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**HETEROLOGOUS PROTEIN PRODUCTION
IN
*KLUYVEROMYCES LACTIS***

by

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requirements for the degree of Doctor of Philosophy
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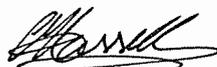
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ABSTRACT

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. *drosophilum* 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 μ F, and ∞ Ω with 0.2 μ g plasmid DNA. Transformation efficiencies were comparable to those obtained using a PS10 Electropulsator, and were in the range 10⁴-10⁵ transformants per 10⁷ cells per μ g 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).

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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 |