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Growth and β-galactosidase synthesis in aerobic chemostat cultures of *Kluyveromyces lactis*

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Growth and β-galactosidase (β-gal) expression were characterized in the yeast *Kluyveromyces lactis* strain NRRL Y-1118 growing in aerobic chemostat cultures under carbon, nitrogen or phosphate limitation. In lactose or galactose-limited cultures, β-gal accumulated in amounts equivalent to 10–12% of the total cell protein. The induced β-gal expression was repressed when cells were grown under N- or P-limitation. In lactose medium, enzyme levels were 4–8 times lower than those expressed in C-limited cultures. A similar response was observed when galactose was the carbon source. These results suggest that a galactose-dependent signal (in addition to glucose) may have limited induction when cells were grown in carbon-sufficient cultures. Constitutive β-gal expression was highest in lactate-limited and lowest in glucose-limited media and was also repressed in glucose-sufficient cultures. Other *K. lactis* strains (NRRL Y-1140 and CBS 2360) also showed glucose repression (although with different sensitivity) under non-inducing conditions. We infer that these strains share a common mechanism of glucose repression independent of the induction pathway. The kinetics of β-gal induction observed in C-limited cultures confirms that β-gal induction is a short-term enzyme adaptation process. Applying a lactose pulse to a lactose-limited chemostat culture resulted in 'substrate-accelerated death'. Immediately after the pulse, growth was arrested and β-gal was progressively inactivated. Yeast metabolism in C-limited cultures was typically oxidative with the substrate being metabolized solely to biomass and CO_2. Cells grown under P- or N-limitation, either with glucose or lactose, exhibited higher rates of sugar consumption than C-limited cells, accumulated intracellular reserve carbohydrates and secreted metabolic products derived from the glycolytic pathway, mainly glycerol and ethanol.

Keywords: *Kluyveromyces lactis*; β-galactosidase; continuous culture; enzyme expression

Introduction

The lactose/galactose (lac/gal) regulon of *Kluyveromyces lactis* consists of a set of genes that encode the enzymes required for lactose and galactose metabolism [8]. The two genes involved in utilization of lactose are *LAC12* and *LAC4* encoding lactose permease and β-galactosidase (β-gal) respectively. Expression of these genes is regulated at the transcriptional level. This regulation is dependent on the function of a positive regulatory protein (Lac9p), that activates transcription by binding to a specific promoter sequence upstream of the target gene. Under noninduced conditions (absence of galactose or lactose in the culture medium), transcription is slowed down because the action of Lac9p is antagonized by the negative regulatory protein Gal80p. On induction with lactose or galactose, Lac9p becomes competent to activate transcription [8,12,26]. Glucose repression is strain dependent as measured by the reduction of enzyme levels in media containing galactose plus glucose as compared with galactose alone. Glucose appears to decrease *LAC9* expression and/or the specific activity of the regulatory protein [3,16,27]. A *LAC9*-independent repression pathway has been proposed as an additional mechanism [28].

Physiological studies complement molecular research and provide useful information for process design. Most of the existing knowledge on enzyme induction and repression of the lac/gal regulon was derived from batch culture studies [7,15]. Surprisingly, few data are available from continuous (chemostat) cultures, which offer the advantage of well-defined growth conditions, ie growth rate and nutrient limitation. The aim of this work was to characterize β-galactosidase expression and growth of *K. lactis* strain Y-NRRL 1118 in aerobic chemostat cultures. This strain showed a strong glucose repression phenotype [16].

K. lactis has industrial potential as a source of microbial protein, enzymes such as β-galactosidase and diverse metabolites such as ethanol and flavoring compounds. It is also an attractive microbial host for the expression of foreign genes and protein secretion [2,21]. Therefore, results obtained here would be of relevance to the practical applications of this yeast.

Materials and methods

Microorganisms

The yeast strains used were *K. lactis* NRRL Y-1118, *K. lactis* NRRL Y-1140 (ARS Culture Collection, Peoria, IL, USA) and *K. lactis* CBS 2360 (Centraalbureau voor Schimmelcultures, Baarn and Delft, Netherlands). Stock cultures were maintained on solid medium under vaseline at 4°C. The solid medium contained (g L^{-1}): lactose 20; bactopeptone 5; malt extract 3; yeast extract 3; agar 30.

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Media and culture conditions

Yeast cultures were carried out in a previously described synthetic medium [15] which was modified to be used in chemostat cultures (Table 1). Sugars, lactate and phosphates were sterilized separately by autoclaving them. Vitamins were sterilized by filtration. Chemostat cultures were performed in a 1.5-L fermentor (LH series 210, LH Engineering, UK) with a working volume of 900 ml. The stirring rate was 700 rpm and the air flow rate was adjusted to 30 L h^{-1} (20°C, 101.3 Pa). Under these conditions, the maximum oxygen transfer rate of the reactor, determined by the sulfite method [4], was 92 mmol L^{-1} h^{-1}. The pH was controlled at 4.7 ± 0.1 by the automatic addition of 0.6 M KOH or H_2SO_4. All cultures were carried out at 30°C. Yeast growth was strictly aerobic (dissolved oxygen tension was always above 30% saturation).

As a criterion for the establishment of a steady state, the time elapsed between a change in the culture conditions and sampling corresponded to at least five volume changes. Three samples for analysis were taken at each steady state at intervals equivalent to one or two generation times. The standard errors of mean values of enzyme activities and growth parameters were always less than 10%.

Analytical methods

Samples for analysis were taken from the culture effluent stream. A volume of culture was collected in an ice bath (within 1–5 min) and centrifuged in an Eppendorf microfuge (30 s, 14 000 × *g*) at 5°C. The supernatant medium was filtered through a membrane filter (Millipore, 0.2-μm pore size) and frozen at –20°C until it was analysed for residual nutrients and extracellular products. This sampling procedure gives only an approximate value for the concentration of the growth-limiting substrate. For accurate measurements of the very low residual substrate concentration, rapid sampling and quenching techniques are required in order to avoid interference by the high metabolic activity of the cells [25]. The cell pellet was washed twice with distilled water and frozen for the determination of biomass composition and enzyme activity. Lactose in the feed medium and in the supernatant phase of N- or P-limited cultures was determined with DNS reagent [18]. Glucose was measured by the glucose oxidase-peroxidase method (Glicemia, Wiener, Argentina). Total carbohydrates in whole cells and residual sugar concentrations in carbon-limited cultures were estimated by the anthrone method [13]. Lactate, ethanol, acetic acid, pyruvate and glycerol were measured with specific enzymatic kits (UV methods, Boehringer Mannheim, Germany). Protein in whole cells was determined by the Lowry method, using bovine serum albumin as standard [13].

Growth was monitored by optical density at 625 nm in a Pye Unicam SP6-250 spectrophotometer. Biomass dry weights were also determined by drying culture samples (10–30 ml) at 105°C after centrifugation and washing. The fraction of budded cells was determined with a Neubauer chamber.

β-gal activity was determined with lactose as substrate using permeabilized cells. Pellets were thawed and cells resuspended with buffer PPB-Mn (50 mM potassium phosphate, pH 6.6 containing 0.1 mM Mn^{+2}). Permeabilization was achieved by adding toluene (2% v/v) to the cell suspension and incubating for 15 min at 37°C. At zero time, 1 ml of lactose solution (278 mM in PPB) was added to 1 ml of permeabilized cell suspension. After an appropriate reaction time, the reaction was stopped by heating the sample for 1 min in boiling water. The glucose produced was determined by the glucose oxidase-peroxidase method. The cell concentration in the reaction mixture was adjusted according to the sample activity. The standard assay was carried out at 37°C. At 30°C the reaction rate decreased by a factor of 1.42. One unit of β-gal activity corresponds to the hydrolysis of 1 μmol of lactose per minute. Under the conditions of the assay, 400 units are equivalent to 139 000 Miller units [6] and thus correspond to the activity of 1 mg of pure β-gal. Specific β-gal activity is expressed as units (mg biomass)$^{-1}$. The specific rate of β-gal synthesis (q_e) was calculated as specific activity × dilution rate (at steady state) and expressed as units (mg biomass)$^{-1}$ h^{-1}.

Table 1 Chemostat culture media

Compound	C-limited	N-limited	P-limited
Carbon source	4	16	16
NH_4CI	2.3	0.69	2.3
$NaH_2PO_4 \cdot H_2O$	0.79	0.79	0.1
$MgCl_2 \cdot 6H_2O$	0.6	0.6	0.6
KCl	0.4	0.4	0.4
Na_2SO_4	0.5	0.5	0.5
$CaCl_2$ $2H_2O$	0.14	0.14	0.14
Microelements[a]	1 ml	1 ml	1 ml
Vitamins[b]	1 ml	1 ml	1 ml

The components are expressed in g L^{-1}.
[a]Stock solution (g L^{-1}): $FeSO_4 \cdot 7H_2O$ 15.0; $ZnSO_4 \cdot 7H_2O$ 5.0; $MnSO_4 \cdot H_2O$ 3.0; $CuSO_4 \cdot 5H_2O$ 0.75; $CoCl_2 \cdot 6H_2O$ 0.15; $Na_2MoO_4 \cdot H_2O$ 0.65; H_3BO_3 0.1; KI 0.1; citric acid 50.
[b]Stock solution (g L^{-1}): niacin 12, pantothenic acid (hemicalcium salt) 4.0; pyridoxine 1.0, thiamine-HCl 1.0, folic acid 1.0, *p*-aminobenzoic acid 1.0, biotin 0.06, myo-inositol 60.

Chemicals

Lactose, glucose, galactose, dl-lactate and vitamins were purchased from Sigma Chemical Co (St Louis, MO, USA). Bactopeptone was from Difco (Detroit, MI, USA). Malt extract and yeast extract were from Merck (Darmstadt, Germany). Other medium components were of analytical grade.

Results

Yeast growth and β-gal expression in C-limited cultures

The effect of dilution rate (D) on yeast growth and β-gal expression was examined in C-limited cultures with lactose or galactose as the carbon source (induced conditions). Typical growth parameters with lactose are shown in Figure 1. The culture was stable up to a D of 0.50 ± 0.01 h^{-1}. From the wash-out kinetics, a maximum growth rate of 0.51 h^{-1} was determined, which was somewhat higher than that observed in batch cultures (0.465 h^{-1}). This difference may be ascribed to an insufficient adaptation of the yeast to the batch culture conditions [9]. Carbon balances (data not

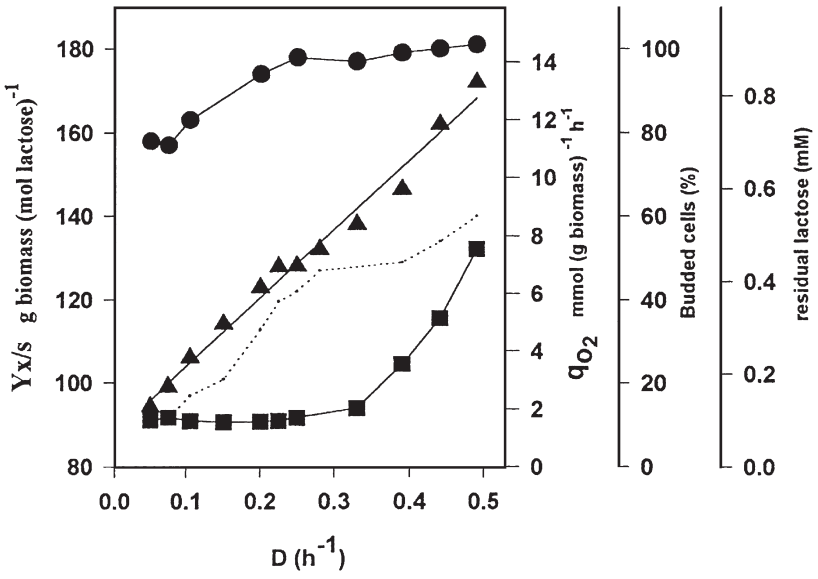

Figure 1 Aerobic growth of *K. lactis* NRRL 1118 in lactose-limited chemostat culture. The lactose concentration in the feed medium was ~11.5 mM. (●) $Y_{x/s}$, biomass yield; (▲) q_{o2}, specific rate of oxygen consumption; (■) residual lactose; (---) budded cells.

shown) indicated a purely oxidative metabolism at all growth rates. The decrease of biomass yields at low growth rates can be attributed to maintenance effects [22]. Residual lactose concentrations were ~0.1–0.5 mM. A similar growth pattern was observed with galactose. Residual galactose concentrations were 12- to 15-fold higher than those observed in lactose cultures (data not shown).

The extent of β-gal expression was analyzed in terms of q_e, the specific rate of enzyme synthesis. The dependence of q_e with D followed a similar pattern in lactose and galactose media. Figure 2 shows that q_e increased in an approximately linear manner to a maximum at an intermediate dilution rate of 0.25–0.35 h^{-1} and declined again at D values above 0.4 h^{-1} as the culture approached wash-out. In lactose medium, the maximum rate of synthesis was 5.0 Units (mg biomass)$^{-1}$ h^{-1} while the highest specific activity corresponded to cells grown at dilution rates between 0.10 and 0.20 h^{-1}. In galactose medium, q_e values at low growth rates were higher than those observed in lactose medium, yielding cells with a higher specific activity. Nevertheless, the maximum q_e was of the same order (5.5 Units (mg biomass)$^{-1}$ h^{-1}). It was estimated that β-gal in lactose or galactose-induced cells accumulated in amounts equivalent to 10–12% of the total cell protein.

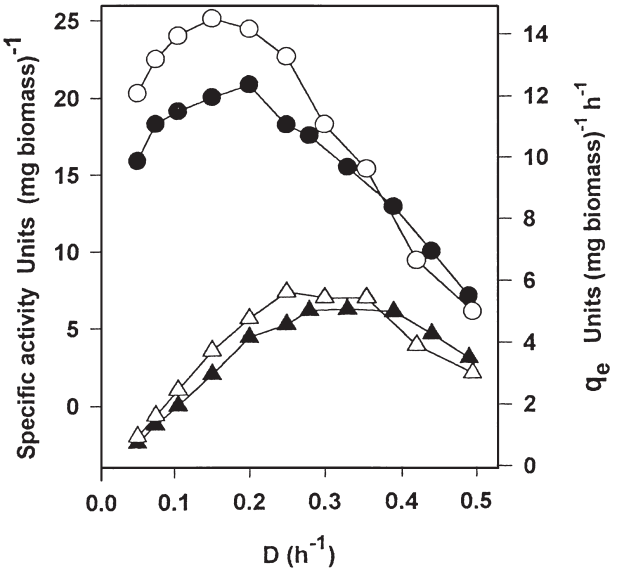

Figure 2 Steady state β-gal expression as a function of growth rate in aerobic lactose and galactose-limited chemostat cultures of *K. lactis* NRRL 1118. Specific β-gal activity: (▲) galactose; (●) lactose; q_e (specific rate of β-gal synthesis): (△) galactose; (○) lactose.

β-gal expression under noninducing conditions was determined at D values of 0.1 and 0.25 h^{-1} with glucose or lactate as carbon source. As can be seen, constitutive enzyme expression was repressed by glucose (lactate-grown cells showed β-gal levels ~8–10-fold higher than cells grown on glucose) and also responded to changes in the growth rate (with both carbon sources q_e values were higher at D = 0.25 h^{-1}: Table 2). Fructose gave similar enzyme levels as glucose (data not shown). A common intermediate of glucose and fructose metabolism, rather than the sugars themselves, might be the effector molecule that exerts the repressive effect. Alternatively, both glucose and fructose might be active in repressing enzyme expression.

Constitutive and induced β-gal levels were determined in two other *K. lactis* strains, CBS 2360 and NRRL Y-1140, that have been described as non-repressible and weakly repressible by glucose, respectively [3,16,27]. The results obtained at D = 0.26 h^{-1} showed that, in these strains, synthesis of β-gal was also higher in lactate-grown cells than cells grown with glucose (Table 2). The highest noninduced activity in both glucose and lactate media corresponded to the non-repressible strain.

The three strains gave similar growth yields with the

Table 2 Steady state β-gal expression of different *K. lactis* strains grown in aerobic chemostat cultures

Yeast strain	GRP	D (h^{-1})	NL	β-gal specific activity: Units (mg biomass)$^{-1}$		
				glucose	lactate	lactose
K. lactis NRRL 1118	+	0.10	C	0.22 ± 0.09 [4] (0.022)	1.88 ± 0.35 [2] (0.19)	17.7 ± 1.47 [4] (1.77)
			N	4.75×10^{-3} [2]	–	2.97 ± 0.18 [2] (0.297)
			P	0.13 ± 0.04 [3] (0.013)	–	4.40 ± 0.65 [3] (0.44)
		0.25	C	0.15 ± 0.034 [4] (0.037)	1.54 ± 0.28 [2] (0.385)	18.3 ± 2.23 [8] (4.6)
			N	5.8×10^{-3} [2]	–	2.15 ± 0.3 [3] (0.54)
			P	0.11 ± 0.05 [2] (0.027)	– –	4.6 ± 0.28 [3] (1.15)
K. lactis NRRL 1140	±	0.26	C	0.73 ± 0.036 [2] (0.19)	1.69 ± 0.03 [2] (0.44)	8.25 ± 0.21 [2] (2.14)
K. lactis CBS 2360	–	0.26	C	2.1 ± 0.05 [2] (0.55)	3.5 ± 0.3 [2] (0.91)	10.5 ± 0.27 [2] (2.73)

Values in the table indicate the mean value ± standard deviation of β-gal specific activity obtained in [N] independent chemostat cultures. Numbers in parenthesis are q_e values (Units (mg biomass)$^{-1}$ h^{-1}).
GRP: Glucose repression phenotype. + repressible, ± weakly repressible, – non-repressible (according to references [3], [16] and [27]).
NL: nutrient limitation.

three carbon sources tested indicating a common metabolic pattern in C-limited chemostats. Growth parameters with glucose were similar to those with lactose (Table 3). All the strains used dl-lactate efficiently. At D = 0.25 h^{-1} the residual lactate concentration was less than 1 mM and $Y_{x/s}$ was about 40 g biomass (mol lactate)$^{-1}$ (data not shown).

Yeast growth and β-gal expression in carbon-sufficient cultures

Yeast cultures were carried out under nitrogen or phosphate limitation with lactose or glucose as carbon sources. Table 3 summarizes the results of yeast growth, biomass composition and extracellular products formed under the different growth conditions tested. Cells grown under P- or N-limitation exhibited higher rates of sugar consumption and also higher respiration rates as compared to C-limited cells. The enhanced carbon flux resulted in the accumulation of intracellular reserve carbohydrates, particularly in N-limited cultures at low growth rates, and also in the secretion of metabolic products derived from the glycolytic pathway, mainly glycerol and ethanol. The latter predominated in P-limited cultures. Minor amounts of acetate (only at D = 0.25 h^{-1}) and pyruvate were also detected in the culture media under N- or P-limitation (~0.25–2.0 mM). In all cases cell yield decreased as a consequence of carbon overflow. Carbon balances in N- and P-limited cultures either with lactose or glucose showed 90% and 70% of carbon recovery at D = 0.1 and 0.25 h^{-1}, respectively.

The induced and constitutive β-gal expression was repressed when cells were grown under N- and P-limitation (Table 2). In lactose medium, enzyme levels were reduced 4–8 times compared to those expressed in C-limited cultures. A similar response was observed when galactose was the carbon source, ie at D = 0.1 h^{-1} the β-gal activities in C- and N-limited cells were 24.5 and 1.98 Units (mg biomass)$^{-1}$, respectively (data not shown). It is noteworthy that N-limited cells exhibited lower β-gal levels than cells grown under P-limitation. β-gal was strongly repressed in glucose media under N-limitation and only slightly in P-limited cultures.

Kinetics of β-galactosidase induction

β-gal induction was examined in C-limited cultures (D ~0.22 h^{-1}) by switching the feed medium containing glucose to a feed medium containing lactose. As the data indicate (Figure 3), cells were immediately induced for β-gal (the first sample was taken 15 min after the change of the feed medium) and 20% of the fully induced level was expressed after one doubling time (0.66 volume changes). The specific β-gal activity increased continuously during the entire period of transition and reached steady state levels after five volume changes. As expected from the behavior observed in other inducible systems, the transient period was accompanied by an initial accumulation of lactose and a fall in the cell population because induction of the lactose transporter and the lactose catabolic enzymes is a prerequisite for full growth on the carbon source [1,5]. When the shift was carried out with a mixture of lactose and glucose or lactose and lactate (2 g L^{-1} of each carbon source), similar induction kinetics were observed (data not shown). The repressive effect of glucose on β-gal expression was also observed with the mixed substrates. The steady state specific activities of the induced cultures were 8.9 and 17 Units (mg biomass)$^{-1}$ in lactose/glucose and lactose/lactate media, respectively.

When the feed medium with lactose was switched to a feed medium containing glucose, the specific β-gal activity decreased (~50% per doubling time) until basal levels were

Table 3 Steady state metabolic parameters of *K. lactis* NRRL 1118 grown in aerobic chemostat culture under different nutrient limitations

D (h^{-1})	Limitation	C-source	Biomass (g L^{-1})	S_{res} (mM)	$Y_{x/s}$ (g mol^{-1})	TC (%)	Protein (%)	q (mmol (g biomass)$^{-1}$ h^{-1})				
								Sugar	O_2	CO_2	Glycerol	Ethanol
0.1	C	lactose	1.72	<0.05	163	30	43.5	0.614	3.75	4.0	nd	n.d
		glucose	1.76	<0.05	79.2	30	42	1.26	4.05	4.1	nd	nd
	N	lactose	3.9	7.6	112	52	29	0.87	5.6	5.9	0.25	0.039
		glucose	4.32	0.6	48.6	56	32	2.05	5.8	6.1	0.31	0.03
	P	lactose	3.86	11	107	43	32	0.93	5.7	6.2	0.29	0.067
		glucose	4.05	0.78	43.2	46	37	2.31	5.85	6.5	0.52	0.038
0.25	C	lactose	1.89	<0.05	178	28.5	45	1.4	6.95	7.8	<0.02	<0.02
		glucose	1.84	<0.05	83	29	42	2.9	7.3	7.45	<0.02	<0.02
	N	lactose	3.05	8.7	89	39	32	2.8	9.0	9.1	0.71	0.38
		glucose	3.45	19	38	37	34	5.35	8.2	8.8	0.93	0.22
	P	lactose	2.21	10	65	29	44	3.8	10.0	12.1	1.35	2.5
		glucose	2.17	35	29	33.5	47	6.6	11.0	11.95	1.13	1.75

S_{res}: residual sugar concentration. $Y_{x/s}$: biomass yield with respect to carbon source. TC: total carbohydrates. Percentages are relative to dried biomass.
Data represent the mean of values obtained in the same independent chemostat cultures as indicated in Table 2. The standard errors of these means were all less than 10%.

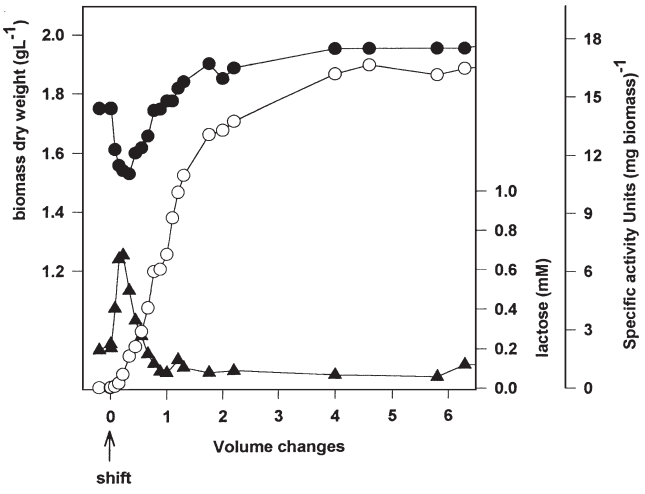

Figure 3 Kinetics of β-gal induction in chemostat cultures of *K. lactis* NRRL 1118. At zero time (zero volume changes), the feed medium containing glucose (~22 mM) was changed to a feed medium containing lactose (~11.5 mM). Steady state cultures were C-limited, D ~0.22 h^{-1}. (●) Biomass dry weight; (○) specific β-gal activity; (▲) lactose concentration.

reached (data not shown). This indicates that induction was quickly arrested after the shift and β-gal was not further inactivated.

Inactivation of β-gal upon a sudden change in lactose concentration

When a culture growing in steady state under lactose limitation was pulsed with 5 mM lactose, the concentration of biomass started to decrease and lactose accumulated (Figure 4). The decrease of biomass concentration followed a wash-out kinetics indicating that growth ceased immediately after the pulse (the estimated D value was 0.22 h^{-1}). The specific β-gal activity decreased during the transient indicating enzyme inactivation. The half life of β-gal was about 4.4 h.

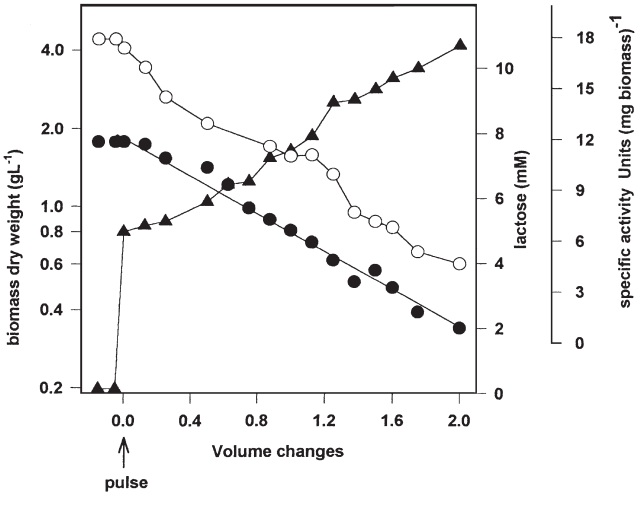

Figure 4 Transient changes in the biomass and sugar concentration and of the specific β-gal activity following the sudden addition (pulse) of lactose to a lactose-limited aerobic chemostat culture of *K. lactis*. The pulse of lactose was 5 mM. The lactose concentration in the feed medium was ~11.5 mM. D ~0.25 h^{-1}. (●) Biomass dry weight; (○) specific β-gal activity; (▲) lactose concentration.

Discussion

From a dynamic point of view, enzyme expression is best analyzed in terms of q_e, the specific rate of enzyme synthesis. Our results on the expression of β-gal in chemostat cultures showed that q_e varied in a wide range (over 10^4-fold) in response to the environmental conditions, confirming that the lac/gal regulon of *K. lactis* is tightly regulated. The maximum q_e was reached in both lactose- and galactose-limited cultures at intermediate growth rates. When cells were grown in lactose-sufficient cultures (either N- or P-limited), repression of β-gal may have been caused by an increased concentration of the internally released glucose. However, a galactose-dependent signal, which limited or slowed down induction under these culture conditions, may have also existed. This became apparent when the carbon source was galactose (12-fold decrease of β-gal levels in N-limited cells compared with C-limited cells). This negative regulation could explain the decrease of q_e observed in galactose-limited cells growing at high D values (above 0.4 h^{-1}), a condition which was coincident with a marked increase in the levels of the carbon source in the culture medium. The same galactose effect, in addition to the repression exerted by glucose (or a derivative), probably contributed to the repression of β-gal synthesis in lactose media under similar growth conditions. Alternatively, submaximal q_e values at low growth rates in lactose and galactose-limited cultures may be attributed to the low effective level of the inducer [12].

Constitutive β-gal expression was repressed by glucose. The weakly repressive strain NRRL Y-1140, and even the non-repressive strain CBS 2360, also showed glucose repression under non-inducing conditions in our chemostat cultures. Although it is difficult to compare these results with those obtained in batch cultures, where the glucose repression phenotype had been studied in the presence of the inducer, we can infer that the *K. lactis* strains tested share a common mechanism of glucose repression (although with different sensitivity) that is independent of the induction pathway. This possibility has been invoked to explain the fact that glucose repression also occurred in mutants defective in the Lac9p activator [16,28]. Constitutive β-gal expression also responded to the external concentration of the carbon source. This was particularly evident in N-limited cultures where β-gal was repressed 30-fold as compared to glucose-limited cells. Again, this may be attributed to an increased internal concentration of glucose when cells were grown in glucose-sufficient cultures.

In lactose-limited cultures, β-gal accumulated in amounts equivalent to 10% of the cell protein. In C-limited cultures, especially at low growth rates, the intracellular substrate concentration is usually in the μM range [17]. The kinetic properties of β-gal (K_m = 18–40 mM, V_{max} ~0.33 mmol lactose min^{-1} (mg protein)$^{-1}$, 30°C, PPB-Mn), indicate that the enzyme is not an efficient catalyst under these physiological conditions. Thus, the beneficial effect of synthesizing high enzyme levels is easy to infer. The microorganism can optimize its growth rate by sustaining the appropriate metabolic flux. In nature this has an obvious ecological advantage [19]. Direct evidence for this situation can be obtained by comparing the *in vivo* and *in vitro* β-

gal activity of cells grown in lactose media. The V_{max} displayed by permeabilized cells (30°C, PPB-Mn) ranged between 100 to 900 mmol lactose (g biomass)$^{-1}$ h^{-1} (the K_m values of β-gal for lactose were the same irrespective of the growth rate, carbon source or nutrient limitation), whereas the enzyme activity *in vivo* (q_s in steady state conditions) was in the range 0.5–3.5 mmol lactose (g biomass)$^{-1}$ h^{-1}. Similar expression levels occurred when cells were grown in galactose medium, a condition in which the function of β-gal is not required, albeit for the metabolism of the substrate. This resulted from the fact that all the structural genes of the lac/gal regulon depended on the same regulatory pathway.

The kinetics of β-gal induction observed in C-limited cultures confirm that β-gal induction is a short-term enzyme adaptation process [7].

When a lactose-limited chemostat was pulsed with an excess of lactose, growth ceased immediately and β-gal was progressively inactivated. A similar effect was observed in maltose-limited chemostat cultures of *Saccharomyces cerevisiae* after a pulse of maltose [20]. This phenomenon, known as substrate-accelerated death, was explained in terms of an uncontrolled uptake of sugar into the cell after the pulse. This sugar uptake led to an osmotic stress, which in combination with a possible decrease in internal pH due to rapid proton influx, resulted in cellular death. It is most likely that a similar effect occurred after the lactose pulse, considering that lactose uptake in *K. lactis* is also mediated by a sugar/proton symport system [5]. It has been demonstrated that enzyme inactivation under stress conditions is mediated by mechanisms of phosphorylation and subsequent proteolytic degradation [11,14]. Further research is needed in order to elucidate the mechanisms involved in the inactivation of the lac-gal gene products triggered by lactose stress.

Yeast growth was characterized under different culture conditions. Cells grown in C-limited cultures displayed a net respiratory metabolism with the substrate being metabolized solely to biomass and CO_2. This behavior was expected from previous batch culture experiments and from data reported for similar yeasts such as *K. fragilis* grown in chemostats [15,24]. When cells were grown under conditions of N- or P-limitation, there was an increase of the specific rate of sugar uptake, indicating that the rate of carbon metabolism was not strictly regulated to match the imposed rate of biomass biosynthesis (the rate observed under C-limitation). The increased rate of carbon uptake resulted in the accumulation of reserve carbohydrates and also in the secretion of glycolytic metabolites (mainly ethanol and glycerol). These metabolic changes which occurred in response to the nutrient status and the growth rate have been commonly observed in microbial and cell cultures [10,19]. In order to obtain a better insight on the physiology of the yeast strain used, other study strategies such as metabolic network stoichiometry analysis or metabolic control analysis should be considered [23].

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