# CLONING AND EXPRESSION OF THE BACILLUS LICHENIFORMIS a-AMYLASE GENE IN SACCHAROMYCES CEREVISIAE

a thesis presented for the degree of Ph.D.

*by*

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August 1995

I hearby certify that this material which I now submit for the assessment on the programme of studies leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that this work has been cited and acknowledged within the text of my work.

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Date: 31/7/1995

**Acknowledgements**

I would like to thank my supervisor Dr. Thecla Ryan for her constant advice and encouragement, her patience when things were not going so well and her infectious enthusiasm when they were. I would also like to thank her for all her help in the preparation of this thesis, she made the distance between us seem short.

I would like to thank Sharon for introducing me to the practicalities of Yeast genetics and big Maggie for always being there to lend a helping hand. I will always be grateful for all the help which they have given me.

My thanks to Fiona for introducing me to PCR technology and for always having spare electroporation cuvettes hidden in her press. John for his computer oriented brain, Peter for his help with yeast chromosomal DNA and Aidan and the rest of the good time club, Sue-Anne, Brian, Maura, Dan, Seamus and Ger. Their friendship, support and humour made my time in the John Barry a time I will always remember.

I would also like to thank John Mac for introducing me to immunology and all the technical staff at DCU for their help, especially Robert, Brian, Joe and Monica.

Lastly and most importantly I would like to thank my wife Geraldine for her constant help, support, encouragement and monumental patience. I couldn't have done this without her.

## Dedication

This thesis is dedicated to my wife Geraldine and to my parents and extended family in appreciation for their constant support and encouragement.

ABSTRACT

Cloning and expression of the *Bacillus licheniformis* α-amylase gene in *Saccharomycs cerevisiae.*

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The α-amylase gene from *Bacillus licheniformis* together with its signal peptide sequence was cloned into a yeast expression vector under the control of the yeast ADH1 promoter and terminator. The resulting construct, pAAMY, when introduced into *Saccharomyces cerevisiae* cells which when subsequently grown under pH-controlled conditions, produced active α-amylase enzyme. At least 95% of the recombinant amylase is located extracellurarly. Temperature sensitive yeast mutants defective in the specific steps in the secretory pathway were used to show that the native *Bacillus* signal peptide is capable of effeciently directing the α-amylase through the yeast secretory pathway.

The extracellular amylase produced by the yeast was found to have similar pH and temperature profiles to the native *Bacillus* enzyme. Using Western blotting and activity gels, this extracellular α-amylase fraction was found to be heterogeneous with respect to molecular weight, varying from approximately 55 to 71 kilodaltons. After treatment with endoglycosidase$H_f$ the amylase specific bands were resolved to two bands, one major and one minor. The major band corresponded in size to the the control native bacterial enzyme, indicating that most of the heterogeniety in the band size originally observed was due to glycosylation. The second faint amylase band was approximately 3KD larger. This form of the enzyme is likely to represent the unprocessed form of the enzyme with its signal peptide still attached.

Several approaches were taken in an attempt to improve both the plasmid stability and the quantity of the active enzyme produced by the yeast cells. These included the isolation of yeast mutants using ethyl methane sulphonic acid(EMS), the targeted integration of the α-amylase gene into single and reiterated regions of the yeast chromosomal DNA, and finally the removal of part of the 3′ untranslated region of the DNA fragment carrying the *Bacillus* α-amylase gene.

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**Overview**

This project was undertaken to characterise the expresssion and secretion of the α-amylase of *Bacillus licheniformis* from *Saccharomyces cerevisiae* under the control of the α-amylase bacterial signal peptide. The project also set out to analyse the effect of changes in media composition, plasmid copy number and plasmid stability on the level of secretion of the amylase. The *Bacillus licheniformis* α-amylase was chosen for use as a reporter gene in this project as it is easily assayable and had not previously been expresssed in yeast. The information gained from this project could then be used for the optimisation of the secretion of other proteins of commercial interest.

The introduction covers the vectors available for the expression of foreign genes in Yeast and the factors which can affect the level of expression and secretion of these genes. It also discusses the various methods available for the optimisation of the levels of expression and secretion of heterologous genes from yeast.

The final two sections of the introduction deal with the expresssion and secretion of foreign genes from other yeasts and a review of the current literature on α-amylase expression in yeast.

# CHAPTER 1

# INTRODUCTION

## 1.1. General introduction.

The yeast *Saccharomyces cerevisiae* has been used by man for over two thousand years in food and beverage manufacture. It is now recognized and established as an ideal eukaryotic microorganism for microbiological and genetic studies. Although genetically more complex than bacteria, it shares many of the technical advantages that allowed rapid progress in the molecular genetics of prokaryotes. It possesses a rapid growth rate and can be grown to high cell densities on well defined inexpensive media. It has a well defined genetic system and possesses a versatile DNA transformation system which has made *S. cerevisiae* particularly amenable to gene cloning and genetic engineering techniques. Mutant isolation can be carried out easily and structural genes can be identified by complementation from plasmid libraries. Plasmids can either be introduced as autonomously replicating molecules or integrated into the yeast genome via homologous recombination, thereby allowing for targeting of plasmid DNA to specific sites within the yeast genome.

Although itself a unicellular organism, yeast, being eukaryotic, possesses much of the complex cell biology typical of higher multicellular organisms including a highly compartmentalised intracellular organisation and an elaborate secretory pathway which mediates the secretion and post-translational modification of many host proteins. The presence of this complex eukaryotic cell biology permits the use of yeast as a model organism for studying eukaryotic gene regulation, structure-function relationships of proteins, chromosome structure, gene expression and translation as well as basic aspects of metabolism. Mammalian genes are routinely introduced into yeast for analysis of the function of the corresponding gene products. Thus yeast can be readily manipulated and is seen as the eukaryotic equivalent of the prokaryote *Escherichia coli.*

## 1.2. Heterologous gene expression.

Since *Saccharomyces* is one of a small group of GRAS (Generally Regarded As Safe) organisms recognized by the FDA and since well developed methods exist for its large scale production, it is suited for the production of recombinant proteins for pharmaceutical applications, food processing and the nutritional enhancement of yeast based foods. Yeast cells can perform eukaryotic post-translational modifications such as glycosylation (Tanner and Lehle, 1987; Kukuruzinska *et al.*, 1987), fatty acylation (Towler and Glaser, 1986) and proteolytic processing (Julius *et al.*, 1983) that may be essential for protein activity thus displaying certain advantages over currently used bacterial systems when recombinant proteins derived from plants, animals or other fungi need to be produced. Manufacturers of pharmaceuticals intended for injection must be aware of possible endotoxin contamination since the cell wall of bacteria like *E. coli* contains a glycolipid component which can cause fever and shock if introduced into a patient's blood stream. Yeast as a GRAS organism does not possess this toxic cell wall component making it acceptable for the production of both food and pharmaceutical products and also reduces the rigourous screening that must be carried out on pharmaceutical products produced in *E. coli*. As a eukaryotic organism, yeast also has a well defined secretory pathway and can be used as a suitable host organism for the production of secreted proteins. Secretion by yeast is often the preferred route of production for many proteins as correct folding of proteins with disulphide bonds appears to occur more readily in the secretory pathway, glycosylation of proteins coincides with secretion and the secretion of proteins removes them from the bulk of the yeast proteins and proteases thus facilitating easier purification of the recombinant protein product.

Although *S. cerevisiae* has proved to be very useful for the

production of recombinant proteins it has some disadvantages, when compared for example to *E. coli*. *E.coli* can grow to 120 g/l in two days under vigourous fermentation conditions while it usually takes yeast about twice as long to reach the same density. There are many available regulated promoters in *E.coli* but fewer are known in yeast. The hyperglycosylation of proteins in yeast is also a major problem as it can lead to loss of activity of the recombinant protein and also to an immunogenic response from patients as the outer chain glycosylation in yeasts differs from that in mammals, (Melnick *et al.*, 1990, Kurkurzinska *et al.*, 1987).
Recombinant protein expression in *S. cerevisiae* was first described by Hitzeman *et al.* in 1981 and the first genetically engineered vaccine licensed by the FDA for administration to humans, hepatitis B surface antigen, was produced in this organism (Valenzuela *et al.*, 1982).

### 1.3. Vectors for use in *Saccharomyces cerevisiae*.

To achieve optimal expression of recombinant genes, two important properties of vectors are, for the most part, stable propagation and high-copy number. Ideally a vector should be maintained in most or all cells in a population, even without selective pressure, and should be present in each cell in high-copy numbers. In yeast, a number of vectors and strategies have been explored to achieve these goals but in most cases one of these criteria is filled at the expense of the other.
In yeast, extra-chromosomal replicons are based either on plasmids containing yeast autonomous replication sequences (ARS), (Campbell, 1983), which function as origins of replication, or on the native 2$\mu$ circle of *S.cerevisiae* (Hartley and Donelson, 1980).

#### 1.3.1. Autonomous replication sequences, ARS.

ARSs are yeast chromosomal origins of replication, (Williamson, 1985) and ARS vectors are present in multiple

copies per cell (1 to 20) due to segregational bias even though they only replicate once per cell cycle (Fangman *et al.*, 1983). They are mitotically unstable and plasmid-free cells accumulate at a rate of up to 20% per generation without selection due to the inefficient transmission of plasmids to daughter cells during cell division, (Murray and Szostak, 1983). Obviously such plasmid behaviour is not optimal for efficient heterologous gene expression and in practice ARS vectors are hardly ever used for large-scale foreign gene expression. ARS vectors can be stabilized by including yeast centromeric sequences (CEN) but then the copy number is reduced to one to two plasmids per cell (Clark and Carbon, 1980). Thus ARS/CEN vectors are only used where one requires low-level expression, as in the case where the protein product is toxic to the yeast cell.

1.3.2. 2$\mu$ (micron) circle based vectors.

To date, the most straightforward approach to achieving both high-copy number and stable propagation has been to use vectors derived from the yeast plasmid 2$\mu$ circle. The 2$\mu$ yeast plasmid is a 6.3 kb plasmid present in the nuclei of most *Saccharomyces* strains at about a hundred copies per haploid genome (Futcher, 1988). Four genes are encoded by the plasmid; FLP, REP1, REP2, and D. It contains an origin of replication OR1, which behaves as a typical ARS element, the STB locus (needed *in cis* for stabilisation) and two 599 bp inverted repeat sequences. FLP encodes a site-specific recombinase which catalyzes recombination at specific sites lying near the centre of the inverted repeats. This promotes flipping about the targets in the inverted repeats so that cells contain two forms of 2$\mu$, A and B (Broach and Hicks, 1980). In spite of the fact that the 2$\mu$ circle confers no apparent selective advantage or no known phenotype to cells in which it is resident and might be somewhat disadvantageous to the host cell, it is nevertheless stably inherited (Futcher and Cox, 1983; Mead *et al.*, 1986; Walmsley *et al.*, 1983). The stability of the plasmid is due largely to its ability to ensure its

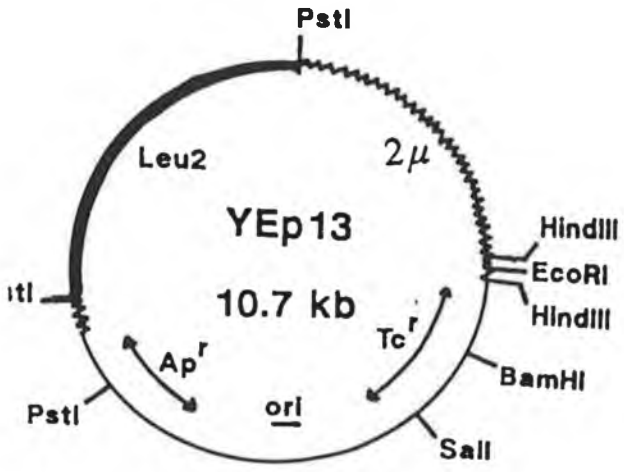
transmission to both daughter cells during division and to amplify its relative copy number level in cells which have received a reduced number of copies (Murray and Szostak, 1983; Kikuchi, 1983; Volkert and Broach, 1986). Efficient segregation is promoted by the *trans*-acting products of genes REP1 and REP2 (Kikuchi, 1983; Cashmore *et al.*, 1986) and by having the STB locus *in cis* which appears to act as a centromere-like element in the partitioning process (Kikuchi, 1983). Amplification appears to depend on the inverted repeats and the FLP gene product and it overcomes host regulation which restricts each replication origin to one initiation per cycle (Volkert and Broach, 1986). Futcher (1988) proposed a model whereby FLP promotes recombination between replicated and unreplicated DNA so that inversion occurs and two replication forks can follow each other around the circle spinning off an increasing catenane of plasmid genomes. Replication would terminate only when a second recombination event reinstated the bidirectional orientation of the forks.

The most basic 2$\mu$ vectors contain the 2$\mu$ origin of replication, the STB locus, a yeast selectable marker and bacterial plasmid sequences and are used in a 2$\mu^+$ host strain supplying REP1 and REP2 proteins (Kikuchi, 1983). These vectors have a small size and are easily manipulated, are ten times more stable than ARS plasmids and are present in 10 to 40 copies per cell. Most constructs carry the STB locus as the 2.2 kb *Eco*R 1 fragment or the *Hin*d III fragment from the B form of the 2$\mu$ circle. Both these fragments encompass the plasmid origin of replication and one of the inverted repeats with its FLP recombination target site. In order to limit recombination with 2$\mu$, the inverted repeat can be removed but adjacent STB sequences can not be removed as they seem to have an important role in protecting STB from transcriptional inactivation (Murray and Cesarini, 1986).

More complex 2$\mu$-based shuttle vectors contain the REP1 and REP2 genes in addition to the ORI-STB and can be used in 2$\mu$-free host strains (Armstrong *et al.*, 1989). Fig 1.1

shows a restriction map of plasmid pYE which has been used for construction of both expression and secretion vectors. The plasmid includes the *E. coli* plasmid pBR322 with an intact origin of replication and a β-lactamase gene (conferring resistance to ampicillin) allowing propagation and selection in *E. coli*. The plasmid also includes the yeast TRP1 gene allowing for selection in yeast. In addition, the entire 2μ plasmid is cloned in pYE thus negating the need to use $2\mu^+$ strains and avoiding recombination between the plasmid and an endogenous 2μ plasmid which could restructure the expression vector. Also, by using 2μ-free hosts, the copy number of the recombinant expression vector may be increased as it represents the only 2μ plasmid in the cell and it has been shown that there appears to be some competition between exogenous 2μ vectors and native 2μ such that the copy number of both is depressed (Futcher and Cox, 1984).

Figure 1.1




Restriction map of plasmid Yep13.

1.3.3. High copy number vectors.

A number of ultra-high copy number vectors are based on plasmid pJDB219 which contains the entire 2$\mu$ circle genome cloned into the *Eco*R 1 site of the bacterial plasmid pMB9 with disruption of FLP, (Hartley and Donelson, 1980). The plasmid also contains a sequence of yeast DNA encompassing the LEU2 gene inserted in the *Pst*1 site of the D gene. This LEU2 gene lacks its promoter and is cloned in pJDB219 in the opposite orientation to D and it is thought that its transcription is driven by a promoter located near REP3 (Sutton and Broach, 1985). The expression of the truncated LEU2 gene is several orders of magnitude less than that of the chromosomal LEU2 gene (Erhart and Hollenberg, 1983) and so the gene on the pJDB219 plasmid has been designated leu2-d (for diminished activity). Plasmid pJDB219 and its derivatives are present in yeast cells at approximately 200- 300 copies per haploid genome which is an order of magnitude higher than most standard 2$\mu$ based vectors (Futcher and Cox, 1984). It is thought that this high copy number is due to the requirement for high copies of the leu2-d allele to achieve leucine prototrophy in a leu2$^-$ strain. The presence of a normal LEU2 gene on a pJDB219 based plasmid abolishes the high-copy properties of the plasmid (Broach, 1983). In 2$\mu$-free cells pJDB219 is very stable due to its high copy number and in addition the plasmid persists in most cells in the population for many generations following a shift to non-selective medium. Both properties make the plasmid ideal for use in large scale fermentation (Futcher and Cox, 1984; Walmsley *et al.*, 1983) where continuous selection is not always practical.

1.3.4. Centromeric plasmids.

The vectors described above are capable of stable maintenance at high copy numbers. However it may be required, in some instances, to maintain expression vectors at low copy numbers. This would be desirable in cases where expression of the heterologous gene is toxic to the yeast cell. As mentioned above, addition of centromere (CEN)

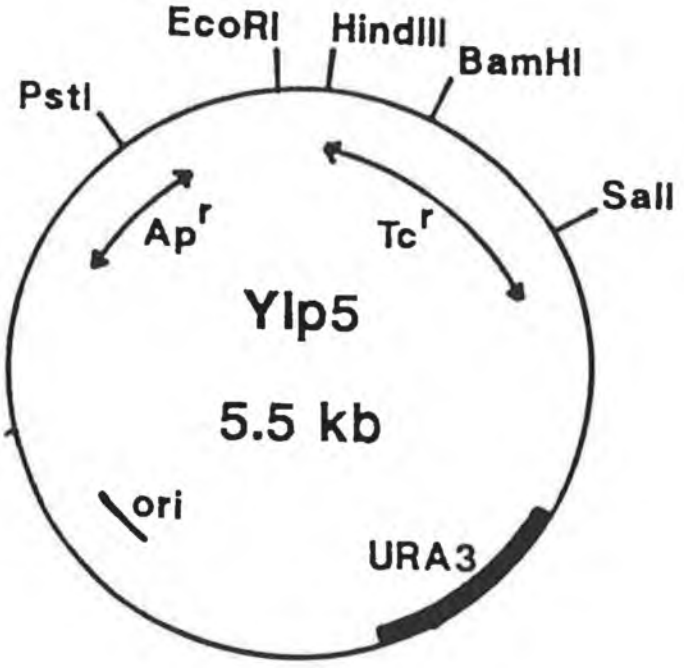
sequences to unstable plasmids can reduce loss through segregation to 1-3% per generation under non selective conditions. The copy number of CEN-containing plasmids, including those containing 2$\mu$ replicons is controlled at one to two per haploid genome (Clarke and Carbon, 1980). It has also been shown that in certain cases heterologous protein secretion may be more efficient if the expression system is stably maintained at low copy numbers (Smith *et al.*, 1985).

1.3.5. Integrating vectors.

The most stable way to maintain introduced genes is by integration of the entire plasmid into the chromosome by homologous recombination. The gene is thus normally present in only one copy (in haploids) and is very stable. Continuous selection to maintain the gene is not generally required unless expression is deleterious to the cell. Integrating vectors, (YIp, for yeast integration) contain yeast chromosomal DNA to target integration to a specific location, a selectable bacterial or yeast marker and a bacterial replicon but lack yeast origin of replication sequences (see Fig 1.2). The vector containing the desired heterologous gene is usually restricted at a unique restriction site in the yeast homologous DNA to linearise the plasmid as this gives approximately a 10-fold higher transformation efficiency compared to transformation with a circular plasmid. The linearisation of the plasmid DNA targets integration as DNA will integrate almost exclusively at the chromosomal sites that are homologous to the cut ends of the molecule. This single cross-over integration results in duplication of the chromosomal target sequence which means that the vector can be excised by recombination (fig.1.3). However, loss of vector occurs typically at a rate of less than 1% per generation in the absence of selection pressure (Hinnen *et al.*, 1978). If a high concentration of integrating vector is used in the transformation, tandem multicopy inserts can occur, most likely due to repeated recombination events (Orr-Weaver and

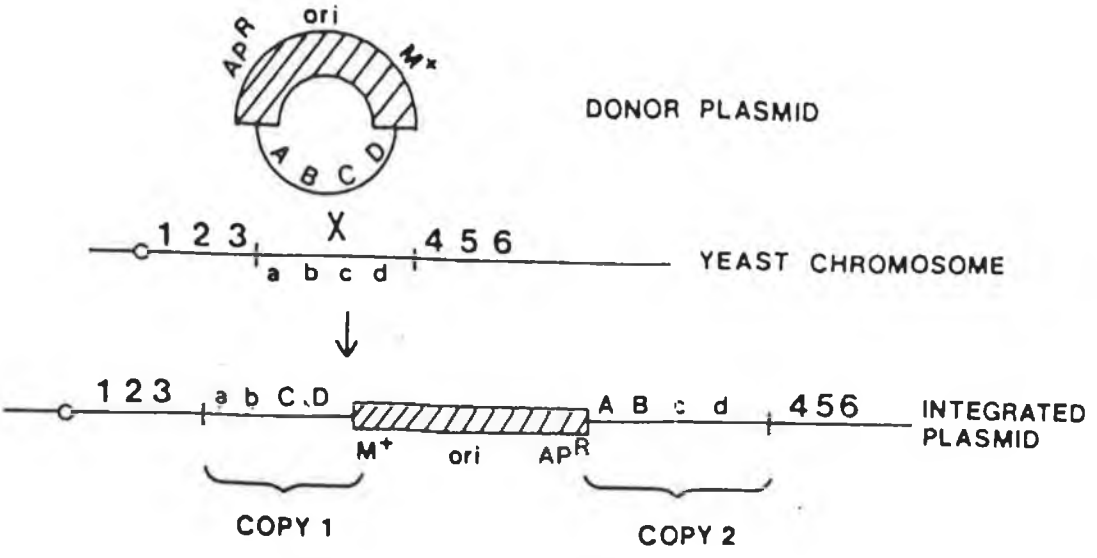
Szostak, 1983). These multicopy integrants are relatively stable and can be used, for instance, in gene dosage studies.

Fig.1.2.




Yeast integrating vector YiP5.

Fig.1.3.




Single cross over integration.

multiple integration into rDNA in yeast) which contains a portion of the rDNA unit and the LEU2-d marker has been constructed by Lopes *et al.*, 1989, 1990. Transformation with pMIRY2 restricted at the *Sma* 1 or *Hpa* 1 site resulted in $Leu^+$ transformants with 100-200 copies integrated into the rDNA cluster at a non-transcribed region. They found that use of the LEU2-d or other promoter defective markers was important for the isolation of high copy integrants. The transformants were very stable, with 80-100% of the integrated copies retained after 70 generations. They also found that the levels of foreign protein produced using the PGK promoter were as high as from 2$\mu$ vectors.

The transposable element Ty, present in 30-40 copies per genome in most *Saccharomyces* strains can also be used as a target for integration. The use of a transplacement vector targetted to replace Ty, where the vector copy number could be amplified using the LEU2-d selection marker was described by Kingsman *et al.* (1985). They found that levels of interferon produced from the amplified transformants were several times higher than from single-copy ARS/CEN vectors but almost 10 fold less than with 2$\mu$ vectors. Vectors that integrate by single cross-over into delta ($\delta$) elements, which exist either alone or as part of Ty throughout the *S.cerevisiae* genome, have more recently been used by Shuster *et al.* (1990). They constructed a vector expressing the *E. coli lacZ* gene with the LEU2 and CUP1 markers. Following transformation with vector restricted by *Xho* I, which cuts in the $\delta$ element with the LEU2 marker fragment, $leu^+$ transformants were selected. Multi copy integrants were then selected by increasing the copper concentration in the media. These multi-copy integrants gave up to 10-fold the $\beta$-galactosidase level of single-copy strains.

**1.4. Yeast Promoters.**

To obtain efficient transcription of foreign genes in yeast, the use of yeast promoters is essential. This conclusion was reached when it was shown that in general

foreign transcriptional promoters gave aberrant initiation, for example *Drosophila* ADE8 (Henikoff and Furlong, 1983), or were totally inactive, for example herpes simplex virus thymidine kinase (Kiss *et al.*, 1982). The first use of a yeast promoter was when a 1500 bp fragment 5′ of the ADH1 (alcohol dehydrogenase) gene was used for the intracellular expression of leukocyte α-interferon (Hitzeman *et al.*, 1981).

Sequential 5′ deletion analysis carried out for a number of yeast genes indicated that yeast promoters are larger in base pair size when compared to their prokaryotic counterparts (Struhl, 1981; Beier and Young, 1982; Guarente and Mason, 1983). In every case, sequences of more than 80 bp upstream from the mRNA start are critical for wild-type level of transcription. Yeast promoters consist of at least three elements which regulate the efficiency and accuracy of initiation of transcription (Struhl, 1989). These are TATA elements, initiator elements and upstream activation sequences (UASs). Transcription of a gene depends on a TATA box sequence (consensus TATAA) in the 5′ flanking region. They are found 40 to 120 bp upstream of the initiation site, they bind TFIID and general transcription factors (Hahn *et al.*, 1989) and are needed to elicit mRNA initiation by RNA polymerase II. The initiator element which is poorly defined, directs mRNA initiation at closely adjacent sites (Struhl, 1989). UASs bind to regulatory proteins which, under appropriate conditions for the particular gene, transmit to RNA polymerase II the signal to initiate transcription (Schneider and Guarente, 1991). The study of well defined cases made it clear that many UASs are mapped to short regions of DNA i.e., the cyc1 (Guarente *et al.*, 1984), gal 1, 10 (Giniger *et al.*, 1985), and leu2 (Martinez-Arias and Casadaban, 1984) upstream elements all map within a short stretch of DNA, 15-40 bp long.

### 1.4.1. Glycolytic promoters.

The first promoters used were those of genes encoding the

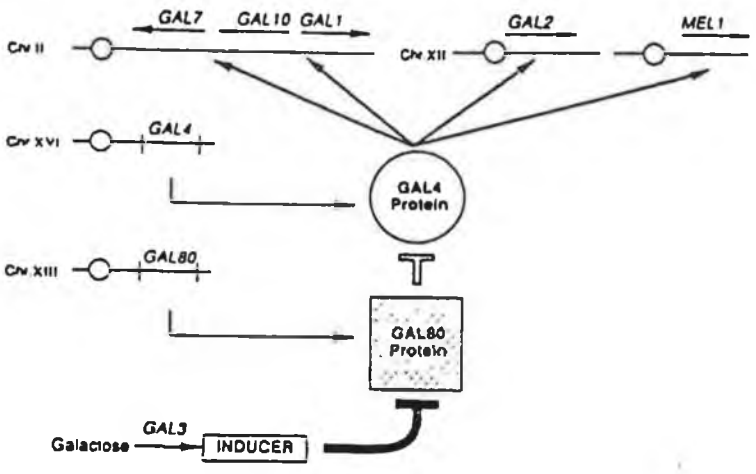
abundant glycolytic enzymes, alcohol dehydrogenase I (ADH1) (Hitzeman *et al.*, 1981), phosphoglycerate kinase (PGK) (Hitzeman *et al.*, 1983) and glyceraldehyde-3-phosphate dehydrogenase (GAP) (Tuite *et al.*, 1982). These genes are expressed at very high levels and glycolytic promoters are the most powerful promoters of *S.cerevisiae*, PGK mRNA accumulating to 5% of the total for example. These promoters are thus widely used for high-level expression of cloned yeast or foreign genes. The ADH1, PGK and GAP genes are often considered as constitutive but they can be induced by addition of glucose. Expression of α-interferon using the PGK promoter was induced 20- to 30-fold by the addition of glucose to a culture grown on acetate as the carbon source (Tuite *et al.*, 1982).

1.4.2. Regulatable Promoters.

A major problem with the expression of a number of genes cloned in yeast is that accumulation of the foreign protein may be toxic to the cell. Thus it can be extremely difficult or even impossible to grow the cells to the high densities needed for protein production on a commercial scale. One way to circumvent these problems is to use regulated transcription systems that allow the cells to be grown to a high-density under conditions where the protein is not expressed. One of the most commonly used regulated promoters is outlined below.

The regulation of the genes required for metabolism of galactose has been extensively studied and the GAL promoters of the GAL1, GAL7 and GAL10 genes represent the most powerful tightly-regulated promoters of *S. cerevisiae*. There are many genes involved in the regulation of GAL promoters but the main interaction is between the *trans*-activator encoded by GAL4, the repressor encoded by GAL80 and the GAL UAS (fig. 1.5) The GAL4 gene product binds to sites within the GAL UAS thereby activating transcription while GAL80 binds to the carboxy-terminal of GAL4 thus acting as repressor unless galactose is added which is necessary for the action of the GAL4 protein.

Addition of glucose to cells growing in galactose causes immediate repression of transcription (Schneider and Guarente, 1991). The tight regulation of expression by the carbon source makes the GAL promoters highly suitable for manipulating the expression of cloned genes. The extremely strong induction of transcription by galactose is useful for expressing high levels of a protein. GAL1, GAL7 and GAL10 mRNAs are rapidly induced greater than 1000-fold to approximately 1% of total mRNA on addition of galactose (St. John and Davis, 1981).
Figure 1.5.




Galactose regulation in yeast.

The genes involved in regulation and metabolism and their chromosomal location are shown. Activation is indicated by bold lines with arrows and inhibition is indicated by lines with bars.

Another method to regulate gene expression has been to use promoters that are activated when a nutrient is depleted or removed from the growth medium. The ADHII promoter is repressed under conditions where glucose is present in the growth medium (Shuster,1987). As glucose levels in the growth medium drop, as in the later stages of fermentation, the gene is derepressed and transcription is activated. In addition, expression vectors have been engineered to produce hybrid promoters made up of constitutive promoter elements (for strength) combined with the UASs of regulated genes (Velati-Bellini *et al.*, 1986). The controlled expression of human interferon (gamma) using a hybrid promoter consisting of the GPD (glucose-6-phosphate dehydrogenase) promoter combined with the UAS of the GAL 7 promoter has been described by Fieschko *et al.*, 1987.
While mention has been made of the most commonly used promoters in yeast, selection and screening is ongoing to identify new promoters from genomic libraries (Goodey *et al.*, 1986; Santangelo *et al.*, 1988, Duffy, M., 1994).

1.4.3. Transcription, translation and optimisation.
In addition to the type of promoter used, a number of factors affect the level of expression of a gene in yeast, in particular, the relative abundance of its mRNA, mRNA stability and the efficiency with which the mRNA is translated. Genes that are highly expressed, for example, alcohol dehydrogenase I (ADH1), histone genes, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), generally have mRNA levels that can range from 0.5-6% of the total cellular mRNA (Bennetzen and Hall, 1982), while genes that are more poorly expressed may have mRNA levels that are 2-3 orders of magnitude lower. The base sequence of the mRNA and hence the 3-D structure formed can also be important in determining the rates at which translation is initiated and protein synthesis occurs (Stanssens *et al.*, 1985; Wong and Chang, 1986; Lee *et al.*, 1987).
In general, when a yeast promoter is used to express a foreign protein, the yield of product is much lower than

the yield obtained when the homologous protein is produced using the same promoter. For example, when the PGK promoter is used on multi-copy vectors, foreign proteins such as i.e. α-interferon accumulate to 1-2% of the total cellular protein whereas the homologous protein, phosphoglycerate kinase, accumulates to over 50% (Chen *et al.*, 1984; Mellor *et al.*, 1985). It was shown that the α-interferon mRNA was not unstable but was initiated at a six-fold lower rate (Mellor *et al.*, 1987).

In yeast the optimal expression of a gene can also be affected by DNA sequences at the 3′ end of the gene, in particular the transcriptional termination sequence. It is usual to have yeast transcriptional terminators present in expression vectors to form mRNA 3′ ends and to avoid read through into vector sequences adjacent to the 3′ end of the gene. Terminators from genes such as ADH1, GAP and 2μ have been used. It has been shown that transcription termination may be coupled with polyadenylation, as in higher eukaryotes, and that a poorly conserved tripartite sequence may be part of the mRNA terminator (Zaret and Sherman, 1982). This sequence, identified in an analysis of 15 yeast genes has been shown to be tolerant to large sequence alterations (Osborne and Guarente, 1989). Thus fortuitous sequences in foreign genes causing termination can affect the yield of full-length transcripts resulting in low yields or complete lack of foreign gene expression in yeast. This problem has been observed in a number of genes with a very high AT content (Romanos *et al.*, 1992), one example being the tetanus toxin fragment C of *Clostridium tetani*. At least six fortuitous polyadenylation sites were identified in this gene resulting in truncated mRNA and no expression of protein. These sites were eliminated by increasing the GC content from 29% to 47% (Romanos *et al.*, 1991) by chemical synthesis which resulted in efficient production of fragment C. At present, chemical DNA synthesis along with site directed mutagenesis appears to be the only solution to this problem.

The efficiency with which mRNA is translated will also determine the success of foreign gene expression in yeast. The rate of initiation is of primary importance in translational efficiency and in eukaryotes it appears that an initiation complex assembles at the 5′ end of the mRNA and moves in the 3′ direction seeking out the first AUG codon, whereupon initiation occurs (Kozak, 1989). The structure of the 5′ untranslated leader sequence of a mRNA molecule affects the rate of initiation of translation and gene expression can be adversely affected when foreign 5′ leaders, possessing sequences giving rise to deleterious structures are present. An example of this is the poor expression achieved with the hepatitis B core antigen when the viral 5′ sequence (approx. 100bp) was retained (Kniskern *et al.*, 1986). When the 5′ viral sequence was removed the yield of protein increased from 0.05% to 26% of soluble cell protein. Yeast mRNA leaders are generally about 50 bp long, are A-rich and have very little secondary structure. Yeast genes with higher than average leader lengths (greater than 80 nucleotides) are capable of forming stable stem-loop structures within the 5′ non-coding region. The result of this is decreased gene function most likely because the ribosome cannot interact with a free 5′ end or scan toward the AUG codon. The optimal action of leader sequences can by affected by inserting a long sequence of G residues which inhibits translation completely or by insertion of a sequence of U residues which partially inhibits translation (van den Heuvel *et al.*, 1990). However, secondary structure is the most important factor affecting function and sequences giving rise to such secondary structures as hairpins have been shown to drastically inhibit translation of the HIS4 and CYC1 mRNA in yeast cells (Baim and Sherman, 1988; Cigan *et al.*, 1988). Therefore, where possible, the inclusion of foreign untranslated 5′ leaders in yeast expression vectors should be avoided and where it is not possible their sequence should be examined for secondary structures or long runs of G and U residues.

Another factor contributing to the poor expression of some foreign proteins in yeast may be the difference between yeast and higher eukaryotic leader sequences adjacent to the AUG start codon. In yeast there is a bias in nucleotide distribution of 5′ A/YAA/UAAUGUCU 3′ (where Y is a pyrimidine U or C) (Cigan and Donahue, 1987), whereas in higher eukaryotes it is 5′ CACCAUGG 3′.
In addition to the above sequence bias, there also seems to be a codon bias in yeast leader sequences with highly expressed yeast genes having leader regions which are A rich and G deficient (Cigan and Dohahue, 1987). If sequences around the translation initiation region of foreign genes are optimized, this can have a very marked effect on their expression in yeast. For example, an increase in the expression level of the hepatitis B surface antigen was obtained by replacing the natural untranslated leader sequence with an optimized leader sequence (Bitter *et al.*, 1987). Similarly, expression of human immune interferon $\gamma$ was achieved in yeast using an optimized leader sequence (Fieschko *et al.*, 1987).

**1.5. The secretory pathway in yeast.**

Protein secretion takes place in almost all cell types. Bacterial cells are compartmentalised to some degree, the cytoplasm being surrounded by an inner and an outer membrane separated by the periplasm. Proteins are transported from the cytoplasm to both membranes and to the periplasm by what is thought to be a common export pathway (Ito *et al.*, 1981). In eukaryotes, the generally accepted pathway for soluble and membrane proteins is as follows: endoplasmic reticulum(ER) $\longrightarrow$Golgi $\longrightarrow$vesicle $\longrightarrow$subcellular membrane or cell surface. This pathway was elucidated by using autoradiography and cell fractionation to follow pulse-labeled proteins through their intracellular route in pancreatic exocrine cells (Jamieson and Palade, 1967a, 1967b, 1968). Transport events include the insertion of newly synthesised proteins into the nucleus, mitochodrial

plasma membrane, lysosomes and the vacuole as well as assembly of the cell surface and secretion of proteins into the extracellular space.

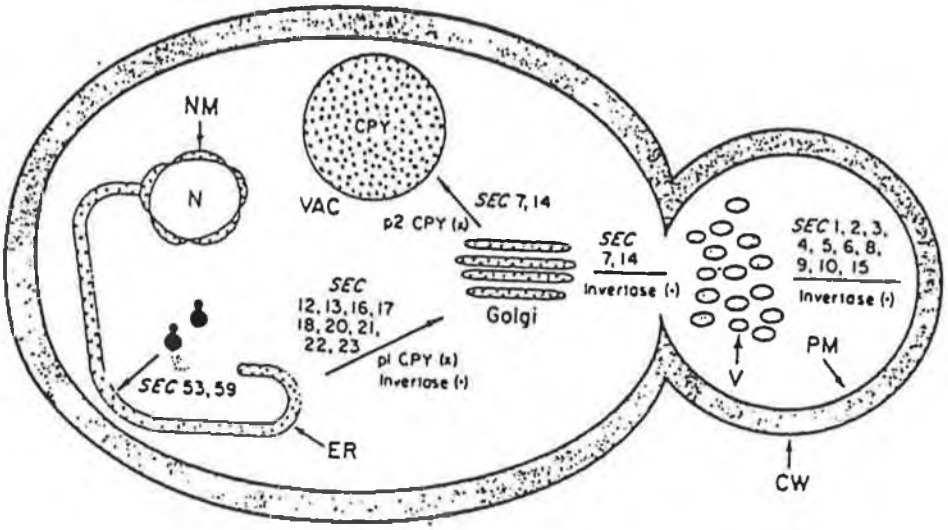
In *S.cerevisiae*, the cell surface consists of three layers, the cell wall consisting of mannoproteins and structural polysaccharides, glucan and chitin, a periplasm containing mannoproteins and a plasma membrane. Most of the secreted enzymes such as invertase and acid phosphatase are located somewhere between the plasma membrane and the cell wall where they are accessible to low molecular weight substrates. Other smaller non-glycosylated proteins such as α-factor and killer toxin are secreted through the cell wall into the culture medium (Schekman, 1982).

In yeast, secretion is correlated with cell surface growth, invertase and acid phosphatase being secreted into the bud portion of a growing cell which represents the point of cell surface addition during most of the division cycle (Tkacz and Lampen, 1973; Field and Schekman, 1980). The yeast secretory pathway was dissected by the isolation of a series of secretion defective mutants (Novick and Shekman, 1979). Given the correlation between secretion and cell growth it was assumed that secretory mutants would be lethal. To circumvent this problem a collection of temperature sensitive growth mutants were screened to identify strains which were defective in the export of the periplasmic proteins invertase and acid phosphatase at the restrictive temperature (37$^{o}$C) but which did export them at the permissive temperature (25$^{o}$C). Two classes of *sec* mutants were identified. Class A *sec* mutants, 192 in total, accumulate active secretory enzymes in an intracellular pool due to a block in export (Novick *et al.*, 1980). Class B *sec* mutants, 23 in total, do not secrete or accumulate active secretory proteins at the restrictive temperature even though protein synthesis occurs at an almost normal rate for several hours at 37$^{o}$C. Through complementation analysis, 23 *sec* loci have been identified in Class A mutants (Novick *et al.*, 1980) while 4 *sec* loci have been identified in class B mutants (Ferro-Novick *et al.*, 1984).

When cells are returned to the permissive temperature many of the class A *sec* mutants secrete the enzymes that have been accumulating at 37°C. Similarly, class B *sec* mutants, of which *sec*53 and *sec*59 are the best characterised complementation groups, are thermoreversible. In class A *sec* mutants use of cycloheximide to inhibit protein synthesis does not block this thermoreversibility, implying that continued protein synthesis is not required for secretion. However, use of energy inhibitors abolishes the reversibility indicating that energy-requiring steps are needed in the secretory process (Novick *et al.*, 1981). In addition to blocking export, Class A mutants accumulate secretory organelles in the stage in the pathway where the block occurs. For example, members of nine of the complementation groups develop an exaggerated endoplasmic reticulum while members of two groups (*sec*7 and *sec*14) accumulate structures related to that of Golgi vesicles. These exaggerated vesicles can be observed in electron micrographs of thin sections of the cells (Novick *et al.*, 1980; Esmon *et al.*, 1981).

To order the events in the yeast secretory pathway, Novick *et al* (1981) generated a number of double secretory mutants and characterised these with respect to invertase accumulation and organelle morphology. The experiments were based on the fact that in most of the cases only one of three different organelles accumulated in the *sec*A mutants and if these organelles represent stages in the route of secretory proteins, then a double mutant should accumulate the organelle that corresponds to the earliest block. In this way it was shown that the yeast secretory pathway closely resembles that of higher eukaryotes. Analysis of the class A, *sec* mutants showed that the ER accumulating phenotype is epistatic to the golgi body and vesicle accumulating phenotypes while a golgi mutant is epistatic to all vesicle-blocked mutants. Figure 1.5 shows the order of the events in the pathway as determined by this type of *sec* mutant analysis.

Figure 1.6.




Order of events in the yeast secretory pathway.
N: nucleus; NM: nuclear membrane; ER: endoplasmic reticulum; SEC: wild-type gene; VAC: vacuole; V: vesicle; PM: plasma membrane; CW: cell wall; CPY: carboxypeptidase; p1CPY and p2CPY: pro-enzyme forms of CPY.

**1.6. Signal peptides.**

Most eukaryotic and prokaryotic secretory proteins are synthesized *in vitro* as precursors with N-terminal extensions of 15 to 30 amino acids (Briggs and Gierasch, 1985). In yeast, as in higher eukaryotes, protein secretion is directed by an N-terminal signal sequence which mediates co-translational transfer into the ER. The signal hypothesis was proposed by Blobel and Dobberstein in 1975. According to their model it is the signal peptide which is responsible for selecting certain proteins for processing through the secretory pathway. The signal, as it emerges from the ribosome, is recognized and bound by specific receptors in the ER membrane which direct the protein across the membrane. The signal peptide is removed by a signal peptidase either during or after transfer.

Two essential components of the transport machinery identified were a signal recognition protein (srp) and an srp receptor or docking protein (Walter and Blobel,

1980,1981; Meyer *et al.*, 1980). It was shown that the srp binds the signal peptide on its emergence from the ribosome thus stopping further translation. The srp-polypeptide ribosome complex was then proposed to interact with the membrane-bound srp receptor or docking protein where translation resumes, the signal peptide is cleaved and the protein is translocated. The srp and docking protein are released and the processed form of the secretory protein is now in the lumen of the ER.

Biochemical and genetic studies of both prokaryotic and eukaryotic signal peptides have revealed that at least three structurally and functionally distinct regions exist in all signal peptides: a positively charged amino or N-terminal region (n-region) 1-5 residues long, a hydrophobic core region (h-region) 7-15 residues long and a more polar carboxy or C-terminal region 5-6 residues long (c-region) (Benson *et al.*, 1985; von Heijne, 1985; Oliver, 1985). In general, the introduction of charged or polar residues in the h-region often greatly reduces export, whereas alterations in the c-region which contains the cleavage site influence the efficiency of cleavage of the signal sequence from the mature protein (von Heijne, 1984a; von Heijne, 1986). Decreasing the net N-terminal charge also severely inhibits export (Vlasuk *et al.*, 1983).

Similarities between prokaryotic and eukaryotic signal sequences were further emphasized when it was observed that they are functionally interchangeable. For example, the *Bacillus amylofaciens* $\alpha$-amylase enzyme is secreted in yeast using its own signal peptide (Ruohonen *et al.*, 1987). $\beta$-Lactamase, the *E.coli* secretory protein can be synthesised in a eukaryotic cell-free translation system and can be translocated efficiently into pancreatic microsomes (Muller *et al.*, 1982). Similarly, in bacterial cells, some eukaryotic proteins can be secreted (Talmadge *et al.*, 1980).

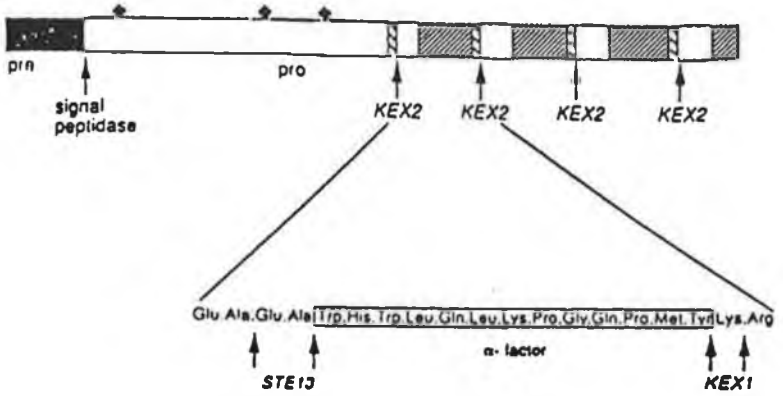
Signal peptides can also be used to direct cytosolic proteins into the secretory pathway. When the eukaryotic globin protein was fused to the $\beta$-lactamase signal peptide

it was translocated into microsomal vesicles (Lingappa *et al.*, 1984). Thus signal peptides play an extremely important role in protein export and in directing proteins to bacterial and ER membranes.

1.6.1. α-factor signal sequence.

The prepro region from α-factor (MFα1) is the most frequently used signal sequence for heterologous protein secretion. In many cases it is used with the MFα1 promoter. The protein, prepro-α-factor, encoded by MFα1 is 165 amino acids long, which comprises a signal sequence of 19 amino acids (pre region) and a pro region followed by four tandem repeats of the mature 13 amino acid α-factor sequence. During the export of the α-factor percursor through the golgi apparatus, the prepro region is cleaved by the KEX2 gene product, a protease (Julius *et al.*, 1984). The α-factor repeats are separated by spacer peptides of the sequence Lys-Arg $(Glu\text{-}Ala)_{2\text{-}3}$ or Lys-Arg-Glu-Ala-Asp-Ala-Glu-Ala and the KEX2 protease also cleaves here on the carboxyl side of arginine. The Glu-Ala or Asp-Ala dipeptides are exised from the $NH_2$ terminus of each repeat by the STE13 gene product, a dipeptidyl amino peptidase, while the KEX1 carboxypeptidase removes the Lys and Arg residues at the carboxyl terminus of the first three repeats (fig 1.6). These proteolytic events yield the fully processed, mature α-factor peptide.

Figure 1.7.




Cleavage of the prepro region of α-factor by KEX2.
The product of the MFα1 gene is shown schematically. The peptide product from the cleavage of prepro-α-factor by KEX2 is indicated and sites for further processing by STE13 and KEX1 are shown.

The cleavage of the prepro region by KEX2 has been utilised to direct the release of several correctly-matured polypeptides into the medium. Fusions of the MFα1 prepro to genes encoding mature human α1-IFN (Singh *et al.*, 1984), β-endorphin (Bitter *et al.*, 1984) and human EGF (Brake *et al.*, 1984) resulted in efficient secretion of the heterologous protein. Processing of the prepro region took place, but the Glu-Ala spacer at the N-terminus of the secreted protein was not always removed. Loison *et al.*, 1988, used the prepro sequence of the MFα1 gene to secrete biologically active leech hirudin, the most potent antithrombic factor found in nature. Initially they found that although a protein of the expected molecular weight was synthesised and secreted, it was inactive. This was shown to be due to incomplete N-terminal maturation of the precursor as a result of inefficient processing by the dipeptidyl aminopeptidase. Biologically active hirudin was secreted only when the precursor harboured a KEX2 cleavage site immediately before the first amino acid of the mature sequence. This result is not unexpected as it has been observed that when alpha mating type yeast cells are

transformed with a multicopy plasmid bearing the α-factor gene, the normal pathway of synthesis and processing is overloaded, Julius *et al.*, (1983). Cleavage of the Glu-Ala sequences by a dipeptidyl aminopeptidase appears to be a rate-limiting step as a large proportion of secreted α-factor retained the Glu-Ala -Glu-Ala sequence at its $NH_2$ terminus (Carter *et al.*, 1986). Instead of interposing an additional Lys-Arg between the Glu-Ala repeats and the mature foreign gene, as was done by Loison *et al.*, an alternative way to overcome this problem is to incorporate the STE13 gene on a multicopy plasmid (Julius *et al.*, 1984).

When the first strategy, incorporation of a KEX2 site immediately before the mature protein, was employed to produce human EGF, 80% of the secreted material was processed correctly but 20% retained the Glu-Ala repeats at the amino terminus (Brake *et al.*, 1984). The result indicated that the KEX2 protease cleaves at either of the Lys-Arg sequences but that a portion of molecules are not cut at both. The solution to this problem was to remove the Glu-Ala repeats from the secretion construct, whereupon 100% of the secreted material was cleaved after the Lys-Arg sequence. Despite problems such as those outlined above, the MFα1 prepro sequence has been used to direct the synthesis of other biologically active heterologous proteins including human interleukin-6 (Guisez *et al.*, 1991), human nerve growth factor (Kanaya *et al.*, 1989) and human erythropoietin (Elliot *et al.*, 1989) and the system has been demonstrated to be generally applicable.

### 1.6.2. Invertase SUC2 signal.

The yeast invertase SUC2 signal has also been used for foreign protein secretion although not to the same extent as the MFα1 signal. Using this signal, human α-2 interferon was secreted (Chang *et al.*, 1986) with the signal correctly cleaved, unlike the native signal which is not cleaved correctly in yeast (Hitzeman *et al.*, 1983). Mouse-human

chimeric antibodies have been produced using the invertase signal to secrete immunoglobulin heavy and light chains (Horwitz *et al.*, 1988) and human single-chain urinary plasminogen activator has also been efficiently secreted using this signal (Melnick *et al* ., 1990).

1.6.3. Cytosolic versus secreted proteins.

Yeast has been used for the expression and secretion of many foreign proteins and often secretion is the preferred option compared to intracellular production. Once translated, the protein must fold correctly to be biologically active but often the intracellular environment does not favour the correct folding of heterologous proteins unless they are naturally cytosolic. Thus intracellularly produced proteins often aggregate in an insoluble form especially at high rates of synthesis and at higher temperatures (Kiefhaber *et al.*, 1991). Low temperature growth or decreasing the induction rates can increase product solubility in yeast as has been shown for *E. coli* (Schein and Noteborn, 1988; Kopetzki *et al.*, 1989). Examples of naturally secreted proteins which are insoluble when produced intracellularly are prochymosin (Golf *et al.*, 1984; Smith *et al.*, 1985), human serum albumin (Quirk *et al.*, 1989), HIVgp120 (Barr *et al.*, 1987) human tissue plasminogen activator (Pennica *et al.*, 1983) and human $\gamma$-interferon (Simons *et al.*, 1984). These naturally secreted proteins are subjected to an abnormal environment in the cytoplasm where formation of disulphide bonds is not favoured nor can glycosylation take place.

Another feature of intracellular production is that proteins produced will have methionine at the $NH_2$ end. Naturally secreted proteins have specific $NH_2$ termini (rarely methionine) and therefore the intracellularly expressed protein represents an analog of the natural material and may have reduced activity or other undesirable properties. Production in a secretion system provides a method for the generation of native $NH_2$ termini (Bitter *et al.*, 1987).

However not all naturally secreted proteins form insoluble aggregates when expressed intracellularly, and α-interferon (Hitzeman *et al.*, 1981), α1-antitrypsin (Rosenberg *et al.*, 1984), tumour necrosis factor (Sreekrishna *et al.*, 1989) and Factor XIIIa (Rinas *et al.*, 1990) are examples of some that are soluble and biologically active. Nevertheless, many pharmacologically important proteins are naturally secreted and more often than not have to be processed through the secretory pathway to be correctly folded. On an industrial scale another advantage of secretion over intracellular production is the ease of continuous fermentation and processing and the relatively high initial purity of the desired protein product (Smith *et al.*,1985). In addition the protein product is removed from the bulk of yeast proteins, most importantly the proteases. Foreign proteins may be unstable or toxic when expressed intracellularly and here again secretion could overcome such problems.

Although seemingly an ideal solution to many problems, secretion of foreign proteins is not without its own drawbacks and glycosylation is becoming regarded more and more as a major drawback to the secretion of therapeutic glycoproteins from yeast. As these proteins process through the secretory pathway, asparagine-linked glycosyl structures may be added, the signal for which is the same for yeast and mammalian glycoproteins. The proteins then move on, in vesicles, to the Golgi where modification to the glycosyl structures take place. This is where problems can occur because modifications made in yeast differ from those made in higher eukaryotes and thus the fidelity of post-translational modifications may be compromised. In addition, the yeast proteins which assist in folding and disulphide bond formation are different than those in higher eukaryotes and this can lead to malfolding which in turn can result in retention in the ER and degradation (Romanos *et al.*, 1992). However, when such problems are encountered in *S. cerevisiae* for example, they can often be overcome by expressing the proteins in another yeast. This

will be discussed in a section 1.7.4.

Some of the promoters described above (1.4) have been used in secretion vectors (generally based on the 2μm plasmid) but very often a promoter and signal from the same gene are used together in vector construction, for example MFα1, PHO5 or SUC2. *S.cerevisiae* can however, tolerate considerable flexibility in the secretion signal sequence and this has been shown by Kaiser *et al.*, (1987) for the yeast invertase enzyme. Several investigators have used the signal sequence of the foreign gene of interest in association with a yeast promoter but results from these studies have been variable. For instance, no secretion into the medium was observed using the signal sequence of calf prochymosin (Mellor *et al.*, (1983); Smith *et al.*,(1985) or human α1-antitrypsin (Carbezan *et al*,.1984). When the signal sequence of human α- and γ-interferon was used only a portion of the protein synthesised was secreted and degradation of the preprotein took place (Hitzeman *et al.*, 1983). In contrast, the signal sequences of barley α-amylases 1 and 2 (Sogaard and Svensson, 1990), human serum albumin (Sheep *et al.*, 1990) and *Aspergillus niger* glucose oxidase (De Baetselier *et al.*, 1991) result in secretion of protein into the culture medium. When an α-amylase from *Bacillus amyloliquefaciens*, using its own signal sequence, was successfully secreted from yeast (Ruohonen *et al.*, 1987) it illustrated that a prokaryotic signal sequence can function in yeast to direct secretion.

Despite some successes using heterologous signal sequences, most secretion vector constructions use either the secretion signal from yeast α-factor or from yeast invertase to direct foreign proteins out of the cell.

## 1.7. Improving levels of heterologous protein expression and secretion.

The secretion capacity of *S. cerevisiae* for homologous proteins appears quite low when compared to other organisms. *Aspergillus niger*, for example, secretes grams of glucoamylase per litre of culture broth (Cullen *et al.*,

1987), *Bacillus subtilis* secretes similar levels of α-amylase (Yamazaki *et al.*, 1983) and *Yarrowia lipolytica* secretes an alkaline extracellular protease at high levels (Ogrydziak and Scharf, 1982). By comparison, *S.cerevisiae* secretes less than 100mg of invertase, mating factor or acid phosphatase per litre of culture broth (Bostian*et al.*, 1982; Julius *et al.*, 1983; Williams *et al.*, 1985). However, it appears that severe limitations do not exist in the secretion machinery and increased synthesis and secretion of invertase, up to 2% of soluble cell protein, has been achieved by addition of invertase gene copies on extrachromosomal plasmids (Esmon *et al.*, 1987).

The question which interests many researchers however, is not how efficiently *S. cerevisiae* secretes homologous proteins but how efficiently it secretes heterologous proteins, particularly mammalian proteins. While there have been some successes, for example the P28-1 antigen of *Schistoma mansoni* (Loison *et al.*, 1989), superoxide dismutase (Hallewell *et al.*, 1987), *Mucor pusillus* rennin (Hiramatsu *et al.*, 1989), hepatitis B virus core antigen (Kriskern *et al.*, 1986) and *Aspergillus niger* glucose oxidase (De Baetselier *et al.*, 1991), there are also several documented cases of poor secretion efficiencies of mammalian proteins in *S.cerevisiae*; calf prochymosin (Smith *et al.*, 1985), human α-1-antitrypsin (Moir and Dumais, 1987), human tissue plasminogen activator (Lemontt *et al.*, 1985), α-interferon (Hitzeman *et al.*, 1983), anchor-minus influenza haemagglutin (Jabbar and Nayak, 1987) and human lysozyme (Jigani *et al.*, 1986). In all these cases, at least as much protein remained inside the cell as was secreted to the outside culture broth or the periplasm. Explanations offered for these observed low secretion efficiencies are the large size of the proteins, the presence of proteolytic enzymes in the culture broth, or incorrect signal sequence processing.

One approach towards improving this low-level secretion of heterologous proteins in *S.cerevisiae* has been the isolation of mutants that result in increased secretion of

the foreign protein from the yeast, i.e. supersecretion mutants. The rationale for this approach was the idea that any complex, multistep, biochemical pathway must have one or more rate-limiting steps and that it should be possible to isolate chromosomal mutations which affect these rate-limiting steps. It was predicted that all mutations initially should occur at a single locus, namely that which codes for the most rate-limiting step in the pathway but that by repetitively isolating mutations in a strain already carrying one or more hypersecreting mutations it should be possible to sequentially alter the rate-limiting steps and produce dramatic increases in the amount of a secreted foreign protein (Wood and Brazell, 1987). The most often quoted example of a progressive yield improvement from a microbial system is the production of penicillin from the organism *Penicillium chrysogenum*. Over a period of twenty years the penicillin yields from production strains have been improved 55-fold as a result of a stepwise mutational program including treatment of the organism with radiation and chemical mutagens (Aharonowitz and Cohen, 1981).

1.7.1. Classical mutagenesis.

The best mutagens for most purposes are those that induce high frequencies of base-pair mutations and little lethality. The widely used alkylating agents N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethylmethane sulphonate (EMS) fulfill these criteria but are highly specific in their action, almost exclusively producing transitions at G.C sites (Kohalmi and Kunz, 1988). This specificity is not a problem for most purposes although it could be disadvantageous where in some types of reversion, specific mutations at specific sites are required. Ultraviolet light (UV) is also a reasonably good mutagen, producing a greater range of substitutions, most occurring in runs of pyrimidines, particularly T-T pairs (Lee *et al.*, 1988). UV also induces a significant frequency of frameshift mutations, almost exclusively of the single

nucleotide variety.
Choosing an optimal mutagen dose usually requires balancing the need for a high mutation frequency with reasonably high survival and avoidance of multiple mutations. The highest proportion of mutants per treated cell is usually found at doses giving 10 to 50% survival (Lawrence, 1991).
After treatment with mutagens, cell cultures have to be allowed to grow for several generations under nonselective conditions to enhance the production and expression of mutations, to allow full expression of mutant phenotypes and to allow recovery from mutagen damage which can cause some cells to stop growing temporarily or to grow more slowly. Plating dilutions of treated cells on solid medium, to get colonies for screening, has the advantage that each induced mutation identified is of independent origin. If outgrowth in liquid medium is required, independent mutations can be isolated by dividing the mutagenised culture before outgrowth and taking a single mutant from each subculture.

### 1.7.2. Super-secreting mutants of *S. cerevisiae.*

An additional advantage of *S. cerevisiae* as a host organism for protein secretion is the ease with which mutants can be generated and screened for useful phenotypes. Several *S.cerevisiae* strains with a super-secreting phenotype have been isolated and all were isolated by screening or selecting for more of a particular product outside the cell.
Bussey *et al*. (1983), identified mutations that resulted in increased secretion of a yeast protein. They described mutations denoted *ski*5 and *kre*1 which caused yeast cells to accumulate more killer toxin and other small secreted yeast proteins in the medium. However, neither *ski*5 nor *kre*1 affect the secretion pathway itself. The increased accumulated levels of secreted homologous proteins appear to result from loss of a cell surface protease (*ski*5) or from perturbations in the cell membrane affecting distribution between the periplasm and the culture broth

(*kre*1). Thus these mutations are affecting secretion levels indirectly.

Smith *et al.*, 1985, using calf prochymosin as a model for yeast secretion of heterologous proteins describe the isolation of two super-secreting mutants. They achieved secretion of prochymosin from yeast cells by using the secretion signal from yeast invertase but found that the yeast cells had a limited capacity for prochymosin secretion, with less than 1% of the prochymosin made being secreted. Even when a five-fold increase in production of prochymosin was achieved through the use of a stronger promoter only one tenth more prochymosin was secreted. Thus a mutagenesis approach coupled with a suitable rapid screening assay was adopted to isolate super-secreting mutants.

The assay was based on the fact that when a plate, on which yeast colonies secreting prochymosin have been growing, is overlaid with a mixture of milk and molten agarose, opaque regions of clotted milk form and their size and intensity are related to the amount of chymosin secreted by individual colonies. The assay identified 39 super-secreting strains all producing the same total amount of prochymosin as the parent strain but some secreting as much as eight or ten times the wild-type amount. Complementation analysis revealed that at least four genes can lead to a super-secreting phenotype but mutations in two genes in particular, *ssc*1 and *ssc*2, (for super secretion) were identified as the strongest and most easily manipulated alleles. Haploid yeast strains constructed to contain both the *ssc*1 and *ssc*2 mutations were found to secrete prochymosin more efficiently than either *ssc*1 or *ssc*2 single mutants, indicating that the effects were additive. It was also observed that the *ssc*1 mutation increased the secreted yield of bovine growth hormone by at least ten-fold. A model proposed to account for the super-secreting mutations is that they open up new bypass routes around a single rate-limiting step in the secretion of heterologous proteins. The *ssc*1 gene was subsequently

found to be identical to *prm*1 encoding a P-type ATPase which is believed to be a calcium pump situated in the secretory pathway (Rudolph *et al.*, 1989). Mutation of this *pmr*1 gene increased levels of scuPA by five to fifty-fold (Turner *et al.*, 1991) but not of α1-antitrypsin which was efficiently secreted by the wild-type strain. This was thought to indicate that the *pmr*1/*ssc*1 mutation may have the greatest effect when proteins are poorly secreted by wild-type strains.

Wood and Brazzell, 1987, used secretion from yeast of the cloned *Stahphylococcus aureus* nuclease protein (Shortle, 1983) as a model system for isolating super-secretion mutants. In an initial screen four mutants were isolated out of $10^4$ colonies which showed higher levels of nuclease activity than the parent strain. These mutants were crossed with a Matα strain to determine whether the mutations would cause similar increases in nuclease activity in the media of Matα cells as in the Mata in which they were isolated. From crosses with of the mutants, Matα segregants were isolated with increased nuclease activity. One of these segregants, or level 1 mutants, judged to be the best, was mutagenized again whereupon two more mutants were identified giving a 173-fold and 62-fold increase in the secreted protein. These two mutants were again mutagenized to look for level 3 mutants and two were identified both from the level 2 mutant giving the 173-fold increase. Both these mutants gave a 405-fold increase in protein secretion. The results support the idea that the amount of a protein secreted is subject to rate-limiting steps which can be successively altered to produce higher and higher levels of secretion. The authors compared their results to those of Smith *et al.* (1985) and found that the mutants, including the hybrid strain, isolated by Smith *et al.* did not give as large an increase in secretion as their highest level 3 mutants. In addition the *ssc* mutants only altered the internal and external distribution of prochymosin whereas the mutants isolated in the above study increased the total amount of nuclease made. An alternative model was

proposed that was judged to be consistent with the above data and that of Smith *et al*. The mutations could represent alterations in enzymes or protein structures used in secretion of native proteins which could then perform their function on a foreign protein at a faster rate. The additive effect of mutations was explained by the fact that when the most rate-limiting step in a biochemical pathway is increased, some other step becomes rate-limiting and can be subjected to similar mutation. The specificity of the mutations effect on secretion of different foreign proteins was thought to be due to the possibility that different foreign proteins could impose different constraints on the secretion machinery due to size, tertiary structure, disulphide bond formation, glycosylation sites and processing requirements.

Sakai *et al*., 1988, isolated mutants responsible for an oversecretion phenotype in *S.cerevisiae* using a promoter of SUC2 and the gene coding for α-amylase of mouse as marker of secretion. Prior to mutagenesis the chimeric gene was integrated into the yeast chromosome and a single-copy integrant selected for use. α-amylase secretion was detected by plating colonies on starch-containing plates and staining with a solution of potassium iodide (Nishizawa *et al*., 1987). Out of a screen of 3,500 colonies, five strains were identified exhibiting the oversecretion phenotype. Out of these, two recessive mutations, *ose*1 (for oversecretion) and *rgr*1 (for resistant to glucose repression) were identified. The *ose*1 mutant secreted α-amylase 12-15 times more than the parent cells but this did not arise from an increase in transcription as mRNA levels were the same in both mutant and parent cells. When β-endorphin was used as a marker for secretion, the *ose*1 mutant showed a 10-fold oversecretion indicating that the oversecretion phenotype was not specific for amylase. The *rgr*1 mutant showed an increased level in SUC2-amylase chimeric gene transcription resulting in the amylase oversecretion phenotype. Transcription was resistant to glucose repression. The *rgr*1 mutant also exhibited a

temperature-sensitive lethality which was tightly linked to the oversecretion phenotype. Suzuki *et al.*, 1989, described the identification of yeast mutant strains secreting large amounts of human lysozyme. Colonies were screened using an agar medium containing bacterial cells. Nine mutants secreting over 10 times more lysozyme than the wild-type strain were identified. Three of these mutants showed reduced carboxypeptidase Y (CPY) activity. When one of these mutants was further characterized it was found that a single mutation, *ssl*1 was causing both the supersecretion of lysozyme and deficiency of CPY activity. This was the first report showing that heterologous protein secretion could be enhanced by a mutation in a protease in yeasts. Although the exact mechanism by which this was occurring was not known, the possibility that it was due to a mutation in the structural gene for CPY, *prc*1, was discounted because *prc*1 maps quite far from the centromere on chromosome XIII (Mortimer and Schild, 1985) while *ssl*1 was found to be closely linked to a centromere. In addition, CPY deficiency in itself does not lead to supersecretion. Because the *ssl*1 mutation affects the processing of CPY but does not elevate the transcription of the lysozyme gene it was assumed that the oversecretion could be caused by a mutation of genes related to either protein transport and localization or to protein degradation. In a subsequent study, Jigami *et al.*, 1990, isolated another human lysozyme oversecretion yeast mutant showing a deficiency in the degradation of intracellular lysozyme. This mutant, designated *ldd*1 (for lysozyme degradation) was thought to be caused by a recessive single gene mutation which is deficient in the component of ubiquitin dependent protein degradation system.

Sleep *et al.*, (1991), described the use of a widely applicable antibody-dependent screening procedure that identified clones secreting elevated levels of a heterologous protein with no measurable enzymatic activity. This screen is suitable for any protein for which antibodies are abundantly available. The screening

procedure was developed for recombinant human albumin. When colonies secreting the human recombinant albumin were allowed to grow on agar plates containing a polyclonal antibody to human serum albumin, opaque halos appeared around the colonies. After successive rounds of mutation and selection the secretion of recombinant human albumin was enhanced 6-fold to levels in excess of 1% cell dry weight. When α1-antitrypsin and human plasminogen activator inhibitor 2 genes were transformed, on appropriate plasmids, into the series of mutant yeast strains, the levels of the internally expressed proteins increased to 40% and 20% respectively of the total soluble protein. This result demonstrated that the antibody screening procedure could be applicable to other proteins even if they are internally expressed as it was observed that sufficient of the above proteins was released as a result of autolysis of colonies to produce precipitin halos in solid media.
The examples detailed above demonstrate that the screening approach has often been successful in the isolation of super-secreting strains and where high yield is required this method should be considered.

1.7.3. Integration.
Apart from screening for super-secreting phenotypes, there are other methods used to increase the secretion and expression of foreign proteins in yeast. Smith *et al.* (1985), made the surprising observation that the efficiency with which prochymosin was secreted from yeast was considerably greater when the gene for prochymosin fused to a yeast promoter and secretion signal sequence was integrated into the yeast chromosome. Strains were constructed with several integrated copies of invertase-prochymosin genes and although such strains produced almost the same total amount of prochymosin as strains containing the same construction on a multicopy plasmid, the integrated strains secreted at least four times as much. The authors presumed that the improved secretion efficiency observed on integration of prochymosin

would also be observed for other gene products. However this is not always the case as was observed by Sakai *et al.*, 1988. When they integrated their SUC2-amylase chimeric gene into the yeast chromosome no increase in expression or secretion was seen.

Lopes *et al.* (1989), as already mentioned in section 1.3.5, designed a vector capable of integrating multiple copies of the region of interest into the yeast genome. The vector, pMIRY2, was present in 100-200 copies per cell. When the heterologous gene for thaumatin, the sweet tasting protein from *Thaumato coccusdanelli*, was cloned into the plasmid, transformants carrying about 140 copies of the gene were obtained. The level of thaumatin in the transformants was found to be approximately 100 times the expression level observed in a *S. cerevisiae* transformant carrying a single thaumatin gene integrated in chromosome IV. The level of thaumatin in the pMIRY2-thaumatin transformants was comparable to levels achieved with 50 extrachromosomal copies of the gene on a YEp vector. Thus the pMIRY2 system is an attractive one for the high-level expression of heterologous proteins. Sakai *et al.*, 1991, described the use of a δ-integration system to obtain enhanced secretion of human nerve growth factor (hNGF) from *S.cerevisiae*. They had previously developed the δ-integration system Sakai *et al.* (1990) which allowed multicopy and multisite integration based on homologous recombination between the δ (delta) sequences of the yeast retrotransposon Ty element on yeast chromosomes and those subcloned on YIp-type plasmids. However they found that the copy number of the integrated plasmids was relatively low (3-5) because each δ-integration was occurring only on a particular chromosome. Using two selectable markers, haploid yeast strains were constructed with approximately 20 copies of a δ-integrated hNGF expression cassette on four chromosomes by transmission of δ-integrated chromosomes by standard genetic crosses. Expression and secretion of hNGF was under control of the PGK promoter and the MFα1 prepro signal.

Secretion of hNGF from these strains was about 3-4-fold that from cells carrying the 2$\mu$-based plasmid, pSSE9 carrying the hNGF gene (Kanaya *et al.*, 1989). Oversecretion was caused by an increased amount of mRNA as was determined by Northern and Western analyses. It was felt by the authors that this $\delta$-integration system should have applications for industrial purposes for a number of reasons: using four or more selectable markers 50-100 copies of the heterologous gene expression cassette could be introduced into the yeast chromosomes, $\delta$-integration is mitotically and meiotically stable even in rich media allowing continuous culture and since different genes can be integrated into different chromosomes it could be possible to produce products that require the sequential action of several proteins in one-step culture.

1.7.4. Use of non-saccharomyces yeasts.

The majority of recombinant proteins produced in yeast have been expressed and secreted using *S.cerevisiae* as a host system. However, it could be said that *S.cerevisiae* is not an ideal host due to certain limitations and drawbacks with regard to heterologous protein expression. In general, product yields are usually low, except for some notable exceptions already mentioned, and even using a strong promoter, the maximum yields obtained is 1-5% of total protein. There are difficulties in scaling up production because of the observed poor plasmid stability of autonomously replicating plasmids during production runs. Even when inducible promoters have been used, plasmid stability has been found to drop significantly (Da Silva and Bailey, 1991). In addition several reports have noted the hyperglycosylation of secreted glycoproteins in *S.cerevisiae* (van Arsdell *et al.*, 1987; Moir and Dumais, 1987; Lemontt *et al.*, 1985) which may cause dramatic differences in immunogenicity and diminished activity. Over the past ten years or so much effort has gone into modifying and improving *S.cerevisiae* as a host system for expression but many researchers are now evaluating

alternative yeast hosts for stable high-level production of recombinant proteins.

Development of efficient transformation and selection systems is pivotal to the use of alternative yeasts as expression systems and to this end transformation systems have been described for in excess of a dozen yeasts (Reiser *et al.*, 1990). However, only a few have been developed for commercial expression of heterologous proteins and these include the methylotrophs *Pichia pastoris* and *Hansenula polymorpha*, the fission yeast *Schizosaccharomyces pombe*, the alkaline-utilizer *Yarrowia lipolytica*, the lactose-producing *Kluveromyces lactis* and the amylolytic yeast *Schwanniomyces occidentalis*. The methylotrophic yeasts *P.pastoris* and *H.polymorpha* have been used for the expression of over twenty proteins of commercial interest and some of the proteins expressed have also been expressed in *S.cerevisiae*, allowing some comparisons to be made. For example, both *P.pastoris* and *S.cerevisiae* have been used for the expression of murine epidermal growth factor and the human proteins insulin-like growth factor 1 and serum albumin. In each case a comparison of the productivity in the two species shows that yields were greater from *P.pastoris* than from *S.cerevisiae*. Comparing levels of EGF, *S.cerevisiae* carrying over 50 copies of the gene under control of the GAL7 promoter produced 0.6 mg/l of culture medium while *P.pastoris* carrying 1 and 13 copies under control of the AOX1 promoter produced 1.9 mg/l and 22.4 mg/l of EGF respectively (Buckholz and Gleeson, 1991). When the expression of prochymosin in *S.cerevisiae* (Smith *et al.*, 1985) and *K.lactis* (van den Berg *et al.*, 1990) each containing an integrated copy of the gene, was compared, the total yield of prochymosin for *K.lactis* was almost 20-fold higher than for *S.cerevisiae*. Similarly, yields of secreted HSA from *K.lactis* were higher than those achieved using *S.cerevisiae* (Fleer *et al.*, 1991).

To circumvent problems such as toxicity to the cell by the product being produced, regulated promoters are commonly used to drive the expression of proteins in *S. cerevisiae*.

Regulated promoters are also available in these alternative yeast hosts. In *P. pastoris* and *H. polymorpha* the regulated promoters are from the AOX1 (alcohol oxidase 1) gene (Ellis *et al.*, 1985) and the MOX (methanol oxidase) gene respectively (Ledeboer *et al.*, 1985). In both hosts, methanol induces expression of foreign proteins and with limited methanol feeds, very high levels of both the natural product alcohol oxidase and heterologous proteins under the AOX1 or MOX promoters can be achieved (Gleeson and Sudbery, 1988). The AOX1 promoter is repressed when cells are growing in glucose or glycerol (Tschopp *et al.*, 1987). The MOX gene is significantly derepressed during glucose limitation or in the absence of glucose, as when substrates such as glycerol, sorbitol or ribose are used (Egli *et al.*, 1980). This means that tight regulation of the promoter is lost in the conditions normally used for high-biomass fermentations (Gellison *et al.*, 1991). The GAM promoter in *S.occidentalis*, which is tightly repressed by glucose and induced by maltose has also been used to express heterologous proteins (Piontek *et al.*, 1990). In *K.lactis* and *Y.lipolitica* the Lac4 (van den Berg *et al.*, 1990) and XPR2 (Franke *et al.*, 1988) genes are also tightly regulated by medium components.

Stability of the production strains is an essential feature of an expression system. Taking *K. lactis* as an example, both plasmid and integration based expression cassettes have been successfully used. Multicopy plasmids isolated from *K. drosophilarum*, whose *ori* is a 2$\mu$-like origin of replication, were used to develop recombinant *K. lactis* strains expressing heterologous proteins. The *ori* was shown to be stable in both shake-flask and pilot scale cultures under nonselective conditions (Buckholz and Gleeson, 1991). When *K. lactis* was used to secrete prochymosin, cells contained several integrated copies of the expression plasmid. This system was scaled up to 41,000 litres with no loss in production capacity (van den Berg *et al.*, 1990). Similar levels of stability have been observed with *P. pastoris* (Cregg *et al.*, 1987) and *H. polymorpha* (Janowicz

*et al.*, 1991).

Many of the heterologous proteins expressed in non-saccharomyces yeasts have been secreted and in many cases the proteins were secreted into the culture medium. In *H. polymorpha*, for example, the 150 kd glucoamylase from *S. occidentalis* was secreted into the culture medium at levels of up to 1.4 mg/l (Gellison *et al.*, 1991) whereas in *S. cerevisiae* proteins in excess of 30 kd are generally retained within the cells or in the periplasmic space (De Nobel and Barnett, 1991). Prochymosin is secreted from *K.lactis* with an efficiency of more than 95% (van den Berg *et al.*, 1990) compared to 10% for wild-type *S.cerevisiae* (Smith *et al.*, 1985). Similarly, factor XIIIa from *S.pombe* (Bröker and Bauml, 1989) and cellulase from *S.occidentalis* (Piontek *et al.*, 1990) are both secreted into the culture medium. Thus it seems that, unlike *S.cerevisiae*, secretion of proteins through the cell wall is a common feature of these alternative yeast hosts.

Regarding glycosylation, the average chain length of glycoproteins from *P.pastoris* was found to be $mannose_{8-14}$ (Grinna and Tschopp, 1989) compared to $mannose_{>40}$ from *S. cerevisiae* (Tarentino *et al.*, 1974). Invertase expressed in *P. pastoris* (Tschopp *et al.*, 1987) was glycosylated in a similar manner to invertase from an *S. cerevisiae sec*18 mutant blocked in the transit of proteins from the ER to the golgi (Esmon *et al.*, 1984). Thus, *P. pastoris* does not hyperglycosylate secreted invertase, unlike *S. cerevisiae*. Similarly, *S. occidentalis* doesn't hyperglycosylate secreted proteins (Deibel *et al.*, 1988). It has been reported, however, that recombinant α-galactosidase expressed in *H. polymorpha* is over-glycosylated (Sierkstra *et al.*, 1991).

Accurate processing of the N-terminal signal sequence directing nascent peptide chains into the ER is an extremely important feature of a secretion system. *S. cerevisiae* possesses the endopeptidase KEX2, located in the golgi, which proteolytically matures the mating pheromone α-factor (Julius *et al.*, 1984). *P. pastoris* has been shown

to possess endopeptidase activity analogous to KEX2 and can efficiently process small peptides under the control of theα-factor leader sequence (Siegel *et al.*, 1989; Vedvick *et al.*, 1991). *H. polymorpha* was also found to accurately process an α-factor leader fusion to HSA (Hodgkins *et al.*, 1990), *Y. lipolitica* accurately processed prochymosin by a KEX2-like activity (Franke *et al.*, 1988) and secretion of prochymosin from *K. lactis* proceeded efficiently using the α-factor leader (van den Berg *et al.*, 1990). Thus alternative yeasts possess many advantageous characteristics and are a valuable addition to the selection of yeast expression systems available for protein production and should be considered when high levels of expression and secretion of foreign proteins are required.

1.7.5. Other approaches.

One novel approach to obtaining hyperproducing mutants of yeast is to use flow cytometry and cell sorting (FCCS). This method, used with the yeast *Phaffia rhodozyma* was described by An *et al.*, 1991, to isolate mutants overproducing the carotenoid, astaxanthin, an important constituent of aquaculture feedstuffs. Astaxanthin is produced as a secondary metabolite of *P.rhodozyma* and the isolation of mutants producing increased quantities is limited by the lack of genetic selections. In this study, experimental conditions were developed that gave a quantitative correlation of fluorescence and carotenoid content. Distinctive differences were detected by FCCS in fluorescence and forward scatter values of mutant and wild-type populations of yeast cells. Results showed that in mutated populations a 10,000-fold enrichment of carotenoid-overproducing strains could be obtained through cell sorting. It should be possible to use this system to isolate mutants hyperproducing other secondary metabolites although attempts to isolate metabolite hyperproducing mutants have often been unsuccessful due to low fluorescence by the desired compounds. Nevertheless, FCCS is a powerful technique capable of quantitatively analyzing

more than 5,000 cells per second thus requiring much less time for enrichment compared to random screening.

Another method that has been used to obtain increased expression of heterologous proteins in yeast is gene codon optimization. Yeast utilizes predominantly only 25 out of the 61 possible codons for its many proteins (Bennetzen and Hall, 1981; Ikemura, 1982) and this very asymmetric codon usage correlates with the relative abundancy of the corresponding tRNAs. Researchers disagree on the importance of the role played by codon usage in protein production. When 39% of the major codons in the yeast phosphoglycerate kinase gene were changed to minor ones, a 10-fold reduction in protein levels was observed (Hoekema, *et al.*, 1987). However, impressive production of some heterologous proteins in yeast has been achieved without optimizing the codon usage pattern, for example hepatitis B virus core antigen (Kriskern *et al.*, 1986), while the complete synthesis of human epidermal growth factor using preferred yeast codons only resulted in a low level of protein (Urdea *et al.*, 1983). However no comparison was made between the mutated and the normal gene. A study by Kotula and Curtis, 1991, compared the rate of synthesis of a synthetic murine immunoglobulin light (kappa) chain gene containing only yeast-preferred codons with that of the murine-derived cDNA. Overall, greater than 53% of the codons were replaced by synonymous yeast-preferred codons. A greater than 5-fold increase in the rate of protein synthesis and a larger increase in the steady state level of the polypeptide was observed using the codon optimized cDNA. Although the invertase signal sequence was used in the vector construction the kappa chain was poorly secreted into the culture medium. It was proposed that this could have been because of an exposed hydrophobic surface normally interacting with the heavy chain as it has already been shown that co-expression of light and heavy chains results in efficient secretion of functional antibody (Better and Horwitz, 1989). The results in the above study demonstrate the potential value of converting the coding sequence of a

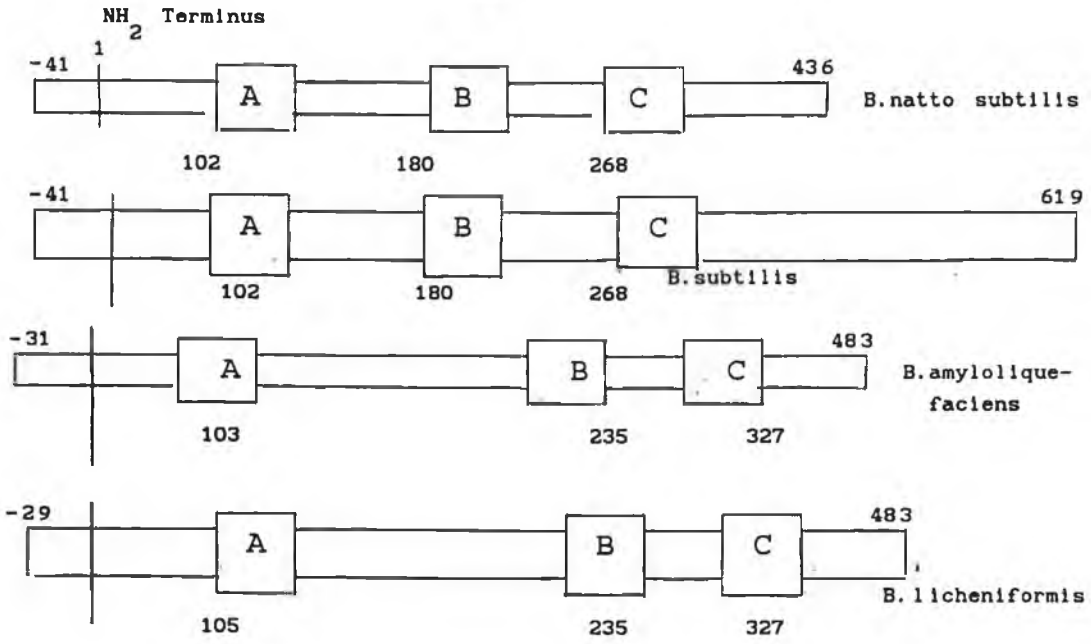
foreign gene to yeast-preferred codons.

**1.8. Alpha amylase in Yeast.**

The α-amylases, α-1,4-glucan-4-glucanohydrolases, catalyse the hydrolysis of 1,4-glucosidic linkages in starch resulting in the release of short oligosaccharides and α-limit dextrins. Many kinds of fungi, bacteria, plants and animals produce α-amylases and the fungal α-amylases are widely used in industry in the production of bread, maltose syrups and many alcoholic beverages such as beer and spirits (Hesseltine, 1983). *S.cerevisiae*, the main agent in alcoholic fermentation, lacks amylolytic activity and in the alcoholic beverages industry external α-amylase has to be added to liquefy raw materials. The commercial enzymes used for starch degradation represent a significant cost in the production of alcoholic beverages.

In general, amylases may be divided into three functional domains constituting the regions vital for the activity of the enzyme. The three common amino regions are located approximately 100, 200 and 300 amino acids from the amino terminal of the mature enzyme (Yamane *et al.*, 1988). Fig 1.7 shows a schematic diagram of four *Bacillus* α-amylases.

Figure 1.8.




Schematic diagram of four *Bacillus* amylase genes.

The numbers at the COOH-terminus of each enzyme refer to the amino acid length. The numbers under the boxes lettered A, B and C indicate the positions of His residues in the regions. Domains A and B have been assigned to substrate binding and domain C to the catalytic domain (Svenson and Sogaard, 1992). Although *S.cerevisiae* does not possess amylolytic activity, several yeasts of other genera assimilate starch and a number of these organisms secrete enzymes with α-amylase or debranching activity or sometimes both (Mc Cann and Barnett, 1986). However, among the amylolytic yeasts, complete hydrolysis of starch is rare (De Mot *et al.*, 1984a; De Mot *et al.*, 1984b). The most efficient starch degrading yeasts are among strains of the genera *Schwaniomyces* and *Saccharomycopsis* i.e. *Schwanniomyces occidentalis* and *Saccharomycopsis fibuligera*. Starch degradation can also be achieved by representatives of the genus *Lipomyces* (*L.starkey*, *L.kononekoae*, *L.tetrasporus*). In addition, some non-ascoporogenous yeasts produce amylolytic enzymes, strains of *Leucosporidium capsuligenum*, *Filobasidium*

*capsuligenum*, *Candida silvanorum*, *Candida tsukubaensis*, *Cryptococcus flavus*, and *Torulopsis inteniosa* to name some. *Schwanniomyces* strains produce α-amylase and glucoamylases and exhibit alpha-1,6-linkage cleaving activity (Simoes-Mendes, 1984; De Mot *et al.*, 1984c). In *S.occidentalis* two types of glucoamylase differing in molar mass were found (Simoes-Mendes, 1984). These enzymes are secreted under aerobic conditions during exponential growth phase at a low rate with maximum activity in stationary phase. Both enzymes were found to be thermolabile being inactivated in five minutes at 60°C (Calleja *et al.*, 1984). *S.fibuligera* produces an α-amylase (optimum temp. 40°C, optimum pH, 5.9) and a glucoamylase (optimum temp. 50°C, optimum pH, 6.0) with a relatively high debranching activity (Ulda and Saha, 1983; Steverson *et al.*, 1984). The genes encoding these enzymes were identified on two independent plasmids isolated from *S.cerevisiae* which had acquired starch degrading ability after being transformed with a gene library of *S.fibuligera* (Yamashita *et al.*, 1985a; Yamashita *et al.*, 1985b). *L. kononenkoae* also produces α-amylase (optimum temp. 40°C, optimum pH, 5.5) and glucoamylase (optimum temp. 50°C, optimum pH, 4.6) (Spencer-Martins and van Uden, 1979; Estrela *et al.*, 1982). However some of these amylolytic yeasts are not suitable for alcoholic production because they have a low tolerance for ethanol and exhibit slow fermentation rates (De Mot *et al.*, 1986).

One amylolytic yeast, *Saccharomyces diastaticus*, on the other hand, does exhibit high tolerance for alcohol and high fermentation rates (De Mot *et al.*, 1986; Laluce and Mattoon, 1984). *S.diastaticus* is very closely related to *S.cerevisiae* genetically and in fact according to a recent classification (Barnett *et al.*, 1990) the *S.diastaticus* starch utilizing strains, previously considered as a different species, have now been included in *S.cerevisiae*. Thus the primary difference between the two is that *S.diastaticus* secretes a glucoamylase, whereas *S.cerevisiae* lacks this ability (Laluce and Mattoon, 1984; Tamaki,

1978). A variety of *S.diastaticus* strains were evaluated for direct conversion of starch and dextrins to ethanol (Laluce and Mattoon, 1984) and through a combination of strain selection, hybridization and systematic optimization of fermentation conditions, up to 80% conversion of Litner starch was attained. The residual 20% carbohydrate represented some type of limit dextrin refractory to hydrolysis by *S.diastaticus* glucoamylase and could only be eliminated by prior treatment of starch with α-amylase. When this was done, 97% conversion of starch was achieved with *S.diastaticus*. The glucoamylase is produced towards the end of exponential phase and in stationary phase (Searle and Tubb, 1981). Synthesis of the enzyme is regulated by catabolite repression and there is also some control of expression by the mating-type locus (Yamashita *et al.*, 1985a). Enzyme production is determined by the genes *STA*1, *STA*2 and *STA*3 and homology between sequences in the genome of *S.cerevisiae* and some sequences in the *STA* genes has been observed (Yamashita *et al.*, 1985b; Pretorius *et al.*, 1986). Although *S.cerevisiae* cannot ferment starch, it has been found that during sporulation a glucoamylase and a debranching enzyme of the α-1,6-glucosidase transferase type are synthesised (Colonna and Magee, 1978; Clancy *et al.*, 1982). It may be that the region showing homology to the *STA* genes of *S.diastaticus* is responsible for these activities.

Despite the existence of yeast species which secrete α-amylases, glucoamylases and debranching enzymes, much work has gone into engineering *S.cerevisiae* strains which secrete α-amylase. Manipulating *S.cerevisiae* to synthesize and secrete functional α-amylase potentially could result in achieving an economical one-step bioconversion of starch as *S.cerevisiae* is the main agent used in brewing, baking and ethanol producing industries.

1.8.1. Expression of α-amylase in *S.cerevisiae*.

One of the first reports of the expression and secretion of an α-amylase in *S.cerevisiae* was by Rothstein *et al.*

(1984), who reported on the secretion of a wheat α-amylase. The experiment was not carried out with the aim of genetically improving *S.cerevisiae* but to investigate whether a protein, α-amylase, from wheat, could be processed and secreted in an active form from *S.cerevisae* using the plant signal sequence, which was located internally and not at the N-terminal end of the polypeptide. An expression vector containing the PGK promoter was used for the cloning. Two main points were clear from the results about the secretion of the wheat α-amylase. Firstly, that the wheat α-amylase signal peptide was recognized by the yeast processing apparatus and secondly, that the α-amylase was secreted into the medium in an active form. While the result points mainly to the general similarity between eukaryotic secretory signals it was also noted by the authors that the result was likely to be of interest to the brewing industry. However it was suspected that the efficiency of the plant signal peptide recognition in yeast might be reduced because of its location internally between part of the N-terminus of PGK and the mature α-amylase protein. It was judged, by the authors, that if recombinant DNA techniques were to be used by the brewing industry to produce an amylolytic *S.cerevisiae*, it would be a barley or other cereal α-amylase that would be preferred for legislative and customer preference reasons and so Rothstein *et al.*, 1987, reported on the improvement of the efficiency of the wheat α-amylase production and secretion in *S.cerevisiae*. The wheat signal peptide was repositioned to the N-terminus of the α-amylase which resulted in a significant improvement in its recognition in the yeast secretory pathway, but, as in the previous report, expression of the α-amylase was low, approximately 0.1% of the total cell protein and the amount of α-amylase secreted was influenced greatly by the type of culture medium used. On selective medium no starch degradation occurred while on rich medium containing starch areas of clearing were visible on staining with iodine vapour, but secretion was low. It was not clear why the

efficiency of secretion was so low but it could have reflected a deficiency of a necessary sequence or structure in the plant signal sequence resulting in a problem in the transport of the α-amylase from the ER across the cell membrane but more likely reflected the fact that the authors did not use buffered minimal medium (see Results section of this thesis). This result however, may indicate the importance, in many cases, of using yeast signal peptides to secrete heterologous proteins.
However, the high level expression and secretion of a rice α-amylase, by *S.cerevisiae*, using its own signal peptide under control of the yeast enolase promoter was described by Kumagi *et al.*, 1990,. Rice α-amylase is inactive until its signal peptide is removed (Miyata and Akazawa, 1982) and the appearance of starch-hydrolysis halos around colonies transformed with the α-amylase suggested that the rice α-amylase signal peptide was recognized and efficiently processed by the yeast secretory pathway even though attempts to determine the amino acid sequence of the N-terminus of the recombinant rice α-amylase, failed. The principal difference between this and Rothstein's results is the high level of expression and secretion of the rice α-amylase compared to the wheat enzyme and this difference may be due to the signal peptide as it has been shown that expression of human serum albumin in yeast is greatly influenced by the choice of signal peptide (Sleep *et al.*, 1990).

The expression and secretion of the GAI form of the *Aspergillus awamori* glucoamylase enzyme in *S.cerevisiae* (using the promoter and termination regions from a yeast enolase gene), while representing further improvements in the production of an efficient amylolytic *S.cerevisiae*, is another example of a natural leader sequence being recognized by the yeast secretory pathway (Innis *et al.*, 1984). The glucoamylase from *A.awamori* was chosen because it can attack α-1,4 and α-1,6 glycosidic bonds unlike the glucoamylase of *S.diastaticus* which can only attack α-1,4

bonds. Thus the *A.awamori* glucoamylase can efficiently hydrolyse highly polymerized starch to glucose. It was observed, however, that when the entire glucoamylase gene was introduced into the yeast on an autonomously replicating plasmid, YEp13 (Broach *et al.*, 1979) no enzymatic activity, immunoreactive peptide or glucoamylase mRNA sequences could be detected. When the glucoamylase was modified by excision of four intron sequences and cloned into the expression shuttle vector pAC1, the recombinant glucoamylase gene efficiently secreted more than 90% of the glucoamylase into the medium. Thus the putative control sequences of the *Aspergillus* gene for mRNA transcription and/or processing did not function in *S.cerevisiae* and the glucoamylase intervening sequences were either inefficiently or incorrectly spliced.

Nakamura *et al.*, 1986, reported on the expression of a human salivary α-amylase gene in *S.cerevisiae* and its secretion, again using the natural leader sequence. The salivary α-amylase was chosen because it is one of the most potent endoglycosidases that can act on raw starch and amylases from human saliva have sometimes been employed as the enzyme source for the hydrolysis of starch. A yeast strain was constructed that carried the salivary α-amylase cDNA under the control of the yeast PHO5 promoter in the yeast-*E.coli* shuttle vector pAM82 (Miyaohara *et al.*, 1983). Synthesis of the enzyme was induced by deprivation of inorganic phosphate, appearing approximately 6 hours after induction and reaching a maximal level at 30 hours. The cells produced about $7.9 \times 10^5$ active molecules of α-amylase, of which almost 50% were secreted to the medium. The amylase was found to retain 95% of its activity after storage for one week at 30°C indicating that it was stable. It also had the same specific activity as the authentic α-amylase in human saliva. It was concluded in this paper that if the efficient enzyme secretion reflected a unique property of the salivary α-amylase signal sequence, then it could also be useful for the secretion of other foreign proteins. In a subsequent study (Sato *et al.*, 1986) the

secreted α-amylase was characterised by determination of the N-terminal amino acid sequence to show whether the α-amylase signal sequence was correctly recognized and processed by the yeast secretory pathway. Determination of the N-terminal amino acid sequence revealed that the 15 amino acid signal sequence had been cleaved from the secreted enzyme and that the N-terminal residue, glutamine, the $16^{th}$ amino acid had been modified into pyroglutamate as is commonly observed with the mammalian salivary α-amylase. This result was in contrast to the results obtained by Hitzeman *et al.*, 1983, where examination of the N-terminus of the secreted human interferon provided evidence that the precursor was processed at several unnatural sites and that the efficiency of secretion was poor. Sato *et al.*, also confirmed that secretion and expression of the human α-amylase depended on the signal sequence. When a plasmid carrying the signal-minus α-amylase cDNA was transformed into yeast, the α-amylase was expressed but the amount of enzyme produced was only one thousandth of that from the signal-plus construct. The authors did not distinguish whether this was due to lower transcriptional and translational efficiency reflecting the minor 34 nucleotide difference in front of the ATG initiation codon in the signal-minus α-amylase construct or whether it was due to non-secreted α-amylase being rapidly attacked by protease. In any case, no enzymatic activity was detected in the culture medium indicating that the signal sequence was necessary for secretion of the α-amylase.

The efficient secretion by *S.cerevisiae* of the *Bacillus amyloliquefaciens* thermostable α-amylase (under the control of the ADH1 promoter) using its own signal peptide (Ruohonen *et al.*, 1987) was a very important study indicating that a prokaryotic signal sequence could function in yeast, a eukaryotic host. The promoter of the α-amylase gene, contained in plasmid pKTH10 (Palva, 1982) was removed by *Bal*31 digestion and fragments carrying the

gene, heterologous at their 5′ ends were put into the expression vector pAAH5 (Ammerer, 1983) after it was found that only low levels of α-amylase activity were detected using the expression vector pAAR6. Thus, three forms of the α-amylase gene were constructed: the *Bacillus* signal sequence in complete form (YEpαa1), in partial form (YEpαa2) or missing (YEpαa3). Secretion of α-amylase was only observed with the complete signal sequence. Through immunoblotting of proteins isolated from the *S.cerevisiae* strain grown in the presence and absence of tunicamycin (an enzyme which prevents N-glycosylation), (Mahoney and Duksin, 1979), it was determined that the α-amylase was glycosylated, strongly suggesting that the enzyme was being secreted through the yeast secretory pathway. To confirm this, YEpαa1 was transformed into *sec*7 and *sec*1 strains. In these strains secretion of protein was blocked at 37$^{o}$C so that the proteins accumulate at the golgi and in the secretory vesicles respectively (Novick *et al.*, 1980; Novick *et al.*, 1981). Secretion of the α-amylase was blocked in both mutants at the restrictive temperature but occurred normally at the permissive temperature indicating that the α-amylase progressed through the normal secretory pathway. Secretion of the α-amylase was very efficient, 10μg/l/hour.

Pretorius *et al.*, 1988, also reported on the successful expression and secretion of the *B.amyloliquefaciens* α-amylase in *S.cerevisiae*. This time however, neither yeast promoter nor yeast secretory signals were used and the enzyme was expressed using the original amylase promoter and signal peptide. The α-amylase was introduced into several *Saccharomyces* strains and *S.cerevisiae* transformants were shown to secrete high levels of biologically active α-amylase, strain JM2773-15B secreting 123u/ml. In light of the previous studies mentioned where expression, processing and export of α-amylase or glucoamylase required yeast regulatory or secretory signals it was felt by the authors that the promoter, termination and secretion signals of this amylase gene would have

potential application for the expression of foreign genes in yeast.

While the above studies demonstrate that native (non-yeast) signal peptides can direct efficient secretion of heterologous proteins these signal peptides must be evaluated on a case by case basis and in many cases it is necessary to use a yeast signal peptide to obtain efficient secretion.

The expression and secretion in *S.cerevisiae* of an α-amylase encoded by cloned mouse pancreatic cDNA (Filho *et al.*, 1986), was under the control of the MFα1 promoter and secretion signals instead of the natural leader sequence. The shuttle vector, p69A (Kurjan and Herskowitz, 1982) modified to contain the MFα1 signal sequence giving rise to the new plasmid pES, was used to clone the pancreatic α-amylase which was inserted after the preproleader of the MFα1 gene. The transcription termination and polyadenylation signals of the 2μ FLP gene were present at the 3′ flanking region of the insert. After transformation with recombinant DNA, 16 out of 600 *S.cerevisiae* strain GRF-18 cells were shown to secrete biologically active α-amylase. One of the starch digesting clones was chosen (pESA) and used to retransform strains GRF-18 and DC79. When selective pressure was relaxed, stable transformants of both strains were isolated suggesting that the plasmid had been integrated into the chromosomal DNA. It has been shown that cells transformed with p69A, the parent plasmid of pES, tend to lose or integrate the plasmid rapidly (Julius *et al.*, 1984). One of the stable strains of GRF-18 was analysed for its ability to hydrolyse starch. It was shown to be able to degrade all the starch (1%) in the medium after approximately 48 hours and the α-amylase activity was measured at 153u/ml which is comparable to the activity of 187u/ml found for the amylolytic yeast *Schwanniomyces alluvius* (now *occidentalis*) (Wilson and Ingledew, 1982). It was also concluded that the preproleader of the α-factor precursor must be responsible

for directing the efficient export of α-amylase in the yeast transformants as it was determined by sequence analysis that only two codons from the mouse pancreatic α-amylase signal peptide were retained in the plasmid construct.

In the hydrolysis of starch, α-amylase produces primarily maltose, maltotriose and oligosaccharides (De Mot and Verachtert, 1985) while glucoamylase acts on the nonreducing ends of starch chains to release free glucose (Mc Cann and Barnett, 1986). Kim *et al.*, 1988, constructed a yeast strain which secreted both α-amylase and glucoamylase by transforming *S.diastaticus* derivatives with the mouse salivary α-amylase plasmid, pMS12, previously constructed by Thomsen, 1983, which contains the α-amylase cDNA under control of the ADH1 promoter. The *S.diastaticus* derivatives, which contained both STA (glucoamylase) genes and the transformation selection marker *trp*1, were constructed by crossing *S.cerevisiae* SHU32a with *S.diastaticus* 5301-17B and *S.cerevisiae* SHU32α with the hybrid strain CL1-17B where the genome is derived from both *S.cerevisiae* and *S.diastaticus*. After the diploids were sporulated, segregants KK1-R1 and KK2-R1 exhibiting both tryptophan auxotrophy and glucoamylase secretion were selected. When strains KK1-R1 and KK2-R1 were transformed with plasmid pMS12, starch hydrolysis was almost complete, exceeding 97% by the end of 5 days in transformants producing both α-amylase and glucoamylase, thus making it possible to attain almost the same efficiency of conversion in one step as was previously attained in two (Laluce and Mattoon, 1984; Mattoon *et al.*, 1987).

Because *S.cerevisiae* and not *S.diastaticus*, is the organism widely used in the process of brewing, baking and ethanol production, Hollenberg and Strasser, 1990, constructed an amylolytic *S.cerevisiae* strain harbouring the genes for α-amylase and glucoamylase from *S.occidentalis*. The amylolytic system of this yeast is irreversibly inactivated at 60$^{0}$C making it especially advantageous in brewing and

baking processes. Producing an *S.cerevisiae* strain with both α-amylase and glucoamylase activity was judged to be potentially useful in the production of light beer. In brewing the fermentable sugars are provided from barley by partial hydrolysis of starch during the malting process. This results in a considerable amount of unfermentable carbohydrate, like dextrins, which contribute to the high calorific content of traditionally brewed beer. These have to be removed in the production of light beer by adding glucoamylase. *Aspergillus* glucoamylase is widely used for this purpose but it cannot be inactivated at temperatures normally employed to pasteurize beer (Tubb, 1986) and this can result in an unstable beer which changes its chemical properties on storage. An expression cassette containing both the α-amylase and glucoamylase genes of *S.occidentalis* under the control of the GAL 10 and GAL 1 promoters respectively was cloned into a centromere plasmid resulting in one copy per haploid genome in the transformants. Expression of both genes was found to be inducible with galactose. The levels of enzymes produced by the transformants were almost the same as for the donor strain, *S.occidentalis*, indicating that the amylolytic system was as efficient in *S.cerevisiae*.

Shibuya *et al.*, 1992a, described the expression and secretion, in *S.cerevisiae*, of the cDNAs of α-amylase and glucoamylase from *Aspergillus shirousamii*, which is widely used in the Japanese alcoholic beverages industry particularly in the making of shochu. The α-amylase and glucoamylase cDNAs were cloned independently into the plasmid pYcDE1 (Mc Knight and Mc Conaughty, 1983) downstream of the ADH1 promoter. Both enzymes were expressed using their own signal peptides which were cleaved off at the same positions as those of the native enzymes. The efficiency of secretion of both enzymes was high, 38 and 20mg/l for α-amylase and glucoamylase respectively. In a subsequent study (Shibuya *et al.*, 1992b), a fusion gene encoding a polypeptide of 1116 amino acids was constructed using the same genes. The sequential

order of the fusion was as follows: the 5′-non-coding region of the α-amylase cDNA, the α-amylase-encoding cDNA, the glucoamylase-encoding cDNA minus its signal peptide and the 3′ non-coding region of the glucoamylase cDNA. In addition Lys-Pro was inserted as a spacer region between the two cDNAs. The fusion protein was cloned into pYcDE1 to give plasmid pYF2. A fragment containing the ADH1 promoter, the fusion gene and the CYC1 terminator was then isolated from pYF2 and cloned into the integrating vector YIp5 to give plasmid pIF2. Both plasmids were transformed into *S.cerevisiae*. Plasmid pIF2 integrated into the genome as was confirmed by Southern blot analysis and higher enzyme activities in the culture supernatant were observed with this plasmid than with pYF2. One pIF2 transformant, F6-5, showed the highest enzyme activity, 2.8u/ml for α-amylase and 0.43u/ml for glucoamylase. The molecular weight of the fusion protein secreted by F6-5 was 145 kd which was very close to the sum of the α-amylase (54 kd) and the glucoamylase (94 kd) individually expressed by *S.cerevisiae* in the previous study (Shibuya *et al.*, 1992). In addition, the N-terminal amino acid sequence of the fusion protein was identical to that of the α-amylase indicating that the glucoamylase domain added to the C-terminus of the α-amylase did not affect cleavage of the signal peptide. Although F6-5 secreted a relatively small amount, 2.3mg/l, of the fusion protein, the authors expect that practical use of this fusion protein will be made although greater overproduction levels would be necessary for it to be commercially viable.

# CHAPTER 2

# MATERIALS AND METHODS.

**2.1 Yeast and bacterial strains and plasmids.**

The bacterial and yeast strains and plasmids used in this study are described in Table 2.1 and 2.2.

Table 2.1

| Strain and Genotype | Source or Reference |
|---|---|
| *S.cerevisiae* | |
| DBY746<br>Mat$\alpha$,*his*-$\Delta$1,*leu*2-3,-112,*ura*3-52,*trp*1-289a. | D.C.U. stocks |
| RSY11<br>Mat$\alpha$,*sec*18-1,*ura*3-52,*leu*2-3,-112,*suc*2. | R. Schekman, U. of California, Berkeley, U.S.A. |
| RSY12<br>Mat$\alpha$,*sec*53-6,*ura*3-52,*leu*2-3,-112. | " |
| RSY45<br>Mat$\alpha$,*sec*1-1,*ura*3-52,*leu*2-3,-112,*trp*1-289. | " |
| RC631<br>Mata,*sst*2-1,*rme*,*ade*2-1,*ura*1,*his*6,*met*1 | V.Bugeja, Maynooth College. |
| MD50<br>Mat$\alpha$,*pep*4-3,*leu*2-3,*leu*2-112. | J.R.Dickinson, Univ. of Wales, Cardiff. |
| DBY746::pFAMY 1-13<br>DBY746 transformants harbouring the integrating plasmid pFAMY. | This study |
| DBY746::pMIAMY1-5<br>DBY746 transformants harbouring the ribosomal DNA integrating vector pMIAMY. | This study |

Throughout this thesis the following notation is used to describe strains harbouring an integrating plasmid, and strains harbouring an episomal plasmid.

DBY746/pAAMY = Strain DBY746 transformed with the episomal vector pAAMY.
DBY746::pMIAMY = Strain DBY746 transformed with integrating plasmid pMIAMY.

*E.coli*

JA221 D.C.U. stocks
F',*rec*A1,*leu*B6,*trp*Δ5,*hsd*MT,*hsd*R$^-$,*lac*Y,*xyl*.

Table 2.2

| Plasmid | Relevant Characteristics | Source or Reference. |
|---|---|---|
| Yeast expression vectors: | | |
| pAAH5 | Adh1 promoter and terminator, LEU marker unique *Hin*dIII cloning site. | Ammerer, G. (1983) |
| pAH9<br>pAH10<br>pAH21 | pAAH5 derivatives, ATG start sites in 3 frames, no yeast terminator sequences. | Ammerer, G. (1983) |
| pSL5 | pUC8 with *B. licheniformis* amylase on *Bam*H1 - *Hin*d111 fragment. | O'Kane *et al.* 1983 |
| pSL5$_2$ | pSL5 with a single *Hin*d111 8mer linker (CAAGCTTG) in *Bam*H1 site. | This study |
| pSL5$_3$ | pSL5 with single *Hin*d111 8mer linker inserted in the *Pst*1 site giving a *Hin*d111 signal minus amylase fragment. | This study |

| | | |
|---|---|---|
| pAAMY | pAAH5 containing the amylase *Hind*111 fragment from pSL5₂ inserted in the correct orientation for expression in the unique *Hind111* site between the Adh1 promoter and terminator. | This study |
| Yepαa1 | pAAH5 containing entire *Bacillus amylofaciens* α -amylase gene | Ruohonen *et al.* 1987 |
| pUAMY19 | pUC19 containing the *Hind*111 amylase fragment from pSL5₂, | This study |
| pUAMY18 | pUC18 containing the *Hind*111 amylase fragment from pSL5₂ | This study |
| pMC312 | BAL 31 deletant of pUAMY18 with inserted *Hind*111 8mer linker (see Appendix #5 for sequence data) | This study |
| pMC612 | BAL 31 deletant of pUAMY18 with inserted *Hind*111 8mer linker. | This study |
| pUC312 | pUC19 containing the *Hind*111 amylase fragment from pMC312. | This study |
| pAA312 | pAAMY with the shortened *Hind*111 amylase fragment from pMC312 cloned into it's unique *Bam*H1 site. This plasmid was constructed by Marten Olssen. | |
| pFL34 | Yeast integrating vector ura3 marker, unique *Bam*H1 site for inserts and unique *Stu*1 site for linearisation prior to integration. | Bonneaud et al (1991) |
| pFAMY | pFL34 with the *Bam*H1 partial fragment from pAAMY containing the entire amylase gene and Adh1 promoter and terminator. | This study |

| | | |
|---|---|---|
| pMIRY2 | Ribosomal DNA integrating vector leu 2d marker, unique *Bam*H1 cloning site, unique *Sma*1 site for linearisation. | Lopes et al (1989) |
| pMIAMY | pMIRY2 with the *Bam*H1 partial fragment from pAAMY containing the entire amylase gene and the Adh1 promoter and terminator. | This study |
| pJG317 | β-glucanase gene plus full bacterial signal in pAAH5. | DCU stocks |

## 2.2 Materials

Except where stated all chemicals were from Sigma Corporation, BDH and Reidel de Haen and were of AnalaR grade or equivalent. Restriction enzymes, T4 DNA ligase, klenow polymerase, calf intestinal phosphatase, pUC19 and pBR322 plasmid DNA were from Boehringer Mannheim and Bethesda Research Labs. (BRL). Endoglycosidase $H_f$ was supplied by New England Biolabs.

## 2.3 Microbiological Media.

Solid complex media contained 15g/L Oxoid No. 3 Technical agar. Solid minimal media contained 12g/L Oxoid No. 1 Bacteriological agar. All media components were from Oxoid or Difco unless otherwise stated. All media were sterilized by autoclaving at 15lb/in$^2$ at 121$^0$C for 20 minutes.

**Luria Bertani (LB) medium.**

Used for routine culturing of *E. coli.*

| | |
|---|---|
| Tryptone | 10g |
| Yeast extract | 5g |
| NaCl | 10g |

Distilled water ($dH_2O$)to 1 Litre.

**SOC medium for electroporation** of *E.coli.*

| | |
|---|---|
| Tryptone | 2% |
| Yeast Extract | 0.5% |
| NaCl | 10mM |
| KCl | 2.5 mM |
| $MgCl_2$ | 10mM |
| $MgSO_4$ | 10mM |
| Glucose | 20mM |

**Synthetic complete (SC orminimal medium, MM).**

Used for the selective growth of yeast auxotrophic strains carrying plasmids.

| | |
|---|---|
| Difco yeast nitrogen base | 6.7g |
| Glucose | 20g |

$dH_2O$ to 1 litre, autoclave.

Appropriate supplements were added from the stock solutions given below after autoclaving.

| Supplement stock solutions | volume added per litre | Final conc. mg/ml. |
|---|---|---|
| L-histidine (2% w/v) | 1.0ml | 20.0 |
| Uracil (0.25% w/v) | 8.0ml | 20.0 |
| L-tryptophan (0.4% w/v) | 5.0ml | 20.0 |
| L-leucine (1.5% w/v) | 2.0ml | 30.0 |
| L-lysine (3% w/v) | 1.0ml | 30.0 |

For integrative transformation it was necessary to supplement the above media with 0.75 g/l of the mix listed below.

Supplemental mix for integrative transformation.

| | |
|---|---|
| Adenine | 1g. |
| Uracil | 1g. |
| Tryptophan | 1g. |
| Histidine | 1g. |
| Arginine | 1g. |
| Methionine | 1g. |
| Tyrosine | 1.5g. |
| Leucine | 3.0g. |
| Lysine | 1.5g. |
| Phenylalanine | 2.5g. |
| Threonine | 10g. |
| Aspartic Acid | 5g. |

compounds were omitted as required, ie for selection on Ura-minus media uracil was omitted from this mix.

**YEPD (complex medium for routine yeast culturing).**

| | |
|---|---|
| 10 g | Yeast extract. |
| 20 g | Bacteriological Peptone. |
| 20 g | Glucose. |
| $dH_2O$ | to 1 Litre. |

For enzyme activity studies on the α-amylase constructs it was necessary to buffer the synthetic complete and YEPD media as follows: 10 g/l succinic acid and 6 g/l NaOH were

added to broth media while solid media was made up in 0.1M phosphate buffer pH 6.9. It was necessary to buffer the liquid media with succinic acid/NaOH as autoclaving of the phosphate buffered media resulted in a precipitate which interfered in O.D. measurements.

**MYGP**

Complex media for the α-factor plate assay.

| | |
|---|---|
| Malt extract | 3g |
| Yeast extract | 3g |
| Peptone | 5g |
| Glucose | 20g |

$dH_2O$ to 1 litre.

**2.4 Antibiotics.**

For selective growth of *E.coli* strains, antibiotics were added as appropriate after autoclaving and cooling media to 55°C. The ampicillin stock solution (50mg/ml) was made up freshly in sterile water and used at a concentration of 50μg/ml for solid media and 40μg/ml for broth. Tetracycline (10mg/ml) was dissolved in 50% ethanol, stored in the dark at -20°C and used at a working concentration of 10μg/ml. Chloramphenicol (50mg/ml) was made up in ethanol and stored at - 20°C and used at a working concentration of 20μg/ml.

**2.5 Growth conditions and strain storage.**

All *E.coli* strains were grown overnight at 37°C in LB medium. All *S. cerevisiae* strains were grown overnight at 30°C in complex media or for 36 hours in MM medium. Both yeast and bacterial strains were stored as glycerol stocks. An equal volume of 80% glycerol was added to 1ml of a late log phase culture and stored at - 70°C. Working stocks were stored on plates at 4°C.

**2.6 Buffers and solutions.**

All buffers and solutions used for DNA manipulations were autoclaved and stored at room temperature unless otherwise stated.

**General buffers.**

**Phosphate Buffered Saline (PBS).**

| | |
|---|---|
| $KH_2PO_4$ | 1.09g |
| $Na_2HPO_4$ | 2.14g |
| NaCl | 9.0g |

$dH_2O$ to 1 Litre.

**Tris borate buffer (10X) (TBE).**

| | |
|---|---|
| Tris-HCl | 108g |
| $Na_2$-EDTA | 9.3g |
| Boric acid | 55g |
| $H_2O$ | to 1 litre |
| pH | 8.3 |

**Tris acetate buffer (50X) (TAE).**

| | |
|---|---|
| Tris-HCl | 2M |
| Glacial acetic acid | 242g |
| $Na_2$-EDTA (0.5M) | 100ml |
| $H_2O$ | to 1 litre |
| pH | 8.0 |

**Buffers and solutions for DNA manipulations.**

**TE Buffer**

| | |
|---|---|
| Tris-HCl | 10mM |
| $Na_2$-EDTA | 1mM |
| pH | 8.0 |

**2 x BAL 31 Buffer**

| | |
|---|---|
| Tris-HCl (pH 7.2) | 40mM |
| $MgCl_2$ | 25mM |
| $CaCl_2$ | 25mM |
| EDTA | 2mM |
| NaCl | 1.2M |

**Klenow (Nick Translation) Buffer.**

| | |
|---|---|
| Tris-HCl (pH 7.2) | 500mM |
| $MgSO_4$ | 100mM |
| dTT | 10mM |
| BSA | 5mg/ml |

**STE Buffer.**

| | |
|---|---|
| Tris.Hcl (pH 8.0) | 10mM |
| NaCl | 100mM |
| EDTA | 10mM |

**Ligation Buffer (10X).**

| | |
|---|---|
| Tris.Hcl (pH 7.6) | 0.5M |
| $MgCl_2$ | 100mM |
| dTT | 150mM |
| Spermidine | 10mM |
| BSA | 500µg/ml |
| ATP | 10mM |

**DNAse free RNAse.**

10mg/ml RNAse was dissolved in 10mM Tris.HCl (pH 7.5) and 15mM NaCl and heated to 100°C for 15 minutes, allowed to cool to room temperature and stored at -20°C.

**Lithium acetate solutions for yeast transformations.**

**Lithium acetate (0.1M) in TE.**

5.1g LiAc were added to TE and the volume was made up to 500ml. The solution was filter sterilized or autoclaved.

**PEG 4000 (40%) in 0.1M LiAc/TE.**

40g of Polyethylene Glycol were dissolved to 100ml in 0.1M LiAc\TE solution.

**Solutions for plasmid DNA minipreps from *E.coli*.**

**Solution 1**

| | |
|---|---|
| 0.5M glucose | 1.0ml |
| 0.1M EDTA pH 8.0 | 1.0ml |
| 1M Tris-HCl pH 8.0 | 0.25ml |
| $dH_2O$ | 7.75ml |

**Solution 2**

| | |
|---|---|
| 1N NaOH | 2.0ml |
| 10% SDS | 1.0ml |
| $dH_2O$ | 7.0ml |

This solution was made freshly each month.

**Solution 3**

To 60ml 5M potassium acetate 11.5ml, glacial acetic acid and 2.85ml $dH_2O$ were added. The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

**STET buffer.**

| | |
|---|---|
| Sucrose | 8.0g |
| Triton-X-100 | 5.0ml |
| 1M Tris-HCl pH 8.0 | 5.0ml |
| 0.5M EDTA ph 8.0 | 10ml |

made up to a final volume of 100ml with $dH_2O$.

**Triton mix**

| | |
|---|---|
| 20% Triton-x-100 | 5.0ml |
| 0.25M EDTA (pH 8.0) | 12.5ml |
| 1M Tris-HCl | 2.5ml |
| $H_2O$ to | 50ml |

**Phenol/Chloroform mix.**

500g of powdered phenol were dissolved in 500ml of chloroform. 4g of 8-hydroxyquinoline and 20ml of isoamyl alcohol, were then added and the solution was stored at $4^oC$ under 0.1M Tris.Hcl, pH 7.5.

**Solutions for DNA fragment isolation from agarose gels: (Geneclean procedure).**

**Sodium Iodide solution**

| | |
|---|---|
| NaI | 90.8g |
| $dH_2O$ to | 100ml |

The solution was stirred for 30 minutes and then filtered through Whatman No. 1 filter paper. 15g of $Na_2SO_4$ were added and the solution stored in the dark at $4^oC$.

**Ethanol wash solution.**

| | |
|---|---|
| Ethanol | 50% |
| NaCl | 100mM |
| Tris-HCl pH 7.5 | 10mM |
| EDTA pH 7.5 | 1mM |

The solution was stored at - $20^oC$.

**DNS assay solutions.**

| | |
|---|---|
| Dinitrosalicyclic acid | 10g. |
| Sodium Potassium Tartarate | 300g. |
| NaOH | 16g. |

The above contents were made up to 1 litre with distilled water.

**Solutions for SDS polyacrylamide gel electrophoresis.**

**Acrylamide/Bisacrylamide solution (30/1)**

| | |
|---|---|
| 3.2g | BisAcrylamide |
| 120g | Acrylamide |
| $dH_2O$ | to 400ml |

This solution was stored in the dark at 4°C.

**Solubilization buffer**

| | |
|---|---|
| DTT | 100mM |
| Tris.HCl pH 6.9 | 80mM |
| Glycerol | 10% |
| SDS | 2% (w/v) |
| Bromophenol blue | 0.2% |

**SDS running buffer**

| | |
|---|---|
| Tris.HCl, pH 8.3 | 25mM |
| Glycine | 192mM |
| SDS | 0.1% |

**Coomassie Stain**

| | |
|---|---|
| Methanol | 400ml |
| $dH_2O$ | 500ml |
| Glacial acetic acid | 50ml |
| Coomassie brilliant blue | 0.5% |

The destain solution was as above but contained no comassie dye.

**Solutions for α-amylase activity gels.**

**Renaturation buffer.**

| | |
|---|---|
| Triton-X-100 | 2.5% |
| $CaCl_2$ | 2mM |
| NaCl | 50mM |
| HEPES pH 6.9 | 0.1M |

The activity buffer was identical to the renaturation buffer but without the Triton-X-100.

**Solutions for preparation of yeast extracts for β-glucanase assays.**

**Lysis buffer (1X).**

| | |
|---|---|
| Sodium phosphate pH 7 | 50mM |
| EDTA | 10mM |
| Triton-X-100 | 0.1% |
| Sarcosyl | 0.1% |
| β-mercaptoethanol | 10mM |
| PMSF | 1mM |

PMSF was made freshly as a 10mM stock in isopropanol and used as required.

**Solutions for $EndoH_f$ treatment of glycoproteins.**

**Denaturing buffer.** 5% SDS
10% β mercapto-ethanol
made up to volume with $dH_2O$.

**$EndoH_f$ activity buffer (10X).**

| | |
|---|---|
| Sodium citrate pH 7.4 | 0.5M |

**Buffers and Solutions for Western blotting:**

Transfer buffer: (per litre)
3.03g Tris. base
14.4g glycine
200ml methanol

TBST buffer: 150mM NaCl
10mM Tris. HCl, pH8.0
0.05% Tween-20

Blocking solution: TBST with 1% (w/v) BSA

AP (alkaline phosphatase) buffer:
100mM Tris. HCl, pH9.5
100mM NaCl
5mM $MgCl_2$

NBT stock: NBT (nitroblue tetrazolium), 50mg/ml in 70% N,N-dimethylformamide.

BCIP stock: BCIP (5-bromo-4-chloro-3-indolyl phosphate), 50mg/ml in 100% N,N-dimethylformamide.

AP colour development solution: 10ml AP buffer
66ul NBT stock
33ul BCIP stock

NBT was added to the AP buffer and mixed. BCIP was then added and mixed. The solution was made up freshly from stocks before use.

**Buffers and solutions for Southern blotting.**

**Denaturing solution.**

| | |
|---|---|
| NaCl | 87.66g |
| NaOH | 20g |
| $dH_2O$ to | 1 Litre. |

**Neutralizing Solution.**

| | |
|---|---|
| NaCl | 87.66g |
| 1M Tris-Hcl, pH 8.0 | 500ml |
| $H_2O$ to | 1 Litre. |

**Transfer buffer. (20 X SSC).**

| | |
|---|---|
| NaCl | 175.83g |
| sodium citrate | 88.2g |

Adjust the pH to 7.0 with a few drops of 5M NaOH.

| | |
|---|---|
| $dH_2O$ to | 1 Litre. |

**Denhardt's solution.**

| | |
|---|---|
| Ficoll | 5.0g |
| Polyvinyl Pyrrolidine | 5.0g |
| Bovine Serum Albumin | 5.0g |
| $dH_2O$ to | 500ml. |

**Prehybridization solution.**

6 x SSC

5 X Denhardt's

0.5% S.D.S.

100 µg/ml denatured salmon sperm DNA

**Hybridization solution.**

This is the same as prehybridization solution but in addition it contains 10mM EDTA and the $^{32}P$-labelled DNA probe.

**Denatured salmon sperm DNA.**

The salmon sperm DNA (Sigma type-111 sodium salt) was dissolved in water at a concentration of 10mg/ml by stirring at room temperature for 6 hours. To shear the DNA it was then passed through an 18-guage hypodermic needle several times. The DNA was then aliquoted into microcentrifuge tube, boiled for 10 minutes and stored at -20°c. Before use in Southern blotting the the DNA was boiled for 5 minutes and chilled quickly in an ice bath.

**Solutions for ELISA.**

**Carbonate buffer.**

| | |
|---|---|
| Sodium carbonate | 0.150g |
| Sodium bicarbonate | 0.293g |
| $dH_2O$ to | 100ml |
| pH | 9.6 |

**Solutions for DNA sequencing.**

**40% acrylamide solution.**

| | |
|---|---|
| Acrylamide | 380g |
| N,N'-methylbisacrylamide | 20g |
| $dH_2O$ to | 600ml |

The solution was heated to 37°C to dissolve the acrylamide and the volume was then adjusted to 1 litre with distilled water. The solution was then filtered through a Gelman filter (0.45μm) under vacuum and stored in the dark at 4°C.

**6% Acrylamide/urea stock solution.**

| | |
|---|---|
| 10 x TBE | 100ml |
| Urea (ultrapure) | 460g |
| 40% Acrylamide solution | 75ml |
| $dH_2O$ to | 1 litre |

The solution was filtered through a Gelman filter (0.45μm) under vacuum and was stored at 4°C in a dark bottle.

**2.7 Transformation of *E.coli*.**

100ml of LB were inoculated with 1ml of an overnight culture of *E.coli* and incubated in a shaking water bath at 37°C to an $O.D._{600}$ of 0.3 - 0.6. 10ml of cells were then chilled on ice for 10 minutes and spun down at 5000 rpm for 5 minutes. The cells were resuspended in 5ml of 0.1M $MgCl_2$ and spun down as before. The cells were then resuspended in 5ml 50mM $CaCl_2$ and put on ice for 60 minutes. The cells were centrifuged as above and resuspended in 1ml $CaCl_2$. 200μl of cells were then added to 2μl plasmid DNA or 20μl of ligation mix and the reaction left on ice for 60

minutes. The cells were then heat shocked at 42°C for exactly 2 minutes and put back on ice immediately. 0.7ml LB was then added and the cells were incubated at 37°C for 60 minutes and plated out onto appropriate selective media.

**2.8 Preparation of electrocompetent *E. coli* cells.**

1 litre of prewarmed LB was inoculated with 10ml of a fresh overnight culture. The culture was grown at 37°C with vigourous shaking to $O.D._{600}$ 0.6 - 0.8. The flasks were then chilled on ice for 30 minutes and harvested by centrifugation at 4000rpm, for 15 minutes. The cells were resuspended in 1L of cold sterile water by gentle shaking in an ice water bath. The cells were then harvested by centrifugation at 4000rpm for 5 minutes, gently resuspended in 500ml of sterile water, harvested as above and resuspended in 20ml of 10% sterile glycerol. The cells were then centrifuged at 4000rpm for 15 minutes, gently resuspended in 2ml of 10% glycerol and aliquoted into microcentrifuge tubes in volumes of 100µl and stored at -70°C for further use.

**2.9 Electroporation of competent *E. coli* cells.**

The electroporation cuvettes were chilled on ice for 5 minutes. 1-10µl of the ligation mix/plasmid were added to the electrocompetent cells, mixed gently with a pipette tip and kept on ice for 5 minutes. The cells were then transferred to the electroporation cuvette by running them gently down the side of the cuvette to prevent air bubbles from forming. The cuvette was tapped off the desk once, dried with tissue and immediately placed into the electroporation apparatus. The cells were electroporated using the following parameters: 2.49kV, a resistance of 200 Ohms, the capacitance extender set at 125µFD, and a capacitance of 25µFD. The observed time constant was generally 3.5-4.5. 1ml of SOC media was then added immediately to the cuvette, the cells were transferred to a sterile microcentrifuge tube and incubated at 37°c for 60 minutes. 2µl, 20µl and 200µl of the transformation mix was

plated out onto appropriate selective media.

**2.10. Lithium Acetate Yeast transformation.**

Ito *et al*, (1980)

The yeast strain was grown up overnight at 30°C in YEPD to $O.D._{600}$ 0.8-1.2. A volume of 100 ml of cells was spun down at room temperature for 5 mins at 15,000 rpm , the supernatant decanted off and the cells washed once with 20 ml sterile TE. Cells were centrifuged as above, and the supernatant was decanted off carefully. The cells were gently resuspended in 1.5 ml of 0.1 M LiAc/TE and incubated at 30°C for 1 hour with gentle shaking. For each transformation, 200µl of cells were used and the following were added: 10µl of 10 mg/ml salmon sperm DNA as carrier, 1 to 5 µg plasmid DNA (linearized for integrative transformation) and 800µl of 40% PEG 4000 in 0.1 M LiAc/TE. The cells were then gently mixed and incubated at 30°C for one hour. The cells were then heat shocked at 42°C for 5 minutes and centrifuged at room temp for 2 minutes at 5,000 rpm. The supernatant was removed gently with a pipette and the cells were washed with 1 ml sterile water, spun down for 2 minutes at 5,000 rpm and the supernatant carefully decanted off. The cell pellet was resuspended in 200µl sterile $dH_2O$ and plated out on appropriate selective media. The plates were incubated at 30°C for 2 to 4 days and the transformants were then streaked out for single colonies.

**2.11. Rapid small scale isolation of plasmid DNA from *E.coli*.**

Two methods were routinely used for the small scale isolation of plasmid DNA. The first method was used when the DNA isolated was used in DNA manipulations and the second when the DNA was used for restriction analysis. The solutions used are described in sect. 2.6.

**Method 1.** (Birnboim and Doly 1979)

1ml of an overnight culture grown up in selective media was pelleted in a microcentrifuge at 5,000 rpm for 5mins. The

supernatant was decanted off and the cells were resuspended in 100μl of solution 1. After 5 minutes at room temperature 200μl of solution 2 were added and mixed by inversion. The microcentrifuge tube was then placed on ice for 5 minutes. Then 150μl of solution 3 were added, the solution mixed by inversion and put on ice for 10 minutes. The cell debris and chromosomal DNA was pelleted by centrifugation as above, and 400μl of the supernatant were removed to a fresh microcentrifuge tube. 400μl of phenol/chloroform were added, mixed by vortexing and centrifuged for 2 minutes at 10,000 rpm. The aqueous layer was removed to a new microcentrifuge tube and 800μl of cold absolute ethanol were added and mixed by inversion. After 10 minutes at room temperature, the plasmid DNA was pelleted by centrifugation for 10 minutes at 12,000 rpm. The pellet was washed twice with 200μl 70% ethanol, once with ether and allowed to dry for 10 minutes at 55°C. The pellet was resuspended in 60μl TE and 1μl of 10mg/ml DNase free RNase.

**Method 2.** STET preparation (Holmes and Quigley, 1981).
A patch of cells from a plate grown overnight on selective media was put in a sterile microcentrifuge tube using a sterile cocktail stick. The cells were resuspended by vortexing in 300μl of STET buffer and 20μl of 10mg/ml lysozyme were added, mixed by vortexing and left at room temperature for 10 minutes. The microcentrifuge tube was then placed in a boiling water bath for 60 seconds and the cell debris was then pelleted by centrifugation at 12,000 rpm for 5 minutes. The supernatant was removed and added to an equal volume of isopropanol. After 30 minutes at - 20°C the plasmid DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes. The pellet was washed twice with 70% ethanol, once with ether, allowed to dry for 10 minutes at 55°c and resuspended in 60μl TE and 1μl 10mg/ml DNase free RNase.

**2.12 Large scale isolation of plasmid DNA from *E.coli*.**
250 ml of LB were inoculated with 2.5ml of a fresh overnight culture. The cells were then grown up to an $OD_{600}$

of 0.8, at which point 1ml of chloramphenicol (50mg/ml in ethanol) was added for plasmid DNA amplification. After overnight incubation at 37$^{o}$C, the cells were harvested by centrifugation at 5000 rpm for 10 mins. The cell pellet was resuspended to 2ml with sucrose (25% in 0.25M Tris.Hcl pH 8.0). The cell suspension was then transferred to a Beckmann plastic screw capped ultracentrifuge tube and 0.4ml of lysozyme (20mg/ml in 0.25M Tris-HCl, pH 8.0) was added and the suspension incubated on ice for 5 minutes. 0.8ml of EDTA (0.25M, pH 8.0) was then added and the cell suspension was mixed by vortexing and incubated on ice for 10 minutes. 3.2ml of Triton mix (sect 2.6) were then added to the tube, mixed and after 15 minutes on ice the cell suspension was centrifuged at 40,000 rpm for 40 minutes at 4$^{o}$C. The supernatant was then transferred to a sterile plastic tube containing 6.9g of caesium chloride. The salt was dissolved by gently inverting the tube several times and the solution was then transferred to a Beckmann quickseal polyallomer ultracentrifuge tube. 180$\mu$l of ethidium bromide (10mg/ml) was then added to the tube and the solution weight was made up to 14.1g with 10mm EDTA. The tube was filled with mineral oil, balanced and then sealed using a Beckmann heat sealer. The caesium chloride gradient was formed by centrifuging the tube at 50,000 rpm for 24 hours at 18$^{o}$C. After this time the plasmid DNA had separated from the chromosomal band and was visualized using an ultraviolet light source. The plasmid DNA was removed by inserting a needle just below the plasmid band (lower band) and carefully drawing the plasmid containing fluid into the syringe. The ethidium bromide was then removed by extracting the DNA four times with an equal volume of isopropanol saturated with 20 X SSC (sect 2.6). The caesium chloride was removed by placing the DNA solution in dialysis tubing and dialysing against several changes of distilled water. The dialysis tubing was boiled in 10mM EDTA and rinsed in distilled water prior to use.

**2.13. Large scale isolation of plasmid DNA using Qiagen columns.**

Columns were supplied by Qiagen Inc. and used according to the manufacturers instructions. Yields of 25-40μg of plasmid DNA were obtained using this preparation method.

**2.14. General DNA Manipulations.**

All enzymes used for plasmid and chromosomal DNA digests were used in accordance with the manufacturers instructions. Digests were run on 0.7% agarose gels which were stained for 30 minutes in an ethidium bromide bath (100μl of a 10mg/ml solution in 1 litre of distilled water). Gels were then destained for 5 minutes in distilled water and visualized on an ultraviolet light box.

**Klenow reaction.**

After restriction digests and prior to recircularisation or the addition of linkers to plasmids, the klenow reaction was used to fill in cohesive termini. Following digestion the restriction enzyme was heat inactivated (65$^{o}$C for 10 mins.) and the DNA was cleaned by passage through a sepharose CL4B column. The volume was made up to 80μl with $dH_2O$.

**Klenow reaction mix:**

| | |
|---|---|
| DNA | 80μl. |
| 10 x klenow buffer | 10μl. |
| 2mM dNTPs | 8μl. |
| Klenow enzyme | 2μl. |

The reaction was incubated at 22$^{o}$C for 30 minutes and the klenow enzyme was then heat inactivated by incubation at 65$^{o}$C for 10 minutes.

**Addition of Linkers.**

After the klenow reaction DNA linkers were ligated using the following reaction mixture.

| | |
|---|---|
| Klenowed DNA | 100µl. |
| 100mM ATP | 1µl. |
| 100mM Spermidine | 1µl. |
| 500mM dTT | 3µl. |
| Linker | 2µl. |
| 10 x klenow buffer | 1µl. |
| T4 DNA ligase | 1µl. |

The reaction was incubated at 22°C for 60 minutes, after which a further 3µl of 500mM dTT and 1µl of T4 DNA Ligase were added and the reaction incubated overnight at 14°C.

**BAL 31 Digests.**

Approximately 10µg of plasmid DNA were linearized, phenol/chloroform extracted twice, ether extracted once and then precipitated with 2 volumes of ethanol and 1/10th volume of 3M sodium acetate. The DNA was then resuspended in 60µl TE (pH 7.2). 60µl of 2x BAL 31 buffer and 1µl of BAL 31 were then added and the reaction incubated at 30°C. At given time points 15µl of the reaction mixture were removed and added to 2µl 0.25M EGTA on ice. The number of base pairs removed by the exonuclease activity was then worked out by digesting the BAL 31 treated plasmid with a suitable restriction enzyme and running the digests on a 0.7% agarose gel.

**Dephosphorylation of digested DNA.**

0.5µl of Calf Intestinal Phosphatase (CIP) and 1.5µl CIP buffer were added to 13µl of linearized plasmid DNA and incubated at 37°C for 20 minutes. The following were then added to the reaction:

| | |
|---|---|
| 10 x STE | 10µl. |
| 10mM EDTA | 10µl. |
| 20% SDS | 25µl. |
| $dH_2O$ | 60µl. |
| TE | 100µl. |

The reaction was incubated for 10 minutes at 65°C to heat inactivate the enzymes, extracted twice with phenol/chloroform, once with ether and then ethanol precipitated overnight at -20°C.

**DNA ligations.**

Ligations were carried out in the presence of 1 unit of T4 DNA ligase and vectors (CIP treated) and insert fragments were routinely ligated in a ratio of 1:10. Ligations were incubated overnight at 12 -14°C or at 22°C for 6 hours.

**Isolation of DNA from agarose gels- Geneclean procedure.**

The required band was cut from the gel and placed in a sterile microcentrifuge tube. The fragment was weighed and 2ml/g of the sodium iodide solution was added. The microcentrifuge tube was then incubated at 50°C for 2 minutes to dissolve the agarose and after vortexing, the microcentrifuge tube was incubated at 50°C for 5 minutes. 1µl of the glass milk suspension was added, mixed by vortexing for 10 seconds and the microcentrifuge tube was placed on ice for 5 minutes. The glass milk DNA complex was then spun down in an minifuge for 5 seconds and the supernatant discarded. The pellet was washed 3 times with the ethanol wash solution. The DNA was then eluted by resuspending the pellet in 10µl TE, incubating for 3 minutes at 50°C, spinning down the pellet and removing the supernatant to a clean microcentrifuge tube. The pellet was then resuspended as above and the procedure repeated giving a total volume of 20µl of isolated fragment.The DNA was stored at - 20°C for future use.

**Column cleaning procedure for cleaning up DNA using Sepharose CL6B columns.**

A hole was made in the bottom of a 0.75ml microcentrifuge tube with a sterile needle. Approximately 50µl of 40 mesh glass beads were added to the microcentrifuge tube to prevent the sepharose gel from leaking through the hole. 0.5ml of 70% sepharose CL6B in TE were then added to the

tube and the column was washed through 4 times with sterile $dH_2O$ and once with TE using a volume equal to the sample to be applied. After each wash the column was spun down for 2 minutes exactly at 1500 rpm. The sample was then applied to the column and spun down for 2 minutes at 1500 rpm. The DNA was now ready for use.

**2.15 Isolation of total Yeast DNA**

5ml of a culture grown overnight in YEPD to $O.D._{600}$ 1- 1.5 were pelleted, washed with 5ml of water, repelleted and washed with 2ml of SPM buffer. The cells were then pelleted and resuspended in 0.5ml of SPM buffer (sect. 2.6). 50µl of the cell suspension was diluted to a volume of 5ml in $dH_2O$. This sample was used as a negative control when working out the spheroplasting efficiency. 100µl of lyticase were added to the cell suspension and incubated at 37°C and the spheroplasting monitored until an $O.D._{600}$ of 5 - 20% of the negative control was reached, usually 40 - 60 minutes. 100µl of Proteinase K (Merck, 10mg/ml freshly made up in 150mM NaCl), 50µl EDTA (0.5M, pH 8.0), and 70µl 25% SDS were added to the spheroplasts and incubated for 30 minutes at 37°c. 700µl of phenol/chloroform mix were then added, mixed by inversion and spun for 10 minutes at 10,000rpm. The upper phase and the interphase were transferred to a fresh eppendorf with a wide bore 1ml micropipette tip. This step was repeated, but only the upper phase was taken and transferred to a fresh microcentrifuge tube and extracted once with an equal volume of chloroform. 1.5 volumes of cold ethanol (-20°C) were then added and the DNA was allowed to precipitate for 10 minutes and then pelleted for 15 minutes at 12000 rpm. The DNA was resuspended in 300µl of sterile TE buffer. 15µl of RNase (10mg/ml) were added and the reaction incubated at 37°C for 30 minutes and then extracted once with 100µl phenol/chloroform. The aqueous layer was then precipitated with 1.5 volumes of cold ethanol and spun down at 12000 rpm for 15 minutes. The pellet was carefully resuspended in 50 - 100µl of TE. 5µl of the DNA was then run on a 0.7%

agarose gel with lambda size markers (Type 11 from BRL) to check the chromosomal DNA size range.

**2.16. Isolation of plasmid DNA from yeast.**

5ml of cells from an late exponential phase culture were harvested by centrifugation (5000rpm/5mins)and then resuspended in 0.5ml 1M sorbitol/0.1M EDTA. 50µl of lyticase (8000u/ml) were then added and the cells were incubated for 60 minutes at 37°C. The cells were then spun at 1000rpm for 1 minute and resuspended in 0.5ml 50mM Tris-HCl,pH 7.4/20mM EDTA. 50µl of 10%SDS was now added and the mixture was now incubated for 30 minutes at 65°C. 200µl of 5M potassium acetate was added and the mix was left on ice for 60 minutes, spun for 5 mins/5000rpm after which the supernatant was removed and precipitated with an equal volume of isopropanol. After 5 minutes at room temperature the DNA was pelleted by spinning for 10 seconds. The pellet was then dried at 55°C and resuspended in 300µl T.E. pH 7.4. 15µl of RNase (1mg/ml)was added to the resuspended DNA and incubated for 30 minutes at 37°C after which the DNA was precipitated by the addition of 30µl of 3M sodium acetate and 300µl of cold isopropanol. The DNA was pelleted by centrifugation (5mins/5000rpm) and resuspended in 50µl of T.E. This volume of DNA was then used for transformation of *E.coli*.

**2.17. Plate assays for detecting α-amylase activity.**

In order to visualize α-amylase activity on plates, amylase secreting strains of *E.coli* or *S.cerevisiae* were grown up overnight on LB (for *E.coli*), YEPD or minimal medium supplemented with 2% lintners starch. The amylase activity was visualized by incubating the plates at 4°C for 24 hours, after which the undegraded starch had precipitated leaving clear haloes corresponding to areas of amylase activity.

Alternatively, the plates were inverted over iodine crystals for 60 seconds. Areas where the starch had been

broken down appeared as clear haloes on a dark blue background. Plates were also made up containing 0.003% bromophenol purple and 2% lintners starch, the amylase activity was visualised on these plates after 48 hours growth followed by precipitation of the undigested starch overnight.

**Preparation of Cibacron Blue-starch (CS) for the detection of amylase production:**

Cibacron blue starch was made by a modification of the method of Klein *et al.*, (1969). 100g of Litner's starch were suspended in 900ml of distilled water and heated to 53$^{o}$C. 10g of Cibacron Blue F3GA (Sigma) in 50ml of distilled water were added with constant stirring. 200g of $Na_2SO_4$ and 10g of $Na_3PO_4$ were then added gradually (in small amounts) over a period of 15 minutes. The mixture was stirred at 50-55$^{o}$C for 75 minutes. The suspension was collected by filtration and washed with deionized water with vigourous stirring until the supernatant was colourless. The blue suspension was washed with distilled water, then with methanol, collected by filtration and dried in a vacuum desiccator. To detect amylase production, minimal medium plates containing 2% starch were overlaid with 5ml of minimal medium containing 1% CS. Plates were incubated for 2 days and examined for zones of clearing.

**2.18. DNS assay for measuring α-amylase activity.** (Miller *et al.* 1960).

The following reaction mix was set up in a test tube:

| | |
|---|---|
| 0.1M $NaHPO_4$ buffer pH 6.9 | 1.0ml |
| 1% Lintners starch | 1.0ml |
| 0.5M NaCl | 0.2ml |

The reaction mix was preincubated at 93$^{o}$C for 10 minutes. 0.5ml of culture supernatant or extract (diluted as necessary) were then added to the reaction mix and mixed by vortexing. The reaction was incubated for 30 minutes at 93$^{o}$C. 1ml of DNS solution was then added to the tube and the reaction was boiled for 15 minutes, allowed to cool,

and the O.D. measured at 540nm. One unit of enzyme activity was defined as that amount of enzyme producing 0.15μmol of reducing sugar in 30 minutes in the standard assay.

**2.19. Starch breakdown assay for measuring α-amylase activity.**

(Laoide et al, 1989)

The standard reaction mix contained the following:

| | |
|---|---|
| 0.1% Lintners starch | 1.0ml. |
| 5M NaCl | 0.2ml. |
| 0.1M $KHPO_4$ Buffer | 1.0ml. |

The reaction mix was prewarmed at 93°C for 15 minutes. 0.5ml of supernatant or extract (diluted as necessary) were added, mixed by vortexing and the reaction incubated for 30 minutes at 37°C. The reaction was then stopped by the addition of 1ml of iodine solution (0.1% KI,0.01% $I_2$ in 1M HCl) and allowed to cool for 15 minutes before reading the O.D. at 620nm.

**2.20. SDS Polyacrylamide gel electrophoresis (PAGE).** All protein samples were routinely run on 10% SDS polyacrylamide gels. The gels were prepared as follows.

**Separating gel:**

| | |
|---|---|
| Acrylamide/Bisacrylamide solution (30:1) | 10.5ml |
| 1.87M Tris.HCl, pH 8.8 | 6.3ml |
| $dH_2O$ | 14.1ml |
| 10% SDS | 180μl |
| 10% Ammonium Persulphate | 150μl |
| TEMED | 30μl |

**Stacking gel:**

| | |
|---|---|
| Acrylamide/Bisacrylamide solution (30:1) | 3.75ml |
| 0.5M Tris.HCl, pH 6.8 | 1.8ml |
| $dH_2O$ | 11.5ml |
| 10% SDS | 150μl |
| 10 % Ammonium persulphate | 150μl |
| TEMED | 150μl |

The separating gel was poured, covered with a layer of isopropanol and allowed to set for 4 hours. The stacking gel was then poured and allowed to set for one hour prior to use. All protein samples were boiled for 5 minutes in solubilisation buffer before being loaded on the gel. After loading, gels were routinely run for 12 hours at 100 volts in SDS running buffer, stained for 12 hours and then destained and stored in 7% acetic acid.

**2.21. α-amylase activity gels.**

For the direct visualization of α-amylase activity in gels the running gel was made up with 0.25% lintners starch in place of water. The lintners starch was boiled for 15 minutes prior to use in the gel. After the gel had run, the amylase was renatured by incubation in renaturation buffer (sect. 2.6) for 2 hours with fresh buffer added after the first hour. The gel was then washed 3 times with distilled water and incubated at 55$^{o}$c for 6 hours in activity buffer (sect. 2.6).

**2.22. Preparation of yeast cell extracts for assaying α-amylase activity and for PAGE.**

10ml of cells were spun down at 10,000 rpm for 5 mins, washed once in water and once with 1.2 M sorbitol, 50mM potassium phosphate pH 7.5 and spun as before. The cells were then resuspended in 950μl of 1.2M sorbitol containing the following: 50 mM potassium phosphate pH 7.5, 14mM mercaptoethanol and 1mM PMSF. 50μl of lyticase (8000 units/ml) in 50 mM potassium phosphate pH 7.5) were then added, and spheroplasting was followed microscopically until complete, usually 20 - 30 minutes. The spheroplasts were spun down at 4,000 rpm for 5 minutes, resuspended and washed twice with 1 ml 1.2M sorbitol, 50mM potassium phosphate pH 7.5. The spheroplasts were then lysed by resuspending them in 1ml 50mM potassium phosphate pH 7.0, 1mM PMSF and 0.2% triton-x-100. The cell debris was spun out for 10 minutes at 10,000 rpm and the supernatant was used to assay for internal α-amylase activity. An equal

volume of 2 x solubilisation buffer was added to aliquots of the supernatant for PAGE.

**2.23. Preparation of yeast cell supernatants for α-amylase activity gels.**

500ml of the yeast culture supernatant were concentrated down to 10ml using an Amicon ultrafiltration system. The concentrated supernatant was then dialysed against 4 changes of 10mM phosphate buffer, pH 6.9, over a period of 16 hours to remove excess salt. The dialysed supernatant was aliquoted into microcentrifuge tubes and freeze-dried overnight. Aliquots were resuspended in appropriate buffers just prior to use.

**2.24. Preparation of yeast cell supernatants for determination of α-amylase and β-glucanase activity.**

Yeast cultures were centrifuged at 5,000 rpm for 5 minutes. The supernatants were then dialysed against PBS for 16-24 hours at $4^{o}C$ with 3 changes of buffer prior to quantification of α-amylase and β-glucanase activity using the DNS assay.

**2.25. Preparation of yeast cell extracts for determination of β-glucanase activity.**

5 ml of cells were harvested by centrifugation at 5,000 rpm for 5 minutes and resuspended in 5 ml of 1X lysis buffer. 1g of glass beads were added and the cells were vortexed for 3 minutes (6 x 30 second bursts, with 30 seconds on ice between each burst). The cell debris was spun out and the cleared lysate removed to a fresh tube. Cell breakage was followed microscopically.

**2.26. Treatment of α-amylase with Endoglycosidase$H_f$.**

This treatment was carried out to remove N-linked core carbohydrate from the protein sample prior to loading onto SDS polyacrylamide gels. A freeze-dried aliquot as prepared in section 2.22 was denatured by boiling for 10 minutes in denaturing buffer (sect. 2.6). One tenth the reaction

volume of $EndoH_f$ reaction buffer (0.5M Sodium Citrate) and 1 $\mu$l of $EndoH_f$ were then added to the reaction and incubated for 60 minutes at 37°C. An equal volume of 2 x solubilisation buffer was then added and samples applied to the polyacrylamide gel after boiling for 5 mins.

**2.27. Western blotting.**

**Transfer of proteins to nitrocellulose.**

After electrophoresis, the SDS polyacrylamide gel was equilibrated in transfer buffer for 20 minutes. Two fibre pads, nitrocellulose sheets and Whatman filter paper were then soaked in transfer buffer. Proteins were transferred using a Bio-Rad transfer cell and the transfer unit was assembled as follows: A moist fibre pad was placed on the cathode panel of the transfer unit followed by a piece of Whatman # 2 filter paper cut to the size of the gel. This was followed by the gel, a sheet of nitrocellulose membrane, another piece of filter paper and the second fibre pad. The unit was then closed and placed in the transfer tank which was filled with transfer buffer. Transfer was allowed to proceed for 4 hours at 60V, 0.21A or overnight at 30V, 0.1A. A small stirring bar was used to circulate the buffer to prevent localized heating. The efficiency of transfer was monitored by the use of prestained molecular weight markers (Sigma). The nitrocellulose membrane was then either used immediately or stored in a sealed plastic bag at 4°C to keep it moist.

**Binding of primary and secondary enzyme-conjugated antibodies.**

All incubation and washing steps were carried out at room temperature. The nitrocellulose membrane was submerged and rinsed in TBST until evenly wet. The TBST was then replaced with blocking solution and incubated with shaking for 30 - 60 minutes. The blocking solution was decanted off and replaced with TBST (0.1ml/$cm^2$) containing an appropriate concentration of the primary antibody and incubated for 60 minutes. The membrane was then washed 3 times for 10

minutes each time with TBST, transferred to TBST containing a 1/7500 dilution of the anti-rat IgG alkaline phosphatase conjugate, incubated for 60 minutes and again washed 3 times for 10 minutes each time in TBST.

**Development of alkaline phosphatase (AP)colour reaction.**
The nitrocellulose membrane was blotted dry on filter paper and transferred to 10ml of the AP colour development solution (sect. 2.6). The colour was allowed to develop until bands of the desired intensity appeared. The reaction was stopped by washing the membrane several times in distilled water. The membrane was then blotted dry and stored in a sealed plastic bag.

**2.28. Southern blotting.**
The technique used was that described by Southern (1975) After electrophoresis, the gel was stained with ethidium bromide and photographed. The gel was then transferred to a tray containing several volumes of denaturing solution and incubated with shaking at room temperature for 45 minutes. The denaturing solution was then decanted off and replaced with neutralizing solution and the gel was shaken for one hour. The DNA was then transferred bidirectionally as follows: a stack paper towels 6cm high was placed on a glass plate and 3 sheets of Whatman 3MM filter paper presoaked in 20 X SSC were then placed on top of the paper towels. A sheet of nitrocellulose the exact size of the gel and presoaked in 20 X SSC was then placed onto the filter paper and the gel was carefully placed on top of the nitrocellulose. This sandwich was then then repeated on top of the gel. The entire arrangement was weighted down with a five kilogram weight. After 24 hours transfer was complete and the nitrocellulose was removed carefully, soaked in 6 x SSC at room temperature for five minutes, allowed to air dry and then baked for 2 hours at $80^{o}$C.

**2.29. Preparation of radioactive probe.**

The probe was prepared by random priming using the Promega labeling system based on the method developed by Feinberg and Vogelstein (1983).

The reaction mix contained the following:

| | |
|---|---|
| 5 X labelling buffer | 10μl |
| Mix of unlabelled dNTPs (final conc 20μM each) | 2μl |
| Linear DNA template | 25ng |
| 1mg/ml acetylated nuclease free BSA | 25μl |
| [α $-^{32}$P] dNTP (3000 Ci\mM) | 4μl |
| Klenow enzyme | 5 Units |

the final reaction volume was made up to 50μl with sterile $dH_2O$.

The components were mixed gently and the reaction tube was incubated at room temperature for 3 hours. The reaction was then terminated by the addition of EDTA to a final concentration of 20mM and then boiled for 2 minutes. The reaction tube was placed directly onto ice and used immediately or was stored at -20°C until required.

**2.30. Hybridisation of probe to baked filters.**

The baked filters were soaked for 2 minutes in 6 X SSC and then put into a heat sealable bag. Prehybridization mix (0.2ml/$cm^2$ of nitrocellulose filter) was warmed to 68°C and added to the bag. The excess air was then squeezed from the bag and the bag was sealed and incubated on a shaking table at 68°C for 4 hours. The prehybridization mix was then replaced with hybridisation solution (50μl/$cm^2$) containing the labelled denatured probe. After overnight incubation (16 - 20 hours) at 68°C the fluid was removed from the bag and the filter was transferred to a solution of 2 X SSC and 0.5% SDS and incubated at room temperature for 15 minutes. This solution was then decanted off and replaced with 2 X SSC and 1% SDS and the filter was incubated for a further 15 minutes at room temperature. The filter was then incubated for 2 hours at 68°C in a solution of 0.1% SSC and 0.5% SDS. The buffer was decanted off and replaced with fresh buffer and incubated for a further 30 minutes. The

filter was then air dried at room temperature on a sheet of 3MM Whatman paper, placed in a plastic heat sealable bag and exposed to Kodak x-ray film. After exposure the film was developed and fixed using Kodak DX-80 developer and FX-40 x-ray developer.

**2.31. Mutagenesis of *S. cerevisiae* with E.M.S.**

Cells (100ml) were grown to stationary phase in minimal media and were centrifuged at 5,000 rpm for 5 mins. Cells were washed once and resuspended in 10ml of 0.1M phosphate buffer, pH 7.0. The cells were then transferred to a 50ml screw top tube and 0.3ml of EMS added. The tube was vortexed vigourously as EMS is poorly miscible in this buffer. The tube was then incubated in a shaking water bath at $30^{o}C$ and samples were taken at intervals of 15 minutes and added to an equal volume of freshly made, filter sterilised, 10%(w/v) sodium thiosulphate. Samples were then spun at 5,000 rpm for 5 minutes, washed twice with 5 ml of 0.1M phosphate buffer, pH 7.0 and plated on buffered minimal medium, pH 6.9, containing 2% starch. Various dilutions of the samples were plated to obtain less than 200 colonies per plate. Plates were counted after 2 days growth at $30^{o}C$.

**2.32. Curing *S. cerevisiae* of plasmid DNA.**

In order to cure plasmid containing cells the strains were grown up in YEPD for 2 days and then replica plated onto YEPD and minimal media. The plasmids which grew on YEPD and not on minimal media were considered cured.

**2.33. Plasmid stability studies.**

Strains were inoculated into 5ml of minimal media lacking the amino acid used for selection and grown to stationary phase. The cell number was determined by direct cell counting and $10^{6}$cells were inoculated into 100ml of YEPD. After overnight incubation at $37^{o}C$ the cell counting and inoculation were repeated, this procedure was followed for up to 7 days. The number of generations per day was between

9 and 11. Each day after the cell count had been determined suitable dilutions were plated onto YEPD plates in duplicate. After 2 days at 30°C. 100 colonies from the YEPD plates were replica plated onto YEPD and minimal media. The percentage of colonies retaining plasmid (percentage plasmid stability) was determined from the number of colonies growing on the minimal media plate.

**2.34. Elisa assay.**

The antigen was dissolved (40μg/ml) in carbonate buffer pH 9.6 and 100μl were added to each well of a 96 well plate. The plate was incubated for 2 hours at 37°C or overnight at 4°C. The antigen was then decanted off and the plate was washed 4 times with PBS Tween-20 (0.1%) and finally with PBS. The plate was then inverted and the excess liquid shaken off onto tissue. 200μl of the blocking solution (1% w/v BSA in PBS, pH 9.0) was added to each well and the plate was incubated for 1 hour at 37°C. The blocking solution was decanted off and the plate washed as before. 100μl of the serum dilutions (1/10 to 1/256,000) were added to the plate in duplicate. Controls (positive and negative) and blank samples (containing PBS only) were also added to the plate in duplicate. The plate was incubated for 1 hour at 37°C or overnight at 4°C and washed as before. 100 μl of the secondary antibody was added to each well and the plate incubated for 1 hour at 37°C. The washing was repeated as before and 100μl of freshly prepared substrate solution was added to each well. As an additional control a set of blank wells containing only the substrate were also prepared. After 30 minutes the colour began to develop and the concentration of the antibody in the serum could be quantified from the plate.

**2.35. α-factor plate assay.**

An overnight culture of RC631 cells (sect. 2.1) were grown to an $O.D._{600}$ of 1.0. 1ml of a $10^{-2}$ dilution of the cells was then added to 15ml of molten MYGP agar (sect. 2.3),

mixed gently and poured into a sterile petri dish. The agar was then allowed to set at room temperature and 9mm wells were cut in the agar using a sterile 1ml blue tip. 100µl of sample was added to each well and the plates were incubated overnight at 30°C.

**2.36. Photography.**

All photographs were taken with a Nikon FG-20 camera loaded with Kodak TMAX-100 film. DNA gels were photographed using a Cokin # A.003 ultraviolet filter. Where necessary Vivitar 49mm close up lenses were used. All developing and printing supplies were from Kodak. Negatives were developed for 9 minutes at 20°C in D76 developer and fixed in a 1:3 dilution of Unifix in distilled water until all the background colour had disappeared from the negatives. The negatives were printed onto Kodak 11RC, F4 high contrast paper, developed in a 1:10 dilution of Dektol developer for 2 minutes and then fixed for 10 minutes in a 1:3 dilution of Unifix in distilled water.

**2.37. DNA sequencing.**

Preparation of plates.

The plates, spacers and combs were washed in tap water containing 2% RBS and rinsed in distilled water. The plates were allowed to air dry and were washed with ethanol, air dried and treated with 1ml of Repel Silane (Dimethyldichlorosilane solution 2% (w/v) in 1,1,1-trichloroethane, LKB, Sweden) and air dried. The plates were then given a final polishing with ethanol. The spacers were wiped with ethanol, placed between the plates and the plates were then taped up.

**Preparation of sequencing gel.**

The gel was prepared in a clean 100ml plastic beaker. 75µl of TEMED and 196µl of a freshly prepared solution of Ammonium persulphate (10% w/v in $dH_2O$) were added to 60ml

of stock acrylamide/urea solution. The acrylamide solution was then gently mixed using a 20ml syringe, and then carefully poured using this syringe. In order to help prevent leakage and the formation of air bubbles the plates were held at a slight angle during the pouring of the gel. The gel combs were placed, straight edge down, in the top of the gel to form an even surface for loading of the DNA samples. The gel was allowed to set for 3 hours and stored in the sequencing rig covered in TBE (sect. 2.6) or on the bench covered in tissue soaked in TBE and sealed with parafilm to prevent drying.

**Preparation of DNA for sequencing.**

The DNA used for sequencing reactions was prepared by pooling 6 mini (STET or Alkaline lysis) preps of the plasmid DNA and resuspending in 400µl of sterile $H_2O$. The DNA solution was then extracted twice with an equal volume of phenol/chloroform to remove contaminating protein. The DNA was then freeze dried and resuspended in 10µl of $H_2O$. To estimate the DNA concentration, 1µl of this sample was run on a 0.7% agarose gel and compared to 1µl of commercial pBR322 (0.25µg/µl).

**Annealing Reaction.**

The DNA was denatured by adding 2µl of 1M NaOH to 8µl of DNA and incubating at room temperature for 5 minutes. The denatured DNA was then column cleaned into an microcentrifuge tube containing 2µl of annealing buffer and 2µl of primer (both supplied in the T7 polymerase sequencing kit, Pharmacia). After incubation at 37°C for 20 minutes and standing at room temperature for at least 10 minutes, the sequencing reactions were carried out immediately or the samples were stored at -20°C.

**Sequencing Reaction (T7 polymerase, Pharmacia).**

The enzyme T7 polymerase was diluted to 1.5u/µl and stored on ice until required. The dNTP's were aliquoted into microcentrifuge tubes (2.5µl) labelled A,C,G, and T and

left on ice. Labelling mix was prepared according to the manufacturer's instructions, however, 0.5µl of [$\alpha$-$^{35}$S] dATP instead of the recommended 1µl were used. The dNTP's were heated at 37°C for at least 1 minute and 4.5µl of the template/enzyme mix was added to each of the dNTP tubes. The reaction was then further incubated at 37°C for 5 minutes at which time the reaction was stopped by the addition of 5µl of Stop solution containing bromophenol blue. The sequencing reactions could be stored for up to one week at -20°C.

**2.38. Polymerase Chain Reactions (PCR).**

PCR reactions were carried out using a Perkin Elmer PCR apparatus. Each PCR reaction contained the following reaction components:

| | |
|---|---|
| 10x Polymerase buffer | 10µl |
| dNTPs | 10µl |
| Primers | 2µl of each |
| Target DNA | 3µl |
| $dH_2O$ | 72.5µl |
| Taq polymerase | 0.5µl |

Each reaction volume was overlaid with 60µl of sterile mineral oil and the PCR reactions were carried out using the following program.

1. 95°C - 10 minutes (to dissociate total DNA).
2. 95°C - 90 seconds.
3. 45-50°C Annealing temperature.
4. 72°C 2 minutes (elongation step, under these conditions approximately 1 kb of DNA is synthesised per minute). Steps 2 to 4 were repeated for 30 cycles and the products were then refrigerated.

**3.39. Sucrose density gradient centrifugation.**

Sucrose solutions (10 to 40% w/v) were made in a buffer containing:

| | |
|---|---|
| NaCl | 1mM |
| Tris.HCl (pH 8) | 20mM |
| EDTA | 5mM |

The gradients were prepared by sequential addition of

sucrose solutions (2 ml of each in a polypropylene tube) beginning with 40% sucrose and freezing at -20$^{o}$C between each addition. Before use the gradients were allowed to thaw at room temperature for 1 hour. DNA was then layered onto the gradient which was spun at 22,000rpm for 18 hours in a swing bucket rotor. Samples were then collected by piercing the base of the tube and every second sample was run on an agarose gel to size the samples. Pooled fractions were diluted 1:2 with water and the DNA was recovered by precipitation with ethanol.

# Chapter 3

## Results 1.

**Cloning of *Bacillus licheniformis* α-amylase; characterisation and analysis of the heterologous amylase produced by *Saccharomyces cerevisiae*.**

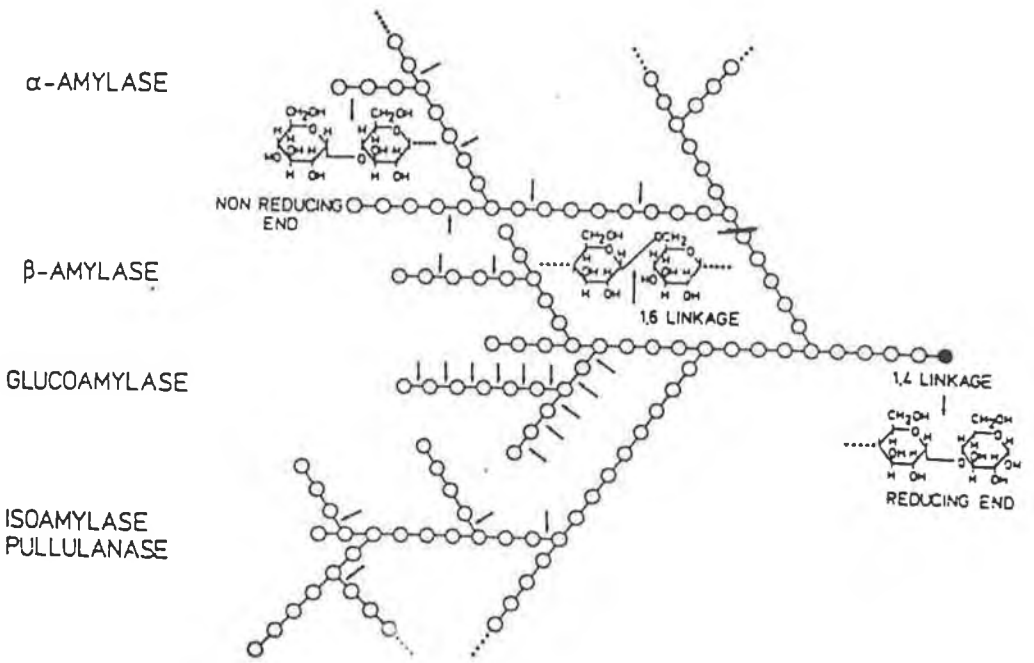
## 3.1 Introduction.

α-amylase (1,4-α-D-glucan glucanohydrolase E.C. 3.2.1.1.) which catalyses the hydrolysis of the α-1,4-glucosidic linkages of starch is widely distributed in nature occurring in many species of plants, animals, fungi and bacteria. It is an endoamylase which liberates poly and oligosaccharide chains of varying lengths from starch. α-amylases have many commercial applications, the largest volume being used in the liquefication of starch, the products of which are used widely in the fermentation industry. α-amylases are also used in the desizing of fabrics, in the baking industry, in the production of adhesives, pharmaceuticals, detergents, in sewage treatment and in animal feed (Vihinen and Mäntsäla 1989).

In the brewing industry, *Saccharomyces cerevisiae*, the main agent in alcoholic fermentation, lacks amylolytic activity. In order to overcome this problem, during the mashing phase of the brewing cycle, malted barley is steeped in warm water and α-amylase is added to aid in the breakdown of the starch present in the barley to lower molecular weight compounds which can then which can be utilised by yeast for growth and fermentation.

Starch is composed of amylose and amylopectin (see Fig.3.1). Amylose is a mainly linear polysaccharide which is composed of α-D-glucose units joined primarily by α-1,4 linkages and to a lesser extent by α-1,6 linkages. Amylopectin has a highly branched tree like structure. The proportion of branches is an important property of the substrate as different enzymes hydrolyse different substrates with different specificities. The relative content of amylose and amylopectin varies with the source of substrate. The average chain length of amylose is about 1000 glucose units. The chain profile of amylopectin usually has a bimodal distribution with longer and shorter chains having average lengths of 40 to 60 and 11 to 25 D-glucosyl residues respectively.

Fig 3.1

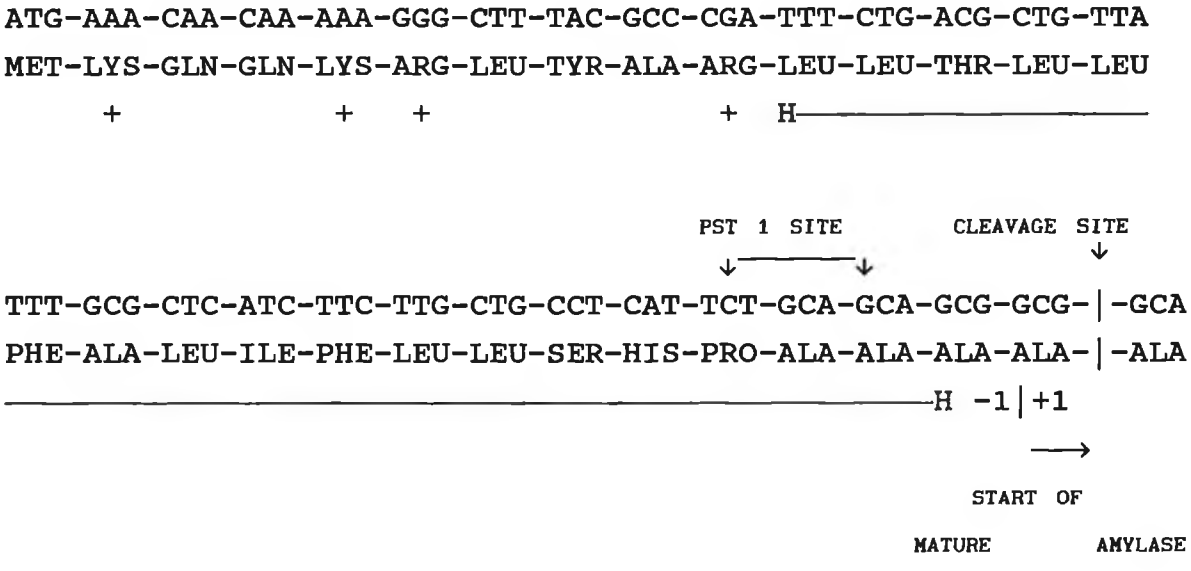



Schematic structure of amylopectin and action pattern of amylolytic enzymes. The circles represent glucose units and the arrows represent sites at which the amylolytic enzymes can hydrolyse.

Several amylolytic enzymes hydrolyse starch to its degradation products. The actions of these enzymes can be divided into two categories. Endoamylases split linkages in random fashion in the interior of the starch molecule. Exoamylases hydrolyse from the non-reducing end (see Fig.3.1), successively resulting in shorter end products. α-amylase is an endo enzyme which hydrolyses internal α-1,4-bonds and can bypass 1,6 linkages. β-amylase is an exoenzyme which liberates maltose by hydrolysing 1,4 linkages from the nonreducing end. Glucoamylase produces glucose and can degrade both 1,4- and 1,6 linkages. Isoamylase and pullulanase are debranching enzymes which hydrolyse both 1,4 and 1,6 linkages.
α-amylase (1,4 α-D-glucanohydrolase EC 3.2.1.1) which catalyses hydrolysis of the α-1,4-glucosidic linkages of starch is widespread amongst microbes. The α-amylase from

*Bacillus licheniformis*, is a calcium dependent, thermophillic enzyme which hydrolyses the α-1,4 linkages of starch molecules, is used widely in the food processing and brewing industry. The cloned gene has a coding sequence of 1,536 nucleotides (Appendix 1) which is translated to a protein of 512 amino acids (Yukki *et al*. 1985) including a leader or signal peptide of 29 amino acids. This signal sequence is characteristic of most signal peptides in that it includes a short cluster of 4 positively charged amino acids (2 lysine and 2 arginine residues) within the first 10 amino acids, followed by an extensive hydrophobic domain containing 19 amino acids before the coding region of the mature α-amylase. The signal ends with a potential cleavage site as shown below (fig. 3.2) (Stephens *et al* 1984). A useful feature of this signal peptide is that it has a *Pst*1 site adjacent to the cleavage site which allows for the convenient separation and manipulation of the signal peptide and mature α-amylase.

Figure 3.2.




Features of the *B. licheniformis* α-amylase signal peptide.
+ indicates positively charged residues.
H-H indicates the long hydrophobic region.

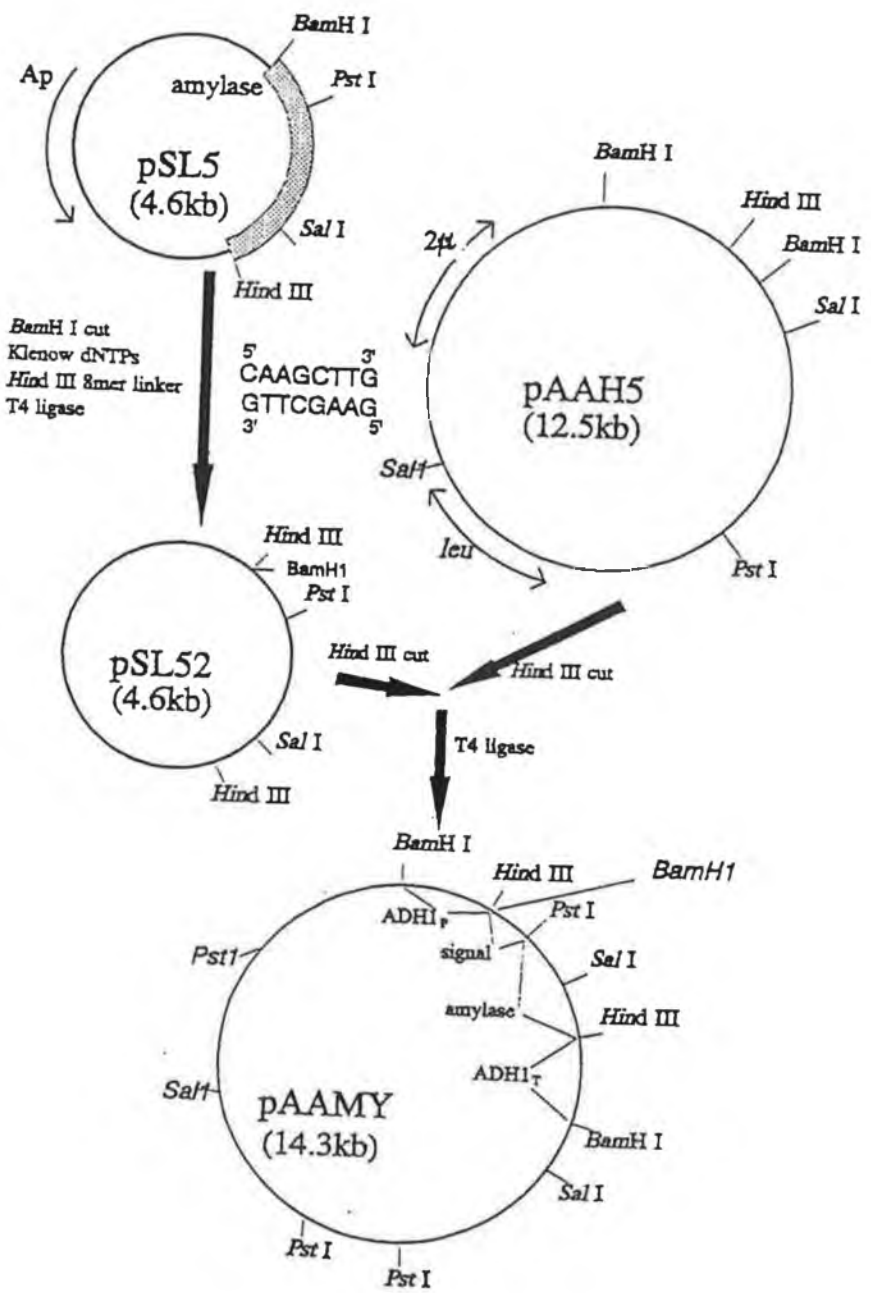
The entire α-amylase gene, including its signal peptide and transcriptional terminator sequence, (see appendix # 1 for sequence) was inserted into pUC8 on a *Bam*H1-*Hin*d111 fragment (O' Kane et al, 1986). This plasmid, pSL5, (Fig 3.3) was used as a starting point for the plasmid constructs in this work which was undertaken to examine the production and secretion of the *B. licheniformis* α-amylase in *Saccharomyces cerevisiae*. The α-amylase from *B. licheniformis* was selected for this study as it is a well characterised, easily assayable enzyme of industrial importance and had not previously been expressed in *S.cerevisiae*. This project was undertaken firstly to characterise the production of *B. licheniformis* α-amylase by *S.cerevisiae*. This system could then be used as a model system for examining the effect of changes in media composition, vector constructs used, and mutagenesis of the host organism on the levels of heterologous protein produced. The knowledge obtained from this study could then be applied to the production of other heterologous proteins of industrial importance. A number of other heterologous amylases and other starch degrading enzymes have been previously expressed in *S. cerevisiae* (section 1.8).

**3.2 Construction of a yeast episomal vector containing the *Bacillus licheniformis* α-amylase gene.**

The *Bacillus licheniformis* amylase was cloned into the yeast episomal vector pAAH5 (Ammerer 1983) as outlined below and in fig. 3.3. Firstly the plasmid pSL5 was linearised at its unique *Bam*H1 site and the cohesive termini were filled in with deoxynucleotides using the klenow fragment of DNA polymerase 1 (2.14). The plasmid was then religated in the presence of *Hin*d111 8-mer linkers (5' CAAGCTTG 3') resulting in the vector pSL52. The filling in of the cohesive terminii of the *Bam*H1 linearised plasmid followed by the ligation of the *Hin*d111 linker resulted in the recreation of the the *Bam*H1 site at the 3' end of the inserted *Hin*d111 linker. The amylase containing *Hin*d111 fragment of this vector was then isolated from a 0.7%

agarose gel using the Geneclean procedure (2.14) and ligated into pAAH5 which had previously been linearised at its unique *Hind*111 site and CIP treated (2.14).

Figure 3.3



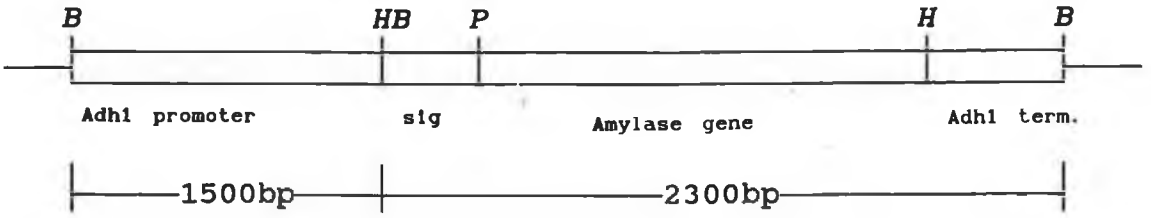

Construction of pAAMY.

The ligation mix was transformed into *E.coli* strain JA221 (2.7) and transformants were plated on LB media containing 2% lintners starch and screened for amylase activity (sec 3.3). Although expression of amylase in *E.coli* would not normally be expected from this vector as it lacks a prokaryotic promoter at the cloning site, previous work (Hunter, 1991) with a glucanase gene inserted into the vector pAAH5 resulted in expression of the glucanase in *E.coli*. To check if this was also the case with the amylase gene the initial screening for amylase containing plasmid was carried out on starch containing plates. After transformation of the ligation mixture the resulting colonies were replica plated onto starch containing LB medium and assayed for amylase activity. A number of these transformants had amylase activity. DNA was isolated from these amylase positive transformants and the orientation of the amylase gene was confirmed by restriction digest analysis.

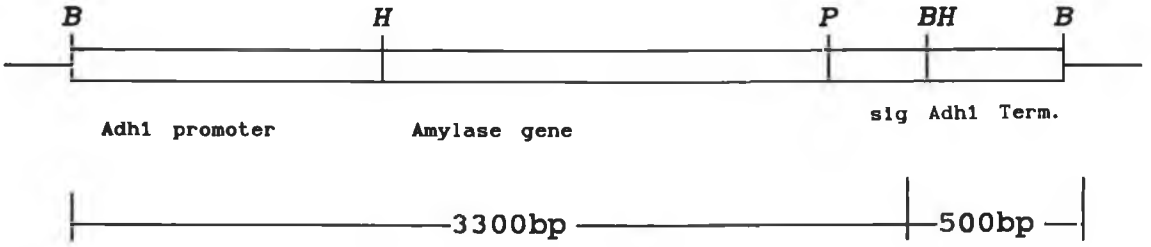
A plasmid which contained the amylase fragment in the correct orientation between the ADH1 promoter and terminator (fig.3.3) was identified and named pAAMY. The orientation was confirmed by the sizes of the *Bam*H1, *Pst1* and *Sal*1 fragments obtained. In the case of the *Bam*H1 fragments there are *Bam*H1 sites at either end of the ADH1 promoter, terminator cassette. The distance from the promoter to the unique *Hin*d111 cloning site is 1500bps, and the distance from the terminator to the unique *Hin*d111 cloning site is 500bps. If the amylase had been inserted in the correct orientation ie Adh1 promoter-5'amylase3'-Adh1 terminator, a *Bam*H1 restriction would give fragments of 1.5, 2.3 and 10.5. However if it was inserted in the opposite orientation it would give band sizes of 0.5, 3.3 and 10.5. (see Fig.3.4 a,b). Similiarly if the fragment was present in the correct orientation the fragment sizes should have been as follows, *Pst1* 5.8, 4.2, 3.9, 0.5, *Sal*1 6.7,6.2,1.5. As these correspond to the sizes obtained in the gel shown in Fig 3.5 it is apparent that the fragment has been inserted in the correct orientation.

Figure 3.4

(a).Correct orientation of amylase fragment




(b).Incorrect orientation of amylase fragment.

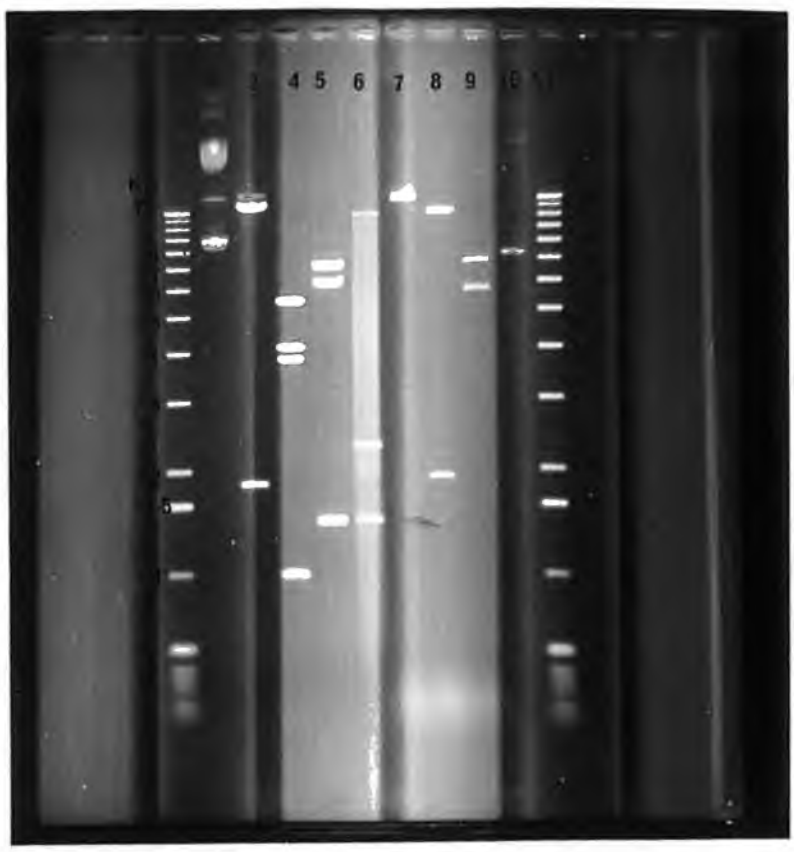



Orientation of amylase gene in pAAMY, fragment sizes after *Bam*H1 digest.

This plasmid was transformed into *S.cerevisiae* DBY746 (2.10) and transformants were transferred to minimal media (sec 2.3) containing 2% lintners starch to screen for amylase activity. None of the transformants appeared to have any amylolytic activity but when some were grown on YEPD (sec 2.3) containing 2% lintners starch, all were found to be amylase positive. Growth of *S.cerevisiae* for 48 hours at 37$^{o}$C in unbuffered minimal media broth resulted in a drop in pH from 6.2 to 2.3. In YEPD broth the drop in pH is from 6.3 to 5.8. The lower buffering capacity of minimal media was thought to be the reason for a lack of detectable amylase activity in the minimal media as the amylase is inactive at the final pH of 2.3 obtained in minimal media. To ascertain whether or not the lower buffering capacity of the minimal media was responsible for the failure to detect amylase activity, minimal media supplemented with 2% lintners starch was buffered to pH 6.9 with 0.1M sodium phosphate buffer, the optimium pH for the

*B. licheniformis* amylase activity (Ortlepp *et al.*, 1983). When buffered medium was used it was possible to detect amylase activity.

Figure 3.5



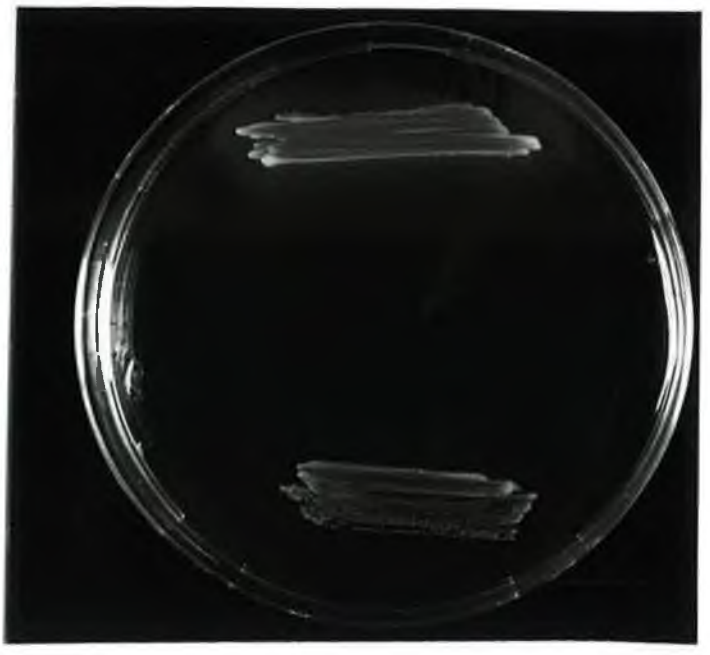

Restriction analysis of plasmid pAAMY.
The approximate size of each of the restriction fragments is shown in kilobases in brackets below. The relevant sizes of the λ 1 kb ladder are as indicated.

Lane 1 : λ1KB DNAladder, 12,11,10,9,8,7,6,5,4,3,2,1.5,1.0.5
Lane 2 : pAAMY uncut.
Lane 3 : pAAMY *Hind*111 (12.5,1.8)
Lane 4 : pAAMY *Pst*1(5.8,4.2,3.9,0.5)
Lane 5 : pAAMY *Sal*1(6.7,6.2,1.5)
Lane 6 : pAAMY *Bam*H1(10.5,2.3,1.5)
Lane 7 : pAAMY *Hind*111(12.5)
Lane 8 : pAAH5 *Bam*H1 (10.5,2)
Lane 9 : pAAH5 *Sal*1(6.7,5.7)
Lane 10: pAAH5 uncut
Lane 11: λ1KB DNA ladder.

**3.3. Detection of α-amylase activity using plate assays.**

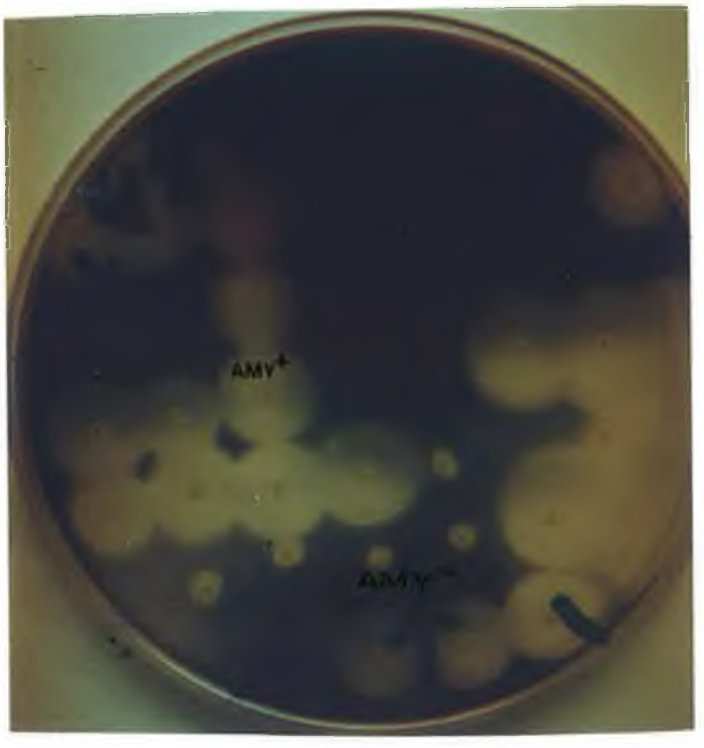
Two plate assays were established to screen *E.coli* strains producing the *B.licheniformis* α-amylase enzyme. The first method (2.17) involved using LB medium supplemented with 2% Litner's starch and buffered to pH 6.9 with 0.1M sodium phosphate. Amylolytic colonies were easily detectable by growing the cells overnight at 37°C and then incubating the plates at 4°C overnight to precipitate the undegraded starch. Colonies producing α-amylase were surrounded by clear haloes where the starch had been broken down (fig. 3.6). The second method involved incubating the LB plate overnight at 37°C and then exposing it to iodine vapour for 20-30 seconds. The amylase-producing colonies were surrounded by distinct clear haloes while non-amylolytic colonies were stained blue by the iodine vapour (fig. 3.7). This assay was non-toxic to *E.coli* over short incubation times. The two assay procedures were used as the iodine assays facilitated easy photography of the plates under study, as the contrast between the dark background and the clear haloes was better than that seen in the precipitation assay. This assay was also used as the haloes could be visualised directly after staining with iodine and did not require overnight incubation for the precipitation of the undegraded starch. When large numbers of plates were being screened the starch precipitation assay was preferable as it protected the user from prolonged exposure to hazardous iodine vapour. Both assay procedures had similiar sensitivity, but the definition of the haloes was superior with the iodine assay.

Figure 3.6.



Detection of amylase positive *E.coli* colonies using starch precipitation assay.

Figure 3.7



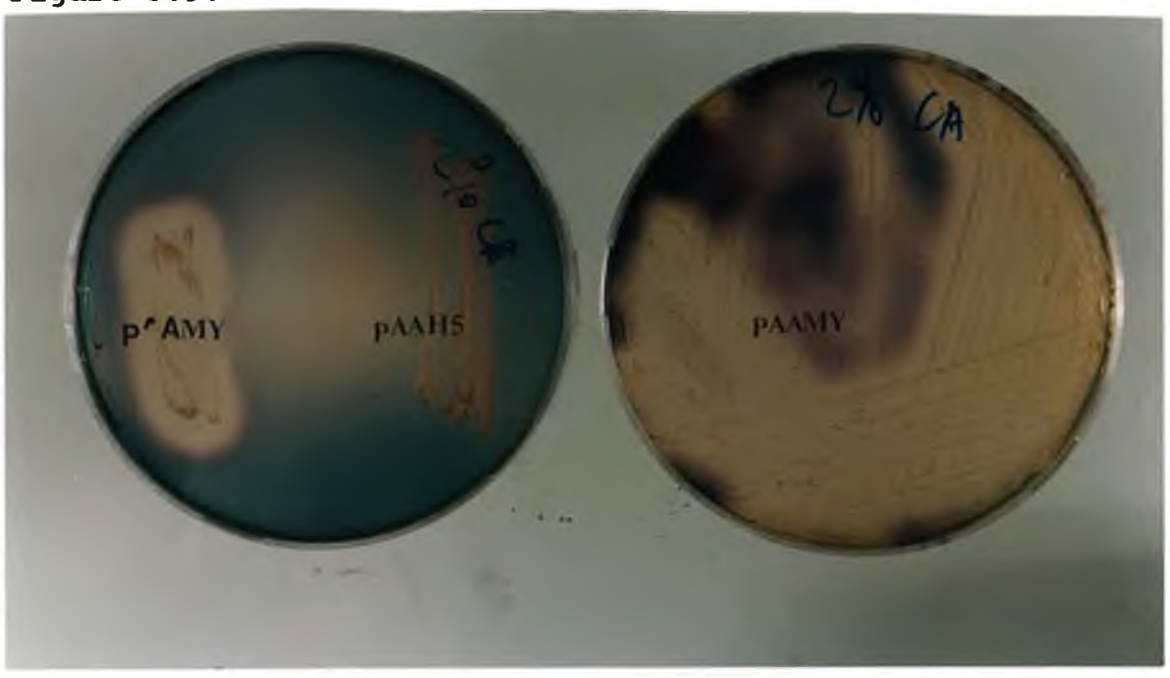

Detection of amylolytic activity in *E.coli* strains using iodine vapour.

Two assays were successfully established for the detection of amylolytic yeast strains. The first assay was identical to the precipitation assay used for *E.coli* except that buffered yeast minimal medium pH 6.9 (Sec. 2.3) was used instead of LB media and the yeast strains were grown at 30$^{0}$C for 48 hours, followed by incubation at 4$^{0}$C for 24 hours. The most clearly defined haloes were obtained when the 2% glucose of the minimal medium was added prior to autoclaving the medium, as the slight caramelisation of the glucose provided a better contrast to the clear haloes surrounding amylase positive colonies, this is illustrated in figure 3.8. The second method involved exposing the plates to iodine vapour. Similar to the *E.coli* strains on LB, the amylolytic yeast strains on minimal medium were surrounded by clear haloes while the rest of the plate was stained blue (fig. 3.9).

Figure 3.8.


Detection of amylolytic yeast strains using the starch precipitation assay.

Figure 3.9.




Detection of amylolytic yeast strains using iodine vapour.

**3.4. Quantitative detection of α-amylase.**

The starch degradation assay of Laoide *et al.*, (1987) (2.19) was initially used for the quantitative determination of amylase activity. However, it was found that it was not possible to assay yeast cell extracts using this assay as both the protease inhibitor PMSF and β-mercaptoethanol used in the preparation of yeast cell extracts resulted in the complete loss of colour in the assay. Therefore all assays of amylase activity were carried out using the dinitrosalicylic acid or DNS assay developed by Miller (1960) which quantifies the reducing sugars produced as a result of the digestion of starch by α-amylase. The optimium assay conditions for the *B. licheniformis* α-amylase are a pH of 6.9 and a temperature of 93$^{o}$C (Ortlepp *et al.*, 1983 , also see Sec. 3.5 below). Figure 3.10 shows a typical standard curve used for the determination of amylase activity. One unit of enzyme activity was defined as that amount of enzyme which liberates glucose or other reducing carbohydrates with a reduction power corresponding to 0.15μmoles D-glucose in 30 minutes in the standard assay.

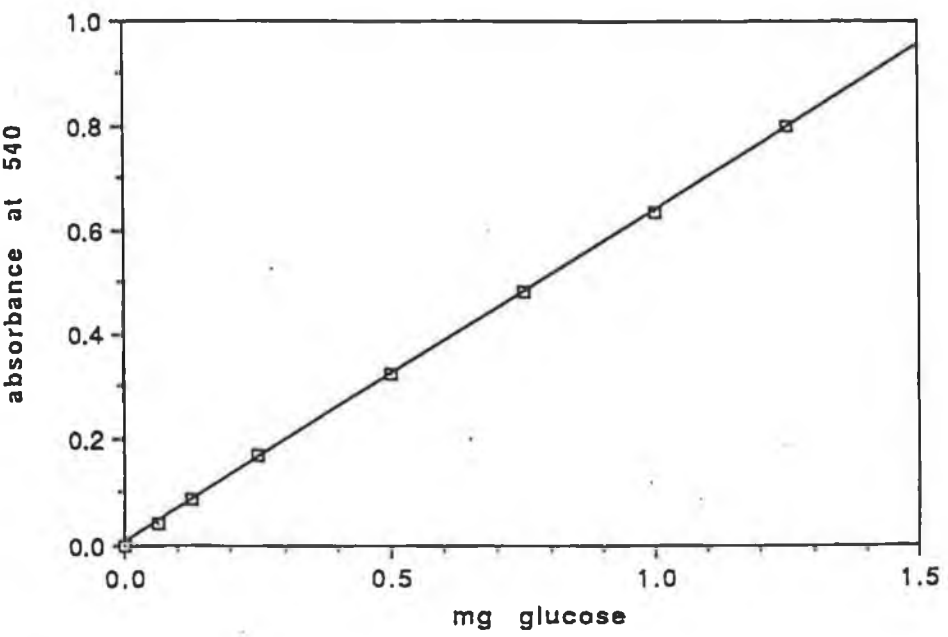
The units of amylase activity were calculated as follows.

$$\text{Units of enzyme activity} = \frac{\text{mg/glucose/ml per 30mins X D X RV}}{180.2 \times 10^{-6}}$$

Where D = Dilution factor.
R.V.= Total reaction volume.
180.2= Mol WT glucose.

Figure 3.10

Standard curve used for DNS assay.



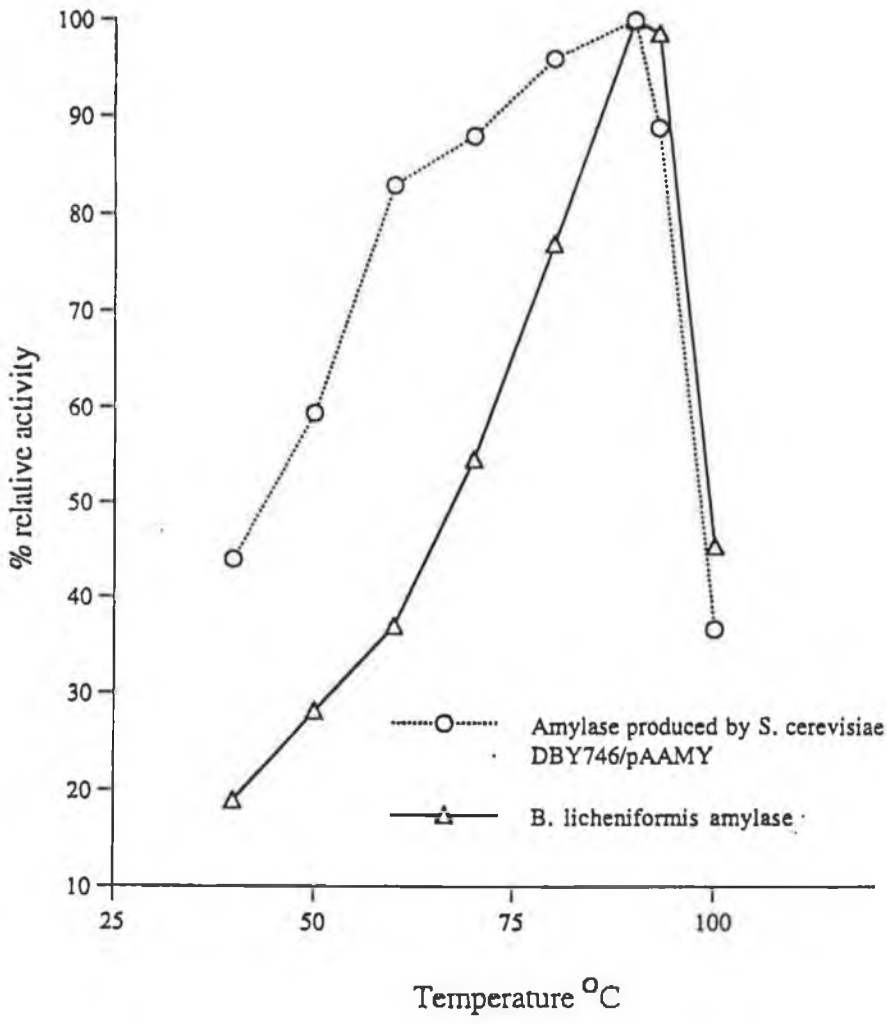

Standard curve for DNS assay.

### 3.5 Comparison of pH and temperature profiles of the native *B. licheniformis* amylase and the recombinant amylase produced by *S. cerevisiae.*

The pH and temperature profiles of both the commercial *B. licheniformis* amylase and the amylase produced by *S. cerevisiae* DBY746/pAAMY were determined and compared as shown in figs. 3.11 and 3.12. Amylase activity was quantified using the DNS assay. Culture supernatants of stationary phase (36 hours) DBY746/pAAMY were dialysed against PBS prior to being assayed to remove any reducing sugars which would otherwise have interfered with the assay. The pH profile was determined over the range pH 2 to 10 and the temperature profile over the range 35$^{o}$C to 100$^{o}$C. All assays were carried out in duplicate and the mean values are expressed as the relative percentage activity with the optimal conditions giving 100% activity. The two graphs show that the temperature and pH profiles of the two amylases are very similiar indicating that the *B. licheniformis* amylase secreted by *S. cerevisiae* retains similiar physiochemical characteristics to that of the native bacterial amylase.

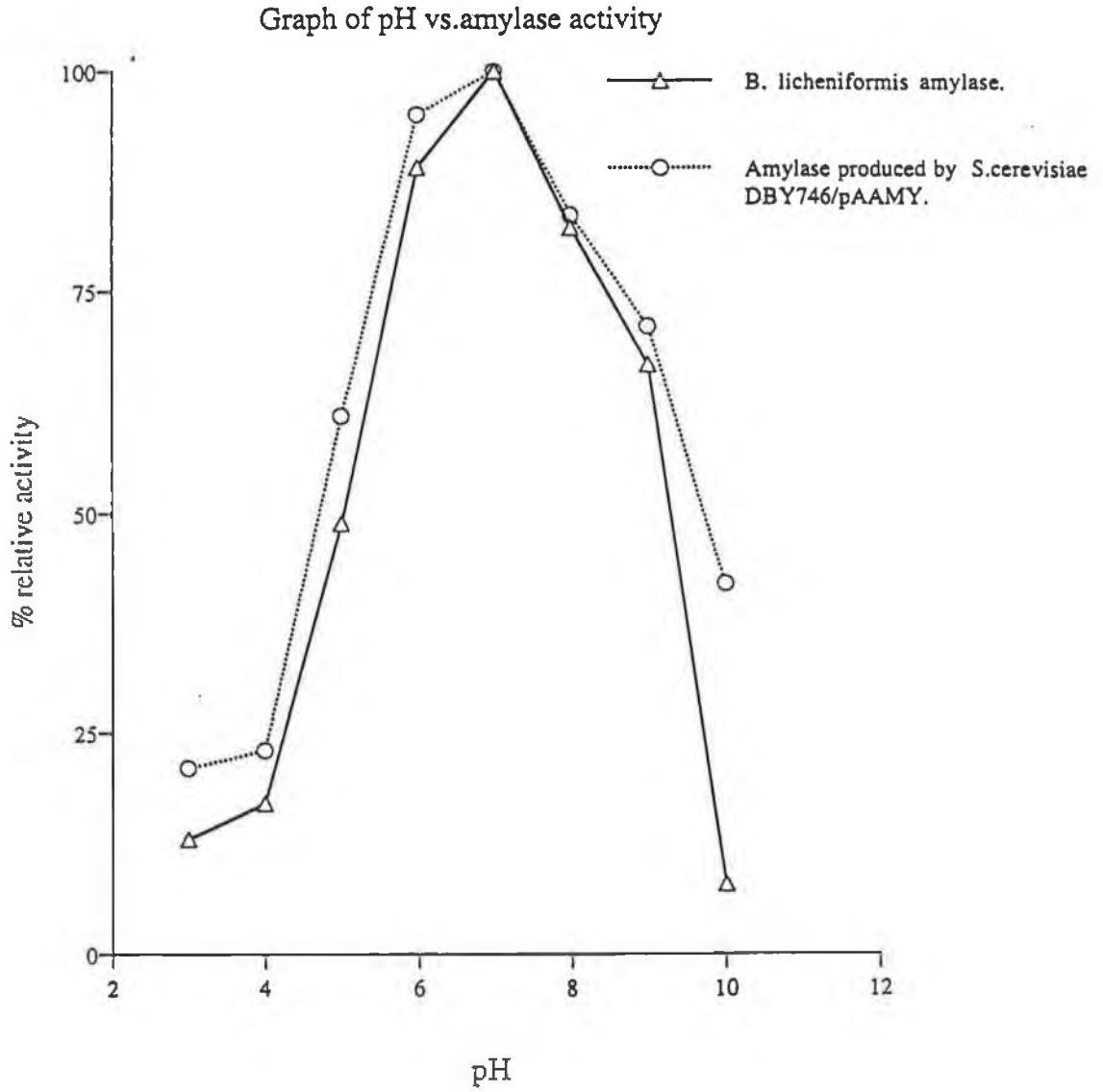
Figure 3.11.

Graph of temp.vs.amylase activity.




Comparison of the temperature profile of commercial and yeast produced *B. licheniformis* α-amylase.

Figure 3.12.



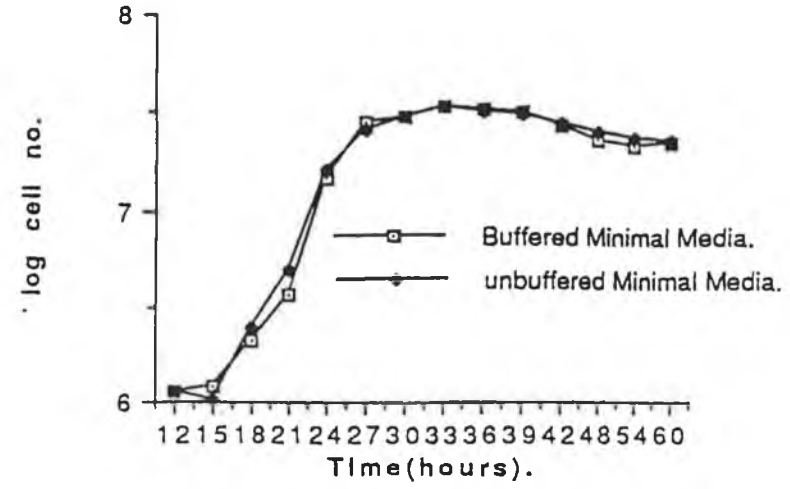

Comparison of the pH profiles of commercial and yeast produced *B. licheniformis* α-amylase.

**3.6. Effect of pH on the activity of the α-amylase produced by *S. cerevisiae.***

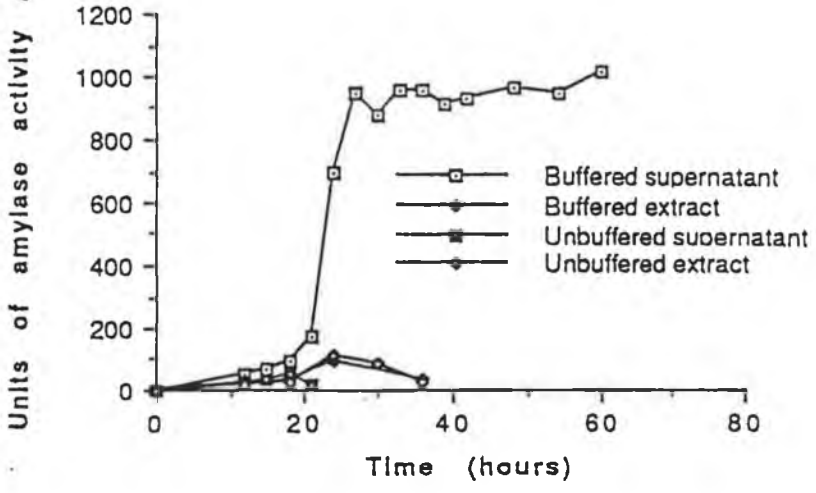
Initially when an attempt was made to measure the extracellular amylase activity produced by DBY746/pAAMY in unbuffered minimal media (sec 2.3) broth cultures no amylase activity could be detected. However when the medium was buffered to pH 6.2 with succinic acid and sodium hydroxide it was possible to detect amylase activity throughout the growth cycle. (Sodium phosphate buffer could not be used in liquid media as it results in a precipitate in the broth after autoclaving.)

Cell extracts for measuring the internal levels of amylase activity were prepared as in section 2.22. The levels of amylase activity were measured throughout the growth cycle, all activity determinations were carried out in duplicate and all cell counts were measured by direct cell counting in a haemocytometer. Figure 3.13 shows comparisons of both cell growth, pH and the levels of internal and external amylase present in buffered and unbuffered minimal media. It is significant to note that the level of internal amylase is very similiar in both the buffered and unbuffered media, and reaches a maximium of approximately 5% of the total amylase activity (supernatant plus extract) present in the buffered media. This data suggests that the majority of the amylase produced by the yeast is successfully transported out of the cell. The peak of internal amylase activity occurs at 24 hours and that of the external amylase is reached at 27 hours. From the graph it can be seen that the decrease in pH is concomittant with the loss of amylase activity in the supernatant of the unbuffered cultures. The buffering of the media does not significantly affect cell growth. All future amylase determinations were therefore carried out using buffered minimal media.

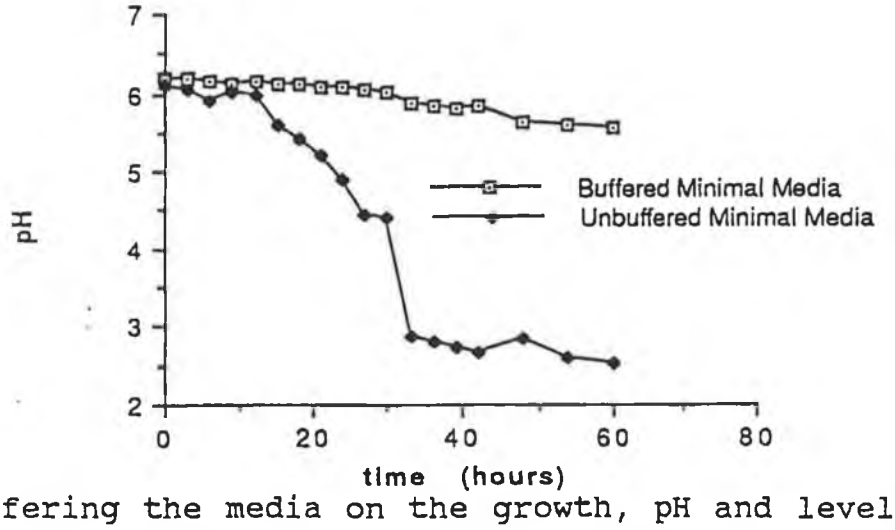
Figure 3.13. Graph of log cell no. versus time.




Graph of Amylase activity versus time —




Graph of decrese in pH against time. —




Effect of buffering the media on the growth, pH and levels of amylase activity in culture supernatants of DBY746/pAAMY.

**3.7. Comparison of the levels of amylase produced by DBY746/pAAMY when grown on YEPD and minimal media.**

The extracellular amylase produced in both rich (YEPD) and minimal media was determined throughout the growth cycle. Only the extracellular levels were determined as it had been shown previously that approximately 95% of the amylase produced by *S. cerevisiae* is located externally. The results are compared in figure 3.14(a). which shows that higher levels of amylase were produced by DBY746/pAAMY when grown in rich media. In the stationary phase (36hours) the amylase produced in rich media was approximately 5 times greater than that present in minimal media broth. Fig 3.14 (a) and (b) show that the higher level of amylase present in the rich media is due mainly to the increased cell population in the richer media. The specific activities (ie number of units of amylase activity per cell) at 36 hours in the growth cycle was 3.45 $x10^{-5}$ in minimal media and 2.45 x $10^{-5}$ in YEPD. Thus while the overall activity per ml was higher in YEPD the specific activity was highest in minimal media. To exclude the possibility that the low level of amylase detected internally may have been due to the procedure used to prepare cell extracts, the same procedure was carried out on DBY746 transformed with the plasmid YEpα1 (Ruohonen *et al.*, 1987). This plasmid, shown in appendix 2, contains the amylase gene from *Bacillus amylofaciens* cloned into the unique *Hin*d111 site of pAAH5. After transforming this plasmid into DBY746 the authors found that 25% of the amylase activity was in the cell extract. This plasmid could therefore be used as a control to check the validity of the results obtained using the above methods. The plasmid YEpα1 was transformed into DBY746 and extracts were prepared and assayed using the same procedures as those used for the *B. licheniformis* amylase, with the exception of the assay temperature which was 65°C. The internal level of amylase obtained for the *amylofaciens* amylase was 27% which is within 2% of that obtained by Ruohonen *et al.*, 1987 indicating that the methods used for the preparation and assay of cell extracts

were not responsible for the low levels of internal *B. licheniformis* amylase activity.

Figure 3.14.(a).

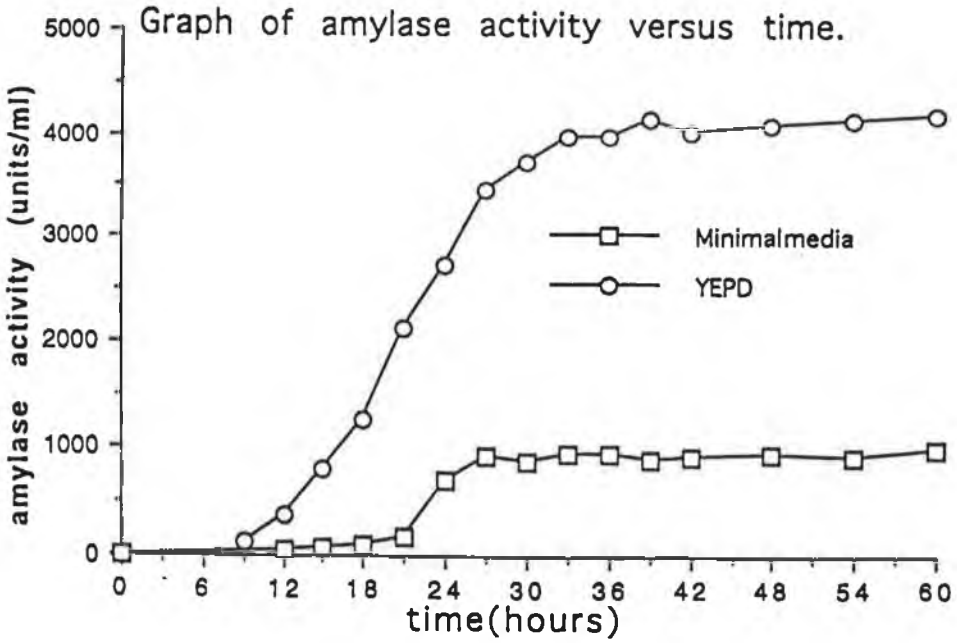
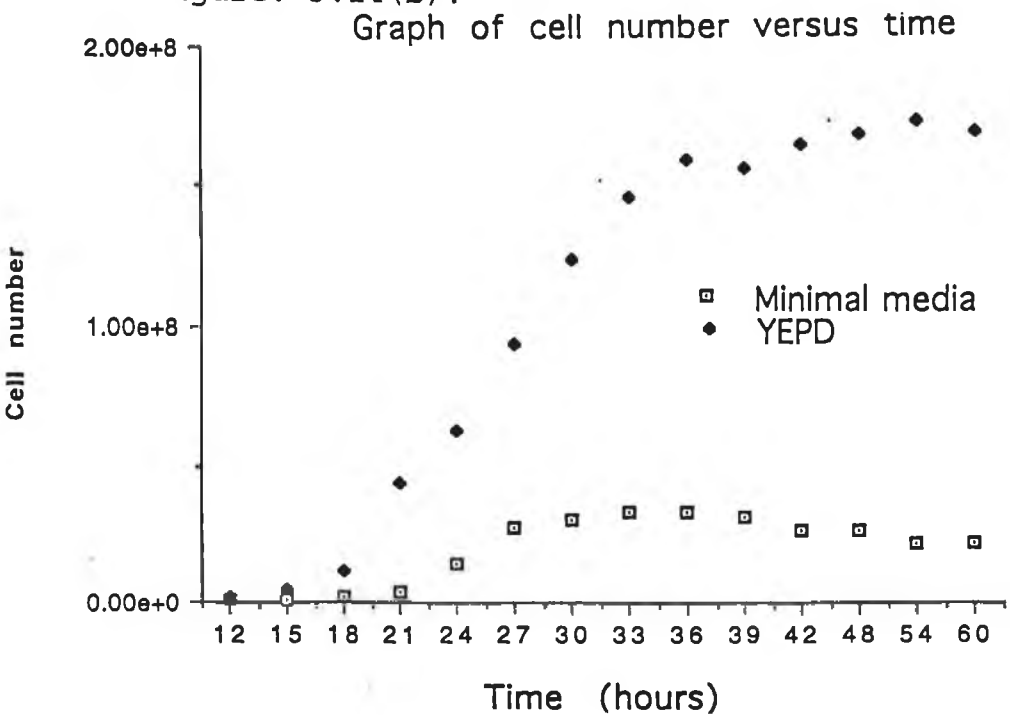



Figure. 3.14(b).



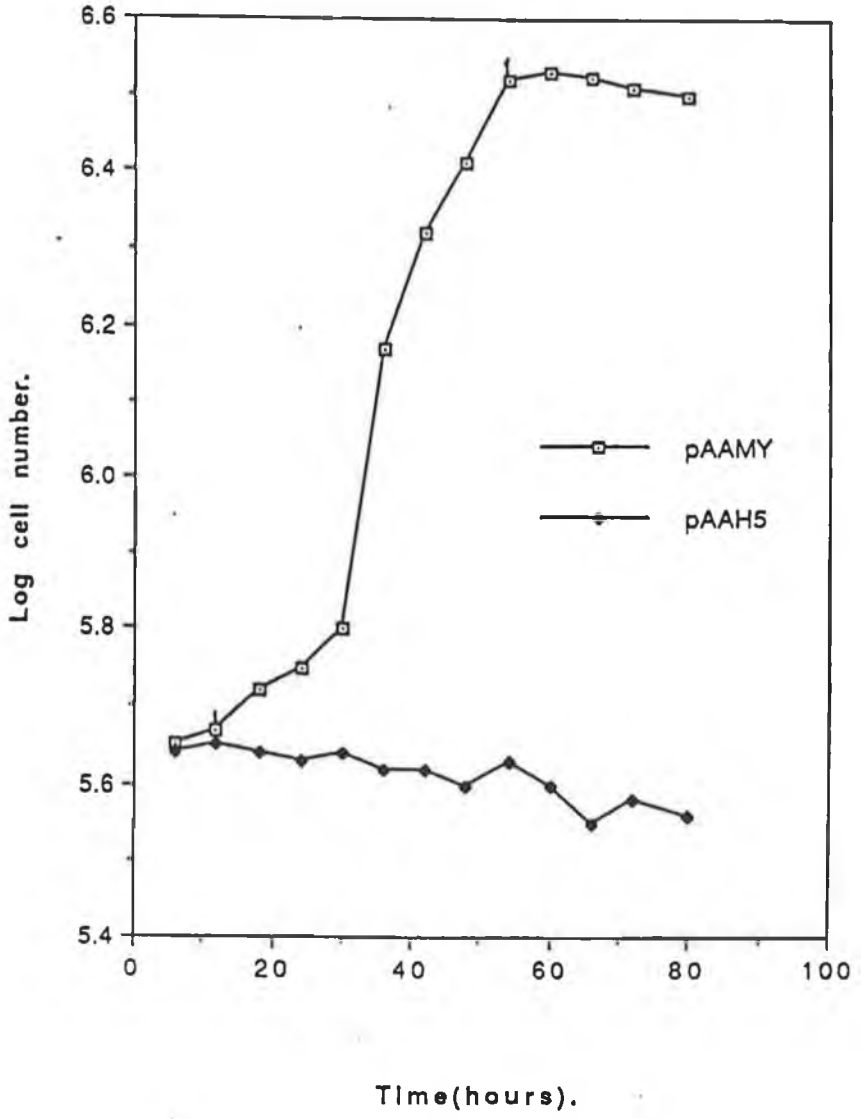

Comparison of the levels of amylase and cell numbers of DBY746/pAAMY when grown on YEPD and minimal media.

**3.8. Growth of DBY746/pAAMY using starch as the sole carbon source.**

To establish whether the yeast strain DBY746/pAAMY was capable of growth on starch as its sole carbon source the strain was grown up in buffered liquid minimal media which contained no glucose and 1% soluble starch (as higher levels of starch proved difficult to dissolve). The growth of the amylase producing strain DBY746/pAAMY and a control amylase minus strain DBY746/pAAH5 was monitored by plating out appropriate dilutions of each strain on YEPD. The use of a plate counting procedure was neccessary due to the presence of starch in the liquid media which made the media quite viscous and therefore difficult to count directly with a haemocytometer. Fig 3.15 shows that while there was no growth of the control strain DBY746/pAAH5 there was substantial growth of DBY746/pAAMY. The growth of DBY746/pAAMY on starch alone is characterised by a long exponential phase of 33 hours duration compared to an exponential phase of 15 hours when grown on minimal media containing 2% glucose. Cells grown in minimal media containing 2% glucose reached stationary phase after 27 hours growth (see Fig. 3.15(b)) whereas cells grown on minimal media containing 1% starch reached stationary phase after 50 hours growth. This longer exponential phase and the lower final cell number of the strain in this starch containing minimal media compared to growth in minimal medium containing 2% glucose may be attributed to the fact that before growth can take place there must be some amylase produced by this strain to digest the starch thereby releasing low molecular weight carbohydrates which can be taken into the cell and utilised as a carbon source by the yeast. The inoculum contained a small amount of amylase activity which is sufficient to breakdown a small quantity of starch which in turn leads to an increase in amylase secretion eventually leading to a delayed exponential phase. The lower final cell number can be attributed to the fact that only 1% starch was used in this media and α-amylase only catalyses the hydrolysis of

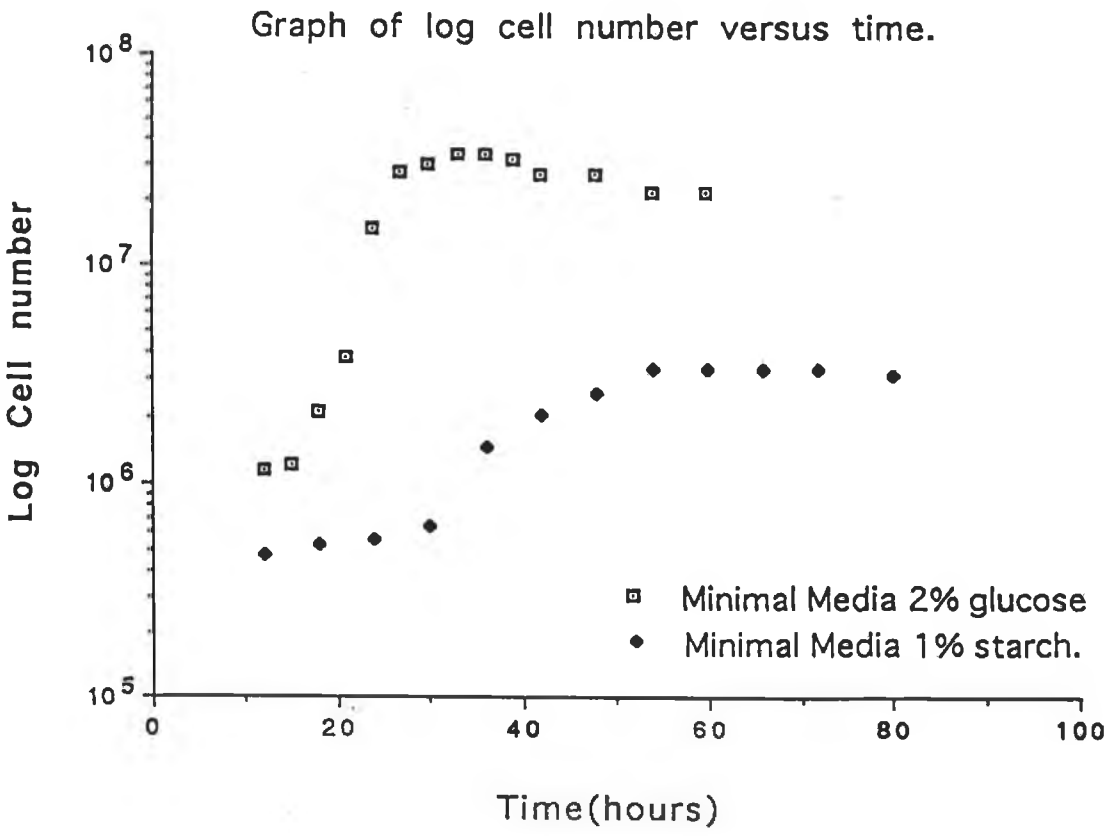
1,4-glusodic linkages in starch and does not therefore result in its complete hydrolysis. All the carbohydrate present in the starch was therefore not available in a substrate utilisable form. (See Fig.3.15(a).

Figure 3.15(a).




Growth of DBY746/pAAMY and DBY746/pAAH5 in minimal media containing 1% soluble starch as the sole carbon source.

Figure 3.15.(b).




**Comparison of the growth of DBY746/pAAMY when grown on minimal media with 2% glucose and 1% starch.**

**3.9. Analysis of the secretion of the *Bacillus lichenformis* α-amylase in *S.cerevisiae* secretion defective mutants.**

To establish if the α-amylase expressed in *S.cerevisiae* was directed through the normal yeast secretory pathway and not through a non-specific pathway or leakage from the cell, the ability of the *B.licheniformis* signal peptide to transport the α-amylase through the conventional pathway was examined in three secretion defective (*sec*) mutants. The characteristics of each of these *sec* mutants are given in table 3.1.

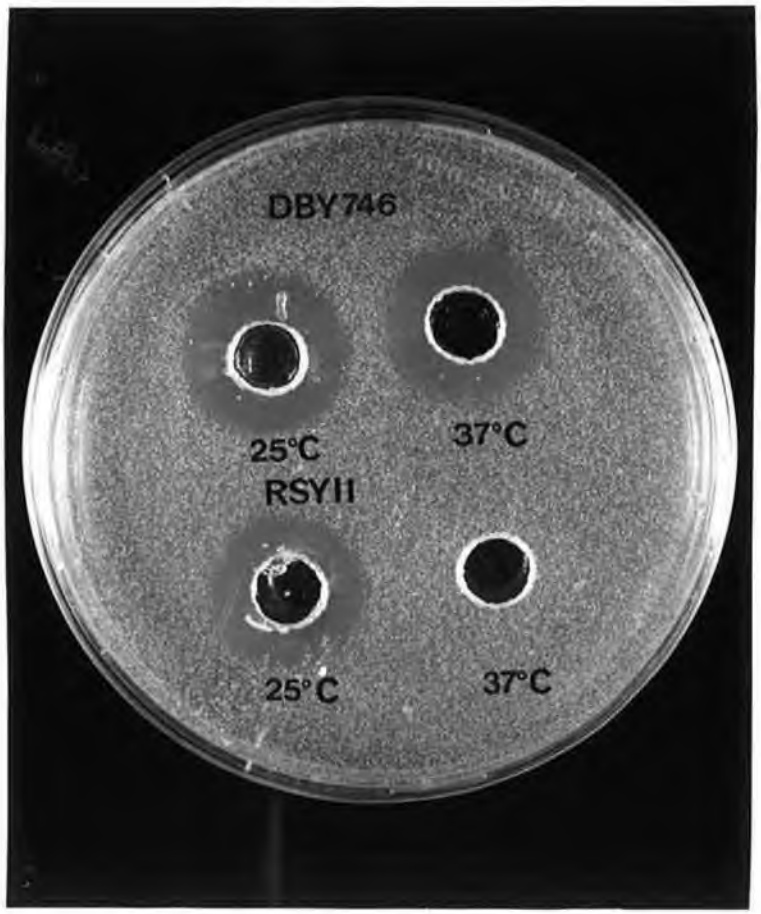
Table 3.1. Summary of *sec* mutants.

| Strain | Mutation | Block in secretory pathway |
|---|---|---|
| RSY12 | *sec* 53 | ER, glycosylation mutant |
| RSY11 | *sec* 18 | ER to Golgi (lacks protein involved in vesicle fusion) |
| RSY45 | *sec* 1 | Secretory vesicles between golgi and cell surface |

These mutants block the secretion of proteins via the normal yeast secretory pathway at the non-permisive temperature of 37$^{o}$C, while at the permissive temperature of 25$^{o}$C protein secretion is not blocked. To ensure that the *sec* defective phenotype was being induced in the three strains the levels of α-factor pheronome secreted in these mutants at the permissive and non-permissive temperature were determined. α-factor, produced by mat α strains, is one of the few homologous secreted proteins in yeast and it inhibits the growth of strains of the opposite mating type, i.e. mat a. RC631 is a mat a strain containing the *sst*-2-1

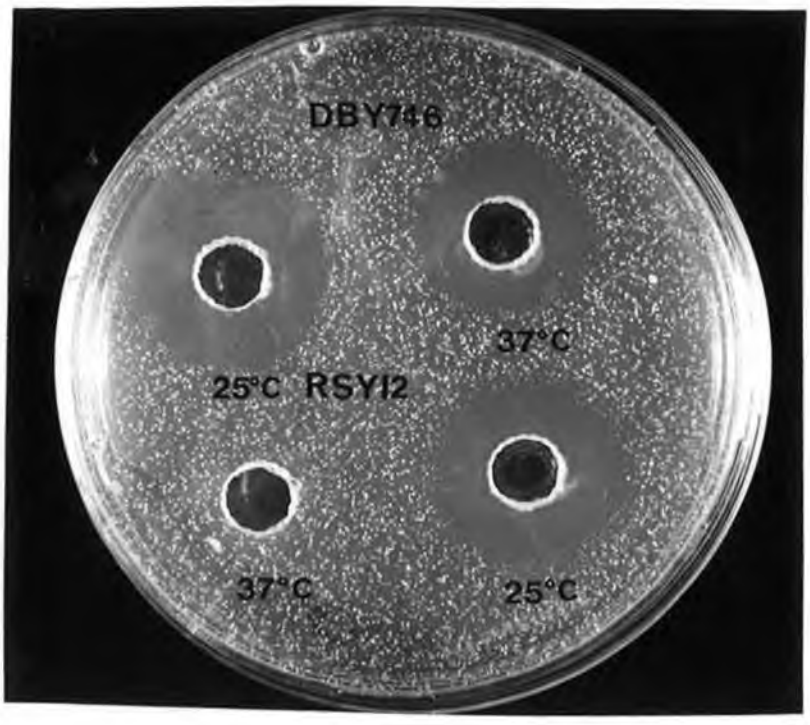
mutation, making it extra sensitive to the inhibitory effect of the α-factor mating pheromone (Julius *et al.*, 1983). RC631 was incorporated into MYGP agar as described in section 2.35. Wells (6mm in diammeter) were cut in the agar and 150μl of supernatant from strains RSY11, RSY12, RSY45 and DBY746 (as a negative control) grown at the permissive and non-permisive temperatures were placed in the wells. The plates were incubated at 30°C for 48 hours to allow the RC631 to grow. Results of this assay are shown in fig. 3.16 Clear haloes represent areas where the growth of RC631 was inhibited by the presence of α-factor in the supernatants.

Figure 3.16.




(a) Block in secretion of α-factor in RSY11.

Figure 3.16




(b) Block in secretion of α-factor in RSY12.

A similar result was obtained for *sec* 45 but is not shown. These results indicate that the mutant phenotype was being induced in the three *sec* strains as no inhibition of RC631 was observed at the non-permissive temperature of 37°C. No blockage occured in DBY746 as expected.

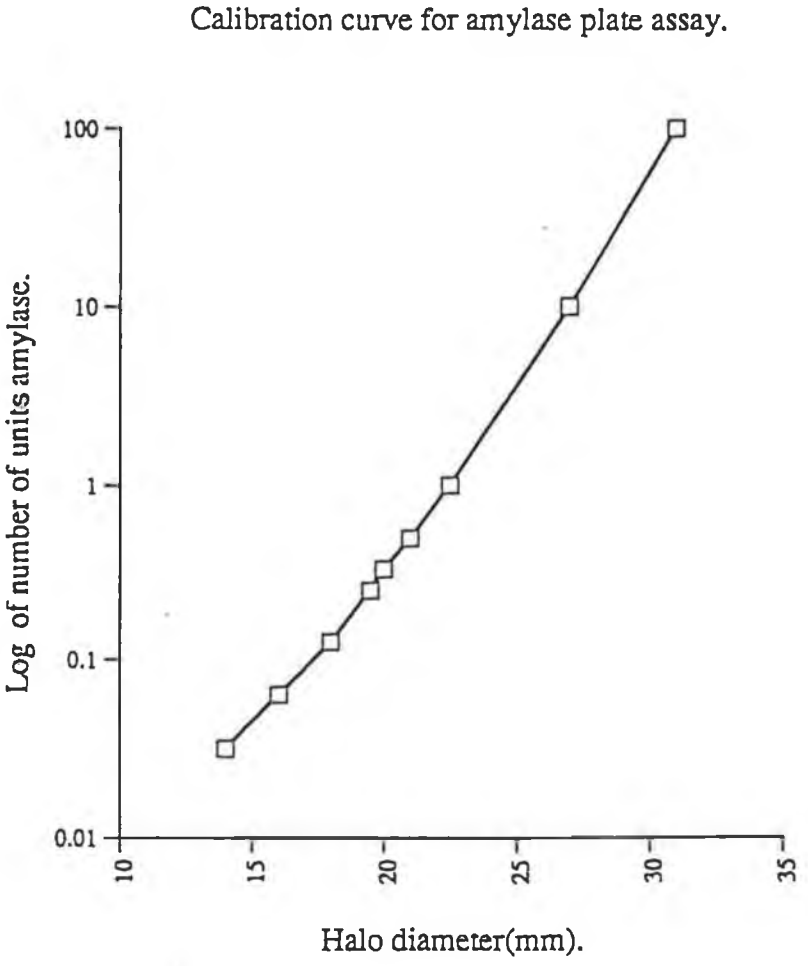
The α-amylase plate assay (2.17) was used for the analysis of amylase activity in the *sec* mutants. In order to show that the halo size was proportional to the level of α-amylase present, a calibration curve was constructed by analysing the size of haloes produced in the plate assay over the amylase concentration range 0.0625 units to 100 units of amylase activity. (Sigma *B.licheniformis* amylase Type X11A). One unit of amylase activity is defined as the amount neccessary to liberate 1mg of maltose from starch in 3 minutes at pH 6.9 and 20°C. Figure 3.17 shows the well assays used for the construction of the calibration curve which is shown in figure 3.18. The calibration curve is linear over this range but becomes non-linear above this range. The α-amylase plate assay could therefore be used as

a semi-quantitative tool for the measurement of α-amylase activity.

Figure 3.17.


Plate assays used for the construction of the standard curve for the α-amylase plate assay.

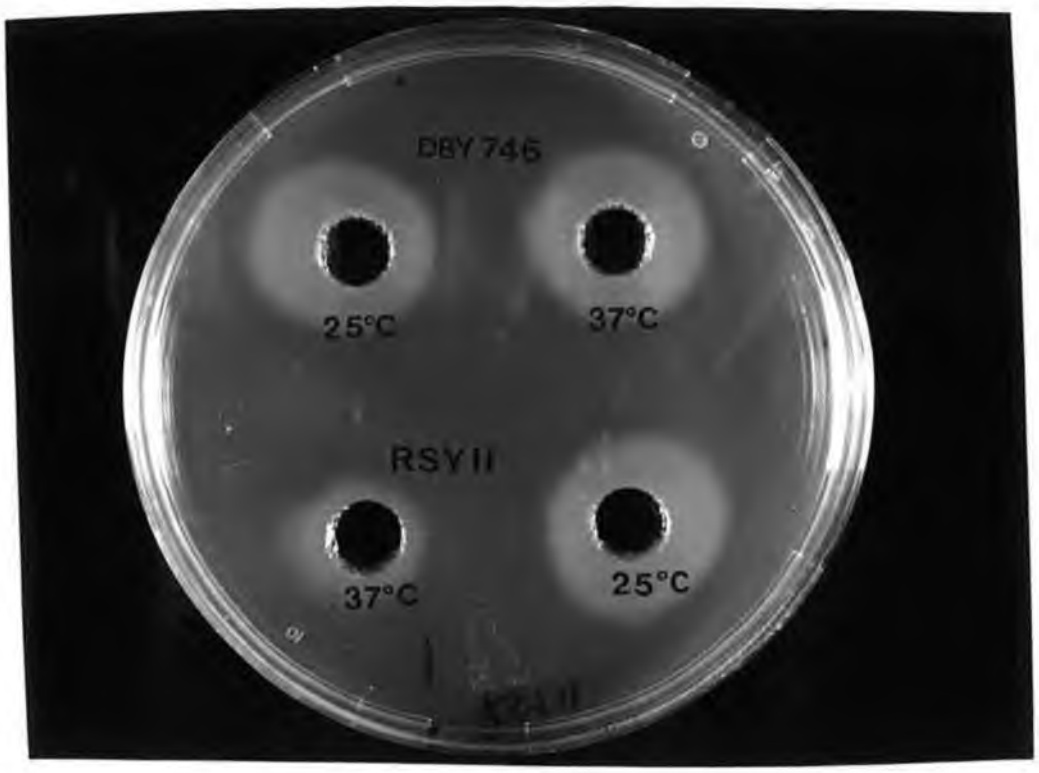
Figure 3.18.




Calibration curve for the a-amylase plate assay.

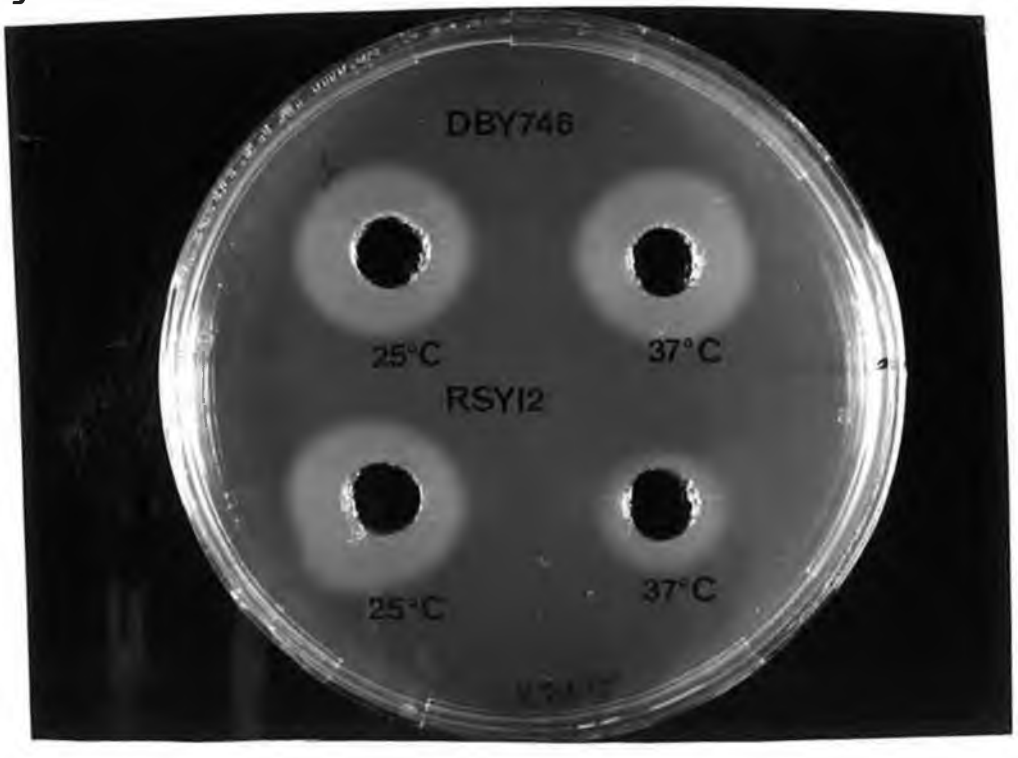
To analyse the secretion pattern of the *B.licheniformis* α-amylase in the secretion mutants, the plasmid pAAMY, containing the ADH1 promoter-α-amylase-ADH1 terminator was transformed into strains RSY11, RSY12 and RSY45. The transformed strains where grown in minimal medium at the permissive ($25^{o}C$) and the non-permissive ($37^{o}C$) temperatures and the supernatants were prepared as outlined in section 2.24. The supernatants were assayed for α-amylase activity using the above assay. Wells, 6mm in diammeter, were cut from buffered agar (pH 6.9) containing 2% Lintner's starch and 150μl of the supernatants were placed in the wells. Plates were incubated for 24-48 hours at $37^{o}C$ and clear haloes indicating α-amylase activity could be visualised surrounding the wells after a further incubation at $4^{o}C$ for 24 hours. Alternatively, haloes could be visualised immediately by inverting the plate over iodine crystals for 20-30 seconds. DBY746 transformed with pAAH5 was used as a negative control. The results in figs. 3.19 and 3.20 show that a significant block in secretion of α-amylase was observed in RSY11 and RSY 12 at the non-permissive temperature while at the permissive temperature α-amylase secretion was normal. This result suggests that the bacterial α-amylase signal is capable of directing the α-amylase through the normal yeast secretory pathway.

Fig 3.19




Block in secretion of α-amylase in RSY11/pAAMY.

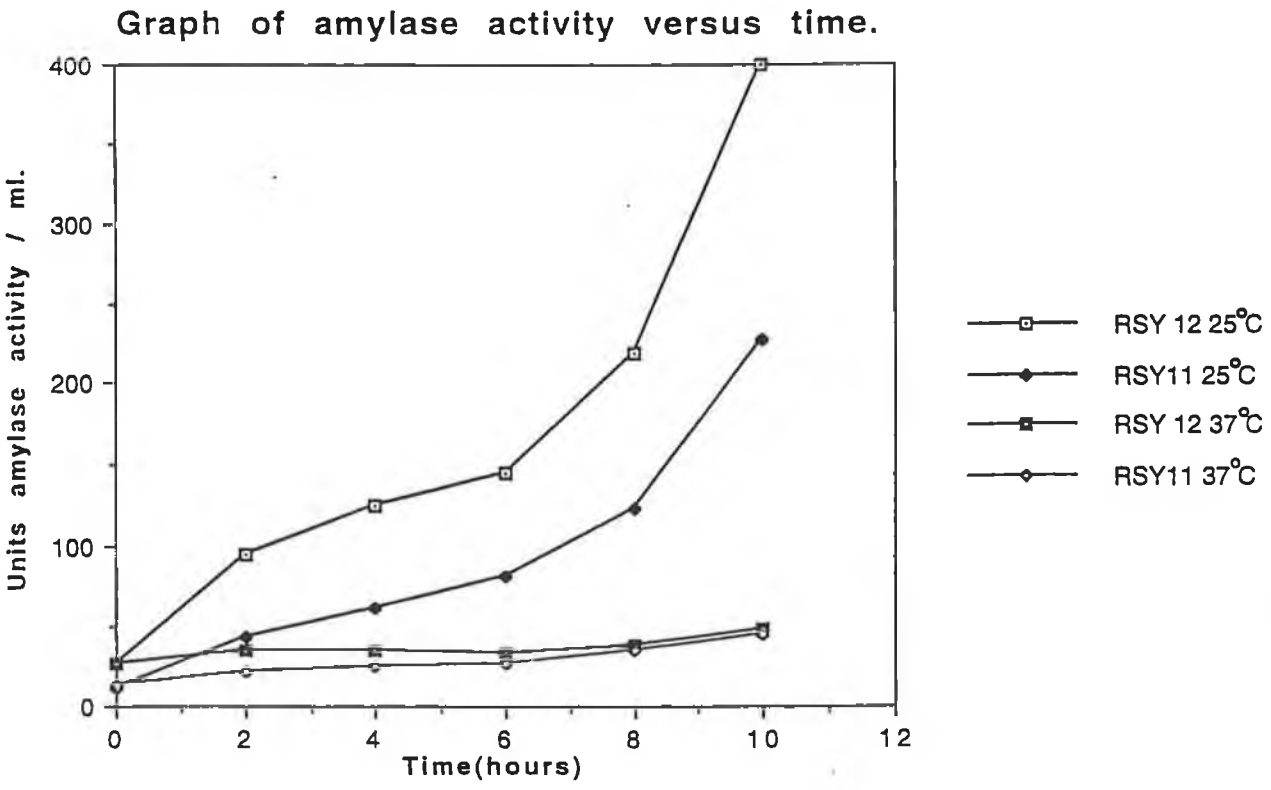
Figure 3.20.




Block in secretion of α-amylase in RSY12/pAAMY.
A similiar result was obtained for RSY45 but is not shown.

A quantitative assay of the levels of amylase secreted by the *sec* mutants RSY11 and RSY12 was also carried out. RSY11 and 12, transformed with pAAMY, were grown up to $O.D._{600}$ 0.15 in two 100ml flasks for each strain. One of each of the cultures was then transferred to 37°C and all cultures were allowed to grow for a further 10 hours. Samples were taken every two hours and the level of α-amylase activity was determined using the DNS assay as described in section 2.18. The graph shown in fig. 3.21 further supports the conclusion that the bacterial amylase signal is capable of being recognized by the normal yeast secretory pathway, as secretion of the amylase is greatly reduced after incubation at 37°C.

Figure 3.21.




Block in secretion of α-amylase at 37°C in RSY11/pAAMY and RSY12/pAAMY.

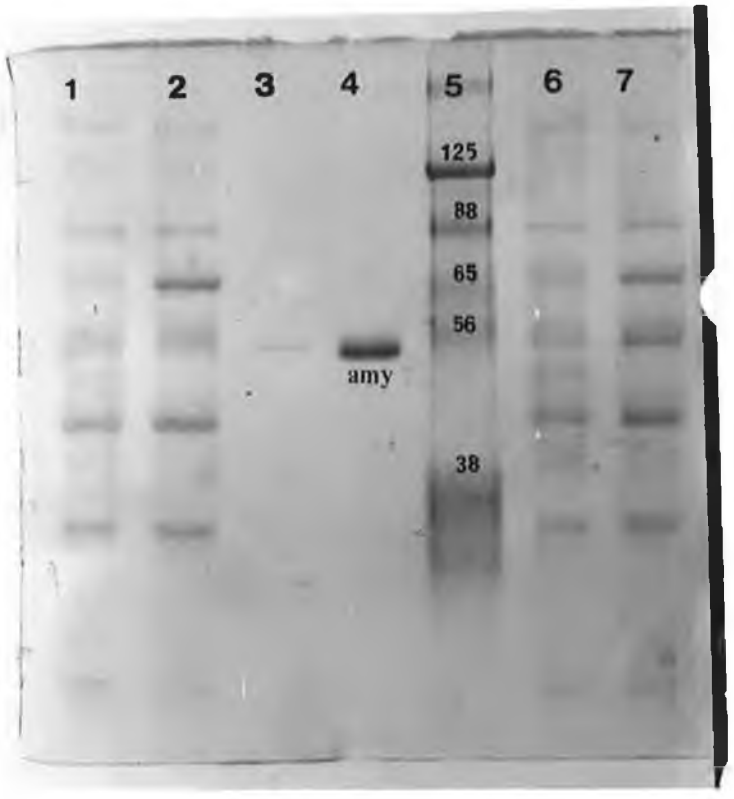
**3.10. Analysis of *B.licheniformis* α-amylase expressed in *S.cerevisiae* using SDS polyacrylamide gel electrophoresis (PAGE).**

In order to further characterise the amylase expressed in *S.cerevisiae*, the supernatants of DBY746, transformed with pAAMY (containing the amylase gene of *B.licheniformis)* and pAAH5 (the parent vector of pAAMY as a negative control) were analysed on SDS PAGE gels. DBY746, transformed with the plasmids was grown to stationary phase ($O.D._{600}$ of 1.15) and 500ml of the supernatants were harvested and concentrated to 5ml, aliquoted in 100μl samples and freeze-dried as described in section 2.23. Samples were then resuspended in 100μl of 1X solubilisation buffer (2.6). This sample, which was equivalent to 10ml of the original culture, was loaded on a 10% polyacrylamide gel and electrophoresed for 12 hours at 100 volts. After overnight staining of the gel in coomassie brillant blue, the gel was destained and photographed (fig.3.22).

The result showed that it was not possible, using coomassie staining, to localise a specific band in the pAAMY supernatant corresponding in size to the native α-amylase from *B.licheniformis*. The native *B. licheniformis* amylase has a molecular weight of 55,200 (Yukki *et al,* 1986), the bands shown in lanes 3 and 4 are the predicted size for the amylase. While there are bands present at approximately the same molecular weight as the *B.licheniformis* α-amylase, these bands are also present in the control pAAH5 supernatant. An identical gel to the one shown was also silver stained (not shown) but this staining procedure produced a high level of background bands and was not helpful in determining the molecular weight of the secreted amylase. Therefore it was necessary to take another approach to estimate the size of the recombinant amylase produced by *S. cerevisiae*. The protein could either be purified to homogeneity or a polyclonal antibody could be raised to the native *B.licheniformis* α-amylase. The latter method was chosen as it was felt that the antibody raised would be more useful for characterising the recombinant

α-amylase and that Western blots would be a more sensitive way of detecting the amylase present in the supernatants and extracts of strains expressing the amylase.

Figure 3.22




Commassie stained gel of the proteins present in the culture supernatants of DBY746/pAMMY and DBY746::pAAH5.

Lane 1 :DBY746/pAAH5 supernatant 1:3 dilution.
Lane 2 :DBY746/pAAH5 supernatant.
Lane 3 :Commercial *B.licheniformis* amylase (1 unit).
Lane 4 :Commercial *B.licheniformis* amylase (10 units).
Lane 5: Prestained molecular weight markers.
Lane 6 :DBY746/pAAMY supernatant 1:3 dilution.
Lane 7 :DBY746/pAAMY supernatant.

**3.11. Raising a polyclonal antibody to *B. licheniformis* α-amylase.**

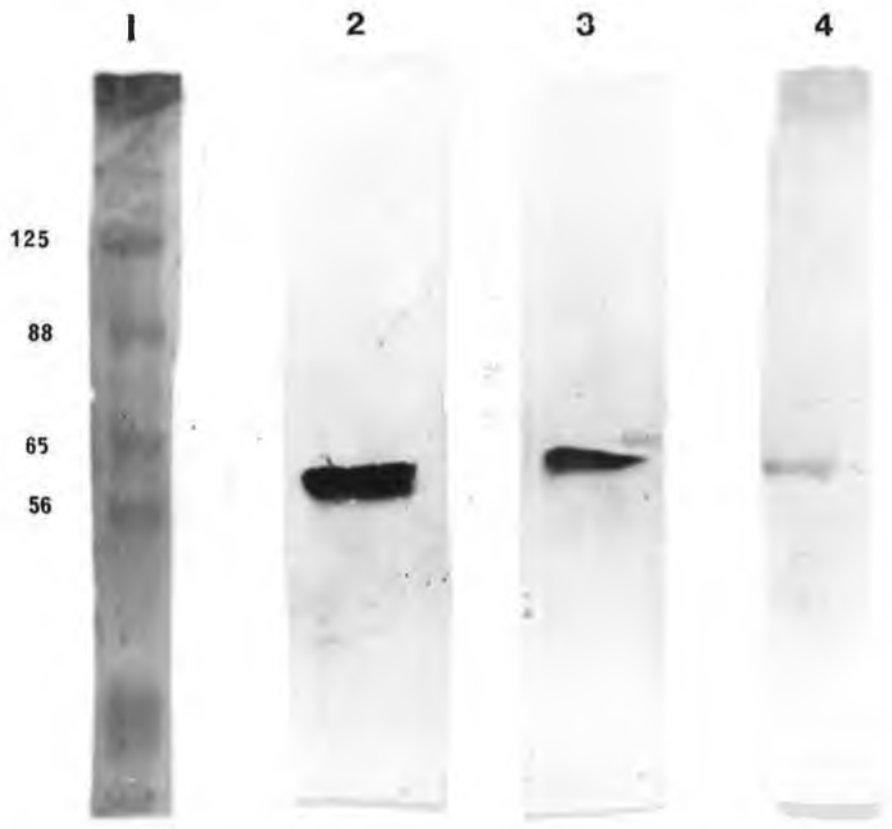
Commercial *B. Licheniformis* α-amylase (250μl) (Sigma, type X11A, units), shown to be largely free of contaminating proteins (coomassie gel not shown), was made up to 500μl with sterile PBS. Freunds complete adjuvant (0.5ml) was added and the solution sonicated until it had emulsified. This emulsion was then injected subcutanenously into a Wister rat. After 4 weeks the process was repeated, this time using Freunds incomplete adjuvant. Two further immunizations were carried out at 2 week intervals using Freunds incomplete adjuvant. One week after the last injection, 1ml of serum was removed, allowed to clot overnight at $4^{o}C$ and then cleared by centrifugation at 10,000 rpm for 10 minutes. The serum was tested for reactivity to the commercial α-amylase by Western blotting. When the reactivity was established, aproximately 15ml of blood was extracted from the rat and treated as above. Approximately 9ml of antiserum was recovered and the antibody preparation was titered by both ELISA and Western blotting.

3.11.1. Titering of the α-amylase polyclonal antibody.

Western blot analysis:

One unit of *Bacillus Licheniformis* α-amylase (Sigma type X11A) was electrophoresed in each of 3 separate lanes on a 10% SDS polyacrylamide gel. After electrophoresis the protein was transferred to nitrocellulose as described in section 2.27. The nitrocellulose was then cut in 3 and titred against 1/100, 1/1,000, and 1/10,000 dilutions of the primary α-amylase antibody by Western blot analysis. The result of this analysis is shown in fig. 3.22. The Western blot analysis of the antibody showed that it bound to a band of molecular weight of approximately 55,000, which corresponds to the size of the *B. licheniformis* amylase and was present in a high titre as even at the 1/10,000 dilution of antibody a strong positive band is visible.

Figure 3.23.




Western blot used for titering the polyclonal antibody raised to the commercial *B.licheniformis* amylase.

1 : prestained molecular weight markers.

2 : 1/100 dilution of amylase antibody.

3 : 1/1000 dilution of amylase antibody.

4 : 1/10000 dilution of amylase antibody.

3.11.2. ELISA of the α-amylase antibody.

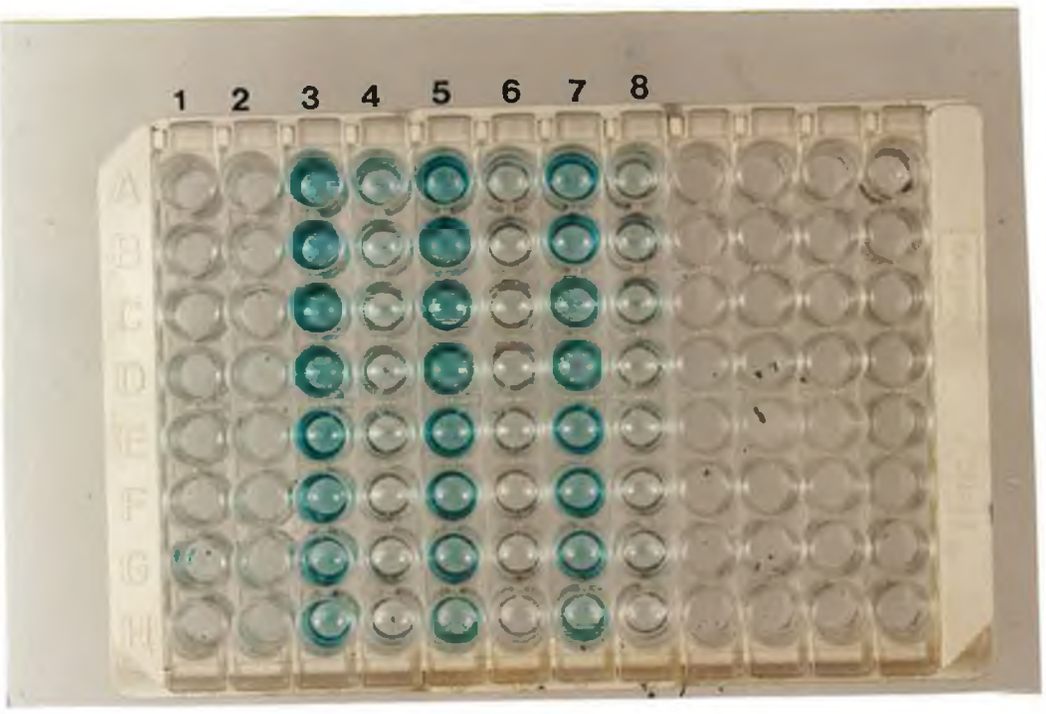
The antibody titre was also estimated by the use of an ELISA (Enzyme Linked Immuno Sorbant Assay) which was carried out as outlined in section 2.34. Table 3.2 is a representation of the 96 well plate used in the assay. Reagents added to each well are indicated. Figure 3.24. shows the results of the ELISA showing that there was a strong positive signal up to a dilution of 1/16000 of the primary amylase antibody. It also showed that there was no positive signal present in any of the negative controls.

Table 3.2. Samples loaded on microtitre plate for ELISA.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| A | PBS | PBS | 1/100 | 1/32K | 1/100 | 1/32K | 1/100 | 1/32K |
| B | PIS | PIS | 1/250 | 1/64K | 1/250 | 1/64K | 1/250 | 1/64K |
| C | MM | MM | 1/500 | 1/128K | 1/500 | 1/128K | 1/500 | 1/128K |
| D | $H_2O$ | $H_2O$ | 1/1000 | 1/250K | 1/1000 | 1/250K | 1/1000 | 1/250K |
| E | YEPD | YEPD | 1/2000 | 1/512K | 1/2000 | 1/512K | 1/2000 | 1/512K |
| F | PIS | PIS | 1/4000 | 1/1024K | 1/4000 | 1/1024K | 1/4000 | 1/1024K |
| G | MM | MM | 1/8000 | PIS | 1/8000 | PIS | 1/8000 | PIS |
| H | PBS | PBS | 1/16000 | MM | 1/16000 | MM | 1/16000 | MM |

PBS = Phosphate buffered saline.
MM = buffered minimial media.
PIS = Preimmunised serum.
YEPD = Yeast extract peptone broth.

Figure 3.24.




ELISA assay of the α-amylase antibody.

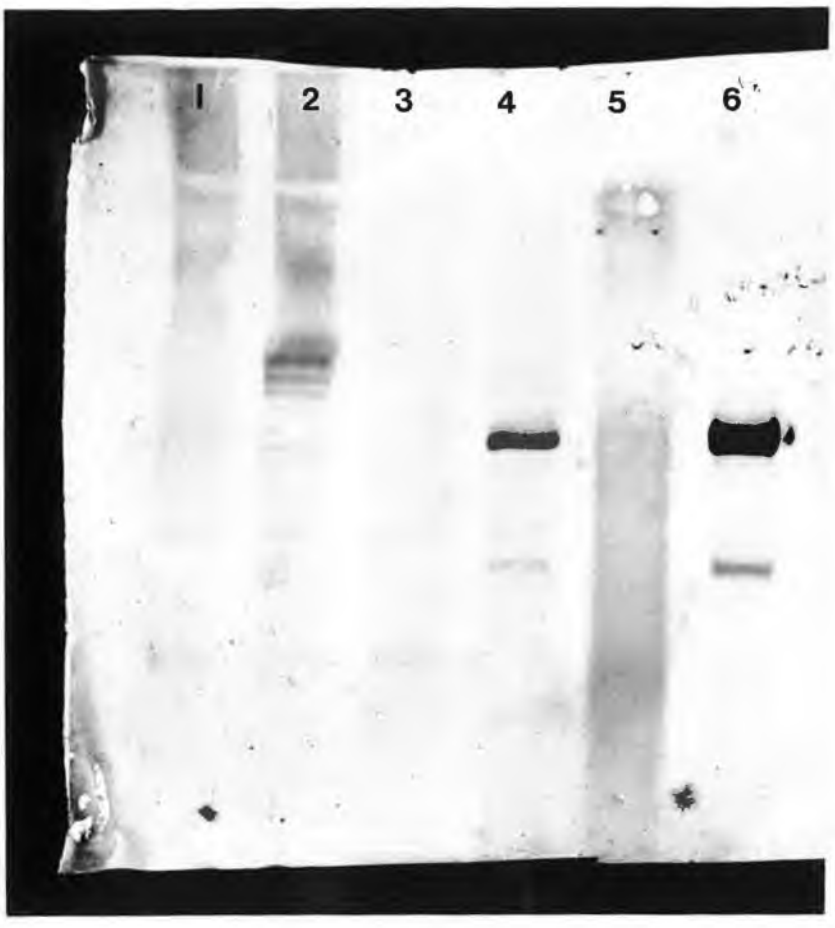
**3.12. Western blot analysis of the *B. licheniformis* α-amylase produced by *S.cerevisiae*.**

Extracts and supernatants of DBY746 transformed with pAAMY and pAAH5 (as a negative control) were prepared as outlined in sections 2.23 and 2.24. The quantity of sample loaded on the polyacrylamide gel was prepared from approximately 3 x $10^8$ cells taken at a time interval of 30 hours into the growth cycle (see fig 3.14). After electrophoresis the proteins were transferred to nitrocellulose and probed with the amylase antibody (2.27). Fig. 3.25 shows the result of this Western blot. The protein sizes were estimated from prestained molecular weight markers which were run on the same gel and subsequently cut off and stained with commassie blue. (not shown in figure).

The result showed that there was no amylase band present in the supernatant of DBY746 transformed with pAAH5 (lane 3, the amylase negative plasmid), while in the supernatant of DBY746 transformed with pAAMY (lane 2) a faint positive band corresponding in size to the molecular weight (55,200)

of the native amylase from *B.licheniformis* was visible. An additional series of stronger bands recognised by the antibody were also observed in the supernatant of DBY746 transformed with pAAMY. These bands, at molecular weights 6-16 kd higher than the *B.licheniformis* amylase could have resulted from inefficient processing of the signal peptide and/or core glycosylation of the amylase as it proceeded through the yeast secretory pathway or a combination of both. The amylase signal peptide is 29 amino acids long and if the signal peptide had not been cleaved it would lead to an increase in molecular weight of the amylase of approximately 3kd. The *B. licheniformis* amylase while not glycosylated in its native environment contains 7 potential glycosylation sites (see appendix 1). Each core glycosylation event leads to an increse in molecular weight of approximately 3kd (Melnick *et al*, 1991). The largest increase in molecular weight would therefore be 24kd (7 core glycosylations plus the uncleaved signal peptide). No amylase positive bands were visible in the extract of DBY746/pAAMY (lane 1). This is probably due to the fact that only approximately 5% of the total amylase activity is located intracellularly (Fig 3.13).

Figure 3.25




Western blot showing the multiple amylase bands present in the supernatant of DBY746/pAAMY.

Lane 1 : DBY746/pAAMY extract.
Lane 2 : DBY746/pAAMY supernatant.
Lane 3 : DBY746/pAAH5 supernatant.
Lane 4 : 1 unit native *licheniformis* amylase.
Lane 5 : Prestained molecular weight markers.
Lane 6 : 10 units native *licheniformis* amylase.
The size markers were stained separately and used to determine the size of the bands present on the western blot. (not shown in photo).

**3.13. Development of a sensitive assay for the detection of amylase activity in polyacrylamide gels.**

Initially, a zymogram/overlay gel was used to analyse whether or not the additional bands observed in the Western blot (fig. 3.25) were active amylase bands. Samples of supernatant and extract were electrophoresed on a 10% SDS polyacrylamide gel which was then overlaid with a thin 1% agarose gel containing 0.05% starch. The two gels were covered with foil and incubated at 37$^{o}$C for time periods ranging from 20 minutes to 12 hours. The starch-containing overlay gel was then stained with 0.01% $I_2$/0.1% KI in 1N HCl to localise the position of the amylase positive bands. Bands were visible as clear areas against a dark violet background, however the bands observed were very diffuse and it was not possible to determine which of the multiple amylase bands seen on the Western blot were active. It was therefore necessary to develop an activity gel capable of resolving active amylase bands which differed in molecular weight by as little as 3kd.

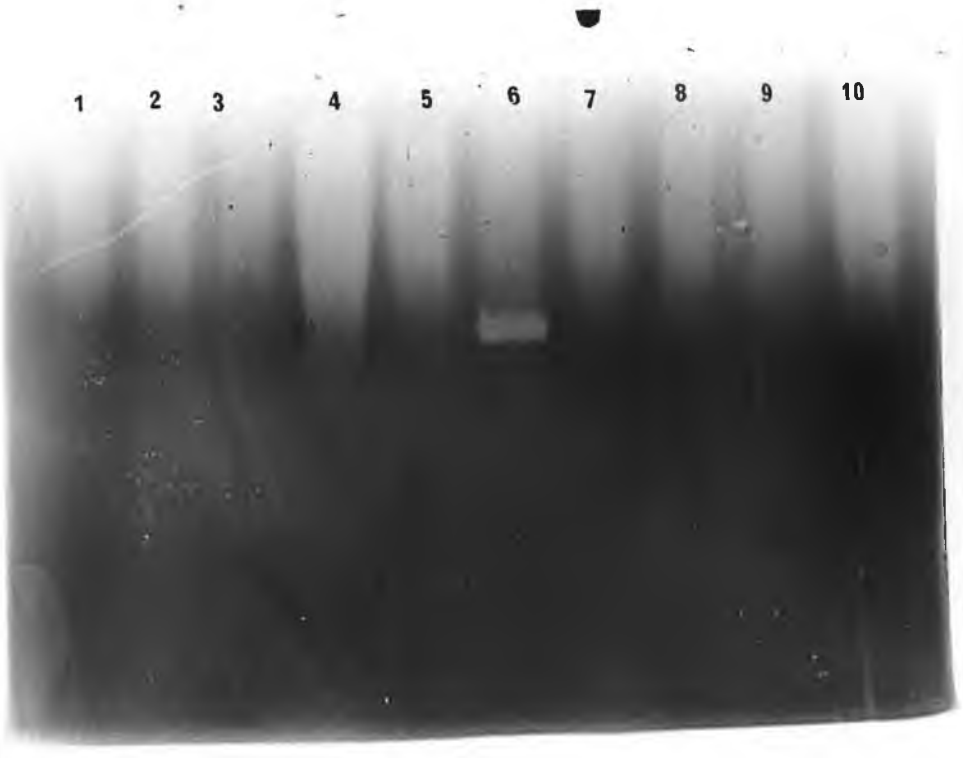
Initially, the activity gel (10% SDS polyacrylamide) was made in a 1% starch solution in place of distilled water but the running time of this gel was approximately 25% longer than the ordinary gel and resulted in overheating of the gel apparatus. Subsequently, gels were made in a starch concentration of 0.05% as used in the zymogram above and gels ran normally. Comparing the ordinary and starch-containing gels by coomassie staining, the inclusion of starch in the gels was found to have no adverse effect on the protein banding pattern (not shown). To visualise amylase bands on the activity gel a renaturation step had to be carried out followed by an incubation to allow the renatured amylase to degrade the starch incorporated in the gel. Optimisation of the clarity and intensity of the amylase bands was carried out by varying the renaturation conditions and the conditions used for degradation of the starch. In the renaturation step HEPES and phosphate buffers were used at pH 6.9 and the concentration was varied from 0.01M to 0.1M; Triton-X-100 was varied from

0.025% to 1%; the incubation temperature was varied from 20°C to 65°C and the incubation period from 20 minutes to 6 hours, replacing the incubation buffer with fresh buffer midway for the longer incubations.

For the degradation step, HEPES and phosphate buffer (pH 6.9) were again used and the concentration was varied from 0.01M to 0.1M; the incubation temperature was varied from 20°C to 65°C and the incubation period from 20 minutes to 24 hours. The calcium chloride concentration in the degradation buffer was varied from zero to 20mM. After the renaturation and degradation steps the gels were stained with 0.1% KI/0.01% $I_2$ in 1N HCl to visualise the active amylase bands. Optimum conditions established were as follows: gels (10% polyacrylamide containing 1% starch) were renatured by two 1 hour incubations at 20°C in 0.1M HEPES (pH 6.9), 0.25% Triton-X-100 with subsequent incubation of the gel for 8 hours at 37°C in 0.1M HEPES (pH 6.9), 5mM $CaCl_2$. The improvement in the visibility of the amylase bands in the presence of calcium was expected as the *B.licheniformis* α-amylase is a calcium-dependent enzyme. Fig. 3.27 shows a gel run under the above conditions.

It can be seen from the gel that there was a smear of amylase activity running from the top of the gel to the position of the amylase band. This smearing was most likely due to a small percentage of the amylase retaining activity after solubilisation, as the enzyme is highly thermostable with a temperature optimum of 93°C. During electrophoresis, heat generated in the system was sufficient to allow this active amylase to degrade the starch in the gel, resulting in the pattern observed in fig. 3.26. This smearing may have masked the amylase bands present in the samples prepared from DBY746/pAAMY which can be clearly seen on subsequent activity gels (Figs 3.27, 3.28).

Figure 3.26.




Amylase activity gel showing the smearing of the amylase activity.

Lane 1 : DBY746/pAAMY extract.
Lane 2 : DBY746/pAAH5 extract.
Lane 3 : DBY746/pAAH5 supernatant.
Lane 4 : DBY746/PAAMY supernatant.
Lane 5 : Prestained molecular weight markers.
Lane 6 : 10 units native *licheniformis* amylase.
Lane 7 : 1 unit native *licheniformis* amylase.
Lane 8 : 0.1 unit native *licheniformis* amylase.
Lane 9 : DBY746/PAAMY supernatant 1/10 dilution.
Lane 10: DBY746/PAAMY extract 1/10 dilution.

In order to try to minimise this background amylase activity subsequent gels were run at 4°C (60V, 18 hours), all buffer components were precooled to 4°C before use and amylase samples were boiled for 10 minutes prior to loading on the gels. This approach was successful, as can be seen in fig. 3.27. Reducing the quantity of the standard *B.licheniformis* amylase loaded was also shown to reduce the level of background amylase activity (fig. 3.27). An attempt was made to remove all background amylase activity by including 5% urea in the gels, solubilisation buffer and running buffer but it proved impossible to detect any amylase activity subsequent to renaturation, incubation and staining. The gel in fig 3.27 also contains the supernatant from DBY746 transformed with pAAMY in lane 5. An amylase band identical in size to the native *B.licheniformis* amylase is clearly visible. In addition, 5-6 active higher molecular weight forms (indicated by the arrow) of the amylase were also visible on the gel (not clearly visible in the photograph, shown more clearly in fig 3.28). These bands correspond in size to the higher molecular weight bands observed on the Western blot (fig. 3.25). Thus the procedure developed was shown to be effective in clearly resolving all the active amylase bands.

Figure 3.27.

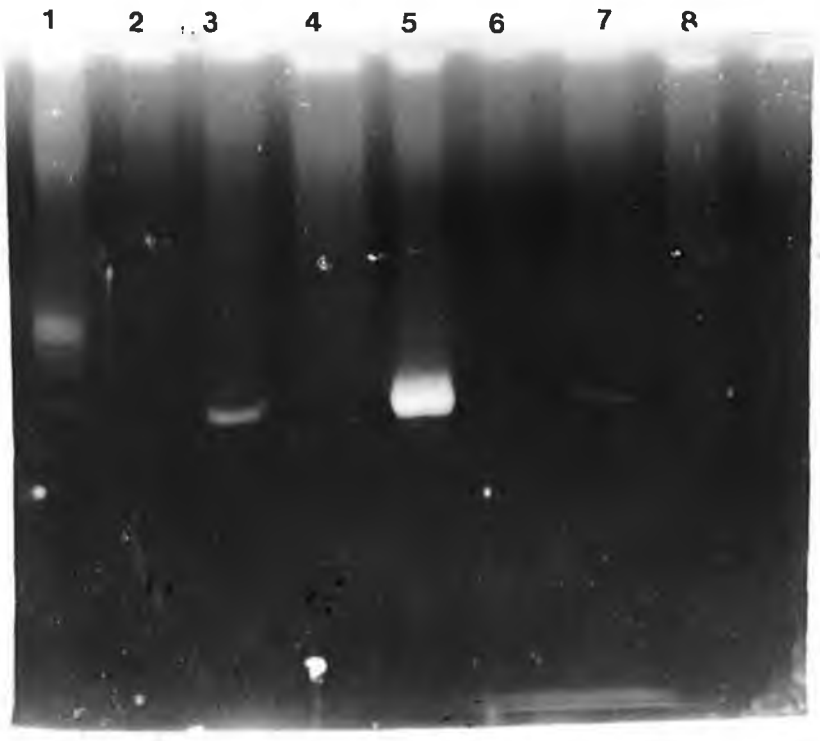

Amylase activity gel run at 4°C showing decrease in smearing. (The additional active amylase bands are also visible indicated by the arrow).

Lane 1 : 10 units native *B.licheniformis* amylase.
Lane 2 : 1 unit native *B.lichemiformis* amylase.
Lane 3 : 0.1 units native *B.licheniformis* amylase.
Lane 4 : Prestained molecular weight markers.
Lane 5 : DBY746/pAAMY supernatant.

**3.14. EndoH$_f$ treatment of the *B.licheniformis* α-amylase expressed in *S.cerevisiae.***

As incomplete processing of the signal peptide would lead to an increase of only 3kd in the size of the amylase, all of the multiple amylase bands detected in the supernatant samples of DBY746/pAAMY (figs. 3.25, 3.27) could therefore not be due to the incomplete processing of the signal peptide. To ascertain whether the increases in molecular weight were due to core glycosylation of the amylase as it was translocated through the secretory pathway, samples of the extract and supernatant isolated from DBY746/pAAMY (grown in buffered minimal medium) were treated with EndoH$_f$. The enzyme EndoH$_f$ removes core glycosylation from glycoproteins. Supernatant and extract prepared from 3 x $10^8$ cells of a 30 hour culture (see fig 3.13) (2.23, 2.24). were resuspended in 200μl of denaturation buffer (2.6) and boiled for 10 minutes. 100μl of the supernatant and extract suspensions were then aliquoted into two fresh eppendorfs and treated with EndoH$_f$ (sec 2.6). The samples were incubated at 37$^0$C for 60 minutes. The samples were boiled for 10 minutes prior to loading. Both the treated and untreated samples were divided in two and loaded on an activity gel (fig. 3.28) and an SDS polyacrylamide gel for Western blotting (fig. 3.29). The supernatant from DBY746/pAAH5 was treated in an identical way and served as a negative control while the native *B.licheniformis* α-amylase was included as a positive control.

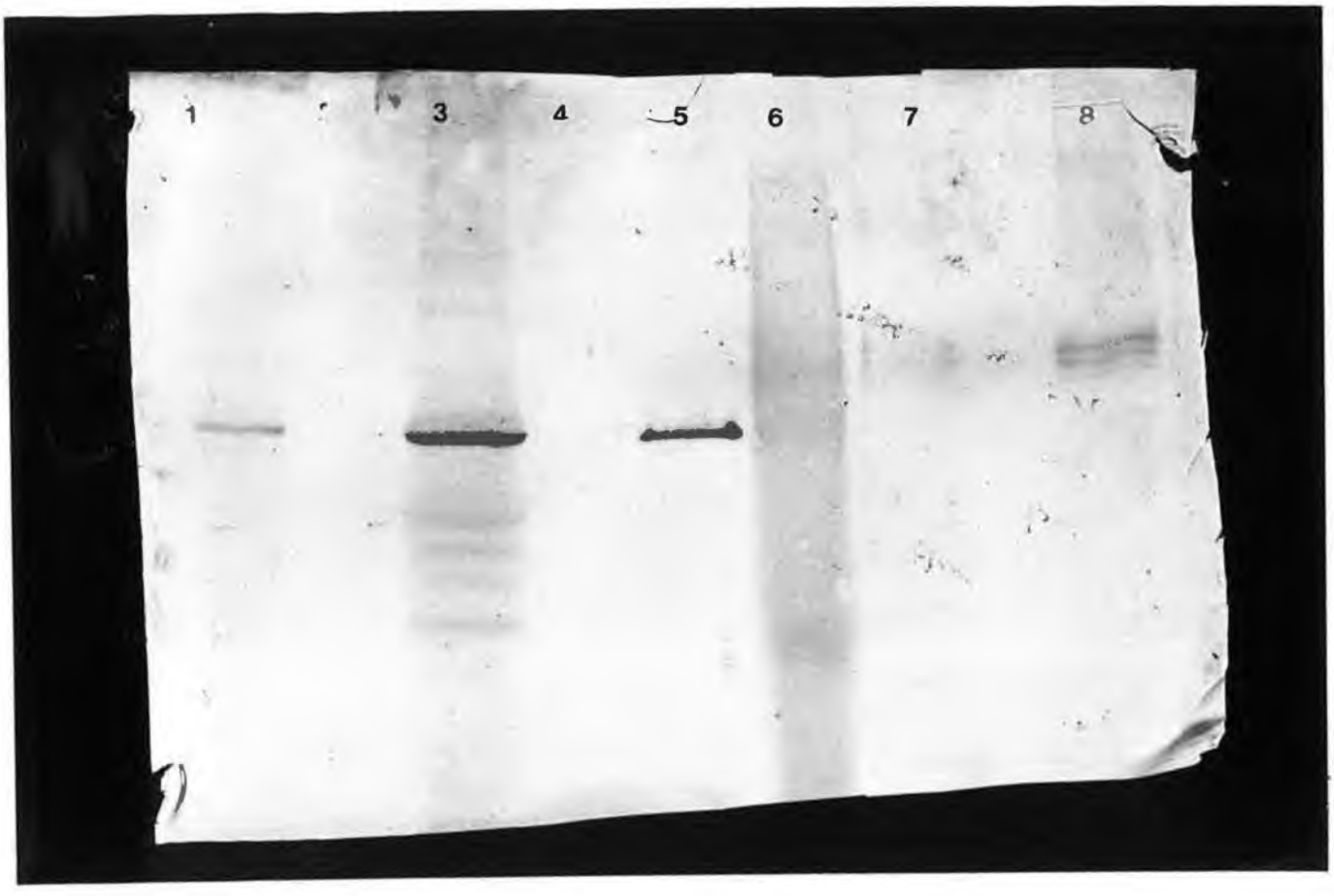
Figure 3.28.




Amylase activity gel after treatment with endo$H_f$.

Lane 1 : Supernatant from DBY746/pAAMY.
Lane 2 : Extract from DBY746/pAAMY.
Lane 3 : DBY746/pAAMY supernatant treated with Endo$H_f$.
Lane 4 : Extract treated with Endo$H_f$.
Lane 5 : 1 unit of the *B.licheniformis* amylase.
Lane 6 : Prestained molecular weight markers.
Lane 7 : 1/10 dilution of supernatant of DBY746/pAAMY, Endo$H_f$ treated
Lane 8 : 1/10 dilution of supernatant DBY746/pAAMY.

Figure. 3.29.




Western blot of DBY746/pAAMY supernatant and extract after treatment with $EndoH_f$.

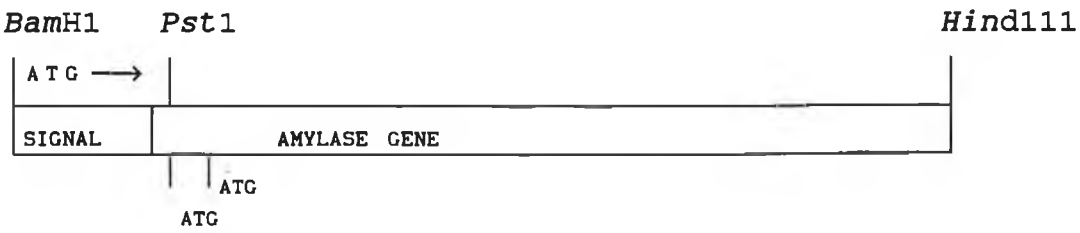
Lane 1 : 1/10 dilution of supernatant from DBY746/pAAMY End treated.
Lane 3 : DBY746/pAAMY Supernatant treated with $EndoH_f$.
Lane 4 : DBY746/pAAH5 supernatant.
Lane 5 : 1 unit of the *B.licheniformis* amylase.
Lane 6 : Pprestained molecular weight markers.
Lane 7 : 1/10 dilution of SN of DBY746/pAAMY.
Lane 8 : Supernatant of DBY746/PAAMY.

It can be seen from figs. 3.28 and 3.29 that the bands on the Western blot correspond to those present in the activity gel. It was observed that after treatment with $EndoH_f$ the disappearance of the higher molecular weight forms of the amylase was accompanied by an increase in the intensity of the amylase band corresponding in size to the native *B.licheniformis* α-amylase in both the activity gel and the Western blot. In the activity gel (fig 3.28) the amylase band corresponding to a 1/10 dilution of DBY746/pAAMY supernatant (lane 7) only becomes visible after $endoH_f$ treatment. In lane 3 in both fig 3.28 and 3.29 there is also a faint band which is present just above the main amylase band. It is approximately 3kd bigger than the size of the main amylase band, this band may represent a fraction of the amylase whose signal peptide had not been cleaved off. The disappearance of all but one of the higher molecular weight bands after $EndoH_f$ treatment demonstrated that these higher molecular weight forms of the amylase were active and result from different levels of core glycosylation of the amylase as it proceeded through the yeast secretory pathway. In the Western blot shown in Fig 3.29 in lane 3 in addition to the bands discussed above there are a number of lower molecular weight bands. These bands may be due to the presence of proteolytic degradation products of the amylase, and these bands are not active amylase bands as they do not appear in the equivalent lane (lane 3) in the activity gel (Fig 3.29).

**3.15. Construction of a vector containing a signal-minus α-amylase.**

In order to demonstrate conclusively that the α-amylase bacterial signal peptide was responsible for the translocation of the *B.licheniformis* amylase through the yeast secretory pathway, an attempt was made to construct a vector which contained the sequence coding for the mature α-amylase but which lacked the signal peptide. This vector called pAAMYS (for signal minus) is outlined below. The entire amylase gene is shown schematically in fig. 3.30.

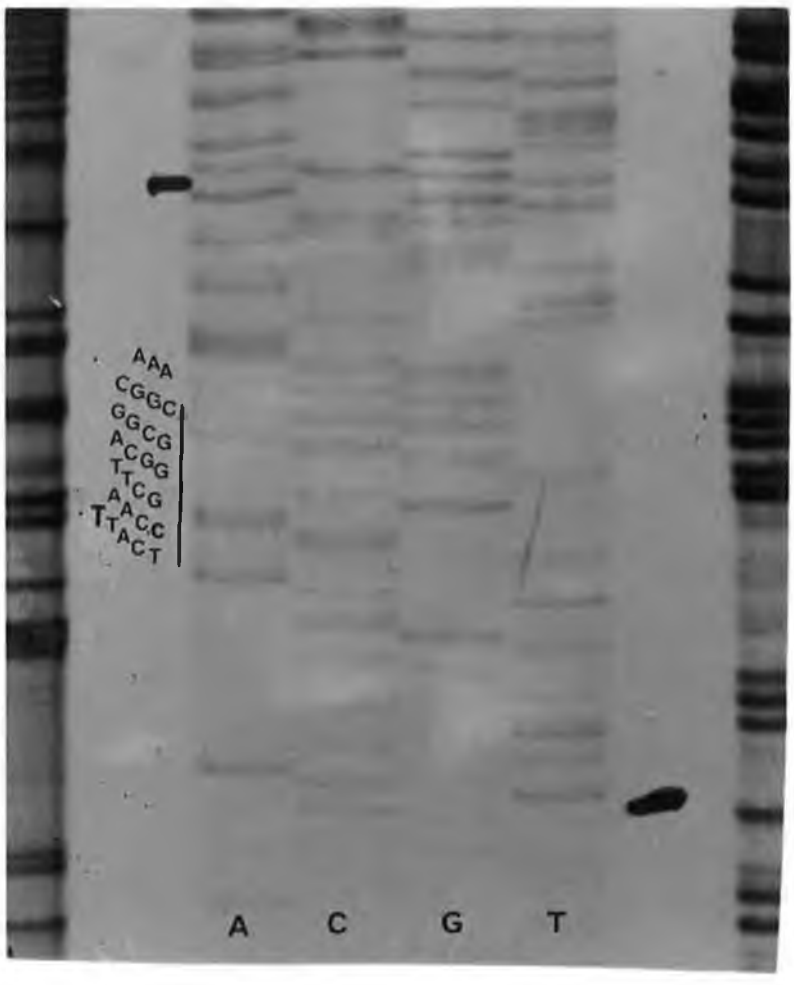
Figure 3.30




Schematic representation of the entire *B. licheniformis* amylase gene.

To facilitate separation of the signal peptide and mature amylase and the subsequent manipulation of the signal-minus gene, a *Hind*111 linker was inserted at the *Pst*1 site located at the 3'end of the signal peptide. Firstly the plasmid pSL5 (fig. 3.3) was restricted with *Pst*1 and the 3' overhangs generated were removed with the klenow fragment of DNA polymerase 1. The plasmid was then religated in the presence of 8 mer *Hind*111 linkers, (5' CAAGCTTG 3') giving the plasmid pSL5$_3$. To ensure that the klenow enzyme had fully digested the 5'-3' overhangs and that only a single copy of the linker had successfully ligated to the blunt ends thereby generated, this region of the construct was sequenced by first subcloning the *Bam*H1-*Hind*111 fragment into pUC19 and then sequencing by the dideoxy chain termination method (sec 2.37) using the universal primer. A section of the sequencing gel showing the junction site spanning the inserted linker is shown in figure 3.31.
This sequence data confirms that the *Hind*111 8mer linker had been correctly inserted into the unique *Pst 1* site in pSL5.

Figure 3.31.




Section of the sequencing gel confirming the sequence around the inserted *Hind*111 8mer linker.

The sequence read from bottom to top is as follows:

*Hind*111 linker

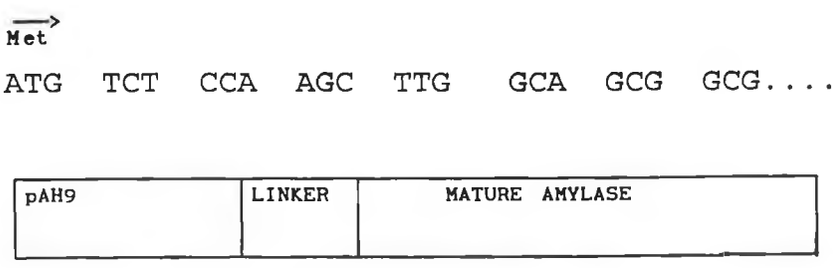
↓

CTCATCTTCTTTGCTGCCTCATTCCAAGCTTGGCAGCGGCGGCAAATCTTAAT

GGGACGCTGATGCACTATTTTGAA

Although there are two ATG start sites at the 5′ end of the mature amylase gene both of these start sites are out of frame and would give rise to nonsense transcripts. Therefore it is necessary to provide an in frame start site at the 5′ end of the gene. This can be achieved by using one of the pAH series of vectors (Ammerer, 1983). These three yeast expression vectors contain the yeast ADH1 promoter and an ATG start site in three different reading frames proximal to the unique *Hin*dIII cloning site. The plasmid pAH9 was chosen as insertion of the *Hin*dIII amylase fragment from pSL5$_3$ into this vector would give an in frame fusion between the start site and the gene (fig. 3.32).

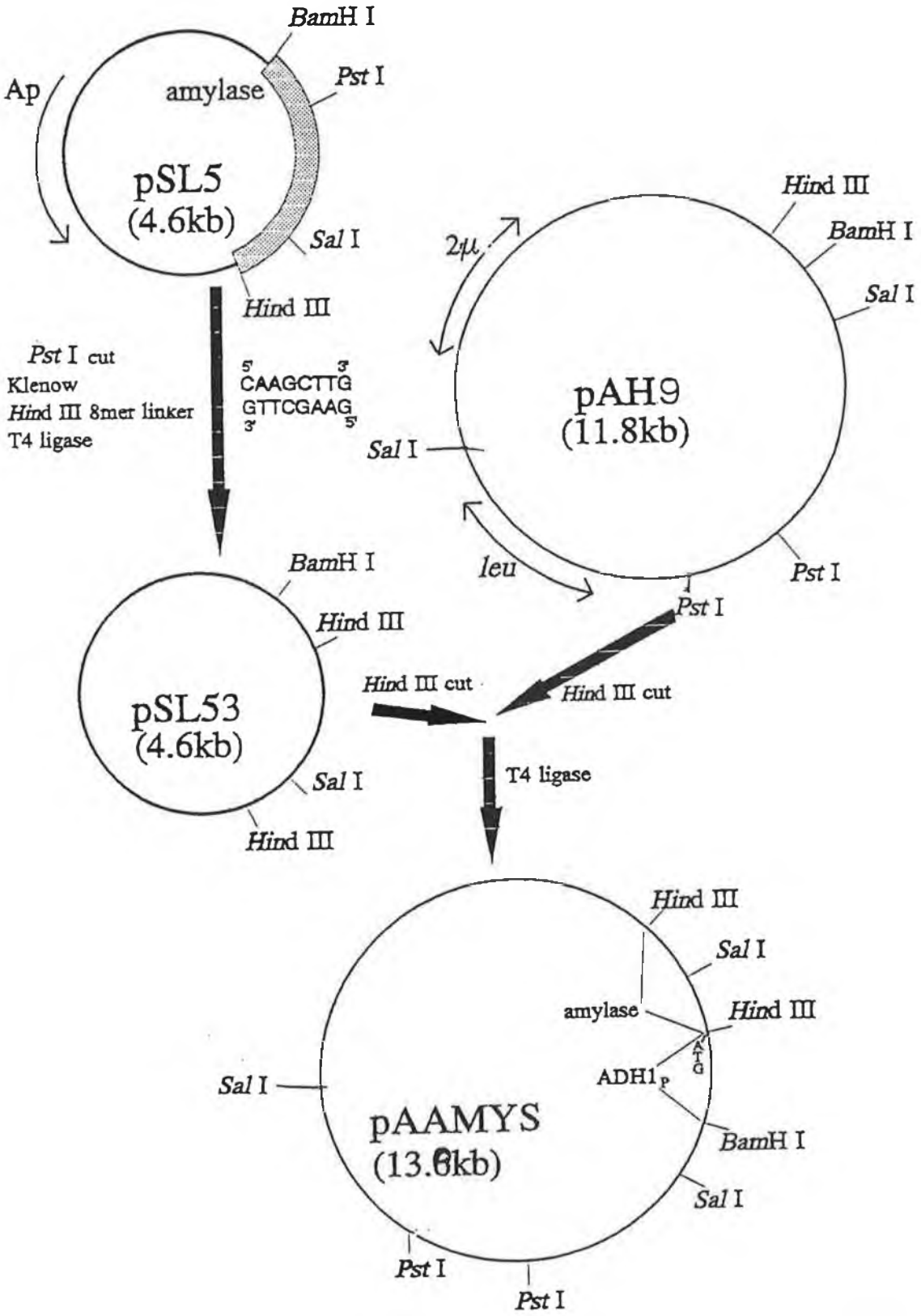
Figure 3.32.




Schematic diagram of the sequence at the junction of the vector pAH9 and the *Hin*dIII fragment containing the signal minus amylase gene.

Plasmid pSL5$_3$ was restricted with *Hin*dIII and the *Hin*dIII amylase-containing fragment was isolated from a 0.7% agarose gel (2.14). Several attempts were made to ligate this fragment into the unique *Hin*dIII site in pAH9 (fig. 3.33) to give plasmid pAAMYS but all attempts were unsuccessful. Control ligations of this fragment into pBR322 were carried out without difficulty. The data from the analysis of amylase secretion in *sec* mutants and the fact that the amylase is glycosylated, demonstrates, however, that the signal peptide directs the amylase through the yeast secretory pathway.

Figure 3.33.




Schematic diagram for the construction of plasmid pAAMYS.

# CHAPTER 4.

## Results 2.

## Strategies to increase the levels of *B. licheniformis* α-amylase produced by *S. cerevisiae*.

**4.1. Isolation of supersecreting (SSC)mutant strains.**

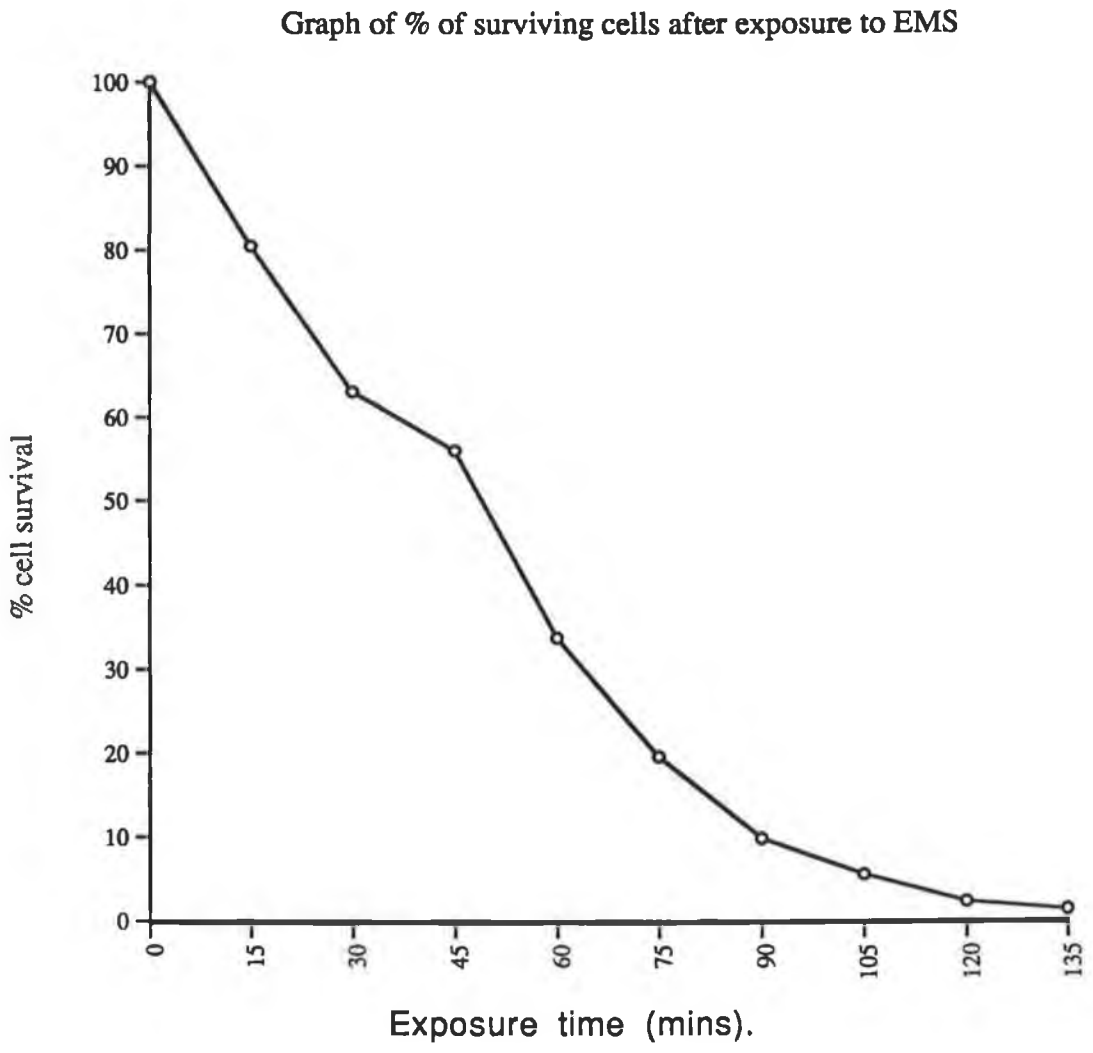
The rationale for this method was based on the assumption that any complex, multistep pathway, such as the process of protein secretion in yeast, must have one or more rate-limiting steps which, if altered by mutation, could lead to an increase in secretion. It is assumed that initially these mutations would occur at the most rate-limiting step in the pathway and that by repetitively isolating mutations in a strain already carrying one or more supersecretion chromosomal mutations, it should be possible to sequentially alter the rate-limiting steps and affect substantial increases in the levels of heterologous proteins secreted. It has also been shown (Wood and Brazell, 1989, Sakai *et al.*, 1990) that mutagenesis of yeast strains expressing heterologous proteins can lead to an increase in the overall, i.e. intracellular plus extracellular, quantity of heterologous protein produced in the cell.

Ethyl methane sulphonate (EMS) was selected as the mutagenic agent as it is highly efficient at inducing mutations, up to $5 \times 10^{-4}$ to $10^{-2}$ per gene without substantial killing (Cold Spring Harbor, Methods in Yeast Genetics, 1986), it does not produce clusters of closely linked mutations and had been used previously for the successful mutation of *S.cerevisiae* (Smith *et al.*, 1985). EMS is also safer to handle than powders such as NGG (N-methyl-N'-nitro-N-nitrosoguanidine).

In terms of the most efficient operation of a random screen, the optimum dose of mutagen is that which gives rise to the highest proportion of mutants in the surviving population. Generally, an increase in the dose of mutagen will result in a decrease in the survival rate. The optimum rate of survival for the isolation of mutants is in the range of 10-30% with lower survival rates leading to an increase in the number of multiple mutations and higher survival rates leading to a decrease in the number of mutated cells in the overall population.

The initial step in the isolation of mutants was the generation of a survival curve to determine the dosage of EMS required to give a cell survival rate of 20%. The protocol used for mutagenesis was as follows. Cells were grown to stationary phase in minimal media and were centrifuged at 5,000 rpm for 5 mins. Cells were washed once and resuspended in 10ml of 0.1M phosphate buffer, pH 7.0. The cells were then transferred to a 50ml screw top tube and 0.3ml of EMS added. The tube was vortexed vigorously as EMS is poorly miscible in this buffer. The tube was then incubated in a shaking water bath at 30$^{0}$C and samples were taken at intervals of 15 minutes and added to an equal volume of freshly made, filter sterilised, 10%(w/v) sodium thiosulphate (to inactivate the EMS). Samples were then spun at 5,000 rpm for 5 minutes, washed twice with 5 ml of 0.1M phosphate buffer, pH 7.0 and plated on buffered minimal medium, pH 6.9, containing 2% starch. Various dilutions of the samples were plated to obtain less than 200 colonies per plate. Plates were counted after 2 days growth at 30$^{0}$C. Using this procedure a survival curve was generated as shown in fig 4.1. The survival curve shows that exposure of the yeast cells to EMS for 75 minutes gave a survival rate of 20% which is the recommended survival rate for recovering a high proportion of mutant colonies in the surviving cell population. As the cells were plated onto minimal and not rich media the survival rate also includes the percentage of colonies which had lost their plasmid during the growth of the culture prior to mutagenesis. However as the cells were grown to stationary phase on minial media, the % of cells retaining the plasmid after this time would be close to 100%, in plasmid stability studies after 30 hours growth on selective minimal media 97-98% of cells still retained the plasmid after 30 hours growth. This plasmid loss was regarded as insignificant when compared to the overall cell death of approximately 80%.

Figure 4.1.




Survival curve showing the percentage of cells surviving after exposure to EMS.

### 4.1.1 Screening of potential mutants.

A suitable procedure was established for screening surviving cells for the mutant phenotype, i.e., increased external levels of α-amylase. Generally when dealing with large numbers of colonies the screening procedure needs to be sensitive, easy to use, capable of screening a large number of colonies per plate and inexpensive. A number of different plate assays for amylase activity were examined for their suitability for use in the initial screening procedure. These were the starch precipitation assays as described in section 3.3, a bromophenol blue based assay and an assay using chemically synthesized Cibacron blue starch. The method for the detection of amylolytic yeast strains using bromophenol purple involved the incorporation of 0.003% bromophenol purple into bufffered minimal medium (containing 2% starch). Again, amylase-producing colonies are surrounded by clear halos against a blue background (fig. 4.2).

Figure 4.2

Detection of amylolytic yeast strains using Bromophenol purple.

The final method evaluated for the detection of amylase-producing strains was the use of Cibracron Blue-starch. The Cibacron Blue dye was synthesized (2.17) by binding the dye chemically to the starch, at a ratio of 1 molecule of dye per 20-30 glucose units; as the amylase breaks down the starch, the dye bound to the smaller glucose units can diffuse through the medium. This diffusion of the solubilised unit carrying the dye creates a zone of clearing against a blue background (fig 4.3).

Figure 4.3


Detection of amylolytic yeast strains using Cibacron Blue starch.

Each of the above assays were assessed as a potential initial screening procedure for the detection of mutagenised colonies secreting elevated levels of α-amylase. The optimal assay was assessed on the basis of halo clarity, i.e. clear definition of halo perimeter, sensitivity i.e. halo size relative to size of colony, simplicity, reproducibility, and the number of colonies assayable per plate. Incorporation of bromophenol purple into plates although producing halos around amylolytic

colonies and not around control strains, was not used because as a pH indicator it is not specific for starch degradation and more specific assays which also gave good halos were available. Cibacron blue starch was not used because although it gave well defined haloes both the chemical synthesis and pouring of the overlay plates were difficult and time consuming. It was also difficult to get a uniform distribution of the dye throughout the plate. The most suitable assay was that using buffered yeast minimal medium (pH 6.9) containing 2% Litner's starch. However, a lower level of glucose, 0.5%, compared to 2% in the previously described assay, was used as the higher level gave rise to large colonies and halo sizes. Because this is a zone-based assay it suffers from the problem of the decreasing exponential relationship between the product titre and the zone diammeter such that a large increase in the quantity of product gives rise to only a relatively small increase in zone diammeter. The use of a low level of glucose in the plates kept the colony size and therefore the zone diammeter to a minimum which made it easier to see increases in halo size and also had the added advantage of allowing more colonies to be screened per plate. Two alternative methods of decreasing the halo size were also tried. The pH of the medium was lowered to pH 4.2 at which the amylase activity was 25% of its maximum activity at pH 6.9 and the starch concentration was increased up to 5%. The use of a lower level of glucose was selected as the best method as the lower pH gave rise to large colony sizes with relatively small haloes and the higher levels of starch were difficult to work with due to problems with insolubility at higher concentrations. An attempt was also made to grow the mutagenised colonies on starch as the sole carbon source but this was unsuccessful.

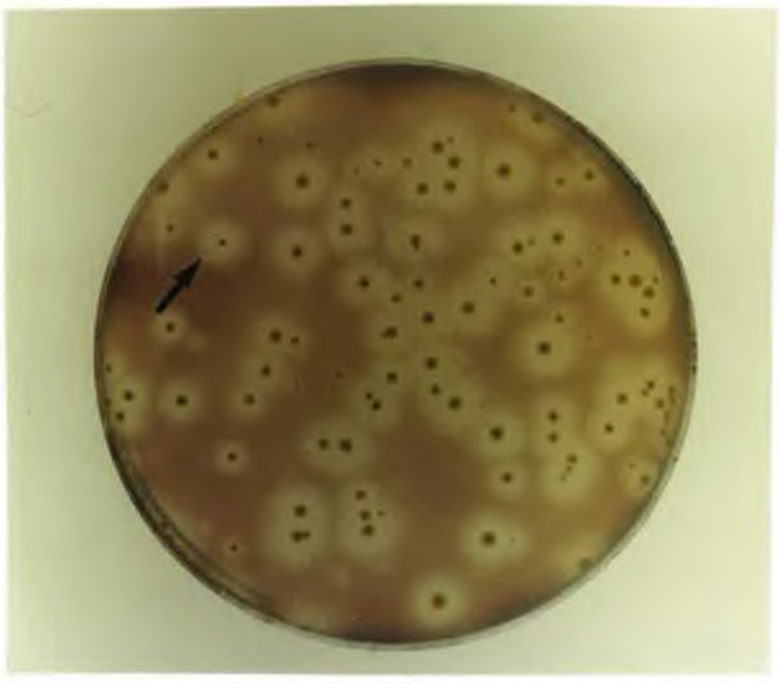
Following mutagenesis the surviving colonies were plated out to give 100-200 colonies per plate (fig 4.4). After two days growth the colonies were assayed for increases in

amylase secretion either by staining with iodine vapour or incubation at 4°C overnight. There were substantial interplate and intraplate differences both in colony and halo size, so potential mutants were selected by comparing potential mutants to those colonies nearby on the same plate. Unmutagenised cells plated on identical media had more uniform colony and halo sizes. Potential mutants were then replated (100 colonies per plate) grown for 48 hours and screened as before (fig. 4.5) In both fig 4.4 and 4.5 potential mutants are indicated with arrows.

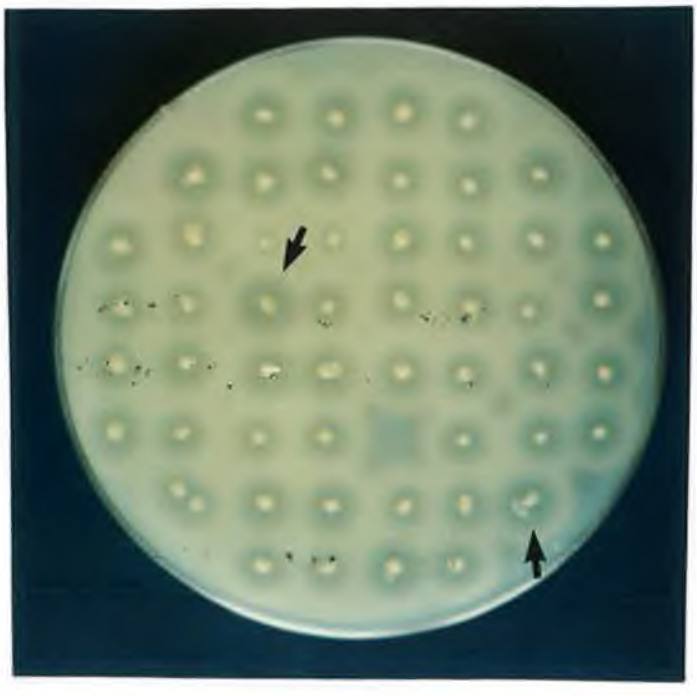
Figure 4.4

(a) Initial screen of surviving colonies after precipitation of starch by incubation overnight at 4°C.

Figure 4.4

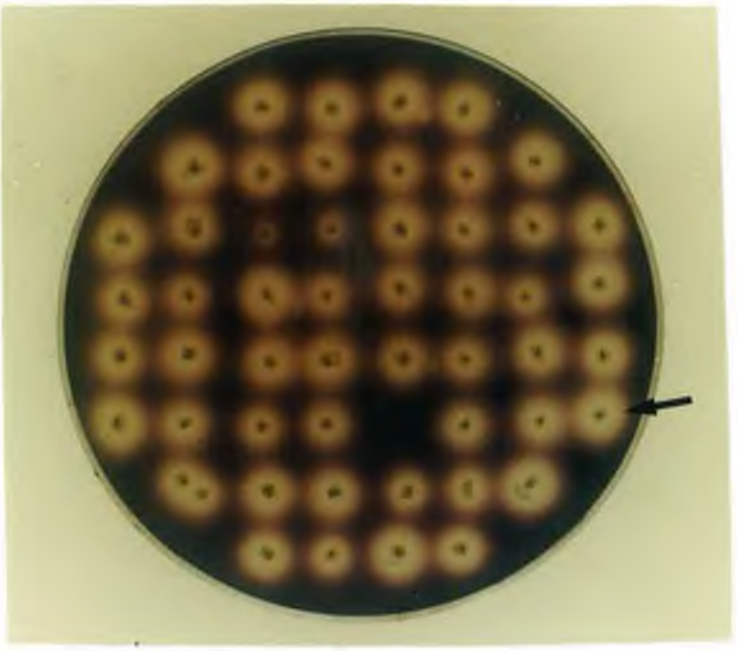


(b). Initial screen after exposure to iodine vapour.
Figure 4.5.



(a) Secondary screen after precipitation of starch by overnight incubation at $4^{o}C$.

Figure 4.5



(b) Secondary screen after exposure to iodine vapour.
Those colonies which appeared to have larger halo sizes compared to surrounding colonies were plated out and grown to stationary phase in 5ml of buffered yeast minimal broth and the supernatants were assayed for amylase activity using the plate assay described in section 3.3 (fig. 4.6). Potential mutants are indicated with arrows. Strains giving larger halo sizes than the control DBY746/pAAMY supernatant were again grown to stationary phase in yeast minimal broth and the amylase secreted was quantified by the DNS spectrophotometric assay (2.18).

Figure 4.6.

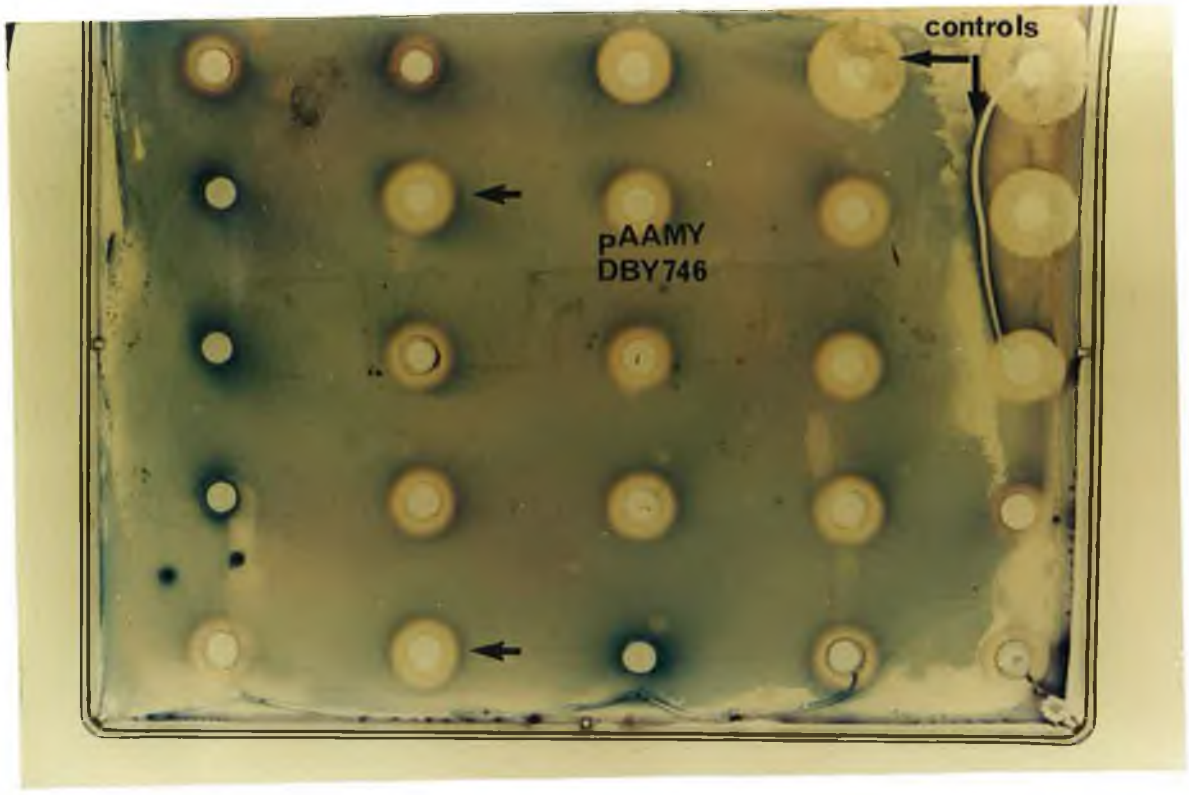



Plate assay of supernatants from potential supersecreting mutants.

**4.1.2 Characterisation of supersecreting (SSC) strains.**

Out of an initial screen of approximately 30,000 surviving colonies, approximately 1,000 were replated and 97 were assayed by the plate assay. Of these, 25 were assayed using the DNS assay and the specific amylase activity of each of these strains is shown in Table 4.1. The amylase activity of the strains was determined after 36 hours growth at 30$^{o}$C, and cell counts were determined directly using a haemocytometer. Specific activities i.e. activity per cell were determined by dividing the activity obtained in the DNA assay by the number of cells present.

Table 4.1. Amylase activity per cell in SSC strains.

| Strain | Act/ml | Spec.Act | Strain | Act/ml | Spec.Act. |
|---|---|---|---|---|---|
| DBY746 | 958 | $2.9x10^{-5}$ | mut13 | 111 1 | $3.1x10^{-5}$ |
| mut1 | 1779 | $2.6x10^{-5}$ | mut14 | 442 5 | $1.6x10^{-4}$ |
| mut2 | 1023 | $3.3x10^{-5}$ | mut15 | 977 | $2.7x10^{-5}$ |
| mut3 | 1344 | $2.7x10^{-5}$ | mut16 | 1211 | $3.5x10^{-5}$ |
| mut4 | 998 | $2.2x10^{-5}$ | mut17 | 2673 | $8.1x10^{-5}$ |
| mut5 | 2520 | $8.4x10^{-5}$ | mut18 | 789 | $2.8x10^{-5}$ |
| mut6 | 3069 | $9.3x10^{-5}$ | mut19 | 881 | $2.8x10^{-5}$ |
| mut7 | 967 | $2.6x10^{-5}$ | mut20 | 1001 | $3.3x10^{-5}$ |
| mut8 | 1201 | $3.1x10^{-5}$ | mut21 | 1359 | $3.9x10^{-5}$ |
| mut9 | 901 | $2.1x10^{-5}$ | mut22 | 867 | $3.2x10^{-5}$ |
| mut10 | 1322 | $3.7x10^{-5}$ | mut23 | 2250 | $7.5x10^{-5}$ |
| mut11 | 1011 | $2.4x10^{-5}$ | mut24 | 912 | $2.5x10^{-5}$ |
| mut12 | 837 | $3.2x10^{-5}$ | mut25 | 839 | $1.9x10^{-5}$ |

The 5 strains showing the largest increases in specific activity were selected for further characterisation. In these strains the increases in the levels of amylase secreted varied from 2.3 to 5.4 fold. These 5 strains were named SSC1 to SSC5 and are shown it table 4.2.

Table 4.2

| Strain | Cell No. | Act. SN/ml. | Spec.Act | Fold increase |
|---|---|---|---|---|
| DBY746 | $3.3x10^{7}$ | 958 | $2.9x10^{-5}$ | |
| SSC1 | $3.0x10^{7}$ | 2250 | $7.5x10^{-5}$ | 2.6 |
| SSC2 | $3.3x10^{7}$ | 2673 | $8.1x10^{-5}$ | 2.8 |
| SSC3 | $3.0x10^{7}$ | 2520 | $8.4x10^{-5}$ | 2.9 |
| SSC4 | $3.3x10^{7}$ | 3069 | $9.3x10^{-5}$ | 3.2 |
| SSC5 | $2.95x10^{7}$ | 4425 | $1.6x10^{-4}$ | 5.4 |

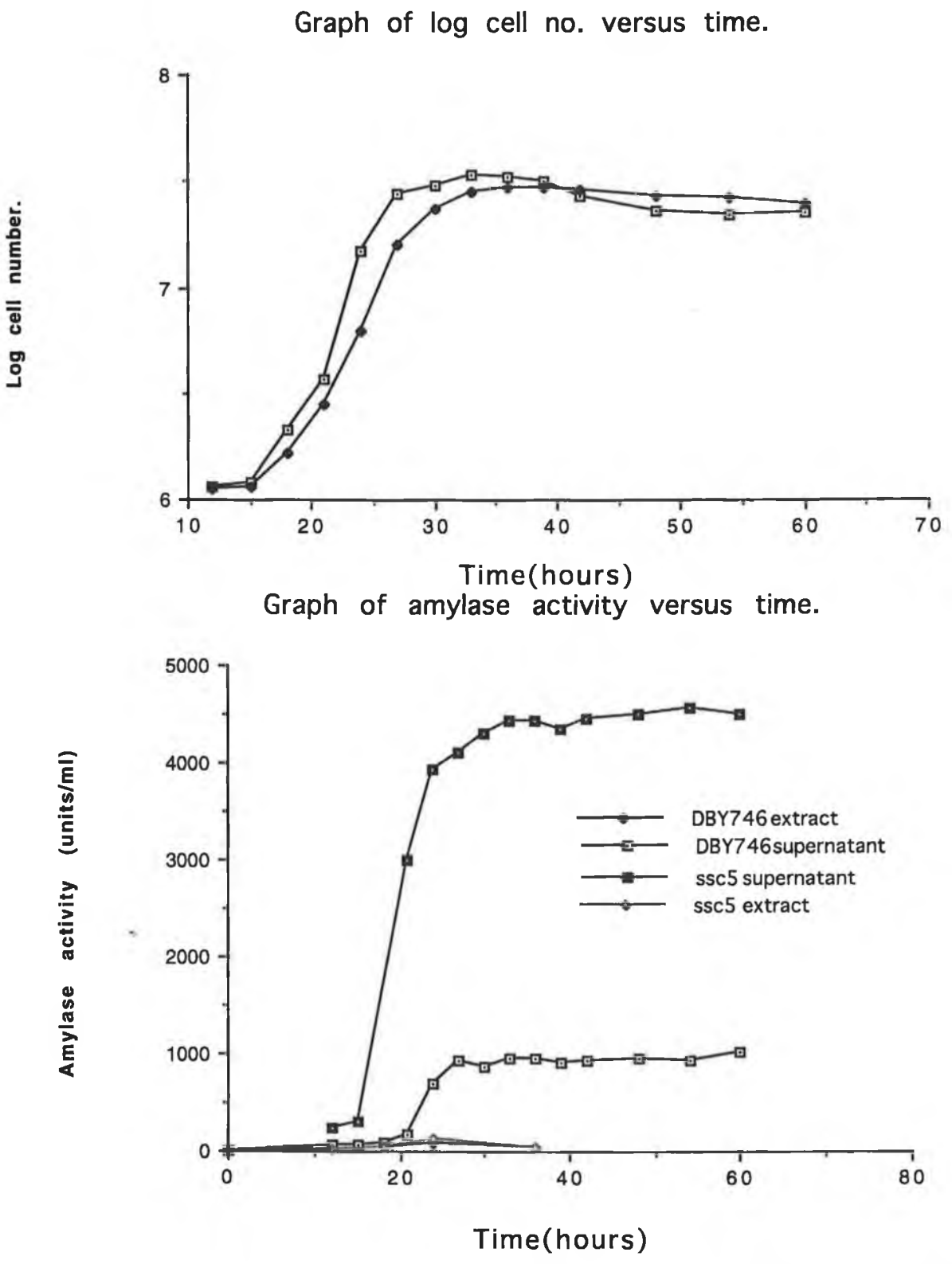
Increases in specific amylase activity in SSC1-SSC5.
The figures are for the fold increase in amylase activity and represent the average of two determinations.
To ensure that the mutations responsible for the observed

increases in amylase activities were chromosomal, i.e. host mutations and not plasmid borne mutations each of the SSC strains outlined above were first cured (2.32) of the amylase-containing plasmid (pAAMY) and then retransformed with an unmutagenised source of the plasmid. Following this experiment it was established that the retransformed SSC strains retained the increased levels of amylase secretion indicating that the mutations responsible for the increase in amylase secretion were chromosomally located. As a second means of checking that the mutations were not on the plasmid, plasmid DNA was also isolated from each of the SSC strains (2.16) amplified in *E.coli*, checked by restriction digest with *Bam*H1 and retransformed into DBY746. No increase in the secretion of amylase was observed in these strains confirming the chromosomal location of the mutations. The plasmid DNA isolated from each of the mutants gave an identical restriction pattern to the original plasmid indicating that no gross rearrangement of the plasmid had taken place as a result of the mutagenesis carried out.

The levels of amylase activity produced throughout the growth cycle of DBY746/pAAMY and SSC5/pAAMY were determined using the DNS assay (2.18). The result of this analysis is shown in fig. 4.7. The result shows that while there is a substantial increase in the external levels of amylase present the levels of internal amylase are similiar. The plot also shows that the growth rate and overall cell number of SSC5 are very similiar to that of the original unmutagenised strain DBY746.

In an attempt to further increase the levels of amylase produced the mutagenesis protocol as outlined above was carried out using SSC5/pAAMY as the starting strain in an attempt to isolate secondary mutants with further increases in amylase secretion. However these attempts were unsuccessful.

Figure 4.7.




Graph of cell growth and amylase production by DBY746/pAAMY and SSC5/pAAMY.

To determine whether the mutations in SSC4 and SSC5 (the mutants which showed the highest increase in amylase activiity) responsible for the increased levels of amylase production were specific for the amylase or would result in an increased level of other heterologous proteins DBY746, SSC4 and SSC5 were transformed with the plasmid pJG317 (appendix 2 (b)) which contains the entire *Bacillus subtilis* β-glucanase gene i.e. the region coding for the signal peptide and the mature β-glucanase. The internal and external levels of β-glucanase were determined for each of the strains as described in section 2.24 and 2.25. Table 4.3. shows the results of these assays.

Table 4.3. Glucanase activity units per cell.

| Strain | Internal | External | Total | % increase |
|---|---|---|---|---|
| DBY746/pJG317 | $8.2x10^{-6}$ | $1.0x10^{-5}$ | $1.8x10^{-5}$ | ---- |
| SSC4/pJG317 | $8.1x10^{-6}$ | $1.06x10^{-5}$ | $1.9x10^{-5}$ | 5.5 |
| SSC5/pJG317 | $9.3x10^{-6}$ | $1.15x10^{-5}$ | $2.1x10^{-5}$ | 16.6 |

Although both SSC strains showed a slight increase in the overall levels of β-glucanase produced, these levels were insignificant relative to the increases observed for α-amylase suggesting that the mutations were specific for the amylase.

The α-factor assay (2.35) was used to determine if the chromosomal mutations in SSC4 and SSC5 would lead to the increased secretion of the yeast α- factor pheremone. The Strains DBY746, SSC3, SSC4 and SSC5 were cured of the amylase plasmid and grown to stationary phase in minimal medium and assayed for the levels of α-factor produced. Fig. 4.8 shows the results of the assays. As can be seen from the plates there was no apparent increase in the levels of α-factor secreted by the SSC strains, however as the haloes in these plates were poorly defined it is difficult to measure accurately what, if any increase in

**4.3 Integration of the amylase gene into the yeast chromosomal DNA using Yeast integrating (YIP) vectors.**

A number of reports have suggested that by decreasing the copy number of plasmids containing heterologous genes in *S.cerevisiae* an increase in the levels of heterologous proteins produced can be achieved (Smith *et al.*, 1985, Cole *et al.* 1988). It is argued that this increase is due to the decrease in stress placed upon the cell compared to a cell harbouring multiple copies of the gene. In cells with a reduced plasmid copy number there is a decrease in the levels of intracellular protein present, which may then be more readily secreted as the secretory pathway does not become clogged with excessive levels of the protein.

In contrast with the reports of Smith *et al.*,1985 and Cole *et al.* 1988), there are also a number of reports on nerve growth factor (Sakai *et al.*, 1991) and thaumatin (Lopes *et al.*, 1989) which show that the use of vectors which are targeted to integrate at reiterated DNA elements in the genome, either delta elments or rDNA results in an increased copy number. This higher copy number results in an increase in the level of protein produced relative to that produced by either centromeric or low copy integration plasmids.

The *B.licheniformis* α-amylase was cloned into both high and low copy integrating vectors (see section 1.3.5) to analyse the effect on α-amylase production of low copy number integration and high copy number integration on the overall amount of amylase produced by strains transformed with these vectors.

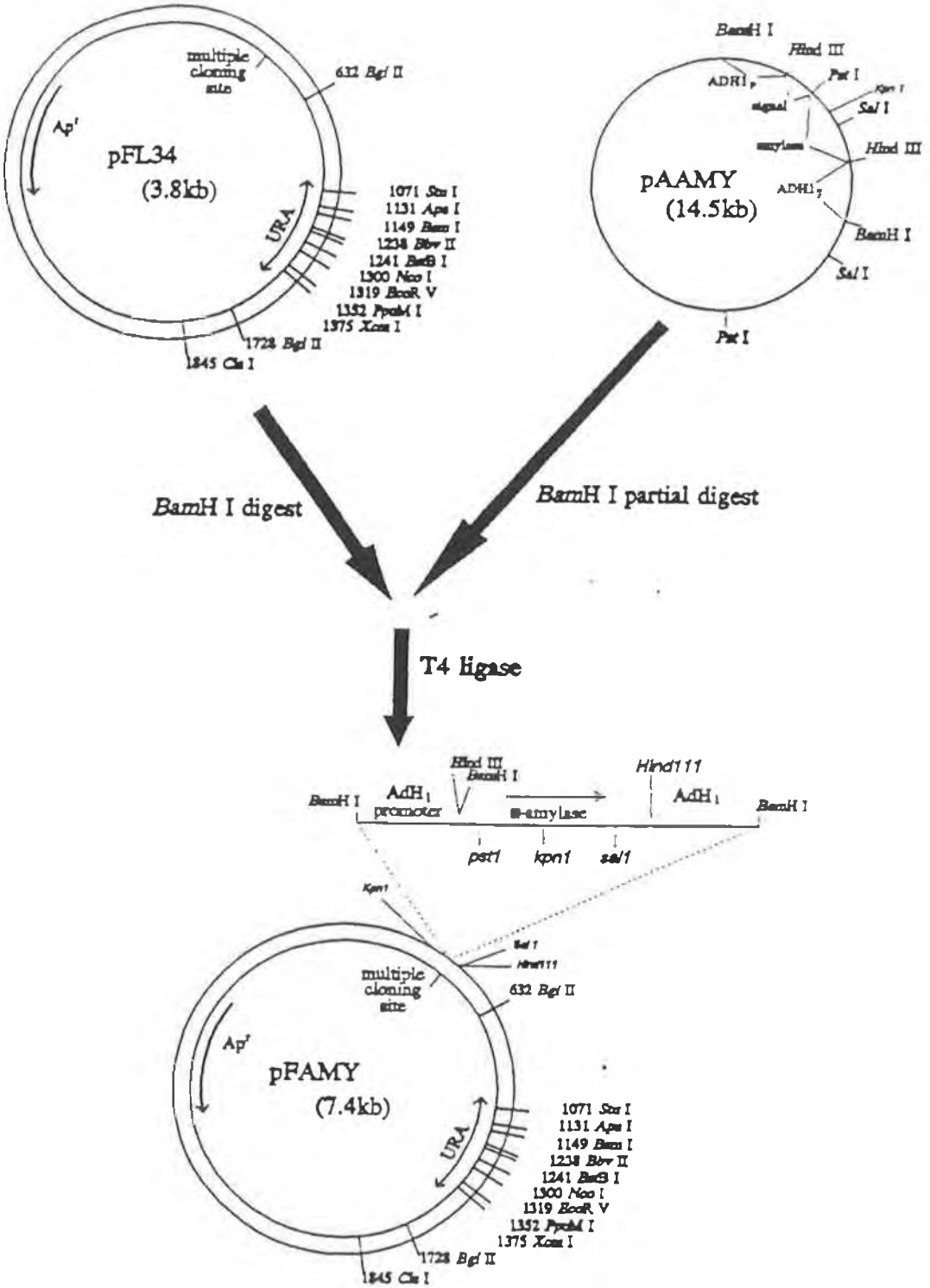
Both Centromeric vectors and integrating vectors (See sections 1.3.4,1.3.5) have a low copy number but integration has the added advantage of stability as integrated transformants do not require selective pressure for the maintenance of the heterologous gene. Strains harbouring integrated plasmids may therefore be grown to higher cell densities on rich media.

For the low copy number integration expermient the yeast integrating plasmid, YIp5 (Struhl 1989), was initially

chosen. The ADH1 promoter-amylase-ADH terminator cassette was isolated from a partial digest of pAAMY (Fig 4.9) and repeated attempts were made to subclone this *Bam*H1 fragment into the unique *Bam*H1 site of YIp5 were unsuccessful. Several strategies were tried including, isolation of the *Bam*H1 fragment from an agarose gel by the geneclean procedure (2.14) prior to ligation with phosphatase treated YIp5 vector DNA (2.14), isolation of the *Bam*H1 fragment by sucrose density gradient centrifugation (2.39) and subsequent ligation with both CIP treated and untreated vector DNA (the latter in the ratios of fragment:plasmid from 1:1 to 1000:1) but in all cases only religated YIp5 was obtained from the transformations. To rule out the possibility that this problem was due to the vector, a second source of YIp5 was obtained and the above strategies were repeated, again with no success. A control ligation with CIP treated YIp5 and lambda DNA digested with *Bam*H1 was successful and showed that the YIp5 vector used was capable of stably maintaining inserted DNA sequences and that the reaction conditions used had been optimised for the successful ligation of DNA fragments into YIp5. As a result of the repeated unsuccessful ligations using YIp5 the use of the YIp5 vector was abandoned.

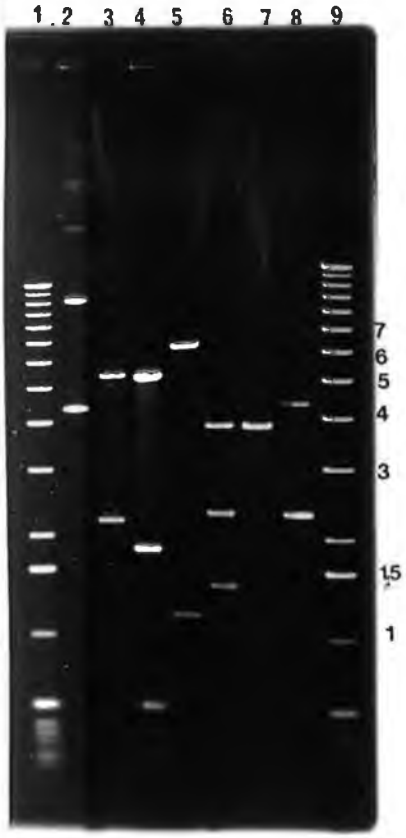
Instead the yeast integrating vector pFL34 (Bonneaud *et al.*, 1991) was then obtained and the amylase-containing fragment was successfully subcloned into the unique *Bam*H1 site of the polylinker of pFL34 at the first attempt giving the plasmid pFAMY (fig. 4.9). The orientation of the fragment in the vector was confirmed by restriction analysis (fig. 4.10). As the same sample of insert fragment DNA, i.e., that isolated from the sucrose density gradient, and the same ligation conditions were used as for YIp5, the success of this ligation at the first attempt supports the possibility that some inherent problem in the *Bam*H1 amylase gene-containing fragment from pAAMY does not allow it to be stably maintained in YIp5.

Figure 4.9.




Construction of pFAMY.

Figure 4.10




Restriction digests of pFAMY showing the orientation of the *Bam*H1 fragment insert. The sizes of the restriction fragments are given below (kb).

Lane 1 :λ 1 kb DNA ladder 12,11,10,9,8,7,6,5,4,3,2,1.5,1Kb
Lane 2 :Uncut pFAMY
Lane 3 :pFAMY *Kpn*1 5.8, 2.1
Lane 4 :pFAMY *Hin*d111 5.56, 1.8, 0.53
Lane 5 :pFAMY *Sal*1 6.68, 1.22
Lane 6 :pFAMY *Bam*H1 3.8 2.3, 1.5
Lane 7 :pFL34 *Bam*H1 3.8
Lane 8 :uncut pFL34
Lane 9 :λ 1 kb DNA ladder (important sizes indicated in Kb on figure).

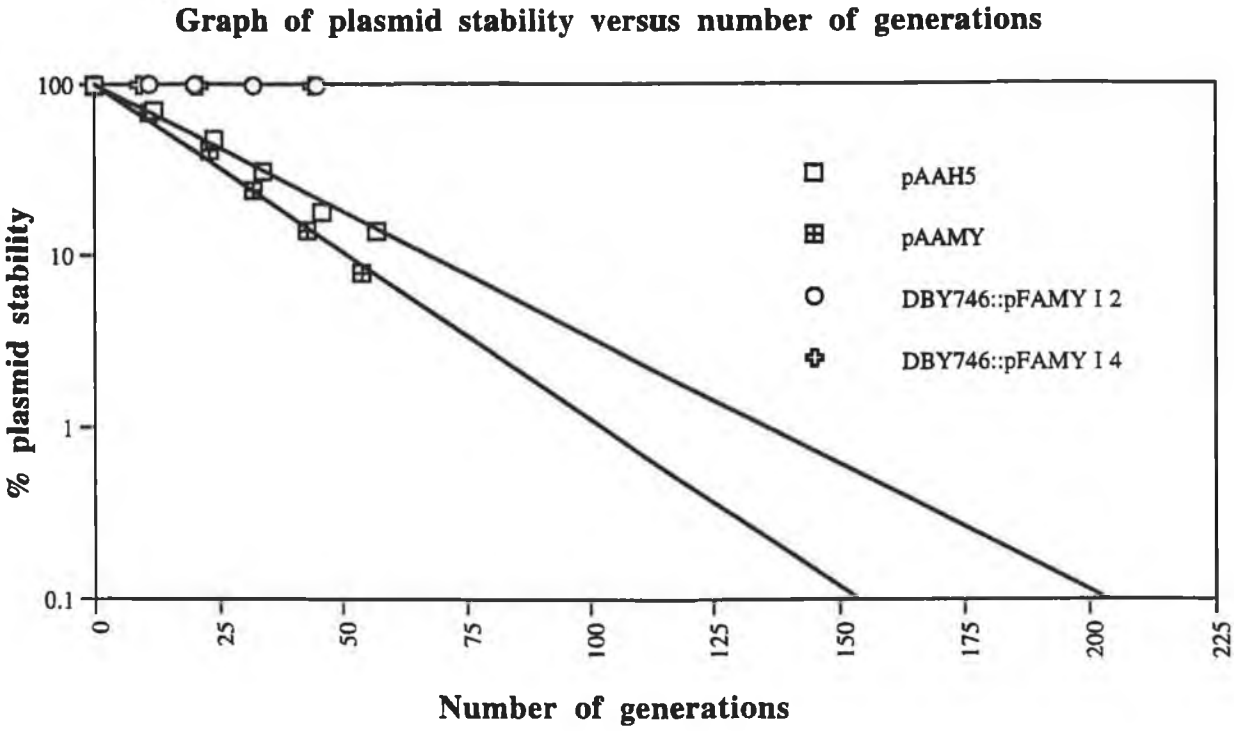
Plasmid pFAMY was then linearised at its unique *Stu*1 site and transformed into DBY746. DBY746 contains the *ura*3-52 mutation (Rose and Winston, 1984) due to the presence of a Ty element within the *ura* 3 gene. Only those transformants complemented with the *ura* 3 gene from plasmid pFAMY are capable of growth on uracil minus minimal media. Initially the transformation mix was plated on uracil minus minimal medium but no transformants were observed. To obtain transformants it was found to be neccessary to supplement the minimal medium with the supplementary mix described in section 2.3. Transformants obtained on this medium were replated onto minimal medium containing Litner's starch and screened for amylase activity. 10 amylase positive transformants were selected and the levels of amylase secreted were determined for 5 of them using the DNS assay (2.18) (Table 4.3). The results of this determination show that the levels of amylase secreted by these strains ranged from 22-57% of the level secreted by DBY746 transformed with the episomal plasmid pAAMY. No amylase activity could be detected intracellularly. Thus it is apparent that the use of a low copy number integrating plasmid does not result in an increase in the levels of $\alpha$-amylase in the supernatant. The use of the second integration method i.e. the use of a integrating plasmid which is targeted to reiterated sites in the genome resulting in a high plasmid copy number was therefore undertaken.

Table 4.3. Levels of amylase expressed by DBY746 transformed with pFAMY.

| Strain | Units of secreted Amylase. | Cell No. | Units/Cell |
|---|---|---|---|
| $I_1$ | 363.7 | $3.10 \times 10^{7}$ | $1.17 \times 10^{-5}$ |
| $I_2$ | 207.0 | $3.43 \times 10^{7}$ | $7.80 \times 10^{-6}$ |
| $I_3$ | 407.2 | $3.43 \times 10^{7}$ | $1.19 \times 10^{-5}$ |
| $I_4$ | 552.3 | $2.93 \times 10^{7}$ | $1.88 \times 10^{-5}$ |
| $I_5$ | 228.5 | $2.88 \times 10^{7}$ | $7.90 \times 10^{-6}$ |
| pAAMY | 952.8 | $3.30 \times 10^{7}$ | $2.89 \times 10^{-5}$ |

Plasmid stability studies (2.33) were carried out for strains $I_1$, $I_2$, and $I_4$ and compared to the plasmid stability of pAAMY and pAAH5 and the results are shown in figure 4.15. It can be seen from the graph that the strains carrying the integrating plasmid were almost 100% stable with only 2% of the cells losing their plasmids over approximately 45 generations indicating that the plasmid had successfully integrated into the *S.cerevisiae* chromosome. In addition attempts to isolate plasmid DNA (2.16) from these strains were unsuccessful while it was possible to isolate pAAMY from DBY746.

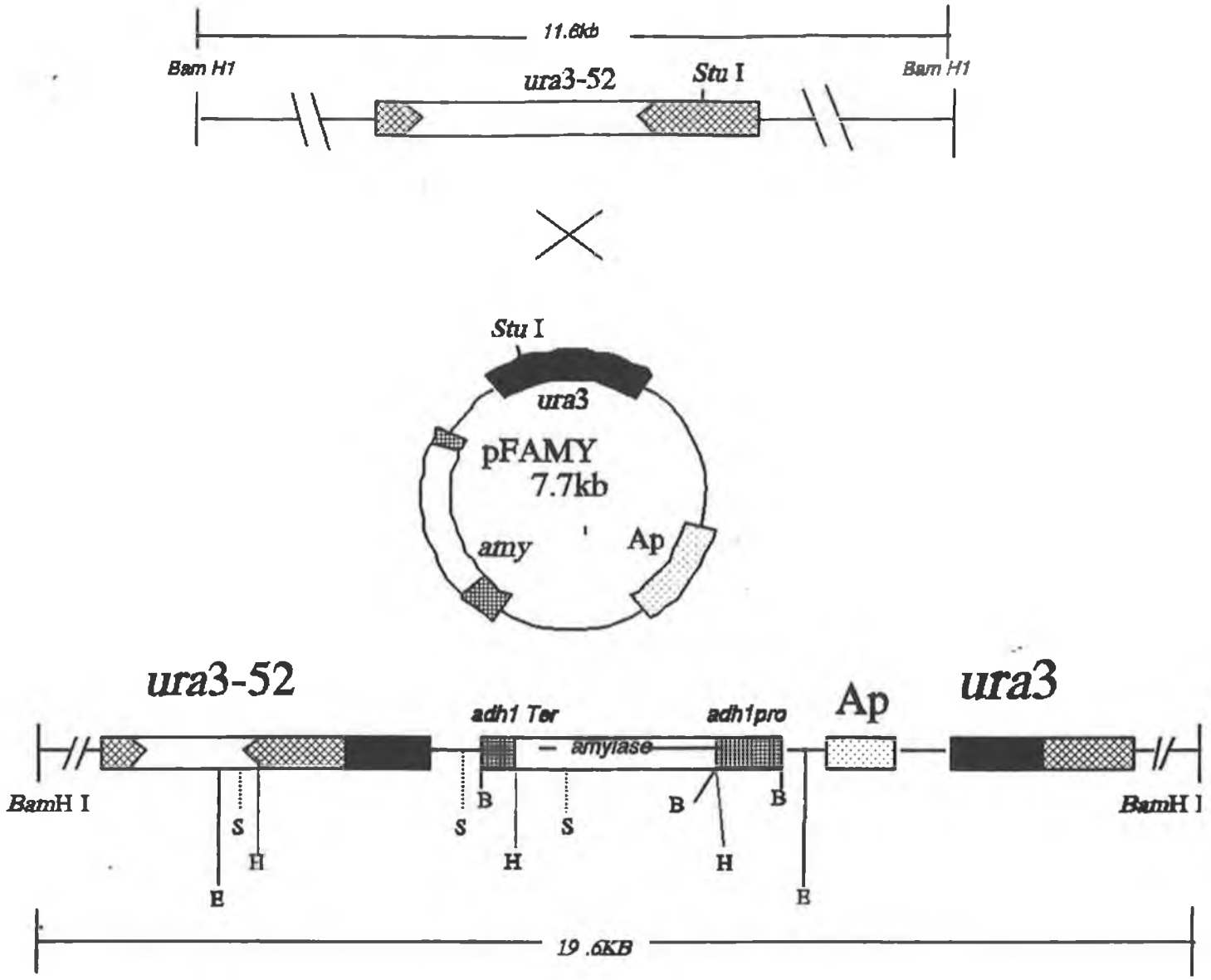
Figure 4.11.




Graph of plasmid stability versus number of generations.

From the graph it can be seen that the plasmid pFAMY was stably maintained in strains $I_2$ and $I_4$.
To confirm that the integration had taken place, total DNA was isolated from 13 transformants (2.15), DBY746::pFAMY$I_1$ to $I_{13}$, digested overnight with *Bam*H1, electrophoresed, Southern blotted (2.28) and probed with *Stu*1 linearised $^{32}$p-labeled pFAMY (2.29). *Hin*d111 and *Bam*H1 digested pFAMY were included on the gel both as positive controls and as size markers. Assuming that the integration had taken place by a single cross-over event (1.3.5) the resulting DNA restriction pattern, after restriction with *Bam*H1, at the site of integration should be that shown in fig. 4.16.

Figure 4.12.




Schematic diagram of probable integration event.

This DNA region, when digested with *Bam*H1 and probed with pFAMY should give fragment sizes of 1.5, 2.3 and 2 other fragments totaling 15.5kb. The blot of the gel is shown in fig. 4.13. It can be seen that where complete digestion of the chromosomal DNA samples has ocurred in the lanes containing *BamH1* digests of total DNA from DBY746::pFAMY transformants 1-13, three prominent *Bam*H1 bands of sizes

3.8, 2.3 and 1.5 kb are present, the other bands present are assumed to be due to incomplete digestion of total DNA. The three prominent *Bam*H1 bands however compare exactly in size to the bands obtained when the parental vector, pFAMY, is digested with *Bam*H1 (fig 4.10.) thus suggesting that the plasmid had not integrated into the yeast chromosome. Unfortunatly the expected 11.6Kb *Bam*H1 control *ura*3-52 fragment (Rose and Winston 1984) did not light up in the lane(#16) containing the chromosomal digest of DBY746.

Figure 4.13 on the following page shows the blot of *Bam*H1 digests of total DNA isolated from DBY746::pFAMY transformants 1-13, probed with $^{32p}$labelled *Stu*1 linearised pFAMY.

The samples were loaded as follows.

Lanes:1-8 DBY746::pFAMY transformants 1 -8 total DNA *Bam*H1 cut

Lane :9 Plasmid pFAMY *Hin*d111 cut

Lane :10 Plasmid pFAMY *BamH*1 cut

Lanes:11-15 DBY746::pFAMY transformants 9-13 total DNA *BamH*1 cut

Lane 16 DBY746 total DNA *Bam*H1 cut

Fig 4.13


Blot of *Bam*H1 digests of total DNA isolated from DBY746::pFAMY transformants 1-13, probed with $^{32p}$labelled *Stu*1 linearised pFAMY.

Considering the fact that the plasmid pFAMY contains no yeast origin of replication and that strains $I_1$, $I_2$ and $I_4$ were shown to stably maintain the amylase when grown on non-selective media for 40 generations (fig 4.11), it was assumed that the plasmid had successfully integrated into the yeast chromosome. However the data on this blot suggests that the plasmid had not intregrated and was being maintained extrachromasomally.

The exact location of the plasmid therefore required further investigation. In an attempt to define the location of the plasmid pFAMY in these strains total DNA was isolated from $I_2$, $I_4$ and DBY746 (as a negative control) and was digested with *Hind*111, *Eco*R1, *Sal*1 and *Kpn*1 followed by electrophoresis and Southern blotting and was then probed with *Eco*R1 linearised $^{32}$p-labeled pFAMY. These 4 enzymes were selected as unlike the *Bam*H1 digestion, *Eco*R1, *Hind*111 and *Sal*1 enzymes cut both within the plasmid and within the TY element(see Fig.4.13). *Kpn*1 was chosen as it cuts within the plasmid and not in the TY element. The presence of junction fragments of the predicted sizes would therefore provide unequivocal proof of the integration of the plasmid at the *ura*-3-52 locus. The plasmid pFAMY digested with the above enzymes was included on the gel as a positive control. A 1kb DNA ladder was also included as a size marker. The gel is shown in fig.4.14. and the blot in fig. 4.19. If the integration had taken place by a single crossover event as outlined in fig. 4.12, the following fragment sizes (in base pairs) should be observed for each of the enzymes used after probing with labeled pFAMY:

| | Plasmid Fragments | Junction fragments |
|---|---|---|
| *Hind*111 | 1800, 530 | 2750,and a larger fragment* |
| *Sal*1 | 1200, | 2740 and a larger fragment * |
| *Eco*R1 | | 6600 and a larger fragment * |
| *Kpn*1 | 2099 | two larger fragments. * |

*These larger fragments are of unknown size as the sequence 3′ to the integration site has not been mapped (fig. 4.13). However, the sizes of the bands observed after probing with

linearised labeled pFAMY were as follows:

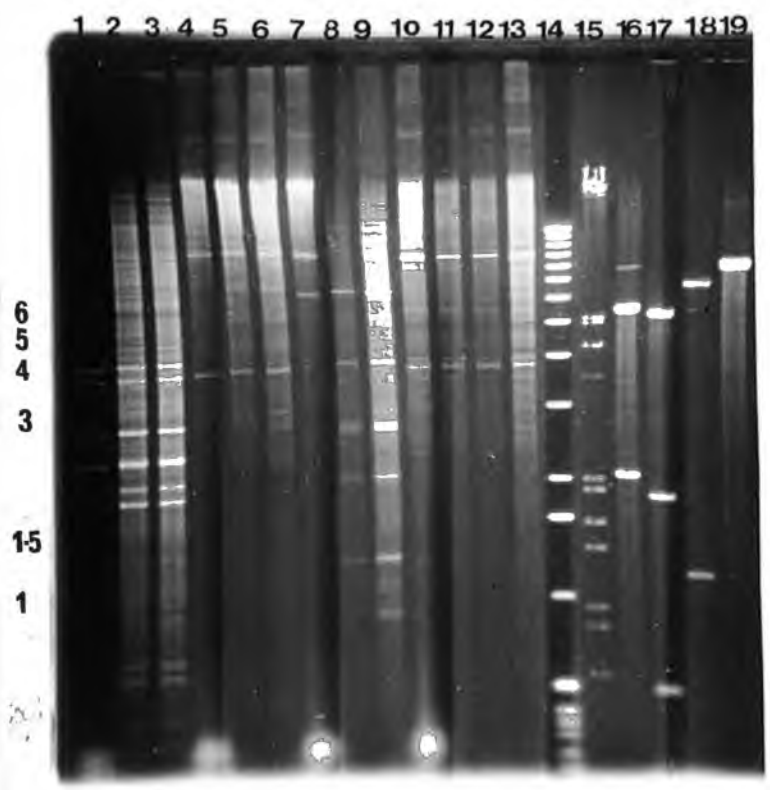
*Hind*111 :1800, and 5.56.

*Sal*1 :1.22 and 6.68.

*Eco*R1 :7600.

*Kpn*1 :2100 and 5800.

Additional bands were also observed but these bands also occured in the control digests of DBY746 and are therefore assumed not be not of plasmid origin. Other bands present may be due to incomplete digestion of the chromosomal DNA. These sizes are identical to the sizes obtained on digestion of the parental plasmid as seen in lanes 15-19. Figure 4.14




Digests of total DNA from DBY746::pFAMY, $I_2$ and $I_4$ and plasmid pFAMY. Approximate fragment sizes are given in kb for the plasmid digests on the following page..

Lane 1 :DBY746 *Eco*R1.

Lane 2 :DBY746::pFAMYI2 *Eco*R1.

Lane 3 :DBY746::pFAMYI4 *Eco*R1.

Lane 4 :DBY746 *Sal*1.

Lane 5 :DBY746::pFAMYI2 *Sal*1.

Lane 6 :DBY746::pFAMYI4 *Sal*1.

Lane 7 :DBY746 *Hin*dIII.

Lane 8 :DBY746::pFAMYI2 *Hin*dIII.

Lane 9 :DBY746::pFAMYI4 *Hin*dIII.

Lane 10:DBY746 *Kpn*1.

Lane 11:DBY746::pFAMYI2 *Kpn*1.

Lane 12:DBY746::pFAMYI4 *Kpn*1.

Lane 14:λ 1 Kb DNA ladder 12,11,10,9,8,7,6,5,4,3,2,1.5, 1,0.5kb important sizes are indicated on picture.

Lane 15:λ *Hin*dIII/*Eco*R1.

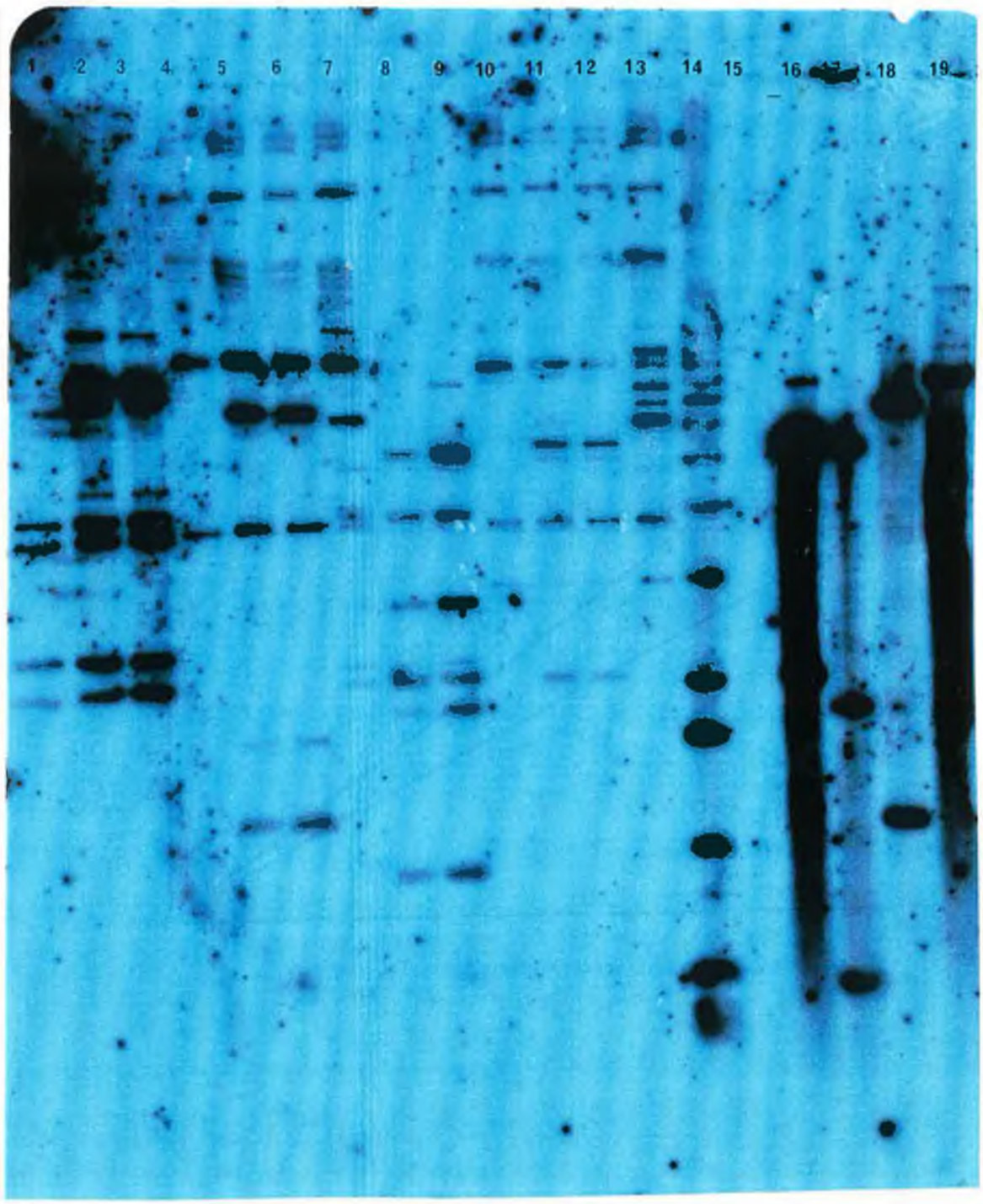
Lane 16:pFAMY *Kpn*1. 5.8, 2.1.

Lane 17:pFAMY *Hin*dIII. 5.6, 1.8, 0.5.

Lane 18:pFAMY *Sal*1. 6.7, 1.2.

Lane 19:pFAMY *Eco*R1. 7.9.

Fig 4.15.




Blot of the gel shown in fig. 4.14. The blot was probed with *Eco*R1 linearised $^{32}$p-labeled pFAMY. The contents of each lane and the approximate fragment sizes are as listed on the following page.

Lane 1 :DBY746 *Eco*R1.
Lane 2 :DBY746::pFAMYI2 *Eco*R1.
Lane 3 :DBY746::pFAMYI4 *Eco*R1.
Lane 4 :DBY746 *Sal*1.
Lane 5 :DBY746::pFAMYI2 *Sal*1.
Lane 6 :DBY746::pFAMYI4 *Sal*1.
Lane 7 :DBY746 *Hind*111.
Lane 8 :DBY746::pFAMYI2 *Hind*111.
Lane 9 :DBY746::pFAMYI4 *Hind*111.
Lane 10:DBY746 *Kpn*1.
Lane 11:DBY746::pFAMYI2 *Kpn*1.
Lane 12:DBY746::pFAMYI4 *Kpn*1.
Lane 14:λ 1 kb DNA ladder.
Lane 15:λ *Hind*111/*Eco*R1.
Lane 16:pFAMY *Kpn*1. 5.8, 2.1.
Lane 17:pFAMY *Hind*111. 5.6, 1.8, 0.5.
Lane 18:pFAMY *Sal*1. 6.7, 1.2.
Lane 19:pFAMY *Eco*R1. 7.9.

The blot shown in fig. 4.15. supports the evidence from the initial blot (fig. 4.13.) indicating that the plasmid pFAMY had not integrated into the chromosomal DNA. The data obtained could not rule out the possibility of multiple integration events in tandem. If this had occured junction fragments should also be present in the blot. It is possible that the system was not sensitive enough to detect these fragments. The other evidence i.e., plasmid stablilty, the lack of a yeast origin of replication in pFAMY and the inability to isolate plasmid DNA from the strains transformed with pFAMY, which suggested that integration had taken place. As these results conflicted with each other it was neccessary to try to design an experiment which would unequivocally prove the location of the plasmid DNA in the transformed strains.

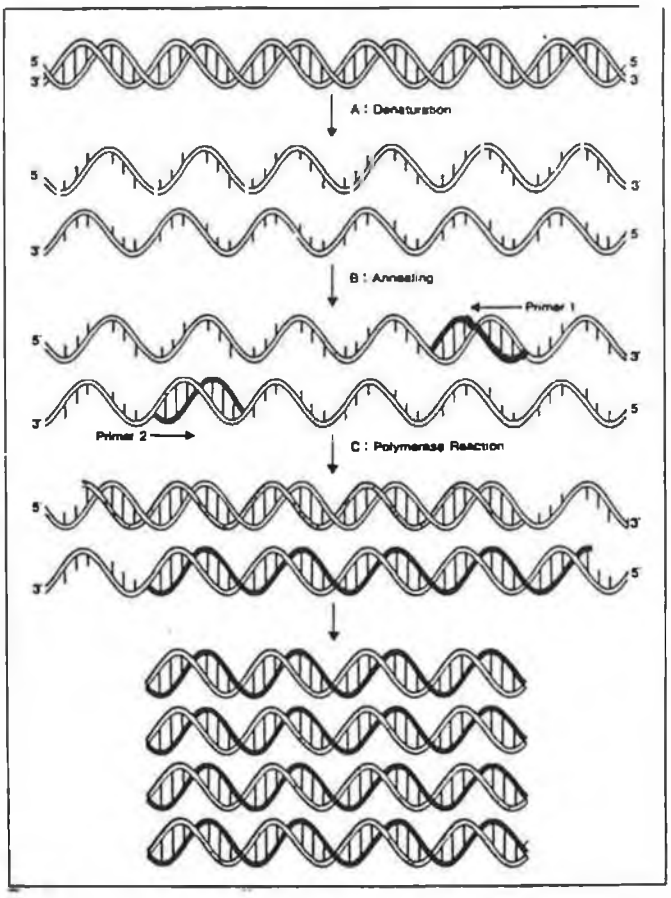
In an attempt to conclusively demonstrate whether the plasmid pFAMY had integrated into the *S.cerevisiae* chromosome a set of experiments were designed based on the polymerase chain reaction.

**4.2.1. The use of the Polymerase chain reaction (PCR) to define the chromosomal position of the plasmid pFAMY.**

As both attempts at defining the position of the plasmid by chromosomal digestion followed by Southern blotting had been unsuccessful, an alternative method was chosen to try and define the position of the plasmid pFAMY in the transformed yeast strains. The method chosen was the Polymerase Chain Reaction (PCR). The PCR reaction should be capable of differentiating between integrated and non-integrated plasmid as the specific primers chosen for the experiment were designed so that in one reaction only strains in which the plasmid was located extrachromosomally would result in the amplification of a target sequence and in a second reaction only plasmid which had successfully integrated into the host genome would result in the amplification of the target sequence.

The polymerase chain reaction results in the *in vitro* amplification of specific DNA sequences by a process of repetitive cycling between DNA denaturation, primer annealing and then extension by *Taq* DNA polymerase. The DNA of interest is first heat denatured, then primer molecules specific for the flanking regions of the DNA sequence which is to be amplified bind to this single stranded DNA. The primer thus acts as a starting point for the synthesis of a new strand of DNA which is synthesised by *Taq* DNA polymerase using the single stranded DNA as a template. After synthesis of this new strand the DNA is again denatured the primers allowed to anneal and the process is repeated for a given number of cycles. The primers are chosen so that when each is extended the newly synthesised strands will overlap the binding site of the opposite oligonucleotide primer thereby resulting in an exponential increase in the number of target sequences. A schematic diagram of a typical PCR reaction is shown in fig. 4.16. PCR is a very powerful technique sensitive enough to pick up and amplify single DNA molecules and is therefore ideally suited to the identification of inserts within chromosomal DNA.

Figure 4.16.




Schematic diagram of a typical PCR reaction.

The 3 primers selected for use in the PCR reactions shown in fig. 4.17(a).

Figure 4.17(a).

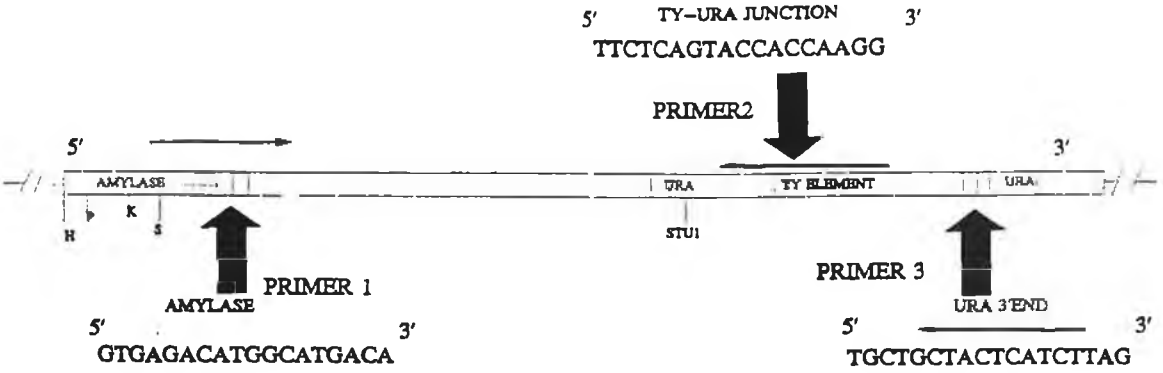
Primer 1: 5′-GTGAGACATGGCATGACA-3′ Amylase primer.
2: 5′-TTCTCAGTACCACCAAGG-3′ TY*ura* junction primer
3: 5′-TGCTGCTACTCATCCTAG-3′ ura 3′ primer.

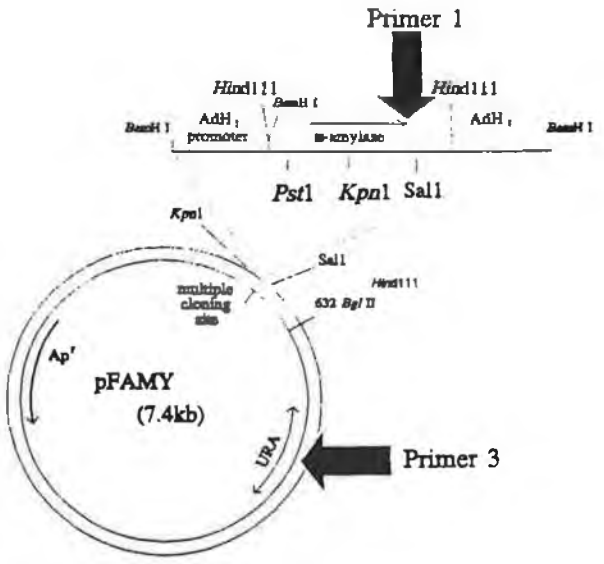
Sequence of primers used in PCR reactions.

The location of these specific sequences is shown in figure 4.17(b), Primer 1 is at the 3′ end of the amylase gene, primer 2 is at the junction of the *ura* 3 gene and the TY element in the DBY746 chromosome and primer 3 is at the 3′

end of the *ura* 3 gene present in both the DBY746 chromosome and the plasmid DNA (fig 4.17(b)). If the integration of plasmid pFAMY had occurred, a reaction with primers 1 and 2 was expected to amplify a fragment of 1880 bp from total DNA isolated from DBY746::pFAMYI$_2$ and DBY746::pFAMYI$_4$ strains while no fragment amplification was expected if integration had not taken place. Likewise a reaction using primers 1 and 3 was expected to amplify a fragment of 1950 bp if the plasmid had not integrated and no fragment if the plasmid had successfully integrated and was being maintained extrachromosomally. Thus by using a combination of the three primers it should be possible to show whether or not the plasmid had successfully integrated at the *ura*-3-52 locus of the yeast chromosomal DNA.

Figure 4.17(b).




Schematic diagram of the plasmid integration site and expected sizes of fragments after PCR amplification using Primers 1 and 2 and 1 and 3.

For the PCR experiments total DNA was isolated from DBY746::pFAMY $I_2$, $I_4$ and DBY746 (as a negative control) (2.15). All annealing reactions were carried out at 45°C unless otherwise stated. Fig. 4.18 shows the results of the preliminary PCR reactions carried out. Lane 1 shows that the amylase primer (primer 1) and the *ura* 3′ primer (primer 3) resulted in amplification of a band of 1.9 kb. Lane 2 shows that amylase and TY*ura* primer (primer 2) resulted in the same size band being amplified from pFAMY plasmid DNA. This result was unexpected as only the amylase and *ura* 3′ primers should have given this size fragment. Lanes 1 contained a band of approximately 300bps and lane 2 contained a band of approximately 400bps. Neither of these bands were expected. Lane 3 contains DBY746 total DNA incubated with the amylase and TY*ura* primers and lane 4 contains DBY746 total DNA incubated with the amylase and *ura* 3′primer. No 1.9 kb band was observed in these lanes. However, lane 3 shows a band of approximately 300bps identical in size to that seen in lanes 1, suggesting that this is a nonspecific band. In an attempt to ascertain the reason for the appearance of these bands of 3-400bps in lanes 1-3 each of the 3 primers was checked against the GENBANK sequence data base for sequence homology using the BLAST program, but only homology to the expected DNA sequences was found. It is not known why the amplification of these bands occured. Lane 5 contains total DNA from DBY746::pFAMY$I_4$ incubated with the amylase and TY*ura* primers and this gives the expected 1.9 kb fragment indicating that the plasmid has successfully integrated into the chromosomal DNA at the *ura*3-52 locus. This data is supported by the fact that when the same total DNA is incubated with the amylase and *ura* 3′ no fragment is amplified as expected. This conclusion is however undermined by the fact that in lane 1 and 2 a fragment of 1.9 kb results when plasmid pFAMY is incubated with both sets of primers. Further experiments were carried out in an attempt to clarify the situation.

Figure 4.18.

Results of PCR amplification Gel 1.

| Lane | DNA | Temp | Primers | Frag. Size |
|---|---|---|---|---|
| Lane 1: | pFAMY 1/100 dil | 45°C | Amy + Ty*ura* | 1.9, 0.3kb |
| Lane 2: | pFAMY 1/1000 dil | 45°C | Amy + *ura* 3′ | 1.9, 0.4kb |
| Lane 3: | DBY746 | 45°C | Amy + TY*ura* | 0.3kb |
| Lane 4: | DBY746 | 45°C | Amy + *ura* 3′ | ---- |
| Lane 5: | pFAMYI4 | 45°C | Amy + TY*ura* | 1.9, 0.3kb |
| Lane 6: | pFAMYI4 | 45°C | Amy + *ura* 3′ | ---- |
| Lane 7: | λ 1kb DNA ladder | | | |
| Lane 8: | No DNA, neg. ctrl | 45°C | Amy + TY*ura* | ---- |

The results of the above PCR reactions suggested that the 12 bps of the TY*ura* primer which are homologous to the 3'end of the *ura* gene were sufficient to bind to the *ura* gene and act as a starting point for the extension by *Taq* polymerase. In PCR reactions, increasing the annealing temperature enhances discrimination against incorrectly annealed primers and increases the specificity of the amplified bands. The annealing temperature of the 12bps specific to the 3' end of the ura3 gene is 38$^{o}$C as opposed to 53$^{o}$C for the entire Ty-*ura* primer. It should therefore have been possible to dissociate the TY*ura* primer from the pFAMY plasmid DNA by increasing the annealing temperature while leaving the TY*ura* primer bound to the TY*ura* region of chromosomal DNA. This should have occured as only 12 bp of the TY*ura* primer are homologous to plasmid pFAMY while it is 100% homologous to the TY*ura* region of chromosomal DNA. The annealing temperature of the reactions was increased to 50$^{o}$C and the reactions were carried out as in fig. 4.22 but resulted in no bands being amplified. As this temperature appeared to be too high for the PCR reactions they were repeated using a temperature of 47$^{o}$C but again no bands were amplified. The reason for the temperature sensitivity of the reactions is not known. Attempts to increase the stringency of the reaction by reducing the concentration of pFAMY plasmid DNA resulted in amplification of the bands previously observed.

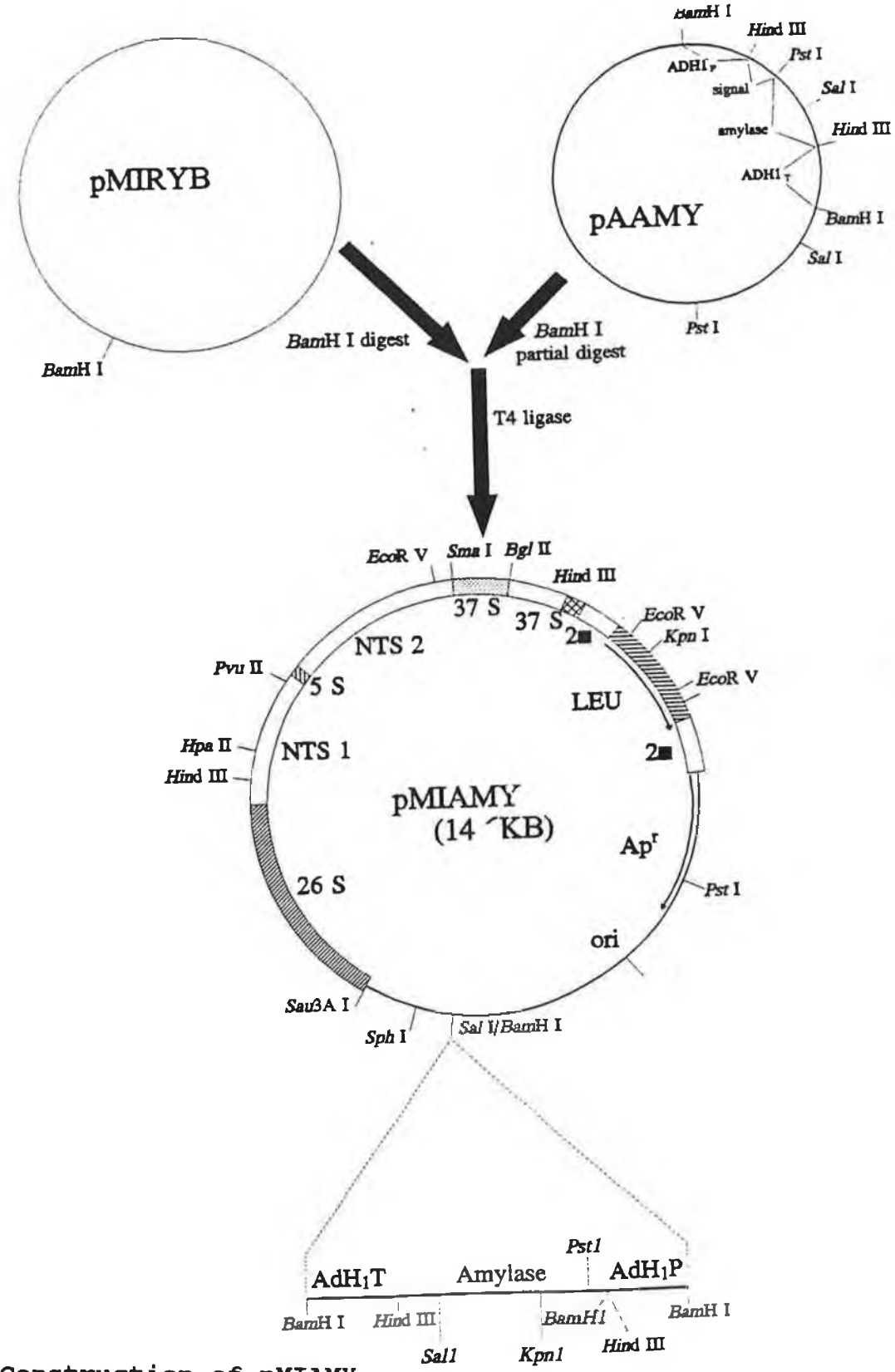
Figure 4.19 shows the results of reactions carried out using DBY746::pFAMYI2 total DNA and also the result of increasing the annealing temperature from 45$^{o}$C to 46$^{o}$C. The gel shows that using both the amylase and TY*ura* primers and the amylase and *ura* 3' primers gave a band of about 1.9KB when incubated with DBY746::pFAMYI2 DNA. An attempt to remove the the 1.9 kb band present in the reaction using the amylase and the *ura* 3' primer were unsuccessful as raising the annealing temperature to 46$^{o}$C resulted in the loss of this size fragment for both sets of primers (lanes 6 and 7). Lanes 4 and 5 show that when PCR reactions with pFAMY were carried out at 46$^{o}$C this resulted in the

While it was hoped that these PCR experiments would confirm whether the plasmid pFAMY had successfully integrated into the yeast chromosomal DNA, the results obtained were not clear cut. The presence of a 1.9 kb fragment when DBY746::pFAMYI$_4$ total DNA is amplified using the amylase and TY*ura* primers and the absence of this fragment in reactions using the amylase and *ura* 3′ primers indicates that this strain harbours the plasmid integrated at the *ura*-3-52 locus. Amplification of total DNA from strain DBY746I$_2$ resulted in a 1.9 kb fragment with both sets of primers. This result would be compatible with an integration event of two or more tandemly repeated copies of the plasmid. The conclusion that integration had successfully occured is supported by the stability of the plasmid, the inability to recover plasmid pFAMY DNA from these two strains and the lack of a yeast origin of replication on the plasmid.

**4.3. Expression of *B.licheniformis* α-amylase from a ribosomal DNA multicopy integrating vector.**

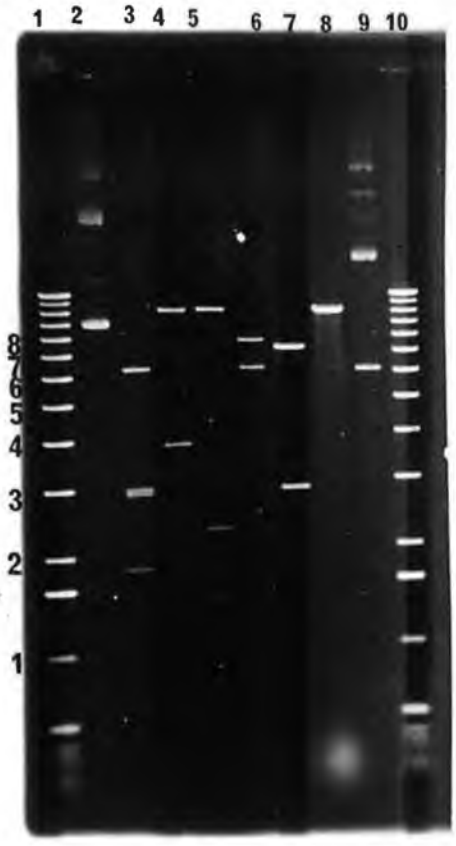
A number of strategies based upon integration into reiterated chromosomal DNA have been used to generate stable multicopy integrants; integration into TY elements (Kingsman *et al.*, 1985), delta elements (Shuster *et al.*, 1990; Sakai *et al.*, 1991) or reiterated ribsomal DNA (rDNA) sequences (Lopes *et al.*, 1989). At present the best results in terms of copy number and expression are obtained using integration into the rDNA cluster which consists of about 140 tandem repeats of a 9.1 kb fragment on chromosome X11 (Lopes *et al.*, 1989, 1990). They used the plasmid pMIRY2 containing a portion of the rDNA unit and the *leu*2d marker to integrate genes encoding PGK (phosphoglycerate kinase), SOD (superoxide dismutase) and thaumitin. Transformation with these plasmids linearised at the *Sma*1 or *Hpa*1 site gave $leu^+$ transformants with 100-200 copies integrated into a non-transcribed spacer region of the reiterated rDNA. The transformants were highly stable with 80-100% of the copies being retained after 70 generations. The levels of the heterologous proteins produced in these integrated strains were similar to those produced by 2μm episomal vectors.
Based upon the above results, integration into the reiterated rDNA cluster was the final method chosen to attempt to increase the level of the *B.licheniformis* α-amylase produced in *S.cerevisiae*. The integrating vector was constructed as shown in fig. 4.20. The *Bam*H1 fragment from pAAMY containing the ADH1 promoter and terminator and the α-amylase gene was isolated from a 0.7% agarose gel after partial digestion with *Bam*H1 and cloned into the unique *Bam*H1 site of pMIRY2 giving pMIAMY. The orientation of the insert was confirmed by restriction digest analysis (fig. 4.21).

Figure 4.20.




Construction of pMIAMY.

Figure 4.21.




Restriction digests of pMIAMY showing orientation of the inserted *Bam*H1 fragment.Approximate sizes are shown in kb.

Lane 1 :λ 1kb Dna ladder 12,11,10,9,8,7,6,5,4,3,2,1.5,1kb.
Lane 2 : pMIAMY uncut
Lane 3 : pMIAMY *Hind*111 cut 6.5, 3.0, 2.8 ,1.8
Lane 4 : pMIAMY *Pst*1 cut 10.3, 4.0
Lane 5 : pMIAMY *Bam*H1 cut 10.3, 2.0, 1.8
Lane 6 : pMIAMY *Kpn*1 cut 7.5, 6.5
Lane 7 : pMIRY *Hind*111 cut 7.5, 2.8
Lane 8 : pMIRY *Bam*H1 cut 10.3
Lane 9 : pMIRY uncut
Lane 10:λ 1kb Dna ladder 12,11,10,9,8,7,6,5,4,3,2,1.5,1kb.

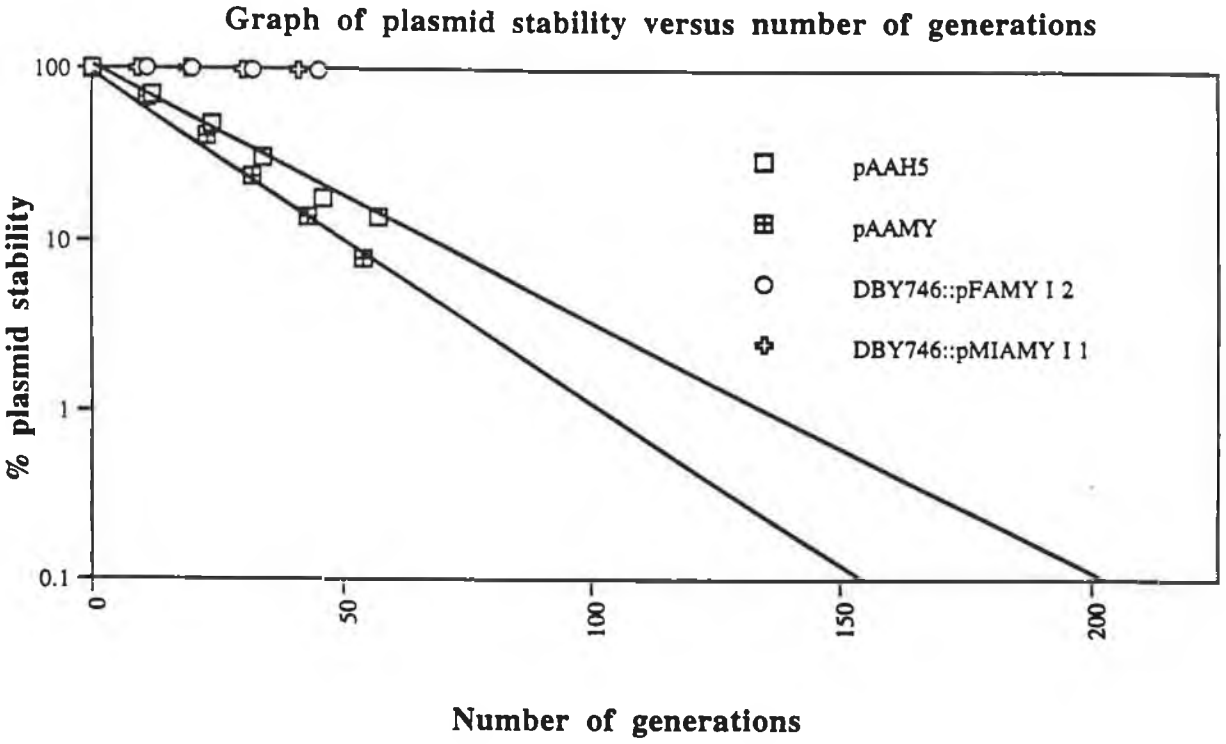
Plasmid pMIAMY was transformed into *S.cerevisiae* DBY746 after linearisation at the unique *Sma*1 site and $leu^+$ transformants (from $leu^-$ minimal medium) were analysed for amylolytic activity. Three of the transformants were selected for further study and the levels of amylase secreted were analysed after growth of the strains for 36 hours at 30°C on minimal medium. Table 4.4 gives the level secreted by these strains compared to the levels secreted by DBY746/pAAMY.

Table 4.5. Comparison of α-amylase activity in the supernatants of DBY746 transformed with pAAMY and the ribosomal integrating vector pMIAMY

| Plasmid | Cell Number | Activity U/ml | Activity/cell. |
|---|---|---|---|
| pAAMY | $3.3 \times 10^7$ | 958. | $2.9 \times 10^{-5}$ |
| pMIAMY1 | $3.11 \times 10^7$ | 1231 | $3.9 \times 10^{-5}$ |
| pMIAMY2 | $3.21 \times 10^7$ | 888 | $2.76 \times 10^{-5}$ |

From the above table it can be seen that the levels of amylase secreted by the strains containing the multiple integrating plasmid were similar or greater to those secreted by DBY746 containing the episomal plasmid. Preliminary plasmid stability studies, where strain DBY746::pMIAMY1 was grown over approximately 45 generations (fig. 4.22.) indicated that there was only 1% plasmid loss suggesting that the plasmid had been stably integrated into the non-functional spacer region in the reiterated rDNA sequence in DBY746. Attempts to isolate plasmid DNA from DBY746::pMIAMY1 and 2 were unsuccessful while plasmid was isolated from DBY746/pAAMY used alongside these strains as a control, further indicating that the plasmid pMIRYAMY was not episomally located in these strains.

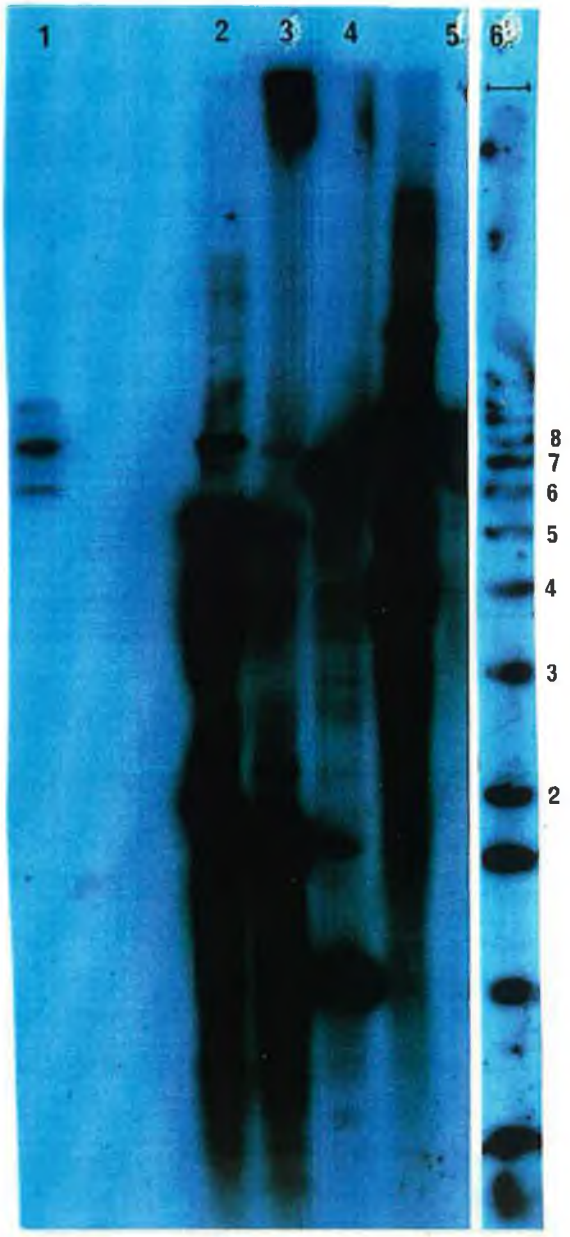
Figure 4.22.




Comparison of plasmid stabilities of various strains harbouring the amylase gene.

To confirm the chromosomal location of the plasmid, total DNA was isolated from DBY746::pMIAMY1 (as a representative of the multiple integrated strains) and digested with *Kpn*1, run on an agarose gel, Southern blotted and probed with the $^{32}$p-labeled *Hin*dIII amylase fragment of $pSL5_2$. The result of this blot is shown in fig. 4.23.

Figure 4.23.

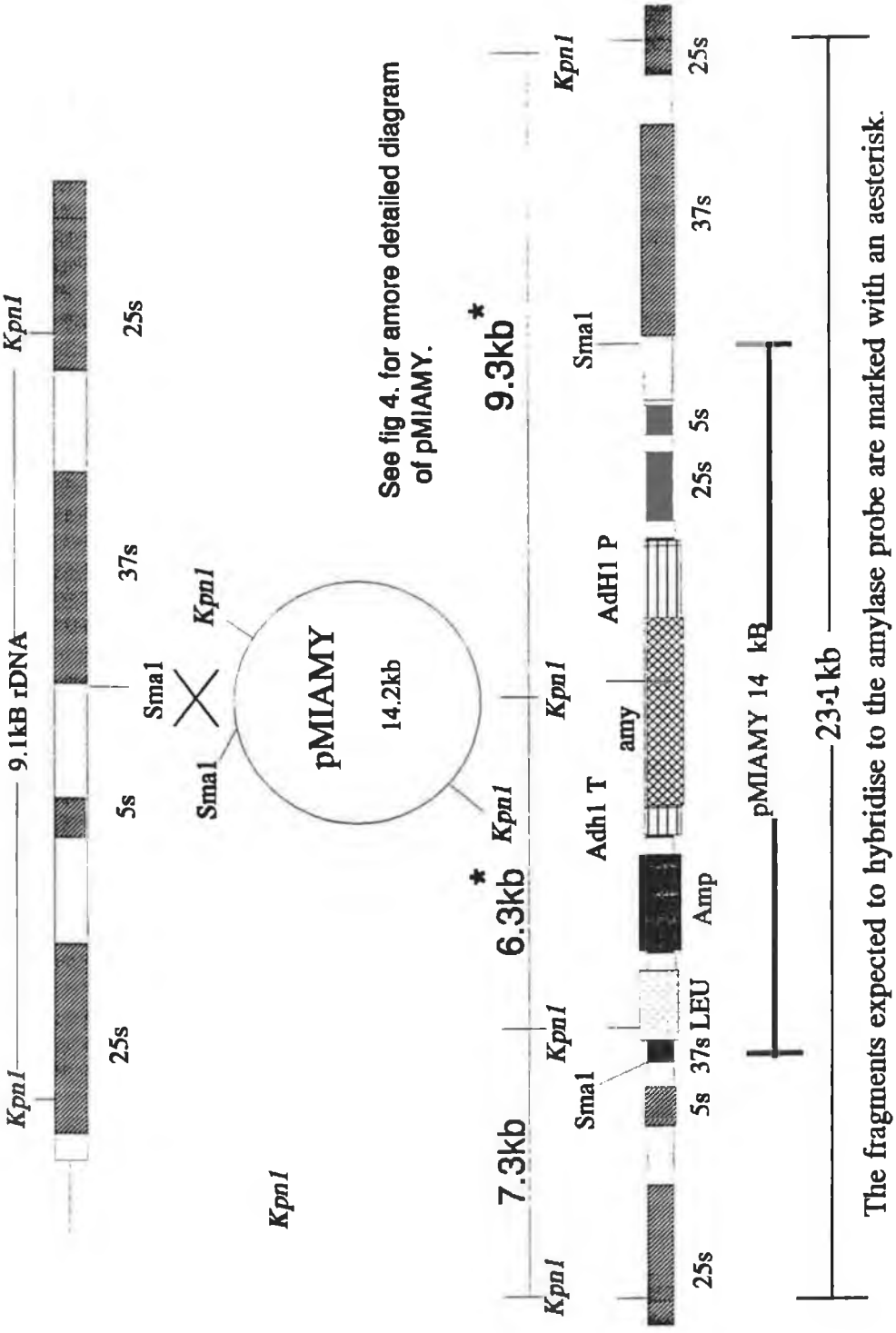



Southern blot probed with the *Hin*dIII amylase fragment from pSL52.

Lane 1 : pMIAMY I1 *Kpn*1
Lane 2 : pFAMY *Kpn*1 5.8,2.1
Lane 3 : pFAMY *Hin*dIII 1.8
Lane 4 : pFAMY *Sal*1 6.7,1.2
Lane 5 : pMIAMY *Kpn*1 7.5,6.5
Lane 6 : 1kb DNA ladder

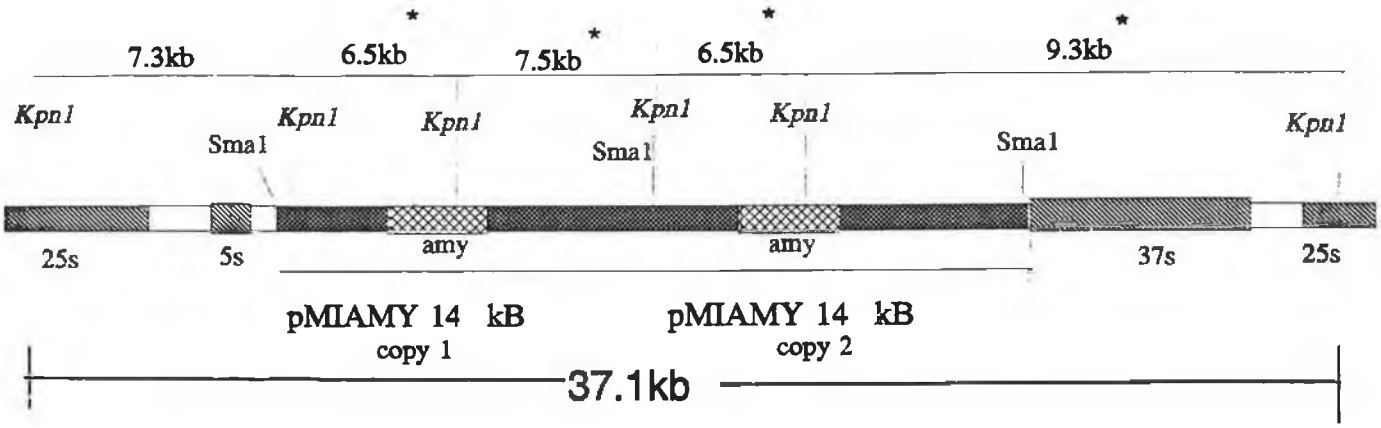
Lane 5 contains the plasmid pMIAMY DNA digested with *Kpn*1 giving fragments of 7.5 and 6.5 kb. If the plasmid had successfully integrated into the the rDNA, which consists of a cluster of about 140 copies of a 9.1 kb *Kpn*1 fragment, digestion of total DNA from these integrants should give bands on the Southern gel equivalent to the total size of this repeat plus the plasmid, ie 14 + 9.1 =23.1 kb. Alternatively if the plasmid had integrated in tandem the total size of the DNA region would be 14+(14n)+9.1. where n = the number of plasmids integrated in tandem. A probable mechanism for both the single copy and multiple integration is shown in figure 4.24 (a) and (b).Linearising the plasmid with *Sma*1 would result in the restriction pattern shown in Figure 4.24(a) and a single crossover event wouldresult in the section of DNA shown. A *Kpn*1 digest of total DNA should therefore produce 3 DNA fragments, the amylase probe would be expected to hybridise to 2 of these fragments as shown. If two or more copies of the plasmid had integrated in tandem the resulting size of the restriction fragments after *Kpn*1 digestion would be as shown in Figure 4.24(b). In this case the amylase would be expected to hybridise to 3 of the fragments as shown. The presence of three bands of the approximate sizes expected supports the conclusion that integration of 2 or more copies of the plasmid had occured at the rDNA locus. The conclusion that integration had successfully taken place is supported by the plasmid stability study (fig. 4.22) and by the levels of amylase produced by these integrants (table 4.4).

**Figure 4.24(a).**




Probable arrangement of DNA after single crossover integration of the plasmid pMIAMY at the 9.1Kb rDNA repeat.

Figure 4.24(b)




**The fragments expected to hybridise to the amylase fragment are marked with an aesterisk.**

Probable arrangement of DNA after the integration in tandem of 2 or more copies of the plasmid pMIAMY at the 9.1Kb rDNA repeat.

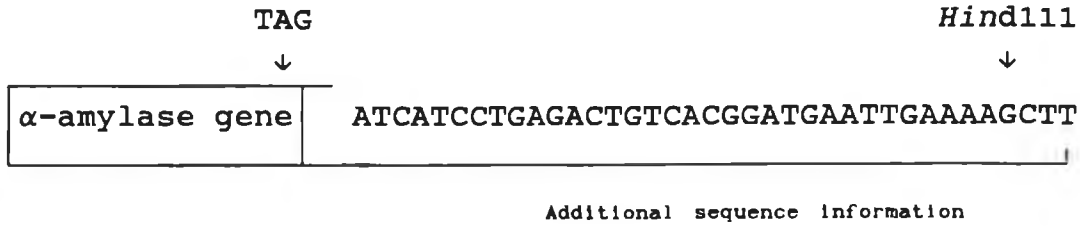
**4.4 Bal 31 deletions of the 3' end of the *B.licheniformis* α-amylase gene containing fragment.**

In a recent paper by Demouder *et al*,1992, it was shown that deleting the major part of the untranslated 3'end of the murine interleukin2 (mIL2) gene substantially increased the levels of mIL2 produced by S.cerevisiae. The authors attributed this increase to the removal of a destabilising sequence in the 3' untranslated region which may have been responsible for the rapid degradation of the mIL2 mRNA.

The α-amylase-containing fragment used in this study contained a palindromic motif characteristic of a transcriptional termination sequence at the 3' end of the amylase containing DNA fragment (see appendix no.1). This experiment was designed to analyse what effect if any the disruption of this palindromic sequence would have on the levels of α-amylase produced by both *E.coli* and *S.cerevisiae*.

Before any deletions could be carried out it was neccessary to know the entire sequence of the amylase containing DNA fragment. Restriction site analysis showed that the α-amylase gene containing fragment was longer than published sequence of Yukki *et al*., 1985. In order to have complete sequence information before Bal 31 deletions were carried out, the 3' end of amylase-containing fragment was sequenced by the dideoxy chain termination method (2.37). The 3' end of the fragment in pSL5 was sequenced using the universal primer. Sequence analysis showed that there was an additional 37 base pairs present which had not previously been sequenced (fig. 4.25).

Figure 4.25.

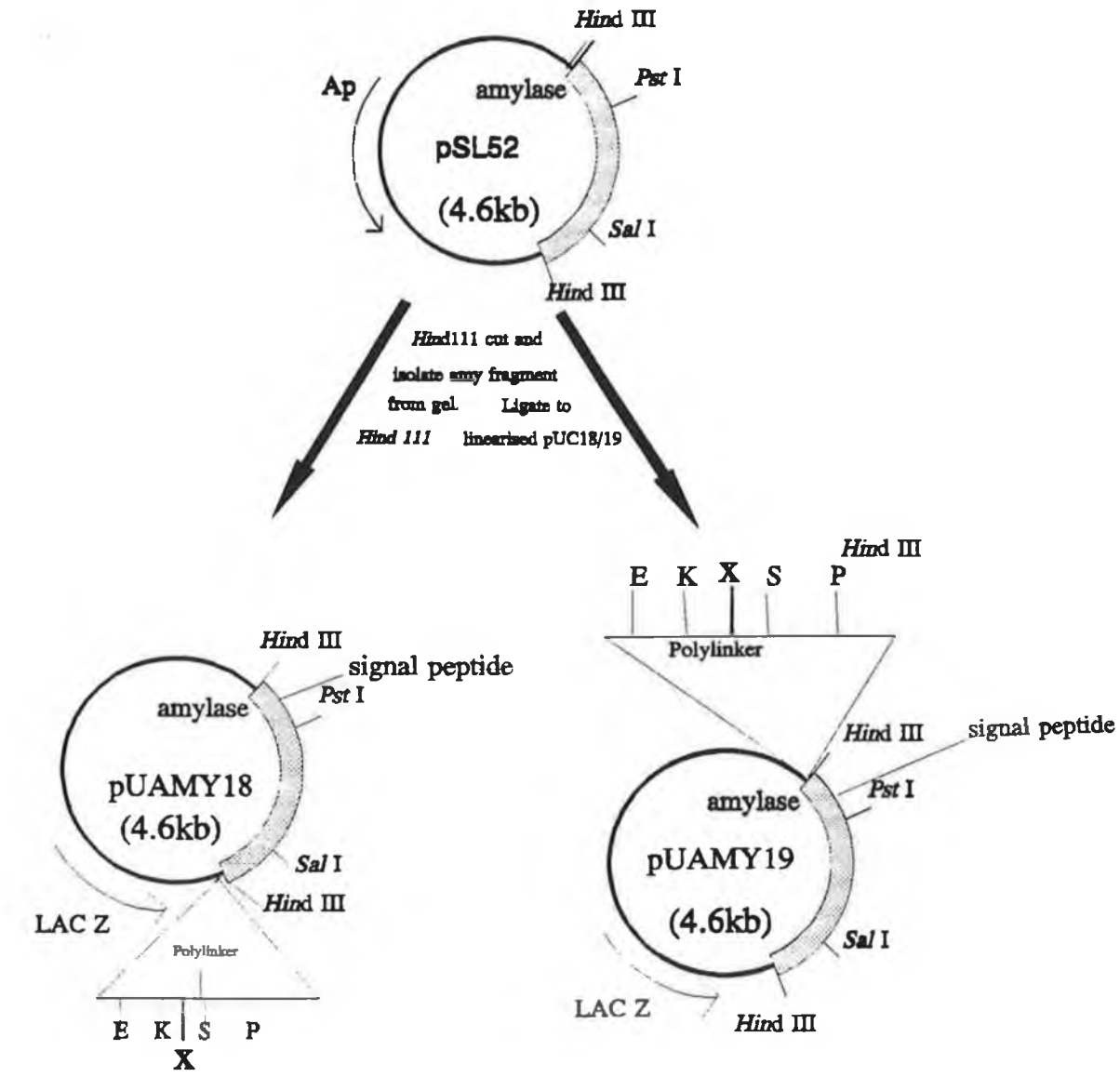



Additional sequence at 3′ end of the amylase containing *Hind*111 fragment from pSL5₂.

Deletion of the 3′ end of a DNA fragment is carried out by first linearising the plasmid at a suitable site proximal to the 3′ end of the DNA fragment, followed by BAL 31 digestion of the linearised plasmid. The vector pSL5₂ contains only one restriction site at the 3′end of the amylase containing fragment, a *Hind 111* site. However this is not a unique site and it was therefore not possible to use this plasmid for the BAL 31 deletions. Therefore before the BAL 31 deletion could be carried out the *Hind*111 amylase containing fragment from pSL5₂ had to be subcloned into either pUC18 or 19 which would then allow the plasmid to be linearised at a number of sites close to the 3′ end of the amylase containing gene fragment. Initially the *Hind*111 fragment from pSL5₂ was subcloned into pUC19. However, when DNA was isolated from 20 amylase positive clones, it was found that in each case the amylase fragment was in the opposite orientation to the one required, which is the 5′ end of the gene adjacent to the *lacZ* promoter. Other amylase positive clones secreting very large quantities of α-amylase were observed on the ligation plates but it proved impossible to isolate DNA from these strains. It is possible that these strains contained the amylase in the required orientation but were unstable due to the large quantities of amylase expressed by the plasmid. In plasmid pUAMY19 (fig. 4.26), containing the fragment in the opposite orientation to the one required, there is amylase expression even though the gene is

inserted in a reverse orientation relative to the *lacZ* promoter, indicating that some promoter activity is present in the amylase gene-containing fragment or that read through is occuring from the *amp* promoter.

Figure 4.26.

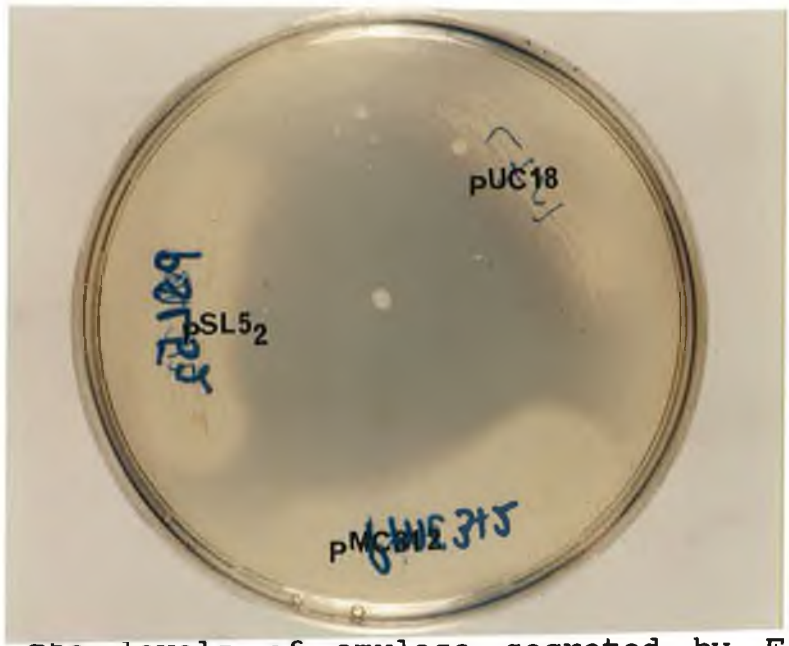



K =Kpn1
E =EcoR1
S = Sal1
P = Pst1
X=*Xba1*

Plasmid pUAMY19 and pUAMY18 containing the *Hind 111* amylase fragment from pSL52

As pUC19 proved to be unsuitable, the *Hin*d111 amylase containing fragment from pSL5$_2$ was subcloned into pUC18 giving plasmid pUAMY18 (fig. 4.26) which expressed amylase. As with pUC19 the amylase fragment could only be cloned in one direction i.e., the 3′ end of the gene adjacent to the *lac*Z promoter but this was the orientation required for deletion with Bal 31, as in this construct (Fig 4.26) there are a number of unique sites available for linearisation of the plasmid at the 3′ end of the α-amylase gene containing fragment. Plasmid pUAMY18 was linearized with *Xba*1, column cleaned (2.14) and digested with Bal 31. The Bal 31 treated samples were column cleaned and religated in the presence of 8mer *Hin*d111 linkers (5′-CAAGCTTG-3′) and transformed into *E.coli*. A number of amylase positive colonies were isolated and one, pMC312 (for 3 minutes Bal 31 treatment), was chosen for further analysis as it resulted in a large increase in amylase production in *E.coli*. PMC316 ( 6 minutes Bal 31 treatment) was also analysed as it resulted in a decrease in the levels amylase produced in *E. coli* relative to the parental vector pUAMY18. Fig. 4.27. shows a comparison of the levels of amylase produced by *E.coli* transformed with pMC312, pSL5$_2$ and a negative control pUC18.

Figure 4.27.



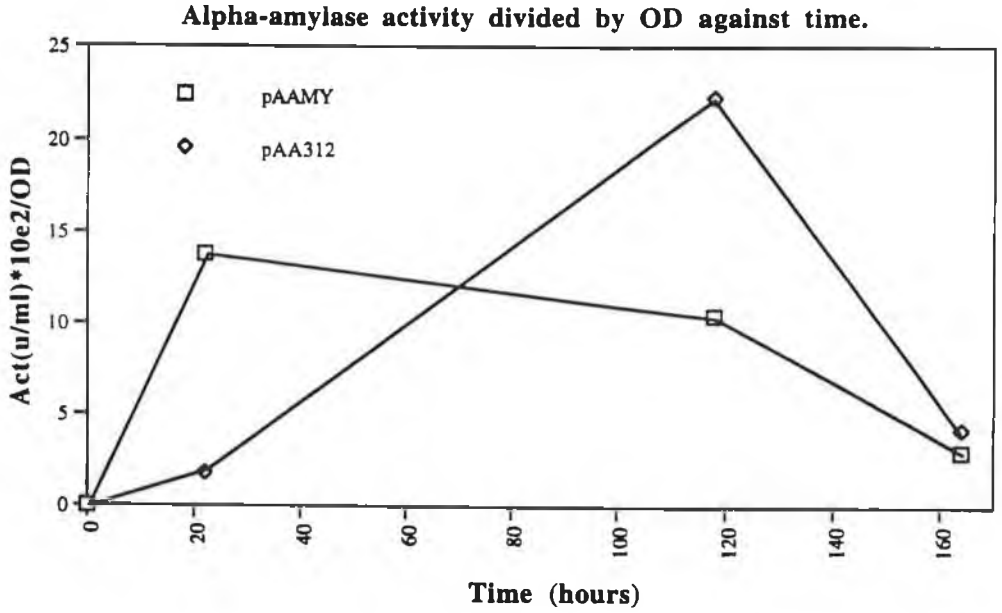

Comparison of the levels of amylase secreted by *E.coli* harbouring the plasmids pSL5$_2$, pUC18 and pMC312.

Restriction digest analysis showed that the amylase fragment in pMC312 was approximately 250bp shorter than the parental amylase-containing fragment. To find out the exact extent of the Bal 31 deletion, the shortened *Hind*111 amylase fragment from pMC312 was subcloned into pUC19 and sequenced using the universal primer. The exact extent of the deletion was determined by comparing the sequence of the 3′ end of the BAL 31 treated amylase containing fragment with the original entire sequence using the SEQAID sequence analysis program. The extent of the deletion is shown in appendix no. 1.

Comparison of the two sequences showed that 237 base pairs had been deleted from the parental fragment. Deletion of this number of base pairs disrupted the putative palindromic transcriptioal termination site (see appendix # 1) but left the entire $\alpha$-amylase gene including its translational stop site intact. To check whether the increase in the secretion of amylase seen in *E.coli* with the deleted fragment would also occur in yeast, the shortened *Hind*111 fragment was subcloned from pMC312 into the unique *Hind*111 site of pAAH5 Giving pAA312. The orientation of the amylase fragment was confirmed by restriction analysis and the plasmid was transformed into DBY746.

The levels of amylase produced in minimal media by DBY746/pAA312 were analysed over a period of 160 hours and compared to the levels of amylase produced by DBY746/pAAMY(the plasmid with the full amylase gene containing fragment). The result of this study is shown in figure 4.28.The graph clearly shows that there is a substantial increase in the levels of amylase present in the supernatant of DBY746/pAA312. However this increase is only observed after an extended period of growth.

Figure 4.28




Comparison of amylase activity in DBY746/pAA312 and DBY746/pAAMY.

Although the deletion had not actually proceeded into the actual structural amylase gene it did delete a section of the palindromic sequence as shown below. Palindromic sequences at the 3′ end of prokaryotic genes are characteristic motifs of transcriptional termination sequences. The disruption of this palindromic sequence would have allowed transcription to proceed through this site and into the Lac Z gene. It is possible that the larger mRNA resulting from this is more stable than the original amylase only mRNA and this may be the reason for the increase in amylase activity observed in *E. coli.*
In yeast the disruption of this palindromic sequence may have allowed transcription to continue through to the Yeast ADH1 terminator. This may have had the effect stabilising the mRNA in yeast and therefore have resulted in an increase in the quantity of amylase produced.

# Chapter 5.

# Discussion.

**Secretion of the of the heterologous α-amylase.**

The α-amylase gene from *B.licheniformis* was successfully expressed and efficiently secreted from *S.cerevisiae*. Since in the construct used, the α-amylase gene is under the control of its own native signal peptide, this would strongly suggest that the *bacillus* signal peptide is being recognised by and processed through the yeast secretory pathway. The *B. licheniformis* α-amylase signal peptide is shown diagrammatically in Fig 5.1.

Fig. 5.1.

MET-LYS-GLN-GLN-LYS-ARG-LEU-TYR-ALA-ARG-LEU-LEU-PHE-ALA
LEU-ILE-PHE-LEU-LEU-SER-HIS-PRO-ALA-ALA-ALA-ALA-ALA
-1/+1

The signal sequence contains the characteristic motifs of both eukaryotic and prokaryotic secreted proteins. It includes a short cluster of 4 positively charged amino acids within the first 10 amino acids followed by an extensive hydrophobic domain which precedes a more polar region of 6 amino acids at the C terminal domain which contains the signal cleavage site (von Heijne, 1988). The signal cleavage site conforms to the general rule that the border between the hydrophobic and carboxyl regions falls between residues -7 and -6 and is signaled by a proline residue, an amino acid known to produce B turns in polypeptides. It also obeys the -3 -1 rule whereby the amino acids in positions -3 and -1 must be small and uncharged (von Heijne, 1988). The position of this cleavage site has been confirmed by N terminal amino acid sequencing (Kuhn, et al. 1982). In this study, both Western blotting and activity gel analysis, following treatment of the yeast derived amylase with endoHf, showed a single band corresponding to a molecular weight of approximately 53kd indicating that the signal peptide had been correctly processed by the signal peptidase during translocation of the amylase through the yeast secretory pathway. Although cleavage may have occurred at more than one site, due to

the large size of the amylase protein, this was not detectable on either the Western blots or activity gels. Protein sequence analysis would be required to confirm this.

Although post translational glycosylation does not occur in *Bacillus*, the *B. licheniformis* amylase gene contains a total of seven potential glycosylation sites (appendix 2). Core glycosylation of the amylase at each of these sites would result in an increase in molecular weight of the amylase from 55 to 76 kd, as each unit of core gylcosylation is estimated to increase the molecular weight of a protein by 3 kd (Melnick et al., 1990). From the Western blots and activity gels it is apparent that several different forms of the amylase are present ranging from approximately 52 kd to 68kd. A contributing factor to this increased molecular weight could have been due to a percentage of the secreted protein retaining its signal peptide. Retention of the signal peptide would increase the molecular weight of the protein by 3.3 kd and it would therefore be impossible to distinguish between glycosylation and signal peptide retention. Treatment of the secreted amylase with endo$H_f$ resolved the amylase bands present to one very intense band plus a much fainter band approximately 3kd higher in molecular weight. This result showed that the multiple forms of the amylase observed were due to varying levels of glycosylation. The appearance of only a faint band approximately 3kd larger than the native amylase indicates that the majority of the amylase present in the supernatant has had its signal peptide cleaved off.

Although there appears to be heavily glycosylated forms of the amylase on activity gels this smearing observed at the top of the gel is also seen in the lanes containing the native *B. licheniformis* amylase and controls and is therefore likely to be an artifact of the activity gel system.

The highly efficient secretion of the *B.licheniformis* amylase from *Saccharomyces cerevisiae* (approximately 95%) contrasts with lower levels of secretion of the *Bacillus amylofaciens* amylase (approximately 75%) reported by Ruohonen *et al.*, 1987 while Pretorius *et al.*, 1988,

reported no detectable *Bacillus* amylase in the supernatant. However, this is most likely due to the fact that the medium used was not buffered which has been shown in this study to be essential for the detection of amylase activity. The difference between the levels of the *B. licheniformis* amylase and the *B.amylofaciens* amylase secreted into the medium is not due to the parental plasmid as in both cases the plasmid pAAH5 (Ammerer *et al.*, 1983) was used and must therefore be due to differences in the amylase proteins, either in the signal peptides, the mature proteins or both. There may also be significant differences in the 3′ and 5′ flanking non coding regions of the two amylase genes, however these differences would only result in differences in the transcription and translation levels and not in the ratio of secreted protein. A comparison of the signal peptides of the two sequences is shown in fig. 5.2.

This comparison shows that there are significant differences in the signal peptides of the two amylases with only a few short regions of homology being present giving an overall 30% homology. The percentage homology of the mature proteins is 80.3% (Yukki *et al.*, 1985). It is likely therefore that the significant difference in the secreted levels of the two amylases is due to differences in their signal peptides. The efficient secretion of the *B.licheniformis* amylase directed by its signal peptide indicates that the *B.licheniformis* signal peptide may be useful for the efficient secretion of other heterologous proteins in yeast. Recently, the *B.licheniformis* amylase has been successfully expressed in transgenic tobacco using its own signal peptide (Pen *et al.*, 1992). The amylase was found to be present in two distinct forms due to glycosylation of the protein. The signal peptide was recognized by the plant secretory machinery and the amylase was efficiently secreted further supporting the idea that the *B. licheniformis* signal peptide could be useful as a general signal peptide for the efficient secretion of heterologous proteins from both prokaryotic and eukaryotic organisms.

Fig. 5.2.

```
B.l.

-29                                                    -20
MET LYS GLN GLN LYS ARG LEU THR                  ALA ARG LEU LEU
              *   *   *                              *   *
    MET ILE GLN LYS ARG LYS ARG THR VAL SER PHE ARG LEU VAL
B.a.  -31                                          -20

                                    -10<————————— SIGNAL
B.l.
THR LEU LEU PHE ALA LEU ILE PHE LEU LEU PRO HIS SER ALA ALA
                          *                 *   *
LEU MET CYS THR LEU LEU PHE VAL SER LEU PRO ILE THR LYS THR
           B.a.

B.l  -1    |  +1  MATURE AMYLASE ————————>
  ALA ALA  |  ALA ASN LEU ASN GLY THR LEU MET GLN TYR PHE GLU
           |                *   *   *   *   *   *   *   *   *
  SER ALA  |          VAL ASN GLY THR LEU MET GLN TYR PHE GLU
           |
           B.a.              +1
```

Comparison of the amino acid sequences of the α-amylase genes of *B.licheniformis* (B.l.) and *B. amylofaciens* (B.a.). Homology is denoted with an asterisk (*).

**Characterisation of the yeast produced amylase.**

Initially when SDS-PAGE coomassie stained gels were used to try to detect a specific protein band corresponding to the α-amylase, there appeared to be no specific band corresponding to the amylase present on the gel as the banding patterns from the amylase positive supernatant and the control amylase negative appeared to be identical. (fig 3.22). In an attempt detect the amylase band the more sensitive silver staining procedure was used, however while this resulted in the appearance of a number of extra bands in the gels again the banding patterns of the amylase

containing and control supernatants were indistinguishable. This inability to detect a specific band, on either Coomassie or the silver stained SDS-PAGE gels, corresponding to the molecular weight of the native amylase may be due to the fact that the amylase was subsequently shown to be present in multiple forms resulting in a lower concentration of each form thus making it difficult to visualise. It is also possible that the amylase band is masked by the homologous secreted protein glucan-1,3 B-glucosidase which has a molecular weight of approximately 55 kd (Ramirez *et al.*, 1989). A band corresponding to this molecular weight is seen in the supernatants of DBY746/pAAH5 and DBY746/pAAMY (fig 3.22). α-amylase specific bands were subsequently identified using activity gels and Western blotting.

A comparison of the molecular weights (from western blots and activity gels) and the pH and temperature profiles of the native *B.licheniformis* amylase and the amylase produced by yeast show that the two forms are almost identical. The slight changes apparent in the pH and temperature profiles may be due either to experimental error or to changes in the physiological characteristics of the amylase possibly due to its glycosylation in yeast.

The temporal production of the *B.licheniformis* amylase is significantly different from that observed by Ruohenen et al, 1991 for the *B.amylofaciens* amylase. They found that on minimal media the maximal level of amylase present in the supernatant was at approximately 20 hours followed by a gradual decrease in the level of amylase present. In this study the amylase activity was shown to be directly related to cell growth with the maximal level of amylase observed in the supernatant at 36 hours. This growth related production of heterologous protein has also been found in the case of β-glucanase, β-glucuronidase (Hunter, 1991 and several other proteins expressed in yeast. The observed differences in the activity of the *B. licheniformis* amylase and the amylase from *B.amylofaciens* was unexpected as both genes were subcloned into the same vector and were therefore under the control of identical promoter and terminator sequences from the yeast alcohol dehydrogenase 1

gene. The observed differences may therefore reflect the relative stabilities of the two amylases in minimal media, the *licheniformis* amylase being more stable than the *amylofaciens* amylase. The activity profiles for the two amylases when grown on YEPD were very similiar.

**Cloning of the *Bacilus* α-amylase gene.**

Throughout the course of this work considerable difficulty was encountered in making constructs which contained the *B. licheniformis* α-amylase gene. In the plasmid constructs pUAMY18 and pUAMY19 for example it was only possible to clone the amylase gene in one orientation, with the start site in reverse relative to the lacZ promoter. Fig 4.26. It also proved impossible to insert the amylase expression cassette into the integrating vector YIP5. A possible reason for this may be due to the plasmid instability as a result of cells which express large levels of the amylase protein. O'Kane *et al* (1986) showed that the *B.licheniformis* amylase gene was a highly sensitive indicator of promoter activity. It is possible therefore that when the amylase gene is cloned in the correct orientation relative to the lac Z promoter that this leads to very high cellular levels of the amylase which may destabilise the host cell. In the case of both pUAMY18 and pUAMY19 after transformation of the ligations the majority of the transformants gave small amylase haloes and after plasmid isolation and restriction analysis all the plasmids were shown to contain the amylase gene in the reverse orientation relative to the lac z promoter. Several other colonies were present on the transformation plates which had barely visible cell growth and very large amylase haloes. However attempts to grow these transformants to cellular densities suitable for plasmid isolation failed. It is likely that these transformants contained the plasmids with the amylase gene in the same orientation as the lacZ promoter, resulting in very high levels of expression and cellular instability.

The reason for the difficulties encountered in the insertion of the amylase expression cassette into YIP5 are unclear. The problem was not with the vector or the

ligation conditions used as *BamH*1 digested lambda DNA fragments were successfully ligated into the vector, nor was it with the DNA fragment containing the amylase expression cassette as the same fragment preparation was used and successfully ligated into pFL34 at the first attempt. The reason must therefore lie in the combination of the specific sequences present in the YIP5 plasmid and the amylase expression cassette. In pFL34 the amylase gene was inserted into the unique *BamH*1 cloning site in the polylinker down stream from the Lac Z promoter, whereas in YIP5 the amylase expression cassette would have been inserted into the unique *BamH*1 site located proximal to the tetracycline promoter. It is possible that expression of the amylase from the tetracycline promoter led to instability of cells containing this vector and resulted in the inability to isolate transformants containing the vector. This possibility is supported by the fact that Laoide and Mc Connell (1989) have previously shown that the amylase could be successfully expressed under the control of a promoter greater than 1500 bps away.

**Characterisation of potential integrants by Southern blotting.**

The initial data, obtained from southern blots of *BamH*1 digests of total DNA isolated from 12 strains transformed with pFAMY, showed that only bands corresponding to the *BamH1* digested plasmid were present (Fig 4.13). This result was unexpected for two reasons; firstly the analysis of two transformants selected for plasmid stability studies showed that the rate of plasmid loss was less than 0.1% per generation, and secondly the plasmid pFAMY lacks a yeast origin of replication and should therefore only be able to stably maintain itself if it had been successfully integrated into the yeast chromosome, both these results indicate that the plasmid had successfully integrated into the yeast chromosomal DNA. To further investigate the cellular location of the plasmid pFAMY, a series of enzyme digests of total DNA isolated from DBY746::pFAMYI2 and

DBY746::pFAMYI4 were carried out. Southern blots of these digests again showed only bands which corresponded to the digested plasmid DNA, supporting the conclusion that the plasmid was not integrated. One alternative explanation is that the plasmid integrated in tandem in multiple copies, with visible bands on the blots corresponding to the internal fragments present in the tandemly integrated fragments, if the number of copies present was sufficiently high it is possible that only these internal fragments may be visible whilst the junction fragments at the site at the extremities of the tandemly integrated plasmids may not be visible due to their single copy number.

This proposal is supported by the fact that the single copy 11.6kb *BamH1* band from the untransformed strain DBY746 failed to show on either of the two blots mentioned above.

The plasmid stability studies and the fact that the plasmid lacks a yeast origin of replication support the conclusion that the plasmid is chromosomally located. Further characterisation of these transformants would be neccessary to show conclusively the location of the plasmid.

**PCR characterisation of the potential integrants.**

As Southern blotting of total yeast DNA digests failed to show conclusively whether or not the plasmid pFAMY had successfully integrated into the yeast chromosomal DNA another approach was taken to try to show conclusively the intracelluar location of the plasmid in these transformants. An experiment was designed based on the polymerase chain reaction (PCR). PCR was chosen as it is an extremely powerful method capable of amplifying genes which are present in single copies in total cellular DNA. For the purposes of this experiment three primers were selected. (see Fig 4.21(a). One primer was selected from the amylase gene, one primer was selected from the 3'end of the ura3 gene and the third primer was selected from the junction of the TY element and ura3 gene. The amylase primer was used in both reactions in conjunction with one of the other

primers. When the amylase and the TY-ura primers are used to amplify total DNA isolated from the pFAMY transformants, a 1.9kb band should only be present if the plasmid had successfully integrated into the yeast chromosomal DNA. Similarly when the amylase and the ura3 primers are used to amplify the total yeast DNA isolated from these transformants they should only give a 1.9kb band if the plasmid had not successfully integrated into the yeast chromosomal DNA (See Fig 4.17(b). Therefore combinations of the three primers should have clarified the ambiguity as to whether or not the plasmid pFAMY had successfully integrated into the chromosome at the ura3-52 locus in the transformants pFAMYI2 and pFAMY I4.

Unfortunately however the results obtained from these PCR experiments were inconclusive.
The reason for the inconclusive results is most likely due to the fact that the sequence selected for the TY-ura primer was at the junction of the ura3 gene and the inserted TY element and therefore contained a short (12bps) region of homology with the ura3 gene. The reason for this choice of sequence was that it should have led to the unambiguous identification of the cellular location of the plasmid pFAMY, as only chromosomal yeast DNA sequences which contained both the integrated amylase gene and the TY-ura3 junction should have resulted in the amplification of a 1.9kb fragment. It was reasoned that with only 12bps of the primer sequence homologous to the plasmid DNA that the primer would not stay bound at annealing temperatures above 30$^{o}$C. Experimentally however the 12 base pairs present which are homologous to the ura3 gene seem to have been sufficient to stay bound to the plasmid DNA even at an annealing temperature of 45$^{o}$C, resulting in the amplification of a band of approximately 1.9KB from the plasmid pFAMY when this primer was used in conjunction with the amylase primer. Attempts to remove this primer from the plasmid DNA ura3 site by increasing the annealing temperature were unsuccessful as increasing the temperature above 45$^{o}$C also resulted in the loss of the required fragment from amplified chromosomal isolates of pFAMY2 and

pFAMYI4. A possible way to overcome this problem would be to select a primer with total homology to the TY element and none to the ura3 gene.
This ambiguity with regard to whether the plasmid had successfully integrated or not could be removed by the use of pulsed gel electrophoresis followed by Southern blotting.

**Super Secretion (SSC) Mutants.**

The procedure used for the isolation of supersecreting mutants was shown to be successful and several mutants were isolated which secreted enhanced levels of the amylase. The highest increase obtained in the levels of secreted amylase was approximately five times that of the wild type. This result cannot be explained by a change in the ratio of intracellular to extracellular amylase as only approximately 5% of the amylase was located internally. The reason must therefore have been due to either an increase in the total level of amylase produced or to a decrease in the level of proteolytic degradation of the amylase produced. Alternatively the speed at which the amylase was translocated through the secretory pathway may have been increased leading to a decrease in the time where the protein was susceptible to degradation by intracellular proteases. Wood and Brazill, (1987) and Sakai et al (1988) reported similiar increases in the levels of overall protein production with one of their mutants giving an 10 fold increase in the combined levels of intracellular plus extracellular protein produced, whereas the work carried out by Smith *et al* (1985) only resulted in mutants with an altered internal and external distribution of prochymosin.

In this study the observed increase in the levels of extracellular amylase present may have been due to one of the following:
1. A higher ability in specific mRNA production due to either gene amplification or more efficient transcription.
2. Increased efficiency of translation of the amylase specific mRNA.

3. Increased levels of general protein production due to more efficient energy utilisation for protein synthesis at the expense of cell growth.
4. Production of a higher cell mass resulting in an increased level of protein production.
5. Reduction in the levels of specific protein production.
6. Mutations in regions coding for proteins involved in the secretory pathway or in regions controlling the expression of genes involved in the secretory pathway.

As the growth pattern of each of the SSC strains closely resembled that of the parent strain it is unlikely that the increased amylase present in the supernatants is due to either increased cell mass or to the redirection of energy utilisation to protein production from cell. The failure of a number of attempts to isolate strains with a further increase in amylase secretion following mutagenesis of SSC5 may have been due to the fact that the initial screen had been optimised and further increases may not have been detectable as the plate assay is less sensitive to increases in amylase activity above a certain level. While mutants with a higher level of amylase may have been present the limitations of the plate assay may have made them undetectable. It is probable that if a sufficient number of mutagenised colonies were screened using the DNS spectrophotometric assay that second level mutants may be obtained. Sleep *et al* (1991) devised a generally utilisable plate screen which uses antibodies to the heterologous protein being produced to detect increases in the level of protein production amongst mutagenised colonies. It is likely that this assay could be adapted to successfully screen for second level amylase mutants as they showed that the halo size was directly proportional to the level of heterologous protein present. This system is also a more widely usable one as it does not depend upon the protein having an enzyme activity which is easily assayable by a specific plate assay. It does however depend upon having access to large quantities of specific antibody.
In hindsight had the data showing the high percentage of amylase secretion been available prior to the initiation of

attempts to generate super secreting mutants, a more useful approach would have been to select a heterologous protein which was very poorly secreted by *S.cerevisiae*. This approach would have been more likely to isolate general super secretion mutants rather than ones which appear to be specific for the α-amylase.

**Multiple integration of the *B. licheniformis* α-amylase by the use of a rDNA integrating vector.**

Previous experiments have shown that depending upon the heterologous gene being expressed, substantial increases in heterologous protein production can be achieved by either reducing the plasmid copy number to 1 or 2 copies per cell or increasing the copy number to more than 100 copies per cell see section (4.3). In the case of the *B. licheniformis* amylase gene integration at the ura3-52 locus results in a low plasmid copy number as the ura3-52 locus is present in only one copy in the strain DBY746. When integration was targeted to this locus by using the *Stu1* linearised vector pFAMY the resulting transformants produced less amylase than strains transformed with the episomal vector pAAMY (see table 4.3).
The second method of increasing heterologous protein production was then tried i.e. multiple integration targeted to the ribosomal DNA locus, resulting in a high copy number that is stably maintained even in the absence of selective pressure. The rDNA vector pMIAMY was successfully constructed and integrated into the yeast ribosomal DNA locus. The plasmid was stably maintained and produced similiar levels of amylase to the episomal vector pAAMY. (Table 4.4) It is likely therefore that optimal levels of secreted amylase would be produced using a combination of the ribosomal integrating vector and one of the SSC strains, or by the mutagenesis and subsequent screening of one of the strains harbouring multiple copies of the amylase gene in the ribosomal DNA. The rDNA integrating vector offers the two advantages of high stability plus high levels of amylase production due the presence of multiple copies of the amylase gene integrated

into the yeast rDNA. Further improvements in the levels of amylase produced could possibly be achieved by the optimisation of the codons in the amylase gene to those preferred by *S.cerevisiae*. Using this method Kotula and Curtis (1991) increased the rate of an immunoglobulin gene 5 fold and increased the steady state concentrations of the protein by at least 50 fold.

**BAL 31 deletion of the 3'untranslated region or the *B.licheniformis* amylase gene.**

In a recent paper by Demoulder *et al,* 1992, it was shown that by deleting the major part of the untranslated 3'end of the murine interleukin 2 (mIL2) gene substantially increased the levels of mIL2 produced by *S. Cerevisiae.* They attributed this increase in mIL2 production to the removal of a destablising sequence in the 3'untranslated region which may have been responsible for the rapid degradation of the mIL2 mRNA.
A similiar experiment was designed for this study to ascertain whether an increase in the levels of extracellular amylase produced could be obtained by deleting the 3' untranslated region of the amylase gene.
The Bal31 deletion of the 3' untranslated region resulted in a large increase in the levels of extracellular amylase produced by *E.coli* and to a significant increase in the levels of extracellular amylase produced by *S.cerevisiae.* This increase may have been due to the removal of a destablising sequence in the 3' untranslated region or alternatively it may have been as a result of the disruption of the putative palindromic transcriptional termination sequence. The disruption of this putative transcriptional termination sequence may have led to increased stability in the mRNA in *E.coli* as read through would have given an amylase-lacZ transcriptional fusion which may have been more stable then the original mRNA. In *S.cerevisiae* disruption of this sequence would have resulted in an amylase-ADH1 mRNA fusion. The ADH1 terminator contains the correct transcriptional termination sequences for mRNA stability in *S. cerevisiae.*

**Concluding remarks.**

This work has shown the the α-amylase from *B. licheniformis* is efficiently expressed and secreted from *S. cerevisiae* under the control of the bacterial signal peptide with 95% of the total amylase activity present in the supernatant. This highly effective signal peptide could be used either fused directly to other heterologous proteins to improve their secretion from yeast or to stabilise heterologous proteins which are found to be unstable in yeast. It is also possible that the production of a fusion protein with the amylase may stabilise the protein and result in a large percentage of the protein being secreted. This hypothesis is supported by the work of Ward *et al* (1990) which showed that the fusion of the chymosin gene to the glucoamylase gene from *Aspergillus awamori* they were able to significantly improve the production of Chymosin. Ecker *et al*. 1989 also showed that fusion of ubiquitin to heterologous proteins also resulted in a significant increase in the levels of the heterologous protein produced. Transformation of the supersecreting strains isolated in this study with the vector containing an amylase fusion protein may also result in an increase in the levels of the fusion protein secreted. The inclusion of a suitable cleavage site in the amylase-protein fusion would allow for the easy recovery of the protein of interest after affinity purification of the fusion protein on a starch based resin.

More generally from the work presented in this thesis the construction of an integrating vector system comprising the rDNA locus together with a yeast expression system (i.e. promoter, terminator, selective marker) including the *B. licheniformis* α-amylase signal peptide could be useful for the secretion of high levels of other heterologous proteins. It is possible that the levels of secretion could be further increased by the use of a selective screen for supersecretors after mutagenesis with EMS. Studies involving the removal of or the addition of additional glycosylation sites in the α-amylase gene (or gene of interest) would provide useful information on the effect of glycosylation on protein secretion and stability.

Previously Moir *et al* 1987 showed that the addition of glycosylation sites in the heterologous protein by site directed mutagenesis of the heterologous gene resulted in a increase in the levels of secreted protein. However this glycosylation may also have an undesired effect on the activity and/or stability of the protein.

The use of this easily assayable amylase gene both as a reporter gene and as a promoter probe has already been successfully demonstrated in *S.cerevisiae* (Duffy, 1995). Disappearance of the characteristic amylase haloes on starch containing media could also be used for the detection of successful ligations by insertional inactivation after optimisation of the cloning sites available by site directed mutagenesis.

# CHAPTER 6

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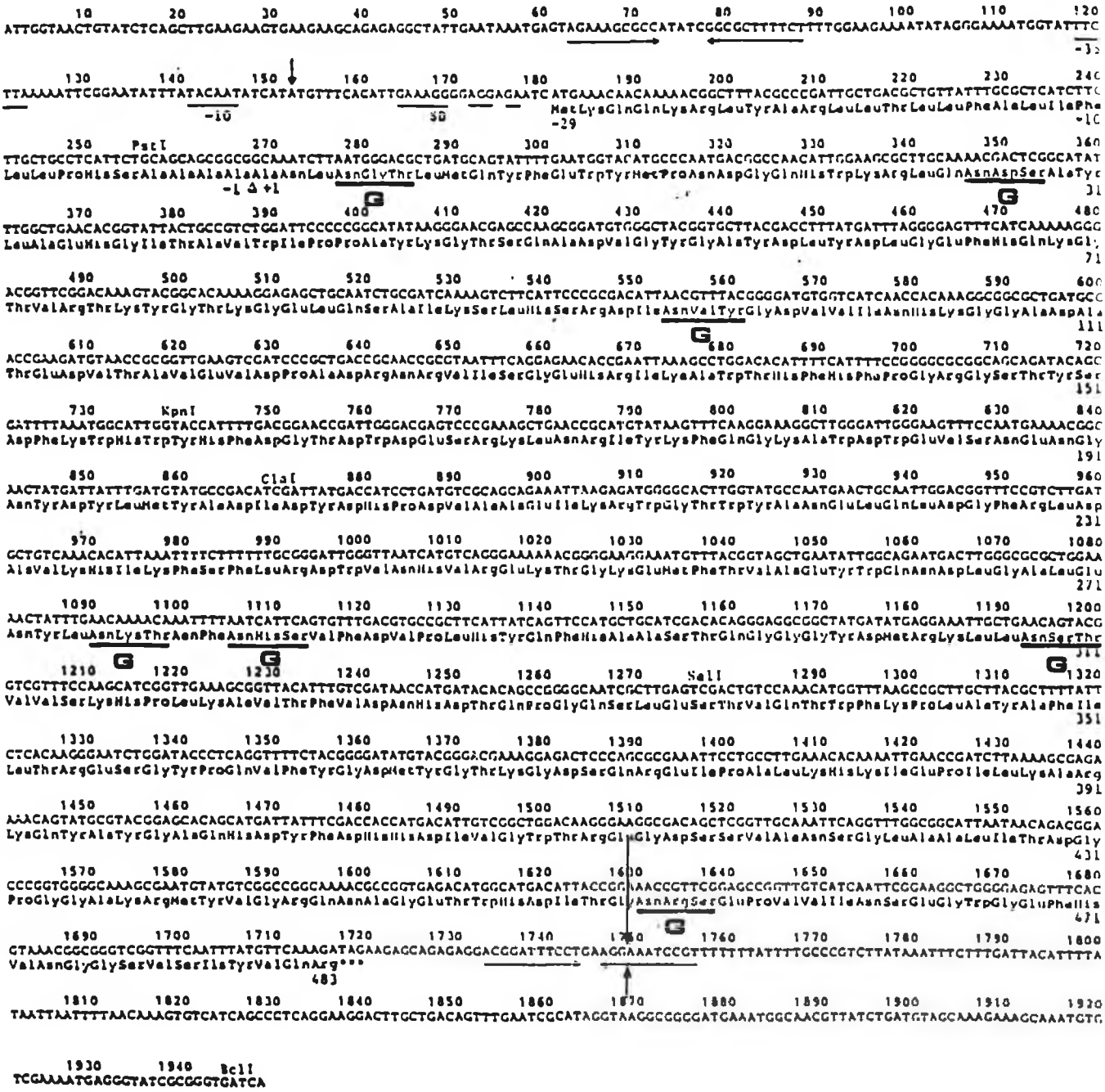
Appendix 1.

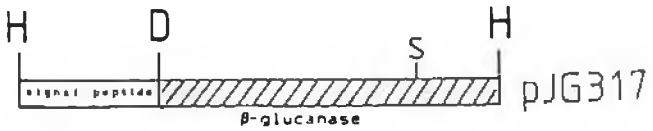
Nucleotide and amino acid sequence of *Bacillus Licheniformis* α-amylase gene.

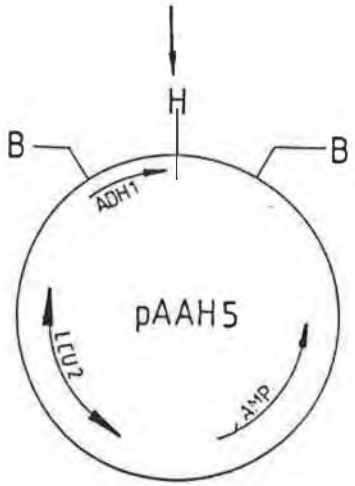


1. G :potential glycosylation sites.
2. ↓ :start of α-amylase gene fagment in pSL5.
3. ⟶⟵ :palindromic sequence at 3' end of amylase containing gene fragment.
4. ↑ :extent of Bal31 deletion.

Appendix 2(a).

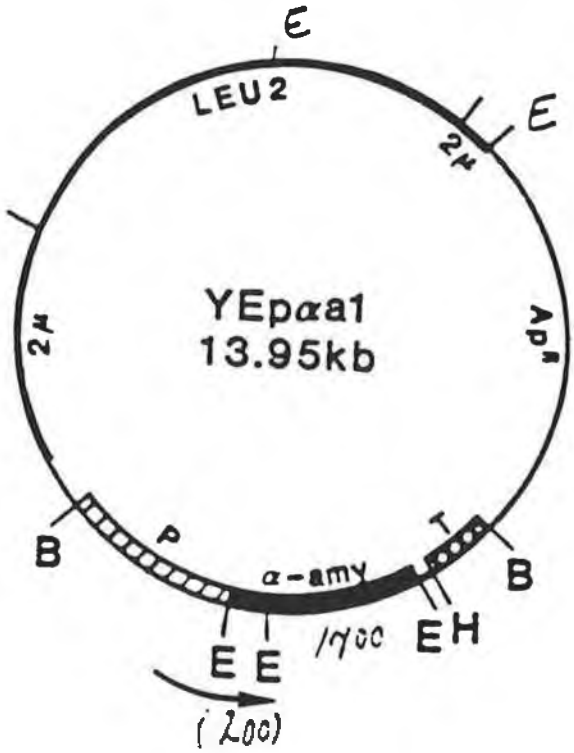
Plasmid diagram of pJG317.




Appendix 2(b).

Plasmid diagram of YEpαa1.




AMY=amylase gene, P=ADH1 promoter, T=ADH1 terminator