Regulation of β-D-Galactosidase Synthesis in Candida pseudotropicalis

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Regulation of lactose (β-D-galactosidase) synthesis in the lactose-utilizing yeast Candida pseudotropicalis was studied. The enzyme was inducible by lactose and galactose. When grown on these sugars the enzyme level of the yeast was 20 times or higher than when grown on glycerol. The $K_m$ and optimal pH were similar for the lactase induced either by lactose or galactose. The hydrolysis of o-nitrophenyl-β-D-galactopyranoside by the lactase was inhibited by galactose and several analogs and galactosides, but not by glucose. Lactose uptake activity observed in lactose-grown cells was very reduced in cells grown on glucose or galactose. Glucose repressed the induction of lactase, but not the metabolic system for galactose utilization. In continuous culture on lactose medium at dilution rates below 0.2 h$^{-1}$ the specific lactase activity was higher than in batch cultures and decreased with increases in dilution rate. Lactase was induced by pulses of lactose and galactose in cells growing on glucose, but only at low dilution rates where the steady-state concentration of glucose was very low.

The yeast Candida pseudotropicalis grows efficiently on lactose and may be a good potential source for the commercial production of lactase (β-D-galactosidase, EC 3.2.1.23) (8, 11, 27). In a previous study growth and extraction conditions for maximal production of the enzyme by one C. pseudotropicalis strain (NCYC 744) were determined, and it was found that the concentration of lactose in the medium affects the amount of enzyme produced (8).

In contrast with the lactose operon in Escherichia coli (4), the available data on the mechanisms regulating the synthesis of lactase and other enzymes involved in the catabolism of lactose by yeasts are rather limited (1, 6, 7, 9, 22, 23, 25), and C. pseudotropicalis has not been included in these studies. The objective of this work was to gain information on these mechanisms operating in C. pseudotropicalis NCYC 744. Lactase in this yeast was found to be inducible and subject to catabolite repression.

The information obtained here is important, among other things, for the design or modification of a process aimed at the production of the enzyme.

MATERIALS AND METHODS

Microorganism and culture media. C. pseudotropicalis NCYC 744 was obtained from the National Collection of Yeast Cultures, Surrey, England. It was maintained on slants made of five different carbon sources. Each consisted of 2% (wt/vol) lactose, glucose, galactose, lactic acid, or glycerol (all from Merck & Co.) in addition to 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, and 2% agar (all from Difco Laboratories). The slants were kept at 4°C and subcultured monthly.

Yeast nitrogen base (YNB, Difco) at 6.7 g/liter supplemented with different concentrations of one or two carbon sources (lactose, glucose, galactose, lactic acid, or glycerol) was used throughout for liquid media.

Growth conditions. Batch cultivations were done in cotton-plugged 300-ml Erlenmeyer flasks containing 45 ml of medium and 5 ml of a 12- to 24-h-old inoculum grown in the medium specified for each experiment. The flasks were incubated in a reciprocating shaker bath (120 strokes per min). For continuous cultivation a Bioflo C30 fermentor (New Brunswick Scientific Co.) with a set working volume of 280 ml was used. Impeller speed and aeration rate were fixed at 500 rpm and 400 ml/min, respectively. In all cases the temperature was maintained at 30 ± 1°C. The cells were harvested by centrifugation at 5,000 × g for 5 min.

Enzyme extraction and assays. The yeast cells were treated with 2% chloroform under the conditions previously established (8). After treatment, cell debris was removed from the supernatants by filtration through 0.45-μm membranes (Gelman), and the filtrates were assayed for lactase activity as described below. All assays were done at 37°C by using as substrate 10 mM o-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma Chemical Co.) in 0.1 M potassium phosphate buffer (pH 6.2) supplemented with 0.5 mM MgSO4 and 0.1 mM MnCl2 (phosphate buffer). Assay mixtures contained 0.1 to 0.5 ml of enzyme extract and

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4 ml of ONPG. The reactions were stopped after 1 to 5 min by the addition of 1 ml of 0.5 M Na2CO3. The amounts of o-nitrophenol liberated were determined by relating absorbancy at 420 nm to o-nitrophenol concentration with a calibration curve. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of o-nitrophenol in 1 min. Specific activity was expressed as the number of enzyme units per milligram (dry weight) of cells.

**Lactose uptake determinations.** Two different lactose uptake experiments were done. In the first experiment the yeast was grown for 18 h (to stationary phase) on three different media containing 6.7 g of YNB per liter supplemented with 2.5 g of lactose, glucose, or galactose. Each culture was washed three times and suspended in 0.1 M phosphate buffer (pH 7) to a concentration of 0.6 mg of cells per ml. Five milliliters of each suspension was added to 5 ml of B-glucose-[1-14C]lactose (Amersham Corp.; 57.7 mCi/mmol) solutions containing 2 μCi/ml (approximately 0.035 mM lactose). The mixtures were kept at 30°C with agitation (120 strokes per min), and 1-ml samples withdrawn at different times. The samples were filtered through 0.45-μm membranes (Gelman) and washed thrice with cold buffer in the same filters. The membranes with the retained cells were transferred to vials containing scintillation liquid (Aquasol; New England Nuclear Corp.), and radioactivity was measured in a scintillation counter (Tricarb 3385; Packard Instrument Co.).

In a second experiment the yeast was grown for 3 h in five different media containing YNB plus 2.5 g of lactose, glucose, galactose, lactose plus glucose, or lactose plus galactose per liter. The cells from each culture were harvested, washed, and incubated with [14C]lactose (approximately 17.5 μM lactose, final concentration) for 5 min, after which they were handled and the radioactivity was measured as described above.

**Miscellaneous procedures.** Yeast concentrations were estimated by relating absorbance readings in a Klett-Summerson colorimeter (no. 54 filter, 520 to 580 nm) to dry weight with a standard curve previously established. Before the readings were taken, the cell suspensions were always diluted to give turbidity values lower than 120 Klett units. A reading of 50 Klett units was equivalent to a yeast concentration of 0.15 mg of cells per ml.

Specific growth rates (μ) were calculated during exponential growth according to the equation: μ = (lnX2 - lnX1)/t2 - t1, where X1 and X2 represent cell concentrations at times t1 and t2, respectively. Generation times (T) were determined by the equation: T = 1/μn.

Total sugars were determined by the antrone method of Scott and Melvin (21).

Quantitative and qualitative analyses of mixtures of sugars were done in a modified aminoacid analyzer (JEOL 3BC) by using the reagent of Mopper and Gindler (15).

The Michaelis constant (Km) of the lactase was determined by using ONPG and lactose as substrates (0.5 to 50 mM in phosphate buffer [pH 6.2]) at 37°C and plotting the results by the method of Lineweaver and Burk (14) as modified by Dixon (10). The glucose liberated when lactose was used as substrate was measured by the glucose oxidase-peroxidase method recommended by Sigma (technical bulletin no. 510).

**RESULTS**

**Yeast growth and enzyme levels on different carbon sources.** *C. pseudotropicalis* strainNCYC 744 grew well on the five carbon sources tested, with the lowest generation time on galactose (88 min), followed by glucose (95 min), lactic acid (99 min), lactose (106 min), and glycerol (238 min) (Fig. 1). The specific lactase activity fluctuated during growth with a low point during mid-log phase and decreased steadily after reaching stationary phase. It was up to 20 times higher in cells growing on lactose and galactose than in those growing on the other carbon sources.

**Properties of lactase induced by galactose and lactose.** Some properties of the lactase produced by strain 744 grown on galactose and lactose were compared. With ONPG as substrate, the Km were 3.4 × 10⁻³ and 3.2 × 10⁻³ M, respectively, and with lactose as substrate the Km were 33.7 × 10⁻³ and 32.6 × 10⁻³ M, respectively. Peak enzyme activity in both cases was ob-

FIG. 1. Growth curves and specific lactase activities of *C. pseudotropicalis*NCYC 744 growing in YNB media containing (per liter) 2.5 g of galactose (○), glucose (●), lactic acid (△), or lactose (▲) and 1.25 g of glycerol (□). The cells were precultured twice on the same media.
TABLE 1. Effect of different compounds on ONPG hydrolysis by lactases induced by lactose and galactose

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concn (mM)</th>
<th>ONPG hydrolysis (% of control) by extracts of cells grown on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lactose</td>
</tr>
<tr>
<td>IPTG</td>
<td>10</td>
<td>117</td>
</tr>
<tr>
<td>Fucose</td>
<td>30</td>
<td>110</td>
</tr>
<tr>
<td>Glucose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ribose</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Melibiose</td>
<td>100</td>
<td>86</td>
</tr>
<tr>
<td>MG</td>
<td>100</td>
<td>76</td>
</tr>
<tr>
<td>Galactose</td>
<td>100</td>
<td>64</td>
</tr>
<tr>
<td>Lactose</td>
<td>30</td>
<td>71</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>30</td>
<td>49</td>
</tr>
</tbody>
</table>

a IPTG, Isopropyl-l-thio-β-D-galactoside; MG, methyl-β-D-galactoside.

served at pH 6.2 and between 45° and 47.5°C. The effect of different compounds on the ONPG hydrolysis was also similar in both cases (Table 1): melibiose, methyl-β-D-galactoside, galactose, lactose, and galactosamine inhibited ONPG hydrolysis.

Effect of adaptation to different carbon sources on subsequent induction. Cells grown on glycerol, glucose, or lactose were immediately induced for lactase production when transferred to media containing lactose or galactose (Fig. 2A, B, and C). Cells preincubated on galactose were induced when transferred to medium with lactose; however, when transferred to medium with lactose, neither enzyme induction nor growth took place for several hours (Fig. 2D). With this single exception, the period of adaptation from one carbon source to another was relatively short.

Effect of carbon source on lactose uptake. Since the cells grown on galactose had a high lactase specific activity, but had difficulties starting growth on lactose, the possibility of insufficiency in the transport mechanism for the disaccharide in these cells was explored. The uptake of [14C]lactose by cells grown to stationary phase on galactose, lactose, or glucose is shown in Fig. 3. Uptake of [14C]lactose was much higher by lactose-grown cells than by those grown on galactose or glucose. The activity of the transport was also affected when galactose or glucose was present in lactose medium. Cells growing in mixtures of glucose plus lactose or galactose plus lactose incorporated significantly less [14C]lactose than did cells growing on lactose alone (Table 2).

Effect of glucose on lactase synthesis. When the yeast was grown on mixtures of galactose and lactose, both sugars were utilized simultaneously, and the enzyme was induced early and synthesized throughout growth (Fig. 4A). When placed in a mixture of glucose and lactose the

FIG. 2. Effect of adaptation to different carbon sources on subsequent lactase induction. C. pseudotropicalis NCYC 744 was grown on YNB medium containing glycerol (A), glucose (B), lactose (C), or galactose (D) until the sugars were exhausted. The cells were collected, washed and transferred to media containing galactose (O), lactose (Δ), glucose (Δ), glycerol (△), or lactic acid (□), and the lactase specific activity was determined at indicated times. Glycerol was used at 1.25 g/liter; all other carbon sources were used at 2.5 g/liter.
yeast had diauxic growth, utilizing lactose only after the glucose was dissimilated. Likewise, the enzyme was induced only after the glucose was utilized (Fig. 4B). Galactose and glucose were utilized simultaneously, but induction of the enzyme took place only at low levels and toward the end of growth, once the concentration of glucose was greatly reduced (Fig. 4C).

The previous experiments demonstrated that lactase could not be induced in the presence of glucose. The effect of different concentrations of this sugar on induction was studied; yeast cells grown on glycerol were placed on mixtures of lactose (1.25 g/liter) and glucose at different concentrations (0.02 to 1.25 g/liter) in phosphate buffer, and the levels of lactase were determined after 3 h. Although variability was observed in several experiments, the enzyme was not induced when glucose was present at concentrations higher than 0.04 g/liter (data not shown).

**Lactase induction and synthesis in continuous culture.** Yeast cultures maintained up to 15 days at a fixed dilution rate (0.1 h⁻¹) did not exhibit changes in the lactase specific activity. At low dilution rates (i.e., <0.2 h⁻¹) the values of specific activity were higher than those obtained in batch cultures and decreased with increases in dilution rate (Fig. 5). When the lactose medium was changed to glucose the levels of specific activity decreased slowly (ca. 50% per generation time [6.93 h]) until basal levels were reached (Fig. 6).

The enzyme was rapidly induced in glycerol cultures when pulses of lactose were added (Fig. 7). However, when the same lactose pulses were added together with the different concentrations of glucose, induction did not occur (Table 3).

When lactose pulses were added to cultures growing on glucose, the enzyme was induced only at low dilution rates. The lower the dilution rate, the higher the response obtained (Fig. 8A). Similar results were obtained with galactose pulses (Fig. 8B).

**DISCUSSION**

The lactase of *C. pseudotropicalis* NCYC 744 was induced by galactose and lactose as occurs in the other lactose-utilizing yeasts *Saccharomyces* (Klyuyveromyces) *lactis* (25) and *Kluyveromyces fragilis* (6). Galactose was a better inducer than lactose, which suggests that although it remains to be identified, the natural inducer may be a galactose analog. The fluctuations in specific activity observed are not understood at this point, but may reflect the interactions among factors controlling induction and repression of lactase synthesis as discussed below. *Streptomyces violaceus* and *Neurospora crassa* have two types of lactases, one inducible by lactose and the other inducible by galactose (2, 3, 18-20). The results presented here do not suggest differences in the lactase induced by lactose or galactose in strain NCYC 744. The ONPG hydrolysis by lactase from cells grown on either galactose or lactose was inhibited by galactose, but not by glucose; the same occurs with the lactases produced by *Aspergillus foetidus* (5) and *Aspergillus oryzae* (17), but is opposite to the response of the enzyme produced by *K. fragilis* (26).

Induction of lactase by galactose and lactose was rapid and not affected by the carbon source

**TABLE 2.** Uptake of [¹⁴C]lactose by *C. pseudotropicalis* NCYC 744 grown on different sugars*⁸*

<table>
<thead>
<tr>
<th>Cells grown on:</th>
<th>[¹⁴C]lactose uptake (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>115,481</td>
</tr>
<tr>
<td>Glucose</td>
<td>1,067</td>
</tr>
<tr>
<td>Galactose</td>
<td>21,499</td>
</tr>
<tr>
<td>Lactose + glucose</td>
<td>18,664</td>
</tr>
<tr>
<td>Lactose + galactose</td>
<td>30,114</td>
</tr>
</tbody>
</table>

* Each sugar at 2.5 g/liter in YNB. The cells were grown for 3 h, washed, and incubated with [¹⁴C]lactose for 5 min.
FIG. 4. Growth curves and specific lactase activity of *C. pseudotropicalis* NCYC 744 growing on mixtures of sugars (1 g/liter each) in YNB. A, Mixture of galactose plus lactose; B, mixture of lactose plus glucose; and C, mixture of galactose plus glucose. Symbols: concentrations of yeast (○), galactose (●), lactose (△), and glucose (■); lactase specific activity (□).

FIG. 5. Continuous culture of *C. pseudotropicalis* NCYC 744 on YNB medium containing 2.5 g of lactose per liter. Symbols: effect of dilution rate on lactase specific activity (○); and cell concentration (●).

FIG. 6. Decrease of lactase specific activity in *C. pseudotropicalis* NCYC 744 growing in continuous culture (dilution rate fixed at 0.1 h⁻¹) when the medium was shifted from YNB containing lactose to YNB containing glucose.
used for previous growth, with the exception of the unexpected lag in growth and induction suffered by galactose-grown cells when transferred to lactose (Fig. 2). Although $^{14}$C]lactose was readily taken up by lactose-grown cells, it was not the case with galactose-grown cells. Therefore, when transferred to lactose these cells took little of the disaccharide which, in addition, was rapidly hydrolyzed by the lactase. The internally released glucose and galactose may have repressed or inhibited the lactose transport (or both). As a consequence, further penetration of lactose remained low, and growth remained slow. It was only after several hours, when the specific activity of lactase decreased, that enough lactose could enter the cells and normal growth ensued.

The low uptake of $^{14}$C]lactose by cells grown on a mixture of galactose and lactose (Table 2) is indicative that galactose may have repressed (16) or inhibited the lactose transport (or both). When strain NCYC 744 was grown on glucose until all the sugar was used, subsequent induction of lactase was not affected, but when glucose was present, even at low concentrations (e.g., 20 µg/ml), the enzyme could not be induced. This indicates the existence of catabolite repression as occurs in K. fragilis (6) and is in contrast with the response of K. lactis where glucose does not cause permanent repression of lactase synthesis or lactose utilization (9).

The simultaneous utilization of galactose and glucose by strain NCYC 744 and the glucose repression of the induction of lactase by galactose (Fig. 4) indicates that; (i) the enzymes required for galactose catabolism were not repressed by glucose, and (ii) lactase was not required for galactose dissimilation.

During growth in batch culture, and as a consequence of metabolism, a microorganism continuously changes the characteristics of the medium; in term, these changes in the environment have influence over the physiological state of the cells, including the control of enzyme synthesis. By growing the microorganism in continuous culture, steady states are achieved that facilitate the study of the effect of variables on enzyme synthesis. The absence of significative changes in yeast concentrations or specific lactase activity when strain NCYC 744 was maintained for several days at a fixed dilution rate indicates genetic homogeneity and stability of the population. However, it may be possible to select spontaneous mutants or hyperproducers of lactase by maintaining the culture at the appropriate dilution rate for longer times (13).

The decrease in specific lactase activity with increases in dilution rate (Fig. 5) was similar to that reported for K. fragilis growing on 0.2% lactose (6) and suggests a dependence of lactase synthesis on growth rate. However, continuous cultivation in this work was done with only one concentration of lactose, and it is possible that the specific activities of the enzyme may be different when different concentrations of the sugar are used. This was observed in batch cultures (8), and it has been reported that K. fragilis produced a constant level of lactase independently of dilution rate when grown on media with 1% lactose (6).

Glucose does not inactivate (12, 24) or inhibit intracellular lactase, as shown by the slow decrease in activity observed when the lactose medium was changed to glucose (Fig. 6). The decrease corresponded well with a halt in lactase activity.

![Graph](http://aem.asm.org/)

**FIG. 7.** Induction of lactase by lactose pulse (1 mg/ml, final concentration, in the reactor at 0 time) in C. *pseudotropicalis* NCYC 744 growing in continuous culture on glycerol. Dilution rate was fixed at 0.09 h⁻¹.

Symbols: lactase specific activity (O); lactose concentration (●).

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**TABLE 3.** Continuous culture of *C. pseudotropicalis* NCYC 744 on glycerol and effect of glucose on the lactase induction by pulses of lactose

<table>
<thead>
<tr>
<th>Sugar concn in reactor immediately after pulse (mg/ml)</th>
<th>Lactase sp act (U/mg of cells)</th>
<th>Before pulse</th>
<th>1 h after pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>Glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.3</td>
<td>1.23</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.25</td>
<td>0.23</td>
</tr>
<tr>
<td>1</td>
<td>0.38</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>1</td>
<td>0.25</td>
<td>0.1</td>
<td>0.15</td>
</tr>
<tr>
<td>1</td>
<td>0.125</td>
<td>0.03</td>
<td>0.045</td>
</tr>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Dilution rate fixed at 0.09 h⁻¹. The feed medium contained 1.25 g of glycerol per liter.
synthesis due to repression, followed by dilution of the enzyme among daughter cells.

The glucose effect was again evidenced in continuous culture when glucose at concentrations as low as 0.05 mg/ml (50 times less than lactose) repressed the enzyme induction by lactose in cells growing in glycerol (Table 3). The enzyme was induced by lactose and galactose in cultures growing on glucose, but only at low dilution rates where the concentrations of glucose in the reactor were low (Fig. 8). The level of enzyme could be the result of an equilibrium between the effects of internal inducer (whether lactose, galactose, or a derivative) and the repression caused by the internal supply of glucose.

Work is in progress to elucidate the causes for different levels of enzyme activity in several lactose-utilizing yeasts and to optimize conditions for lactase production in continuous culture.

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LITERATURE CITED


