HETEROLOGOUS PROTEIN PRODUCTION IN *KLUYVEROMYCES LACTIS*

by

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A thesis presented in partial fulfilment of the
requirements for the degree of Doctor of Philosophy
in Biotechnology and Bioprocess Engineering
at Massey University, Palmerston North,
New Zealand

1993

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ABSTRACT

In this study, the recombinant yeast *Kluyveromyces lactis* CBS 683 : pCR1 was investigated as a model system for the production of a heterologous protein in a whey-based medium.

The plasmid pCR1 has been constructed to express a wheat α-amylase enzyme in *K. lactis* strains. The construct is based on the vector pCXJ-kan1, which has been derived from pKD1, a native plasmid of *K. lactis* var. *drosophilarum* containing the essential regions for plasmid replication and stability. Construct pCR1 produces an α-amylase from DNA isolated from a wheat cDNA clone which is controlled by a *Saccharomyces cerevisiae* PGK promoter.

An electroporation method using a Bio-Rad Gene Pulser has been optimized for introducing heterologous DNA into *K. lactis* yeasts. Selection of transformants can be made using either the biosynthetic marker *URA*A or the G418 resistance gene, depending on whether the yeast is an auxotrophic mutant or a wild-type strain, respectively. Transformation was optimal at 4500 V cm^{-1}, 25 μF, and ∞ Ω with 0.2 μg plasmid DNA. Transformation efficiencies were comparable to those obtained using a PS10 Electropulsator, and were in the range 10^4-10^5 transformants per 10^7 cells per μg DNA. Twenty-nine *Kluyveromyces* strains were examined for efficiency of transformation and fermentation performance on rich glucose and rich lactose media under high and low aeration in batch culture. Of these, *K. lactis* CBS 141 and CBS 683 were chosen for recombinant studies.

The transformed yeasts *K. lactis* CBS 141 : pCR1 and CBS 683 : pCR1 were qualitatively shown to produce an active α-amylase enzyme. The α-amylase was produced at a low level but could be measured using a modified starch-iodine assay. A typical yield of 6 U ml^{-1} was obtained for batch growth of *K. lactis* CBS 683 : pCR1 in a rich lactose medium, where one unit is the amount of enzyme that will hydrolyze 0.1 mg starch in 30 minutes at 40°C when 4.0 mg starch is

present.

Both batch and continuous cultivation were used to investigate growth of the recombinant yeasts and, in particular, plasmid stability and protein production were examined. Three methods for measuring the stability of plasmid pCR1 in recombinant *K. lactis* were statistically analyzed and compared, and two, the plate ratio and clearing zones methods, were chosen for use in the fermentation studies. Initial batch fermentation studies indicated plasmid pCR1 to be extremely unstable in *K. lactis* CBS 141 : pCR1 and so only *K. lactis* CBS 683 : pCR1 was investigated further. Plasmid instability was also high in this latter yeast, with 50 - 60 % of cells becoming plasmid-free after 10 generations of non-selective growth in high aeration batch culture using a whey-based medium. In batch culture the stability of the plasmid pCXJ-kan1 was much higher, with minimal plasmid loss detected, and this indicated that the low stability of the plasmid pCR1 was probably due to the PGK-α-amylase DNA insert.

The stability of plasmid pCR1 was shown to improve by using low aeration conditions, selective medium, or a growth temperature of 20°C in both batch and continuous culture. The use of a selective medium and a lower temperature also allowed the level of α-amylase to be maintained for an increased fermentation time, and the latter also gave an increased specific yield of α-amylase in continuous culture.

Thus, this study has demonstrated the successful production of a wheat α-amylase from a *K. lactis* strain grown in a whey-based medium.

LIST OF PUBLICATIONS

This work has been published or presented in part in the following papers.

1. Russell, C.M., Mawson, A.J., and Yu, P.-L. (1991). Production of recombinant products in yeasts: a review. Australian J. Biotechnol. 5:48-55.

2. Russell, C.M., Mawson, A.J., Yu, P.-L., Jarvis, A.W., and Smart, J.B. (1991). Screening *Kluyveromyces* strains for potential use in a strain improvement programme. Presented at The First Congress of U.K. Biotechnology, Leeds, U.K. September 24-27.

3. Mawson, A.J., Russell, C.M., Wongso, D., Grubb, C.F., and Yu, P.-L. (1991). The development of whey fermentation processes using *Kluyveromyces* strains. Presented at The 4th Specialist Meeting on Biology of *Kluyveromyces*, Dusseldorf, Germany, September 21-22.

4. Russell, C.M., Jarvis, A.W., Yu, P.-L., and Mawson, A.J. (1993). Optimization of an electroporation procedure for *Kluyveromyces lactis* transformation. Biotechnol. Techniques 7:489-494.

5. Russell, C.M., Jarvis, A.W., Yu, P.-L., and Mawson, A.J. (1993). Expression and secretion of wheat α-amylase in *Kluyveromyces lactis*. Appl. Micro. Biotechnol. (in press).

ACKNOWLEDGEMENTS

I wish to thank my chief supervisor, Dr John Mawson, for his constant encouragement, advice, and guidance throughout this thesis. It was a pleasure to work with him.

I am indebted to Dr Audrey Jarvis and Dr John Smart, my supervisors at the New Zealand Dairy Research Institute (NZDRI), for being available when needed and for challenging, encouraging, and supporting the research ideas.

I also wish to thank my co-supervisor, Dr Pak Lam-Yu, for his interest in this project and support of funding.

I gratefully acknowledge the NZDRI and the Department of Process and Environmental Technology (PET, formerly the Biotechnology Department), Massey University, for both financial support and facilities which enabled this study. In addition, the NZDRI funding of the European trip in my second year of studies is greatly appreciated. I am also grateful to the Ministry of Research, Science and Technology (MoRST) for further personal financial support through a MoRST Study Award (formerly the DSIR Women's Study Award).

I wish to give special thanks to an exceptional woman friend, Tania Ngapo, who has incredible strength, enough to see the two of us through the past three-plus years' rollercoaster, and in addition teach me generosity and open up new interests.

Other people that deserve my thanks and thoughts are:
Dr Noemi Gutierrez and Assoc Prof Ian Maddox for their motivation and for being special friends,
Dr Catherine Day for discussion of work and for being a close friend,
Dr Wilhemina Cambourn for encouragement,
Dr Micheline Wesolowski-Louvel and Dr Hiroshi Fukuhara for the opportunity to work at the Institut Curie, Paris,
The PET technicians, Anne-Marie Jackson, Judy Collins, John Sykes, Janice Naulivou, Mike Sarahayam, and also John Alger, Bruce Collins, and Wayne Mallet for help and some memorable fun-times,
The other post-graduate students that have come and gone over the past three years and the present students,

Dr Grahame Manderson for interesting chats,
And my Biotechnology class - in particular Carolyn, Mary, Tracy, Barbs, and Ian S.

Thanks is also extended to my Mother, Father, and Grandmother for their confidence in me and encouragement, regardless of comprehending what it is I'm doing.

Finally, I give my love and gratitude to my partner, Tony, for coping with the traumas, planning weekend escapes, enlightening life, and encouraging me to live outside of my thesis.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Abbreviations of units

°C	degrees celcius
g	gram(s)
h	hour(s)
kb	kilobases
kDa	kilodaltons
l	litre(s)
M	molar
min	minute(s)
m	metre(s)
rpm	revolutions per minute
%(w/v)	percentage weight by volume
% (v/v)	percentage volume by volume
µF	microfarads
Ω	resistance ohms
s	second(s)
V	volts
yr	year

Other abbreviations

Ap	ampicillin
ARS	autonomous replicating sequence
A_x	absorbance at x nm
BOD	biological oxygen demand
cfu	colony forming units
D	dilution rate
DO	dissolved oxygen
DTT	dithiothreitol
DWM	diluted whey medium
EDTA	ethylenediaminetetraacetic acid
GC	gas chromatography
GRAS	generally recognised as safe
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HSA	human serum albumin
IPTG	isopropyl thiogalactopyranoside

IR	inverted repeat
Kan	kanomycin
LB	Luria Bertani broth
MM	minimal medium
MW	molecular weight
OD	optical density
ORF	open reading frame
ori	origin of replication
PEG	polyethylene glycol
R	rate of generation of plasmid-free cells
SDS	sodium dodecyl sulphate
WPC	whey protein concentrate
WYP	optimized whey medium
YPD	rich glucose medium
YPL	rich lactose medium
X-gal	5-bromo-4-chloro-3-indoyl galactopyranoside

CHAPTER 1

INTRODUCTION

This research extends ongoing programmes within the Department of Process and Environmental Technology aimed at efficient utilization of whey produced from the New Zealand dairy industry. Whey is a by-product of the cheese and casein industries and the volume produced annually is increasing, with the most recent New Zealand season (1992/93) producing over 3 x 10^6 m^3 yr^{-1} (Barnett, 1993). While much of this is used in the manufacture of products such as lactose, whey powders, and whey protein concentrates, a significant quantity, particularly of deproteinated whey, remains to be utilized. Some of it is used as fertilizer, with the remainder going to disposal via, for example, biological treatment. Whey disposal is undesirable since whey has a high biological oxygen demand (BOD), typically 40,000 mg l^{-1}. Whey is also a potential substrate for fermentation processes and many examples are described in the literature, recently reviewed by Sienkiewicz and Riedel (1990). The fermentation of whey to ethanol by the yeast *Kluyveromyces marxianus* has proved successful in New Zealand. This is now an established technology which has satisfied the local market for both potable and industrial alcohol and has a limited export market. There is little prospect for further plant expansion in the industry as anticipated increases in local demand can be met by the existing distilleries (Mawson, 1987). However, increasing quantities of whey are being produced, and with the introduction of the Resource Management Act in New Zealand in 1991, alternative and improved processes to utilize whey will have to be sought.

Recombinant DNA technology can be used to produce both industrially and/or medically important proteins. The ability to express heterologous genes in microorganisms allows the large-scale production of proteins which cannot be

produced in significant amounts from natural sources. Since the discovery that yeast cells could be transformed with exogenous DNA (Beggs, 1978; Hinnen *et al.*, 1978) there has been much interest in genetic manipulation of yeasts for the synthesis of new proteins and to both improve existing, and develop new strains, for industrial fermentations. Yeasts are eukaryotes that can be manipulated both biochemically and genetically in a similar manner to prokaryotes. However, the advantages yeasts offer over prokaryotes include their past and present use in the large scale production of food and beverage products, their general non-pathogenicity, and their ability to secrete, and to carry out simple post-translational modifications of proteins. Food yeasts are also on the FDA GRAS (Generally Recognised As Safe) list which facilitates the approval for their use for the production of recombinant food and medical products that are free from toxins. *Saccharomyces cerevisiae* is now established as one of the few organisms which have so far been successful in producing commercially viable heterologous products. Recently, other yeast hosts have been studied for the production of heterologous proteins. Commercial recombinant products currently manufactured by yeasts include a hepatitis B vaccine, insulin, and chymosin (Heslot and Gaillardin, 1992).

The aim of this project was to investigate the production of heterologous proteins from recombinant *Kluyveromyces* yeasts in whey-based media. The enhancement of the whey to ethanol fermentation by the co-production of a heterologous protein and ethanol was of particular interest. This offers the prospect of processing larger quantities of whey with higher economic return in addition to the continued success of the existing process. The economic returns for the existing operation is limited by both the low value of the product (ethanol) and the saturated New Zealand market.

The production of α-amylase by *K. lactis* was selected as a model system for studying the expression and stability of foreign genes in *Kluyveromyces*. The enzyme α-amylase is used in such industries as starch processing, baking, fruit and vegetable processing, and fuel alcohol production (Gerhartz, 1990).

Heterologous α-amylase from a variety of sources has been successfully produced in *S. cerevisiae*. To date there have been no reports of heterologous protein production from *Kluyveromyces* yeasts using whey as the substrate.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Of equal importance in considering heterologous protein production are the host-vector system, the genetic stability of the vector and the gene coding for the protein of interest, the choice of protein to be produced, and the substrate availability. Each of these points will be discussed in this review, with particular reference to yeasts, and the *Kluyveromyces* genus when possible.

2.2 HETEROLOGOUS PROTEIN PRODUCTION IN YEASTS

2.2.1 INTRODUCTION

Interest in heterologous protein production in yeasts can be considered to be two-fold: to extend the capabilities of existing yeast strains in, for example, bioconversion processes, and to produce medium or high-value proteins. The stages involved in developing a viable heterologous protein production system in yeasts include: transforming a suitable yeast host with an appropriate vector containing the exogenous DNA; selecting for the transformants using a marker; and analyzing for expression, secretion, and post-translational modifications of the final protein. Each of these aspects will be discussed with emphasis on the systems available for *Kluyveromyces* yeasts.

2.2.2 YEAST HOSTS

The success or failure of a process often depends on the initial choice of host organism. The requirements of a suitable host listed by Shuler and Kargi (1992)

include a high growth rate, the availability of vectors and promoters, good expression levels with protein secretion, and the ability to carry out post-translational modifications such as protein folding and glycosylation. The ability to use low cost media and media availability should also be considered. Organism safety is also significant, especially for those products destined for use in foods.

Most genetic manipulation studies of yeasts have been conducted using strains of *Saccharomyces cerevisiae* since it is presently the most commonly used in industry and a great deal of genetic information on this organism already exists (Schekman and Novick, 1982; Kingsman *et al.*, 1985; 1987; 1988; Bitter *et al.*, 1987; Bitter, 1987). *S. cerevisiae* strains are widely used, for example, in the baking, brewing, wine-making, and distilling industries. *S. cerevisiae* is on the FDA GRAS list which may facilitate obtaining regulatory approval for a new product.

However, Romanos *et al.* (1992) listed several disadvantages of using *S. cerevisiae* for protein production including the lack of very strong, tightly regulated promoters, the need to use fed-batch fermentation to attain high cell densities, and hyperglycosylation of proteins. Some of these problems have been addressed using molecular genetics by developing, for example, new and improved vectors and promoters. However, an alternative has been to develop expression systems in other yeasts to take advantage of, for example, promoter strength, secretion efficiency, and ease of growth to high cell density. These so-called 'non-conventional' yeasts offer unique systems which comprise a rich, largely unexploited, reservoir of biological and biochemical diversity (Weber, 1988; Weber and Barth, 1988).

Until recently, the genetics of most other non-*Saccharomyces* yeasts were poorly understood. This has been due to a number of factors, including the lack of a sexual cycle in many species, aneuploidy of these strains, and the limited number of investigators working on any one species. In the last few years more work has

been performed with non-*Saccharomyces* yeasts that have been selected for their particular growth characteristics or other favourable intrinsic properties. Some of these yeasts, such as *K. lactis* and *Yarrowia lipolytica*, are already commercially important at present. As more characterization studies are completed for other yeasts, these too may become important. A review by Weber (1988) discussed a specialized symposium on the genetics of non-conventional yeasts, which evaluated the trends in genetic, molecular biology, and biotechnological aspects. Klein and Zaworski (1990) noted in a review of transformation and cloning systems in non-*Saccharomyces* yeasts that differences exist between the alternative yeasts and *S. cerevisiae* that necessitate modifications of existing recombinant DNA protocols. However, they recognized that the wealth of knowledge gained in the development of *S. cerevisiae* provides an insight into what might be expected in the development of protein production systems in non-*Saccharomyces* yeasts.

There are already several examples of heterologous protein production systems developed for non-*Saccharomyces* yeasts, which are summarized in Table 2.1. A number of recent reviews describe the development of cloning systems for non-*Saccharomyces* yeasts, including Weber and Barth (1988), Klein and Zaworski (1990), Reiser (1990), Heslot and Gaillardin (1992), and Romanos *et al*. (1992). For example, *Pichia pastoris* uses an efficient and tightly-regulated promoter, and straightforward techniques for high-biomass cultivation. Comparative studies suggest it can be used to avoid limitations on transcription that are sometimes encountered with *S. cerevisiae*, and it appears to have the advantages of high-density growth and scale-up without a reduction in specific productivity (Romanos *et al*., 1992). *Hansenula polymorpha* has similar properties to *P. pastoris* in that it can be easily grown to high cell densities, resulting in high volumetric yields of secreted proteins. The expression system developed is also similar to that of *P. pastoris*. *Schizosaccharomyces pombe* is one of the most intensely studied and well-characterized of the non-conventional yeast species and the available cloning techniques have been reviewed by Russell (1989). However, *S. pombe* has mainly been used in the isolation and study of homologous mammalian

Table 2.1 Examples of heterologous proteins produced in non-*Saccharomyces* yeasts. (Adapted from Romanos *et al.* (1992)).

Yeast	Protein	Reference
Pichia pastoris	β-galactosidase	Tschopp *et al.* (1987)
	HBsAg	Cregg *et al.* (1987)
	Tetanus toxin fragment C	Clare *et al.* (1991a)
	Pertactin	Romanos *et al.* (1991)
	TNF	Sreekrishna *et al.* (1989)
	Streptokinase	Hagenson *et al.* (1989)
	SOD	Thill *et al.* (1990)
	S. cerevisiae invertase	Tschopp *et al.* (1987)
	Bovine lysozyme	Digan *et al.* (1989)
	Human EGF	Brierly *et al.* (1990)
	Murine EGF	Clare *et al.* (1991b)
	Aprotinin	Thill *et al.* (1990)
Hansenula polymorpha	β-lactamase	Janowicz *et al.* (1988)
	HBsAg	Shen *et al.* (1989)
	PreS1-S2-HBsAg	Janowicz *et al.* (1991)
	α-galactosidase	Fellinger *et al.* (1991)
	Glucoamylase	Gellison *et al.* (1991)
	HSA	Hodgkins *et al.* (1990)
	S. cerevisiae invertase	Janowicz *et al.* (1988)
	Pre-S2 HBsAg	de Roubin *et al.* (1991)
Kluyveromyces lactis	Prochymosin	van den Berg *et al.* (1990)
	IL-1β	Fleer *et al.* (1991a)
	HSA	Fleer *et al.* (1991b)
	tPA	Yeh *et al.* (1990)
	TIMP	Yeh *et al.* (1990)
	HBsAg	Martinez *et al.* (1992)
Yarrowia lipolytica	β-galactosidase	Gaillardin and Ribet (1987)
	S. cerevisiae invertase	Nicaud *et al.* (1989)
	Bovine prochymosin	Franke *et al.* (1988)
	Porcine IFN	Heslot *et al.* (1990)
Schizosaccharomyces pombe	Polyoma middle-T Ag	Belsham *et al.* (1986)
	β-galactosidase	Kudla *et al.* (1988)
	CAT	Toyama and Okayama (1990)
	Human epoxide hydrolase	Jackson and Burchell (1988)
	Factor XIIIa	Broker and Bauml (1989)
	IBD virus VP3	Jagadish *et al.* (1990)
	E. coli β-gluronidase	Pobjecky *et al.* (1990)
	Single-chain Ab	Davis *et al.* (1991)
	Bacterio-opsin	Hildebrandt *et al.* (1989)
	STP1 glucose transporter	Sauer *et al.* (1990)
	S. cerevisiae invertase	Moreno *et al.* (1985)
	S. diastaticus glucoamylase	Erratt and Nasim (1986)
	S. cerevisiae α-mannosidase	Kuranda and Robbins (1987)
	S. cerevisiae exoglucanase	Kuranda and Robbins (1987)
	S. cerevisiae endochitinase	Kuranda and Robbins (1987)
	Antithrombin III	Broker *et al.* (1987)
	S. occidentalis α-amylase	Strasser *et al.* (1989)

genes, rather than for protein production.

K. lactis and *Y. lipolytica* have both been examined largely because of their capacity for high level protein secretion. *Y. lipolytica* has the ability to grow to high cell density at industrial scale and has been investigated for use in a number of industrial processes, for example, the bioconversion of alkanes and fatty acids into alcohol, and the production of single cell protein from *n*-paraffins (Romanos *et al.*, 1992). *K. lactis* has been used in the food industry for many years in the production of β-galactosidase (lactase). Therefore, its large scale cultivation has been extensively studied, and it is well accepted for the production of proteins for human use. The ability to grow on cheap substrates, such as lactose and whey, further increases its potential as a host for the production of heterologous proteins, especially for lower value products. *K. lactis* has well-developed molecular genetics and, unlike most other non-*Saccharomyces* yeasts studied to date, it has the advantage of highly stable episomal vectors (Romanos *et al.*, 1992). *Kluyveromyces* as a host organism for heterologous protein production has been found to be superior to *S. cerevisiae* both for intracellular and extracellular production of proteins (Rietveld *et al.*, 1988).

2.2.3 YEAST TRANSFORMATION

Transformation of yeast cells is more complicated than transformation of prokaryotes because the cell wall, which is mainly composed of glucans and mannoproteins, presents an additional barrier to DNA insertion (Schekman and Novick, 1982; Zoltnik *et al.*, 1984). Consequently, established protocols for DNA-mediated transfer either involve removal of the cell wall or pretreatment to render the cell wall "leaky" to DNA. The three main methods for yeast transformation are spheroplast transformation, chemical treatment with alkali cations or thiol compounds, and electroporation. These will be described below and then their application to *Kluyveromyces* yeasts will be discussed.

The efficiency or frequency of transformation depends on the genetic background

of the host, the plasmid genetics, and the type of selection applied, as well as the method of transformation employed (Heslot and Gaillardin, 1992). However, the use of recombinant DNA technology in yeasts does not necessarily manipulate the genome in a precise and completely defined way, and it may be necessary to analyze a number of transformants when optimizing expression. Transformation may also be mutagenic, both for the host cell (Shortle *et al.*, 1984) and for the introduced DNA (Clancy *et al.*, 1984), but the frequency of mutation has been shown to be low enough that it is not a major concern. More recently, Danhash *et al.* (1991) have reported that transformation introduces a heritable slow-growth phenotype in *S. cerevisiae*, and Higgins and Strathern (1991) have reported that electroporation of *S. cerevisiae* cells stimulates recombination events. However, the mechanism is not understood and the extent of plasmid-chromosome recombination has not been determined.

2.2.3.1 The spheroplast method

Spheroplast transformation of *S. cerevisiae* was first achieved by Hinnen *et al.* (1978) who removed the cell wall with the enzyme β-glucanase, exposed the spheroplasts to Ca^{2+} and DNA, and finally treated them with polyethylene glycol (PEG). The transformed cells were then washed and embedded in a selective, isotonic agar for regeneration of the cell wall. Transformation protocols based on this work have since been modified by fusion of yeast spheroplasts with DNA-containing liposomes (Russel and Stewart, 1985) or with *E. coli* minicells harbouring yeast-*E. coli* shuttle plasmids (Gyuris and Duda, 1986). This has improved the transformation frequency from 10^3-10^4 transformants per μg DNA to 10^6-10^7 transformants per μg DNA in some cases, as noted by Esser and Kaemper (1988).

2.2.3.2 Chemical pretreatments with alkali cations or thiol compounds

The use of alkali cations or thiol compounds (for example, 2-mercaptoethanol) in the presence of PEG permits transformation of intact yeast cells with plasmid

DNA (Ito *et al.*, 1983; 1984). Several alkali metal ions such as Ca^{2+}, Li^{+}, Na^{+}, K^{+}, Cs^{+}, and Rb^{+} have been shown to be effective. The cation requirement (i.e., monovalent versus divalent) was found to be strain specific, some strains being transformable only in the presence of Ca^{2+}, others only with Li^{+} (Bruschi *et al.*, 1987). The method has been reviewed by Kimura (1986) and is widely used despite the fact that it generally gives lower transformation frequencies than the spheroplast method, typically about 10^3 transformants per µg DNA (Becker and Guarente, 1991). However, a modification has recently been reported which uses dimethyl sulfoxide to increase the frequency about 25-fold (Hill *et al.*, 1991). As with spheroplast transformation, the mechanisms of transformation using chemical pretreatment are poorly understood (Heslot and Gaillardin, 1992).

2.2.3.3 Electroporation

Transformation by electroporation involves application of an electric pulse to a suspension of protoplasts or cells in a buffer containing DNA. The high voltage electric field causes the temporary formation of pores in the cell membrane, thereby allowing the exogenous DNA to enter the cells. Electroporation has been used to transform higher plant protoplasts (Fromm *et al.*, 1985), mammalian cells (Neumann *et al.*, 1982), filamentous fungi (Ward *et al.*, 1989), yeast protoplasts (Karube *et al.*, 1985) and intact yeast cells (Hashimoto *et al.*, 1985; Delorme, 1989; Gysler *et al.*, 1990; Simon and McEntee, 1990; Becker and Guarente, 1991).

Kimura (1986) reported that the transformation frequency obtained by electroporation was lower than the above two methods, however, Meilhoc *et al.* (1990) described an electroporation procedure which has a high efficiency of transformation. They reported transformation frequencies of 10^7 transformants per µg DNA for *S. cerevisiae* and 10^4 transformants per µg DNA for *Y. lipolytica*. The main difference between this and the previous reports for intact yeast cells is that Meilhoc *et al.* (1990) used a square wave electric pulse whereas the other studies used electric pulses with exponential decay. It was suggested that the

square wave pulses allowed greater control over the electric field strength and pulse duration, which appeared to be two of the crucial parameters for successful transformation of yeasts.

2.2.3.4 Transformation of *Kluyveromyces*

Transformation of *K. lactis* was first accomplished by Das and Hollenberg (1982) using a 2μ-based vector and a modification of the spheroplast protocol reported by Beggs (1978). The transformation efficiency was extremely low (4 transformants per μg DNA) which was found to be due to inefficient spheroplast regeneration. Several isosmotic stabilizers were screened and the replacement of sorbitol with 0.6 M KCl increased the regeneration efficiency by a factor of three. Whole cell transformation using the LiCl method has been adapted for *K. lactis* (Mizukami and Hishinuma, 1988), and was also used by van den Berg *et al.* (1990) to obtain a recombinant *K. lactis* strain to commercially produce chymosin. Recently, there have been four reports of transformation by electroporation of *K. lactis* (Meilhoc *et al.*, 1990; Bolen and McCutchan, 1992; Russell *et al.*, 1993; Sanchez *et al.*, 1993).

2.2.3.5 Summary of transformation methods

At present the spheroplast method appears to be that most commonly used for transformation of yeasts, since it generally gives the highest transformation efficiency and is well-established. However, it is time-consuming and can be limited by the regeneration step (Heslot and Gaillardin, 1992). Transformation by chemical pretreatments is quick and simple, and does not require spheroplast formation or regeneration in agar. The advantages of electroporation for transformation of intact yeast cells with foreign DNA include ease and efficiency without removal or chemical pretreatment of the cell wall. The techniques have been developed for *S. cerevisiae* and, as noted for electroporation, each does not transform the same or different yeast strains to the same efficiency. Transformation efficiencies typically range from 10^2 to 10^5 transformants per μg

plasmid DNA (Higgins and Strathern, 1991), and it is generally accepted that the methods need to be adapted for the yeast strain of interest to obtain optimum transformation conditions.

2.2.4 YEAST VECTORS

There are many different types of plasmids which have been used to express heterologous genes in yeasts. These have already been described in many reviews including Botstein and Davis (1982), Struhl (1983), Kingsman *et al.* (1985), Sturley and Young (1986), Carter *et al.* (1987), Bitter *et al.* (1987), Esser and Kaemper (1988), Heslot and Gaillardin (1992), and Romanos *et al.* (1992).

Plasmid vectors available for yeast transformation generally contain an *E. coli* origin of replication (ori) and a drug-resistant marker (usually an antibiotic). These inclusions allow propagation and manipulation of plasmid DNA in *E. coli* before introduction of the plasmid into the desired yeast recipient. Such plasmids are named shuttle vectors as they may be 'shuttled' between *E. coli* and yeasts. They must also include a yeast marker to permit selection in yeasts, unique multiple cloning sites for insertion of heterologous DNA, and DNA sequences for efficient replication in yeasts. Figure 2.1 shows a generalized scheme of a typical shuttle vector.

2.2.4.1 Selection systems

Selection for *S. cerevisiae* transformants is generally via biosynthetic genes which complement the corresponding auxotrophy in the recipient. Ratzkin and Carbon (1977) complemented the *E. coli LEU*B mutant with a fragment of yeast DNA, which was shown to complement the yeast *LEU*2 gene (Hinnen *et al.*, 1978). Other genes have been directly selected from the yeast genome by their ability to complement specific auxotrophic mutations in *E. coli* and some of these are listed in Table 2.2. *S. cerevisiae* genes have been widely used for complementing auxotrophs of other yeast species (Weber and Barth, 1988), for example, *URA*4

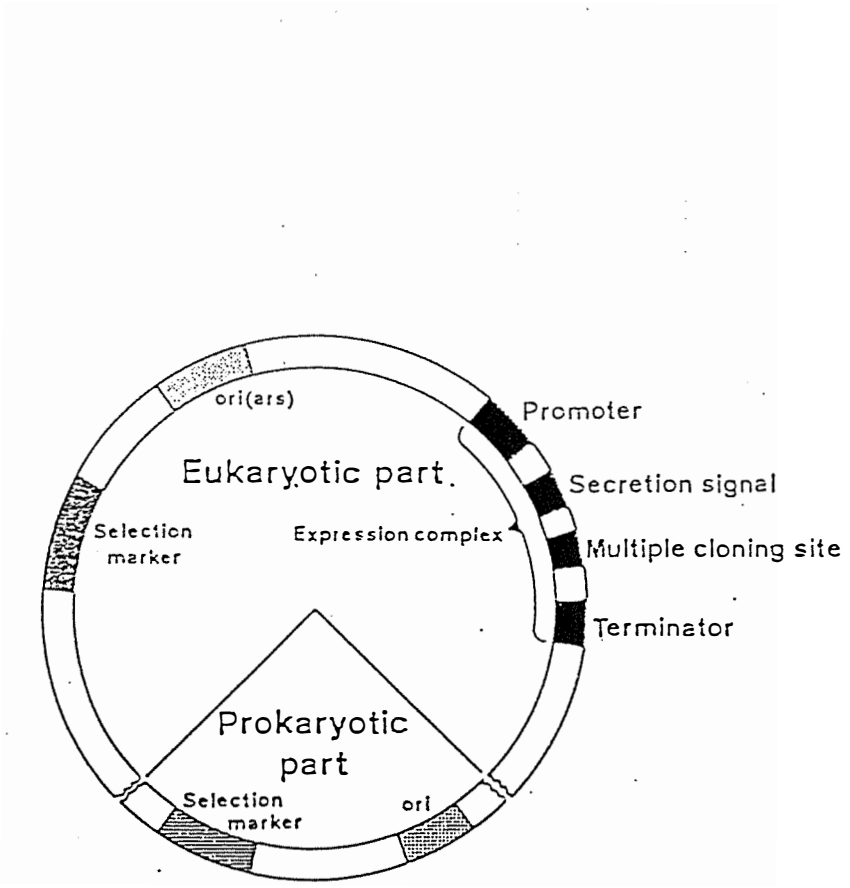


Figure 2.1 Generalized scheme of a shuttle vector for eukaryotic cloning. (From Esser and Kaemper (1988)).

Table 2.2 Examples of marker genes for yeast vectors. (From Russell *et al.*, 1991).

Biosynthetic pathways complementation marker genes		
Marker gene	**Gene product**	**Reference**
HIS3	imidazole glycerol phosphate dehydratase	Struhl *et al.* (1977)
LEU2	β-isopropyl malate dehydrogenase	Ratzkin and Carbon (1977)
URA3	orotidine-5'-phosphate decarboxylase	Bach *et al.* (1979)
TRP5	tryptophan synthetase	Walz *et al.* (1978)
ARG4	arginosuccinate lyase	Clarke and Carbon (1978)

Resistance marker genes		
Marker gene	**Resistance to**	**Reference**
chloramphenicol acetyltransferase	chloramphenicol	Hadfield *et al.* (1986)
dihydrofolate reductase	methotrexate	Zhu *et al.* (1985)
phleomycin resistance gene	phleomycin	Gaillardin and Ribet (1987)
aminoglycoside phosphotransferase	G418	Jimenez and Davies (1980) Sreekrishna *et al.* (1984)
hygromycin phosphotransferase	hygromycin	Gritz and Davis (1983) Kaster *et al.* (1984)
metallothionein	copper ions	Butt *et al.* (1984)
TCM1	trichodermine	Fried and Warner (1981)
CYH2	cycloheximide	Fried and Warner (1982)
methylgloxal resistance gene	methylgloxal	Murata *et al.* (1985)
zinc resistance gene	zinc ions	Kimura (1986)
cadmium resistance gene	cadmium ions	Kimura (1986)
thymidine kinase	sulphanomide amethopterin	Zhu *et al.* (1984) McNeil and Friesen (1981)
RIM-C	cycloheximide	Takagi *et al.* (1986)

in *S. pombe*, *LEU2* in *Y. lipolytica*, *HIS4* in *P. pastoris*, and *ADE2* in *Candida albicans*. A number of selection markers are available for *K. lactis*. Examples include *S. cerevisiae TRP1* (Das and Hollenberg, 1982) and *URA3* (De Louvencourt *et al.*, 1983), and *K. lactis TRP1* (Stark and Milner, 1988), *URA3* (Shuster *et al.*, 1987), and *LAC4* (Das and Hollenberg, 1982). This selection technique is the most common method to identify yeast transformants but can only be used with yeast strains with defined auxotrophic mutations. The selection medium must lack the amino acid, for example, leucine, uracil, tryptophan, or histidine, to identify the transformants. Unless the plasmid is highly stable, a selective pressure must also be applied by cultivating the transformed strain on a medium lacking the corresponding metabolite.

Industrial yeast strains may be polyploid, which makes it difficult or impossible to obtain appropriate auxotrophs. For that reason, dominant selectable markers have been developed. Although yeasts are relatively insensitive to antibiotics some dominant selectable drug resistance markers which are available are also listed in Table 2.2. These resistance markers can be used for selection in wild-type and/or industrial yeast strains. For example, resistance to G418 is encoded by the *E. coli* aminoglycoside phosphotransferase gene, part of the Tn601 transposon. G418 is a 2-deoxystreptamine antibiotic structurally related to gentamycin but inhibits protein synthesis in a much wider range of prokaryotes and eukaryotes (Jimenez and Davies, 1980). The G418-resistance gene has been used successfully in *K. lactis* (Das and Hollenberg, 1982; Sreekrishna *et al.*, 1984) but was claimed to transform *S. cerevisiae* with only 10 % the efficiency of an auxotrophic marker (Jimenez and Davies, 1980). More recently, Macreadie *et al.* (1991) have developed a copper-resistance selection system for *K. lactis* based on the *CUP*1 gene encoding metallothionein of *S. cerevisiae*.

2.2.4.2 Types of vectors

There are two types of transformation events that occur in most yeasts - integration and autonomous replication. Integration involves the homologous recombination of DNA into the yeast genome, so that the DNA is under control of the replication mechanisms of the yeast itself. If the DNA can be maintained and replicated as an episome, separate to the yeast genome, then the vector is capable of autonomous replication. Several different types of vectors have been developed for *S. cerevisiae* that transform yeasts by either of these mechanisms. The general properties and uses of these plasmids are shown in Table 2.3.

The first yeast-cloning vector described by Hinnen *et al.* (1978) was a yeast integrating vector (YIp) which consisted of a bacterial cloning vector carrying a yeast selection gene (in this case *LEU*2). YIp vectors lack a yeast origin of replication so to survive they must integrate into the yeast genome where they can replicate and avoid being degraded.

Table 2.3 Properties of the more common yeast vectors used in yeast transformation. (Adapted from Esser and Kaemper (1988), Struhl (1983), and Old and Primrose (1989)).

Vector	Origin of replication	Transformation frequency[a]	Copy number per cell	Stability[b] mitosis	Stability[b] meiosis	Advantages	Disadvantages
YIp	non-autonomous	10^0-10^1	1-10	0.1	1-10	- high stability of cloned genes - integrated YIp behaves as ordinary genetic marker - useful for surrogate yeast genetics (e.g. changes in chromosome via deletions, inversions, transpositions)	- low transformation frequency - cannot recover original vector
YRp	ARS	10^2-10^4	10-20	30	90	- high copy number - high transformation frequency - can readily be recovered from yeast - useful in complementation studies - can integrate into the chromosome	- low stability of transformants
YCp	ARS	10^2-10^4	1-10	30	90	- low copy number useful if heterologous gene product harmful to yeast - high transformation frequency - useful in complementation studies	- low copy number therefore recovery from yeast more difficult than YRp and YEp
YEp	2μ	10^3-10^5	100	30	90	- high copy number - high transformation frequency - can readily be recovered from yeast - useful in complementation studies	- generation of novel recombinants *in vivo* by recombination with endogenous 2μ plasmid

[a] transformants per μg DNA

[b] loss in non-selective medium, % per generation

Some YIp vectors were demonstrated by Stinchcombe *et al.* (1979) to be maintained extrachromosomally. They contained an autonomously replicating sequence (ARS) which functioned as a replication origin for the plasmid in yeasts. These plasmids were named yeast replicating plasmids (YRp). However YRp vectors were shown to be exceedingly unstable and segregated unevenly upon cell division (Murray and Szostak, 1983b). This unstable nature was exploited by Clarke and Carbon (1980) to identify plasmids which contain yeast centromeres (CEN). The centromeres stabilized the yeast construct, and the vectors were called yeast centromeric plasmids (YCp).

The most commonly used vectors for the expression of heterologous proteins in yeasts are the yeast episomal plasmids (YEp). YEp vectors usually contain part of the 2μ native plasmid of *S. cerevisiae*, which is made up of both a CEN sequence and an ARS sequence. There are several other sequences on the 2μ plasmid which are involved in plasmid maintenance, including the genes A-D, which code for FLP, REP1, REP2, and gene product D (Caunt *et al.*, 1988; Romanos *et al.*, 1992; Heslot and Gaillardin, 1992). Other yeast species, such as *Kluyveromyces*, also have their own native plasmids with sequences which may be used to construct other YEp vectors. *Kluyveromyces* vectors will be discussed further in the following section (Section 2.2.4.3).

Other vectors which may be used include expression, linear, and promoter plasmids (Parent *et al.*, 1985). Expression plasmids (YXp) have translational promoters and terminators, and sequences that direct post-translational processing and secretion. Yeast telomeres (ends of chromosomes) have been combined with a centromere and ARS sequence on a linear plasmid (YLp) (Murray and Szostak, 1983a). Yeast promoter plasmids (YPp) have easily-assayed protein-coding sequences that allow the fusion and analysis of homologous and heterologous promoters (Bitter *et al.*, 1987). These were also reviewed extensively by Heslot and Gaillardin (1992).

2.2.4.3 Vectors for *Kluyveromyces*

Transformation systems for *K. lactis* were initially developed by isolating ARS sequences from this organism, since neither the *S. cerevisiae ARS*1 nor 2μ sequences were found to replicate in *K. lactis* (Das and Hollenberg, 1982; Sreekrishna *et al.*, 1984). However, as with *S. cerevisiae*, *K. lactis* ARS vectors were found to be highly unstable and so have limited use in expression systems. Potential alternative vector systems are provided by both linear and circular plasmids which have both been isolated from *Kluyveromyces* strains.

Two cytoplasmic linear plasmids, k1 (8.9 kb) and k2 (13.4 kb) have been discovered in killer strains of *K. lactis* and have been reviewed by Gunge (1986), Stark *et al.* (1990), and Heslot and Gaillardin (1992). These plasmids are stably maintained at 50 to 100 copies per cell. The regions of k1 that encode killer toxin can be deleted without affecting maintenance. Attempts have been made to use the linear killer DNA plasmids as cloning vectors in *K. lactis* (de Louvencourt *et al.*, 1983). However, their 5'-termini are covalently linked to protein, hindering manipulation *in vitro* and amplification in *E. coli*, and they encode their own cytoplasmic transcription system which does not recognize nuclear promoters (Kaemper *et al.*, 1989a; 1989b). The difficulties in manipulation can be overcome by targeted integration of foreign DNA into native k1. If conventional selection markers are used, for example *LEU2*, this results in linear nuclear plasmids containing telomeres (Kaemper *et al.*, 1989b). By fusing such markers to k1 promoters, recombinant linear plasmids which are cytoplasmic and stable can be generated (Kaemper *et al.*, 1989b; Tanguy-Rougeau *et al.*, 1990). It should be possible to use this system for foreign gene expression, although the k1/k2 promoters appear to be rather weak and further development may be necessary.

The most successful episomal vector system developed to date for *K. lactis* transformation is based on stable high-copy *K. lactis* expression vectors which have been constructed from the *K. drosophilarum* plasmid, pKD1 (Falcone *et al.*, 1986; Bianchi *et al.*, 1987). This plasmid, shown in Figure 2.2, is a 1.6μm circle

of 5,757 base pairs that comprises roughly 2 % of the total cellular DNA. There are three open reading frames (ORFs) (A, B, and C) and a pair of inverted repeats (IR1 and IR2), with unique restriction sites available in each of the ORFs. Two isomers exist, form A differs from form B by an inversion of the relative orientation of the two parts separated by the inverted repeats. Although there is little sequence similarity, pKD1 is organizationally very similar to the *S. cerevisiae* 2μ plasmid in that it encodes analogous replication, amplification, and segregation functions (Chen *et al.*, 1986). Several different vectors based on pKD1 have been constructed which behave similarly to the 2μ vectors, such as pCXJ-kan1 shown in Figure 2.3. These vectors have recently been used to produce recombinant human interleukin-1β (Fleer *et al.*, 1991a) and human serum albumin (HSA) (Fleer *et al.*, 1991b) in *K. lactis*.

An integrating vector system was used by van den Berg *et al.* (1990) to produce bovine prochymosin from *K. lactis*. The vectors contained the promoter and terminator of the β-galactosidase (lactase) gene, the prochymosin encoding DNA and signal sequence, the *E. coli* vector pUC19, and the marker conferring resistance to G418. The prochymosin produced was found in the culture medium and on acid treatment was converted into active chymosin indistinguishable from the natural enzyme from calf rennet. For large-scale production of chymosin, a well-characterized *K. lactis* strain was selected and transformed with the appropriate plasmid, in such a way that several copies of the plasmid were integrated. The recombinant strain appeared quite stable, with no decrease in chymosin production and no DNA rearrangement being detected after 45 generations. These results have led to the development of a process at the 41,000 litre scale, with the chymosin successfully tested in cheese trials (van den Berg *et al.*, 1990).

Integration has also been used by Martinez *et al.* (1992), who inserted the hepatitis B surface antigen into the *K. lactis* genome and controlled its expression using β-galactosidase regulatory sequences.

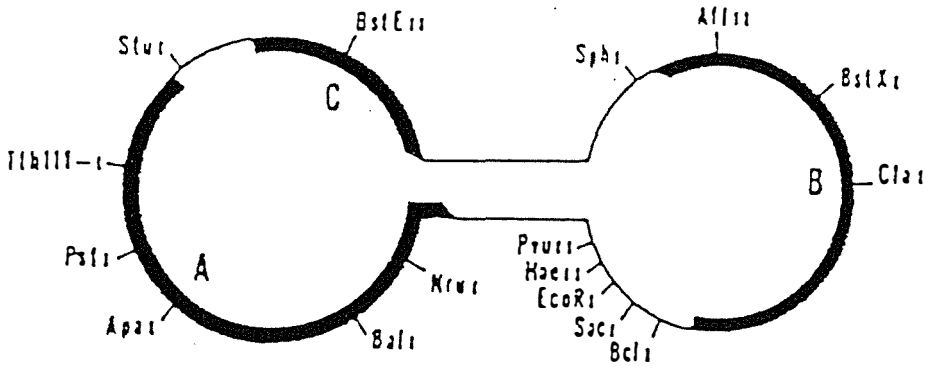


Figure 2.2 **The structure of plasmid pKD1, form B. The three open reading frames are indicated by Gene A, B, and C. The central stem represents the inverted repeats. Some of the unique restriction sites are shown. (From Bianchi *et al.* (1987)).**

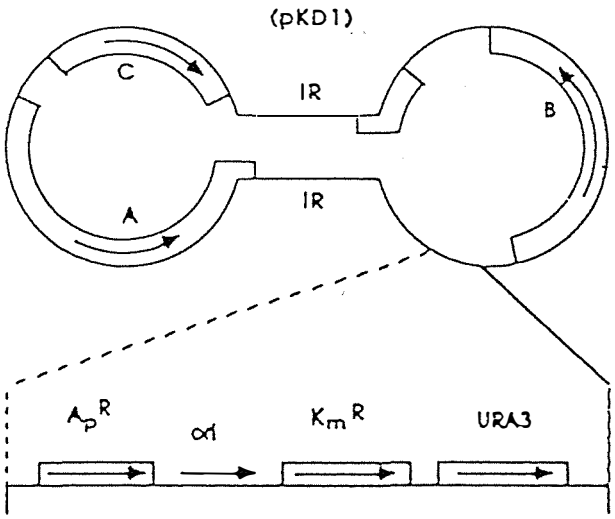


Figure 2.3 **The structure of plasmid pCXJ-kan1, a cloning vector based on pKD1 and used for *Kluyveromyces lactis* transformation. Bacterial plasmid and marker sequences were introduced into a neutral region of pKD1. (From Wesolowski-Louvel *et al.*, 1990).**

2.2.5 EXPRESSION, SECRETION, POST-TRANSLATIONAL MODIFICATIONS

2.2.5.1 The expression complex

For a transformed yeast strain to express the heterologous gene, the vector must contain various DNA sequences and signals which regulate the processing of an introduced DNA sequence into a protein. These collectively are named the "expression complex". The expression complex comprises a promoter and a terminator surrounding the multiple cloning sites for heterologous gene insertion, and it may also contain signals for protein secretion.

2.2.5.2 Promoters

Promoters originating from yeast genes are used since promoters are usually host-specific and those from higher eukaryotes tend to have a lower efficiency of initiation of transcriptions when used in yeasts (Beggs *et al.*, 1980; Rothstein *et al.*, 1984). Table 2.4 gives some common examples of promoter elements derived from yeasts.

Table 2.4 Some of the more common promoter elements used for heterologous gene expression vectors in yeasts.

Promoter element	Abbreviation	Reference
acid phosphatase	*PHO5*	Kramer *et al.* (1984) Meyhack *et al.* (1982)
alcohol dehydrogenase	*ADH1*	Hitzeman *et al.* (1981) Bennetzen and Hall (1982)
galactokinase	*GAL1*	Stepien *et al.* (1983) Goff *et al.* (1984)
glyceraldehyde-3-phosphate dehydrogenase	*GAP3*	Bitter and Egan (1984) Holland and Holland (1980)
mating factor-α	MFα	Bitter *et al.* (1984) Kurjan and Herskowitz (1982)
phosphoglycerate kinase	PGK	Dobson *et al.* (1982a) Tuite *et al.* (1982)
triose phosphate isomerase	TPI	Alber and Kawasaki (1982)

Promoters for glycolytic enzymes are widely used as these enzymes are among the best represented proteins in a yeast cell. Each can represent 1-5% of total cell protein, even though they are often encoded by single copy genes. It was assumed that this high-level expression was due to the association of strong promoters with these genes (Goodey *et al.*, 1987). Some of these promoters are inducible or repressible (Brent, 1985). This trait is used to achieve regulated expression of heterologous proteins in yeasts. As an example, the transcription of the acid phosphatase gene is strongly repressed when inorganic phosphate is present in the growth medium and is induced by depletion of inorganic phosphate. Other examples have been discussed by Kingsman *et al.* (1985), Goodey *et al.* (1987), Heslot and Gaillardin (1992), and Romanos *et al.* (1992). Inducible/repressible systems will also be discussed further when considering stability (Section 2.3.3.3.6).

Only a small number of promoters have been used in *K. lactis* expression vectors, the best characterized being the *K. lactis LAC4* gene, encoding β-galactosidase (lactase), which is induced up to 100-fold by lactose or galactose. Several *S. cerevisiae* promoters are active in *K. lactis* and have been used for foreign gene expression, for example *PHO5* and PGK (Fleer *et al.*, 1991a). In future, promoters from the recently isolated *K. lactis* genes encoding glyceraldehyde-3-phosphate dehydrogenase (Shuster, 1990) and alcohol dehydrogenase (Saliola *et al.*, 1990; Shuster, 1990) may be used.

2.2.5.3 Protein secretion

A number of recent reviews cover the subject of protein secretion including Heslot and Gaillardin (1992), and Romanos *et al.* (1992). Generally it is useful to have the protein product of heterologous genes secreted from the yeast cell, since many proteins expressed internally may be toxic to the cell, and secretion facilitates recovery of the protein (Goodey *et al.*, 1987). In addition, many eukaryote proteins of industrial interest are normally secreted in the native system, with some produced as precursor proteins containing signal sequences

that are proteolytically removed to produce the mature proteins. In the larger precursor form, the NH_2-terminal amino acid of the protein will usually not be methionine. Direct expression of the corresponding genes in yeasts will require a methionine codon be put in front of the mature coding sequence, giving a protein produced with a NH_2-terminal methionine. If this methionine is not removed by a yeast enzyme, the resulting analogue of the protein may have reduced activity (Heslot and Gaillardin, 1992). Also, many proteins contain internal disulphide bonds. The cytoplasm of yeasts is a reducing environment which prevents the accurate formation of these bonds leading to incorrect folding of the heterologous protein and reduced activity. However, when a protein with disulphide bonds passes through the yeast secretory pathway, the disulphide bonds appear to be correctly formed (Heslot and Gaillardin, 1992). The mechanism of secretion in yeasts appears to be similar to that of higher eukaryotes (Schekman and Novick, 1982). However, heterologous gene products derived from higher eukaryotes and produced in yeasts are not secreted in high amounts, indicating that the secretion signals for these genes are not correctly recognized in the yeast cell. Therefore signal sequences from proteins naturally secreted from yeasts are usually used, such as that for acid phosphatase (Perlman and Halvorsen, 1983; Smith *et al.*, 1985), the α-mating type factor (Julius *et al.*, 1984), and invertase (Perlman and Halvorsen, 1983).

Heslot and Gaillardin (1992) and Romanos *et al.* (1992) noted that *K. lactis* is more efficient at secreting foreign proteins than *S. cerevisiae*. Signal peptides derived from HSA, the *K. lactis* killer toxin α-subunit, and the prepro peptides of α-factor from both *K. lactis* and *S. cerevisiae* have been used (Romanos *et al.*, 1992). For example, in marked contrast to *S. cerevisiae*, prochymosin was efficiently secreted by *K. lactis* in a fully soluble, acid-activated form, using a single-copy integration vector carrying the *LAC*4 promoter (van den Berg *et al.*, 1990). About 80% of the prochymosin produced was secreted even when expressed without a signal peptide, although the overall level was reduced. The highest levels were obtained using the native leader peptide or the α-factor prepro sequences from either *K. lactis* or *S. cerevisiae*.

Other heterologous proteins have been secreted using pKD1-derived vectors. Fleer *et al.* (1991b) described the secretion of HSA using its own secretion signal and that of the *K. lactis* killer toxin α-subunit. The level of HSA produced was highly strain-dependent and this was largely due to differences in stability of the vector. Using the *S. cerevisiae* promoter, the highest-expressing strain produced about 300 mg l^{-1} HSA in shake-flasks. In high density (80-90 g l^{-1} dry weight of cells) fed-batch fermentations several g l^{-1} HSA were produced from cultures of up to 1000 litres. The secretion of interleukin 1β (IL-1β) by *K. lactis* has also been reported by Fleer *et al.* (1991a) using the toxin α-subunit signal. The signal peptides were accurately and efficiently processed, but only about 20% of the product was secreted (80 mg l^{-1} in shake-flasks) in this case.

2.2.5.4 Post-translational processing and glycosylation

Recent studies for *S. cerevisiae* have indicated that yeasts are capable of carrying out *in vivo* post-translational modifications of eukaryotic proteins. Some of these modifications, which include acetylation, phosphorylation, glycosylation (addition of carbohydrate chains), proteolytic cleavage of signal sequences, and tertiary structure folding to give the correct three-dimensional conformation, are essential for the stability of the expressed heterologous proteins, for solubility, or for the expression of biological activity. Many reviews discuss these points (Smith *et al.*, 1985; Kingsman *et al.*, 1985; 1987; 1988; Bitter *et al.*, 1987; Romanos *et al.*, 1992; Heslot and Gaillardin, 1992).

2.2.6 SUMMARY

Development of heterologous protein production systems in yeasts have concentrated on the well-defined *S. cerevisiae*. However, some alternative yeasts have recently been assessed as having more suitable properties for heterologous protein production, and so the knowledge developed for *S. cerevisiae* has been used as a base to exploit these yeasts. Of particular interest to this study, *Kluyveromyces* yeasts have been found to have improved secretional properties

compared with *S. cerevisiae*, and already some heterologous protein production systems have been reported.

The three methods used to transform yeasts are the spheroplast method, chemical pretreatment with alkali cations, and electroporation. The latter is the most recently reported method and has the advantages of ease and efficiency over the others. In addition, the reported transformation efficiencies are as high as for the spheroplast method, the most commonly used method due to its high transformation efficiencies and well-established protocol. All three methods have been used for *Kluyveromyces* transformation, however electroporation of *K. lactis* has only been reported very recently.

The 'vehicle' used to carry the heterologous DNA into the yeast cell is the vector, of which a number of types have been developed. The vector usually has shuttle characteristics allowing manipulation of DNA in *E. coli*, and DNA coding for the gene of interest, selection mechanism(s), and processing and/or secretion of the heterologous DNA. Depending on the type of vector, the heterologous DNA may be integrated into the host genome, or may be maintained and replicated as an episome. Vectors have been available for *S. cerevisiae* since 1978, and since then much development of these has occurred. However, these vectors have met with little success when trialled in non-*Saccharomyces* yeasts. A more effective route has been to find native plasmids of the non-*Saccharomyces* yeast of interest and develop these into viable vectors. This path has been successful for *Kluyveromyces* yeasts, with the native pKD1 plasmid of *K. drosophilarum* being developed for the production of heterologous proteins from *K. lactis*.

Finally, yeasts, being eukaryotes, have advantages over prokaryotic systems for producing heterologous proteins in that they can bring about post-translational modifications, such as glycosylation, of the protein. The successful production of many proteins which require these modifications for full biological activity has been demonstrated in *Saccharomyces*, and more recently, non-*Saccharomyces* yeasts.

2.3 PLASMID STABILITY IN YEASTS

2.3.1 INTRODUCTION

Plasmid stability is one of a number of factors influencing productivity of fermentations employing recombinant bacteria and yeasts. Plasmid stability can be defined as the ability of transformed cells to maintain a multi-copy plasmid unchanged during cell growth, manifesting its phenotypic characteristics (Imanaka and Aiba, 1981). The stability of a plasmid must be considered in the scale-up of a fermentation involving recombinant micro-organisms. Recently, researchers have begun to study and model the fermentation processes with respect to relationships between microbial physiology, plasmid copy number, gene expression for host/vector systems, and plasmid stability. Studies of stability of recombinant yeasts are at the early stage when compared to recombinant bacteria, so in the following discussion reference will be made to bacterial systems where there is little or no information for yeasts in a particular area. Also, unless otherwise noted, stability studies in yeasts refer to *S. cerevisiae*.

2.3.2 TYPES OF STABILITY

The stability of a plasmid is a function of the genetics of the plasmid and is strongly influenced by the genetics and physiology of the host. In the same host, different plasmids can exhibit different degrees of stability and the same plasmid can show different levels of stability in different hosts (Zabriskie and Arcuri, 1986). Plasmid stability is often measured and defined in terms of its antonym, i.e. plasmid instability. Plasmid instability can be subdivided into two major types: structural instability and segregational instability.

2.3.2.1 Structural instability

Structural instability is the change in plasmid structure due to deletions, insertions, or rearrangements of DNA. These are physical changes and are

sometimes difficult to characterize as they can result in the loss of a desired gene function, yet the DNA coding for the selectable markers may be retained. As a result, the effects of structural instability are harder to define and this type of stability appears not to be as common as segregational instability (Bailey *et al.* 1986).

2.3.2.2 Segregational instability

Caunt *et al.* (1988) defined segregational instability as the loss of the complete plasmid due to defective partitioning between the parent and daughter cells during cell division. Until recently, segregational instability has been measured only as a fall in the number of plasmid-containing cells due to plasmid-free cells being formed at a low frequency. An improved definition for apparent segregational instability incorporates two factors: the probability of generating a plasmid-free cell from a plasmid-bearing cell and the growth rate differential between these two different types of cells (Impoolsup *et al.*, 1989a). Plasmid-bearing cells allocate a portion of their resources to support plasmid-related activities, which include maintenance and replication of plasmid DNA, and expression of plasmid genes. There is a growing body of evidence that this additional metabolic burden results in slower growth of plasmid-bearing cells than that of plasmid-free cells under comparable cultivation conditions (for example: Imanaka and Aiba, 1981; Walmsley *et al.*, 1983; Zabriskie and Arcuri, 1986; Parker and DiBiasio, 1987; Wittrup and Bailey, 1988). Further, the growth rate differential between these two cell types usually increases with increased plasmid DNA content and/or increased expression of plasmid genes. This growth rate advantage of plasmid-free cells allows them to outgrow plasmid-containing cells and eventually take over the entire population, causing a decline in productivity of the plasmid-encoded products (Ryan and Parulekar, 1991).

2.3.3 FACTORS AFFECTING STABILITY AND METHODS OF INCREASING STABILITY

2.3.3.1 Introduction

Zabriskie and Arcuri (1986) have reviewed the effects of plasmid replication, plasmid stability, and heterologous gene expression, together with other factors, on the fermentation process and product yield in recombinant bacterial systems. They also noted the possible relationship between plasmid stability, plasmid copy number, and growth rate of recombinant yeasts. Caunt *et al.* (1988) reviewed the major factors affecting the stability of yeast strains carrying multi-copy recombinant plasmids and described strategies for overcoming stability problems. It is usual to remedy structural instability at the genetic level by careful construction of the plasmid to reduce the likelihood of recombination events. The effects of apparent segregational instability may be reduced in a number of ways. Factors which must be considered are plasmid copy number, physiological consequences of plasmid gene expression, and environmental stresses imposed on host cells, including the conditions of growth and rate of cell growth. Each of these points will be considered below.

2.3.3.2 Genetic factors

2.3.3.2.1 Host genetics

Studies of the plasmid pBR325 in *E. coli* suggested that differences in host genotype may affect plasmid stability (Noack *et al.*, 1981). Jones and Melling (1984) reported different rates of loss of the same plasmid in different *E. coli* strains in chemostat cultures. Incompatibility of plasmids is of primary concern with recombinant bacteria and can show itself in one of two ways (Caunt *et al.*, 1988):

(1) the mutual exclusion of two distinct but related plasmids from a cell following non-selective growth; or

(2) when two plasmids cohabit a cell, the number of copies of either plasmid in the cell is less than the number of copies of either plasmid when it is the sole occupant of the cell.

Livingston (1977) observed that complete incompatibility of native 2μ plasmids from different sources does not occur in yeasts. However, 2μ plasmids in yeasts have been shown to exhibit the second aspect of bacterial incompatibility in up to three ways:

(1) the presence of an introduced 2μ plasmid in a cell reduces the number of copies of endogenous 2μ plasmids (Gerbaud and Guerineau, 1980);
(2) the presence of endogenous 2μ plasmids in yeasts reduces the copy number of the introduced 2μ plasmid (Jarayam *et al.*, 1983); and
(3) certain introduced plasmids can convert ('cure') strains containing endogenous 2μ plasmids into 2μ-free cells (Dobson *et al.*, 1980).

The presence or absence of endogenous 2μ plasmids in yeasts was also found to have an important influence on the segregational stability of 2μ-based vectors (Beggs, 1978). For example, Blanc *et al.* (1979) showed that stability of introduced plasmids was increased in *S. cerevisiae* strains containing no endogenous 2μ plasmids. Walmsley *et al.* (1983) discovered that the presence of 2μ sequences represented a selective advantage in the host cells used. It appears as if a certain level of 2μ-type molecules or gene products is advantageous, whilst some higher level is a burden to the cell (Caunt *et al.*, 1988). The presence of certain 2μ sequences on either the hybrid vector or the native 2μ is necessary for the propagation of plasmids in the cells (Caunt *et al.*, 1988; Romanos *et al.*, 1992; Heslot and Gaillardin, 1992).

Bianchi *et al.* (1987) have constructed several different types of vector based on pKD1 (shown in Figure 2.2) for *K. lactis* which behave similarly to the 2μ vectors. Vectors carrying just the *cis*-acting replication element, located near one of the inverted repeats, can be maintained in host strains which have resident pKD1. However, vectors containing the entire pKD1 plasmid are significantly more stable

and can be maintained in a *K. lactis* host strain as shown by Chen *et al.* (1989). Such vectors are highly stable in pKD1-free strains, even in the absence of selection, although stability is somewhat reduced in hosts containing pKD1, perhaps due to incompatibility or competition.

Mead *et al.* (1986a) reported that a 2μ-based plasmid showed enhanced stability in a diploid yeast strain when compared to its haploid parents. The endogenous 2μ plasmid is also more stable in diploid compared to haploid strains (Mead *et al.*, 1986b). This was suggested to be due to an increased copy number in the diploid and reduced selective burden of the plasmid on the host.

Transformation and curing techniques in yeast may cause some deleterious effects to the transformed cells, as discussed in Section 2.2.3. Mead *et al.* (1987) found transformants to have a slower growth rate than either of the parental strains, and Danhash *et al.* (1991) have suggested that transformation introduces a slow-growth phenotype in *S. cerevisiae*. This effect on phenotype may be directly consequential of detrimental effects on genotype, therefore these effects must be considered for transformed cells.

2.3.3.2.2 Plasmid genetics

There is much interest in improving stability of vectors for yeasts in order to improve protein production systems. Jones and Melling (1984) showed that some insert sequences increased stability while others decreased stability in bacteria. Therefore, stability does not appear to be related to size alone but also to both the presence and expression of foreign DNA. Gerbaud *et al.* (1979) and Futcher and Cox (1984) found that 2μ-based plasmids were much less stable when inserts were included. It is not certain whether this was due to increased size, a higher copy number, or an increased metabolic load on the cell. For *K. lactis*, the unique *Eco*R1 site adjacent to one of the inverted repeats of the plasmid pKD1 can be used to insert foreign DNA without interruption of plasmid functions (Chen *et al.*, 1989). This also has been shown to have no effect on the high stability of

the natural plasmid. However, the insertion of some protein coding sequences have been found to affect the stability, as noted by Fleer *et al*. (1991b), but this was attributed to increased metabolic burden rather than increased plasmid size.

Yeast integrating plasmids (Ylp) may be used to increase stability. Survival of the DNA requires integration into the host genome by homologous recombination (Parent *et al*., 1985). Very stable transformants can be obtained but the copy number of the DNA is usually only one thus giving low expression levels. Also, excision and subsequent loss of integrated genes may still occur.

Plasmid loss may be overcome using a selective mechanism, for example, the use of plasmids which code for antibiotic resistance in hosts which are normally sensitive to the antibiotic. Plasmid-free cells which do not carry the antibiotic gene are not viable in a selective medium containing the antibiotic. An advantage of this system is that contaminant cells are also less likely to survive (Stephens and Lyberatos, 1988). However, significant disadvantages include the cost of the antibiotic and as a result, there is increasing interest in other methods of overcoming plasmid instability which allow the use of cheaper, less well-defined media. Other problems are the probability of contaminating the final product with trace amounts of the antibiotic and that the plasmid may confer antibiotic resistance to the host genome by insertion mechanisms, enabling plasmid-free cells to survive (Rowlands, 1984; Chang and Lim, 1987).

Another selection method is the use of auxotrophic mutants and plasmids that encode for the essential amino acid or nutrient that the host lacks. This also has problems in development of a useful process due to the higher costs of a defined medium. As for antibiotic selection systems, there may be some structural instability problems such as the cell losing the capability of expressing the foreign DNA whilst retaining the selective mechanism.

Using the auxotrophic basis of selection, Marquet *et al*. (1986) and Loison *et al*. (1986) developed a *S. cerevisiae* double mutant strain to increase plasmid

stability. The strain was defective in two separate places in the uracil biosynthesis pathway and was transformed with a plasmid which corrected for one of these mutations. This resulted in plasmid-containing cells being able to grow in non-selective medium, whilst plasmid-free cells were non-viable. Marquet *et al.* (1986) concluded from their culture studies that plasmid loss had been eradicated in the double mutant host. However, Das and Campbell (1990) interpreted the results differently - the double mutant host does not prevent plasmid loss but renders plasmid loss undetectable. Since, by design, plasmid loss is lethal, plasmid-free cells must eventually cease to proliferate and to synthesize the heterologous protein. However, before this occurs, cells may continue to multiply for several, or even many, generations (Murray and Szostak, 1983a; Futcher and Cox, 1984). This mechanism may be explained by Srienc *et al.* (1986), who noted that it is not the selection gene itself which endows the host cell with the selective growth phenotype but the product of that gene, either RNA or protein, which performs the function essential for growth in selective medium. Therefore, if the cell loses the plasmid carrying the selection gene it may still survive as the essential nutrient can be stored in the cell or may be extracellularly expressed by other cells, leading to plasmid-free cells being formed which contain complementing product. Plasmid-free cells have been shown to survive up to six generations after plasmid loss (Murray and Szostak, 1983a), as depicted in Figure 2.4. Thus, the use of selective media may only reduce plasmid instability rather than eliminate it completely (Kleinman *et al.*, 1986).

A further potential selection system incorporates features of the killer strains of *S. cerevisiae*, which harbour a virus-based killer system, reviewed by Wickner (1986) and Tipper and Bostian (1984). Killer strains kill sensitive yeast strains by secreting a protein toxin (zymocin) to which they are themselves immune (Bussey 1988). It appears that the same gene codes for both the toxin and the immune system. These genes could be incorporated into a plasmid, enabling plasmid-containing cells to be immune to the toxin whereas plasmid-free cells would be sensitive. However there are disadvantages in the use of such a system, for example zymocins are heat-labile, subject to protease digestion and active at a

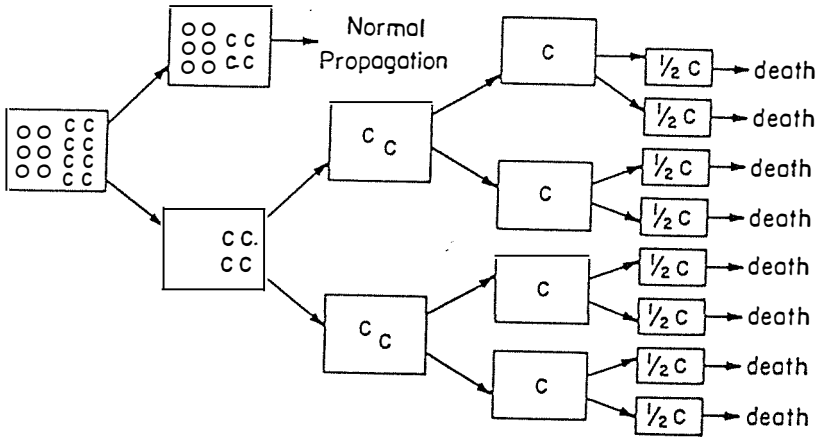


Figure 2.4 **Reduction of complementing factor in plasmid-free cells. Newly generated plasmid-free cells usually contain sufficient complementing factor (C) to withstand killing by a selective agent or starvation from the lack of a growth factor. In this case, the plasmid-free cell undergoes three divisions before the complementing factor is reduced to an ineffective level. (From Shuler and Kargi (1992)).**

pH less than five (Caunt *et al.*, 1988). A killer system based on DNA plasmids also exists in *K. lactis* strains as described previously (Section 2.2.4.3).

Das and Campbell (1990) noted that generally such strategies may appear to improve the efficiency of protein production, although plasmid instability is not completely eliminated by these approaches.

2.3.3.2.3 Effect of copy number

The factors which control copy number and its effect on stability appear to be complex and relate to the specific growth rate and level of recombinant protein production. These, in turn, are dependent on both the genetics of the plasmid and host, and the growth conditions. Theoretically, a high copy number, which causes a large decrease in the growth rate, is expected to give higher instability (Caunt *et al.*, 1988). Since the growth rate differential between plasmid-free and plasmid-bearing cells is expected to increase with increasing plasmid DNA content and enhanced production of plasmid-encoded product(s), plasmid-bearing cells with high copy numbers will therefore be at a disadvantage while competing with their plasmid-free counterparts for the necessary nutrients. Furthermore, excessive foreign DNA content, as well as over-production of non-essential products such as recombinant proteins, may be deleterious to the host and the host itself may self-impose constraints on the extent of plasmid-related activities (Cheah *et al.*, 1987; Ryan and Parulekar, 1990; 1991).

Walmsley *et al.* (1983) investigated the maximum specific growth rate, μ_{max}, for a yeast containing three plasmids with different copy numbers. The plasmid with the highest copy number caused the largest decrease in μ_{max}, whilst the plasmid present at one copy per cell had no effect on μ_{max}. Theoretically, they expected high instability from a large decrease in μ_{max} caused by increased copy numbers. However, experimentally it was found that increased copy number enhanced stability. Others have also shown that if the copy number of a 2μ plasmid is high then the stability is high (Jarayam *et al.* 1983; Futcher and Cox, 1984). Hinnen

and Meyhack (1982) also indicated that segregational instability in yeasts was lower with higher copy number. These results may be explained by the probability of plasmid transfer to daughter cells: if a copy number is high then there is an increased chance of at least one plasmid transferring during cell division. A few studies have reported the existence of an optimum plasmid copy number for expression of cloned genes for recombinant *S. cerevisiae* systems (Seo and Bailey, 1986; Ryan and Parulekar, 1990)

2.3.3.2.4 Effect of heterologous gene expression

Plasmid stability in yeasts has also been observed to be inversely related to gene expression. For example, increased expression of a plasmid acid phosphatase gene lowered plasmid stability (Parker and DiBiasio, 1987), and plasmid stability was also found to be low when a plasmid-encoded toxin was highly expressed (Lee and Hassan, 1987). Gopal *et al.* (1989) found that expression of heterologous prochymosin in *S. cerevisiae* imposed a large energy drain on the host and suggested it to be a limiting factor in recombinant systems. A possible explanation for this is that increased transcription interferes with plasmid replication, hinders segregation, and/or overburdens the DNA repair mechanism of the cell. In addition to gene expression and copy numbers, it is also important to consider the effect of growth rate on stability. This will be discussed under environmental factors (Section 2.3.3.3.1).

2.3.3.3 Environmental factors

The manipulation of environmental conditions may result in increased or decreased stability of a plasmid in a particular host. Simulations integrating gene expression and fundamental biological parameters, such as plasmid copy number, transcription efficiency and translation efficiency, have shown the importance of growth rate and reactor operating conditions on plasmid stability (Caunt *et al.*, 1988).

2.3.3.3.1 Effect of growth rate

A number of workers have shown that the specific growth rate has an effect on plasmid stability. Kleinman *et al.* (1986) showed that in selective medium the stability of a 2μ plasmid increased with increasing growth rates. DiBiasio and Sardonini (1986) also found increased 2μ plasmid loss at low growth rates using a selective medium. It was suggested that yeasts divide with larger buds at faster growth rates, and these larger buds increase the chance of plasmid transfer to the daughter cell. Da Silva and Bailey (1991a) also investigated the influence of dilution rate in selective continuous fermentations of recombinant yeasts and also found increased plasmid stability with increased dilution rates. Similarly, Caulcott *et al.* (1987) found that the apparent stability of a low copy number plasmid in *E. coli* was higher at faster growth rates. Prediction of the segregational instability and the differences in the growth rates showed that the former was the primary cause of plasmid loss, rather than the growth rate differences. However, Impoolsup *et al.* (1989a) found results contrary to all of these in that the apparent plasmid stability decreased with increasing growth rate in non-selective media in continuous culture. They postulated that the larger bud theory was unlikely and suggested that when cells are growing faster they make more mistakes during each cell division. Recombinant 2μ plasmids are also thought to have defective amplification systems, so at faster growth rates the cells will have less time to correct mistakes made during any one division, leading to higher levels of segregational instability.

Therefore stability is affected by two types of growth rate effects: the overall growth rate of the culture, and the difference in growth rate between the plasmid-bearing and plasmid-free cells, as discussed in Section 2.3.2.2. However, the effect of growth rate on a given yeast-plasmid combination is not yet resolved. With more studies on different systems and investigations such as the quantitation of plasmids contained in buds at various growth rates, the effect of growth rate may be determined.

2.3.3.3.2 Effect of medium composition

Jones and Melling (1984) have shown plasmid stability in recombinant cells to decrease during phosphate or glucose-limited growth, and Ensley (1986) used nitrogen-limited growth to stabilize a recombinant culture of *Pseudomonas*. In a study by Caulcott *et al.* (1987), the primary reason for loss of plasmid from *E. coli* was the segregational instability of the plasmids rather than the growth rate differences, and this instability could be reduced by growth under phosphate or nitrogen limitation. Other studies of recombinant *E. coli* were inconclusive as to the effects of glucose, ammonia, or phosphate limitation on stability (Godwin and Slater, 1979; Jones *et al.*, 1980; Noack *et al.*, 1981; Caulcott *et al.*, 1985), but Shoham and Demain (1990) attributed this to the failure in these studies to differentiate between effects on segregational instability, structural instability, or on the growth rates of plasmid-free and plasmid-bearing cells.

Little work has been reported on optimizing heterologous protein production in yeasts by manipulating the medium composition. de Roubin *et al.* (1991, 1992) investigated production of *H. polymorpha* biomass and pre-S2 antigen and found that the nutritional conditions for good antigen expression were different from those for good biomass production. In particular, antigen production was greatly enhanced by the addition of complex organic nitrogen sources. Thus, the authors noted that the level of heterologous protein produced may not be proportional to the yield of biomass.

2.3.3.2.3 Effect of dissolved oxygen (DO)

Oxygen tension has significant effects on the metabolism of micro-organisms, and so it may be expected that it may also have an effect on plasmid stability. This has been confirmed by Hopkins *et al.* (1987) who caused an exponentially-growing recombinant *E. coli* system to become unstable by a DO shock, in which the air was turned off for 30 minutes causing a fall in the air saturation value from 80% to 10%.

Caunt *et al.* (1990) showed that plasmid stability was slightly higher when recombinant yeast cells were grown in air alone compared to in oxygen-enriched air, and considerably higher than in nitrogen-enriched air. This is consistent with a previous report by Lee and Hassan (1987), who used a 2μ-based plasmid in *S. cerevisiae* to discover that transformants grown in air gave the highest plasmid stability when compared to growth in nitrogen and pure oxygen. However, plasmid stability increased as a function of dilution rate, regardless of the oxygen tension used (Lee and Hassan, 1987).

2.3.3.3.4 Effect of growth temperature

Temperature appears to affect not only cell growth but also plasmid copy number and plasmid stability in recombinant cultures. Aiba and Koizumi (1984) showed that plasmid stability in *Bacillus stearothermophilus* was high at temperatures under 50°C, but became progressively less stable as the temperature was increased above 50°C. Son *et al.* (1987) studied the effect of temperature on the stability of a plasmid and expression of a cloned cellulase gene in *Bacillus megaterium* . Results from batch culture showed stability fell at temperatures above 30°C. However, plasmid copy number and efficiency of gene expression increased with temperature elevation.

2.3.3.3.5 Effect of cycling environmental parameters

Weber and San (1988) showed that cycling of growth rate using square-wave oscillations resulted in an increased lag before plasmid-free cells appeared in a recombinant culture. Impoolsup *et al.* (1989b) and Caunt *et al.* (1990) improved the stability of a yeast plasmid using deliberately induced cyclic changes in the DOT during continuous culture in a non-selective, undefined medium. They showed the system to be completely stabilized at lower growth rates using this technique. Stephens *et al.* (1992) demonstrated that, when starting with a mixed culture of plasmid-free and plasmid-bearing cells, it was both possible to stabilize and even increase the proportion of plasmid-bearing cells by cycling. Substrate

cycling has also been used by others to increase stability (Gu *et al.*, 1989b; Parker and DiBiasio, 1987).

Stephens and Lyberatos (1988) modelled the use of cycling substrate levels to manipulate growth rates in order to stabilize recombinant organisms. They proposed that plasmid-containing cells are slower to adapt to changes in substrate concentration than the plasmid-free cells due to the recombinant cells having an extra burden on cell metabolism. During the periods of substrate limitation in a cycling regime, plasmid-free cells are growing below their maximum growth rate and therefore have a lower mean growth rate. By manipulating the cycle times and amplitudes, the normal growth rate differences between plasmid-free and plasmid-containing cells can be reduced, leading to an increased stability. If the plasmid-free cells are faster in adapting to environmental changes, then it may be possible to give a competitive edge to the plasmid-containing population through cycling.

2.3.3.3.6 Separation of growth and product formation

High instability has been attributed to energy expenditure on both plasmid maintenance and high expression levels. Greater stability may therefore be conferred by separating these functions, and such control of heterologous gene expression may also be advantageous when the synthesized product is deleterious to the host cell, or when gene expression significantly reduces cell growth. Separation of growth and gene expression may be achieved using a two-phase culture, where cultures are grown rapidly to high cell density in the uninduced state, then synthesis of the heterologous protein is induced by a change in temperature or by the removal of the repressor or addition of inducer.

A number of studies have used temperature-shift techniques to examine gene expression from recombinant *E. coli* cultures (Siegel and Ryu, 1985; Lee *et al.*, 1988; Hortascu and Ryu, 1990; Park and Ryu, 1990). Temperature-sensitive control of expression in *E. coli* allows high productivity for longer periods than in

conventional systems. Sledziewski *et al.* (1988) have described the construction of a temperature-regulated recombinant yeast promoter. Different levels of expression of β-galactosidase were obtained from a recombinant *S. cerevisiae* yeast by adjusting the growth temperature. At the permissive temperature (25°C) β-galactosidase was produced at high levels, and at the restrictive temperature (35°C) transcription of *LACZ* was repressed. Between these two temperatures intermediate levels of β-galactosidase were produced, indicating the level of heterologous product could be altered by adjusting the temperature. Potential disadvantages of temperature-shift induction are the possible deleterious effects of the temperature change on the host cell. Da Silva and Bailey (1989b) indicated that a non-optimal temperature for the yeast host may increase proteolysis and give a lower overall efficiency of growth, with lower growth rate, lower yield, and increased maintenance requirements.

2.3.3.3.7 Immobilization

Immobilization of recombinant cell cultures onto a solid surface offers potential as an alternative for achieving plasmid stability. Marin-Iniesta *et al.* (1987) and Huang *et al.* (1989) showed immobilized *E. coli* cultures to have both increased stability and specific growth rate in continuous culture when compared with free cells. Walls and Gainer (1989) showed that surface immobilization of plasmid-bearing yeast increased plasmid stability in continuous culture. It has also been shown that immobilization of recombinant cells can increase their productivity with respect to heterologous product formation (Okita *et al*, 1985; Doran and Bailey, 1986; Groom *et al.*, 1988; Walls and Gainer, 1991).

2.3.4 MODELS OF RECOMBINANT FERMENTATION SYSTEMS

The factors influencing plasmid stability in recombinant cultures are varied and complex, as described in Section 2.3.3. Models may be used to develop a greater understanding of the biological interactions and suggest possible routes to optimize operating conditions necessary to maximize protein production in

recombinant cell cultures. Cooper *et al.* (1987) stated that models may be used to predict in advance how unstable a plasmid may be, however the actual recombinant systems are far too complex for this approach to be reasonable and therefore the proposed models have not been used by many workers in the field. This is because there is a lack of experimental information for determining the validity of some of the models, and there can be many combinations of host, plasmid, and fermentation conditions possible (Satyagal and Agrawal, 1989).

Growth models can generally be classified as (Zabriskie and Arcuri, 1986):

(1) structured, when various molecular mechanisms are included;
(2) unstructured, when modelling details are limited to the cellular level;
(3) segregated (or single cell models), where cell to cell variations are considered;
(4) non-segregated (or population models), in which all cells are assumed to be homogeneous.

The frequently used Monod equation (Monod, 1949), for modelling continuous culture or the exponential phase of batch growth, is of the following form:

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad (2.1)$$

where:

μ = specific growth rate (h^{-1})
μ_{max} = maximum specific growth rate (h^{-1})
S = limiting substrate concentration (g l^{-1})
K_s = substrate concentration when $\mu = 1/2\ \mu_{max}$ (g l^{-1})

The model is both unsegregated and unstructured and assumes balanced growth. This is only true in the exponential growth phase and when the population is not too large and growth is relatively slow. This means the model has limitations as many fermentation systems fall outside these criteria. However, because of its simplicity, the model, or modified equations based on it, are still frequently used.

General models based on the Monod equation were developed over thirty years ago by Moser (1958) for modelling genetic stability during continuous culture of micro-organisms. More recently, a number of investigations have attempted to analyze quantitatively the growth of recombinant micro-organisms by introducing key kinetic parameters. These have included the frequency of generation of plasmid-free daughter cells from plasmid-containing parent cells and the specific growth rate difference between plasmid-containing and plasmid-free cells. Examples include the studies of Imanaka and Aiba (1981), Kim and Ryu (1984), Cooper *et al.* (1987), San and Weber (1989), and Syamsu *et al.* (1992). However, little experimental data have been reported, and Mosrati *et al.* (1993) noted that the meaning of the kinetic parameters is probably not fully understood due to their empirical nature. It is also difficult to experimentally measure these kinetic parameters.

Models based on the Monod model and most other unstructured and nonsegregated methods at modelling unstable recombinant cell populations only partially satisfy the need to predict accurately the properties of the system. As a result of plasmid segregation and cell size distribution in a population the plasmid distribution profile varies with different growth conditions (Kim and Shuler, 1990). However, almost all experimental measurements of plasmid copy numbers and protein production are based on average values. Satyagal and Agrawal (1990) observed that there is a need to develop expressions that relate experimental determinations of plasmid concentrations with other measures of this variable used in theoretical studies of recombinant cells. However, an exact determination of the plasmid distribution profile is necessary for predicting the time-dependent features of the population, plasmid stability, and productivity of the recombinant system (Kim and Shuler, 1990). Also, a relationship between the plasmid content to the amount of cloned gene and its expression is deemed necessary in obtaining estimates of the recombinant cell cultures (Satyagal and Agrawal, 1990). Structured and segregated models give a greater knowledge of the fermentation allowing aspects of the organism or plasmid to be modified to improve the fermentation. However, again the experimental data to validate these

models are very difficult to obtain.

2.3.5 SUMMARY

Plasmid stability is a major concern when investigating heterologous protein production in recombinant yeasts. Instability can be brought about by genetic or environmental effects, or a combination of both. By addressing these areas, stability of a recombinant plasmid may be improved. In the last decade, many possible causes and means of improving plasmid stability have been reported. Initially, the choice of plasmid and host must be made, since plasmid stability is both a function of the plasmid genetics and host genetics and physiology. This will usually tackle the problem of structural instability, however to improve segregational instability the fermentation conditions must be modified to enhance growth of plasmid-containing cells and reduce the population of plasmid-free cells.

Defined media, containing an antibiotic or deficient in a growth supplement, are most commonly used to support only the growth of plasmid-containing cells or inhibit the growth of plasmid-free cells. However, this method may only reduce plasmid stability, rather than eliminate it completely, and for large-scale production the media costs may be high. Alternative techniques for improving plasmid stability involve manipulation of other environmental conditions, including growth rate, nutrient limitation, dissolved oxygen tension, and growth temperature. Plasmid stability has also been improved by separating the stages of growth and product formation by using inducible or repressible promoters, or by temperature-controlled promoters. Immobilization of recombinant cell cultures also offers potential as an alternative for increasing plasmid stability.

Mathematical models have been suggested to simulate and increase understanding of recombinant cell systems. However, to date, the proposed models are not yet adequate to completely describe the process, primarily because the many combinations of plasmid, host, and fermentation conditions

possible produce different effects on plasmid stability and heterologous protein production.

The study of recombinant plasmid stability in yeasts is still very much in its infancy. As more experimental information becomes available, it is likely that more stable heterologous protein production systems will be developed by improving and manipulating the host, vector, and environmental conditions.

2.4 PRODUCTION AND USE OF α-AMYLASE

2.4.1 INTRODUCTION

The production of heterologous α-amylase from *K. lactis* was the model system chosen for this research study. This section concerns the utilization of α-amylase in industrial processes and describes previous studies of α-amylase production by recombinant organisms.

2.4.2 α-AMYLASE

Amylases are enzymes that catalyze the hydrolysis of starch. α-Amylases (E.C. 3.2.1.1., 1,4 α-D-glucan glucanohydrolase, endo-amylase) belong to the class of endo-amylases which are also known as dextrinogenic or liquefying α-amylases (Fogarty and Kelly, 1990). Specifically, α-amylase catalyzes the hydrolysis of α-1,4 glucan linkages to produce maltose, dextrins, and larger oligosaccharides from starch, amylose, amylopectin, and glycogen. Most α-amylases are single polypeptides of molecular weight around 50 kDa and they are generally found in the digestive tract of animals, in germinating seeds, and in some micro-organisms (Thomsen, 1987).

Fogarty and Kelly (1990) have reviewed the general characteristics of α-amylases. The enzymes are generally stable in the pH range 5.5 - 8.0 and to extremes of pH in the presence of calcium. Optimal activity normally occurs

between pH 4.8 and 6.5, but there are differences in the shapes of the pH activity curves of the different enzymes and also in the values of the pH optima. Most purified α-amylases lose activity rapidly above 50°C, but this inactivation may be retarded in the presence of calcium. α-Amylases do not contain co-enzymes, but they are calcium metallo-enzymes with at least one atom of calcium per molecule of enzyme. The strength of the binding is dependent on the source of the enzyme and such binding is required for catalytic activity.

A large number of methods have been described for measuring α-amylase activity and are based on one of the following events observed in the enzyme digests (Bernfield, 1955):

(1) Increase in reducing power of a solution of amylopectin or soluble starch;
(2) Decrease of the viscosity of a starch paste; and
(3) Change in the iodine-staining properties of the substrate.

2.4.3 USES OF α-AMYLASE

α-Amylase is an enzyme of major importance since starch and products derived from starch contribute to essential human nutrition and have many industrial uses. Major applications have been reviewed by Gerhartz (1990) and include its use in the brewing and baking industries, and in the production of sweeteners and fuel alcohol. For example, α-amylase is often deficient in the wheat grain and may be supplemented to obtain the desired profile of minimal dextrins, fermentable sugars for yeast metabolism, and carbon dioxide formation in the baking industry. In the manufacture of syrups and sweeteners, α-amylase is added to corn and other grains to reduce the viscosity and partially hydrolyze the starch to produce glucose. Starch conversion to produce ethanol is analogous to the conversion of syrups and sweeteners in that glucose is the final product sought. α-Amylase is used to convert the feedstock, for example, corn, cassava, or sugar cane, to a material which can be fermented by yeasts to produce ethanol.

2.4.4 PRODUCTION OF HETEROLOGOUS α-AMYLASE

The production of a cloned α-amylase may have commercial applications either as a purified enzyme or by extending the substrate range of a useful microorganism. For example, the introduction of an α-amylase gene into *S. cerevisiae* could provide the ability to degrade and utilize high molecular weight polysaccharides as a carbon source. Thus, for example, the addition of α-amylase may not be necessary in the fuel alcohol industries, thereby reducing the costs (Astolfi-Filho *et al.*, 1986; Ribeiro do Santos *et al.*, 1986). α-Amylase genes have been cloned and expressed in *S. cerevisiae* from the following sources: human salivary gland (Nakamura *et al.*, 1986; Sato *et al.*, 1986), mouse (Tokunaga *et al.*, 1987; Astolfi-Filho *et al.*, 1986; Thomsen, 1987), wheat (Rothstein *et al.*, 1984; 1987), the yeast *Saccharomycopsis fibuligera* (Tanaka *et al.*, 1988), and *B. amyloliquefaciens* (Zubay, 1988; Ruohonen *et al.*, 1991).

In the food and beverage industries the use of recombinant DNA techniques to produce an amylolytic yeast is likely to require a barley, or other cereal, α-amylase for both legislative and customer preference reasons. For this reason, Rothstein *et al.* (1984; 1987) sought to produce a wheat α-amylase from *S. cerevisiae*. They cloned a wheat α-amylase cDNA clone fused to a PGK promoter in *S. cerevisiae* and efficiently processed and secreted into the medium an α-amylase enzyme identical in size to the wild-type α-amylase.

It has also been noted by Chen *et al.* (1993a) that α-amylase is a useful marker for evaluating the stability of recombinant yeast, since most yeast strains lack the ability to utilize starch. They used a mouse α-amylase to examine plasmid stability in a *S. cerevisiae* strain.

2.4.5 SUMMARY

The system of heterologous α-amylase production lends itself as an excellent model for studying the expression of a foreign gene in yeasts other than *S.*

cerevisiae, and for studying the secretion and release of the heterologous product. This is because the enzyme is widely used in industry, there is already an experience base for heterologous α-amylase production from *S. cerevisiae*, and the activity of α-amylase is easily assayed.

2.5 WHEY AS A SUBSTRATE FOR FERMENTATION PROCESSES

2.5.1 INTRODUCTION

Whey is an abundant byproduct of the dairy industry and can present a disposal problem due to its high BOD of approximately 40,000 mg l^{-1}. It has been estimated that 47 % of the whey produced worldwide remains unused (Wessinger *et al.*, 1990). While for smaller processing plants the return of whey to agriculture may be economically favourable, for larger plants processes such as drying, protein recovery, whey fermentation, and lactose production may be more advantageous (Sienkiewicz and Riedel, 1990). As the production of whey is constantly increasing, established processes for whey utilization must be improved and new solutions for processing the excess sought.

2.5.2 TYPES OF WHEY

Whey is the fluid obtained by separating the coagulum from milk, cream, and/or skim milk (Short, 1978). Different types of whey are produced with 'sweet whey', having a pH value greater than 5.5, derived from the manufacture of cheese or rennet casein, and 'acid whey', with a pH value less than or equal to 5.0, being obtained from cottage cheese, lactic acid casein, or mineral acid casein. Whey contains lactose, serum proteins, mineral salts, rennet, lactic or mineral acid, and water. It is a dilute material containing about 6 %(w/v) total solids, of which the major constituent (> 70 %) is lactose (Sienkiewicz and Riedel, 1990). The serum proteins are usually recovered as a whey protein concentrate (WPC). They show a range of functional properties when soluble and have a high nutritive value in both the soluble and insoluble form (Short, 1978). Table 2.5 gives typical

compositions of deproteinated sweet and acid wheys.

Table 2.5 Composition of deproteinated sweet and acid wheys of various sources. (Adapted from Short, 1978).

Parameter	Whey source			
	Cheddar cheese	Cottage cheese	Lactic casein	Sulphuric casein
total solids (g kg^{-1})	57.0	58.0	56.8	56.4
total nitrogen (g kg^{-1})	0.26	0.33	0.64	0.37
non-protein nitrogen (g kg^{-1})	0.24	0.30	0.46	0.32
ash (g kg^{-1})	5.0	5.6	5.7	7.9
lactose (g kg^{-1})	49.0	43.0	44.8	46.0
lactic acid lactate (g kg^{-1})	1.4	4.4	3.4	-
ash/TS (%)	8.8	9.7	10.0	14.0
lactose/TS (%)	86.0	74.0	79.0	82.0
pH	5.5	5.0	5.0	5.0

2.5.3 UTILIZATION OF WHEY

Table 2.6 details the volumes of whey (processed and non-processed) produced in two recent seasons in New Zealand. A breakdown of the usage of whey for these two seasons is also shown in Table 2.6.

In New Zealand, the major products from whey processing are whey protein concentrate (WPC), lactose and lactalbumin (Barnett, 1993) and examples of end-uses are products such as infant formulae, meat sausages, biscuits, chocolate drinks and cheese spreads. Sienkiewicz and Riedel (1990) also list many other properties and uses.

Sienkiewicz and Riedel (1990) noted a number of options for the use of deproteinated whey in fermentation processes, with examples shown in Table 2.7. Whilst many processes are proposed only a limited number have been commercialized, and of these only ethanol is produced in New Zealand. The first

Table 2.6 Summary of quantities of whey processed in New Zealand over the 1991/92 and 1992/93 seasons (Jim Barnett, 1993).

	1991/92 Season		1992/93 Season	
	Volume (m^3 yr^{-1})	% of whey produced	Volume (m^3 yr^{-1})	% of whey produced
Whey produced	3023981		3118770	
Whey processed	1821874	60.3	2289105	73.4
Non processed whey	201233	39.7	829664	26.6
Processed whey		**% of processed whey**		**% of processed whey**
WPC		42.0		38.3
Whey powder		5.6		5.1
Whey cheese		1.0		0.5
Lactose		20.9		29.7
Ethanol		5.8 (26.8)[a]		4.7
Infant formula		7.1		7.9
Lactalbumin		17.6		13.9
Non processed whey		**% of non processed whey**		**% of non processed whey**
Fertilizer		46.5		46.5
Biological treatment		0.1		0.2
Animal		8.1		11.7
River/sea		17.7		3.7
Irrigation		27.6		37.9

[a] Value in brackets is ethanol produced from the deproteinated whey of WPC and lactalbumin.

Table 2.7 Examples of fermentation products from whey or whey permeate.

Fermentation products	Examples
solvents	ethanol, butanol
biogas	methane
polysaccharides	xanthan
organic acids and their derivatives	amino acids, lactic, acetic, and citric acids
enzymes	β-galactosidase
others	caretenoids, flavour compounds, fats and oils, starter cultures, and whey yeast

distillery was commissioned by the New Zealand Co-operative Dairy Co. Ltd (NZCDC) at Reporoa in 1980. The four distilleries now in operation at Reporoa, Tirau, Clandeboye, and Edgecumbe, supply the New Zealand market for both industrial and potable ethanol with an increasing proportion also exported, primarily to Japan. Mawson (1987) recently reviewed the whey to ethanol industry of New Zealand.

2.5.4 USE OF RECOMBINANT ORGANISMS FOR WHEY FERMENTATION

There has been interest in using *S. cerevisiae* to ferment whey, however this yeast does not ferment lactose since it lacks both lactose permease and β-galactosidase activity (Chen and Chiger, 1985). To overcome these problems a number of techniques have been used, including the coimmobilization of *S. cerevisiae* cells and β-galactosidase to ferment whey permeate or deproteinated whey (Marwaha and Kennedy, 1984; Hahn-Hagerdal, 1985; Roukas and Lazarides, 1991). However, disadvantages include the relatively high cost of β-galactosidase, and the diauxic growth of *S. cerevisiae* on glucose and galactose (Roukas and Lazarides, 1991).

An alternative to techniques such as coimmobilization is to use recombinant DNA technology to construct β-galactosidase-positive strains of *S.cerevisiae*. Sreekrishna and Dickson (1985) have cloned and expressed both the lactose permease and β-galactosidase of *K. lactis* in *S. cerevisiae*, and Porro *et al.* (1991; 1992) and Compagno *et al.* (1993) have expressed an *E. coli* β-galactosidase in *S. cerevisiae*. The former recombinant strain grew on lactose as a carbon source, but the rate of growth was low. The transformant produced by Porro *et al.* (1991) had a high growth yield in rich lactose media and also showed high production of ethanol. Farahnak *et al.* (1986) have constructed fusion strains but such strains remained sensitive to inhibition by lactose. Alternatively, whey could be used as a substrate for production of other heterologous proteins. Harlander (1989) has noted that, for economic reasons, whey will probably be used to produce heterologous proteins using yeasts. To date no literature exists

for the production of a heterologous protein in *K. lactis* using whey as the medium.

2.5.4 SUMMARY

Significant developments in the field of whey utilization have taken place over the last few decades. Whey in liquid form has limited use and so the concentration, drying and fermentation of whey, as well as the isolation of individual whey constituents have become increasingly important. As dairy processing plants in New Zealand become more centralized and therefore larger, the problem of disposing of excess non-processed whey also increases. It is therefore desirable to find higher-returning products to increase the amount of whey processed. With recent advances in molecular biology techniques the yeast *K. lactis* could be engineered to produce additional products to ethanol from whey and a new process developed.

Ideally, as a result of increased whey utilization, there would be less pollution, increased sales in presently manufactured whey products, and markets for improved and new whey products.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Microbiological media

The following media were used in this study:

(1) Luria Bertani broth (LB) (Miller, 1972)
Composition (g l^{-1}): tryptone, 10; yeast extract, 5; NaCl, 5; pH 7.2.

(2) Rich glucose medium (YPD) (Bianchi *et al.*, 1987)
Composition (g l^{-1}): glucose, 20; Bactopeptone, 20; yeast extract, 10.

(3) Rich lactose medium (YPL) (adapted from YPD)
Composition (g l^{-1}): lactose, 20; Bactopeptone, 20; yeast extract, 10.

(4) Minimal medium (MM) for selection of *K. lactis* MW98-8C transformants (adapted from Bianchi *et al.*, 1987)
Composition (g l^{-1}): glucose, 20; yeast extract without amino acids, 6.7; arginine, 0.02; lysine, 0.02.

(5) Starch agar for *E. coli* (Gatenby *et al.*, 1986)
Composition: soluble starch 10 g l^{-1} (boiled to dissolve); Bactopeptone, 15 g l^{-1}; glucose, 2 g l^{-1}; NaCl, 5 g l^{-1}; $CaCl_2$, 1 mM.

(6) YPL-starch and MM-starch (adapted from Rothstein *et al.*, 1984)
Composition: YPL or MM; starch, 10 g l^{-1}; $CaCl_2$, 1 mM.

(7) Enriched whey media (WYP; developed in this study)
Composition (g l^{-1}): lactose (by dissolving sulphuric acid casein whey permeate powder) 20; Bactopeptone, 2.5; yeast extract, 2.5.

Hard agar contained 15 g l^{-1} agar and soft agar contained 7.5 g l^{-1} agar. Selective media was supplemented as required with ampicillin (Ap, 50 μg ml^{-1}) or kanamycin (Kan, 50 μg ml^{-1}) for *E. coli* transformant selection, and G418 (200 μg ml^{-1}) for *Kluyveromyces* transformant selection. All antibiotics were supplied by Sigma Chemical Company (St. Louis, USA). Spray-dried sulphuric acid whey permeate was obtained from the New Zealand Dairy Research Institute (Palmerston North, New Zealand). Yeast extract, tryptone, Bactopeptone, agar, and yeast extract without amino acids were obtained from the following sources: Gibco Ltd (Paisley, Scotland, UK), Ajax Chemicals (Sydney, Australia), Oxoid Ltd (Basingtonstoke, England), and Difco Laboratories (Detroit, Michigan, USA).

3.1.2 Enzymes and chemicals

Restriction enzymes, RNase A, T4 DNA ligase, calf alkaline phosphatase, and Klenow enzyme were obtained from Boehringer Mannheim GmbH (Germany), Bethesda Research Laboratories (BRL) (Gaithersburg, USA), or Promega (Wisconsin, USA), and were used according to the instructions of the suppliers.

All other chemicals used were of analytical grade. Their sources were: BDH Chemicals Ltd (Dorset, UK), Bevaloid Chemicals Ltd (Levin, New Zealand), Polychem (Auckland, New Zealand), and Sigma Chemical Company (St. Louis, Missouri, USA).

3.1.3 Gases and other materials

Oxygen-free nitrogen and hydrogen gas were supplied by New Zealand Industrial Gases Ltd (Palmerston North, New Zealand). Pyroneg detergent was supplied by Diversey-Wallace Ltd (Papatoetoe, New Zealand).

3.1.4 Bacteria, yeast strains, and plasmids

Bacterial and yeast strains and plasmids used in this study are summarized in Table 3.1.

Bacterial cultures were maintained on selective LB plates. For long-term storage, all cultures were stored in 30 %(v/v) glycerol in LB at -70°C. *E. coli* was cultured aerobically in LB broth at 37°C and exponential stage cells were harvested at an absorbance of 0.3 - 0.7 at 600nm (A_{600}). Solid or liquid media was supplemented as required with the appropriate antibiotics, ampicillin or kanamycin.

Selective and non-selective YPL, YPD, or MM plates were used to maintain yeast cultures. For long-term storage, all cultures were stored in 30 %(v/v) glycerol in YPL at -70°C. Yeast inoculum was prepared by inoculating a 5 ml broth of selective or non-selective rich medium from a single colony grown on maintenance plates. The broth was incubated at 30°C overnight with vigorous shaking. For all fermentation studies using recombinant yeasts, the inoculum was developed in the selective rich medium YPL-G418. For continuous culture studies an 8 h culture grown in this selective medium (5 ml) was used to inoculate a 50 ml broth; this was grown overnight and used to inoculate the fermenter.

3.2 STERILIZATION AND CLEANING

3.2.1 Media sterilization

All culture media were sterilized by autoclaving at 121°C for 15 min, except that fermentation media prepared in volumes in excess of 4 l which were sterilized for 30 min. For minimal media, the amino acids were sterilized separately by membrane filtration (0.2 μm), and aseptically added to the other medium components prior to inoculation. All stock solutions of antibiotics (25 mg ml^{-1}) were also filter-sterilized and added to the media before inoculation.

Table 3.1 Bacterial strains, yeast strains, and plasmids used in this work.

	Relevant features	Source or reference
Bacterial strains		
E. coli DH5α™ competent cells	F⁻, *END*A1, *HSD*R17(r_k^-,m_k^+), *SUP*E44, *THI*-1, λ-, *REC*A1, *GYR*A96, *REL*A1, Δ(*ARG*F-*LAC*zya)U169, ϕ80d*LACZ*ΔM15	BRL product sheet
Yeast strains		
K. lactis		Obtained from Hiroshi Fukuhara (Institut Curie, Paris, France)
CBS 141		
CBS 683		
CBS 762		
CBS 845		
CBS 1065		
CBS 1067		
CBS 1797		
CBS 2359		
CBS 2360/7		
CBS 2619		
CBS 2621		
CBS 2896		
CBS 5618		
CBS 6315		
CBS 6747		
MW 98-8C		
MW 108-8B		
pM 6-7A		
pM 44-B		
K. marxianus		
ATCC 1179		
ATCC 1195		
ATCC 36907		
CBS 397		
CBS 712		
CBS 4572		
CBS 5795		
CBS 6556		
Y113		Process and Environmental Technology culture collection (Massey University) (from the NZ Distillery Co Ltd, Edgecumbe)
Plasmids		
pMA230-520	Ap^R, PGK-α-amylase fragment	Rothstein *et al.* (1984)
M13 bluescript	cloning vector, *LAC*zya, Ap^R, ColE1 ori	Stratagene Cloning Systems (San Diego, California, USA)
pCXJ-kan1	pKD1 with Kan^R, Ap^R, *URA*3, *LAC*zya, ColE1 ori	Chen *et al.* (1989); obtained from Hiroshi Fukuhara (Institut Curie, Paris, France)
pCXJ	pCXJ-kan1 without Kan^R	this study
pCXJ-PGK-α-amylase	pCXJ with PGK-α-amylase	this study
pCR1	pCXJ-PGK-α-amylase with Kan^R	this study

Pipettes, test-tubes, bottles, and glass wool gas filters were sterilized by dry heat at 160°C overnight. The fermenter vessel containing the medium and associated components, except the pH probe, were sterilized in an autoclave for 20 min at 121°C. The pH electrode for insertion into the continuous fermenter was sterilized in 50 %(v/v) aqueous ethanol for 18 h and rinsed with sterile distilled water immediately before use.

3.2.3 Cleaning of glassware

All glassware was washed in hot Pyroneg^R solution, rinsed in tap water, and distilled water, and hot air dried.

3.3 MOLECULAR BIOLOGY METHODS

3.3.1 Isolation of DNA

3.3.1.1 Isolation of plasmids from *E. coli*

The alkaline lysis (modified from Birboim and Doly, 1979 as reported by Sambrook *et al.*, 1989) and rapid boil (Holmes and Quigley, 1981, described by Sambrook *et al.*, 1989) methods were used to extract DNA from *E. coli* for small-scale preparations. For large-scale preparations two alkaline lysis methods were used with different DNA purification procedures. The first was a modified method of that reported by Sambrook *et al.* (1989) and was used in conjunction with a cesium chloride purification (Day, 1991). The other was a method supplied with a Circleprep^R kit (BIO 101, CA, USA). The former method took several days to complete while the Circleprep^R protocol took less than 2 h. All water used in these preparations was deionized distilled water. Volumes less than 1.5 ml were centrifuged in a Biofuge 13 Centrifuge (Heraeus, Germany), and larger volumes in a Sorvall RC5C-SS34 Centrifuge (Du Pont, USA), unless otherwise noted.

3.3.1.1.1 Rapid boil DNA isolation

Cells were grown in LB + Ap broth (5 ml) overnight and harvested from a 1.5 ml volume by centrifugation (13,000 rpm, 1 min). The pellet was drained well, resuspended in STET buffer (350 µl: 8 % sucrose; 5 % Triton-X-100; 50 mM EDTA, pH 8; 50 mM Tris-HCl, pH 8), and freshly prepared lysozyme (25 µl: 10 mg ml^{-1}) was added. The contents were mixed by inversion, boiled for 40 sec and immediately centrifuged (13,000 rpm, 10 min). The supernatant was transferred to a fresh tube, extracted once with phenol/chloroform (phenol:chloroform:isoamyl alcohol, 25:24:1), and precipitated with ethanol (2.5 vol) for 30 min at -70°C. The DNA pellet was recovered by centrifugation (4°C, 13,000 rpm, 10 min), washed with 70 % ethanol, dried under vacuum, and resuspended in water or TE buffer (30 µl: 10 mM Tris-HCl, pH 8; 1 mM EDTA).

3.3.1.1.2 Small-scale alkaline lysis plasmid DNA preparation

Cells were grown overnight in LB + Ap broth (5 ml), and 1.5 ml was transferred to a microfuge tube. After centrifugation (13,000 rpm, 1 min) the supernatant was discarded, leaving the cell pellet as dry as possible. The cells were resuspended by vortex mixing in ice-cold solution I (100 µl: 50 mM glucose; 10 mM EDTA; 25 mM Tris-HCl, pH 8) and stored at room temperature for 5 min. Freshly-prepared solution II (200 µl: 0.2 M NaOH; 1 % sodium dodecyl sulphate (SDS)) was added to the mixture, inversion mixed, and stored on ice for 5 min. An ice-cold solution of potassium acetate (150 µl: 60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml deionized distilled water, pH 4.8) was added and the tube was vortexed gently in an inverted position for 10 sec. After centrifugation (4°C, 13,000 rpm, 5 min) the supernatant was transferred to a fresh tube and mixed by inversion with an equal volume of phenol/chloroform. The tube was centrifuged (4°C, 13,000 rpm, 2 min) and the supernatant was again transferred to a fresh tube. Ethanol (2 vol) was added and the mixture was allowed to stand at room temperature for 5 min. After centrifugation (13,000 rpm, 5 min) 70 % ethanol (1 ml) was added. The tube was recentrifuged, the supernatant was carefully

removed, and the pellet was dried under vacuum and resuspended in water or TE buffer (30 µl).

3.3.1.1.3 Large-scale alkaline lysis plasmid DNA preparation and cesium chloride purification

A 2 l flask containing 500 ml LB + Ap was inoculated with 5 ml of an overnight LB + Ap *E. coli* culture and shaken vigorously for 4 - 6 h until A_{600} was approximately 0.6. Chloramphenicol (2.5 ml: 34 mg ml^{-1} prepared fresh in ethanol) was added and the flask was incubated at 37°C with vigorous shaking for 12 - 16 h. The cells were harvested by centrifugation (4°C, 5,000 rpm, 10 min), washed in ice-cold STE (100 ml: 0.1 M NaCl; 10 mM Tris-HCl, pH 7.8; 1 mM EDTA), resuspended in solution I (10 ml) containing lysozyme (5 mg ml^{-1}), and left at room temperature for 5 min. The suspension was mixed gently by inversion with freshly made solution II (20 ml), and held on ice for 10 min. Ice-cold potassium acetate (15 ml) was added, mixed by inversion, and left on ice for 10 min. The mixture was centrifuged (4°C, 18,000 rpm, 20 min) and the supernatant was transferred to clean centrifuge tubes. Isopropanol (0.6 vol) was added to each tube, mixed well, and left at room temperature for 15 min. The tubes were centrifuged (12,000 rpm, 30 min), and the pellet was washed with 70 % ethanol at room temperature. The ethanol was carefully removed and the tubes were dried under vacuum. The pellets were dissolved in TE buffer (4 ml).

Cesium chloride (1 g for every 1 ml DNA preparation) was added and mixed gently until the salt dissolved. Ethidium bromide (80 µl of 10 mg ml^{-1} for every 1 ml CsCl solution) was added, the solution was mixed well and left for 1 - 2 h. After centrifugation (18,000 rpm, 10 min) the supernatant was transferred to crimp-seal Sorvall tubes, balanced, and topped up with parafilm oil if necessary. The tubes were sealed and spun in a Sorvall TV 865 centrifuge (58,000 rpm, overnight). The plasmid bands were removed into sterile tubes and mixed well with an equal volume of 1-butanol saturated water. Phases were allowed to separate and the upper phase was discarded. This was repeated several times

until both phases were colourless. The solution was diluted 1:2 or 1:3 depending on the increase in volume from the butanol washes. An equal volume of 95 % ethanol was added and the mixture was held at -70°C, 1 h. After centrifugation (4°C, 18,000 rpm, 10 min) the pellet was washed 3 times with 70 % ethanol and recentrifuged. The pellet was dried and resuspended in TE buffer (2 ml).

3.3.1.1.4 Large-scale DNA preparation and purification using the Circleprep^R kit

All reagents were supplied with the Circleprep^R kit. *E. coli* was grown in LB + Ap (100 ml) overnight. The culture was centrifuged (5,000 rpm, 5 min) and the pellet was vortexed with prelysis buffer (4 ml). Alkaline lysis reagent (4 ml) and neutralizing solution (4 ml) were added and the solution was mixed by inversion for 5 min. The cell debris was removed by centrifugation (5,000 rpm, 5 min) and isopropanol (12 ml) was added to the supernatant. After centrifugation (4°C, 12,000 rpm, 5 min), the supernatant was discarded and the pellet was dissolved in 0.5 ml water. The mixture was boiled for 5 min, and immediately held on ice for 1 min. Saturated LiCl (300 µl) was added and after 5 min at room temperature the solution was centrifuged (12,000 rpm, 2 min) and the pellet was dissolved in 0.5 ml water. LiCl (300 µl) and glass milk (75 µl) were added, and mixed by inversion for 5 min. After centrifugation (12,000 rpm, 10 sec) the pellet was washed twice with binding buffer (1 ml) and twice with wash solution (1 ml). The pellet was resuspended in water (100 - 300 µl), incubated at 60°C for 5 min, and centrifuged (12,000 rpm, 30 sec) to remove the glass milk. The supernatant was transferred to a fresh tube, 100 % ethanol was added and the tube was left at -70°C for 30 min. The tube was centrifuged (12,000 rpm, 30 min) and the pellet was washed with 70 % ethanol, dried and resuspended in water or TE buffer (100 - 200 µl).

3.3.1.2 Isolation of plasmids from *K. lactis*

The method used for yeast plasmid isolation was supplied by Ibba (1991) and

was based on a Qiagen plasmid midi kit designed for *E. coli* plasmid isolation (Qiagen Inc., CA, USA). Qiagen buffers P1, QBT, C, and QF, and Qiagen-tip 100 midi-columns were supplied with the kit.

Recombinant strains of *K. lactis* were grown in selective YPL-G418 medium (10 ml) overnight. The culture was centrifuged (4,000 rpm, 5 min), and the pellet washed in 0.9 % NaCl and recentrifuged. The supernatant was discarded and the pellet was resuspended in Zymolyase buffer (0.5 ml: 1 M sorbitol; 75 mM β-mercaptoethanol; 25 mM sodium phosphate; pH 6.5). The A_{600} was measured for the suspension (10 µl) plus 1 % SDS (1 ml). The suspension was transferred to a microfuge tube, zymolase enzyme (Zymolyase 5000T, 125 µl: 20 mg ml^{-1}) added, and the A_{600} was monitored for the suspension plus SDS until it reached 10 % of the original value. After centrifugation (13,000 rpm, 30 sec) the pellet was gently resuspended in modified P1 and held on ice for 5 min. It was gently mixed with NaOH-SDS (0.2 M NaOH; 1 % SDS) and left at room temperature for 5 min. Potassium acetate (0.3 ml: 2.55 M, pH 4.8) was added and after gentle mixing the tube was centrifuged (4°C, 13,000 rpm, 30 min) and the supernatant was carefully removed and applied to Qiagen midi column, previously equilibrated with QBT buffer (4 ml). The column was washed twice with QC buffer (10 ml) and the DNA was eluted with QF buffer (5 ml). Isopropanol (0.5 vol) was added to the DNA fraction and the mixture was held at room temperature for 20 min. After centrifugation (4°C, 13,000 rpm, 30 min) the supernatant was carefully removed and the pellet was washed in 70 % ethanol (-20°C) and recentrifuged (4°C, 13,000 rpm, 5 min). The pellet was dried and resuspended in TE buffer (50 µl).

3.3.2 DNA manipulations

Reaction conditions for DNA manipulations, including restriction digests, dephosphorylation, blunt-ending, and ligation of DNA, were carried out according to the manufacturers' recommendations (Boehringer Mannheim GmbH, Germany; Bethesda Research Laboratories (BRL), Gaithersburg, USA; or, Promega, Wisconsin, USA).

3.3.3 Agarose gel electrophoresis

DNA was mixed with gel-loading buffer (0.25 % bromophenol blue, 40 % sucrose in water) in a ratio 9:1 and was analyzed by electrophoresis on horizontal agarose (0.5 - 1.2 %(w/v)) gels immersed in TAE buffer (40 mM Tris-acetate; 1 mM EDTA, pH 8). The gels were stained with ethidium bromide (0.5 $\mu g\ ml^{-1}$) and DNA was detected with a short wavelength UV transilluminator.

3.3.4 DNA purity and concentration

The DNA was analyzed on agarose gels by restriction enzyme digests. If the DNA was not digested satisfactorily, the sample was further purified by phenol/chloroform, extracted once, and the DNA was precipitated using ethanol and 0.3 M sodium acetate solution. The DNA concentration was found by measuring the A_{260} and multiplying the obtained value by 50 to give concentration in units of $\mu g\ ml^{-1}$.

3.3.5 Elution of DNA from agarose gels

DNA was separated on agarose gels (SeaKem, GTG grade, FMC Co., Rockland, ME, USA). The band of interest was cut out under long-wave UV light. The gel block was trimmed into tiny pieces and frozen at -70°C for 30 min in parafilm. The gel was squeezed and the liquid fraction transferred to a clean microfuge tube where the DNA was extracted once with phenol/chloroform and precipitated with ethanol.

3.3.6 DNA-DNA hybridization using agarose gels

After electrophoresis in 1 % agarose gels, the DNA was transferred to nylon filters using a modified protocol of Southern (1975) as detailed by the ECL non-radioactive gene detection system (Amersham International, UK). The DNA was labelled with the ECL system, and hybridization, washing, and signal steps were

completed according to the instructions of the supplier. Details of the method are described below.

3.3.6.1 Processing the gel

The gel was covered with depurination solution (250 mM HCl) and agitated for 15 min. After rinsing 3 times with distilled water it was covered with denaturation solution (1.5 M NaCl; 0.5 M NaOH) and agitated again for 15 min, followed by rinsing 3 times with water. The gel was then covered with neutralization solution (1.5 M NaCl; 0.5 M tris-HCl, pH 7.5) and agitated for a further 15 min.

3.3.6.2 Southern blotting

A glass dish was partially filled with 20x SSC (0.3 M sodium citrate; 3 M NaCl; pH 7). A blotting stand was placed in the middle of the dish and covered with a wick made from 3 sheets of filter paper (Whatman™-3MM) saturated with 20x SSC. The gel was placed on the 3MM paper and surrounded with cling film to prevent the SSC being absorbed directly by paper towels. A sheet of nylon membrane (Hybond-N^+) was cut to the size of the gel and placed on top without trapping air bubbles. Three sheets of 3MM paper were cut to size, wetted with 20x SSC and placed on top of the membrane. A 5 - 7 cm stack of absorbent paper towels was placed on top of the 3MM paper, and a glass plate and weight (approximately 0.5 kg) were placed on top of the towels. This arrangement was left overnight.

3.3.6.3 Processing the blot

The blotting stack was dismantled and the membrane and gel removed together. The membrane was placed side down on a clean piece of 3MM paper, and the gel peeled off, stained and checked for DNA transfer under UV light before being discarded. The membrane was marked so the face carrying the DNA was distinguishable. The membrane was placed on a pad of 2 - 3 pieces of 3MM

paper soaked in NaOH (0.4 M). The membrane was rinsed briefly (1 min) by immersion in 5x SSC with gentle agitation.

3.3.6.4 Preparation of labelled probe

The DNA to be labelled was diluted to 10 ng μl^{-1}, with at least 20 µl available for probing. The double-stranded DNA was boiled for 5 min followed by immediate cooling on ice for 5 min. An equal volume of DNA labelling reagent was mixed thoroughly with the cooled DNA, followed by an equal volume of the provided glutaraldehyde solution. The microfuge tube was centrifuged briefly to settle the reaction mixture in the bottom of the tube, and incubated for 10 min at 37°C before the hybridization steps.

3.3.6.5 Hybridization

NaCl (0.5 M) was added to the supplied hybridization buffer. The buffer was heated to 65°C and blocking agent (5 %(w/v)) was added gradually with stirring. Once prepared the buffer was stored at 4°C until required. For pre-hybridization the membrane blot was placed in the buffer (0.25 ml cm^{-2}) and agitated at 42°C for at least 15 min. The labelled DNA probe was mixed with 1 ml prehybridization buffer before addition to the bulk of the buffer for hybridization. Incubation at 42°C with agitation was continued overnight.

3.3.6.6 Washing the membrane

The blot was removed from the hybridization medium, placed in a clean container, covered with an excess of primary wash buffer (360 g urea; 4 g SDS; 25 ml 20x SSC; made up to 1 l, and kept for up to 3 months at 4°C) and incubated at 42°C with agitation. The wash buffer was discarded, replaced with the equivalent volume of fresh primary wash buffer, and incubated for a further 20 min at 42°C with agitation. The wash buffer was discarded and the blot was placed in a fresh container and washed 2 times with fresh secondary wash buffer,

2x SSC, for 5 min at room temperature with agitation.

3.3.6.7 Signal generation and detection

The signal generation and detection steps were carried out in a dark room. An equal volume of detection reagent 1 was mixed with detection reagent 2 to give sufficient solution to cover the blot (0.125 ml cm^{-2}). The excess buffer was drained from the blot and the blot was placed in a fresh container. The detection buffer was added directly to the blot face carrying the DNA and incubated for precisely 1 min at room temperature. Excess detection buffer was drained off and the blot was wrapped carefully in cling-film to ensure no air-pockets. The blot was quickly placed, DNA side up, in an X-ray film cassette. The lights were switched off and a sheet of autoradiography film (Cronex^R X-ray film R1, Du Pont Ltd, Sydney, Australia) was placed on top of the blot. The cassette was closed and exposed for 1 min before removing the film and developing using an X-ray film developer (Agfa Curix 60, Type 9462). Further exposures for differing lengths of time were carried out until the appearance of the developed film was satisfactory.

3.3.6.8 Reprobing blots

Blots that required reprobing were stored in cling-film. For reprobing the same steps as for the first hybridization were carried out. However, a different DNA probe, labelled the same way as the original probe, was used.

3.3.7 Transformation of *E. coli*

E. coli was transformed as described by the BRL product sheet for DH5α^{TM} competent cells (BRL). Competent cells (50 µl) were mixed with DNA (1 - 3 µl, 1 - 10 ng) and the tube was tapped gently to mix. The cells were incubated on ice for 30 min before being heat shocked at 37°C for 20 sec. After incubation on ice for 2 min, LB (0.95 ml) was added and the mixture was shaken (225 rpm, 1 h, 37°C) to allow expression. The treated cells (100 µl) were added to soft LB

agar, with IPTG (0.5 mM) and X-Gal (40 μg ml^{-1}) added for when blue/white colour screening used to identify interruptions in the β-galactosidase gene was required, and the mixture was spread on LB + Ap plates and incubated at 37°C for up to 48 h.

3.3.8 Electroporation of *K. lactis*

K. lactis strains were transformed using an electroporation method received from Fukuhara (1991) for a PS10 Electropulsator (JOUAN, France) or using the procedure developed in this study for a Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA, USA). The PS10 and Bio-Rad electroporators are shown in Figures 3.1 and 3.2 respectively. Cells were grown in YPD broth (100 ml) aerated at 30°C until mid-log phase (A_{600} 0.6 - 1.0), harvested by centrifugation (4°C, 8,000 rpm, 5 min) and washed with ice-cold electroporation buffer (EB; 10 mM Tris-HCl, pH 7.5; 270 mM sucrose; 1 mM $MgCl_2$). The cells were resuspended in YPD broth (10 ml) containing dithiothreitol (DTT; 25mM) and HEPES buffer (20 mM, pH 8), and incubated at 30°C for 30 min. They were recentrifuged, washed in EB, and resuspended in EB (1 ml). An aliquot (100 μl) of the cell suspension was mixed with DNA (0.2 μg) and placed on ice for 15 min. The mixture was transferred to either a sterile tissue culture plate with the 4 mm electrodes lowered into the cell-DNA suspension (PS10 Electropulsator) or a sterile Gene Pulser cuvette (Bio-Rad, 2 mm electrode gap). For the PS10 Electropulsator one pulse at a setting of 1000 V (2500 V cm^{-1}) and 20 ms was used, and for the Bio-Rad Gene Pulser one pulse at 900 V (4500 V cm^{-1}), 25 μF, and ∞ Ω (optimal conditions as described in Chapter 5) was applied. Cells were resuspended in YPD broth (1 ml) and incubated at 30°C for 1 h before diluting and plating on MM agar, or incubated for varying times before diluting and plating on selective YPD-G418 plates. The plates were incubated at 30°C for 2 - 3 days before counting colonies and examining transformants.

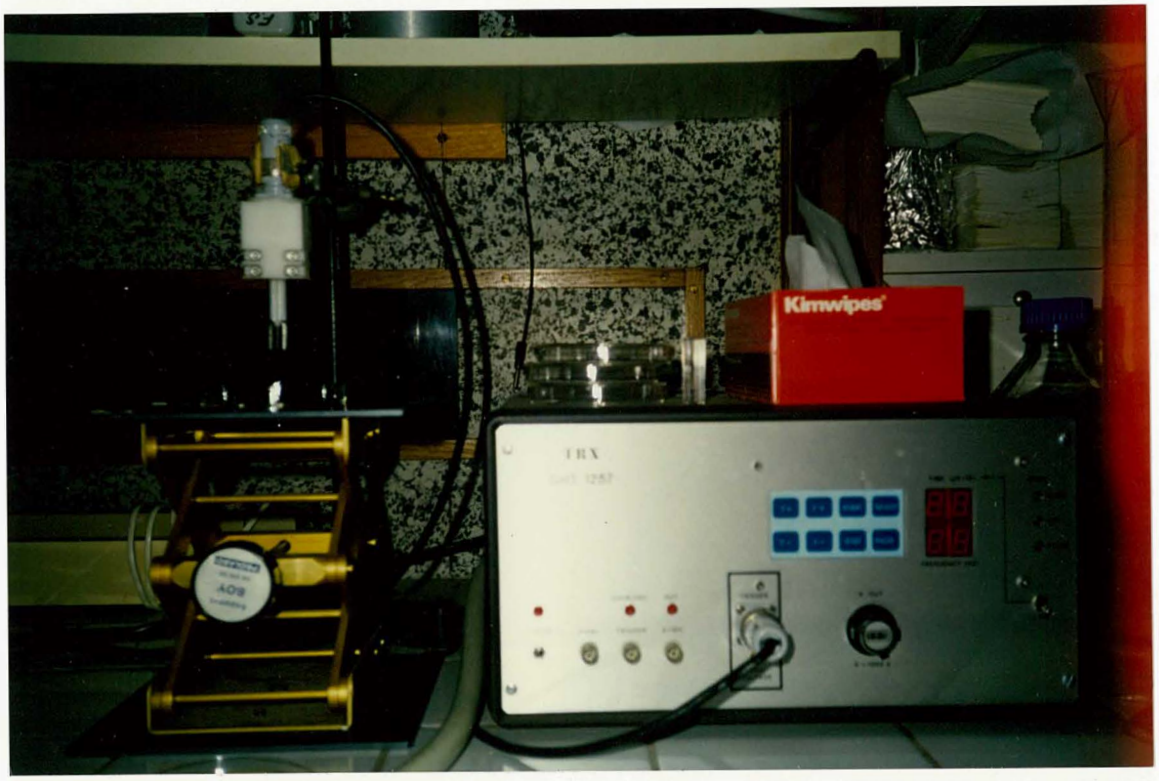


Figure 3.1 The PS10 electropulsateur (Institut Curie, Paris) used to transform *Kluyveromyces* yeasts.

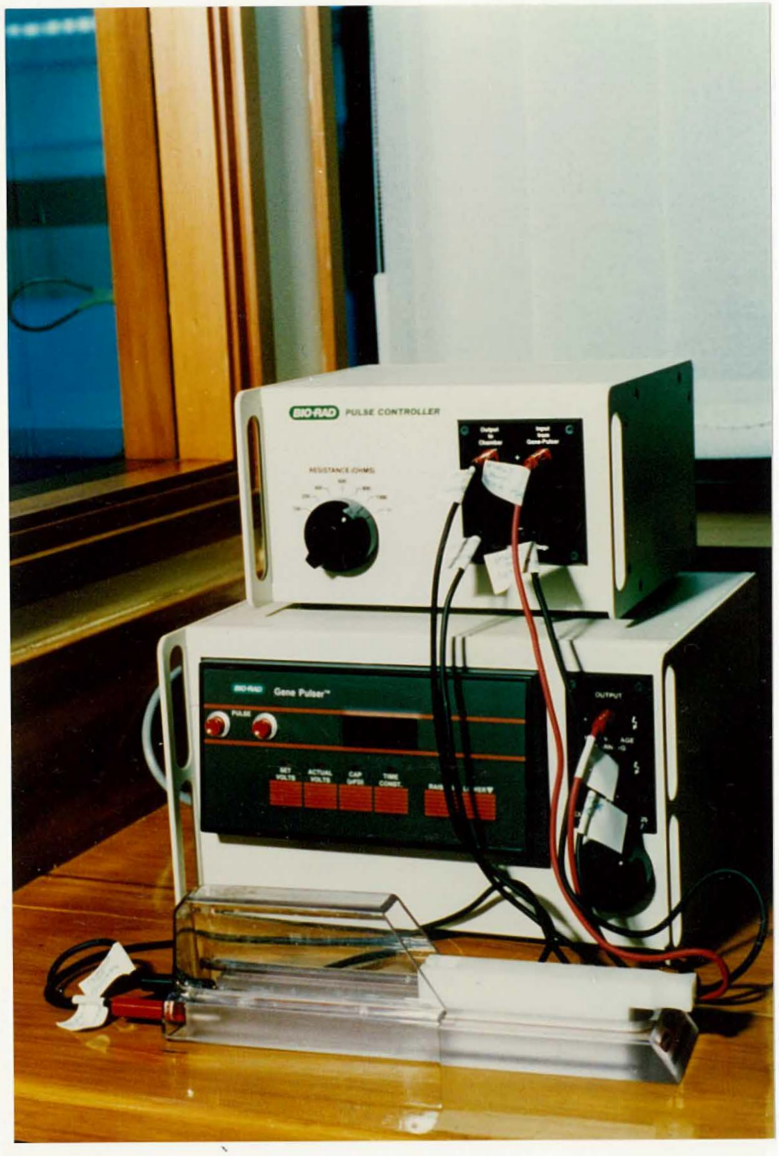


Figure 3.2 **The Bio-Rad Gene Pulser (New Zealand Dairy Research Institute, Palmerston North) used to transform *Kluyveromyces* yeasts.**

3.4 ANALYTICAL AND BIOCHEMICAL METHODS

3.4.1 pH measurement

All pH measurements, apart from those for continuous fermentations, were made using an Orion Research Digital Ionanalyzer (Model 701A; Watson Victor Ltd, New Zealand) which was calibrated prior to use with pH 4.0 and 7.0 buffers.

3.4.2 Total cell count

Total cell counts for yeast cultures were performed using a standard haemocytometer (Assistant, Germany) under 400x magnification.

3.4.3 Determination of biomass dry weight

A calibration curve was constructed for determining yeast biomass dry weight from A_{600} values using a Philips spectrophotometer (PU 8625 Series UV/VIS, Philips Scientific, Cambridge, England). The yeast *K. lactis* CBS 683 was grown aerobically in batch shake-flask culture for 48 h. At different stages of growth flasks were removed, A_{600} for different dilutions was determined, and dry weight was estimated using the following method. A known volume (20 ml) of fermentation culture was centrifuged (4,000 rpm, 10 min), and the cell pellet was washed in water, recentrifuged, and resuspended in water (8 - 10 ml). The suspension was placed in a pre-weighed pre-dried container and dried in a hot air oven at 105°C for 24 h. After drying, the containers were placed in a desiccator for at least 2 h before being weighed. *K. lactis* CBS 683 harbouring plasmid and *K. lactis* CBS 141 were also grown in shake-flask for 24 and 48 h and absorbance and dry weight results showed the calibration curve for *K. lactis* was also valid for these yeasts. The calibration curves for absorbance at different dilutions versus dry weight are shown in Figure 3.3, and were linear up to about an absorbance of 0.6. Linear regression gave values shown in Table 3.2 and allowed calculation of dry weights from absorbance measurements.

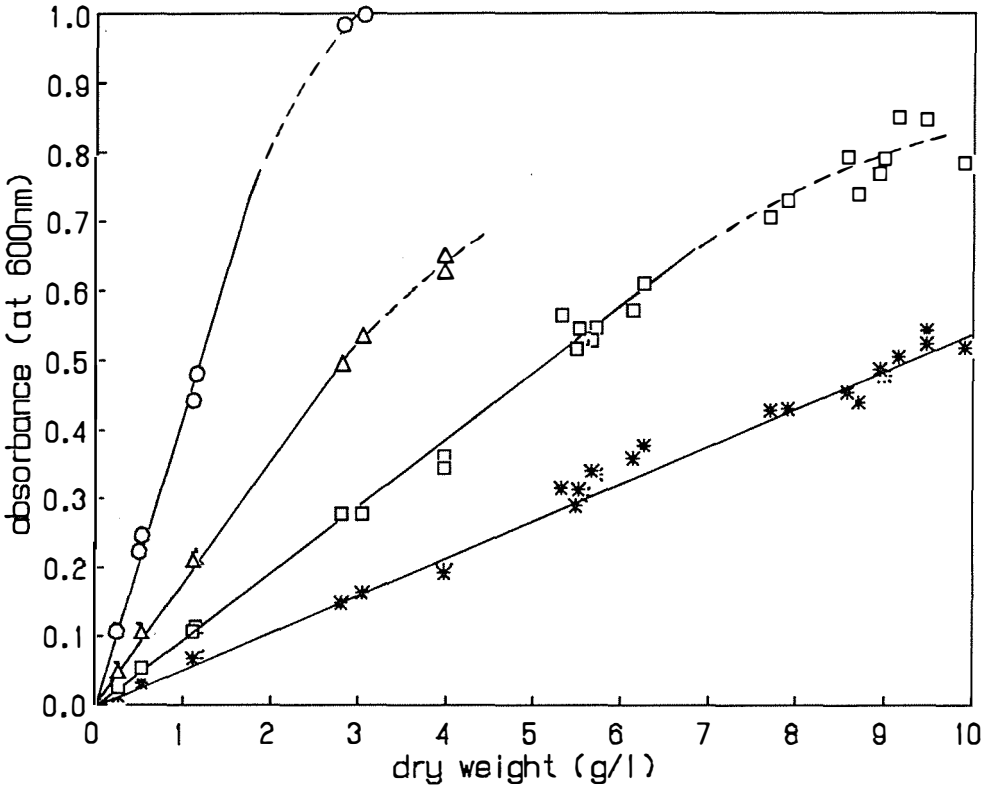


Figure 3.3 **Calibration curve for A_{600} at different dilutions versus dry biomass weight for *K. lactis* CBS 683. Dilutions: 1:10 (— o —); 1:25 (— ▵ —); 1:50 (— □ —); 1:100 (— * —).**

Table 3.2 Equations used to calculate dry weight from absorbance measurements at different dilutions.

Dilution factor	Dry weight calculation	Regression coefficient (R^2)
1:10	dry weight = 2.47 x A_{600}	0.994
1:25	dry weight = 5.74 x A_{600}	0.999
1:50	dry weight = 10.40 x A_{600}	0.993
1:100	dry weight = 18.35 x A_{600}	0.991

3.4.4 Analysis of sugars

Glucose and lactose were measured using an enzymatic method based on that described by van der Zee (1991). Buffer A contained potassium phosphate buffer (0.1 M, pH 7), 4 amino-antipyrine (0.77 mM), glucose oxidase (9300 units l^{-1}; β-D-glucose:oxygen 1-oxido-reductase; EC 1.1.3.4, Type X-S, Sigma Co.), and peroxidase (1500 units l^{-1}; EC 1.11.1.7, Type VI-A, Sigma Co.). Solution B was prepared by adding magnesium chloride (0.5 ml: 1 M) and phenol (2.5 ml: 11 mM) to every 100 ml freshly-made buffer A. For lactose analysis β-galactosidase (1 ml to every 100 ml solution B; Maxilact LX-5000, Gist-Brocades Ltd, Delft, The Netherlands) was also added. The sample containing the sugar was diluted as appropriate to give a sugar concentration in the range 0 to 1 g l^{-1}. The diluted sample (0.2 ml) was mixed with solution B (2 ml), incubated at 30°C for 4 h, and the absorbance of the solution was measured at 510 nm. A standard curve was prepared using either lactose or glucose solutions in the concentration range 0 to 1 g l^{-1}.

3.4.5 Protein concentration

Protein concentration was measured by the Coomassie blue binding assay (Sedmak and Grossberg, 1977) using bovine serum albumin as standard. Equal volumes of sample or serial diluted sample and 0.06 % Coomassie blue G-250 in 3 % perchloric acid (twice filtered with Whatman No.1 filter) were mixed

together and immediately read by spectrophotometer at an absorbance of 640nm.

3.4.6 Determination of ethanol

Two gas chromatographs fitted with a flame ionization detector were used: a Shimadzu gas chromatograph (Model GC-APF, Shimadzu Corporation, Kyoto, Japan) and a Carlo Erba gas chromatograph (Model GC 6000 vega series 2, Carlo Erba strumentazione, Milan, Italy). The methods were very similar for each but operating conditions varied. Isopropanol (10 g l^{-1}) was used as an internal standard. Standard ethanol solutions, in the range 0 to 10 g l^{-1}, were added to an equal volume of internal standard. The sample volume injected was 2 μl.

For the Shimadzu, a 1 m x 0.15 cm ID column containing Poropak Q was used at a carrier gas (nitrogen) flow rate of 60 ml min^{-1} and a column temperature of 200°C. The injector and detector temperatures were 220°C. Quantitation of the sample ethanol concentration was accomplished using a Varian integrator data system III C (Varian, California, USA) to measure the peak areas. The peak spectrum was also recorded using a Seconic SS 250 G recorder (Japan). Ethanol concentration was calculated by measuring the relative areas of the ethanol and isopropanol peaks and comparing this with the standard curve prepared from values for the known standard solutions.

For the Carlo Erba, a 2 cm x 0.4 cm ID column was used at a carrier gas (nitrogen) flow rate of 40 ml min^{-1} and the same column temperatures as above. Ethanol concentration was determined using a Hitachi Chromato-integrator (Hitachi Ltd, Tokyo, Japan, Model D-2500) as for the procedure above.

3.4.7 Determination of α-amylase activity

Transformed yeasts were streaked onto YPL-starch agar and incubated at 30°C for 2 - 3 days. After colony growth, the plates were inverted over beakers containing solid iodine at 50°C (Rothstein *et al.*, 1984). Clearing zones indicated

starch degradation by the α-amylase enzyme, while the remaining starch stained a deep blue-black colour.

Quantitative determination of α-amylase activity was performed using a modification of the Wilson and Ingledew (1982) assay. Cell-free supernatant (200 µl) was added to starch substrate (400 µl; 0.2 %(w/v) soluble starch boiled to dissolve in 0.05 M KH_2PO_4 - NaOH buffer (pH 6) and cooled to 40°C) and the mixture was incubated at 40°C for 30 min instead of 10 min, as for the Wilson and Ingledew (1982) method. The reaction was stopped by adding sample-starch mixture (200 µl) to iodine reagent (5 ml; stock solution: 0.5 %(w/v) I_2 in 5 %(w/v) KI: 200 µl stock solution plus 1 ml 5 M HCl was added to 100 ml water), and the absorbance was read immediately at 620nm. Standard α-amylase enzyme, *Bacillus subtilis* A-6505 Type III-A (67 (units)(mg solid^{-1})), was obtained from Sigma Chemical Co. (St Louis, USA). One unit of α-amylase is defined as the amount of enzyme that hydrolyzed 0.1 mg of starch in 30 minutes at 40°C when 4.0 mg of starch was present.

3.4.8 Disruption of yeast cell walls

The method used for the small-scale disruption of yeasts to obtain the internal cell fraction was based on that described by Hummel and Kula (1989). The grown culture (50 ml) was centrifuged (4000 rpm, 10 min) and washed in buffer (0.1 M KH_2PO_4, pH 7.5; 0.01 % mercaptoethanol; 0.1 % polyethylene glycol 4000). Fresh buffer (4 ml) was added to approximately 1 g wet cells, and the suspension (0.6 ml) was placed in a microfuge tube with 1.2 g glass beads (0.5 mm diameter). The mixture was vortexed for 15 minutes and, after centrifugation (13,000 rpm, 10 min), the clear supernatant was used for enzyme and protein analysis.

3.5 PLASMID STABILITY MEASUREMENT

Three methods were investigated for measuring the plasmid stability of recombinant cultures. An analysis and comparison of the plate ratio, clearing

zones, and transfer colony method is given in Chapter 6, with the former two chosen for batch and continuous culture studies. When values were obtained with both methods the mean plasmid stability was taken. Colony forming units (cfu) were determined using the spread plate technique on selective YPL-G418-starch and non-selective YPL-starch agar. Dilutions of the sample were prepared using peptone water (5 g l^{-1}). After incubation at 30°C for 2 - 3 days, the colonies were counted using a Colony Counter (Model 560, Suntex Instruments Co. Ltd, Taipei, Taiwan), and α-amylase production was determined via clearing zones.

3.6 FERMENTATION CULTURE CONDITIONS

3.6.1 Batch shake-flask culture

High aeration conditions comprised 50 ml medium, shaking at 250 rpm (gyratory shaker, New Brunswick Scientific Co., New Jersey, USA) in 250 ml Erlenmeyer flasks , at 30 °C. Low aeration conditions were 200 ml medium, shaking at 50 rpm, at 30°C, with a gas trap fitted. A shaking waterbath (Model SW 20-C, Julabo Labortechnik, Germany) was used for temperatures of 15, 20, and 25°C. A cooling water system (Model F10-UC/5, Julabo Labortechnik, Germany) was set-up to maintain the low temperature of 15°C. Samples of the fermentation broth (3 ml) were analyzed for cell growth, pH, plasmid stability, and α-amylase activity. The remainder of the sample was centrifuged (8,000 rpm, 5 min) and the supernatant was stored at -20°C for lactose and ethanol analyses.

3.6.2 Continuous culture

Two similar fermenters were used: a culture apparatus constructed in the Process and Environmental Technology Department workshop (Massey University, New Zealand) with electronic circuitry by Designer Electronics (Palmerston North, New Zealand) for fermentations investigating dilution rate effects, and a Multigen F2000 Benchtop fermenter (New Brunswick Scientific Co., New Jersey, USA) for studying the other effects. The change was made because mechanical problems

arose with the equipment. For both fermenters, the same 2 litre pyrex glass vessel of 1 litre working volume was used, with a polyethylene-polypropylene head containing holes for the insertion of probes and other sensors. The vessel was unbaffled, with the various probes and tubes assumed to provide sufficient baffling. A photograph of the fermenter apparatus is shown in Figure 3.4, and a schematic diagram of the fermenter apparatus and fermenter vessel are displayed in Figures 3.5 and 3.6, respectively.

Agitation was provided by a single 6-bladed disc-turbine impeller mounted 5 cm above the base of the fermenter on the central impeller shaft. This was driven from the base of the fermenter using indirect magnetic coupling to turn the impeller. High aeration agitation was achieved at 600-650 rpm, and low aeration agitation at 175-200 rpm.

The fermenter temperature was usually maintained at 30 ± 0.2°C which was controlled by means of a heating element, inserted into one of the closed-ended metal tubes in the fermenter head, and an electronic thermostat. A digital thermocouple was used to check the temperature and the temperature was also continuously monitored using a calibrated thermocouple and a Honeywell Versaprint Multipoint chart recorder (Amiens, France). For one of the runs the temperature was controlled at 20 ± 0.2°C by combining the heating element system with the refrigeration unit previously used for batch culture at 15°C, from which cooling water at 19.0°C was recycled through internal coils within the fermenter.

Air was supplied to the fermenter vessel from the laboratory compressed air line, through a pressure regulator and filter (Norgren Martonair, Warwickshire, England). A Gap meter (Gap Basingstoke, England) regulated the flowrate. Two Gap meters were used: the first controlled the airflow to the fermenter over the range 0 to 0.6 l min^{-1} for high aeration; and the other controlled the airflow over the range 0 to 5 ml min^{-1} for low aeration. The measured air was passed through a sterile glass wool-packed filter, and a sterile Millipore filter (Millex-FG_{50}, 0.20

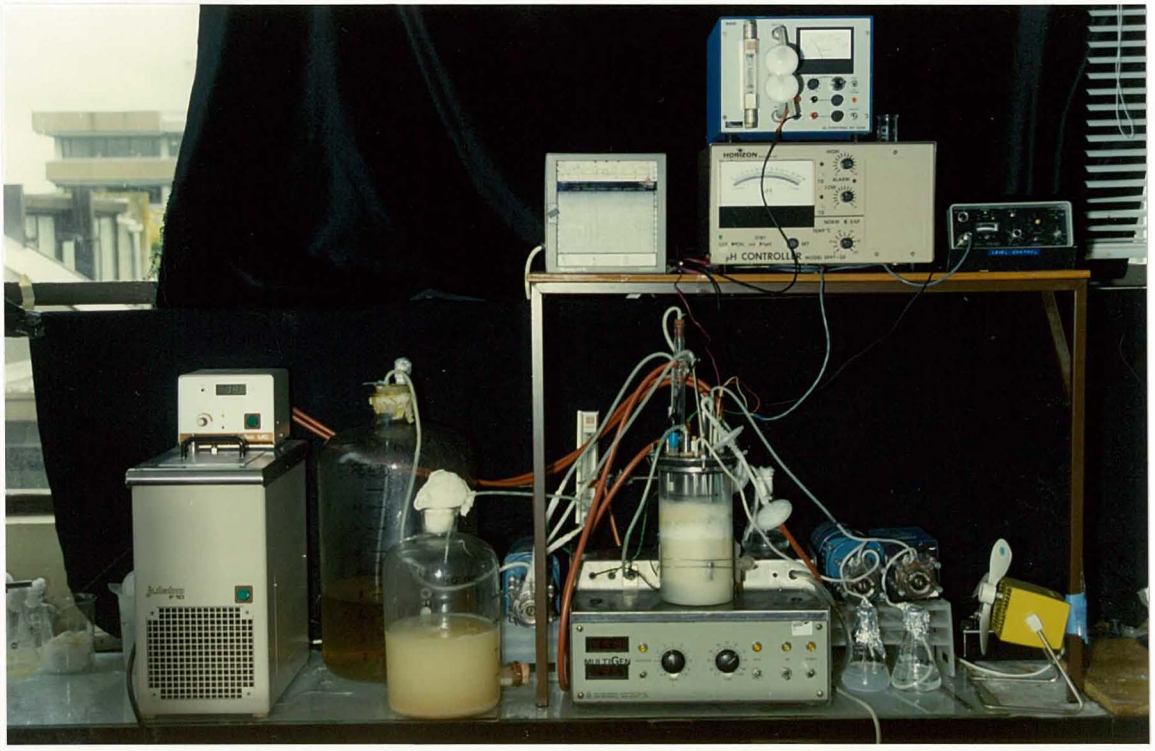


Figure 3.4 **Photograph of the fermenter apparatus used for continuous culture experiments. Major items from left are: (bottom) refrigeration unit, feed-medium vessel, culture outlet collection vessel, fermenter vessel, antifoam reservoir, pH reservoir; (top) recorder, pH and DO recorders and controllers, and antifoam controller. Further detail of apparatus is shown in Figure 3.5.**

LEGEND

A compressed air line
AF1 air-filter on air-line
AF2 air-filter on exhaust air-line
AF3 air-filter on feed-medium vessel
C1 foam detector
C2 pH controller
C3 temperature controller
CD condenser for air outlet
G gap meter
MD magnetic drive motor
P1 feed-medium pump
P2 culture-outlet pump
P3 antifoam pump
P4 NaOH pump
PR pressure regulator on air-line
R1 DO recorder
R2 pH recorder
R3 temperature recorder
RU refrigeration unit (for 20°C run only)
V1 fermenter vessel
V2 feed-medium vessel
V3 culture outlet collection vessel
V4 NaOH reservoir
V5 antifoam reservoir
WT water-trap

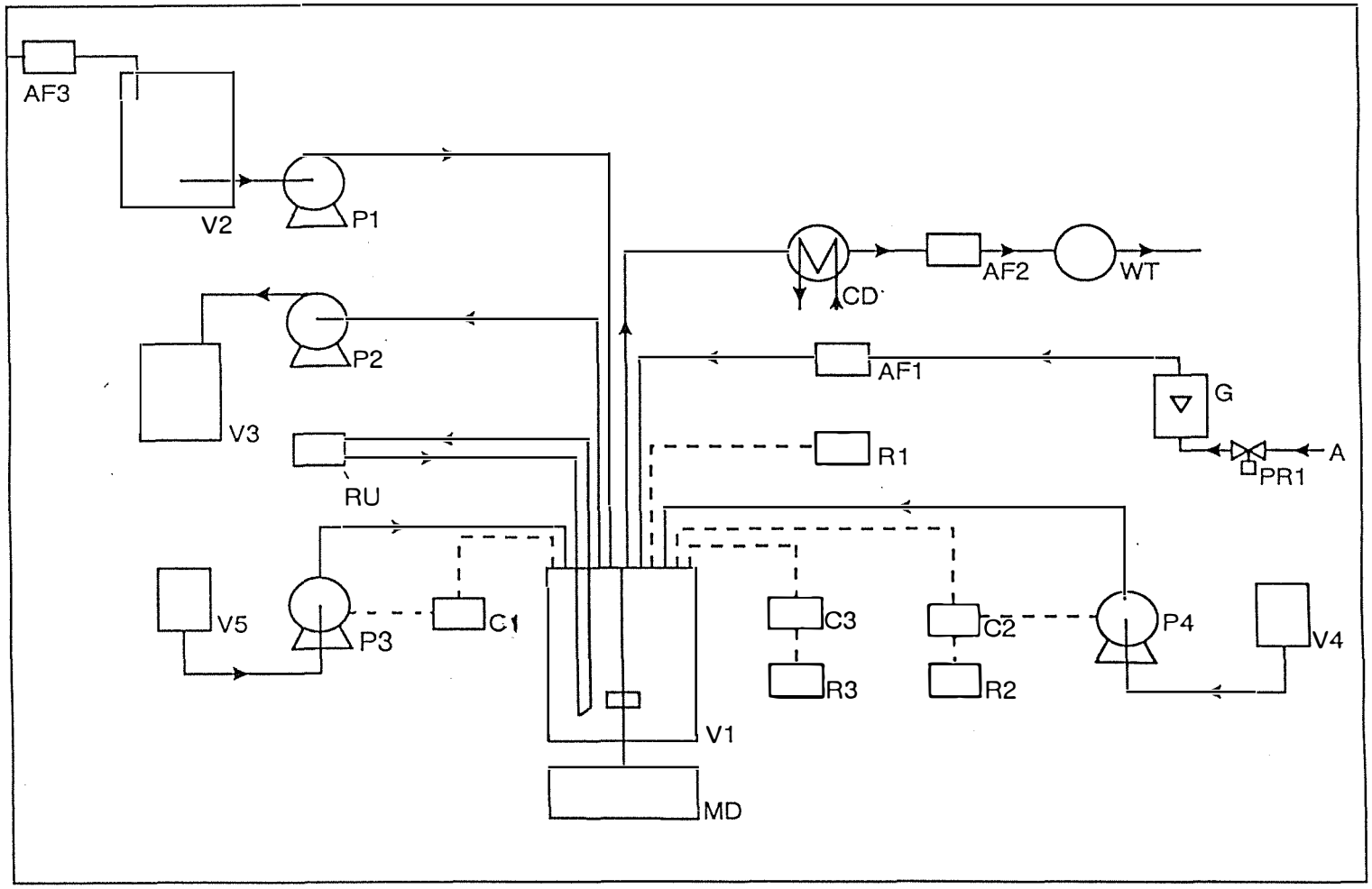
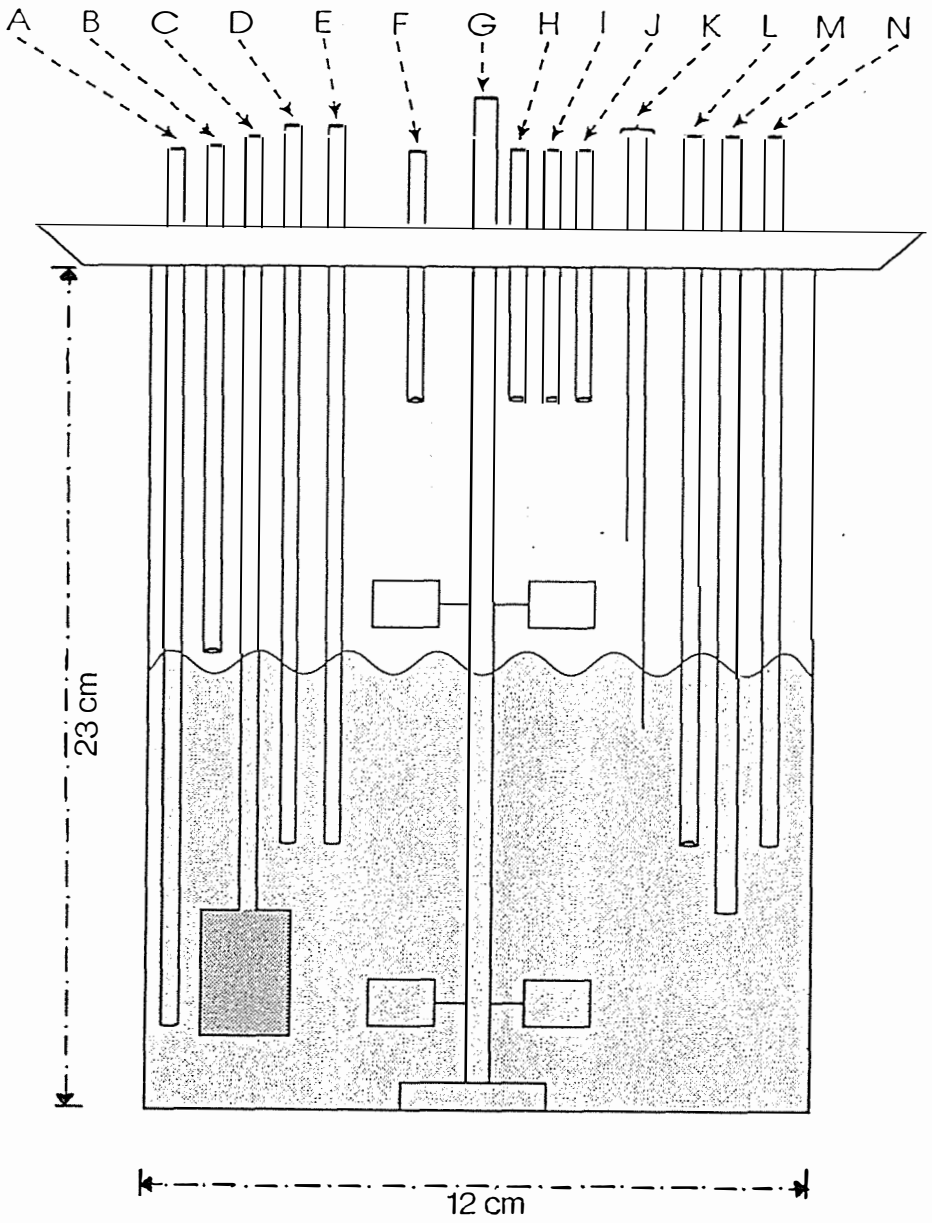


Figure 3.5 **Schematic diagram of the fermenter apparatus used for continuous culture experiments.**



LEGEND

A	heating element	H	medium feed inlet
B	culture outlet	I	antifoam addition inlet
C	air diffuser	J	NaOH addition inlet
D	thermocouple port/temperature recorder	K	foam detector
E	thermometer port	L	DO probe
F	air outlet	M	pH probe
G	Impeller drive shaft	N	thermocouple port/temperature control

(D and E for refrigeration control only)

Figure 3.6 Schematic diagram of the fermenter vessel used for continuous culture experiments.

μm, Millipore Co., Molsheim, France) before entering the culture vessel through a 4 cm stainless steel sintered metal plate. Exhaust air was vented through a water cooled condenser to minimize the loss of fermentation liquid by evaporation. From the condenser the gas passed through a second sterile Millipore filter and bubbled through a water trap. Dissolved oxygen tension was measured using a galvanic oxygen electrode (Type G2 Steam Sterilizable, Uniprobe Instruments Ltd, Cardiff, UK) which was connected to a dissolved oxygen module (Code 508, L.H.Fermentation, Stoke Poges, UK) and continuously monitored using the Honeywell Versaprint multipoint chart recorder. Prior to inoculation, the probe was calibrated *in situ* by sparging oxygen-free nitrogen gas through the air supply system to obtain zero saturation conditions, followed by vigorous agitation and aeration to give conditions of 100 % air saturation (p_O = 0.21 atm). High aeration conditions required an air flowrate of 0.15 l min^{-1} to maintain the air above 20 %, and an air flowrate of 3 - 5 ml min^{-1} was used to obtain low aeration conditions.

The culture pH was measured using a combination pH electrode (Broadley James Co., Santa Ana, USA) connected to a Horizon pH Controller Model 5997-20 (Ecology Co., Oak Park Avenue, Chicago, Illinois, USA). The pH electrode was calibrated using pH 4.0 and pH 7.0 buffer solutions prior to each fermentation. The pH controller was connected to the Honeywell Versaprint multipoint chart recorder which continuously recorded the pH. A Masterflex fixed speed (1 rpm) peristaltic pump (Cole-Parmer Instrument Co., Chicago, Illinois, USA) was connected to the controller to automatically maintain the culture at pH 5.0 ± 0.2 using 1 M NaOH dosing.

Foam was detected by conductivity between two probes, one submerged in the culture, and the other 2.5 cm above the culture fluid. A foam controller (Electronics Workshop, Process and Environmental Technology Department, Massey University, New Zealand) connected to a Masterflex fixed speed (1 rpm) peristaltic pump dosed sterile Bevaloid 6009D antifoam (10 g l^{-1}) to control foaming problems. A mechanical foam breaker, consisting of a 6-bladed disc-

turbine impeller, mounted 2 cm on the central agitation shaft above the surface of the culture fluid, also helped to maintain low foam levels.

Yeast growth and foam residue on the walls of the vessel were periodically dislodged using a teflon coated bar magnet inside the fermenter vessel which was secured and moved by a horse-shoe magnet from outside the vessel.

The feed medium, WYP or WYP-G418, was delivered by a Masterflex peristaltic pump (5 - 100 rpm, Model 7546-10, Head model 7016, Cole-Parmer Instrument Co., Chicago, Illinois, USA). The dilution rate was controlled by a solid state Masterflex controller attached to the pump. Initially, the fermentation was operated in batch mode (1.05 litre culture volume). Sampling was achieved by switching the culture outlet pump on (the same as the peristaltic pump controlling the feed but with a larger head, Model 7015) and after allowing approximately 10 ml to run through, a sample of 3 ml was taken. After 8 - 10 h incubation, on checking that cell growth was in the exponential phase of growth, the delivery of feed medium was commenced at the required dilution rate. Samples during continuous culture were also taken from the culture outlet line. It was assumed, due to vigorous fermenter mixing and the short distance between the fermenter and outlet, that the culture outlet fluid would be representative of the fermenter contents. The outlet tube was positioned at the 1 litre liquid level in the fermenter vessel and this allowed control over the level of fluid.

Fermentation samples were immediately analyzed for cell growth via the A_{600} calibration, diluted and plated onto selective and non-selective media for plasmid stability measurement, and analyzed for α-amylase activity. The remainder of the sample was centrifuged (8,000 rpm, 5 min) and the supernatant stored at -20°C for ethanol and lactose analyses.

3.7 DISCUSSION OF METHODS

Four methods were used for preparing plasmid DNA from *E. coli*. Depending on how much DNA was required for the subsequent work either the small-scale or large-scale methods were used. Initially, the small-scale rapid boil DNA method was used for quick preparations of plasmid DNA that were used in restriction enzyme digests to check restriction sites. The other small-scale method, the alkaline lysis method, was that used regularly at Institute Curie, Paris, and although it took longer and was more complicated than the rapid boil method, it gave cleaner DNA preparations that were also more concentrated. Therefore, alkaline lysis was chosen as the routine method used for small-scale DNA preparations in this study.

The two methods for large-scale plasmid preparations gave a ten-fold difference in DNA concentration. The large-scale alkaline lysis method produced the larger quantity of DNA, but was extremely time-consuming, requiring a period of three days for completion. This method was used only once in order to prepare a sufficient quantities of DNA for use at Institute Curie for both plasmid construction work and electroporation studies. The Circleprep^{R} method required only a few hours for the preparation of plasmid DNA. Even though the quantity prepared was lower than that of the alkaline lysis method this method was routinely used as the large-scale protocol for plasmid DNA isolation, and the DNA prepared in this way was used for plasmid constructions and yeast and *E. coli* transformation procedures.

CHAPTER 4

CONSTRUCTION OF THE VECTOR pCR1 FOR EXPRESSION OF A WHEAT α-AMYLASE IN *KLUYVEROMYCES* YEASTS

4.1 BACKGROUND AND AIM

The plasmid pKD1, shown in Figure 2.2, has been used to construct novel vectors in *Kluyveromyces lactis*, which have exhibited an efficiency and stability comparable to the 2μ-based *Saccharomyces cerevisiae* system (Bianchi *et al.*, 1987). One of these recombinant vectors is pCXJ-kan1, presented in Figure 2.3. This is a shuttle vector containing the A, B, and C open reading frames of pKD1, a pair of inverted repeats, the *URA*A gene for selection in Ura$^-$ mutant yeasts, and the vector pUC19 which includes an *E. coli* ORI, an ampicillin resistance gene, and *LAC* genes (Falcone *et al.*, 1986). It also includes a G418 resistance gene for selection of *Kluyveromyces* transformants. The plasmid encodes a number of proteins that are required for the stable maintenance of the plasmid at high copy number during cell division.

Kluyveromyces strains offer an advantage in secretion capacity over *S. cerevisiae* for production of heterologous proteins (Macreadie *et al.*, 1991), and vectors based on pKD1 have recently been used to secrete interleukin 1β (Fleer *et al.*, 1991a) and human serum albumin (Fleer *et al.*, 1991b).

The production of α-amylase by *K. lactis* was selected as a model system for studying the expression and stability of foreign genes in *Kluyveromyces*. The enzyme degrades α-1,4 glycosidic bonds in compounds such as starch and glycogen, and has been produced from *S. cerevisiae* by a number of workers. Rothstein *et al.* (1984) used an isolated cDNA clone from a wheat α-amylase gene and *S. cerevisiae* PGK promoter to secrete a functionally active enzyme

from *S. cerevisiae* and this cassette was used in this study. An advantage of using α-amylase is that it is easily detected by a simple assay based on the binding of iodine to high molecular weight starch.

The aim of this work was to construct a plasmid encoding the α-amylase enzyme from the pCXJ-kan1 vector, and, using this, to investigate heterologous α-amylase protein production in *K. lactis* yeasts.

4.2 MATERIALS AND METHODS

Full details of the materials and methods are given in Chapter 3. Specifically, the media used for the work described in this chapter were: LB broth and agar with appropriate antibiotics for *E. coli*; YPD broth, and YPD-G418 and MM selective agar for yeast; and starch, YPD-starch-G418, and MM-starch agar for α-amylase activity detection. Competent cells of *E. coli* DH5α were used for plasmid manipulations, with final plasmid constructions transformed into *K. lactis* MW98-8C, CBS 141, and CBS 683 for α-amylase determination. Details of the original plasmids and vectors derived in this study are given in Table 3.1. The plasmid construction methods, *E. coli* and *K. lactis* transformation, and other molecular biological protocols are described in Section 3.3. The qualitative and quantitative methods for α-amylase analysis are given in Section 3.4.7, and the protein assay and yeast cell disruption method are described in Sections 3.4.5 and 3.4.8, respectively.

4.3 RESULTS

4.3.1 Construction of pCR1

The cloning vector pCR1 was constructed as outlined in Figure 4.1. Plasmid pMA230 was digested with *Hin*d III and *Pst* I to produce fragments carrying the PGK (*Hin*d III -*Pst* I, 1.5 kb) and the α-amylase (*Pst* I - *Pst* I, 1.5 kb) genes. The 1.5 kb doublet was recovered from an agarose gel by freeze-thawing and the two

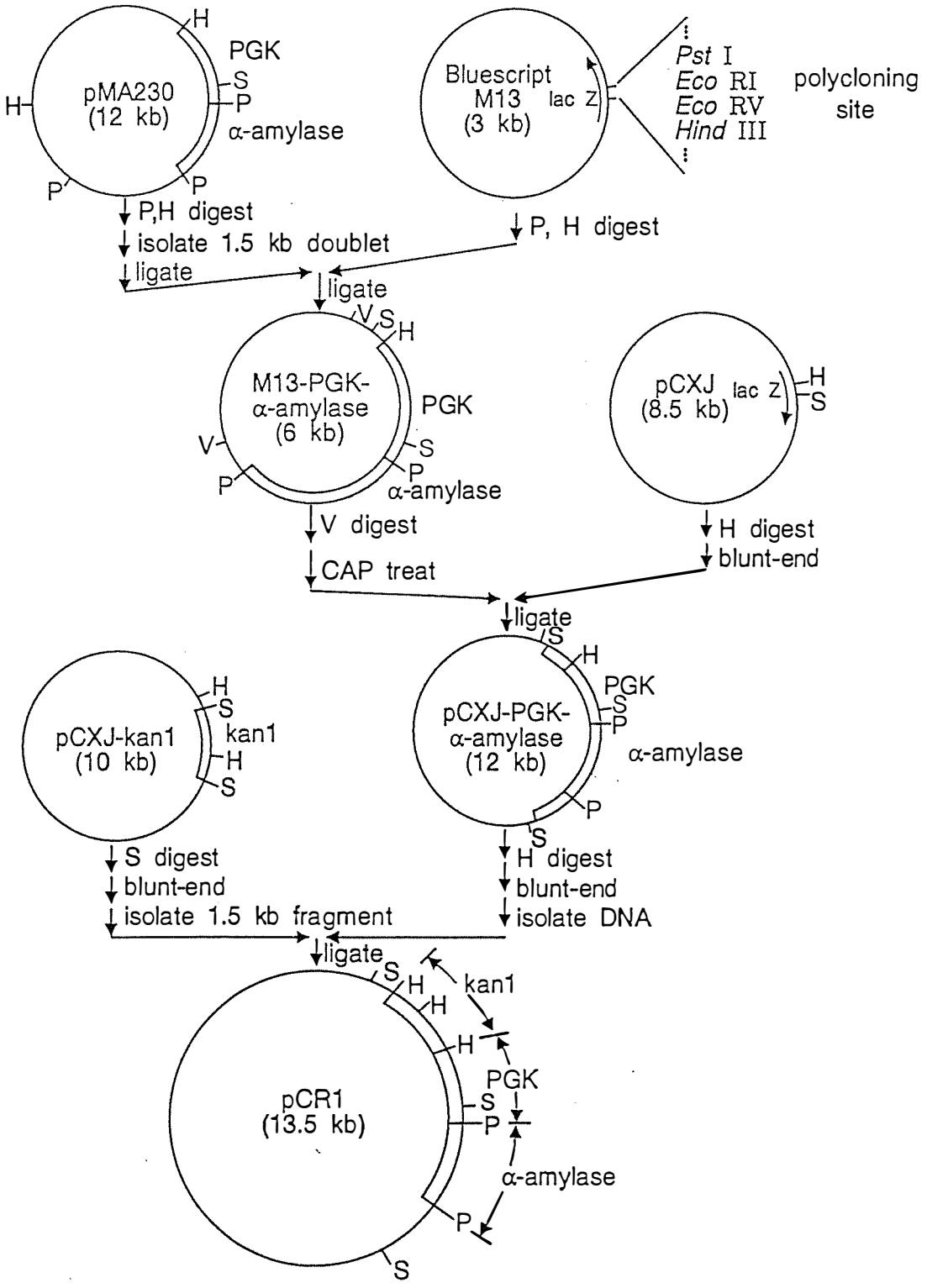


Figure 4.1 Schematic diagram of plasmid pCR1 construction. Sizes are not to scale. Relevant restriction sites and structural genes are shown on each plasmid. (abbrev: H = *Hin*d III; P = *Pst* I; S = *Sal* I; V = *Pvu* II)

DNA fragments ligated together. This ligation mixture was ligated with a *Pst* I/*Hin*d III double-digested Bluescript-M13 vector and used to transform *E. coli* DH5α competent cells. Transformants were selected as white (Lac^-) colonies on LB + Ap agar containing IPTG and X-gal. Analysis of isolated DNA by restriction digests showed that the PGK and α-amylase fragments were cloned into Bluescript-M13 in the desired orientation with the PGK gene ligated to the start of the α-amylase gene. The M13-Bluescript-PGK-α-amylase plasmid was cut with *Pvu* II to give two blunt-ended fragments. These were treated with calf alkaline phosphatase to prevent self-ligation, and then ligated to pCXJ, which was linearized at the unique *Hin*d III site and treated with Klenow enzyme to fill in the recessed ends. The blunt-ended ligation yielded transformant *E. coli* colonies distinguishable by disruption of the *LACZ* gene with blue/white selection on LB + Ap agar. White colony transformants were chosen and examined for plasmid sizes and restriction sites. Approximately half contained the PGK-α-amylase fragment rather than the M13 fragment. At this stage the expression of the α-amylase in *K. lactis* MW98-8C containing the vector pCXJ-PGK-α-amylase was confirmed with the qualitative α-amylase analysis.

The plasmid pCXJ-PGK-α-amylase can only be selected in Ura^- auxotrophic mutants. To enable use of the vector in other strains the *KAN*1 gene coding for G418 resistance was inserted. The pCXJ-kan1 vector was digested with *Sal* I to yield *KAN*1, and pCXJ-PGK-α-amylase was linearized with *Hin*d III. Both fragments of DNA had their recessed ends filled in with Klenow and were run on a 1 % agarose gel. The required bands were eluted from the gel by freeze-thawing. Blunt-ended ligations gave transformant *E. coli* colonies on LB + Kan agar. The composition of the final recombinant plasmid pCR1 was confirmed initially by restriction digests with *Hin*d III and *Sal* I, and later by appropriate hybridization.

As a further check to ensure pCR1 contained the correct genes, Southern hybridization was used to confirm the *KAN*1 gene and the PGK-α-amylase cassette in pCR1. Also, the plasmid pCR1 was probed for in the yeast *K. lactis*

CBS 683 : pCR1, which showed α-amylase activity. The results of the hybridizations are shown in Figures 4.2 and 4.3, respectively. Figure 4.2 shows that the *KAN*1 gene hybridized with the appropriate bands of pCXJ-kan1 and pCR1. The PGK-α-amylase fragments were shown to occur in the pMA230 1.5 kb doublet band and the pCR1 bands. The plasmid pCR1 contained both the *KAN*1 gene and PGK-α-amylase promoter-gene sequence, and the transformed *K. lactis* producing α-amylase did indeed contain the pCR1 plasmid, as shown in Figure 4.3. It was interesting to note that in the final stage of construction, upon ligating the blunt-ended *Sal* I and *Hin*d III sites of the *KAN*1 insert and pCXJ-PGK-α-amylase vector, two *Hin*d III sites were generated.

4.3.2 Expression of α-amylase in *Kluyveromyces*

4.3.2.1 Qualitative α-amylase detection

An intermediate vector in the construction, pCXJ-PGK-α-amylase, was inserted into *K. lactis* MW98-8C using the optimized transformation procedure for the Bio-Rad Gene Pulser. Selected *K. lactis* MW98-8C Ura$^+$ transformants containing either pCXJ-kan1 or pCXJ-PGK-α-amylase were plated onto YPD agar containing starch and after growth the plates were tested for starch degradation. The controls, *viz.* the *K. lactis* MW98-8C parent strain and the transformants containing pCXJ-kan1, showed no clearing zones on inversion over iodine vapour (Figure 4.4). The transformants which harboured pCXJ-PGK-α-amylase gave large clearing zones around the colonies, indicating the starch had been degraded by the heterologous α-amylase enzyme.

The constructed plasmid pCR1 was similarly tested in *K. lactis* CBS 141. G418-resistant colonies were selected following transformation and compared with those containing pCXJ-kan1 and non-transformed CBS 141. The transformants carrying pCR1 exhibited the same clearing zones as for pCXJ-PGK-α-amylase on YPD-starch agar, whereas the controls did not.

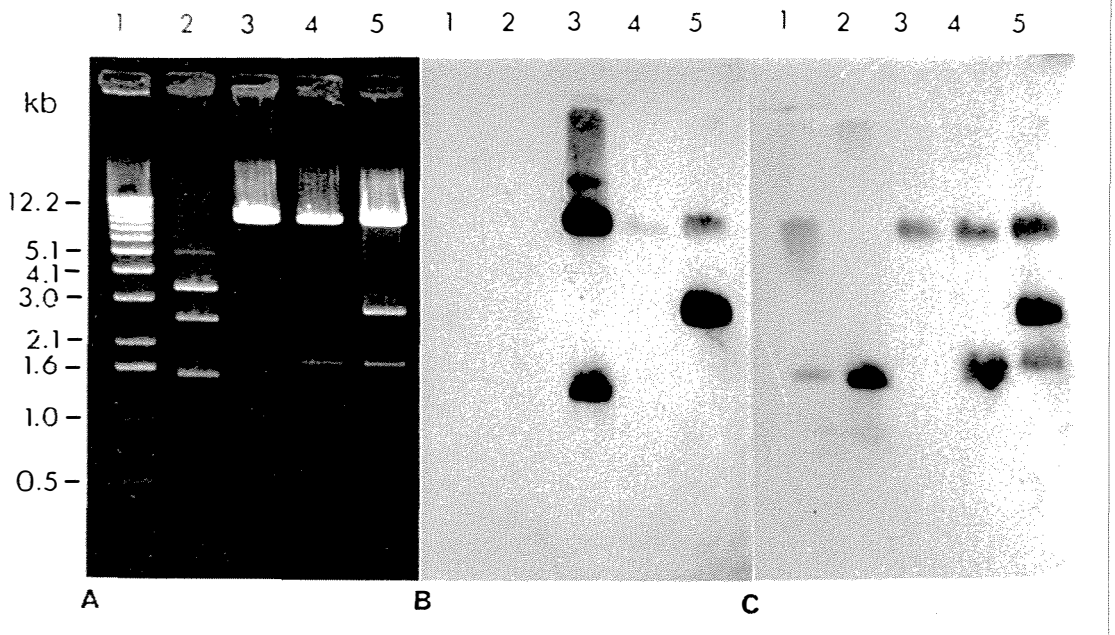


Figure 4.2 **Southern-blot analysis of constructed plasmids pCXJ-PGK-α-amylase and pCR1 to show the occurrence of the *KAN*1 gene and PGK-α-amylase cassette. Panel A shows the original gel, Panel B was probed with *KAN*1 DNA, and Panel C was probed with PGK-α-amylase DNA. Lanes: 1, 1 kb DNA ladder (BRL); 2, pMA230 digested with *Pst* I and *Hin*d III (the 1.5 kb band is a doublet of PGK and α-amylase DNA); 3, pCXJ-kan1 digested with *Sal* I; 4, and 5, pCXJ-PGK-α-amylase and pCR1 digested with *Sal* I, respectively.**

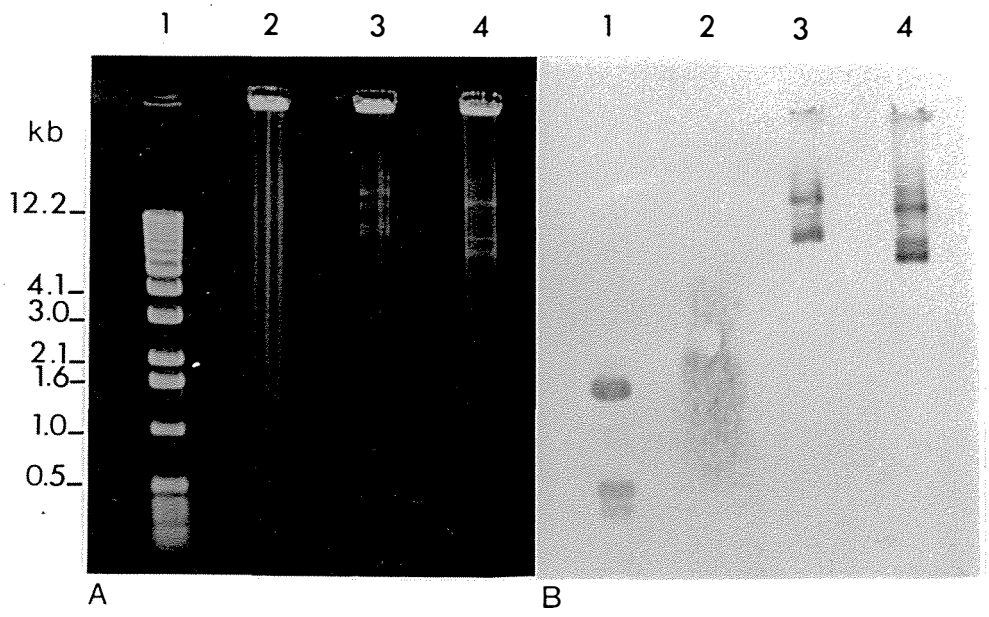


Figure 4.3 **Southern-blot analysis of plasmid DNA derived from *K. lactis* CBS 683 containing either no plasmid (pl⁻), or plasmids pCR1 or pCXJ-kan1. Panel A shows the original gel, and Panel B was probed with pCR1 DNA. Lanes: 1, 1 kb DNA ladder (BRL); 2, 3, and 4, plasmid DNA isolated from *K. lactis* CBS 683 : pl⁻, : pCR1, and : pCXJ-kan1, respectively.**

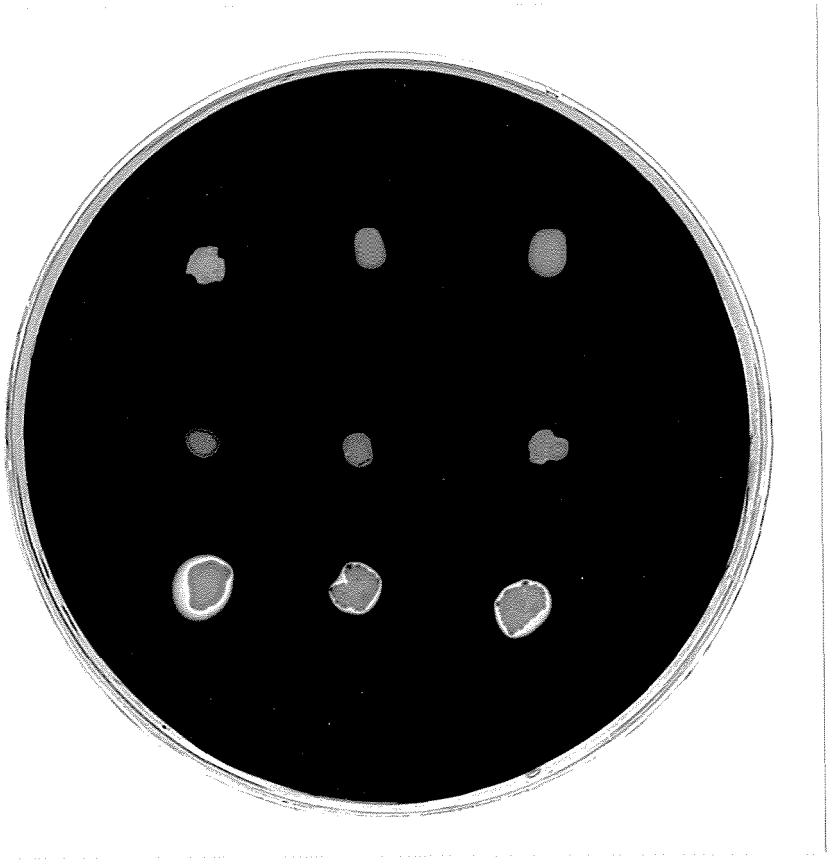


Figure 4.4 **Plate showing α-amylase-iodine clearing zones. Top row: *K. lactis* MW 98-8C parent strain; middle row: *K. lactis* MW 98-8C : pCXJ-kan1; and, bottom row: *K. lactis* MW 98-8C : pCR1.**

Another intermediate vector, M13-PGK-α-amylase, was tested for α-amylase expression in *E. coli* DH5α by plating the transformants onto starch agar. After incubation at 37°C for 48 h no starch utilization was observed for *E. coli*. Construct pCR1 was also tested for α-amylase secretion in the yeasts *K. lactis* MW98-8C and CBS 141 on MM-starch plates. No clearing zones were observed indicating no starch degradation.

4.3.2.2 Quantitative α-amylase determination

The modified starch-iodine assay of Wilson and Ingledew (1982) was used to measure α-amylase production levels for *K. lactis* CBS 683 : pCR1. Supernatant samples from high aeration shake-flask cultures grown in YPL broth gave a typical value of 0.4 μg ml^{-1} (2.7 Sigma U ml^{-1}) equivalent Sigma standard (*Bacillus subtilis* Type IIIA). This was equivalent to 6 U ml^{-1}, where one unit is the amount of enzyme that hydrolyzed 0.1 mg starch in 30 minutes at 40°C when 4.0 mg starch was present (modified from Wilson and Ingledew, 1982).

A range of temperatures for incubation of the sample and starch substrate was investigated with results shown in Table 4.1. The samples used came from a continuous fermentation (using selective medium as described in Chapter 7).

Table 4.1 Modified α-amylase method showing effect of incubating the sample (ex continuous fermentation) and starch-iodine mixture at different temperatures.

Temperature (°C)	α-Amylase (U ml^{-1})[a]
20	2.3
30	8.5
40	11.8
50	15.6
60	14.6

[a] One unit is the amount of enzyme that hydrolyzed 0.1 mg starch in 30 minutes at 40°C when 4.0 mg starch was present.

It was found that the assay temperature giving the highest values was closer to 50°C than the recommended 40°C. However, the assay at 40°C had already been used for a number of fermentations in this study, and it is the temperature used in most reported starch-iodine assays. Therefore for all α-amylase assays, the conditions for incubation of the sample and starch substrate mixture were 40°C and 30 minutes.

Using the cell disruption method the internal α-amylase and protein were determined for *K. lactis* CBS 683 : pCR1 grown in WYP-G418. The results are shown in Table 4.2.

Table 4.2 Results of secreted (supernatant) and internal α-amylase and protein measurements for *K. lactis* CBS 683 : pCR1 (ex high aeration WYP-G418 shake flask cultures).

Sample	Total protein (mg ml^{-1})	Original culture volume (ml)	Concentrated sample volume (ml)	α-Amylase (U per ml conc. sample)[a]	α-Amylase (U per ml original culture)[a]	α-Amylase (µg per ml original culture)[b]
supernatant	-	1	1	6.1	6.1	$3.7x10^{-1}$
internal	36.4	50	7	7.9	1.1	$6.8x10^{-2}$

[a] One unit is the amount of enzyme that hydrolyzed 0.1 mg starch in 30 minutes at 40°C when 4.0 mg starch was present.

[b] µg Sigma standard α-amylase per ml original culture volume.

4.4 DISCUSSION

Two plasmids constructed in this work were shown to express the α-amylase enzyme in *K. lactis*. The plasmid pCXJ-PGK-α-amylase can be used to obtain transformants producing α-amylase from Ura^- mutant strains of *K. lactis*, whereas plasmid pCR1 can be used for both Ura^- yeasts and industrial-type strains using either the Ura^+ genotype or the G418-resistance phenotype for selection. The

PGK-α-amylase cassette for production of the α-amylase enzyme was inserted within the functionally neutral point of pKD1.

The negative starch utilization results of *E. coli*, harbouring an intermediate vector coding for α-amylase, indicated the α-amylase enzyme was not expressed in this bacterium. There are two possible reasons for the apparent non-expression. Firstly, *E. coli* may not recognize the plant secretion signal encoded by the PGK-α-amylase cassette. Foreign signal peptide recognition in *E. coli*, whether from eukaryotic or prokaryotic genes, can vary considerably (Gatenby *et al.*, 1986). Secondly, *E. coli* may not recognize the yeast PGK promoter and/or the cassette may not be in frame with the *LACZ* coding region which contains the lac promoter (Lazarus, 1991), so that the α-amylase gene is not translated or transcribed by *E. coli*. The latter explanation is more likely since Gatenby *et al.* (1986) showed that the wheat α-amylase signal peptide was recognized in *E. coli*, resulting in secretion of the precursor enzyme into the periplasmic space, although the efficiency of recognition of the wheat signal peptide in *E. coli* was low.

Negative starch results for *K. lactis* containing pCR1 were observed on minimal media. This agreed with results by Rothstein *et al.* (1984, 1987). Rothstein *et al.* (1987) showed that the secretion of α-amylase by *S. cerevisiae* into the medium was efficient in rich medium but barely detectable in a minimal medium or selective minimal medium, although the α-amylase was still being synthesized in an equivalent amount. They found intracellular α-amylase which was not secreted and suggested that a fraction of protein may have been diverted to some organellar compartment, and therefore not secreted, or the process of transporting the α-amylase from the endoplasmic reticulum across the cell membrane was slow due to a deficiency for a necessary sequence or structure.

There are two simple colorimetric methodologies for determining α-amylase activity - those based on the disappearance of the blue-black colour of the starch-iodine complex, and those based on the appearance of glucose on starch degradation. In this work the former method was used, since glucose already

present in the growth medium (YPD) would complicate an analysis carried out using the latter. The modified method of Wilson and Ingledew (1982) allowed an estimate of α-amylase production to be made for *K. lactis* CBS 683 : pCR1. A typical value obtained from shake-flask cultures of *K. lactis* CBS 683 : pCR1 assayed for α-amylase was 6 U ml^{-1}, where one unit is defined as the amount of enzyme that hydrolyzed 0.1 mg starch in 30 minutes at 40°C when 4.0 mg starch was present.

Comparison of CBS 683 : pCR1 with other recombinant amylolytic yeasts was complicated by the fact that different assay conditions for the determination of α-amylase were used in each case. Sigma (1991) noted of α-amylase quantitation that "so many different assay procedures and 'units' are being used around the world that it is almost impossible to evaluate various offers". Aside from this, the level of α-amylase activity produced by *K. lactis* CBS 683 : pCR1 was low. Examples of other reported α-amylase enzyme levels are shown in Table 4.3. The value obtained in this study is approximately 10-fold less than those values for mouse and *S. alluvius* α-amylases obtained using the 'equivalent assay' but incubated for 30 minutes instead of 10 minutes.

Rothstein *et al.* (1987) noted that the expression of α-amylase protein was low for their constructs in *S. cerevisiae* (including the construct used in this work). They did not cite α-amylase activity levels but noted they were approximately 0.01- 0.05 % of the total cell protein. Another of their constructs, with the signal peptide deleted, gave ten-fold higher production of α-amylase, however the protein was in a non-secreted form. In addition, *S. cerevisiae* transformed with pMA230 containing the PGK-α-amylase cassette was observed to grow slowly both on agar and liquid media. In this work, the total (internal and supernatant) α-amylase was 0.44 μg ml^{-1} of Sigma standard α-amylase, with a internal protein concentration of 36 mg ml^{-1}. This gave a total α-amylase level of approximately 0.001 % of the total cell protein. The internal fraction of α-amylase was found to be about 15 % of the total α-amylase indicating that the greater proportion was secreted out of the cell.

Table 4.3 Examples of levels of α-amylase produced from various yeasts.

Yeast	Origin of gene	α-Amylase activity	Reference
S. cerevisiae	*Schwanniomyces occidentalis*	20.2 mU ml^{-1}[a]	Strasser *et al.* (1989)
Schizosaccharomyces pombe	*S. occidentalis*	10.3 mU ml^{-1}[a]	Strasser *et al.* (1989)
K. lactis	*S. occidentalis*	30.5 mU ml^{-1}[a]	Strasser *et al.* (1989)
S. cerevisiae	mouse pancreas	153 U ml^{-1}[b]	Astolfi-Filho *et al.* (1986)
Schwanniomyces alluvius	native	187 U ml^{-1}[b]	Wilson and Ingledew (1982)

[a] One unit is defined as the amount of enzyme that liberated 1 μmol 2-chloro-4-nitrophenol per minute at 37°C.

[b] One unit is defined as the amount of enzyme that hydrolyzed 0.1 mg starch in 10 minutes at 40°C when 4.0 mg starch was present.

4.5 SUMMARY

The plasmid pCR1 was constructed to express and secrete a wheat α-amylase enzyme in *K. lactis* strains. The construct was based on the vector pCXJ-kan1, which was derived from pKD1, a native plasmid of *K. lactis* var. *drosophilarum* containing the essential regions for plasmid replication and stability. Construct pCR1 produced an α-amylase through incorporation of a *S. cerevisiae* PGK promoter and DNA isolated from a wheat cDNA clone. The α-amylase was produced at low concentration but was easily measured with a starch-iodine assay. A typical yield of 6 U ml^{-1} was obtained for batch growth in rich lactose medium for *K. lactis* CBS 683 : pCR1, with one unit defined as the amount of enzyme that hydrolyzed 0.1 mg starch in 30 minutes at 40°C when 4.0 mg starch was present.

CHAPTER 5

SCREENING AND TRANSFORMATION BY ELECTROPORATION OF *KLUYVEROMYCES* STRAINS

5.1 BACKGROUND AND AIM

This chapter describes the optimization of an electroporation method using a Bio-Rad Gene Pulser for *Kluyveromyces lactis* transformation. The electroporation method for transforming *K. lactis* yeasts was obtained from Fukuhara (1991), and is similar to that reported by Meilhoc *et al.* (1990) for *Saccharomyces cerevisiae* transformation. However, the conditions recommended in these methods could not be replicated on the Bio-Rad Gene Pulser and so the effect of varying the parameters on the transformation efficiency was determined.

The second aim of the work reported in this chapter was to screen selected strains of *Kluyveromyces* yeasts as potentially suitable recipients for plasmids carrying heterologous DNA. The strains were evaluated for successful transformation, lactose and glucose utilization, and ethanol production, when grown under high and low aeration. Desirable strains would fall into one or a number of the following categories:

(1) those which were transformed with high efficiency;

(2) those which aerobically utilized glucose and/or lactose, and achieved both a high cell yield and a high growth rate;

(3) those that utilized glucose and/or lactose to give a high yield of ethanol with high productivity under low aeration conditions.

It was proposed to choose at least two strains for further fermentation and plasmid stability studies.

The work described in this chapter was performed concurrently with that of Chapter 4.

5.2 MATERIALS AND METHODS

Full details of the materials and methods used for screening and the optimal transformation methods by electroporation of *K. lactis* strains are given in Chapter 3. The 28 *Kluyveromyces* yeasts chosen for screening work are listed in Table 3.1.

Growth characteristics were determined by shake-flask experiments (Section 3.6.1) using rich glucose and lactose media (YPD and YPL broths) under high and low aeration. Samples were taken at 0, 24, and 48 h. Each inoculum was prepared as described in Section 3.1.4, in which yeast taken from a single colony on YPD agar was transferred to YPD or YPL broth (5 ml) and incubated with shaking at 30°C overnight. Following a total cell count, a measured aliquot of inoculum was transferred to each shake-flask to give an initial cell count of approximately $5x10^5$ - $1x10^6$ cells ml^{-1}. The methods used to characterize the yeast growth were:

(1) Total cell count via haemocytometer (Section 3.4.2);
(2) Analysis of residual sugar, glucose or lactose (Section 3.4.4); and,
(3) Determination of ethanol (Section 3.4.6).

Electroporation screening of *K. lactis* strains was conducted with the method described in Section 3.3.8, using the PS10 Electropulsator at Institut Curie, Paris. YPD broth was used for growing the yeast prior to electroporation and for resuspending the electroporated cells, and YPD agar and selective YPD-G418 agar were used for selection of plasmid-containing transformants. The plasmid used for screening was pCXJ-kan1.

The conditions recommended for the PS10 Electropulsator (2500 V cm^{-1}, 20 ms; Figure 3.1; Fukuhara, 1991) could not be replicated on the Bio-Rad Gene Pulser

(Figure 3.2). Therefore, the effects of voltage, resistance, capacitance, concentration of plasmid DNA, inclusion of carrier DNA, and time of implementing G418 selection on the transformation of two yeast strains with the Bio-Rad Gene Pulser were investigated. The machine settings were varied in the following ranges: voltage, 2500 - 6500 V cm^{-1}; resistance, 200 - ∞ Ω (maximum setting); and capacitance, 3 - 25 μF (maximum setting). The yeast strains used were *K. lactis* MW 98-8C and CBS 141, and the plasmids used were pCXJ-kan1 for all investigations, and pCR1 for plasmid comparison studies. The carrier DNA used was denatured salmon sperm DNA. The plasmids were prepared using both the large-scale DNA preparations as described in Section 3.3.1.1.3 and 3.3.1.1.4. The media used included that described for electroporation screening work, as well as selective Ura^- MM agar. *K. lactis* MW 98-8C transformants were selected using both MM and YPD-G418 agar, whereas *K. lactis* CBS 141 transformants were selected only with the latter.

5.3 RESULTS

5.3.1 Screening *Kluyveromyces* strains for potential use in a strain improvement programme

In total, 28 *Kluyveromyces* strains were screened for their potential as hosts for heterologous protein production based on transformation efficiencies, glucose and lactose utilization, biomass yields, and ethanol production. Table 5.1 summarizes the transformation efficiencies obtained using the PS10 Electropulsateur at the Institut Curie. Table 5.1 also summarizes, in qualitative terms, the biomass growth with high aeration, and ethanol production for low aeration, attained from shake-flask cultures grown on YPD and YPL broth. Full details of the results are given in Appendix 1 (Tables A1.1 - A1.3).

Table 5.1 Summary of screening results for *Kluyveromyces* yeasts[a].

Yeast	Transformation efficiency[b]	Biomass[c]		Ethanol[d]	
		glucose	lactose	glucose	lactose
K. lactis					
CBS 141	+++	+++	+++	0	+++
CBS 683	+++	++	+++	+++	++++
CBS 762	+++	++	++	+++	++++
CBS 845	+++	++	+++	+	++++
CBS 1065	++	+++	++	+++	++++
CBS 1067	+	+++	+++	++++	++++
CBS 1797	++	+++	++	++++	++++
CBS 2359	++	+++	+++	+++	+++
CBS 2360/7	++	+++	++	+	++++
CBS 2619	+	+++	+++	+++	++++
CBS 2621	++	+++	+++	+++	++++
CBS 2896		+++	++	++++	0
CBS 5618	++	+++	+++	+	+++
CBS 6315	++	++	++	++++	++++
CBS 6747	+++	++	++	+++	++++
MW 98-8C	++	+++	+	0	0
MW 108-8B	++	++	++	++	+++
pM 6-7A	++	+++	++	+++	++++
pM 44-B	++	++	++	++	++++
K. marxianus					
ATTC 1179	0	++	++	++++	++++
ATTC 1195	0	++	+++	++++	++++
ATTC 36907	+	+++	+	++++	0
CBS 397	0	++	++	++++	++++
CBS 712	+	+++	+++	++++	++++
CBS 4572	0	++	+	+++	0
CBS 5795	0	++	+++	++++	++++
CBS 6556	+	+++	+++	++++	+
Y113	0	++	+++	++++	++++

[a] Derived from data shown in Appendix 1, Tables A1.1 - A1.3.

[b] Key for transformation efficiency

0	$\leq 10^3$	G418 transformants per 10^7 cells per µg pCXJ-kan1 DNA
+	$> 10^3$	
++	$> 10^4$	
+++	$> 10^5$	

[c] Key for cell biomass at 48 h

+	$1x10^8$ -≤ $4x10^8$	cells ml^{-1}
++	$4x10^8$ -≤ $8x10^8$	
+++	$8x10^8$ -≤ $2x10^9$	

[d] Key for ethanol production at 48 h

0	≤ 0.5	g l^{-1}
+	0.5 -≤ 2.0	
++	2.0 -≤ 4.5	
+++	4.5 -≤ 7.0	
++++	7.0 -≤ 9.0	

5.3.2 Transformation of *K. lactis* using the Bio-Rad Gene Pulser

5.3.2.1 Optimization of Bio-Rad Gene Pulser variables

The transformation efficiencies obtained with *K. lactis* MW 98-8C and plasmid pCXJ-kan1, for various parameter settings of the Bio-Rad Gene Pulser are shown in Table 5.2. The efficiency of transformation increased with increasing resistance to the maximum setting (∞ Ω) and with increasing voltage to 900-1100 V. A capacitance setting of 25 μF gave better results than a setting of 3 μF. The combination 900 V (4500 V cm^{-1}), 25 μF, and ∞ Ω gave high transformation efficiencies for both selection systems and so was used in all subsequent work.

Table 5.2 Effects of voltage, resistance, and capacitance on transformation efficiency for *K. lactis* MW 98-8C and pCXJ-kan1 using the Bio-Rad Gene Pulser.

Voltage (V)	Voltage (V cm^{-1})	Resistance (Ω)	Capacitance (μF)	Transformation efficiency[a] Ura⁻	G418
500	2500	∞	25	3.0×10^3	2.5×10^3
700	3500	∞	25	1.3×10^4	4.0×10^3
900	4500	∞	25	3.5×10^4	3.0×10^4
1100	5500	∞	25	1.7×10^4	3.2×10^4
1300	6500	∞	25	4.0×10^3	1.4×10^4
1100	5500	200	25	1.1×10^5	9.5×10^2
1100	5500	600	25	1.7×10^5	1.4×10^3
1100	5500	1000	25	3.0×10^5	9.8×10^3
1100	5500	∞	25	2.5×10^5	5.8×10^3
1100	5500	600	3	1.3×10^3	$< 10^2$
1100	5500	1000	3	2.5×10^3	$< 10^2$
1100	5500	∞	3	5.5×10^3	$< 10^2$

[a] Transformation efficiency is expressed as number of transformants per 10^7 cells per μg plasmid DNA.

5.3.2.2 DNA concentration

The transformation efficiency at the optimal settings for both yeasts, *K. lactis* MW 98-8C and CBS 141, was examined as a function of the plasmid concentration in the range 0 - 2 μg DNA in a standard 20 μl volume. The total number of

transformants increased with increasing plasmid concentration as shown in Table 5.3. At levels greater than 0.2 µg increasing the amount of DNA did not increase the total numbers of transformants and the transformation efficiency per µg of DNA was greatest at low concentrations. It was decided that 0.2 µg DNA gave a satisfactory combination of a high number of transformant colonies with a high transformation efficiency. The addition of 5 µg sheared salmon sperm DNA to 0.2 µg plasmid DNA had no effect on the transformation efficiency.

Table 5.3 Effect of plasmid pCXJ-kan1 concentration on number of transformants and transformation efficiency for *K. lactis* MW 98-8C with Ura⁻ selection using the Bio-Rad Gene Pulser.

DNA (µg)[a]	number of transformants per ml	transformation efficiency[b]
0	0	-
0.02	1.1×10^4	5.7×10^5
0.05	1.6×10^4	3.3×10^5
0.2	4.0×10^4	2.0×10^5
0.35	3.9×10^4	1.1×10^5
0.5	3.3×10^4	6.7×10^4
0.75	3.8×10^4	5.1×10^4
1.0	2.4×10^4	2.4×10^4
2.0	2.3×10^4	1.1×10^4
0.2[c]	3.4×10^4	1.7×10^5

[a] The quantity of DNA was added in a standard 20 µl volume.

[b] Transformation efficiency is expressed as number of transformants per 10^7 cells per µg plasmid DNA.

[c] Plus 5 µg Salmon sperm DNA.

5.3.2.3 Different plasmids

The plasmid used also appeared to influence transformation efficiency. For pCXJ-kan1 and *K. lactis* CBS 141 the transformation efficiency was 1.3×10^5 G418-resistant transformants per 10^7 cells per µg DNA compared to only 1.9×10^4 G418-transformants per 10^7 cells per µg DNA for pCR1 in the same strain. It was also noted that the latter transformant colonies were much smaller than the pCXJ-kan1 transformants after 2-3 days growth at 30°C.

5.3.2.4 Optimization of G418 selection time

The time required for growth in YPD broth before plating onto YPD-G418 plates to obtain optimum transformation efficiency was investigated. The results for both yeasts are shown in Figure 5.1. The number of G418-resistant transformants obtained increased with time up to 20 - 25 hours and this was chosen as the standard incubation time for selection of the transformants.

5.3.2.5 Comparison of electroporators

The two electroporators were compared using *K. lactis* MW98-8C and pCXJ-kan1. With the PS10 Electropulsator, which emits a square wave pulse, and using 2500 V cm^{-1} and 20 ms, the transformation efficiency obtained was 10^4-10^5 G418-resistant transformants per 10^7 cells per μg DNA. The Gene Pulser, generating an exponential decay voltage wave, also gave 10^4-10^5 transformants per 10^7 cells per μg DNA at the optimal settings.

5.4 DISCUSSION

In total, 28 *Kluyveromyces* strains were examined. The electroporation summary in Table 5.1 indicated that *K. marxianus* strains have very low transformation efficiencies with the plasmid pCXJ-kan1. Most of the strains, including Y113, which is used commercially for whey ethanol production in New Zealand, gave no transformants on a selection plate (this equates to < 10^3 transformants per 10^7 cells per μg DNA). In contrast, the transformation efficiencies for *K. lactis* strains ranged from 10^3 to 10^5 transformants per 10^7 cells per μg DNA. Therefore, in general, *K. lactis* strains were able to be transformed with plasmid pCXJ and its derivatives using electroporation whereas *K. marxianus* strains were not transformed.

Kluyveromyces yeasts have been divided into three groups based on modern taxonomy studies, which integrated traditional and phenotypic properties of

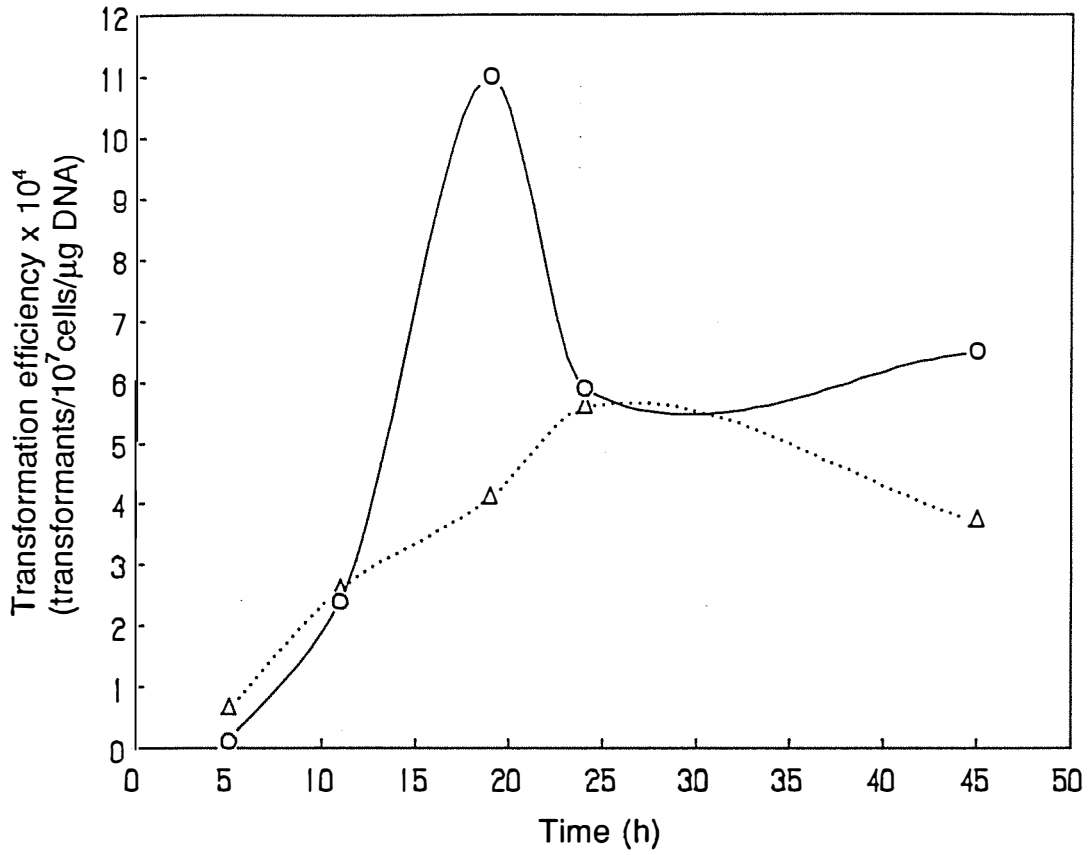


Figure 5.1 **Effect of G418 selection time on G418 transformation efficiency of yeasts *K. lactis* MW98-8C (--o--) and CBS 141 (--Δ--) with plasmid pCXJ-kan1. Electroporation conditions used were 900 V, 25 µF, and ∞ Ω, on the Bio-Rad Gene Pulser.**

morphology and physiology with characteristics derived from biochemistry, molecular biology, and ecology (Smith, 1991). The *Kluyveromyces* genus was initially divided into 18 species on the basis of morphological and physiological characteristics. Subsequent studies on genetics, isoenzyme patterns, and DNA-DNA homologies reduced the number of species to 13. These 13 species were demonstrated to have distinct chromosomal DNA patterns, and their relationships were examined on the basis of restriction mapping between rDNA. The differentiation of the species into three groups was based on all these studies. Between these groups species behaviour did not occur, that is, on genetic crossing there was no interchange of genes or recombination of chromosomes (Smith, 1991). The species of interest in this study fell into two of the groups: *K. lactis* and *K. drosophilarum* were in the first group (Group 1), and *K. marxianus* was in Group 2. Since there is a closer relationship between the former two species rather than between *K. marxianus* and *K. drosophilarum*, the plasmids and derived vectors originating from *K. drosophilarum* could be expected to be more readily transformed into, and more stable, in *K. lactis*. As seen from the transformation efficiencies reported in Table 5.1, this was indeed the case.

Chen *et al.* (1989) also compared a number of *Kluyveromyces* strains for transformation efficiency using a spheroplast method. Their results showed that for those yeast species of Group 1, the transformation frequency for plasmid pCXJ-kan1 was greater than 1.4×10^3 transformants per 3×10^6 protoplasts per 5 µg DNA, with the highest transformation frequencies being obtained for *K. drosophilarum*. Of the other strains tested, only 3 of the so-called 14 different *Kluyveromyces* species showed any reasonable level of transformation, these being *K. vandudenii*, *K. dobzhanskii*, and *K. phaseolosporus*. These species have also been shown to be closely related to *K. lactis* (Sor and Fukuhara, 1989), and can be included in Group 1 (Smith, 1991). Transformation frequencies were reported as being very low or 0 for the remaining 11 species, for which a total of 18 strains were tested, including 3 strains of *K. marxianus*.

In this study the *Kluyveromyces* strains which gave the highest transformation

efficiencies (greater than 10^5 transformants per 10^7 cells per µg DNA) were *K. lactis* CBS 141, CBS 683, CBS 762, CBS 845, and CBS 6747.

For growth under high aeration on rich media containing either glucose or lactose the performances of all yeasts were generally similar, with little difference noted between *K. lactis* and *K. marxianus* strains. The cell population at 48 h ranged from 4.9×10^8 to 1.5×10^9 cells ml^{-1} for glucose, and 1.2×10^8 to 1.9×10^9 cells ml^{-1} for lactose media. Correspondingly, the residual sugar concentration at 48 h was undetectable, although six strains did not grow as fast on lactose and hence the final residual sugar concentration was higher.

Under low aeration conditions, the final population values were again similar for most strains investigated, but were about ten-fold lower than for high aeration. The yeasts grew more slowly, and this was reflected in the residual sugar concentrations. Cell growth, sugar utilization, and ethanol production were all faster in *K. marxianus* strains on glucose compared to *K. lactis*. Half of the *K. marxianus* strains were also faster than *K. lactis* on lactose. Four strains produced very little ethanol (< 1 g l^{-1}) on lactose with lower growth and higher residual sugar. Two of these were *K. marxianus* strains.

For the glucose medium, five strains, all *K. lactis*, gave low ethanol production and used glucose only slowly. However, surprisingly, cell numbers were similar to the other strains. Two of these strains, *K. lactis* CBS 141 and MW98-8C, have been shown to be *RAG*1 and are therefore deficient in the low affinity glucose transport system (Wesolowski-Louvel *et al.*, 1990; Wesolowski-Louvel, 1991). There are at least two transport systems for glucose in *Kluyveromyces* strains, one with a high affinity for glucose while the other is a low affinity system. In *RAG*1 yeasts only the former operates effectively, and since high affinity systems generally have a lower maximum specific growth rate than low affinity systems the glucose is taken up by the yeast cells more slowly with only the high affinity system. Therefore, it is probable that, under low aeration, almost all the ethanol produced is used as an energy source for growth. Consequently, the *RAG*1

yeasts are fermentation defective (Fukuhara, 1991). All *K. marxianus* strains that have been tested for this gene have been shown to be Rag^+ and so have both glucose transportation systems (Wesolowski-Louvel, 1991).

On the basis of these selection tests two *K. lactis* strains were chosen for further fermentation and stability studies. Although *K. marxianus* strains are used industrially for yeast or ethanol production from whey, they were not considered further as they were difficult to transform with pKD1 derived plasmids and stability of the plasmid pCXJ-kan1 was shown to be low (Chen *et al.*, 1989). *K. lactis* CBS 141 was chosen because the cell growth on lactose was comparable to other *K. lactis* strains and ethanol production was reasonable, and it was also used in this study for developing electroporation and expression systems and so was a familiar yeast. The yeast *K. lactis* CBS 683 was also chosen and had similar growth and ethanol production. It has previously been used by Fleer *et al.* (1991b) for production of recombinant HSA. Significantly, they reported high plasmid stability of their pKD1-derived plasmid in *K. lactis* CBS 683 after 40 generations of growth on non-selective media. Although *K. lactis* MW 98-8C had been used for the development of the optimized electroporation procedure and was a Ura^- strain, this yeast was not chosen for further studies because of its poor performance, with respect to both biomass and ethanol production, on lactose.

An electroporation procedure was developed for transformation of *K. lactis* yeasts, for a Bio-Rad Gene Pulser, based on the methods of Fukuhara (1991) and Meilhoc *et al.* (1990). Optimum transformation efficiency was obtained with 4500 V cm^{-1}, 25 μF, and ∞ Ω, and overall the voltage used appeared to be the most important machine variable. The voltage must be high enough to create pores in the cell membrane through which exogenous DNA can enter the cell, yet low enough to avoid excessive cell death (Delorme, 1989). The method recommended by Fukuhara (1991) used a low voltage of 2500 V cm^{-1}. Other workers have also reported the best voltage for the Bio-Rad Gene Pulser to be lower than that found in this investigation: Delorme (1989) cited 3600 V cm^{-1} for

S. cerevisiae, while Bolen and McCutchan (1992) reported 3000 V cm^{-1} for *K. lactis*. These workers observed transformation efficiencies of 1-4.5x10^3 and 2x10^3 transformants per µg of DNA respectively for their particular plasmids and strains used. The transformation efficiency achieved in this work for both *K. lactis* strains MW98-8C and CBS 141 was more than ten-fold higher at 10^4-10^5 transformants per 10^7 cells per µg plasmid DNA. The method developed in this work also differs from these earlier studies (Delorme 1989; Bolen and McCutchan 1992) in that the cells were pretreated before electroporation with 25 mM DTT (dithiothreitol), which reduces disulphide bonds and so weakens the cell wall. The cell wall is seen as a physical barrier which may affect the transformation (Schekman and Novick 1982). Meilhoc et al. (1990) reported that viability of *S. cerevisiae* cells was not affected when they were incubated with DTT for 5 to 30 minutes and that DTT increased the number of transformants by at least five-fold. The results obtained for yeast transformation in this work provide further support for the routine use of DTT.

Meilhoc et al. (1990) reported that the square wave pulses used in their work allowed greater control over the electric field strength and pulse duration, and also suggested that this may increase the transformation efficiency. However, in this work similar transformation efficiencies of 10^4-10^5 transformants per 10^7 cells per µg DNA were achieved for both the PS10 Electropulsator (square wave pulse) and the Gene Pulser (exponential decay pulse) for the same plasmid/host combinations with G418 selection. The transformation efficiencies were lower than those obtained with a square wave pulse for *S. cerevisiae* strain, at 10^7 transformants per µg DNA, but were comparable to the results obtained for *Yarrowia lipolytica* of 5x10^4 transformants per µg DNA (Meilhoc et al. 1990).

The transformation efficiencies reported here for the optimized electroporation method are at least 10-fold superior to those reported for the routine use of a spheroplast method using the same plasmid/host combinations. Chen *et al.* (1989) reported transformation efficiencies of 3x10^3 and 5x10^3 transformants per 10^6 protoplasts per µg DNA for *K. lactis* MW 98-8C and CBS 141, respectively,

for the plasmid pCXJ-kan1 and G418 selection.

The transformation efficiency was examined as a function of plasmid concentration. Transformation efficiency can be expressed as transformants per μg DNA or transformants per electroporation. As the DNA concentration is increased so too should the number of transformants, but there appears to be a threshold point where any further increase in DNA concentration will not markedly increase the number of transformants. This effect was seen in this work and is supported by the results of Delorme (1989). Delorme (1989) suggested that this outcome was due to the plasmid DNA being present in saturating amounts for the cell density used. The transformation efficiency, when reported per μg of DNA, was found to be higher at low quantities of DNA, another trend also in agreement with the results of Delorme (1989). A satisfactory compromise should be found between the number of transformants per electroporation and transformation efficiency per μg of DNA. The value of 0.2 μg plasmid DNA was selected as this gave the greatest number of transformants per plate, and a reasonable efficiency.

The inclusion of carrier DNA in yeast electroporation was investigated since it may have the potential to improve transformation efficiencies. To date, three reports described the use of carrier DNA in electroporation of *S. cerevisiae*, and these showed conflicting results. Delorme (1989) found a slight positive effect on transformation efficiency at low levels of carrier DNA (0.5 - 5.0 μg) and a negative effect at higher values (10 - 20 μg). Bolen and McCutchan (1992) reported no enhancement of transformation efficiency at low concentrations of carrier DNA and a reduced efficiency at higher concentrations, and Rohrer and Picataggio (1992) found increased transformation efficiencies with 5 μg carrier DNA. In this work it was found that carrier DNA had no apparent effect on the transformation efficiency (Table 5.3). In conclusion, the effect of carrier DNA is inconclusive and may depend on the particular yeast host, plasmid, and electroporation method utilized.

Transformation efficiency is known to be affected by the particular plasmid and

strain used in the electroporation. In this study, transformation efficiencies were compared for the plasmids pCXJ-kan1 and pCR1, and it was found that the former consistently gave higher values. This could be due to the difference in plasmid size (10 versus 13.5 kb), the plasmid stability, or a combination of both. Results generated by Delorme (1989) for three different sized plasmids also agree with the work here in that the transformation efficiencies were lower for the larger plasmids, which were also shown to have reduced plasmid stabilities in *S. cerevisiae*. The transformation efficiency obtained for the largest plasmid (11.5 kb) was only half that of the smallest (5.8 kb).

Two types of selection were used to obtain transformants. A negative selection system based on the uracil biosynthetic pathway was used with the yeast MW98-8C, which is a Ura$^-$ mutant, while positive selection based on G418 sensitivity was used for both MW98-8C and CBS 141. *K. lactis* strains transformed with either pCXJ-kan1 or pCR1 can be selected with either system depending on the yeast genotype. Incubation of electroporated cultures in YPD broth at 30°C for 20 - 25 hours before plating onto YPD-G418 agar provided the best conditions for the G418 resistance gene to be expressed before the selection was applied. This method gave transformation efficiencies that were comparable to the Ura$^-$ results, but the number of Ura$^-$ transformants was always slightly higher than the number of G418 transformants.

Since the completion and publication (refer Publication list) of this work, the results of a parallel study of *K. lactis* electroporation have also been reported (Sanchez *et al.*, 1993). This work also used a Bio-Rad Gene Pulser and obtained similar results. The optimized machine conditions Sanchez *et al.* (1993) reported were 1000 V, 25 μF, and 400 Ω, and they also used DTT in their pre-electroporation buffer. They obtained higher transformation efficiencies than those reported in this study, at 10^6 - 10^7 transformants per μg DNA, but used a plasmid that did not allow positive antibiotic selection and did not produce a heterologous protein.

5.5 SUMMARY

An electroporation method was optimized using the Bio-Rad Gene Pulser for the transformation of *K. lactis* yeasts with pKD1-derived plasmids. This yielded 10^4-10^5 transformants per 10^7 cells per µg DNA and so provided much higher efficiencies compared to most other methods reported for transformation of *K. lactis* strains. Based on the transformation efficiencies obtained for *Kluyveromyces* yeasts, and performance on rich glucose and lactose media with respect to growth and ethanol production, two strains were chosen for further fermentation and plasmid stability studies. The selected strains were *K. lactis* CBS 141 and *K. lactis* CBS 683.

CHAPTER 6

PLASMID STABILITY ANALYSIS AND BATCH FERMENTATION STUDIES OF RECOMBINANT *KLUYVEROMYCES LACTIS* EXPRESSING WHEAT α-AMYLASE

6.1 BACKGROUND AND AIM

The goals of research and development programmes for recombinant fermentations are basically the same as those for other fermentations, that is, to obtain high productivity and yield of the required product. Additionally, a viable heterologous protein production system requires high stability of the expression apparatus in the organism of interest, and preferably the heterologous protein should be secreted from the cell. However, although high expression levels are desired from a recombinant culture, the synthesis of a cloned gene product places additional demands on the metabolism of the cells. This can result in a lower growth rate and biomass yield, and hence can reduce overall productivity (Zabriskie and Arcuri, 1986; Stouthaner and van Verseveld, 1987).

This study aimed to characterize the host-vector systems previously developed, that is, *K. lactis* strains containing pCR1 which secrete α-amylase, with respect to growth properties, plasmid stability, and heterologous protein production, for different batch fermentation conditions. In order to achieve this, it was necessary first to evaluate various methods of measuring plasmid stability and choose an appropriate method for use in conjunction with the fermentation studies. Thus, initial investigations focused on plasmid stability, but in later experiments both stability and enzyme production were quantified. Investigations were performed in shake-flask culture and the specific objectives were to:

(1) compare the plasmid stability of two recombinant yeast strains;

(2) compare the stability of transformants of a single yeast strain containing different plasmids;
(3) investigate growth rate differences between plasmid-free and plasmid-containing cells;
(4) compare recombinant yeast growth, ethanol fermentation, plasmid stability, and protein production under conditions of high and low aeration;
(5) investigate recombinant yeast growth characteristics on selective media;
(6) optimize recombinant yeast growth in a whey-based medium; and,
(7) examine the effect of temperature on recombinant yeast growth and protein production.

6.2 MATERIALS AND METHODS

6.2.1 Plasmid stability measurement

For the development of plasmid stability measurement techniques *K. lactis* CBS 683 : pCR1 was grown in rich glucose media (YPD) in high aeration shake-flask culture as described in Section 3.6.1. Samples were diluted in peptone water (5 g l^{-1}) and then spread-plated onto either non-selective YPD or YPD-starch, selective YPD-G418 or YPD-G418-starch agar, or a combination of selective and non-selective agar, depending on the method under investigation.

Plasmid stability was measured as the percentage of cells retaining plasmid after a specified time of incubation or number of generations of cell growth. The stability was also calculated as the normalized change in stability, f/f_0, where the fraction, f, at a given time is defined as:

$$f = \frac{n^+}{n^+ + n^-} \qquad (6.1)$$

where n^+ represents the number of plasmid-containing cells and n^- is the number

of plasmid-free cells; f_0, therefore, measures the initial stability status of the culture (Impoolsup *et al.*, 1989a).

Three methods for measuring stability were investigated. The first method was termed the 'plate ratio' technique and was based on the studies of Hopkins *et al.* (1987), Weber and San (1988), and Tottrup and Carlsen (1990). A diluted sample of fermentation broth was plated onto both non-selective YPL agar and selective YPL-G418 agar. After incubation at 30°C for 48 h the number of colonies were counted and the stability, as the percentage of plasmid-containing cells, was calculated from the following formula:

A. Plate ratio stability

$$stability\ (\%) = \frac{number\ of\ cfu\ on\ YPL\text{-}G418}{number\ of\ cfu\ on\ YPL} x100 \qquad (6.2)$$

The second method was named the 'clearing zone' technique and was similar to that described by Impoolsup *et al.* (1989a). The method was based on the presence of clearing zones around plasmid-containing colonies grown on starch (Section 3.5.7). This permitted visual differentiation between colonies which arose from plasmid-free and plasmid-containing cells. Serially diluted fermentation broth containing the recombinant culture was plated onto non-selective YPL agar, and following incubation at 30°C for 48 h, the colonies were analyzed for clearing zones upon inversion over iodine vapour. Plasmid stability was calculated as follows:

B. Clearing zone stability

$$stability\ (\%) = \frac{number\ of\ cfu\ with\ starch\ clearing\ zones}{total\ number\ of\ cfu} x100 \qquad (6.3)$$

The final method, the 'transfer colony' procedure, has previously been described by Da Silva and Bailey (1991a), and Patkar and Seo (1992). In this method

colonies were initially grown on non-selective agar (YPL), and following growth, a set number of colonies (100) was transferred to the selective agar, YPL-G418. After the second set of plates was incubated at 30°C for 48 h, the number of colonies that grew were counted and the stability of the plasmid was calculated as follows:

C. Transfer colony stability

$$stability\ (\%) = \frac{number\ of\ cfu\ on\ selective\ medium}{total\ number\ of\ cfu\ transferred} x100 \qquad (6.4)$$

6.2.2 Batch investigation studies

Batch investigations of the recombinant and non-recombinant yeasts were also carried out in shake-flask culture. The yeasts studied were *K. lactis* CBS 141 and CBS 683, which were either non-transformed and contained no plasmid (pl^-), or transformed by electroporation and contained either pCXJ-kan1 or pCR1. Details of the yeasts and plasmids can be found in Table 3.1. The broth media used for fermentation studies were YPL and YPD, as described in Section 3.1, and diluted whey medium (DWM) comprising sulphuric acid whey permeate powder (approximately 80 %(w/w) lactose; New Zealand Dairy Research Institute, Palmerston North) made up to 20 g l^{-1} lactose in deionized water. Different levels of yeast extract, peptone, and/or NH_4Cl were added to the diluted whey medium as appropriate to the investigation. The following measurements were used to characterize the yeasts:

(1) pH (Section 3.4.1);
(2) cell biomass (Section 3.4.3);
(3) residual glucose or lactose (Section 3.4.4);
(4) ethanol (Section 3.4.6);
(5) α-amylase production (Section 3.4.7); and,
(6) plasmid stability (as described in this chapter).

6.2.3 Estimation of the maximum specific growth rate

The rate of microbial growth is characterized by the specific growth rate, which is defined as:

$$\mu = \frac{1}{x}\frac{dx}{dt} \qquad (6.5)$$

where:

μ = specific growth rate (h^{-1})

x = cell biomass concentration (g l^{-1})

t = time (h)

With the initial conditions, $x = x_0$ at $t = t_0$, equation (6.6) is obtained:

$$\mu = \frac{lnx - lnx_0}{t - t_0} \qquad (6.6)$$

For the exponential phase of batch growth a plot of lnx against time will yield a slope equal to μ_{max}, the maximum specific growth rate. The values estimated for μ_{max} were obtained with linear regression analysis using QUATTRO.PRO (Boland International Inc., USA) for the exponential stage of cell growth. In all cases at least four data points were used.

6.3 RESULTS

6.3.1 Development and investigation of methods for plasmid stability measurement

The three methods for plasmid stability measurement were compared using an overnight culture of *K. lactis* CBS 683 : pCR1 grown in selective YPD-G418 broth. For each method 12 samples were taken and plated as appropriate. Examples of agar plates used for Methods A and B are shown in Figure 6.1. These display the clearing zones around the plasmid-containing colonies on YPL and YPL-G418 agar plates, upon inversion over iodine vapour. Raw data and statistical analyses for comparison of the three methods are given in Appendix

2 with final results shown in Table 6.1. Calculations were based on the 12 sets of data, however standard deviation and confidence values for a set of triplicate results were also determined, since this lower number of plates was used in subsequent studies for routine stability analysis.

Table 6.1 Comparison of stability values for the three different methods used to measure stability[a].

Method	Number of cfu[b]	Average stability (%)	Standard deviation[c]	Confidence interval 95 %[c]
A. Plate ratio	300	79.1	10.2 (11.8)	6.8 (29.3)
	30	74.9	13.1 (15.1)	8.7 (37.5)
B. Clearing zones	30	75.7	6.1 (7.0)	4.1 (17.4)
C. Transfer colony	100	63.8	6.6 (7.6)	4.4 (18.9)

[a] Calculations were based on a sample size population of 12 plates. Raw data and statistical analyses are given in Appendix 2.

[b] The approximate number of colonies per non-selective YPL agar plate.

[c] Values in brackets are the calculated standard deviations and 95 % confidence intervals for a sample size of 3 plates.

The mean stability values calculated for the different methods were compared using the small sample test for a difference in means, as described in Mendenhall and Ott (1980). Details of the calculations are also given in Appendix 2. At the 95 % confidence level there was no difference between the results for methods A and B, however C was found to differ significantly from both A and B.

The stability values of inocula for the individual fermentations was lower than 100 %, that is, even after growth in selective media less than 100 % of the cells contained plasmid. Typical values obtained for the initial plasmid stability of *K. lactis* CBS 683 : pCR1 in batch culture ranged from 70 to 90 %.

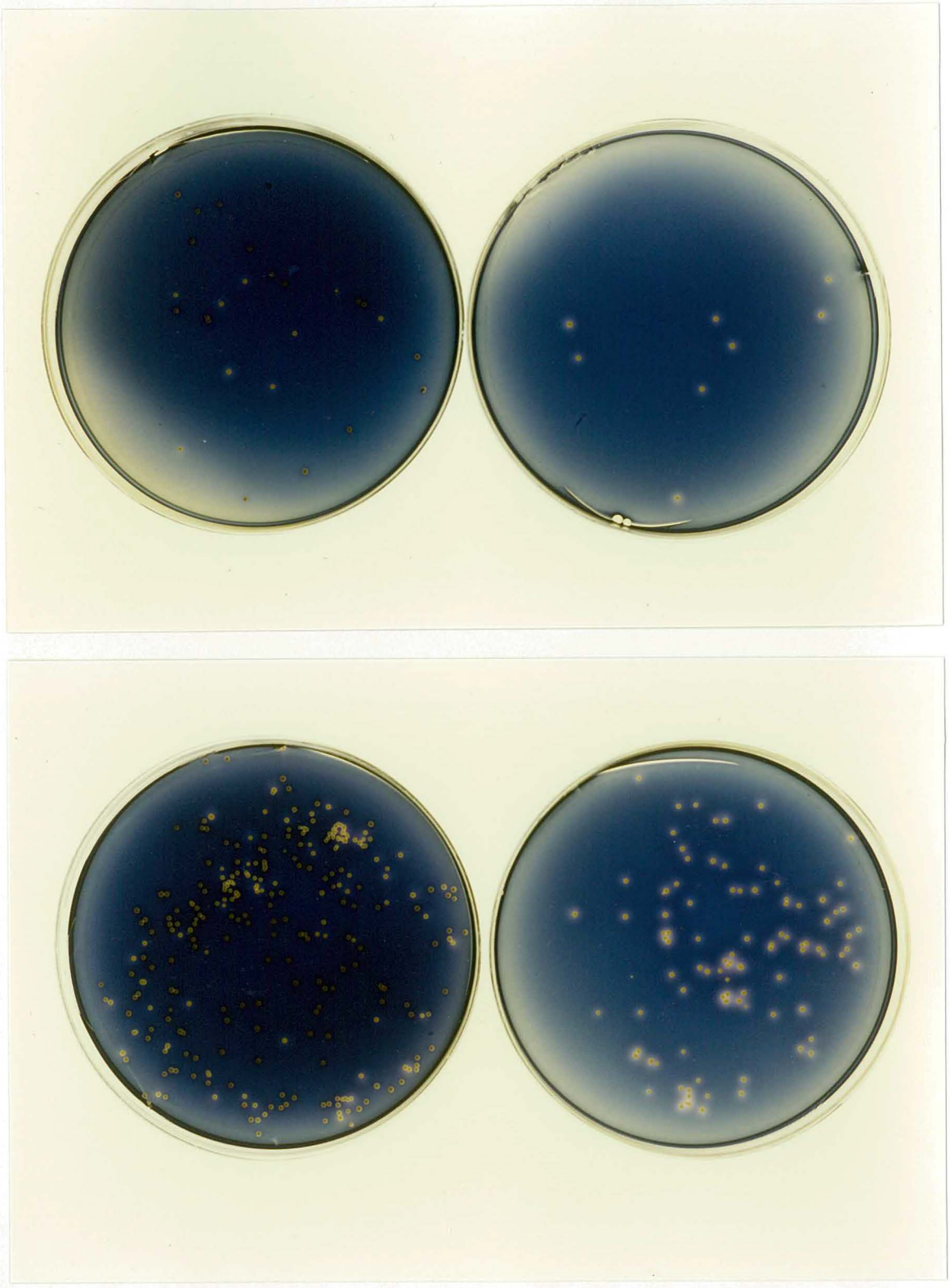


Figure 6.1 Plates showing α-amylase clearing zones for *K. lactis* CBS 683 : pCR1 as used for plasmid stability calculations. YPL agar is on the left and YPL-G418 on the right. The top photo shows a ten-fold higher dilution than the bottom photo.

6.3.2 Batch fermentation studies

6.3.2.1 Comparison of plasmid stability in two recombinant *K. lactis* strains

Growth and plasmid stability of both recombinant and non-recombinant strains of *K. lactis* CBS 141 and CBS 683 were evaluated in batch culture. The summarized results for biomass and ethanol production, residual lactose and plasmid stability for high and low aeration on rich glucose (YPD) and rich lactose (YPL) media are given in Table 6.2. Cell growth for both the untransformed and recombinant pCR1-containing strains followed very similar profiles on both media with similar final concentrations achieved in each case. However, the apparent biomass yield under high aeration was 0.4 (g biomass)(g lactose)$^{-1}$ for *K. lactis* CBS 141 and 0.49 (g biomass)(g lactose)$^{-1}$ for *K. lactis* CBS 683 for YPL medium. During high aeration ethanol was first produced and then consumed with the maximum concentration of ethanol achieved, and sample time at which this was reached, reported in Table 6.2.

Table 6.2 Summary of batch growth of non-recombinant (pl$^-$) and recombinant strains of *K. lactis* CBS 141 and CBS 683 in YPD and YPL broths (see text).

Yeast	Medium	Biomass at 48h (g l^{-1})	Residual sugar (g l^{-1})	Ethanol[a] (g l^{-1})	Plasmid stability[b] (%)	Normalized change in plasmid stability (f/f_0)
HIGH AERATION						
CBS 141	YPD	8	<0.5 at 48h	2 at 36h	49 - 7	0.14
	YPL	8	<0.5 at 48h	4.5 at 36h	49 - 8	0.16
CBS 683	YPD	8	<0.5 at 24h	5 at 24h	69 - 44	0.64
	YPL	9 - 10	<0.5 at 24h	6 at 24h	71 - 41	0.58
LOW AERATION						
CBS 141	YPD	0.2	9.4 at 48h	0.8	49 - 16	0.33
	YPL	0.8	4.8 at 48h	7.6	49 - 12	0.24
CBS 683	YPD	3	<0.5 at 48h	7.0	69 - 47	0.68
	YPL	1.5	<0.5 at 48h	8.2	69 - 45	0.65

[a] If values are reported with times then the ethanol is produced and consumed (for high aeration).

[b] Initial stability value at 0 h; final stability value at 48 h.

Plasmid stability results are also summarized in Table 6.2. The stability of plasmid pCR1 appeared to be strain dependent, with the plasmid stability in *K. lactis* CBS 141 markedly lower than in CBS 683. Since plasmid stability is one of the factors that determines the productivity of heterologous protein production, the *K. lactis* CBS 683 strain was chosen for further investigations.

6.3.2.2 Comparison of stability of plasmids pCXJ-kan1 and pCR1

Recombinant *K. lactis* CBS 683 strains harbouring one of two different pKD1-derived plasmids, pCXJ-kan1 or pCR1, were characterized under high and low aeration in batch culture. Results are summarized in Table 6.3. The apparent biomass yield for the two strains was equivalent at 0.49 (g biomass)(g lactose)$^{-1}$ under high aeration and 0.077 (g biomass)(g lactose)$^{-1}$ under low aeration, and the latter conditions also yielded 0.42 (g ethanol)(g lactose)$^{-1}$. However, the strain carrying pCXJ-kan1 appeared to lead the strain carrying pCR1 in cell growth, lactose utilization, and ethanol production and consumption compared to *K. lactis* CBS 683 : pCR1. This difference, however, was slight as reflected by essentially the same specific growth rates being calculated for both.

Table 6.3 Summary of batch growth of recombinant *K. lactis* CBS 683 containing either plasmid pCXJ-kan1 or pCR1 in YPL broth.

Plasmid	Biomass at 48h ($g\ l^{-1}$)	μ_{max} (h^{-1})	Residual lactose ($g\ l^{-1}$)	Ethanol[a] ($g\ l^{-1}$)	Plasmid stability[b] (%)	Normalized change in plasmid stability (f/f_o)
HIGH AERATION						
pCR1	9 - 10	0.41	<0.5 at 24h	6 at 24h	90 - 69	0.77
pCXJ-kan1	9 - 10	0.40	<0.5 at 24h	6 at 24h	91 - 87	0.96
LOW AERATION						
pCR1	1.5	0.26	<0.5 at 48h	8.2	90 - 72	0.80
pCXJ-kan1	1.5	0.27	<0.5 at 48h	8.2	91 - 86	0.95

[a] If values are reported with times then the ethanol is produced and consumed (for high aeration).

[b] Initial stability value at 0 h; final stability value at 30 h.

Plasmid stability results are shown in Figure 6.2 A and B versus time and biomass, respectively. Although the data show some scatter, the overall trends are clear: plasmid pCXJ-kan1 was considerably more stable than pCR1 over approximately 10 generations of batch growth in rich YPL media. The normalized change in stability over 30 h was 1.0 for pCXJ-kan1 and 0.8 for pCR1 for both high and low aeration, with the largest drop in stability of plasmid pCR1 occurring over the first 24 h.

6.3.2.3 Differences in growth characteristics between plasmid-free and plasmid-containing cells and effect of aeration

Further high aeration batch experiments were conducted in triplicate with cultures of plasmid-free (non-transformed; pl^-) *K. lactis* CBS 683 and plasmid-bearing *K. lactis* CBS 683 : pCXJ-kan1 and CBS 683 : pCR1 strains. The averaged results for cell biomass and pH are presented in Figures 6.3 A and B, respectively, and for ethanol and residual lactose in Figures 6.4 A and B, respectively. The same yield coefficients were achieved for the untransformed strain as in previous runs (Sections 6.3.2.1 and 6.3.2.2).

The fermentation profile for *K. lactis* CBS 683 : pCR1 alone is shown in Figure 6.5 A. As previously noted, the yeast displayed diauxic growth with the general trend as follows: the first phase of exponential growth originated from sugar consumption over the first 20 h. When the sugar was near depletion, at 22 - 25 h, a high concentration of ethanol, 5 - 6 g l^{-1}, had accumulated via the fermentative pathway of growth. At lactose depletion, the second phase of exponential growth commenced with ethanol being used as the energy source, and this continued until the ethanol was depleted at about 40 h. For high aeration, the pH dropped from 6.8 to 5.0 in the first 20 h and increased over the remainder of the fermentation to reach a maximum of about 8 at 40 h. The initial fall corresponded to the phase of ethanol and CO_2 production, and the pH increase coincided with the second phase of diauxic growth. The final biomass concentration reached 9 - 10 g l^{-1}, with an apparent yield of 0.49 (g biomass)(g

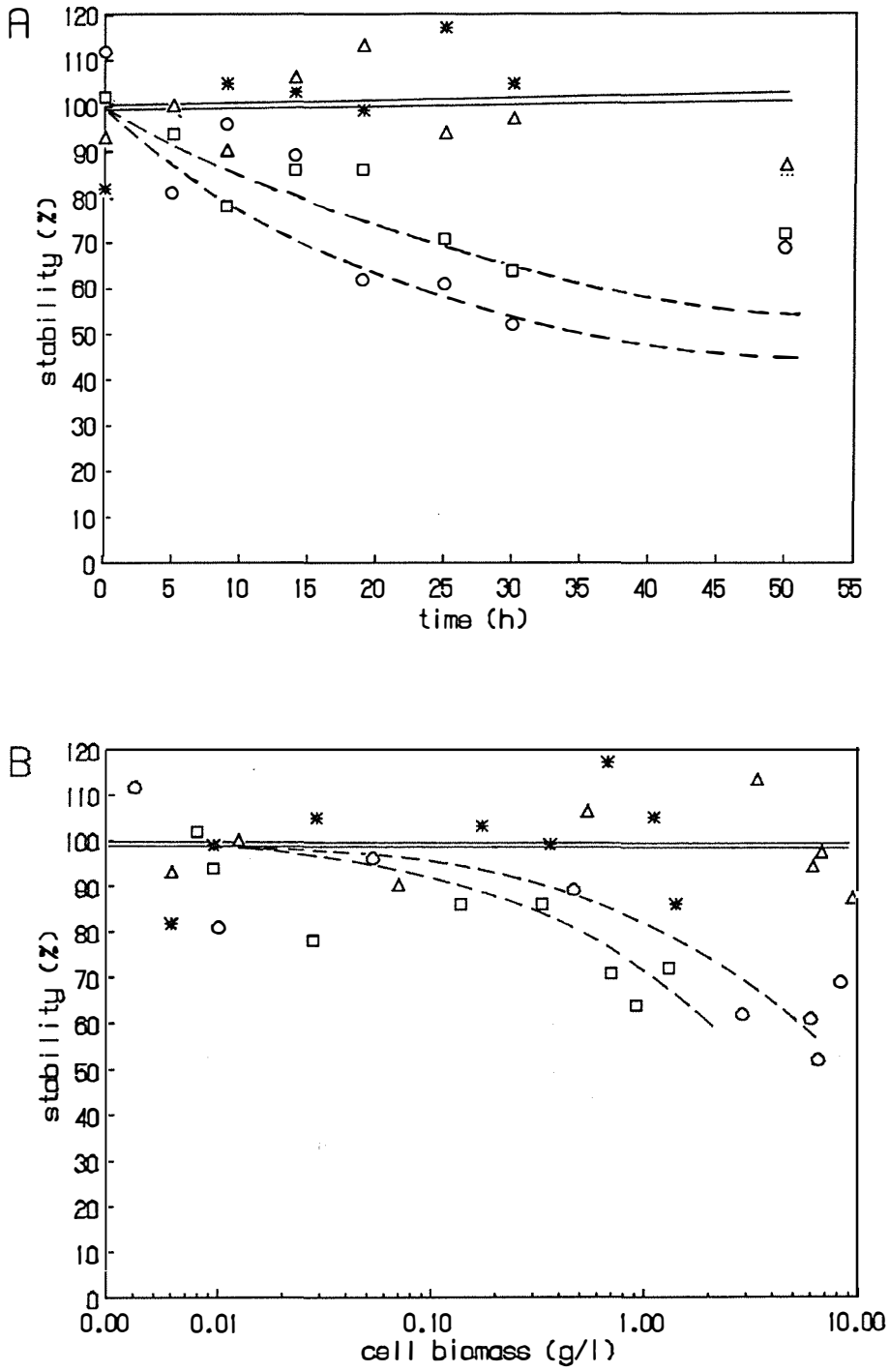


Figure 6.2 **Plot of plasmid stability versus time (A) and cell biomass (B) for recombinant strain *K. lactis* CBS 683 : pCR1 under high (- - o- -) and low (- - □- -) aeration, and strain CBS 683 : pCXJ-kan1 under high (—Δ—) and low (—*—) aeration batch culture in YPL broth.**

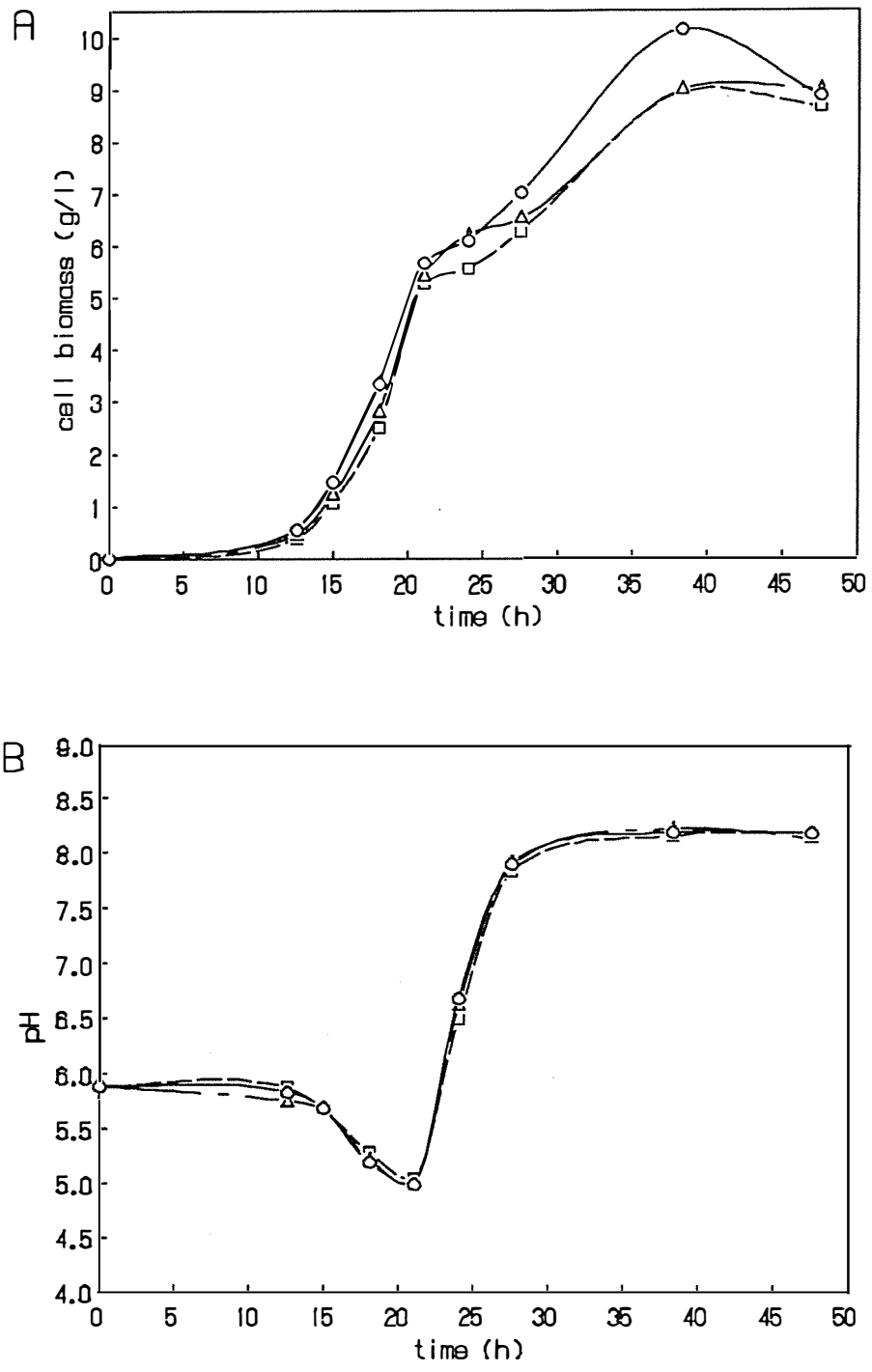


Figure 6.3 **The time course of cell biomass (A) and pH (B) for non-recombinant *K. lactis* CBS 683 (—○—) and recombinant strains CBS 683 : pCXJ-kan1 (- ·- Δ- ·-) and CBS 683 : pCR1 (- - □- -) under high aeration batch culture in YPL.**

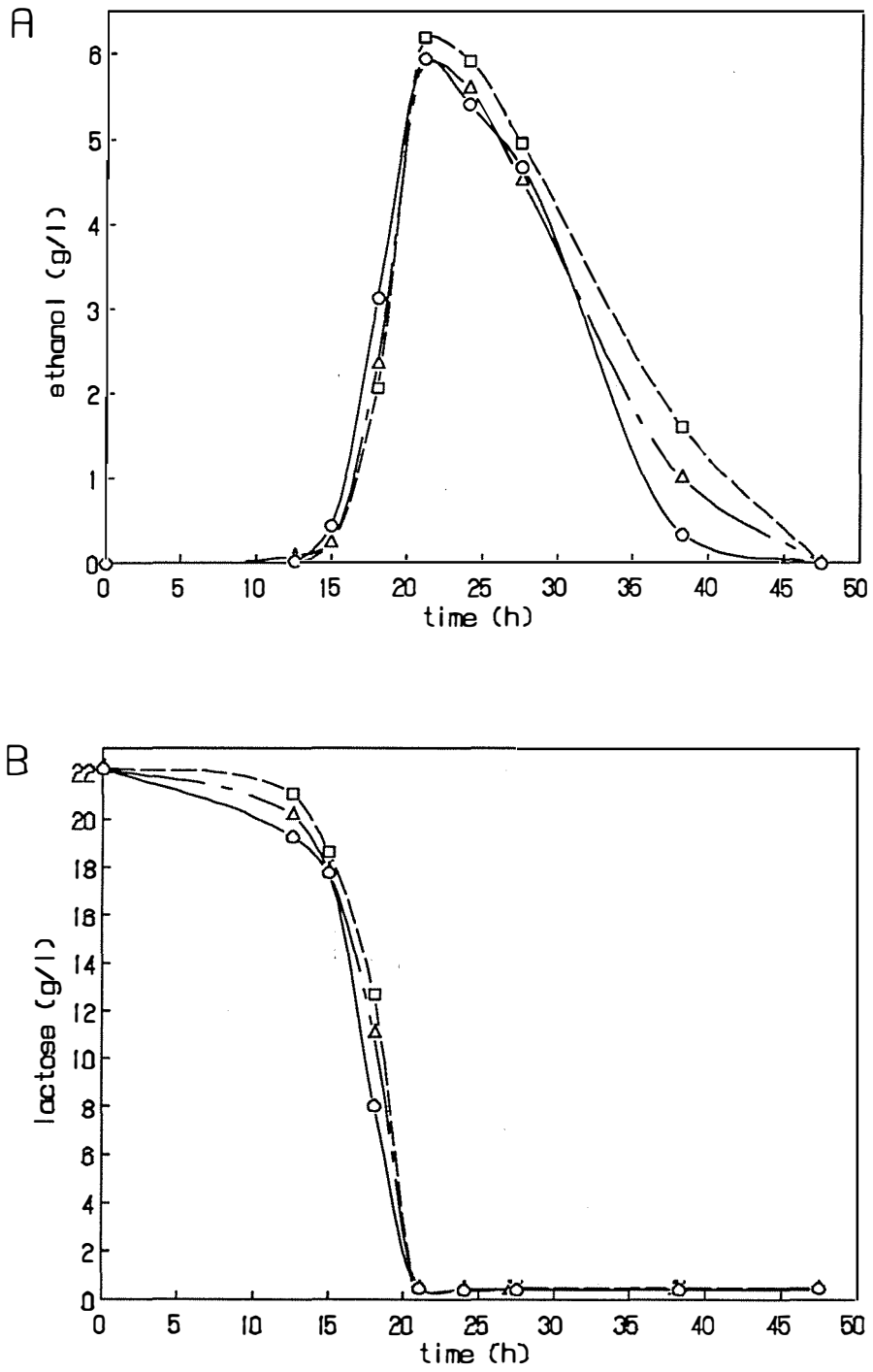


Figure 6.4 **The time course of ethanol (A) and lactose (B) for non-recombinant *K. lactis* CBS 683 (—○—) and recombinant strains CBS 683 : pCXJ-kan1 (- ·- Δ- ·-) and CBS 683 : pCR1 (- - □- -) under high aeration batch culture in YPL.**

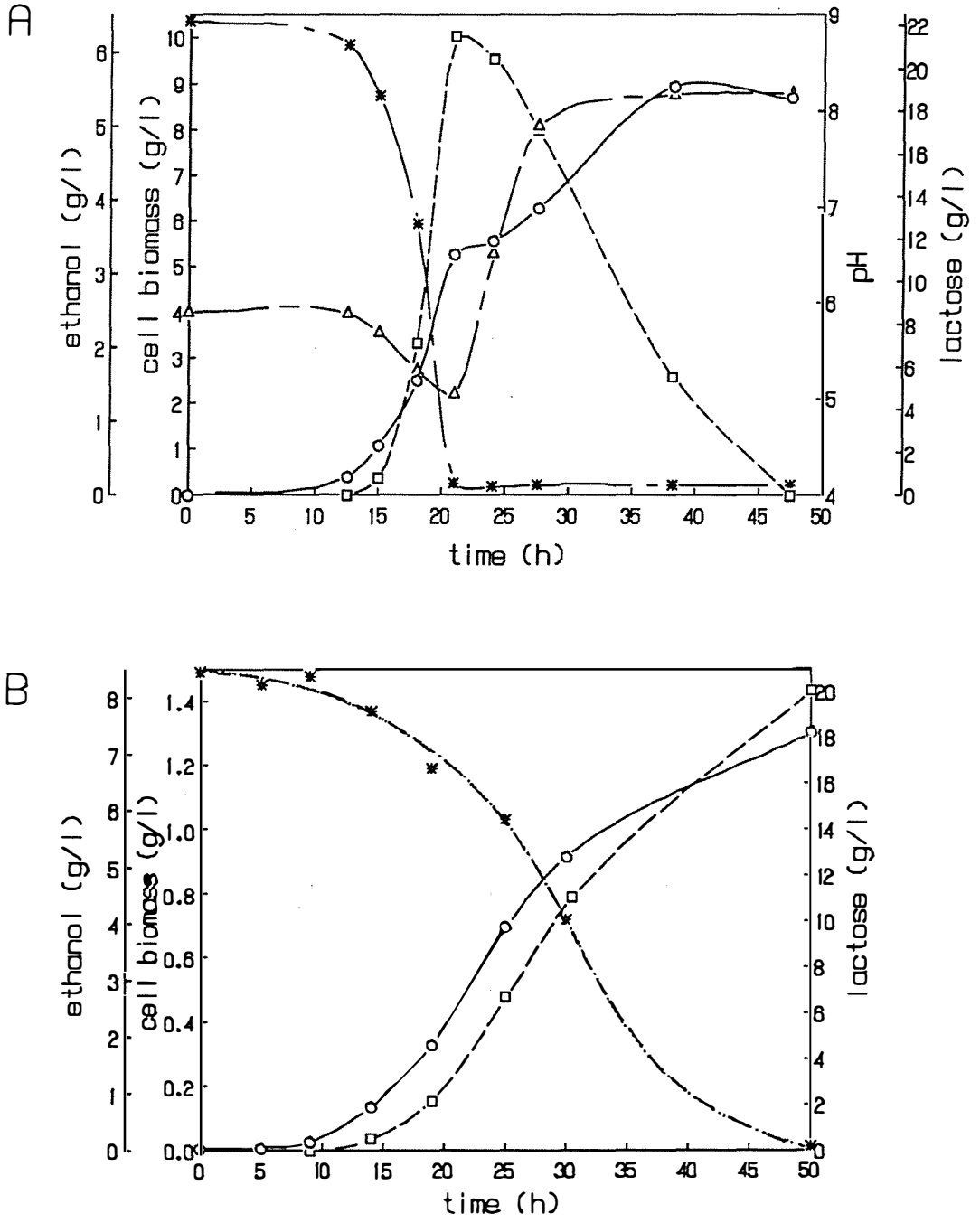


Figure 6.5 **The time course profiles of recombinant *K. lactis* CBS 683 : pCR1 under high (A) and low (B) aeration batch culture in YPL broth: (—o—) cell biomass; (- · - Δ - · -) pH; (- - □ - -) ethanol; (-·-*-·-) lactose.**

lactose)$^{-1}$. At this concentration the yeast cells tended to aggregate and settle very quickly after sampling, indicating that cell flocculation was a feature of the yeast *K. lactis* CBS 683.

The maximum specific growth rates calculated for the lactose and ethanol phases for the non-recombinant and recombinant strains on YPL medium are given in Table 6.4.

Typical results for cell biomass, lactose utilization, and ethanol production under low aeration conditions on YPL broth are shown in Figure 6.5 B, and agreed with previous data given in Table 6.3.

Table 6.4 Maximum specific growth rates calculated for batch fermentation of non-recombinant (pl$^-$) and recombinant *K. lactis* CBS 683 strains in highly aerated YPL broth.[a]

Plasmid	μ_{max} on lactose (h^{-1})	μ_{max} on ethanol[b] (h^{-1})
pl$^-$	0.357	0.035
pCXJ-kan1	0.349	0.033
pCR1	0.352	0.027

[a] Maximum specific growth rates calculated from the results shown in Figure 6.4 A.

[b] Estimated for only three data points.

6.3.2.4 Investigation of effect of selective media

Table 6.5 summarizes the effects of addition of G418 to YPL for *K. lactis* strains CBS 683 : pCR1 and CBS 683 : pCXJ-kan1 under high and low aeration. There were no observable differences in the growth characteristics on YPL and YPL-G418 media. The major effect of addition of G418 to the medium was to stabilize the plasmid pCR1 by a positive selection mechanism. Plasmid pCXJ-kan1 was stable irrespective of the medium used.

Table 6.5 Summary of batch growth of recombinant *K. lactis* CBS 683 strains in selective YPL-G418 and YPL broths.

Plasmid	Medium	Biomass at 48h ($g\ l^{-1}$)	μ_{max} (h^{-1})	Residual lactose ($g\ l^{-1}$)	Ethanol[a] ($g\ l^{-1}$)	Plasmid stability[b] (%)	Normalized change in plasmid stability (f/f_0)
HIGH AERATION							
pCR1	YPL	9 - 10	0.41	<0.5 at 24h	6 at 24h	90 - 69	0.77
	YPL-G418	9 - 10	0.40	<0.5 at 24h	6 at 24h	94 - 93	0.99
pCXJ-	YPL	9 - 10	0.40	<0.5 at 24h	6 at 24h	91 - 87	0.96
kan1	YPL-G418	9 - 10	0.39	<0.5 at 24h	6 at 24h	91 - 93	1.02
LOW AERATION							
pCR1	YPL	1.5	0.26	<0.5 at 48h	8.2	90 - 72	0.80
	YPL-G418	1.5	0.28	<0.5 at 48h	8.2	90 - 82	0.91
pCXJ-	YPL	1.5	0.27	<0.5 at 48h	8.2	91 - 86	0.95
kan1	YPL-G418	1.5	0.31	<0.5 at 48h	8.2	91 - 91	1.00

[a] If values are reported with times then the ethanol is produced and consumed (for high aeration).

[b] Initial stability value at 0 h; final stability value at 48 h.

6.3.2.5 Optimization of a whey-based medium for *K. lactis* CBS 683 : pCR1

The lactose concentration of whey, before dehydration, is 40 - 45 g l^{-1}. However, for this study the whey powder was dissolved to give a 20 g l^{-1} lactose solution as used in the other types of media investigated. Results for both high and low aeration batch culture of untransformed *K. lactis* CBS 683 and transformed CBS 683 : pCR1 on the diluted whey (DWM) are shown in Figure 6.6 A and B for high and low aeration, respectively, and summarized in Table 6.6. Under high aeration the growth rate on DWM was slower than on YPL, with an apparent biomass yield of 0.22 (g biomass)(g lactose)$^{-1}$ compared to 0.49 (g biomass)(g lactose)$^{-1}$ for YPL.

Under low aeration a slower growth rate was again observed on DWM compared to YPL. Ethanol production and lactose utilization rates were consequently slower, however the final ethanol concentration was similar to that obtained on YPL at approximately 8 g l^{-1}. The apparent yields of biomass and ethanol were 0.067 (g biomass)(g lactose)$^{-1}$ and 0.44 (g ethanol)(g lactose)$^{-1}$, respectively.

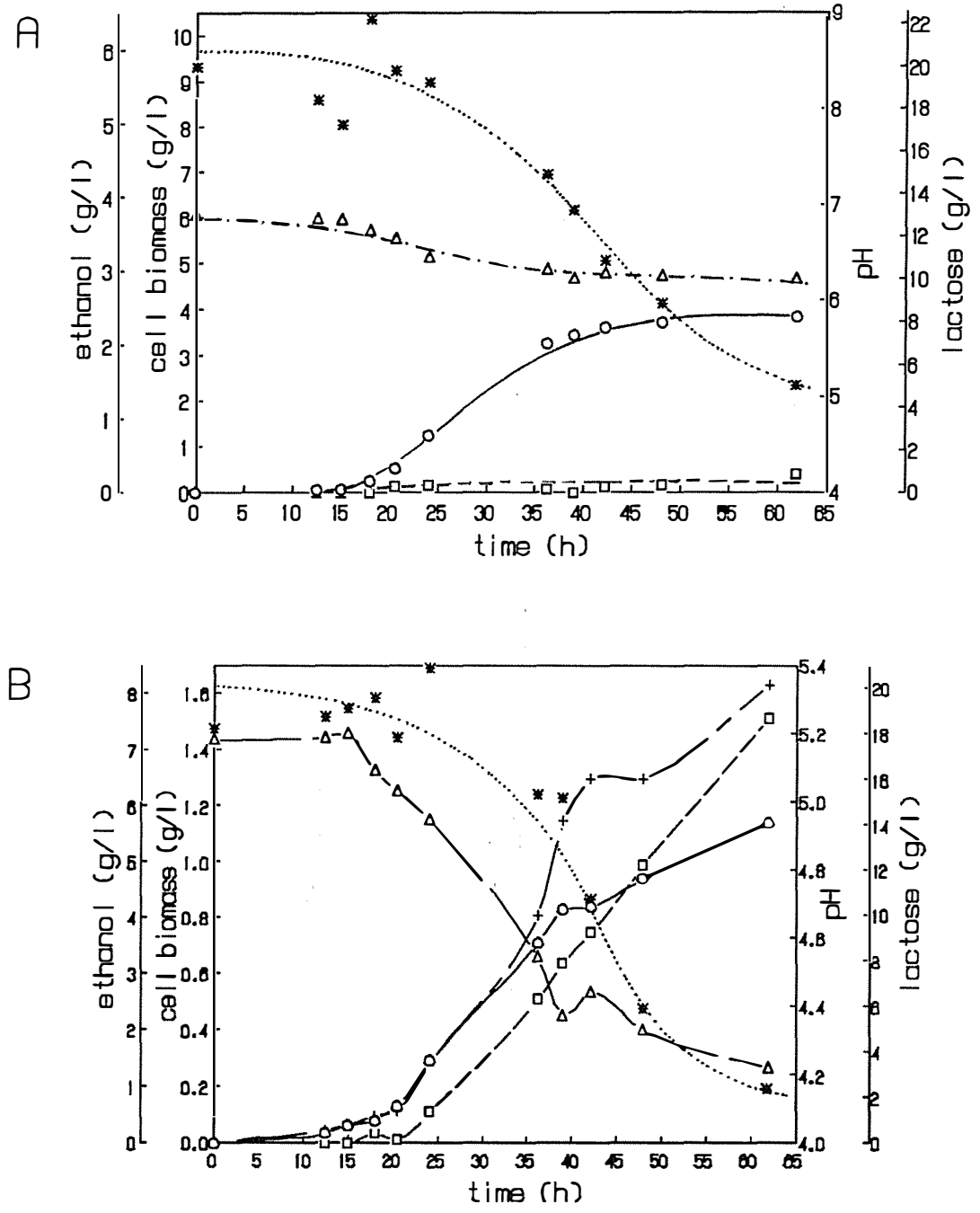


Figure 6.6 **The time course profiles of recombinant *K. lactis* CBS 683 : pCR1 under high (A) and low (B) aeration batch culture in DWM broth ((—○—) cell biomass; (- · - △ - · -) pH; (- - □ - -) ethanol; (·····*·····) lactose; (—+—) cell biomass for non-recombinant strain).**

Table 6.6 Summary of batch growth of recombinant *K. lactis* CBS 683 : pCR1 grown on DWM and YPL broths.

Medium	Biomass at 48h ($g\ l^{-1}$)	μ_{max} (h^{-1})	Residual lactose ($g\ l^{-1}$)	Ethanol[a] ($g\ l^{-1}$)	Plasmid stability[b] (%)	Normalized change in plasmid stability (f/f_0)
HIGH AERATION						
YPL	9 - 10	0.36	<0.5 at 24h	6 at 24h	72 - 36	0.50
DWM	4	0.30	2 at 48h	<0.5 at 24h	72 - 49	0.68
LOW AERATION						
YPL	1.5	0.25	<0.5 at 48h	8.2	72 - 49	0.68
DWM	1.2	0.18	2 at 60h	8.0	72 - 56	0.78

[a] If values are reported with times then the ethanol is produced and consumed (for high aeration).

[b] Initial stability value at 0 h; final stability value at 48 h.

The plasmid stabilities in DWM under both high and low aeration were higher than those for YPL. Also, plasmid stability for both media appeared to be slightly higher under low aeration compared to high aeration (Table 6.6).

Addition of nutrients was investigated in order to improve growth of *K. lactis* CBS 683 on DWM. Initially, the addition of 0.1 %(w/v) yeast extract to DWM was studied. A comparison of the growth characteristics of *K. lactis* CBS 683 : pCR1 on YPL, DWM, and DWM + 0.1 % yeast extract is given in Table 6.7 and shows that addition of yeast extract improved the yeast growth on DWM.

The effect of increased concentrations of yeast extract on growth of *K. lactis* CBS 683 : pCR1 in DWM were examined. Yeast extract was added at 0, 0.01, 0.1, 0.5, 1.0, 2.0, and 5.0 %(w/v) and the results are summarized in Table 6.8. As shown previously, yeast extract addition improved whey utilization. The apparent biomass yield increased from 0.22 (g biomass)(g lactose)$^{-1}$ for DWM to 0.5 (g biomass)(g lactose)$^{-1}$ for DWM + 2 - 5 % yeast extract, and the maximum specific growth rate improved from 0.36 h^{-1} to 0.40 - 0.42 h^{-1}, respectively. Plasmid pCR1 stability results, also shown in Table 6.8, generally decreased with increased concentrations of yeast extract.

Table 6.7 **Summary of batch growth of recombinant *K. lactis* CBS 683 : pCR1 grown on YPL, DWM, and DWM + 0.1 % yeast extract broths.**

Medium	Biomass at 48h ($g\ l^{-1}$)	μ_{max} (h^{-1})	Residual lactose ($g\ l^{-1}$)	Ethanol[a] ($g\ l^{-1}$)	Plasmid stability[b] (%)	Normalized change in plasmid stability (f/f_0)
HIGH AERATION						
YPL	9 - 10	0.36	<0.5 at 24h	6 at 24h	72 - 36	0.50
DWM	4	0.30	2 at 48h	<0.5 at 24h	72 - 49	0.68
DWM+0.1% yeast extract	6	0.33	<0.5 at 48h	<0.5 at 24h	72 - 43	0.60
LOW AERATION						
YPL	1.5	0.25	<0.5 at 48h	8.2	72 - 49	0.68
DWM	1.2	0.23	2 at 60h	8.0	72 - 56	0.78
DWM+0.1% yeast extract	1.4	0.25	2 at 48h	8.0	72 - 56	0.78

[a] If values are reported with times then the ethanol is produced and consumed (for high aeration).

[b] Initial stability value at 0 h; final stability value at 48 h.

Table 6.8 **Summary of high aeration batch growth of recombinant *K. lactis* CBS 683 : pCR1 on DWM plus different levels of yeast extract.**

Medium	Biomass at 48h ($g\ l^{-1}$)	μ_{max} (h^{-1})	Residual lactose[a] ($g\ l^{-1}$)	Ethanol[b] ($g\ l^{-1}$)	Plasmid stability[c] (%)	Normalized change in plasmid stability (f/f_0)
YPL	11	0.40	<0.5 at 24h	6 at 24h	-	-
DWM	4	0.36	2 at 48h	<0.5 at 24h	72 - 63	0.86
DWM + 0.01% yeast extract	4	0.36	2 at 48h	<0.5 at 24h	72 - 57	0.79
DWM + 0.1% yeast extract	6	0.38	<0.5 at 48h	<0.5 at 24h	72 - 57	0.79
DWM + 0.5% yeast extract	7.5	0.36	<0.5 at 24h	5 at 24h	72 - 59	0.82
DWM + 1% yeast extract	9	0.40	<0.5 at 24h	5 at 24h	72 - 53	0.74
DWM + 2% yeast extract	11	0.40	<0.5 at 24h	5.5 at 24h	72 - 50	0.69
DWM + 5% yeast extract	11	0.42	<0.5 at 24h	5.5 at 24h	72 - 46	0.64

[a] Initial lactose concentration was 22 $g\ l^{-1}$.

[b] If values are reported with times then the ethanol is produced and consumed (for high aeration).

[c] Initial stability value at 0 h; final stability value at 48 h.

The addition of NH_4Cl and peptone was also investigated as alternative nitrogen sources and the results are shown in Figure 6.7. The combination of 0.25 % yeast extract and 0.25 % peptone (WYP) gave the best apparent cell growth rate and yield, at 0.44 h^{-1} and 0.42 (g biomass)(g lactose)$^{-1}$, respectively. Doubling the concentrations of the extra nutrients (0.5 % yeast extract and 0.5 % peptone) gave a 10 % increase in cell biomass compared to WYP. However, WYP was the final medium chosen for further investigations, including the continuous culture studies, since the addition of twice the amount of nutrients only had a slight effect. Also, economic consideration must be given to minimizing the level of additives in larger-scale fermentations.

6.3.2.6 α-Amylase production and plasmid stability by *K. lactis* CBS 683 : pCR1 in the optimized whey medium.

α-Amylase production, yeast growth characteristics and plasmid stability were compared in WYP and 2.5x WYP (50 g l^{-1} lactose, 6.25 g l^{-1} yeast extract, 6.25 g l^{-1} peptone) media under high and low aeration. The results for high aeration fermentation characteristics are shown in Figure 6.8 A and B for WYP and 2.5x WYP, respectively, and results for both high and low aeration are summarized in Table 6.9.

The calculated specific growth rate of *K. lactis* CBS 683 : pCR1 on 2.5x WYP was similar to that for WYP, however the biomass yield was lower at 0.30 (g biomass)(g lactose)$^{-1}$ compared to 0.43 (g biomass)(g lactose)$^{-1}$ for WYP. The α-amylase concentration in the cell-free supernatant was determined to be 6 U ml^{-1} in WYP. However, in 2.5x WYP the level was almost double this at 11 U ml^{-1}.

Stability of the plasmid pCR1 was very similar for both WYP and 2.5x WYP media.

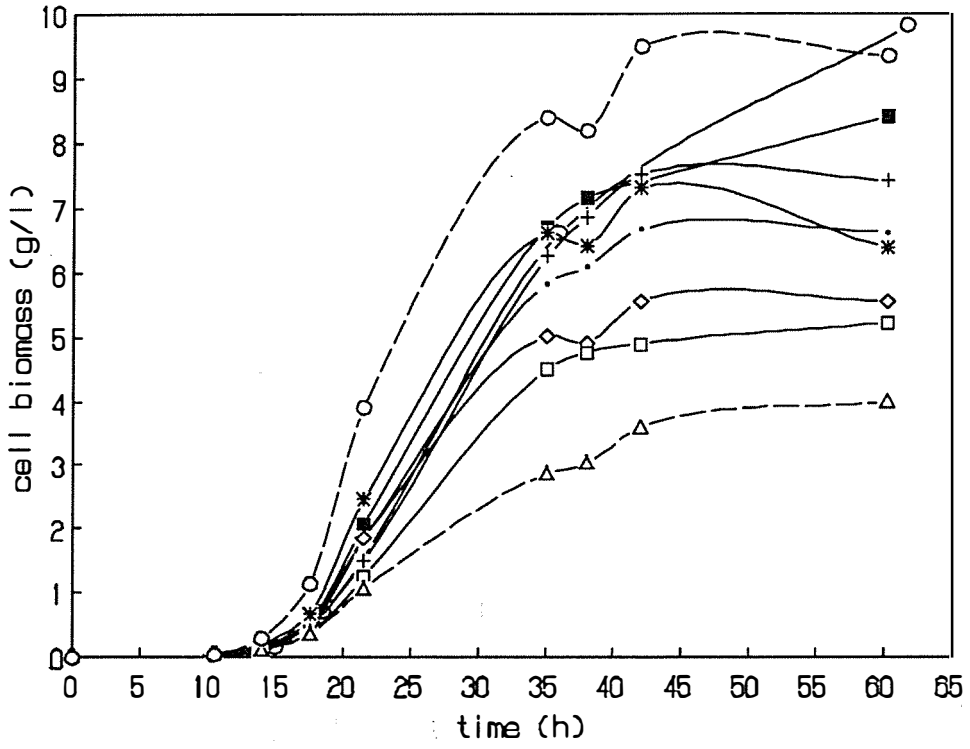


Figure 6.7 **The time course of cell biomass under high aeration batch culture for recombinant *K. lactis* CBS 683 : pCR1 in YPL (- - o- -), DWM (- - Δ- -), and DWM + different levels of nutrient sources. Type and level of nutrient addition: (—+—) 0.5 % yeast extract; (—*—) 0.5 % peptone; (—□—) 0.5 % NH_4Cl; (—■—) 0.25 % peptone + 0.25 % yeast extract; (—•—) 0.25 % NH_4Cl + yeast extract; (—◇—) 0.25 % NH_4Cl + 0.25 % peptone; (—o—) 0.5 % yeast extract + 0.5 % peptone.**

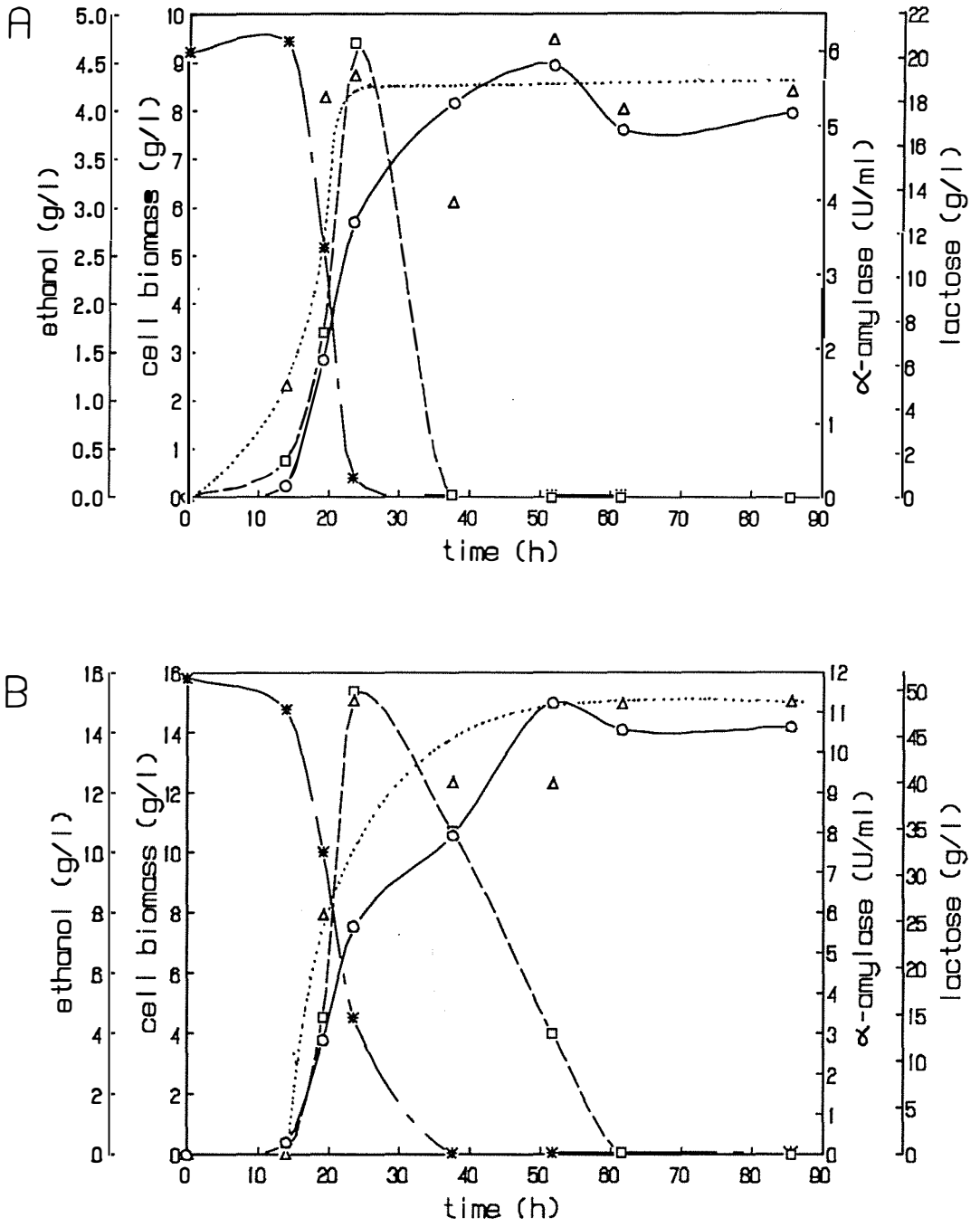


Figure 6.8: **The time course profiles of recombinant *K. lactis* CBS 683 : pCR1 under high aeration batch culture in WYP (A) and 2.5x WYP (B) broths ((—○—) cell biomass; (-···- Δ -···-) α-amylase; (- - □ - -) ethanol; (-···*···-) lactose).**

Table 6.9 Comparison of batch growth for recombinant *K. lactis* CBS 683 : pCR1 on WYP and 2.5x WYP broths under high and low aeration.

Medium	Biomass at 48h ($g\ l^{-1}$)	μ_{max} (h^{-1})	Residual lactose ($g\ l^{-1}$)	Ethanol[a] ($g\ l^{-1}$)	α-Amylase ($U\ ml^{-1}$)	Plasmid stability[b] (%)	Normalized change in plasmid stability (f/f_0)
HIGH AERATION							
WYP	8.4	0.44	<0.5 at 36h	5 at 24h	6	73 - 56	0.77
2.5x WYP	14.9	0.46	<0.5 at 36h	15 at 24h	11	73 - 56	0.77
LOW AERATION							
WYP	1.3	0.29	<0.5 at 60h	8	1	73 - 53	0.73
2.5x WYP	2.4	0.20	<0.5 at 60h	21	2	73 - 57	0.78

[a] If values are reported with times then the ethanol is produced and consumed (for high aeration).

[b] Initial stability value at 0 h; final stability value at 48 h.

For low aeration, the growth rate on WYP was greater than on 2.5x WYP, and again, the yield of biomass was lower on 2.5x WYP with 0.067 and 0.048 (g biomass)(g lactose)$^{-1}$ calculated for WYP and 2.5x WYP, respectively. The yield of ethanol was similar at 0.41 (g ethanol)(g lactose)$^{-1}$ for WYP and 0.42 (g ethanol)(g lactose)$^{-1}$ for 2.5x WYP. The plasmid stability was similar to that for high aeration, however α-amylase production was much lower at 1 and 2 U ml^{-1} for WYP and 2.5x WYP, respectively.

6.3.2.7 Effect of temperature on α-amylase production and plasmid stability

Growth characteristics of, and α-amylase production by, recombinant *K. lactis* CBS 683 : pCR1 were examined at temperatures of 15, 20, 25, and 30°C. Profiles of the results for cell biomass and α-amylase are shown in Figure 6.9 A and B, respectively, and plasmid stability results are shown in Figure 6.10. A summary of the results is also given in Table 6.10.

Table 6.10 Summary of high aeration batch growth of recombinant *K. lactis* CBS 683 : pCR1 on WYP broth at different temperatures.

Temperature (°C)	Biomass (g l^{-1})	μ_{max} (h^{-1})	Residual lactose (g l^{-1})	Ethanol[a] (g l^{-1})	α-Amylase (U ml^{-1})	Plasmid stability[b] (%)	Normalized change in plasmid stability (f/f_0)
15	8 - 9 at 100h	0.19	<0.5 at 100h	-	12	73 - 34	0.47
20	8 - 9 at 48h	0.21	<0.5 at 48h	5 at 40h	18	81 - 63	0.78
25	8 - 9 at 36h	0.31	<0.5 at 36h	5 at 24h	12	75 - 60	0.80
30	8 - 9 at 24h	0.44	<0.5 at 24h	5 at 20h	7	81 - 45	0.56

[a] If values are reported with times then the ethanol is produced and consumed (for high aeration).

[b] Initial stability value at 0 h; final stability value at 60 h (160h for 15°C).

At the lower temperatures the initial lag phase was longer, being, for example 50 h at 15°C compared to 3 h at 30°C. The specific growth rate also decreased with temperature, however final biomass concentration and apparent biomass yield were the same for all cases, at 0.44 (g biomass)(g lactose)$^{-1}$. The production of α-amylase was optimum around 20°C with 18 U ml^{-1} produced, and stability of the plasmid pCR1 also appeared to be greatest at 20 - 25°C.

The growth rate varied with temperature and so the results were fitted to the Arrhenius equation (Shuler and Kargi, 1992):

$$\mu_{max} = Ae^{-\frac{E}{RT}} \qquad (6.7)$$

where μ_{max} = maximum specific growth rate (h^{-1})

A = numerical constant characteristic for growth

e = base of natural logarithm

E = activation energy for growth (J mol^{-1})

R = gas constant in energy units (8.314 J mol^{-1} deg^{-1})

T = temperature (°C)

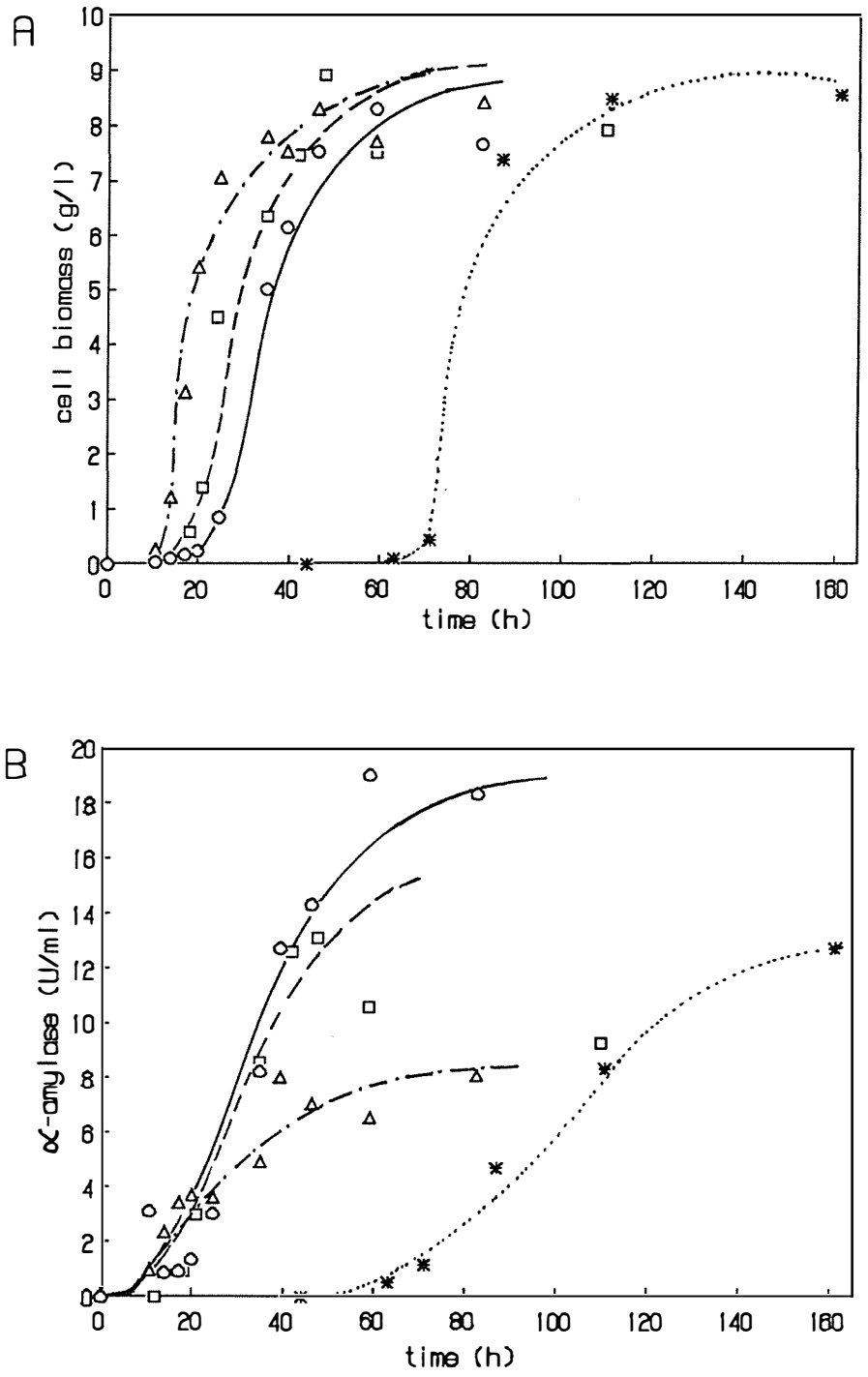


Figure 6.9 **The time course of cell biomass (A) and α-amylase (B) for high aeration batch culture of recombinant *K. lactis* CBS 683 : pCR1 in WYP broth at 15 (·····*·····), 20 (—o—), 25 (- - □- -), and 30°C (- · - Δ- · -).**

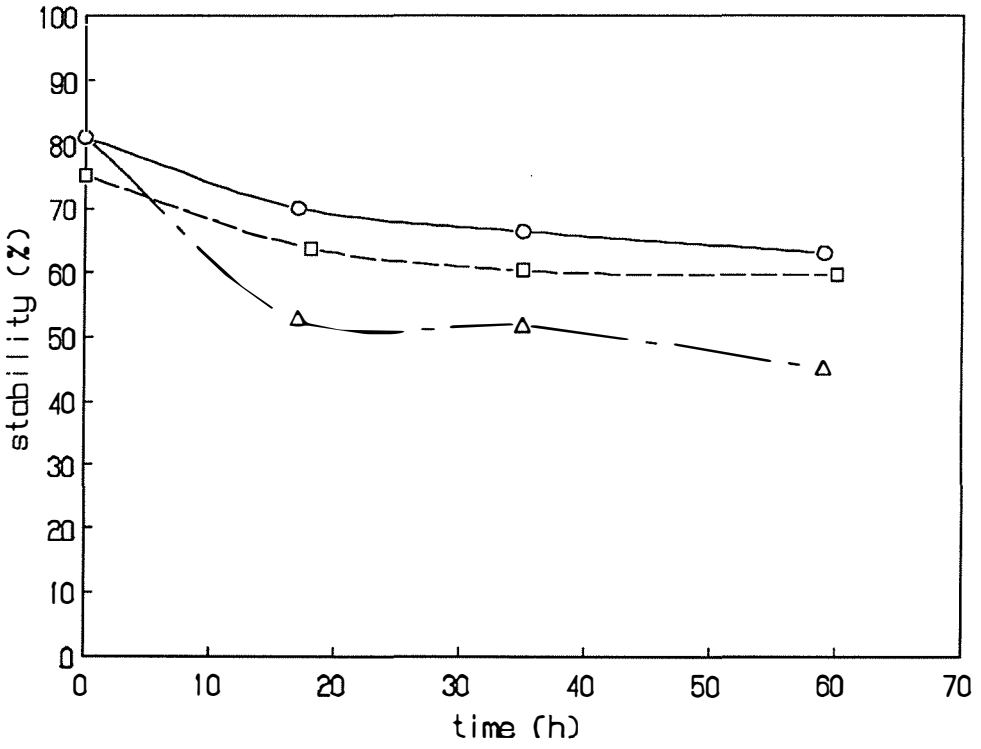


Figure 6.10 **The time course of plasmid stability for high aeration batch culture of recombinant *K. lactis* CBS 683 : pCR1 in WYP broth at 20 (—o—), 25 (- - □ - -), and 30°C (- · - △ - · -).**

Taking the natural logarithm of both sides of the equation gives:

$$\ln\mu_{max} = \ln A e^{-\frac{E}{RT}} \qquad (6.8)$$

To obtain values for ln A and E/R a linear regression analysis using QUATTRO.PRO (Boland International Inc., USA) was performed for $\ln\mu_{max}$ and 1/T. The following result was obtained:

$$\ln\mu_{max} = -0.150 - \frac{24.29}{T} \qquad (6.9)$$

with a correlation coefficient (R^2) of 0.838.

Therefore, the effect of temperature on growth rate of *K. lactis* CBS 683 : pCR1 fits the following Arrhenius equation:

$$\mu_{max} = 0.861\, e^{\frac{-24.29}{T}} \qquad (6.10)$$

6.4 DISCUSSION

Three methods to measure plasmid stability were investigated, with the advantages and disadvantages of each shown in Table 6.11. There appear to be no other reports in the literature which compare plasmid stability measurement techniques.

Method B, which is based on clearing zones around colonies on starch plates, appeared to be the best for measuring plasmid stability in *K. lactis* CBS 683 : pCR1. The major advantages of this method were that it was the most accurate relative to the number of colonies grown on a plate, and that the plasmid-free and plasmid-containing colonies could be counted on the same plate.

Table 6.11 Advantages and disadvantages of plasmid stability measurement methods.

Method	Advantages	Disadvantages
A. Plate ratios	- simple and quick - most representative of time of sample taken - can use <400 colonies per plate - can check structural stability with non-selective agar	- least accurate method relative to the number of colonies - number of colonies varies per test
B. Clearing zones	- simple and quick - can be used in conjunction with method A - most representative of time of sample taken - plasmid-free and plasmid-containing colonies counted on same plate - most accurate method relative to the number of colonies	- requires accurate estimation of colonies (<150 colonies per plate) - number of colonies varies per test
C. Transfer colony	- uses the same number of colonies per test - reasonably accurate method relative to the number of colonies	- very time-consuming - requires two incubation periods - requires more resources, ie. plates - least representative of time of sample taken

The plate ratio method (A) had the advantage that the requirement for a certain number of colonies per plate was not as sensitive as for method B. In addition, a check can be made for structural stability of the pCR1 plasmid using the selective agar. When measuring stability, checks were regularly made for clearing zones around G418-resistant colonies on selective agar. It was found that all G418-resistant colonies degraded starch, indicating structural stability of the G418 and α-amylase genes of pCR1 in *K. lactis*. Method A, however, was the least accurate method with respect to the number of colonies and this was probably due to the errors associated with counting two different plates for each measurement.

Since the plate ratio method used both non-selective and selective agar, and the clearing zones method required only the former, both methods were used to measure plasmid stability in the batch (this chapter) and continuous (Chapter 7) studies. No significant difference was detected between the results of methods A and B at the 95 % confidence level (Appendix 2).

Method C, the transfer colony method has been widely used by, for example, Da Silva and Bailey (1991a; 1991b) and Patkar and Seo (1992). However, this method was found to be extremely time-consuming and required two incubation periods before the results were generated. The time required for colony transfer could be reduced using replica plating techniques, however more accurate estimation of colony numbers would be required for this to be feasible. The stability results were found to be significantly lower than the other two methods at the 95 % confidence level (Appendix 2). This difference was probably due to the effects of the second plating and incubation period. Shuler and Kargi (1992) noted that, from a single cell, it may take up to 25 generations to form an easily observable colony, and therefore plasmid loss can occur in a colony. If plasmid loss occurred early in the colony growth then the colony could be essentially plasmid-free, and would be counted as such despite originating from a plasmid-containing cell. Measured plasmid stabilities were therefore not truly representative of the time when the sample was taken, but reflected the effects of the incubation periods. This appears to be a significant disadvantage of the method, in addition to the points noted above, and it was not used further.

It was noted that for all fermentations the initial plasmid stability value was less than 100 %. This was in contrast to reports citing initial stability values of 100 %, such as Son *et al.* (1987) for recombinant *Bacillus megaterium* producing cellulase, and Parker and DiBiasio (1987) and Walmsley *et al.* (1983) for recombinant *S. cerevisiae* yeasts. In particular, stability of pKD1-derived plasmids in *K. lactis* strains has been reported to be practically 100 %, after growth in selective medium and at the beginning of a fermentation (Chen *et al.*, 1989; Fleer *et al.*, 1991a; 1991b). However, other reports for plasmid stability in bacteria and yeasts noted initial stabilities of less than 100 %. For example, Impoolsup *et al.* (1989a) reported that plasmid stability in *S. cerevisiae* was only 65 - 70 % at the start of a fermentation, even with an inoculum grown in selective medium. Park *et al.* (1993) reported initial plasmid stabilities of around 85 % for a *S. cerevisiae* strain producing heterologous α-amylase, and Kleinman *et al.* (1986) reported approximately 85 % initial plasmid stability in another recombinant *S. cerevisiae*

strain. The lower stability may have been due to the ability of the plasmid-free cells to grow in the presence of selection, for example, by retention of an essential growth factor or resistance product. In this study, it appears the G418-resistance product was either retained by the plasmid-free cells or obtained from plasmid-containing cells, thus permitting the survival of the plasmid-free cells in selective medium. Murray and Szostak (1983a) have shown that plasmid-free *S. cerevisiae* cells can survive up to six generations in selective media after plasmid loss. An associated factor is that none of the methods used is exactly representative of the sample time taken, because a number of generations of cell growth are required to form a colony. Therefore, it may be possible that the plasmid stability at the beginning of a fermentation will be less than 100 %.

The stability of the expression vector pCR1 was found to vary between strains *K. lactis* CBS 141 and CBS 683. Within 10 generations of highly aerated non-selective batch growth only 15 % of the *K. lactis* CBS 141 : pCR1 culture still contained plasmid, compared to 60 % for *K. lactis* CBS 683 : pCR1. Therefore, the CBS 683 strain was chosen over CBS 141 for the continuing investigations. Previous studies have also shown that plasmid stability is species and strain dependent. For example, Chen *et al.* (1989) reported that plasmid stability of pCXJ-kan1 transformed into various *Kluyveromyces* species was found to differ markedly between species and strains. *K. drosophilarum*, the yeast pKD1 originates from, and *K. lactis* strains had plasmid stabilities of greater than 90 % after 10 generations of non-selective growth, whereas those *K. marxianus* strains that were able to be transformed were poorer at less than 40 % plasmid stability. More recently, Fleer *et al.* (1991b) constructed a pKD1-based expression vector to produce HSA and found greater than 90 % of *K. lactis* CBS 683 transformants harboured the plasmid after 10 generations of non-selective growth compared to 70 % for *K. lactis* strain MW 98-8C and only 20 % for *K. marxianus* ATCC 12424 transformants. Fleer *et al.* (1991b) also showed that greater than 90 % of *K. lactis* CBS 683 transformants harboured the HSA expression vector, as compared to 45 % for *K. lactis* strain MW98-8C, after 40 generations of non-selective growth. The genetic background of the host may influence the efficiency of transcription

and translation, the components of the secretory pathway for heterologous protein production, and the plasmid stability and copy number of pKD1-derived vectors. However, as noted by Fleer *et al.* (1991b), the exact cause of these host-dependent variations remains to be established.

The stabilities of two plasmids, pCR1 and pCXJ-kan1, were compared in *K. lactis* CBS 683. The high stability of pCXJ-kan1 agreed with the observations of Chen *et al.* (1989), who showed that insertion of foreign DNA into a unique *Eco*R1 site adjacent to one of the repeats of the plasmid pKD1 did not affect the high stability of the natural plasmid in *K. lactis* and *K. drosophilarum* strains. However, Fleer *et al.* (1991a) reported that the insertion of some protein-coding sequences were found to affect the stability. They noted that the plasmid stability was a function of the transcriptional activity of the promoter which directed the production of recombinant interleukin-1β (IL-1β), with a decrease in stability observed with increasing promoter strength. When interleukin-1β was constitutively expressed with a stronger PGK promoter, only 44 % of the *K. lactis* MW 98-8C cells retained the plasmid after 40 generations of growth. This compared poorly to 75 % stability for inducing, and greater than 90 % for repressing growth conditions when an inducible PHO promoter was used. The PGK plasmid exhibited a pronounced reduction in copy number compared to pCXJ-kan1, and neither plasmid construction proved to be as stable as the control plasmid, pCXJ-kan1, for which they reported 100 % stability after 40 generations of growth. Plasmid stability of the pKD1-derived vector had been reduced by insertion of foreign DNA coding for a heterologous protein.

In this study, the lower stability of pCR1, when compared to pCXJ-kan1 is most probably due to the PGK-α-amylase cassette, although it has been inserted into the neutral point of pKD1. The constitutive PGK promoter alone may have had a large effect on stability.

There appeared to be a small difference in the batch fermentation profiles for plasmid-free and plasmid-containing *K. lactis* CBS 683 strains. On rich YPL

medium, under high aeration, the maximum specific growth rates calculated during the lactose utilization phase of growth for *K. lactis* CBS 683 : pCXJ-kan1 and CBS 683 : pCR1 were approximately 2 % lower than that for the non-recombinant strain (Table 6.4). However, replicate fermentations would be required to determine whether this difference is statistically significant. A reduced growth rate would reflect the expected metabolic burden imposed by the plasmid-related activities of the host as noted by a number of studies including Imanaka and Aiba (1981), Walmsley *et al.* (1983), Zabriskie and Arcuri (1986) Parker and DiBiasio (1987), Wittrup and Bailey (1988), and Impoolsup *et al.* (1989a). Ryan and Parulekar (1991) reported that recombinant *E. coli* cells harbouring a plasmid had a much lower growth rate and overall mass yield compared to the plasmid-free cells. However, the model of Mead *et al.* (1986b) predicted that plasmid-free yeast cells may only have a 2.5 % growth rate advantage over plasmid-containing cells, and that the difference would be difficult to detect from fermentation data. They also noted that it would be difficult to accurately measure the maximum growth rate of plasmid containing cells, particularly in non-selective medium, since the population contains a transient mixture of plasmid-containing and plasmid-free cells. In this work, the growth rate advantage of plasmid-free cells was most likely higher than that calculated because of the relatively low stability. In addition, the pCR1 plasmid was less stable and so the recombinant culture comprised more plasmid-free cells compared with the strain containing pCXJ-kan1. Thus, this would probably indicate that the growth rate had a larger influence on the apparent segregational stability of pCR1 compared to that of pCXJ-kan1.

In addition to the difference between growth rates for non-recombinant and recombinant *K. lactis* CBS 683 yeast strains, the growth rates on ethanol were found to be ten-fold less than those on lactose in YPL medium in the two stages of diauxic growth. Ethanol utilization generates energy less efficiently than lactose utilization. The medium may also be poorly buffered and therefore buffering may need to be considered. There was not enough data to determine whether the two-phase growth or metabolism switching mechanism had an effect on plasmid

stability. Further work could be performed to investigate the effect of pH on plasmid stability.

The whey permeate powder used was produced by spray-drying sulphuric acid whey containing 40 - 50 g l^{-1} lactose. This was dissolved to give 20 g l^{-1} lactose and consequently all other whey components, such as the nitrogen sources, were also diluted relative to the original whey. The growth of recombinant *K. lactis* CBS 683 on DWM was poor compared to growth on the rich YPL medium and so the effects of nutrient supplementation were investigated. Additionally, it is clear that a carbon balance could not be made for the fermentation of DWM, and it may be possible that another product, such as glycerol, was formed in high concentrations.

Addition of 0.25 % yeast extract and 0.25 % peptone to the diluted whey (WYP) gave a satisfactory improvement in growth characteristics for minimum addition of extra nutrients. The level of additives should be kept low due to the costs entailed for a larger-scale fermentation.

Cell flocculation was associated with high biomass concentration (> 9 - 10 g l^{-1}) for *K. lactis* CBS 683. The could result in a less-representative sample being taken and may explain the increased scatter in the cell biomass results. However, this yeast may have an advantage over non-flocculating yeasts in a commercial process in that this property could be used to achieve high-cell densities, and the economic separation of the downstream processing of fermentation products would be facilitated (Stratford, 1992). Flocculation is also an important factor to consider in fermentation culture mixing.

One of the aims of this thesis was to investigate the feasibility of co-production of ethanol and a heterologous protein by whey fermentation. Commercially, in New Zealand ethanol is produced via an anaerobic fermentation as a means of whey disposal. However, the level of aeration may affect both the plasmid stability and heterologous protein production of a recombinant system (Lee and

Hassan, 1987; Hopkins *et al.*, 1987; Caunt *et al.*, 1990). Therefore, it was of interest to compare the effects of two levels of aeration on the recombinant yeast *K. lactis* CBS 683 : pCR1. Results indicated that plasmid stability was slightly lower with high aeration compared to low aeration. This may have been due to the increased number of cell divisions corresponding to a higher number of generations of cell growth for high aeration, typically 10-11 generations compared to approximately 8 generations for low aeration under the batch culture conditions investigated. Additionally, it was noted that there appeared to be a larger difference in growth rate between plasmid-free and plasmid-containing cells using low aeration, indicating the growth rate difference had a greater influence on the apparent stability under low aeration compared to high aeration conditions. The plasmid-containing strain *K. lactis* CBS 683 : pCR1 appeared to be a slower ethanol producer compared to the plasmid-free strain under low aeration. However, the yield of ethanol at the end of fermentation was similar for both. This difference would require consideration in studies of co-production of ethanol and heterologous protein from whey with the slower ethanol production rate making the ethanol process less efficient.

Media may be formulated to favour the growth of plasmid-carrying cells over plasmid-free cells, or selective pressure may be employed using auxotrophic mutants or antibiotic-resistant genes. The addition of G418 to media was shown to fully stabilize plasmid pCR1 in recombinant *K. lactis* CBS 683 in batch culture. However, the values obtained for plasmid stability were less than 100 %. This was probably due to the plasmid-free cells either retaining the G418-resistance product for a number of generations or obtaining it from other plasmid-containing cells, as noted earlier. The addition of antibiotic to the medium did not appear to have any other effect on cell characteristics.

Reports have shown that an increased temperature can lead to decreased stability, increased plasmid copy number, and increased protein production (Aiba and Koizumi, 1984; Son *et al.*, 1987; Tulin *et al.*, 1991). This study has shown that a decrease in temperature from that providing both higher biomass

concentration and rate of cell growth gave increased stability and, in contrast to the other reports, increased protein production. The effect of temperature (15 - 30°C) on cell growth was found to fit an Arrhenius relationship. In batch culture, 20°C was found to give the greatest plasmid stability and α-amylase protein production, but the specific growth rate was markedly slower compared to growth at 30°C. In contrast to this lower temperature the optimum ethanol production temperature is higher, in the range 30 - 40°C (Sienkiewicz and Riedel, 1990). These results suggest that it may be more feasible to have a separate operation, conducted at the lower temperature, for heterologous protein production, rather than a process devised to co-produce ethanol and the protein.

6.5 SUMMARY

Three methods available to measure plasmid stability were compared and two, the plate ratio and the clearing zones techniques, were chosen for use in fermentation studies. The plasmid pCR1 was found to be more stable in *K. lactis* CBS 683 than in *K. lactis* CBS 141 and so the former was chosen for the remainder of investigations. The recombinant yeast *K. lactis* CBS 683 : pCR1 was studied using batch culture to investigate yeast growth, ethanol production, and plasmid stability for different environmental conditions. Plasmid pCXJ-kan1 was found to be considerably more stable than pCR1 with the low stability of the latter probably due to the PGK-α-amylase insert. The utilization of whey was improved with addition of nitrogen sources, in particular yeast extract. The plasmid stability and α-amylase levels were lower than would be acceptable for commercial production but both were improved by either using a selective medium or a lower temperature of 20°C compared to that providing an increased growth rate. For example, at 30°C the level of α-amylase was approximately 7 U ml^{-1} compared to 18 U ml^{-1} at 20°C. Thus, overall the work has demonstrated the successful production of a wheat α-amylase from a *K. lactis* strain grown in whey media.

CHAPTER 7

CONTINUOUS FERMENTATION STUDIES OF RECOMBINANT *KLUYVEROMYCES LACTIS* EXPRESSING WHEAT α-AMYLASE

7.1 BACKGROUND AND AIM

Continuous fermentations can be employed to examine recombinant yeast culture characteristics over a larger number of generations and under more controlled environmental conditions than for batch culture. At steady-state the cells are in a state of balanced growth with an average specific growth rate equal to the dilution rate. In batch culture, balanced growth is only attained, if at all, during the relatively short period of exponential growth (Da Silva and Bailey, 1991a).

Continuous culture techniques have been used by other investigators to examine different characteristics of recombinant microorganisms, including the effects of dilution rate (Impoolsup *et al.*, 1989a; Jang *et al.*, 1990; Da Silva and Bailey, 1991a), induction mechanisms (Da Silva and Bailey, 1989a; 1989b; 1991a), cyclic changes (Weber and San, 1988; Impoolsup *et al.*, 1989b; Caunt *et al.*, 1990; Stephens *et al.*, 1992), aeration (Caunt *et al.*, 1989; Jang *et al.*, 1990), media and nutrient limitation (de Roubin *et al.*, 1991; 1992; Chen *et al.*, 1993b), and plasmid origin and promoter strength (Da Silva and Bailey, 1991b) on, for example, the kinetics of growth, plasmid stability, and heterologous protein production.

In this study continuous culture was used to examine the effects of growth rate, aeration, selective medium, and temperature on the recombinant yeast *K. lactis* CBS 683 : pCR1.

7.2 MATERIALS AND METHODS

Continuous culture investigations were carried out as described in Section 3.6.2 for the transformed yeast *Kluyveromyces lactis* CBS 683 : pCR1. The inoculum was prepared in WYP-G418 broth as described in Section 3.1.4, and the media used in the fermenter was WYP or WYP-G418 for non-selective and selective runs, respectively. The following methods were used to characterize the yeast culture:

(1) cell biomass (Section 3.4.3);
(2) residual lactose (Section 3.4.4);
(3) ethanol determination (Section 3.4.6);
(4) α-amylase production (3.4.7); and,
(5) plasmid stability (plate ratio and clearing zones methods, Section 3.5 and 6.2.1) and the rate of generation of plasmid-free cells (see below).

7.2.1 Determination of the rate of generation of plasmid-free cells from plasmid-containing cells

In this section the three models that were used to determine the kinetics of plasmid loss from *K. lactis* CBS 683 : pCR1 in continuous culture are presented. One of the simplest models for predicting plasmid stability has been described by Shuler and Kargi (1992) which was adapted from work by Cooper *et al.* (1987). This first model is termed Model A. A simple modified version of the Shuler and Kargi (1992) model is also presented and has been derived in this work (Model B). The third model (Model C) is that described by Syamsu *et al.* (1992) and the basis of this is also detailed.

All the models consider only two cell types: plasmid-free (n^-) and plasmid-containing (n^+), where n^- and n^+ are the concentrations of plasmid-free and plasmid-containing cells, respectively. According to the model proposed by Imanaka and Aiba (1981), the dynamics of plasmid-containing and plasmid-free

cell populations in a continuous culture of recombinant micro-organisms can be described by a material balance on each cell type:

$$\frac{dn^+}{dt} = \mu^+ n^+ - Dn^+ - Rn^+ \qquad (7.1)$$

$$\frac{dn^-}{dt} = \mu^- n^- - Dn^- + Rn^+ \qquad (7.2)$$

where:

R	=	specific rate of generation of plasmid-free cells (h^{-1})
μ^-, μ^+	=	specific growth rate of plasmid-free and plasmid-containing cells, respectively (h^{-1})
D	=	dilution rate (h^{-1})
t	=	time (h)

For the Shuler and Kargi (1992) model the situation can be simplified if it is assumed that the total number of cells (n_T) is constant after a few generations in continuous culture with constant operating conditions (i.e., the culture is at steady-state). Shuler and Kargi (1992) noted that this approximation is acceptable in many cases as long as the metabolic burden imposed by plasmid-encoded functions is not too drastic and D is less than 80 % of either μ^+_{max} or μ^-_{max}. Addition of equations 7.1 and 7.2 gives:

$$\frac{dn^+}{dt} + \frac{dn^-}{dt} = \mu^+ n^+ + \mu^- n^- - D(n^+ + n^-) \qquad (7.3)$$

Since n_T is constant and $n_T = n^+ + n^-$, equation 7.3 becomes:

$$0 = \mu^+ n^+ + \mu^- n^- - D(n_T) \qquad (7.4)$$

or:

$$0 = \mu^+ f^+ + \mu^- f^- - D \qquad (7.5)$$

where f^+ is the fraction of the total cell population that contains plasmid and f^- is the fraction of plasmid-free cells. Since $f^+ + f^- = 1$, then:

$$D = \mu^{+}f^{+} + \mu^{-}(1 - f^{+}) \tag{7.6}$$

and substituting equation 7.6 into equation 7.1, after the latter is divided by n_T, yields:

$$\frac{df^{+}}{dt} = \mu^{+}f^{+} - f^{+}[\mu^{+}f^{+} + \mu^{-}(1 - f^{+})] - Rf^{+} \tag{7.7}$$

After rearrangement, the following equation is obtained:

$$\frac{df^{+}}{dt} = f^{+2}\Delta\mu - f^{+}(\Delta\mu + R) \tag{7.8}$$

where:

$$\Delta\mu = \mu^{-} - \mu^{+} \tag{7.9}$$

Assuming a constant $\Delta\mu$ and R the equation can be solved. However, these assumptions are valid only if the copy number distribution of plasmid-containing cells remains constant during the experiment. To account for this, the model assumes that all plasmid-containing cells are identical in both growth rate and the probability of plasmid loss, since it assumes that all cells have exactly the same plasmid copy number. The analysis is best applied to situations where $\Delta\mu$ is not extremely large. The solution is:

$$\frac{1}{f^{+}} = \frac{1}{1 - f^{-}} = \frac{\Delta\mu}{\Delta\mu + R} + ce^{(\Delta\mu + R)t} \tag{7.10}$$

where c is the constant of integration. The initial condition, $f^{-} = f_0^{-}$ at t = 0 gives c as:

$$c = \frac{1}{1 - f_0^{-}} - \frac{\Delta\mu}{\Delta\mu + R} \tag{7.11}$$

and once c is evaluated, equation 7.11 can be rearranged to yield:

$$f^- = \frac{(f_0^-\Delta\mu+R)e^{(\Delta\mu+R)t}-R(1-f_0^-)}{(f_0^-\Delta\mu+R)e^{(\Delta\mu+R)t}+\Delta\mu(1-f_0^-)} \qquad (7.12)$$

If the assumption that the initial fraction of plasmid-free cells is small ($f_0^- << 1$) is made, equation 7.12 becomes:

$$f^- = \frac{(f_0^-\Delta\mu+R)e^{(\Delta\mu+R)t}-R}{(f_0^-\Delta\mu+R)e^{(\Delta\mu+R)t}+\Delta\mu} \qquad (7.13)$$

The limiting case that segregational instability is dominant over growth rate dependent stability, $\Delta\mu \leq R$, will give $f_0^-\Delta\mu << R$, and with a binomial expansion of equation 7.13 the following equation is obtained:

$$f^- \approx 1-(1+\frac{\Delta\mu}{R})e^{-(\Delta\mu+R)t}+\frac{\Delta\mu}{R}(1+\frac{\Delta\mu}{R})e^{-2(\Delta\mu+R)t} \qquad (7.14)$$

For $t > 1/(\Delta\mu + R)$:

$$f^- \approx 1-(1+\frac{\Delta\mu}{R})e^{-(\Delta\mu+R)t} \qquad (7.15)$$

Thus:

$$\ln f^+ = \ln(1-f^-) = \ln(1+\frac{\Delta\mu}{R})-(\Delta\mu+R)t \qquad (7.16)$$

The intercept of a plot of $\ln f^+$ versus time can be used to evaluate f_i^+, the initial estimate of the number of plasmid-containing cells, and the slope m is:

$$m = -(\Delta\mu + R) \qquad (7.17)$$

These values allow estimates of $\Delta\mu$ and R to be made using the following two equations:

$$\Delta\mu = m(f_i^+ - 1) = mf_i^- \quad (7.18)$$

$$R = mf_i^+ = -m(1 - f_i^-) \quad (7.19)$$

This model has been labelled Model A and linear regression analysis with QUATTRO.PRO (Boland International Inc., USA) was used to estimate f_i^+ and m.

In many cases estimated values of μ^+ and μ^- are close to D, suggesting that $\Delta\mu$ is small (Syamsu *et al.*, 1992). With the assumption that $\Delta\mu << R$, equation 7.12 becomes:

$$f^- = \frac{Re^{Rt} - R(1 - f_0^-)}{Re^{Rt}} \quad (7.20)$$

Rearrangement of equation 7.20 gives:

$$\ln\left(\frac{1 - f_0^-}{1 - f^-}\right) = Rt \quad (7.21)$$

and as previously noted $f^- + f^+ = 1$, then:

$$\ln\left(\frac{f_0^+}{f^+}\right) = Rt \quad (7.22)$$

Thus, a plot of $\ln(f^+_0/f^+)$ versus time will yield a slope equal to R, the rate of generation of plasmid-free cells. This method of analysis is termed Model B, and linear regression with QUATTRO.PRO was used to estimate R.

The model described by Impoolsup *et al.* (1989a) and Syamsu *et al.* (1992) can also be used to calculate the growth rates of plasmid-free and plasmid-containing cells and the probability of plasmid loss. This model is also based on equations 7.1 and 7.2, and assumes that the cells are in a state of exponential growth and biomass is the only product. With a limiting and constant substrate concentration and if the probability of plasmid loss, p, is equal to μ^+/R, the change in plasmid-containing cells in continuous culture can be rewritten as:

$$\frac{dn^+}{dt} = ((1-p)\mu^+ - D)n^+ \tag{7.23}$$

$$\frac{dn^-}{dt} = (\mu^- - D)n^- + p\mu^+ n^+ \tag{7.24}$$

Equation 7.23 is integrated, with the initial condition $n^+ = n^+_0$ at $t = t_0$, to give:

$$n^+ = n_0^+ e^{(((1-p)\mu^+ - D)(t-t_0))} \tag{7.25}$$

Integration of equation 7.24 requires a complementary function and particular integration techniques (Syamsu, 1993), and with the initial condition that $n^- = n^-_0$ at $t = t_0$ the following equation is obtained:

$$n^- = (n_0^- - \frac{p\mu^+ n_0^+}{((1-p)\mu^+ - \mu^-)}) e^{(\mu^- - D)(t-t_0)} + (\frac{p\mu^+ n_0^+}{(1-p)\mu^+ - \mu^-}) e^{((1-p)\mu^+ - D)(t-t_0)} \tag{7.26}$$

For estimation of the three parameters μ^+, μ^-, and p simultaneously, the total number of cells in the reactor (n_T) and plasmid loss must be normalized (as in equations 7.27 and 7.28, respectively) before being used since the dimensions and magnitudes of these are different.

$$\frac{n_T}{n_0} = \frac{(n^+ + n^-)}{(n_0^+ + n_0^-)} \tag{7.27}$$

$$\frac{f^+}{f_0^+} = \frac{(n^+/n_T)}{(n_0^+/n_{T0})} \tag{7.28}$$

Equations 7.25 to 7.28 provide a relationship between the experimental data and the parameters μ^+, μ^-, and p, and have collectively been termed Model C. Estimates of the three parameters were obtained by non-linear regression using MATLAB (The Mathworks Inc., South Natick, MA, USA).

7.2.2 Estimation of biological parameters of yeast growth

Biomass data from continuous culture were analyzed using a Langmuir plot as described in standard texts (for example, Moser, 1988). In this method the Monod equation (equation 2.1) is rearranged to give:

$$\frac{S}{\mu} = \frac{K_s}{\mu_{max}} + \frac{S}{\mu_{max}} \qquad (7.29)$$

A plot of S/μ versus S will give a slope of $1/\mu_{max}$ and y-intercept of K_s/μ_{max}. Linear regression analysis with QUATTRO.PRO was used.

7.3 RESULTS

7.3.1 Effect of dilution rate

The maximum specific growth rate of *K. lactis* CBS 683 : pCR1 was estimated from batch growth as approximately 0.44 h^{-1} (Chapter 6.3.2.5). Dilution rates of 0.08, 0.21, and 0.31 h^{-1} were thus chosen for studying the effects on cell growth characteristics and plasmid stability of recombinant *K. lactis* CBS 683 : pCR1 in non-selective WYP media. Duplicate runs were performed for the dilution rates of 0.31 h^{-1} and 0.21 h^{-1} with a further run at 0.20 h^{-1}. The steady-state results for the duplicate runs were in agreement. Continuous culture proceeded for at least 40 generations, apart from the fermentation at 0.08 h^{-1} which only reached 18 generations. The profile for the fermentation at the dilution rate of 0.20 h^{-1} is shown in Figure 7.1. The steady-state values for biomass, ethanol, and residual lactose are given in Table 7.1.

As expected, the biomass concentration decreased with increasing dilution rate. At the high cell concentration obtained at the lower dilution rate the yeast cells tended to aggregate, as seen in batch conditions when cell growth attained > 9 - 10 g l^{-1}. This caused problems with blockages in fermenter outlet lines which led to increased fermenter liquid volume and loss of steady-state and so limited the

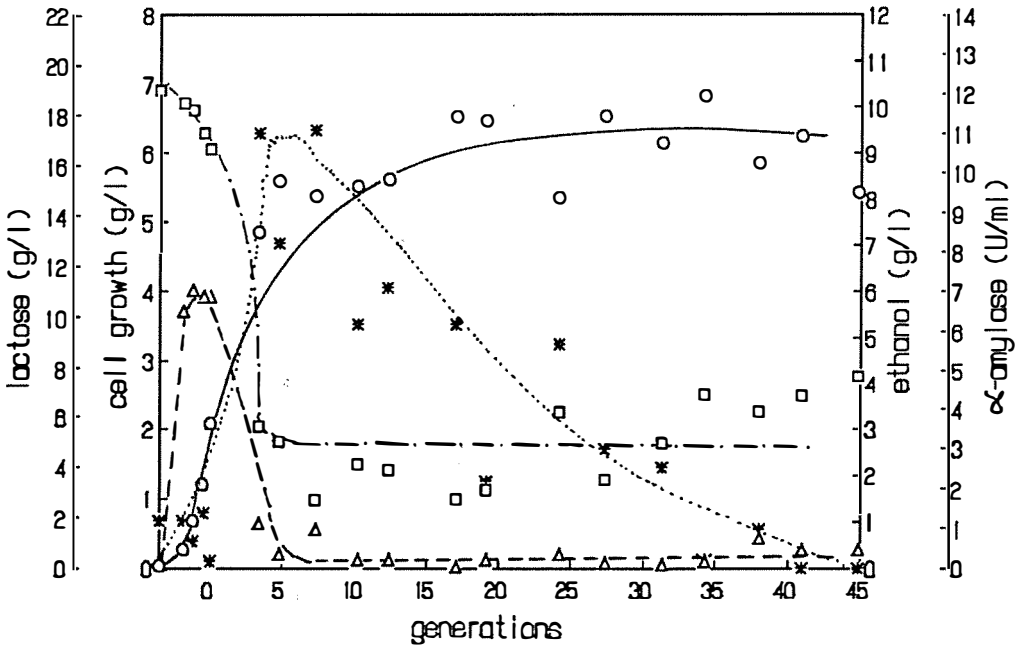


Figure 7.1 **The fermentation profile for continuous culture at D = 0.20 h^{-1} for recombinant *K. lactis* CBS 683 : pCR1 ((—o—) cell biomass; (- · - □ - · -) lactose; (- - Δ - -) ethanol; (·····*·····) α-amylase).**

time for which the fermentations could be conducted. One further run at 0.08 h^{-1} was performed but for the reasons cited this was also terminated after only 8 generations (70 h).

Table 7.1 Summary of the steady-state concentrations and plasmid stability results for *K. lactis* CBS 683 : pCR1 grown in continuous culture at different dilution rates.

Dilution rate	Number of generations of continuous culture	Steady-state values[a]			Plasmid stability[b]	Normalized change in plasmid stability (f/f_0)[d]	Rate of generation of plasmid-free cells (R)	
		Cell biomass	Ethanol	Residual lactose				
(h^{-1})		($g\ l^{-1}$)	($g\ l^{-1}$)	($g\ l^{-1}$)	(%)		(h^{-1})	(gen^{-1})
0.08	18	9.5	0.04	0.3	75 - 25[c]	-	0.006	0.051
0.21 (a)	41	6.5	0.25	3.4	77 - 5	0.06	0.021	0.071
0.21 (b)	43	6.5	0.22	4.8	68 - 6	0.09	0.018	0.058
0.20	44	6.0	0.27	4.9	88 - 4	0.05	0.021	0.073
0.31 (a)	45	3.9	0.80	9.7	64 - 12	0.19	0.023	0.052
0.31 (b)	43	3.5	0.84	9.7	68 - 9	0.13	0.023	0.051

[a] Steady-state values were calculated as the average results of the continuous culture after 2 - 3 residence times. Results for D = 0.08 h^{-1} are given but steady-state was not maintained.

[b] Initial stability value at the start of continuous conditions. Final stability value at 40 generations of growth.

[c] Final stability value at 18 generations.

[d] Calculated for 40 generations of cell growth.

The results for this group of continuous fermentations were analyzed using a Langmuir plot, as shown in Figure 7.2. Linear regression analysis gave values for μ_{max} of 0.369 h^{-1} and K_s of 2.52 g l^{-1} with a regression coefficient (R^2) of 0.91.

Plasmid stability results for all dilution rates are shown in Table 7.1 and results for plasmid stability as a function of number of generations for the dilution rates of 0.08, 0.20, and 0.31(a) h^{-1} in Figure 7.3. These indicated that the stability of plasmid pCR1 was poor. The fraction of plasmid-containing cells when plotted against generations of growth followed an exponential decay pattern. In most fermentations a short initial stage that had a different slope to the remainder of

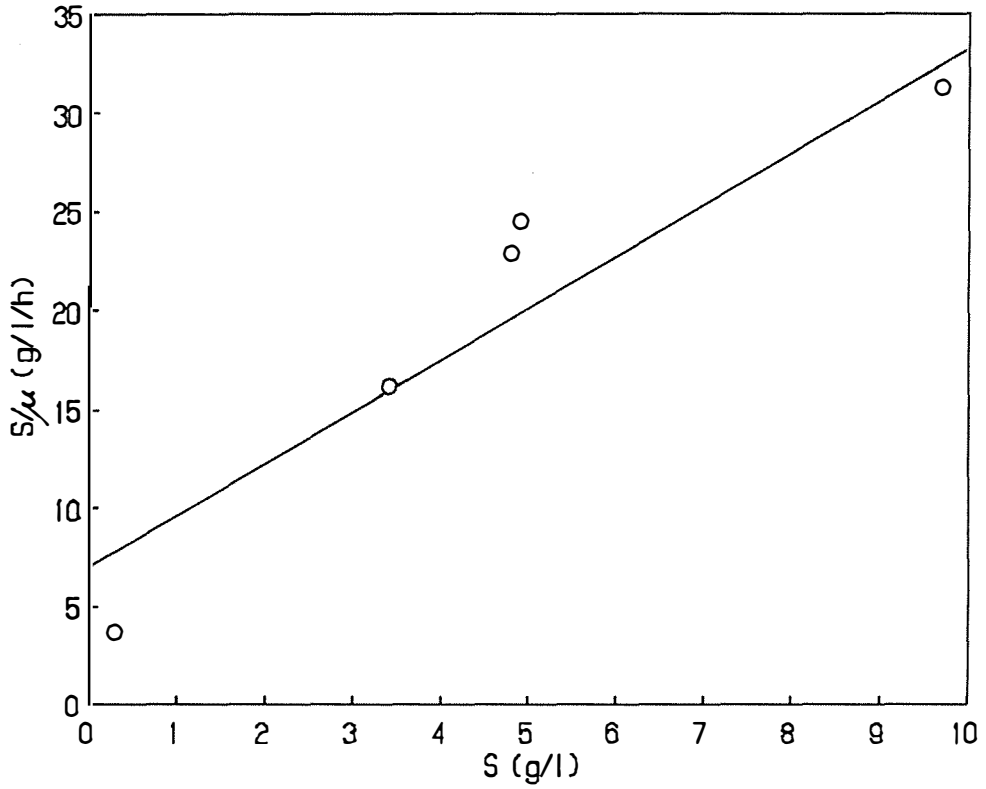


Figure 7.2 **The Langmuir plot for continuous culture of recombinant *K. lactis* CBS 683 : pCR1.**

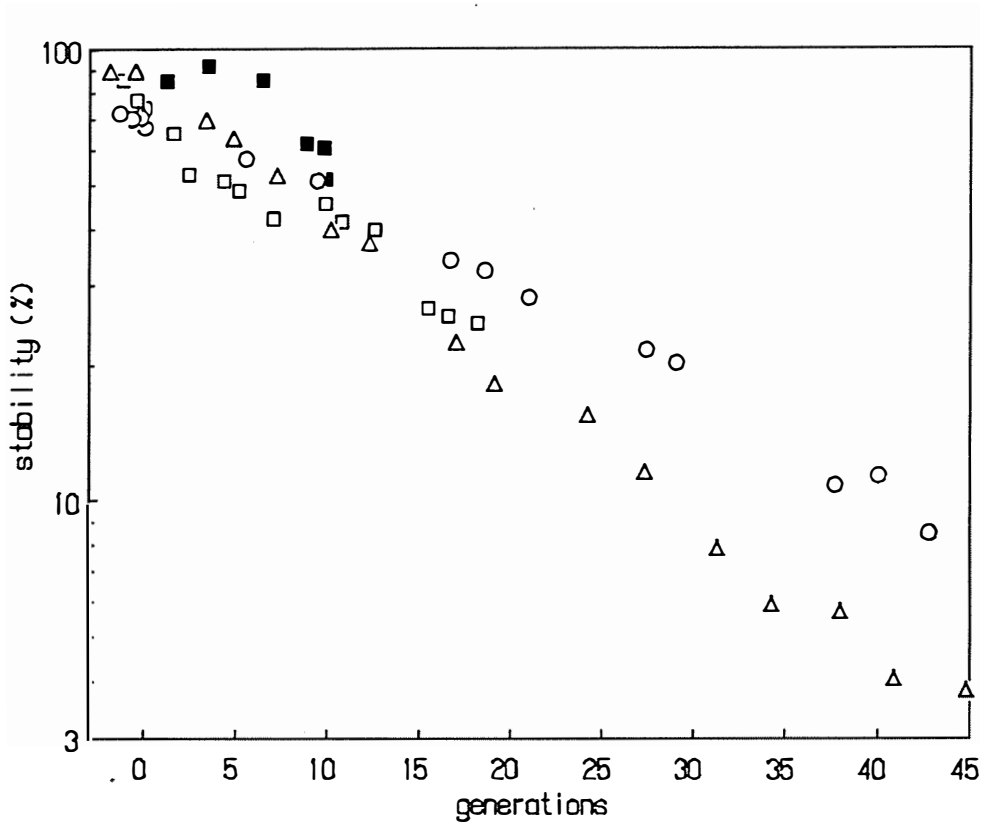


Figure 7.3 **The plasmid stability profiles of recombinant *K. lactis* CBS 683 : pCR1 in continuous culture at different dilution rates, and in batch culture. ((—□—) 0.08 h^{-1}; (—Δ—) 0.20 h^{-1}; (—○—) 0.31 h^{-1} (b); (—■—) batch).**

the fermentation was observed. This different plasmid-loss rate corresponded to the fermenter batch phase and start of continuous culture, and was therefore not incorporated in the calculations for rates of generation of plasmid-free cells in continuous culture, R. Model B was used to calculate the R values shown in Table 7.1. The values for R indicated that the rate of plasmid loss was higher at a dilution rate of 0.20 - 0.21 compared to 0.31 h^{-1}. The results of the fermentation at 0.08 h^{-1} were doubtful due to the difficulty in maintaining steady-state. The normalized change in plasmid stability also supported this, with values obtained for 0.2 and 0.31 h^{-1} of 0.05 - 0.09 and 0.13 - 0.19, respectively, for 40 generations of cell growth. Batch results are also plotted in Figure 7.3. The stability results of a batch culture of *K. lactis* CBS 683 : pCR1 in YPL broth under high aeration (Figure 6.3 A and B) were analyzed with Model B. The value obtained for R was comparable to the continuous results at 0.057 gen^{-1}.

7.3.2 Effect of aeration

The effect of aeration was considered further with continuous fermentation. Two levels of aeration only were considered, high DO (DO greater than 20 % of air saturation), resulting from an impeller speed of 600 - 650 rpm and air flowrate of 0.15 l min^{-1}, and low (less than 5 % DO), resulting from 175 - 200 rpm and 3 - 5 ml min^{-1}, with a dilution rate of 0.20 h^{-1}. The resultant steady-state and plasmid stability data are given in Table 7.2, and the fermentation profile for low aeration is shown in Figure 7.4 A. The DO level for low aeration continuous culture was not as low as that for comparable batch cultures, as indicated by the biomass concentration, which reached about 3 g l^{-1}, compared to 1.4 g l^{-1} for batch culture. However, cell biomass was only half that of the 6 g l^{-1} reached in high aeration continuous culture, which indicated that oxygen was limiting. This was also reflected in the ethanol production.

In contrast to the batch results, in which there was little difference in plasmid stability between high and low aeration conditions, plasmid stability under low aeration in continuous culture was much greater than for high aeration, as shown

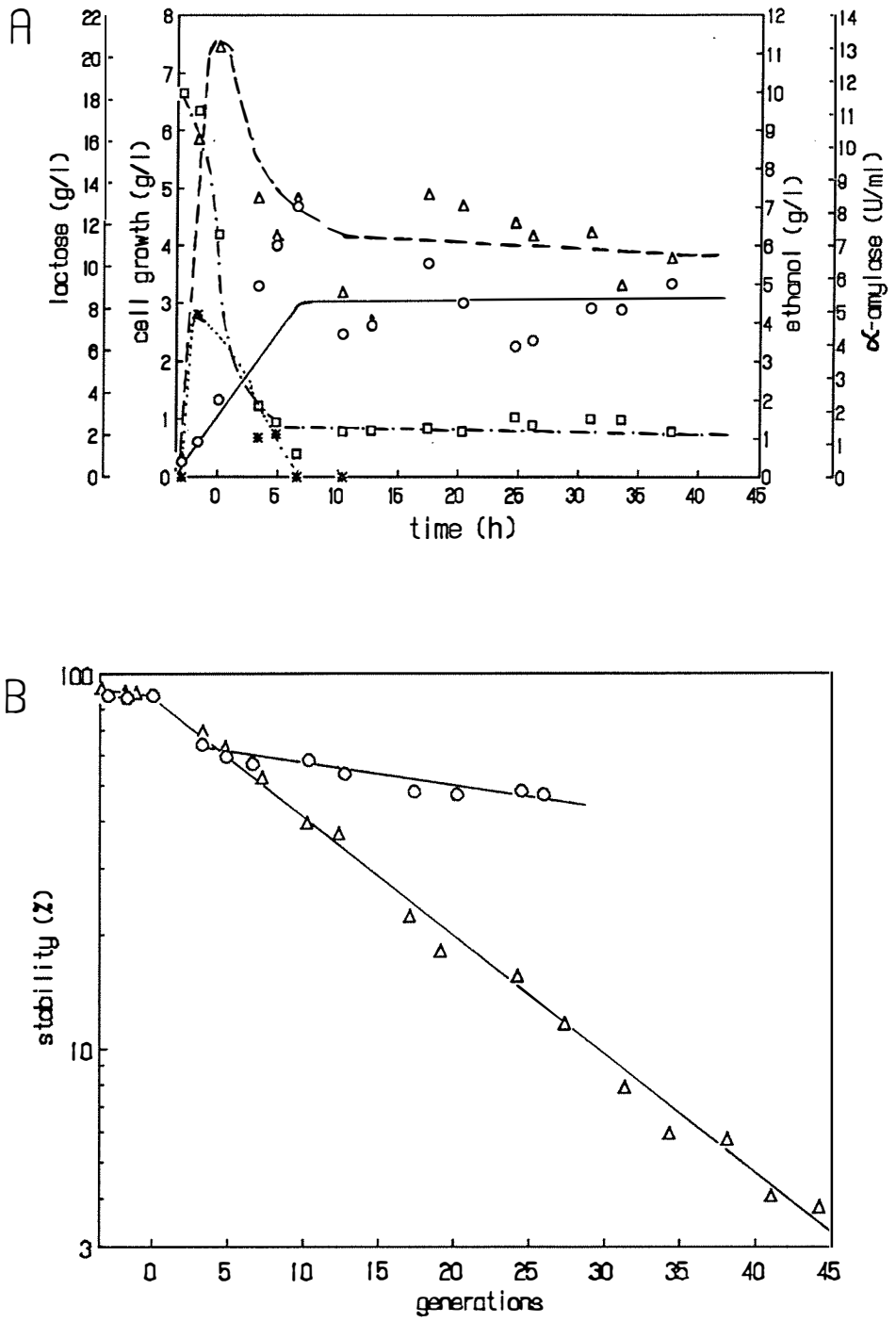


Figure 7.4 **A. The fermentation profile for low aeration continuous culture at D = 0.20 h^{-1} for recombinant *K. lactis* CBS 683 : pCR1 ((—o—) cell biomass; (- · - □ - · -) lactose; (- - Δ - -) ethanol; (·····*·····) α-amylase).**
B. The plasmid stability profiles for low aeration (—o—) and high aeration (—Δ—) continuous culture.

in Figure 7.4 B. The rate of generation of plasmid-free cells under low aeration was 6-fold lower compared to that for high aeration. Despite this higher stability, the production of α-amylase was minimal and the enzyme could not be detected after 17 h (5 generations).

Table 7.2 Summary of the steady-state concentrations and plasmid stability results for *K. lactis* CBS 683 : pCR1 grown in continuous culture at different levels of aeration (dilution rate = 0.20 h^{-1}).

Level of aeration	Number of generations of continuous culture	Steady-state values[a]			Plasmid stability[b]	Normalized change in plasmid stability (f/f_0)[c]	Rate of generation of plasmid free cells	
		Cell biomass ($g\ l^{-1}$)	Ethanol ($g\ l^{-1}$)	Residual lactose ($g\ l^{-1}$)	(%)		(h^{-1})	(gen^{-1})
high	44	6.0	0.27	4.9	88 - 16	0.18	0.021	0.073
low	26	3.1	6.02	2.3	87 - 47	0.54	0.003	0.012

[a] Steady-state values were calculated as the average results of the continuous culture after 2 - 3 residence times.

[b] Initial stability value at the start of continuous conditions. Final stability value at 25 generations of growth.

[c] Calculated for 25 generations of cell growth.

7.3.3 Effect of selective media

Continuous growth in selective medium was also investigated to confirm the increase in plasmid stability observed in batch culture. The selective medium used was WYP-G418 and the fermentation was performed under high aeration with a dilution rate of 0.20 h^{-1}. The results are shown in Figure 7.5 A, and the steady-state values are given in Table 7.3.

As seen in batch culture, the selective medium stabilized the plasmid in *K. lactis* CBS 683 : pCR1 (Figure 7.5 B). In the selective medium the rate of generation of plasmid-free cells was 10-fold lower than in the non-selective medium. Correspondingly, α-amylase production also remained high at approximately 10 U ml^{-1} throughout the fermentation.

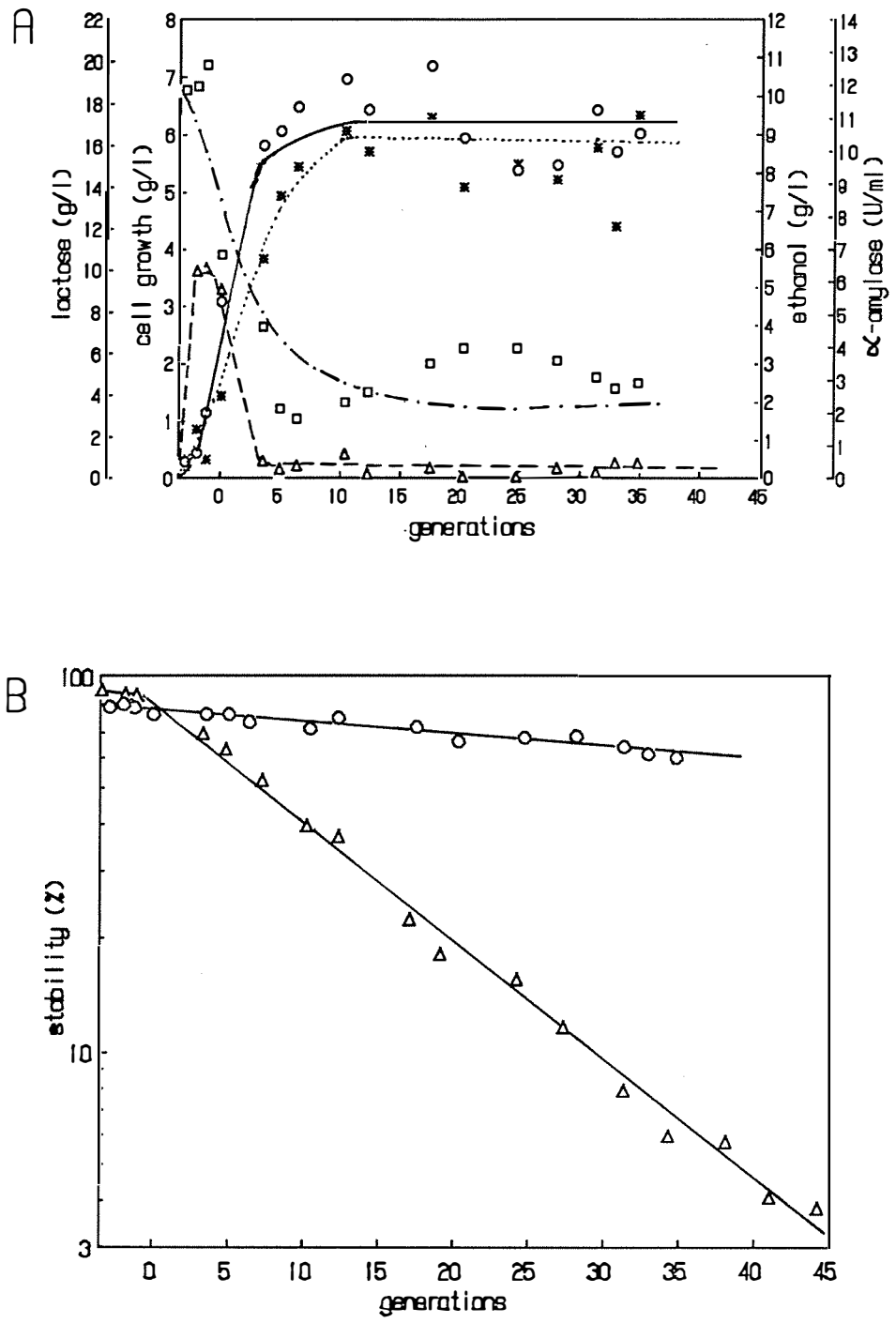


Figure 7.5 **A. The fermentation profile for continuous culture using selective medium at D = 0.20 h^{-1} for recombinant *K. lactis* CBS 683 : pCR1 ((—o—) cell biomass; (- ·- □- ·-) lactose; (- - Δ- -) ethanol; (·····*·····) α-amylase).**
B. The plasmid stability profiles for continuous culture using selective WYP-G418 medium (—o—) and non-selective WYP medium (—Δ—).

Table 7.3 **Summary of the steady-state concentrations and plasmid stability results for *K. lactis* CBS 683 : pCR1 grown in continuous culture in selective and non-selective media (dilution rate = 0.20 h^{-1}).**

Medium	Number of generations of continuous culture	Steady-state values[a]			Plasmid stability[b]	Normalized change in plasmid stability (f/f_0)[c]	Rate of generation of plasmid-free cells	
		Cell biomass	Ethanol	Residual lactose				
		(g l^{-1})	(g l^{-1})	(g l^{-1})	(%)		(h^{-1})	(gen^{-1})
WYP	44	6.0	0.27	4.9	88 - 6	0.07	0.021	0.073
WYP-G418	35	6.2	0.22	4.6	79 - 60	0.76	0.002	0.007

[a] Steady-state values were calculated as the average results of the continuous culture after 2 - 3 residence times.

[b] Initial stability value at the start of continuous conditions. Final stability value at 35 generations of growth.

[c] Calculated for 35 generations of cell growth.

7.3.4 Effect of temperature

In batch culture the temperature for both optimum plasmid stability and protein production was found to be about 20°C and so the effect of this temperature was examined further in continuous culture. The fermentation was conducted at a dilution rate of 0.20 h^{-1} with high aeration in non-selective WYP medium at 20°C. The steady-state results and stability data are presented in Table 7.4. The fermentation profile for 20°C is shown in Figure 7.6 A and the plasmid stability results, for both 20 and 30°C, in Figure 7.6 B.

Biomass concentration was only about half of that at 30°C, and the residual lactose and ethanol concentrations were two-fold higher at the lower temperature. The production of the heterologous α-amylase was at the same level at 10 U ml^{-1}, however the loss of enzyme product was not as rapid as at the higher temperature and the specific yield of α-amylase was clearly higher. Plasmid stability was greater at 20°C, as was observed in batch culture, with the rate of generation of plasmid-free cells four-fold lower.

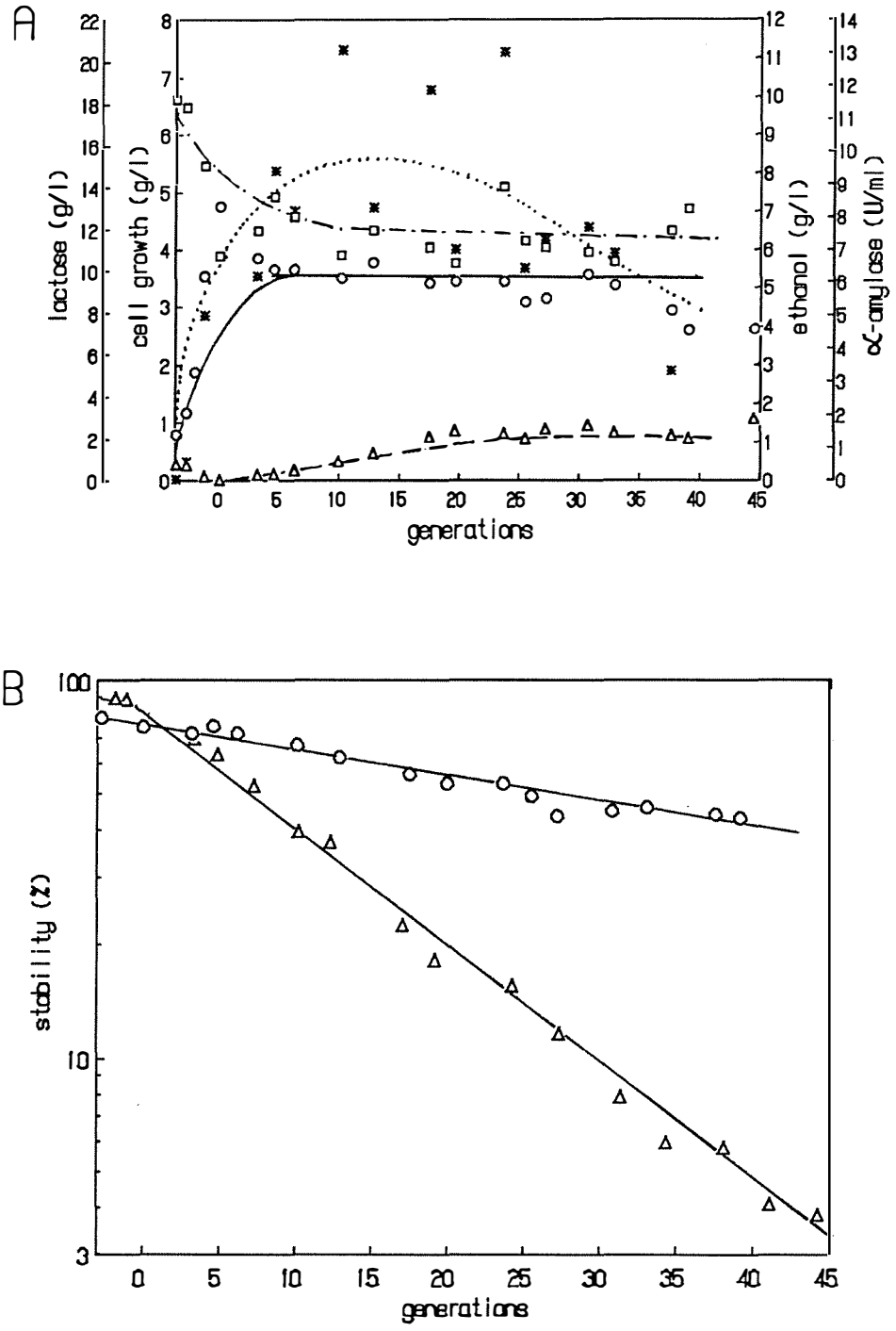


Figure 7.6 **A. The fermentation profile for continuous culture at 20°C and at D = 0.20 h^{-1} for recombinant *K. lactis* CBS 683 : pCR1 ((—o—) cell biomass; (- ·- □- ·-) lactose; (- - Δ- -) ethanol; (·····*·····) α-amylase).**

B. The plasmid stability profiles for continuous culture at 20°C (—o—) and 30°C (—Δ—).

Table 7.4 **Summary of the steady-state values and plasmid stability results for *K. lactis* CBS 683 : pCR1 grown in continuous culture at different temperatures (dilution rate = 0.20 h^{-1}).**

Temperature	Number of generations of continuous culture	Steady-state values[a]			Plasmid stability[b]	Normalized change in plasmid stability (f/f_0)[c]	Rate of generation of plasmid-free cells	
		Cell biomass	Ethanol	Residual lactose				
(°C)		(g l^{-1})	(g l^{-1})	(g l^{-1})	(%)		(h^{-1})	(gen^{-1})
20	40	3.4	1.02	11.8	75 - 43	0.57	0.005	0.017
30	44	6.0	0.27	4.9	88 - 6	0.07	0.021	0.073

[a] Steady-state values were calculated as the average results of the continuous culture after 2 - 3 residence times.

[b] Initial stability value at the start of continuous conditions. Final stability value at 40 generations of growth.

[c] Calculations based on 40 generations of cell growth.

7.3.5 Comparison of models

The three models, Model A as described by Shuler and Kargi (1992), Model B as derived in this study, and Model C, that reported by Syamsu *et al.*, (1992), were compared for the continuous fermentations investigated in this work. Table 7.5 gives examples of the estimates calculated for R, p, μ^+ and μ^- for selected runs.

The values obtained for R with Model A were slightly lower than those of Model B, although both models showed the same trends in R. The estimates obtained for R using Model C were higher than those of Models A and B. However, Model C did not always yield meaningful results with respect to the growth rates calculated.

Table 7.5 **Examples of estimated values obtained from three models for: R, rate of generation of plasmid-free cells; p, probability of plasmid loss; and μ^+ and μ^-, growth rates of plasmid-containing and plasmid-free cells, respectively.**

Fermentation run[a]	Model A				
	R^b		p	μ^+	μ^-
	(h^{-1})	(gen^{-1})	(= R/μ^+)	(g l^{-1})	(g l^{-1})
D = 0.2 h^{-1}	0.0183	0.063	0.092	0.199	0.20
D = 0.2 h^{-1} low aeration	0.0024	0.008	0.012	0.199	0.20
D = 0.2 h^{-1} temperature = 20°C	0.0036	0.013	0.018	0.199	0.20
	Model B				
	R^b				
	(h^{-1})	(gen^{-1})			
D = 0.2 h^{-1}	0.021	0.073			
D = 0.2 h^{-1} low aeration	0.003	0.012			
D = 0.2 h^{-1} temperature = 20°C	0.005	0.017			
	Model C				
	R^b (= $p\mu^+$)		p	μ^+	μ^-
	(h^{-1})	(gen^{-1})		(g l^{-1})	(g l^{-1})
D = 0.2 h^{-1}	0.025	0.087	0.125	0.20	0.20
D = 0.2 h^{-1} low aeration	0.009	0.031	0.045	0.21	0.20
D = 0.2 h^{-1} temperature = 20°C	0.010	0.035	0.052	0.21	0.20

[a] Conditions for continuous culture were as described in Section 3.7.2.

[b] R, in units of h^{-1}, is changed to units of number of generations, gen^{-1} by multiplying by ln2/D.

7.4 DISCUSSION

No true steady-states were attained in these continuous fermentations since a mixed population of plasmid-free and plasmid-containing cells was always present in the reactor, but the measured biomass, residual substrate, and ethanol concentrations did approach what could be considered constant values in a pseudo-steady-state. This can be explained by the growth rates of the two strains being similar. The difference in the growth rates between the plasmid-containing and plasmid-free strains in continuous culture was calculated to be less than 3 % using Model A, the model of Cooper *et al.* (1987). This agrees with the apparent difference in growth rates found in batch culture of approximately 2 % (Section 6.3.2.3). No other physiological differences between the plasmid-containing and plasmid-free strains were apparent, apart from the presence of the plasmid in the recombinant strain.

The normalized stability data ($\ln(f/f_0)$) when plotted against time or generations of growth, followed a linear relationship. Thus, the number of plasmid-containing cells followed an exponential decay pattern, and plasmid stability per generation remained constant for a particular set of conditions. Three models were compared for predicting the plasmid loss of the recombinant culture *K. lactis* CBS 683 : pCR1. The initial stage of batch culture (approximately 10 hours) and the phase during which the pseudo-steady state was being achieved (approximately two residence times) were not used to calculate the rate of plasmid loss, since these periods would not be representative of the conditions prevailing at the pseudo-steady-state.

Model A (Shuler and Kargi, 1992) divided the stability into segregational and growth rate effects and assumed the initial plasmid stability to be 100 %. Model B assumed that the difference in growth rates was negligible and, unlike Model A, took into account the initial stability. The values obtained for R with this method were slightly greater than those calculated with Model A. This was expected since Model A took into account the growth rate effects of the apparent

segregational stability. Irrespective of this slight difference, Model B showed the same trends in R as Model A. The third model, Model C (Syamsu *et al.*, 1993), did not assume a negligible difference in growth rates or 100 % initial plasmid stability and gave less meaningful estimates of R due to a wide variation in the growth rates determined for plasmid-free and plasmid-containing cells.

The model chosen to estimate the rate of plasmid loss was Model B, the modified version of that described by Shuler and Kargi (1992). A more complex model was not used since only the general trend of plasmid loss rate data was required in order to compare the different conditions under investigation. Model B incorporated a large number of assumptions to arrive at its simple form and hence drastically simplified the real case. However, it was adequate for this study since more complex models would require much more data, such as plasmid copy numbers, and the analytical expressions would be difficult to develop or obtain.

The stability of the culture under batch fermentation was compared with the stability under continuous fermentation using the modified model. The results indicated that the rate of generation of plasmid-free cells was similar for both culture techniques, with R values of 0.05 - 0.07 gen^{-1} calculated for non-selective continuous culture compared to 0.057 gen^{-1} for the batch culture used for the comparison.

The plasmid stability of the culture *K. lactis* CBS 683 : pCR1 was low, with less than 20 % of cells containing plasmid after 40 generations of growth at 0.31 h^{-1} in WYP medium, and less than 10 % at a dilution rate of 0.2 h^{-1}. The low stability of the pCR1 plasmid contrasted with the stabilities reported for other pKD1-derived plasmids for extended growth. For example, the pKD1-derived vectors for the production of recombinant human serum albumin had greater than 90 % stability in *K. lactis* CBS 683 after 40 generations of non-selective growth (Fleer *et al.*, 1991b). Possible reasons for this have already been discussed in Chapter 6 (Section 6.4).

The rate of generation of plasmid-free cells was slightly higher at 0.2 h^{-1} compared to 0.31 h^{-1}. The rate value for 0.08 h^{-1} appeared to fall somewhere between the dilution rates of 0.2 and 0.31, but since problems were encountered in maintaining the pseudo-steady-state it was uncertain whether the results represented the true rate of decline. Further fermentations at other dilution rates would be necessary to confirm that plasmid stability may be improved using increased dilution rates.

Other studies that have investigated the effect of growth rate or dilution rate have used recombinant *S. cerevisiae* yeasts expressing heterologous proteins with much greater plasmid stability than that used here. For example, Impoolsup *et al.* (1989a) examined dilution rates of 0.15 to 0.42 h^{-1}, and found the fraction of plasmid-bearing yeast cells to be close to 1 for low dilution rates of < 0.23 h^{-1} up to 100 generations. Da Silva and Bailey (1991a) used an inducible protein production system and also found, after induction and 40 - 50 residence times, that the fraction of plasmid-containing cells was still around 0.8 - 0.9 for dilution rates of 0.1 - 0.26 h^{-1}. However, although there was a decrease in stability with low dilution rates, Da Silva and Bailey (1991a) found an increase in specific enzyme activity and biomass concentration with increased productivity as the dilution rate was lowered. This may be attributed to an increase in plasmid copy number.

Ryan and Parulekar (1991) have reviewed the relationship between copy number, growth rate, plasmid stability and protein production. Generally, except at very low growth rates, there is an increase in plasmid copy number with a decrease in specific growth rate. Depending on the plasmid DNA content in a host, an increase in plasmid copy number accompanying a reduction in dilution (growth) rate may either increase or decrease plasmid stability and hence increase or decrease heterologous protein production. Thus several recombinant systems exhibit improved plasmid stability with an increase in dilution rate (Aiba and Koizumi, 1984; Siegel and Ryu, 1985; Sayadi *et al.*, 1989; Wei *et al.*, 1989), but in other cases an opposite trend has been observed (Weber and San, 1988;

Impoolsup *et al.*, 1989a). Although not all the reports give copy number data, Ryan and Parulekar (1991), who also showed an improved stability with increased dilution rates, noted that this trend is normally associated with high copy number systems. Although the copy number of pCR1 has not been determined in *K. lactis* CBS 683 : pCR1, related plasmids have been reported to have medium to high copy numbers (Chen *et al.*, 1989; Fleer *et al.*, 1991b). Therefore the observation of increased stability at the higher dilution rate of 0.31 compared to 0.2 h^{-1} appears to hold with the trend described by Ryan and Parulekar (1991).

Previously, the level of aeration was shown to only slightly affect the stability of plasmid pCR1 in batch culture, with the plasmid stability lower at high aeration. In these continuous culture experiments two levels of aeration were studied. In contrast to the batch results aeration had a marked effect, with the plasmid being considerably more stable under low aeration conditions. The rate of generation of plasmid-free cells was six-fold lower at low aeration compared to high aeration.

These results differ to those reported by Caunt *et al.* (1989) who showed that recombinant *S. cerevisiae* strains exhibited high plasmid stability in air alone (DO 80 % of air saturation) compared to in nitrogen-only (equivalent to low aeration; DO < 10 %) and oxygen-only conditions (175 % DO). These same trends were shown by Lee and Hassan (1987). Despite the improved plasmid stability of pCR1 at low aeration, α-amylase production was minimal (< 2 U ml^{-1}), probably because of the lower cell biomass. Again, in contrast, Lee and Hassan (1987) showed that production of heterologous killer toxin for a recombinant *S. cerevisiae* strain was enhanced in nitrogen-only conditions compared with production in air or oxygen-alone. They suggested the higher production was due to an increase in promoter activity or toxin processing and secretion or both. The conflicting results shown here may be a consequence of the different metabolic state of the recombinant *K. lactis* CBS 683 : pCR1 yeast at different levels of aeration, or the level of aeration may affect the plasmid copy number and lead to the stability difference observed. Lee and Hassan (1987) noted that little is

known about the relationship between expression and stability of the plasmid and the oxygen demand/supply to the host organism.

The addition of G418 to the WYP medium was shown to stabilize plasmid pCR1 in recombinant *K. lactis* CBS 683 : pCR1 in batch culture and was studied further in continuous culture. This confirmed the plasmid could be stabilized for extended periods of growth, with the rate of generation of plasmid-free cells estimated to be ten-fold less in selective compared to non-selective media. This increased plasmid stability allowed α-amylase production to be maintained, although the level of α-amylase was not any higher than the highest level in non-selective medium. This may indicate that the copy number of the plasmid was not affected by the selection mechanism, since increased copy numbers generally lead to increased heterologous protein production. This could be confirmed with plasmid copy number analysis.

Batch investigations also showed that both plasmid stability and α-amylase protein production were enhanced at a culture temperature of 20°C compared to at 30°C. The lower temperature was investigated in continuous culture and was also found to increase the plasmid stability over a larger number of generations of cell growth, with the rate of generation of plasmid-free cells estimated to be approximately four-fold lower. In addition, the specific yield of α-amylase protein was nearly two-fold greater than that at 30°C, the temperature giving the greatest cell biomass, and enzyme production was maintained for a longer period due to the increased plasmid stability. In batch culture at 20°C, the level of α-amylase obtained was 18 U ml^{-1}. In contrast, the level of α-amylase obtained in continuous culture at 20°C with a dilution rate of 0.2 h^{-1} was lower at 10 U ml^{-1}. This can be explained by the temperature effect on the dilution (growth) rate. In batch culture, μ_{max} was found to be lower at 20°C compared to the higher temperatures of 25 and 30°C. In continuous culture the growth rate is equal to the dilution rate and therefore is independently controlled. With a dilution rate of 0.2 h^{-1} the continuous culture at 20°C was operating closer to μ_{max} compared to at 30°C, and therefore there was a lower cell biomass at the lower temperature. Thus, it is expected that

with a decrease in dilution rate at the lower temperature, cell biomass would increase and correspondingly the α-amylase level would be higher, and closer to the batch results. An increased concentration of heterologous protein would allow recovery costs to be reduced. A balance needs to be achieved between this and the rate of production of the protein, with a higher rate allowing lower fermentation costs.

7.5 SUMMARY

Plasmid stability and heterologous α-amylase production of the recombinant yeast *K. lactis* CBS 683 : pCR1 were investigated using continuous culture to extend the preliminary batch culture studies. Plasmid stability was found to increase with increased dilution rates, the use of a selective medium, a culture temperature of 20°C, and a low level of aeration. Low aeration was detrimental to heterologous enzyme production. However, the use of selective medium and a lower temperature allowed the level of α-amylase to be maintained for an increased length of time and the latter also gave an increased specific yield of α-amylase protein. Therefore, methods effective for increasing both the plasmid stability and heterologous protein production have been demonstrated.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

Yeasts have major potential for the production and secretion of heterologous proteins. Commercial products currently manufactured and sold include a recombinant hepatitis B vaccine and insulin from *Saccharomyces cerevisiae*, and chymosin from *Kluyveromyces lactis*. Until recently, *S. cerevisiae* was the preferred yeast host due to the volume of information concerning molecular and genetic aspects of this organism. However, several problems have been encountered with *S. cerevisiae*, such as the lack of very strong, tightly regulated promoters and hyperglycosylation of proteins, suggesting it may not be as useful as other yeasts for the development of commercial products and processes (Reiser *et al.*, 1990; Heslot and Gaillardin, 1992; Romanos *et al.*, 1992).

The yeast chosen for this study was of the genus *Kluyveromyces*. *Kluyveromyces* yeasts have been used in the food industry for many years in the production of, for example, β-galactosidase, cell biomass, and ethanol, and are classified as GRAS organisms (Sienkiewicz and Riedel, 1990). In New Zealand, ethanol production from whey is a well-established technology. The large-scale cultivation of *Kluyveromyces* has been extensively studied and its ability to grow on cheap substrates, such as lactose and whey, further increases its potential as a host for the production of, particularly, lower value heterologous proteins. In addition, *Kluyveromyces* yeasts are similar to *S. cerevisiae* in having well-understood molecular genetic systems, they have the capacity for high-level secretion, and have native episomal vectors, similar to the well-studied 2μ plasmid of *S. cerevisiae*, which can be used for plasmid constructions (Romanos *et al.*, 1992). For example, the native plasmid of *K. drosophilarum*, pKD1 (Falcone *et al.*, 1986) has been used to construct *Kluyveromyces* episomal vectors (Bianchi *et al.*, 1987).

The major aim of this thesis was to investigate the effect of production of a heterologous protein on a *Kluyveromyces* yeast, and, within this, examine the possibility of co-production of ethanol and a recombinant protein from whey to improve the economics of an established industrial fermentation. Initial work involved insertion of a gene, encoding a protein of interest, into a pKD1-based vector. The chosen protein was a wheat α-amylase enzyme which had previously been produced using a PGK promoter in *S. cerevisiae* by Rothstein *et al.* (1984). Their PGK-α-amylase cassette was used in the construction of the vector pCR1, which was subsequently shown to secrete an active α-amylase from *K. lactis* yeasts. At the same time, an electroporation method for transformation of *Kluyveromyces* yeasts with pKD1-based vectors was optimized for the Bio-Rad Gene Pulser. *K. lactis* strains can be successfully transformed by this method, but *K. marxianus* strains have very low efficiencies of transformation. The latter are more efficient ethanol producers and are used in the New Zealand whey-ethanol industry, however most *K. lactis* strains produce comparable quantities of ethanol, albeit over a longer time. To date all heterologous proteins produced from *Kluyveromyces* yeasts are in a *K. lactis* host, including the commercial production of recombinant chymosin (Heslot and Gaillardin, 1992). Presently, no such system is available for *K. marxianus* strains, although van Djiken *et al.* (1991) noted that these strains also have potential for large-scale production of heterologous proteins. The main obstacle at the moment is the availability of a suitable vector system and until this is addressed by means of, for example, the discovery of native *K. marxianus* or closely-related yeast vectors, recombinant *K. marxianus* strains will be difficult to develop.

The recombinant *K. lactis* CBS 683 strain containing the vector pCR1 produced an active α-amylase enzyme in measurable quantities. However, the production level was low compared to values reported for both heterologous α-amylase expressed from *S. cerevisiae* using alternative α-amylase encoding genes (Strasser *et al.*, 1989; Astolfi-Filho *et al.*, 1986), and a yeast producing native α-amylase (Wilson and Ingledew, 1982). Possibly, the low level of enzyme expression may be due to non-yeast derived flanking sequences of DNA on the

vector pCR1. Broker *et al.* (1991) discussed the importance of eliminating this non-yeast DNA from heterologous genes in expression plasmids, since they may contain certain elements that negatively influence the protein expression.

The PGK-α-amylase cassette chosen for this work has been shown to express higher quantities of protein in *S. cerevisiae* upon deletion of the signal peptide, however the protein was not secreted. Also, repositioning the signal peptide was shown to improve α-amylase secretion in *S. cerevisiae* (Rothstein *et al.*, 1987). Further rearrangements or deletions may also lead to improvements in α-amylase expression and secretion from this particular PGK-α-amylase cassette.

In future studies, replacement of the constitutive PGK promoter with an inducible promoter would allow the uncoupling of cell growth and heterologous protein production, and permit regulation and increased production of heterologous proteins by manipulation of the culture environment. While this work was being undertaken, Fleer *et al.* (1991a) compared a constitutive PGK promoter with an inducible PHO promoter in a recombinant *K. lactis* strain, and observed that, with the increased plasmid stability and increased copy number of the plasmid using the latter, interleukin-1β protein production was also increased. Ideally, the promoter used to regulate the production of heterologous proteins would be derived from a *Kluyveromyces* yeast. Recently, Meacock and Walsh (1991) investigated stress and phase-induced genes from *K. lactis* with the intention of developing induction systems for this yeast.

The wheat α-amylase enzyme was chosen since it is probable that an α-amylase enzyme derived from a barley or other cereal would be preferable to one from a microbial source for both regulatory bodies and consumers. In addition, there was already an experience base for heterologous α-amylase production from *S. cerevisiae*, and the activity of α-amylase is easily assayed. So, although the level of α-amylase expressed by the recombinant *Kluyveromyces* strain is low, it is still practical for studying the effect of heterologous protein production in *K. lactis* as a model enzyme.

In future work it may be desirable to choose a protein which would be useful to the dairy industry. There may be markets developed for products such as proteinases and lipases produced as heterologous enzymes. For example, chymosin is already commercially produced from a recombinant *K. lactis* (van den Berg *et al.*, 1990). Recently, some important industrial applications for lipases have been identified such as in the hydrolysis of milk fat to yield flavour agents, in cheese ripening, and in the modification of butter fat (Godtfredsen, 1990). Such proteins are already being produced using recombinant bacteria and fungi, for example, *Aspergillus oryzae*. However, the advantages offered by yeast hosts, and in particular *K. lactis*, may allow the generation of a more desirable operation.

The recombinant yeast *K. lactis* CBS 683 : pCR1 was studied in both batch and continuous culture to investigate the effect of various environmental parameters on plasmid stability and α-amylase protein production. The co-production of ethanol and heterologous protein from a whey-based medium was of particular interest. However, it was found that low aeration, whilst achieving a high concentration of ethanol, was not optimal for α-amylase production, with the latter appearing to be related to the quantity of cell biomass. Therefore, the anaerobic conditions used for ethanol production will not enhance protein production, and highly aerobic conditions, giving increased biomass and hence protein production are not optimal for ethanol production. Co-production could be considered if the specific expression yield of heterologous protein could be increased. However, it may be more economically favourable to consider a separate process for the heterologous protein production, or consider the production of yeast and enzyme rather than ethanol and enzyme, for the utilization of whey.

Other studies have used fed-batch cultivation to improve heterologous protein production, including Bitter *et al.* (1988), Hsieh *et al.* (1988), Gu *et al.* (1989a; 1989b), and Patkar and Seo (1992) for recombinant *S. cerevisiae*, and Fleer *et al.* (1991b) for recombinant *K. lactis*. Fed-batch processes give high cell densities in a short culture time (or generation number), and since heterologous protein

production is usually related to the amount of cell biomass, and stability is higher at a lower number of generations, this technique should also improve the level of α-amylase produced by *K. lactis* CBS 683 : pCR1. This method of cultivation also allows the separation of the cell growth and cloned gene expression phases when using inducible promoters, thus improving the overall product yield.

As with the choice of protein, the remainder of the expression complex and vector also requires careful consideration. Once the chosen vector is constructed, if it is required to increase the plasmid stability and hence protein production, investigations could then involve the environmental parameters. For example, in this study it was shown that temperature had a large effect on both plasmid stability and heterologous protein production. At a temperature of 20°C both plasmid stability and the specific yield of heterologous enzyme were much improved compared to at 30°C, which provided an increased growth rate. Other environmental parameters may also be found to improve the recombinant system, for example, pH, mixing, a more controlled DO level, and addition or limitation of various nutrients (Zabriskie and Arcuri, 1986; Caunt *et al.*, 1988). Caunt *et al.* (1988) noted that cyclic changes in various parameters and immobilization techniques have been shown to increase plasmid stability in recombinant *S. cerevisiae* yeasts and these could also be investigated for recombinant *K. lactis*. These last two methods operate by manipulating the different growth rates of plasmid-free and plasmid-containing cells, however they are only suitable for use in continuous culture. In addition, copy number data would allow more insight into the effects of plasmid stability and heterologous protein production in *K. lactis* since the relationship between these parameters appears to be dependent on the system under investigation. The level of heterologous protein production depends on the copy number and the stability of the plasmid utilized.

Once the recombinant yeast has been developed on a small-scale, consideration needs to be given to scale-up for commercial production. If low volume production is required, for example, for high value products such as recombinant human therapeutic proteins, then scale-up is not so much an issue. Lower value

products usually require a high volume of production to be economically viable and so scale-up becomes important. This would be so for a process based on the fermentation of whey in New Zealand, such as a co-production process for a heterologous protein and ethanol or yeast. Scale-up needs careful consideration because conditions in a large vessel are much more heterogeneous than in a small vessel. Caunt *et al.* (1988) noted that scale-up often reduces the yield of heterologous protein, with this effect probably due to poor mixing and oxygen transfer. It was also shown in this study that the yeast *K. lactis* CBS 683 cells aggregate. Flocculation may be advantageous to the recovery of a recombinant product, especially if it is a secreted protein, in removal of the cells from the fermentation broth that contains the protein. However, a disadvantage of this characteristic is that a higher degree of mixing would be required to keep the cells suspended during the fermentation. These as well as techniques to recover the heterologous protein will need to be dealt with for a more economically viable system.

In conclusion, a wheat α-amylase enzyme has successfully been produced from a recombinant *K. lactis* yeast grown on a whey-based medium. As a model system, investigations of the effects of various environmental parameters on the heterologous protein production and plasmid stability have been made in both batch and continuous culture. Finally, this study has illustrated that the choice of heterologous protein, plasmid, and host is of vital importance to a commercially viable heterologous protein production system.

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APPENDIX 1

Screening results for *Kluyveromyces* strains are given in Tables A1.1, A1.2 and A1.3 for transformation efficiencies, performance on glucose and performance on lactose, respectively.

Table A1.1 Transformation efficiencies for *Kluyveromyces* yeasts.

Yeast	Transformation efficiency (transformants per 10^7 cells per µg DNA)	Transformation efficiency[a]
K. lactis		
CBS 141	1.3×10^5	+++
CBS 683	1.2×10^5	+++
CBS 762	2.2×10^5	+++
CBS 845	2.3×10^5	+++
CBS 1065	2.6×10^4	++
CBS 1067	6.5×10^3	+
CBS 1797	4.0×10^4	++
CBS 2359	6.0×10^4	++
CBS 2360/7	2.7×10^4	++
CBS 2619	2.6×10^3	+
CBS 2621	6.8×10^4	++
CBS 2896		
CBS 5618	7.6×10^4	++
CBS 6315	1.1×10^4	++
CBS 6747	1.1×10^5	+++
MW 98-8C	7.0×10^4	++
MW 108-8B	5.8×10^4	++
pM 6-7A	4.9×10^4	++
pM 44-B	1.3×10^4	++
K. marxianus		
ATTC 1179	$< 10^3$	0
ATTC 1195	$< 10^3$	0
ATTC 36907	2.4×10^3	+
CBS 397	$< 10^3$	0
CBS 712	1.2×10^3	+
CBS 4572	$< 10^3$	0
CBS 5795	$< 10^3$	0
CBS 6556	4.5×10^3	+
Y113	$< 10^3$	0

[a] key for transformation efficiency
$> 10^5$ +++
$> 10^4$ ++
$> 10^3$ +
$\leq 10^3$ 0

Table A1.2 Screening results for *Kluyveromyces* yeasts for rich glucose media (YPD) under high and low aeration.

Yeast	HIGH AERATION						LOW AERATION								
	cell growth (cells ml^{-1})			glucose conc. (g l^{-1})			cell growth (cells ml^{-1})			glucose conc. (g l^{-1})			ethanol (g l^{-1})		
	0h	24h	48h	0h	24h	48h	0h	24h	48h	0h	24h	48h	0h	24h	48h
K. lactis															
CBS 141	$8.6x10^5$	$1.0x10^9$	$1.5x10^9$	20.0	14.8	1.5	$9.1x10^5$	$1.1x10^7$	$3.2x10^7$	20.0	20.3	9.3	0.0	0.1	0.4
CBS 683	$6.5x10^5$	$5.6x10^8$	$6.5x10^8$		0	0	$1.2x10^6$	$4.2x10^7$	$5.4x10^7$		13.5	3.7		2.2	6.2
CBS 762	$1.1x10^6$	$6.8x10^8$	$6.3x10^8$		0	0	$9.5x10^5$	$4.5x10^7$	$5.8x10^7$		10.2	1.0		2.9	6.9
CBS 845	$6.6x10^5$	$1.8x10^7$	$5.3x10^8$		0	0	$8.8x10^5$	$5.9x10^7$	$6.5x10^7$		11.8	3.0		2.3	1.1
CBS 1065	$7.3x10^5$	$8.2x10^8$	$1.0x10^9$		0	0	$1.0x10^6$	$5.3x10^7$	$8.6x10^7$		13.0	2.2		2.7	5.0
CBS 1067	$8.9x10^5$	$1.1x10^9$	$1.2x10^9$		0	0	$1.2x10^6$	$4.2x10^7$	$7.2x10^7$		10.4	0.4		3.3	7.5
CBS 1797	$8.8x10^5$	$9.3x10^8$	$1.2x10^9$		0	0	$1.0x10^6$	$4.7x10^7$	$2.7x10^8$		10.4	0.4		1.5	7.5
CBS 2359	$9.3x10^5$	$9.8x10^8$	$1.1x10^9$		0	0	$1.1x10^6$	$5.1x10^7$	$6.2x10^7$		10.2	3.4		2.9	5.7
CBS 2360/7	$6.0x10^5$	$9.1x10^8$	$1.0x10^9$		0	0	$8.8x10^5$	$2.5x10^7$	$6.2x10^7$		17.9	9.3		0.5	1.4
CBS 2619	$7.9x10^5$	$7.4x10^8$	$8.4x10^8$		0	0	$1.1x10^6$	$4.1x10^7$	$6.3x10^7$		8.6	1.5		2.3	5.7
CBS 2621	$1.3x10^6$	$7.7x10^6$	$8.1x10^8$		0	0	$8.1x10^5$	$5.7x10^7$	$6.9x10^7$		8.3	0.5		4.2	5.9
CBS 2896	$9.1x10^5$	$7.9x10^8$	$1.4x10^9$		1.4	0	$1.7x10^6$	$6.8x10^7$	$7.2x10^7$		6.5	1.7		5.1	7.8
CBS 5618	$4.6x10^5$	$1.1x10^9$	$1.1x10^9$		1.3	0	$3.0x10^5$	$9.2x10^7$	$3.4x10^7$		16.1	12.2		0.2	1.4
CBS 6315	$6.1x10^5$	$6.2x10^8$	$7.8x10^8$		0	0	$1.2x10^6$	$4.7x10^7$	$8.5x10^7$		11.3	0.1		3.9	7.1
CBS 6747	$8.3x10^5$	$6.8x10^8$	$4.9x10^8$		0	0	$6.5x10^5$	$2.7x10^7$	$4.7x10^7$		11.4	4.0		2.4	5.9
MW 98-8C	$8.4x10^5$	$5.5x10^8$	$9.1x10^8$		15.9	5.5	$8.0x10^5$	$1.2x10^7$	$1.9x10^7$		20.7	21.0		0.1	0.0
MW 108-8B	$8.6x10^5$	$5.5x10^8$	$6.7x10^8$		0	0	$6.1x10^5$	$8.0x10^7$	$1.3x10^8$		9.3	0.3		3.8	3.4
pM 6-7A	$5.6x10^5$	$8.0x10^8$	$9.1x10^8$		0	0	$8.9x10^5$	$7.8x10^7$	$1.2x10^8$		11.4	0.2		2.0	4.9
pM 44-B	$1.4x10^6$	$6.3x10^8$	$7.5x10^8$		0	0	$1.2x10^6$	$1.0x10^7$	$1.2x10^8$		10.0	0.4		3.2	4.3
K. marxianus															
ATTC 1179	$1.3x10^6$	$6.5x10^8$	$5.9x10^8$		0	0	$1.2x10^6$	$1.1x10^8$	$8.8x10^7$		0.5	0.1		7.4	7.9
ATTC 1195	$1.0x10^6$	$7.1x10^8$	$7.3x10^8$		0	0	$1.2x10^6$	$9.0x10^7$	$8.6x10^7$		0.8	0.1		7.7	8.3
ATTC 36907	$7.1x10^5$	$9.9x10^8$	$1.5x10^9$		0	0	$1.1x10^6$	$1.3x10^8$	$1.3x10^8$		3.0	0.1		6.1	8.1
CBS 397	$8.6x10^5$	$6.7x10^8$	$6.7x10^8$		0	0	$6.8x10^5$	$9.9x10^7$	$1.2x10^8$		1.1	0.1		7.8	7.8
CBS 712	$1.1x10^6$	$8.8x10^8$	$1.1x10^9$		0	0	$1.3x10^6$	$8.7x10^7$	$7.2x10^7$		5.6	0.1		5.5	8.1
CBS 4572	$6.4x10^5$	$8.4x10^8$	$7.8x10^8$		0	0	$1.0x10^6$	$5.4x10^7$	$7.7x10^7$		5.2	1.0		5.7	6.3
CBS 5795	$1.0x10^6$	$6.1x10^8$	$7.5x10^8$		0	0	$1.0x10^6$	$1.1x10^8$	$9.4x10^7$		1.8	0.1		7.4	8.5
CBS 6556	$8.9x10^5$	$9.6x10^8$	$1.3x10^9$		1.5	0	$7.1x10^5$	$1.3x10^8$	$1.9x10^8$		4.6	1.9		6.6	7.7
Y113	$1.2x10^6$	$5.1x10^8$	$7.8x10^8$		0	0	$6.8x10^5$	$8.6x10^7$	$1.1x10^8$		4.4	0.1		7.5	8.5

Table A1.3 Screening results for *Kluyveromyces* yeasts for rich lactose media (YPL) under high and low aeration.

Yeast	HIGH AERATION						LOW AERATION								
	cell growth (cells ml^{-1})			lactose conc. (g l^{-1})			cell growth (cells ml^{-1})			lactose conc. (g l^{-1})			ethanol (g l^{-1})		
	0h	24h	48h	0h	24h	48h	0h	24h	48h	0h	24h	48h	0h	24h	48h
K. lactis															
CBS 141	$7.9x10^5$	$7.4x10^8$	$1.6x10^9$	20.0	15.0	1.4	$5.9x10^5$	$2.3x10^7$	$9.0x10^7$	20.0	15.8	4.5	0.0	0.7	5.8
CBS 683	$1.0x10^6$	$5.3x10^8$	$8.0x10^8$		0.2	0.1	$9.9x10^5$	$3.1x10^7$	$6.8x10^7$		11.4	3.0		2.9	7.5
CBS 762	$1.3x10^6$	$7.7x10^8$	$6.5x10^8$		0.1	0.1	$8.4x10^5$	$4.0x10^7$	$1.1x10^8$		9.5	0.6		3.0	8.4
CBS 845	$1.1x10^6$	$1.5x10^7$	$1.2x10^9$		12.4	0.2	$6.3x10^5$	$4.9x10^7$	$1.3x10^8$		10.8	0.2		2.4	9.1
CBS 1065	$9.1x10^5$	$8.4x10^8$	$9.1x10^8$		0.1	0.1	$1.2x10^6$	$5.7x10^7$	$9.2x10^7$		9.5	0.3		3.6	8.3
CBS 1067	$4.6x10^5$	$9.0x10^8$	$1.1x10^9$		0.1	0.1	$9.5x10^5$	$3.4x10^7$	$8.2x10^7$		9.4	0.3		3.4	8.0
CBS 1797	$1.1x10^6$	$8.0x10^8$	$6.8x10^8$		0.1	0.1	$1.1x10^6$	$6.9x10^7$	$9.0x10^7$		12.0	2.0		2.3	7.6
CBS 2359	$1.4x10^6$	$9.8x10^6$	$1.3x10^9$		0.1	0.1	$1.2x10^6$	$4.8x10^7$	$9.3x10^7$		9.5	0.6		3.4	6.6
CBS 2360/7	$6.4x10^5$	$4.4x10^8$	$6.6x10^8$		0.1	0.1	$7.9x10^5$	$2.8x10^7$	$7.4x10^7$		12.6	2.7		1.8	7.3
CBS 2619	$1.3x10^6$	$1.1x10^9$	$1.0x10^9$		0.1	0.1	$1.3x10^6$	$4.2x10^7$	$7.6x10^7$		9.2	0.5		3.8	8.1
CBS 2621	$1.0x10^6$	$9.5x10^8$	$1.1x10^9$		0.1	0.1	$9.1x10^5$	$4.3x10^7$	$6.7x10^7$		7.8	0.3		4.4	8.5
CBS 2896	$6.0x10^5$	$3.6x10^8$	$5.5x10^8$		0.1	0.1	$1.6x10^6$	$3.2x10^7$	$2.6x10^7$		18.8	19.1		0.1	0.1
CBS 5618	$5.6x10^5$	$1.2x10^9$	$1.1x10^9$		0.1	0.1	$8.5x10^5$	$7.2x10^7$	$2.2x10^8$		11.2	0.4		2.5	6.1
CBS 6315	$8.0x10^5$	$6.8x10^8$	$5.9x10^8$		0.1	0.1	$9.6x10^5$	$2.9x10^7$	$4.2x10^7$		9.0	1.0		4.3	8.2
CBS 6747	$5.5x10^5$	$5.7x10^8$	$5.0x10^8$		0.1	0.1	$7.8x10^5$	$3.3x10^7$	$5.0x10^7$		10.0	1.1		3.0	7.8
MW 98-8C	$9.0x10^5$	$4.8x10^7$	$3.4x10^8$		20.1	11.2	$1.1x10^6$	$6.9x10^6$	$7.3x10^7$		20.1	21.0		0.0	0.1
MW 108-8B	$6.9x10^5$	$7.2x10^8$	$8.9x10^8$		0.1	0.1	$7.8x10^5$	$4.1x10^7$	$6.7x10^8$		10.3	0.9		3.3	6.0
pM 6-7A	$8.6x10^5$	$8.4x10^8$	$9.0x10^8$		0.1	0.1	$1.2x10^6$	$6.5x10^7$	$1.4x10^8$		10.3	0.1		3.2	8.4
pM 44-B	$8.9x10^5$	$7.2x10^8$	$8.7x10^8$		0.1	0.1	$6.8x10^5$	$2.7x10^7$	$9.0x10^8$		11.6	3.0		2.2	7.5
K. marxianus															
ATTC 1179	$8.3x10^5$	$4.8x10^8$	$8.0x10^8$		0.1	0.1	$6.4x10^5$	$6.8x10^7$	$7.7x10^7$		1.1	0.1		9.1	8.9
ATTC 1195	$8.6x10^5$	$7.6x10^8$	$1.1x10^9$		0.1	0.1	$9.4x10^5$	$7.1x10^7$	$1.4x10^8$		0.9	0.1		8.5	8.4
ATTC 36907	$6.0x10^5$	$9.2x10^7$	$1.2x10^8$		13.0	9.4	$5.3x10^5$	$1.2x10^7$	$4.4x10^7$		15.8	18.0		0.1	0.4
CBS 397	$8.9x10^5$	$6.6x10^8$	$7.1x10^8$		0.1	0.1	$1.0x10^6$	$7.1x10^7$	$6.7x10^7$		2.9	0.1		8.5	8.5
CBS 712	$8.0x10^5$	$8.3x10^8$	$1.1x10^9$		12.2	3.2	$6.3x10^5$	$9.7x10^6$	$2.4x10^7$		16.9	18.0		0.1	8.3
CBS 4572	$1.3x10^6$	$1.7x10^8$	$1.3x10^8$		19.7	13.5	$1.0x10^6$	$1.2x10^7$	$4.7x10^7$		14.5	18.0		0.1	0.1
CBS 5795	$7.1x10^5$	$6.0x10^8$	$1.2x10^9$		0.1	0.1	$6.6x10^5$	$1.1x10^8$	$1.3x10^8$		0.5	0.1		8.5	8.8
CBS 6556	$9.0x10^5$	$1.4x10^9$	$1.9x10^9$		0.1	0.1	$9.6x10^5$	$3.3x10^7$	$5.8x10^7$		18.1	16.6		0.4	1.5
Y113	$1.0x10^6$	$5.6x10^8$	$9.3x10^8$		0.1	0.1	$5.9x10^5$	$7.5x10^7$	$1.1x10^8$		5.6	1.0		7.0	8.5

APPENDIX 2

Raw data for plasmid stability measurements are shown in Table A2.1.

Table A2.1 Raw data for plasmid stability of *K. lactis* CBS 683 : pCR1 grown in YPD-G418 broth.

Sample	Method used			
	A. Plate ratio[a]		B. Clearing zones[b]	C. Transfer colony[c]
	10^{-2}	10^{-3}	10^{-3}	
1	293/327	30/34	25/34	50/100
2	274/291	24/37	29/37	70/100
3	244/295	32/38	29/38	71/100
4	261/329	22/30	24/30	62/100
5	225/330	26/36	31/36	67/100
6	229/326	18/29	19/29	67/100
7	218/329	22/28	23/28	63/100
8	205/329	22/42	33/42	70/100
9	227/285	27/33	23/33	66/100
10	226/279	27/30	24/30	66/100
11	261/299	23/25	18/25	60/100
12	282/320	16/27	18/27	53/100
mean (x)	0.791	0.749	0.757	0.638
std dev (n=12)	0.102	0.131	0.061	0.066
std dev (n=3)[d]	0.118	0.151	0.070	0.076

[a] Values are represented as x_1/x_2 where x_1 is the colony count on selective agar and x_2 is the colony count on non-selective agar, at either the 10^{-2} or the 10^{-3} dilution.

[b] Values are represented as x_1/x_2 where x_1 is the number of colonies with clearing zones and x_2 is the total number of colonies, at the 10^{-3} dilution.

[c] Values are given as x_1/x_2 where x_1 is the number of colonies on selective agar and x_2 is the total number of colonies transferred from non-selective agar.

[d] Standard deviations for 3 samples were calculated from the standard deviation for 12 samples using the following formula (Morton, 1993):

$$s_{n=3} = \frac{2 s_{n=12}}{\sqrt{3}} \qquad \text{(A1.1)}$$

Estimation of the 95 % confidence interval (CI) was performed using the following formula:

$$x \pm \frac{ts}{\sqrt{n}} \qquad \text{(A2.2)}$$

An example calculation for Method A 10^{-2} dilution, for n = 12 (n = 3), is as follows:

n	=	12 (3)
a	=	0.025
df	=	12 - 1 = 11 (2)
t	=	2.201 (4.303)
95 % CI:		79.1 ± 2.201 x 10.21/$\sqrt{11}$

Therefore the 95 % confidence interval for n = 12 is 79.1 ± 6.78 (n = 3, 79.1 ± 29.3).

The three methods were compared using a small sample test of a difference in means, and $\alpha = 0.05$. It was assumed a normal distribution occurred for the conditions investigated. The equations were obtained from Mendenhall and Ott (1980). An example calculation for the comparison of methods A (10^{-2} dilution) and B (10^{-3} dilution) is given below.

null hypothesis, $H_0 : x_A - x_B = 0$

alternative hypothesis, $H_a : x_A - x_B \neq 0$

estimate for common population standard deviation,

$$s_{AB} = \sqrt{\frac{(n_A-1)s_A^2 + (n_B-1)s_B^2}{n_A+n_B-2}} \qquad \text{(A2.3)}$$

gives $s_{AB} = 13.47$

Calculation of t-value,

$$t = \frac{x_A - x_B}{s_{AB}\sqrt{\frac{1}{n_A} + \frac{1}{n_B}}} \qquad \text{(A2.4)}$$

gives t = 0.764

Rejection rate for $\alpha = 0.05$ uses t-value with

a	=	0.025
df	=	(12+12-2) = 22
t	=	2.074

Reject H_0 if $-2.074 > t > +2.074$

Calculated t-value for comparison of methods A and B is 0.764, therefore there is insufficient evidence to indicate difference in mean stabilities.

Comparison of methods A and C gives a calculated t-value = 4.362, therefore there is sufficient evidence to indicate a difference in mean stabilities.

Comparison of methods B and C gives a calculated t-value = 4.590, therefore there is sufficient evidence to indicate a difference in mean stabilities.