STUDIES ON THE PLASMID STABILITY, PLASMID COPY NUMBER AND ENDO (1,3)(1,4) β-GLUCANASE PRODUCTION BY FREE AND ALGINATE IMMOBILISED RECOMBINANT *SACCHAROMYCES CEREVISIAE* CELLS

A thesis presented for the
degree of Ph D

by

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June 1994

I hereby certify that this material, which I now submit for assessment on this programme of study leading to the award of PhD is entirely my own work and has not been taken from the work of others save to the extent that such work has been cited and acknowledged within the text of my work

Signed Peter Canavan

Peter D Canavan

Date 29-May 1994

All men dream but not equally
Those who dream by night in the dusty
recesses of their minds wake in the day to
find that it was vanity but the dreamers
of the day are dangerous men, for they
may act their dreams with open eyes, to
make it possible.

T E Lawrence
"The Seven Pillars of Wisdom"

This Thesis is dedicated to my parents for all their love and encouragement.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those people who helped me throughout this project, and especially

My supervisors Dr Thecla Ryan and Dr Padraig Walsh, for their advise, encouragement and guidance It is said that "*two's company but three's a crowd*", I am grateful for the way *this* crowd worked so well together A special word of thanks to Thecla and Padraig for their co-operation in the preparation of this thesis

Professor Martin Clynes, the staff and technicians of the School of Biological Sciences, for their help and advise Thank you to Dr Michael Parkinson for his help with the statistical analysis

The postgrads in the "John Barry", especially Sharon, Margaret, Fiona and Ger, for their lively discussions and helpful contributions in the recombinant DNA work

The many postgrads in Biology, for making the years spent in DCU so memorable and enjoyable, especially the "Good-Time Club" and Jacqui and Ron for the memorable times in Shannard, Charlemont and Shanliss

My parents, to whom I owe a very special thank you, for their continuous interest, support and encouragement throughout my education

And last, but by no means least a very special thank you to Geraldine, for encouraging, supporting and being so patient, as only she can

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ABSTRACT

Studies on the plasmid stability, plasmid copy number and endo (1,3)(1,4) β-glucanase production by free and alginate immobilised recombinant *Saccharomyces cerevisiae* cells.

A recombinant yeast strain, *Saccharomyces cerevisiae* DBY746, containing the plasmid pJG317, was grown in a variety of fermentation modes including batch, serial batch and chemostat culture incorporating a wide range of media types Plasmid pJG317 consists of a 2μ-derived yeast episomal plasmid containing the gene which encodes for the bacterial enzyme endo (1,3)(1,4) β-glucanase The concentration of enzyme produced appears to be proportional to the number of plasmid copies per cell Specific enzyme activities were found to be in the range of 1 4 × 10^{-6} to 4 8 × 10^{-6} U/cell for free cell culture, with a corresponding plasmid copy number of 8±0 5 to 40±6 3 copies per cell respectively

A procedure for measuring the copy number of pJG317 in *S cerevisiae* was developed, tested and optimised The procedure is based on Southern hybridisation and measured the relative intensities of hybridisation of a probe to the single copy yeast chromosomal actin gene and to the multicopy plasmid pJG317

Plasmid pJG317 is quite unstable under non-selective conditions and its copy number and stability are influenced by both growth rate and nutrient supply By immobilising cells in calcium alginate gel beads, the plasmid could be stabilised and high volumetric productivities of up to 3 8 U/ml-h attained Although radial gradients in biomass concentration and in percentage of plasmid-containing cells in the alginate gel beads were confirmed, no significant difference was found between the plasmid copy number of cells in the centre of the gel beads (36 5±6 8) and cells close to the surface of the gel beads(32 4± 3 3)

NOMENCLATURE

C_p	Number of copies of plasmid per cell	[-]
D	dilution rate	[h-1]
D_{act}	Relative area under β-actin peak	[-]
D_p	Relative amount of plasmid DNA	[-]
D_{plas}	Relative area under plasmid peak	[-]
E	Enzyme concentration	[U/ml]
E_t	Final enzyme concentration	[U/ml]
E_{av}	Average enzyme concentration during a continuous fermentation	[U/ml]
dO_2	Dissolved oxygen	[% saturation]
M_{act}	Size of β-actin DNA	[kb]
M_{plas}	Size of plasmid DNA	[kb]
S	Plasmid positive cells	[%]
t_d	Doubling time	[h]
t	Time	[h]
t_f	Duration of batch fermentation	[h]
X_t	Total cell concentration	[cell/ml]

Symbols

α-INF	alpha interferon
2μ	yeast native 2μm circle
cir^0	yeast strain not containing 2μm circle
cir^+	yeast strain containing 2μm circle
P+	Plasmid-containing cells
P-	Plasmid-free cells

Abbreviations

ARS	Autonomously Replicating Sequences
CCC	Covalently Closed Circle
CIP	Calf Intestinal Phosphatase
DNS	Dinitrosalicylic acid
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
EDTA	Ethylene Diamine Tetracetic Acid
ER	Endoplasmic Reticulum
GRAS	Generally Regarded As Safe
HSA	Horse Serum Albumin
ICL	Initial Cell Loading
LB	Luria Bertani
OD	Optical Density
ORI	Origin Of Replication
PBS	Phosphate Buffered Saline

PCN	Plasmid Copy Number
SPM	Sorbitol-Phosphate-Mercaptoethanol
TAE	Tris-Acetic acid-EDTA
TE	Tris-EDTA
UAS	Upstream Activation Sequences
YEPD	Yeast-Extract-Peptone-Dextrose
YNB	Yeast Nitrogen Base

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CHAPTER ONE

INTRODUCTION

1.1 HETEROLOGOUS PROTEIN PRODUCTION IN YEAST

The yeast *Saccharomyces cerevisiae* has been increasingly employed as a host for heterologous gene expression and protein secretion This interest is due both to the ease and favourable economics of yeast fermentation, developed over the years of industrial experience, and to the rapid progress made in the molecular genetics of the organism *Saccharomyces* is one of a small group of GRAS (generally recognised as safe) organisms recognised by the FDA (Martin and Scheinbach, 1989) Yeasts, in general, have a rapid growth rate and can be grown to very high cell densities They can be propagated on simple defined media and can be transformed with a variety of either self-replicating or integrating plasmid vectors A wide range of genetic, molecular and biochemical techniques have been developed for use in yeast The eucaryotic yeasts possess much of the complex cell biology typical of multicellular organisms, including a highly compartmentalised intracellular organisation and an elaborate secretory pathway which mediates the secretion and modification of many host proteins (Emr, 1990) The utilisation of the yeast expression system allows a broader range of potential applications than is possible with bacterial expression systems Expression levels similar to the highest levels seen in *E coli* have been achieved in yeast *S cerevisiae* has been successfully used to express high levels of several different types of proteins, including soluble cytoplasmic proteins, membrane proteins and secreted proteins (Hitzemann et al , 1983, Oberto and Davidson, 1985, Sleep et al , 1990)

1.1.1 Introduction of cloned genes

Several methods are presently used to transform yeast cells with cloned DNA The most widely used procedures are the spheroplast method (Beggs, 1978), in which exogenous DNA is incorporated into cells during cell fusion, and an alkali cation treament that renders intact cells permeable to DNA (Ito et al , 1984) The spheroplast method consists of removing the yeast cell wall by enzymatic treatment in an osmotically buffered medium The medium contains mercaptoethanol, normally used to break disulphide

bonds in cell wall proteins The inclusion of mercaptoethanol enhances spheroplast formation DNA is incorporated into the spheroplasts during cell fusion Transformed spheroplasts are then allowed to regenerate cell walls in osmotically buffered, selective solid medium High transformation efficiencies have been attained with this method The alkali salt procedure (Ito et al , 1984) allows transformation of intact cells Metal ions including Li^+, Na^+, K^+, Cs^+, Rb^+ and some thiol compounds, such as 2-mercaptoethanol, can produce competent cells for DNA uptake Lithium acetate treatment appears to be the most widely used method (Martin and Scheinbach, 1989) Cells are incubated in lithium to render them competent, followed by incorporation of DNA in the presence of polyethylene glycol 4000 Although this method is convenient and fast, transformation frequencies are somewhat lower than those attained with the spheroplast method More recently, another approach, electroporation, has been used and a highly efficient method has been reported by Meilhoc et al (1990) DNA is electrically introduced into intact yeast cells by applying electric field pulses in a simple and rapid procedure Pretreatment of yeast cells in the early phase of exponential growth, with dithiothreitol (DTT) increases the transformation efficiency

1.1.2 Yeast plasmid vectors

Yeast cells are generally transformed with plasmid DNA containing selectable marker genes The selectable marker is used to select transformed cells that are auxotrophic for amino acids such as Leucine (LEU2), Tryptophan (TRP1), Uracil (URA3) and Histidine (HIS3) Continued selection requires the use of minimal growth media lacking the relevant nutrient TRP1 and URA3 vectors can be selected in the presence of acid protein hydrolysates such as casaminoacids These protein hydrolysates lack tryptophan and uracil and are often used in semidefined media to enhance growth rates (Martin and Scheinbach, 1989) The introduction of the leucine defective gene (LEU2-d) as a selectable marker maintains higher than average plasmid copies per cell The gene has a truncated promoter which reduces transcription levels of the gene High copy number is required to allow cell growth in LEU2 auxotrophic strains (Erhart and Hollenberg, 1983) Dominant markers such as CUP1 (copper resistance) or

neo (g418 resistance) are particularly useful when introducing plasmids into yeast strains, such as brewing strains, which are polymorphic. They can also be used for selection of plasmid bearing cells in rich medium (Kingsman et al., 1987). A number of "autoselection systems" have been developed to ensure that plasmid selection is maintained, irrespective of culture conditions. Bussey and Meaden (1985) showed that expression of DNA encoding the yeast killer toxin and immunity gene, could be used for self-selection of transformants of laboratory or industrial yeasts, since plasmid free cells are killed by plasmid containing (P+) cells.

Yeast plasmid vectors are usually classed as episomal vectors or integrating vectors. Episomal vectors exist as extrachromosomal replicons within the cell and are based on either plasmids containing yeast autonomously replicating sequences (ARS) which function as origins of replication, or on the native 2μm circle (2μ) plasmid. Integrating vectors are incorporated directly onto the yeast chromosomes by exploiting the unusually high frequency of homologous recombination found in *S. cerevisiae*. Once integrated, the genes replicate and segregate with the chromosomes during mitosis.

1.1.2.1 *Episomal vectors*

(i) *ARS Vectors*

The ARS incorporated into these vectors are chromosomal origins of DNA replication which allow a single round of DNA synthesis during each cell division cycle (Murray and Szotak, 1983). Since there are no sequences present on the plasmids that allow for efficient segregation to daughter cells, and replication is not amplified, plasmid copy number (PCN) is usually dependent on the initial number of plasmids introduced into the cells during transformation. Plasmid amplification may occur under strong selection pressure, but plasmids can be lost at a relatively high rate if selection pressure is withdrawn. In practice ARS vectors are seldom used for foreign gene expression (Martin and Scheinbach, 1989).

(ii) *CEN Plasmids*

CEN vectors are plasmids that contain cloned yeast centromere sequences in addition to 2μ origin of replication or ARS The presence of the centromere allows the plasmid to be segregated from mother to daughter cells, during mitosis, as highly stable single copies per cell CEN-ARS plasmids are used where low-level expression is desired A related type of yeast episomal vector with regulated copy number has been described by Chlebowicz-Sledziewska and Sledziewski (1985) Vectors with regulatable centromeres were constructed containing the glucose-repressible ADH2 promoter adjacent to CEN3 In the ARS vectors, copy number could be increased from 1-2 to 5-10 by a switch from glucose to ethanol as the carbon source The CEN element was inactivated by transcription, leading to the increase in PCN In a 2μ-based vector, the PCN could be increased from 1-2 to about 100 and was maintained with high stability

(iii) *2μ-Based vectors*

The most commonly used expression vectors for yeast are *E coli* - yeast shuttle vectors based on the 2μ circle The 2μ is a 6 3kb plasmid present in most *Saccharomyces* strains at about 50 to 100 copies per haploid genome (Hartley and Doneldson, 1980, Futcher, 1988) It has no known function and is stably inherited The plasmid encodes four genes FLP, REP1, REP2 and REP3 The primary role of the REP system is maintenance of high copy number, which in turn is responsible for stability (Jayaram et al , 1983) In addition, 2μ contains an origin of replication (ORI), which behaves as a typical ARS element Efficient segregation of 2μ depends on having ORI and REP3 loci in *cis*, together with the gene products of REP1 and REP2 The simplest 2μ-based vectors contain the 2μ ORI and REP3, a yeast selectable marker, and bacterial plasmid sequences They are used in cir$^+$ (2μ containing) strains which supply REP1 and REP2 gene products, in *trans* These vectors are the most convenient to use routinely, due to their small size and ease of manipulation and they generally exist in 10 to 40 copies per cell

More complex 2μ based vectors contain the REP1 and REP2 genes in addition to the ORI and REP3 loci and can be used in cir^0 (2μ free) host strains By propagating the vector in cir^0 strains, high copy numbers may be achieved without the potential complications caused by recombination with endogenous 2μ plasmids Furthermore, by using cir^0 hosts, the PCN of the recombinant expression vector may be increased, since it represents the only 2μ plasmid in the cell There is evidence that 2μ and 2μ based vectors display incompatibility (Broach, 1983, Jayaram et al 1983) The presence of endogenous 2μ in a yeast strain may reduce the potential PCN of a 2μ based vector introduced into that particular strain (Gerbaud and Guerineau, 1980) In seeking to optimise the PCN of a cloned gene, strains devoid of endogenous 2μ (cir^0) should be employed in conjunction with the appropriate 2μ-derived vectors These more complex 2μ based vectors, although cumbersome, are more stable and better suited for scale-up than the simpler 2μ-based vectors containing just the REP3 and ORI loci

1.1.2.2 *Integrating vectors*

Chromosomal integration offers a more stable alternative to episomal maintenance of foreign DNA Integrating vectors normally contain a selectable marker, but lack yeast ORI Since *Saccharomyces* strains are highly recombinogenic, a DNA molecule containing yeast DNA sequences once transformed into the cells, will recombine with its homologous chromosomal sequences with high frequency Furthermore, plasmid DNA that has been linearised will integrate almost exclusively at the chromosome sites that are homologous to the cut ends, thus providing a mechanism for introducing cloned DNA sequences to specific sites in the genome This phenomenon is the basis of gene disruption, where specific native yeast genes can be inactivated (Rothstein, 1983) or replaced with altered genes that contain modified regulatory or structural regions (Winston et al , 1983) When high DNA concentrations of integrating vectors are used in transformations, tandem multicopy inserts can result due to repeated recombination events (Orr-Weaver and Szostak, 1983) Multicopy

integrants are relatively stable and have been used, for example, in gene dosage studies (Cashmore et al., 1986).

The ribosomal DNA (rDNA) cluster was the target of integration of an integrating vector constructed by Lopes et al. (1989,1990). The integrating vector contained some rDNA sequences and the LEU2-d marker. The rDNA cluster consists of about 140 tandem repeats of a 9.1kb unit on chromosome XII. Transformation with the vector gave 100 to 200 copies of LEU+ transformants integrated into the rDNA. The transfomants were highly stable, with 80-100% of the integrated copies being retained after 70 generations. The levels of foreign protein produced using the PGK promoter was as high as that produced in 2μ based vectors. Other DNA sequences that may be used as targets for integration include the transposable element *Ty*, which is present in 30 to 40 copies per genome in most *Saccharomyces* strains. Kingsman et al. (1985) described the use of a vector targetted to replace *Ty* and whose copy number could be amplified using the LEU2-d selection marker. Shuster et al. (1990) used vectors that integrated into delta (δ) elements, which exist alone or as part of *Ty* throughout the *S. cerevisiae* genome. The integration vector contained the *E. coli* lac Z gene with the LEU2 and CUP1 markers. The β-galactosidase level achieved was up to ten fold that of single copy strains.

1.1.3 Expression of heterologous genes

Gene expression is most frequently regulated at the level of transcription and it is generally assumed that the steady state mRNA level is a primary determinant of the final yield of a foreign protein (Romanos et al., 1992). Most strategies used to express foreign genes in yeast have focussed on the production of high mRNA levels, in order to maximise gene expression. This has usually been accomplished through the use of multiple copy plasmids in order to increase the number of gene sequences per cell by fusing coding sequences to strong yeast promoters to enhance transcription. Present strategies rely on choosing the most appropriate yeast vector and choosing from a large variety of native or engineered promoters which may be constitutive and/or regulated, along with the inclusion of an appropriate

transcription terminator The correct choice is critical for any one application, especially where a process is to be scaled-up

1.1.3.1 *Promoters*

Yeast genes are regulated in a manner similar to higher eucaryotes Yeast promoters consist of at least three elements which regulate the efficiency and accuracy of initiation of transcription (Struhl, 1989) upstream activation sequences (UAS), TATA elements and initiator elements UAS are short sequences of DNA that determine the activity and regulation of the promoter through specific binding to transcriptional activators They work in both orientations, at long distances from the transcription initiation site TATA elements are found 40 to 120bp upstream of the initiation site and provide a window within which initiation of transcription can occur The initiator element, which is poorly defined, directs mRNA initiation at closely adjacent sites Yeast promoters may be highly complex, extending over 500bp, containing multiple UAS, negative regulatory sites and multiple TATA elements associated with different initiation sites

Both constitutive and regulated promoters are used for heterologous protein production In attempts to generate high levels of mRNA for the heterologous gene, early work centered on the use of strong constitutive promoter elements from genes involved in glycolysis (Martin and Scheinbach, 1989) Although these promoters are amongst the most powerful of *S cerevisiae* and produce high levels of homologous proteins, yields are generally lower when they are utilised to produce heterologous proteins The most widely used glycolytic promoters are alcohol dehydrogenase (ADH1), phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate (GAP) (Romanos et al , 1992) Use of regulated promoters allow the recombinant cells to be grown to high cell densities, under conditions where the heterologous gene is not actively expressed Once the desired cell mass has been reached, fermentation conditions can be altered so that the gene is derepressed and the heterologous protein is highly expressed Promoters for genes involved in galactose metabolism, such as GAL1 and GAL10, are glucose repressed and are activated by the addition of galactose to the growth medium (Johnston, 1987) The GAL promoters are tightly regulated Other promoters require

the removal or depletion of a nutrient such as phosphate or glucose from the growth medium, the PHO5 promoter (Kramer et al , 1984) and the alcohol dehydrogenase II - ADH2 promoter (Shuster, 1987) The promoter of the copper resistance gene CUP1, encoding copper metallothionen, has been used in expression vectors The promoter is tightly regulated and independent of culture parameters The concentration of Cu^{2+} for induction depends on the copper resistance of the host strain (Henderson et al , 1985, Etcheverry, 1990)

Fusion of the GAL10 promoter region to the α-interferon (α-INF) structural gene has been used to attain high cell densities under glucose repressed conditions, with high yields of α-IFN following induction with galactose (Fieschko et al , 1987) By contrast, when α-IFN was fused to a constitutive PGK promoter, early expression, coupled with an unstable episomal expression vector resulted in poor cell growth and low yields of protein Expression vectors have been engineered to produce hybrid promoters which take advantage of strong constitutive promoter elements combined with the UAS of regulated genes (Velati-Bellini et al , 1986) Most promoters are regulated to some extent, but the most prowerful glycolytic promoters are poorly regulated This makes them undesirable for use in large-scale culture, where there is more opportunity for the selection of non-expressing cells, and unsuitable for expressing proteins toxic to the cell In such cases it is preferable to use a tightly regulated promoter, so that the growth and expression phases can be separated

1.1.3.2 *Terminators*

Yeast transcriptional terminators are usually present in expression vectors for efficient mRNA 3' end processing Efficient termination is probably required for maximal expression (Romanos et al , 1992) In higher eucaryotes mRNA 3' end processing involves cleavage and polyadenylation and it appears that yeast mRNAs follow the same pattern of termination, processing and polyadenylation of pre mRNA (Butler et al , 1990) Terminators from a number of genes have been used in expression vectors and include ADH1, TRP1, GAP1 and the FLP from 2μ

1.1.3.3 *Transcription and translation*

Such factors as the relative abundance of mRNA, mRNA stability, and the efficiency with which the mRNA is translated all influence the level of expression of a heterologous gene in yeast (Martin and Scheinbach, 1989) The mRNA level is determined by both the rate of initiation and its stability Initiation of transcription is governed by the type of promoter used The elongation of transcripts is not thought to affect the overall rate of transcription, but the yield of full length transcripts (mRNA) could be affected by fortuitous sequences in foreign genes which cause pausing or termination (Romanos et al , 1992) The half-lives of yeast mRNAs range from 1 to 100 minutes and can therefore have a major effect on the steady-state mRNA level (Brown, 1989) An inverse relationship between mRNA length and stability has been found Ribosome attachment may also contribute to mRNA stability in some cases, but this may be oversimplified as a complex relationship between mRNA stability and translation exists Translational efficiency is thought to be controlled primarily by the rate of initiation This is affected by the structure of the 5' untranslated leader of a mRNA Initiation in eucaryotes is thought to follow a scanning mechanism, whereby the 40S ribosomal subunit plus cofactors bind the 5' cap of the mRNA and then migrate down the untranslated leader, scanning for the first AUG codon Any part of this process which is affected by the structure of the leader could limit the translation initiation rate (Romanos et al , 1992) Codon usage is known to affect the translational elongation rate and although translational elongation is not normally thought to affect the yield or quality of the polypeptide, the codon content of a foreign gene may influence the yield of protein, where the mRNA is produced at very high levels This may be more likely to occur in growth on minimal medium, when the cell produces a wide variety of biosynthetic enzymes, encoded by genes containing rare codons (Sharp and Cowe, 1991)

1.1.3.4 *Post-Translational processing*

The ultimate yield of heterologous protein is equally affected by both the rate of synthesis and the rate of degradation Correct polypeptide folding is essential to ensure that proteins adopt their functionally-active conformation Similarly, processing such as amino-terminal modifications of the

polypeptides, must be specific and accurate Amino-terminal modifications are the most common processing event and occur on most cytosolic proteins (Kendall et al , 1990) Vacuolar proteolysis is influenced by culture conditions and increases several fold during nitrogen or carbon starvation, or in stationary phase Generally, very low yields are obtained with proteins which are naturally short-lived or with some polypeptides which are naturally secreted One of the best ways to improve protein stability is by segregating the protein product from intracellular proteases, via secretion Furthermore secretion also tends to result in a protein of greater quality

1.1.4 Secretion

Most recombinant proteins produced from heterologous genes remain trapped in the cell and must be released by cell fracture or enzyme digestion This requires that the recombinant protein be purified from other proteins present in the cell Yeast cells normally export only a small portion of their total protein (0 5% in the case of *S cerevisiae*) (Romanos et al , 1992) and the cell wall appears to constitute a formidable barrier for secretion Secretion of foreign proteins therefore can lead to relatively pure mature proteins in the medium Homologous proteins secreted by yeast include invertase, which is secreted through the plasma membrane, but is trapped in the periplasmic space Other proteins actually pass through the cell wall and are released into the culture medium, such as the mating hormones α and *a* factors, which are small, 11 to 13 amino acid polypeptides (Martin and Scheinbach, 1989)

Proteins destined for export are synthesized containing an N-terminal signal sequence that routes the protein to the endoplasmic reticulum (ER) membrane, where it is translocated into the ER lumen Cleavage of signal sequences by signal peptidases, N-linked glycosylation of asparagine residues, disulphide bond formation catalysed by disulphide isomerase, and other proteolytic processing and protein folding steps take place here The proteins are then routed through the golgi apparatus Transport from the ER to the golgi has been shown to be rate limiting (Romanos et al , 1992) Further protein processing, such as trimming and addition of carbohydrate residues, can take place here Movement through the golgi is followed by

packaging of secreted proteins into secretory vesicles for export from the cell

The addition of carbohydrate moieties to the processed protein is termed glycosylation and is both organism and cell type specific Expression of a protein in a heterologous system, will almost certainly result in a product with modifications which differ from the native material Oligosaccharides may be either N-linked to asparagine or O-linked to serine or threonine residues O-Linked oligosaccharides synthesised by yeast are very different from those of higher eucaryotes, being composed of mannose residues N-Linked glycosylation in yeast and higher eucaryotes is more conserved, and involves the addition of a core oligosaccharide unit in the ER (Romanos et al , 1992) This core oligosaccharide consists of two N-acetylglucosamine, nine mannose and three glucose residues The glucose residues are subsequently trimmed from the side chain and one mannose residue is also removed These steps are common to yeasts, plants and higher eucaryotes Processing of the chain may take place through a stepwise addition of further mannose residues which comprise the outer chain, which can be up to 75 residues long, with many branch chains (Kukuruzinsha et al , 1987) Addition of the outer chain to heterologous proteins is regarded as "hyperglycosylation", because it results in more extensive glycosylation than is found in higher eucaryotic glycoproteins

As many commercially important proteins undergo further modifications after synthesis, directing a protein through the yeast secretion pathway may also be desirable in terms of the post-translational processing and folding that may be essential to its function Most of the secretion systems have been constructed by fusing the leader or pre-pro sequences of the α-mating factor, or the invertase signal sequence, to the N-terminal portions of the foreign gene Recombinant proteins secreted from yeast include Interleukin 2, Epidermal growth factor (EGF) (Brake et al , 1984), *Aspergillus awamori* glycoamylase (Innis et al , 1985), wheat α-amylase (Rothstein et al , 1984) and *Bacillus subtilis* β glucanase (Cantwell, 1986a)

This approach offers certain advantages over intracellular production Many pharmacologically important proteins are naturally secreted and can often only adopt their correct conformation by folding within the secretory pathway Secretion can be a solution to the accumulation of toxic

cytoplasmically-expressed proteins However, the modifications made to a secreted protein along the secretion pathway may differ from those made by higher eucaryotic cells and, as a result, glycosylation is increasingly regarded as a drawback to the secretion of therapeutic glycoproteins from yeast The yeast proteins which assist in folding and disulphide bond formation differ from their counterparts in higher eucaryotes and this may affect folding of heterologous proteins, which can result in protein retention in the ER and degradation In addition to problems of transport, other undesirable events such as aberrant processing or hyperglycosylation may take place during the secretory process Despite these facts, secretion of heterologous proteins is still a most productive and advantageous method of recovery Use of glycosylation mutants has allowed the production of more homogeneous proteins with limited glycosylation (Melnick et al , 1990) Similarly, the use of "super secreting" mutants, which bypass the rate limiting step in the secretory process *i e* transfer of polypeptides from the ER to golgi, has resulted in high yields of core-glycosylated proteins (Smith et al , 1985) The choice of appropriate leader or signal sequences used to direct secretion has been found to influence the quality and quantity of the secreted product (Sleep et al , 1990) The choice of leader sequences and their relationship to the structural protein, is crucial to the successful secretion of high quality product

One of the major disadvantages of utilising yeasts instead of *E coli* for heterologous protein production, has been the generally lower yield of product, often due to the difficulty in obtaining high level transcription of foreign genes This problem appears to have been addressed in *S cerevisiae* in such ways as overexpressing transcriptional *trans*-activators (e g ADH2) (Price et al , 1990), constructing glycolytic promoters with superimposed regulation (Walton and Yarranton, 1989), and by random screening for super-expressing and super-secreting mutant strains (Sleep et al , 1991) The use of yeasts such as *Pichia pastoris*, which naturally have powerful tightly-regulated promoters, has provided an alternative solution There have been many successes in the production of therapeutic proteins from yeast, for example, the recombinant subunit vaccine against hepatitis B virus, human proinsulin, EGF and Human Serum Albumin (HSA) There have also been developments in the food industry, such as the experimental use of recombinant yeast secreting glucoamylase in brewing (Hammond, 1991), and the production of chymosin from *Kluyveromyces lactis* (Van der

Berg et al , 1990) Problems encountered with using *S cerevisiae* as a host for heterologous protein production, may be partially or completely circumvented by the use of other yeasts such as *Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis, Yarrowia lipolytica,* and *Schizosaccharomyces pombe* (Romanos et al , 1992) Most of these alternative systems are based on commercially important yeasts, that have been selected for their favourable growth characteristics at industrial scale, or on yeasts which have other favourable intrinsic properties, such as high level secretion

1.2 β-GLUCANASES

1.2.1 Substrates

The glucans, polymers of glucose linked by glucosidic bonds, are the most abundant class of polysaccharides found in nature Structurally they may be relatively simple, consisting of linear macromolecules, with the linkages between the glucose residues all of the same type, as in cellulose [(1,4) β-] and laminaran [(1,3) β-] Alternatively, they may be of greater complexity, possessing more than one type of glucosidic bond, either in linear chains, such as in lichenan with (1,3) β- and (1,4) β-linkages, or in having branched chains, as in the case of barley glucan with (1,3) β- and (1,4) β-glucosidic bonds, or yeast glucan with (1,3) β- or (1,6) β-linkages (Haliwell, 1975, Shiota et al , 1985, McClear and Glenmc-Holmes, 1985, Yalpani, 1988)

Glucans have great importance and potential application in the chemical, pharmaceutical and food industries, due to their unique chemical and physical properties, such as the capacity to alter flow characteristics of fluids, and to beneficially interact in the hydrated state with other dispersed or dissolved molecular species, which they may bind, chelate, complex, emulsify, encapsulate, flocculate, stabilise or suspend (Yalpam, 1988) However, besides those commercially utilisable properties, glucans play an important role in biological systems, where they are found in microorganisms and higher plants, as structural entities of the cell wall, as

cytoplasmic and vacuolar reserve materials, and as extracellular substances (Bielecki and Galas, 1991, Bull and Chesters, 1963) Recent advances in the industrial application of polysaccharides and the demand for improved or unique product properties, have contributed to the growing interest in enzymes which are involved in the biosynthesis, modification and degradation of these materials Among the substrates for β-glucanases is barley glucan, a high molecular weight, mixed (1,3) (1,4) β-glucan, which comprises 75% of the cell wall in barley endosperm (Fincher, 1975) This β-glucan has significant effects on the industrial exploitation of barley grain It forms highly viscous solutions, and in the brewing industry causes gels, hazes and precipitates in beer (Eukenlund, 1972) β-Glucanase action on the barley in malting and mashing processes during brewing, degrades the β-glucan and allows access of other hydrolytic enzymes to the starch and protein reserves in the endosperm Other substrates of importance for various β-glucanases include yeast cell walls, laminarin, pustulan, curdlan, scleroglucan, lentinan and schizophyllan (Bielecki and Galas, 1991)

1.2 2 Distribution, properties and applications

β-Glucanases are produced by many different microorganisms The yeasts *Saccharomyces* sp , *Candida* sp , *Hansenula* sp and *Schizosaccharomyces* sp , all produce the enzyme The genus *Bacillus* is a rich source of β-glucanases (Martin et al , 1980) β-Glucanases produced by filamentous fungi or actinomycetes have also been characterised *Penicillium* sp , *Aspergillus* sp , *Mucor* sp , and *Trichoderma* sp , all produce β-glucanases (Bielecki and Galas, 1991)

The variety of β-glucanases amongst microorganisms refers not only to their properties and modes of action, but also to differentiated regulation of their production The enzyme synthesis can be regulated in both positive and negative directions by such control mechanisms as induction, feed-back inhibition, and catabolite repression Since each of these mechanisms is influenced by environmental conditions, factors such as pH, temperature, medium composition, aeration and the stage of growth, are all important Enzymes may be inducible, semi constitutive or constitutive β-Glucanases have different substrate and product specificities They can be exo and endo

enzymes which produce a broad range of oligosaccharides Some of the β-glucanases have the capability to hydrolyse only (1,3) β-glucosidic bonds, while others can hydrolyse glucans with mixed glucosidic bonds such as in the case of many *Bacillus* species, which can produce an enzyme which splits both (1,3) β- and (1,4) β-glucosidic bonds (Lloberas et al , 1988)

β-Glucanases may be used directly in cell lysis for the release of intracellular or wall associated material, or in conjunction with other techniques to improve the rate and yield of product extraction (Kobayashi et al , 1982) The enzymes are used in the production and preparation of yeast extracts and compounds with adhesive properties, which can be useful for packaging in the food industry In the laboratory, β-glucanases play an important role in lytic enzyme systems used for the production of protoplasts β-Glucanases of known specificity and mode of action are used as a tool for the determination of the structure, localisation and isolation of specific biopolymers of microbial cell walls (Pastor et al ,1984, Gopal et al , 1984) Lentinan, isolated from the Japanese edible mushroom *Lentinus edodes* , is rich in β-glucans and has some immunomodulator activity, which exerts an inhibitory action on different types of tumour The use of (1,3) β-glucanase is of great importance in the brewing industry to improve wort filtration and quality of finished beer by degradation of barley β-glucan The enzyme is also used for the quantification of (1,3) (1,4) β-glucan in barley and malt (McClear and Glennic-Holmes, 1985)

1.2.3 Molecular cloning

The problems associated with excess of β-glucans in brewing wort can be alleviated by the application of commercial enzyme preparations or by construction of a yeast strain capable of hydrolysing the mixed linkage (1,3) (1,4) β-glucans found in barley (Borriss et al , 1985) Only *Bacillus subtilis* is known to produce an extracellular enzyme which has the same recognition site, cleaves the same linkages and produces a similar range of disaccharides from barley β-glucan as the malt β-glucanase (Cantwell et al , 1986a)

Efficient expression of a cloned β-glucanase gene in yeast required not only a yeast promoter and terminator sequence for the correct initiation and

termination of transcription, but also the removal of intervening *Bacillus* DNA sequences upstream of the initiation codon (Cantwell et al , 1986a, 1986 b) Progressive deletion of DNA sequences in the 5' region, resulted in increasing levels of expression of the β-glucanase gene in yeast under yeast promoter control Further increases in yield were obtained by using the alcohol dehydrogenase (ADH1) promoter Cantwell et al (1986 b) constructed two improved β-glucanase secretion plasmids, one with the β-glucanase gene fused to its complete signal sequence under ADH1 promoter and terminator control (plasmid pJG317), and the second with the β-glucanase structural gene, minus its signal sequence, under the control of α-factor mating pheromone promoter, its down stream leader sequence and the α-factor (plasmid pJG314) Using these plasmids, successful secretion of active (1,3) (1,4) β-glucanase by a recombinant yeast, was attained

Endo β-glucanase of *Trichoderma reesei* has been produced on laboratory and pilot-scale using recombinant strains of "bottom-fermenting" *S cerevisiae* (Zurbriggen et al , 1991) The purpose of work by Demolder et al (1993) was to develop a recombinant yeast strain in which the cell wall could be degraded in a controlled manner This was accomplished by expressing the (1,3) β-glucanase of *Nicotiana plumbaginifolia,* in *S cerevisiae* under the control of the yeast GAL1 promoter β-Glucanases have also been cloned into bacteria Lee and Pack (1987) transformed a *Bacillus megaterium* strain with a plasmid containing the endo (1,4) β-glucanase from *Bacillus subtilis* The gene encoding endo (1,4) β-glucanase in thermophilic *Bacillus* sp PDV was cloned into *E coli* by Sharma et al (1987) Louw and Reid (1993) cloned and sequenced the gene for (1,3) (1,4) β-glucanase from *Bacillus brevis* and characterised the thermostable enzyme's biochemical properties Finally, Wolska-Mitaszko (1985) cloned and expressed *Arthrobacter* endo (1,3) β-glucanase in *E coli* cells (Bielecki and Galas, 1991)

1.3 PLASMID COPY NUMBER

1.3.1 Introduction

An important parameter in the study of recombinant microbial systems is the number of molecules of plasmid in each cell, referred to as the plasmid copy number (PCN) Procedures for determining PCN have been developed and applied to various recombinant systems to measure both high and low copy number plasmids The procedures may differ in the level of sensitivity and accuracy, depending on whether they are developed to measure either high or low copy number plasmids The limiting factor in measuring low copy number plasmids is the DNA detection limit PCN has been measured in studies which investigate the influence of cell growth rate on PCN in bacteria (Seo and Bailey 1985, 1986) and in yeast (Bugeja et al , 1989), nutritional requirement effects on plasmid stability and plasmid content (Sayadi et al , 1989), the influence of PCN on the relative levels of many individual proteins and ribosome components (Birnbaum and Bailey, 1991), the influence of protein over production on cell physiology and plasmid stability (Van der Aar et al , 1992), and control and regulation of 2μ copy number (Jayaram et al , 1983)

1.3.2 Indirect methods of measuring PCN

Indirect methods of determining PCN do not require measurement of plasmid DNA directly, but instead involve measuring the levels of expression of particular proteins For R1 plasmid derivative, resistance to ampicillin in agar plates is proportional to β-lactamase gene dosage (Uhlin and Nordstrum, 1978) Methods based on indirect protein measurements all require primary proof that the phenotype being analysed is linearly proportional to gene dosage, and are therefore limited to plasmids that contain one or other of the few genes that show this proportionality Such factors as production kinetics, turnover rate and denaturation of the protein being qualified, must be taken into account

1.3.3 Direct methods of measuring PCN

Direct methods for measuring PCN all involve the physical separation of plasmid DNA from cell lysates, chromosomal and ribosomal DNA Caesium chloride (CsCl) gradient centrifugation (Lovett and Helinski, 1975), HPLC (Coppella et al , 1986, 1987) and gel electropheresis (Projan et al , 1983) have all been employed to separate plasmid DNA Another characteristic of direct methods is a detection system to measure the levels of plasmid DNA present and comparison to the relative levels of a reference DNA, such as chromosomal DNA, ribosomal DNA or individual genes CsCl gradients contain ethidium bromide and can be visualised under UV light DNA separated by HPLC is also detected by UV Ethidium bromide fluorescence densitometry of electropheretic gels is one of the most common methods for measuring PCN High and low copy number plasmids require different degrees of sensitivity, in that more sensitive methods of detection are employed for measuring the PCN of low copy number plasmids DNA hydridisation methods, which include hybridisation in solution, sandwich hybridisation and Southern hybridisation, are perhaps the most commonly used, techniques for detecting DNA for the purpose of determining PCN

1.3.3.1 *Caesium chloride gradients*

Centrifugation of cell lysates or cleared cell lysates in CsCl gradients containing ethidium bromide (EtBr), offers a simple method of separating plasmid DNA from chromosomal DNA The separated DNA may be visualised under UV illumination and quantified by gel electrophoresis The relative intensities of fluorescence of chromosomal to separated plasmid DNA or of a standard plasmid DNA preparation to separated plasmid DNA, may be measured, giving an indication of the PCN CsCl gradients offer minimal estimates of PCN, as all plasmid DNA extracted will not be in supercoiled form Nicking of supercoils by shearing, the action of non-specific endonucleases, or disruption of plasmid relaxation complexes, causes plasmid DNA to band with chromosomal DNA in EtBr-bouyant density gradients (Lovett and Helinski, 1975)

1.3.3.2 *HPLC*

The Nucleogen DEAE 4000-10 HPLC column has been shown to isolate transfer RNA (tRNA), ribosomal RNA (rRNA) and DNA from crude cell lysates (Coplan and Riesner, 1984) Coppella et al (1986, 1987) applied this HPLC system for PCN determination in both recombinant yeast and *E coli* systems Chromosomal DNA and plasmid DNA coeluted and retention was independent of size Therefore, the chromosomal DNA has to be removed, by denaturing during isolation of DNA from cells The PCN was calculated from the ratio of plasmid DNA to rRNA, and calculated on a per cell basis, using published data and measured constants Where the rRNA content was not constant, the plasmid peak area (from the chromatogram), together with the cell concentration, was used to calculate the PCN per cell

The HPLC technique for measuring PCN is direct and quick but relies heavily on constants and variables obtained from published data, which may be specific to one particular recombinant system In recombinant yeast, the 2μ circle cannot be separated from expression plasmids and therefore assumptions on the number of copies of 2μ must be made, in order to calculate the PCN of the expression vector (Coppella et al , 1987) Another disadvantage is that, some plasmid DNA may also be removed with the chromosomal DNA, leading to an underestimate of PCN

1.3.3.3 *Gel electrophoresis*

Gel electrophoresis is widely used to separate plasmid DNA to determine the PCN (Projan et al , 1983) For high copy number plasmids, direct fluorescence densitometry of ethidium bromide-stained electrophoretic agarose gels has been employed (Projan et al , 1983, Moser and Campbell, 1983, Seo and Bailey, 1985, 1986) For low copy number plasmids, where the PCN may be as low as one or two copies per cell, more sensitive techniques are employed to detect and quantify the DNA separated by gel electrophoresis Such techniques include DNA hybridisation (Bitter et al , 1987, Korpela et al , 1987)

1.3.3.3.1 *Ethidium bromide fluorescence densitometry*

High copy number plasmids may be visualised and hence stained by direct fluorescence densitometry of EtBr-stained electrophoretic agarose gels By comparing the fluorescence intensity of plasmid bands to some reference DNA of known concentration or quantity e g chromosomal band, ribosomal DNA (rDNA), or lambda (λ) DNA, the number of molecules per reference DNA equivalent can be calculated From this the number of plasmid copies per cell may be calculated

Projan et al (1983) developed a simple and straight-forward procedure for measuring the PCN of *S aureus* based on direct fluorescence densitometry of EtBr - stained electrophoretic gels The PCN measurement procedure consists of separating whole cell lysates by agarose gel electrophoresis, staining the gels with EtBr and analysing the stained gel by scanning densitometry Typical results are calculated according to the equation

$$Cp = \frac{Dp \times Mc}{Dc \times Mp}$$

where ***Cp*** indicates plasmid copies per cell, and ***Dp*** and ***Dc*** are the relative amounts of plasmid and chromosomal DNA respectively in the gel as determined from densitometer scans, ***Mp*** is the molecular weight of the plasmid and ***Mc*** is the total chromosomal DNA per cell In order to establish the reproducibility and repeatability of the method, Projan et al (1983) investigated several parameters influencing the procedure, which included the degree of trapping of plasmid DNA in the linear chromosomal fraction, the kinetics of staining-destaining, as a function of EtBr and DNA concentration, the fluorescence signal as a function of the amount of DNA present, and the effect of molecular topology on EtBr binding to DNA in agarose The procedure was used to determine the PCN of plasmids ranging in size from 4 2 to 27kb and from 12 to 880 copies per cell The repeatability of this technique was estimated to be ±20%

Direct densitometry of EtBr fluorescence is perhaps one of the simplest methods for measuring PCN However, many variables must be standardised, if large errors of up to 20% are to be minimised (Projan et al , 1983) These variables include gel staining and destaining times, polaroid

film development conditions (*i e* linearity of polaroid film response), DNA concentration *etc* The method is only applicable for high copy number plasmids

1.3.3.3.2 *DNA hybridisation*

In the case of low copy number plasmids (as is often the case in recombinant yeast), an attractive and accurate alternative for measuring PCN is DNA hybridisation Such techniques as hybridisation in solution, Sandwich hybridisation and Southern hybridisation have all been employed to quantify PCN and differ in the degree of complexity of the procedure (Gerbaud and Guerineau, 1980, Jayaram et al , 1983, Korpela et al , 1987)

Hybridisation in solution

DNA hybridisation in solution, when employed to measure PCN, involves extraction and denaturation of total DNA and radioactive labelling and denaturation of probe DNA A renaturation or reassociation reaction between probe DNA and total cellular DNA is monitored using hydroxyapatite chromatography, in which the single stranded DNA and the reannealed DNA, are eluted at different ionic strengths The radioactivity is counted in a scintillation counter and the kinetics of reassociation determined The PCN is calculated from the relative quantities of single stranded and reannealed DNA (Gerbaud and Guerineau, 1980) DNA hybridisation in solution is fast and accurate Reassociation takes place at faster rates than in filter bound hybridisation techniques and the technique is used to detect minute quantities of DNA (Gelb et al , 1971, Sharp et al , 1974) However, the technique is time consuming, involved and more difficult than filter hybridisation methods (Shepard and Polisky, 1979)

Sandwich hybridisation

A method for detection and quantification of DNA was developed by Ranki et al (1983) and modified to measure PCN by Korpela et al , (1987) The method is based on a three-DNA-component sandwich hybridisation and

involves the cloning of two non-overlapping restriction fragments of sample DNA into two vectors, the plasmid pBR322 and M13 phage The single stranded recombinant phage DNA is immobilised on 1cm diameter filter discs, while the pBR322 DNA is radioactively labelled, and used as a probe When the two reagents are incubated under annealing conditions, no radioactivity becomes filter bound Only if denatured sample DNA is added as the third reagent, will the radioactive probe become attached to the immobilised M13 DNA Many vectors in *E coli* and yeast and other cells contain an antibiotic resistance marker, such as the ampicillin or tetracycline resistance genes The genes are also present in pBR322, which is the radio-labelled probe, and therefore the ampicillin or tetracycline resistance genes are being quantified After hybridisation, the filter discs are washed and the radioactivity bound to the filters is determined in a counter To convert counts per minute into plasmid molecule numbers, standards with known amounts of plasmid molecules or chromosomal DNA are analysed

Hybridisation efficiency is dependent on both the filter bound and probe DNA concentrations and also on the hybridising conditions (Korpela et al , 1987) A major attraction of the Sandwich hybridisation technique is its suitability for use with crude DNA samples, thus alleviating the need for tedious and rigorous DNA isolation procedures

Southern hybridisation

Southern hybridisation involves the binding of a radioactively-labelled specific DNA probe to size-fractionated DNA immobilised on nitrocellulose or Nylon filters To quantify PCN, a hybridisation probe is utilised which is complementary to both plasmid DNA and a native chromosomal gene Such DNA probes may derive from either the promoter, transcription-terminator, or auxotrophic selectable marker of the expression vector, since these sequences are all also represented in the genome (Bitter et al , 1987) Whole cell DNA is isolated, digested with an appropriate restriction enzyme and size-fractionated by agarose gel electrophoresis The DNA is then transferred to nitrocellulose and hybridised to the radioactive probe (Maniatis et al , 1982) Knowledge of the genomic and plasmid restriction maps allows identification of the fragment representing either the chromosomal gene or the recombinant plasmid The chromosomal fragment

in each sample is an internal standard for quantification and generally represents one (haploid strains) or two (diploid strains) genes per cell Comparison of the amount of probe hybridised to the plasmid fragment to that hybridised to the chromosomal fragment (of defined copy number) allows calculation of the PCN per cell Transfer or binding efficiency of DNA fragments in Southern blot analyses is size dependent, and therefore it is essential that the plasmid and chromosomal restriction fragments be of similar size (Bitter et al , 1987) Calculation of PCN must take into account the relative lengths of the fragments and the extent to which the fragments are covered by the labelled probe (Broach, 1983) Finally, a more accurate PCN is obtained by dividing the PCN by the fraction of cells containing the plasmid

Southern hybridisation is an accurate and direct method for measuring PCN The procedure must be standardised in order to avoid errors in the values of PCN determined Southern hybridisation as a method of determining PCN is theoretically precise, but is rather difficult to perform and is time consuming It contains inherent flaws that can be avoided only with care (Projan et al , 1983)

1.3.3 Application of methods

In one of the most widely used techniques for measuring the PCN, physical separation of plasmid from chromosomal DNA was performed by CsCl gradient centrifugation followed by detection of the plasmid bands by gel electrophoresis Quantification of PCN was by scanning densitometry (Moser and Campbell, 1983) The basic procedure involves mixing equal concentrations of cells in which the PCN is to be measured, with a second cell culture containing a different plasmid of known PCN This addition of cells containing a reference plasmid, is to provide an internal standard Cleared cell lysates are then prepared and supercoiled plasmid DNAs are isolated by equilibrium centrifugation in CsCl-EtBr density gradients The plasmid DNAs are then fractionated by gel electrophoresis The relative amounts of plasmid DNA are quantified by scanning densitometry This procedure yielded a minimum value of PCN, and was employed to estimate the PCN of high copy number bacterial plasmids

Determination of the PCN in yeast cells has employed variations of the method developed by Projan et al (1983) *i e* direct fluorescence densitometry of EtBr stained electrophoretic gels One method for determining the copy number of a high copy number plasmid in yeast is to compare the relative intensity of ethidium bromide staining of a plasmid specific restriction fragment to a fragment corresponding to the repeated ribosomal DNA (rDNA) sequences in restriction digestions of total DNA, isolated from an appropriate transformed strain (Broach, 1983) By restricting total genomic yeast DNA with the enzyme *Kpn* I, a 9 0kb rDNA repeat fragment in 100 to 150 copies per cell results (Warner, 1989) Both rDNA restriction fragments and plasmid specific restriction fragments stand out sharply against the background of staining of random genomic DNA fragments, after fractionation of the digested DNA by electrophoresis on agarose gels The relative amount of plasmid DNA to rDNA can be quantified from densitometer scans of photographic negatives of the gel The absolute value of PCN is calculated from the relative intensities of the plasmid and rDNA bands, taking into account the relative sizes of the fragments which generated the bands and using the estimate of 100 to 150 copies of rDNA per haploid genome

Futcher and Cox (1984) modified this procedure to measure the PCN of 2μ circle-based plasmids in *S cerevisiae* Total genomic DNA was digested, run on agarose gels and then stained with ethidium bromide The most prominant bands seen were those due to plasmid restriction fragments and those due to the 2 7, 2 23 and 1 82kb *Eco* RI restriction fragments of rDNA By comparing the relative intensities of the peaks due to rDNA with those due to plasmid fragments, and by taking into account the relative sizes of the fragments causing the peaks, and using an estimate of 140 copies of rDNA per haploid genome (Schweizer et al , 1969), an estimate of the copy number of various plasmids was made The technique developed by Futcher and Cox (1984) has been employed by Zealey et al (1988) and more recently by Porro et al (1991), to measure the PCN of high copy number plasmids in yeasts

Relative to bacterial systems, yeast recombinant plasmids are of low copy number. The theoretically precise, accurate and more sensitive techniques of DNA hybridisation are most commonly employed to determine the PCN of yeast systems Gebaud and Guerineau (1980) used hybridisation in

solution to determine the amount of 2μ DNA in different yeast strains. A PCN of 10 of the *S. cerevisiae* expression vector pAAH5 was evaluated using Sandwich hybridisation (Korpela et al., 1987). Jayaram et al. (1983) investigating 2μ control and regulation employed Southern hybridisation to determine the PCN of 2μ derived plasmids.

The yeast integrating vector YIp5 was employed as a probe (Jayaram et al., 1983) which consists of pBR322 plus a 1.1kb fragment encoding one of the uracil biosynthetic genes (URA3). Total genomic yeast DNA was digested and fractionated by gel electrophoresis, transferred to nitrocellulose and probed with labelled YIp5. Two fragments appeared on the autoradiogram; one representing the single copy chromosomal URA3 gene and the other; the plasmid containing pBR322 sequences. The PCN was calculated from densitometer scans of the autoradiogram which yielded the relative intensities (*i.e.* quantities) of single copy URA3 DNA to multicopy plasmid DNA. It was shown using this procedure, that 2μ circle and 2μ-based yeast plasmids encode certain components required for high copy propagation. The existence of a copy control system for 2μ circle and 2μ-based plasmids that overides normal cellular restriction on plasmid replication and amplifies the plasmid when copy number is low, was hypothesised.

Bugeja et al. (1989) used several probes in their measurements of yeast PCN, during glucose limited chemostat culture. The probes included; YIP1 (Struhl et al., 1979) which is homologous to a plasmid DNA fragment and an area of yeast chromosomal DNA around the single copy histidine (HIS3) gene; pYIrA12 (Petes et al., 1978) which carries the multi-copy yeast ribosomal DNA (rDNA) genes. For 2μ plasmid copy numbers, blots were hybridised with YEP6 (Struhl et al., 1979), which is homologous to 2μ sequences and the chromosomal HIS3 gene. In the case of single copy HIS3 probes, it was found necessary to cut the bands from the filter and determine radioactivity by liquid scintillation counting to ensure reproducible results.

Instead of using a probe containing two different DNA fragments; one homologous to the plasmid DNA sequences and the other homologous to the reference chromosomal DNA sequences, Van der Aar et al. (1992) used the gene for phosphoglycerate kinase (PGK1) as a probe. The episomal plasmids being studied all contained the PGK1 gene, and the yeast genome also contains the same single copy PGK1 gene. Southern blotting and

hybridisation with a single stranded ^{32}P-labelled PGK-specific probe was performed after digestion of total yeast DNA The PCN was calculated from the relative intensity of the hybridising bands on the autoradiogram Van der Aar et al (1992) used this procedure in studies on the effects of phosphoglycerate kinase overproduction in *S cerevisiae* on cell physiology and plasmid stability

In conclusion , for bacterial systems with high plasmid content, methods employing CsCl gradients and/or gel electrophoresis have been employed to measure PCN EtBr-fluorescence densitometry has been used to calculate the PCN of such high copy number systems These methods are quick and relatively easy to perform Although not very accurate, with errors of up to 20%, (Projan et al , 1983), they have been employed to measure large PCN, where accuracy is not so critical Variations of these techniques have been successfully employed to determine the PCN of recombinant yeast systems where the PCN was high Where the PCN is low, more sensitive techniques such as DNA hybridisation have been employed While these techniques are tedious, time consuming and difficult to perform, they are accurate and sensitive enough to measure PCN of 1 or 2 per cell

Most methods which quantify PCN utilise reference DNA Techniques for measuring high copy number plasmids have reference DNAs of large denominations such as total chromosomal DNA (Projan et al , 1983), multicopy plasmids (Moser & Campbell, 1983) or multicopy rDNA (Futcher & Cox, 1984) Techniques for measuring low copy number plasmids have reference DNAs of low denominations such as single chromosomal markers or genes (Bugeja et al , 1989, Van der Aar et al , 1992)

1.4 THE INFLUENCE OF ENVIRONMENTAL CONDITIONS ON PLASMID STABILITY AND COPY NUMBER

Plasmid stability may be defined as the ability of transformed cells to maintain a multicopy plasmid unchanged during cell growth, manifesting its phenotypic characteristics (Caunt et al , 1988) Several factors influence the

instability of a plasmid and include structural instability of the plasmid itself, segregation of the plasmid to daughter cells during cell division, and the physiological consequences due to plasmid gene expression Environmental stresses also play a major role in plasmid stability and include such variables as host cell growth rate, nutritional limitations or requirements, oxygen concentration and cultivation conditions such as the presence or absence of selection pressure, pH and temperature This combination of factors may result in the instability of the plasmid becoming an important factor in the use of a particular recombinant strain in large scale production

Two major types of plasmid instability have been identified in recombinant cells Segregational instability is due to a failure to transmit the recombinant DNA to progeny cells, and structural instability is due to mutation or changes in the structure of the plasmid DNA caused by events such as recombination (Caunt et al , 1988) Of the various factors influencing plasmid stability, environmental stresses are perhaps the easiest to control and hence serve as one route to improving or optimising the plasmid stability for a particular host-plasmid bioreactor system The various environmental factors or growth conditions and their effect on plasmid stability are discussed below

1.4.1 Growth rate

The stability of a 2μ-based plasmid encoding β-galactosidase was measured in *S cerevisiae* grown in continuous culture in non-selective medium by Impoolsup et al (1989) The apparent stability of the plasmid was found to decrease with increasing growth rate An increase in segregational instability was the dominant factor in the increased plasmid loss Recombinant 2μ plasmids are thought to have defective amplification systems and at faster growth rates, the cells have less time to correct mistakes made during any one division (Futcher 1988) The PCN appeared to be stable

In contrast to the above, Kleinman et al (1986) and Futcher and Cox (1984) reported that the stability of 2μ-based plasmids increased with increasing

growth rate when the strain was grown in defined medium Plasmid instability per generation remained constant for a particular growth rate Kleinman et al (1986) reported that a possible reason for increased stability at higher growth rates was that cell division results in larger buds at fast growth rates, which increased the chances of transfer of plasmids to the daughter cell A major factor involved could be more intense selection under extreme nutrient limitation of low dilution rate conditions Parker and Di Biasio (1987) suggested that the amount of DNA repair may overburden the cells repair capabilities, resulting in plasmid loss due to DNA degradation This is possibly one reason why at low growth rates, where the enzymes essential for DNA repair are synthesised less often, the plamid stability is lower The results obtained indicated that the stability of the recombinant yeast system used could be improved at high dilution rates and also that increased plasmid expression levels resulted in decreased stability Walls and Gainer (1989), Da Silva and Bailey (1991), Porro et al (1991) also concluded that for recombinant yeasts grown in continuous culture, the plasmid loss experienced by the yeast population varies with dilution rate, with the greatest loss occuring at low dilution (growth) rates

Compared with bacterial systems, reported findings on the influence of growth rate on PCN in yeast systems are far fewer Porro et al (1991) reported that the PCN remained relatively unchanged at different dilution rates Bugeja et al (1989), investigating the stability and copy number of a 2μ-based yeast plasmid in *S cerevisiae* in glucose limited culture reported that the average PCN of the cells retaining the plasmid remained constant at approximately 50 in high dilution rate culture, whereas it rose to almost 600 in the low dilution rate culture The plasmid was considerably more stable at a high dilution rate than at a lower dilution rate

Growth rate effects in bacterial systems have been much more extensively studied Seo and Bailey (1986), investigating the influence of growth rate on plasmid stability and copy number in free cell *E coli* continuous culture, reported that plasmid content exhibits a maximum near the lowest dilution rates investigated and decreases as dilution rate increases Throughout this study plasmid stability remained high Reinikainen and Virkasarvi (1988), working with a recombinant *E coli* strain and Koizumi et al (1985), working with a recombinant *Bacillus* sp reported that, in contrast to Seo and Bailey (1986), the plasmid content increased with increasing growth

rate These observations of different trends show the absence of a general rule and indicate the importance of different plasmids, hosts and growth conditions in determining the PCN- plasmid stability - growth rate relationships

Siegel and Ryu (1985), De Taxis du Poet et al (1987), Sayadi et al (1989) concluded that, as a general observation in recombinant bacterial systems, the dilution rate of free cell continuous cultures influences both the plasmid stability and PCN Plasmid stability was found to be greatest at the highest dilution rates, where the PCN was lowest It was suggested that faulty partitioning of the plasmid is not the primary cause of the declining number of plasmid-containing cells in the population It seems likely that the increased plasmid content at lower dilution rates makes the plasmid-bearing cells less competitive compared with the plasmid free segregants, leading to a more rapid increase in the plasmid-free fraction of the population (Siegel and Ryu, 1985) Factors such as increased expression of cloned gene or increased plasmid content, which increase the metabolic burden placed on the host cell, result in a more rapid increase in the fraction of plasmid free cells in the population De Taxis du Poet et al (1987) reported that for the same range of dilution rates, the PCN differed depending on which medium was used PCN is not only influenced by growth rate, but can also be affected by the nature of the growth limiting nutrient, usually glucose for minimal media and which is not determined in more complex media (Kleinman et al , 1986), De Taxis du Poet et al , 1987, Reinikainen and Virkajarvi, 1988, Bugeja et al , 1989)

1.4.2 Nutritional limitations

The nutritional requirements of P+ and P- cells are different and the particular type of nutrients supplied may influence the plasmid stability and the PCN of the cells (Klemperer, 1979, Sayadi et al , 1989) In batch culture, *E coli* cells 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 P- cells was greater than P+ cells, and differed little in a simple salts medium where phosphate was not limiting Jones and Melling (1984) studied the stability of pBR322 and

related plasmids in *E coli* grown in chemostat culture Experiments were performed in glucose-, phosphate- or magnesium- limited chemostats The majority of the plasmids studied exhibited the highest stability under phosphate limitation The kinetics of plasmid loss varied from plasmid to plasmid and was influenced by the imposed nutrient limitation Sayadi et al (1989), investigating the effects of nutritional limitations on plasmid stability in *E coli,* reported that when the culture was Mg^{2+}-depleted, the rate of plasmid loss was accentuated and the percentrage of plasmid-containing cells declined Phosphate, ammonium and glucose limitations also caused plasmid instability, but the effect was not as pronounced as with magnesium depletion The PCN determined after batch culture depended on the nature of the growth limiting factors and increased for ammonium and glucose limitations and decreased when phosphate and magnesium were limited All nutritional limitations led to a decrease in biomass production and cloned gene product concentration Caulcott et al (1987), reported that the stability of a low-copy number plasmid was very low in batch culture, but was significantly stabilised by growth in continuous culture with phosphate, nitrogen or potassium limitation However, the plasmid was quite unstable when grown in continuous culture with sulphate limitation It appears that the small plasmid size and low PCN contributed to the reported findings

Matsui et al (1990) reported that the amino acids cystine, proline, threonine and glutamate influence plasmid stability in *E coli* Similary, Kuriyama et al (1992) reported that the addition of a high concentration of L-histidine was effective in decreasing the appearance of P- segregants in *S cerevisiae* , harbouring a 2μ-based plasmid, encoding the gene for Hepatitis B virus surface antigen Few studies on the direct effects of nutritional limitations on plasmid stability have been conducted in yeast, but the influence of nutritional limitations on cloned gene product production has been documented in several cases Coppella and Dhurjati (1989) found that the production of human EGF was highly dependent on the medium used, as a chemically defined, non-enriched medium had a significantly lower yield than did enriched or complex media PCN and the physiological state of the yeast cells did not change with media type

1.4.3 Oxygen concentration

Changes in the oxygen supply to micro-organisms can have effects on the growth rate and metabolic activity of the organism In continuous culture studies the plasmid stability of *S cerevisiae* was studied in complex media sparged with air, oxygen or nitrogen (Lee and Hassan, 1987) The plasmid contained the yeast gene for killer toxin production and the corresponding immunity gene Plasmid stability was highest with air sparging but expression of the cloned gene product was highest in an anaerobic environment (N_2 sparging)

The introduction of a dissolved oxygen (dO_2) shock caused a sharp decrease in plasmid stability in *S cerevisiae* (Caunt et al , 1989) and 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 brought about by dO_2 shock, rendered plasmid maintenance even more unfavourable An *E coli* culture in which the dO_2 was allowed to drop to 5% of maximum saturation and then increased to 100% saturation immediately, showed similar detrimental effects on plasmid stability (Hopkins et al , 1987) In batch culture in the presence or absence of selection pressure (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 to 1%, even in the presence of ampicillin

It seems likely that oxygen plays an important role in plasmid loss Changes in dO_2 are very important when large scale processes are considered Imperfect mixing or micromixing problems are more likely to occur in large scale bioreactors, producing pockets of medium where the dO_2 may be lower than the average bulk concentration (Huang et al , 1990)

1.4.4 Cultivation conditions

In cultivation of recombinant strains, optimal microbial growth, plasmid stability, PCN and heterologous protein production may all require different fermentation conditions Changes in chemical, physical or physiological

conditions may be necessary during fermentation *E coli*, harbouring a recombinant plasmid, was grown in a fermenter to study the effects of selected process parameters on the growth of the host strain and plasmid amplification with chloramphenciol treatment (Reinikainen et al , 1989) Eighteen fermentations were performed, in which the fermentation temperature, pH and turbidity of the culture at the onset of amplification were selected as the independent process parameters It was reported that PCN could be influenced by controlling fermentation temperature and pH The maximal copy number during growth and the optimal plasmid production were found to require fermentation conditions different from those needed for optimal host growth and cell division

Aiba and Koizumi (1984) showed that plasmid stability in *B stearothermophilus* was high at temperatures under 50°C, but decreased as the temperature was increased above 50°C Son et al (1987) studied the effect of temperature on the stability of a particular recombinant plasmid and expression of the cloned gene product, cellulase, in *Bacillus* strain They found that although both high PCN and efficient gene expression were favoured by higher temperatures, the plasmid was stably maintained only at temperatures below 30 °C in batch culture

Recombinant plasmids in yeasts and bacteria are unstable to varying degrees, unless a selective pressure is applied to the growing organisms With bacteria, antibiotics are commonly used where the host is senstive to the drug and the plasmid encodes resistance to the antibiotic (Reinikainen et al , 1989) Nutritional selection is employed with yeasts, where the host is auxotrophic for a particular amino-acid, brought about by a specific mutation in one of the genes involved in the synthesis of that amino acid The plasmid contains the unaltered gene that has been mutated in the host strain thereby compensating for the auxotrophic phenotype of the host Continued selection thus requires the use of minimal growth medium lacking the relevant amino acid (Impoolsup et al , 1989)

Selective media generally will not prevent segregational and structural plasmid loss, but will inhibit the growth of plasmid-free segregants Under nutritional selection it may be possible for plasmid-free cells to replicate, due to pools of the missing precursor or amino acid being present in the cell cytoplasm or in the bulk medium (Mason, 1991) If selection pressure is

extreme (*e g* high antibiotic concentration), then even low copy number plasmid containing cells will be inhibited, forcing a population of high PCN cells (Dennis et al , 1985) Growth media incorporating either nutritional or antibiotic selection usually maintain a high plasmid stability However, these media are unecomonical for large-scale processes

Futcher and Cox (1984) summarising on the wide variety of environmental factors which influence PCN and plasmid stability in yeasts, stated that,

> *"The wide variation in the behaviour of different plasmids in various hosts implies that the control of copy number and instability of plasmids is complex, finely balanced and easily upset It seems unlikely that any simple rule could explain the array of results obtained "*

Several strategies can be used to improve plasmid stability, such as selective methods, including maintaining selection for antibiotic resistance by use of antibiotics in the growth medium, complementation of host auxotrophy by incorporating auxotrophic markers on plasmid vectors, resistance to heavy metals such as copper and the incorporation of suicide proteins Non-selective methods include incorporation of partition loci to obtain controlled partitioning of the plasmid to the daughter cells during cell division (Kumar et al , 1991) As an alternative integrating vectors which are non-autonomously replicating plasmids that contain yeast DNA and transform yeast at low frequencies, may be utilised (Parent et al , 1985) The plasmid then undergoes integration into the chromosome by homologous recombination Other non-selective methods or strategies for improving plasmid stability include the separation of growth and gene expression phases by employing a temperature sensitive expression mutant or controlled promoter (Sledziewski et al , 1988) Once cells have grown to a high density, the temperature can be changed or the repressor removed, allowing gene expression The use of immobilisation of whole living recombinant cells is an attractive alternative for stabilising plasmids under non-selective growth conditions (De Taxis du Poet et al , 1987) The gel beads are seen to act as a reservoir of plasmid-containing cells and over-growth of plasmid-free segregants does not occur

1.5 IMMOBILISATION OF LIVING CELLS

Cells may be immobilised by a variety of methods including, (i) physical attachment to an inert carrier such as silica or glass, (ii) entrappment methods using various hydrogels, (iii) methods which cause aggregation or flocculation and (iv) physical containment of cells behind membranes (Nagashima et al , 1984) Entrapment methods are the most commonly employed means of immobilising cells In addition to natural polymers such as alginate, carrageenan, cellulose, gelatin and agar, synthetic polymers such as polyacrylamide, hydrophobic monomers and photo-cross-linkable resins have been used (Fukui and Tanaka, 1982)

A cell suspension is usually mixed with the viscous polymer and charged dropwise to a gelating solution where gel beads are formed The gel beads are incubated in nutrient media where the entrapped cells grow to form microcolomes Eventually after continued growth the microcolonies near the surface begin to merge together and reduce the diffusion of nutrients to and inhibitory products from the gel beads As a result, sharp radial gradients in growth rate, cell density and cell activity arise When the cell density near the bead surface reached the maximum that the carrier can accommodate, cells are pushed from the bead surface at a rate commensurate with continued immobilised cell growth (Monbouquette et al , 1990)

When cell immobilisation occurs naturally, as in waste water treatment and in vinegar fermentation, processes seem to benefit from it This tends to suggest that if other microorganisms could be encouraged to immobilise naturally, then they too could be exploited to advantage In fermentation processes where immobilisation has been induced, there is considerable evidence that the operational stability has been enhanced compared to free cell systems (Dervakos and Webb, 1991) Immobilisation influences the physiology and performance of cells through a number of parameters which may act in opposite directions Immobilised cells grow and exist in an environment, which is not only governed by intraparticle diffusion limitations, but also by physical boundaries Diffusion limitations may result in substrate limitation and product inhibition, while the physical boundaries allow the separation of extracellular metabolites which can result in increased productivities compared to free cell systems On the other

hand, the very nature of physical boundaries may restrict cell growth and therefore inhibit the metabolic activity of immobilised cells (Hahn-Haegerdal, 1990) The methods employed for immobilising cells are many and varied and have been reviewed extensively (Rosevear et al , 1987, Tampion and Tampion, 1987) Many of the techniques have been taken directly from enzyme immobilisation technology and the choice for a particular application is largely governed by the required physiological state of the cells and their application

1.5.1 Advantages

The merits of using immobilised cells in place of free cells in suspension depend on the characteristics of the particular system The major advantages include

1.5 1.1 *Enhanced biological stability*

The stability of immobilised cells may be substantially higher than that of freely suspended cells The storage and operational stability of viable cells can be greatly extended by immobilisation (Nagashima et al , 1984, Mugnier and Jung, 1985) When the cells are not viable, the increased catalytic stability of intracellular activities can be attributed to the protective effect of the immobilisation matrix against such physio-chemical challenges as temperature, pH, and organic solvents (Bajpai and Margaritis, 1987, Barros et al , 1987) In the case of viable non-growing or slow-growing cells, increased extracellular enzyme biosynthesis (which compensates for enzyme denaturation or deactiviation) the capability of cells to assimilate products released from lysed cells for growth and maintenance, and the possibility of reduced cell mass degradation rates, all contribute to the enhanced stability experienced by immobilised cells (Koshcheenko et al , 1983, Toda and Sato, 1985) When the cells are growing, a balance between cell growth and cell deactivation (or leakage) is maintained and becomes important in maintaining a constant metabolic activity over long periods of time Enhanced stability for recombinant immobilised cells in the absence of selection pressures has been ascribed to the decreased number of cell

divisions within the matrix, which prevents the appearance and take-over of plasmid free cells (de Taxis du Poet et al , 1986, Sayadi et al , 1989)

1.5.1.2 *High biomass concentration*

The relatively high biomass concentrations observed within immobilised cell particles can lead to enhanced reaction rates and thus, reduced reactor volumes Dhulster et al (1984) observed a very high *E coli* cell density within the cavities of κ-carrageenan gel of at least two orders of magnitude higher than the cell density in free cell suspension (1 7 x 10^{11} cells/ml-gel compared to 8 x 10^{8} cells/ml) The ability to operate chemostat cultures at dilution rates in excess of the organism's maximum specific growth rate becomes feasible with immobilised cells, thus increasing the volumetric productivities and reducing the risk of microbial contamination

1.5.1.3 *Improved product yields*

One of the most common factors for increased product yields in immobilised cell systems is through channelling the flow of energy away from cell mass synthesis In most of these systems excessive cell growth is undesirable and hence a medium is used such that growth rate and thus biomass is reduced (Foerberg et al , 1983) Other factors contributing to improved product yields include the extended use of the catalytic activities of the cells in the immobilised state and advantageous metabolic changes within the immobilised cell (Doran and Bailey, 1986, 1987)

1.5.1.4 *Integration with down stream processing*

The ability to treat cells as a discrete phase facilitates cell-liquid separation Recovery of cells for further processing or disposal can be achieved by draining the fermenter In this way the first downstream processing step is integrated with fermentation The recovery and purification of products may also be affected by cell immobilisation κ-Carrageenan immobilised cells of *Myxococcus xanthus* secreted foreign proteins out of the beads while native

proteins were predominantly retained within the gel The beads represented the first purification step for protein recovery (Younes et al , 1987)

1.5.1.5 *Advantageous partition effects*

The use of an appropriate matrix may aid in establishing a favourable microenvironment for the immoblished cells Yeast cells immobilised in κ-carrageenan produced a very high concentration of ethanol at a very high value of glucose conversion (Wada et al , 1981) It was suggested that the ethanol shunned the very polar gel phase and thus reduced the extent of product inhibition within the aggregate Fukui and Tanaka (1982) demonstrated the effectiveness of hydrophobic gels in bioconversions involving poorly water-soluble organic substrates such as steroids

1.5.1.6 *Cell proximity*

Reactions which are normally performed in a series of steps, can be carried out in the same vessel with significant productivity/cost advantages The conversion of starch to ethanol, which involves an enzymatic step followed by a fermentation step using co-immobilisation of glucoamylase and *S cerevisiase* is one such example (Fukushima and Yamade, 1984)

1.5 2 Metabolic changes

Immobilised cells show various modifications in physiology and biochemical composition compared to free cells Altered morphological forms as well as higher or lower metabolic activity, growth rates and product yields have been observed (Hilge-Rotmann and Rehm, 1990) The effects of the microenvironment on yeast metabolism were studied by Chen and Wu (1987), who found that yields of various metabolic products and the utilisation ratios of various specific amino acids differed from those of free cells in suspension culture

Disturbances in the growth pattern of immobilised cells due to contact with the immobilisation matrix or other cells were proposed by Doran and Bailey (1986), in order to explain various metabolic changes of yeast cells immobilised on crosslinked gelatin These metabolic changes included reduced biomass yields, decreased specific growth rates and increased rates of glucose consumption and ethanol production The observed changes were attributed to disturbances to the yeast cell cycle by cell attachment, which may have caused alterations in the normal pattern of bud development, DNA replication and synthesis of cell wall components Other studies observed that the intracellular pH of immobilised cells was lower than that of suspended cells (Galazzo and Bailey, 1989) Hilge-Rotmann and Rehm (1990) measured increased specific activities of the glycolytic enzymes hexokinase and phosphofructokinase in immobilised yeast cells and suggest that the alterations in physiology are apparently connected with growth of cells in aggregates Changes in the cell membrane permeability caused by interactions between cells and the immobilisation matrix may allow the passage of enzyme sustrates which cannot enter the normal cell Enhanced product excretion may also be a result of altered membrane permeability (Dervakos and Webb, 1991)

It has also been hypothesised that changes in cell morphology caused by immobilisation may be responsible for enhancement in the metabolic activities of cells Chlorotetracycline production by immobilised *Streptomyces aureofaciens* was correlated with the morphological development of cells in the gel (Mahmoud and Rehm, 1986) It was observed that micropellets were formed within alginate beads which were never found in free cell culture of the mycelia These micropellets may have enlarged the active biological surface and hence, may be the reason for the higher productive capacity of immobilised cells

Compounds present in an immobilisation matrix may contribute to a more favourable medium composition Immobilising *Saccharomyces bayanus* cells on celite or entrapment within κ-carrageenan resulted in higher ethanol production (Vieira et al , 1989) The increase was due to medium supplementation with the compounds present in the immobilisation supports Reduced water activity and/or oxygen deficiency was proposed by Mattiasson and Hahn-Haegerdal (1982) in order to explain changed yields or new metabolic behaviours in immobilised cells Changes in osmotic

pressure and surface tension were suggested by Vijayalakshmi et al (1979) in order to explain a three fold increase in the oxygen uptake rate of yeast cells when immobilised onto a cross-linked support

Finally, under conditions of mass transfer limitation, cells immobilised at certain locations within a matrix may be exposed to concentrations of substrates and products which promote a particular pathway for the flow of mass within the cell and therefore, the production of metabolites associated with this pathway It is possible that such conditions of concentration are not achievable in free cell culture due to the stoichiometrical or operational constraints imposed by the bioreactor type and mode of operation (Dervakos and Webb, 1991)

1.5.3 Operational, biological and diffusional considerations of cell immobilisation

Immobilisation of living cells may lead to operational, biological or diffusional problems The mechanical properties of the immobilisation carrier or matrix are important in determining the operational life-time of an immobilised cell bioreactor Mechanically stable carriers can withstand the pressure exerted by the entrapped growing cells, by intraparticle gas formation and are also able to resist harsh hydrodynamic conditions, as well as compaction in packed beds The choice of the bioreactor and its operating conditions may be restricted by the mechanical stability of the carrier Certain nutrient concentrations in media can cause disruption or dissolution of carriers, for example, the presence of cation chelating agents, such as phosphate, can dissolve calcium alginate gel beads (Dainty et al , 1986) Factors that have been reported to stabilise immobilisation carriers include the use of alternative counterions like Ba^{2+} and Sr^{2+} in alginate gelation (Tanaka and Irie, 1988, Ogbonna et al , 1989), or media supplementation with calcium ions

Cell leakage from the immobilisation matrix is usually undesirable Wang et al (1982) reported that increased shaking speed/agitation or local fluid shear greatly increased the rate of cell leakage Uncontrolled growth of immobilised cells, apart from decreasing product yields, may also result in a

number of operational problems Fermenter plugging is often observed as a result of cell overgrowth The stability and integrity of the carrier is also affected by the concentration of gaseous products within the matrix which may lead to the flotation and/or rupture of the beads This has been observed repeatedly in ethanol fermentations where carbon dioxide is one of the main products Other problems introduced by cell immobilisation include increased substrate limitations caused by diffusion resistances This is especially relevant in the case of oxygen, leading to many attempts to increase the oxygen supply to immobilised cells (Ghommidh et al , 1981, Adlercreutz and Mattaisson, 1982) Finally, as the product concentration increases with distance from the carrier surface, the effects of product inhibition become more pronounced and may eventually lead to a decrease in reaction rate

Cell immobilisation, with its many advantages over free cell systems, can lead to many changes in the behaviour of immobilised microorganisms An immobilised cell system is a heterogeneous system as there are at least two phases, a solid gel matrix and a liquid culture medium Cell distribution within the gel matrix is non uniform The cell population in terms of morphology, age and metabolic activity, may vary depending on nutrient supply within the matrix The immobilisation matrix itself may affect cell metabolism, cell division or budding The greatest benefit is derived in systems where some, but not too much growth occurs This may occur when growth is limited by diffusional effects Even so, viable immobilised cell systems can achieve remarkably high cell densities There is clearly a balance to be reached between increased volumetric productivity, due to greater numbers of cells, and reduced specific productivities due to increasingly severe diffusional limitations

1.6 IMMOBILISATION OF RECOMBINANT CELLS

1.6.1 Plasmid stability

High plasmid stability and biomass productivity are important factors for the development and commerical exploitation of genetically engineered cells It can be shown mathematically that immobilisation may stabilise a plasmid bearing population (Bailey and Ollis, 1986) By immobilising the recombinant cells in a solid matrix, such as calcium alginate or κ-carrageenan gel beads, growth of cells is usually retarded (De Taxis du Poet et al , 1987, Walls and Gainer, 1989) This slowing down of growth combined with the physical constraints of a solid matrix, restricts the number of cell divisions, which in turn reduces the chances of a plasmid containing cell losing its plasmid and hence its ability to produce the recombinant protein of interest Immobilisation stabilises the plasmid and greatly improves productivity Inloes et al (1983), Mosbach et al (1983), De Taxis du Poet et al (1987), Nasri et al (1987a) investigating immobilisation of whole living cells for recombinant protein production concluded that improved plasmid stability in immobilised cell systems cannot be explained by just a single factor In general, it is thought that higher plasmid stability is due, in part, to the absence of competition between P+ and P- cells within the gel matrix, or the restriction on the number of cell divisions within the matrix It may also be due in part to increased PCN Furthermore, the microenvironment of immobilised cells may also play an important role in higher plasmid stabilities

Plasmid bearing *E coli* were immobilised in κ-carrageenan beads and cultivated in a chemostat (De Taxis du Poet et al , 1986) Plasmid inheritability profiles were different for free and immobilised cell systems The initial percentages of plasmid-containing cells were equal After 18 generations, the percentages of P+ cells remaining were 21% and 65% respectively for free and immobilised cells Cells released from gel beads when grown in subsequent batch culture, did not maintain a better plasmid inheritability then that of free cells The mechanical properties of the gel beads allows only a limited number of cell divisions (10 to 16) in each clone of cells before the clone escapes from the gel bead This number of generations is not sufficient for the cells to appear within the cavities or

microcolonies In a free cell system P- segregants could only be detected after approximately 25 to 30 generations (Nasri et al , 1987a)

Nasri et al (1987a) extended this analysis to three genetically different *E coli* hosts, using the same plasmid as before, and again reported that, in the absence of selection pressure, the fraction of cells in the beads containing the plasmid was greater than in free cell cultures In free cell culture, all strains exhibited varying degrees of plasmid instability However, when immobilised, all three strains showed stable plasmid maintenance for the duration of the culture When P+ and P- cells were co-immobilised P- cells did not overun the culture, which illustrates the absence of competition between P- and P+ cells It also suggests that increased plasmid stability is not due to plasmid transfer between cells

Nasri et al (1987a, 1987b) suggested a mechanism for increased plasmid stability in immobilised recombinant *E coli* cells The gel beads may be regarded as a reservoir of cells carrying the plasmid, with the slower growing cells in the core of the bead exhibiting a higher percentage of P+ cells than the faster growing cells at the surface of the bead The recombinant cells grow in the outer layer for 10 to 16 generations, where they form microcolonies After prolonged incubation, the gels lose much of their mechanical rigidity and, consequently, a sequential cell leakage from the disrupted gel is observed This sequential cell leakage in turn exposes those cells in the inner sections of the gel to increased accessibility to oxygen and nutrients The lost recombinant cells are continuously being replenished by newer cells of higher plasmid stability The number of generations the cells undergo in a gel bead (10 to 16) is not sufficient for P- cells to segregate in large enough numbers and take over the culture In an attempt to relate this increased plasmid stability with PCN, it was hypothesised that as the microcolonies enlarge, growth of recombinant cells inside the microcolomes represents a period of gradually decreasing plasmid copy number up to a level at which instability becomes apparent It is thought that a gradient in both plasmid stability and PCN may exist throughout the gel beads

Walls and Gainer (1989, 1991) investigated the effects of immobilising a recombinant strain of *S cerevisiae* on the plasmid stability and specific productivity of secreted α-amylase The yeast cells were immobilised via

gluteraldehyde to the gelatin beads and used in both fluidised and packed bed configurations Plasmid stability increased for the immobilised cells during continuous culture at dilution rates both above and below washout Continuous free cell suspension cultures were not stable and rapidly lost the plasmid Immobilisation resulted in increases in both specific and volumetric productivity during continuous culture, with a packed bed design resulting in the highest specific productivity

In a study of (1,3) (1,4) β-glucanase production by a recombinant yeast strain immobilised in calcium alginate gel, Cahill et al (1990) reported a 20-fold increase in the enzyme productivity of a continuous immobilised cell bioreactor system compared with a free cell batch fermentation Immobilised plasmid stability was consistently higher than free cells The percentage of plasmid containing cells in the inner part of the beads, the outer part of the beads and those cells leaked from the matrix was measured It was concluded that the gel beads act as a pool of P+ cells, with the plasmid stability of cells in the bead core exceeding that of cells immobilised at the bead surface

1.6.2 Factors affecting plasmid retention, expression of cloned genes and biomass production

1.6 2.1 *Inoculum size*

Inoculum size or initial cell loading (ICL) influences the plasmid stability of immobilised cell cultures Berry et al (1988), investigating the effect of inoculum size on plasmid stability, selected different ICL in the range of 4 7 x 10^3 to 2 1 x 10^{10} cells/ml-gel With a low ICL, the number of cells in the gel beads increased dramatically, with giant colonies observed near the gel surface and in the centre of the gel beads This illustrated that with a low ICL, oxygen and nutrient mass transfer limitations were minimised In contrast, very high ICL resulted in dense growth limited to the outer 50 to 150 μm of the gel beads The cells of a high ICL undergo only 3 to 5 cell divisions to reach the same biomass which result after 26 generations from the low ICL Higher plasmid stability was obtained with high ICL because fewer cell divisions took place before the recombinant cells leaked from the

gel matrix In contrast, cells of a low ICL undergo many more cell divisions, increasing the probability of plasmid loss and increasing competition between P- cells and P+ cells, resulting in a lower proportion of immobilised cells bearing the plasmid Berry et al (1988) reported that expressed gene product activity increased with increasing ICL, as a result of slightly higher biomass concentration High cell densities within gel beads have resulted in increased production of proinsulin (Birnbaum et al , 1988) Several studies have shown that the inoculum size does not however, affect the final biomass concentration in the matrix which is primarily controlled by the mechanical properties of the matrix (Simon, 1989, Walsh, 1993)

1.6.2.2 *Matrix effects*

Few studies have been reported on the effect of using different matrices to immobilise recombinant cells As outlined in Section 1 5 1, the use of an appropriate matrix may aid in establishing a favourable microenvironment for immobilised cells, such as polar effects which may play a role in preventing product inhibition in ethanol production It was reported that ethanol shunned the very polar κ-carrageenan gel beads thus leading to high production yields (Wada et al , 1981) Hydrophobic gels have been used in bioconversions involving poorly water-soluble substrates such as steriods (Fukui and Tanaka, 1982) Certain compounds present in the immobilisation matrix may contribute to a more favourable medium composition or, the reduced water activity or oxygen deficiency in many immobilisation matrices can alter the metabolic behaviour of immobilised cells (Mattiasson and Hahn-Haegerdal, 1982)

Among the agarose, alginate and polyacrylamide gels tested for immobilisation of recombinant *E coli* cells for proinsulin production, agarose was reported to be the most effective, since it allowed rapid release of entrapped labelled insulin Alginate and polyacrylamide beads released only 15 to 20% of entrapped insulin (Mosbach et al , 1983) Birnbaum et al (1988) chose agarose to immobilise insulin producing cells due to its high porosity Agar, agarose, alginate, κ-carrageenan, polyacrylamide gels have all been used to immobilise recombinant microrganisms However, other matrices used for immobilisation of recombinant cells include silicone

polymer, silicone foam, cotton cloth and Cyclodex 1 microcarriers (for mammalian cell immobilisation) (Kumar and Schugerl, 1990)

Expressed gene product productivites have been shown to increase with increasing gel bead volume fraction (Berry et al , 1988, Birnbaum et al , 1988) By maintaining a high gel bead volume in the bioreactor, volumetric productivities were increased due to the increased total biomass in the bioreactor However, plasmid stability was independent of gel bead volume (Birnbaum et al , 1988) Proinsulin production was studied using different gel bead volumes In the case of beads prepared from low inoculum, the proinsulin concentration was found to increase with increasing number of gel beads in the medium (Birnbaum et al , 1988) Higher volumetric productivities were observed with an increased microcarrier concentration, for production of human immune interferon by recombinant mammalian cells Specific productivities remained constant for different microcarrier concenetrations (Smiley et al , 1989)

Furui and Yamashita (1985) reported that diffusion coefficients of solutes in immobilised *E coli* cells, decreased with increasing gel concentration Gosmann and Rehm (1986) reported that increasing alginate concentration led to a decrease in the specific oxygen uptake rate by immobilised cells The findings suggest that low gel concentrations minimise mass transfer limitations and maximise biomass production Berry et al (1988) studied the effects of κ-carrageenan concentration on growth and plasmid stability of recombinant *E coli* cells and reported a slight increase in biomass concentration with lower gel concentrations There was no significant difference in plasmid stability resulting from changes in gel concentration

1.6.2 3 *Nutritional limitations*

Although there are several reports on the effects of nutritional limitations on plasmid stability and cloned gene expression in free cell recombinant cultures (Coppella and Dhurjati, 1989, Turner et al , 1991), only a few studies have been reported on the effects of nutritional limitations of immobilised recombinant cells The effects of nutritional limitations on plasmid stability and cloned gene product activity in free and immobilised *E coli* cells were investigated by Sayadi et al (1989) Glucose, nitrogen,

phosphate, and especially magnesium limitation affected the plasmid stability in immobilised cell systems In all limited medium conditions, concentrations of plasmid-free cells increased in both free and immobilised cell systems, but the effect was considerably reduced in the latter Phosphate and magnesium limited conditions influenced the plasmid stability significantly In the case of magnesium depleted culture the plasmid was shown to be relatively unstable and a decrease in viable cell number during immobilised continuous culture was observed In contrast to free cell systems, the cloned gene product activity increased in immobilised cells under all culture conditions It was suggested that the greater plasmid instability was due to the increased requirement of plasmid-containing cells for several nutrients, particularly magnesium, compared to plasmid-free cells

Sode et al (1988) investigated the effect of medium cycling on cloned gene expression in immobilised yeast cell culture *S cerevisiae* cells were transformed with an α-peptide secreting vector and continuous production of the α-peptide was performed using immobilised recombinant cells in a column reactor In an attempt to improve productivity, the feed to the column was alternated between minimal and complex medium This medium cycling method resulted in 1 4 times higher α-peptide being produced during a 150 hour period compared with that achieved by feeding minimal medium

One of the few studies on nutritional requirements for immobilised yeast involved step changes in feed medium composition in a plug flow reactor employing immobilised *Kluyveromyces fragilis* cells, followed by a return to a basal medium (Chen et al , 1990) A defined medium containing a mixture of essential nutrients with an inorganic nitrogen source, maintained 90% of the productivity in the reactor compared to the usual complex medium This defined medium was unable to promote growth of the immobilised cells during reactor startup Experiments on reduced ammonium sulphate concentration in minimal medium and reduced yeast extract and peptone concentrations in complex medium, indicated that stable productivity could be maintained for extended periods in the absence of any nutrients with the exception of a few salts Productivity rates decreased by 35 to 65% as nitrogenous nutrients were eliminated and in the absence of limited nitrogenous nutrients, growth rates reduced by 75 to 95%

Nutritional deficiencies can lead to a decoupling of growth and productivity of immobilised yeast (Chen et al , 1990) Parascandola et al (1993) studied the effects of medium composition on the production of two yeast cell wall enzymes, invertase and acid phosphatase, in immobilised cells of *S cerevisiae* Rich and minimal media, various concentrations of carbon source (raffinose or glucose) and mode of nutrient supply (batch or continuous systems) were all investigated The above two studies were performed with non-recombinant yeast systems, but illustrate nevertheless possible changes in nutrient requirements upon immobilisation Further evidence of the influences of medium composition on plasmid stability in free cell systems has been included in section 1 4 2

1.6.2.4 *Incubation conditions*

Sayadi et al (1987) studied the effect of temperature on plasmid stability At 31^{0}C plasmid pTG201 was stable, but as the temperature increased to 42^{0}C, the stability decreased in both free and immobilised cell systems However, cells grown in the immobilised cell system showed higher plasmid stability during the temperature increase The production of cloned gene product increased with increasing temperature due to the presence of a temperature sensitive promoter system residing on the plasmid A two-stage continuous immobilised cell culture system was employed in order to separate the cell growth phase from production of cloned gene product In the first stage immobilised cells were grown in the repressed state at the lower temperature As the cells were immobilised, the plasmid stability was maintained high The leaked cells from the first stage were fed continuously into a second stage where the higher temperature derepressed the cells leading to higher productivity A very similar bioreactor system was developed by Berry et al (1990) using the same host and cloned gene product, catechol 2,3-dioxygenase Transcription of the gene was controlled by the *trp* promoter In the first stage of a two-stage chemostat an immobilised culture was grown in the presence of tryptophan which acted as repressor A high plasmid stability was maintained The cells released from the gel beads were continuously transferred into the second stage reactor where expression was induced by 3 β-indolyl acrylic acid leading to efficient production of catechol 2,3-dioxygenase

Proinsulin production was shown to be improved between 25 to 30 ^{0}C with an optium pH of 7 0 The stability of the plasmid in this optimal range was not reported (Birnbaum et al , 1988)

1.6.2.5 *Aeration and agitation*

By supplying pure oxygen to an immobilised cell reactor, Marin Iniesta et al (1988) reported that the immobilised cells maintained a higher plasmid stability The recombinant cells were shown to grow faster in air than in pure oxygen However, final biomass concentrations were higher from immobilised cells grown in pure oxygen Furthermore, beads cultured in pure oxygen contained larger microcolomes in the centre The PCN and proportion of plasmid containing cells were maintained with little change for more than 200 generations under pure oxygen supply Huang et al (1989) undertook similar studies and showed that increased plasmid stability with pure oxygen was the result of lower growth rates and decreased expression of cloned gene product In a further study by Huang et al (1990), the effect of agitation on plasmid stability, biomass production and cloned gene product activity was investigated The growth rate of immobilised cells was independent of agitation in complex medium and increased with increasing agitation in minimal medium This is probably due to increased external transport of oxygen and nutrients It was suggested that the growth rate of immobilised cells attained a maximum in complex medium due to nutrient effects which are possibly more important

Agitation rate could not exert any effect on immobilised cell growth rate even if external diffusion of nutrients was improved with higher agitation Increased agitation caused increases in shear stress at the bead surface, in which cells residing on the outer layer of the beads leaked more rapidly into the medium Plasmid stability decreased with increasing agitation rate both for free and immobilised cells in minimal medium, but varying the agitation rate did not appear to influence the plasmid stability of immobilised cells in complex medium This was explained by the fact that the rate of plasmid loss was constant, owing to a constant growth rate of cells in complex medium In conclusion, mild agitation is favourable for the maintenance of plasmid stability and higher immobilised biomass concentration and hence higher productivities

1.6.3 Heterologous protein production

Expression of cloned gene product by immobilised recombinant cells has been employed for an increasing number of hosts and vector systems Increased catechol 2,3-dioxygenase resulted after fermentations incorporating both immobilised and free *E coli* in a two-stage chemostat (Berry et al , 1990) A relatively high production rate of β-lactamase was maintained successfully for more than three weeks in a hollow fibre membrane bioreactor under continuous operation (Inloes et al , 1983) Recombinant *E coli* cells were immobilised in silicone foam for the production of the thermostable amylase and a five-fold increase in enzyme levels was achieved in a semi-continuous culture compared to free cell cultures (Oriel, 1988) Higher concentrations of human proinsulin could be produced with immobilisation of cells at higher cell concentrations (Birnbaum et al , 1988) Long term stability of a continuous immobilised *S cerevisiae* bioreactor system was reported by Karkare et al (1986) in which α-human chorionic gonadotropin was produced

CHAPTER TWO

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* 1-289a) was utilised in all experiments For convenience, the organism will be referred to simply as pJG317 or DBY746 (pJG317) throughout this work

2.2 PLASMIDS

Plasmid pJG317 is a derivative of pAAH5 (Ammerer, 1983)and encodes the endo β-(1,3) (1,4)-glucanase gene from *Bacillus subtilis* under the control of the yeast ADH1 promoter

Plasmid pRB149 which was a gift from Dr David Botstein, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusettes, USA, and consists of the 3 8kb yeast chromosomal β-actin gene (Ng and Abelson, 1980) cloned into the yeast integrating vector YIp5

2.3 BUFFERS AND SOLUTIONS

2.3.1 DNS (3,5 - Dinitrosalicylic acid) (for measuring β-glucanase activity and glucose concentration)

3,5 - Dinitrosalicylic acid	10 g/l
Potassium Sodium tartrate	300 g/l
Sodium hydroxide	16 g/l

2.3.2 PBS (Phosphate buffered saline) (Dialysing buffer for β-glucanase assay)

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

2.3.3 Solutions for the isolation of total genomic DNA from yeast

SPM	Sorbitol	0 9M
	Sodium phosphate buffer (pH7 5)	0 05M
	2-mercaptoethanol (freshly added)	0 14M
TE buffer	Tris Cl	10mM
	Na_2-EDTA	1mM
	pH	8 0

Lyticase — Lyticase enzyme (Sigma) was dissolved in 0 05M potassium phosphate buffer (pH 7 5) to a final concentration of 8,000 U/ml

RNase (DNase free) — 10 mg/ml RNase A (Sigma) was dissolved in 10mM Tris Cl (pH7 5), 15mM NaCl, heated to 100°C for 15 minutes, allowed to cool slowly to room temperature and stored at -20 °C

Phenol/chloroform mix — 100 g phenol was dissolved in 100 ml chloroform with 4 0 ml isoamylalcohol and 0 8 g 8-hydroxyquinolone The phenol mix was stored under 100mM Tris Cl (pH7 5) at 4°C in the dark

2.3.4 **Solutions for maxi-preps of plasmid DNA** (large-scale isolation of plasmid DNA from *E coli*)

Triton mix	20% Triton X-100		5 0 ml
	0 25M EDTA		12 5 ml
	1 0M Tris Cl (pH8 0)		2 5 ml
	H_2O	to	50 ml

2.3.5 **Solutions for mini-preps of plasmid DNA** (small-scale isolation of plasmid DNA from *E coli*)

Solution 1	0 5M Glucose	1 0 ml
	0 1M EDTA	1 0 ml
	1 0M Tris Cl (pH8 0)	0 25 ml
	H_20	7 75 ml
Solution 2	(Made freshly every month and stored at room temperature)	
	1 N NaOH	2 0 ml
	10% SDS	1 0 ml
	H_20	7 0 ml
Solution 3	(3M Potassium acetate, pH 4 8) To 60 ml of 5M potassium acetate, 11 5 ml of glacial acetic acid and 2 85 ml of H_20 were added	

2.3.6 **Buffers for DNA manipulations**

Restriction buffers	Buffers were supplied by BRL and were used according to the manufacturer's instructions

Ligation buffer	(10×)	Tris Cl (pH7 6)	200mM
		$MgCl_2$	100mM
		ATP	10mM
		DTT	100mM
		DTT was stored as a 500mM stock at -20°C and added separately to ligation reactions	
STE buffer		Tris Cl (pH 8 0)	10mM
		NaCl	100mM
		EDTA	10mM

2.3.7 Buffers for agarose gel electrophoresis

50× TAE	Tris	2 0M
	Glacial acetic acid	242 g
	0 5M EDTA	100 ml
	pH	8 0
5× Bromophenol blue	Bromophenol blue	0 25%(w/v)
	Ficoll (type 400)	25%

2.3.8 Solutions for isolation of DNA from agarose

Sodium iodide solution	90 8 g NaI was dissolved in 100 ml H_20 The solution was filtered through Whatman no 1 filter paper, 15 g of Na_2SO_4 was added and the solution stored at 4°C in the dark	
Ethanol wash solution	Ethanol	50%
	NaCl	100mM
	Tris HCl (pH7 5)	100mM
	EDTA	1mM
	This solution was stored at -20°C	

2.3.9 Solutions for Southern hybridisation

Depurination solution	HCl		0 25M
Denaturing solution	NaCl		87 66 g/l
	NaOH		20 g/l
Neutralising solution	NaCl		87 66 g/l
	1M Tris Cl	(pH 8 0)	500 ml
Transfer buffer (20× SSC)	NaCl		175 83 g/l
	Trisodium citrate		88 2 g/l
	pH		7 0
Denhardt's solution	Ficoll		10 g/l
	Polyvinylpyrrolidone		10 g/l
	BSA (Fraction V)		10 g/l

Prehybridisation solution
6× SSC
5× Denhardt's solution
0 5% SDS
100 μg/ml denatured salmon sperm DNA

Hybridisation solution as for prehybridisation solution except it contains in addition EDTA (0 1M) and the ^{32}P-labelled DNA

2.3.10 Solutions for Dig-DNA (non-radioactive) hybridisation

Hybridisation solution	5× SSC	
	Blocking reagent	1 0%
	N-lauroylsarcosine, Na-salt	0 1%
	SDS	0 02%

Buffer 1	(Maleate buffer)	Maleic acid	0 1M
		NaCl	0 15M
		pH	7 5

Buffer 2 (Blocking solution) 1% solution of blocking reagent dissolved in Buffer 1

Buffer 3	Tris Cl	0 1M
	NaCl	0 1M
	$MgCl_2$	0 05M
	pH	9 5
Colour Solution	NBT solution	45 μl
	X-phosphate solution	35 μl
	Buffer 3	10 ml

2.4 MEDIA

All media was sterilised by autoclaving at 121^{0}C (15 psi) for 15 minutes

2.4.1 Bacteriological

Luria Bertani (LB) medium contains the following components per litre

Tryptone (Oxoid)	10 0 g
NaCl	10 0 g
Yeast extract	5 0 g

2.4.2 Selective

Selective medium (minimal medium) contained the following components

Yeast nitrogen base (without amino acids)	6 7 g/l
Glucose	20 0g/l
Uracil	20 0 mg/l
Succinic acid	10 0mg/l
Sodium hydroxide	6 0 g/l

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 0g/l Oxoid bacteriological agar no 1 was added to the above for solid medium preparation

2.4.3 Non-selective

Non-selective medium yeast-extract-peptone-dextrose(YEPD) contained the following components

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

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

2.4.4 Salts

All salts media were supplemented with amino acids, as required

Amino Acid	*Supplement*	*Final concentration*
Histidine	1 ml/l of 2 0% soln	20 mg/l
Trypophan	5 ml/lof 0 4% soln	20 mg/l
Leucine	2 ml/l of 1 5% soln	30 mg/l
Uracil	8 ml/l of 0 25% soln	20 mg/l

All media were adjusted to pH 5 0 Succinic acid (10 mg/l) sodium hydroxide (6 g/l) buffering system was used

Salts A — Basal salts medium (Meth Microbiol , Vol 4) generally used for carbohydrate or nitrogen assimilation studies

Glucose	20 0 g/l
Yeast extract	1 0 g/l
KH_2PO_4	1 0 g/l
$MgSO_4\ 7H_2O$	0 5 g/l
$(NH_4)_2SO_4$	5 0 g/l

Salts B — Davis's yeast salt medium (Meth Microbiol , Vol 4)

Glucose	20 0g / 1
Yeast extract	1 0 g/l
KH_2PO_4	2 0 g/l
NaCl	2 0 g/l
$(NH_4)_2SO_4$	1 0 g/l
$(NH_4)_2NO_3$	1 0 g/l
NA_2HPO_4	4 0 g/l

Salts C	Non-nutritious salt medium (Chen et al , 1990)	
	Glucose	20 0 g/l
	$MgSO_4$ $7H_20$	0 5 g/l
	KH_2PO_4	1 36 g/l

2.4.5 Yeast Extract optimisation

This medium is a modification of YEPD (Section 2 4 2), where the peptone has been replaced by an inorganic nitrogen source $(NH_4)_2SO_4$ All media were adjusted to pH5 0 and contained the succinic acid sodium hydroxide buffering system The media were also supplemented with histidine (20 mg/l), tryptophan (20 mg/l), leucine (30mg/l) and uracil (20mg/l)

All media contained	Glucose	20 0 g/l
	$(NH_4)_2SO_4$	1 0 g/l

Media no 1 contained 0 g/l Yeast extract
Media no 2 contained 0 5 g/l Yeast extract
Media no 3 contained 1 0 g/l Yeast extract
Media no 4 contained 3 0 g/l Yeast extract
Media no 5 contained 6 0 g/l Yeast extract

2.4 6 Phosphate optimisation

This medium is a modification of the Basal Salts medium (Section 2 4 4) with varying concentrations of KH_2PO_4 As with previous optimisation media, it was supplemented with the amino acids tryptophan, leucine, uracil and histidine, and incorporated the succinic acid sodium hydroxide buffering system The media were adjusted to pH 5 0

All media contained	Glucose	20 0 g/l
	Yeast extract	1 0 g/l
	$(NH_4)_2SO_4$	5 0 g/l
	$MgSO_4\ 7H_20$	0 5 g/l

Media no 1 contained 0 g/l KH_2PO_4
Media no 2 contained 1 0 g/l KH_2PO_4
Media no 3 contained 2 5 g/l KH_2PO_4
Media no 4 contained 5 0 g/l KH_2PO_4
Media no 5 contained 10 0 g/l KH_2PO_4

2.4.7 Nitrogen optimisation

As with previous optimisation media, these media was supplemented with histidine, tryptophan, uracil and leucine, adjusted to pH 5 0 and incorporated the succinic acid sodium hydroxide buffering system

All media contained	Glucose	20 0 g/l
	Yeast Nitrogen base (without amino acids and ammonium sulphate)	1 7 g/l

Media no 1 contained 0 g/L $(NH_4)_2SO_4$
Media no 2 contained 2 5 g/L $(NH_4)_2SO_4$
Media no 3 contained 5 0 g/L $(NH_4)_2SO_4$
Media no 4 contained 10 0 g/L $(NH_4)_2SO_4$
Media no 5 contained 15 0 g/L $(NH_4)_2SO_4$

2.5 CULTIVATION CONDITIONS

The organism was maintained on selective medium agar plates grown for 72 hours at 30°C and then stored for one month at 4°C before transferring to fresh selective medium

2.5.1 Inoculum preparation

The yeast strain was inoculated from an agar plate into a 500 ml Erylenmeyer flask containing 200 ml selective medium This "seed" culture was incubated at 30 ^{0}C on an orbital shaker at 150 rpm for 24 to 48 hours Cell counts and/or Optical Density (OD_{600}) were measured and the required volume added to either flasks of media for free cell culture or a given quantity of sodium alginate for immobilisation

In the case of immobilised cell fermentations where high cell loads were required, the seed culture was centrifuged and the pellet resuspended in 10 ml of 0 01% peptone water and the cell concentration measured The required volume of concentrated cell suspension was then added to sodium alginate for immobilisation

2.5.2 Cell immobilisation

Cells were immobilised in 3% calcium or strontium alginate gel A solution of sodium alginate from *Laminaria Hyperborea* (71% guluronic acid and 29% Manuronic acid - Lennox Chemicals, Dublin, Ireland) was prepared at a concentration of 3 0 g per 70 ml The solution was boiled to help dissolution, and autoclaved at 121^{0}C for 15 minutes When cooled, a calculated amount of inoculum yeast suspension was added to the alginate solution to yield the desired initial cell loading (ICL) and the volume was adjusted to 100 ml using sterile distilled water

The suspension was mixed well and pumped through sterile silicone tubing (outside diameter 4 mm) The desired alginate volume/weight was expelled dropwise from a height of approximately 20 cm into sterile 0 2M $CaCl_2$ or 0 1M $SrCl_2$ Beads of 5 mm diameter were formed and allowed to harden for 2 hours in this solution at room temperature The beads were then washed in sterile 0 01% peptone water and inoculated into growth media All preparation work was carried out in a laminar flow cabinet

2.5.3 Shake flask cultivation

Shake flask cultures were performed in either (a) 200 ml of medium in 500 ml Erylenmeyer flasks or (b) 400 ml of medium in 1 litre Erylenmeyer flasks The flasks were incubated at 30°C, on an orbital shaker at 150 rpm

2.5.4 Batch fermentation

Batch fermentations were perfomed in a 2 Litre bioreactor (Life Sciences Laboratories Ltd , Luton, England) Operating parameters were as follows,

Termperature	30°C
pH (controlled at)	5 0
Agitation	400 rpm
Aeration	1 0 v/v/min
Liquid volume	1 3 l
pH controlled with	1M H_2SO_4 / 2M NaOH

Silicone anti-foam (7000 K) was added to medium at a rate of 0 1 ml per litre prior to autoclaving The fermentation vessel and medium were autoclaved at 121°C for 30 minutes Air supplied to the bioreactor was filtered through two 0 45μm Sartorius PTFE air filters Temperature was monitored using a resistance thermometer and 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 bioreactor was agitated by two sets of four-blade turbine impellers of diameter 45 mm and spaced 55 mm apart The vessel contained two 120 mm x 20 mm baffles The vessel diameter was 160mm

Some batch fermentations were performed in a 16 litre Microgen fermenter (New Brunswick Scientific Co Inc , Edison, N J , USA) using YEPD medium Operating parameters were as follows

Temperature	30°C
Agitation	600 rpm
Aeration	1 0 v/v/min

Liquid volume 10 0 l

Silicone antifoam (7000 K) was added to the medium at a dosage rate of 0 1ml per litre prior to autoclaving The fermentation vessel and medium were sterilised *in situ* at 121^{0}C for 30 minutes Temperature was monitored using a resistance thermometer and controlled to within 0 1 ^{0}C The vessel of diameter 220 mm was agitated by three sets of six-blade turbine impellers of diameter 720 mm and spaced 100 mm apart The vessel contained four 450mm x 22 mm baffles

2.5.5 Serial batch cultivation (free cells)

A flask of YEPD medium (pre-shaken at 30^{0}C) was inoculated to give a cell concentration of 10^4 cells/ml and incubated at 30^{0}C and 150 rpm for 24 hours From this a fresh pre-warmed, pre-aerated flask was inoculated to yield an initial cell concentration of 10^4cells/ml This flask was then incubated as above for 24 hours and so on Samples were taken at the end of each batch for enzyme activity, cell number, plasmid stability and PCN measurement

2.5.6 Repeated batch cultivation (Immobilised cells)

Immobilised cell gel beads were prepared and inoculated to a fresh flask of pre-warmed, pre-aerated medium which was either YEPD medium or in cases of pre-incubation, selective medium The beads were incubated for 24 hours at 30^{0}C and 120 rpm after which the medium was decanted The beads were then washed twice in sterile 0 01% peptone-water, and transferred to a flask containing fresh YEPD medium The number and types of media changes (*e g* salts media, low nitrogen media or high phosphate media instead of YEPD media) depended on the particular experiment The ratio of immobilised cells to medium volume and vessel volume also varied between the different experiments Samples of beads and medium were taken at the end of each batch for enzyme activity, cell concentration, plasmid stability and PCN

2.5.7 Continuous fermentation

Continuous fermentations were carried out in a 1 litre glass bioreactor with a working volume of 0 5 litre YEPD medium was used in all cases, and silicone anti-foam (7000k) was added to medium reservoirs at a dosage of 0 1 ml per litre prior to autoclaving The fermentation vessel (and medium), tubing, air filters, inlet and outlet connections, 8 litres of medium (contained in a 10 litre medium-reservoir vessel) were autoclaved at 121^{0}C for 30 minutes Air supplied to the bioreactor at a rate of 2 v/v/min, was filtered through two 0 45μm Sartorius PTFE air filters The pH of the medium was set at pH5 0 prior to autoclaving and was found not to fluctuate to any great extent during the course of the fermentation The medium in the bioreactor was agitated by a magnetic stirrer and the air was introduced via a ring sparger The chemostat apparatus (including medium reservoirs) was incubated in a 30^{0}C warm room Dilution rates are specified for each individual experiment The bioreactor was inoculated from a seed culture at a high cell density The cells were grown in batch mode for 16 to 20 hours until the desired cell concentration was reached The high initial cell density minimised the number of generations (and hence the fraction of P- cells) before stationary-phase was reached The fermentation was then switched to continuous mode Depending on the required dilution rate, the medium was supplied to the bioreactor by means of a Watson Marlow peristatic pump adjusted to give the desired flow rate The exit line was set at a level which ensured that a constant volume of 500 ml was maintained in the bioreactor and passed through a second Watson Marlow pump set at maximum flow rate Thus the flow rate was set by the feed pump while the exit pump acted to maintain the fermenter volume The flow rate was periodically checked by measuring the flow to the spent medium reservoir Samples were taken throughout the fermentation and analysed for enzyme activity, cell number, plasmid stability and PCN measurement

2.6 SAMPLING AND MEASUREMENT OF FERMENTATION PARAMETERS

2.6.1 Bead dissolution

2.6.1.1 *Total dissolution*

Three to five immobilised cell beads were weighed accurately before dissolving them in 10 to 15 ml of 10% sodium citrate solution at 30°C by shaking on an orbital shaker for 2 hours

2 6.1.2 *Multistep dissolution* (Boross et al , 1990, Walsh et al , 1993)

This technique was used to progressively dissolve successive layers from the surface of calcium alginate gel beads 20 beads were placed on Whatman no 1 filter paper, to remove any surface liquid and then weighed on an analytical balance The average diameter of a single gel bead could then be calculated The 20 beads were placed in 10 ml of dissolving solution (1 - 10% sodium citrate) and placed on a magnetic stirrer for 5 minutes at room temperature The gel beads were removed, dried on filter paper and weighed again, yielding a new average bead diameter The fraction of dissolving solution contained those cells which had been removed from the outer layer of the alginate beads The beads were placed in a fresh 10 ml fraction of dissolving solution and the process repeated In this way successive layers of alginate were dissolved and the cells present in those layers could be counted and assayed for plasmid stability and PCN In order to remove very thin layers of alginate, the dissolution time or concentration of sodium citrate was reduced When required, the procedure was scaled up (60 beads in 30 ml fractions of dissolving solution) so that sufficient cells could be harvested from the inner sections of the alginate beads, where cell numbers were about one tenth that of the outer sections

2.6.2 Cell enumeration

2.6.2.1 *Free cells*

Total cell counts were performed using a Neubauer haemocytometer All counts were performed in duplicate Standard curves of cell number versus OD_{600} were constructed for cells in YEPD and selective medium During the course of a fermentation in either of these media, the cell number could be obtained from the samples OD_{600}

2.6.2.2 *Immobilised cells*

To calculate total cell numbers per bead or per g alginate, total dissolutions were performed on the beads as in Section 2 6 1 1 The OD_{600} was measured and from a standard curve of yeast cell number in sodium citrate versus OD_{600} the cell number was calculated To calculate cell number per section of bead, multistep dissolutions were performed on the beads, as described in Section 2 6 1 2 The OD_{600} was measured and using the cell number versus OD_{600} standard curve the number of cells per section of bead could be calculated

2.6 3 Cell viability

Cell viability was measured using surface colony counts by the spread plate method Samples were serially diluted on 0 01% peptone Aliquots of 0 1 ml were dispersed evenly over the surface of YEPD agar plates using a sterile glass spreader Duplicate spread plates were made and the particular dilutions used were chosen so as to ensure that approximately 10, 100 and 1000 colonies per plate grew on incubation at 30°C for 48 hours Viability was expressed (on a percentage basis) as the number of viable cells divided by the total cell number (measured as outlined in Section 2 6 2 above)

2.6 4 Glucose analysis

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

2.6.5 β-Glucanase activity assay

The procedure used was that developed by Cahill (1990) 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 media 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 10mM 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

Samples were dialysed overnight at 4^{0}C, against phosphate buffered saline (PBS) with three changes of buffer at least three hours apart The enzyme substrate was freshly prepared before each assay A solution of 1% β-glucan was prepared in 0 1M phosphate buffer (pH 7 0) by boiling Upon cooling 1 ml quantities were aliquoted into the reaction test tubes using a Gilson P-1000 micro-pipette, for accurate dispensing without solution droplets adhering to the dispenser The tubes were allowed to attemperate for 10 minutes at 50^{0}C in a waterbath The reaction was initiated by adding enzyme and allowed to proceed for precisely 10 minutes Samples were quenched with 2 ml DNS

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^{0}C As the presence of the DNS reagent inhibited enzyme action, the blanks were a measure of the residual sugar in the samples after dialysis

After incubation, the 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 absorbance on a PYE unicam UV/VIS spectrophotometer at 540nm was measured Subtracting the blank values from the sample values, the concentration of glucose equivalents released from the β-glucan substrate solution could be calculated using glucose as the standard Units of β-glucanase activity are expressed as μg of glucose equivalents, released per minute per ml of sample

2.6.6 Plasmid stability measurement

2.6.6.1 *Free cells*

Samples were suitably diluted and spread plated so as to ensure that 10, 100 and 1000 colonies grew on duplicate sets of YEPD agar plates After incubation at 30^{0}C for 48 hours, 200 colonies were randomly replica plated onto selective media and onto YEPD agar plates 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

2.6.6.2 *Immobilised cells*

Plasmid stability of cells immobilised throughout the alginate gel beads was measured by performing total dissolutions on the beads using 2% sodium citrate (see Section 2 6 1 1) After suitable dilution the samples were spread plated onto YEPD plates and treated similarly to the free cell samples (Section 2 6 6 1) Plasmid stability of cells immobilised in different regions of the gel beads were measured by performing multistep dissolutions on the beads using 2% sodium citrate (see Section 2 6 1 2) Suitable dilutions were made and samples were spread on YEPD plates The samples were then treated similarly to free cell samples (Section 2 6 6 1)

2.7 RECOMBINANT DNA TECHNIQUES

2.7.1 Transformation of *E Coli*

Prepartion of competent cells

A flask containing 100 ml LB medium was inoculated with 1 0 ml of an overnight culture of JA221 and incubated at 37°C until OD_{600} of 0 3 to 0 4 was reached The culture was chilled on ice for 30 minutes before 10 ml was harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C The cells were washed in 5 ml ice-cold 50mM $MgCl_2$ and resuspended in 5 ml of ice-cold 50mM $CaCl_2$ After 30 minutes on ice, the cells were centrifuged as before and resuspended in 1 ml of 50mM $CaCl_2$

Transformation of competent cells

Plasmid DNA (up to 250ng) was added to 200μl aliquots of competent cells in an Eppendorf tube and incubated on ice for 1 hour The transformation mix was heat shocked by incubating at 42°C for exactly 2 minutes and returned to ice immediately A 0 8 ml quantity of LB medium was added and the samples incubated at 37°C for 1 hour to allow expression of the plasmid encoded antibiotic resistant marker gene Cells were plated on LB agar containing the appropriate selective antibiotic and incubated overnight at 37°C

2.7 2 Isolation of plasmid DNA from *E Coli*

2.7.2.1 *Small scale* (Birnboim and Doly 1979)

Cells were grown overnight at 37°C in LB medium incorporating the appropriate antibiotic Samples of 1 5 ml were pelleted by centrifugation and the supernatent discarded The pellet was resuspended in 100μl of Solution 1 and left on ice for 5 minutes Solution 2 (200μl) was then added and mixed by inversion Again the sample was left on ice for 10 minutes

150μl of Solution 3 was added, mixed by inversion and the sample left on ice for 5 minutes After centrifugation at 10,000 rpm for 5 minutes in order to pellet chromosomal DNA, 400μl of supernatent containing plasmid DNA was removed to a fresh tube An equal volume of phenol/chloroform was added and vortexed After centrifugation the aqueous layer was removed to a new tube and 0 8 ml of ice cold ethanol was added The sample was allowed to stand at room temperature for 10 minutes before the DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes The pellet was washed twice in 70% ethanol dried under vacuum, and resuspended in 30-50μl TE buffer containing 5μl RNase

2.7.2.2 *Large scale*

Cells from a late log phase culture (250 ml) were harvested by centrifugation and resuspended in sucrose (25% in 50mM Tris Cl, pH8 0) to a volume of 2 ml The cell suspension was transferred to a screw cap polycarbonate 50iTi tube (Beckman) and 0 4 ml of lysozyme (20 mg/ml in 0 25M Tris Cl, pH8 0) was added Upon incubation on ice for 5 minutes, 0 8 ml of 0 25M EDTA was added, the cells mixed and incubated on ice for a further 10 minutes A 3 2 ml quantity of Triton lysis mix (Section 2 3) was added, mixed and left on ice for 15 minutes When cell lysis was evident (indicated by an increase in viscosity of the mixture) the sample was centrifuged at 40,000 rpm for 40 minutes at 4^{0}C Exactly 6 9 g of caesium chloride was dissolved in the cleared lysate This solution was transferred to a Quick Seal polyallomer ultracentrifuge tube (Beckman) and 0 18 ml of an ethidium bromide solution (10 mg/ml) was added The total solution weight was adjusted to 14 1 g with 10mM EDTA, pH8 0 The tube was filled with mineral oil, heat sealed and centrifuged at 50,000 rpm for 24 hours at 18^{0}C to form a density gradient The plasmid band, which had separated from the chromosomal band was visualised by ultra violet transillumination and extracted from the tube using a sterile 18 gauge needle and 1 ml syringe Ethidium bromide was removed from the sample by extracting with isopropanol saturated with 20×SSC The DNA was dialysed against TE buffer (pH8 0) for at least 12 hours, with 3 changes of buffer to remove the caesium chloride

2.7.3 Isolation of total DNA from *S. cerevisiae*

This procedure is a modification of that of Cryer et al. (1975).

2.7.3.1 *Free cells*

A total of $1x10^9$ cells were harvested by centrifugation. The pellet was washed once with 10 ml distilled water and once with 2 ml SPM (Section 2.3). The cells were resuspended in 0.5 ml SPM. A 20 μl sample was taken and diluted in 5 ml distilled water and the OD_{600} measured. This value represented time zero OD value for evaluation of protoplasting efficiency. 100 μl of Lyticase enzyme was added and mixed. The sample was incubated at 30°C for 1-2 hours, or until a reduction of OD_{600} to 10-30% of the original OD_{600}, when a 20 μl sample was diluted in 5 ml distilled water. 100 μl Proteinase K, 50 μl of 1mM EDTA : 0.25M sodium phosphate (pH8.0) and 70 μl of 25% (w/v) SDS were added to the yeast spheroplasts. This lysis mixture was incubated for 30 minutes at 37°C. On completion of incubation, 500 μl of phenol/chloroform was added and mixed by inversion. The sample was left on ice for 10 minutes, before centrifugation at 12,000 rpm for 5 minutes. The upper aqueous phase was carefully removed using a wide bore pipette (cut-off tip of 1 ml blue Gilson Tip) to a fresh tube. The lower phase (including the interphase) was back extracted with 200 μl of TE buffer, centrifuged as before and the supernatent pooled with the aqueous phase of the previous extraction. Two further phenol/chloroform extractions were perfomed as above except with no back extraction of the interphase to ensure complete deproteinisation and extraction of lipids. The aqueous phase was extracted once with 700 μl chloroform at room temperature. 1 ml of ethanol (-20°C) was added and the sample mixed by inversion and incubated at -20°C for at least 2 hours. The total DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes. The DNA pellet was dried at 50°C washed twice with 70% ethanol and carefully dissolved overnight in 300 μl TE buffer. 15μl of RNase was added to the dissolved DNA, and incubated for 30 minutes at 37°C. The DNA solution was again deproteinised, precipitated with ethanol and washed in 70% ethanol as before. Finally, the DNA pellet was carefully dissolved in 100 μl TE buffer.

2.7.3.2 *Immobilised cells*

Depending on whether DNA was to be isolated from cells from whole alginate beads or cells located in different regions of the bead, total dissolutions (Section 2 6 1 1) or multistep dissolutions (Section 2 6 1 2) were carried out respectively A total of $1x10^9$ cells were harvested by centrifugation and washed three times in 20 ml distilled water The washed cells were treated as in Section 2 7 3 1 above

2.7.4 GENERAL DNA MANIPULATIONS

2.7.4.1 *Restriction digestions*

Restriction buffers were supplied by the manufacturers and used under the recommended conditions For restriction of 15-20 μl of total yeast DNA, 2 μl of restriction enzyme (Sal I) and 2 5 μl of the appropriate buffer were added The solution volume was then adjusted to 25 μl with sterile distilled water After 2 hours incubation at 37°C a further 1 μl of restriction enzyme was added and the mixture incubated overnight at 37°C

2.7.4 2 *Ethanol precipitations*

Precipitation of DNA was achieved by the addition of 1/10th the volume of 3M sodium acetate and 2 volumes of ethanol After incubation at -20°C for at least 1 hour, the DNA was recovered by centrifugation at 10,000 rpm for 20 minutes, the pellet washed once with 70% ethanol, dried under vacuum and dissolved in TE buffer

2.7.4.3 *Dephosphorylation of DNA*

1 unit of calf intestinal phosphatase (CIP) was added at the end of a restriction digest and the sample incubated at 37°C for 20 minutes The reaction was stopped by heating to 65°C for 5 minutes after the addition of

1/10th volume 10× STE, 10mM EDTA and 1/20th volume 10% SDS, (added to denature the enzyme) The DNA was extracted with phenol/chloroform and ethanol precipitated

2.7.4.4 *Ligation*

Ligations were performed in 1× ligation buffer using 1 unit of T4 DNA ligase Vector and insert DNAs were mixed in a ratio of approximately 1 10

2.7.5 Agarose gel electrophoresis

Agarose gels (0 9%) were prepared in a horizontal gel apparatus (Atto) and run at a constant voltage of 35 V in 1× TAE buffer overnight A concentration of 5× bromophenol blue dye was added to samples before loading Gels were stained in ethidium bromide (5 μg/ml) for 30 minutes, destained in water for 5 minutes and the DNA visualised by UV transillumination Gels were photographed using Kodak TriX-pan 35mm film which was developed with Kodak Universal developer (1/8 v/v in distilled water) for 10 minutes at 20^{0}C and fixed in Kodafix (1/4 v/v in distilled water) Printing was on Kodak F4 photographic paper using Kodak Dektol developer and Kodak Unifix fixer

2.7.6 Isolation of DNA fragments from agarose gels - gene clean procedure

After agarose gel eletrophoresis, the DNA band of interest was cut from the gel and weighed Two to three volumes of sodium iodide solution were added to the agarose The sample was vortexed and incubated at 55^{0}C for 5 minutes or until the agarose had completely dissolved A 2-5 μl quantity of glass bead slurry - Silica 325 mesh glass beads (which were prepared by T. Ryan, Dublin City University) was added, the sample vortexed and left on ice for 5 minutes The beads were centrifuged at 12,000 rpm for 5 seconds,

and the supernatent discarded The glass bead pellet was washed three times with 150 μl ice-cold ethanol wash solution, centrifuging for 5 seconds between each wash The DNA was eluted from the pelleted beads by resuspending in 10-20 μl TE buffer, and incubating for 3 minutes at 55°C The beads were then centrifuged and the eluate collected The elution step was repeated to ensure maximum recovery of DNA

2.7.7 Determination of DNA concentration

Serial 1 1 DNA dilutions were prepared by mixing 5 μl of DNA sample with 5μl of TE buffer After addition of 1 μl of bromophenol blue dye, 5 μl of each dilution was loaded onto a 1 2% agarose gel In the same way dilutions of DNA (of known concentration) were made and loaded onto the same gel Electrophoresis was performed at 100 V for 20 minutes The gel was then stained with ethidium bromide and destained in water The concentration of sample DNA was estimated by comparing the intensity of fluorescence of the samples with that of the standard DNA samples

2.7.8 Southern blotting - transfer of DNA to nitrocellulose filters

The technique used is that described by Southern (1975) and modified by Smith and Summers (1980), whereby nucleic acids are transferred bidirectionally by diffusion to produce two blots which are essentially identical

After electrophoresis, the gel was placed in several volumes of 0 25M HCl solution, gently agitated for 15 minutes at room temperature This incubation was performed twice The gel was next rinsed well with water and incubated in several volumes of denaturing solution with agitation for 1 hour The denaturing solution was decanted and the gel was soaked for 1 hour in neutralising solution again with constant shaking To transfer DNA fragments from agarose gels to nitrocellulose, a sheet of nitrocellulose (Schleicher and Schnell) presoaked in 6X SSC was placed on top of three sheets of Whatman 3MM filter paper saturated in the same buffer The

filter paper and nitrocellulose sheets were the same size as the gel Next the gel was place on top of the nitrocellulose sheet Another nitrocellulose sheet was added on top of the gel and three more buffer saturated Whatman 3MM filter papers were added A stack of paper towels (5-8 cm high) was placed underneath and on top of the filter papers and a light weight was placed on top to ensure even contact Transfer was allowed to proceed overnight During the first 2 hours of transfer the gel stack was inverted every 20 minutes to ensure even transfer The nitrocellulose sheets were then removed, soaked in 6X SSC at room temperature for 5 minutes, air dried, and baked for 2 hours at 80^{0}C

2.7.9 Prepartion of radioactive probe

Random primer labelling of probe DNA was performed using a "Prime a gene" kit (Promega), based on the method developed by Feinberg and Vogelstein (1983)
25ng of DNA was linearised and denatured by heating to 100^{0}C for 5 minutes and transferred to ice The reaction consisted of

5× labelling buffer	10 μl
Mixture of 3 unlabelled dNTPs	20 μl each
Denatured linear DNA template	25 ng
Acetylated BSA (1 mg/ml)	2 μl
[^{32}P] dATP (3,000 Ci/mM)	5 μl
Klenow polymerase	5 μl
Sterile water to	50 μl

The components were mixed gently and the reaction tube incubated at room temperature for 1 hour The reaction was terminated by heating to 100^{0}C for 2 minutes and chilled on ice 2μl of 0 5M EDTA was added and the labelled, denatured probe DNA was used directly for hybridisation to nitrocellulose filters

2.7.10 Preparation of non-radioactive probe

Using a "DIG -DNA labelling and detection kit" (Boehringer Mannheim), DNA was labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate The dUTP was linked via a spacer arm to the steroid hapten digoxigenin, 100 ng of DNA was linearised and denatured as in Section 2 7 9
The reaction consisted of

Freshly denatured probe DNA	100 ng
hexanucleotide mixture	2μl
dNTP labelling mixture	2μl
(containing dATP, dCTP, dGTP (1mMol/L), dTTP	
(0 65mMol/l), DIG-dUTP (0 35mMol/l)	
Klenow polymerase	2μl

The components were mixed gently and incubated at 37°C for at least 1 hour The reaction was quenched by addition of 2 μl EDTA (0 2 mol/l) and the labelled DNA precipitated by addition of 2 5 μl LiCl (4 mol/l) and 75 μl ethanol (-20°C) The mixture was left to stand at -20°C for 2 hours The DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes, washed with 70% ethanol, dried under vacuum and dissolved in 50 μl TE buffer Labelled DNA could be stored at -20°C or used directly for hybridisation to nitrocellulose filters

2 7.11 Hybridisation of Southern filters

2.7.11.1 *Radioactive labelled probe*

The baked filter was soaked in 6× SSC for 2 minutes and placed into a heat-sealable plastic bag Prehybridisation fluid (0 2 ml/cm^2 of nitrocellulose filter) pre-warmed to 68°C was added to the bag After squeezing air from the bag it was sealed and incubated at 68°C for 4 hours with constant shaking After prehybridisation, the fluid was replaced with hybridisation solution (50 μl/cm^2 filter) containing the ^{32}P- labelled denatured probe The

bag was again incubated at 68°C with constant shaking for 16-20 hours After hybridisation the filter was washed in the following solutions 5 minutes at room temperature with 2× SSC/0 5% SDS, 15 minutes at room temperature with 2× SSC/0 1% SDS, 2 hours at 68°C with 0 1× SSC/0 5% SDS, this last wash was repeated for a further 30 minutes The filter was air dried, placed in a heat sealable plastic bag and exposed to Kodak X-ray film After exposure, the film was developed and fixed using Kodak DX-80 developer and Kodak FX-40 X-ray liquid fixer

2.7.11.2 *Non-radioactive labelled probe*

The nitrocellulose filter was prehybridised in a sealed plastic bag in hybridisation solution (0 2 ml/cm² filter) at 68°C for at least 1 hour After prehybridisation the fluid was replaced with fresh hybridisation solution (25 µl/cm² filter) containing freshly denatured labelled probe DNA The filter was incubated at 68°C overnight The filter was then washed in the following solutions 2× 5 minutes at room temperature with 2× SSC/0 1% SDS and 2× 15 minutes at 68°C with 0 1× SSC/0 1% SDS

For immunological detection the "DIG-DNA labelling and detection" kit (Boehringer Mannheim) was used The filter was washed briefly in buffer 1 (maleate buffer) before incubating for 30 minutes in buffer 2 (blocking solution) The filter was incubated for a further 30 minutes in 20 ml of diluted antibody *i e* 1/5000 dilution or 150 mU/ml of anti digoxigenin AP conjugate Unbound antibody conjugate was removed by washing for 2× 15 minutes with 100 ml buffer 1 (maleate buffer) The filter was equilibrated for 2 minutes in 20 ml buffer 3 For colour development the filter was incubated in 10 ml colour solution in the dark The reaction was stopped by washing the filter in TE buffer for 5 minutes

2.8 DETERMINATION OF PLASMID COPY NUMBER

Total yeast DNA was isolated, digested with an appropriate restriction endonuclease, fractionated electrophoretically, transferred to nitrocellulose

and probed with pPC64 Plasmid pPC64 consists of 2 7kb puC-19 DNA plus a 1 2kb fragment of the yeast chromosomal β-actin gene Autoradiography was performed at room temperature using Kodak X-ray film

In each lane of the resultant autoradiogram,two fragments showed hybridisation, the chromosomal β-actin and the plasmid PJG317 fragment homologous to the pBR322 sequences of puC-19 The plasmid copy number was determined from the relative intensities of the single copy β-actin band to the multicopy plasmid band

Using a GS-300 Transmittance/Reflectance Scanning Densitometer coupled to a GS-350 Data System (Hoefer Scientific Instruments, San Francisco), the autoradiogram was scanned and the area under each peak measured The plasmid copy number (PCN) was calculated from the following equation

$$\mathbf{PCN} = \frac{D_{plas}}{D_{act}} \frac{M_{act}}{M_{plas}} \frac{1}{S}$$

where

D_{plas}	area under plasmid peak
D_{act}	area under β-actin peak
M_{plas}	2 7
M_{act}	1 2
S	percentage plasmid positive cells

M_{plas} and M_{act} are the sizes of the puC-19 and β-actin DNA fragments on the probe available for hybridisation and represent the degree of homology of probe to plasmid and to chromosomal DNA

Each autoradiogram contained a "reference" DNA sample (*i e* a sample of yeast total DNA of known copy number), to which all measurements of PCN were adjusted proportionally to ensure that the reference DNA sample maintained a constant PCN In this way, PCN was determined relative to the single copy chromosomal β-actin gene and to a reference sample of known copy number, allowing comparisons of PCN calculated from different autoradiograms

CHAPTER THREE

INTRODUCTION TO RESULTS

3.1 INTRODUCTION

One of the problems associated with using recombinant microorganisms in large scale fermentation is plasmid instability, where the plasmid encoding the gene for the formation of a product such as an extracellular enzyme is lost as the fermentation progresses (Imanaka and Aiba, 1981, Noack et al , 1981, Srienc et al , 1986) One way of alleviating this and hence greatly improving productivity is to slow down the growth of the organism This may be accomplished by immobilising the plasmid-containing cells in a solid matrix, such as a calcium alginate gel bead and using these gel beads as heterogenous catalysts in a bioreactor Immobilisation also allows continuous processing in the case of extracellular protein production and also has the advantage of retaining cells in the bioreactor (Dervakos and Webb, 1991) Figure 3 1 illustrates the increased plasmid stability and improved β-glucanase production by immobilised *S cerevisiae* cells, harbouring the plasmid pJG317, which contains the gene which encodes for the enzyme

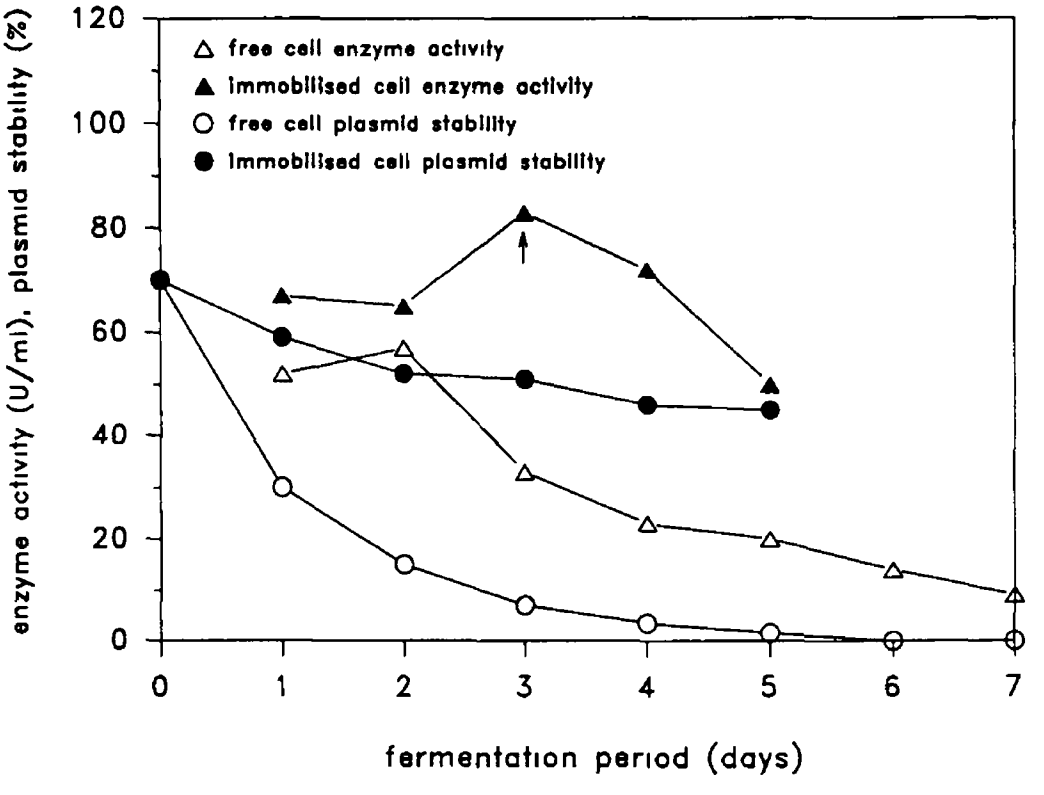


Figure 3 1 Plasmid stability and β-glucanase profiles in free and immobilised yeast cell fermentations
(arrow indicates increase in glucose concentration from 2 to 5%)

The improved plasmid stability in immobilised cell systems can be explained by a combination of the following reported findings (Mosbach et al , 1983, De Taxis du Poet et al , 1987, Nasri et al , 1987a, Dervakos and Webb, 1991)

(i) The restriction in the number of cell divisions that occur before the cells escape from the gel beads and are washed out of the reactor

(ii) The allowance of only a few generations (10 to 16) of plasmid minus (P-) cells to compete with plasmid containing (P+) cells before they detach from the gel

(iii) The absence of competition between P+ and P- cells inside the gel beads due to the mechanical properties of the gel bead system

(iv) The maintenance of stable PCN in the cells for longer periods

(v) The avoidance of genetic modifications or fluctuations which normally occur after prolonged growth of mircroorganisms in free cell cultures

(vi) P+ cells are continuously replenished by new P+ cells which reside deeper in the gel bead and whose growth was retarded by nutrient and/or oxygen depletion Thus gel beads can be a dynamic reservoir of concentrated P+ cells

It has been shown that cell growth within an immobilisation matrix is heterogeneously distributed due to diffusional resistances (Gosmann and Rehm, 1986, Godia et al , 1987, Marin-Iniesta et al , 1988) The surface microcolonies merge to form a dense layer of cells, reducing the diffusion of nutrients to and inhibitory products from the gel beads As a result sharp radial gradients in growth rate, cell density and cell activity arise (Monbouquette et al , 1990) It is likely that the heterogeneous distribution of cell growth is responsible for the gradient in plasmid stability observed within the beads Whether or not this increased stability is due, in part, to a higher PCN has yet to be conclusively established Indeed, for immobilised recombinant bacterial cells containing multicopy plasmids, it has been shown that immobilised cells contain a higher PCN than free cells (De Taxis du Poet et al , 1987, Sayadi et al , 1989) The measured PCN of immobilised cells was an average of high PCN corresponding to the internal cell population which grew at a low growth rate due to restrictions in oxygen and nutrients, and low PCN corresponding to the external cell population which had a higher growth rate closer to that of free cells It was hypothesised that

due to diffusion limitations, a gradient in cell growth rate and PCN exist The compartmentalisation of cell growth may also be responsible for the higher plasmid stabilities obtained in immobilised cell systems There are no reported cases of PCN measurements for immobilised yeast, although data from free cell recombinant yeast fermentations indicates that growth rate may influence PCN in certain cases (Bugeja et al , 1989)

A bioreactor system has been developed which employed immobilised recombinant *S cerevisiae* cells and continuously produced the enzyme endo (1,3) (1,4) β- glucanase with little sign of decline for periods of up to 240 hours in non selective medium The enzyme productivity of this reactor was over 20 fold that of a batch fermentation employing selective media (Cahill et al , 1990) The percentage of P+ cells in the core of the beads, the bead surface and those cells leaked from the matrix was measured by making cross sections of the gel beads and isolating cells from the appropriate sections A gradient of plasmid containing cells was found to exist throughout the gel beads with the cells on the surface having a lower stability than cells in the bead core

Harvesting cells from specific locations wthin a gel bead is difficult Most procedures employed for measuring PCN e g ethidium bromide fluorescence densitometry, CsCl gradient centrifugation and DNA hybridisation, are tedious and time consuming For these reasons, the PCN of cells at different locations throughout the gel bead (such as the inner core or the outer layer) has not been directly measured

This study set out to develop, test and optimise an accurate and reproducible procedure for measuring the PCN of recombinant yeast The recombinant system chosen was the system of Cahill et al (1990) *S cerevisiae* DBY746, transformed with the plasmid pJG317 acted as the model Plasmid pJG317 is 13kb in size and carries the gene encoding the enzyme endo (1,3) (1,4) β-glucanase pJG317 is a derivative of plasmid pAAH5 except that it carries the glucanase gene from *Bacillus subtilis* under the control of ADH1 promoter The PCN of pAAH5 was measured as 10 (Korpela et al , 1987) and it is reasonable to assume that pJG317 is also a low copy number plasmid For this reason a procedure for measuring the PCN had to be both accurate and sensitive After rejecting the ethidium

bromide fluorescence technique, a procedure based on Southern hybridisation was developed

The technique was used to investigate the influence of growth rate on PCN and plasmid stability in free cell yeast fermentations Free cell growth rates, plasmid stabilities, PCN and enzyme specific productivities were established The growth rate of immobilised cells depends on their location in the gel beads Consequently the PCN may vary, giving rise to a radial gradient in PCN in immobilised cells Using recently developed and refined multistep dissolution techniques (Walsh et al , 1993) the plasmid stability gradients obtained by Cahill et al (1990) were confirmed and the PCN of cells was directly measured at three different locations throughout the gel beads Finally, the effect of environmental growth conditions on plasmid stability, PCN and β-glucanase productivity in immobilised and free cells were investigated

CHAPTER FOUR

DEVELOPMENT OF A REPRODUCIBLE AND ACCURATE PROCEDURE FOR MEASURING PLASMID COPY NUMBER

4.1 ELEMENTARY WORK ASSOCIATED WITH USING SOUTHERN HYBRIDISATION AS A MEANS FOR MEASURING PLASMID COPY NUMBER

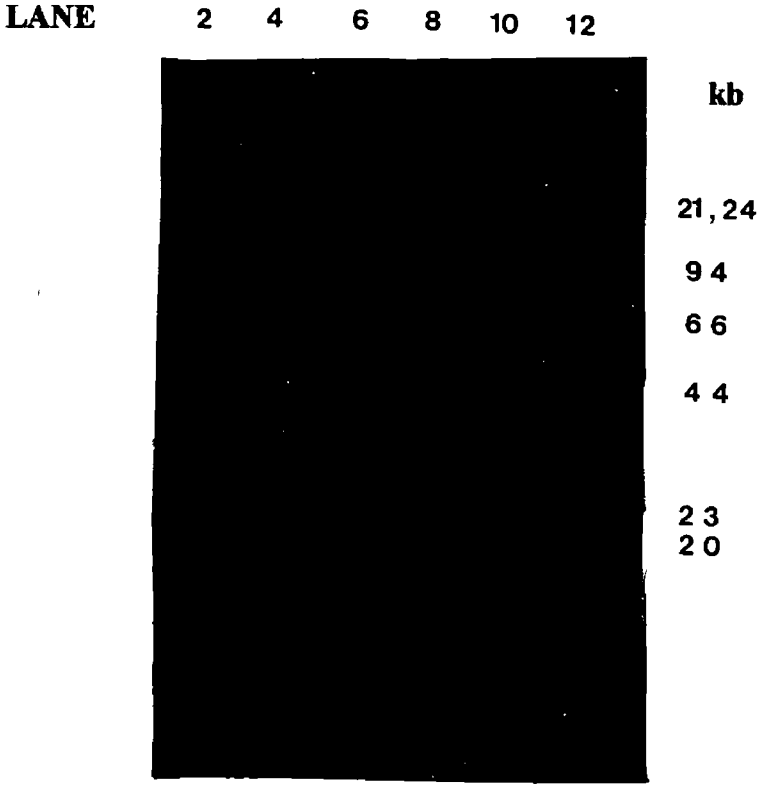
4.1.1 Restriction map of plasmid pJG317

Many methods used to measure PCN involve restricting the chromosomal and plasmid DNA A detailed restriction map of the plasmid is necessary to provide information on the sizes and numbers of DNA fragments obtained upon restriction with a particular enzyme

Using single and double restriction digestion of plasmid pJG317 DNA, a comprehensive restriction map of the plasmid was established Figures 4 1 and 4 2 are the restriction patterns after gel electrophoresis Table 4 1 is a summary of the fragment sizes determined relative to λ DNA size markers Finally Figure 4 3 represents a restriction map of plasmid pJG317

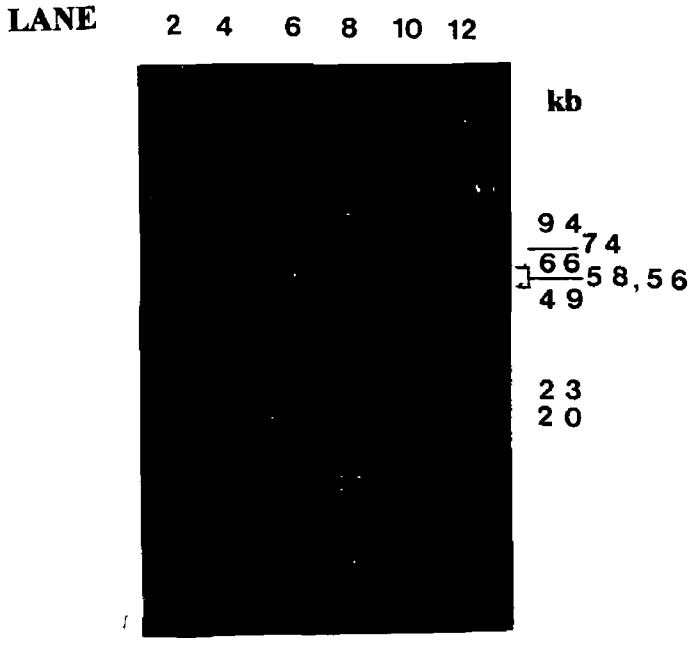
Table 4 1 DNA fragment sizes resulting from restriction digestion of pJG317 DNA

Restriction Enzyme	Fragment size (kb)
EcoR I	11 5, 2 2
Sal I	6 6, 4 7, 1 1, 0 5
Pst I	4 5, 4 0, 3 6, 1 0
Kpn I	11 2, 2 5
Bam HI	11 2, 1 6, 1 0
Hind III	12 3, 0 8
Xba I	13 2
EcoR I + Kpn I	9 1, 4 8, 4 4, 2 1, 1 8
EcoR I + Sal I	4 7, 3 7, 2 2, 1 1, <1 0
Pst I + Sal I	6 6, 4 7, 4 2, 2 8, 2 6, 1 3, 1 0
Kpn I+ Xba I	9 8, 2 5, 1 4
Xba I + Sal I	6 5, 2 5, 2 3, 1 1



LANE	SAMPLE
1	λ Hind III + EcoR I
2	λ EcoR I
3	λ Hind III
4	Bam HI
5	Kpn I
6	Xba I
7	Sal I
8	Pst I
9	Hind III
10	EcoR I
11	pJG317 uncut
12	λ EcoR I
13	λ Hind III

Figure 4 1 Restriction digestion of plasmid pJG317



LANE	SAMPLE
1	λ Hind III + EcoR I
2	pJG317 uncut
3	EcoR I + Kpn I
4	Kpn I
5	Kpn I +Xba I
6	Pst I
7	Pst I + Sal I
8	Sal I
9	Sal I + EcoR I
10	Xba I + Sal I
11	EcoR I
12	EcoR I
13	λ Hind III + E coR I

Figure 4 2 Single and double restriction digestions of plasmid pJG317

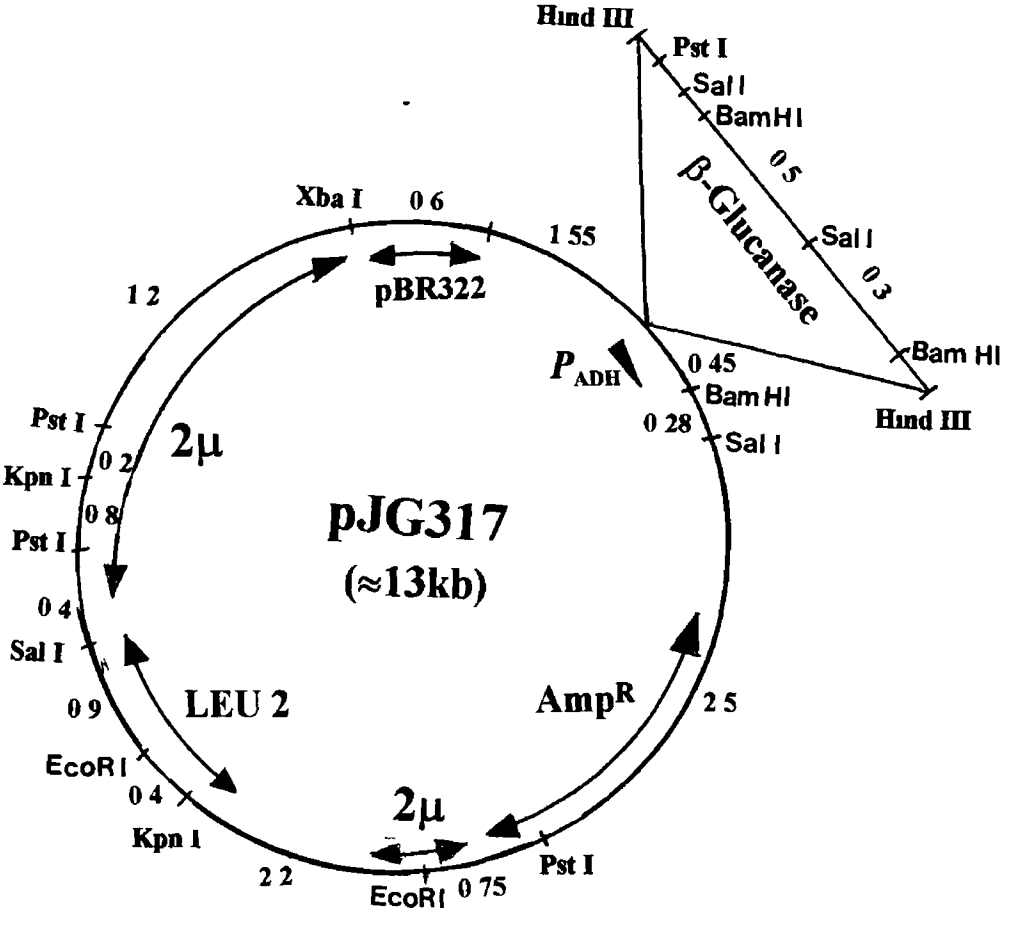


Figure 4 3 Schematic diagram of plasmid pJG317 restriction sites (EcoR I, Hind III, Kpn I, Pst I, Bam HI, Sal I)

4.1.2 Optimisation of procedures

The procedure used for isolating total DNA was that of Cryer et al (1975) However, because of the difficulty in restricting yeast DNA the procedure was modified by incorporating extra phenol/chloroform extractions, longer ethanol precipitations at -20°C and by adding a step which involved washing the DNA pellet with 70% ethanol The procedure outlined in Section 2 7 3 incorporates the modification steps

The cell lysis procedure used Lyticase (Sigma) instead of Zymolyase This step was optimised with respect to quantity of enzyme used, incubation time and quantity of cells used Optimisation of the DNA isolation procedure ensured that a high yield of clean DNA could be obtained The measurement of PCN by DNA hybridisation required complete digestion of total yeast DNA The restriction conditions were modified to include extra restriction enzyme and overnight incubations The fully modified DNA isolation procedure (including cell lysis) is outlined in Section 2 7 3 and the optimised restriction conditions are outlined in Section 2 7 4

4.2 EVALUATION OF THE SUITABILITY OF SEVERAL PROBES AND CHARACTERISATION OF DBY746 FOR THE PRESENCE OF 2μ DNA

To measure the PCN by DNA hybridisation a probe must be constructed in such a way that it contains two essential fragments of DNA One of these fragments must bind or hybridise to the plasmid DNA while the other fragment must bind to some reference DNA which usually resides on the chromosome Examples of such reference DNA fragments include the single copy Uracil gene (Jayaram et al , 1983), the β-actin gene (Apostol and Greer, 1988) and the histidine gene (Bugeja et al , 1989) or the multicopy ribosomal DNA (Broach, 1983) PCN is calculated from the intensity of hybridisation to the plasmid specific fragment, relative to the intensity of hybridisation to the reference DNA

The vector DNA pUC-19 was chosen as the plasmid specific portion of the probe since both pUC-19 and pJG317 contain homologous pBR322 DNA sequences Also by inserting a reference DNA fragment into the vector pUC-19 and cloning the resultant plasmid a plentiful supply of probe DNA is ensured

The Uracil (URA3) gene and the β-actin gene which both reside as single copies on the yeast chromosome were chosen as reference DNA fragments Finally the particular *Saccharomyces cerevisiae* DBY746 strain being used was characterised by confirming the presence of 2μ DNA and determining the 2μ restriction map

4.2.1 Restriction analysis of β-actin and URA3 regions within the yeast genome

Measuring PCN by Southern hybridisation partly involves restricting total genomic DNA and later hybridising a probe to one particular segment of the genomic DNA *i e* the reference DNA fragment To assess the potential for β-actin or URA 3 to be used in the probe and as the reference DNA fragment, the number and sizes of the restriction fragments relative to those of pJG317 DNA had to be determined

Total yeast DNA was isolated from the parent strain DBY746 and restricted with the enzymes, EcoR I, Hind III, Kpn I, Sal I, Pst I, Xba I After gel eletrophoresis the DNA was immobilised on nitrocellulose filters (Section 2 7 7) which were probed with radioactively labelled β-actin DNA or URA3 DNA

Both the β-actin and URA3 genes were obtained by restriction digestion of the plasmid pRB149 with EcoR I (Figure 4 4) This plasmid was a gift from Dr David Botstein (Shortle et al , 1982) and consists of the 3 8kb β-actin gene inserted into the yeast integrating vector YIp-5 EcoR I restriction of pRB149 DNA yielded two fragments a 3 8kb β-actin gene fragment and the 5 5kb YIp-5 fragment which were separated by gel electrophoresis (Section 2 7 5) and purified by the gene clean procedure (Section 2 7 6)

The 3 8kb β-actin DNA fragment contained the whole β-actin gene (Ng and Abelson, 1980) and was labelled with ^{32}P-dATP (Section 2 7 9) and hybridised to the nitrocellulose filters containing restricted DBY746 DNA Figure 4 5 is the resultant autoradiogram, showing restricted DNA fragments of the DBY746 chromosomal β-actin single copy gene (Ng and Abelson, 1980) Table 4 2 shows the β-actin restriction fragment sizes obtained from the autoradiogram and compares them with the predicted restriction fragments sizes of Ng and Abelson (1980) It may be concluded that the restriction fragment sizes obtained agree very closely to those predicted of Ng and Abelson

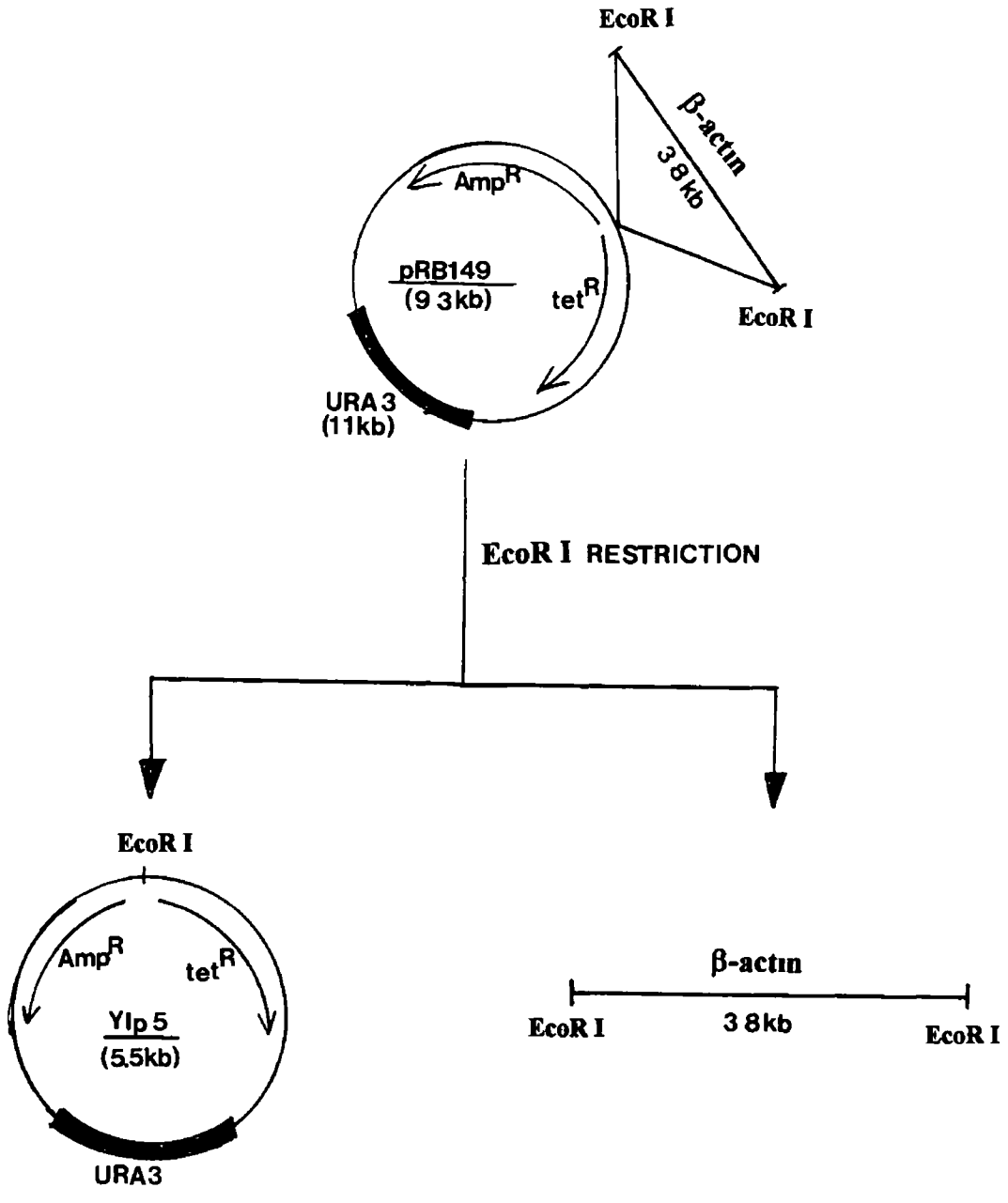


Figure 4 4 Schematic diagram of plasmid pRB149 and restriction to yield two probes (i) 3 8kb β-actin gene and (ii) 5 5kb YIp5 vector containing a 1 1kb URA3 DNA fragment

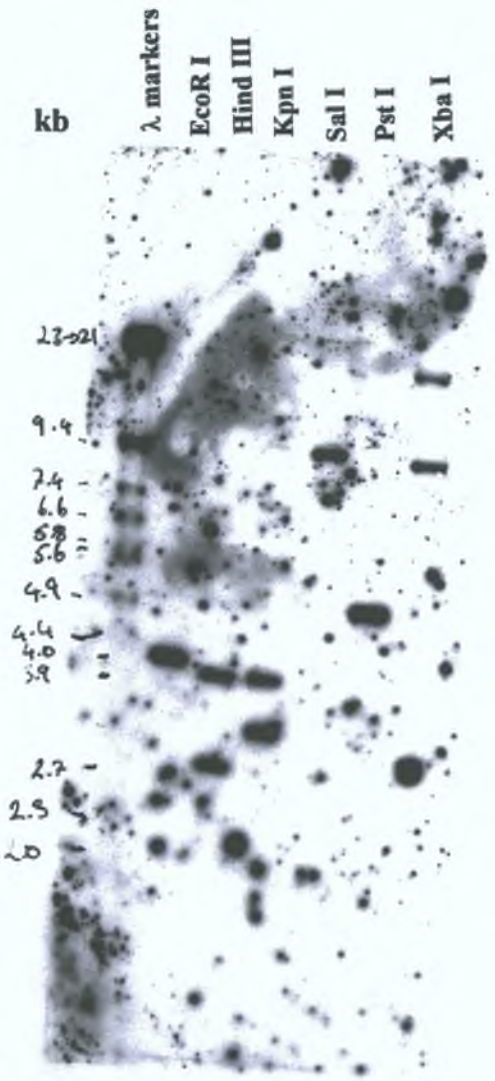


Figure 4.5 Autoradiogram of the restriction fragments of the single copy chromosomal β-actin gene of DBY746

Table 4 2 *Restriction pattern of chromosomal β-actin gene fragments from autogradiogram in Figure 4 5 (#1) and that of Ng and Abelson (1980) (#2)*

Restriction Enzyme	Fragment size #1 (kb)	Fragment size #2 (kb)
HindIII	3 9 & 2 7	3 7 &2 5
E coR I	4 0	3 8
Sal I	9 4	>3 8
Xba I	9 4 & 20	>3 8
Kpn I	3 9 & 3 0	>3 8 & >2 3
Pst I	4 8	4 6

Note > implies that the fragment sizes are greater than the value listed As the values taken from the literature were from diagrams/restriction maps the exact sizes are unknown

The 5 5kb YIp5 DNA fragment was isolated from pRB149 along with the β-actin DNA fragment and also used as a probe and hybridised to restricted DBY746 genomic DNA YIp5 consists of pBR322 DNA plus a 1 1kb fragment spanning the yeast URA3 gene (Struhl et al , 1979) As no pJG317 DNA was present in the DBY746 genomic DNA, only the URA3 DNA fragment hybridised to DBY746 URA3 gene fragments (Figure 4 6) Table 4 3 compares the restriction fragment sizes of those obtained from the autoradiogram with those of Struhl et al (1979)

Table 4 3 *Restriction pattern of chromosomal URA3 gene fragments from autoradiogram in Figure 4 6 (#1) and that of Struhl et al (1979) (#2)*

Restriction Enzyme	Fragment size #1 (kb)	Fragment size #2 (kb)
HindIII	4 0 & 2 2	(5 0) 4 1 & 2 3
E coR I	10 0 & 8 0	>3 3 & >3 3
Sal I	23 0 & 6 6	>4 3 & >2 1
Xba I	20 0	>5 9
Kpn I	7 0 & 8 0	>5 9
Pst I	7 4 & <1 0	>4 8 & 0 6

Note > implies that the fragment sizes are greater than the value listed As the values taken from the literature were from diagrams/restriction maps the exact sizes are unknown

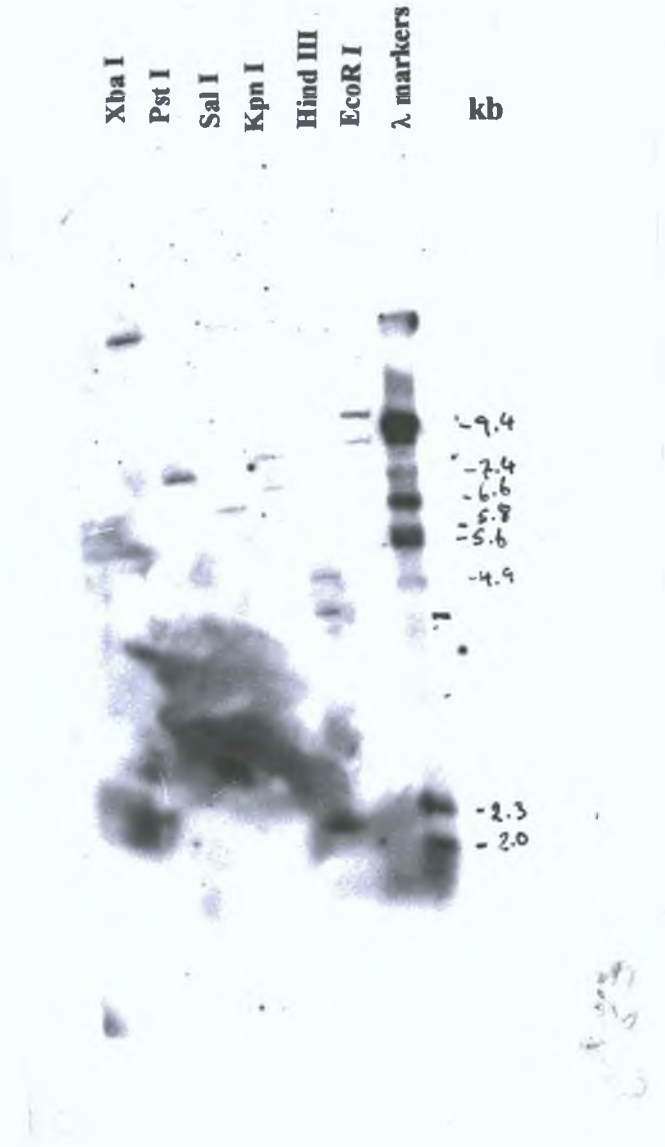


Figure 4.6 Autoradiogram of the restriction fragments of the single copy chromosomal URA3 gene of DBY746.

A comparison of both the β-actin and URA 3 regions of the DBY746 genome, with respect to the number of fragments and the particular sizes of the fragments resulting from restriction digestion, led to the conclusion that β-actin had the greatest potential as the reference portion of a probe to be employed for measuring PCN

4.2.2 Characterisation of DBY746 for the presence of 2μ DNA

The 2μ circle is a 6318bp double stranded DNA plasmid present in most *Saccharomyces* strains (including DBY746) at 60 to 100 copies per haploid genome (Clarke-Walker and Miklos, 1974, Hartley and Donelson 1980) As complete restriction of DBY746 genome is a prerequisite for PCN determination by DNA hybridisation in order to separate out the reference DNA fragment from the total chromosomal DNA, it was necessary to determine the position of the 2μ restriction fragments relative to restricted chromosomal and pJG317 DNA fragments In this way any interference due to the presence of 2μ restriction fragments on probe hybridisation or PCN calculation can be predicted and/or prevented

Total yeast DNA was isolated restricted electrophoresed and blot-transferred to nitrocellulose filters as described in Section 4 2 1 By restricting plasmid pJG317 with Xba I and Kpn I a 1 4kb 2μ DNA fragment was cut out and separated by gel eletrophoresis and purified by the gene clean procedure (Section 2 7 6), labelled (Section 2 7 9) and hybridised to the nitrocellulose filter containing restricted genomic DBY746 DNA (Figure 4 7)

The restriction fragments obtained agree very well with those of Jayarem et al (1983) The different intensity of bands (*i e* faint and dark) are due to different degrees of homology between the 1 4kb 2μ probe DNA and the various restriction fragments As expected neither of the restriction enzymes Sal I nor Kpn I cut the 2μ DNA

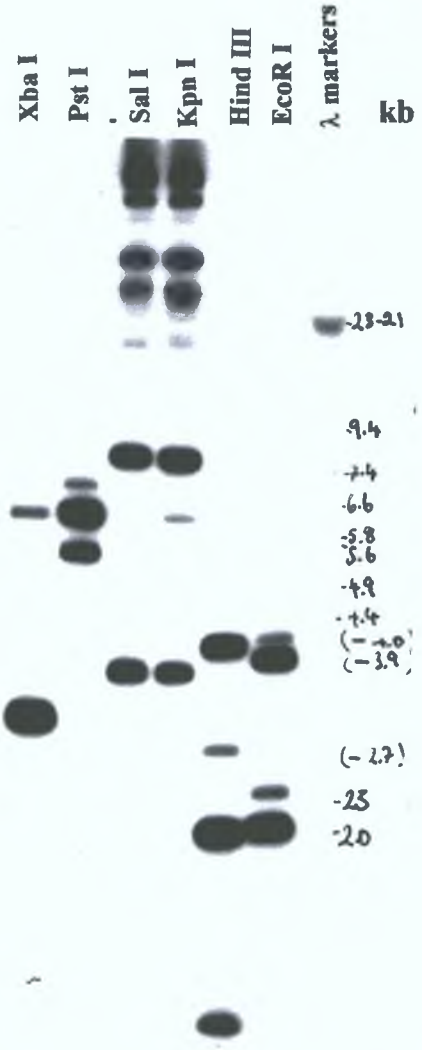


Figure 4.7 Autoradiogram of the restriction fragments of the 2μ circle

4.2.3 Evaluation of pUC-19 as plasmid specific-portion of the probe

A potential probe used to measure PCN needs to contain sequences that will hybridise to pJG317 pUC-19 and pJG317 have the ampicillin resistant gene (Amp^R) of pBR322 pUC-19 can hybridise to pJG317 and hence could be used as the plasmid pJG317 specific portion of a probe

Total DBY746 DNA with and without plasmid pJG317 DNA was isolated, restricted with the restriction enzymes, EcoR I, Hind III, Xba I, Sal I and fractionated by agarose gel electrophoresis The DNA was transferred to nitrocellulose filters and probed with labelled pUC-19 DNA (Figure 4 8) pUC-19 bound to those pJG317 restriction fragments containing pBR322 sequences and futhermore did not bind to any DBY746 parent DNA However, on close examination of the autoradiogram faint bands can be seen on the DBY746 parent strain DNA By comparing the pattern of these bands with those in Figure 4 7 (2μ restriction pattern), it can be seen that they are very similar It may be concluded that pUC-19 may hybridise non-specifically to some 2μ circle DNA, but this is unlikely to interfere with the measurement of PCN

Reviewing the results of the above hybridisations a suitable probe for measuring PCN can be planned The probe should consist of the 3 8kb β-actin gene inserted into the vector pUC-19 On restriction of total genomic DBY746 DNA including plasmid pJG317 with the endonuclease Sal I and hybridising with the β-actin/pUC-19 probe, 2 clear bands would result on an autoradiogram The pUC-19 part of the probe would hybridise to the 6 6kb Sal I restriction fragment of pJG317 while the β-actin part of the probe would hybridise to the 9 4kb Sal I restriction fragment containing the chromosomal β-actin gene in one copy per genome The PCN can then be calculated from the intensity of hybridisation to the 6 6kb plasmid specific fragment relative to the intensity of hybridisation to the 9 4kb β-actin DNA fragment

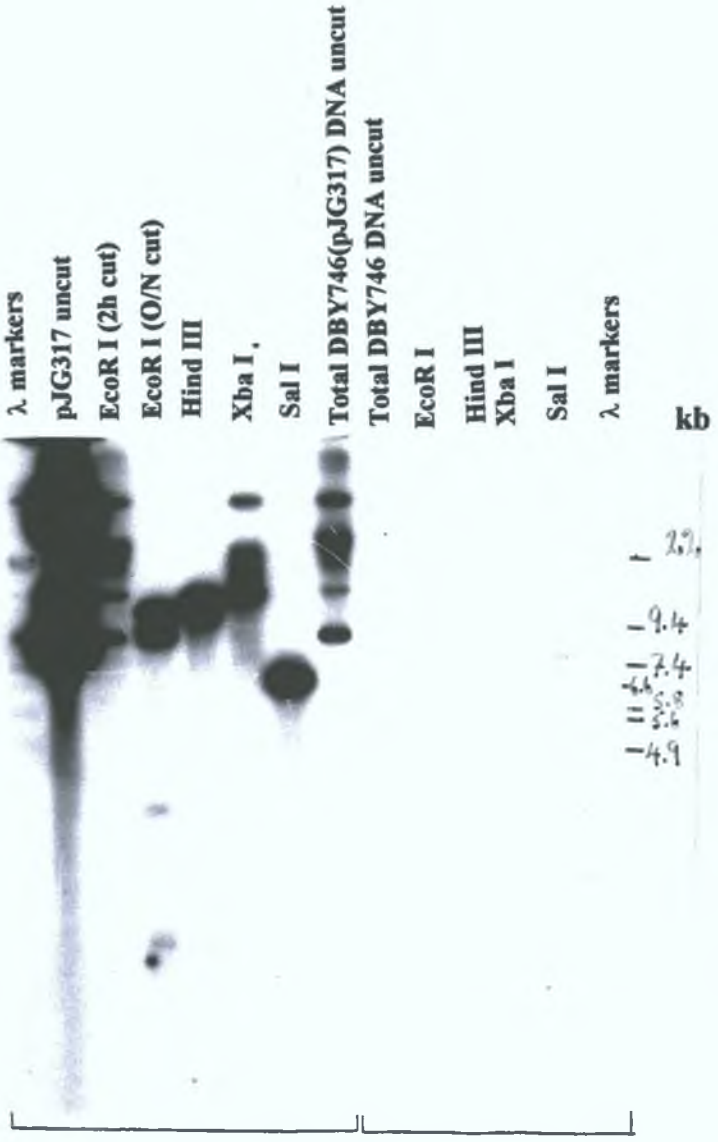


Figure 4.8 Autoradiogram of pJG317 restriction fragments homologous with puC-19 DNA, and non-specific binding of puC-19 to genomic DBY746 DNA.

4.3 CONSTRUCTION AND CLONING OF PROBE DNA

The 3 8kb β-actin gene was excised from plasmid pRB149 by EcoR I restriction After fractionation of the DNA fragments by gel electrophoresis (Section 2 7 5) the β-actin DNA was purified by the gene clean procedure (Section 2 7 6) Commercial pUC-19 vector DNA (Boehringer Mannheim) was restricted with EcoR I and dephosphorylated using calf-intestinal phosphatase (Section 2 7 4) A ligation reaction involving the dephosphorylated vector DNA (pUC-19) and the insert DNA (β-actin gene) was performed (Section 2 7 4) *E coli* JA221 cells were then transformed with the new 6 5kb (2 7kb pUC-19 + 3 8kb β-actin) plasmid (Section 2 7 1) Finally for confirmation, DNA was isolated from positive transformants (Section 2 7 2 1) and restricted with EcoR I The presence of a 2 7kb and 3 8kb band indicated that the cloning had been successful The new plasmid consists of the β-actin gene inserted into pUC-19 and is referred to as pPC64 This is the probe which will be used to measure PCN A large quantity of pPC64 DNA was prepared by the maxi prep procedure (Section 2 7 2 2)

4.4 TESING THE NEW PROBE FOR ABILITY TO MEASURE PCN

Total DNA was isolated from yeast, restricted and electrophoresed, before being transferred to nitrocellulose filters The newly constructed probe (pPC64) was linearised and labelled Hybridisation of probe to the DNA immobilised on the filters was allowed to proceed at 68^{0}C overnight (Figure 4 9 (a)) The following points can be made

(i) Lanes 3 to 10 contain total DNA restricted with Sal I As expected two bands are visible and represent the 9 4kb restriction fragment of the single copy chromosomal β-actin

gene, and the 6 6kb restriction fragment of the multicopy plasmid pJG317

(ii) Lanes 11 to 13 contain total DNA restricted with Pst I As predicted three bands are visible and represent the 4 6kb chromosomal β-actin restriction fragment, the 4 0kb pJG317 restriction fragment and a very small chromosomal β-actin restriction fragment

(iii) Lane 2 shows total parent strain (DBY746) DNA (*i e* no plasmid present) restricted with Sal I Only the 9 4kb β-actin band is visible Figure 4 9 (b) shows lanes 2 and 3 with a longer exposure time The presence of the single β-actin band in lane 2 is more visible here

(iv) Lane 1 contains unrestricted probe pPC64 DNA which acts as a positive control

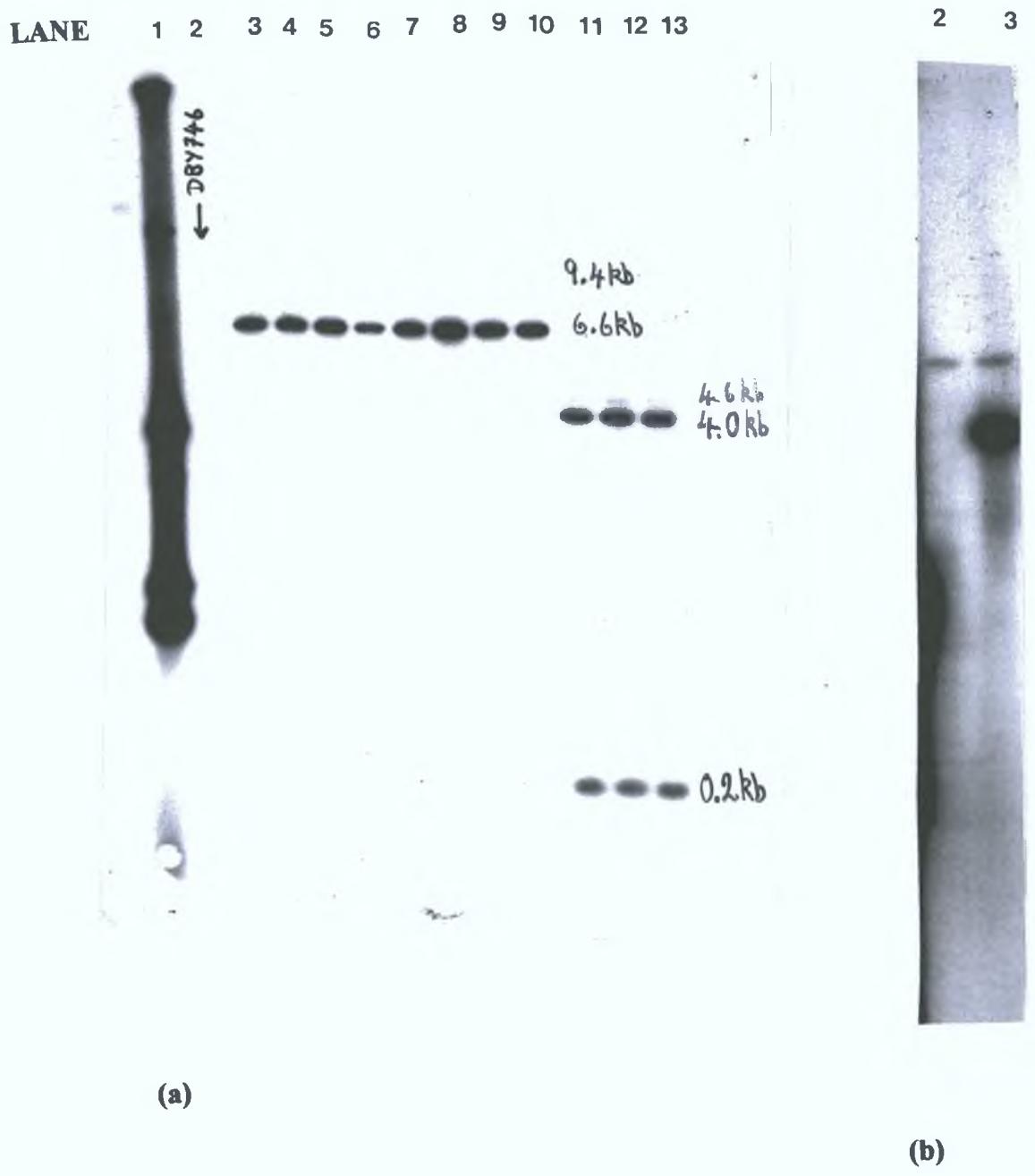


Figure 4.9 (a) Autoradiogram of total genomic DBY746 (pJG317) DNA probed with probe pPC64 (puC-19 + β-actin).
(b) Lanes 2 and 3 after longer exposure time.
Note the single chromosomal β-actin band of DBY746 DNA only in lane 2 and the β-actin band plus the plasmid pJG317 band of DBY746 (pJG317) DNA in lane 3.

4.5 CHARACTERISATION AND CONFIRMATION OF HYBRIDISATION

Plasmid copy number is calculated as described in Section 2.8

$$PCN = \frac{D_{plas}}{D_{act}} \frac{M_{act}}{M_{plas}} \frac{1}{S}$$

where

D_{plas}	:	area under plasmid peak
D_{act}	:	area under β-actin peak
M_{plas}	:	2.7
M_{act}	:	1.2
S	:	percentage plasmid positive cells

M_{plas} and M_{act} are the sizes of the pUC-19 and β-actin DNA fragments on the probe (pPC64) available for hybridisation to filter bound DNA. These values represent the degree of homology of the probe with plasmid and chromosomal DNA. In the construction and cloning of this probe the 3.8kb β-actin gene was inserted into the 2.7kb pUC-19 vector yielding a plasmid of size 6.5kb. This was confirmed by restriction analysis.

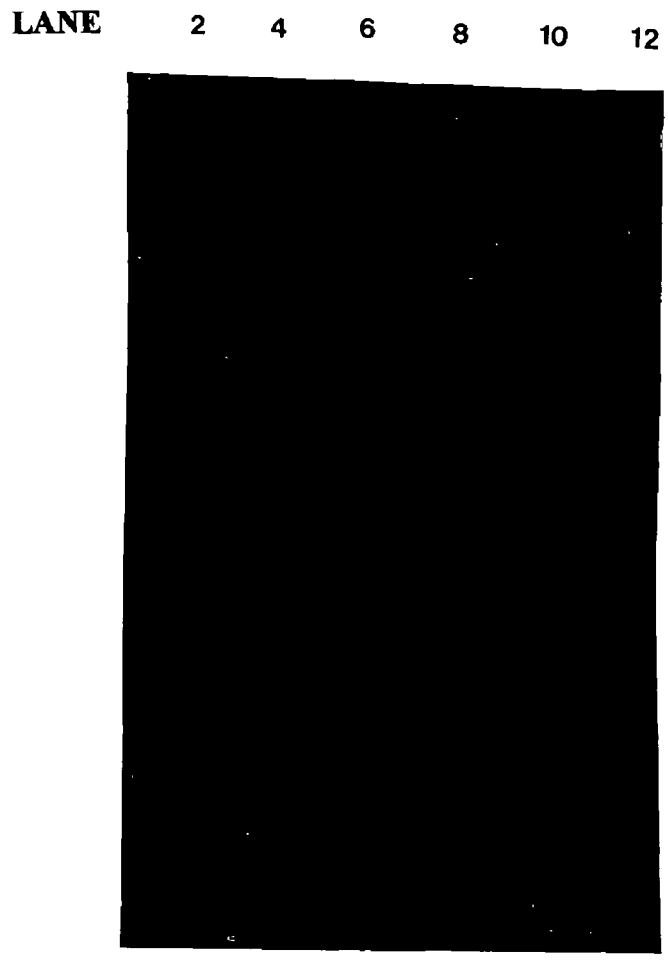
However, evidence from Figure 4.9 and detailed restriction analysis of pPC64 DNA (Figure 4.10) revealed that the deletion of 2.6kb had occurred. Table 4.4 lists the various fragment sizes obtained by digestion of pPC64 with the different restriction enzymes. Figure 4.11 is a schematic of the β-actin gene as mapped by Ng and Abelson (1980) and a restriction map of pUC-19 DNA (Yanisch Perron et al., 1985). From results of Figure 4.10 and Table 4.4 the position of the 2.6kb deletion in plasmid pPC64 was established and is represented in Figure 4.11

Despite the deletion of 2.6kb from pPC64, 1.2kb of the β-actin gene remains in 2.7kb pUC-19 vector. All that is required for measuring PCN is a fragment of DNA that will hybridise to the reference DNA (in this case the β-actin chromosomal gene) and to the multicopy plasmid DNA. Furthermore, the sizes of the fragments which hybridise to the reference and plasmid DNA sequences must be known. Figure 4.9 shows that pPC64 with

a 2 6kb deletion can be successfully applied to measure PCN For calculation of PCN, M_{plas} is 2 7 while M_{act} is 1 2

Table 4 4 DNA fragment sizes resulting from restriction digestion of plasmid pPC64 DNA (Figure 4 10)

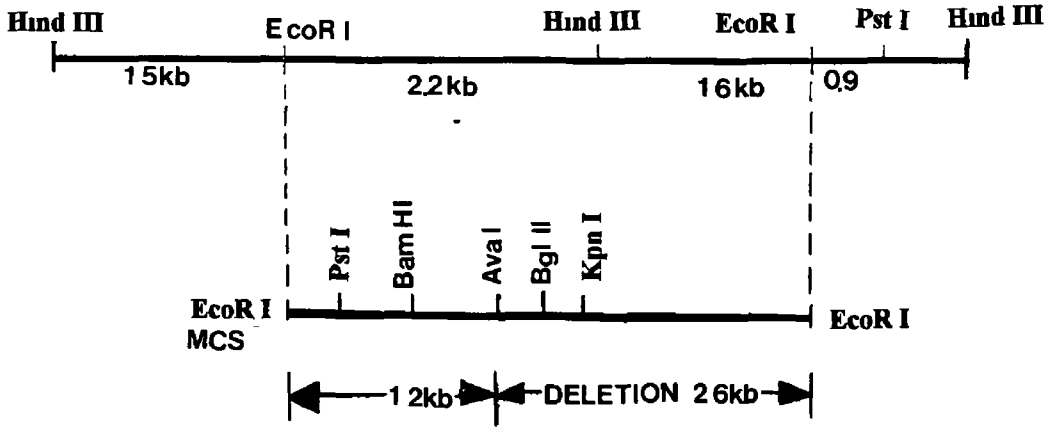
Restriction Enzyme	Fragment size (kb)	Conclusions
EcoR I	4 0	One EcoR I site missing
Bam HI	3 2 & 0 8	β-actin Bam HI site present
Pst I	3 8 & 0 2	β-actin Pst I site present
Bgl II + EcoR I	4 0	Bgl II site missing
Pvu II	1 6 & 2 4	1 2kb insert into pUC-19
Ava I	4 0	Ava I site missing



LANE	SAMPLE
1	3 8kb B-actin gene
2	pUC-19 DNA uncut
3	1kb λ ladder 12 to 1kb
4	pPC64 DNA uncut
5	EcoR I
6	Bam HI
7	Pst I
8	Bgl II + EcoR I
9	Pvu II
10	Ava I
11	pPC64 DNA uncut
12	100bp λ ladder

Figure 4 10 Restriction digestion of plasmid pPC64 (probe)

(a) β-actin Gene



(b) pUC-19

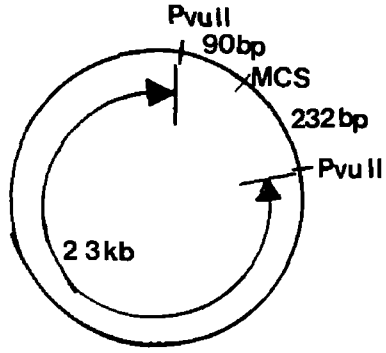


Figure 4 11 Schematic diagrams of (a) restriction map of β-actin gene (Ng and Ableson,1980) showing the 2 6kb deletion and (b) simplified restriction map of puC-19 (Yanisch-Perron et al , 1985)

MCS Multiple cloning site

4.6 OPTIMISATION OF PCN MEASUREMENT PROCEDURE

Figure 4 12 shows the influence of X-ray film exposure time on PCN Longer exposure times result in darker bands After long exposure times the intensity of the band on the X-ray film and the measured PCN are not linearly related, resulting in an underestimate of PCN Figure 4 12 represents the linearity of response of the X-ray film Figure 4 13 illustrates the effect of DNA concentration on PCN DNA concentration was varied by loading different quantities of DNA to the agarose gel, resulting in various quantities of DNA being immobilised onto the nitrocellulose filters Linearity of DNA concentration was measured with respect to plasmid band intensity on the autoradiogram From Figure 4 13 it may be concluded that any quantity of DNA above 10μl of a standard total DNA isolate solution (approx 2μg total DNA) has no effect on PCN as measured by plasmid band intensity on the autoradiogram The quantity of cells harvested, from which total DNA was isolated was thus decreased and standardised at 1×10^9 cells The quantity of DNA used in restriction reactions and the amount of DNA loaded to agarose gels was also standardised In this way the quantity of DNA isolated, restricted and used to measure PCN was standardised and maintained within the linear range of X-ray film response, ensuring a reproducible PCN result The reproducibility of the procedure was tested by harvesting 4 sets of cells from the same fermentation and isolating the DNA from each cell sample The PCN of each sample was measured yielding the following results

Sample 1	PCN	=	11
Sample 2	PCN	=	9
Sample 3	PCN	=	10
Sample 4	PCN	=	9

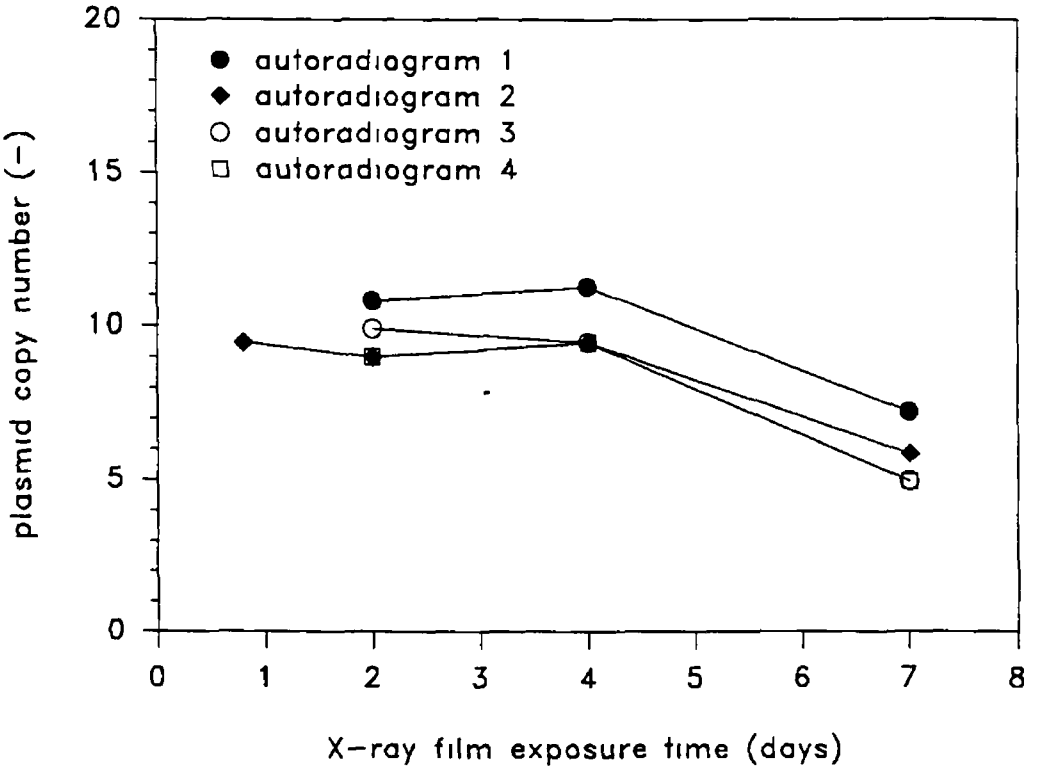


Figure 4 12 Calculated plasmid copy number versus X-ray film exposure time

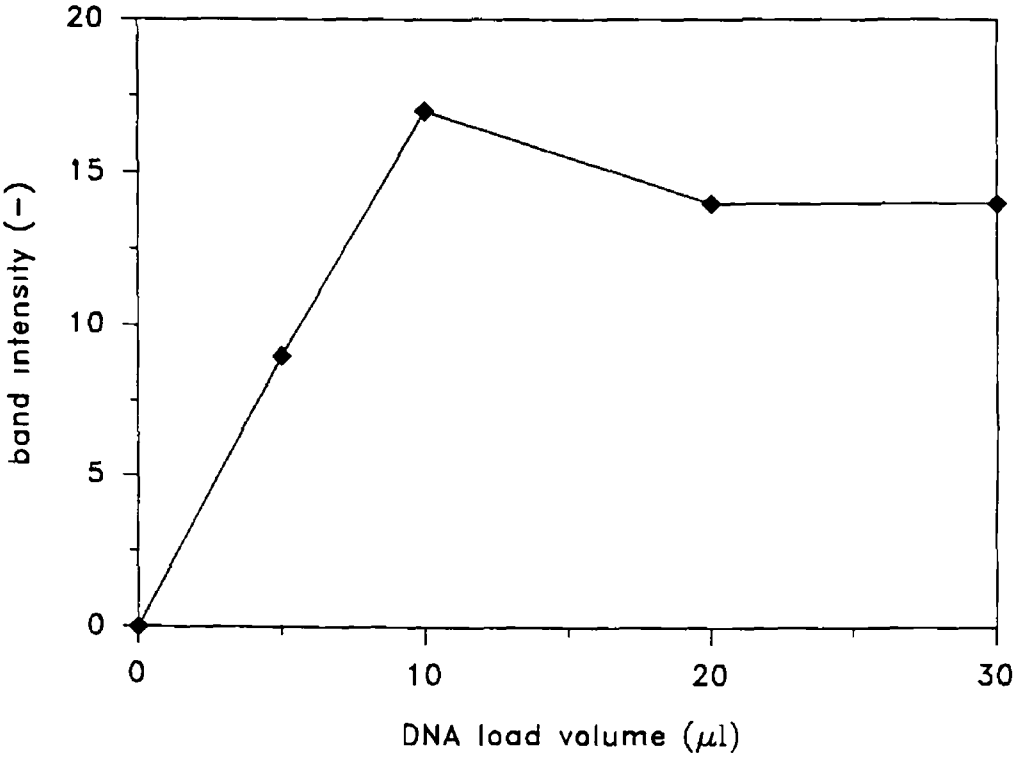


Figure 4 13 Band intensity on X-ray film versus DNA load volume

4.7 DISCUSSION

Methods of measuring PCN based on caesium chloride separation of plasmid from chromosomal DNA (Shepard and Polisky, 1979, Moser and Campbell, 1983) are inherently uncertain in that they depend on the plasmid having a covalently closed circular (CCC) configuration and are not easily correctable for occurrence of non-CCC forms or for loss of plasmid DNA during preparative procedures These methods give minimum estimates of PCN The use of HPLC to measure PCN (Copella et al , 1986, 1987 (a)) could also be classed as a physical separation method This procedure, although attractive due to its reliability, speed and ease of performance, contains inherent flaws DBY746 contains the endogenous yeast 2μ circle However, the HPLC method fails to separate this from other plasmid DNA This procedure relies heavily on constants and variables obtained from the literature, which may be specific to one particular recombinant system Hybridisation methods (Gerbaud and Guerineau, 1980, Jayaram et al , 1983, Bitter et al , 1987, Korpela et al , 1987) are theoretically precise, but are difficult to perform and are time consuming Indirect methods based on gene dosage all require primary proof that the phenotype being analysed is linearly proportional to gene dosage (Uhlin and Nordstrom, 1976) and are therefore limited to plasmids that contain genes that show this proportionality

Fluorescence densitometry of agarose electrophoretic gels (Projan et al , 1983, Futcher and Cox, 1984) presents a quick, easy and relatively reliable procedure for determining PCN This method was unsuccessfully applied to the DBY746 (pJG317) recombinant system (data not shown) The plasmid DNA band (CCC-form) was very faint and barely visible on ethidium bromide stained electrophoretic gels When total yeast genomic DNA (including plasmid pJG317) was restricted the background level of restricted DNA fragments was too great to pick out the plasmid DNA bands Korpela et al (1987) measured the PCN of the expression vector pAAH5 using the precise and accurate sandwich hybridisation procedure and found this vector to exist in 10 copies per cell, when grown on selective medium The PCN of pAAH5 was unchanged when viral genes were inserted Plasmid pJG317 consists of the expression vector pAAH5 with the gene encoding (1,3) (1,4)

β-glucanase inserted. It was assumed that pJG317 also exists in approximately 10 copies per cell. This may be the reason why on ethidium bromide stained gels, the pJG317 DNA band was barely visible compared with the large chromosomal band, the multicopy 2μ circle or rDNA restriction fragments. This procedure was used by Projan et al. (1983) and by Futcher and Cox (1984) to measure the PCN of medium to high copy number (30 to 880) plasmids. It was concluded that this procedure cannot be employed to measure the PCN of the low copy number plasmid pJG317.

Hybridisation methods although difficult to perform and time consuming are exact, accurate and if performed carefully are very reproducible (Bitter et al., 1987). Several hybridisation methods of varying complexity may be employed to measure PCN, such as Southern hybridisation, Sandwich hybridisation and hybridisation in solution. Of these methods it was decided to develop a procedure for measuring PCN based on Southern hybridisation as this procedure is more straight forward and less difficult to perform.

Essential requirements in the development of a procedure for measuring PCN based on Southern hybridisation include :

- a restriction map of the plasmid
- a restriction map of the region of the genome containing the reference DNA.
- an effective and reproducible procedure for isolating restrictable genomic DNA.

Early work concentrated on achieving the above. As potential reference DNA, the loci of URA3 (uracil) and β-actin were investigated. Standard procedures for the isolation and restriction of total genomic yeast DNA were modified and are as outlined in Chapter Two.

A most important element of any PCN method using hybridisation is the probe used. This is usually complementary to both plasmid DNA and a native chromosomal yeast gene. Such DNA probes may derive from either the promoter, transcription terminator or selectable marker of the expression vector, since these sequences are all also represented in the yeast genome (Bitter et al., 1987). The chromosomal fragment on each probe acts as an internal standard for PCN quantification and generally represents one gene per haploid genome. The following chromosomal gene fragments have been utilised in probes for measuring PCN in yeast: URA3, β-actin, HIS, rDNA

and PGK (Jayaram et al , 1983, Apostol and Greer, 1988, Bugeja et al , 1989 Broach 1983, Van der Aar et al ,1992)

Of the two loci investigated as possible reference DNA fragments in a probe to measure PCN, β-actin proved to have the most promise Restriction of total genomic yeast DNA with Sal I produced a 9 4kb fragment containing the whole β-actin gene, which meant that autoradiograms had only a single band when probed with DNA containing β-actin DNA For quantification of PCN scanning densitometry of the autoradiograms is required and the fewer the bands present, the more accurate the calculation of intensity and the lower the chance of background interference

The probe also needs to contain sequences that will hybridise to plasmid pJG317 These are provided by the presence of pBR322 sequences in both pUC-19 and pJG317 Probing total genomic DBY746 (pJG317) DNA with pUC-19 yielded a single band on the autoradiogram in the case of Sal I restriction This band represents the 6 4kb Sal I restriction fragment of pJG317 The possibility of pUC-19 cross reaction (*i e* non-specific binding to 2μ DNA) was investigated and found to be possible but unlikely

Using recombinant DNA techniques, probe pPC64 was constructed and cloned Despite the fact that a deletion of 2 6kb in the β-actin gene occurred, the probe was successfully employed to measure the PCN of DBY746 (pJG317) The probe was characterised by detailed restriction analysis and the portion of pUC-19 and β-actin sequences available for hybridisation determined

PCN is calculated from the relative intensities of the single copy β-actin band to the multicopy plasmid pJG317 band, obtained from densitometer scans of the autoradiograms The calculations are based on those of Jayaram et al (1983) and Projan et al (1983) Filter hybridisation depends on two processes, diffusion of probe to the filter and hybridisation of the probe at the filter (Anderson and Young, 1985) Assuming diffusion effects to be constant (identical hybridisation reaction conditions which maximise diffusion such as high incubation temperature, low reaction volume and agitating the reaction mixture) and also assuming to be constant or ignoring such factors as probe DNA reassociation kinetics or steric hindrance effects, hybridisation is a function of the concentration of filter bound DNA The

probe pPC64 hybridises to the plasmid band and the β-actin band in proportion to their concentrations The different rates of hybridisation of probe DNA, to the plasmid and β-actin DNA is driven by the concentration of homologous DNA available for hybridisation on the probe A correction factor of 1 2/2 7 = 0 44 compensates for the increased tendency of the probe DNA to bind to filter bound plasmid DNA as opposed to β-actin DNA due to the differences in homologous sequences The probe pPC64 contains 1 2kb of the β-actin gene and 2 7kb of pUC-19 DNA Finally, the PCN calculation takes into account the percentrage of cells that contain the plasmid so that the final PCN calculated is the PCN of plasmid bearing cells in the population

The procedure for measuring PCN was standardised and optimised by investigating such factors as

- quantity of cells harvested
- quantity of DNA loaded onto the gels (Figure 4 13)
- exposure time of the autoradiograms (Figure 4 12)

Only by maintaining identical procedures such as DNA isolation, restriction, Southern blotting, probe labelling and hybridisation reaction conditions in addition to noting the results from optimisation studies will the PCN procedure be accurate and reproducible The repeatibility of the procedure was tested by obtaining PCN values that differed very little m identical fermentation samples The PCN of pAAH5 of which pJG317 is a derivative was measured as 10 to 11 copies per cell using the newly developed and optimised PCN procedure (data not shown) Korpela et al (1987) measured the PCN of pAAH5 as 10 copies per cell using the Sandwich Hybridisation procedure

Various probes have been used to measure the PCN in yeast, each containing different reference DNA fragments, usually derived from selectable markers or rDNA fragments Apostol and Greer (1988) used a probe containing the 3 8kb β-actin gene inserted into the vector pBR322 This probe was used to investigate the copy number and stability of yeast 2μ circle based plasmids carrying a transcription conditional centromere Apostol and Greer(1988) isolated total genomic yeast DNA and restricted it with the enzyme Hind III The labelled probe detected two single copy chromosomal fragments corresponding to the 3 7kb and the 2 5kb Hind III β -actin restriction fragments (Fig 4 5) The probe also detected two plasmid

bands at 10 5 and 8 6kb Each lane on the resulting autoradiogram had four bands which makes scanning densitometry difficult The system developed in the present work could have employed restriction of total genomic DNA with enzymes such as EcoR I, Pst I, Hind III or Kpn I, all of which would have resulted in multiple (3 or more) chromosomal β-actin or plasmid bands on the autoradiogram Restriction of total genomic DNA with Sal I results in a single chromosomal β-actin band and a single plasmid pJG317 band which makes scanning densitometry and hence PCN calculation easier Also, throughout this study, in conjunction with PCN, the plasmid stability of free and immobilised cells under various growth and environmental conditions is investigated Plasmid instability can be either structural or segregational The added advantage of having a minimum number of restriction fragments on the autoradiogram is that any large plasmid deletions or rearrangements can be easily detected, thus ascertaining whether structural instabilities occur

CHAPTER FIVE

INVESTIGATION OF THE INFLUENCE OF GROWTH RATE ON PLASMID STABILITY, PLASMID COPY NUMBER AND ENZYME PRODUCTION IN FREE CELL CULTURE

5.1 INTRODUCTION

Immobilisation of recombinant cells results in an increase in the apparent plasmid stability Gradients in plasmid stability exist throughout a particular gel bead, with the slow growing cells on the inside having a higher percentage of plasmid-containing cells than the faster growing cells on the outside of the gel beads (De Taxis du Poet et al , 1987, Cahill et al , 1990) The gradient in plasmid stability is a consequence of the gradient in immobilised cell growth rate, which in turn is caused by increased diffusional resistances to essential nutrients and oxygen from the outer to the inner regions of the gel beads, as cell growth proceeds (Gosmann and Rehm, 1986, Marin Iniesta et al , 1988, Sayadi et al , 1989) In order to quantify the gradient in plasmid stability in immobilised cells, it was necessary to measure the PCN of immobilised cells and determine whether a gradient in PCN exists across the gel beads However, before this can be accomplished, a study on the influence of cell growth rate on plasmid stability and PCN had to be performed, since growth rate is one of the major factors governing plasmid stability and PCN in recombinant cells This study was conducted using free cells

The relationship between free cell growth rate, plasmid content and cloned gene product was also studied Increased PCN and cloned gene expression can lead to an increased metabolic load or burden on recombinant cells, leading to a decrease in the percentage of plasmid-bearing cells

Finally, since the PCN of free cells in various different fermentation types was to be measured this study would serve as a good test of the newly developed procedure for PCN measurement This procedure was developed using yeast cells grown in batch culture in selective medium In this study, batch, serial batch and continuous cultures in selective and complex media were performed

5.2 FREE CELL BATCH FERMENTATIONS

Figure 5 1 represents the complete fermentation profile (cell growth, enzyme production and sugar consumption) of free cells grown on selective medium in a 2 L Life Sciences bioreactor (Section 2 5 4) Using a 15 L Microgen fermenter with a working volume of 10 L (Section 2 5 4) the fermentation profile of free cells grown on YEPD medium was followed and is illustrated in Figure 5 2

In order to measure plasmid copy number (PCN) of cells total chromosomal and plasmid DNA was isolated from the cells (Section 2 7 3) and restricted with endonuclease Sal I (Section 2 7 4) The DNA was fractionated electrophoretically (Section 2 7 5) and transferred to nitrocellulose (Section 2 7 7) The DNA probe pPC64 containing β-actin sequences in the vector pUC-19 was labelled with ^{32}P (Section 2 7 9) and hybridised to the nitrocellulose filter (Section 2 7 11) Autoradiography using X-ray film was then performed The autoradiogram was scanned using a densitometer and the area under each peak was determined (Section 2 8) The plasmid copy number of each sample was calculated from the relative intensities of hybridisation of the probe DNA to the single copy β-actin chromosomal gene and the multicopy plasmid pJG317 (Section 2 8) Figure 5 3 shows the eletrophoretic pattern of total yeast DNA restricted with Sal I Figure 5 4 is the resulting autoradiogram when this DNA was hybridised to pPC64, and Figure 5 5 shows examples of densitometer scan tracings of Lanes 2 and 8 of the autoradiogram Finally, Table 5 1 shows the values of PCN calculated

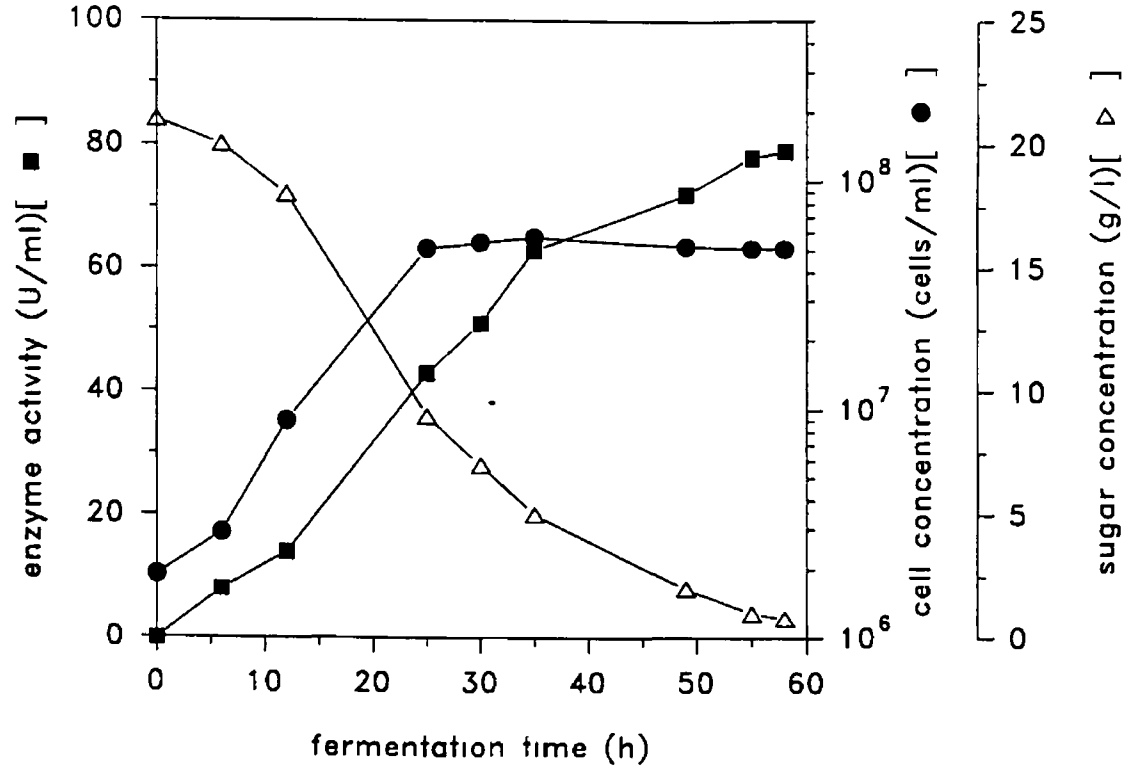


Figure 5 1 Fermentation profile for free cells in selective medium

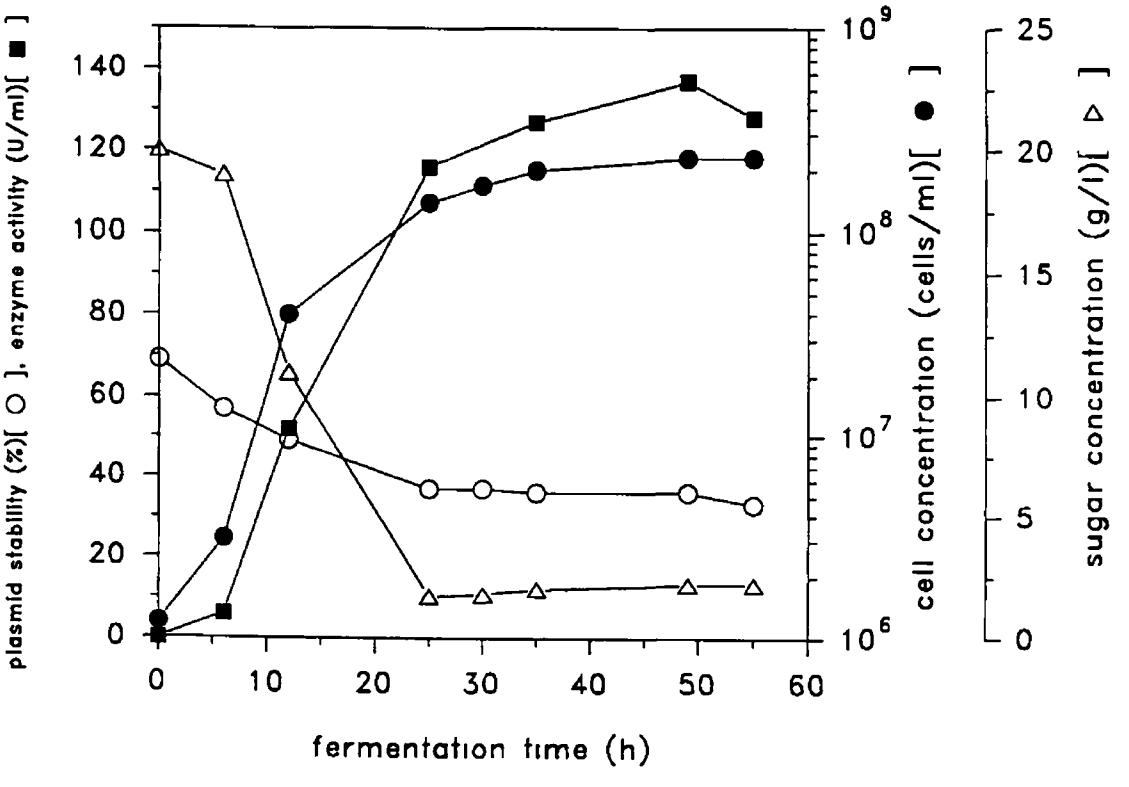
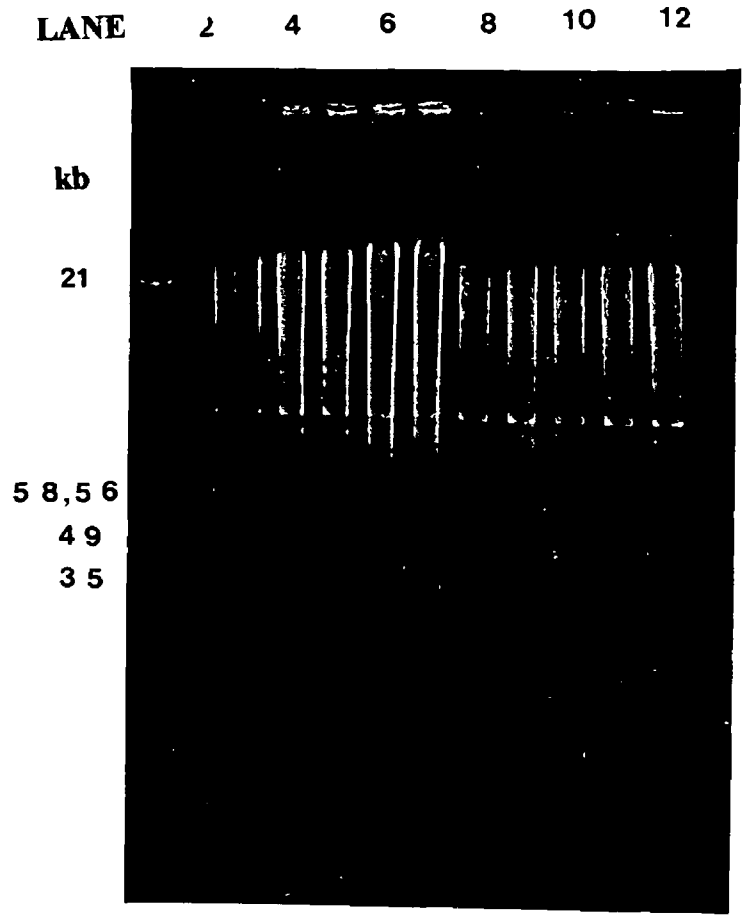


Figure 5 2 Fermentation profile for free cells in YEPD medium



LANE	SAMPLE
1	λ size markers
2	Standard reference DNA
3	Batch fermentation (YEPD) 49 h
4	Batch fermentation (YEPD) 30 h
5	Batch fermentation (YEPD) 25 h
6	Batch fermentation (YEPD) 12 h
7	Batch fermentation (YEPD) 6 h
8	Batch fermentation (Selective) 58 h
9	Batch fermentation (Selective) 49 h
10	Batch fermentation (Selective) 35 h
11	Batch fermentation (Selective) 25 h
12	Batch fermentation (Selective) 12 h
13	Probe DNA (pPC64) {+ve control}

Figure 5 3 Restriction pattern of total genomic DBY746 (pJG317) DNA cut with endonuclease Sal I

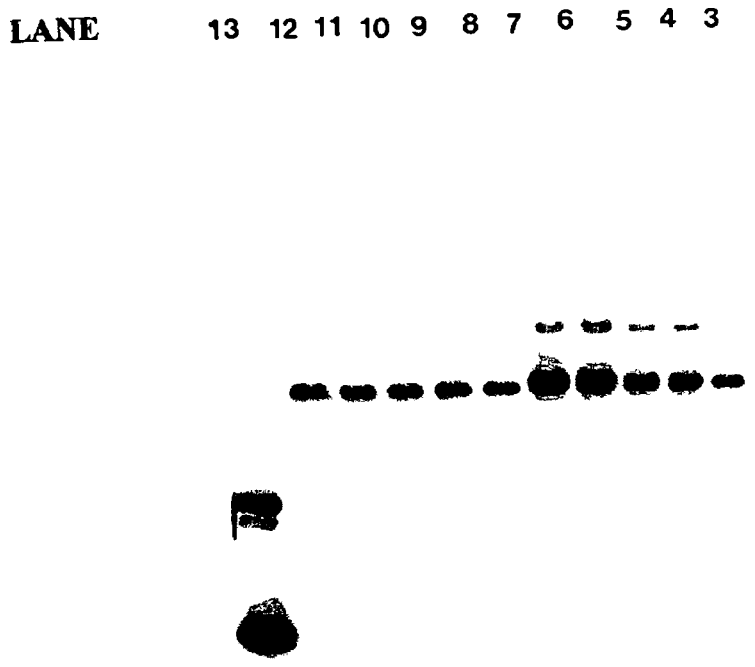
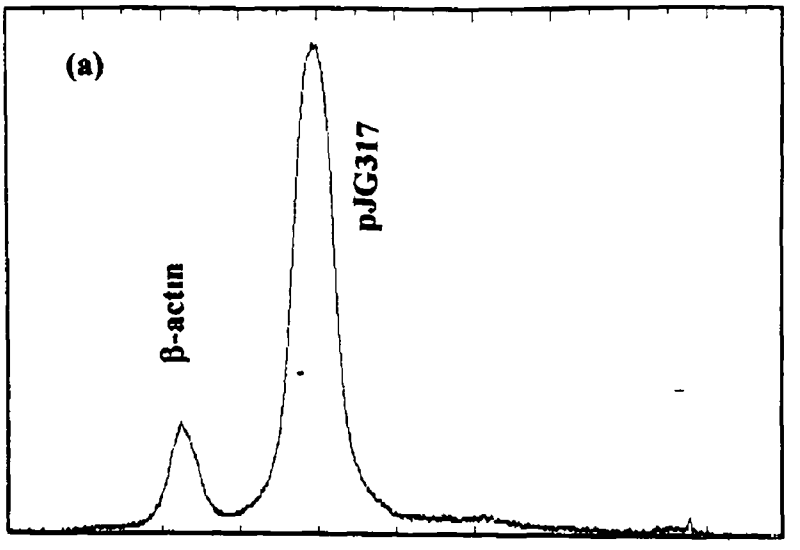
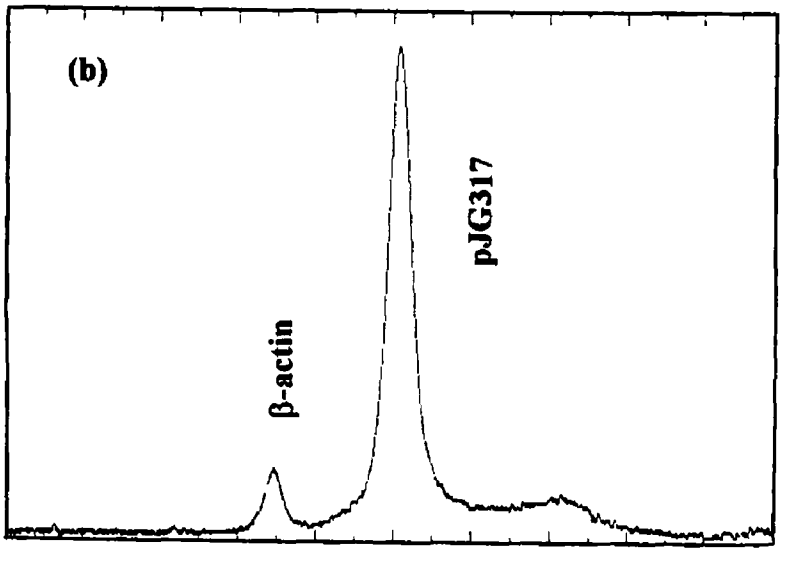


Figure 5 4 Autoradiogram of the single copy chromosomal β-actin band and multicopy plasmid pJG317 DNA band, of cell samples during batch fermentation on selective (Lanes 8-12) and YEPD (Lanes 3-7) media

(Samples in each lane are as in Figure 5 3)



LANE 6 12h fermentation on YEPD medium



LANE 11 25h fermentation on selective medium

Figure 5 5 Densitometer scan tracings of autoradiogram in Figure 5 4
Note (a) Lane 6 = 12 hours growth on YEPD
(b) Lane 11 = 25 hours growth on selective medium
PCN is calculated from the relative peak areas

Table 5 1 *PCN values for free cell fermentations on selective and non selective media*

SELECTIVE MEDIUM			
Time (h)	**Gen**	**Plasmid Stability (%)**	**PCN**
12	2 2	100	8
25	4 9	100	8
35	5	100	8
49	5	100	9
58	5	100	9
YEPD MEDIUM			
Time (h)	**Gen**	**Plasmid Stability (%)**	**PCN**
6	1 5	57	25
12	5	49	25
25	7	37	33
30	7 1	37	27
55	7 5	33	34

5.3 SERIAL BATCH FERMENTATIONS

In order to follow the total loss of plasmid from a cell population and to determine if a corresponding decline in enzyme production occurred, serial batch fermentations using YEPD were performed (Section 2 5 5) Figure 5 6 shows that the decrease in plasmid positive cells is accompanied by a decrease in enzyme activity These decreases follow an exponential decay pattern

When measuring PCN of samples, no plasmid band was detected on the autoradiograms after generation number 63 at which time only 1 5% of the cell population were plasmid harbouring cells This result confirms that when plasmid stability equals zero percent as measured by the replica plate method (Section 2 6 6), no plasmid can be detected by DNA hybridisation Figure 5 7 illustrates the loss of plasmid from the cell popluation Here, densitometer scan tracings of the cell population after 12 generations (26% plasmid positive cells) are compared with that of the same cell population after 63 generations (1 5% plasmid positive cells) However as Table 5 2 shows, there is little or no change in the PCN of the cells with a mean value of 25±5 5 copies per plasmid bearing cell

Table 5 2 PCN values for free cells in serial batch fermentations

Time (h)	Generation	Plasmid Stability (%)	PCN
24	12	26	33
48	25	15	18
72	38	7	23
96	50	3 5	25
120	63	1 5	27
144	76	0	-

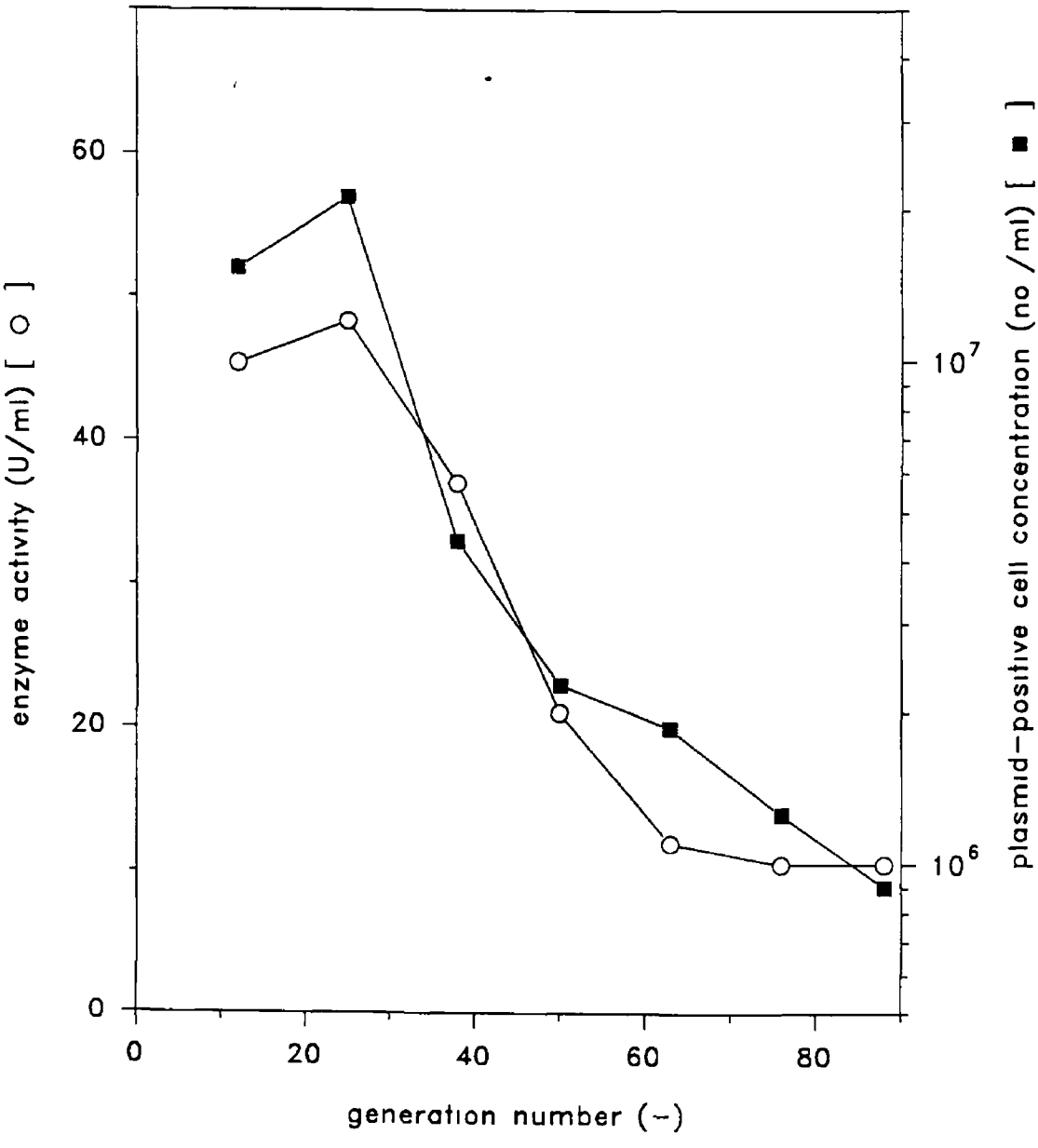
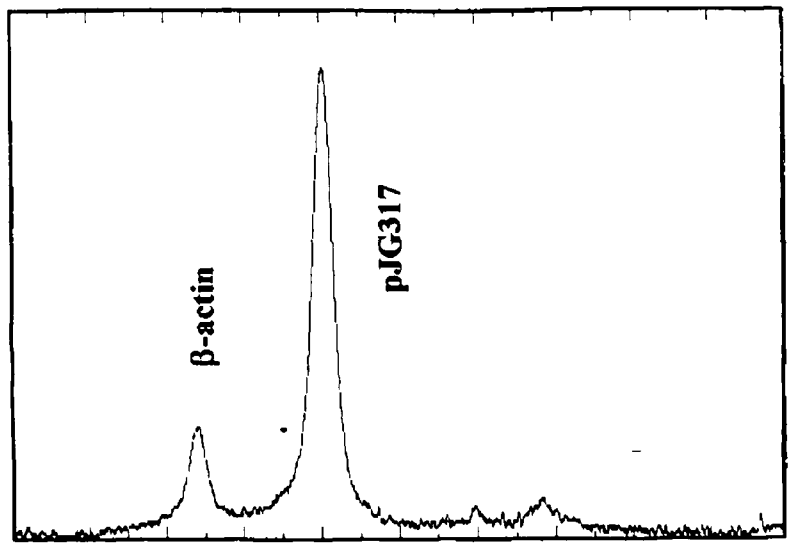
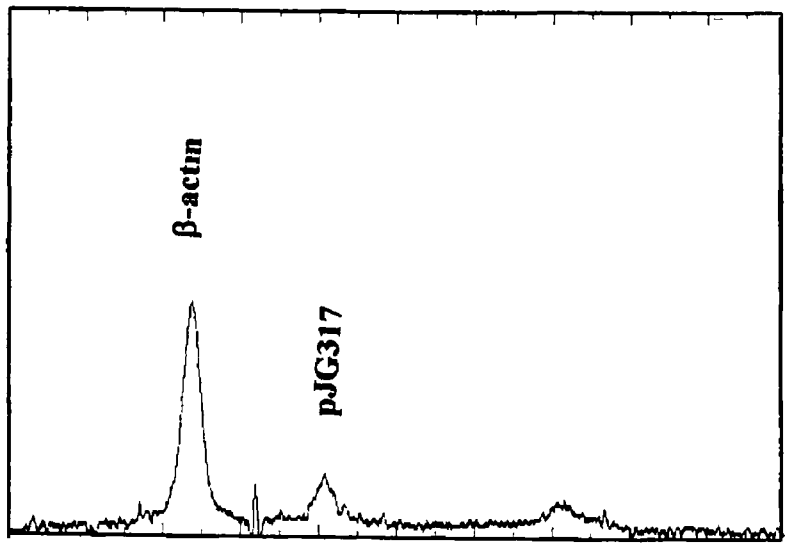


Figure 5 6 Enzyme activity and plasmid stability profiles in free cell serial batch culture



(a) Generation no. 12 (plasmid stability = 26%)



(b) Generation no. 63 (plasmid stability = 1.5%)

Figure 5 7 Densitometer scan tracings of cells in serial batch fermentation at (a) Generation no 12 and (b) Generation no 63

5.4 CONTINUOUS CULTURE

The previous studies examined plasmid stability, PCN and cloned gene expression in batch culture In order to evaluate environmental effects in more well defined conditions, a continuous culture system was used (Section 2 5 7) and dilution rate was altered in order to obtain different specific growth rates The parameters determined as a function of dilution rate include plasmid stability, PCN and β-glucanase activity

The value for the maximum specific growth rate (μ_{max}) of DBY746(pJG317) has been previously shown to be 0 8h^{-1} (Cahill, 1990) A dilution rate of 0 67h^{-1} was chosen as an example of a fast growing culture However, washout of the chemostast occurred indicating that D= 0 67h^{-1} was close to or possibly greater than μ_{max} Therefore a dilution rate of 0 5h^{-1} was chosen as an example of a fast growing culture (doubling time, t_d =1 5 h) and a dilution rate of 0 1h^{-1} was chosen to represent a slow growing culture (t_d =6 9 h) The number of generations in the chemostat was calculated from $D\ t$ / ln2 , where D is the dilution rate and t is the time (De Taxis du Poet et al 1987)

Figures 5 8 and 5 9 show the fermentation profiles of continuous cultures at dilution rates of 0 5h^{-1} and 0 1h^{-1} respectively Unlike at the lower dilution rate (Figure 5 9), there is a slight downward trend in cell numbers and an upward trend in sugar concentration at D=0 5h^{-1} (Figure 5 8), indicating that D=0 5h^{-1} is quite close to μ_{max} Any slight fluctuations in the system, especially in flow rates, could result in a loss of cells

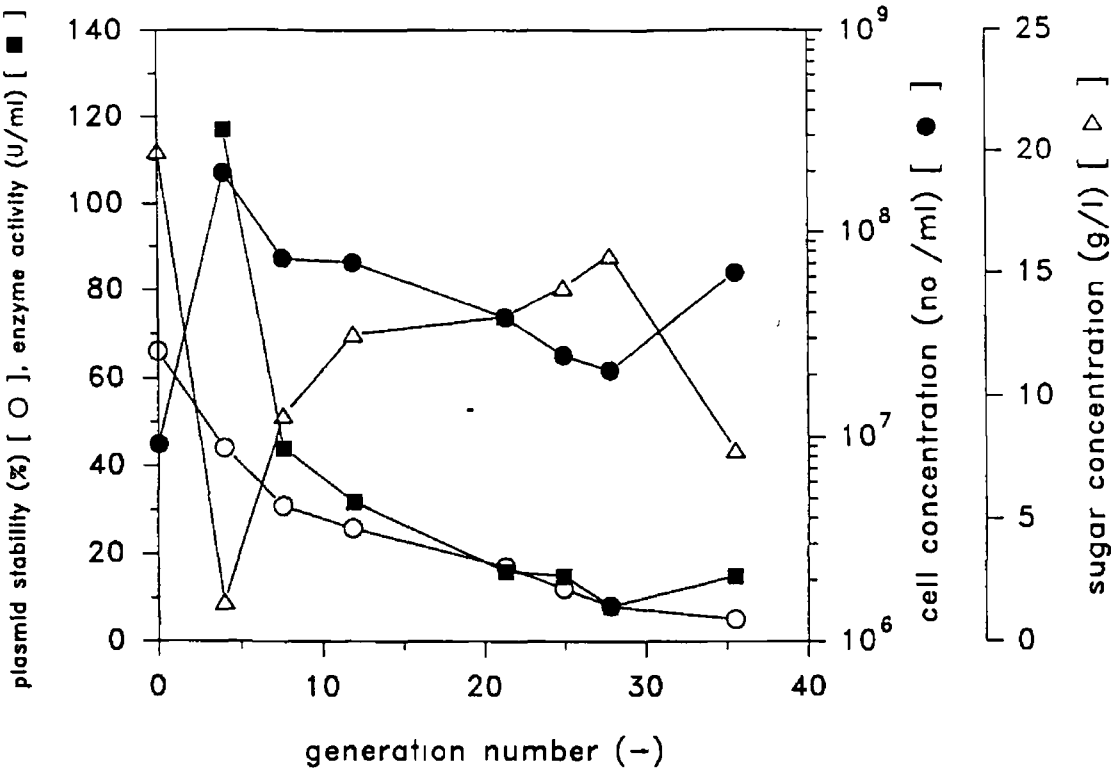


Figure 5 8 Fermentation profile for free cell continuous culture ($D = 0\,5\ h^{-1}$)

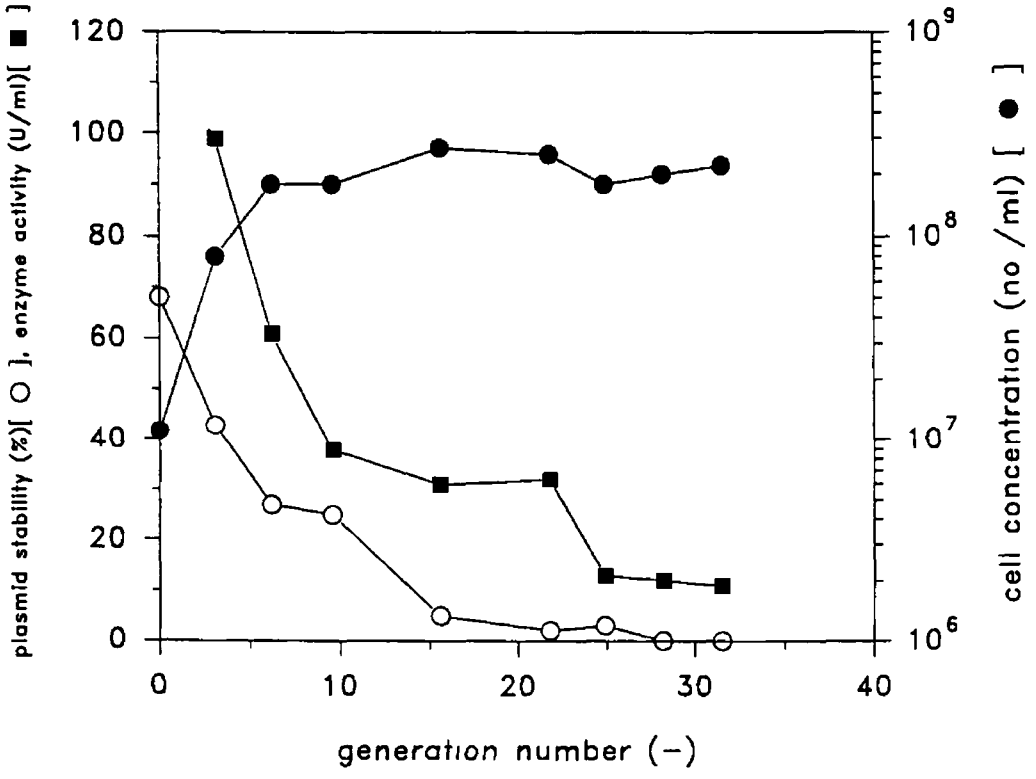


Figure 5 9 Fermentation profile for free cell continuous culture ($D = 0\,1\ h^{-1}$)

5.5 THE INFLUENCE OF GROWTH RATE ON PLASMID STABILITY AND PCN

The operation of a glucose limited chemostat enabled direct control over growth rate of the cells A fast (D = 0 5h^{-1}) and slow (D =0 1h^{-1}) growing cultivation was achieved Serial batch cultivation (Section 5 3) represents a cell population growing in excess nutrients with no limitations in other environmental factors such as oxygen supply In other words, the cells growing in serial batch grew at a maximum or near maximum specific growth rate before slowing down when nutrients became exhausted The cells were soon after reinoculated into fresh medium and continued to grow at their maximum specific growth rate When investigating the influence of growth rate on plasmid stability and PCN, data from a slow and fast growing culture was obtained from chemostat studies, and data from an assumed maximum specific growth rate was obtained from serial batch fermentations The influence of growth rate on plasmid stability is shown in Figure 5 10 while growth rate effects on PCN are presented in Figure 5 11

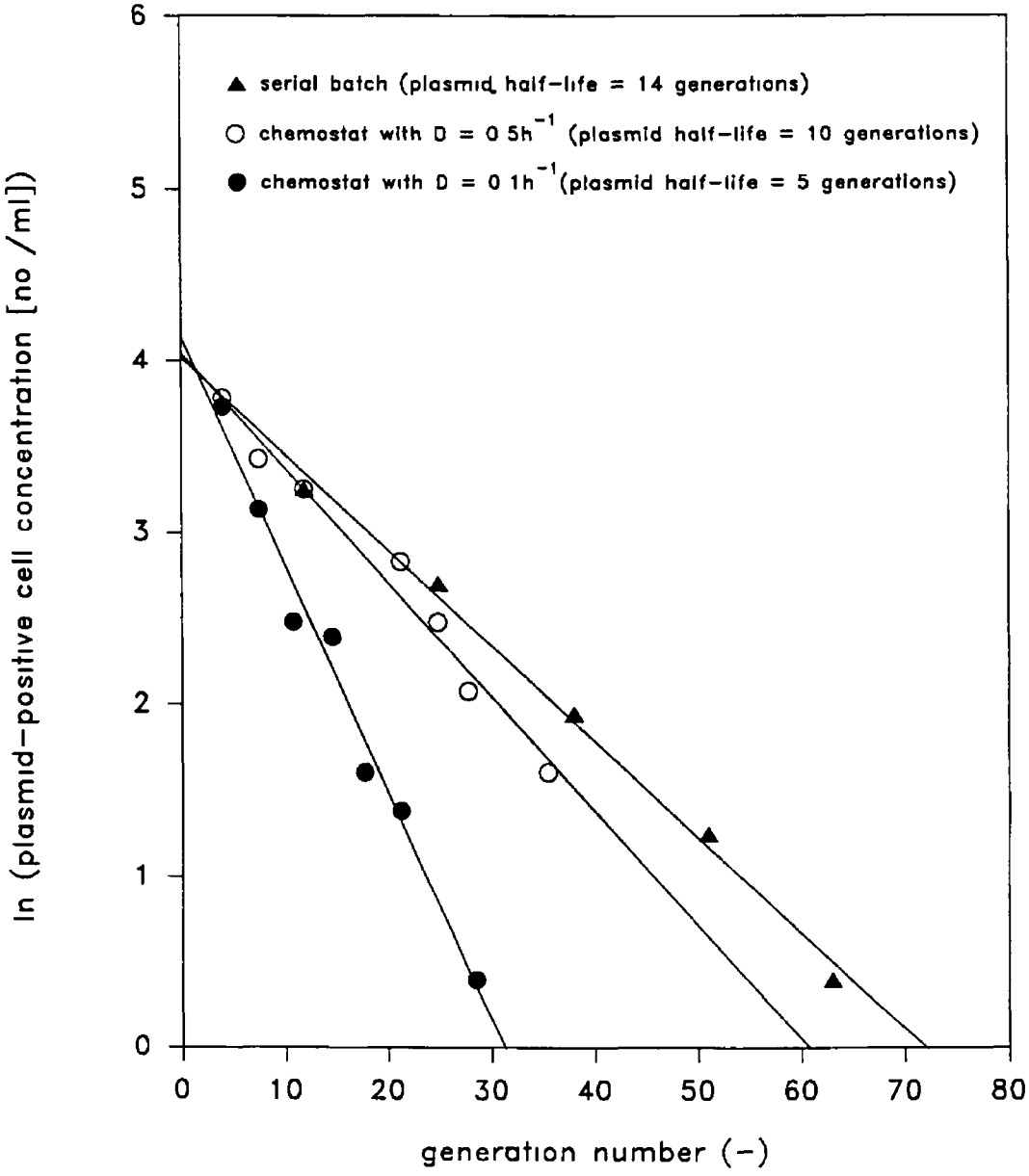


Figure 5.10 Plasmid stability profiles for free cells in serial batch and chemostat cultures

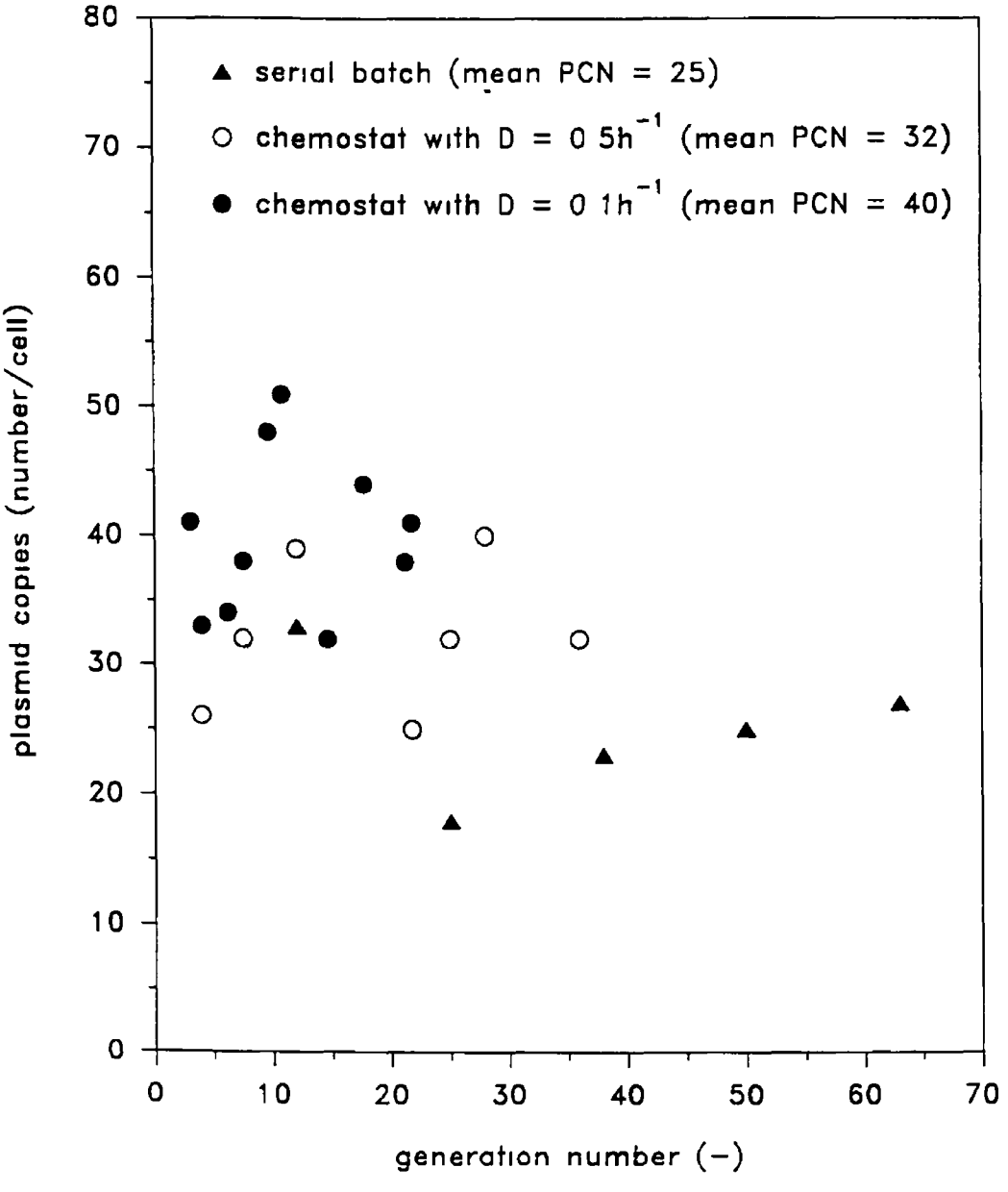


Figure 5 11 Plasmid copy number profiles for free cells in serial batch and chemostat cultures

5.6 THE RELATIONSHIP BETWEEN GROWTH RATE, PCN AND ENZYME PRODUCTIVITIES

By reviewing all free cell fermentations *i e* batch fermentations in shake flasks and in fermenters, using both selective and YEPD media, and continuous cultures operated at high and low dilution rates, a relationship between growth rate, PCN and enzyme activity and enzyme productivity was established and is presented in Table 5 3 Where

apparent specific activity (U/cell) = E/X_t

specific enzyme activity (U/plasmid positive cell) = $(E/X_t)(100/P+)$

volumetric enzyme productivity (U/ml-h) = E_f/t_f {for batch systems}

volumetric enzyme productivity (U/ml-h) = $E_{av}\,D$ {for continuous systems}

Table 5 3 *Relationship between growth rate, plasmid copy number, enzyme activity and enzyme productivity for different free cell fermentations*

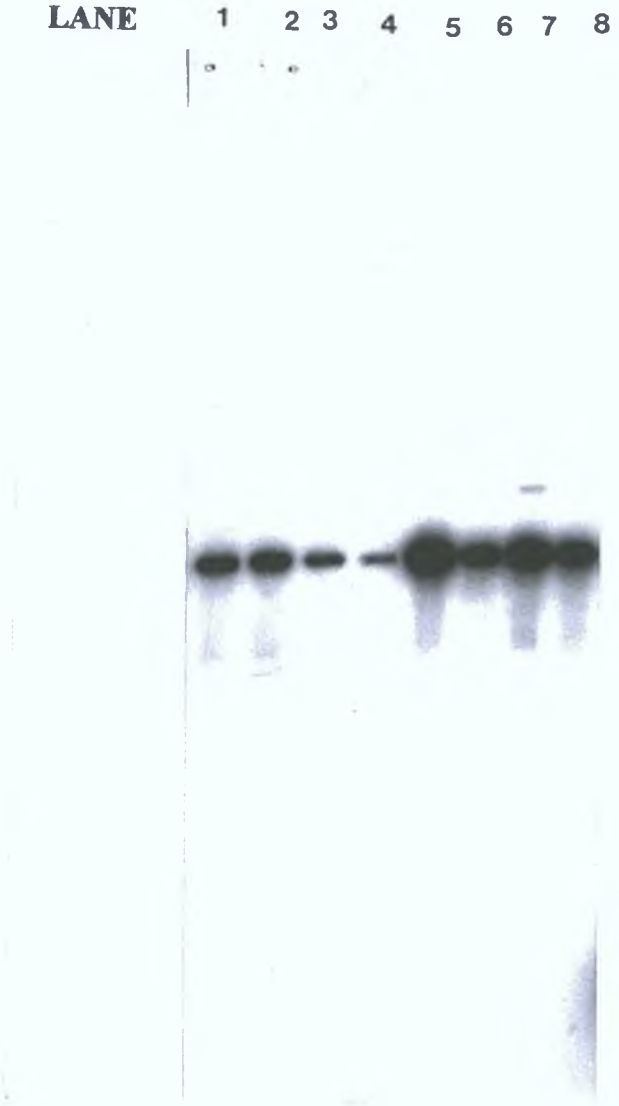
Fermentation	Plasmid copy number	Specific activity (10^{-6} U/cell)	Enzyme productivity (U/ml-h)	Plasmid stability (%)
Selective medium 2 l bioreactor	8	1 4	1 7	100
YEPD flask[a] (fast μ)	27	2 9	2 2	30[b]
Chemostat $D = 0\ 5\ h^{-1}$	32	3 4	22 0	high
Chemostat $D = 0\ 1\ h^{-1}$	40	4 8[c]	10 0	low

[a] mean value from several different flask scale fermentations
[b] final plasmid stability
[c] mean value obtained from two continuous fermentations

5.7 PCN CORRECTION FACTORS

The procedure for determining PCN is intricate and involves many variables which are difficult to control Such variables include quantity of DNA transferred to nitrocellulose, degree of incorporation of radioisotope into probe DNA, degree of hybridisation of particular sequences to filter bound DNA, autoradiogram exposure time *etc* These factors cause the estimation of PCN to vary from one autoradiogram to another

In order to standardise the PCN values obtained, two samples were selected from each of the free cell fermentations (batch, serial batch and continuous) and the PCN determined from a single autoradiogram (Figure 5 12) From these data the relative PCN values of the different fermentations was calculated Furthermore any of these DNA samples used in this autoradiogram could be used as a "standard reference" in future PCN measurements Each "standard reference" has an accepted PCN value as determined from this autoradiogram (Figure 5 12) When used with other samples in other autoradiograms the final PCN value can be adjusted according to the relative values of PCN of the standard refrence sample in the new autoradiogram and the accepted PCN determined from this autoradiogram In this way a PCN value becomes reproducible from one autoradiogram to the next and also takes into account the relative differences in PCN from one fermentation type to another In practice usually two different standard reference samples were included in all agarose gels and hence autoradiograms, so as to minimise errors in PCN correction factors



LANE	SAMPLE
1	Continuous culture (D=0.67 h^{-1}) Gen no. 9
2	Continuous culture (D=0.5 h^{-1}) Gen no. 5
3	Continuous culture (D=0.1 h^{-1}) Gen no. 8
4	Serial batch culture Gen no 25
5	Batch culture (YEPD) T=6 h
6	Batch culture (YEPD) T=49 h
7	Batch culture (Selective) T=12 h
8	Batch culture (Selective) T=58 h

Figure 5.12 Comparison of the PCN of cells from different fermentations for the purpose of establishing "standard references" and correction factors.

5.8 DISCUSSION

Growth of the yeast with or without plasmid pJG317 is greater in YEPD (Complex) medium than in selective medium with final cell numbers in the former ten times that in the latter This corresponds to about two and a half more generations Since the cloned gene product, (1,3) (1,4) β-glucanase production is growth associated (Cahill, 1990), enzyme production is greater in YEPD than in selective medium Despite the fact that there is a loss in the number of plasmid containing cells and hence those cells which can produce the β-glucanase enzyme when the cells are grown non-selectively, the volumetric enzyme productivities and specific enzyme activities are higher for YEPD batch culture than for selective batch culture (Table 5 3) Plasmid pJG317 is quite unstable under non-selective conditions, with the percentage plasmid containing cells falling to 37% after only 24 hours The PCN of cells grown in both media did not change drastically throughout the fermentations There was greater variation in the PCN values of cells grown in YEPD as opposed to selective medium (Table 5 1) The average PCN for the two batch fermentations were 8 4±0 5 copies and 29 8±4 4 copies per cell for selective medium and YEPD respectively Although the growth rate of cells affects PCN (Bugeja et al , 1989, Walls and Gainer, 1989) and the growth rate of cells in YEPD is greater than that in selective medium, other factors such as medium composition or nutrient requirements/limitations must also play a role in determining PCN

Serial batch fermentation was performed until plasmid pJG317 was totally lost from the cell population Figure 5 6 clearly shows a decrease in enzyme activity with plasmid loss Cloned gene product activity is proportional to the concentration of plasmid in the cells This result is in agreement with results from Kingsman et al (1985) and Romanos et al (1992) for a constitutively expressed plasmid encoded gene product During the measurement of PCN, the autoradiogram clearly showed the 6 6kb Sal I restriction fragment becoming fainter as the fermentation progressed, until it disappeared altogether (Figure 5.7) The β-actin band intensity remained relatively constant for each sample This result confirms the loss of plasmid from the cells as the measured plasmid stability decreases Therefore, it may be assumed that segregational instability rather than structural instability

is the dominant factor in the increased pJG317 loss Futcher and Cox (1984), Impoolsup et al (1989) and Porro et al (1991) also reported segregational instability as the major reason for plasmid loss in recombinant yeast cell fermentations The PCN of the cell population decreased as the plasmid was lost However, the PCN per plasmid containing cell remained relatively constant with an average PCN value of 25 2±5 5 copies per cell (Table 5 2) This compares well with YEPD batch culture

In order to assess the effects of growth rate on plasmid stability, PCN and enzyme activity, better defined and controlled growth conditions were necessary For this reason chemostat culture was performed Low (D=0 1h^{-1}) and high (D=0 5h^{-1}) dilution rates were investigated Serial batch culture represents a cell population growing in excess nutrients with little or no limitations in other environmental factors such as oxygen supply Although it was very difficult to exercise control over cells in serial batch, it is assumed that the cells grow at a maximum or near maximum growth rate Figure 5 10 illustrates the effect of growth rate on plasmid stability It can be seen that the plasmid has a higher stability at higher growth rates than lower growth rates Kleinman et al (1986) suggested that a possible reason for increased plasmid stability at higher growth rates could be that cell division results in larger buds at high growth rates, thus increasing the chances of plasmid transfer to the daughter cells From Figure 5 10, it can also be seen that the plasmid loss per generation remains constant for the different growth rates In this way plasmid loss kinetics are similar to some enzyme decay kinetics (*i e* exponential decay) and as in the case of enzymes the half-lives of the plasmid may be calculated Half the number of plasmid bearing cells lose plasmids every 14 generations at near maximum growth rates (serial batch) while at low dilution rates, the same number of cells lose their plasmids every 5 generations Futcher and Cox (1984) also reported that for several recombinant yeast systems, plasmid instability per generation remained constant for a particular growth rate Bugeja et al (1989), Walls and Gainer (1989), Porro et al (1991) and De Silva and Bailey (1991) all reported that for recombinant yeasts grown in continuous culture, the loss of the plasmid varied with dilution rate In all cases the greatest loss occurred at the lower dilution rates The results obtained indicate that the stability of the recombinant yeast system may be improved by operating at high growth (dilution) rates

Porro et al (1991) found the PCN not to change at different dilution rates while Coppella et al (1989) reported that PCN showed no dependence on the physiological state of the cell In the DBY746(pJG317) recombinant yeast system, the PCN of fast growing cultures (serial batch) which had the highest plasmid stability had the lowest PCN of 25 2±5 5 copies per cell The next fastest growing culture (D=0 5h^{-1}) had an average PCN value of 32 3±5 7 copies per cell, while the slow growing culture (D=0 1h^{-1}) which had the lowest plasmid stability had the highest PCN of 40 0±6 3 copies per cell A one way analysis of variance least significant difference test with significance level 0 05, which tests whether the means of samples are significantly different from each other was performed, and showed the average PCN values at all three growth rates to be significantly different from each other

Siegel and Ryu (1985) reporting on a kinetic study of instability of a recombinant plasmid in *E coli* in continuous culture draw conclusions which may be applied to this recombinant *S cerevisiae* system in order to explain the influence of growth rate on plasmid stability and PCN It is suggested that the increased plasmid content (or PCN) at low growth rates makes the plasmid-carrying cells (P+) less competitive with those cells which have lost the plasmid (the plasmid free segregants, P-) *i e* a difference in growth rate between P- and P+ cells exists This would lead to a more rapid increase in the P- fraction of the population Plasmid stability would be lower in the slow growing cultures due to the metabolic burden placed on the cells as a result of high plasmid content and increased expression of the cloned gene The converse would apply to cells growing at fast growth rates

Several findings suggest that factors other that growth rate influence PCN, such as the nature of the growth limiting nutrient or the more intense selection that occurs under extreme nutrient limitation of low dilution rate conditions (Kleinman et al , 1986, De Taxis du Poet et al , 1987, Reinikainen and Virkajarvi, 1988, Bugeja et al , 1989) The influence of nutrient requirements and / or limitations is investigated in the present study and is presented in Chapter Seven

Throughout the free cell fermentations enzyme activity, plasmid stability and PCN were measured Table 5 3 outlines the relationship between these parameters Slow growing cultures have a higher plasmid content (PCN)

and also tend to have a greater specific enzyme activity The greater the number of plasmid copies present in the cell, the greater the cloned gene expression leading to a higher enzyme production This finding reinforces the possibility of a greater metabolic burden occurring in those cells with higher PCN (and hence higher enzyme production), leading to a greater loss of plasmid from the cells *i e* a low plasmid stability This is observed in slow growing cultures The opposite is found in fast growing cultures Low PCN results in lower specific enzyme activity and therefore a lower stress or metabolic burden on the recombinant cells, which leads to a higher plasmid stability Table 5 3 shows this to be the case for DBY746 (pJG317) free cell fermentations

That such a correlation may be made is testimony to the reproducibility and effectiveness of the method for measuring PCN Having been developed, tested and optimised with cells grown in the simplest, fermentation type *i e* batch culture in selective medium, the procedure was applied to a complete range of multiple samples for various different cultivation conditions using complex media The consistency of results for a given fermentation and the difference in results between different fermentations implies that the procedure may be confidently employed to measure the PCN of recombinant yeast systems The lowest PCN value measured was 8 copies per cell for selective batch culture and the highest PCN value measured was 40 copies per cell for continuous culture at a dilution rate of D=01 h^{-1} The values obtained are comparable to those of yeast episomal plasmids which is typically about 20 to 40 per genome and the stability is less than that of the native 2μ circle (Futcher and Cox, 1984, Bugeja et al , 1989)

The use of selective pressure in all seed cultures should ideally provide a population consisting of 100% plasmid containing cells However, this is not the case, as can be seen from Figures 5 2, 5 8 and 5 9 where the starting cell population contains about 70% plasmid containing cells Various experiments on seed cultivation conditions were unable to increase this value, although younger seed cultures did result in slightly higher plasmid stabilities The selective pressure is not totally effective because the growth rate of plasmid-free cells in selective medium is not zero (Syamsu et al , 1992) The reason for the relative ineffectiveness of the selective medium is thought to be leakage of the gene product or some component which forms the basis of the selective mechanism Leakage, whether directly into the plasmid-free daughter cells or

into the bulk medium itself, followed by uptake by the plasmid-free segregants, enables them to grow in conditions which were expected to select against them (Srienc et al , 1986 Satyagal, 1989, Mason, 1991)

CHAPTER SIX

DETERMINATION OF GROWTH AND PLASMID STABILITY PROFILES IN IMMOBILISED CELL CULTURE AND MEASUREMENT OF PLASMID COPY NUMBER

6.1 INTRODUCTION

The technique developed for measuring PCN was successfully applied to free cell fermentations involving different media and differing fermentation modes The relationships between growth rate, plasmid stability, PCN and enzyme activity have also been investigated Using the multistep dissolution technique of Walsh et al (1993) immobilised cell cultures of DBY746 (pJG317) were studied by isolating and analysing cell samples from various locations throughout the gel beads The heterogeneous nature of cell growth in the gel beads was illustrated It was also established that the plasmid stability (averaged over the whole bead) of immobilised DBY746 (pJG317) cells was greater than for free cells Using the multistep dissolution technique the evolution of a gradient in plasmid stability throughout the gel beads during a fermentation was detected The PCN of immobilised recombinant yeast has not been reported although it has been hypothesised that a difference in PCN might exist in cells immobilised in different locations within the gel beads, and is a contributing factor to the higher plasmid stability and the gradient in plasmid stability which exists in immobilised cells In order to test this hypothesis, the PCN of cells immobilised in various locations throughout the gel beads was measured

Diffusion resistances give rise to a heterogeneous growth pattern in immobilised cell gel beads, which in turn give rise to populations of cells with differing plasmid stabilities (Nasri et al , 1987a) This is due to cells growing faster in the outer sections of the gel beads and other cells growing slower in the inner regions of the beads The influence of growth rate on PCN for free cell cultures has been described in the previous chapter and this study is extended to determine if growth rate differences affect the PCN in an immobilised cell system

6.2 ENZYME PRODUCTION AND PLASMID STABILITY

Immobilised cells were prepared as in Section 2 5 2 with an ICL of 10^6 cells/g-gel Repeated batch cultivation (Section 2 5 6) was performed with 50g gel beads in 250ml medium in a 0 5 1 Erylenmeyer flask and three medium changes in YEPD A similar immobilised cell fermentation was performed with the first batch incubation in selective medium rather than YEPD, with subsequent batch incubations in YEPD The purpose of this was to assess the effect of first incubating the immobilised cells in selective medium (a term referred to as "Preincubation") on enzyme production and plasmid stability On preincubation of the beads, the cells grew from 10^6 to 10^8 cells/g-gel, and as selective medium was used, the whole population maintained the plasmid pJG317 To contrast this method of fermentation a control of ordinary repeated batch fermentations were carried out in YEPD only *(i e* no preincubation in selective media) Preincubation thus selected for a large population of plasmid bearing immobilised cells at the beginning of a particular fermentation

Table 6 1 and 6 2 show that preincubation does result in higher plasmid stabilities of immobilised cells and hence higher enzyme activity, compared to immobilised cells grown in YEPD only Final cell numbers are quite close to each other for the different fermentations

Table 6 1 Plasmid stability, enzyme activity and cell growth in repeated bacth fermentation with preincubation in selective medium

Batch	Cell concentration (no /g-gel)	Plasmid Stability (%)	Enzyme activity (U/ml)	Leaked cell concentration (no /ml)
Selective	9 4 x 10^7	65	33	0
YEPD #1	9 1 x 10^8	54	97	3 0 x 10^7
YEPD #2	9 4 x 10^8	53	97	3 3 x 10^7
YEPD #3	1 3 x 10^9	46	105 [a]	9 4 x 10^7

[a] 48 h incubation

Table 6 2 *Plasmid stability, enzyme activity and cell growth in repeated batch fermentation with no preincubation*

Batch	Cell concentration/ (no /g gel)	Plasmid Stability (%)	Enzyme activity (U/ml)	Leaked cell concentration (no /ml)
YEPD #1	6 5 x 10^8	28	54	5 2 x 10^6
YEPD #2	1 3 x 10^9	25	59	5 1 x 10^7
YEPD #3	1 5 x 10^9	22	49	5 4 x 10^7
YEPD #4	1 7 x 10^9	22	64 [a]	9 2 x 10^7

[a] 48 h incubation

6.3 GROWTH PROFILES IN ALGINATE BEADS

Using the multistep dissolution technique (Section 2 6 12) cell numbers throughout the alginate gel beads were calculated for the above repeated batch fermentation Figure 6 1 illustrates the development of a gradient in cell growth as the fermentation progresses A similar gradient also developed in those beads in the repeated batch culture with no preincubation

6.4 PLASMID STABILITY PROFILES IN ALGINATE BEADS

During the course of the above repeated batch fermentations beads were sampled at each media change and multistep dissolutions performed in order to measure the plasmid stability of cells throughout the beads (Section 2 6 6 2) Total dissolutions (Section 2 6 1 1) were also performed to determine the average plasmid stability of all cells immobilised at that particular time during the fermentation

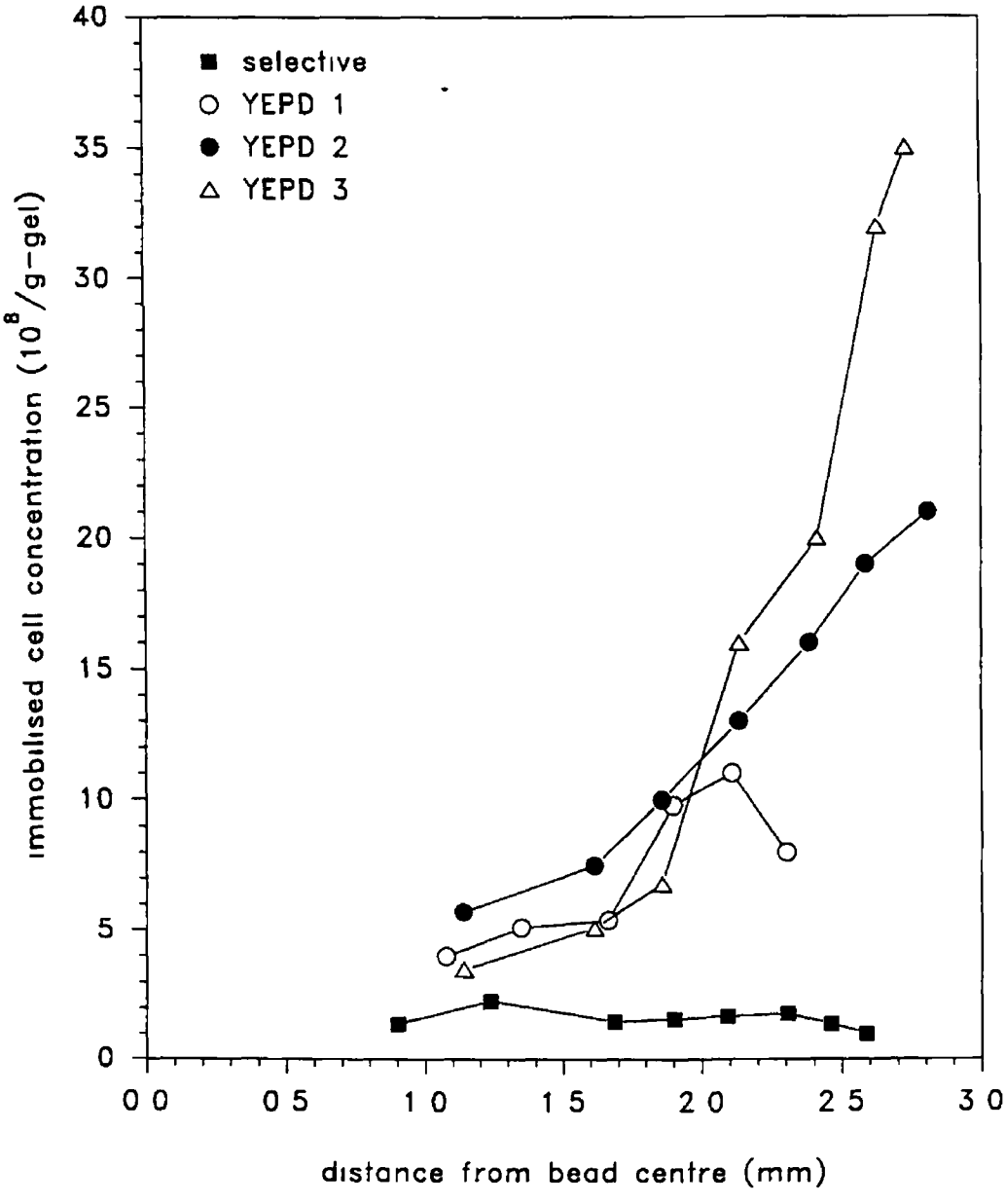


Figure 6 1 Immobilised cell concentration profiles determined using multi-step dissolution of gel beads (repeated batch fermentation with pre-incubation in selective medium)

Multistep dissolutions were carried out in such a way so as to liberate cells from the outer 200μm of the gel beads (referred to as "outer cells") and cells from the inner 2 0mm of the gel beads (referred to as "inner cells") Therefore, plasmid stability could be determined for inner cells, outer cells, cells from the total bead (referred to as "total cells") and also cells which had leaked from the gel matrix Table 6 3 shows that a gradient in plasmid stability does exist throughout the gel beads and Figure 6 2 illustrates the progressive loss of plasmid from cells in the inside and outside of the beads and also from leaked cells over the course of a fermentation run

Table 6 3 Plasmid stability profiles (%) for immobilised cells during repeated batch culture (with preincubation)

Batch	Total cells (%)	Outer cells (%)	Inner cells (%)	Leaked cells (%)
Selective	65	-	-	-
YEPD #1	53	53	69	54
YEPD #2	54	52	65	46
YEPD #3	46	46	63	29

6.5 MEASUREMENT OF PLASMID COPY NUMBER

In order to measure the PCN of cells immobilised throughout the alginate gel beads and of cells located in the inner and outer regions of the beads, total and multistep dissolutions (Section 2 6 1) were performed Beads were sampled throughout the repeated batch fermentations described in Section 6 2 and dissolutions were performed so as to liberate the cells from throughout the beads ("total cells"), from the inner 2 0mm ("inner cells") and from the outer 200μm ("outer cells") Total DNA was isolated from the cells (Section 2 7 3), restricted, electrophoresed, blot transferred and hybridised to probe pPC64 so that the PCN could be determined Due to differences in cell numbers throughout the gel beads, different numbers of beads had to be progresively dissolved in order to harvest enough cells from the outside and inside of the gel beads In the case of outer cells, multistep dissolutions were performed on a sample size of 6 beads, so as

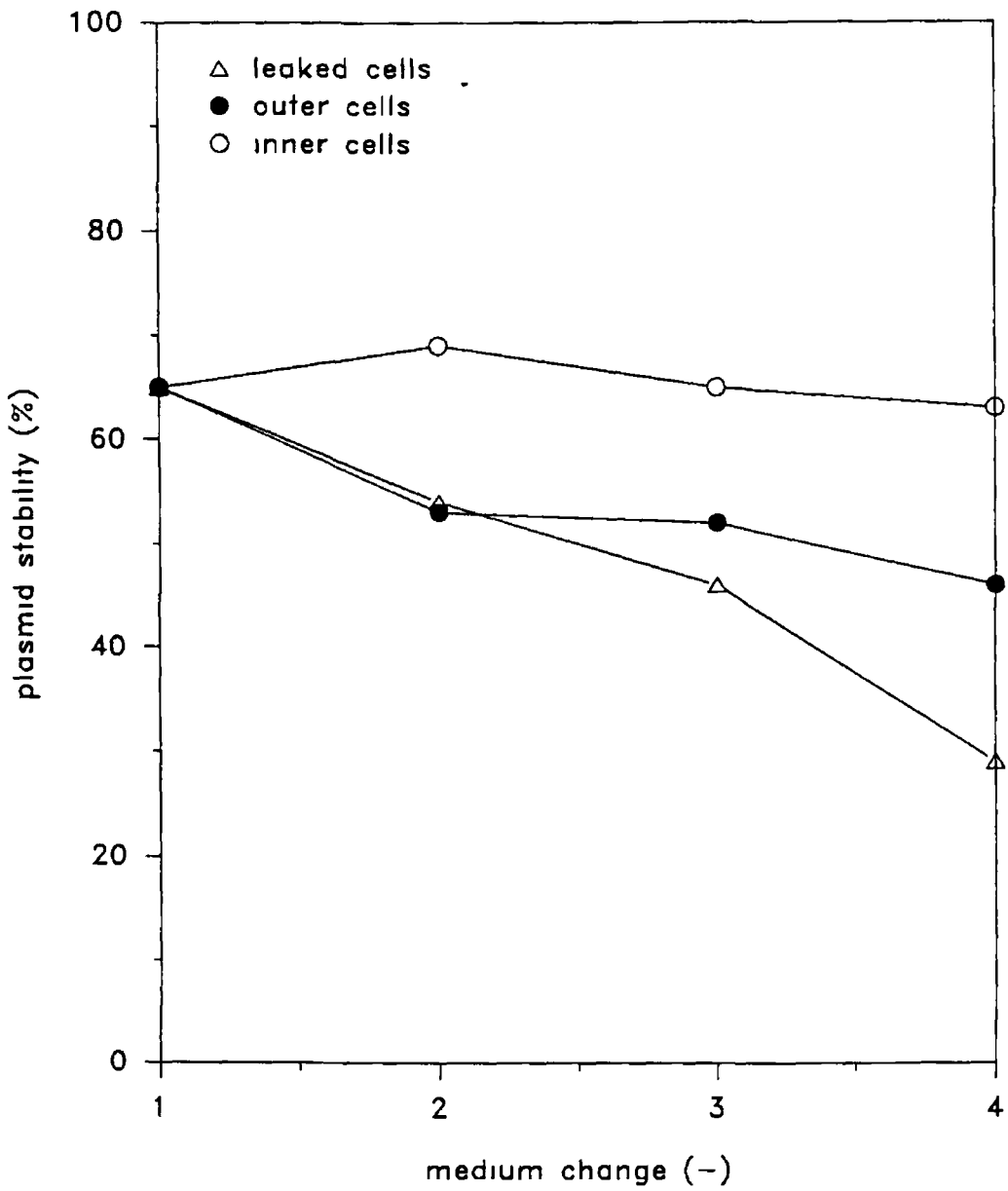


Figure 6 2 Plasmid stability profiles of immobilised cells (repeated batch fermentation with pre-incubation in selective medium)

to isolate a sufficient quantity of DNA to measure the PCN of one sample In the case of inner cells, where cell numbers were of the order of ten times less than on the outside of the bead, up to 60 beads had to be progressively dissolved, so that the PCN of one sample of inner cells could be measured

On development of the autoradiograms, it was noticed that instead of the usual two bands that appear (*i e* the chromosomal β-actin band and the plasmid pJG317 band), there was a third band present This extra band was located between the two former bands and was approximately 8 0kb in size Several characteristics associated with the appearance of this extra band included the fact that it was independent of hybridisation temperature (64°C or 68°C), it was reproducible, it appeared much more frequently in immobilised cell DNA samples than in free cell DNA samples It was noticed that, the longer the cells were immobilised the darker or more intense the extra band was and it only appeared faintly in free cell samples where plasmid stability (and hence plasmid content per cell) was high Figure 6 3 is an example of an autoradiogram showing the β-actin band, the pJG317 band and the extra band

It was unlikely that the DNA isolation procedure was responsible, as samples isolated together and using the same reagents showed different results *i e* DNA from leaked cells (where no extra band was present) was isolated together with DNA from immobilised cells (where the extra band was present) It is possible that the appearance of this extra band was the result of partial digestion of the yeast total DNA and in particular pJG317 As shown in Figure 6 4, complete digestion of pJG317 by the restriction enzyme Sal I yields four fragments, of sizes 6 6, 4 7, 1 0 and 0 5kb However, a partial digestion could yield extra fragments of 7 6 and 5 2kb These fragments would contain sections of the β-glucanase gene The extra band appearing on the autoradiograms could be the 7 6kb partial fragment from pJG317

In order to test this hypothesis, the following experiment was performed The β-glucanase gene was cut from plasmid pJG106 (β-glucanase gene in pUC-19 vector), gene cleaned and labelled non-radioactively (Section 2 7 10) A duplicate nitrocellulose filter to one that had been previously probed with ^{32}P- labelled pPC64 and showing the extra band was then probed with this new β-glucanase probe (Section 2 7 11 2) The results in

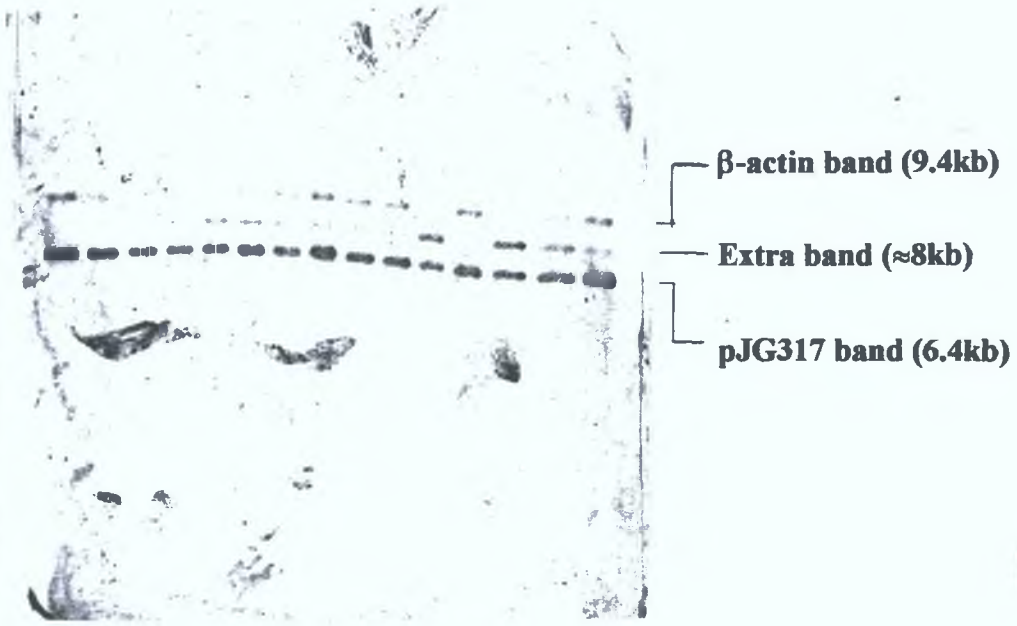


Figure 6.3 Filter bound genomic DNA isolated from immobilised cells and hybridised to a non-radioactively labelled pPC64 probe.

Note the appearance of a third "extra" band of approx. 8kb, between the usual chromosomal β-actin band (9.4kb) and plasmid pJG317 band (6.6kb) in some lanes.

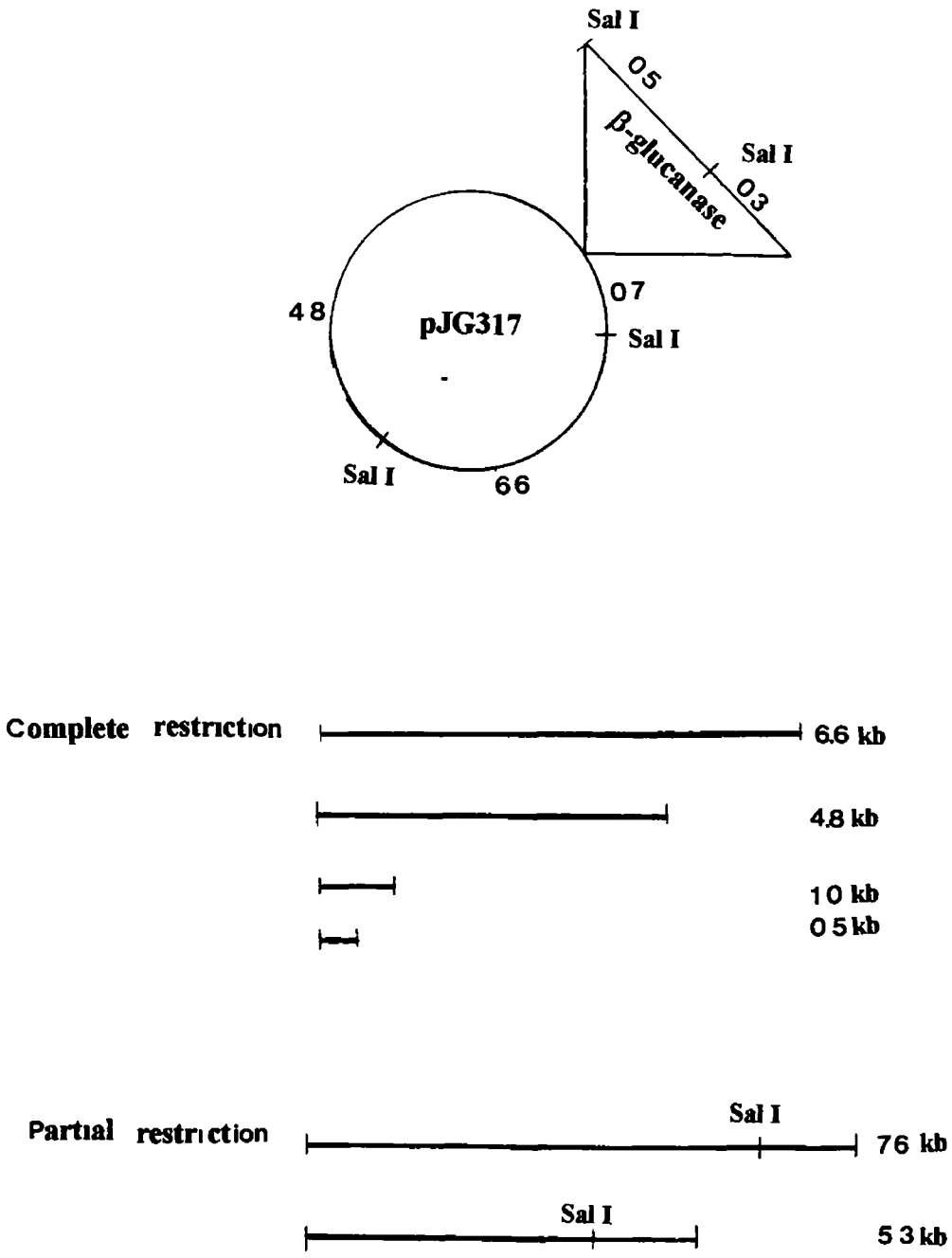


Figure 6 4 Schematic diagram of plasmid pJG317 showing the resultant fragments of complete Sal I restriction digestion and partial digestion (The 7 6kb partial restriction fragment is the "extra band")

NOTE The β-Glucanase gene, when used as a probe hybridises to the completely restricted 0 5 and 1 0kb fragments and hybridises to the 7 6 and 5 3kb partially restricted DNA fragments The β-Glucanase probe also hybridises to linear open circular, covalently closed circle and multimer forms of the unrestricted plasmid that would be present in a partially restricted total yeast DNA sample

Figure 6 5 show that the extra band and multimer bands did hybridise to the β-glucanase probe Therefore, it was concluded that DNA from immobilised cells was not restricting completely Without complete restriction of the DNA the procedure for measuring PCN is not accurate

To circumvent the problem of incomplete DNA restriction of immobilised cell samples, the DNA was diluted 2 fold, and extra restriction enzyme was used, (4μl enzyme per 20μl diluted DNA) This improved the restrictability of the DNA samples and the PCN was measured Some lanes on the autoradiogram had a faint extra band and the PCN was calculated from the relative intensities of the single copy β-actin band to the sum of the 6 6kb pJG317 band and the faint 7 6kb partially cut pJG317 band The resultant value was regarded as an estimated rather than an absolute PCN value Each immobilised cell sample was assayed for PCN up to 4 different times yielding absolute values or a mixture of absolute and estimated values An average figure yielded the final PCN for that particular sample Figure 6 6 illustrates autoradiograms for which absolute PCN values (complete digestion) and estimated PCN values (partial digestion) were calculated Also shown are samples from which the partial restriction of DNA was too great to yield an accurate figure for PCN and hence these sample were rejected Table 6 4 shows the calculated PCN for immobilised cells including those values estimated in the case of partial digestion of the DNA Table 6 5 is a summary of the results for PCN of immobilised cells during repeated transfer fermentations with and without preincubation

Finally, a second repeated batch fermentation was performed in YEPD (with no preincubation) Starting with 10^6 cells/g-gel the immobilised cells were grown to 1 2 x 10^9 cells/g-gel over three batch incubations Multistep dissolutions were performed to remove cells from the outer 0 5mm ("outer cells") inner 3 0mm ("inner cells") and cells throughout the whole bead ("total cells") PCN was measured as before on samples from the final batch fermentation The results were very similar to the first set of repeated batch experiements The PCN values obtained were as follows total cells 33, inner cells 29, outer cells 39, leaked cells 36 plasmid copies per cell

From the above results, it may be concluded that the PCN of immobilised DBY746 (pJG317) cells does not vary significantly throughout a gel bead

Unlike growth or plasmid stability no detectable gradient in PCN exists throughout an immobilised cell gel bead

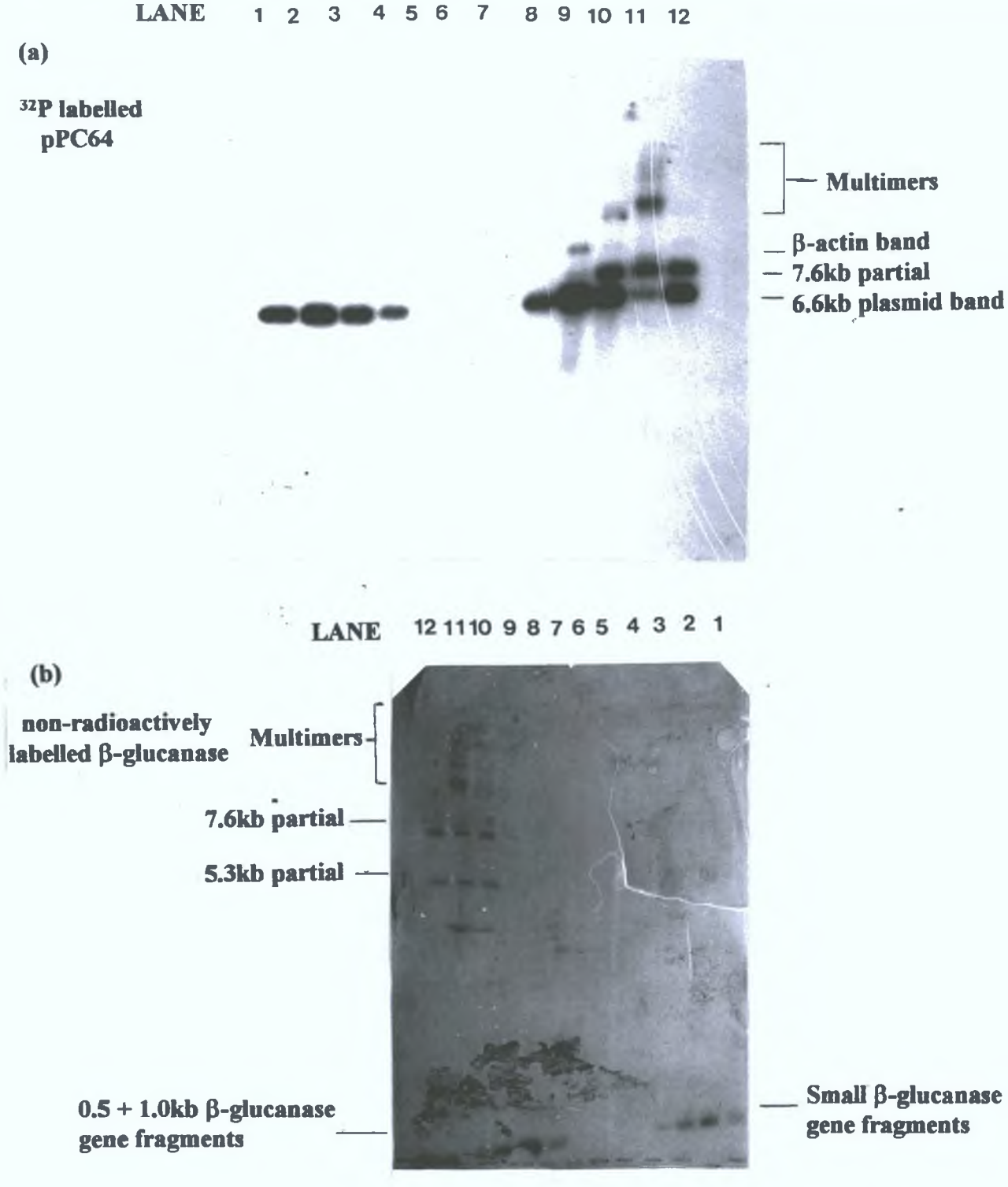
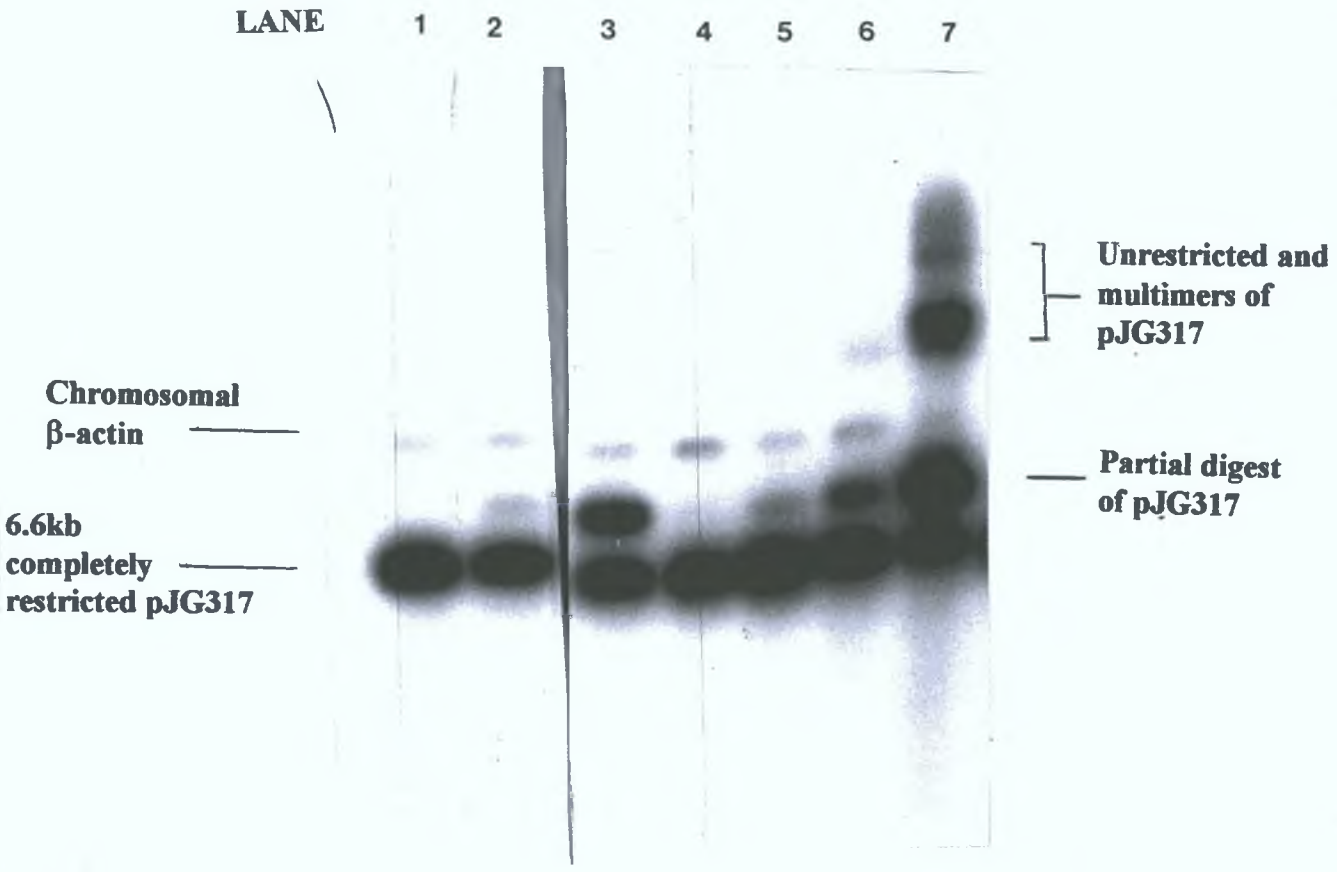


Figure 6.5 Duplicate filters showing the "extra" band probed with (a) pPC64 and (b) β-glucanase gene.

Note: On complete restriction only the small β-glucanase gene DNA fragments which are cut out of plasmid pJG317 on Sal I restriction should be visible (Lanes 1-7).
The presence of multimer bands, and bands at 7.6 and 5.3kb in only those lanes showing an extra band (i.e. lanes 10-12) indicates the presence of β-glucanase DNA in these bands, leading to the conclusion of partial digestion of pJG317 being responsible for the "extra" band.



LANE	OPTION
1	Complete restriction; absolute value of PCN
2	Slight partial restriction; estimated value of PCN
3	Much partial restriction; impossible to accurately measure PCN
4	Complete restriction; absolute value of PCN
5	Slight partial restriction; estimated value of PCN
6	Much partial restriction; impossible to accurately measure PCN
7	Much partial restriction; impossible to accurately measure PCN

Figure 6.6 Autoradiogram in which total genomic DNA from immobilised cells was probed with pPC64.

Note: PCN was calculated normally in cases of complete restriction (Lanes 1 and 4). An estimated value of PCN was obtained in cases where slight partial restriction fragments occurred (Lanes 2 and 5). However, partial restriction was too extensive in other cases (Lanes 3, 6, 7) for any accurate value of PCN to be calculated.

Table 6 4 Calculated PCN for immobilised cells during repeated batch fermentations with and without preincubation

SAMPLE	FERMENTATION	PCN	AVERAGE PCN
Total cells	Selective	18, 22❖	20
	YEPD 1	32, 24❖, 29❖	28
	YEPD 2	30, 29❖	30
	YEPD 1a	25, 26, 21, 25	24
	YEPD 2a	29, 36❖, 30❖, 38❖	33
	YEPD 3a	29, 37❖	33
	YEPD 4a	32, 32	32
Inner cells	YEPD 1	28❖, 33	31
	YEPD 1a	33	33
	YEPD 2a	46, 33	40
	YEPD 3a	29, 34❖, 43	35
	YEPD 4a	34, 48, 41	41
Outer cells	YEPD 1	34❖	34
	YEPD 1a	30❖, 36	28
	YEPD 3a	28❖	28
	YEPD 4a	34	34
Leaked cells	YEPD 1	34	34
	YEPD 2	27, 30	29
	YEPD 3	30, 36	33
	YEPD 2a	32, 37, 34	34
	YEPD 3a	20, 33, 30	28
	YEPD 4a	34, 34, 45	38

Note (i) Selective, YEPD 1, YEPD 2, YEPD 3 Repeated batch fermentation involving preincubation of alginate beads in selective medium, followed by 3 consecutive incubations in YEPD

(ii) YEPD 1a, YEPD 2a, YEPD 3a, YEPD 4a Repeated batch fermentation involving 4 consecutive batch incubations in YEPD

(iii) ❖ indicates estimated value of PCN, calculated from the relative intensities of single copy chromosomal β-actin DNA and the sum of the faint partial pJG317 7 6kb fragment and the normal 6 6kb pJG317 completely restricted plasmid fragment

Table 6 5 PCN of immobilised cells in YEPD - summary of results

Location of cells	Mean PCN	Number of data points
Inner	36 5±6 8	11
Outer	32 4±3 3	5
Leaked	32 6±5 6	14

Note The statistical analyis performed on the three samples above plus the total dissolutions was a one way analysis of variance Least significant difference test with significance level 0 05, which tests whether the means of samples are significantly different from each other

6.6 DISCUSSION

Two distinct phases of immobilised cell growth have been reported (Monbouquette et al , 1990) Early in a typical immobilised cell fermentation, the cells throughout the gel matrix rapidly divide and grow at approximately equal rates The result of this first phase is an increasing bioreactor volumetric productivity and very low leaked biomass concentrations in the fermenter The cells continue to grow and form spherical micro-colonies (Gosmann and Rehm, 1988) After a number of generations a second phase in immobilised cell growth becomes apparent The microbial population becomes increasingly non-uniform and the more rapidly growing surface colonies consume substrates and nutrients to the detriment of cells located in the interior of the gel beads Eventually a dense outer layer of biomass forms around a relatively sparsely populated core (Wada et al , 1980, Monbouquette et al , 1990, Walsh et al , 1993) Figure 6 1 illustrates the development of the heterogeneous distribution of cells within the calcium alginate gel beads Throughout the course of a repeated batch fermentation, relatively uniform growth takes place during the first batch culture Cell numbers increase in both selective and YEPD media (Table 6 1 and 6 2) corresponding to 6 5 and 9 3 generations respectively As growth progresses, a gradient in biomass concentration develops with an approximate 10 fold higher cell concentration developing on the bead outer

surface compared with the bead interior Cells on the surface of the bead undergo 11 to 12 generations, while cells in the core of the bead undergo 8 to 9 generations The characteristic feature of this second phase of immobilised cell growth is the appearance of pseudo steady-state levels of leaked cells, residual nutrients and product in the bulk medium surrounding the gel beads (Monbouquette et al , 1990)

Plasmid pJG317 is unstable and is rapidly lost from a population of growing DBY746 (pJG317) cells This has been illustrated in free cell batch and continuous fermentations (Chapter Five) Usually cells are immobilised at high concentrations and they quickly grow and attain the pseudo steady-state, characteristic of the second phase of an immobilised cell fermentation Only a few generations occur which prevents excessive loss of the recombinant plasmid from the cell population (Berry et al , 1988, Sayadi et al , 1989, Walls and Gainer, 1989, 1991) Investigations into the influence of environmental conditions on immobilised cell growth and enzyme productivity were conducted and the results obtained from varying the cell load (inoculum) indicated that a medium cell loading of 10^6 cell/g-gel yielded higher enzyme production and greater stability of the immobilised cell system compared with low (10^3 cell/g-gel) and high (10^8 cells/g-gel) cell loadings These results are discussed in greater detail in Chapter Seven The production of cloned gene product *i e* β-glucanase, in immobilised cell culture is also discussed in Chapter Seven This is the reason for immobilising the cells at a cell load of 10^6 cells/g-gel in the above experiments During the first stage of a repeated batch culture between 6 and 9 generations take place in the immobilised cells (Table 6 1 and 6 2) in selective and YEPD media respectively In the case of YEPD (where there is an absence of selective pressure), this amount of growth and number of cell divisions is sufficient for a substantial number of the plasmid containing cells to lose the plasmid Repeated batch cultivation in YEPD results in a rapid decrease in plasmid stability (Table 6 2) In an effort to circumvent this problem, the first incubation in the repeated batch cultivation was performed in selective medium Immobilised cells were able to grow and divide rapidly and attain a high biomass concentration while still maintaining the plasmid due to selective pressure Once a high cell density had been reached, the immobilsed cells were transferred into YEPD where they continued to grow and produce the β-glucanase enzyme Due to the physical restraint imposed by the gel matrix and the hetergeneous nature of biomass

distribution in the gel beads, the cells grew at what appeared to be a slower rate and the plasmid was not lost from the population at any appreciable rate

The successful application of this preincubation in selective medium is reflected in the increased plasmid stability of the immobilised cells during repeated batch cultivation compared with the relatively low plasmid stability of immobilised cells in batch fermentations in YEPD only This increased plasmid stability results in an increased enzyme production in the immobilised cell fermentation (Tables 6 1 and 6 2) The stability of the preincubation system is reflected by the smaller quantities of released or leaked cells appearing in the bulk medium during the course of the repeated batch culture The half life of penicillin G produced by immobilised *P chrysogenum* was found to be nine fold greater than with free, cells when the immobilised cells were periodically placed into a minimal production medium (Deo and Gaucher, 1984) Sode et al (1988), in an attempt to improve productivity in an immobilised recombinant yeast bioreactor, found that alternating the feed to the column bioreactor, between minimal and complex media resulted in 1 4 times greater α-peptide production compared with that achieved only by feeding minimal medium

Nutrient depletion and physical boundaries limit the growth of immobilised cells (Hahn-Haegerdal, 1990) This has successfully been exploited to enchance the stability of plasmids in immobilised recombinant cell systems (De Taxis du Poet et al , 1987, Inloes et al , 1983, Sayadi et al , 1989, Cahill et al , 1990, Walls and Gainer, 1991) This enhanced stability has also been demonstrated for immobilised DBY746 (pJG317) cells in the present work Repeated batch cultivations were performed over a four day period The plasmid stability decreased to 46% yet the enzyme was still produced in high levels (80 to 100 U/ml) A free cell fermentation over the same time period resulted in the plasmid stability decreasing to less than 10%, with a corresponding decline in enzyme activity Figure 3 1 illustrates this point very clearly Here an immobilised cell fermentation is conducted in the usual manner - high cell loadings and repeated batch fermentation with no preincubation in selective medium These results are graphically compared to a standard free cell serial batch fermentation over the same time period The results clearly show that the bead-averaged plasmid stability of

immobilised cells is greater than the plasmid stability of free cells This results in greater β-glucanase production in the immobilised cell system

Using the multistep dissolution technique of Walsh et al (1993), immobilised cells from different locations in the gel beads were isolated Cell numbers were calculated per weight of gel bead dissolved, and plotted versus distance from the centre of the gel (Figure 6 1) When the gel beads were exposed to a sodium citrate solution, the bead diameter decreasd linearly with time, and is a function of the $CaCl_2$ concentration used for gel formation (Walsh et al , 1993) By calculating the linear rate of reduction of the gel bead diameter, dissolution times required to dissolve specific fractions of the gel beads could be predicted Cells from the outer 200μm of the gel beads and the inner 2 0mm were isolated The plasmid stability of the inner cells, outer cells and the cells which had leaked from the gel beads, were measured Figure 6 2 shows that a gradient of plasmid stability exists throughout the beads Cells residing on the outside of the gel beads grow faster (Figure 6 1) and lose the plasmid at a greater rate than slower growing cells which reside in the inside of the beads Leaked cells lose the plasmid at rates comparable with free cells The rate of plasmid loss is more pronounced during the early stages of immobilised cell fermentation due to the outer cells growing at a faster rate because of access to nutrients In the latter stages of the fermentation, when a pseudo steady-state exists, the rate of plasmid loss of cells on the surface of the beads approaches that of the cells in the inner core This is because cells on the outside, although still growing at faster growth rates are quickly shed from the bead surface This is reflected in the sharp decrease in plasmid-containing cells in the leaked cell population in the latter stages of the fermentation (Figure 6 2) Cahill (1990) detected and measured a gradient in plasmid stability in immobilised cells of DBY746 (pJG317) by partitioning cross sections of gel beads Plasmid stability gradients were measured in both gel cylinders and gel beads Using the multistep dissolution method in the present work, the standard deviation of the PCN was lower than that measured by Cahill (1990), resulting in greater accuracy in the plasmid stability values

In an attempt to explain the gradient in plasmid stability in immobilised cells, the PCN of cells on the outside and inside of the gel beads were measured in addition to cells isolated by dissolving entire beads and also cells leaked from the gel matrix It has been shown that recombinant

immobilised bacterial cells containing multicopy plasmids have higher PCN than free cells (De Taxis du Poet et al , 1987, Sayadi et al , 1989) However, the PCN of these immobilised cells is a weighted average of high PCN of the internal cell population and low PCN of the external cell population It has already been estabilished that PCN varies with cell growth rate (Chapter Five), in agreement with the findings of Bugeja et al (1989) also working with a recombinant *S cerevisiae* strain Results from growth profiles of immobilised DBY746 (pJG317) cells show that a gradient in growth (Figure 6 1) and plasmid stability (Figure 6 2) does indeed exist These gradients are caused by the resistance to oxygen and nutrient diffusion into and through the gel beads, and by the physical nature of the gel matrix It was of interest therefore to examine if a difference in PCN could be detected between slow growing internal cells and faster growing external cells

Measurement of PCN of immobilised cells was quite difficult due to incomplete digestion of total genomic DNA in some immobilised cells samples This led to a decrease in the accuracy in calculating PCN The problem was largely overcome by diluting the DNA and using extra restriction enzyme It was quite difficult to restrict the DNA isolated from cells that had been immobilised for long periods of time while DNA isolated from leaked cells was easier to restrict The fact that the total DNA was only partially restricted appeared to be due to immobilisation This could be due to the polyploid nature of some immobilised cells (Doran and Bailey, 1986) The cellular composition of *S cerevisiae* cells is affected by immobilisation Measurements of intracellular polysaccharide levels showed that immobilised yeast stored larger quantities of reserve carbohydrates and contained more structural polysaccharide than free cells (Doran and Bailey, 1986) The DNA content of immobilised cells may be several times greater than that of exponential phase suspended cells Other findings on the change in metabolism in immobilised yeast cells include increased glycolytic enzyme activities (Hilge-Rotmann and Rehm, 1990) , lower internal pH of immobilised cells (Galazzo and Bailey, 1989), altered membrane permeability (Devakos and Webb, 1991) The fact that the problem of partial digestion of total genomic DNA was circumvented to a large degree by diluting the DNA indicates that some inhibitory intracellular substance present in the DNA solution may have been diluted to below some threshold concentration where it no longer interferred with DNA restriction Because

DNA from cells immobilised for shorter periods of time and DNA from leaked cells (which probably underwent some generations in suspension) were restricted with little-or no problem suggests that some metabolic change occurs in immobilised cells, rather than the immobilisation matrix or dissolution system (alginate and/or sodium citrate) being responsible for the difficulties in estimating PCN

Table 6 6 Mean PCN of cells immobilised throughout the gel beads and for free cell fermentations

Fermentation type	Mean PCN	Number of data points
Inner cells (immobilised)	36 5±6 8	11
Outer cells (immobilised)	32 4±3 3	5
Leaked cells (immobilised)	32 6±5 6	14
Bead-averaged (immobilised)	29 6±4 6	14
Serial batch (free)	25 2±5 5	5
Batch (free)	28 8±4 4	5
Continuous D=0 5h^{-1} (Free)	32 3±5 7	7
Continuous D=0 1h^{-1} (Free)	40 0±6 3	10

The statistical analysis performed on the samples in Table 6 6 was a one way analysis of variance Least significant test with significance level 0 05, which test whether the means of samples are significantly different from each other No significant gradient in PCN of immobilised cells can be detected It has been shown for free cells that PCN is affected by growth rate However, it must be noted that extremes of growth rate were tested Slow growing cultures (D=0 1h^{-1}) had a doubling time of almost 7 hours while fast growing cultures (D=0 5h^{-1}) had a doubling time of 1 5 hours Yet despite these wide differences in growth rate, a relatively small difference in mean PCN was measured, *i e* mean PCN=32 3±5 7 for the fast growing culture and a mean PCN=40±6 3 for the slow growing culture In immobilised cells a difference in growth rate exists, yielding beads where the biomass concentration on the outside of the gel beads is 10 times greater than that in the inside This difference in growth rate is enough to bring about a gradient in plasmid stability It is possible that the difference in growth rate between inner and outer cells does cause the evolution of a cell population with differing average PCN However, it may be that as the

difference in growth rate is not very large, the difference in PCN is also not so large, and the technique employed for measuring the PCN is not sensitive enough to accurately detect these small differences Although biomass concentration in immobilised cells differs by a factor of 10, this represents a difference of approximately 3 to 4 generations between cells on the inside and outside of the gel beads This indicates that although growth rate differences exist, it is not nearly as large as the difference in growth rate found in free cell continuous cultures, where a difference in PCN was detected

In summing up, the results obtained here support some of the reported advantages of immobilising recombinant cells Biomass concentrations are higher by a factor of 10 for immobilised cells compared with free cells Also, it was observed in several fermentation cases throughout this study, that irrespective of the initial cell loading or whether preincubation in selective medium was performed, the final cell concentration of immobilised cells was always approximately equal This will be demonstrated further in Chapter Seven This phenomenon has been reported by several investigators (Mitani et al , 1984, Godia et al , 1987, Simon, 1989, Walsh, 1993) It has been shown that the apparent plasmid stability of DBY746 (pJG317) cells is improved by immobilisation, which is in agreement with the findings of Sayadi et al (1989), Cahill et al (1990) and Walls and Gainer (1991) A gradient in plasmid stability was measured in the immobilisation matrix which was brought about by nutrient limitation and physical boundaries causing a gradient in biomass concentration throughout the gel beads PCN of immobilised cells was measured but as summarised in Table 6 6, no gradient in PCN was detected This is possibly due to the small differences in PCN that would arise in immobilised cell populations from various locations throughout the gel beads, growing at rates which are not large enough to produce significant differences in PCN

CHAPTER SEVEN

EFFECT OF ENVIRONMENTAL GROWTH CONDITIONS ON PLASMID STABILITY, PLASMID COPY NUMBER AND β-GLUCANASE PRODUCTION IN FREE AND IMMOBILISED CELL CULTURE

7.1 INTRODUCTION

The DBY746 (pJG317) recombinant yeast system has been investigated in both free and immobilised cell culture Growth rate effects on plasmid stability, PCN and biomass concentration have been studied and are important for the stability of β-glucanase production Due to its increased plasmid stability and increased enzyme production, an immobilised DBY746 (pJG317) cell system is perferrable and hence many of the investigations into improving enzyme production concentrated on the immobilised cell system

Immobilisation factors such as the effects of cell loadings and type of counter-ion used in immobilisation matrix gelation have been reported to influence cloned gene productivities (Berry et al , 1988, Tanaka and Irie, 1988, Ogbanna et al , 1989) Such investigations were performed for the DBY746 (pJG317) immobilised cell system It has also been reported that various nutritional requirements and/or limitations can affect both the productivity and the genetic and biological stability of a recombinant cell system (Sode et al , 1988, Sayadi et al , 1989, Turner et al , 1991) The above work was conducted with free cell bacterial and yeast systems Reports on the nutritional requirements of immobilised yeast are scarce (Chen et al , 1990) and even fewer reports on the effect of nutritional requirements/limitations on recombinant immobilised yeast cell systems exist In the present study the influence of nutrient availability on the immobilised recombinant yeast system was investigated and the final section of this project attempted to relate nutrient effects with the PCN of the cells Previous results on PCN showed that the greatest difference in PCN occurred in different media (Chapter Five) The PCN of cells grown in YEPD was up to 5 times greater than the PCN of cells grown in selective medium Due to the difficulties encoutered in measuring the PCN of immobilised cells (Chapter Six), all media influences on enzyme productivities were first performed with immobilised cells but the actual relationship between nutritional requirements and/ or limitations and PCN was performed with free cells

Of the various factors which can influence the genetic stability and productivity of a recombinant cell system, environmental stresses are

perhaps the easiest to control and hence serve as one route to improving or optimising the particular recombinant cell bioreactor system The various environmental stresses which influence β-glucanase production by immobilised cells were investigated and some of these stresses were explained on a genetic level by measuring the PCN of the cells

7.2 INVESTIGATION INTO THE USE OF STRONTIUM AS THE COUNTERION IN THE GELATION OF ALGINATE BEADS

In attempts to improve the stability of the immobilised cell system, the calcium ions used as the counter-ion in alginate gelation may be replaced with strontium or barium to yield more chemically and physically stable gel beads (Tanaka and Irie, 1988) Cells were immobilised in both calcium alginate and strontium alginate gel beads at a cell load of 10^6cells/g-gel The cells used for immobilisation were from the same seed fermentation The beads were preincubated for 36 hours in selective media before being transferred to YEPD media Repeated batch cultivation involving three transfers was performed for each sample of immobilised cells (Section 2 5 6) Figure 7 1 shows the fermentation profile for calcium alginate and strontium alginate immobilised cells No real difference in growth, sugar assimilation, and plasmid stability was detected Enzyme production was slightly higher for strontium alginate beads However this advantage was negated by the fact that the strontium ion caused precipitation in selective medium Further investigations established that some component of the yeast nitrogen-base used in selective media was being precipitated during incubation Because of this, no further experiments were performed with strontium alginate gel beads

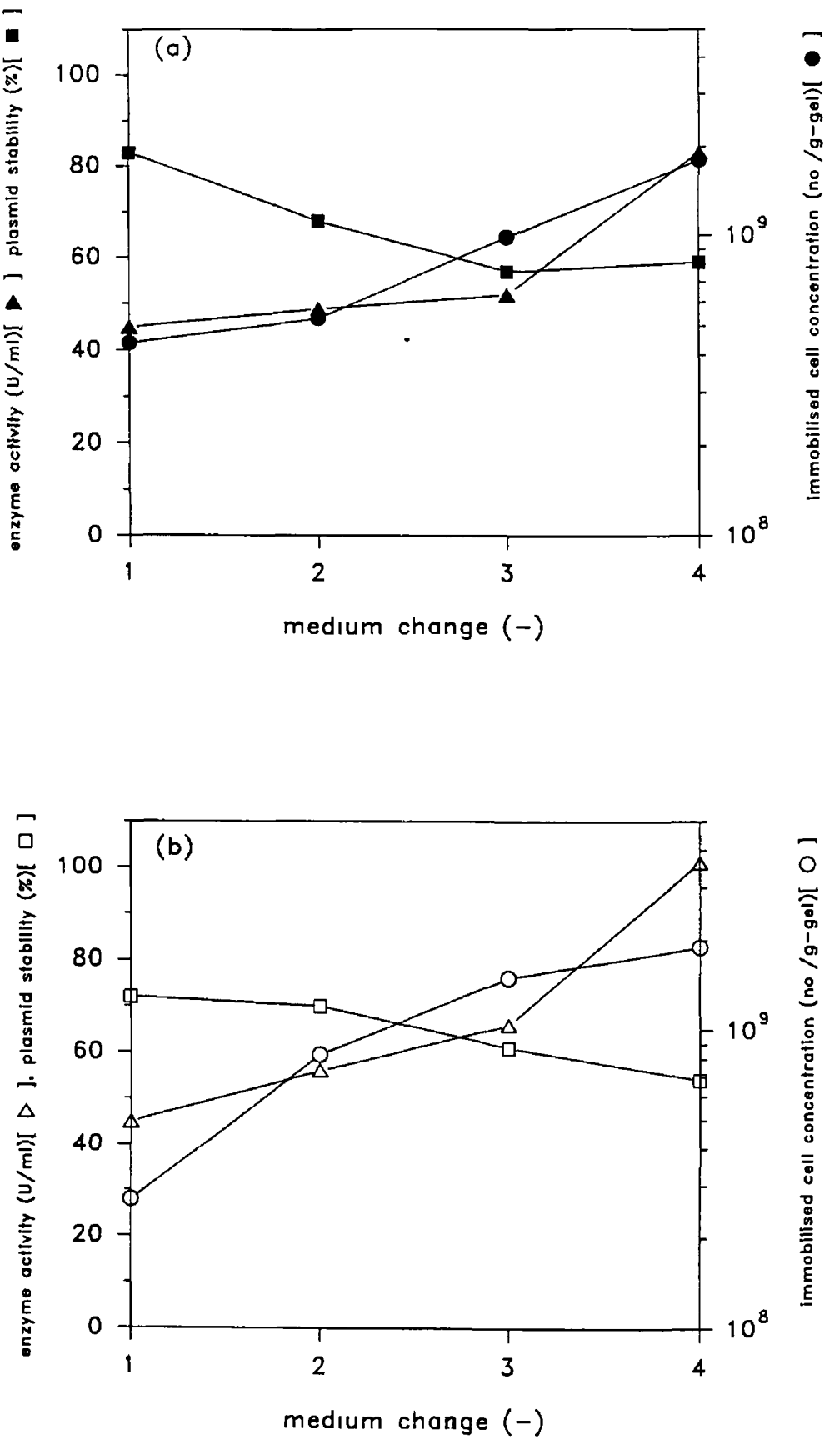


Figure 7 1 Fermentation profiles of immobilised cell gel beads (a) Calcium alginate (b) Strontium alginate

7.3 THE INFLUENCE OF CELL LOAD ON PLASMID STABILITY AND ENZYME PRODUCTIVITY

Calcium alginate gel beads were prepared at three different initial cell loadings (ICL) High - 10^8 cells/g-gel, Medium - 10^6 cells/g-gel and Low - 10^3 cells/g-gel The gel beads were preincubated in selective media for 36 hours before being transferred to YEPD media Repeated batch fermentations were performed with five media changes taking place YEPD incorporating 2% glucose was used for the first three media but this sugar concentration was increased to 5% for media change numbers four and five Each immobilised cell system was exmained for overall enzyme production, biomass production, plasmid stability and bead stability i e bead diameter, leaked cell concentration and bead hardness By preincubating the gel beads in selective media , the immobilised cells were allowed to reach a high and equal biomass concentration of approximately 10^8 cells/g in each system and by maintaining selection pressure, a large proportion of the cells were plasmid positive at the beginning of fermentation in YEPD media Preincubation of the gel beads allowed each immobilised cell system to reach comparable levels of biomass concentration and enzyme producing potential (*i e* approximately equal plasmid stabilities) at the start of the repeated batch cultivation Figure 7 2 shows the effect of cell load on enzyme activity, plasmid stability, biomass production and bead diameters This last parameter was measured as alginate gel beads have been shown to swell over the course of a fermentation as a result of cell growth (Walsh et al , 1993) Table 7 1 outlines the mean volumetric and specific enzyme activities for each immobilised cell system

Table 7 1 Mean volumetric productivities and specific enzyme activities of immobilised cells at various ICL

ICL	Mean volumetric productivity (U/ml h)	Mean specific activity (U/cell)
High (10^8 cells/g gel)	2 58	3 31 x 10^{-7}
Medium (10^6 cells/g gel)	3 15	3 84 x 10^{-7}
Low (10^3 cells/g gel)	2 58	6 77 x 10^{-7}

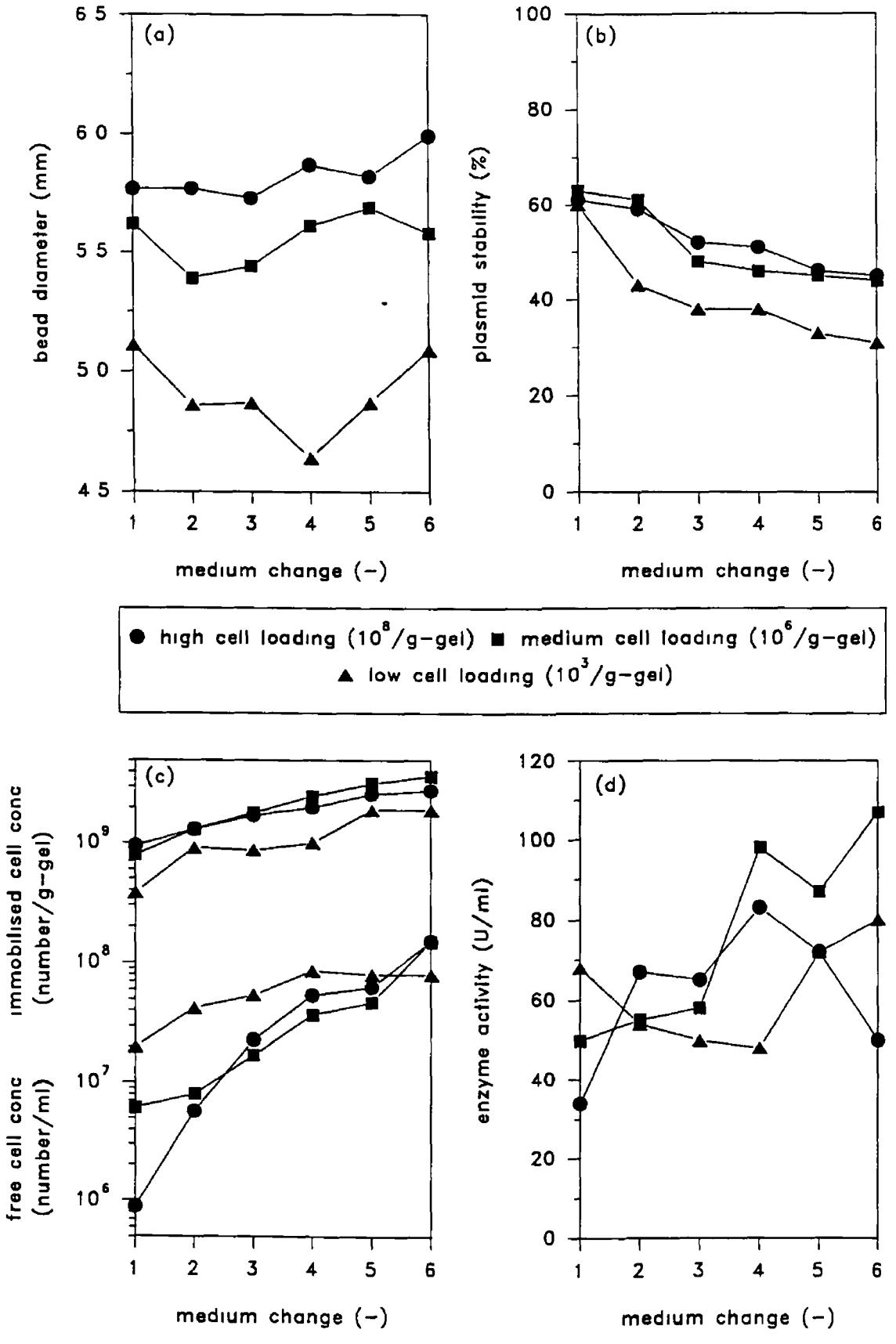


Figure 7 2 The influence of cell loading on (a) bead diameter, (b) plasmid stability, (c) immobilised and leaked cell concentrations, and (d) enzyme activity during calcium alginate immmobilised cell culture

7.4 THE INFLUENCE OF MEDIA COMPOSITION AND NUTRITIONAL LIMITATIONS ON ENZYME PRODUCTIVITY, PLASMID STABILITY AND BIOMASS PRODUCTION IN IMMOBILISED CELL CULTURE

From a seed fermentation incubated in selective medium 40 g quantities of calcium alginate beads were prepared with an ICL of 10^6 cell/g-gel These beads were then incubated in selective media for 36 hours to increase biomass concentration and maintain a high proportion of plasmid-containing cells Repeated batch fermentations with three media changes were then conducted The media used depended on the particular experiment being performed The alginate beads (40 g) were incubated for 24 hours in 200ml medium in a 0 5 l Erylenmeyer flask Samples of beads and fermentation broth were taken before each media change and the following parameters measured leaked cells, cell concentration per bead, enzyme activity, residual sugar concentration and plasmid stability Five experiments were performed as part of this investigation They included

(a) Effect of bulk nutrients (Sections 2.4.1 and 2.4.2)

- selective medium
- selective medium enriched with 6 0 g/l casasmino acids (Difco)
- YEPD medium
- YEPD medium enriched with 6 0 g/l casamino acids
- selective medium and leucine (non-selective minimal medium)

(b) Effect of salts media (Section 2.4.4)

- basal salts medium - Salts A
- Davis's salts medium - Salt B
- non-nutritious salts medium - Salts C

(c) Effect of yeast extract concentration (Section 2.4.5)

- yeast extract concentrations of 0, 0 5, 1 0, 3 0 and 6 0 g/l

(d) **Effect of ammonium/nitrogen concentration (Section 2.4.7)**

- ammonium sulphate concentrations of 0, 2 5, 5 0, 10 0 and 15 0 g/l

(e) **Effect of phosphate concentration (Section 2.4.6)**

- potassium dihydrogen phosphate concentrations of 0, 1 0, 2 5, 5 0 and 10 0 g/l

Figures 7 3 to 7 7 show the results of immobilised cell fermentations in all the above media with results for enzyme activity, biomass production and plasmid stability Table 7 2 represents a summary of results from all the media investigations The maximum enzyme activity, maximum biomass concentration, average specific enzyme activity and average volumetric enzyme productivity for each fermentation are shown

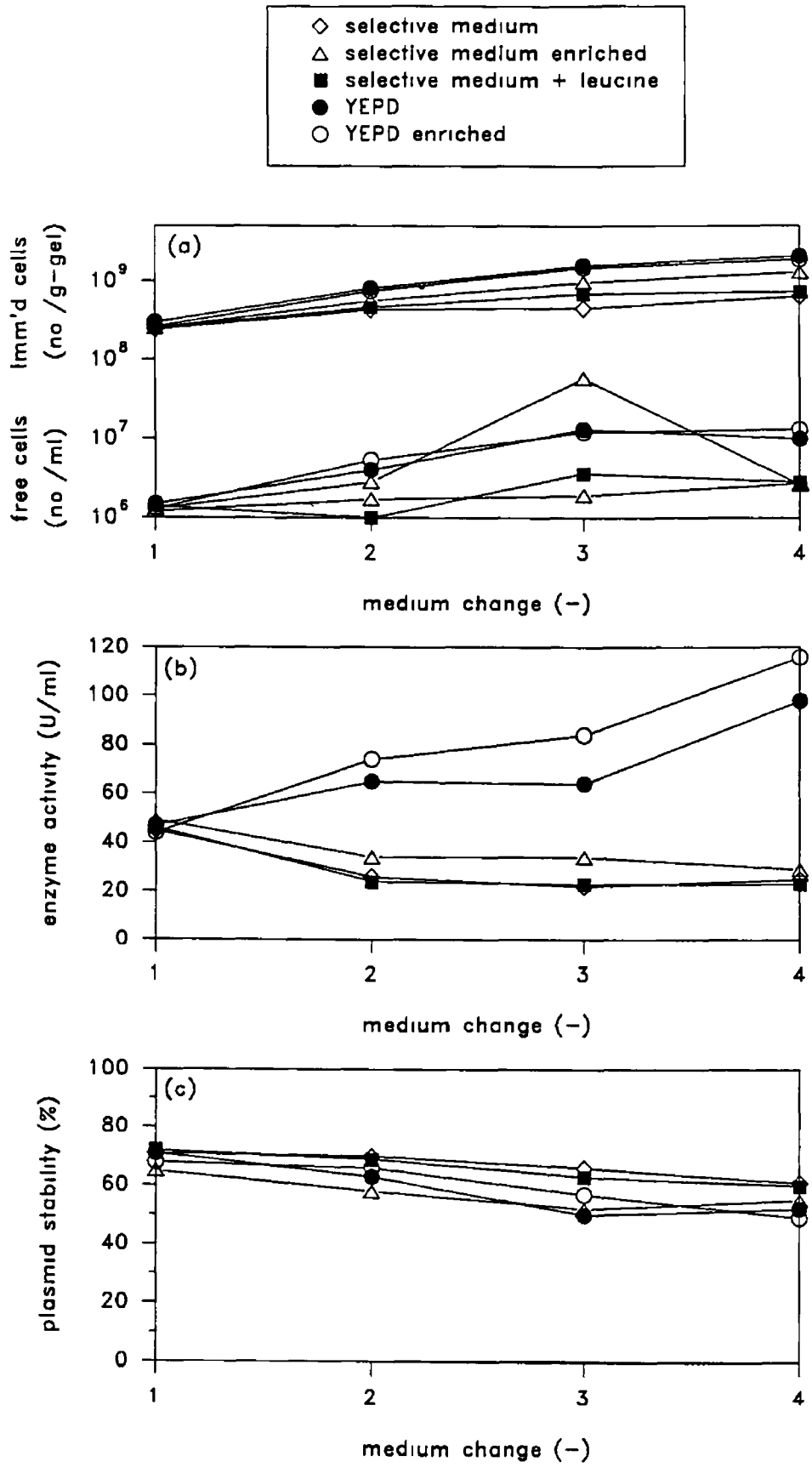


Figure 7 3 The influence of bulk nutrient concentration on (a) immobilised and leaked cell concentrations, (b) enzyme activity, and (c) plasmid stability in immobilised cell culture

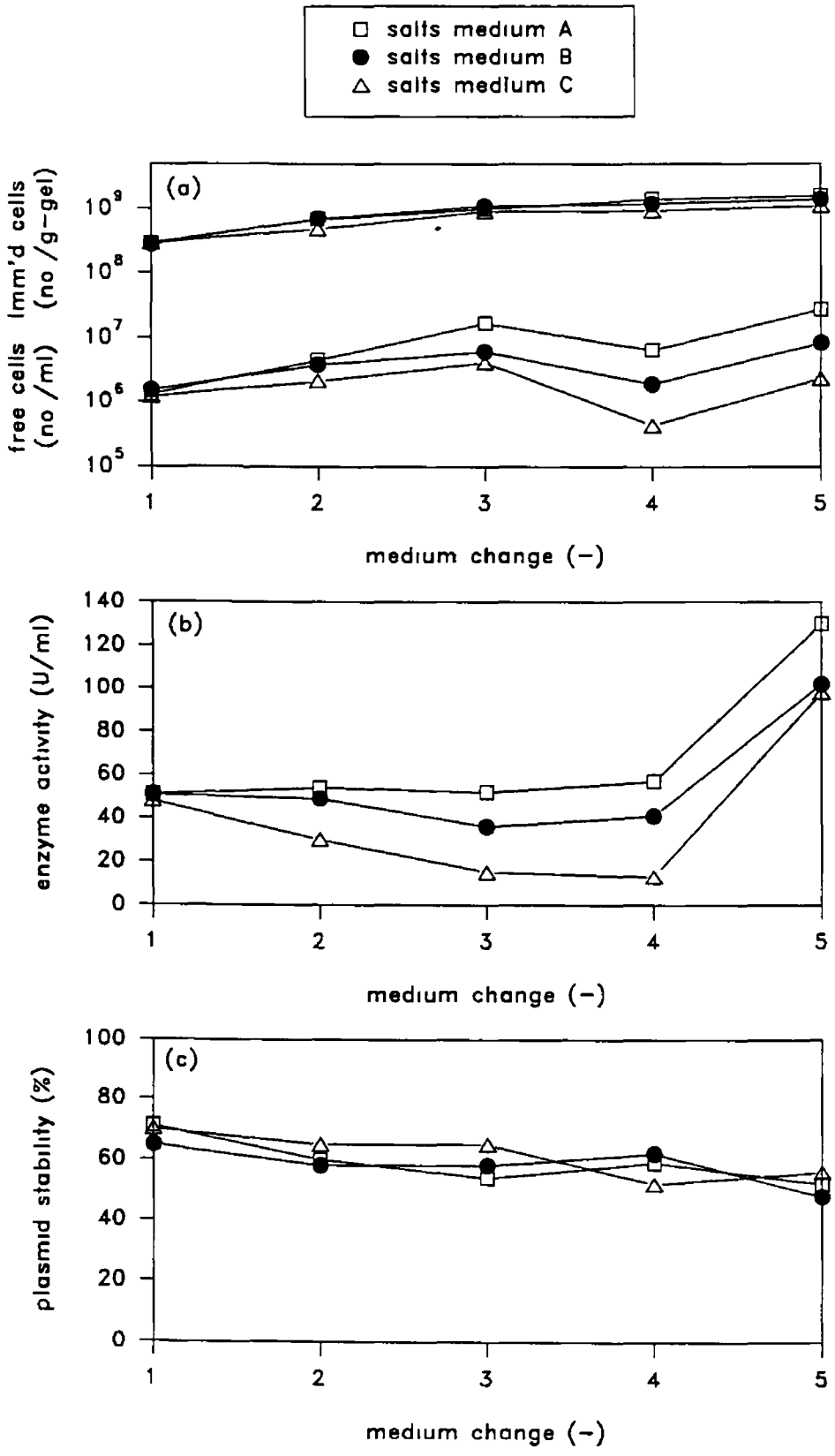


Figure 7 4 The influence of different salts media on (a) immobilised and leaked cell concentrations, (b) enzyme activity, and (c) plasmid stability in immobilised cell culture

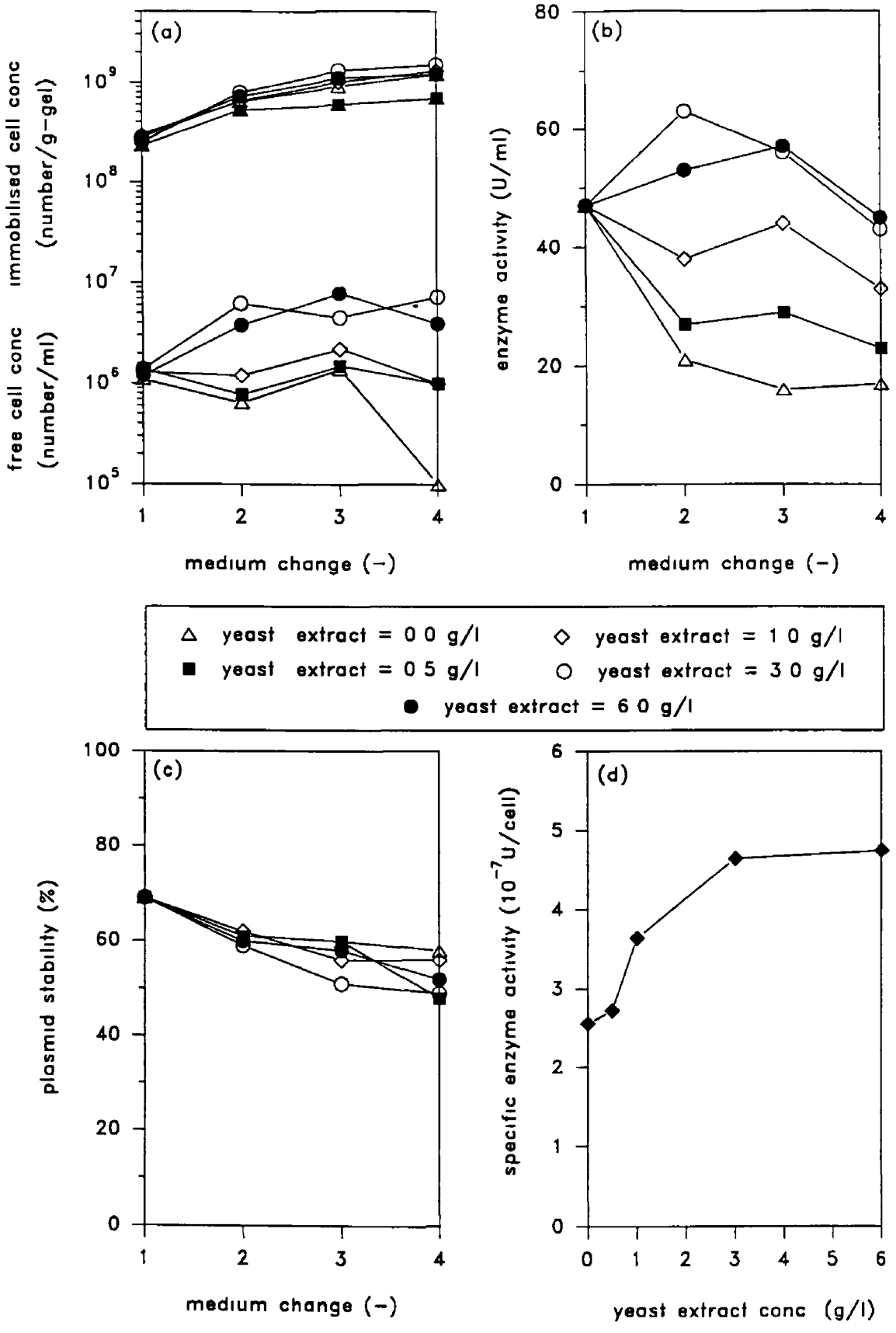


Figure 7 5 The influence of yeast extract concentration on (a) immobilised and leaked cell concentrations, (b) enzyme activity, (c) plasmid stability, and (d) specific enzyme activity during immobilised cell culture

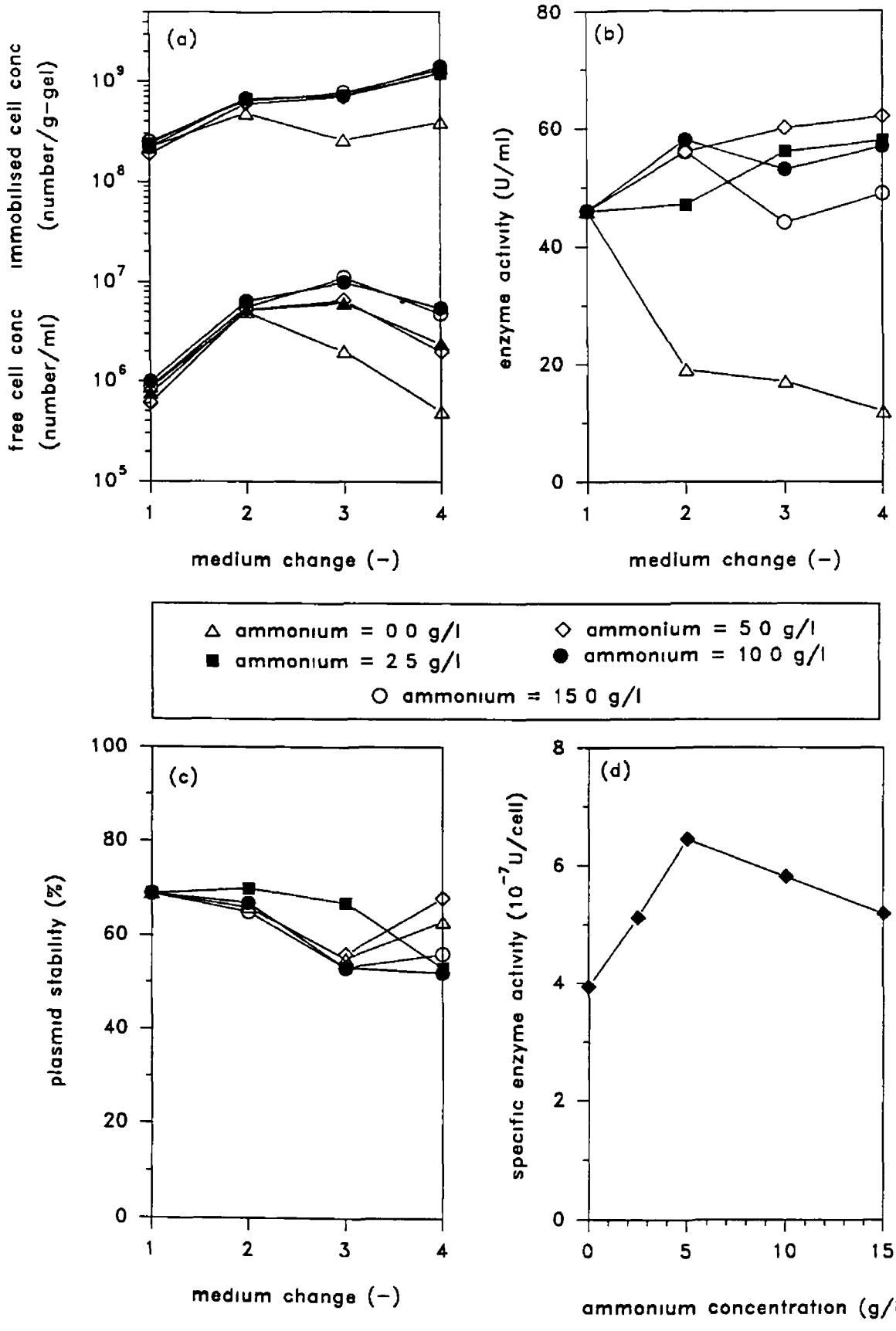


Figure 7 6 The influence of ammonium concentration on (a) immobilised and leaked cell concentrations, (b) enzyme activity, (c) plasmid stability, and (d) specific enzyme activity during immobilised cell culture

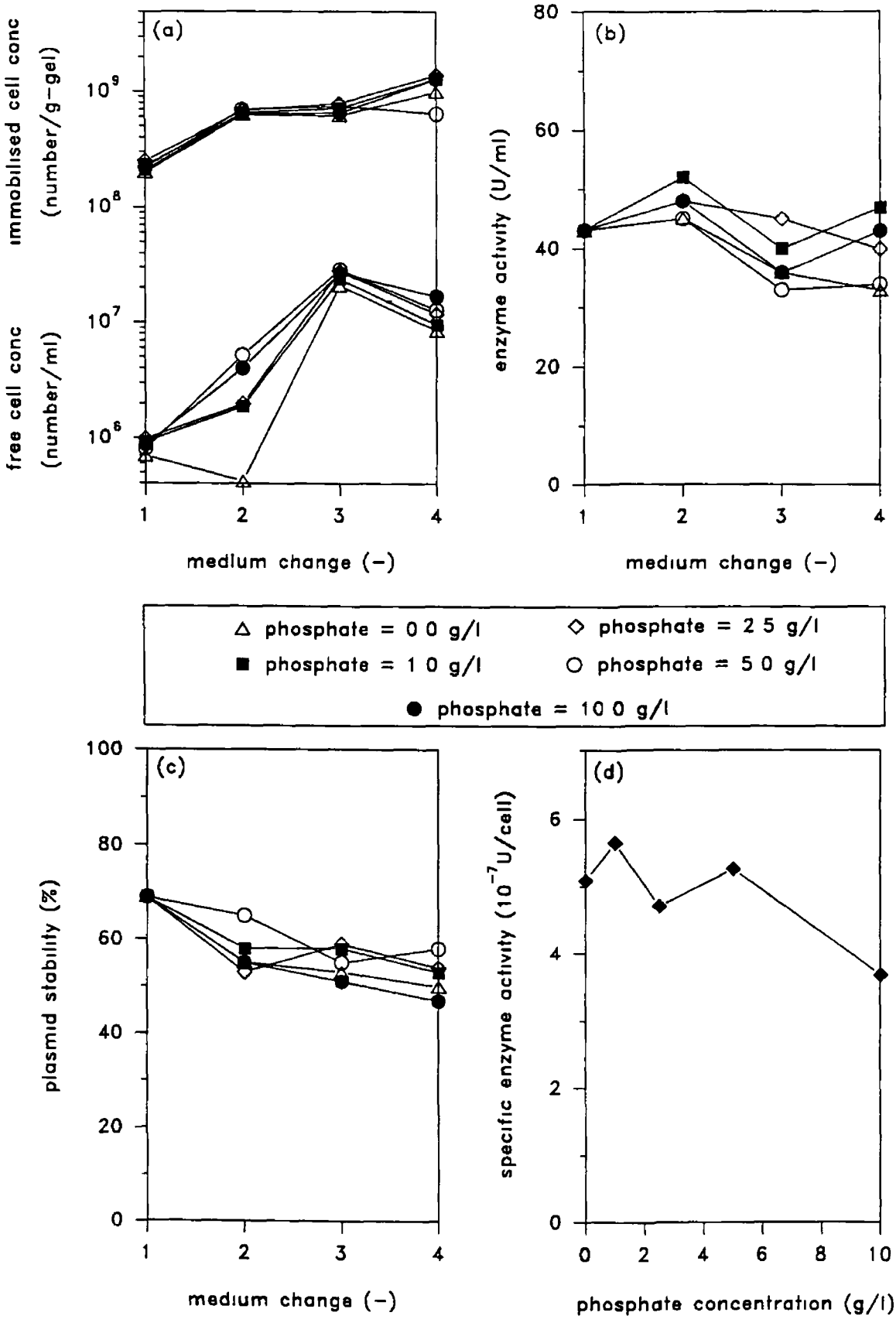


Figure 7 7 The influence of phosphate concentration on (a) immobilised and leaked cell concentrations, (b) enzyme activity, (c) plasmid stability, and (d) specific enzyme activity during immobilised cell culture

Table 7 2 *Enzyme production and biomass concentration of immobilised cell fermentations on different media*

FERMENTATION	Max enzyme activity (U/ml)	Max biomass concentration (cells/g-gel	Average specific enzyme activity (U/cell)	Average volumetric productivity (U/ml-h)
Selective	26	$6\,4 \times 10^{8}$	$3\,78 \times 10^{-7}$	1 01
Enriched selective	34	$1\,3 \times 10^{9}$	$3\,54 \times 10^{-7}$	1 38
Selective + LEU	24	$7\,3 \times 10^{8}$	$3\,02 \times 10^{-7}$	0 97
YEPD	98	$2\,1 \times 10^{9}$	$4\,95 \times 10^{-7}$	3 15
Enriched YEPD	116	$1\,9 \times 10^{9}$	$6\,24 \times 10^{7}$	3 8
Basal salts	57	$1\,4 \times 10^{9}$	$4\,93 \times 10^{7}$	2 27
Davis's salts	49	$1\,2 \times 10^{9}$	$3\,87 \times 10^{-7}$	1 75
Non-nutritious salts	30	$9\,3 \times 10^{8}$	$2\,49 \times 10^{-7}$	0 81
0 g/l yeast extract	21	$6\,9 \times 10^{8}$	$2\,56 \times 10^{7}$	0 75
0 5 " "	29	$1\,2 \times 10^{9}$	$2\,72 \times 10^{-7}$	1 1
1 0 " "	44	$1\,3 \times 10^{9}$	$3\,64 \times 10^{-7}$	1 46
3 0 " "	57	$1\,2 \times 10^{9}$	$4\,74 \times 10^{7}$	2 16
6 0 " "	63	$1\,5 \times 10^{9}$	$3\,64 \times 10^{7}$	2 25
0 g/l ammonium	19	$4\,8 \times 10^{8}$	$3\,94 \times 10^{-7}$	0 67
2 5 g/l "	58	$1\,2 \times 10^{9}$	$5\,12 \times 10^{-7}$	2 24
5 0 g/l "	62	$1\,2 \times 10^{9}$	$6\,44 \times 10^{-7}$	2 47
10 0 g/l "	58	$1\,4 \times 10^{9}$	$5\,81 \times 10^{-7}$	2 34
15 0 g/l "	56	$1\,3 \times 10^{9}$	$5\,19 \times 10^{-7}$	2 07
0 g/l phosphate	45	$1\,0 \times 10^{9}$	$5\,09 \times 10^{7}$	1 59
1 0 g/l "	52	$1\,3 \times 10^{9}$	$5\,65 \times 10^{-7}$	1 93
2 5 g/l "	48	$1\,4 \times 10^{9}$	$4\,71 \times 10^{7}$	1 85
5 0 g/l "	48	$1\,3 \times 10^{9}$	$5\,26 \times 10^{7}$	1 76
10 0 g/l "	48	$1\,4 \times 10^{9}$	$3\,68 \times 10^{7}$	1 57

7.5 THE INFLUENCE OF MEDIA COMPOSITION AND NUTRITIONAL LIMITATIONS ON ENZYME PRODUCTIVITY AND PCN IN FREE CELL CULTURE

From the media effects on enzyme production in immobilised cell culture (Section 7 4), a selection of media that displayed differing specific enzyme activities were chosen for further study The aim of this study was to determine whether media composition and nutritional limitations influence PCN and, if so, to establish a relationship between media composition, specific enzyme activity and PCN The following media were chosen

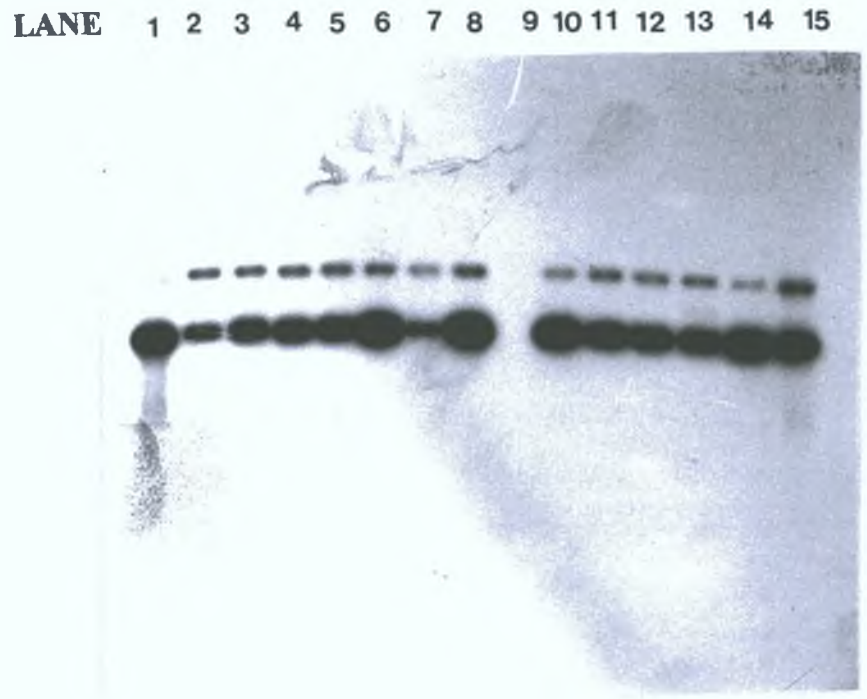
(i) Selective medium (Section 2 4 1)
(ii) Selective medium enriched with 6 0 g/l casamino acids (Section 2 4 1)
(iii) YEPD (Section 2 4 2)
(iv) YEPD enriched with 6 0 g/l casamino acids (Section 2 4 2)
(v) Selective medium with 5 0 g/l ammonium sulphate (Section 2 4 7)
(vi) Selective medium with 15 0 g/l ammonium sulphate (Section 2 4 7)
(vii) Selective medium with 2 5 g/l ammonium sulphate (Section 2 4 7)
(viii) Basal salts medium with 1 0 g/l potassium dihydrogen phosphate (Section 2 4 6)
(ix) Basal salts medium with 10 0 g/l potassium dihygrogen phosphate (Section 2 4 6)
(x) Dextrose, ammonium sulphate medium with 0 5 g/l yeast extract (Section 2 4 5)
(xi) Dextrose, ammonium sulphate medium with 6 0 g/l yeast extract (Section 2 4 5)

Serial batch cultivation (Section 2 5 5) involving three media transfers was performed for each medium with approximately 30 generations taking place Biomass concentration, enzyme activity and plasmid stability were assayed before each transfer Cell pellets were also harvested and the total genomic DNA from each sample was isolated Using this DNA, the PCN for each sample was determined

Table 7 3 outlines the results obtained detailing biomass concentration, plasmid stability, enzyme activity and PCN for each medium over approximately 30 generations of growth The mean specific enzyme activities and mean PCN are also represented Figure 7 8 is an autoradiogram showing the various intensities (and hence numbers of copies) of the plasmid on all 11 different media

Table 7 3 Enzyme production, biomass concentration and PCN of free cells grown on different media

Medium	Sample	Cell conc (cells/ml)	Gen	Plasmid stability (%)	Enzyme Activity (U/ml)	Specific enzyme activity (U/cell)	PCN	Mean spec enzyme activity (U/cell)	Mean PCN
Selective	1	$3\ 7 \times 10^{7}$	8 3	63	48	$2\ 1 \times 10^{-6}$	20		
	2	$4\ 4 \times 10^{7}$	20 4	67	49	$1\ 7 \times 10^{-6}$	14	$1\ 9 \times 10^{-6}$	14
	3	$3\ 7 \times 10^{7}$	28 9	71	52	$2\ 0 \times 10^{-6}$	9		
Selective	1	$8\ 9 \times 10^{7}$	9 8	44	45	$1\ 2 \times 10^{-6}$	36		
enriched	2	$1\ 2 \times 10^{8}$	20	29	34	$9\ 8 \times 10^{-7}$	10	$1\ 1 \times 10^{-6}$	10
YEPD	1	$8\ 2 \times 10^{7}$	9 5	35	85	$3\ 0 \times 10^{-6}$	26		
	2	$7\ 1 \times 10^{7}$	22 2	17	51	$4\ 2 \times 10^{-6}$	22	$3\ 7 \times 10^{-6}$	25
	3	$8\ 3 \times 10^{7}$	31 8	11	35	$3\ 8 \times 10^{-6}$	26		
YEPD	1	$8\ 1 \times 10^{7}$	9 4	38	85	$2\ 8 \times 10^{-6}$	14		
enriched	2	$7\ 0 \times 10^{7}$	22 1	19	48	$3\ 6 \times 10^{-6}$	19	$3\ 5 \times 10^{-6}$	18
	3	$8\ 2 \times 10^{7}$	31 7	10	34	$4\ 2 \times 10^{-6}$	21		
Nitrogen	1	$4\ 4 \times 10^{7}$	8 6	67	47	$1\ 6 \times 10^{-6}$	14		
2 5 g/l	2	$6\ 9 \times 10^{7}$	21 2	74	40	$7\ 8 \times 10^{-7}$	8	$1\ 3 \times 10^{-6}$	11
	3	$4\ 2 \times 10^{7}$	29 8	72	42	$1\ 4 \times 10^{-6}$	11		
Nitrogen	1	$4\ 7 \times 10^{7}$	8 7	59	50	$1\ 8 \times 10^{-6}$	14		
5 g/l	2	$7\ 0 \times 10^{7}$	21 4	62	47	$1\ 1 \times 10^{-6}$	16	$1\ 7 \times 10^{-6}$	16
	3	$5\ 2 \times 10^{7}$	30 7	47	56	$2\ 3 \times 10^{-6}$	17		
Nitrogen	1	$2\ 6 \times 10^{7}$	7 9	59	29	$1\ 9 \times 10^{-6}$	12		
15 g/l	2	$6\ 3 \times 10^{7}$	20 4	73	41	$8\ 9 \times 10^{-7}$	8	$1\ 4 \times 10^{-6}$	10
	3	$2\ 3 \times 10^{7}$	28 2	67	22	$1\ 4 \times 10^{-6}$	9		
Phosphate	1	$7\ 0 \times 10^{7}$	9 2	61	62	$1\ 5 \times 10^{-6}$	8		
1 g/l	2	$4\ 0 \times 10^{7}$	21 2	21	46	$5\ 3 \times 10^{-6}$	12	$3\ 3 \times 10^{-6}$	13
	3	$5\ 6 \times 10^{7}$	30 3	16	28	$3\ 1 \times 10^{-6}$	18		
Phosphate	1	$6\ 9 \times 10^{7}$	9 2	55	55	$1\ 5 \times 10^{-6}$	8		
10 g/l	2	$3\ 9 \times 10^{7}$	21 1	22	46	$5\ 4 \times 10^{-6}$	11	$3\ 0 \times 10^{-6}$	9
	3	$5\ 4 \times 10^{7}$	30 2	25	29	$2\ 2 \times 10^{-6}$	9		
Yeast	1	$1\ 7 \times 10^{7}$	7 2	35	0		18		
Extract	2	$2\ 0 \times 10^{7}$	18 2	12	0		22	-	20
0 5 g/l	3	$1\ 0 \times 10^{7}$	24 8	15	0		21		
Yeast	1	$7\ 3 \times 10^{7}$	9 3	31	33	$1\ 5 \times 10^{-6}$	12		
Extract	2	$2\ 7 \times 10^{7}$	20 6	10	0		13	$1\ 5 \times 10^{-6}$	13
6 g/l	3	$2\ 0 \times 10^{7}$	28 2	2	0		-		



LANE	SAMPLE	PCN
1	Standard reference DNA	-
2	Yeast extract 0.5 g/l	20
3	Yeast extract 6.0 g/l	13
4	Phosphate 10.0 g/l	9
5	Phosphate 1.0 g/l	13
6	Ammonium sulphate 2.5 g/l	11
7	Standard reference DNA	-
8	Ammonium sulphate 15.0 g/l	10
9	Ammonium sulphate 5.0 g/l	16
10	YEPD enriched with casamino acids	18
11	YEPD	25
12	Selective medium enriched with casamino acids	10
13	Selective medium	14
14	Standard reference DNA	-

Figure 7.8 Autoradiogram showing the different plasmid intensities (and hence content) of free cells grown on various different media.

7.6 DISCUSSION

In order to prolong or improve the stability of immobilised cell bioreactor systems attempts have been made to improve the stability of the calcium alginate gels One approach used is to further crosslink the gel bead with gluteraldehyde (Takata et al , 1977) Other methods include the binding of alginate to polycations (Birnbaum et al , 1988) and the use of various different counter-ions in the gelling process (Tanaka and Irie, 1988) Tanaka and Irie (1988) reported that strontium and barium alginate beads were more chemically and physically stable in electrolyte solutions than calcium alginate beads Ogbonna et al (1989) concluded that $SrCl_2$ is best for immobilisation of aerobic microbial cells in both alginate and carrageenan gel beads It was reported that cell leakage out of the beads was comparitively lower and unlike calcium alginate beads, strontium alginate was relatively stable in the presence of phosphates and citrate Figure 7 1 shows that biomass concentration remains approximately equal for calcium and strontium alginate beads with slightly higher enzyme activities in the case of strontium alginate However, as preincubation in selective medium of strontium alginate beads results in precipitation of some component of the yeast nitrogen base, the use of strontium alginate as an immobilisation matrix was discontinued

Investigations into the effects of cell load on the overall productivity and stability of the immobilised cell bioreactor system shows that a medium ICL of 10^6 cells/g-gel resulted in the greatest enzyme production, and the most stable immobilised cells with respect to genetic (plasmid) stability and physical stability of the beads (illustrated by the least amount of cell leakage) The low ICL (10^3 /g-gel) resulted in the highest specific enzyme activity due to the absence of diffusion resistances which allowed cells in the inner sections of the beads to grow and produce the enzyme However, due to the greater number of generations plasmid stability decreased quicker than in the high (10^8 cells/g-gel) or medium ICL The low ICL resulted in the beads becoming quite unstable as the huge size of the microcolonies exerted very large stresses on the bead structure This physical instability is reflected in the large degree of cell leakage throughout the fermentation and especially in the early stages Berry et al (1988) also reported the presence

of giant microcolonies throughout the gel beads when employing low ICL Plasmid stability was greatest for high ICL due to the reduced number of cell divisions before a steady state condition arose, where leaked cells were being replaced by cells from the inner sections of the gel beads In contrast to the present work, cloned gene product activity reported by Berry et al (1988) was greatest in the case of high ICL due to the higher biomass concentrations reached As mentioned previously (Section 6 6) and in agreement with the findings of Simon (1989) and Walsh (1993), the final immobilised cell concentration reached was approximately equal for high and medium ICL and only slightly lower for the low ICL, resulting in the greatest enzyme productivity occurring with medium ICL

The investigations into the influence of nutritional requirements/limitations yielded the following findings and conclusions (Table 7 2) Enzyme activity, biomass concentration and volumetric productivities increase with increases in bulk nutrient concentrations, especially nitrogenous compounds such as casamino acids (casein hydrolysate) or yeast extract Yeast nitrogen base (YNB) which forms the base of the selective medium, is limiting in inositol (Henry et al , 1977) and it cannot efficiently support respiratory growth It is suitable only for fermentative culture with ethanol being accumulated in batch culture, subject to catabolite repression (Chen et al , 1983) Supplementation of YNB medium with casamino acids permits respiratory growth with the result that higher cell densities can be attained β-Glucanase activity was higher in YNB plus casamino acids than in ordinary YNB as higher biomass concentrations were achieved However, like Chen et al (1993), the higher biomass yields obtained by casamino acids supplementation do not automatically guarantee an enhancement in product yields by secretion There was no increase in the specific enzyme activity The use of YEPD medium enriched with casamino acids resulted in greater specific activities and volumetric productivites compared with YEPD which in turn had greater biomass concentration, enzyme specific activities and volumetric productivities compared to YNB, possibly due to the increased availability of excess nitrogenous compounds Rossini et al (1993) reported that secretion of a recombinant product by *S cerevisiae* was not evident in YNB medium, but could be increased slightly by supplementation with the organic nitrogen sources of yeast extract and/or peptone Supplementation of YNB medium with casamino acids resulted in the production of greater quantities by *S cerevisiae* of the recombiant

human epidermal growth factor However, the product was rapidly degraded by secreted proteases (Coppella and Dhurjati, 1989) It was noted that (like β-glucanase production) the production of hEGF was highly medium dependent as the chemically defined non-enriched YNB medium had a significantly lower yield than enriched medium and YEPD medium, which elicited no measurable extracellular proteolysis

Chen et al (1990) reported that yeast extract and peptone apparently meet similar nutritional requirements for yeast growth and productivity Therefore, to assess the influence of yeast extract on cell growth and enzyme production in the present work, peptone was replaced by 1 0 g/l ammonium sulphate Figure 7 5 shows that enzyme specific activities and volumetric productivities increase with increasing concentrations of yeast extract , although the effect becomes less pronounced at higher yeast extract concentrations This result is very similar to that of Chen et al (1990) also working with immobilised yeast Rossini et al (1993) found that the fraction of secreted β-galactosidase is roughly proportional to the yeast extract and/or peptone content of the growth medium and also that increasing the level of yeast extract or peptone above that found in YEPD medium does not improve β-galactosidase secretion efficiency The results obtained in the present work and those from the afore-mentioned literature indicate the possible existence of some saturation or threshold point of organic nitrogen supply in the form of yeast extract or peptone, above which cloned gene product formation ceases to increase Nipkow et al (1984) reporting on the development of a synthetic medium for continuous cultivation of *Zymomonas mobilis* established that yeast extract could be replaced by a mixture of six mineral salts The nitrogen source in the medium was supplied by increased concentrations of ammonium sulphate Other studies indicate that cell growth and cloned gene product secretion by *S cerevisiae* were relatively insensitive to changes in the concentrations of KH_2PO_4, NaCl, $MgSO_4$, yeast extract and casamino acids However, it was observed that high ammonium sulphate concentrations were necessary for efficient secretion of the heterologous protein (Turner et al , 1991)

The results obtained for nitrogen and phosphate effects are clearly shown in Figures 7 6, 7 7 and 7 4 The absence of a nitrogen source as in the non-nutritious salts medium (Figure 7 4) or 0 0 g/l ammonium sulphate (Figure 7 6) resulted in little or no growth of biomass and very poor enzyme

production Increasing the ammonium sulphate concentration to 5 0 g/l yielded optimum production of β-glucanase Further increases resulted in a decline in the specific enzyme activity and volumetric productivity Comparing the results from the three different salts media (Figure 7 4), it can be seen that Davis's salts medium which is high in phosphates (2 0 g/l KH_2PO_4, 4 0 g/l $NaHPO_4$) has a lower productivity than the Basal salts medium which is high in nitrogenous compounds (5 0 g/l ammonium sulphate) Results suggest that nitrogenous compounds are more influential than phosphates in β-glucanase production by immobilised DBY746 (pJG317) cells There appears to be only a slight difference in productivities when the phosphate concentrations are varied (Figure 7 7) Overall volumetric productivities seem to vary little, with perhaps the enzyme specific activities decreasing slightly with increasing phosphate concentration Chen et al (1990) reported that, although an immobilised cell population could be maintained for extended periods (80 hours) in a bioreactor with reduced ammonium sulphate, the productivity dropped as the nitrogenous nutrients were eliminated The growth rates of biomass also dropped quite rapidly It was concluded that nutritional deficiencies largely decoupled growth and productivity of the immobilised yeast However, this phenomenon is not observed with immobilised DBY746 (pJG317) cells Although plasmid stability and biomass concentration do not change dramatically when the cells are immobilised, any changes (mainly decreases) that do occur, take place in cases where there is a corresponding decrease in β-glucanase activity Continuous patulin production by immobilised *P urticase* was found be to greatly dependent on nitrogen supply in the feed medium (Jones et al , 1983)

Table 7 3 illustrates the effect of nutrient requirements/limitations on PCN As the above results were obtained with immobilised cells, it was very difficult to assess whether the plasmid stability of the organism was affected by the various nutrient deficiencies Previous results (Chapter Five) have indicated a five fold difference in PCN of cells grown in YNB medium (selective medium) and YEPD Several reports suggest that, although growth rate influences PCN, the availability of nutrients may possibly play an equally important role in determining the PCN of cells (De Taxis du Poet et al , 1987, Bugeja et al , 1989) To test this hypothesis and also in an attempt to explain the different specific enzyme activities obtained with the various media used in the above studies the influence of various media types

on PCN was directly investigated There is evidence to support the suggestion that media or specific nutritional requirements influence PCN The PCN measured in different media under different nutritional requirements/limitations varied from a minimum of 9 to a maximum of 25 plasmid copies per cell For a given medium, PCN varies with specific activity - as previously determined in growth rate studies, when it was observed that as specific activity decreased in faster growing cultures the PCN also decreased In the present study, instead of growth rate being the major influencing parameter, it is nutrient availability that causes a change in the PCN and hence a corresponding change in the specific enzyme activity When comparing PCN values for different media, despite the fact that PCN values differ, no such relationship between specific activity and PCN in different media is observed In conclusion, the PCN of cells grown in different media with different nutritional deficencies varies, and for any particular medium, the PCN is proportional to the specific enzyme activity and is thus medium specific Plasmid stability does vary from one medium to the other due to the differing degrees of selection pressure in each medium In some cases, where the PCN is higher, and hence also the specific activity, the plasmid stability is found to be lower This relationship with plasmid stability is somewhat vague and is not so obvious as growth rate influences Few reports on media effects on recombinant yeast PCN exist Coppella and Dhurjati (1989) reported that the PCN did not change as a result of changing the medium Hollenberg (1982) also reported that the PCN of the 2μ circle does not change with the physiological state of the cell Bugeja et al (1989) also found the PCN of the endogenous 2μ circle remained unchanged at different growth rates and yet a large difference in PCN at different growth rates was recorded for the 2μ-derived expression vector It can only be concluded that the normal regulation and control of 2μ copy number and replication does not extend to some 2μ circle-derived plasmids such as the yeast episomal plasmids of which pJG317 is one type This plasmid pJG317 generally exists in low copy numbers (9-40 copies per cell), while the 2μ circle exists in high copy numbers (100-150 copies per cell) This fact reinforces the breakdown in normal 2μ circle replication mechanisms or regulation thereof

As a general observation throughout the course of this work, the specific enzyme activities were higher for free cells than for immobilised cells Although high biomass concentrations are achieved in immobilised cells, not

all cells produce the cloned gene product Due to the heterogeneous nature of growth of immobilised cells, a reduction in growth rate which occurs is accompanied by a reduction in specific fermentation rate (Hahn-Hagerdal, 1990) Finally, enzyme activities in specific media (YNB and ammonium sulphate media) are higher in free cell culture, while activities in general/complex media (YEPD and salts media) are higher in immobilised cell culture

CHAPTER EIGHT

CONCLUSIONS

A procedure for measuring the PCN of recombinant yeast was developed, tested and optimised The procedure is based on Southern hybridisation and measures the relative intensities of hybridisation of a probe to the single copy yeast chromosomal actin gene and to the multicopy plasmid pJG317

Plasmid stability increases with increasing growth rate in free cell culture PCN decreases with increasing growth rate The specific β-glucanase activity is proportional to the plasmid content (PCN) of the cell The only type of plasmid instability that was detected was segregational instability

Immobilised cell culture with medium cell loadings of 10^6 cells/g-gel, which are preincubated in selective medium before being transferred to complex (YEPD) medium, ensures the attainment of high biomass concentrations with a high percentrage of plasmid-containing cells This system of immobilised cell culture ensures highest enzyme production with the highest genetic and physical stability

Using the multistep dissolution technique, the heterogeneous growth patterns of immobilised cells were illustrated A gradient in plasmid stability exists throughout the immobilised cell population with slow growing cells in the inner sections of the gel beads having a higher plasmid stability than faster growing cells residing on the bead surface, which in turn have a higher plasmid stability than cells which have leaked from the immobilisation matrix

Immobilised cells have a higher plasmid stability than free cells Enzyme production over long periods of time is greater for immobilised cells where the volumetric productivity is greater However, the specific enzyme activity is greater for free cells due to the reduction in growth rate of immobilised cells brought about by diffusion resistances

No significant gradient in PCN was detected throughout an immobilised cell gel bead This is possibly due to the relatively small differences in growth rate between cells in the inner and outer regions of the gel beads not being significant or large enough to produce cell populations with large differences in PCN No significant change in PCN occurs in immobilised cell fermentations compared with free cell fermentations

Nutrient requirements and limitations influence the PCN of recombinant yeast The PCN varies with the media used and for any specific medium, the PCN is proportional to the specific β-glucanase activity This may explain why immobilised and free cells have differing enzyme activities Of the nutrients investigated, enzyme production is greatest in complex media Nitrogenous compounds play a major role in enzyme production and also significantly affect cell growth

It may be concluded that the 2μ derived yeast episomal plasmid pJG317 does not behave in a similar fashion to the endogenous 2μ circle The plasmid is quite unstable under non-selective culture conditions and its copy number and stability is influenced by both growth rate and nutrient supply The quantity of cloned gene product (β-glucanase) appears to be proportional to the number of plasmid copies per cell The plasmid may be stabilised and high volumetric productivities attained by employing immobilised cell culture

Recomendations for further work

1 Integrating the β-glucanase gene into the DBY746 chromosome is one means of stabilising the production of the enzyme A study comparing the production of plasmid-encoded β-glucanase and chromosome-encoded β-glucanase in free and immobilised cell culture would provide an insight into the potential of each system for long term continuous enzyme production

2 This study shows that a relationship between the number of plasmid copies per cell and enzyme production does indeed exist Further work on the kinetics of β-glucanase production and degradation, with PCN being monitored could provide details of this relationship

3 To fully assess if a gradient in PCN does exist in cells immobilised throughout the gel beads, a recombinant system with a high PCN and showing a larger difference in PCN at different growth rates should be chosen and the same studies as were performed with the DBY746(pJG317) recombinant system repeated

CHAPTER NINE

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