a higher plasmid stability than free cells which have never been immobilised The results presented here are in agreement with these findings

In order to investigate further whether the compartmentalised nature of growth within the gel is responsible to some degree for improved plasmid maintenance, plasmid stability was measured in different areas of the beads The results presented show a gradient of the percentage of plasmid-containing cells from high at the bead core to low at the bead surface The centre of the bead therefore acting as a reservoir of plasmid-containing cells as suggested by Marin-Iniesta (1988)

In all immobilisation experiments presented here, the initial cell concentration was relatively low (roughly 10^7 cells per gram of gel) Berry (1988), found that, with immobilised *Escherichia coli* cells, the fraction of plasmid-containing cells decreased more rapidly if beads were inoculated at a low cell concentration Beads inoculated at a high cell concentration did not show a decrease in the fraction of plasmid-containing cells at all It is felt that this may well apply to immobilised recombinant yeast also

Table 4 1 compares the enzyme productivities of the

TABLE 4 1 ENZYME PRODUCTIVITIES IN REACTOR SYSTEMS STUDIED

Reactor Type	Dilution Rate (h^{-1})	Mode	Medium	Productivity (U/ml/h)
Shake flask	-	Free	Minimal	2 3
Shake flask	-	Free	YEPD	3 6
Shake flask	-	Immobilised	YEPD	5 6
Continuous	0 36	Immobilised	YEPD	16 2
Continuous	0 60	Immobilised	YEPD	27 2
Continuous	1 20	Immobilised	YEPD	48 0

various free and immobilised cell systems studied

In a free cell system, under non-selective conditions, it is not possible to set up a continuous reactor due to the instability of the clone Maximum productivity is achieved in a batch fermentation using rich non-selective medium as discussed in Section 3 2 8 This productivity is very low at 3 6 U/ml/h With an immobilised system it is possible to continuously produce the β-glucanase enzyme under non-selective conditions An immobilised cell continuous reactor can be operated at high dilution rates, as the cells cannot be washed out of the reactor From the results, there is a corresponding increase in enzyme productivity with increasing dilution rate From the above data there is a 20 fold improvement in enzyme productivity in a continuous reactor (D = 1 2 h^{-1}) compared to the enzyme productivity achieved in a batch system under selective conditions Further increases in enzyme productivity appear likely if the dilution rate is increased

One of the disadvantages of entrapment matrices is the heterogeneous distribution of biomass within the gel and also the diffusional resistances at the surface and within the beads which retard growth in the centre of the beads (Kolot, 1988) This may be a disadvantage in non-recombinant systems where a fraction of the biomass in the reactor is inactive or functioning at a reduced level When a recombinant organism is used, however, this heterogeneous distribution of growth may lead to stable recombinant protein production under non-selective conditions over a prolonged period of time

5 POSSIBLE AREAS OF FUTURE RESEARCH

Further insight into the genetics of immobilised pJG317 cells is necessary Plasmid copy number (PCN) is an important parameter, affecting both plasmid stability and β-glucanase production It is important to estimate the PCN of immobilised and free pJG317 cells

Following on from this, PCN could be measured in different areas within the immobilisation matrix Such research may help clarify findings in this work (and elsewhere), of improved plasmid maintenance by immobilised microorganisms

Plasmid stability of immobilised pJG317 was studied only at one initial cell loading Varying the initial cell concentration may affect the long term stability of enzyme production using non-selective medium

Bead size may affect the overall stability of continuous immobilised pJG317 cell reactors during prolonged periods of operation Very small beads may become 'exhausted' of plasmid-containing cells sooner than larger beads There is a trade off between high productivity (very small beads), and long term stability (large beads)

Budding of yeast cells within the immobilisation matrix may differ from budding of free cells Size of buds may affect the degree of plasmid maintenance By direct microscopic measurement, it may be possible to compare the sizes of immobilised cell and free cell buds

6 REFERENCES

Adlercreutz, P and Mattiasson, B (1982), Oxygen supply to immobilised cells 1 Oxygen production by immobilised *Chlorella pyrenoidosa*, Enz Microb Tech , 4 , 332-336

Anderson, R L , Anderson, M A and Stone, B A (1976), Some structural and enzymological aspects of non-starchy polysaccharides in cereal grains, Proc 14th Conv Inst Brew (Aust Section) Melbourne, 153-160

Austin, S J (1988), Plasmid partition, Plasmid, 20 , 1-9

Bailey, J E and Ollis, D F (1986) Biochemical Engineering Fundamentals, 2nd edition, McGraw-Hill, New York, 595-605

Bamforth, C W (1982), Barley beta glucans, their role in malting and brewing, Brewer's Digest, 57 , 22-35

Bathgate, G N and Dalgliesh, C E (1975), The diversity of barley and malt beta glucans, Proc Am Soc Brew Chem , 33 , 32-36

Berry, F , Sayadi, S , Nasri, M , Barbotin, J N and Thomas, D (1988), Effects of growing conditions on recombinant *Escherichia coli* in carrageenan gel beads upon biomass production and plasmid stability, Biotech Letts , 10 , 619-624

Bitter, G A , Chen, K K, Banks, A R and Lai, P H (1984), Secretion of foreign proteins from *Saccharomyces cerevisiae* by alpha-factor gene fusions, Proc Natl Acad Sci (U S A), 81 , 5330-5334

Bussey, H , Vernet, T and Sdicu, A M (1981), Mutual antagonism among killer yeasts - competition between K1 and K2 killer strains of *Saccharomyces cerevisiae* , Can J Micro , 34 , 38-44

Cantwell, B , Brazil, G , Hurley, J and McConnell, D (1985), Expression of the gene for the endo 1-3, 1-4 -β-glucanase from *Bacillus subtilis* in *Saccharomyces cerevisiae* , Proc 20th Eur Brew Conv Congress Helsinki, 259-266

Caunt, P , Impoolsup, A and Greenfield, P F (1988), Stability of recombinant plasmids in yeast, J Biotech , 8 , 173-192

Caunt, P , Impoolsup, A and Greenfield, P F (1989), The effect of oxygen limitation on stability of a recombinant plasmid in *Saccharomyces cerevisiae* , Biotech Letts , 11 , 5-10

Chang, H N, and Moo-Young, M (1988), Estimation of oxygen penetration depth in immobilised cells, Appl Micro Biotech , 29 , 107-112

Dennis, K , Srienc, F and Bailey, J E (1985), Ampicillin effects in five recombinant *Escherichia coli* strains - Implications for selection pressure design, Biotech Bioeng , 27 , 1490 -1495

Derynck, R , Singh, A and Goeddel, D V (1983), Expression of the human interferon - gamma cDNA in yeast, Nucleic Acids Res , 11 , 1819-1837

De Taxis du Poet, P , Dhulster, P , Barbotin, J N and Thomas, D (1986), Plasmid inheritability and biomass production Comparison between free and immobilised cell cultures of *Escherichia coli* BZ18 (pTG201) without selection pressure, J Bact , 165 , 871-877

Dhulster, P , Barbotin, J N and Thomas, D (1984), Culture and bioconversion use of plasmid-harbouring strain of immobilised *Escherichia coli* , Appl Micro Biotech , 20 , 87-93

DiBiasio, D and Sardonini, C (1986), Stability of continuous culture with recombinant organisms, Ann NY Acad Sci , 469 , 111-117

Doran, P M and Bailey, J E (1986), Effects of immobilisation on growth, fermentation properties and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin, Biotech Bioeng , 28 , 73-87

Doran, P M and Bailey, J E (1987), Effects of immobilisation on the nature of glycolytic oscillations in yeast, Biotech Bioeng , 29 , 892-897

Durand, G and Navarro, J M (1978), Immobilised microbial cells, Process Bio , 13 , 14-25

Edgley, M and Brown, A D ((1978), Response of xerotolerant and non-tolerant yeasts to water stress, J Gen Micro , 104 , 343-345

Eikmeier, H , Westmeier, F and Rehm, H J (1984), Morphological development of *Aspergillus niger* immobilised in calcium alginate and K-carrageenan, Appl Micro Biotech , 12 , 53-57

El-Sayed, A H M and Rehm, H J (1986), Morphology of *Penicillium chrysogenum* strains immobilised in calcium alginate beads and used in penicillin fermentations, Appl Micro Biotech , 24 , 89-94

Engberg, B and Nordstrom, K (1975), Replication of R-Factor R1 in *Escherichia coli* K-12 at different growth rates, J Bact , 123, 179-186

Ensley, B D (1986), Stability of recombinant plasmids in industrial microorganisms, CRC Crit Rev Biotech , 4 , 263-277

Esener, A A , Bol, G , Kossen, N W F and Roels, J A (1981), Effect of water activity on microbial growth In Moo-Young, M , Robinson, C W and Vezina C (Eds), Advances in Biotechnology (Volume 1), Pergamon Press, Toronto, 339

Fincher, G B , Lock, P A , Morgan, M M , Lingelbach, K , Wettenhall, R E H , Mercer, J F B , Brandt, A and Thomsen, K K (1986), Primary structure of the (1-3)(1-4)-β-D-glucan 4-glucanhydrolase from barley aleurone, Proc Natl Acad Sci (USA), 83 , 2081-2085

Fujiyama, A and Tamanoi, F (1986), Processing and fatty-acid acylation of RAS1 and RAS2 proteins in *Saccharomyces cerevisiae* , Proc Natl Acad Sci (USA), 83 , 1266-1270

Futcher, A B and Cox, B S (1984), Copy number and the stability of 2 μm-circle based artificial plasmids of *Saccharomyces cerevisiae* , J Bact , 157 , 283-290

Godia, F , Casas, C , Castellano, B and Sola, C (1987), Immobilised cells behaviour of carrageenan entrapped yeast during continuous ethanol fermentation, Appl Micro Biotech , 26 , 342-346

Gomori, G (1955), Preparation of buffers for use in enzyme studies In Colowick, S P and Kaplan, N O (Eds), Methods in enzymology, Volume 1, Academic Press, New York, 138-146

Gosmann, B and Rehm, H J (1986), Oxygen uptake of microorganisms entrapped in calcium alginate, Appl Micro Biotech , 23 , 163-167

Gosmann, B and Rehm, H J (1988), Influence of growth behaviour and physiology of alginate-entrapped microorganisms on the oxygen consumption, Appl Micro Biotech , 29 , 554-559

Hahn-Hagerdal, B , Larsson, M and Mattiasson, B (1982), Shift in metabolism towards ethanol production in *Saccharomyces cerevisiae* using alternations of the physical-chemical environment, Biotech Bioeng Symp , 12 , 199-202

Harder, A and Roels, J A (1982), Application of simple structured models in bioengineering, Adv Biochem Eng , 21 , 55-107

Hinnen, A , Hicks, J B and Fink, G R (1978), Transformation of yeast, Proc Natl Acad Sci (USA), 75, 1929-1933

Hjortso, M A , Dennis, K E and Bailey, J E (1985), Quantitative characterisation of plasmid instability in *Saccharomyces cerevisiae* using flow cytometry-cell sorting, Biotech Letts , 7 , 21-24

Hopkins, D J , Betenbaugh, B J and Dhurjati, P (1987), Effects of dissolved oxygen shock on the stability of recombinant *Escherichia coli* containing plasmid pKN401, Biotech Bioeng , 29 , 85-91

Impoolsup, A , Caunt, P and Greenfield, P F (1989), Effect of growth rate on stability of a recombinant plasmid during continuous culture of *Saccharomyces cerevisiae* in non-selective medium, J Biotech , 10 , 171-180

Inloes, D S , Smith, W J , Taylor, D P , Cohen, S N , Michaels, A S and Robertson, C R (1983), Hollow fibre membrane bioreactors using immobilised *Escherichia coli* for protein synthesis, Biotech Bioeng , 25 , 2653-2681

Jack, T R and Zajic, J E (1977), Immobilisation of whole cells, Adv Biochem Eng , 5 , 125-145

Jackson, E A , Ballance, G M and Thomsen, K K (1986), Construction of a yeast vector directing the synthesis and release of barley (1-3)(1-4) β glucanase, Carlsberg Res Commun , 51 , 445-458

Jirku, V , Turkova, J and Krumphanzl, V (1980a), Immobilisation of yeast cells on polyphenylene oxide, Biotech Letts , 2 , 451-454

Jirku, V , Turkova, J and Krumphanzl, V (1980b), Immobilisation of yeast cells with retention of cell division and extracellular production of macromolecules, Biotech Letts , 2 , 509-513

Jobses, I M L , Egberts, G T C , Van Baalen, A and Roels, J A (1985), Mathematical modelling of growth and substrate conversion of *Zymomonas mobilis* at 30 and 35°C, Biotech Bioeng , 27 , 984-995

Kennedy, J F , Humphreys, J D , Barker, S A and Greenshields, R N (1980), Application of living immobilised cells to the acceleration of the continuous conversions of ethanol (wort) to acetic acid (vinegar) - Hydrous titanium (IV) oxide-immobilised Acetobacter species, Enz Microb Tech , 2 , 209-216

Kierstan, M P J and Coughlan, M P (1985), Immobilisation of cells and enzymes by gel entrapment In Woodward, J (ed), Immobilised cells and enzymes - A practical approach, IRL Press, Oxford, 39-48

Kingsman, S M , Kingsman, A J and Mellor, J (1987), The production of mammalian proteins in *Saccharomyces cerevisiae* , TIBTECH, 5 , 53-57

Kleinman, M J , Gingold, E B and Stanbury, P F (1986), The stability of the yeast plasmid pJBD248 depends on growth rate of the culture, Biotech Letts , 8 , 225-230

Klemperer, R M M , Ismail, N T A and Brown, M R W (1979), Effect of R plasmid RP1 on the nutritional requirements of *Escherichia coli* in batch culture, J Gen Micro , 115 , 325-331

Kolot, F B (1988), Immobilised microbial systems Principles, techniques and industrial applications, R E Krieger Publishing Co , Malabar, Florida, 1-11

Kosikov, K V , Raevskaya, O G , Khoroshutina, E B and Perevertailo, G A (1975), Polyploid hybrids of industrial yeast Races and prospects for their practical application, Microbiology, 44 , 615-620

Lamptey, J and Moo-Young, M (1987), Immobilisation of yeast cells by adsorption on wood chips, Methods in enzymology, 135 (b) , 333-340

Laretta Garde, V , Thomasset, B and Barbotin, J N (1981), Electron microscopic evidence of an immobilised living cell system, Enz Microb Tech , 3 , 216-218

Lee, F J S and Hassan, H M (1987), Effect of oxygen tension on stability and expression of a killer toxin chimeric plasmid in a chemostat culture of *Saccharomyces cerevisiae* , Appl Micro Biotech , 27 , 72-74

Leedham, P A , Savage, D J , Crabb, D and Morgan, G T (1975), Materials and methods of wort production that influence beer filtration, Proc Eur Brew Conv Congress, Nice, 201-216

Lim, F (1984), Biomedical applications of micro-encapsulation, CRC Press Inc , Boca Raton, Florida, 137-154

Lord, P G and Wheals, A E (1980), Asymmetrical division of *Saccharomyces cerevisiae* , J Bact , 142 , 808-818

Lovitt, R W , Walter, R P , Morris, J G and Kell, D B (1986), Conductimetric assessment of the biomass content in suspensions of immobilised (gel entrapped) microorganisms, Appl Micro Biotech , 23 , 168-173

Luzikov, V N (1988), Some aspects of the processing, transport and preservation of foreign proteins in a cell, Microbiological Sciences, 5 , No 6, 170-173

Marin-Iniesta, F , Nasri, M , Dhulster, P , Barbotin, J N and Thomas, D (1988), Influence of oxygen supply on the stability of recombinant plasmid pTG201 in immobilised *Escherichia coli* cells, Appl Micro Biotech , 28 , 455-462

Martin, C E and Scheinbach, S (1989), Expression of proteins encoded by foreign genes in *Saccharomyces cerevisiae* , Biotech Adv , 7 , 155-185

Mattiasson, B and Hahn-Hagerdal, B (1982), Micro-environmental effects on metabolic behaviour of immobilised cells A hypothesis, Eur J Appl Micro Biotech , 16 , 52-55

McAleer, W J , Buynak, E G , Margatter, R Z , Wampter, D E , Miller, W J and Hilleman, M R (1984), Human hepatitis-B vaccine from recombinant yeast, Nature, 307 , 178-180

Measures, J C (1975), Role of amino acids in osmoregulation of non-halophilic bacteria, Nature, 257 , 398-400

Mellor, J , Dobson, M J , Roberts, N A , Tuite, M F , Emtage, J S , White, S , Lowe, P A , Patel, T , Kingsman, A J and Kingsman, S M (1983), Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae* , Gene, 24 , 1-14

Miller, G L (1959), Use of dinitrosalicyclic acid reagent for determination of reducing sugars, Anal Chem , 31 , 426-428

Monbouquette, H G and Ollis, D F (1988), Scanning microfluorimetry of calcium alginate immobilised *Zymomonas mobilis* , Bio/Technology, 6 , 1076-1079

Mori, A , Matsumoto, N and Imai, C (1989), Growth behaviour of immobilised acetic acid bacteria, Biotech Letts , 11 , 183-188

Nasri, M , Sayadi, S , Barbotin, J N and Thomas, D (1987), The use of immobilisation of whole living cells to increase stability of recombinant plasmids in *Escherichia coli* , J Biotech , 6 , 147-157

Navarro, J M and Durand, G (1977), Modification of yeast metabolism by immobilisation onto porous glass, Eur J Appl Micro Biotech , 4 , 243-254

Nguyen A L and Luong, J H T (1986), Diffusion in K-carrageenan gel beads, Biotech Bioeng , 28 , 1261-1267

Nicaud, J M , Mackman, N and Holland, I B (1986), Current status of secretion of foreign proteins by microorganisms, J Biotech , 3 , 255-270

Noack, D , Roth, M , Geuther, R , Muller, G , Hoffmeier, C and Gaspar, S (1981), Maintenance and genetic stability of vector plasmids pBR322 and pBR325 in *Escherichia coli* K12 strains grown in a chemostat, Mol G Genet , 184 , 121-124

Novo Literature Product specification sheet for 'Cereflo' (*Bacillus subtilis* endo-glucanase)

Oriel, P (1988), Immobilisation of recombinant *Escherichia coli* in silicone polymer beads, Enz Microb Tech , 10 , 518-523

Parent, S A , Fenimore, C M and Bostian, K A (1985), Vector systems for the expression, analysis and cloning of DNA sequences in *Saccharomyces cerevisiae* , Yeast, 1 , 83-138

Parker, C and DiBiasio, D (1987), Effect of growth rate and expression level on plasmid stability in *Saccharomyces cerevisiae* , Biotech Bioeng , 29 , 215-221

Pinches, A , Louw, M E and Watson, T G (1985), Growth, plasmid stability and alpha-amylase production in batch fermentations using a recombinant *Bacillus subtilis* strain, Biotech Letts , 7 , 621-626

Pirt, S J (1975), Principles of microbe and cell cultivation, Blackwell Scientific Publications, London, 29-41

Pu, H T and Yank, R Y K (1988), Diffusion of sucroae and yohimbine in calcium alginate gel beads with and without entrapped plant cells, Biotech Bioeng , 32 , 891-896

Pugsley, A P and Schwartz, M (1985), Export and secretion of foreign proteins by bacteria, FEMS Microbiol Rev , 32 , 3-38

Radovich, J M (1985), Mass transfer effects in fermentations using immobilised whole cells, Enz Microb Tech , 7 , 2-10

Reinikainen, P , Korpela, K , Nissinen, V , Olkku, J , Soderlun, H and Markkane, P (1989), *Escherichia coli* plasmid production in fermenter, Biotech Bioeng , 33, 386-393

Sakaki, K , Nozawa, T and Furusaki, S (1988), Effect of intraparticle diffusion in ethanol fermentation by immobilised *Zymomonas mobilis* , Biotech Bioeng , 31 , 603-606

Sambucetti, L C , Schaber, M , Kramer, R , Crowl, R and Curran, T (1986), The FOS gene-product undergoes extensive post-translational modifications in eucaryotic but not in procaryotic cells, Gene, 43 , 69-77

Sato, K and Toda, K (1983), Oxygen uptake rate of immobilised growing *Candida lipolytica* , J Ferm Tech , 61 , 239-245

Sayadi, S , Nasri, M and Barbotin, J N (1989), Effect of environmental growth conditions on plasmid stability, plasmid copy number and catechol 2,3-dioxygenase activity in free and immobilised *Escherichia coli* cells, Biotech Bioeng , 33 , 801-808

Scott, R W (1972), The viscosity of worts in relation to their content of β-glucan, J Inst Brew , 78 , 179-186

Seo, J H and Bailey, J E (1985), Effects of recombinant plasmid content on growth properties and cloned gene product formation in *Escherichia coli* , Biotech Bioeng , 27 , 1668-1674

Smith, R A , Duncan, M J and Moir, D T (1985), Heterologous protein secretion from yeast, Science, 229 , 1219-1224

Sols, A , Gancedo, C and Dela Fuente, G (1971), In Rose, A H , Harrison, J S (Eds) , The yeasts (Volume 2) , Academic Press, London, 271

Stuart, I M , Loi, L and Fincher, G F (1986), Development of (1-3)(1-4)-β-D-glucan endohydrolase isoenzymes in isolated scutella and aleurone layers of barley (*Hordeum vulgare*), Plant Physiol, 80 , 310-314

Tampion, J and Tampion, M D (1987), Immobilised cells principles and applications, Cambridge University Press, Cambridge, 1-12

Tanaka, H M , Matsumara, J A and Veliky, H (1984), Diffusion characteristics of substrates in calcium alginate gel beads, Biotech Bioeng , 26 , 53-58

Troller, J A and Stinson, J V (1981), Moisture requirements for growth and metabolite production by lactic acid bacteria, Appl Environ Micro , 42 , 682-687

Tyagi, R D and Ghose, T K (1982), Studies on immobilised *Saccharomyces cerevisiae* 1 Analysis of rapid ethanol fermentation in immobilised cell reactor, Biotech Bioeng , 24 , 781-795

Uhlin, B and Nordstrom, K (1977), R-plasmid gene dosage effects in *Escherichia coli* K12 copy mutants of R-plasmid R1DRD-19, Plasmid, 1 , 1-7

Uhlin, B , Molin, S , Gustafsson, P and Nordstrom, K (1979), Plasmids with temperature dependent copy number for amplification of cloned genes and their products, Gene, 6 , 91-106

Ulmanen, I , Lundstrom, K , Lehtovaara, P , Sarvas, M , Ruohonen, M and Palva, I (1985), Transcription and translation of foreign genes in *Bacillus subtilis* by the aid of a secretion vector, J Bact , 162 , 176-182

Van Brunt, J (1986), Fungi - the perfect hosts, Bio/Technology, 4 , 1057-1064

Wada, M , Kato, J and Chibata, I (1980), Continuous production of ethanol using immobilised growing yeast cells, Eur J Appl Micro Biotech , 10 , 275-287

Watson, T G , Pinches, A and Louw, M E (1986), Effect of growth rate on the maintenance of a recombinant plasmid in *Bacillus subtilis* , Biotech Letts , 8 , 687-690

Wei, C J , Tanner, R D and Woodward, J (1981), Elucidating the transition between submerged culture and solid state baker's yeast fermentations, Biotech Bioeng Sym , 11 , 541-553

Wei, D , Parulekar, S J , Stark, B C and Weigand, W A (1989), Plasmid stability and alpha-amylase production in batch and continuous cultures of *Bacillus subtilis* TN106 [pAT5], Biotech Bioeng , 30 , 1010-1020

Williams, D and Munnecke, D M (1981), The production of ethanol by immobilised yeast cells, Biotech Bioeng , 23 , 1813-1825

Zabriskie, D W and Arcuri, E J (1986), Factors influencing productivity of fermentations employing recombinant microorganisms, Enz Microb Tech , 8 , 706-717

APPENDIX A

CALCULATION OF THE MAXIMUM SPECIFIC GROWTH RATE OF DBY746 USING THE WASHOUT TECHNIQUE OF PIRT (1975)

In a continuous reactor, changes in the biomass concentration can be expressed as follows,

$$\frac{dX}{dt} = X(\mu - D)$$

where

t = time

X = biomass concentration

D = Dilution rate of reactor

μ = Specific growth rate of the organism

When the dilution rate of a continuous reactor exceeds the maximum specific growth rate of the organism μ_{max}, the concentration of biomass decreases until the concentration of cells within the reactor reaches zero This is known as washout of a continuous reactor

When $D > \mu_{max}$,

$$\frac{dX}{dt} = X(\mu_{max} - D)$$

$$\frac{dX}{X} = dt(\mu_{max} - D)$$

$$ln(X) = (\mu_{max} - D)\,t + c$$

(c is a constant)

From a plot of $ln(X)$ vs time (t), the slope during washout is,

$$\text{Slope} = (\mu_{max} - D)$$

[Figure 3 29 (in Section 3 3 2 2) is a $\log_{10}$ plot of biomass concentration The slope during washout on a *natural* log plot is - 0 168 h^{-1}]

$$(\mu_{max} - D) = - 0\ 168\ h^{-1}$$

$$\mu_{max} = D - 0\ 168\ h^{-1}$$

$$\mu_{max} = (0\ 967 - 0\ 168)\ h^{-1}$$

$$\mu_{max} = 0\ 799\ h^{-1}$$

The maximum specific growth rate of DBY746 in YEPD is calculated to be 0 8 h^{-1} In order to conduct continuous reactor experiments at a dilution rate of 1 5 μ_{max}, a dilution rate of 1 2 h^{-1} is required See Section 3 3 2 3