HETEROLOGOUS PROTEIN PRODUCTION IN *KLUYVEROMYCES LACTIS*

by

CAROLYN MARGARET RUSSELL

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

Massey University Library
Thesis Copyright Form

Title of thesis:

(1) (a) I give permission for my thesis to be made available to readers in Massey University Library under conditions determined by the Librarian.

~~(b)~~ I do not wish my thesis to be made available to readers without my written consent for ... months.

(2) (a) I agree that my thesis, or a copy, may be sent to another institution under conditions determined by the Librarian.

~~(b)~~ I do not wish my thesis, or a copy, to be sent to another institution without my written consent for ... months.

(3) (a) I agree that my thesis may be copied for Library use.

~~(b)~~ I do not wish my thesis to be copied for Library use for ... months.

C. M. Russell

Signed 

Date 22 Octber 1993

The copyright of this thesis belongs to the author. Readers must sign their name in the space below to show that they recognise this. They are asked to add their permanent address.

NAME AND ADDRESS DATE

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