Journal of Industrial Microbiology, (1995) 15, 45–48

Kinetics of lactose transport in *Kluyveromyces fragilis* grown in a chemostat on diluted whey permeate

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Lactose transport was studied in *Kluyveromyces fragilis* grown in lactose-limited chemostat cultures. Kinetic parameters were determined using a method based on genetic population evolution. Lactose transport was carried out via three carriers characterized respectively by K_m of 0.1 mM, 3 mM and 15.5 mM. The synthesis of these lactose carriers and their capacity (V_{max}) are dependent on the dilution rate (D). At D = 0.12 h^{-1}, the high affinity transporter is prominent. For intermediate dilution rate, only the high and the medium affinity systems are present. In cells growing at D = 0.4 h^{-1}, these carriers are absent but instead, the low affinity transporter is present. The effect on lactose transport of such metabolic inhibitors as CCCP, a proton ionophore, and Antimycin A, an energy inhibitor, were also investigated. The high affinity system is the most sensitive to the effect of these inhibitors. Lactose transport through this carrier is probably a mechanism dependent on the proton motive force.

Keywords: *Kluyveromyces fragilis*; lactose transport; continuous culture

Introduction

Whey is an abundant by-product of the cheese and casein industries. The recovery of whey proteins is a process widely used in industry. The resulting ultrafiltrate, which is whey permeate, must be treated to avoid pollution problems. The transformation of lactose to feed yeast is an interesting process due to the good nutritional qualities of yeasts.

Among yeasts, *Kluyveromyces fragilis* is the most used strain for this purpose [9]. Such processes are usually carried out continuously, hence the rate at which lactose is fed is important to obtain high productivity. However, we noted that the efficiency of the process dropped off as the rate of lactose feeding was increased [6]. In these conditions the oxygen level was not limiting, we also obtained a high level of residual lactose. To understand such behaviour the study of lactose transport can be a useful tool. In fact other studies [3,7] reported that sugar transport in yeasts is dependent on dilution rate. Thus Postma *et al* [7] showed that glucose transport in *Candida utilis* is carried out by three carriers which are dependent on dilution rate. Similar results have been reported by Bisson and Fraenkel [3] and Postma *et al* [8] for glucose transport in *Saccharomyces cerevisiae*.

Lactose transport by *Kluyveromyces fragilis* was mainly studied in batch cultures on a well defined medium [4,5]. Such conditions were not adequate to study lactose transport because sugar concentration, a parameter influencing the synthesis of the transporters, is not constant. The use of continuous cultures to study sugar transport is more advantageous because growth conditions are well defined and easily reproduced.

We have studied lactose transport with the aim of optimizing the continuous production of *Kluyveromyces fragilis* on whey permeate. First we investigated the kinetics of lactose transport in a continuous culture on diluted whey permeate, then determined the nature of the carriers identified.

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Received 25 October 1994; accepted 28 February 1995

Materials and methods

Microorganism: *Kluyveromyces fragilis* was maintained on yeast malt agar slopes. The organism was obtained from the collection of yeasts of the Laboratoire de Microbiologie alimentaire et industrielle. It was isolated from a dairy product.

Growth conditions: The organism was grown in a 2-L Biolafitte fermentor (LSL Biolafitte, Saint-Germain-en Laye, France) with a working volume of 1 L. The dilution rate varied from 0.12 h^{-1} to 0.4 h^{-1}. The yeast was cultivated on whey permeate diluted to a lactose concentration of 10 g L^{-1} and supplemented with all the components necessary for growth: $(NH_4)_2SO_4$, 1.5 g L^{-1}; yeast extract, 0.5 g L^{-1}; $FeCl_3$, 2 mg L^{-1}. Temperature was maintained at 30° C and the pH was regulated at 4.5 with 2 N NaOH. Dissolved oxygen was kept above 30% air saturation.

Analytical techniques

Measurement of residual lactose: Samples obtained during steady state were filtered through a 0.2 μm pore size membrane filter. Lactose was then determined by an enzymatic kit (Boehringer Mannheim, Meylan, France).

Determination of dry weight: Ten milliliters of cell suspension were centrifuged at 7000 *g* for 10 min, washed with distilled water, centrifuged again and then dried at 105° C for 24 h.

Ethanol: It was measured using a Delsi Di 200 gas chromatograph (Argenteuil, France) equipped with a flame

ionization detector and a stainless steel column 2 m × 2 mm packed with Porapak Q 80. The carrier gas was nitrogen. The injector and the detector were set at 220° C and the oven temperature was 170° C. Methanol was used as an internal standard.

Transport assay: Ten milliliters of cell suspension were harvested from the continuous culture, centrifuged, washed twice with distilled water and resuspended in 50 ml of 0.1 M phosphate buffer (pH = 6.5). Cell suspension (2.2 ml) was equilibrated at 30° C and 0.1 ml of the labelled substrate 1 μCi ml^{-1} [D-glucose 1-^{14} C]lactose (Amersham, Les Ulis, France) was added. The final volume was 2.5 ml and the lactose concentration varied from 0.5 mM to 100 mM. Samples (0.5 ml) were taken at 20-s intervals up to 80 s. Under these conditions, lactose uptake was linear for the first 80 s. The samples were immediately diluted in 10 ml unlabelled lactose (200 mM) at 4° C and filtered through a 0.22 μm pore size membrane filter and washed twice with a concentrated lactose solution. The filters were then dissolved with 0.5 ml Soluene 100 (Packard, Rungis, France). Ten milliliters of scintillation liquid (Instagel, Packard) were added to the vials and radioactivity was determined in a Packard tri Carb 460 CD counter. For each lactose concentration tested, the incorporation of labelled substrate was measured in duplicate.

Inhibition of transport by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma Chemical Co, Saint Quentin, Fallavier, France) was assayed by pre-incubating the cells for 10 min at 30° C with 100 μM CCCP added in ethanol (1% v/v) or with ethanol alone (control). Inhibition by 0.2 μM Antimycin A (Sigma Chemical Co) was assayed by pre-incubating cells for 30 min. As in the transport assay, lactose concentration was varied from 0.5 to 100 mM. The percentage of inhibition of lactose uptake by each carrier, was calculated according to:

$$\% \text{ inhibition} = \left(1 - \frac{V_{maxI}}{V_{max}}\right) \times 100$$

where V_{maxI} and V_{max} were, respectively, the capacity of lactose uptake in the presence and in the absence of the inhibitor.

Analysis of transport kinetics: To determine the kinetic parameters of lactose transport, we used a method based on genetic concepts developed by Bicking *et al* [2]. This method considers the experimental values and generates many points from the search space by generating populations of points and tests each point independently. At each iteration, called a generation, a new population is generated and tested. Probabilistic rules are used to guide the search and the algorithm requires only information concerning the quality of the solution.

Results and discussion

Continuous growth of Kluyveromyces fragilis *on diluted whey permeate*

Continuous growth of *Kluyveromyces fragilis* on diluted whey permeate diluted to 10 g L^{-1} of lactose was studied (Table 1). Steady state was achieved within four fermenter renewals. The dilution rate was varied from 0.12 h^{-1} to 0.40 h^{-1}. Under these conditions, the apparent growth yield ($Y_{X/S}$) was practically constant and equal to 0.51 g cells g lactose^{-1}. However, for D <0.25 h^{-1}, the apparent growth yield increased with the dilution rate. This indicated the existence of a non-negligible maintenance coefficient of *Kluyveromyces fragilis* for lactose. We can determine this parameter from the plot $1/Y_{X/S\ (apparent)}$ versus 1/D. Thus we obtained a maintenance coefficient of 90 μmol lactose g cells^{-1} h^{-1} and the value of the real growth yield which was 0.53 g cells g lactose^{-1}. Table 1 also indicates that for D >0.29 h^{-1}, residual lactose was present but the apparent growth yield remained constant.

The residual lactose concentration in the chemostat cultures followed classical Monod kinetics over the entire range of dilution rates. The apparent affinity constant for lactose K_S and μ_{max} of the yeast were determined from an Eadie–Hofstee plot (D S^{-1} versus D). The intercept with the X-axis gives μ_{max} = 0.405 h^{-1} and the slope of the line gives K_S = 82 μM. These results indicate that lactose is the limiting nutrient at all the dilution rates studied.

Kinetics of lactose transport

To study lactose transport, we carried out a continuous culture on whey permeate diluted to 10 g L^{-1} of lactose. Six dilution rates were tested (0.12, 0.16, 0.25, 0.29, 0.34 and 0.4 h^{-1}). For each one, at steady state cells were harvested, washed, then exposed to eight concentrations (0.5–100 mM) of the labelled substrate (S). For each concentration, we determined the rate of lactose transport expressed in μmole g cells^{-1} min^{-1}. From the overall data, we plotted V = f(S) for each dilution rate. The curves obtained show that lactose transport follow Michaelis–Menten kinetics.

In order to determine the kinetic parameters (K_m and V_{max}), we used first Lineweaver–Burk, Hanes and Eadie–Hofstee plots. These representations showed the existence of two lactose transporters at all the dilution rates studied except for D = 0.4 h^{-1}. However these methods were not accurate enough to determine K_m (ie K_{m1} values between 0.1 and 0.16 mM and K_{m2} varied from 2.2 to 3.6 mM). To overcome this problem, we used the method developed by Bicking *et al* [2]. The results obtained are presented in Table 2.

These results indicated that lactose transport in *Kluyveromyces fragilis* is mediated by three carriers: a high affinity

Table 1 Concentration of residual lactose, biomass and cell yield as a function of the dilution rate in lactose-limited chemostat cultures of *Kluyveromyces fragilis* at a reservoir lactose concentration of 10 g L^{-1}

D (h^{-1})	Residual lactose (mM)	X (g cells L^{-1})	$Y_{X/S\ apparent}$ (g cells g lactose^{-1})
0.12	0.04	4.7	0.47
0.16	0.05	4.8	0.48
0.24	0.11	5	0.5
0.29	0.2	5.1	0.51
0.34	1.2	4.9	0.51
0.40	7.3	3.8	0.5

Table 2 Kinetic parameters (K_m and V_{max}) of the three lactose carriers identified in *Kluyveromyces fragilis* grown continuously on diluted whey permeate

D (h^{-1})	Residual lactose (mM)	K_{m1} (mM)	K_{m2} (mM)	K_{m3} (mM)	V_{max1} (μmole g cells^{-1} min^{-1})	V_{max2} (μmole g cells^{-1} min^{-1})	V_{max3} (μmole g cells^{-1} min^{-1})
0.12	0.04	0.1	3	–	44	76	–
0.16	0.05	0.1	3	–	40	96	–
0.25	0.11	0.1	3	–	34	130	–
0.29	0.2	0.1	3	–	24	148	–
0.34	1.2	–	3	15.5	–	44	249
0.4	7.3	–	–	15.5	–	–	400

carrier which has a K_m (K_{m1}) of 0.1 mM, a medium affinity carrier characterized by a K_m (K_{m2}) of 3 mM and a low affinity carrier with a K_m (K_{m3}) of 15.5 mM.

The existence of these lactose transport systems depended on the dilution rate. From D = 0.12 h^{-1} to D = 0.29 h^{-1}, where residual lactose concentration was low, the high and medium affinity carriers were present. For D = 0.34 h^{-1}, corresponding to a higher level of residual lactose, the high affinity carrier was not detected, however the medium affinity transporter was still present. At this dilution rate, the low affinity carrier was present. For D = 0.4 h^{-1}, the level of residual lactose was more important, only the low affinity carrier was detectable.

The capacity, that is the V_{max}, of each uptake system was also affected by the dilution rate. For the high affinity system, the maximum reached 44 μmole lactose g cells^{-1} min^{-1}, was obtained at D = 0.12 h^{-1}. For the medium affinity carrier, the highest V_{max2} (148 μmole lactose g cells^{-1} min^{-1}), was obtained at D = 0.29 h^{-1}, V_{max2} was lower at D = 0.34 h^{-1}. The capacity of the low affinity carrier increased from 249 at D = 0.34 h^{-1}, to 400 μmole lactose g cells^{-1} min^{-1} at D = 0.4 h^{-1}.

From the results presented above it seems that the capacity of each carrier is correlated to the dilution rate and hence the residual lactose concentration in the fermenter. However, we cannot demonstrate if the transport capacity determines the level of residual lactose or *vice versa*. The higher lactose concentration when D is larger than 0.29 h^{-1} apparently triggers synthesis of the low affinity carrier and represses or inhibits the high affinity system. Such a specific induction of carriers in continuous cultures in response to glucose concentration has been reported for *Candida utilis* [7].

The *in situ* rate of transport by each of the three carriers was calculated from the kinetic parameters of the different uptake systems and the residual lactose concentration in steady-state cultures, according to:

$$V_{in\ situ} = \frac{V_{max}\ S_{in\ situ}}{K_m + S_{in\ situ}}$$

The *in situ* rate of lactose uptake in steady-state cultures is the sum of the rates of the individual carriers. In Table 3 the *in situ* lactose uptake rate is compared to the lactose consumption rate calculated from the apparent cell yield and the dilution rate:

Table 3 Variation of *in situ* uptake of lactose (calculated), lactose consumption rate (observed) and ratios of both as a function of the dilution rate in lactose-limited chemostat cultures of *Kluyveromyces fragilis* at a reservoir lactose concentration of 10 g L^{-1}

D (h^{-1})	V calculated (μmol g cells^{-1} h^{-1})	V observed (μmol g cells^{-1} h^{-1})	V calculated/ V observed
0.12	810	750	1.08
0.16	890	970	0.92
0.25	1350	1400	0.96
0.29	1515	1660	0.91
0.34	1830	1950	0.94
0.40	7680	2340	3.28

$$\text{Specific rate of lactose consumption} = \frac{D}{Y_{X/S}}$$

For D <0.4 h^{-1}, the rate of lactose consumption was near the calculated rate of lactose uptake. At D = 0.4 h^{-1}, the calculated rate of lactose uptake was higher than the rate of lactose consumption. Thus, under these conditions, the limiting step in lactose catabolism is not its transport but its metabolism. This can explain the increase of residual lactose in the vessel.

The existence of three lactose transporters which are dependent on dilution rate in this strain of *Kluyveromyces* is not well established for other species of *Kluyveromyces*. In fact, in most studies where shake flask cultures were used, lactose level, a parameter controlling the biosynthesis of lactose carriers, was not controlled. Boze *et al* [4] reported that the transport of lactose into *Kluyveromyces lactis* CBS 2359 is carried out through a unique transporter, a permease with a K_m of 2 mM. Dickson and Barr [5] showed that lactose enters *Kluyveromyces lactis* only through a unique transporter characterized by a K_m of 2.8 mM.

Only Antier [1] found evidence for the existence of two lactose carriers in *Kluyveromyces fragilis* strain 111 in a continuous culture. These lactose carriers had a K_m varying from 0.92 to 1.69 mM and a V_{max} of 12 μmole g cells^{-1} min^{-1} for the first transporter, and a constant affinity ranging from 8.8 to 11.7 mM and a V_{max} of 19 μmole g cells^{-1} min^{-1} for the second transporter. They were also present at all dilution rates.

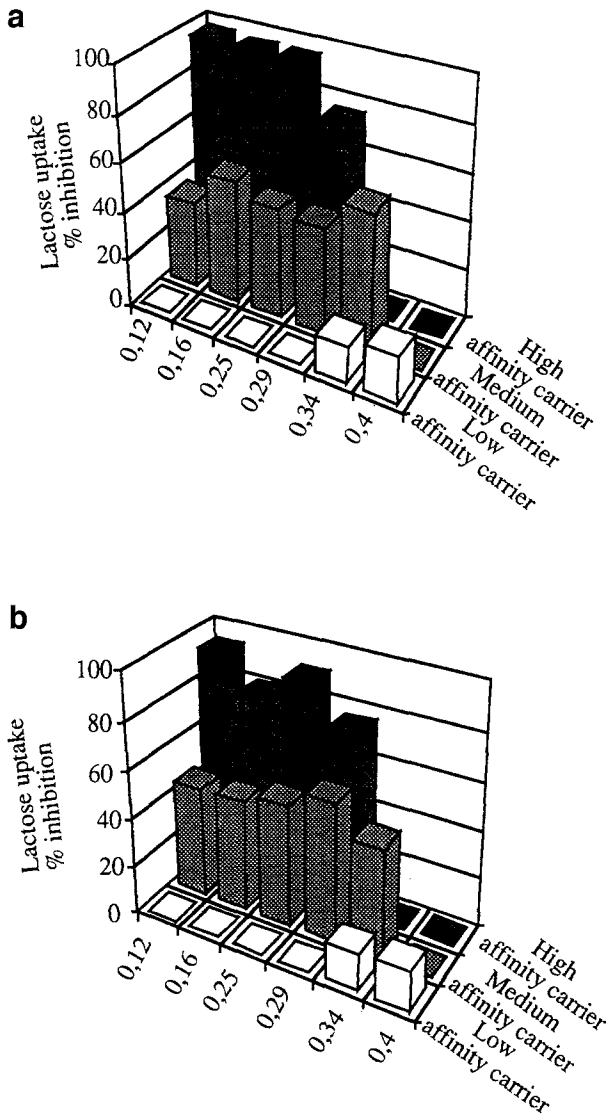

Figure 1 Effects of CCCP (**a**) and Antimycin A (**b**) on the carriers identified in *Kluyveromyces fragilis* grown continuously on diluted whey permeate

Influence of metabolic inhibitors on lactose carriers in Kluyveromyces fragilis

Metabolic inhibitors are useful tools to study the mechanism of transport. We studied the influence of Antimycin A, an energy inhibitor, and CCCP, a proton ionophore, on the three lactose transporters. Cells harvested from continuous cultures grown at different dilution rates were incubated in the presence of Antimycin A or CCCP at lactose concentrations between 0.5 and 100 mM. Percentages of inhibition of lactose transport by each inhibitor for each transporter are presented in Figure 1.

Figure 1a shows that CCCP, at a concentration of 100 μM, inhibited lactose transport via the high affinity transport system from 98 to 82%. The effect of CCCP on the second lactose transporter was lower: inhibition varied from 36 to 56%. Inhibition of lactose transport via the third, low affinity carrier was very low. Since CCCP prevents the formation of a proton gradient across the plasma membrane, the results obtained indicate that lactose transport through the high affinity carrier is certainly coupled to a proton motive force.

The effect of Antimycin A on the different lactose carriers in *Kluyveromyces fragilis* is presented in Figure 1b. The high affinity system is more sensitive to the action of this inhibitor than the two other carriers. For all the dilution rates where the high affinity transporter was present, Antimycin A caused nearly total inhibition of this carrier. For the medium affinity lactose carrier, this percentage was about 45% whereas for the low affinity transporter the percentage of inhibition of lactose transport was very low. It is well established that Antimycin A inhibits electron flux between cytochrome *b* and c_1, hence it prevents the synthesis of ATP. The inhibition of the high affinity carrier in the presence of Antimycin A shows that lactose transport via this carrier requires synthesis of ATP which is necessary for creation of the proton motive force at the plasmic membrane. These results indicate that lactose transport through the high affinity system is probably carried out by active transport through a proton-lactose symporter.

Lactose transport via the medium affinity system is also coupled to utilization of energy, whereas the transport by the low affinity transporter is probably carried out by facilitated diffusion since the effects of the metabolic inhibitors were negligible.

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