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ORIGINAL PAPER

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Effects of cultivation conditions on the production of heterologous α- galactosidase by *Kluyveromyces lactis*

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Abstract Growth conditions relevant for the large-scale production of heterologous proteins with yeasts were studied on a laboratory scale. A strain of *Kluyveromyces lactis*, containing 15 copies of an expression cassette encoding guar α-galactosidase integrated into its ribosomal DNA, was used as a model. By using urea as a nitrogen source, it was possible to produce active extracellular α-galactosidase in shake-flask cultures grown on a defined mineral medium. Inclusion of urea instead of ammonium sulphate prevented unwanted acidification of cultures. With urea-containing mineral medium, enzyme production in shake flasks was comparable to that in complex media containing peptone. In contrast, the presence of peptone was required to achieve high productivity in chemostat cultures. The low productivity in chemostat cultures growing on mineral media was not due to loss of the expression cassette, since addition of peptone to such cultures resulted in an immediate high rate of α-galactosidase production. The discrepancy between the behaviour of shake-flask and chemostat cultures during growth on mineral medium illustrates the necessity of physiological studies for the scaling-up of heterologous protein production from laboratory to production scale.

Introduction

The most extensively studied host for the production of heterologous proteins with yeasts is *Saccharomyces cerevisiae*. However, *S. cerevisiae* has some less favourable characteristics, including hyperglycosylation of excreted proteins (Innis 1989), a limited protein-secretion capacity (Kingsman et al. 1987) and a Crabtree-positive physiology (Petrik et al. 1983; Postma et al. 1988). Therefore, other yeast host systems are currently being developed (for a review see Romanos et al. 1992). A yeast that does not have the above drawbacks is *Kluyveromyces lactis*. Large-scale fermentation technology is available for the production of β-galactosidase (lactase; EC 3.2.1.23) with *K. lactis*. *K. lactis* has the GRAS (generally regarded as safe) status, which is a major advantage for the implementation of large-scale production of heterologous proteins for food and pharmaceutical applications.

Heterologous proteins that have been successfully produced in *K. lactis* include bovine prochymosin (Van den Berg et al. 1990), human serum albumin (Fleer et al. 1991a), human interleukin-1β (Fleer et al. 1991b) and hepatitis-B surface antigen (Martinez et al. 1992). With the exception of prochymosin production, the expression vectors used for heterologous protein production in *K. lactis* were episomal. These systems are characteristically unstable under non-selective process conditions and are therefore less suited for large-scale commercial production. The integrated expression system used for prochymosin production (Van den Berg et al. 1990) was highly stable, but had a low copy number, which may have limited the expression level.

Ideally, vectors for the expression of heterologous genes under non-selective, industrial conditions should combine the high-copy-number characteristic of episomal expression vectors and the high mitotic stability of integrating vectors. A system based on homologous recombination of expression vectors into the iterative ribosomal DNA units of *S. cerevisiae* has been developed by Lopes et al. (1989). This so-called MIRY (multiple integration into the ribosomal DNA of yeast) system enabled expression-vector copy numbers of 100–200. The copy number of the plasmids was stable

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during non-selective growth in shake-flask cultures for 70 generations (Lopes et al. 1989). Recently, a MIRY system has also been developed for *K. lactis* (Bergkamp et al. 1992; Rossolini et al. 1992) and called MIRK (multiple integration in the ribosomal DNA of *K. lactis*).

Guar (*Cyamopsis tetragonoloba*) α-galactosidase (EC 3.2.1.22) has been used as a model protein to study the production of heterologous proteins in a large number of hosts, including *Bacillus subtilis* (Overbeeke et al. 1990), *Hansenula polymorpha* (Fellinger et al. 1991; Sierkstra et al. 1991; Veale et al. 1992; Giuseppin et al. 1993) and *S. cerevisiae* (Verbakel 1991; Giuseppin et al. 1993). Because of the proven expression of α-galactosidase in a variety of hosts, we used this protein as a model to study production of heterologous proteins in *K. lactis* using a MIRK expression system. The natural function of the protein is the removal of (1–6)-α-D-galactose side-chains from storage carbohydrates during germination of guar seeds (Meier and Reid 1982). The use of α-galactosidase to improve the gelling properties of guar gum may be commercially attractive (Bulpin et al. 1990).

Almost invariably, studies on the expression of heterologous genes in yeasts rely on the use of complex media and shake-flask cultures. However, this mode of cultivation is not suited to quantitative studies and bears little resemblance to industrial processes, in which growth is carbon-limited and defined media are generally used (Goodey 1993). To provide a basis for large-scale production, the aim of this study was to investigate the influence of medium composition and cultivation methods on the expression of Guar α-galactosidase in *K. lactis* containing a MIRK-type expression system.

Materials and methods

Host-vector system

Kluyveromyces lactis MSK110 (a *uraA trp1::URA3*) (Stark and Milner 1989) was used as a host strain. The strain was transformed with a MIRY-type plasmid (Lopes et al. 1989) containing sequences for integration into the ribosomal DNA of *K. lactis*, the *Saccharomyces cerevisiae GAL7* promoter, the *SUC2* (invertase) signal sequence, the *C. tetragonoloba* α-galactosidase gene and the *PGK1* terminator of *S. cerevisiae*. A *TRP1-d* marker gene (Lopes et al. 1989) was used to select for multiple integration events. The transformed strain used in the present study (*K. lactis* MSK110-MIRK) was obtained from Dr. Bergkamp, Free University, Amsterdam. This strain contains 15 copies of the expression vector, as indicated by Southern analysis. Detailed descriptions of the construction of the expression cassettes and the transformed strain have been presented elsewhere (Bergkamp et al. 1992). The strain was grown at 30 °C in shake flasks on mineral medium without tryptophan, supplemented with 2% (w/v) glucose. After 24 h, the culture was made 20% (v/v) glycerol and stored in 2-ml vials at − 80 °C. These vials were used as inocula for all subsequent experiments.

Shake-flask cultivation

A frozen stock culture was inoculated in a shake-flask containing 100 ml mineral medium with 2% glucose as the sole source of carbon and energy. After 24 h, cells were diluted ten fold, either in complex medium or in mineral medium. Complex medium contained 10 g l^{-1} yeast extract (Difco, Detroit, USA), 20 g l^{-1} peptone from casein (Merck, Darmstadt, Germany), 20 g l^{-1} galactose (Janssen Chimica, Geel, Belgium). The mineral medium (Verduyn et al. 1992) contained either ammonium sulphate (10 g l^{-1}) or urea (10 g l^{-1}) as the nitrogen source and 20 g l^{-1} galactose as the sole carbon and energy source. Media were heat-sterilized at 110 °C. Cultures were grown in 100 ml medium in 500-ml conical flasks, incubated on a reciprocal shaker at 200 rpm and at 30 °C. Samples taken 24 h and 48 h after inoculation were assayed for biomass dry weight, residual galactose, pH and extracellular α-galactosidase activity.

Chemostat cultivation

K. lactis MSK110-MIRK was grown at 30 °C in Applikon laboratory fermenters (Applikon, Schiedam, the Netherlands), at a stirring speed of 750 rpm. The working volume of the cultures was kept at 1 l by a peristaltic effluent pump coupled to a level controller. An airflow of 1 l min^{-1} was maintained by a Brooks 5876 mass-flow controller (Brooks BV, Veenendaal, The Netherlands). The dissolved-oxygen tension was measured with a polarographic oxygen electrode (Ingold, Urdorf, Switzerland) and was above 50% of air saturation. The pH was automatically controlled at 5.0 by addition of 1 M KOH or 0.5 M H_2SO_4. All experiments were performed at a dilution rate of 0.10 h^{-1}. The mineral medium, supplemented with vitamins, was prepared according to Verduyn et al. (1992). When urea was used as nitrogen source it was added to a final concentration of 2.2 g l^{-1}. Vitamins and, where indicated, L-tryptophan (0.2 g l^{-1} final concentration, Janssen Chimica) were filter-sterilized separately and added to the heat-sterilized medium. Carbon sources were sterilized separately at 110 °C and added to the sterile medium at a final concentration of 5.0 g l^{-1}. Complex medium, consisting of 2.5 g l^{-1} Difco yeast extract, 5 g l^{-1} peptone from casein (Merck), 5 g l^{-1} and galactose (Janssen Chemica), 5 g l^{-1} was heat-sterilized at 110 °C. When peptone or yeast extract was added to mineral media, these components were sterilized separately at 110 °C.

All chemostat cultures were started on mineral medium with ammonium sulphate as the sole nitrogen source and galactose as the carbon source. After five volume exchanges, the reservoir medium was changed to the medium composition that was to be examined. Analyses were performed after five to ten additional volume changes, when the culture dry weight had been stable for at least two volume changes. After the analyses, the fermentation run was terminated.

Transient-state experiments

After a steady-state chemostat culture had been obtained, the medium supply was stopped and galactose and peptone were added to a final concentration of 20 g l^{-1}. Samples were withdrawn at regular intervals for the determination of biomass dry weight and extracellular α-galactosidase.

Dry weight determination

Biomass dry weights were measured after filtering culture samples over pre-dried 0.45 μm polysulphone filters (Gelman Sciences, USA). Cells were washed three times with demineralised water and dried in a microwave oven (Amanda, USA) at 700 W for 15 min.

α-Galactosidase assay

A 50 μl sample of an appropriate dilution of culture supernatant was mixed with 450 μl 22.2 mM *p*-nitro-phenyl α-D-galactopyranoside (Sigma Chemical Co., USA) in 0.1 M Sodium acetate (pH 5.0), pre-equilibrated at 37 °C. After exactly 5 min incubation at 37 °C, the reaction was stopped by addition of 1 ml 10% (w/v) Na_2CO_3. The α-galactosidase activity was calculated by using an absorption coefficient of *p*-nitrophenol at 405 nm of 18.16 cm^2 μmol^{-1}. One unit of α-galactosidase was defined as the amount of enzyme that hydrolyses 1 μmol substrate in 1 min at 37 °C and at pH 5.0. In all assays, the enzyme activity was linearly proportional to the amount of supernatant added. To estimate the amount of α-galactosidase produced by the cultures, a specific activity of 100 units (mg protein)$^{-1}$ was used (Fellinger et al. 1991).

Determination of residual galactose

Samples of shake-flask fermentations were centrifuged at room temperature and the sugar concentration was determined in the supernatant. Measurement of residual sugar in chemostat cultures was performed by rapid sampling into liquid nitrogen as described by Postma et al. (1988). Galactose and glucose were determined with Boehringer Mannheim testkits 176303 and 716251 respectively.

pH-dependent stability of α-galactosidase

K. lactis MSK110-MIRK was grown in shake-flask cultures on mineral medium containing urea as a sole source of nitrogen for 48 h. After centrifugation, the supernatant was passed through a 0.2 μm filter (Acrodisc, Gelman Sciences, USA); 4 ml mineral medium with urea was adjusted with dilute HCl or NaOH to give a final pH of 3, 5 and 7 after mixing with 1 ml supernatant. These solutions were incubated at 30 °C and at regular intervals, samples were withdrawn for α-galactosidase assays.

Results

α-Galactosidase production in shake-flask cultures: complex media

Several *K. lactis* strains containing multiple copies of a guar-α-galactosidase expression cassette, stably integrated into the iterative ribosomal DNA unit have been constructed by Bergkamp et al. (1992). The strain used in the present study, *K. lactis* MSK110-MIRK, contained 15 copies of the expression cassette.

For an initial characterization of α-galactosidase production by *K. lactis* MSK110-MIRK, product formation was studied in shake-flask cultures grown on complex media. Since localization experiments indicated that over 95% of the heterologous protein was excreted (data now shown), only the extracellular α-galactosidase activity was routinely measured in these studies.

In shake-flask cultures grown on complex media, α-galactosidase activities were approximately fourfold higher in cultures supplemented with galactose than in cultures supplemented with glucose (Table 1). This indicated that regulation of the *S. cerevisiae GAL7* promoter in this system is not as tight as in *S. cerevisiae*, where it is almost completely repressed during growth on glucose (Dickson and Riley 1989). Also the native *GAL7* promoter of *K. lactis* is not regulated as strictly as its *S. cerevisiae* homologue (Dickson and Riley 1989).

The amount of heterologous protein produced in the cultures can be calculated by assuming that the specific activity of the *K. lactis*-produced α-galactosidase is the same as that of the *ex planta* enzyme [100 U (mg protein^{-1})]. The α-galactosidase activity observed after 48 h growth in complex media supplemented with galactose then corresponds to about 0.2 g l^{-1} extracellular α-galactosidase. Because the highest α-galactosidase production was obtained with galactose as the carbon source this sugar was used for all further experiments.

α-galactosidase production in shake-flask cultures: defined media

Growth of *K. lactis* MSK110-MIRK in shake-flask cultures on the standard mineral medium resulted in very low α-galactosidase levels (Table 1). Since the virtual absence of α-galactosidase activity in these cultures

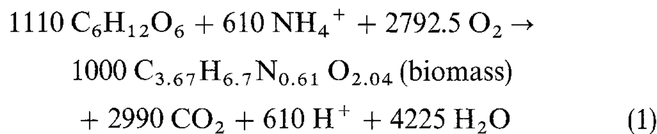
Table 1 Effect of medium composition on extracellular α-galactosidase production, growth and pH in shake-flask cultures of *K. lactis MSK*110–MIRK. All cultures were grown at 30 °C as described in Materials and methods. (*YP* complex medium; yeast extract/peptone, *MM* mineral medium with galactose)

Medium	Time (h)	Biomass (g l^{-1})	α-Galactosidase (kU l^{-1})	α-Galactosidase (kU g biomass^{-1})	pH
YP (galactose)	24	10.9	14.9	1.4	7.0
	48	10.1	18.8	1.9	7.4
YP (glucose)	24	5.3	1.8	0.34	6.1
	48	9.2	4.6	0.50	6.9
MM (NH_4^+)	24	1.7	0.004	0.002	3.0
	48	2.0	0.001	0.001	2.9
MM (urea)	24	6.6	4.5	0.68	5.9
	48	7.2	8.2	1.1	6.9

coincided with a sharp drop of the culture pH (Table 1), the effect of medium pH on the stability of α-galactosidase was investigated.

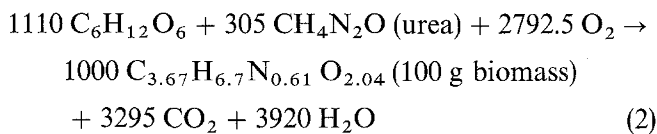
When culture supernatants containing α-galactosidase were incubated at pH 3 (the pH observed after growth on mineral medium with ammonium sulphate), the enzyme was completely inactivated after 1 h (Fig. 1). In contrast, no loss of activity was observed after 24 h incubation of α-galactosidase in mineral medium adjusted to pH 5 or 7. It is as yet unclear whether the apparent instability of the enzyme at low pH is due to intrinsic properties or to extracellular proteases active at low pH.

In mineral medium, the main cause for acidification is the consumption of ammonium salts, which leads to the stoichiometric release of protons, as described by Eq. 1.



$$1110\,C_6H_{12}O_6 + 610\,NH_4^+ + 2792.5\,O_2 \rightarrow 1000\,C_{3.67}H_{6.7}N_{0.61}\,O_{2.04}\,\text{(biomass)} + 2990\,CO_2 + 610\,H^+ + 4225\,H_2O \quad (1)$$

In Eq. 1, all amounts are given in millimoles. The biomass composition has been taken from Roels (1983) and a Y_{sx} of 0.5 g biomass (g sugar)$^{-1}$ has been used (Verduyn 1991). Since, in shake-flask cultures, it is difficult to control pH by titration, we looked for ways to prevent acidification. Since the formation of yeast biomass with urea as the nitrogen source is not associated with acidification (Eq. 2), growth of *K. lactis* MSK110-MIRK with urea as the nitrogen source was investigated.



$$1110\,C_6H_{12}O_6 + 305\,CH_4N_2O\,\text{(urea)} + 2792.5\,O_2 \rightarrow 1000\,C_{3.67}H_{6.7}N_{0.61}\,O_{2.04}\,\text{(100 g biomass)} + 3295\,CO_2 + 3920\,H_2O \quad (2)$$

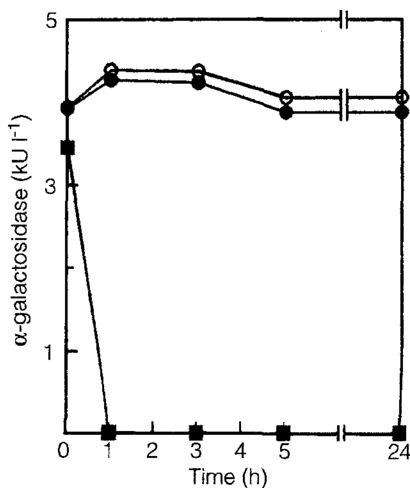


Fig. 1 Stability of heterologous α-galactosidase, produced by *K. lactis* MSK110-MIRK, during incubation at 30 °C in mineral medium adjusted to different pH values. ○ pH 7; ● pH 5; ■ pH 3

Indeed, when *K. lactis* MSK110-MIRK was grown in mineral medium with urea as a sole source of nitrogen, acidification was not observed (Table 1). Furthermore, biomass concentrations were approximately twofold higher than in cultures supplemented with ammonium (Table 1), suggesting that, in the latter cultures, growth was inhibited by the low pH. When the α-galactosidase production in the shake-flask cultures was related to the biomass concentration, α-galactosidase production in cultures grown on a mineral medium with urea was about 50% lower than in cultures grown on complex medium (Table 1).

α-Galactosidase production in chemostat cultures grown on mineral media

The key parameter for comparison of expression levels of proteins is the specific product formation rate q_p [expressed as mg product (g biomass)$^{-1}$ h^{-1}]. In shake-flask cultures, both the growth conditions and the concentration of biomass and product are continuously changing, which makes it laborious to estimate q_p.

A rough estimate of q_p (mg g^{-1} h^{-1}) in the shake-flask experiments presented in Table 1 can be obtained by dividing the α-galactosidase concentration after 48 h incubation by half of the biomass concentration after 48 h and by 48. For the shake-flask cultures grown on galactose as the carbon source in complex medium and mineral medium (with urea as nitrogen source), these estimated values of q_p were 0.8 and 0.5 mg (g dry weight)$^{-1}$ h^{-1}. However, these figures only give an indication of the order of magnitude of q_p in these shake-flask cultures, since biomass and enzyme concentrations were only measured twice.

Chemostat cultivation facilitates the calculation of q_p which, at constant dilution rate, is given by the equation $q_p = D \times c_p \times c_x^{-1}$, in which D is the dilution rate (h^{-1}) and c_p and c_x are the steady-state concentrations of product (α-galactosidase) and biomass, respectively. A further advantage of chemostat cultivation is that product formation can be studied at growth rates below μ_{max}. This is essential for the modelling of large-scale fed-batch fermentations, which are characteristically run at a low and decreasing growth rate (Stouthamer and van Verseveld 1987).

In contrast to the shake-flask cultures, the chemostat cultures were equipped with pH control. Therefore, problems with the stability of α-galactosidase at low pH (Fig. 1) were not anticipated. However, the specific rate of α-galactosidase production in these cultures, grown at pH 5, was extremely low (Table 2) compared to the rates estimated for the shake-flask cultures grown on mineral medium with urea as the nitrogen source. Control experiments in which chemostat cultures were grown on mineral medium with urea as the

Table 2 Effect of medium composition on extracellular α-galactosidase production by *K. lactis* MSK110-MIRK in chemostat cultures. All cultures were grown aerobically (with one exception), at 30 °C, at a dilution rate of $0.10 \, h^{-1}$ and at pH 5, as described in Materials and methods, with a reservoir galactose concentration of $5 \, g \, l^{-1}$. [YP complex medium: yeast extract/peptone, *MM* (NH_4^+) mineral medium with ammonium, *MM* (*urea*) mineral medium with urea, *Y*, yeast extract, *P* peptone]

Medium	α-Galactosidase activity ($kU \, l^{-1}$)	Residual galactose ($g \, l^{-1}$)	Biomass dry weight ($g \, l^{-1}$)	q_p ($mg \, g^{-1} \, h^{-1}$)
MMA(NH_4^+)	0.07	1.1	1.71	0.04
MM(urea)	0.05	0.7	1.44	0.03
MM(NH_4^+) O_2 limitation	0.18	N/D	1.04	0.17
MM(NH_4^+) + tryptophan	0.18	0.01	2.07	0.09
YP	2.4	1.0	1.92	1.3
MM(NH_4^+) + YP	2.9	1.0	2.07	1.4
MM(NH_4^+) + Y	0.47	0.03	3.04	0.16
MM(NH_4^+) + P ($5 \, g \, l^{-1}$)	2.6	1.0	2.00	1.3
MM(NH_4^+) + P ($10 \, g \, l^{-1}$)	3.3	N/D	1.45	2.3

nitrogen source indicated that the low q_p in these cultures was not due to a specific effect of the nitrogen source (Table 2).

High residual galactose concentrations were observed in all steady-state chemostat cultures grown on mineral media (Table 2). A possible explanation for this observation is that the truncated *TRP1-d* promoter caused a partial tryptophan auxotrophy, even though the modified *TRP1-d* gene was present in multiple copies. To test this hypothesis, $0.2 \, g \, l^{-1}$ tryptophan was added to the mineral medium. Indeed, this addition resulted in a substantial decrease of the residual galactose concentration (Table 2). However, q_p did not increase substantially as a result of the addition of tryptophan (Table 2). In theory, a low expression level in this situation might have been due to a poor induction of the *GAL7* promoter due to the low residual galactose concentration. In transient-state experiments, where galactose ($20 \, g \, l^{-1}$) was pulsed to a tryptophan-supplemented culture, no significant increase of the α-galactosidase production rate was observed (data not shown). This experiment eliminated tryptophan deficiency as a possible cause for the low productivity in chemostat cultures grown on mineral media.

Shake-flask cultures of yeasts become oxygen-limited during prolonged growth. To investigate whether the discrepancy between batch and chemostat cultures might be related to oxygen availability, the dissolved-oxygen concentration in the chemostat culture was decreased by diminishing the air supply until ethanol appeared in the culture. In such an oxygen-limited culture, q_p increased approximately fivefold in comparison with aerobic chemostat cultures grown on mineral media (Table 2). The product formation rate in oxygen-limited chemostat cultures was still about threefold lower than the q_p estimated for batch cultures with urea (Table 1). A growth-limiting oxygen supply is not a viable option for large-scale industrial production of α-galactosidase, since it will inevitably lead to alcoholic fermentation. Therefore, this option was not further studied.

Effect of complex medium components on α-galactosidase production in chemostat cultures

To study further the effect of medium composition on α-galactosidase production in *K. lactis* MSK110-MIRK, the organism was grown in aerobic chemostat cultures on the complex medium that gave a high productivity in shake-flask cultures (Table 1). Growth on this medium, composed of yeast extract, peptone and galactose, resulted in a q_p that was higher than the estimated q_p in shake-flask cultures grown on complex medium (Table 2). The difference between the productivity of chemostat cultures grown on complex and defined media can be interpreted in two ways: either as a stimulation of α-galactosidase production by compounds present in the complex medium or as an inhibition by components of the mineral medium. Since addition of all mineral medium components to the complex medium did not negatively influence product formation (Table 2), it was concluded that inhibition by mineral medium components was unlikely.

The addition of $5 \, g \, l^{-1}$ peptone to the mineral medium restored production rates to the level observed in cultures grown on complex medium (Table 2). A further increase of q_p to $2.3 \, mg \, g^{-1} \, h^{-1}$ was observed when the peptone concentration in the reservoir medium was raised from $5 \, g \, l^{-1}$ to $10 \, g \, l^{-1}$. A further increase of the peptone concentration did not result in a higher production rate (data not shown). In contrast to the addition of peptone, addition of only yeast extract ($5 \, g \, l^{-1}$) resulted in much lower increase of α-galactosidase production (Table 2).

Stability of the expression cassette

The stability of the MIRK expression system was studied by prolonged cultivation of *K. lactis* MSK110-MIRK on complex medium with galactose in a chemostat culture. A decrease of q_p to approximately 70% of the initial value was observed within the first 10

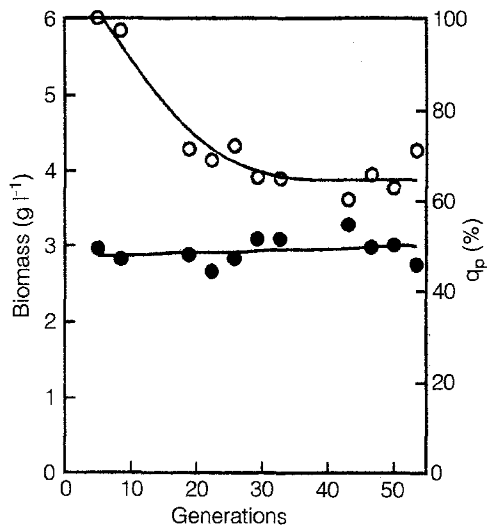


Fig. 2 Stability of α-galactosidase production during prolonged cultivation of *K. lactis* MSK 110–MIRK in chemostat cultures grown on complex medium (yeast extract, peptone and galactose). The specific productivity (q_p) is plotted as a percentage of the value observed five volume changes after the switch to complex medium. Growth conditions: dilution rate = 0.10 h^{-1}, $T = 30\,°C$, pH 5, dissolved oxygen concentration above 50% air saturation. ● Biomass dry weight; ○ q_p

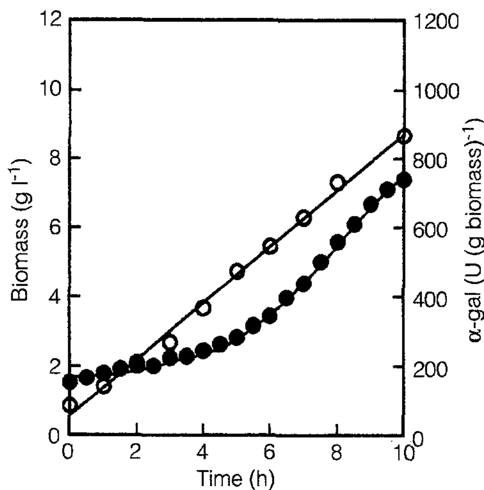


Fig. 3 Growth and specific α-galactosidase production after pulse-wise addition of 20 g l^{-1} galactose and 20 g l^{-1} peptone to a chemostat culture of *K. lactis* MSK110–MIRK, pregrown on a mineral medium with ammonium sulphate as the nitrogen source and galactose as the carbon source. ● Biomass dry weight; ○ α-galactosidase

generations. After this, productivity remained constant for at least 40 generations (Fig. 2).

To investigate whether the low q_p in chemostat cultures grown on a mineral medium might be due to loss of the ability to produce α-galactosidase, these cultures were pulsed with galactose and peptone. The pulses resulted in a rapid formation of α-galactosidase (Fig. 3), indicating that the low α-galactosidase production in the chemostat cultures was not due to loss of the expression vector. The q_p observed during the pulse experiment (approximately 1 mg g^{-1} h^{-1}) was comparable to the q_p observed during growth in chemostat cultures grown on complex medium (Table 2).

When only galactose was pulsed to chemostat cultures grown on mineral media, this did not result in a significant increase in the rate of α-galactosidase production (data not shown).

Discussion

The strong acidification that occurs during growth of yeasts in shake-flask cultures with ammonium salts as a sole nitrogen source is likely to inactivate many heterologous proteins that are excreted in the growth medium. In such cases, use of urea as a nitrogen source is a simple alternative to the use of pH-control systems or the use of high buffer concentrations. This method is likely to be widely applicable: in a screening of 123 yeast strains, 122 strains were positive (La Rue and Spencer 1968). However, it is important to bear in mind that assimilation of urea will only be pH-neutral when the release of ammonia from urea is exactly balanced by its assimilation: when the rate of ammonia release exceeds its rate of assimilation, alkalinization of the medium will occur.

Growth of *K. lactis* MSK110-MIRK on mineral medium with urea as the nitrogen source gave satisfactory α-galactosidase yields in shake-flask cultures (Table 1). However, when the same medium was used for growth in chemostat cultures, α-galactosidase production was very low, even though high residual galactose concentrations, required for induction of the *GAL7* promoter, were present in the cultures (Table 2). For reasons unknown at present, compounds present in peptone were required for optimal productivity in chemostat cultures. Inclusion of peptone in media is not a viable option for large-scale production of heterologous proteins in general. For example, production of human serum albumin, because of its pharmaceutical application, requires the use of defined media (Goodey 1993). Furthermore, peptone may have unexpected side-effects on the physiology of the host. This was evident in the present study, where the addition of casein peptone negatively affected biomass yield (Table 2).

Also when defined mineral media are used, shake-flask cultures differ in many aspects from large-scale fed-batch processes. For instance, it is not possible accurately to control a number of important parameters (concentrations of metabolites and dissolved oxygen, growth rate). The present study clearly demonstrates that even satisfactory productivity in a shake-flask culture on a mineral medium is not a guarantee for efficient production when the process is scaled up. On a larger scale, growth must be carbon-limited in

view of the limited oxygen-transfer and cooling capacities of the bioreactor.

Even after prolonged cultivation on complex medium in chemostat cultures, the MIRK system was stable. This confirms and extends earlier studies with shake-flask cultures of this strain of *K. lactis* (Bergkamp et al. 1992).

It is at present unclear whether the phenomena observed with the multicopy-integration system used in the present study also occur in other MIRY or MIRK expression systems. All shake-flask experiments reported on these systems were performed in complex media containing yeast extract and peptone (Lopes et al 1989; Bergkamp et al. 1992). In studies on *Saccharomyces cerevisiae* strains containing similar multicopy-integration expression vectors (Van der Aar et al. 1990; Verbakel 1991; Giuseppin et al. 1993), the mineral media used were supplemented with significant amounts of yeast extract. Further research, involving other strains and constructs, is needed to assess whether the inability of *K. lactis* MSK110-MIRK to produce α-galactosidase efficiently during growth on defined media in chemostat cultures is an exception or a general characteristic of strains with expression vectors integrated into the ribosomal DNA.

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