Induction of extracellular β -galactosidase (Bga1) formation by D-galactose in *Hypocrea jecorina* is mediated by galactitol

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The ability of *Hypocrea jecorina* (*Trichoderma reesei*) to grow on lactose strongly depends on the formation of an extracellular glycoside hydrolase (GH) family 35 β -galactosidase, encoded by the *bga1* gene. Previous studies, using batch or transfer cultures of pregrown cells, had shown that *bga1* is induced by lactose and D-galactose, but to a lesser extent by galactitol. To test whether the induction level is influenced by the different growth rates attainable on these carbon sources, *bga1* expression was compared in carbon-limited chemostat cultivations at defined dilution (=specific growth) rates. The data showed that *bga1* expression by lactose, D-galactose and galactitol positively correlated with the dilution rate, and that galactitol and D-galactose induced the highest activities of β -galactosidase at comparable growth rates. To know more about the actual inducer for β -galactosidase formation, its expression in *H. jecorina* strains impaired in the first steps of the two D-galactose-degrading pathways was compared. Induction by D-galactose and galactitol was still found in strains deleted in the galactokinase-encoding gene *gal1*, which is responsible for the first step of the Leloir pathway of D-galactose catabolism. However, in a strain deleted in the aldose/D-xylose reductase gene *xy1*, which performs the reduction of D-galactose to galactitol in a recently identified second pathway, induction by D-galactose, but not by galactitol, was impaired. On the other hand, induction by D-galactose and galactitol was not affected in an L-arabinitol 4-dehydrogenase (*lad1*)-deleted strain which is impaired in the subsequent step of galactitol degradation. These results indicate that galactitol is the actual inducer of Bga1 formation during growth on D-galactose in *H. jecorina*.

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INTRODUCTION

β -Galactosidase (EC 3.2.1.23) catalyses the hydrolysis of terminal non-reducing β -D-galactose residues in β -D-galactosides, including the disaccharide lactose (1,4-O- β -D-galactopyranosyl-D-glucose). Such enzymes can be classified as members of four different glycoside hydrolase (GH) families [GH1, GH2, GH35 and GH42; Carbohydrate Active Enzymes database: <http://afmb.cnrs-mrs.fr/CAZY/> (Coutinho & Henrissat, 1999)], indicating a structural and maybe also functional diversity. In biotechnology, β -galactosidases are mainly employed for the hydrolysis of lactose to D-glucose and D-galactose in various products of the dairy industry (Gekas & Lopez-Leiva, 1985; Holsinger & Kligerman, 1991). In addition, β -galactosidases catalyse transgalactosylation reactions, which have attracted considerable interest because of suggested beneficial effects on

human health (Boon *et al.*, 2000; Nakayama & Amachi, 1999).

The filamentous fungus *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) is a potent producer of cellulolytic and hemicellulolytic enzymes (Penttilä *et al.*, 2004; Persson *et al.*, 1991). Lactose induces the formation of these enzymes and is today the major soluble carbon source for their production on a technical scale. Biochemical and genetic evidence is available that *H. jecorina* initiates lactose metabolism by first hydrolysing it extracellularly, and then taking up and metabolizing the hydrolysis products D-glucose and D-galactose, thus placing the extracellular β -galactosidase in a central position for lactose metabolism in this fungus. *H. jecorina* contains one major extracellular GH family 35 β -galactosidase (Seiboth *et al.*, 2005). In $\Delta bga1$ strains, only minor β -galactosidase activities are found, either cell wall bound or in the supernatant, but no intracellular β -galactosidase activity is detectable during growth on lactose.

Abbreviations: D, dilution rate; GH, glycoside hydrolase.

Regulation of Bga1 formation is less well understood. The *bga1* transcript is most abundant during growth of *H. jecorina* on L-arabinose and L-arabinitol. Lower levels are present on lactose, but also on D-galactose and D-xylose, and their corresponding polyols. In addition, *bga1* transcription is subject to Cre1-dependent carbon catabolite repression, which acts at the level of both its basal expression and induction by D-galactose or lactose (Seiboth *et al.*, 2005). The induction by one of the end products of lactose hydrolysis, D-galactose, as well as the hemicellulose monomers D-xylose and L-arabinose, may indicate a cross-talk between the induction of β -galactosidase and hemicellulases, but the mechanisms involved are not yet known.

We have recently provided evidence for a second pathway of D-galactose metabolism in fungi, which proceeds as a stowaway of the fungal D-xylose and L-arabinose catabolic pathway (Fekete *et al.*, 2004; Seiboth *et al.*, 2004; Fig. 1). In this paper, we tested the hypothesis that the operation of this pathway is essential for *bga1* induction by D-galactose.

METHODS

Fungal strains and cultivation conditions. *H. jecorina* strains QM9414 (ATCC 26921), $\Delta bga1$ (Seiboth *et al.*, 2005), $\Delta gal1$ (Seiboth *et al.*, 2004), $\Delta lad1$ (Pail *et al.*, 2004) and $\Delta xyl1$ (B. Seiboth and others, unpublished results) were maintained on malt extract agar. Strains were grown in 500 ml Erlenmeyer flasks on a rotary shaker (250 r.p.m.) at 30 °C in the following medium (pH 5.0): 8 g $\text{NH}_4\text{H}_2\text{PO}_4$ l⁻¹, 7 g Na_2HPO_4 l⁻¹, 4 g KH_2PO_4 l⁻¹, 1 g CaCl_2 l⁻¹, 1 g MgSO_4 l⁻¹, 0.1 g peptone l⁻¹, 20 ml trace elements l⁻¹ (250 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ l⁻¹, 80 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ l⁻¹, 70 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ l⁻¹, 85 mg CoCl_2 l⁻¹) with 10 g l⁻¹ of the respective carbon source.

For chemostat cultivations, cultures were pre-grown with glycerol (10 g l⁻¹) as carbon source, and the content (2 × 100 ml) of two exponential-phase shake-flask cultures was used as inoculum. Mycelia were harvested by filtration after 36 h, washed with cold tap water and then transferred into fresh medium with the respective carbon source without peptone. Chemostat cultivations were carried out in a 2.5 l glass bioreactor (Inel) with a working volume of 2 l, equipped with one six-blade Rushton disc turbine impeller. The operating conditions were pH 5.0, 30 °C, 300 r.p.m. and 0.3 volumes of air per volume of liquid per minute (v.v.m.). To minimize water and substrate losses, the waste gas was cooled in a reflux condenser connected to an external cooling bath (4 °C). The feeding medium contained 3 g l⁻¹ carbon source, a concentration low enough to facilitate carbon-limited cultivation. Glass parts of the reactor were treated with the anti-adhesive agent Sigmacot (Sigma) to avoid fungal growth on reactor walls, and a few drops of the antifoam agent polypropylene glycol 2000 (Union Carbide Chemicals and Plastics) were injected daily into the reactor through a membrane filter (Millipore). Onset of steady state in the culture was established when no changes in biomass dry weight were observed in three successive samples taken over a period of three residence times [defined as the reciprocal of the dilution rate (*D*), e.g. 20 h for *D* = 0.050 h⁻¹].

For transfer cultures, *H. jecorina* strains were pregrown and washed as described above, and transferred into fresh medium supplemented with 10 g l⁻¹ of the respective carbon source without peptone. After 14 h, maximal induction levels of β -galactosidase were found. At this time point, the residual carbon-source concentration in the medium was between 6.5 and 8.0 g l⁻¹.

Biomass determination. Mycelial dry weight was determined by twice withdrawing 20 ml aliquots from the medium, suction filtration through a glass-wool filter, and drying to a constant weight at 80 °C. Data were averaged, and deviated by no more than 14 %. In the case of batch cultures, 5 ml aliquots twice were used.

β -Galactosidase assay. β -Galactosidase activity was determined with ONPG as substrate. The supernatant was diluted to 700 μ l with

Fig. 1. The Leloir pathway and the second (alternative) pathway for D-galactose catabolism. Results from *H. jecorina* (Pail *et al.*, 2004; Seiboth *et al.*, 2004) and *A. nidulans* (Fekete *et al.*, 2004) were combined to draft the second hypothetical pathway for D-galactose degradation. Question marks indicate step(s) which have still to be verified.

0.1 M phosphate buffer (pH 5) and added to 300 μ l 10 mM ONPG. After incubating for 10–60 min at 30 °C, the reaction was stopped by the addition of 2 ml 1 M Na_2CO_3 . A_{405} was measured against a blank in which the reaction had been stopped at $t=0$. Enzyme activities were expressed as nkat ($\text{mg dry weight}^{-1}$). One nanokatal corresponds to the conversion of one nanomole of substrate per second under given conditions.

HPLC analysis. The concentrations of D-galactose, D-glucose, galactitol and lactose in the medium were determined by HPLC analysis, using the H^+ exchange column Aminex HPX- H^+ (Bio-Rad). As mobile phase, 10 mM H_2SO_4 at 55 °C was employed, with isocratic elution and refractive index detection.

Reproducibility. Results are the means of three to five measurements. The data were analysed by Sigmaplot (SPSS) and SDs determined. The SD values were always less than 14 % of the mean.

RESULTS

Extracellular β -galactosidase activity as a reporter for *bga1* gene expression in *H. jecorina*

In our previous work, we have shown that deletion of *bga1* reduces the overall extracellular β -galactosidase activity in the culture supernatant by over 90 % in batch and transfer cultures, thereby providing evidence that the *bga1*-encoded gene product is the main extracellular β -galactosidase in *H. jecorina* on lactose (Seiboth *et al.*, 2005). In order to see whether this conclusion was also valid for the other carbon sources used in this study, we recorded the extracellular β -galactosidase activity [$\text{nkat} (\text{mg dry weight})^{-1}$] of the parental *H. jecorina* QM9414 and a $\Delta bga1$ strain on D-galactose and galactitol over the whole period of a batch cultivation for about 80 h. At each time point, the $\Delta bga1$ mutant exhibited a β -galactosidase activity only 4–8 % of that of QM9414. Similar reductions of β -galactosidase activity for the $\Delta bga1$ strain were later also found in chemostat cultures at $D=0.075$ and 0.030 h^{-1} under D-galactose- and galactitol-limiting conditions, respectively. We therefore consider the appearance of the extracellular β -galactosidase activity in the medium as a reliable reporter for *bga1* expression in *H. jecorina*, and we employed this approach throughout the study.

Growth-rate-dependent β -galactosidase formation on lactose and D-galactose

As the carbon sources (lactose, D-galactose and galactitol) that induce β -galactosidase result in strongly different growth rates in batch cultures, we investigated if these varying growth rates influenced the expression level of the β -galactosidase. Accordingly, chemostat cultivations at five defined dilution rates were performed between 0.075 and 0.015 h^{-1} under D-galactose- or lactose-limiting conditions. These dilution rates were identical to those used in our earlier study on the regulation of cellulases by *H. jecorina* (Karaffa *et al.*, 2006). As reported previously, four to five residence times were needed to achieve steady state under these conditions. The residual steady-state concentrations of

D-galactose and lactose in the medium were 0.10–0.12 mM and 0.09–0.12 mM, respectively, and the steady-state biomass concentration was $1.48 \pm 0.20 \text{ g l}^{-1}$ for both carbon sources. Table 1 shows that D-galactose-supported growth resulted in a significantly higher β -galactosidase activity than lactose-supported growth at the same growth rate ($0.015\text{--}0.042 \text{ h}^{-1}$). Higher dilution rates on lactose led to washing out of the mycelia and were only achievable with D-galactose as carbon source. A D-galactose-grown culture at $D=0.075 \text{ h}^{-1}$ yielded an activity of 100 nkat ($\text{mg dry weight}^{-1}$), which was about a fivefold increase over the activity obtained at the lowest dilution rate. This indicates that β -galactosidase induction by D-galactose is growth-rate associated in *H. jecorina*.

Growth-rate-dependent β -galactosidase formation on galactitol

We have previously found that galactitol also induces *bga1* gene transcription, although to a lower degree than D-galactose (Seiboth *et al.*, 2005). However, galactitol is a poor carbon source for *H. jecorina*, and results in only slow growth rates, thus making a quantitative comparison of these results difficult. Consequently, we performed chemostat cultivations at defined growth rates with galactitol as the limiting carbon source. As previously reported (Karaffa *et al.*, 2006), galactitol only allows the use of dilution rates between 0.03 and 0.05 h^{-1} . The steady-state biomass concentration was somewhat lower ($1.36 \pm 0.17 \text{ g l}^{-1}$) than on D-galactose or lactose, and the residual steady-state concentration of galactitol was 0.08–0.09 mM. At the three dilution rates employed, however, β -galactosidase activities were even higher than on D-galactose, and increased with increasing dilution rate (Table 1), again indicating the growth-rate-associated nature of the extracellular β -galactosidase formation in *H. jecorina*. Performing the same chemostat cultivations with an equimolar mixture of D-galactose and galactitol resulted in slightly reduced β -galactosidase activities at dilution rates

Table 1. Extracellular β -galactosidase activity in the supernatant of carbon-limited chemostat cultures of *H. jecorina* QM9414 at different dilution rates

WO, Washing out of the mycelia; NA, at this dilution rate no stable steady state was achievable.

$D \text{ (h}^{-1}\text{)}$	Extracellular β -galactosidase activity [$\text{nkat} (\text{mg dry weight})^{-1}$]			
	Lactose	D-Galactose	Galactitol	D-Galactose + Galactitol
0.075	WO	100	WO	WO
0.050	WO	93	115	101
0.042	75	87	110	97
0.030	48	67	83	77
0.015	15	27	NA	NA

of 0.03 and 0.042 h⁻¹ than with galactitol alone. We conclude that galactitol, at the same growth rate, can induce β -galactosidase formation at least as well as D-galactose.

Growth-rate-dependent β -galactosidase formation on D-glucose

Growth-rate-dependent β -galactosidase formation could be in conflict with our earlier observations on regulation by the carbon catabolite repressor Cre1. In order to investigate whether carbon catabolite repression in fact influences β -galactosidase formation by *H. jecorina*, we performed chemostat cultivations with D-glucose as the limiting nutrient at low dilution rates. Such a condition has recently been shown to cause carbon catabolite derepression in *H. jecorina* (Karaffa *et al.*, 2006) and *Aspergillus nidulans* (Ilyés *et al.*, 2004). β -Galactosidase activity was found at $D=0.015\text{ h}^{-1}$, which corresponded to about 55 and 30 % of that observed at the same growth rate on lactose and D-galactose, respectively. In contrast to the results obtained with D-galactose or lactose, no β -galactosidase activity was detected with D-glucose as a carbon source at $D=0.03\text{ h}^{-1}$ or higher (Table 2). The residual steady-state biomass concentration was $1.48\pm0.20\text{ g l}^{-1}$ and the D-glucose concentration was 0.08–0.10 mM. Chemostat cultivation on an equimolar mixture of D-glucose and D-galactose resulted in even higher β -galactosidase activities at a dilution rate of 0.015 h^{-1} , and still noticeable activities at 0.03 h^{-1} , but no activity at all at higher dilution rates (Table 2), indicating that D-glucose interferes with the effect of D-galactose. Thus, the presence of D-glucose, consistent with our previous findings (Seiboth *et al.*, 2005), clearly exerts carbon catabolite repression at higher growth rates; however, it aids in the induction by D-galactose at carbon-catabolite-derepressing growth rates.

β -Galactosidase formation is induced via the second pathway of D-galactose catabolism

In theory, D-galactose and galactitol can be catabolized via either the Leloir pathway or the second pathway of D-galactose degradation (Fig. 1). The enzyme catalysing the

reversible reduction/oxidation of D-galactose/galactitol is the *xyl1*-encoded aldose/D-xylene reductase (B. Seiboth and others, unpublished results). In order to investigate whether D-galactose itself is the inducer for β -galactosidase expression or the inducer is formed within one of these two pathways, we tested the induction by D-galactose and galactitol in strains which are defective in the first step of the Leloir pathway ($\Delta gal1$) or the second pathway of D-galactose degradation ($\Delta xyl1$). Since these gene deletions lead to an altered maximal growth rate of the investigated strains on the two carbon sources, and in addition it is to be expected that these deletions will also change the composition of the intracellular accumulating metabolites of the two D-galactose pathways, a quantitative comparison of β -galactosidase activity based on chemostat cultures could not be performed. Therefore, the following experiments were done in transfer cultures. As can be seen from Table 3, in a $\Delta xyl1$ strain induction of β -galactosidase activity during growth on galactitol reached the same level as in the parent strain QM9414, thus ruling out that catabolism of galactitol via the Leloir pathway would be necessary for the induction. In contrast, almost no β -galactosidase activity was produced during growth on D-galactose by the $\Delta xyl1$ strain, indicating that the D-galactose to galactitol conversion is essential for this process. From these results it can also be excluded that the Xyl1 protein – apart from its enzymic conversion of D-galactose to galactitol – is needed for the induction process, because growth on galactitol alone induced β -galactosidase in a $\Delta xyl1$ strain. This effect was also not due to a general interference of this deletion with growth or D-galactose metabolism, because the $\Delta xyl1$ mutant of *H. jecorina* was able to grow on both galactitol and D-galactose. A *H. jecorina* $\Delta gal1$ (galactokinase) strain induced β -galactosidase activity on galactitol and – albeit with lower efficacy – on D-galactose, thus ruling out that the galactokinase itself or a metabolite formed in the Leloir pathway was necessary for the induction. The fact that the β -galactosidase activity formation on D-galactose was decreased in the $\Delta gal1$ strain relative to QM9414 is most likely due to the strongly reduced growth rate of this strain (Seiboth *et al.*, 2004). Taken together, these data indicate that D-galactose

Table 2. Extracellular β -galactosidase activity in the supernatant of D-glucose- and D-glucose+D-galactose-limited chemostat cultures of *H. jecorina* QM9414 at different dilution rates

<i>D</i> (h ⁻¹)	Extracellular β -galactosidase activity [nkat (mg dry weight) ⁻¹]	
	D-Glucose	D-Galactose + D-Glucose
0.075	0	0
0.050	0	0
0.042	0	0
0.030	0	8
0.015	8	32

Table 3. Extracellular β -galactosidase activity formed by *H. jecorina* strains impaired in different steps of the two D-galactose-catabolizing pathways after 14 h of growth in transfer cultures

<i>H. jecorina</i> strains	Extracellular β -galactosidase activity [nkat (mg dry weight) ⁻¹]		
	D-Galactose	Galactitol	D-Glucose
QM9414	68	83	<0.1
$\Delta xyl1$	2	53	<0.1
$\Delta gal1$	20	70	<0.1
$\Delta lad1$	60	115	<0.1

metabolism via the Leloir pathway is dispensable for β -galactosidase induction by D-galactose in *H. jecorina*.

Galactitol is the inducer of β -galactosidase formation in *H. jecorina*

The L-arabinitol 4-dehydrogenase *Lad1* is the second enzyme in the further catabolism of galactitol in the second D-galactose-degrading pathway (Pail *et al.*, 2004). In order to discriminate whether galactitol itself or a further catabolite formed in the later part of this pathway is responsible for the induction of the extracellular β -galactosidase, we made use of a $\Delta lad1$ strain: this strain does not grow on galactitol but exhibits only a slightly reduced growth on D-galactose. Table 3 shows that β -galactosidase activity formation was basically similar to that of QM9414, except that it was even higher on galactitol. This result, together with those obtained with the $\Delta xyl1$ and the $\Delta gal1$ strains, clearly indicates that galactitol itself, formed via the reduction of D-galactose, is able to induce the expression of *bga1* in *H. jecorina*.

DISCUSSION

In this paper, we have provided evidence that induction of β -galactosidase-encoding *bga1* on lactose and D-galactose in *H. jecorina* is in fact caused by galactitol, thereby partially revising and extending some of our earlier findings. By employing deletion strains in the aldose/D-xylose reductase- and galactokinase-encoding genes, we were able to show that D-galactose conversion to galactitol by the *xyl1*-encoded aldose reductase is obligatory for the expression of *bga1*. By the aid of a $\Delta lad1$ strain, which is impaired in the further conversion of galactitol, we additionally showed that the further steps in galactitol catabolism are dispensable for *bga1* induction on D-galactose. The previously reported low efficacy of galactitol to induce β -galactosidase in transfer cultures may therefore be related to the difficulty for the fungal cell to efficiently metabolize this polyol, as evidenced by the higher maintenance energy on galactitol than on D-galactose (Karaffa *et al.*, 2006) and the in general poor biomass formation on galactitol (Druzhinina *et al.*, 2006). In any case, our data show that it is important to use comparable growth rates when comparing different carbon compounds for their inducing potential. Induction of β -galactosidase by galactitol further indicates that the alternative pathway of D-galactose degradation is apparently of major importance for the degradation of lactose by *H. jecorina*. We have recently discussed the fact that this may be due to the necessity of catabolizing the β anomer of D-galactose, which arises from lactose hydrolysis by the action of *Bga1* (Karaffa *et al.*, 2006).

For *A. nidulans* it has previously been shown that induction of the intracellular β -galactosidase activity by D-galactose also occurs in the galactokinase mutant *galE* and therefore does not require D-galactose phosphorylation. Accordingly, Fantes & Roberts (1973) and Fekete *et al.* (2002) speculated that the inducer for intracellular β -galactosidase expression

was produced via an at that time unknown alternative pathway for D-galactose catabolism. It still remains to be proven that galactitol is indeed the actual inducer for this β -galactosidase also, but support for this assumption comes from the fact that the intracellular β -galactosidase activity is not affected in the *A. nidulans* L-arabinitol 4-dehydrogenase mutant *AraA1* during growth on D-galactose (E. Fekete & L. Karaffa, unpublished results). It is noteworthy that the intracellular *A. nidulans* β -galactosidase belongs to the GH2 family, while the *H. jecorina* *Bga1* is a GH35 family member. In contrast to the induction by galactitol, it has been reported that the *Kluyveromyces lactis* GH2 family β -galactosidase is induced by D-galactose (Cardinali *et al.*, 1997). The reason for this might be that yeasts, including *Saccharomyces cerevisiae* and *K. lactis*, use the Leloir pathway exclusively for D-galactose catabolism (Frey, 1996).

β -Galactosidases can also be induced by L-arabinose and L-arabinitol, and this cross-induction is also observed for several glycosidases acting on α - as well as β -linked carbohydrates (de Vries *et al.*, 1999a, 2002; Kristufek *et al.*, 1994; Nikolaev & Vinetski, 1998; Seiboth *et al.*, 2005). Earlier authors have suggested that this may be due to the stereochemical similarity of L-arabinose and D-galactose. However, in view of the present results, it is also likely that the fungus catabolizes the different monomeric components (L-arabinose, D-galactose and D-xylose) released from the heterogeneous hemicelluloses via the same (i.e. the interconnected L-arabinose and D-xylose) pathway, and therefore ensures that the different inducers can be formed within this pathway to induce the full array of enzyme activities needed to degrade these complex hemicelluloses. It should be noted that lactose does not occur in the natural environment of the saprobic ascomycete *H. jecorina*. It is more likely that plant polysaccharides with β -galactosidic linkages such as L-arabino-D-(- β -)-galactans are the original substrates for the β -galactosidase. If true, a cross-induction mechanism such as this would have clear advantages. The ability of galactitol to induce α -galactosidase also (Kristufek *et al.*, 1994) would support this hypothesis. However, while galactitol does induce α -galactosidase, we must admit that the involvement of the second pathway for D-galactose catabolism in this induction has not yet been proven, and may in theory still depend on its oxidation back to D-galactose.

In a similar manner to *A. nidulans*, the growth rate on D-glucose apparently determines carbon catabolite repression in *H. jecorina* also, and below a certain growth rate, carbon catabolite derepression occurs (Ilyés *et al.*, 2004). Importantly, while an equimolar mixture of D-galactose and D-glucose at repressing growth rates resulted in the repression of *bga1*, their combined effect at derepressing (e.g. very low) growth rates resulted in the cumulative formation of extracellular β -galactosidase activity. This observation underlines our previous claim (Seiboth *et al.*, 2005) that the carbon catabolite repressor Cre1 (Strauss *et al.*, 1995) exerts its influence at two distinct levels, e.g. at the basal and the induced level of *bga1* expression.

Interference of carbon catabolite regulation with gene expression at multiple regulatory levels is not unknown in fungi, and such 'double-lock mechanisms' have already been reported in *A. nidulans* for *alcA* regulation (Mathieu & Felenbok, 1994) and β -galactosidase formation (Fekete *et al.*, 2002), and also in *Aspergillus niger* for xylanase biosynthesis (de Vries *et al.*, 1999b). Chemostat-type continuous cultivations (Ilyés *et al.*, 2004; Karaffa *et al.*, 2006; Pakula *et al.*, 2005) are a useful means to investigate such interfering mechanisms.

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