Optimisation of the production of Cathepsin L1 from a recombinant *Saccharomyces cerevisiae.*

A thesis presented for the

degree of M.Sc.

by

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

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Abstract

Optimisation of the production of Cathepsin L1 from a recombinant *Saccharomyces cerevisiae*.

Cathepsin L1 is a cysteine protease that has been previously isolated and functionally expressed in *Saccharomyces cerevisiae*. It has the potential to be employed as a vaccine for liver-fluke disease in cattle and other ruminants. Production of this recombinant enzyme, which is secreted into the media from recombinant yeast, was studied initially in shake flask cultures and subsequently in 5L and 15L fermenters.

In early studies, low productivity and especially variations in Cathepsin L1 production was a significant problem. A standard operating protocol (SOP) has been designed to consistently supply an optimum inoculum for large-scale fermentations. This SOP which involved 'blending' colonies for inoculum cultures in conjunction with sub-culturing starter flasks for two successive cycles of 48 hours, proved to be the most successful for consistently high levels of enzyme production during the ensuing fermentation.

The pH and temperature optima are pH 6.5 and 30^0C respectively for culturing the recombinant yeast to produce both both high biomass levels and high enzyme activity. Addition of casamino acids to the selective media or replacing it with complex YEPD resulted in poor plasmid stability and low Cathepsin L1 production. By supplementing the selective media with extra yeast nitrogen base, using a glucose concentration of 20g/L, enzyme activity increased by 3-4 fold and much higher levels of plasmid stability than observed in non-selective media were sustained. Enzyme activity of 0.74 units/mL were obtained in supplemented media compared to 0.19 units/mL in selective media.

Investigations were performed on the constitutive behaviour of the *ADH1* promoter, which controls the expression of Cathepsin L1 in this recombinant yeast strain. It revealed that enzyme production is repressed at high concentrations of glucose but gradually increases as glucose is utilised. Cathepsin L1 is still expressed during the ethanol consumption phase, albeit at a slower rate than during the latter stages of glucose consumption.

Nomenclature

dO_2	dissolved oxygen concentration	(% saturation)
μ_{max}	Maximum growth rate	(h^{-1})
t_d	Doubling time	(h)
$Y_{P/glu}$	Yield of Cathepsin L1 per unit of glucose metabolised aerobically	$(UmL^{-1}g^{-1})$
$Y_{P/eth}$	Yield of Cathepsin L1 per unit of ethanol metabolised anaerobically	$(UmL^{-1}g^{-1})$
$Y_{x/glu}$	Yield of biomass produced per unit of glucose metabolised aerobically	(gg^{-1})
$Y_{x/eth}$	Yield of biomass produced per unit of ethanol metabolised anaerobically	(gg^{-1})

Abbreviations

2μ	yeast native 2 μm circle
ADH	alcohol dehydrogenase
Cat L	Cathepsin L
DNS	3,5 Dinitro - Salicylic Acid
DTT	1, 4 Dithiothreitol
ER	Endoplasmic Reticulum
GOD-PAP	Glucose Oxidase
GRAS	generally regarded as safe
mRNA	messenger Ribonucleic Acid
NHMec (AMC)	7-amino-4-methyl-coumarin
OD	Optical Density
P+	plasmid-bearing cells
P-	plasmid-free cells
PCN	plasmid copy number
UAS_{RPG}	Upstream activation sequence for ribosomal protein genes
Ura	Uracil
Tris	Tris (hydroxymethyl) methylamine
YEPD	Yeast Extract Peptone Dextrose
YN_2B	Yeast Nitrogen Base
Z-Phe-Arg-AMC.HCl	Z - Phenylalanine - Arginine - NHMec

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Chapter 1 Introduction

1.1 *Fasciola hepatica*

1.1.1 Worldwide Implications of Fascioliasis Infection

Fasciola hepatica is the causative agent of liver fluke disease in mammals in temperate countries like Ireland, while the closely related *F. gigantica,* also from the genus *Fasciola,* is a major infection in ruminants in tropical climates. In agricultural farming, cattle and sheep are the most common hosts for the parasitic trematode *F. hepatica* (Peters and Gilles, 1995). It is estimated that economic losses around the world are in excess of US$2 billion annually as a result of infection from this parasite (Zimmer, 2000). *Fascioliasis* is also appearing as a more prevalent pathogen in humans, with greater than 2 million people affected worldwide, in particular regions such as the Bolivian Altiplano and other parts of South America (Bjorland et al, 1995, O'Neill *et al*, 1999).

1.1.2 Infection of animals with *F. hepatica*

Animals become infected with liver fluke disease when they ingest metacercarial cyst, which may have been deposited on vegetation such as watercress and/or in water. The parasite excysts in the duodenum and the immature worm burrows through the intestinal tract. It then migrates to the liver where it causes extensive haemorrhaging over a two-month period by feeding on host tissue and blood. After approximately eight weeks, the parasites finally traverse to the bile duct where they reside as adult flukes (Andrews, 1999).

1.1.3 Life-Cycle of *F. hepatica*

These mature flukes spawn huge numbers of eggs, which are then conveyed to the intestine with the bile juices, and are passed on to grazing land with the faeces. The eggs hatch releasing the miracadium, and the miracadium uses its spine to penetrate an intermediate mud-snail host, for example *Lymnaea truncatula*. The parasite reproduces asexually within the snail, and after several developmental stages cercariae are produced. The cercariae encyst on vegetation, which result in a metacercaria. One single miracadium can result in as many as 600 metacercariae (Scott, 1987, Andrews, 1999).

1.1.4 Current measures for treatment of *F. hepatica*

Fascioliasis is presently controlled by flukicides such as triclabendazole and closantel, both of which are potent drugs most effective against immature flukes (Meaney *et al*, 2002). At present there is a heavy reliance on using flukicidal drugs to treat diseased animals, as they represent the only available treatment. However there are a number of reasons why farmers will be less likely to rely on these treatments in the future. One of the main deterrents is the cost of these drugs, which in many cases is too expensive for farming communities in developing communities. Another emerging reason is that reports from both Australia and Ireland have shown parasitic resistance in ovine populations (Mulcahy and Dalton, 1998, Spithill and Dalton, 1998). Another major concern with using chemical cures, is the possibility of residues entering the food chain. Thus in recent years, there has been a growing interest in developing a suitable vaccine for the immunisation of cattle and sheep against *F. hepatica*.

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1.2 Potential Liver Fluke Vaccines

Ideally, the vaccine should be proficient in providing immunity against the parasite in both sheep and cattle. One dose of the vaccine should be all that is required to bestow life-long immunity to a calf, and adjuvants should not be a necessity for administering the drug. Vaccine trials in both sheep and cattle have been completed in the last number of years in the UK, USA, Ireland, Australia, Switzerland and Indonesia. Their modes of action and success rates have been reviewed by Spithill and Dalton (1998) and are detailed below.

1.2.1 GST (Glutathione-S-transferase)

GST's in general are isoenzymes that operate in the primary stages of detoxification of both foreign bodies and internal toxic compounds. Vaccine trials on bovine and ovine populations using GST have shown protection levels of greater than 90% in some cases. However, the various adjuvants that were administered along with GST influenced the level of protection induced in the cattle (Spithill and Dalton, 1998).

It was also shown that trial vaccines of GST that were proven to be efficacious against *F. hepatica* were futile against cattle infected with *F. gigantica*. Thus it is worth noting that vaccines that are effective against *F. hepatica* will not necessarily fight against its family member *F. gigantica* (Estuningsih *et al*, 1997).

1.2.2 FABP (Fatty acid-binding protein)

The role of the fatty acid-binding protein (FABP) in *F. hepatica* is thought to be mainly for the transport of fatty acids and their acyl-CoA esters. A native fraction of FABP was isolated from *F. gigantica* and

was tested on cattle, which resulted in a slight reduction (31%) in worm burdens. However when a recombinant protein was created in the laboratory, it did not succeed in protecting against liver fluke disease (Estuningsih *et al*, 1997).

However a 14.8kDa antigen called Sm14 (an FABP isolated from *S. mansoni*), which has a similar genetic make-up to that of Fh12 (isolated from *F. hepatica*) showed 100% protection against *F. hepatica* in mice (Spithill and Dalton, 1998).

1.2.3 Cathepsin L (Cat L)

Cathepsin L proteinases are extracellular cysteine proteases thought to perform a role in the degradation of ingested liver and blood tissues in the gut of the parasite. It has also been suggested that they are involved in other duties such as tissue penetration and immune avoidance. Cathepsin L proteinases have been shown to have the most substantial proteolytic activity secreted from the liver fluke worms (Brady *et al*, 1999). Cathepsin L proteases are distinguished from other Cathepsin categories by their predisposition for substrates such as Z-Phe-Arg-NHMec that contain hydrophobic residues in the P2 position (Dowd *et al*, 1997).

F. hepatica Cathepsin L proteinases have been shown to be extremely stable at 37^0C and at pH 7.0 for up to 20 hours, in comparison to mammalian Cathepsin L which is inactivated completely within 15 minutes under these conditions. They are also active over a broad pH range (5.0 - 9.0), with maximum activity observed at pH 8.0. The parasitic Cat L's are rapidly inactivated upon incubation from 50^0C upwards. The proteinases are exceptionally tolerant of organic solvents, especially dimethylformamide, whereas they are severely

affected in the presence of heavy metal ions, where mercury has multiple toxic effects on the enzyme. The enzyme's stability under a variety of conditions bodes favourably for future use in different industrial settings (Dowd *et al*, 2000).

1.2.3.1 Cathepsin L1 (Cat L1)

This enzyme is thought to be involved in cleaving immunoglobulin at the hinge region, and thus preventing the attachment of immune effector cells to freshly excysted juveniles. The purified active form of the enzyme resolves as a single band with an apparent molecular size of 27kDa on reducing SDS-polyacrylamide gel electrophoresis (Smith *et al*, 1993). Both the purified native form and the recombinant enzyme CatL1 exhibit activity over a broad pH range 4.0 - 8.0, while the optimum pH for activity is at 6.0 or 6.5 (Roche *et al*, 1997, Brady *et al*, 1999). Dalton *et al* (1996) undertook a vaccine trial that involved administering various doses (10 - 500 μg/per injection) of Cat L1 and the mean protection level obtained in cattle was 53.7%. Antibody responses were observed in all groups tested, even those that were only dosed with 10μg of Cat L1 per injection.

1.2.3.2 Cathepsin L2 (Cat L2)

One of the differences between CatL2 and CatL1 is that it can cleave substrates with a proline residue in the P2 position. Also, CatL2 can cleave fibrinogen to form fibrin clots (Dowd *et al*, 1995). CatL2 shares 90% similarity in its amino acid sequence with CatL1 and it has a molecular size of 29.5kDa (Roche *et al*, 1999). The N-terminal sequences of the 27kDa Cat L1 and the 29.5kDa Cat L2 and they diverge only in residue number seven (proline and arginine, respectively) (Dowd *et al*, 1994). Both the purified native form and the

recombinant enzyme CatL2 exhibit activity over a broad pH range 5.0 - 8.0, while the optimum pH for activity is at 6.0 or 6.5 (Dalton *et al*, 1996)

1.2.4 Fluke Haemoglobin (Hb)

Fluke Hb's function is thought to be in Oxygen transport in immature flukes, while in adult flukes, mainly production of eggs. Thus, it was suggested by Dalton *et al* (1996) that Hb vaccination generates antibodies that hinders the molecule's role in Oxygen metabolism in migrating flukes as well as in the reproductive organism and in this manner affects both fluke development and egg production. Trials using Hb alone resulted in a 43.8% protection against further *F. hepatica* infection in comparison to non-vaccinated herds. In addition, the eggs produced showed a significant decline in viability (35%) (Dalton *et al*, 1996).

1.2.5 Combination Vaccines

Wijffels *et al* (1994) tested a combination vaccine comprising of both Cat L1 and Cat L2 in cattle. There was a sizeable reduction (70%) in the volume of eggs produced by the parasites, and those eggs that were formed had a much lower viability than those from non-vaccinated cattle did, however significant decrease in worm burdens was not observed.

Dalton *et al* (1996) in addition to testing Cat L1 and Fluke Hb on their own, the results of which are mentioned on previously, also tried two combination vaccines that incorporated Cat L1/Fluke Hb and Cat L2/Fluke Hb. The Cat L2/ Fluke Hb vaccine was a potent mix and resulted in an outcome of greater than 98% decrease in the eggs' viability, and a 72.4% protection level, while still preserving the liver tissue. The Cat L1/Hb mixture, while still successful in comparison to

the control group, and those vaccines tested singly, was not nearly as effective as the Cat L2/ Fluke Hb (Dalton *et al* 1996)

Mulcahy *et al* (1999) also tested two separate combination vaccines; namely Cat L1/Cat L2 as well as Cat L2/Fluke Hb. Both vaccines produced good antibody responses with high levels of IgG1 and IgG2 resulting. The CatL1/Cat L2 vaccine resulted in 75% reduction on faecal egg counts in comparison to the control group, while the CatL2/Fluke Hb dose had a 25% reduction. The numbers of viable eggs and fluke burdens were both considerably decreased in the vaccinated cattle related to the control group. As mentioned previously, the combination vaccine Cat L2/Hb gave a greater degree of protection that the Cat L1/Hb mixture (Dalton *et al*, 1996). However in furthering these results, Mulcahy *et al* (1999) discovered that the Cat L1/Cat L2 dosage gave the best protection overall.

In the majority of cases, a paucity of available parasitic material prevents large-scale production of these vaccines. Thus this places an important role on both the development and large-scale production of recombinant proteins that have the ability to mimic the protection seen with native purified proteases.

Thus the overall objective of this thesis is to determine a method for producing high levels of the cysteine protease Cathepsin L1 from *Saccharomyces cerevisiae* to make large-scale production of the vaccine both possible in bioengineering terms and economically feasible.

1.3 Heterologous Protein Production

1.3.1 Choice of host

1.3.1.1 Procaryotic

Recombinant DNA technology has been around for almost 20 years. Its inception began with the production of recombinant proteins from the procaryotes *Escherichia coli* and *Bacillus spp*. At that time the genetics of *E. coli* was well established, and manipulation of its DNA was possible, thus this organism was a natural choice as host for this novel technology. Initially, many recombinant proteins were expressed at high levels. Human insulin was the first human health care product derived from rDNA technology, and it was commercialised in 1982 by Eli Lilly and Co (Shin *et al*, 1997).

A number of factors hindered large-scale production of heterologous proteins in *E. coli*. These included the possibility of contamination of the final product from endotoxins, incorrect processing of the desired protein and inefficient secretion of the recombinant protein into the culture medium where it could be harvested (Nicaud *et al*, 1986).

1.3.1.2 Eucaryotic

As a result, research in developing eucaryotic cells as suitable hosts for recombinant protein production was investigated. The yeast *Saccharomyces cerevisiae*, otherwise more commonly known as bakers' or brewers' yeast, was one of the first yeast to be scrutinised. It has a number of advantages over a bacterial system, the main one being that it is 'generally regarded as safe' (GRAS) owing to its long history and extensive knowledge of use in the food industry (Martin

and Scheinbach, 1989). Moreover, unlike some bacteria, yeast do not produce pyrogens or endotoxins.

Eucaryotic cells exhibit many characteristics of cell structure normally found in multicellular organisms such as a nucleus, golgi body, mitochondria and endoplasmic reticulum (ER). Proteins are sorted, altered and often assembled into complexes in the ER and Golgi compartments before being discharged via secretory vesicles across the plasma membrane and out of the cell. Any proteins that are incorrectly folded are either detained in the ER until they are correctly assembled, or are targeted for degradation (Brown and McDonald, 1999).

Yeast unlike procaryotes, are capable of performing post-translational protein modification steps such as processing and glycosylation (Gellissen, 2000). Consequently, *Saccharomyces cerevisiae* has the ability to secrete correctly folded recombinant proteins with disulphide bonds into the culture media (Moir and Davidow, 1991). Biologically active, secreted products are significantly easier to harvest from the media than the denatured, inclusion body types usually produced by recombinant bacteria (Watson *et al*, 1992). It abolishes the need for expensive cell disruption steps, such as sonication, freeze-pressing, agitation with glass beads and the French press and/or refold process steps (Wiseman *et al*, 1987, Eckart and Bussineau, 1996). In addition, yeast generally secrete very few of their own proteins into the culture media (MacKay, 1987), thus the subsequent purification of the desired protein from other contaminating proteins is hugely simplified. Yeast can be grown to very high cell densities on a large-scale and in general have a rapid growth rate. They can be generated on either inexpensive complex media or simple defined media (Goodey *et al*, 1987).

1.1.3.3 Use of other yeast for heterologous protein production

In spite of complete knowledge of the genome of *S. cerevisiae*, and immense understanding of its metabolism, it is not always seen as the ideal host for heterologous protein production. This criticism is partly imputed to lower productivity levels from *S. cerevisiae* compared to *P. pastoris* and other yeast. Lin Cereghino and Cregg (1999) postulate that 'the commonly used 2μ multicopy vectors may be the real culprits.'

In addition, *Saccharomyces cerevisiae* has a tendency to sometimes hyperglycosylate proteins, although glycosylation is not essential for secretion (Eckart and Bussineau, 1996, Hong and Kang, 1998). It is termed as such because the addition of outer chains to heterologous proteins results in more extensive glycosylation than is found in higher eucaryotic glycoproteins. The difference in outer oligosaccharide chain composition of foreign proteins secreted from yeast could affect their folding, stability or activity. This does not present problems for extracellular secretion of the foreign protein nor for the biological activity of the enzyme. The problem arises for immunological reasons where yeast outer chain carbohydrates are not beneficial on mammalian therapeutic proteins (Innis, 1989).

Problems encountered using *S. cerevisiae* as a host for heterologous protein production may be partially or completely circumvented by the use of other yeast as hosts. These yeast include *Pichia pastoris*, (Files *et al*, 2001, Goodrick *et al*, 2001, Scorer *et al*, 1993, Sreekrishna, K *et al*, 1997, Villatte *et al*, 2001, Wang *et al*, 2001) *Pichia stipitis*, (Piontek *et al*, 1998, Passoth and Hahn-Hägerdal, 2000, Shi and Jeffries, 1998) *Hansenula polymorpha*, (de Bruin *et al*, 2000, van Dijk *et al*, 2000) *Kluyveromyces lactis*, (Hensing *et al*, 1995a) *Yarrowia lipolytica* (Chang *et al*, 1998), *Saccharomyces kluyeri* (Müller *et al*, 2001),

Schwanniomyces occidentalis (Piontek *et al*, 1998) and *Aspergillus niger* (Wongwicharn *et al* 1999)

These yeast can utilise a number of cheap, ubiquitous carbon sources. *K. lactis* can grow on lactose recovered from whey processing. *Y. lipolytica* can utilise both ethanol and acetate. *S. occidentalis* is unusual in the *Saccharomyces* family because of its ability to degrade starch completely. *A. niger* also metabolises starch completely. Both *Pichia pastoris* and *P. stipitis* are methylotrophic yeast, and heterologous gene expression is induced when methanol is introduced as the carbon source. *H. polymorpha* can utilise both glycerol and methanol as chief substrates (Gellissen, 2000, Spencer *et al*, 2002).

Of these alternative yeast, *P. pastoris* has probably been the most widely used in industry for the production of a variety of heterologous proteins. It mainly employs a methanol-induced promoter called alcohol oxidase (AOX1), which is tightly repressed by glucose, but enables it to induce extremely high levels of expression when the cells are growing on methanol (greater than 1,000 fold) (Lin Cereghino and Cregg, 1999, Lin Cereghino and Cregg, 2000)

1.3.2 Choice of Plasmid used in yeast

1.3.2.1 Introduction

Bacteria possess large numbers of tiny circular DNA molecules that each holds several thousand base pairs. These mini-chromosomes are known as plasmids, and are not linked to the main chromosome (Watson *et al*, 1992). Plasmids are pieces of double stranded DNA that range in size from 1kb to 300kb (Day, 1982). Because plasmid DNA is so much smaller than the bacterial chromosome (~ 4 million base pairs), it can be easily separated and purified for recombinant DNA purposes.
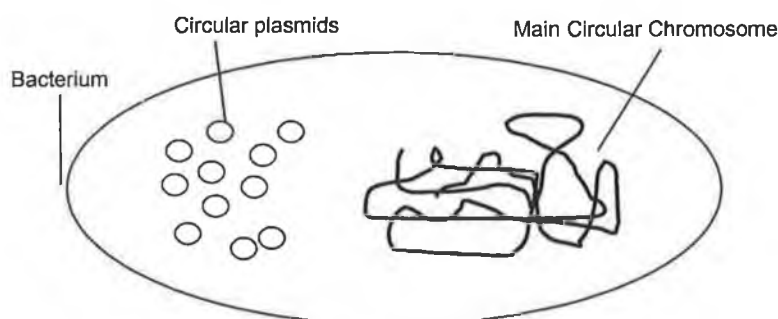
Figure 1.1 Plasmids are small bacterial DNA molecules that replicate independently of, and co-exist with, the host chromosome

Relaxed-control plasmids are used for recombinant DNA work in yeast, as they have high copy numbers (10 - 200 plasmid copies per cell), compared to *stringent-control* plasmids which replicate at the same rate as the host cell's main chromosome, thus only one or a few copies per cell (Watson *et al*, 1992). In the case of *S. cerevisiae*, the plasmids are substantially smaller then the host chromosome, which is approx. 13,800kb in size. These mini-chromosomes can replicate independently of, and co-exist with, the host chromosome. The plasmid must be transferred between cells during cell division, and can

be lost from the host cell, therefore leading to generation of plasmid free cells (Day, 1982). Both a promoter and terminator sequence must be present on the plasmid, if high levels of expression are to be obtained (Goodey *et al*, 1987).

1.3.2.2 Plasmid Instability in yeast

In the commercial production of heterologous proteins the instability of transformed vectors in recombinant organisms represents a significant obstacle. Instability is the inclination of the transformed cells to lose their recombinant characteristics as a result of changes to, or loss of, plasmids. Meanwhile, stability of a plasmid can be described as the ability of transformed cells to maintain a multi-copy plasmid, which is unchanged during cell-growth, and showing its phenotypic characteristics (O' Kennedy *et al*, 1995, Cheng *et al*, 1997).

Three classes of plasmid instability in *S. cerevisiae* cell populations have been mentioned in literature; namely **1)** structural, **2)** segregational and **3)** competitive instability. Structural instability refers to alterations in the plasmid DNA structure, such as, insertion, deletion and rearrangement. Generally, this type of instability is more likely to occur in plasmids with large inserts. This type of instability is often difficult to recognise as the genetic material that is required may be lost from the plasmid, while preserving the bulk of the plasmid DNA, including any selectable markers. This phenomenon causes a major problem in the assessment of plasmid-free cells, as the presence of the selectable markers will give false positive results. In order to diminish the possibility of recombination modifications occurring, caution must be exercised in the construction of the plasmid (Caunt *et al*, 1988, Cheng *et al*, 1997).

Segregational instability is applicable to both bacterial and yeast systems, but is much more destructive in a yeast system, especially when low copy number plasmids are present. This type of instability occurs when a plasmid-bearing host fails to pass on one or more copies of the plasmid to daughter cells during cell division. As bacteria divide by binary fission, there is an equal distribution of plasmids between the daughter cells created. Yeast however, replicate asymmetrically by a process called budding. The mother yeast cell produces a smaller daughter cell, and in the process unequal partitioning of the plasmids occurs. Hence, two cell types reside in the cell population; plasmid-free and plasmid-containing cells. The use of a selective pressure during growth can reduce the effect of this instability (O' Kennedy *et al*, 1995, Caunt *et al*, 1988).

The third type of plasmid instability is referred to as competitive instability. This is in reference to growth of cells in non-selective media, where plasmid-free cells have a growth advantage over plasmid-bearing cells, with an outcome of plasmid-bearing cells outnumbered by their plasmid-free counterparts. The reason is probably due to the additional metabolic loading on the cell caused by the extra plasmid, and during repeated batch cultures or continuous culturing the most competitive cells are selected. Complete takeover by a population of plasmid-less cells would be economically disastrous for any large-scale production of a recombinant protein (Zhang *et al*, 1996, Cheng *et al*, 1997).

The problem of plasmid instability is especially significant with recombinant yeast as almost all yeast plasmid vectors are hybrid plasmids, or 'shuttle vectors', which are relatively unstable. Shuttle vectors contain selectable markers and origins of DNA replication that work in both bacteria and yeast. Other factors such as the

environmental conditions, media composition and the copy number of the plasmid will ultimately affect the stability of the plasmid. This collection of reasons can lead to plasmid instability being a substantial factor in the choice of recombinant strain being used for large-scale production of commercially important enzymes and other proteins.

1.3.2.3 Choice of Plasmid vector for yeast

The choice of plasmid vector systems for heterologous gene expression in *Saccharomyces cerevisiae* is clearly an important one as both stability and copy number must be accounted for. Yeast are transformed with a variety of self-replicating (YRp), episomal (YEp), centromere (YCp) or integrating (YIp) plasmid vectors (Old and Primrose, 1985). For yeast the choices include vectors from a yeast ARS element (Autonomous Replicating Sequence) that can be added together with a yeast chromosomal centromere sequence to limit the copy number of the plasmid vector, or from the endogenous multicopy 2-μm plasmid of yeast. The ARS-based vectors yield high copy levels per cell but poor stability, while the multicopy 2-μm based vectors have high stability but a low copy level per cell. By interlinking the benefits of both, the 2-μm circle based vector was created and it has the attributes of both high copy levels and stable regeneration (Goodey *et al*, 1987).

1.3.3 Choice of Plasmid Promoters for use in yeast

1.3.3.1 Introduction

A promoter site is the site that is recognised by DNA-directed RNA-polymerase as an initiation signal to indicate where transcription to form mRNA begins. The maximum rate of transcription from a particular strand of DNA is controlled by the sequence of bases in the

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promoter (Watson *et al*, 1992). There is an excellent review of the various promoters employed in yeast for heterologous protein production presented by Hensing *et al* (1995b).

1.3.3.2 Inducible Promoters

Inducible promoters can support very high expression levels. They also allow the disassociation of growth and protein production phases. This feature can be invaluable when expression of a foreign gene is detrimental to the growth of a yeast cell, or when the product is toxic. Recombinant cells can be grown to high cell densities under conditions where the heterologous gene is not actively expressed. Once the desired cell mass has been reached, fermentation conditions can be altered and then transcription can be switched on as cells reach stationary phase (Hensing *et al*, 1995b, Moir and Davidow, 1991).

However, inducible expression systems eventually lead to a loss of productivity when growth and product formation stages are separated. Moreover, inducible promoters that work well in a small-scale laboratory scenario do not often transfer well to a large-scale process as some examples are cited below (Hensing *et al*, 1995b).

1.3.3.2.1 GAL promoters

The GAL promoter (e.g. GAL1, GAL4, and GAL10) is repressed in the presence of glucose and when Galactose is added to the culture medium to replace glucose as the carbon source, an increase in expression of 1,000 fold takes place. However, with regards to large-scale production, Galactose is expensive in comparison to Glucose. Porro *et al* (1992) reported a 10-fold induction of expression of foreign protein using a GAL promoter, with the addition of 0.5%w/v Galactose,

even in the presence of Glucose. They achieved this by creating a 'superinducible' vector by cloning the GAL4 gene and the GAL1-10 upstream activating sequence into the same high copy number plasmid (Porro *et al*, 1992).

1.3.3.2.2 Methanol - induced promoters

In methylotrophic yeast such as *Pichia pastoris*, alcohol-oxidase (AOX) and formate dehydrogenase promoters mediate very high expression levels and are tightly regulated. They are repressed during growth on multi-carbon sources such as glucose, and induced during growth on methanol (Lin Cereghino and Cregg, 2000).

1.3.3.2.3 Temperature-regulated promoters

Many temperature-regulated promoters such as MATα2 promoter utilise a mutant protein with a defect in a protein. This deficiency is not required for growth, but used instead to induce heterologous gene expression. Cells are initially grown at a higher temperature at which the temperature sensitive (*ts*) mutation is repressed, with the result that the heterologous gene is silent (Piper and Kirk, 1991, Watson *et al*, 1992). Expression of the foreign protein is then triggered by a down-shift in heat from the repressive temperature of 35^0C to the permissive temperature of 25^0C (Sledziewski *et al*, 1988). However growth of yeast cells above 33-35^{0}C is slower, and thus gives lower biomass yields. In addition, in large-scale fermentations, to decrease the temperature of a culture broth is costly both in terms of time and energy. Other promoters are activated by an increase in temperature in conjunction with the addition of Galactose (DaSilva and Bailey, 1989).

1.3.3.2.4 Phosphate-regulated promoters

PH05 promoters are repressed in the presence of inorganic phosphate. In large-scale fermentation, this derepression cannot be achieved by simply replacing the media; thus it is obtained by decreasing the supply rate of the nutrient. Expression levels can be induced by up to 200-fold with the removal of inorganic phosphate from the media. (Goodey *et al*, 1987) This however is laborious to accomplish in complex media, so defined media is often used, but this is expensive (Hinnen *et al*, 1989).

1.3.3.2.5 Glucose-repressible promoters

Examples of this class of promoters are *ADH2* and *SUC2,* which are repressed by excess sugar. Derepression can occur when the carbon source is replaced with glycerol or ethanol. Pyun *et al* (1999) state that expression of the SUC2 gene is repressed during high glucose concentration, and derepressed at low concentrations of glucose. Cho and Jeffries (1998) confirm that this is the also the case for ADH2. Partial derepression occurs anyway when sugars are utilised throughout the course of the fermentation. Thus glucose-repressed promoters are of limited use when growth and product formation stages have to be strictly isolated.

1.3.3.3 Constitutive promoters

As mentioned earlier there is a loss in productivity in inducible expression systems when the growth and production phases are separate, compared to a constitutive promoter where growth phases and product formation occur simultaneously. Thus if product toxicity,

protein instability or expression cassette instability are not factors, constitutive promoters are the preferred option.

In attempts to generate high levels of mRNA for the heterologous gene, early work centred on the use of strong constitutive promoter elements from genes involved in glycolysis. Although these promoters are amongst the most powerful of *S. cerevisiae* and produce high levels of homologous proteins, yields are generally lower when they are utilised to produce heterologous proteins. The most widely used glycolytic promoters are alcohol dehydrogenase (*ADH1*), phosphoglycerate kinase (*PGK*) and glyceraldehyde-3-phosphate (*GAP*) (Old and Primrose, 1985, Romanos *et al,* 1992)

1.3.3.3.1 *ADH1* Promoter

The original ADH1 is contained on a fragment of about 1500bp, and is considered to be a strong constitutive promoter. Valenzuela *et al* (1982) was one of the first research teams to express a foreign protein (Hepatitis B surface antigen) from *S. cerevisiae* using the ADH1 promoter. The *ADH1* gene has been previously considered to be constitutive, but activity of the *ADH1* promoter is reduced when cells enter the ethanol consumption growth phase or during growth on non-fermentable carbon sources by 2-10 fold (Denis, 1983).

Shiba *et al* (1998) reported on biomass and the production levels of the foreign protein carboxypeptidase Y from *S. cerevisiae*. They tried different expression systems, and discovered that the amount of cell biomass produced was almost identical in both expression systems. However, much higher production levels of the foreign protein were

found using the inducible promoter GAL10, over that of the constitutive promoter ADH1 (Shiba *et al*, 1998).

The ADH1 promoter has also been used in other yeast for example *Schizosaccharomyces pombe* and *Pichia stipitis. Smerdon et al* (1995) reported on the production of the heterologous protein human gastric lipase (hGL) using recombinant *S. pombe* and ADH1 as the promoter. hGL was produced and secreted successfully in a biologically active form, and expression levels were high using this expression system (Smerdon *et al*, 1995, Cho and Jeffries, 1998).

In conclusion, a number of options have to be decided upon in the molecular biology laboratory before proceeding towards large-scale optimisation of heterologous protein production. Once a suitable host has been chosen, a plasmid must be transformed into it. This plasmid should be capable of maintaining stability while also having a high copy number. A selective marker also has to be opted for along with a strong promoter, whether it is constitutive or inducible. All of these will have a significant bearing on the type of media used and the environmental conditions required during fermentation.

1.3.4 Choice of selection marker for yeast

Selective markers are essential in recombinant yeast if plasmid vectors are to be retained. In the case of yeast, the system of selection used most often involves an auxotrophic yeast strain, where a plasmid containing the selective marker is cloned into the auxotrophic mutant. In order to select the yeast bearing the required plasmid, the cells must be grown on media lacking an essential amino acid. This is discussed in more detail in the ensuing section.

1.4 Effect of Media on Plasmid Stability and Protein production

1.4.1 Introduction

As mentioned earlier, one of the main hindrances in utilising *Saccharomyces cerevisiae* or other yeast as hosts for the large-scale production of recombinant heterologous proteins is plasmid stability. If a selective pressure is applied during growth, the effects of segregational instability may be greatly reduced. The general nutrient requirements of recombinant yeast incorporate a Carbon source, a Nitrogen source as well as other macro-nutrients and growth factors, in concentrations favourable to the host organism. However, in a non-selective medium, the plasmid-free cells can outgrow the plasmid-bearing cells and rapidly take-over the culture. Thus the challenge is to design a media with high biomass levels, that allows high plasmid stability and high yields of the desired product.

1.4.2 Selection Pressures

Antibiotics are commonly used as a selection pressure in the case of bacteria, where the host is sensitive to the drug and the plasmid encodes resistance to the antibiotic, as well as containing the gene of interest. However this method is not desirable for large-scale production of recombinant yeast as antibiotic addition is expensive, detrimental to the environment, and complications arise when harvesting the protein of interest (Wang and DaSilva, 1993).

Recombinant plasmids in yeast are unstable to varying degrees, unless a selective pressure is applied to the growing organism to force

them to retain the plasmid. Nutritional selection is often used in the case of yeast, where the host is auxotrophic for a particular amino acid due to a mutation in one of the genes involved in the synthesis of that amino acid. Yeast selectable markers include *HIS3*, *LEU2*, *URA3*, and *TRP1*, which select for transformants growing in media lacking histidine, leucine, uracil and tryptophan respectively (Caunt *et al*, 1988, Watson *et al*, 1992).

For example the host cell could be a double auxotrophic mutant containing a plasmid to correct for one of the mutations. In the case of the yeast strain DBY746, transformed with the plasmid pFheCLY29, the yeast cell is Leucine auxotrophic, which means it cannot make its own leucine. However the plasmid bearing the Cathepsin L proteinase also carries the wild-type LEU2 gene, which encodes the enzyme β-isopropylmalate dehydrogenase in the leucine biosynthesis pathway (Watson *et al*, 1992). Thus yeast that take up the plasmids bearing the LEU2 gene are able to express it at levels sufficient enough to allow them to grow in the absence of added leucine. Culturing the yeast cells in media devoid of leucine will thus isolate yeast bearing the requisite plasmids (Roche *et al*, 1997).

However this restricts the nutrient media that can be used, as the medium has to be defined, thus cultivation of the cells is expensive. It also results in slower growth and a significantly lower yield of cells than can be achieved in non-selective media, thus this method of fermentation may not be feasible for large-scale production (Moir and Davidow, 1991). In addition, selective media does not overcome the problem of plasmid instability completely. Other methods, such as inducing a promoter late in batch culture, can restrict optimisation of the bioreactor operating strategy

1.4.3 Autoselection

In order to circumvent the difficulties associated with plasmid instability, autoselection systems have been developed for both bacteria and yeast. Cells will only survive in the fermentation if the plasmid is retained. This means that any media, be it selective or complex, can be utilised (Wang and Da Silva, 1993).

Wang and Da Silva (1993) investigated the effect of medium enrichment on two strains of *S. cerevisiae*. The autoselective strain had a triple ura3 fur1 urid-k mutant, while the nonautoselective strain had the ura3 gene as a selection marker. Plasmid instability in complex media was eliminated completely when the autoselective strain was examined in batch culture. The autoselective strain also showed exceptional cell growth in the richer medium. In comparison, the nonautoselective strain performed poorly in the complex media. It exhibited plasmid loss of 84%, and the decrease in specific activity of the desired protein invertase was 90%. Further work performed by aforementioned research team confirmed these findings (Napp and DaSilva, 1993).

La Grange *et al* (1997) and Nuyens *et al* (2001) also attempted to increase heterologous expression of two different forms of xylanase in *S. cerevisiae*, by creating an autoselective strain through FUR1 disruption. Significant xylanase activity by both sets of researchers was achieved through medium enrichment. La Grange *et al* (1997) reported a ten-fold increase in specific activity of *T. reesei* xylanase, while Nuyens *et al* (2001) related maximal activity of 8.5nkat/mL of *B. pumilus* xylanase in complex media.

1.4.4 Carbon Source

Yeast are chemoorganotrophic organisms and thus they receive carbon and energy from compounds such as carbohydrates. These carbohydrates are usually sugars, and glucose would be considered the most extensively used by yeast. In terms of *Saccharomyces cerevisiae*, there is a comparatively narrow scope of sugars that can be considered as good growth and fermentation substrates for this yeast. They are glucose, fructose, mannose, galactose, sucrose and maltose (Walker, 1998).

Some yeast can tolerate glucose concentrations of over 200g/L. However, when the concentration of the carbon source is so high, catabolite repression or carbon source repression occurs. This gives rise to the hindrance of one or more of the enzymes used for the synthesis of a secondary metabolite. In addition, cells grown on glucose repress the expression of a large number of genes necessary for the utilisation of other carbon sources. Thus when *S. cerevisiae* is grown on a mixture of glucose and another fermentable sugar, e.g. sucrose, galactose or fructose, the metabolism is di-auxic, and glucose is the first substrate to be metabolised (Greasham and Herber 1997, Walker, 1998, Dynesen *et al*, 1998).

Saccharomyces cerevisiae cannot utilise starch, owing to its deficiency of amylolytic activity. However, Birol *et al* (2000) cloned the genes encoding amylolytic enzymes (*a*-amylase and/or glucoamylase) into recombinant yeast incorporating a YEp plasmid to enable the organism to grow on starch. Five different compositions of media ranging from a minimal selective media to a complex rich media utilising various concentrations of either glucose or starch as its main carbon source were used for the series of experiments.

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Plasmid stability was greater than 95% in both minimal and complex media with glucose. However there was a significant loss of stability when the yeast strain was grown in complex media utilising starch as its carbon source. The growth rate of the recombinant cells was also significantly lower in the runs performed using starch as the carbon source. Despite this, the biomass yield (g biomass/g substrate) and the protein yield (mg protein/g biomass) were significantly higher in the starch-containing medium (Birol et al, 2000).

1.4.5 Selective versus Non-Selective Media

Non-selective or rich media are media where the exact constituents are not known. They contain a wide range of components such as fermentable substrates, peptones and extracts that aid biomass growth. These ingredients are by-products and waste materials from the agriculture and food sectors or extracts of biological materials. These components are relatively inexpensive and high in nutritional value (Greasham and Herber, 1997).

Selective media are the other choice, where every component and its concentration are known. These media are quite costly and cells grown using them tend to have much slower growth rates. However they can be used to get a detailed analysis of the nutritional needs of a micro-organism that is not possible with complex media. The secreted product can also be harvested and purified with a lot less effort and expense in defined than in complex media. Selective media are often used during the development stages for the production of recombinant proteins (Greasham and Herber, 1997).

Chiruvolu *et al* (1996) worked with a yeast strain that was auxotrophic for both uracil and leucine, and various types of media. A series of shake-flask experiments using a number of different media were performed and the results revealed that a relatively high specific growth rate of 0.49h^{-1} was attained in complex YEP media compared with the specific growth rate in the various different minimal media which were between 0.18 and 0.20h^{-1}. Plasmid stability was very high at 95%, when grown in minimal media where both uracil and leucine selection pressures were employed. Surprisingly, plasmid retention in the complex YEP medium was also high at 92%, even though no selection pressures were present. The yield of ovine interferon-τ, the desired protein, was significantly higher in both complex media and Casamino-Acid supplemented media over that of minimal media when large-scale fed-batch fermentations were performed.

Jung *et al* (1991) ascertained that by supplementing a medium whose main component was yeast nitrogen base (without amino acids) with several kinds of vitamins and trace elements, overall productivity increased. The new supplemented media resulted in improved growth rates, plasmid stability, and increased production of Hepatitis B surface antigen (HbsAg). They speculated that lowered growth rates resulted in plasmid instability, which in turn had a knock on effect on the degradation of HbsAg.

Tøttrup and Carlsen (1990) experimented with a recombinant yeast strain that was auxotrophic for Leucine. On transformation with the plasmid pYASI1 it was converted to leucine prototrophy. These investigators performed some plasmid stability experiments in shake-flasks using selective minimal media and rich complex media, supplemented with either glucose or ethanol as the carbon source. Subcultivations of the transformed yeast resulted in higher plasmid

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retention in the richer complex YEP medium compared to the selective minimal medium. They surmised that plasmid stability is affected by growth rate rather than leucine selection, as the growth rate in rich medium was much higher than that of the synthetic selective medium.

Contrary to previous reports that selective media favour plasmid stability, relative to richer complex media, the aforementioned researchers all observed higher productivity, despite lower plasmid stability in complex media or supplemented media. Indeed most researchers will use a selective media for growing the inoculum in order to obtain high initial fractions of plasmid-bearing cells (Buckholz and Gleeson, 1991, Wang and Da Silva, 1993).

1.4.6 Addition of individual components

Defined media often contain some amino acids, but more often than not, they must be supplemented with an amino acid that is necessary for either growth of the recombinant yeast (eg Leucine auxotroph), or expression of the desired protein. Shiba *et al* (1998) supplemented selective thiamine-free media with the amino acid histidine. This in turn raised intracellular levels of the growth factor thiamine and led to a substantial increase in the heterologous protein CPY (carboxypeptidase Y). The combination of identifying the optimal pH, the ideal expression system and altering the histidine concentration they managed a ten-fold increase in CPY production.

Van Dusen *et al* (1997) observed increases in cell dry weight of the yeast with concomitant decreases in heterologous protein expression of HBsAg (hepatitis B surface antigen), when complex media was supplemented with the purine adenine. Adenine is required for both cell viability and maintenance. The concentration of adenine in yeast

extract is for the most part unknown, and most complex media contain yeast extract as a vital ingredient. Van Dusen et al (1997) concluded with the fact that "In the case of recombinant *S. cerevisiae*, adenine concentrations that maximise heterologous protein expression may be quite different from those that maximise biomass and may vary depending on the protein of interest, the expression system and the host background."

1.5 Effect of Environmental Conditions on Plasmid Stability and Protein Production

1.5.1 Introduction

Once a suitable host strain has been developed in the molecular biology laboratory, all that remains is to optimise plasmid stability and production regimes, be that environmental factors or media formulation. Of the various factors influencing plasmid stability and heterologous protein production, environmental parameters are perhaps the simplest to manipulate and regulate. It is particularly pertinent to large-scale cultivation where the value of the protein being expressed must be weighed up against the expense of providing the necessary environment.

1.5.2 Effect of Temperature

Temperature is unquestionably one of the most important environmental factors influencing the growth of S. cerevisiae. Thus in a process where the over expression of a product is necessary, the optimisation of the fermentation temperature is fundamental. *Saccharomyces cerevisiae* is a mesophilic yeast, and the maximum

temperatures for *S. cerevisiae* strains range from 35 - 43^{0}C, with growth limits between 0 and 48^{0}C (Walker, 1998). However the stability of the protein being produced at higher temperatures also has to be taken into account. It has been previously reported that the temperature at which exponential phase cells of *Saccharomyces cerevisiae* were grown before being subjected to heat stress was an important factor in contributing to cell survival (Watson, 1987). Thus careful consideration must be exercised when altering the temperature of the culture broth to induce expression of foreign protein.

Beney *et al* (2001) reported on the coupling effects of increased osmotic pressure and temperature on the viability of *S. cerevisiae* cells. Both osmotic pressure and temperature have a strong influence on the growth and viability of micro-organisms. Increases in osmotic pressure and decreases in temperature generally result in slowdown or cessation of cell growth, and sometimes even cell death. Beney *et al* (2001) determined that yeast resistance to high osmotic stress (99MPa) was enhanced at low temperatures (5 - 11^{0}C).

Tøttrup and Carlsen (1989) demonstrated that by decreasing the fermentation temperature from 30^{0}C to 26^{0}C, in batch and fed-batch experiments, improved yields of human proinsulin were observed. These researchers hypothesized that this may be due to decreased proteolytic activity at the lower temperature. It may also be due to effect temperature had on the glucose repression/derepression of the ADH2-GADPH promoter.

Nagashima *et al* (1994) discovered that by decreasing the cultivation temperature, the production level of the required enzyme doubled compared to conventional methods. They developed a new culture method by initially growing the *S. cerevisiae* cells in selective media for

24 – 48 hrs at 30^0C. They then harvested the yeast by centrifugation techniques, and transferred these to rich complex medium. The batch fermentation was then begun at 15^0C for 48 hours, and the cultivation temperature was then increased to 30^0C for a further 2 days. Using this cold-culture method, the final yields of amylase (28.6mg/L) and human lysozyme (6.1mg/L) were two-fold higher than the traditional methods of culturing at 30^0C.

Cassland and Jönsson (1999) expressed *Trametes versicolor* laccase A in *Saccharomyces cerevisiae* and *Pichia pastoris*, and substantiated results found by previously mentioned experimenters. In the case of *S. cerevisiae*, they found that by decreasing the cultivation temperature from 28^0C to 19^0C during the 72 hours incubation period, there was a 16-fold increase in the level of enzyme activity. Decreased cultivation temperatures in batch fermentations of *Pichia pastoris* transformed with the laccase gene also exhibited much higher levels of expression.

Rossini *et al* (1993) in contrast to the results found by the aforementioned researchers determined that by increasing the culture temperature from 24^0C to 36^0C there was a dramatic increase in enzyme production. They also found that excretion of the foreign protein at 36^0C was twice that seen at 30^0C, the optimal growth temperature of *S. cerevisiae*.

Alvarez *et al* (1994) also discovered that by increasing the cultivation temperature from 24^0C during growth to 37^0C, about 70% of the heterologous protein chloramphenicol acetyl transferase (CAT) was released. However this was based on using thermosensitive mutant yeast strain, which lyses at 37^0C, thus releasing the desired foreign protein.

Temperature is known to affect microbial growth rate, biosynthetic pathways and regulatory systems. These effects are extremely significant in recombinant fermentations. It has been shown here by a number of experimenters that the optimal growth temperature for *S. cerevisiae* of 30^0C does not automatically achieve the highest levels of protein expression.

1.5.3 Effect of pH

As with temperature, the pH of the fermentation broth has to be suitable for growth of the yeast, as well as preventing any degradation of the recombinant protein secreted into the medium. Yeast tend to favour growing when the initial culture medium pH is between 4 - 6 and *Saccharomyces* strains are capable of growth at pH values between pH 2.5 - 8.5 Elemental hydrogen can be found in yeast cellular macromolecules and is obtainable from carbohydrates and other sources. Yeast cell physiology is dependent upon hydrogen ions, as effects on both growth and metabolism of yeast cells can be instigated by discrepancies in both extracellular and intracellular pH, as well as potential denaturation of the heterologous protein being expressed. (Carmelo *et al*, 1996, Walker, 1998)

Kang *et al* (2000) determined that denaturation of the secreted protein, human serum albumin (HSA), correlated with the acidification of extra-cellular pH. The expression of HSA was under the control of the GAL10 promoter, and thus induction of the foreign protein was obtained by periodic feeding with Galactose. However they observed that the pH of the culture broth decreased perceptibly from 6.3 to 4.0 with concomitant HSA degradation. To counteract the acidification of the culture broth owing to galactose addition, the medium was buffered above pH 5.0, or by supplementing with amino acids. They ascertained

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that the protein secreted into the culture medium was extremely vulnerable to cell bound protease(s), and dependent on the pH of the medium.

Similarly, Carmelo *et al* (1996) also ascertained that low pH was detrimental to gene expression. Levels of mRNA from the plasma membrane ATPase encoding gene PMA1 decreased by 50% in *Saccharomyces cerevisiae* cells grown at pH 2.5 compared to the optimal pH of 5.0.

Bae *et al* (1996) also discovered that rising culture medium pH increased the levels of protein harvested. The percentages of recombinant human granulocyte colony-stimulating factor (hG-CSF) recovered from the culture broths were 46, 60 and 76% for pH's 5.0, 5.5 and 6.7 respectively. Shu and Yang (1996) established that although pH did not significantly affect cell growth, it did have a significant bearing on foreign protein production. Both plasmid stability and production of granulocyte-macrophage colony-stimulating factor (GM-CSF) were severely affected in cultures grown at pH 4.0 compared to pH 6.0. This specific result regarding pH was also substantiated by growth of recombinant yeast immobilised in a fibrous bed reactor, and production of GM-CSF (Yang and Shu, 1996).

Shiba *et al* (1998) concluded that pH was the most critical environmental parameter during the cultivation of recombinant yeast. The optimum pH for the production of recombinant CPY (carboxypeptidase) was pH 6.5. Cultivation was also tested at initial pHs of 6.0 and 7.0. The quantity of CPY recovered decreased acutely below pH 6.0, and this was attributed to the presence of acid protease, which denatured the enzyme.

In contrast to previously mentioned results, Rossini *et al* (1993), demonstrated that initial pH at pHs 4.5, 5.5 and 6.7 did not affect either excretion or the activity of the secreted protein, *β*-galactosidase. However, below pH 4.5 *β*-galactosidase is denatured, therefore no experimentation below this initial pH was performed.

Despite the results of Rossini *et al* (1993), it would appear that each yeast system has an optimum pH for growth and production of its foreign protein. The favoured pH for growth of the yeast cell may possibly degrade the protein secreted into its extracellular surroundings. Thus when devising a protocol for production of a novel protein it is advisable to find the pH for maximum productivity.

1.5.4 Effect of Dissolved Oxygen Concentration

In the complete absence of Oxygen, yeast are unable to grow well (Walker, 1998) and in many aerobic fermentations, the rate limiting step is the Oxygen supply as Oxygen is sparingly soluble in aqueous solutions. The Oxygen transfer rate (OTR) governs the provision of Oxygen in the culture medium (Kapat *et al*, 2001). High levels of Oxygen should theoretically increase biomass levels and heterologous protein production. This would increase energy production, which is particularly important in plasmid carrying cells as they have high cellular energy maintenance levels. However, at high pressures, pure Oxygen can be inhibitory towards yeast cells, and there have even been suggestions of Oxygen toxicity (Walker, 1998).

Caunt *et al*, (1989) determined that limitation in dissolved Oxygen tension (DOT) levels has a detrimental effect on plasmid stability yeast during cultivation in a non-selective medium. This team produced *β*-Galactosidase under the control of the CYC1 promoter, which is

maximally expressed under glucose repressed conditions. Once the DOT fell below 10% of air saturation, there was a steep drop of plasmid-bearing cells in the yeast population over time. Further research by Caunt et al (1990) ascertained that by introducing cyclic changes in the DOT, higher plasmid stability resulted. This involved subjecting the recombinant yeast culture to square wave oscillation of the Oxygen content in the air supply.

Pyun *et al* (1999) determined that invertase expression from recombinant *Saccharomyces cerevisiae* under the control of the inducible SUC2 promoter was affected not only by glucose repression, but also levels of Oxygen. They confirmed that maximal expression of the SUC2 gene could only be obtained under anaerobic conditions, when a single-stage continuous culture was performed. By changing conditions from aerobic to anaerobic, a five fold increases in specific invertase activity was achieved. However, cell biomass levels were much lower under anaerobic conditions.

To counteract these affects, they developed a two-stage continuous culture protocol. In order to obtain a high yield of yeast biomass initially, the fermenter was operated under aerobic conditions and a low level of glucose, around 100mg/L. The second stage of the process involved maintaining anaerobic conditions, and lowering glucose concentration to a residual 50mg/L to derepress the SUC2 gene and induce maximal invertase expression. This innovative concept resulted in 2.5 fold increase in invertase productivity compared to the single-stage continuous culture.

Dissolved Oxygen levels in fermentations can be controlled by varying agitation speed and/or airflow rate. The volumetric Oxygen transfer coefficient k_La is altered in correlation with these changes. Kapat *et al*

(2001) determined that production of recombinant Glucose Oxidase (GOD) was reliant on impeller speed. The optimal impeller speed was 420 rev/min, but beyond this speed, no increase in GOD production was observed. It is thought this may be a result of recombinant yeast being sensitive to both the mechanical stress of higher impeller speed (turbulence) and higher aeration rate (bubbles bursting), with an overall effect of lower cell growth and lower productivity.

Caunt *et al*, (1989) and (1990) concluded that plasmid stability is adversely affected by low Oxygen supply, whereas Pyun *et al* (1999) achieved higher heterologous production in anaerobic conditions. It is possible that these conflicting results had much to do with the relevant expression system employed by the two teams. Either way it does emphasise the impact of this environmental parameter and its repercussions for the large-scale production of recombinant proteins.

When scaling a bioprocess from the laboratory to large-scale production, one of the most difficult variables to reproduce is the time profile of dissolved Oxygen. In addition, if mixing is incomplete pockets of depleted Oxygen can occur in large bioreactors, which may have a severe affect on gene expression. There are very few citations on the effect of dissolved Oxygen on heterologous protein production, and thus there is little knowledge in this area at present (Konz *et al*, 1998).

1.5.5 Mode of Cultivation of recombinant yeast

In industry there are four main types of fermentation regimes employed in the production of heterologous protein from recombinant *Saccharomyces cerevisiae*. The pros and cons of each are discussed below.

1.5.5.1 Batch cultures

Batch systems are easy to set-up and are robust closed culture assemblies. Growth follows Monod kinetics, where after an initial lag phase, growth of the cells increases exponentially, until the nutrients are depleted and stationary phase begins. In batch culture, the concentrations of nutrients, substrate, product and biomass are constantly changing. In the industrial application of batch processes, despite strict controlling of environmental parameters such as pH and temperature, differences in product yield occur. When stationary phase has been reached an accumulation of inhibitory by-products and product degradation due to proteolytic activity may occur (Mendoza *et al*, 1994b, Fiechter *et al*, 1987).

1.5.5.2 Continuous cultures

In a chemostat operation, fresh nutrients are constantly supplied to the fermenter with continuous removal of the spent fermentation broth containing some of the cells and desired product. Thus, in a chemostat, steady-state conditions prevail and the concentration of the nutrients and growth limiting substrate are constant. Therefore in continuous culture, the growth rate, 'μ', of the micro-organism is equal to the dilution rate, 'D' (Fiechter *et al*, 1987, Matthews and Webb, 1991). By altering the concentration of the growth limiting substrate the growth rate of the cells can be varied. Continuous culture of recombinant yeast is not usual in pharmaceutical industry because mutation and contamination are more likely to occur (Mendoza *et al*, 1994b, Walker, 1998, Thiry and Cingolani, 2002).

The levels of glucose supplied to a continuous culture are significant because they affect not only metabolism of the yeast which controls

cell concentration and productivity, but also in the case of recombinant yeast, the expression of the protein, whether it is constitutive or inducible. In recombinant systems the critical dilution rate, D_c where glucose effects such as the Crabtree Effect become apparent is lower then in normal *S. cerevisiae* fermentations. *S. cerevisiae* is a glucose-sensitive yeast and ethanol production can occur in the presence of excess glucose. The greater metabolic burden placed on plasmid bearing cells compared to plasmid free cells has been imputed for this phenomenon (Hardjito *et al*, 1993, de Kock *et al*, 2000).

Da Silva and Bailey (1991) studied the effects of dilution rate on plasmid stability and β-Galactosidase production from recombinant *S. cerevisiae* using minimal media in continuous culture. As the dilution rate was lowered, increases in both biomass concentration and β-Galactosidase specific activity were observed. Overall productivity was substantially higher at the lowest dilution rate (D = $0.1h^{-1}$, compared to 0.2 and $0.26h^{-1}$), despite lower plasmid stability and flow rate.

In contrast to this observation, Mendoza-Vega *et al* (1994a) observed consistent plasmid stability at a number of different dilution rates ranging between $0.043h^{-1}$ to $0.3h^{-1}$ in a selective media chemostat. Maximum specific rates of product formation were obtained between dilution rates of 0.15 and $0.2h^{-1}$. At higher dilution rates, a decline of secreted hirudin was seen with concomitant accumulation of both glucose and ethanol.

Hardjito *et al* (1993) observed similar results to those seen by Mendoza-Vega *et al* (1994a) however this time the media was non-selective and discovered that dilution rate had negligible effect on segregational instability. However, in accordance to Mendoza-Vega *et*

al (1994a) an average glucose feed-rate of 1.31g glucose h^{-1}, resulted in both maximal β-Galactosidase and cell production rates.

Plasmid stability of recombinant *Saccharomyces cerevisiae* has been shown to be strongly dependent on growth rate when grown continuously in non-selective medium. Plasmid stability decreased as the growth rate increased (Impoolsup *et al*, 1989). Both Caunt *et al*, (1990) and Zhang *et al*, (1997) also detected that decay rates of the plasmid-bearing fraction of the cell population and protein expression increased with increasing dilution rate in non-selective media.

Bugeja *et al*, (1989) in contrast to the aforementioned results found that the plasmid pJDB248 was far more stable at a higher dilution rate of $0.12h^{-1}$ compared to $0.05h^{-1}$, when recombinant yeast cells were grown in non-selective defined media chemostat culture. However the average plasmid copy number (PCN) in the plasmid-bearing cells was approx. 50 at high dilution rates, compared to almost 600 at low dilution rates. Shu and Yang (1996) and Birol *et al* (2000) also reported that both plasmid retention and specific productivity was significantly increased at higher growth rates.

O' Kennedy *et al* (1995) studied the plasmid loss due to plasmid segregation in both defined and complex glucose-limited media. They ascertained that plasmid loss rates were growth rate dominated in defined cultures, while the reverse was found in non-selective cultures. They prognosticated that plasmid-bearing cells had a growth advantage over plasmid-less cells in complex cultures, despite the metabolic burden of the plasmid.

There are conflicting reports in literature on the effect of dilution rate on heterologous protein production from *S. cerevisiae*. In selective media,

plasmid stability remains high, but because of the slow growth rate, there is low productivity. Alternatively, complex media offers high growth rates at the expense of plasmid stability, but relatively high production rates can occur. Thus it would appear that individual yeast expression systems, with their own vector, selection marker and promoter have different requirements. A dilution rate for one system or fermentation process may not be suitable for another.

1.5.5.3 Fed-Batch Cultures

By applying the principles of batch and continuous processes, the 'Zulauf' process was devised, which later developed into fed-batch culture. In fed-batch mode, high cell density can be reached without the accumulation of ethanol or other inhibitory products, while allowing metabolic control. Exponential feeding of fresh medium extends the batch process, and this allows the cells to be grown at a constant growth rate. There is an increase in the volume and this differs from continuous culture, where it is necessary to keep the volume constant. The production of recombinant proteins in industry often employs fed-batch fermentation (Mendoza *et al*, 1994b, Thiry and Cingolani, 2002).

There are numerous citations in literature on the use of fed-batch processes for the production of foreign protein from recombinant *S. cerevisiae* (Calado *et al*, 2002, Ibba *et al*, 1993b, Lee *et al*, 1994, Gu *et al*, 1991, Hardjito *et al*, 1993, Tøttrup and Carlsen, 1990, Zigova, 2000).

Tøttrup and Carlsen (1990) designed a process for the large-scale production of human Proinsulin using *Saccharomyces cerevisiae* as the host strain. They developed a fed-batch fermentation protocol whereby the expression of the SOD-PI product is repressed during the

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early fermentation period. This was executed by feeding the culture with excess glucose at a constant rate of 1.5g/L/h during the initial 40 - 45 hours. The carbon source was then replaced by ethanol at a constant rate of 1.0g/L/h. The fermentation temperature was also decreased from 30^0C to 26^0C which lead to an overall increase in product yield in both batch and fed-batch processes. The optimised fed-batch protocol resulted in product yields of greater than 1,500mg/L.

Chiruvolu *et al* (1996) developed a similar protocol to that designed by Tøttrup and Carlsen (1990). They were producing oIFNτ (ovine interferon-tau) from recombinant *S. cerevisiae*. The cells were grown at the beginning under glucose limitation to reduce ethanol accumulation. Then, during protein production ethanol was continuously fed to the fermenter @ 1.0g/L/h for fed-batch fermentations. The final biomass and oIFNτ yield improved significantly in 5L fed-batch fermentation.

One research team developed a repeated fed-batch process in order to produce recombinant hirudin using *S. cerevisiae* as the host. This method consisted of harvesting half of the culture every 24 hours, and replacing it with fresh medium. Although the method is similar to continuous culturing, they found that with this novel method, there was over three times as much hirudin produced by the repeated fed-batch process (Ibba *et al*, 1993b).

Lee *et al* (1994) compared batch versus fed-batch fermentations for the production of hirudin. In comparison to Ibba *et al* (1993b), they used galactose as the carbon source as opposed to Glucose. There was greater than four-fold increase in hirudin concentration in the fed-batch compared to the batch fermentation. This team achieved a maximal hirudin concentration of 260mg/L during fed-batch.

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Gu *et al* (1991) also compared batch to fed-batch fermentations for the production of HbsAg (hepatitis B surface antigen). They obtained a 10-fold increase in HbsAg expression in fed-batch compared to batch fermentation. Calado *et al* (2002) also achieved higher expression of cutinases in fed-batch versus batch.

Cheng *et al* (1997) developed a novel method to combat the problem of plasmid loss in non-selective media. This involved growing recombinant cells in selective media in batch phase initially, and then transferring the mode to fed-batch using rich media. By periodically starving the fermentation media of glucose, production of the desired β-Galactosidase was greatly enhanced.

1.5.5.4 Immobilisation

Immobilisation of recombinant yeast cells for the production of foreign proteins is a relatively new method of cultivation. The main aim of immobilisation is to accumulate cells to such an extent that this structure can be retained in the fermenter without washout of cells. It offers several advantages over free cell fermentations including higher cell densities and higher dilution rates, which in turn decrease the risk of contamination. It not necessary to recycle cells, and the yeast cells can be easily segregated from the suspension for down-stream processing and recovering the protein of interest (Matthews and Webb, 1991).

Immobilisation of cells has been used to overcome plasmid stability problems in complex media, and thus increase specific productivity. However because of the lower growth rates of the entrapped plasmid bearing cells, lower productivity may be observed (Yang and Shu, 1996).

Methods of immobilisation include films on solid support, biomass support particles, covalent bonding to a solid support, adsorption to a charged surface and entrapment within bead or disc matrices of gels and resins. Types of fermenter systems employed comprise packed, fluidised and fibrous-bed reactors, sheet and gas mixed bioreactors (Matthews and Webb, 1991, Yang and Shu, 1996, De Alteriis *et al* 2001).

1.6 Conclusion

There are a number of factors to consider when designing a production mechanism for recombinant proteins. Firstly, a number of decisions have to be made in the molecular biology laboratory. This will include which organism to use as a host, whether it is to be procaryotic or eucaryotic. Depending on the organism opted for the expression system has to be decided upon. This will force a decision on the plasmid vector, promoter type; inducible versus constitutive and selectable marker. All of these will have influences during the fermentation of the recombinant organism.

Once the suitable host and expression system have been chosen, tests have to be run to determine optimum fermentation regimes. Parameters such as pH, temperature, dissolved Oxygen concentration, type of media and cultivation mode will all have significant bearings on both heterologous protein production and plasmid stability.

It has been shown here through numerous contrasting citations that each expression system and organism have different reactions to varying fermentation parameters. Thus what may well prove to be the most efficient production protocol for one system, could have a detrimental effect on another. It is of vital importance however, to have a thorough understanding of the expression system and host one is working with.

Chapter 2 Materials and Methods

2.1 Organism

The *Saccharomyces cerevisiae* yeast used was a haploid strain, DBY746 (*Mat α his3-Δ1 leu2-3 leu2-112 ura3-52 trp1-1-289$_a$*) from the Yeast Genetic Stock Centre, Department of Biophysics and Medical Physics, University of California, Berkeley CA. This yeast Genetic Stock Collection has now been moved to ATCC (American Type Culture Collection) and the ATCC number is 204660.

2.2 Plasmid

The plasmid pFheCL1Y29 was transformed into the yeast strain DBY746 by Roche *et al* (1997) using the lithium acetate method. This plasmid is a derivative of the multicopy plasmid pAAH5 (Ammerer, 1983) and encodes the FheCL1 gene under the control of the yeast ADH1 promoter. The selection marker on the yeast vector is Leu2.

2.3 Sterilisation Procedure

All culture media and vessels were autoclaved using a Tomy Autoclave SS-325 (Tomy Seiko Co., Ltd., Japan) at 121^{0}C (15psig) for 15 minutes. The fermenter, used for 3L batch fermentations, was autoclaved for 40 minutes at 121^{0}C.

2.4 Media Preparation

2.4.1 Selective

Table 2.1 Selective Medium (minimal medium) components

Component	Manufacturer	Concentration
Yeast Nitrogen Base	Difco - Code 0392 -15 –9	6.7g/L
D(+)Monohydrate Glucose	Merck	20g/L
Uracil	Sigma	20mg/L

This media was prepared in 0.1M Phosphate Buffer, pH 6.5. Both the Glucose and Uracil were added separately after autoclaving using sterile stock solutions of 40% (w/v) Glucose and 0.2% (w/v) Uracil. Both had also been prepared in Phosphate Buffer.

2.4.2 Non-Selective

Table 2.2 Non-Selective (YEPD) Components

Component	Manufacturer	Concentration
Yeast Extract	Oxoid	10g/L
Bacteriological Peptone	Oxoid	20g/L
D (+) Monohydrate Glucose	Merck	20g/L

Non-Selective Medium - YEPD (Yeast Extract Peptone Dextrose) was made using the above components in distilled water: (Table 2.2) In both cases, solid media was prepared with the addition of 30g/L Oxoid Technical Agar No. 3 (Basingstoke, England) and sterile glucose was added separately after autoclaving.

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2.4.3 Phosphate Buffers

Phosphate buffer was used in all selective media and was prepared at pH 6.5.

Table 2.3 Phosphate Buffer Stock Solutions - Concentration 0.2M

Name	Chemical Formula	Manufacturer	Concentration
Sodium dihydrogen phosphate dihydrate	$NaH_2PO_4*2H_2O$	Merck KGaA, Darmstedt, Germany	31.2g/L in dH_2O
di-Sodium hydrogen phosphate dodecahydrate	$HNa_2PO_4*12H_2O$	Merck KGaA, Darmstedt, Germany	71.7g/L in dH_2O

To prepare 2 Litres of 0.1M Phosphate Buffer, pH 6.5, 680mLs of 0.2M $NaH_2PO_4*2H_2O$ and 320mLs of 0.2M $HNa_2PO_4*12H_2O$ were re-suspended with 1 Litre of distilled water.

2.4.4 Replacement of Carbon Source

Normally the main carbon source in selective media is glucose. Glucose was exchanged with various sugars such as lactose, fructose and mannose. The media prepared is shown in Table 2.4. All monosaccharides (Glucose, Galactose, Fructose and Mannose) were prepared to a stock concentration of 2M, while all disaccharides (Lactose, Maltose and Sucrose) were prepared to a stock concentration of 1M. The sugar stocks were added after autoclaving the other components.

Table 2.4 Breakdown of components in the selective media used in replacing the carbon source.

Component	Conc. Stock	Volume of Stock in Media
Yeast Nitrogen Base	6.7% w/v	10mL
Uracil	0.2% w/v	1mL
Phosphate Buffer pH 6.5		84mL
Single Sugar Stocks		
Glucose (hydrous)	4.0% w/v	5mL
Lactose	3.64% w/v	5mL
Maltose	3.64% w/v	5mL
Galactose	3.64% w/v	5mL
Mannose Stock	3.64% w/v	5mL
Sucrose (anhydrous)	3.45% w/v	5mL
Fructose	3.64% w/v	5mL
Double Sugar		2.5mL of each sugar

2.4.5 Supplementation of Yeast Nitrogen Base and Uracil

Media used in the Supplementation Shake Flask experiment was prepared using Phosphate Buffer and stocks of Uracil (Ura), Yeast Nitrogen Base (YN_2B) and Glucose. Each of the stocks were prepared in Phosphate Buffer as outlined in Section 2.4.4 Glucose was added after autoclaving the other components. Refer to Table 2.5.

Table 2.5 Supplementation of Yeast Nitrogen Base and Uracil in selective media

	YN_2B x1 (6.7g/L)	YN_2B x3 (13.4g/L)	YN_2B x5 (20.1g/L)
	A	**B**	**C**
Ura x1	Ura Stock - 1mL	Ura Stock - 1mL	Ura Stock - 1mL
(20mg/L)	Glu Stock - 5mL	Glu Stock - 5mL	Glu Stock - 5mL
	YN_2B Stock - 10mL	YN_2B Stock -30mL	YN_2B Stock-50mL
	PO_4^- Buffer - 84mL	PO_4^- Buffer - 64mL	PO_4^- Buffer -44mL
	D	**E**	**F**
Ura x3	Ura Stock - 3mL	Ura Stock - 3mL	Ura Stock - 3mL
(60mg/L)	Glu Stock - 5mL	Glu Stock - 5mL	Glu Stock - 5mL
	YN_2B Stock - 10mL	YN_2B Stock -30mL	YN_2B Stock-50mL
	PO_4^- Buffer - 82mL	PO_4^- Buffer - 62mL	PO_4^- Buffer -42mL
	G	**H**	**I**
Ura x5	Ura Stock - 5mL	Ura Stock - 5mL	Ura Stock - 5mL
(100mg/L)	Glu Stock - 5mL	Glu Stock - 5mL	Glu Stock - 5mL
	YN_2B Stock - 10mL	YN_2B Stock -30mL	YN_2B Stock-50mL
	PO_4^- Buffer - 80mL	PO_4^- Buffer - 60mL	PO_4^- Buffer -40mL

2.5 Cultivation Conditions

2.5.1 Culturing on Solid medium

The organism was maintained on solid selective agar plates. The plates were grown for 72 hours in a 30^0C incubator, until single colonies are obtained. The plates were then stored for up to one month at 4^0C, before sub-culturing on to a fresh selective medium plate. This transfer was performed using a single colony transfer with an inoculation loop from a stock plate to a new agar plate.

2.5.2 Shake-Flask Cultures

Unless stated otherwise, all starter cultures were grown in either (a) 250mL Erlenmeyer shake flasks containing 100mL of liquid media, or (b) 1000mL Erlenmeyer shake flasks containing 400mL of liquid media. All shake-flasks experiments were performed in duplicate. The flasks were incubated in a Gallenkamp orbital incubator at 30^0C and agitated at 150rpm for the required length of time. 1mL of the culture was then transferred to a new pre-warmed flask, containing 100mL of media if sub-culturing was required. Sponge bungs were used in preference to cotton wool, as they supplied a consistent Oxygen level to the yeast, in addition to reducing the risk of contamination.

2.5.3 Batch Fermentations

2.5.3.1 5L Fermenter

Batch fermentations were initially performed in a 5L (working volume 3.2L) bioreactor, and controlled using a Biostat B (B. Braun Biotech International GmbH, Melsungen, Germany)

Table 2.6 Operating parameters for 5L fermenter

Parameter	Controlled at:
Temperature	30^0C
Agitation	400 rpm
Aeration	1.0 v/v/m
Liquid Volume	3.0 L

Table 2.7 5L Fermenter Configuration

Items Measured	Dimension(s)
Tank Diameter	160 mm
Tank Depth	305 mm
Number of Impellers	1
Blades per Impeller	6
Impeller Diameter	64 mm
Dimensions of Blade	12 mm x 18 mm
Number of Baffles	4

The fermenter and medium were autoclaved at 121^0C for 40 minutes. The medium was buffered to pH 6.5 using phosphate buffer and Silicone anti-foam (Silcorel AFP20, BDH, Poole, England) was added to the medium at a concentration of 0.1mL/Litre medium prior to autoclaving. Glucose Stock was added asceptically to the medium via a top port after autoclaving had taken place. Air supplied to the

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bioreactor was filtered with two 0.2μm Acrodisc PTFE filters (PALL Gelman Life Sciences). A resistance thermometer sensor (Pt-100 - Mettler Toledo) was used to measure temperaure. A water jacket surrounding the tank regulated temperature to within 0.1^0C. pH was monitored utilising a sterilisable *in situ* pH probe (Mettler Toledo) Dissolved Oxygen was determined using a sterilisable *in-situ* polagraphic electrode with pasteous electrolyte (InPro 6000 Series O2 Probe, Mettler Toledo). The electrode was based on the polargraphic principle, consisting of an Ag-anode and Pt-cathode, and had a gas permeable polymer membrane.

2.5.3.2 15L Fermenter

Further batch fermentations were performed in a 15L (working volume 10L) Biostat C bioreactor (B. Braun Biotech International GmbH, Melsungen, Germany). By scaling up to this volume, frequent sampling could take place without significantly reducing the volume of the liquid culture.

Table 2.8 15L Fermenter Configuration

Items Measured	Dimension(s)
Tank Diameter	187 mm
Tank Depth	580 mm
Number of Impellers	3
Blades per Impeller	6
Inter-impeller distance	140 mm
Impeller Diameter	85 mm
Dimensions of Blade	12 mm x 18 mm
Number of Baffles	4

Silicone anti-foam (Silcorel AFP20, BDH, Poole, England) was again added to the medium at a ratio of 0.1mL per litre medium prior to sterilisation. The fermenter and medium without glucose was sterilised using pre-set control options on the Biostat C controller. The medium was sterilised at 121^0C for 25 minutes. Agitation was set at 100rpm during sterilisation. On cooling to 30^0C, glucose stock and the inoculum were both added asceptically via a top port.

Table 2.9 Operating parameters for 15L fermenter

Parameter	Controlled at:
Temperature	30^0C
Agitation	400 rpm
Aeration	1.0 v/v/m
Liquid Volume	10 L
pH (controlled with)	1.0M NaOH
pH (controlled at)	6.5

The temperature of the fermentation regulated using a closed-loop hot water system with a circulation pump and two stainless steel heat exchangers. Temperature, pH and dissolved oxygen were measured and regulated using the same equipment as in the 5L fermenter.

2.6 Sampling and Measurement of Fermentation Parameters

2.6.1 Enzyme Assay

The fluorimetric assay performed to determine the concentration of Cathepsin L1 secreted into the culture media was an adapted method of that of Dowd *et al* (1997). The reaction was performed in white 96-well plates to a final volume of 100μL. The reaction was quantified in a Perkin Elmer fluorescence spectrophotometer with excitation set at 370nm and emission at 440nm and the slit widths were 10 and 2.5 respectively. Under these conditions, the enzyme reaction is linear for 60 minutes.

Table 2.10 Reaction components of enzyme assay in chronological sequence

Compound	Conc.	Volume	Manuf.	Chemical Formula
Tris Buffer pH 7.0	0.1M	70μL	BDH	$NH_2C(CH_2OH)_3$
DTT	10mM	10μL	Sigma	$C_4H_{10}O_2S_2$
Sample i.e. enzyme		10μL		
Z-Phe-Arg-AMC*(Substrate)	20μM	10μL	Bachem	$C_{33}H_{36}N_6O_6$

*The substrate Z-Phe-Arg-AMC.HCl was stored frozen as a 1mg/100μL (0.016M) stock solution in dimethyl-formamide.

The basis of this assay, is that the enzyme, Cathepsin L1, cleaves off the NH-Mec bond from the substrate Z-Phe-Arg-AMC, which subsequently fluoresces at the given excitation range. A Standard Curve using AMC (7-amino-4-methylcoumarin - $C_{10}H_9NO_2$) (Sigma), was prepared in the range 0 - 10μM. This was stored as a 500μM stock solution in 4% di-methylformamide and distilled water. 100μL of each dilution of AMC was placed in the wells for the standard curve. The plate was incubated at 37^0C for 60mins, and the reaction was then stopped using 20μL of 1.7M Acetic Acid. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of one nmole of NHMec per minute at 37^0C.

2.6.2 - Glucose Analysis - GOD-PAP

A range of standards containing between 0.0g/L and 1.0g/L glucose was prepared. Blanks were prepared using distilled water. 10μL aliquots of each of the standards, blank and of suitably diluted sample were added to test-tubes. 2mL of the GOD-PAP (Randox Labs, Co. Antrim, Ireland) reaction solution was mixed with each of the standards and samples, and incubated at 37^0C for 10mins. The absorbance of the samples was read in a spectrophotometer at 500nm against the blank.

2.6.3 Reducing Sugar Concentration - DNS

The concentration of reducing sugar in the media was determined using an adapted method of the DNS (3,5 Dinitro -Salicylic Acid) Assay (O'Shea 1998). A 0.5mL aliquot of suitably diluted sample was added to 1.5mL of DNS Reagent in a test-tube. The test-tubes were vortexed, and placed in a 100^0C water bath for 10 minutes. 5mL of distilled water was then added to cool the solution. The optical density was read at

540nm on a Unicam 8625 UV/VIS spectrophotometer. The standard curve was prepared using glucose monohydrate with concentrations in the range 0.0g/L to 1.5g/L.

Table 2.11 DNS Reagent - In 100mLs of distilled water

Component	Manufacturer	Concentration	Chemical Formula
3,5 Dinitro - Salicylic Acid	Sigma	10g/L	$C_7H_4N_2O_7$
Sodium Hydroxide	BDH	16g/L	NaOH

2.6.4 Determination of Ethanol Concentration

Ethanol concentration was determined using a Carlo Erba HRGC 5300 Mega Series Gas Chromatographer (Carlo Erba Strumentazione, Milan, Italy) using was a flame ionisation detector and a 2m column packed with 10% Carbowax. Both the injector and detector temperatures were set to 170^0C, while the oven, and thus the column was at 120^0C. The carrier gas was Nitrogen, and its flowrate through the column was approximately 20mL/min. $1\mu L$ of supernatant sample by injected into the column using a microliter® #701 microsyringe (Hamilton Co., Reno, Nevada). A Standard Curve within the range 0 - 2%v/v was prepared using 96%v/v Ethanol. Ethanol concentration was determined from peak areas obtained on a Mega Series integrator chart recorder.

2.6.5 Determination of pH

The pH of the supernatant samples obtained from the dry weight procedure was measured using a Multical pH Meter pH538 (WTW, Weilheim, Germany). The probe was calibrated using pH 7 and pH 4 buffers prior to use.

2.6.6 Cell Enumeration

Cell counts were performed using an improved Neubauer double cell haemocytometer (Weber Scientific international, West Sussex, England). Samples were diluted using 0.2%w/v Saline. All counts were performed in triplicate.

2.6.7 Cell Viability

Methylene Blue (Lancaster Ltd., England) was used to stain the yeast cells. The stain was prepared using 10mg Methylene Blue, 2g of Sodium Citrate Dihydrate in 10mL of distilled water. This was filtered, and the final volume was made up to 100mL using distilled water. 0.5mL of suitably diluted sample was added to 0.5mL of the 0.01%w/v Methylene Blue dye, and incubated for one minute before aliquoting onto a slide. The cells were viewed using a 40x magnification on a microscope. The yeast cells that stained blue were considered non-viable while others were counted as viable. The percentage viability was determined by counting greater than 300 cells. Percentage viability was calculated by dividing viable cells by the total number of cells and multiplying by 100.

2.6.8 Determination of Dry Weight

To determine the dry weight of cells in liquid culture, 20mLs of culture were weighed in a pre-tared plastic universal at room temperature, and centrifuged at 3,500rpm for 25 minutes. The supernatant was then decanted, and the pellet was re-suspended in distilled water. This suspension was re-centrifuged, and the washing step was repeated. The distilled water was removed, and ~5mLs of Ethanol was used to transfer the remaining pellet into a pre-tared glass universal. The ethanol was evaporated in a water bath, set at 100^0C. The universal and pellet were then dried overnight at 100^0C in an incubator. The dry weight was determined by weighing the universal and dried cells at room temperature on an analytical balance (Mettler Toledo College B154 - 4 decimal place).

2.6.9 Plasmid Stability Determination

Samples taken from fermentations were suitably diluted in saline and spread plated on YEPD agar plates. These plates were incubated at 30^0C for 48hours. At this point, 100 colonies were randomly selected and replica plated on Selective and YEPD agar plates using sterile cocktail sticks. These plates were then incubated at 30^0C, for 72hrs (Selective plates) and 48hrs (YEPD plates). Colonies that grew on YEPD plates, but failed to grow on selective agar plates were defined as plasmid-free cells (P-). Whereas colonies that grew on both plates were considered plasmid-bearing cells (P+). The percentage plasmid stability was calculated by counting the number of P+ cells from the 100 colonies plated.

Chapter 3 Review of previous work in producing Cathepsin L1 in batch culture

Laboratory work performed prior to this project involved functionally expressing recombinant Cathepsin L1 in *Saccharomyces cerevisiae* (Roche *et al*, 1997). It was the first Cathepsin L to be expressed and secreted using its own processing signals from *S. cerevisiae*. However only small concentrations of the enzyme were produced in 10L batch fermentations. Roche *et al* (1997) recovered 5mg of purified Cathepsin L1 from a 10L culture.

Up to this point production of the recombinant Cathepsin L1 was only required in sufficient amounts to perform vaccine trials in bovine herds. Successful trials of a combination vaccine comprising of Cathepsin L1 and Cathepsin L2 prompted investigations into increasing the yield of recombinant Cathepsin L1 from *S. cerevisiae* (Dalton *et al*, 1996).

Studies on the stability of the purified native enzyme had also been performed by Dowd *et al* (2000). Cathepsin L proteinases from *F. hepatica* were subjected to experiments at various conditions including different temperatures, pHs and in the presence of organic solvents and heavy metals. The native enzyme's stability in extreme environments lent credence to the potential application of the recombinant proteinase as both a vaccine and its future use in novel bioindustrial settings.

The recombinant yeast system used in this study was one developed by Roche *et al* (1997), and is abbreviated to YCL1 throughout this thesis. The *Saccharomyces cerevisiae* yeast strain DBY746 was transformed with the plasmid pFheCL1Y29. This plasmid is 13.5kb in

size and carries the gene encoding the protease Cathepsin L1 isolated from adult *Fasciola hepatica*.

The plasmid pFheCL1Y29 is a derivative of the multicopy plasmid pAAH5. The plasmid pAAH5 is a shuttle vector that has both the yeast replication region of the 2-μ circle and the *E. coli* replication region of pBR322. It also possesses the LEU2 gene for selection in yeast, and expression of foreign genes such as Cathepsin L1 is under the control of the ADH1 promoter (Ammerer, 1983).

It has previously been shown by using the sandwich hybridisation method that the plasmid copy number of pAAH5 is only 10 (Korpela *et al*, 1987), and thus it is reasonable to assume that its derivative, pFheCL1Y29 is also a low copy number plasmid. Low copy number plasmids in yeast are often subjected to segregational instability during fermentation, where a mother cells fails to pass on any of its plasmids to the daughter cell during cell division (O'Kennedy *et al*, 1995).

In order to alleviate the problem of plasmid instability the YCL1 yeast transformants were regularly cultured in selective media devoid of leucine. The presence of the selection marker LEU2 on the plasmid ensured that only yeast bearing one or more of the inserted plasmids would survive in this selective media. However Roche *et al* (1997) reported both low biomass and low levels of production of Cathepsin L1 from YCL1 when cultured in selective media. Moreover, initial investigations into growth curves and production cycles for this recombinant yeast also revealed significant variation in enzyme activity when fermentations were performed in selective media under similar environmental conditions. No Standard Operating Procedures (SOP's) for the preparation of the initial inoculum, and further scale-up for large fermentations were in place.

Limited characterisation of the growth phase and enzyme production cycle of YCL1 had been established, as most of the work prior to this project concentrated on vaccine trials with the recombinant enzyme. Roche *et al* (1997) mention growth curves of YCL1 cells (i.e. pFHECL1Y29-transformed DBY746 yeast cells) and a doubling time of 3.8 hours. They also claim that expression of Cathepsin L1 from DBY746 yeast had no ill effects on yeast growth, as doubling times for yeast cells with control plasmids pAAH5 and pFheCLY022 were similar. However this data was not presented.

Following a review of previous work, the primary objectives of this project are:

- To devise standard operating protocol to provide a consistent starter culture of viable, high enzyme producing cells. Unless consistent inocula are supplied to large-scale fermentations, no guarantee can be made regarding uniform enzyme production.
- To determine both the growth cycle of YCL1 and the production cycle of Cathepsin L1.
- To identify the optimum environmental parameters such as pH and temperature for the cultivation of YCL1 and production of enzyme.
- To define the optimum media for the growth of YCL1 and the production of recombinant Cathepsin L1 without significantly affecting the plasmid stability.

By collating the data gained from this project, ideal cultivation conditions for the production of Cathepsin L1 can be implemented resulting in an increase in the amount of Cathepsin L1 secreted from YCL1. It is also hoped that the information gleaned from this research can be employed in the production of Cathepsin L2, which is secreted from recombinant *Saccharomyces cerevisiae* in a similar manner.

Chapter 4 Initial experimentation on growth profiles, production cycles and sub-culturing of YCL1

4.1 Introduction

As noted previously, expression of Cathepsin L1 from recombinant yeast was accomplished by Roche *et al* (1997). But little was known about the production regimes of the recombinant yeast. Thus initial experimentation concentrated on collating data relating to its growth and production cycles.

It was important to establish the optimal harvest time of Cathepsin L1 during batch production. This single time-point could also be used during further experimentation for comparative purposes.

It is also essential to define a standard operating protocol for the scaling-up of inocula and starter cultures to be used in fermentations. i.e. to establish the method of transfer from frozen glycerol stock to solid culture (streak or spread plate) to small-scale liquid culture in shake-flasks and finally to large-scale fermenters.

Yeast transformants were cultured in selective media as outlined in Section 2.4.1 comprising glucose, uracil and yeast nitrogen base containing the three amino acids histidine, tryptophan and methionine.

4.2 Initial investigation into YCL1 growth and enzyme production over a 50-hour period in selective media

Initial investigations into YCL1 cell growth and Cathepsin L1 production were performed in shake flasks over a 50hr period. The 50-hour time-point was originally chosen as an end-point for the fermentation because previous work performed with a similar recombinant yeast strain secreting Cathepsin L2 was cultured for a period of two days (Dowd *et al*, 1997). Enzyme production, cell growth and glucose depletion were monitored. All flasks were inoculated from liquid starter cultures. The results of Fermentation 1 can be seen in Figure 4.1
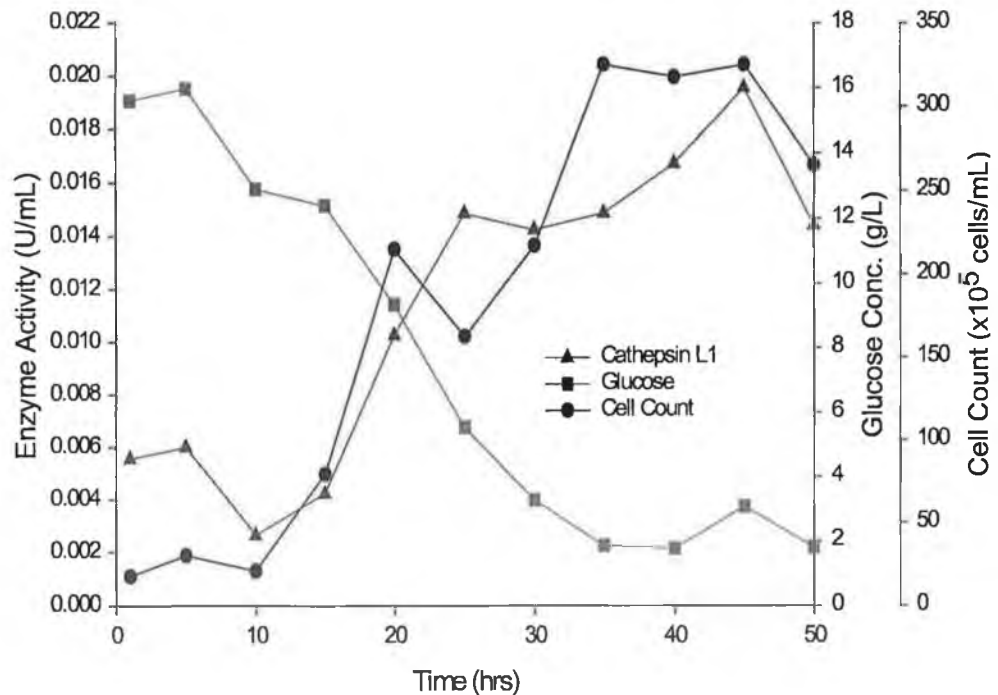
Figure 4.1 Enzyme production, cell growth and glucose utilisation during a period of 50 hours in selective media shake flasks. (Fermentation 1) 1 U enzyme activity = 1 nmole of NHMec released per minute at 37^0C.

The exponential growth phase of YCL1 in selective media is between 10 and 35 hours, in conjunction with the depletion of glucose during this time. Meanwhile there is a concomitant increase in enzyme production with respect to biomass. It would appear that the production cycle of Cathepsin L1 was not completed by 50 hours, thus it was necessary to extend the fermentation time in the ensuing run.

4.3 Further investigation into enzyme production from YCL1 over a 5-day period

The subsequent fermentation run was extended to 120 hours. Again shake flasks contained selective media and were inoculated from liquid starter cultures. Samples were taken every 24 hours, as the growth cycle had been established previously. The results of Fermentation 2 can be viewed in Figure 4.2
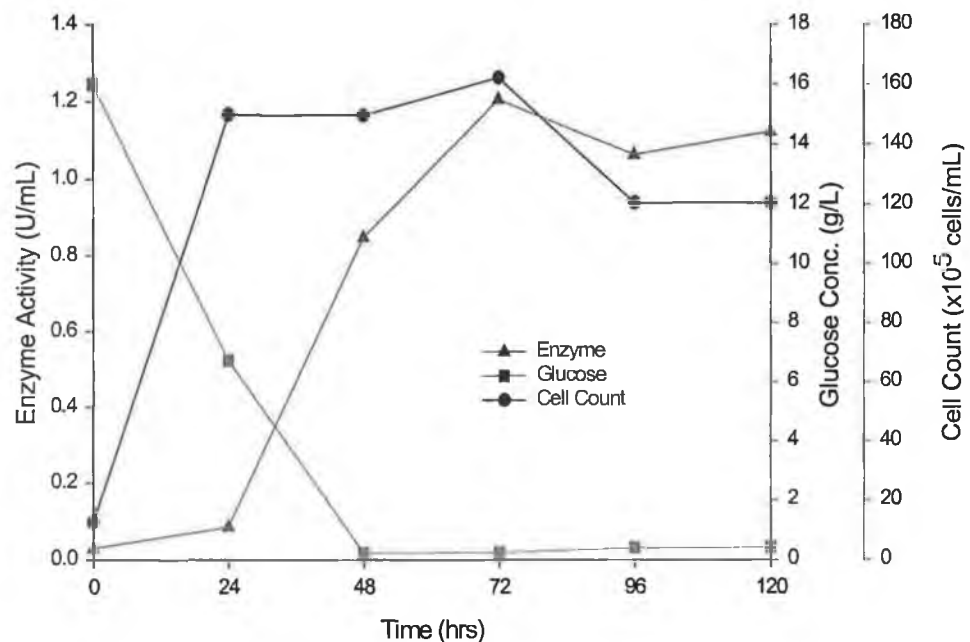
Figure 4.2 Further investigation into the enzyme production cycle in selective media extended to 120hrs. (Fermentation 2)

Cell growth reached stationary phase between 24 and 48 hours, correlating with full consumption of glucose by 48 hours. Meanwhile there was a steady increase in enzyme production where it reached its maximum by 72 hours. After this time-point there was a reduction in both cell numbers and enzyme activity.

4.4 Effect of fermentation time on enzyme activity

The results from five different fermentation runs, that were cultured up to and beyond 96 hours were collated and the enzyme activities at 72 hours and 96 hours were compared. All of these runs were performed in shake flasks or 5L fermenter and cultured in similar environmental conditions as outlined previously.
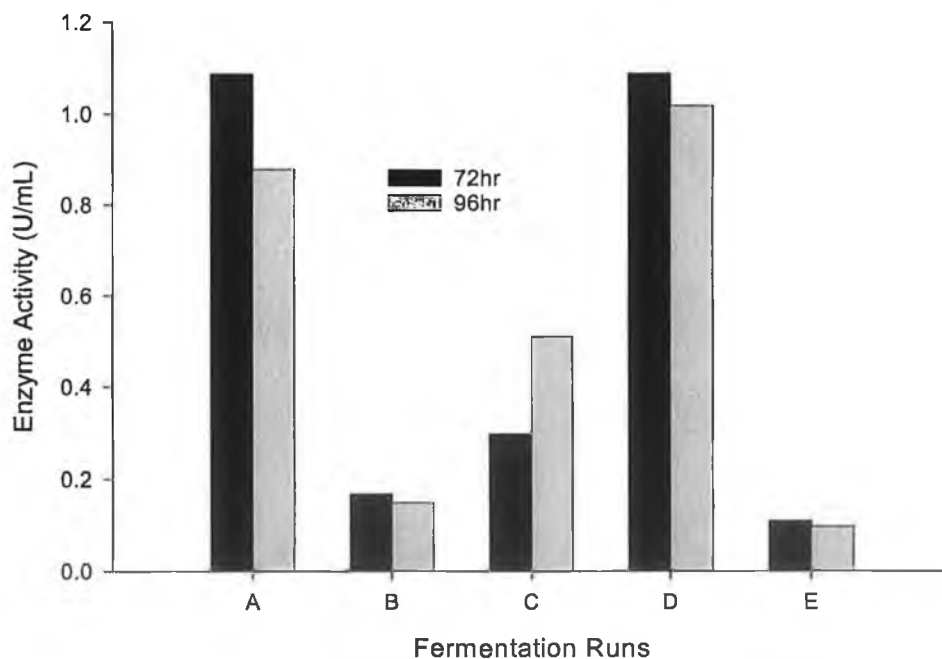
Figure 4.3 Comparison of enzyme activities at 72hours and 96 hours from various fermentations.

With regards to running fermentations for up to and above 96hrs duration, the results from Figure 4.3 indicate that in 4 out of 5 cases,

there was higher activity at 72 hours than at the latter period of 96 hours. Only one of the fermentations, Fermentation C, indicated that it would be conducive to continue fermenting for a further 24 hours. The 72-hour time-point was identified as the juncture at which to stop fermentation and harvest the enzyme. It will also be used for single-point comparisons in examining different environmental parameters.

In addition it appears that there is extreme variability between activities produced in different fermentation runs, despite similar environmental conditions.

4.5 Disparate enzyme activities produced in starter cultures inoculated with various colonies

A typical laboratory method of maintaining *S. cerevisiae* yeast strains involves selecting individual colonies from solid cultures such as streak or spread plates and inoculating them into suitable liquid media. These liquid cultures are typically held in Erlenmeyer shake flasks (e.g. 250mL or 1,000mL, holding 100mL or 400mL of media respectively) and incubated on shaking tables between $20^{0}C$ and $30^{0}C$, until stationary phase has been reached (Kirsop, 1987).

During initial investigations into growth curves and production cycles for this recombinant yeast, the laboratory procedures performed complied with the method described above. However observations from previous experiments indicated that there was variability in Cathepsin L1 production from a number of fermentations performed under similar environmental conditions and selective media. This was perceived as a significant problem and an experiment was performed to determine if starter cultures inoculated with a single colony could be relied upon to produce consistent results.

Seventeen 250mL Erlenmeyer shake flasks containing 100mL of selective media were incubated for a period of 72 hours. Each flask was inoculated with colonies individually selected from streak plates. The 72-hour time-point was again chosen, as it had been shown previously that maximum enzyme activity occurred at this point, and single point comparisons between the starter cultures could be contrasted. The results from this investigation into disparate enzyme activities produced from individual colonies are shown in Figure 4.4.

Individual Starter Cultures
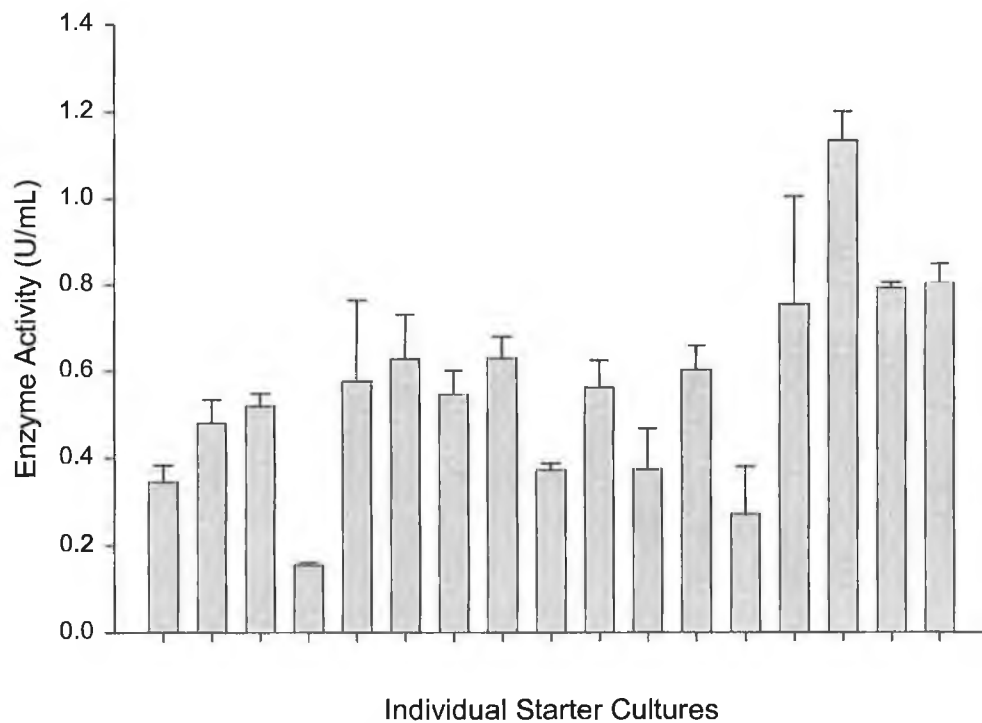
Figure 4.4 Cathepsin L1 activity from individual shake flasks. Each flask contained selective media and was inoculated with a single colony selected from streak plates, and incubated for a period of seventy-two hours in liquid media. The error bars indicate the error in triplicate enzyme assays performed on each of the flasks.

It is apparent that there are indeed large discrepancies in the amount of enzyme activity achieved from starter cultures seeded with individual colonies taken from a streak plate. There is a 7-fold difference in enzyme activity between the lowest and highest producing starter cultures.

Table 4.1 Statistics from shake flasks inoculated with various colonies

Statistics from Shake Flasks	*Enzyme Activity*
Minimum	0.1549 U/mL
Maximum	1.1349 U/mL
Mean	0.5618 U/mL
Standard Deviation	0.2325 U/mL

A new standard operating protocol for the preparation of inocula was devised as a result of these findings. Individual colonies are selected from solid culture and resuspended in an appropriate volume of saline. One mL of this 'blended' suspension inoculates starter cultures, containing 100mL of selective media in 250mL shake flasks.

4.6 Effect of 72-hour cycling in liquid media

As 72 hours was indicated previously as the point where maximum productivity occurred, the effects of sub-culturing YCL1 at 72 hour intervals in liquid broth was monitored. Two sets of 250mL shake flasks, each containing 100mL of selective media were prepared - Flask #1 and Flask #2. Both shake-flask cultures were originally inoculated with separate colonies from a solid culture. Enzyme Activity was monitored.
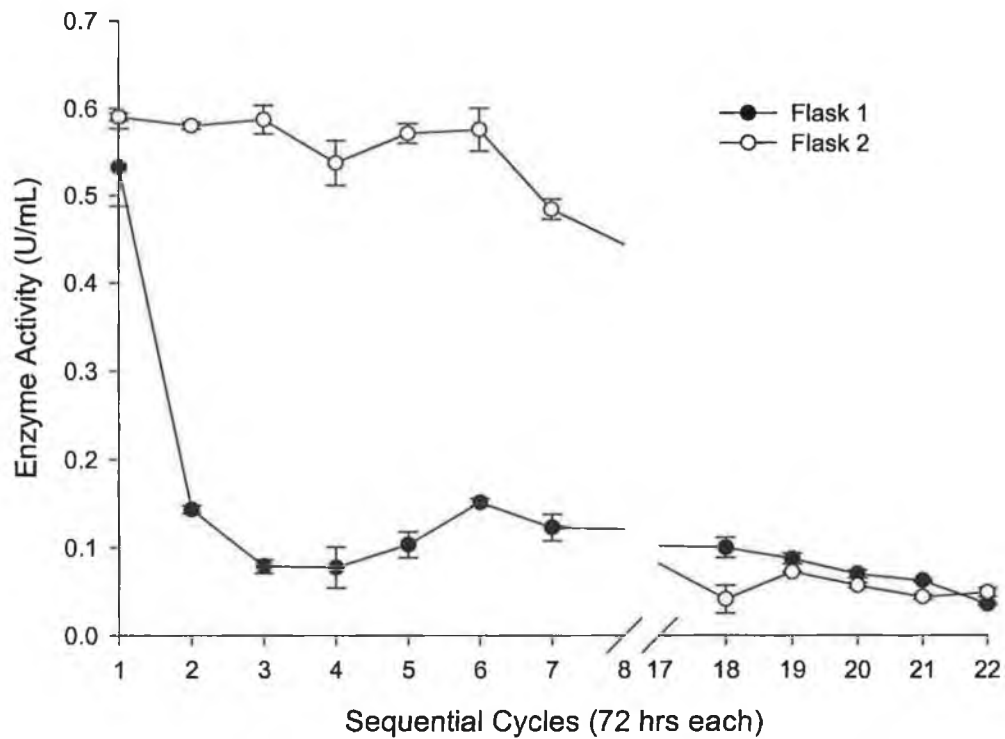
Figure 4.5 72 hour Sub-culturing Experiment. Measurement of Cathepsin L1 activity from a series of shake flasks subcultured in liquid broth at 72hr periods. The error bars indicate the error in triplicate enzyme assays performed on each of the samples. (The break indicates ~ 4-week gap of samples that were not assayed, but flasks were still subcultured.)

Enzyme activity drops considerably in the #1 set of flasks by the second sub-culture cycle, whereas enzyme activity in the #2 set of shake flasks is maintained at a relatively high level for at least seven sub-cultures. No more samples were assayed again until the 18th sub-culture, a period of about four weeks, at which point enzyme activity in both sets of flasks was at trace levels. The main finding from this experiment, as shown in Figure 4.5, is that when YCL1 is sub-cultured

in liquid media for long periods (in this case up to 22 sub-cultures later) it does not maintain a high level of enzyme activity.

4.7 Investigation into the effect of incubation time of starter cultures on enzyme activity

Incubating YCL1 sub-cultures for 72-hour periods is not efficient in maintaining high levels of activity (Section 4.6). An experiment was conducted to identify the appropriate incubation time required for the starter culture to produce high levels of enzyme in subsequent production cultures.

Starter cultures were inoculated from the same 'blend' of colonies as described in Section 4.5, so that each shake flask had the same mixture of cells. Each triplicate set were then grown to four different time-points - 24hrs, 48hrs, 72hrs and 96hrs. When starter cultures were removed at these time-points, they were then used to inoculate production cultures, which were incubated for 72 hours. Again, the 72hr time-point was chosen because maximum enzyme production occurs at this stage in the production cycle and so that a similar time-point for each set of flasks could be contrasted.

The results of the enzyme activity from each set of triplicate shake-flask time-points were averaged and these are presented in Figure 4.6. Enzyme activity in the starter cultures increases with time, reaching a maximum at 96 hours. The production culture that was sub-cultured from the 48-hour starter culture produced the highest overall enzyme activity. The production culture that was inoculated with cells from the 96-hour starter culture was the only sample to see a drop in activity compared to its respective starter culture activity.

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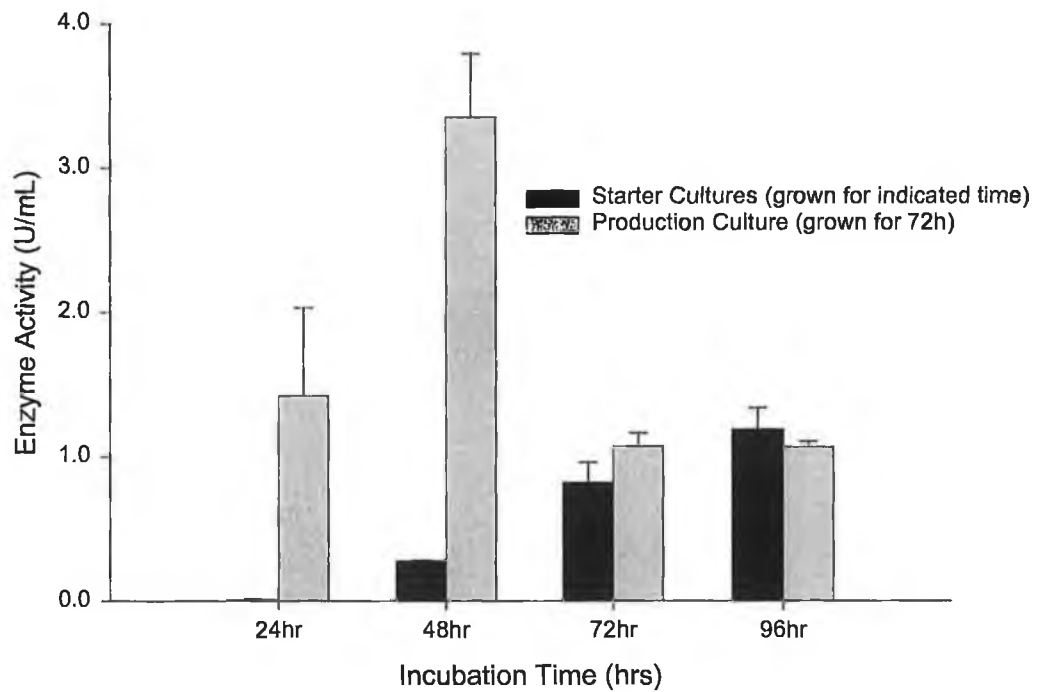
Figure 4.6 Graph showing the average amount of Cathepsin L1 produced in triplicate shake flask samples. Starter cultures were inoculated with a 'blend' of colonies and incubated for each of the indicated times (i.e. 24, 48, 72 and 96 hours). Production cultures were sub-cultured from their respective starter cultures and incubated for 72 hours. Error bars indicate the standard deviation of Cathepsin L1 activity from the three equivalent flasks.

4.8 Further Investigation into the Effect of Sub-Culturing Cycles

When scaling-up for large-scale fermentations, serial sub-cultures are generally required. Cells are initially cultured in small-scale shake-flask cultures usually 250mL - 1L in size. Cells from this culture are then used to inoculate a bench-top bioreactor, which would be in the range of 1 - 2L. The next stage is a pilot-scale bioreactor using a vessel with a capacity of 100 - 1,000L. Finally the industrial scale operation begins in large fermenters (Doran, 1995).

The aim of this experiment was to determine the effect of sub-culturing YCL1 cells for a number of growth cycles as well as identifying the optimum incubation time that the organism can be maintained in liquid broth without considerable loss of production. Figure 4.7 outlines the regime employed to investigate the effect of incubating YCL1 for 24, 48 and 72-hour periods and sub-culturing the organism in liquid broth for these respective incubation times. The 96-hour time point was omitted this time as previous experimentation indicated that it was not worthwhile to continue culturing for this period.

Initially a starter culture was inoculated with a 'blend' of colonies and cultured to 24, 48 and 72 hours. When each of these time-points was reached, 1 mL aliquots were removed from these flasks and were used to inoculate further sets of flasks to continue sub-culturing at 24, 48 and 72 hour periods. This was performed for five cycles of each individual time period (Figure 4.7). These sub-cultures inoculated production flasks that were all cultured for a period of 72 hours so that a consistent time-point could be contrasted. The activities from these production flasks are presented in Figure 4.8, and are in consecutive order from when the first sub-culturing cycle began.

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Initial Starter Culture inoculated

	(24hrs)		(24hrs)		(24hrs)		(24hrs)	
1.24*	→	2.24	→	3.24	→	4.24	‑ ‑ ‑ →	n.24

(72hrs)   (72hrs)   (72hrs)   (72hrs)

1.24.72   2.24.72   3.24.72   (n-1).24.72

	(48hrs)		(48hrs)		(48hrs)		(48hrs)	
1.48*	→	2.48	→	3.48	→	4.48	‑ ‑ ‑ →	n.48

(72hrs)   (72hrs)   (72hrs)   (72hrs)

1.48.72   2.48.72   3.48.72   (n-1).48.72

(72hrs)   (72hrs)   (72hrs)   (72hrs)

1.72* → **2.72** → **3.72** → **4.72** ‑ ‑ ‑→ **(n-1).72**
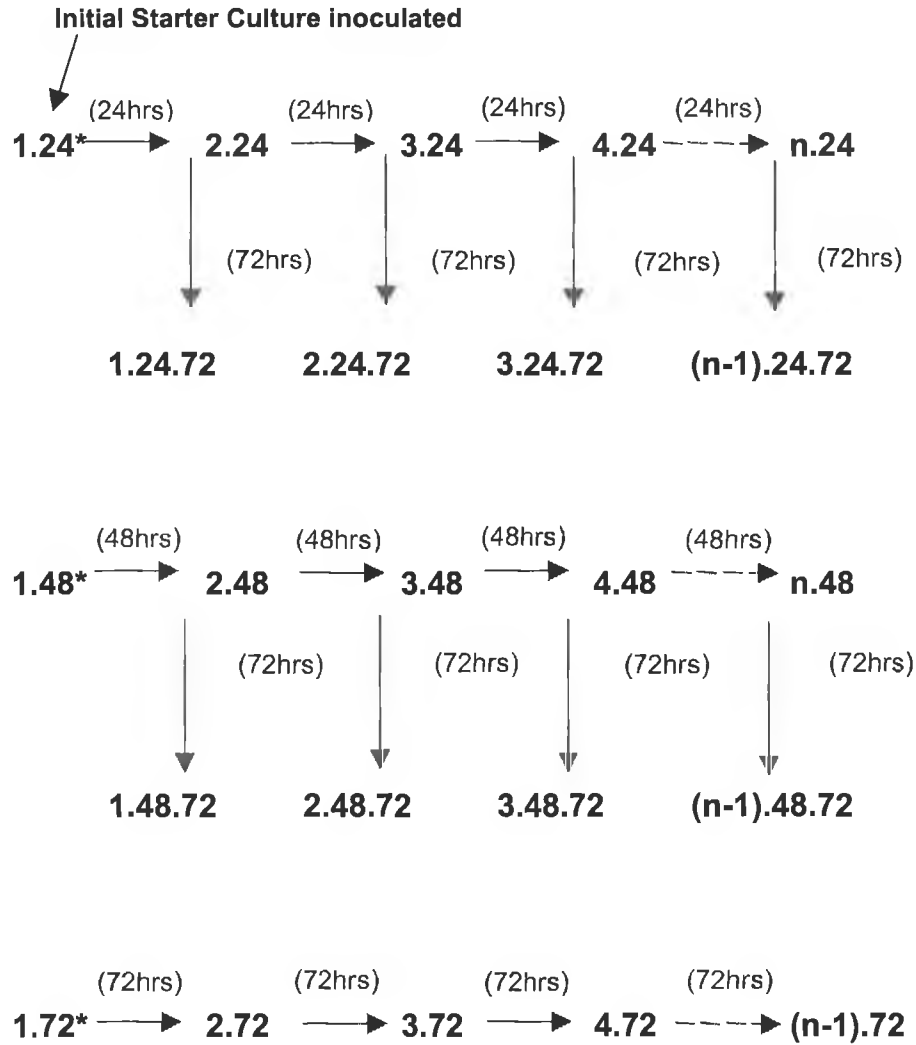
Figure 4.7 Sub-culturing plan. The starter culture was inoculated with a blend of colonies and initially incubated for a period of 24 hours. After 24 hours, the starter culture inoculated both a production culture that was grown for 72 hours (1.24.72), and a sub-culture cycle that was grown for 24 hours (2.24). Sub-culturing from the starter culture also occurred at 48 hours and 72 hours. Each set of time-points (i.e. 24, 48 and 72hr) were sub-cultured for five more cycles of their respective incubation times. In addition, these sub-cultures inoculated production cultures that were incubated for a further 72 hours (x.72) in order to achieve a consistent comparison. * indicates the same initial starter culture grown for 24hrs (1.24), 48hrs (1.48) and 72hrs (1.72).
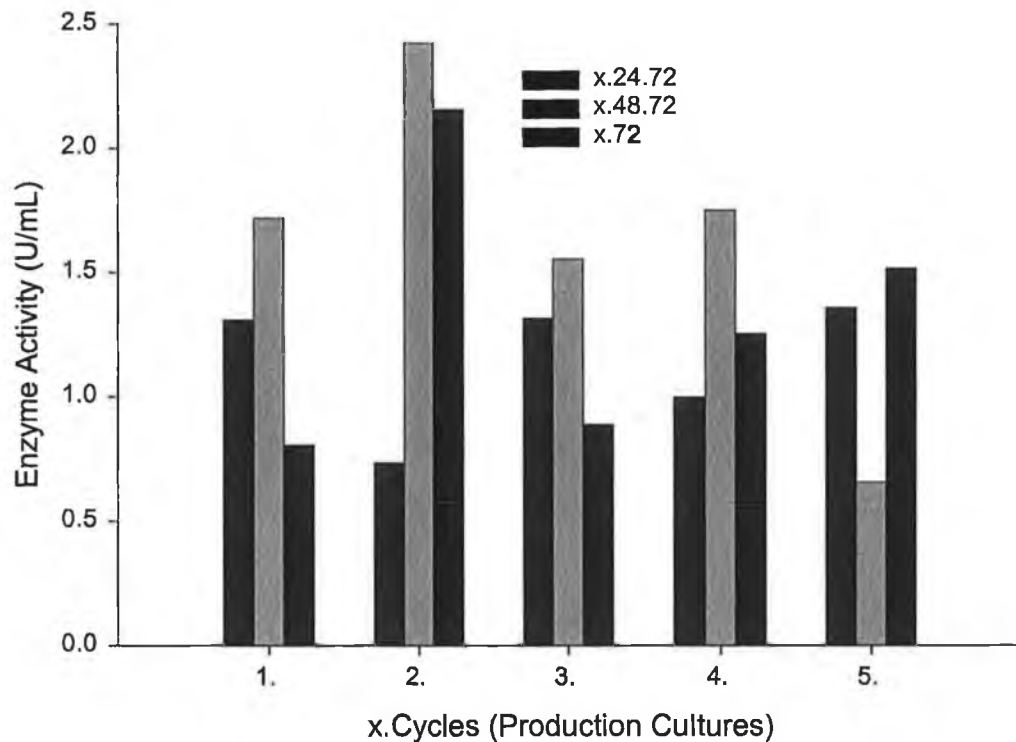
Figure 4.8 Enzyme activity from production cultures in sequential order of the sub-culturing cycles. Each production culture was incubated for 72 hours and was inoculated from sub-cultures that had been incubated for cycles of 24, 48 and 72 hour periods. **'x.'** represents cycle numbers 1→5. e.g. Cycle #1 shows activities for 1.24.72, 1.48.72 and 1.72 production cultures. (Refer also to Figure 4.7)

Overall, the highest enzyme activities were observed in the production cultures from the 2nd sub-culture cycles. (i.e. 2.48.72 had the highest overall activity followed by 2.72) Enzyme activity in the production cultures that were sub-cultured from the 48-hour cycles was higher than both the 24 and 72-hour cycling throughout the first four cycles. Cathepsin L1 production in the 72-hour sub-cultures was highest at the 2nd cycle. The 24-hour cycling achieved mixed results, and never matched the activities from the 48 and 72-hour cycling.

4.9 Discussion

4.9.1 Investigations into the growth and production cycles of YCL1

It would appear, from Figure 4.1 that there is a link between cell growth and enzyme production during the first 50 hours of culturing. Roche *et al* (1997) claimed that the time course of the production of Cathepsin L1 in selective media followed a similar curve to that of cell growth. However, data was not shown to back up this statement in the publication.

Compared to Fermentation 1, shown in Figure 4.1, it can be seen in Figure 4.2 that although enzyme production increased with time, there does not appear to be a direct correlation with the increase in cell biomass when Fermentation 2 was extended to 120 hours. This would suggest that the production cycle of Cathepsin L1 is not directly associated to cell growth, despite indications from Fermentation 1 that this was the case. Cell growth in Fermentation 2 reached stationary phase between 24 and 48 hours, correlating somewhat with what was seen in Fermentation 1, where there was a reduction in cell numbers at 50 hours.

In Fermentation 2 enzyme production reached its maximum of 1.2 U/mL by 72 hours. Whereas, in Fermentation 1, enzyme activity only reached its maximum of 0.0196 U/mL by 45 hours. Production levels of the enzyme were also different between the two fermentations, even at similar time points, i.e. 24 and 48 hrs. Enzyme activities at these time-points are higher in Fermentation 2 compared to Fermentation 1. This is despite similar environmental conditions and media, and the fact that

there is lower cell biomass in the Fermentation 2. This would indicate variability in the cells used to inoculate each fermentation.

Both graphs indicate that there is trace enzyme activity at 24 hours, and Figure 4.2 indicates that significant amounts do not begin to accumulate until 72 hours. This could be for various reasons:

a) The depletion of Glucose to a minimal level (from 20g/L to less than 2g/L) activates other biochemical pathways, and the yeast's energy can be used for the production of its heterologous protein rather than cell doubling.

b) The production and subsequent usage of ethanol somehow activates the ADH1 promoter, which in turn causes the expression of Cathepsin L1.

When enzyme activities at 72 hours and 96 hours from various fermentation runs were compared, indications suggested that harvesting in or around 72 hours for batch cultures would reap the highest levels of Cathepsin L1. (Figure 4.3) There was a decrease in enzyme activity at 96 hours compared to 24 hours previous for all the runs, bar one. This could indicate denaturation of the enzyme at 96 hours or nutrient depletion in the culture. Other reasons could include cells lysing and other proteases interfering with the cysteine protease of interest, or other proteases affecting the detection of Cathepsin L1 when performing the enzyme assay.

4.9.2 Determination of a standard operating protocol for consistent YCL1 inocula

Results from Figure 4.4 show that variability in terms of producing Cathepsin L1 between individual colonies does exist. It is postulated that the reason for these inconsistencies is related to the plasmid copy number of individual yeast cells. It is assumed that the plasmid pFheCL1Y29 has a low copy number as it is a derivative of pAAH5, which is itself a low copy number plasmid (Korpela, 1987).

Low copy number plasmids are more prone to segregational instability, where yeast can divide and the daughter cell has no plasmid(s) passed on to it. Considering this outcome, a standard operating protocol was devised to decrease the variability between starter cultures. To overcome this obstacle a number of options were considered.

A number of initial shake-flasks could be inoculated before the fermentation takes place, and individual shake-flasks that show promising productivity could be used for further sub-culturing purposes. However, this type of approach is time-consuming and also means that shake-flasks would have to be left for 72 hours to screen the best colonies. It would also mean that the yeast cells would be in late stationary phase when high enzyme production is taking place, instead of exponential phase when the cells are ready to adapt and multiply when inoculated in the new fermentation media.

A second approach would be to inoculate a number of flasks again, but rather than choosing one individual flask for further inoculation, the cells from up to 5 different flasks could be 'blended' and used for further inoculation. This in theory would diminish the possibility of selecting low-productivity cells, and enhance the chances of an

average Cathepsin L1 producing cell-population. Again this was considered both time-consuming, and wasteful of consumables and media.

A third option was decided upon. In essence, individual colonies were selected from streak or spread plates and resuspended in an appropriate volume of saline. One mL of this 'blended' suspension was then used to inoculate starter cultures, containing 100mL of media. As with the second option, this choice should in theory average out the number of plasmid bearing and plasmid-less cells, thus allowing a consistent plasmid-bearing population. This option proved worthy of selection, as fermentations from this point provided consistent biomass and activity when cultured in selective media and similar environmental conditions.

8 individual colonies are selected from spread plate

Cells resuspended in saline

Universal containing 8mls of Saline

1mL 'blended' suspension inoculates shake flasks

250mL unbaffled Erlenmeyer shake flask containing 100mL of selective media
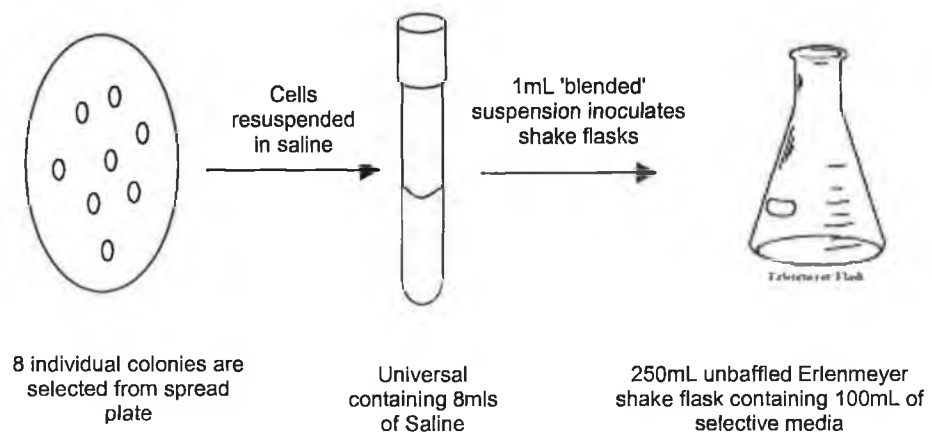
Figure 4.9 Standard Operating Protocol (SOP) for initial inoculation of colonies taken from solid media into liquid media. In this example 8 colonies are resuspended in 8mLs of saline, and 1mL of this suspension is used to inoculate 100mLs of media in a 250mL pre-warmed flask.

As 72 hours was identified as the time-point when maximum enzyme activity occurred, future fermentations can be stopped at this point and the Cathepsin L1 can be recovered. This time-point was also used for single-point comparisons in examining different environmental parameters. In addition, the effects of sub-culturing subsequent *S. cerevisiae* broths at 72-hour intervals on maintaining enzyme activity was examined.

4.9.3 Preparation of an optimum starter culture

The results of the 72 hours continuous sub-culturing experiment, as shown in Figure 4.5 suggest that some selective media cultures tend to lose high activity over time. Therefore extensive sub-culturing in liquid media will lead to a loss of activity over time. The two sets of flasks were initially inoculated with two different colonies and as shown in Section 4.5, different enzyme activities result from individual colonies. This may account for the fact that enzyme activity in #2 set of flasks remains high for up to and beyond 7 sub-cultures, while the activity in #1 set of flasks dramatically drops at the formative stage of the sub-culturing cycles.

It is difficult to speculate about the loss of activity from the #2 set of flasks during the intervening weeks when sub-culturing still took place, although the samples were not assayed for enzyme activity. Whether the activity in the #2 set of flasks dropped gradually or whether it dropped suddenly like what was seen in the #1 set of flasks cannot be declared.

A 'blend' of colonies was employed to inoculate a set of shake flasks to investigate the effect of incubation time on enzyme activity in starter cultures. Enzyme activities from these starter cultures, which were

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incubated for periods of 24, 48, 72 and 96 hours, and the production cultures that were sub-cultured from these starter cultures are shown in Figure 4.6.

Enzyme activity data from the starter cultures confirms previous data about the production cycle of Cathepsin L1 from YCL1 as it increases with time. However, in contradiction to previous experimentation maximum enzyme production occurred at 96 hours as opposed to 72 hours. However, initial experimentation was sub-cultured from liquid to liquid culture, whereas in this experiment, colonies were taken from solid media and transferred into liquid media. The lag phase for these cells was longer as they had to adapt to growing in liquid media. Therefore the stage at which maximum enzyme production occurred would have been much later in the starter culture.

These results also indicate that a starter culture, which has been incubated for 48 hours, will achieve maximum enzyme production when sub-cultured into the ensuing production culture. This is probably due to the fact that the cells taken from solid media and subsequently cultured in liquid media are in exponential phase at around 48hrs. Thus when YCL1 cells in the starter culture are sub-cultured at this time, they adapt easily to the new liquid environment, reducing the lag phase, and entering growth and production phases with a healthy population of yeast cells.

Previously the exponential phase was shown to be between 10 and 35 hours, (Figure 4.1 and 4.2) but cells were sub-cultured from liquid media to liquid media, and the lag phase would have been much shorter. In this case (Figure 4.5) the cells adapting to the liquid media from the solid media caused the longer lag phase. Therefore many more healthy cells in exponential mode would have been generated at

48 hours compared to at 24 hours. Thus when the 24-hour starter culture was used to inoculate its respective 72-hour production culture, although the cells were doubling at the time, it is probable that not enough had been grown to make an impact on the 72-hour sub-culture.

Enzyme activity in the production cultures that were inoculated with cells from the 72 and 96 hours starter cultures were not as high as that observed with the 48 hour starter culutre. Cells would have been in stationary phase or even death phase at 72 and 96 hours in the starter cultures when they were used to inoculate the production sub-cultures, perhaps accounting for the poor performance in the subsequent production cultures. Thus it would appear that 48 hours is the ideal time in which to sub-culture cells in the preparation of a starter culture. In order to develop this procedure for the preparation of starter cultures, further sub-culturing at these incubation times combined with sub-culturing for five cycles was examined to identify the correct number of cycles required for maximum enzyme activity.

The results in Figure 4.8 confirm results observed in Figure 4.6 that maximum enzyme production will be achieved when colonies from solid media are transferred into liquid media and incubated for 48 hours, then sub-cultured for a further 48 hours, before inoculating a production culture and incubating for 72 hours. This information can be used in scaling-up starter cultures for industrial size fermentations. i.e. initially growing cells in a 1L flask for 48 hours, then using this culture to inoculate a bench-top or pilot-scale bioreactor and incubate for a further 48 hours. At this point, enough cells would be generated to inoculate a production culture in a large-scale fermenter that is cultured for 72 hours. See Figure 4.10.

| Starter culture grown for 48 hours in 1L Shake Flask | Second sub-culture grown for 48 hours in bench-top bioreactor | Enzyme Production in Large-Scale Fermenter for 72 hours |
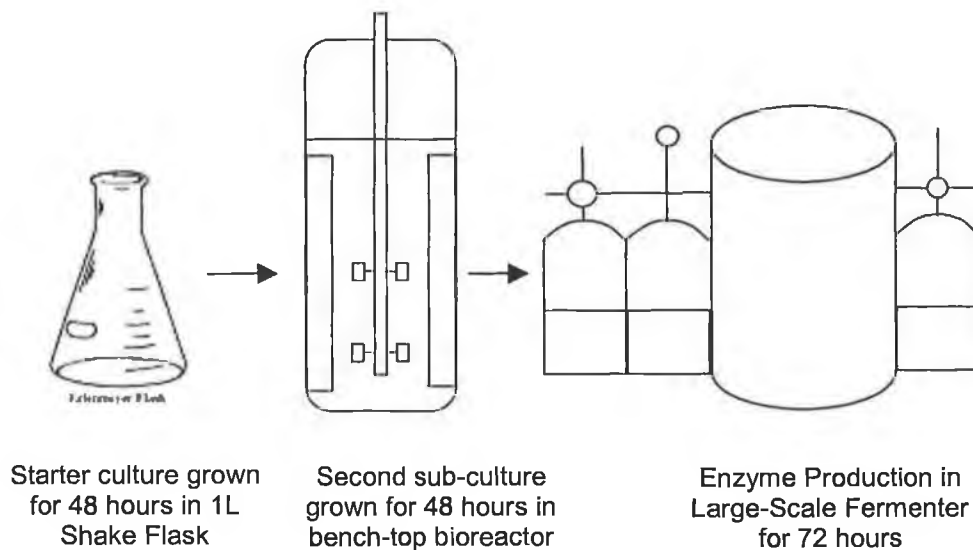
Figure 4.10 Recommended scale-up procedure for maximum Cathepsin L1 production. (Adapted from Doran, 1995)

4.10 Conclusions

Despite initial suspicions, it appears that Cathepsin L1 production is not directly associated with YCL1 growth. There is also variability between colonies in terms of final enzyme production. This may be attributed to the low copy number plasmid. As a result, a standard operating protocol has been devised to provide consistent inocula for the starter culture. In addition, a procedure for the preparation of starter cultures for large-scale fermentations had been identified that is most likely to result in high enzyme activity. Long-term liquid sub-culturing of cells cannot maintain a population of high enzyme producing cells. Seventy-two hours has been identified as the optimal point at which to harvest Cathepsin L1 in batch fermentation.

Chapter 5 The effect of environmental conditions and media composition on Cathepsin L1 production from YCL1

5.1 The Effect of Environmental Conditions on Cathepsin L1 Production

5.1.1 Introduction

It was extremely important to identify critical environmental parameters, such as pH and temperature to ferment YCL1 and maximise Cathepsin L1 production. In terms of yeast cultivation, environmental parameters are perhaps the easiest to alter and monitor. Reports in literature have mentioned that rising culture pH increased enzyme production, and aided stability of the secreted protein (Kang *et al*, 2000, Carmelo *et al*, 1996, Bae *et al*, 1996).

Some heterologous protein expression systems have been reported in literature to have improved productivity through increasing (Rossini *et al*, 1993, Alvarez *et al*, 1994) or decreasing (Tøttrup and Carlsen, 1989, Nagashima *et al*, 1994, Cassland and Jönsson, 1999) the fermentation temperature from a normal culturing temperature of 30^0C.

There is a trade-off in identifying the optimal fermentation pH and temperature for the cultivation of the recombinant yeast, without denaturing the heterologous protein that has been secreted into the surrounding medium (Walker, 1998).

5.1.2 Effect of Culture pH

Shake flasks were prepared at five different pH's ranging between pH 4.0 and pH 8.0. The pH of the media was altered using 2.0M NaOH and 2.0M HCl before sterilisation. After autoclaving , the pH's of each of the flasks had altered. Refer to Table 5.1 for the post-autoclaving pH values. Enzyme production, cell growth and glucose depletion were monitored in the shake flask cultures.

Table 5.1 Effect of autoclaving on the pH of selective media

Initial pH of Shake Flasks	pH after Autoclaving
4.0	4.25
5.0	5.02
6.0	5.97
7.0	6.65
8.0	7.15

Figure 5.1 shows that extreme pH values (i.e. pH 4.0, 5.0 and 8.0) are not favourable for enzyme production from YCL1. The highest enzyme activity recorded over the course of the experiment is seen in the pH 7.0 flask at 72 hrs, followed closely by pH 6.0 flask at 72 hrs. The post-autoclave pH's of these flasks are 6.65 and 5.97 respectively which would confirm previous findings, that buffered medium at pH 6.5 is the optimum pH for culturing YCL1 and producing high enzyme activity (Dowd *et al*, 1997).
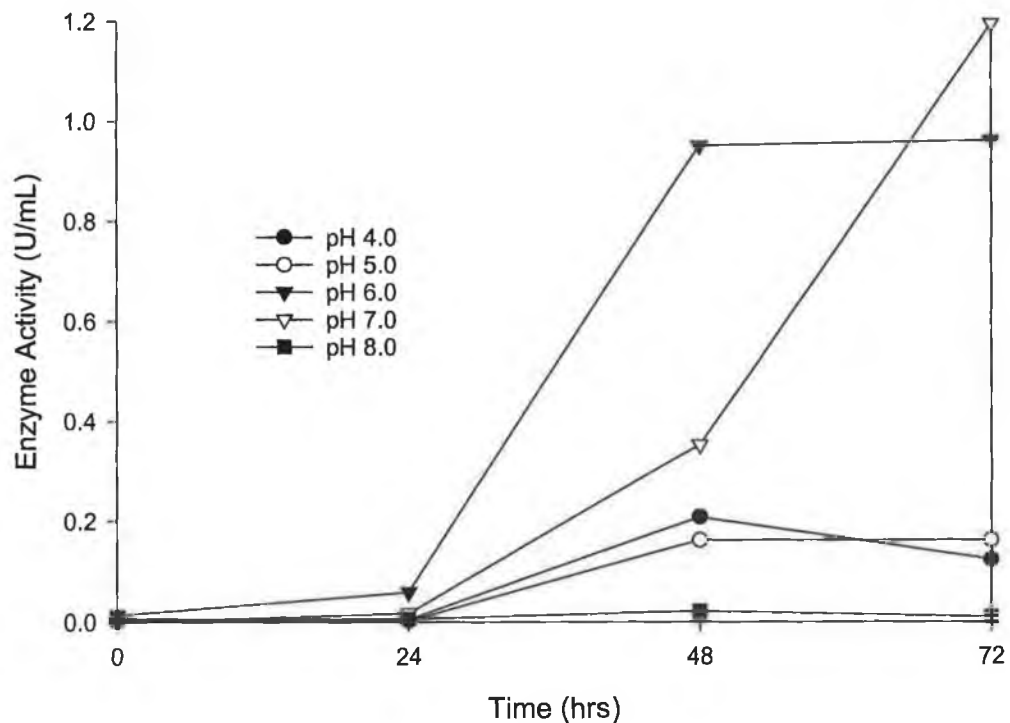
Figure 5.1 Enzyme Production of YCL1 at various initial culture pH's over a time-course of 72hours.

Despite the disparate enzyme activities for the various pH values, cell counts are similar for the final time-point at 72hours, with the exception of pH 8.0. (Figure 5.2) It would appear that this pH value is too extreme for cell growth to occur, thus explaining the poor enzyme activity observed.

Despite similar cell counts throughout the remaining fermentations, disparate amounts of enzyme production were observed. To investigate whether this was as a result of denaturation of the enzyme, or whether these pH values favoured both expression and secretion of the enzyme, the following experiment was conducted.
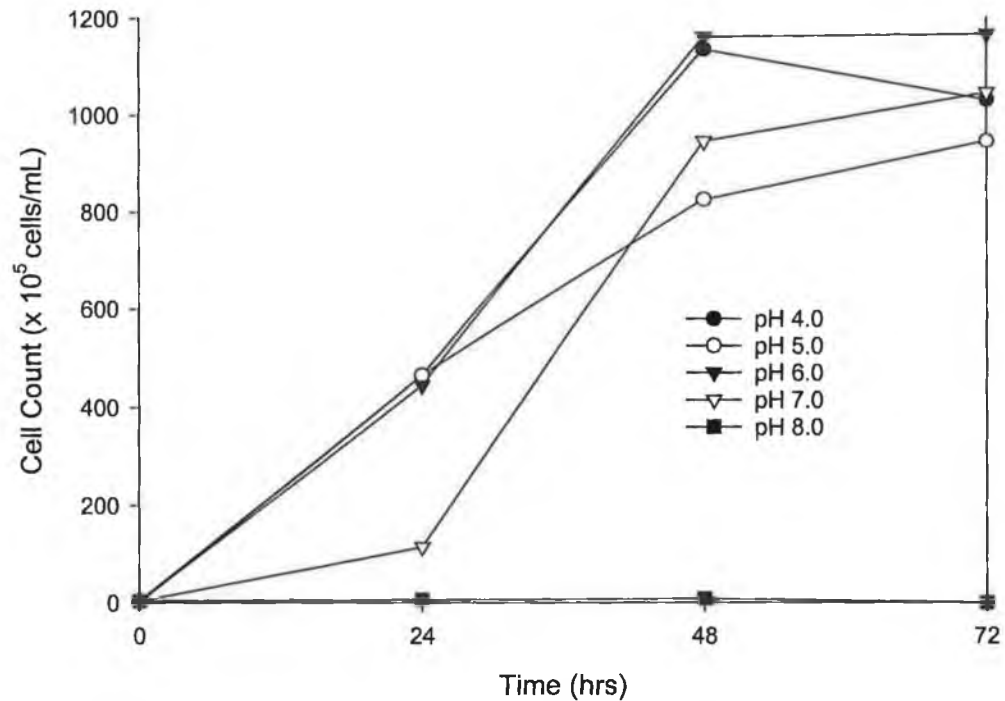
Figure 5.2 Cell Counts for YCL1 at various initial culture pH's over a time-course of 72hours.

5.1.3 Effect of culture pH on enzyme stability

A culture suspension, with a significant concentration of enzyme was centrifuged, and the supernatant was decanted off and separated into individual aliquots of 100mL. The pH of these aliquots was altered by addition of 1.0M NaOH and 1.0M HCl to generate a pH range between pH 4.0 and pH 8.0. Subsequently these aliquots were filtered through 0.45μm sterile filter papers into sterile 250mL shake flasks to remove any remaining yeast cells. The flasks were incuabted at 30^{0}C for a period of 52 hours. The results are shown in Figure 5.3.
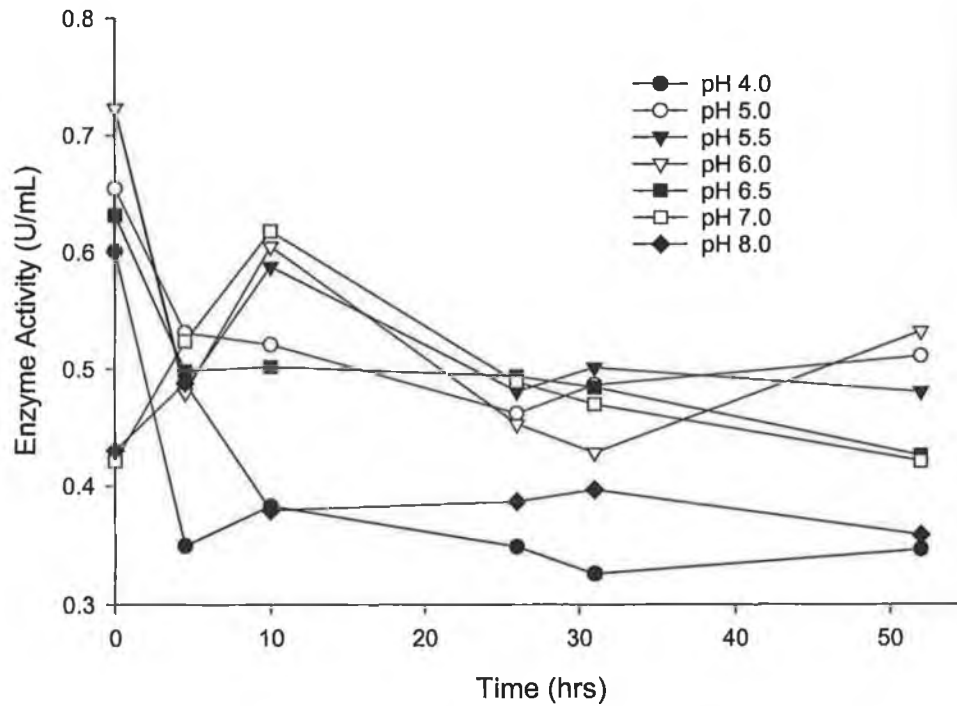
Figure 5.3 Effect of culture pH on enzyme stability.

Enzyme activity in each flask was monitored over a period of 52 hours. The biggest decrease in enzyme activity was seen at the two pH extremes, that of pH 4.0 and pH 8.0. It is apparent that the enzyme is relatively stable at all other pH's. A culture pH of ~6.5 prevents denaturation of the secreted enzyme (Figure 5.3) as well as allowing full expression and secretion of Cathepsin L1 (Figure 5.1).

5.1.4 Effect of incubation temperature

250mL shake flasks containing 100mL of selective media were inoculated from starter cultures and were incubated at three different temperatures - 25^0C, 30^0C and 37^0C. The cultures were grown for a period of 72 hours, and samples were taken every 24 hours.
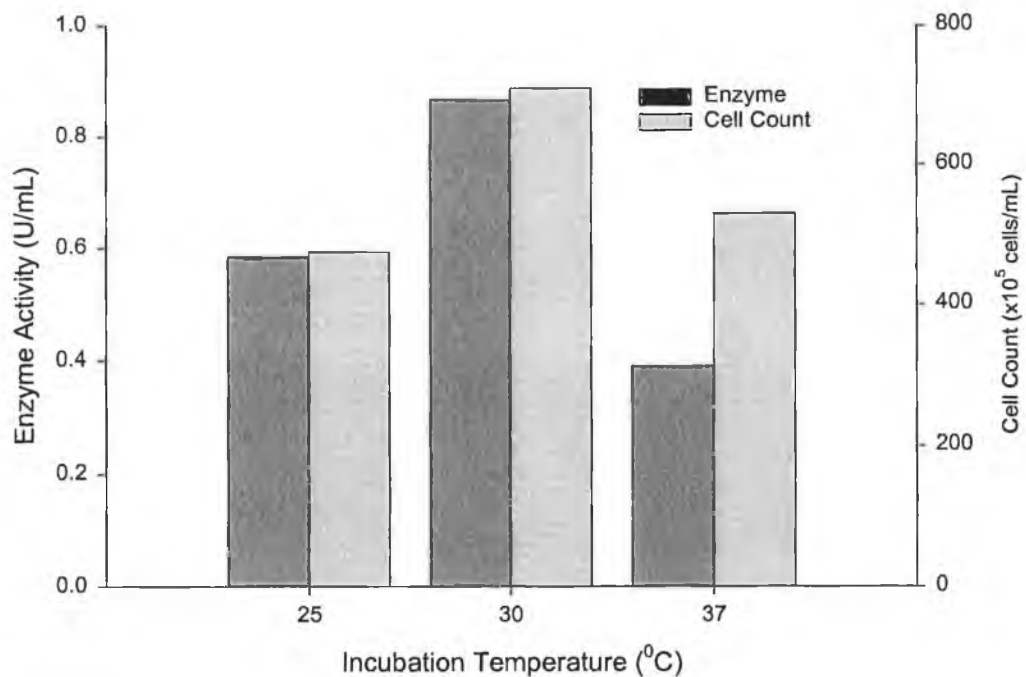
Figure 5.4 Effect of incubation temperature on Cathepsin L1 production and cell counts after incubation for seventy-two hours.

Both enzyme activity and biomass were highest at 30^0C at the end-point of the fermentation, 72 hours. (Figure 5.4) Despite relatively high levels of levels at 72 hours, the 37^0C sample produced a much lower level of enzyme than observed at the other two temperatures. Incubating the culture at 25^0C resulted in similar biomass levels to that observed at 37^0C but its enzyme activity was higher than that seen at 37^0C.
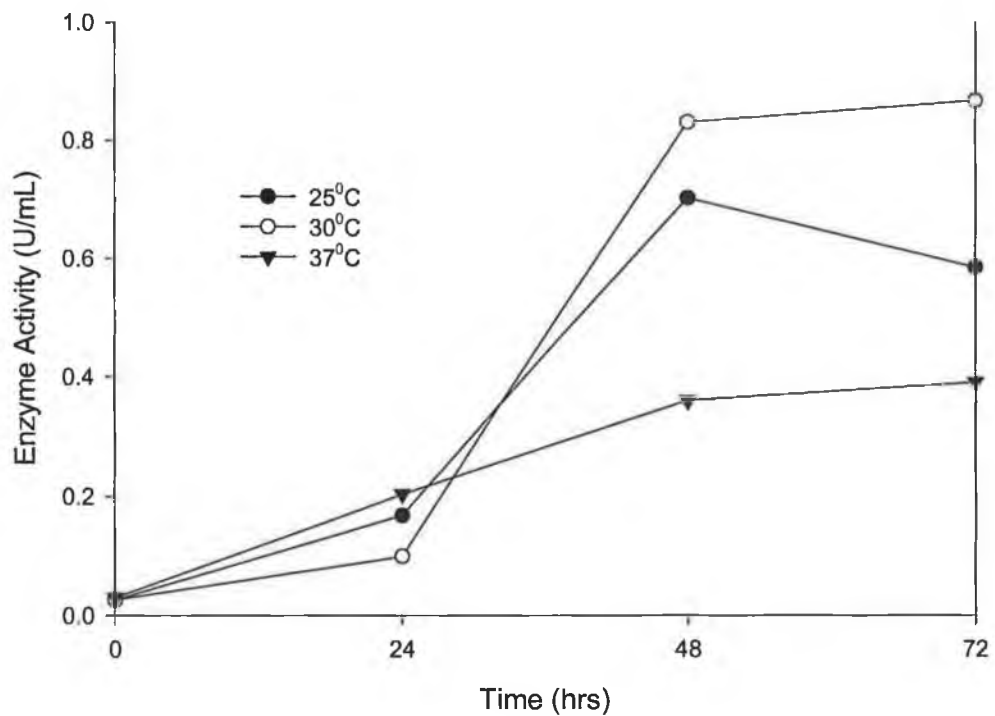
Figure 5.5 Effect of incubation temperature on Cathepsin L1 production over a period of 72 hours.

Figure 5.5 shows that Cathepsin L1 activity was much lower at 37^0C throughout the 72-hour period. The culture incubated at 25^0C seemed to be following a similar pattern as the 30^0C sample, but enzyme activity dropped by the end of the fermentation. Enzyme activity at all three temperatures levelled off by 72 hours. In the case of YCL1, 30^0C proved to be the optimum incubation temperature for both yeast cell growth and expression of Cathepsin L1.

5.2 The influence of media composition on enzyme production

5.2.1 Introduction

There are some citations in literature where heterologous protein production is improved when recombinant *Saccharomyces cerevisiae* is grown in complex media instead of selective media (Tøttrup and Carlsen, 1990, Jung *et al*, 1991). Thus replacing complex media for selective nutrient-limited media to improve Cathepsin L1 production was investigated.

Cheaper, ubiquitous carbohydrate substitutes such as molasses and starch can be used as carbon sources for both heterologous protein and ethanol production from yeast (Birol *et al*, 2000). Molasses has a high content of sucrose (Gough *et al*, 1996), while starch is high in both maltose and non-utilisable glucose polymers (Montesinos and Navarro, 2000). Rather than investigating these carbohydrate sources, individual sugars such as maltose and lactose, that are part of the composition of molasses and starch, were examined instead.

Sterilisation of media is also an important factor to consider in scaling-up from flasks to bioreactors. When autoclaving the media, biochemical alterations can take place in heat-labile vitamins because of the high heat and pressure (Greasham and Herber, 1997). Thus efforts should be made to prepare and sterilise media in bioreactors in a similar manner to that of flasks (Parekh *et al*, 2000). For example, glucose stock solution should be autoclaved separately from the rest of the media to avoid Maillard reactions that occur between reducing sugars and primary amines (Greasham and Herber, 1997).

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5.2.2 Comparison between selective and non-selective media

Experiments were performed in both selective and non-selective (YEPD) media to observe the difference in cell growth, enzyme production and glucose utilisation in both media. Shake flasks were prepared as before, with 100mLs of YEPD and 100mLs of selective media. YEPD consists of bacteriological peptone, yeast extract and glucose. The exact composition of amino acids and nutrients cannot be quantified, but there is leucine present, thus removing any selective pressure for YCL1 in this media.

In Figure 5.6(a), it can be seen that by the end point of the fermentation, 72 hours, enzyme activity in selective media is significantly higher than that observed in YEPD, despite lower cell concentrations (Figure 5.6b). Biomass levels in YEPD are almost ten-fold that seen in selective media at 24 hours. (Figure 5.6b) The cells grown in YEPD have almost reached the end of glucose utilisation phase by 24 hours, and this is verified by the fact that almost all of the glucose has been consumed at this time. (Figure 5.6c) In comparison, the rate of glucose consumption by the cells grown in the selective media is much slower than that of the cells grown in YEPD.

Plasmid Stability from a subsequent YEPD fermentation was examined, and is typical of plasmid instability in non-selective media. At the start of the fermentation plasmid stability was 56%, as the starter culture had been prepared by two liquid sub-cultivations of 48 hours duration, thus some degree of plasmid loss occurred. The plasmid stability subsequently dropped to 40% at 24 hours and 8% at 48 hours in the YEPD media.
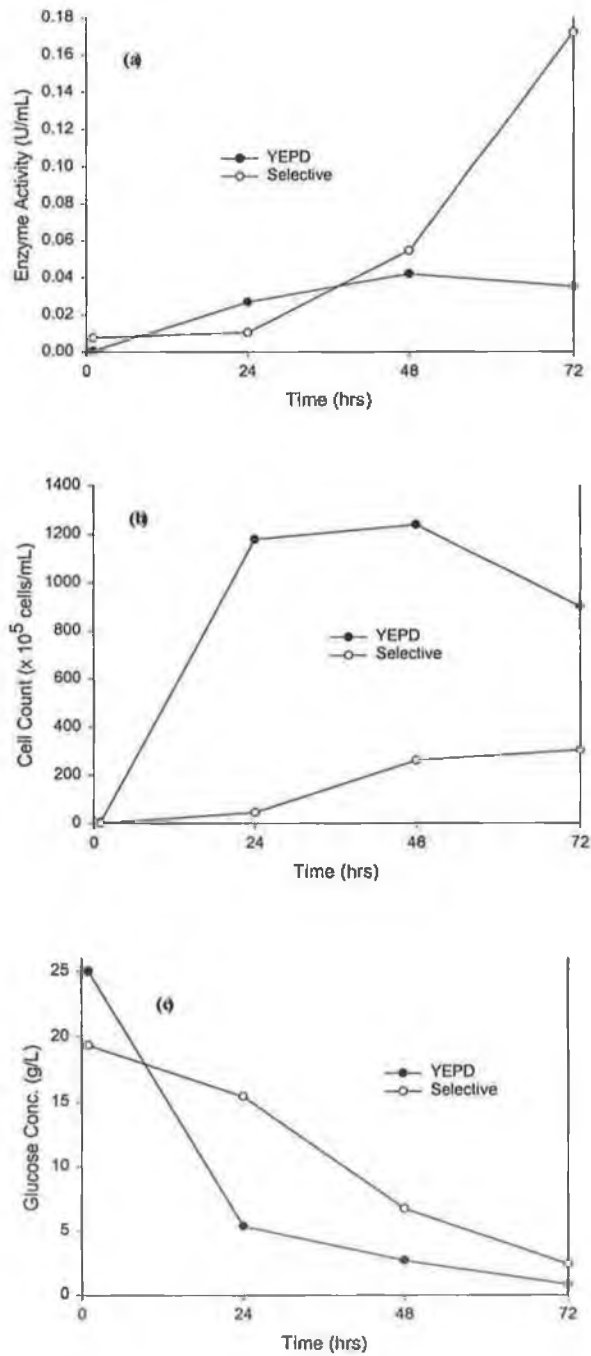
Figure 5.6 Influence of growing YCL1 cells in non-selective YEPD and selective media on **(a)** Enzyme Activity **(b)** Biomass Levels and **(c)** Glucose Concentration over a periods of seventy-two hours.

5.2.3 Supplementation using Casamino Acids

Casamino acids are an acid hydrolysed preparation of the milk protein casein. By supplementing the culture media with casamino acids, the amino acid content of the media will be improved, and thus should increase the yeast cell density. However, the presence of leucine in casamino acids removes the selective pressure. The energy requirements of the organism should be reduced through the addition of amino acids as the yeast cells no longer have to manufacture certain essential amino acids. Chiruvolu *et al* (1996) reported an increase in heterologous protein production when they supplemented their selective media with Casamino acids.
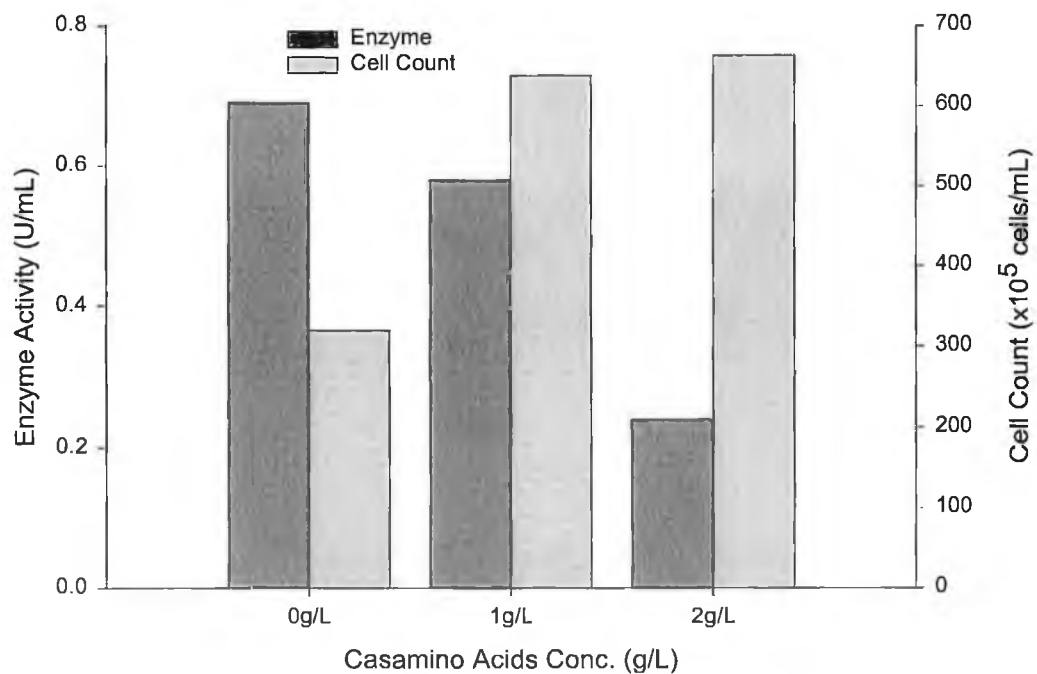
Figure 5.7 Influence of the addition of Casamino Acids on Enzyme production and Cell Proliferation of YCL1

The selective media employed is outlined in Section 2.4.1, with the addition of 1g/L and 2g/L Casamino Acids. Again 100mLs of media was prepared in 250mL Erlenmeyer flasks.

Figure 5.7 shows that as the addition of casamino acids increased, enzyme activity decreased quite considerably. There was an increase by almost two-fold in the cell concentration present in the cultures supplemented with 1g/L and 2g/L Casamino acids, compared to the control sample. Despite this increase in cell numbers, there was a decrease in enzyme activity.

Neither supplementing selective media with casamino acids, nor replacing the media with complex YEPD media resulted in any improvement in Cathepsin L1 production. The most likely explanation for this is plasmid loss through competitive instability, where plasmid free cells have a faster growth rate than plasmid free cells.

Thus optimising the selective media was the next stage in attempting to optimise enzyme production. The three main constituents of this selective media are glucose, yeast nitrogen base (with the amino acids histidine, tryptophan and methionine) and uracil. Additional supplementation of each of these ingredients will be dealt with throughout the rest of this chapter.

5.2.4 Replacement of Carbon Source

Molasses and/or wort are often the principal carbon sources in the industrial fermentation of yeast, and they contain some of the following sugars in varying concentrations. Monosaccharides - Glucose (Glu), Gal (Galactose), Fru (Fructose) and Man (Mannose), as well as some disaccharides - Lac (Lactose), Mal (Maltose) and Suc (Sucrose). These sugars are all considered to have good growth and fermentation characteristics for yeast (Walker, 1998). Previously only glucose had been utilised as the main carbon source, so an investigation into the effect of replacing or supplementing the carbon source with alternative sugars was proposed.
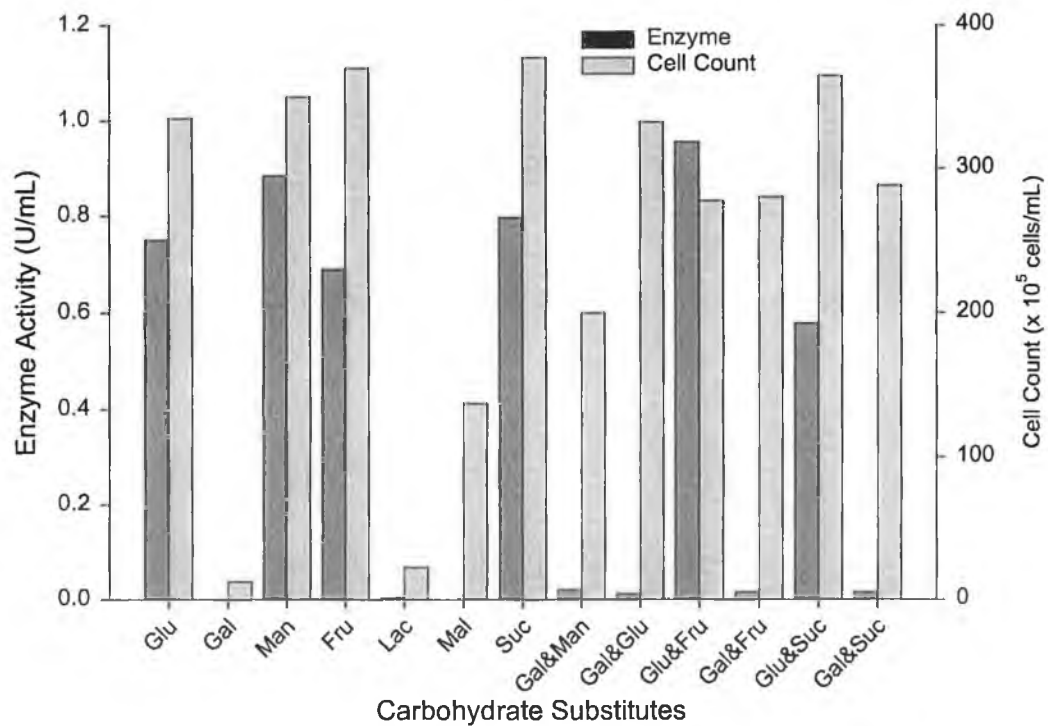
Figure 5.8 Influence on enzyme activity and cell count when the carbon source glucose was replaced with various sugars. The concentration of the single sugars in each flask is 20g/L, and 2 x 10g/L for each of the double sugars.

100mL of media was added into 250mL shake flasks, and the media was prepared as shown in Table 2.4. Glucose was replaced either by a single replacement of another sugar (5mL in 100mL media), or split between two different sugars (2.5mL of each sugar in 100mL media). The most significant results are shown below in Figure 5.8.

While many of the carbon source replacements accomplished high biomass levels, only a few could match or exceed glucose in terms of enzyme production. Mannose and sucrose superseded glucose in both enzyme production and biomass levels, while fructose achieved very higher cell numbers than glucose and enzyme activity almost equivalent to that seen with glucose.

When glucose, mannose, fructose and sucrose were used in conjunction with one another as double sugar replacements, the combined sugars result in similarly high enzyme activity and cell concentrations as seen with the single sugar replacements. As shown in Figure 5.8 a combination of glucose and fructose or glucose and sucrose achieved both high enzyme activity and cell concentrations. The rest of this data is not shown in the graph.

Galactose and lactose both performed poorly in terms of cell growth and along with maltose all showed only trace enzyme activity. Furthermore, when either galactose or lactose was used in conjunction with any other sugar as a double replacement, the outcome was extremely low enzyme activity in spite of relatively good cell growth. Results from the replacements involving galactose along with another sugar are shown in Figure 5.8. The results with lactose and other sugars are not shown, and the low enzyme activity observed with lactose was not to same extent as seen with galactose.

As shown in Figure 5.8 sucrose, mannose and fructose all compared favourably with glucose as a replacement sugar, and ensured high enzyme production from the YCL1 yeast. However, following a cost based analysis on replacing the relatively cheap monohydrate glucose with any of these carbohydrates, the slightly higher enzyme activity achieved did not justify the cost in replacing glucose.

5.2.5 Supplementation of Glucose

As previously noted, various carbohydrate sources were examined, however glucose was shown to be adequate, in terms of yeast cell proliferation, Cathepsin L1 expression and cost. An experiment was performed to determine the effect of supplementing selective media with extra glucose and observing the effect on both cell growth and enzyme production.

Thus a series of shake flasks containing selective media and various concentrations of glucose were prepared. After autoclaving, different volumes of sterile glucose stock were added to the media so that they contained glucose of concentrations 10, 20, 40, 60 and 80 g/L. Previously, all media was prepared using a concentration of 20g/L Glucose. The results at 72 hours can be seen below in Figure 5.9.
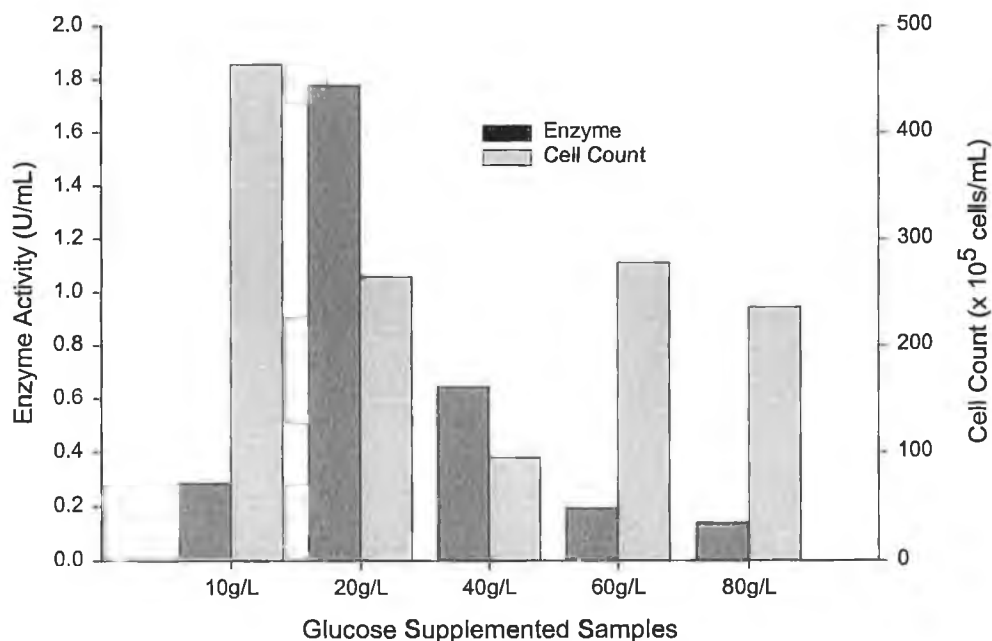
Figure 5.9 Influence on Cathepsin L1 expression and YCL1 cell growth by supplementing shake flasks with extra Glucose after 72 hrs.

The highest enzyme activity was observed in the 20g/L sample, and Cathepsin L1 production decreased as the glucose concentration increased hereafter. Despite the 20g/L sample achieving almost the same concentration of cells as the 60g/L and 80g/L cultures, enzyme activity was far lower in the latter two samples than the 20g/L culture. The residual glucose concentrations in the 40g/L, 60g/L and 80 g/L flasks at 72 hrs were 1.7g/L, 4.9g/L and 5.5g/L respectively compared to less than 0.2g/L for the other two cultures. The highest biomass concentration was seen in the 10g/L glucose sample, but this did not result in high Cathepsin L1 production. As a result of these findings, it would appear that 20g/L Glucose is the optimum concentration for media containing 6.7g/L yeast nitrogen base and 20mg/L uracil, to generate both high cell numbers and high enzyme activity.

5.2.6 Supplementation Using Yeast Nitrogen Base and Uracil

As shown previously, replacement of the selective media with non-selective media did not improve Cathepsin L1 production, mainly because there was significant plasmid loss in non-selective media. However the selective media used in its present concentrations was thought to be insufficient at meeting the nutrional demands of YCL1 during the production of the enzyme. Thus additional supplementation of the two other main components of the selective media, Yeast Nitrogen Base and Uracil, was investigated. (Refer to Section 2.4.5 and Table 2.5 for the concentration of the media components used in preparing this media.)

Stocks of yeast nitrogen base, uracil and glucose were prepared and added to the various shake flasks in different volumes. Shake flasks were prepared containing the recommended concentration of yeast nitrogen base (6.7g/L), as well as three (20.1g/L) and five (33.5g/L) times that concentration. This also applied to Uracil, where 20mg/L is the recommended concentration, but three (60mg/L) and five (100mg/L) times this concentration were also prepared in the shake flasks.

Shake flasks were cultured for 72 hours before analyses were performed on the cultures. In addition to the normal set of tests, i.e. enzyme assays, cell counts, pH and glucose analysis, plasmid stability tests were also performed as part of the examination.

Supplemented Media B that contained 20.1g/L of yeast nitrogen base and 20mg/L Uracil achieved the highest enzyme activity. Enzyme activity from the YN_2B x 3 range of flasks was four-fold that of the

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YN$_2$B x 1 set of flasks and almost three fold that of the YN$_2$B x 5 set of flasks. This can be observed in Figure 5.10. At five times the normal concentration of yeast nitrogen base (YN$_2$B x 5) Cathepsin L1 production is still higher than the 6.7g/L set of flasks, but not to the same extent as the YN$_2$B x 3 samples. High enzyme activity in the YN$_2$B x 3 sample is supported by relatively high plasmid stability, independent of the concentration of uracil added. (Refer to Table 5.3)
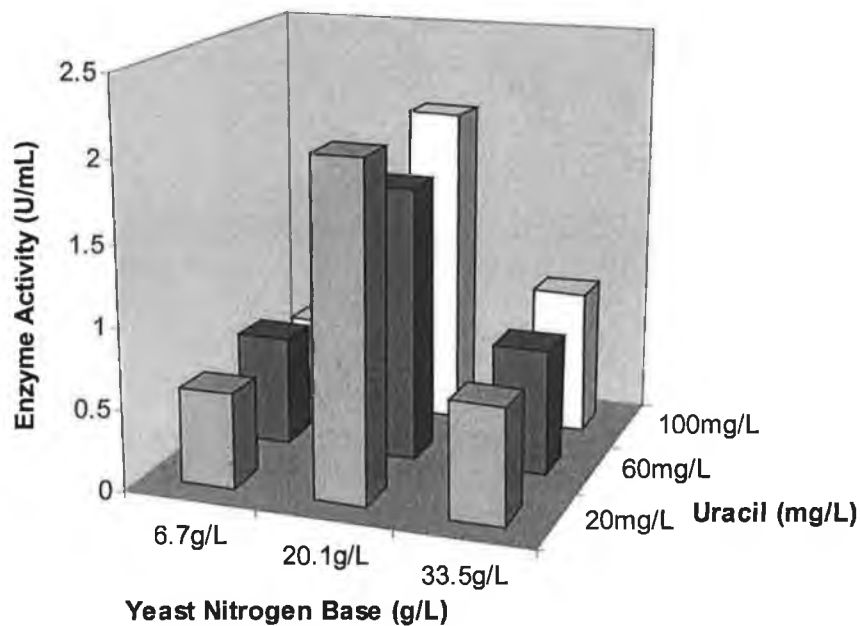
Figure 5.10 Effect of supplementation of yeast nitrogen base and uracil on Cathepsin L1 production.

The biomass concentration is far superior in the 20.1g/L and 33.5g/L yeast nitrogen base supplements than the 6.7g/L sample, which can be seen in Figure 5.11. Biomass levels are all in a similar range for all three concentrations of uracil (i.e. 20mg/L, 60mg/L and 100mg/L) in both the YN$_2$B x 1 and YN$_2$B x 3 range of flasks. In the case of the set of flasks supplemented with 33.5g/L yeast nitrogen base, there was an

increase in biomass in the flasks supplemented with 60mg/L and 100mg/L of uracil compared to the 20mg/L sample.
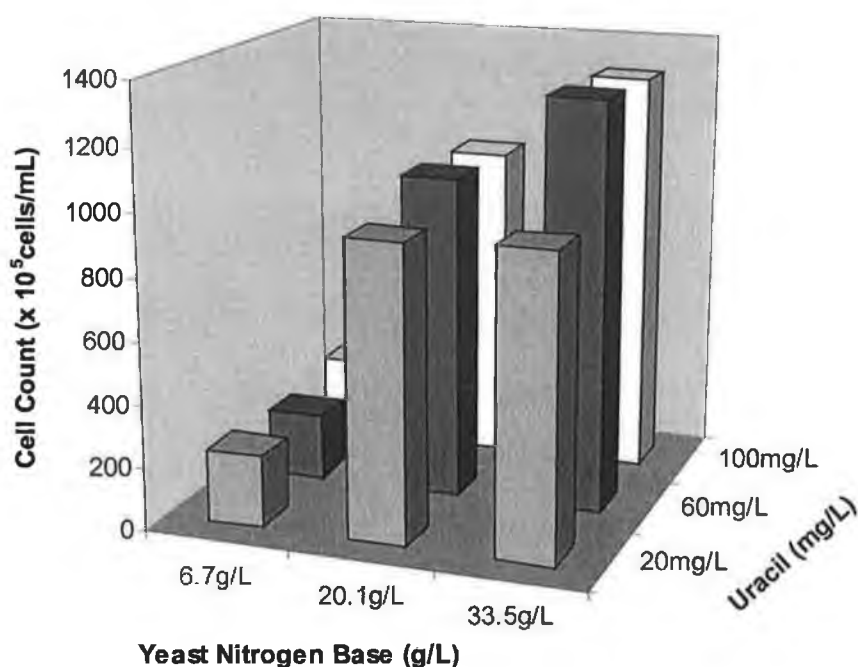
Figure 5.11 Effect of yeast nitrogen base and uracil supplementation on biomass concentration.

The effects of adding uracil can be most easily observed in the YN_2B x 1 (i.e. 6.7g/L) set of flasks. (Figure 5.10) As there was no extra yeast nitrogen base in this array of flasks, any effects that additional uracil would have on the selective media would not be interfered with by any improvements through supplemented yeast nitrogen base. Ura x 3 (60mg/L) reached a slightly higher enzyme activity of 0.68 Units compared to 0.58 Units and 0.54 Units for Ura x 1 (20mg/L) and Ura x 5 (100mg/L) cultures respectively in this array of flasks. Thus, there was no significant increase in enzyme production when the flasks were supplemented with extra uracil. In addition, there was also no notable increase in enzyme production in the other YN_2B supplemented set of flasks when extra uracil was added.

In the YN$_2$B x 1 set of flasks, the plasmid stability of the cultures supplemented with 60mg/L and 100mg/L Uracil dropped considerably by the end-point, 72 hours to 44%. In comparison, the culture containing the recommended uracil concentration of 20mg/L remained at a relatively high level of 70%. (Table 5.2)

Table 5.2 Influence of supplementing selective media with additional yeast nitrogen base (YN$_2$B) and Uracil (Ura) on the plasmid stability of the yeast cells. The plasmid stability of the set of flasks (YN$_2$B x 3) which achieved the highest enzyme activity are highlighted.

		Concentration of Yeast Nitrogen Base (g/L)		
		YN$_2$B x1 (6.7g/L)	YN$_2$B x3 (13.4g/L)	YN$_2$B x5 (20.1g/L)
Conc. of Uracil (mg/L)	Ura x 1 (20mg/L)	70%	*62%*	66%
	Ura x 3 (40mg/L)	44%	*68%*	76%
	Ura x 5 (60mg/L)	44%	*65%*	80%

5.2.7 Batch Fermentations using selective, non-selective and supplemented selective media

In order to substantiate the findings found in shake-flasks regarding the effect of media on Cathepsin L1 production, biomass levels, glucose consumption, ethanol production and subsequent utilisation, the process was scaled-up from shake flasks to 5L and 15L fermenters using selective, YEPD and supplemented media.

Yeast cells were cultured in accordance to the SOP described in Chapter 4. 100mLs of the culture suspension were centrifuged at 2,000rpm for 5 minutes. The supernatant was decanted and the cells were added aseptically to the fermenter. Temperature was controlled at 30^0C and the stirrer speed was at 400rpm. Samples were taken every 3 hours for alternate 12-hour periods. In addition to the normal set of analyses (i.e. enzyme assay, glucose concentration, cell counts and pH) ethanol concentration and dry weights were also performed.

In the case of selective media, 3.0L of media was prepared as outlined in Section 2.4.1 and autoclaved in a 5L fermenter. Non-selective media was prepared with the components outlined in Section 2.4.2 and was also performed in a 5L fermenter. The fermentation run using supplemented media was performed in a 15L fermenter using 10L of media. Supplement Media B was the chosen media for this fermentation because it produced the highest level of enzyme activity in Section 5.2.6. The pH supplemented media was buffered to pH ~6.5

Enzyme activities, biomass, glucose and ethanol concentrations and pH values from the three different media fermentation runs are all compared in the following graphs (Figure 5.12 through 5.16). The

maximum enzyme, biomass and ethanol produced in the three runs are shown in Table 5.3.

Table 5.3 Comparison of enzyme, biomass and ethanol production for all three fermentation runs

	Maximum Cathepsin L1 produced	Maximum Biomass produced	Maximum Ethanol produced
Selective Media	0.1996 U/mL	1.14 g/L	0.41 %v/v
YEPD Media	0.0514 U/mL	3.7 g/L	0.72 %v/v
Supplemented Media	0.74 U/mL	2.29 g/L	1.01 %v/v

Cathepsin L1 production in the supplementation batch run is significantly higher than that seen in the non-selective and selective media. (Figure 5.12) Despite an initial lag period in all three runs, enzyme activity increased concurrently with the latter decrease in glucose in the supplementation run. In non-selective media, enzyme stopped accumulating while the cells were still multiplying. The concentration of Cathepsin L1 in the selective media overall was quite low, but enzyme still accumulated gradually during the ethanol consumption phase.

The lowest number of yeast cells grew in the selective media. (Figure 5.13) YEPD achieved the highest biomass levels overall, but this did not result in higher enzyme production. Cells grew exponentially during the first 12 hours in the supplemented media.

The selective media fermentation showed signs of inefficient utilisation of glucose, as there was not an exponential decrease in its concentration for this run. (Refer to Figure 5.14) Glucose was depleted exponentially in the supplemented batch fermentation and after an initial lag period, glucose decreased rapidly in the YEPD complex media run, compared to the selective fermentation.

Ethanol was produced and subsequently utilised the most efficiently of all the three media in the supplementation media batch run (Figure 5.15). Similar to what was observed with glucose utilisation, ethanol was slow to be consumed in the selective and YEPD media.

The pH of the supplemented media run was phosphate buffered and regulated to ~ 6.5 using NaOH. (Figure 5.16) The selective media run was also phosphate buffered, but the pH of the media was not regulated. It did not drop by more than 0.5 pH units, from an initial value of 6.4. The pH of the YEPD media was neither buffered nor pH regulated, and it dropped from an initial value of pH 6.12 to pH 4.78.
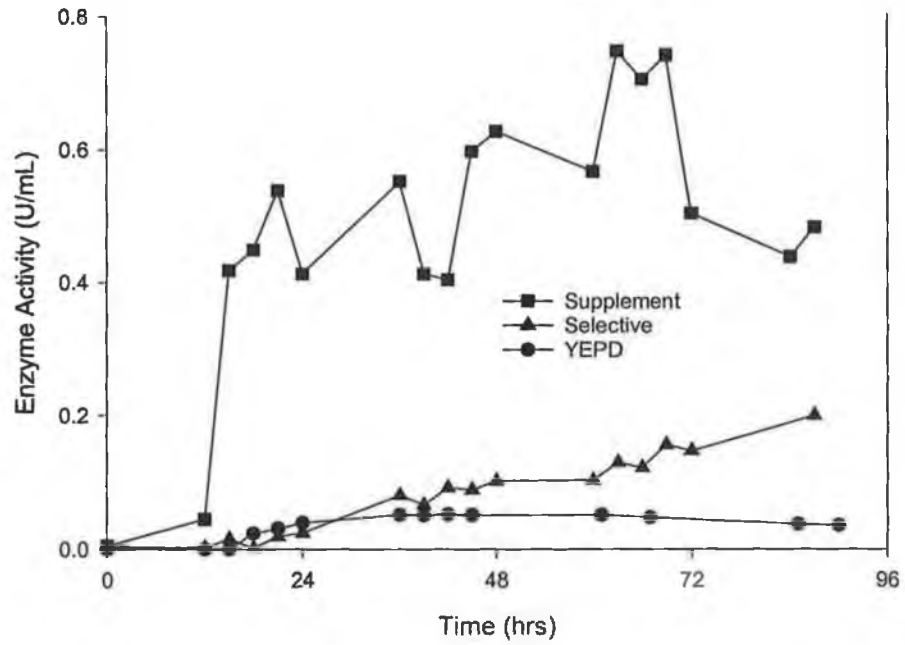
Figure 5.12 Enzyme activity from batch fermentations of YCL1 cultured in selective, YEPD and supplemented media.
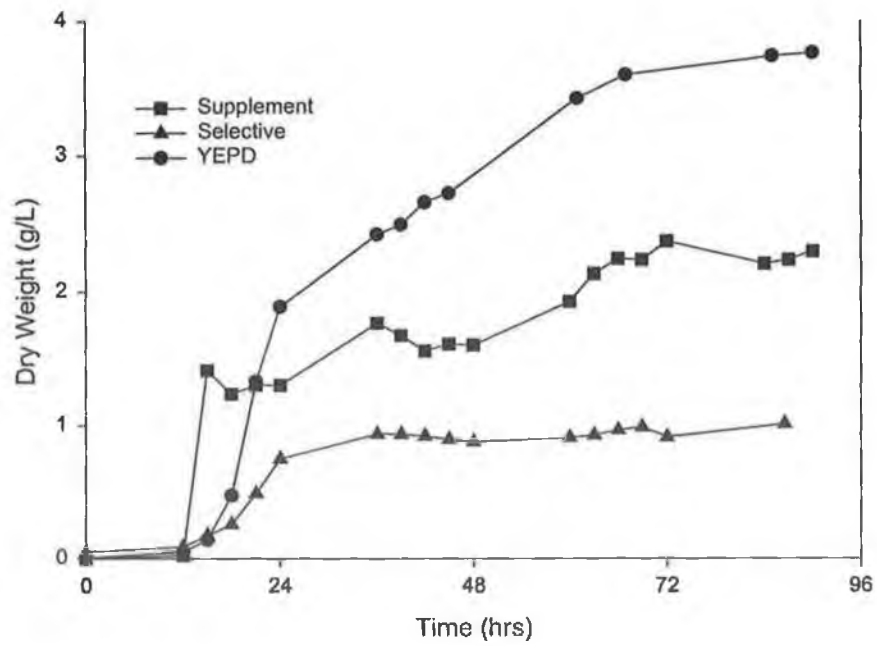
Figure 5.13 Dry weights from batch fermentations of YCL1 cultured in selective, YEPD and supplemented media.
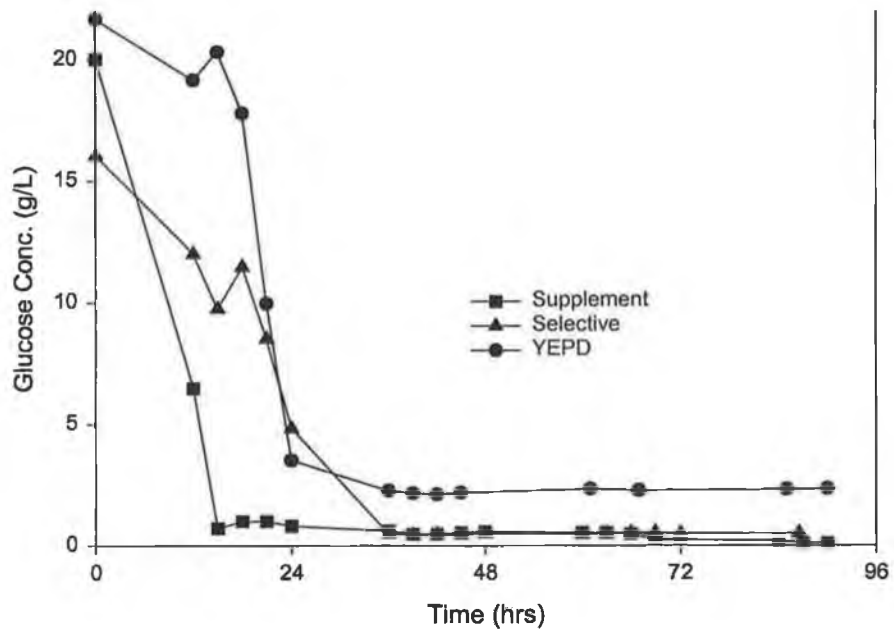
Figure 5.14 Glucose consumption from batch fermentations of YCL1 cultured in selective, YEPD and supplemented media.
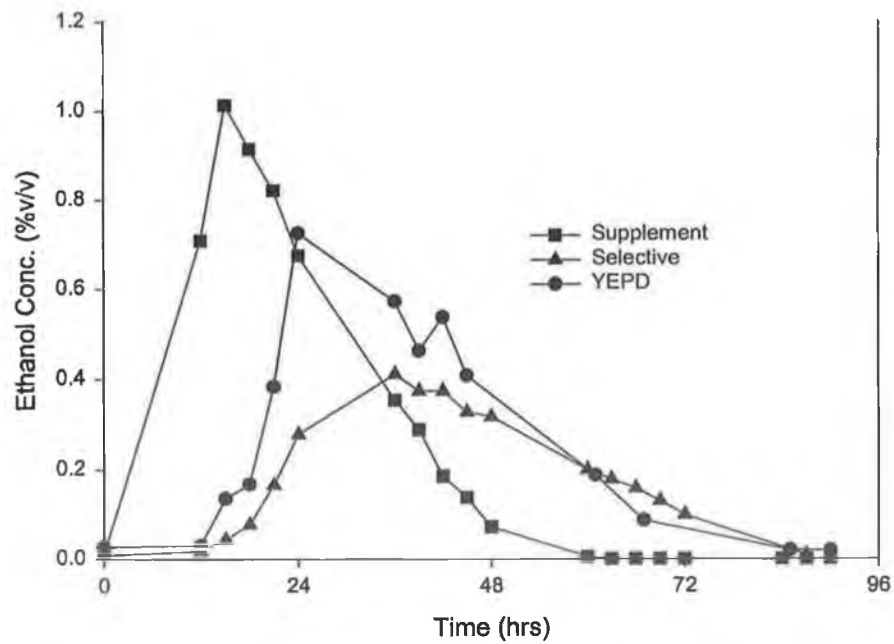
Figure 5.15 Ethanol concentration in batch fermentations of YCL1 cultured in selective, YEPD and supplemented media.
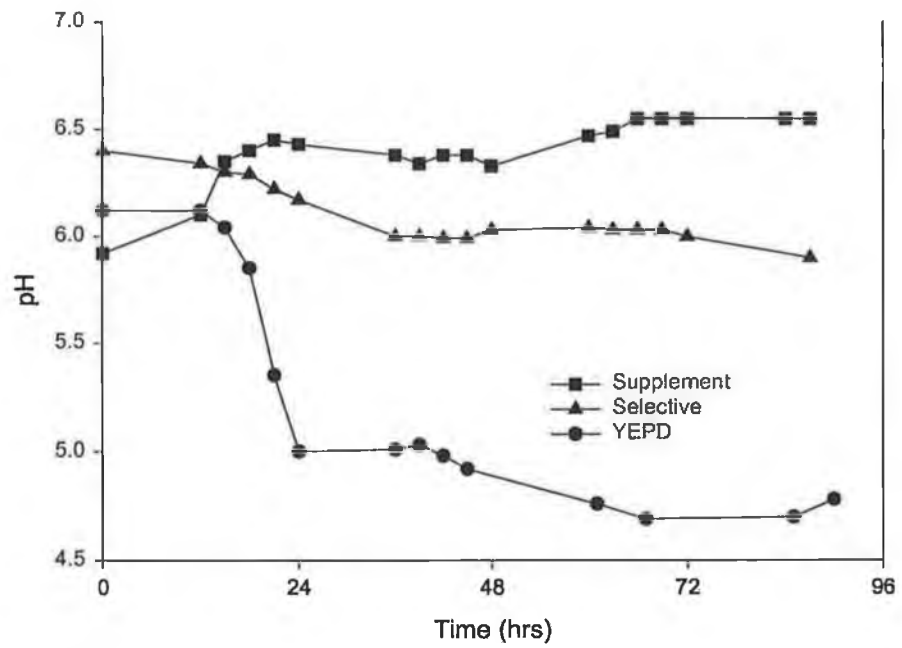
Figure 5.16 pH of the batch fermentations of YCL1 cultured in selective, YEPD and supplemented media.

5.3 Discussion

5.3.1 Effect of pH and temperature on Cathepsin L1 production

The results on enzyme production from recombinant Cathepsin L1 cultured at different pH's confirm the findings of Dowd *et al* (1997). An initial culture pH of ~ 6.5 is optimal both for enzyme expression and secretion as well as biomass concentration. (Figure 5.1 and Figure 5.2) In addition the enzyme is stably maintained at this pH (Figure 5.3). Further investigation into the stability of the enzyme at various pH's confirms that Cathepsin L1 is very stable between the pH range of 5.0 and 7.0 for up to a period of 52 hours as shown in Figure 5.3. Despite pH 5.0 not having an impact on the stability of the enzyme, expression and secretion of Cathepsin L1 may be hampered at this pH (Figure 5.1)

Dowd *et al* (2000) found that the parasitic Cathepsin L was remarkably stable at pH 7.0 - 8.0 over a period of 24 hours in buffered solutions. Roche *et al* (1997) have shown before that the yeast expressed Cathepsin L1 is more stable at pH 7.5 than its *F. hepatica* derived counterpart. Brady *et al* (1999) have reviewed the optimum pH for activity of Cathepsin L-like proteinases from human, *Fasciola hepatica* and *Schistosoma mansoni*. Their review suggest that the Cathepsin L enzymes prefer the acidic pH range, which would substantiate the poor performance at the alkaline pH 8.0 in both pH experiments.

Sledziewski *et al* (1988) reported that through a down-shift in the culture temperature from the repressive 35^0C to the permissive 25^0C, induction of the foreign protein was triggered. DaSilva and Bailey

(1989) in contrast reported improvement in foreign protein expression by an up-shift in temperature.

However these systems had a temperature sensitive (*ts*) mutant promoter, where the protein expression is repressed at certain temperatures. Thus expression of the protein was induced through a change in temperature, whether it be up or down. In the case of YCL1, the promoter is an ADH1 promoter, and thus not induced through a change in temperature. As mentioned previously though, there have been reports in literature, where researchers have found that by increasing or decreasing the culture temperature from a normal temperature of 30^0C, heterologous protein production has been improved (Rossini *et al*, 1993, Alvarez *et al*, 1994, Tøttrup and Carlsen, 1989, Nagashima *et al*, 1994, Cassland and Jönsson, 1999). These expression systems did not have *ts* mutant promoters.

In the case of YCL1, 30^0C was found to be the optimum temperature for both biomass concentration and enzyme production. (Figures 5.4 and 5.5) The biomass concentration and enzyme production were not as high in the 25^0C incubation as observed in the 30^0C incubation. Enzyme activity dropped at the end-point of the fermentation at 25^0C, and this may be due to inactivation of the enzyme during prolonged incubation at this temperature. Previously, Dowd *et al* (2000) showed that when Cathepsin L is incubated between 30^0C and 45^0C for 10 minutes it retains 100% activity, but they have not examined stability of the enzyme below this temperature. Dowd *et al* (2000) also showed that when Cathepsin L is incubated at temperatures between 45^0C to 90^0C, residual activity drops from almost 100% to less than 20%.

Cathepsin L1 production was much lower at 37^0C throughout the 72-hour period. It has been shown before that Cathepsin L is very stable

at 37^0C (Dowd *et al*, 2000), and thus denaturation of the enzyme would not account for the lower activity compared to the other two temperatures. The cell counts during incubation at 37^0C are lower overall than at 30^0C. Growth of *S. cerevisiae* cells above $33\text{-}35^0C$ is slower than at 30^0C (Watson, 1987) and thus a slower generation of cells may account for the lower enzyme activity.

5.3.2 Production of Cathepsin L1 in non-selective media

Once the optimal environmental parameters for the culturing of YCL1 and expression of Cathepsin L1 were established, the ideal media composition of media in which to ferment YCL1 was investigated.

Tøttrup and Carlsen (1990) ascertained that subcultivations in rich, non-selective media resulted in better overall plasmid stability than in selective media because of higher cell growth rates. Ibba *et al* (1993a) reported high plasmid stability and continuous constitutive expression of a protein in non-selective media. They postulated that there was not a detrimental effect on plasmid stability in the complex media, because the protein expressed was not a burden to *S. cerevisiae*.

In contrast to these and other reports (Jung *et al*, 1991, Chriuvolu *et al*, 1996), production of YCL1 dropped considerably when grown in complex YEPD rather than selective media. (Refer to Figure 5.6) The faster cell growth rate seen with YEPD is as expected because there are many more nutrients present in the complex media than in the nutrient limited selective media. Glucose utilisation is also much slower in selective media compared to complex media, because of the slower growth rate of cells in the selective media.

Chiruvolu *et al* (1996) reported improved heterologous protein production when they supplemented minimal media with casamino acids. Despite an increase in biomass levels for casamino-supplemented media, there was no improvement in Cathepsin L1 production compared to the control. (Refer to Figure 5.7)

A potential reason for the outcome in both complex media and casamino- supplemented media is the proliferation of Plasmid free (P-) cells compared to plasmid bearing (P+) cells. The growth rate of the P-cells would be higher than that of the P+ cells, due to the extra metabolic loading of the plasmid bearing cells. This type of plasmid instability is referred to as competitive instability. When extra nutrients from the Casamino acids were added to the media, this population of plasmid-free cells may have outgrown that of the plasmid bearing cells. This could explain why there was much higher cell biomass in the flasks spiked with Casamino Acids, but lower Cathepsin L1 expression.

Despite dramatically increasing the cell growth rate by growing the YCL1 cells in complex media and through supplementation of casamino acids, no improvements in enzyme production compared to the control selective media were observed. Thus further investigation into optimising the selective media had to be examined. This involved investigating additional supplementation of the other main ingredients of selective media - glucose, yeast nitrogen base and uracil. Replacing the main carbon source glucose with another carbohydrate was investigated first.

5.3.3 Effect of replacing glucose as the main carbon source

All yeast metabolise the hexose sugar, glucose. Generally, if yeast ferments glucose, it will also ferment fructose and mannose, but not necessarily galactose (Walker, 1998). This phenomenon was witnessed in this experiment (Refer to Figure 5.8) where fructose and mannose sugar replacements achieved both high levels of enzyme activity and high cell numbers, whereas galactose had poor cell growth and thus low enzyme activity. Mannose and galactose are both epimers of glucose, which means effectively that they differ only in the configuration around one specific carbon atom, which in the case of mannose is carbon atom 2 and in the case of galactose, is carbon atom 4 (Lehninger, 1975).

Sucrose is a disaccharide of both Glucose and Fructose. It is hydrolysed extracellularly and then transported into the yeast cells as glucose and fructose (Kristiansen, 1994). Thus it is not surprising that the Glucose & Fructose mixture had similarly high cell counts and high enzyme activity as the Sucrose replacement.

Walker (1998) mentions that if particular yeast ferments maltose it will not ferment lactose, and vice versa. In this case, YCL1 utilised maltose in preference to lactose for cell growth. However, neither sugar achieved high enzyme activity. Lactose is a disaccharide comprising of galactose and glucose, whereas maltose is a disaccharide of two glucose monomers.

The lactose replacement had similar results to that of galactose i.e. low cell concentration and trace enzyme activity. As lactose hydrolyses into two residues of glucose and galactose, it is not surprising that lactose

had a similar outcome to the galactose replacement. It seems likely therefore that the DBY746 yeast strain cannot metabolise galactose effectively for growth.

It would appear that galactose has an inhibitory effect on the expression of Cathepsin L1. When combined with glucose, mannose or fructose, the enzyme activity dropped significantly compared to each of the sugars on their own, despite relatively high biomass levels in each case.

Yeast cells that are grown on glucose repress the synthesis of a large number of enzymes necessary for the utilisation of other carbon sources. The concentration of glucose drops as it is being consumed, and this derepression occurs at about 0.2%w/v glucose (Dickinson, 1999). Thus when *S. cerevisiae* is grown on a mixture of glucose and another fermentable sugar, e.g. sucrose, galactose or fructose, the metabolism is di-auxic, and glucose is the first substrate to be metabolised (Dynesen *et al*, 1998).

When glucose and galactose were combined, high cell concentrations were achieved. Thus, glucose was effectively the primary sugar to be utilised in this case. Previously it has been shown that once glucose has been fully consumed, enzyme production begins to accumulate gradually. (Figure 4.2) However, in this case, only a small amount of enzyme was produced. This would lend credence to the supposition that galactose has a repressive influence, when used in conjunction with other sugars, on the expression of Cathepsin L1. The repressive effect of galactose will be discussed later.

5.3.4 Supplementation of selective media with extra glucose, yeast nitrogen base and uracil

As glucose was established as the most suitable carbon source, the next step was to determine the optimal concentration of glucose in media. In the case of YCL1, yeast cells were normally fermented in media with a glucose concentration of 2%w/v up to this point.

Some yeast can endure glucose concentrations of up to 20%w/v. However when the carbon source is present in media at high concentrations, catabolite repression occurs. This gives rise to the hindrance of one or more of the enzymes used for the synthesis of a secondary metabolite (Greasham and Herber, 1997).

Despite reasonably high cell numbers in the 6%w/v and 8%w/v glucose supplements, very low enzyme activity was observed. (Refer to Figure 5.9) This observation is most likely due to catabolite repression because of the presence of high concentrations of glucose. The residual glucose concentrations for these cultures at 72 hours were 4.9g/L and 5.5g/L respectively. Much of the glucose would have been utilised for cell growth, however when all other nutrients were fully consumed cell growth ceased and there was a susbstantial concentration of glucose remaining. The presence of large amopunts of glucose may have caused the repression of expression of Cathepsin L1.

When smaller concentrations of glucose were added to the media at the start of the fermetnation (i.e. 10g/L and 20g/L), sufficient glucose was present to maintain cell growth, and when it was fully consumed, the ADH1 promoter would have been induced to express Cathepsin

L1. Therefore in the 2%w/v sample, a reasonably high biomass concentration is accompanied with a considerable quantity of enzyme.

In the 10g/L sample, which has half the concentration of glucose compared to the 2%w/v sample, only modest enzyme activity is achieved, despite a very high cell concentration. In this scenario, it is possible that all of the glucose was consumed before the other nutrients were fully utilised. Thus only sufficient glucose was present to provide the energy requirements for cell division and multiplication and not for the expression of Cathepsin L1.

Once the optimal concentration of glucose for YCL1 cultivation was confirmed (i.e. 20g/L), an investigation into supplementing the selective media with extra yeast nitrogen base and uracil was prompted. The results of this experiment are shown in Figures 5.10 and 5.11, as well as Table 5.3. Jung *et al* (1991) reported that they had to supplement their selective media to improve cell growth, plasmid stability and protein production. Through the addition of sufficient vitamins and trace elements to the media that mainly consisted of yeast nitrogen base, the production of recombinant HbsAg (Hepatitis B surface antigen) was immediately improved.

By tripling the recommended concentration of yeast nitrogen base (YN$_2$B), both biomass concentrations and enzyme production were significantly improved. This would indicate that prior to this supplementation the selective media employed was limited in the amount of vitamins, amino acids and salts. Through supplementing the media with extra YN$_2$B, sufficient nutrients were provided to improve cell growth rates and prevent nutrient deprivation occurring.

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At five times the normal concentration of YN_2B, Cathepsin L1 production is still slightly higher than the 6.7g/L set of flasks, however the level of enzyme production achieved does not justify the addition of this concentration of YN_2B. Despite both high cell numbers and higher plasmid stability, the YN_2B x 5 sample did not produce as much enzyme as the YN_2B x 3 sample. This may have been caused by an overload of nutrients in this set of flasks, where expression may have been repressed.

There was no notable increase in enzyme production in any of the YN_2B supplemented set of flasks when extra uracil was added. Thus the addition of extra uracil does not increase the production of Cathepsin L1 to any great extent.

Biomass levels are all in a similar range for all three concentrations of uracil in the YN_2B x 1 range of flasks, thus supplementing with uracil does not significantly improve cell concentration. There is a slight increase in biomass levels in the 60mg/L and 100mg/L uracil supplements of the YN_2B x 5 set of flasks, but again not enough to justify supplementing future fermentations. Compared to the addition of extra yeast nitrogen base, uracil had no substantial improvement in either biomass or enzyme activities.

5.3.5 Batch fermentations of YCL1

The results from the batch fermentations support the outcome from the shake flask experiments. Dissolved Oxygen was supplied to each of the fermentation runs at a constant rate of 1 v/v/m. Thus any claims that there was an inadequate Oxygen supply to the shake flasks are negated by similar results seen in the batch bioreactor runs.

The pH is regulated using NaOH in the supplemented batch, but not in the selective and non-selective runs. The pH did not drop below 6.35 in the selective run, however the pH is the non-selective batch run dropped to 4.78. Despite Section 5.1.3 proving that pH 5.0 does not affect the stability of the enzyme, it was noted in Section 5.1.2 that an initial culture pH of pH 5.0 results in a low level of expression of Cathepsin L1. Thus the combination of the low pH and the extremely low plasmid stability observed in the non-selective run are attributed to the low levels of enzyme activity detected. In hindsight, the pH of both the selective and non-selective batch runs should have been controlled throughout the fermentation. However the poor plasmid stability of the YEPD media would have contributed to the low enzyme activity.

Cathepsin L1 production is significantly higher in the supplementation media than the other two media, despite a much higher cell concentration in YEPD media. Glucose was fully utilised by 15 hours in the supplemented media, compared to slow consumption of glucose in the selective media. In addition, slow production and subsequent utilisation of ethanol was observed in selective media, and there was a low biomass yield when cells were growing on ethanol. This leans credence to the fact that selective media was nutrient limited before supplementation.

5.4 Conclusion

The optimal pH and temperature for culturing YCL1 and concurrently producing high levels of Cathepsin L1 are pH 6.5 and 30^{0}C respectively. Glucose is the most suitable carbon substrate to employ when fermenting YCL1, and the optimal concentration of glucose is 20g/L. Cathepsin L1 production is curtailed when YCL1 is grown in complex YEPD media or selective media supplemented with casamino acids, most probably due to plasmid loss. Addition of uracil to selective media does not result in any significant improvement in enzyme production. However supplementing minimal media with 20.1g/L yeast nitrogen base increases both cell biomass and Cathepsin L1 levels by 4 fold compared to selective media. The new supplemented media is nutrient sufficient to support the growth of YCL1 and procure high levels of enzyme using 20g/L Glucose.

Chapter 6 Investigation into the factors affecting the constitutive expression of Cathepsin L1

6.1 Introduction

The *ADH1* gene encodes a glycolytic enzyme (ADHI), which is responsible for the last step in the yeast glycolytic pathway (Cho and Jeffries, 1998), that of converting acetaldehyde to ethanol, regenerating NAD^+ (from NADH) in the process (Hong and Kang, 1998). It is expressed in high levels (greater than 1% of total RNA), during growth on glucose. The yeast alcohol dehydrogenase isozyme I (ADHI) is responsible for ethanol production during growth on glucose. The ADH1 promoter activates transcription of the ADH1 enzyme (Tornow and Santangelo, 1990).

The yeast strain YCL1 bears the plasmid pFheCL1Y29 and expresses Cathepsin L1 under the control of the yeast promoter ADH1. The ADH1 promoter was previously thought to be constitutive. However Denis *et al* (1983) showed that alcohol dehydrogenase is repressed when cells are grown on non-fermentative carbon sources such as ethanol. The rate of alcohol dehydrogenase I protein synthesis declined 6-10 fold when yeast were transferred from medium containing glucose to ethanol-containing media, or during growth into stationary phase. This implied that the amount of transcription from the ADH1 promoter decreased during growth on ethanol-containing medium. However during these stages, despite the decrease in transcription, a relatively high level of ADH1 activity is maintained, which gives the appearance of its being synthesised constitutively.

A number of citations have led to the belief that expression from this strong promoter can be enhanced through genetic modification of its upstream sequence (Ruohonen *et al*, 1991, 1995, Vainio, 1994, Onnela *et al*, 1996, Monfort *et al*, 1999). To investigate whether or not Cathepsin L1 was expressed constitutively under the control of the ADH1 promoter on the YCL1 strain, a further batch fermentation was performed. The effect of the presence/absence of ethanol and/or glucose on the expression and secretion of Cathepsin L1 from YCL1 was analysed. In addition suspicions that were highlighted from earlier experimental work about the possible inhibitory effect of galactose on enzyme expression were investigated.

6.2 Investigation into the Constitutive expression of Cathepsin L1 under the control of the ADH1 promoter.

The new improved media identified in Section 5.2.6 was used in a 10L batch fermentation to analyse the factors affecting the constitutive expression of Cathepsin L1 from YCL1. The fermentation was performed using the environmental parameters outlined in Section 5.2.9. The fermentation profile of YCL1 in the supplemented selective media can be seen in Figure 6.1.

Trace amounts of enzyme was expressed in the first 12 hours, but during the later stages of glucose consumption, Cathepsin L1 expression increased rapidly. By 72 hours, enzyme activity had reached its maximum of 0.74 units/mL, and began to decline when the fermentation continued to 96hrs. The biomass levels increased exponentially during growth on glucose and reached a peak biomass of 2.29 g/L. Glucose depleted exponentially from an initial concentration of 2.0%w/v and was fully utilised by 15 hours. Ethanol concentration reached a peak of 1.01%v/v before consumption began.
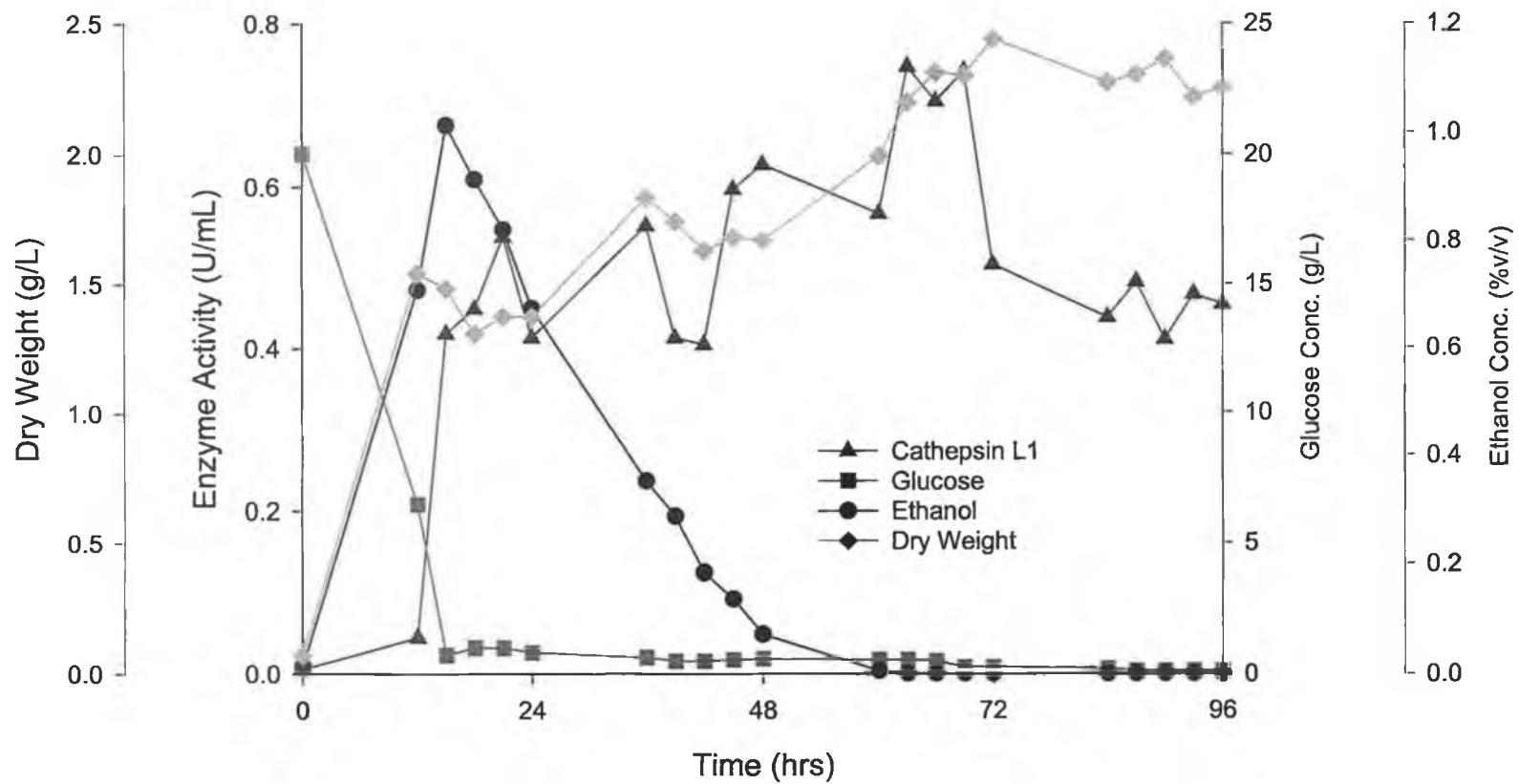
Figure 6.1 Batch fermentation profile for YCL1 cultured in supplemented selective media
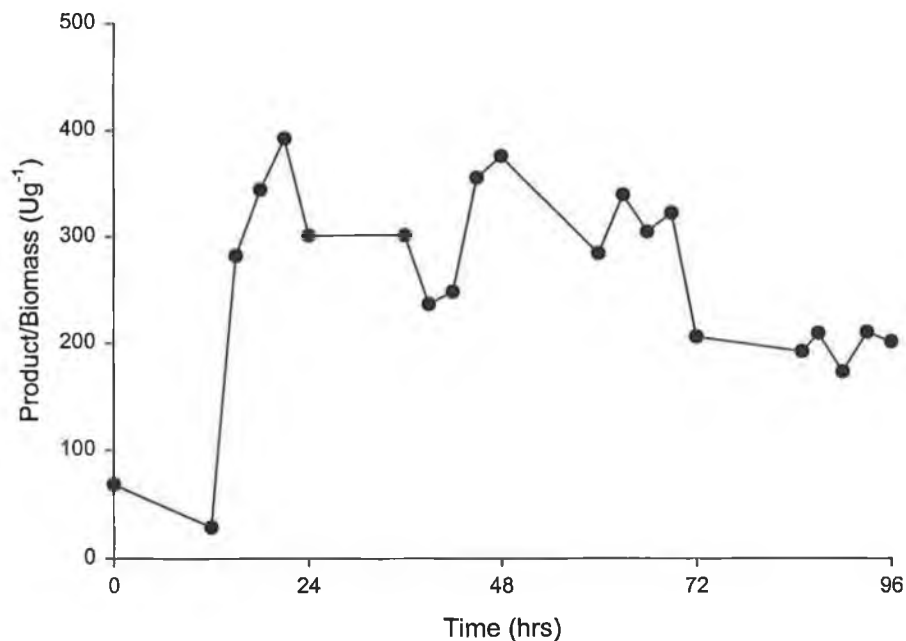
Figure 6.2 Cathepsin L1 activity (UL^{-1}) per gL^{-1} of cell dry weight over the course of 96 hours in supplemented media batch fermentation.

Figure 6.2 confirms that Cathepsin L1 production is repressed during early glucose utilisation phase and exponential cell growth (0 - 12 hrs). It increases substantially during the latter stages of glucose consumption (12 - 18 hrs), when cell growth reached the first stationary phase of di-auxic growth. Expression of Cathepsin L1 is growth-associated during the ethanol consumption phase (18 - 48 hrs) as the amount of enzyme produced per dry weight is proportionately constant.

6.3 Investigation into the Repressive Effect of Galactose on Cathepsin L1 Production from YCL1

As mentioned previously, it was noted that the monosaccharide galactose possibly had an inhibitory effect on Cathepsin L1 expression. To further investigate this observation, a series of 250mL shake flasks were prepared containing 100mL of minimal media. Concentrations of

Galactose ranged from 0 to 10g/L, with a constant concentration of glucose at 20g/L in each flask. The DNS method for calculating the concentration of reducing sugars i.e. both glucose and galactose, was performed in preference to the GOD-PAP method, which selects for glucose only. Reducing sugars are sugars that are capable of oxidising agents such as Cu^{2+} and Ag^+ in alkaline solution (Lehninger, 1975).
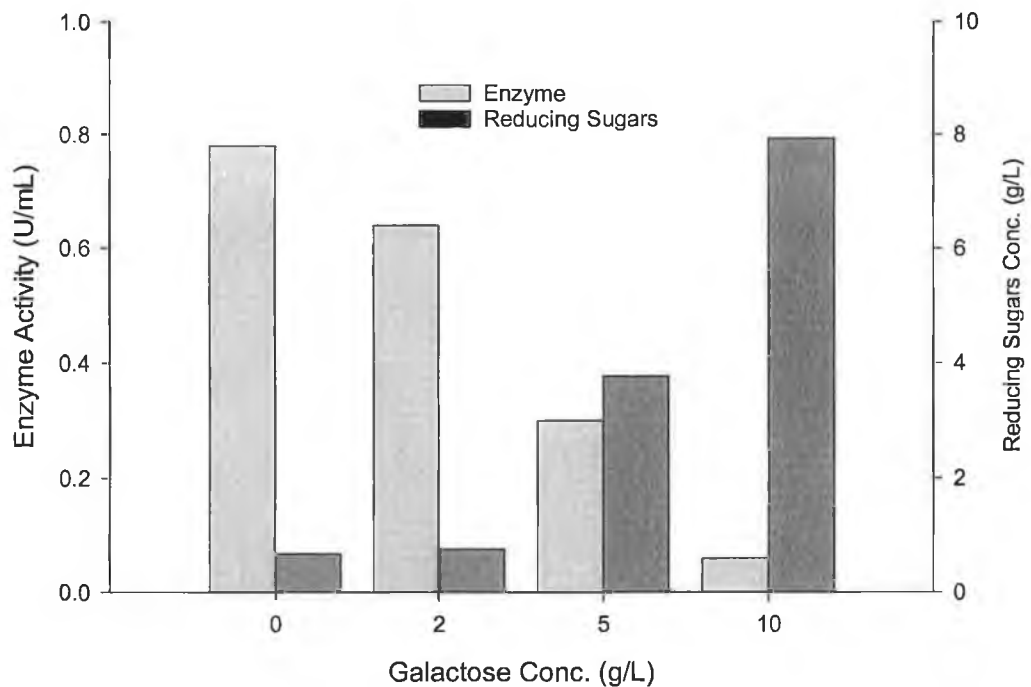
Figure 6.3 The effect of the presence of increasing concentrations of galactose on Cathepsin L1 expression and remaining reducing sugars at the end-point of the fermentation.

The results of the galactose experiment can be seen in Figure 6.3. Analysis was performed on the flasks at the end-point of the fermentation. As the galactose concentration increased, the level of Cathepsin L1 expression decreased. The concentration of reducing sugars remaining at the end-point of the fermentation corresponded similarly to the concentration of galactose that was present in each of the cultures at the beginning. Cell counts were in a similar range for all

cultures, ranging between 2.85 and 3.13 x 10^7cells/mL (Data not shown).

6.4 Discussion

It would appear from literature that to dramatically improve Cathepsin L1 production, genetic modifications of the ADH1 promoter would have to be performed. Ruohenon *et al* (1991) reported a 29-fold increase in α-amylase production from recombinant S. cerevisiae in selective media when a large segment (1,100bp) of the upstream sequence of the ADH1 promoter (1,500bp) was removed. Prior to removal, accumulation of the protein in the medium occurred before stationary phase was reached and it was very low when exponentially growing cells were transferred to ethanol-containing medium from glucose-containing medium. The shortened promoter (~0.4kb) allowed production of α-amylase under all growth conditions. They found that α-amylase was secreted during stationary phase as well as after transferral into ethanol containing medium, but the promoter only becomes activated during ethanol consumption phase. They postulated that the presence of glucose acted as a repressor for activation of the shortened ADH1 promoter, or alternatively ethanol may have had a positive effect.

Vainio (1994) presumed that the short promoter constructed by Ruohonen *et al* (1991) lacked the upstream activation sequence, UAS_{RPG}. Tornow and Santangelo (1990) reported previously that by deleting a 20bp sequence from a UAS_{RPG} consensus sequence in the ADH1 promoter a 25-fold reduction in *ADH1* expression resulted. Thus this *cis*-acting regulatory element, UAS_{RPG} is required for efficient activation of *ADH1* transcription. UAS_{RPG} is found in the promoter of many genes, including glycolytic genes.

In order to rectify this Vainio (1994) constructed both a short promoter that lacked the entire upstream activating sequence (UAS) and a medium-length promoter that contained the putative upstream transcription point but not the UAS. Expression of the foreign protein, which was controlled by the short promoter was delayed by about 10 hours, because of the absence of the UAS. The medium length promoter commenced expression of the protein without delay, but despite this constitutive expression, the short promoter achieved the highest final yield of protein.

Ruohenon *et al* (1995) restored 300bp of the upstream sequence of the truncated ADH1 promoter, and constructed a medium-length ADH1 promoter, which was similar to the promoter that Vainio (1994) constructed. This allowed α-amylase expression during early exponential growth phase on glucose, which had previously been prevented with the short promoter. However, the final yield of α-amylase was again much higher from the yeast controlled by the short promoter than that of the medium and long promoters.

Thus if the timing of the expression of the desired protein is essential, then the short, middle or long promoter could be employed depending on the growth conditions. This middle-length promoter was utilised by Onnela *et al* (1996). They used the shortened promoter (~1.0kb) from Ruohenon *et al* (1995) to express α -acetolactate decarboxylase (α-ald) from a bottom fermenting brewer's yeast. The yeast strain that employed this modified promoter expressed a high level of α-ald throughout the experiment, and as a result no lagering of the beer was required.

Monfort *et al* (1999) compared five different promoters for the production of foreign proteins from baker's yeast. They employed both the shortened ADH1 promoter (Ruohenon *et al,* 1991) and the medium length ADH1 promoter (Ruohenon *et al,* 1995) as well as PGK1, TDH1 and ACT1 promoters. Expression of lipase from yeast controlled by the shortened ADH1 promoter resulted in the highest protein yield at 48 hours when the yeast was cultured in molasses medium. During growth on ethanol, the only promoter to remain active was that of the shortened ADH1 promoter, while all other constructs were turned off.

DeMarini *et al* (2001) replaced a GAL1 promoter with the truncated (medium-length) version of the ADH1 promoter constructed by Ruohenon *et al* (1995). As a result, the plasmid containing the truncated version of the ADH1 promoter is constitutively active in cells grown on glucose. As the starting vectors for this plasmid was a P_{GAL1} plasmid (Longtine *et al*, 1998), it contains the same vector sequence as the GAL1 promoter plasmid. Thus both an inducible (GAL1) and a constitutive (ADH1) promoter can be placed at the same genetic locus using just three primers, because they both possess the same vector sequence. It also means that genes can be overexpressed when cultured in rich medium instead of selective medium, which is required for the maintenance of most multicopy plasmids.

Batch fermentations were performed to investigate the constitutive nature of the *ADH1* promoter. Watson (1976) defines constitutive enzymes as 'enzymes that are synthesised in fixed amounts, irrespective of the growth conditions'. Thus in the case of the *ADH1* promoter, which had previously been considered constitutive, the expression heterologous proteins under its control should theoretically increase as the biomass increases. However Denis *et al,* (1983) questioned the constitutive nature of this promoter.

Batch fermentations were employed in preference to chemostat or fed-batch operations because complete containment can be attained. In addition, the risks of contamination were greatly reduced and there is an overall shorter cycle time compared to the other two modes of cultivation. Moreover all of the citations mentioned previously in relation to the *ADH1* promoter employed batch runs.

The $Y_{x/Eth}$ (Yield of biomass during ethanol consumption phase between 0-12 hrs) was 0.043 g biomass/g ethanol compared to higher yield on glucose. $Y_{x/Glu}$ (Yield of biomass during glucose consumption phase 18-48 hrs) was 0.108 g biomass/g glucose. Although di-auxic growth was exhibited on both glucose and ethanol, the yield of biomass on glucose was far higher than that on the non-fermentable carbon source ethanol. During the glucose consumption stage, the doubling time, t_d, of the yeast cells is 3.18h, and the maximum growth rate, μ_{max} is $0.218h^{-1}$. In comparison, the doubling time on ethanol was 73 hours.

During the first 12 hours of growth the yield of Cathepsin L1 during the glucose consumption phase is only 0.0029 U/mL Enzyme/g/L Glucose ($Y_{P/Glu}$), when three-quarters of the glucose has been consumed. (Refer to Figure 6.1) However during the ethanol consumption phase between 18 and 48 hours the yield of Cathepsin L1 is almost 10 fold that on glucose at 0.0212 U/mL Enzyme/g/L Ethanol ($Y_{P/Eth}$). This behaviour was observed in a number of fermentations where the data is not presented here. Figure 6.2 substantiates these results.

Thus enzyme production would appear to be repressed during the formative stages of cell growth and glucose consumption. During the latter stages of the glucose consumption period, when the glucose

concentration was less than 0.5%w/v, Cathepsin L1 production increased significantly. $Y_{P/Glu}$ for the period between 12 and 15 hours is $0.0649 UmL^{-1}g^{-1}L^{-1}$. Whether the low expression of Cathepsin L1 is due to high concentrations of glucose, or whether the presence of higher levels of ethanol was beneficial is still unknown.

This would indicate that 'semi-constitutive' expression was indeed taking place, as there is higher yield of product during the latter stages of glucose consumption phase (12 - 15 hours) than during the ethanol consumption phase (i.e. $Y_{P/Glu} = 0.0649 UmL^{-1}g^{-1}$ v's $Y_{P/Eth.} = 0.0212 UmL^{-1}g^{-1}$). (Figure 6.1) It would appear that 'derepression' of the promoter occurs when the glucose concentration drops below a certain point, as there is a substantial increase in Cathepsin L1 activity beyond this point in the glucose consumption phase (Figure 6.1 and 6.2).

The original 'long' ADH1 promoter investigated by Denis *et al*, (1983) showed a drop off in expression when cells were growing in ethanol-containing media, which is what is seen in this fermentation run. Cathepsin L1 is still secreted into the medium during the ethanol-consumption phase but the rate of production compared to during the latter stages of glucose utilisation has declined. (Figure 6.1 and 6.2) However, Ruohonen *et al* (1991) noted much higher *α*-amylase expression from the long promoter when exponentially growing *S. cerevisiae* cells utilising the 'long' ADH1 promoter were transferred to ethanol-containing media rather than glucose-containing media, despite a much lower final cell density.

Despite Ruohonen *et al* (1995) claiming the improvements made with middle promoter to instigate constitutive expression during the glucose consumption phase, the level of expression by all three promoters in

the early stages of the fermentation is still extremely small compared to that of the latter ethanol consumption stages.

However the truncated *ADH1* promoter originally constructed by Ruohonen *et al* (1991) increases the level of heterologous protein by almost 30-fold compared to the original long promoter. This shortened ADH1 promoter would appear to be repressed in high concentrations of glucose, thus labelling the new promoter neither constitutive nor inducible. If high yields of the heterologous protein are required, then it would appear that genetic modifications of the ADH1 promoter are required is this expression system is to be used.

When *S. cerevisiae* is grown on a mixture of fermentable sugars, glucose is preferentially metabolised first, and only when it has been exhausted are the other sugars utilised (Dynesen *et al*, 1998). In this case however, once all of the glucose was consumed, it appears that galactose was not metabolised. (Figure 6.3) The concentration of reducing sugars remaining at the end-point of the fermentation, is similar to the concentration of galactose that was present in each of the flask at the start.

It appears that Galactose has an inhibitory effect on Cathepsin L1 production. As the concentration of galactose increased, the level of Cathepsin L1 expression decreased. It is possible that galactose interferes with the biochemical pathways essential for Cathepsin L1 expression. Glucose and galactose are epimers of each other, which means effectively that they differ only in the configuration around one specific carbon atom, in this case carbon atom 4 (Lehninger, 1975).

6.5 Conclusion

The ADH1 promoter that controls the expression of Cathepsin L1 in YCL1 is repressed during the early stages of fermentation when glucose concentration is still high. Expression of Cathepsin L1 is not initiated until most of the glucose has been consumed. Enzyme production continues throughout the ethanol consumption period, albeit at a lower level of transcription than during the latter stages of glucose consumption. In order to increase expression of Cathepsin L1 to a much larger extent genetic modifications on the ADH1 promoter will have to be performed. The presence of Galactose in glucose containing media represses the expression of Cathepsin L1.

Chapter 7 Conclusions and Recommendations

The problem of low productivity and especially variations in Cathepsin L1 production was considered to be a significant obstacle and thus one of the first problems to be investigated in the course of this project. Variations in enzyme activity occurred because starter cultures were inoculated with individual colonies. A standard operating protocol (SOP) was devised to consistently provide an optimum inoculum for large-scale fermentations. Optimising the initial inoculum proved to be an integral part in furthering the development of a production protocol for recombinant Cathepsin L1.

This SOP comprises selecting a number of colonies grown on solid media and re-suspending them in saline. This 'blend' of cells is then used to inoculate a starter liquid culture and is grown for 48 hours. The cells from this culture are then used to inoculate a second sub-culture, which is incubated for a further 48 hours. It is this sub-culture that is used for inoculating a large-scale fermentation.

Long-term sub-culturing of YCL1 cells in liquid media cannot maintain a population of high enzyme producing cells. It is possible that a combination of structural and segregational plasmid instability over time caused this problem. Seventy-two hours was identified as the optimal point at which to harvest Cathepsin L1 in batch fermentation. When fermentations were continued to ninety-six hours and beyond, enzyme production ceased, and progressive decline in activity was noted. Previously, cultures were only grown for up to two days, and as shown throughout these experiements, very small concnetrations of the enzyme are produced by this time in the production cycle.

Investigations into environmental conditions for culturing YCL1 attested the facts that the optimal pH and temperature for culturing YCL1 and concurrently producing high levels of Cathepsin L1 are pH 6.5 and 30^0C respectively.

A number of monosaccharides and disaccharides were examined as potential replacements for glucose. However in terms of Cathepsin L1 production, biomass levels and economical cost, glucose was identified as the most suitable carbon substrate. The optimal concentration of glucose to employ when fermenting YCL1 is 20g/L. Galactose inhibits the growth of YCL1, and has a repressive effect on the expression of Cathepsin L1 when present in glucose-containing media.

Investigations into the original media suggested that the selective media used was nutrient deficient resulting in low cell growth rates. However by growing YCL1 in complex YEPD media, low enzyme production was attained despite very high biomass levels. Further investigation into this media attributed the low performance to significant plasmid loss in this complex media. Examination into supplementing the selective media with casamino acids proved similar to the poor enzyme productivity achieved in YEPD media. In addition the selective media is phosphate buffered, so it does not suffer from acidification like that observed in YEPD media.

When the selective media was supplemented with extra yeast nitrogen base there was almost a four-fold increase in Cathepsin L1 production compared to the nutrient-deficient selective media. There was also a significant increase in biomass levels. One of the other main ingredients of the selective media was uracil. However addition of extra uracil to selective media does not result in any significant improvement

in enzyme production. The new supplemented media is both nutrient sufficient to support the growth of YCL1 using 20g/L Glucose. In addition it has a high plasmid stability relative to that observed in selective media alone.

Expression of Cathepsin L1 from YCL1 is controlled by the *ADH1* promoter. The influence of the *ADH1* promoter had been previously overlooked in previous experimentation using this expression system. By investigating the control of the *ADH1* promoter it helped contribute to greater understanding of the mechanism of production of Cathepsin L1. During the exponential growth phase of YCL1 cells, the production of Cathepsin L1 is repressed. This is supported by the findings of Ruohonen *et al* (1991, 1995).

Enzyme production increases rapidly when cells are at the end of the glucose utilisation phase. Cathepsin L1 continues being expressed during the ethanol consumption phase but at a lower rate than during the latter stages of glucose consumption. It is suggested by Ruohonen *et al* (1991, 1995) that this may be because the presence of ethanol has a positive effect on protein production, or because of the absence of high concentrations of glucose.

If one wants to optimise the large-scale production regimes of a heterologous protein that has expressed in the molecular biology laboratory, it is worthwhile investigating environmental conditions, the correct media and the mode of cultivation. In the course of this project it has been confirmed that every organism has different optima in terms of cultivation conditions, and every expression system has its own favoured prerequisites for foreign enzyme production. In addition the heterologous protein is subject to denaturation when secreted into the surrounding media depending on the environmental conditions.

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Future Recommendations:

- Although supplementing selective media with yeast nitrogen base increased enzyme productivity by more than three fold it is an expensive method for the production of Cathepsin L1. By investigating the components of yeast nitrogen base, individual ingredients could be identified which would aid both cell proliferation and Cathepsin L1 expression.

- Up to this point fermentations of YCL1 have only been performed in batch mode. If fed-batch was employed instead, the feed rate could be controlled to maintain a low glucose concentration and a sufficient ethanol concentration. This should in theory prevent the repression of Cathepsin L1 expression and sustain a high level of enzyme production.

- Ruohonen *et al* (1991, 1995) observed a 29-fold increase in a-amylase production from S. cerevisiae when they truncated the *ADH1* promoter from 1500bp to ~400bp in length. Thus to continue employing this expression system and using *S.cerevisiae* as the host, investigating the possibility of genetically modifying the *ADH1* promoter to improve the yield of Cathepsin L1 should be instigated.

- In numerous citations, heterologous protein production has been improved significantly when *S. cerevisiae* has been replaced with a relatively novel yeast host, *Pichia pastoris*. Gellissen (2000), Cereghino and Cregg (2000), and Spencer *et al* (2002) have all reviewed heterologous protein production in *P. pastoris*. At present, the laboratory researchers who originally expressed recombinant Cathepsin L1 are attempting to express Cathepsin L1 in *P. pastoris*.

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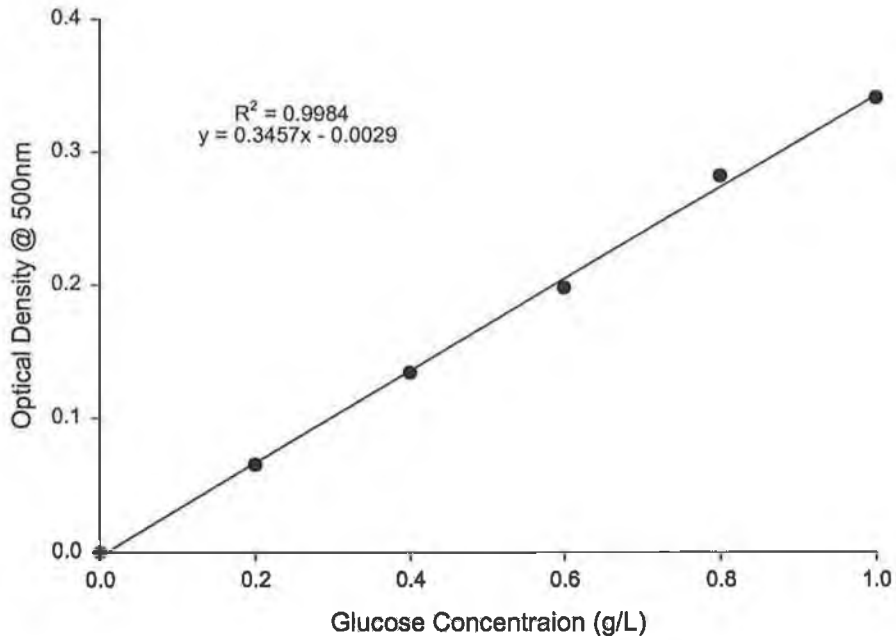
Appendix A - Standard Curves

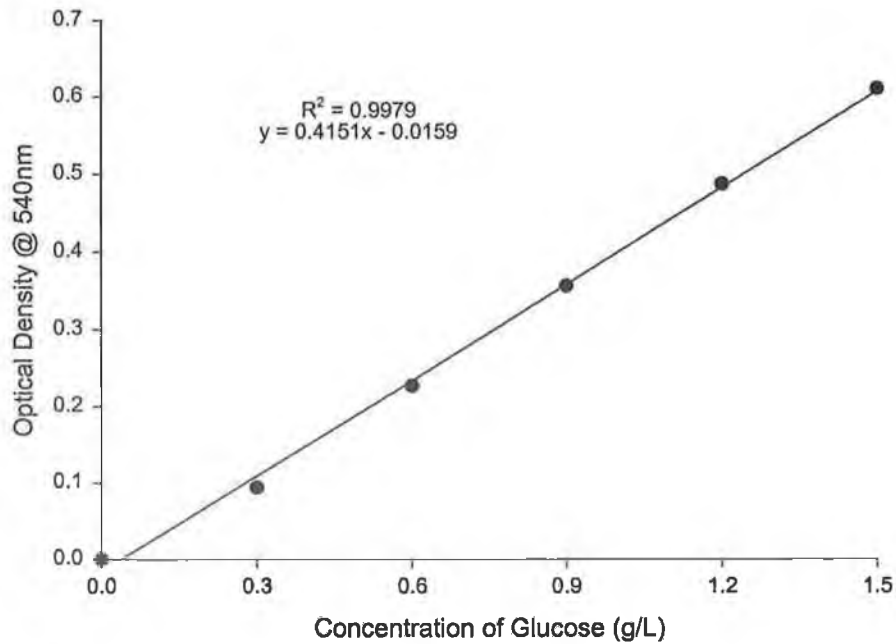**Figure A.1** Standard Curve for GOD-PAP Assay for Glucose

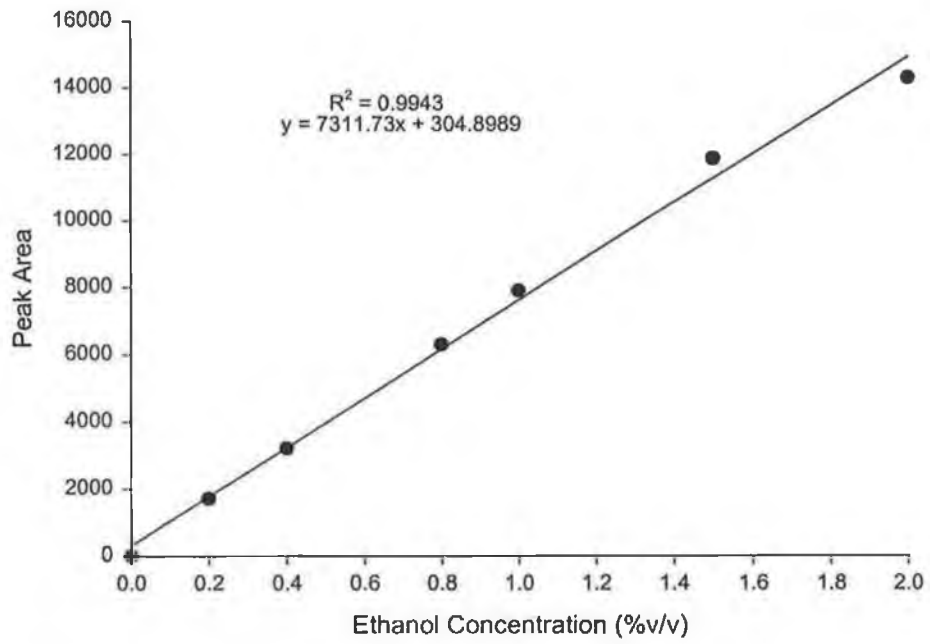**Figure A.2** Standard Curve for DNS Assay for reducing sugars

a

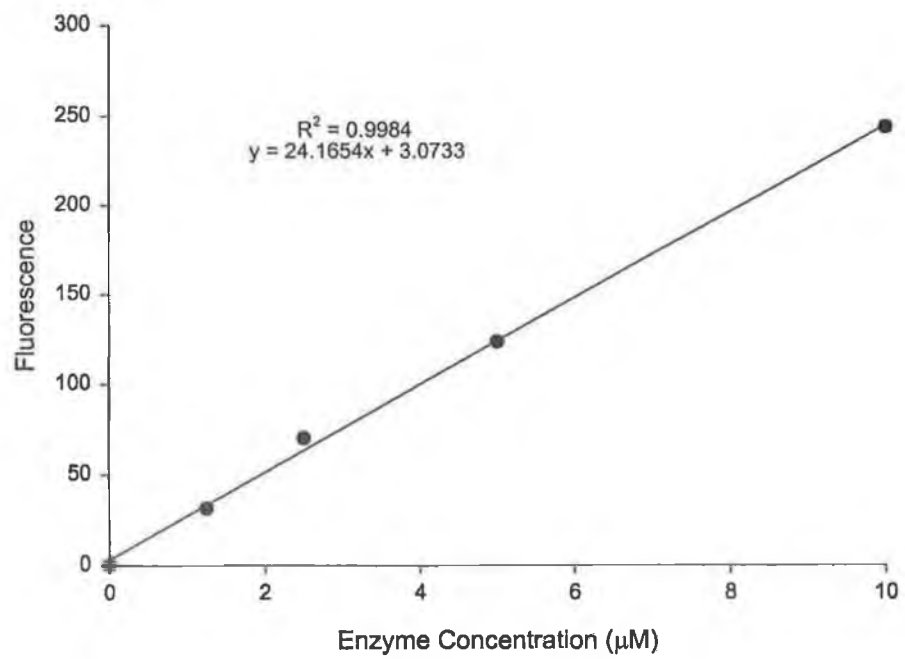**Figure A.3** Standard Curve for Ethanol

R² = 0.9943
y = 7311.73x + 304.8989

Figure A.4 Standard Curve for Enzyme Assay

R² = 0.9984
y = 24.1654x + 3.0733