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ORIGINAL ARTICLE

# The activity of β-galactosidase and lactose metabolism in *Kluyveromyces lactis* cultured in cheese whey as a function of growth rate

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## Abstract

**Aims:** *Kluyveromyces lactis* was cultured in cheese whey permeate on both batch and continuous mode to investigate the effect of time course and growth rate on β-galactosidase activity, lactose consumption, ethanol production and protein profiles of the cells.

**Methods and Results:** Cheese whey was the substrate to grow *K. lactis* as a batch or continuous culture. In order to precise the specific growth rate for maximum β-galactosidase activity a continuous culture was performed at five dilution (growth) rates ranging from 0·06, 0·09, 0·12, 0·18 to 0·24 $h^{-1}$. The kinetics of lactose consumption and ethanol production were also evaluated. On both batch and continuous culture a respirofermentative metabolism was detected. The growth stage for maximum β-gal activity was found to be at the transition between late exponential and entrance of stationary growth phase of batch cultures. Fractionating that transition stage in several growth rates at continuous culture a maximum β-galactosidase activity at 0·24 $h^{-1}$ was observed. Following that stage β-gal activity undergoes a decline which does not correlate to the density of its corresponding protein band on the gel prepared from the same samples.

**Conclusion:** The maximum β-galactosidase activity per unit of cell mass was found to be 341·18 mmol ONP $min^{-1}$ $g^{-1}$ at a dilution rate of 0·24 $h^{-1}$.

**Significance and Impact of the Study:** The physiology of *K. lactis* growing in cheese whey permeate can proven useful to optimize the conversion of that substrate in biomass rich in β-gal or in ethanol fuel. In addition to increasing the native enzyme the conditions established here can be set to increase yields of recombinant protein production based on the LAC4 promoter in *K. lactis* host.

## Introduction

The yeast *Kluyveromyces lactis* van der Walt var. *lactis* (*K. lactis*) is one of the well-known yeast in nature able to assimilate lactose (Dickson and Martin 1980). This property associated to its efficient oxidative metabolism has been exploited for protein and β-galactosidase production. The enzyme is important for the dairy industry because of the benefits derived from lactose hydrolysis such as reduction of lactose intolerance and prevention of lactose crystallization in concentrated milk and ice cream (Bonekamp and Oosteron 1994; Panesar *et al.* 2006). However, the industrial production of β-galactosidase is limited by its low productivity and high costs (Becerra and Siso 1996). To improve productivity it is important to establish the physiological conditions for maximum β-galactosidase production by *K. lactis* culture and in order to to reduce cost it would be interesting to take advantage of a low cost substrate such as cheese whey. In addition to yielding β-galactosidase, the process would

also consume an important by-product, from the dairy industry.

An inducible assimilation system is responsible for lactose metabolism in *K. lactis*: lactose is transported by the Lac12p (permease) through an energy-dependent mechanism transport and then it is hydrolysed by Lac4p (β-galactosidase) to glucose and galactose. Galactose is the intracellular signal that induces the expression of the genes involved in the metabolism of lactose and galactose (Dickson and Barr 1983).

Earlier studies have pointed out that the maximum β-galactosidase activity of the *K. lactis* cultures has been associated to a particular growth phase, a physiological state which corresponds to the entrance in the stationary phase (Dickson and Martin 1980). If the cells are maintained in the stationary phase the β-galactosidase activity per unit of cell mass seems to decline (Dickson and Martin 1980; Inchaurrondo *et al.* 1994). Because the entrance in stationary phase generally comprehend a very short period of time with specific growth rate changing frequently, it is difficult to define it in a batch culture, where the majority of the data has been obtained. However, continuous cultures could be used to investigate a culture at a precise and steady-state physiological condition. In that case the transition between log and stationary phases of a batch culture can be fractionated at various growth rates. Earlier studies (Dickson and Martin 1980) have already anticipated that the β-galactosidase activity of a *K. lactis* culture reaches its maximum after seven to nine cell generations at the log phase, followed by a decline when the cells reach the stationary phase. To our knowledge the β-galactosidase activity of *K. lactis* inducible culture has not been investigated as function of the yeast growth rate. As a variable growth rate can reveal a particular physiological state, it can be associated to the general response of cell to the environment limitation. In this work we propose the use of continuous cultures of *K. lactis* grown in permeate of ultrafiltered cheese whey to examine β-galactosidase activity as function of the growth rate.

## Materials and methods

### Yeast strain, media and culture conditions

The strain of *K. lactis* var. lactis used in this study was a gift from the Food Science Department of the University of California, Davis, USA. The stock culture has been maintained in 200 g $l^{-1}$ glycerol at −80°C. The experimental cultures were performed either as batch or continuous cultures. The nutrient medium in all cases was the whey permeate (WP) autoclaved at 121°C for 10 min after addition of 4 g $l^{-1}$ of sodium citrate. The cells were previously activated by inoculating 1 ml of the −80°C stock into 50 ml of WP and incubated overnight. The control culture for induction was carried out in yeast nitrogen base (YNB) containing glucose as the only carbon source.

### Batch and continuous cultures and time course experiments

Batch cultures were conducted in 1000-ml Erlenmeyer flasks with 300 ml of WP and incubated in a rotary shaker at 30°C and 200 rev $min^{-1}$. Continuous cultures were performed in 1000-ml jacketed flasks (Wheaton®, Millville, NJ, USA) filled with 100 ml of WP and magnetically stirred at 30°C. The culture was fed continuously with WP at flow rates ($F$) that gave dilution rates ($D$) of 0·06, 0·09, 0·12, 0·18 and 0·24 $h^{-1}$, according to the relation $D = F/V$ (Bailey and Ollis 1986), where $V$ is the volume in litres.

### Determination of specific growth rate

A batch culture was initiated with an $A_{600}$ equal to 0·04. The culture was monitored by measuring cell mass ($A_{600}$), substrate and product concentration on samples collected at indicated time intervals. The specific growth rate ($\mu$) was determined by the linear regression of the plot Ln $A_{600}$ unit *vs* time (h), at the initial exponential growth phase.

### Primary metabolites analysis

Concentrations of lactose and ethanol from batch and continuous cultures were carried out by high-performance liquid chromatography (HPLC), using an ion exclusion column Aminex HPX-87H (Bio-Rad, Hercules, CA), kept at 60°C. The eluent for separation was 5 mmol $l^{-1}$ $H_2SO_4$, applied at an elution rate of 0·7 ml $min^{-1}$. The column was coupled to the refractive index detector HP 1047A. External standards for sugar, acids and alcohols were prepared in concentrations ranging from 15 to 30 mmol $l^{-1}$ and samples were diluted when appropriated.

### β-galactosidase activity

The β-galactosidase activity was determined as previously described (Fontes *et al.* 2001; Genari *et al.* 2003), with modifications. Yeast cells were recovered by centrifugation (2840 ***g***, 10 min at 25°C) and re-suspended in Z buffer (0·06 mol $l^{-1}$ $Na_2HPO_4.7H_2O$, 0·04 mol $l^{-1}$ $NaHPO_4.H_2O$, 0·01 mol $l^{-1}$ KCl, 0·001 mol $l^{-1}$ $MgSO_4$ $7H_2O$, pH 7·0). Cells were permeabilized by addition of sodium dodecyl sulfate (SDS) 0·1% (0·1 ml) and absolute chloroform

(0·1 ml). The β-galactosidase activity was measured by taking 0·1-ml sample of the permeabilized cell suspension and mixing with 0·4 ml of a solution containing 4 mg $ml^{-1}$ of *O*-nitrophenil-β-D-galactopyranoside (ONPG; Sigma, St. Louis, USA). After incubation for 10 min at room temperature, the reaction was interrupted by addition of 1 ml of 0·5 mol $l^{-1}$ $Na_2CO_3$. A unit of β-galactosidase activity was expressed as millimoles of *o*-nitrophenol liberated (ONP) per minute according to a standard curve. To take off the effect of cell concentration on enzyme activity, the activity unit was eventually expressed per gram of cell mass.

### SDS-PAGE

Samples collected at indicated time intervals from both batch and continuous cultures were analysed for protein concentration according to Bradford (1976). After breaking the cells with glass beads, same amount of protein from the same amount of cells has been applied and fractionated in denaturing electrophoresis [SDS-polyacrylamide gel electrophoresis (PAGE)] stained with Coomassie Blue as in Laemmli (1970).

## Results

Although a fluctuation on the β-galactosidase activity has been observed during a typical time course batch culture of *K. lactis* in cheese whey (Fig. 1), two maximum peaks of β-galactosidase activity per cell mass can be detected: one at the late exponential growth phase and another at the stationary growth phase (Figs 1, 2). Immediately after both maximum points, enzyme activity has declined. The point of maximum β-galactosidase activity close to the late exponential growth phase agreed with the presence of the inductor (lactose) in the culture medium. However,

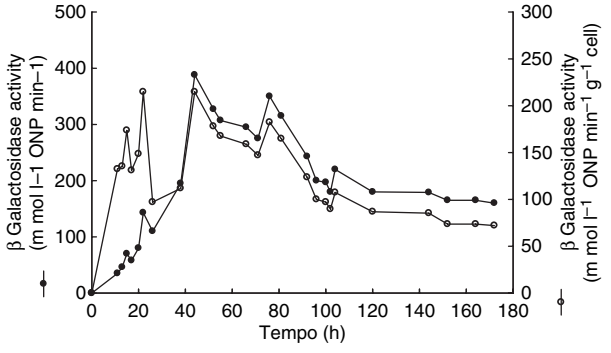


**Figure 1** Time course of *Kluyveromyces. lactis* batch culture on permeate of cheese whey. Specific (per gram of cell mass) (○) and volumetric β-galactosidase activity. Data points represent the average values of three samples collected at the time indicated from a representative batch culture.

the peak of β-galactosidase activity during stationary phase occurred when lactose has been depleted. Ethanol production started later on the exponential growth phase and reached its maximum value after 36 h (stationary phase), when lactose was also completely consumed. Following this period, the consumption of the earlier produced ethanol was observed (Fig. 2).

In order to verify the influence of lactose concentration on β-galactosidase activity, after six generations (12 h) of *K. lactis* cultured in the WP, different lactose concentrations were added to the medium. The results presented in Fig. 3 show that lactose added to the medium only slightly increases β-galactosidase activity compared with the controls (WP without added lactose), however the activity was much lower than at 21 h (before the addition of lactose). Interestingly, the lactose added to the medium never disappeared completely from the medium (data not shown), indicating that the culture aging influences its maximum enzymatic activity.

In order to understand the decline of β-galactosidase activity in the *K. lactis* culture when lactose was still present in the medium, the intracellular protein profile of the culture was analysed by SDS-PAGE. The idea was to associate cell protein to β-galactosidase activity and to any eventual proteolysis.

During the period between 15 and 17 h of *K. lactis* cultured in WP β-galactosidase activity declined according to Fig. 1. However, during the same period the intensity of a protein band associated to the β-galactosidase (absent on the noninduced control) has increased (Fig. 4). From 22 h to 44 h, when β-galactosidase activity has reached its maximal, an additional increase in intensity of the protein band compared to the previous point (17 h) was observed. In spite of the decline in enzyme activity, at 26 h according to Fig. 1, there was an increase in the level of protein at that same time. Although the band intensity seems to be similar at both 44 and 26 h, the β-galactosidase activity was higher at 44 h. At 52 and 66 h the decline in activity was accompanied by a decrease in the protein band intensity. At 76 h an increase in β-galactosidase activity was detected, while a decrease in protein band intensity was observed at the same time. Following this period a decline in β-galactosidase activity was observed accompanied by an apparent decrease in the intensity of the β-galactosidase protein band (Figs 1, 4).

To better precise the results obtained in batch cultures *K. lactis* was cultured in WP at continuous mode. It was evaluated the β-galactosidase activity, lactose consumption and ethanol production at different dilution rates (*D*) corresponded to different specific growth rates. The results are shown in Table 1; specific β-galactosidase activity was higher (341·18 mmol ONP $min^{-1}$ $g^{-1}$) when the growth

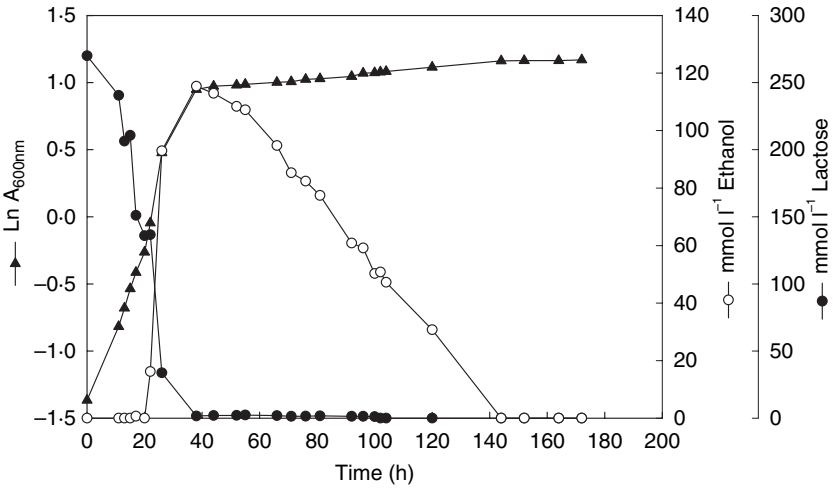


**Figure 2** Time course of *Kluyveromyces lactis* batch culture on permeate of cheese whey. Growth (▲), lactose consumption (●) and ethanol production and consumption (■). Data points represent the average values of three samples collected at the time indicated from a representative batch culture.

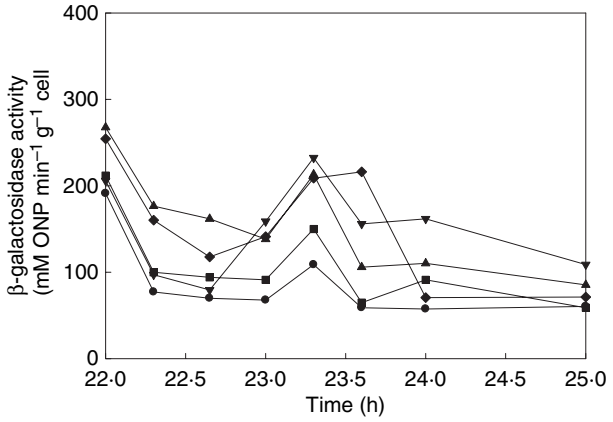


**Figure 3** Specific β-galactosidase activity of *Kluyveromyces lactis* batch cultures per cell mass on whey permeate. After 21 h of growth, lactose was added at concentrations of 0 (●) (control), 7 (▼), 18 (▲), 30 (■) and 60 mmol $l^{-1}$ (♦). Data points represent the average values of three samples collected from a representative batch culture.

rate was 0·24 $h^{-1}$, which corresponds to the late exponential growth phase of a batch culture. However, it is not possible to correlate the specific β-galactosidase activity with the intensity of the protein band (Fig. 5) because at dilution rate 0·24 $h^{-1}$ the β-gal band was weaker than any other dilution rate, while the specific β-gal activity on the contrary was higher. The remaining lactose concentration at each growth rate was coherent with each dilution rate applied. The ethanol yield per cell mass however was apparently independent of the growth rate, which is expected from a more respirofermentative yeast.

## Discussion

The two peaks of β-galactosidase activity during a batch culture of *K. lactis* in cheese whey has been currently detected (Genari *et al.* 2003). The decline in β-galactosidase activity in conditions where lactose is still present in the medium in significant amounts has also been reported earlier (Dickson and Martin 1980). In order to investigate the physiological conditions that lead to the decline in the β-galactosidase activity of *K. lactis* culture, the enzyme activity was followed during the exponential, late exponential and stationary growth phases using batch culture and at the transition between exponential and stationary growth phases using continuous culture. We also analysed the presence or absence of a potential protein band by SDS-PAGE as a tentative to establish a direct relationship between the decline in activity and the presence of the protein.

It has been established that the induction of the Lac/Gal regulon depends on the intracellular concentration of the inductor (Cardinali *et al.* 1997). As expected the results confirmed that the β-galactosidase activity increases during the exponential growth phase after lactose (inductor) has been effectively transported. However, the maximal β-galactosidase activity on the stationary growth phase occurred when lactose was not detected in the culture medium and ethanol has begun to be consumed, showing a typical diauxic growth phase. Furthermore, a decline in β-galactosidase activity at late exponential growth phase was also observed. This decline could be attributed to the low concentration of the inductor in the culture medium. To address this question lactose was added to the culture medium during late exponential growth phase, but only a slight increase in β-galactosidase activity was detected. A reversible glucose inhibition as a result of lactose hydrolysis, as previously established (Fontes *et al.* 2001) should be considered carefully as the activity once performed *in vitro* would have low or any effect of glucose in the reaction mixture. The decline in β-galactosidase activity at earlier stationary

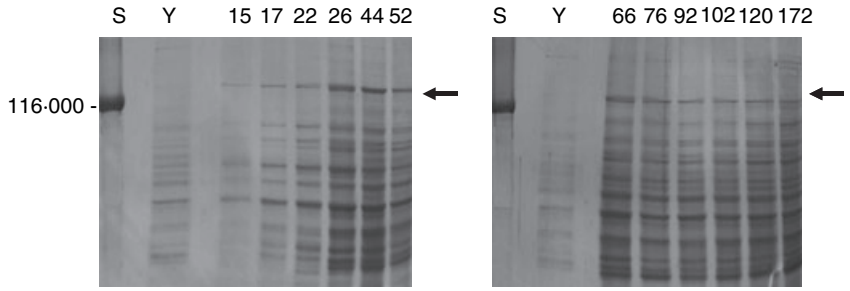


**Figure 4** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of intracellular proteins of *Kluyveromyces lactis* batch culture in yeast nitrogen base (YNB) for 172 h. Lane S is the standard of molecular weight of *Escherichia coli*, β-galactosidase; lane Y is a noninduced control, i.e. *K. lactis* grown to middle log phase in YNB with glucose as the only carbon and energy source; lanes 15, 17, 22, 26, 44, 52, 66, 76, 92, 102, 120 and 172 correspond to samples taken from the culture at the times in hours indicated. Proteins from the same amount of cells were applied to the lanes.

**Table 1** Continuous culture parameters of *Kluyveromyces lactis* on permeate of ultrafiltered cheese whey at different growth rates

| Dilution rate ($h^{-1}$) | 0·06 | 0·09 | 0·12 | 0·18 | 0·24 |
|---|---|---|---|---|---|
| Lactose (mmol $l^{-1}$) | 0·26 | 7·0 | 23·0 | 118·0 | 130·0 |
| Yet/*x* | 4·05 | 4·65 | 4·05 | 1·80 | 1·87 |
| β-gal activity (mmol ONP $min^{-1}$ $g^{-1}$) | 12·50 | 68·10 | 42·65 | 41·20 | 341·18 |
| Generation time (h) | 12 | 8 | 6 | 4 | 3 |
| Cell mass (g) | 3·74 | 3·13 | 2·04 | 1·82 | 0·34 |

Yet/*x* = ethanol yield per cell mass.
ONP, millimoles of *o*-nitrophenol liberated per minute per gram of cell mass.

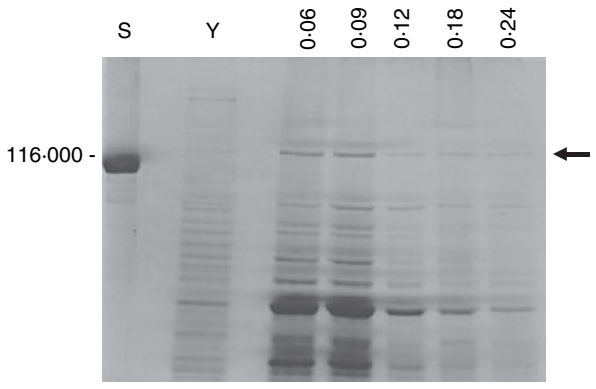


**Figure 5** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of intracellular proteins of *Kluyveromyces lactis* cultured in whey permeate as continuous culture. The lane S is the standard of molecular weight of *Escherichia coli* β-galactosidase, lane Y is a noninduced control, i.e. *K. lactis* grown in yeast nitrogen base with glucose as the only carbon and energy source at a growth rate of 0·09 $h^{-1}$. Lanes 1, 2, 3, 4 and 5 correspond to samples taken from continuous culture in permeate of ultrafiltered cheese whey at steady-state growth rates ($h^{-1}$) of 0·06, 0·09, 0·12, 0·18 and 0·24, respectively.

growth phase could reflect the repression of enzyme synthesis in the absence of the inductor. However, by observing that the protein bands from all samples collected at the stationary phase were present, it can be deduced that the β-galactosidase neither has changed its concentration nor has been degraded. It has been well established that yeasts change its metabolism on low availability of nutrients (Rolland *et al.* 2002). In face of the all undefined possibilities it might be necessary to further investigate the lactose consumption and the b-gal activity on a more defined medium other then the complex cheese whey.

The results obtained with batch culture have shown that the production of β-galactosidase is maximal close to the late exponential growth phase and entrance on the stationary growth phase – the limiting growth phase. However, in batch cultures the environmental condition on this period is not well defined making it difficult to precise the culture physiology where the β-galactosidase production is maximal. On the other hand continuous cultures, by allowing steady-state growth at any specific growth rate (Bailey and Ollis 1986) can reveal where β-galactosidase production is higher. In addition to being uniform and unchangeable with time, the continuous culture allows to investigate the culture at limiting conditions where general stress response occurs. In this work various growth rates were evaluated in continuous culture and it was concluded that the specific growth rate of the yeast cells in cheese whey affected β-galactosidase activity. The maximal β-galactosidase activity is reached with dilution rates close to the exponential growth phase (0·24 $h^{-1}$). This condition may correspond to the maximum peak observed at the late exponential growth phase of the batch culture. However, here again the protein bands associated to the β-galoctosidase does not peak with its higher activity.

On both batch and continuous cultures it was observed that ethanol is always a product of *K. lactis* metabolism, confirming as previously its respirofermentative behaviour


 

(Siso *et al.* 1996). The ethanol yield for cell mass however appears not to obey a unique model. When growth rate approximates to its maximum, ethanol seems to be growth associated. However when growth rate slowed down ethanol yield appeared to be related to cell maintenance. Thus another promising application of *K. lactis* in the dairy industry is the ethanol production from cheese whey permeates. On high substrate concentration and low oxygen level, other yeast of the *Kluyveromyces* genera, *Kluyveromyces marxianus*, reaches ethanol yields close to its theoretical value showing great fermentative potential (Silveira *et al.* 2005). It is possible that on similar conditions *K. lactis* reaches high ethanol yields, because of the similarity of carbon metabolism regulation on both yeasts

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