# Ph.D. Thesis

# CHARACTERIZATION AND REGULATION OF LACTOSE AND GALACTOSE METABOLISM IN THE FUNGUS *ASPERGILLUS NIDULANS*

by

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## INTRODUCTION TO THE SCIENTIFIC PROBLEM

For a vast array of microbes, carbon response mechanisms ensure that catabolism of a rapidly metabolized carbon source like D-glucose results in severe repression of genes encoding enzymes for the degradation of alternative carbon sources. As a consequence, enzymes required for the utilization of less favourable carbon sources are often synthetized at low rates or not at all. The phenomenon is known as carbon catabolite regulation or repression (CCR). The presence of a high concentration of a repressing growth substrate, not restricted to D-glucose, is believed to be necessary to trigger the CCR response.

In filamentous fungi, glucose repression is mediated by the C2H2-type zinc finger protein CreA, encoded by the *creA* gene. Binding specificity of CreA has been evaluated for various promoters, all repressible by glucose. Deletion of *creA* was thought to be letal, but this is now dismissed by more recent reports on viable fungal nullmutants (Shroff et al. 1997).

Apart from the regulation of carbon catabolism, glucose also hinders the transcription of a number of genes including those responsible for the synthesis of commercially important metabolites (antibiotics, amino acids, nucleotides, enzymes). In some cases, application of advanced fermentation technology like limited glucose feed could bypass this effect, but several industrial processes (cellulase, hemicellulase production) are still based on employing a slowly metabolised (derepressing) carbon source in the idiophase.

On technical scale, lactose is the most widely used derepressing carbon source. The disaccharide lactose (1,4-0-ß-D-galactopyranosyl-D-glucose) is a byproduct of cheese production (thus is a renewable carbon source), which accumulates to amounts of 300 000 tons per year worldwide, of which only 15% is used for various microbial fermentations (Roelfsema et al. 1990).

The first step of lactose metabolism is its hydrolysis to glucose and galactose by either an extracellular β-galactosidase or a combination of a lactose permease and an intracellular ß-galactosidase; subsequent galactose metabolism

involves the coordinate operation of at least five enzymes, namely galactokinase, galactose-1-phosphate-UDP-transferase, UDP-glucose epimerase, UDP-glucose pyrophosphorylase and phosphoglucomutase (LeLoir-pathway). The resulting glucose-6-phosphate is further oxidised via the glycolytic pathway.

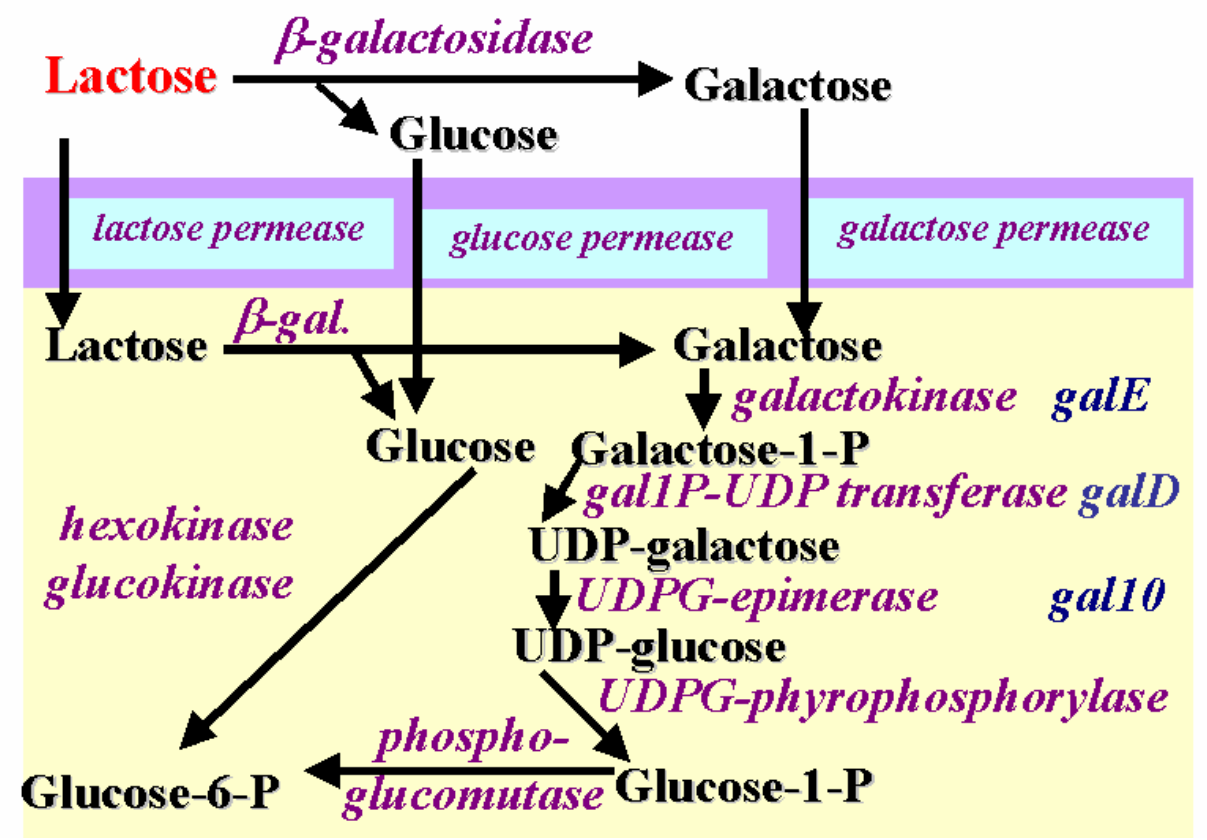


**Figure 1.** General representation of lactose metabolism

Although lactose has been employed in the fermentation industry for over five decades now (penicillin production), no research group has ever studied fungal lactose metabolism in details. Since *Aspergillus nidulans* (teleomorf: *Emericella nidulans*) is long considered as a model species of *Ascomycetes* (Arst and Penalva 2003), this fungus was chosen as a subject of our research.

## MATERIALS AND METHODS

**Maintenance:** Strains were maintained on AMM medium supplemented with the necessary markers (Pontecorvo et al. 1953). Vegetative mycelia were stored at -80 °C in a glycerol solution. For daily use, strains were maintained on agar slopes containing a complex medium that stimulates sporulation (Fekete et al. 2002). Conidiospores were formed at 37 °C, then slopes were sealed and kept at +4 °C. Strains used in this work are listed at **Table I**.

**Table I. List of *A. nidulans* strains used in this study**

| *genotype* (vis~vis lactose metabolism) | phenotype (vis~vis lactose metabolism) | auxotrophy | Colour of conidia | Reference |
|---|---|---|---|---|
| R21 | Wild-type | PABA | yellow | Roberts (1967) |
| A60 *galA1* | Non-inducible galactokinase and gal-1-P uridylyltransferase | Biotin | white | Roberts (1963) |
| A61 *galD5* | gal-1-P uridylyltransferase deficient | Biotin | white | Roberts (1963) |
| A214 *galE9* | galactokinase deficient | Biotin | white | Roberts (1963) |
| A466 g*alD5* | gal-1-P uridylyltransferase deficient | PABA riboflavin Aneurine Piridoxine Lysine | yellow | Käfer (1977) |
| A500 *sbA3* | Deficient in sorbitol utilization | Biotin Methionin Nikotinic acid | green | Käfer (1977) |
| G092 *frA1* | Deficient in fructokinase | Piridoxin | yellow | Roberts (1963) |
| G094 *araA1* | Deficient in arabitol utilization | Biotin | white | Roberts (1963) |
| - *CreAΔ4* | CreA nullmutant | PABA Arginine | yellow | Shroff et al. (1997) |
| - *CreA$^{d}$30* | *creA* mutant | Biotin | green | Arst et al. (1990) |

| | | | | |
|---|---|---|---|---|
| EFES1<br>*galD5, galE9* | A466 x A214 double mutant (G-1-P-UT-ase and galactokinase deficient) | PABA<br>riboflavin<br>Aneurin<br>Pyridoxine<br>Lysine | white | Fekete et al. (2002) |
| EFES2<br>*galE9* | R21 x A214 double mutant (galactokinase deficient) | PABA | Sárga | Fekete et al. (2004) |
| EFES3<br>*frA1, galE9* | A214 x G092 double mutant (galactokinase and fructokinase deficient) | Pyridoxine | Sárga | Fekete et al. (2004) |
| EFES4<br>*araA1, galE9* | EFES2 x G094 double mutant (galactokinase and arabitol utilization deficiency) | Biotin | white | Fekete et al. (2004) |

'**A**' indicates strains from the Fungal Genetics Stock Center (FGSC; University of Kansas Medical Center, USA), '**G**' indicates strains from the Glasgow Center (University of Glasgow, Scotland, UK). **'EFES'** is the acronym for „**E**rzsébet **F**ekete, **E**rzsébet **S**ándor" – those strains were created in our lab.

**Cultivation conditions:** Creation and analysis of double mutants occured on AMM medium solidified with agar. Shake-flask cultivations as well as the batch, fed-batch and continuous flow fermentations happened on AMM2 medium (Fekete et al. 2002). For submerged cultivations, 500 ml Erlenmeyer-flasks or 2 l laboratory fermenters were used. Induction experiments were carried out using mycelia preformed on AMM2 supplemented with glycerol, a carbon source considered „neutral" for the lactose and galactose metabolism (Seiboth et al. 2002).

**Analytical methods**: Growth was followed as Dry Cell Weight (DCW) per medium volume. Determination of carbohydrates (sugars, polyols) were carried out by HPLC using refractive index detection. Intracellular metabolite concentrations were calculated by estimating 2.43 ml intracellular volume for 1 g of mycelial DCW (Slayman and Tatum 1964). The NMR experiments were performed on a Bruker Avance 360 spectrometer with QNP ($^{1}H/^{15}N/^{13}C/^{31}P$)

probe head. When $^{13}C$ NMR spectra were taken on low sample amounts, only the protonated carbons were measured by DEPT135 (90 degree pulse was 17 μses) pulse sequence in Waltz16 composite pulse decoupling mode. For the determination of the configuration of sorbose, optical rotations were measured in water as solvent using the D line of sodium light at 589 nm. For lactose uptake measurements, [D-glucose-1-$^{14}C$] lactose was used with samples being analysed with a scintillation spectrometer. Total protease enzyme activity was determined by the method of Rudolph *et al*. (1949). The first step in the determination of any intracellular enzyme activity is cell disruption, which was performed with an X-press equipment. Beta-galactosidase activity was defined as the potential of o-nitrophenyl-β-D-galactopiranozid hydrolysis. Polyol (L-arabitol, galactitol, D-sorbitol) dehydrogenase activity was measured using the rate-assay technique which defines reaction velocity under the assay conditions. To determine the end-product of galactitol dehydrogenase reaction, a coenzyme-regeneration system of L-lactic dehydrogenase and pyruvate were included in the assay. The supernatant containing the end-product was used for HPLC and NMR analysis. In case of the latter, ten separate 1 ml reactions were made and the pooled supernatants concentrated to dryness by evaporiation. The end-product was confirmed by performing a "reverse" (backward) reaction *in vitro*. Hexokinase activities were determined by HPLC, after incubating the substrate with ATP and the enzyme source. Aldose reductase activity was measured upon using L-arabinose and D-xylose as substrates in the rate assay that based on monitoring NADPH concentration in the reaction mixture. Specific enzyme activities are related to mg protein, which was determined by a modified Lowry method (Peterson 1983), using BSA for calibration.

**Creation of double mutants**: *A. nidulans* EFES2 (a *galE* mutant with a *yA2* colour marker) was obtained by crossing strains A214 (carrying *galE* mutation) and R21 (wild-type). *A. nidulans* EFES3 (carrying a *galE* and *frA1* double mutation), and EFES4 (carrying a *galE* and *araA1* double mutation) were

obtained by crossing strains EFES2 and G092 and strains EFES2 and G094, respectively. Crossing and selection of progenies were performed using standard techniques (Pontecorvo et al. 1953).

**Reproducibility**: All the results presented are means of 3-5 independent experiments. Some data within one experiment were already coming from averaged measurements. The data were analysed and visualized by Sigmaplot (Jandel Scientific), and standard deviations (SDs) for each procedure were determined. The SD values were always less than 14 % of the means, but were between 7-10 % during enzyme activity determinations and below 5 % during HPLC and isotop techniques.

## RESULTS

### I. New method for the determination of galactokinase activity

A major achievement of ours was the development of a novel method for determining specific galactokinase activity. It is based on the quantitative determination of D-galactose-1-P that is released in the following reaction

$$\text{D-galactose} + \text{ATP} \rightarrow \text{D-galactose-1-P} + \text{ADP}$$

Galactose-1-P is determined by HPLC using a Merck Polyspher OA KC column, refractive index detection, 25 mM $H_2SO_4$ as an eluent, 0.5 ml / min elution rate and T=30 °C (Fekete et al. 2002).

### II. Lactose utilization in *Aspergillus nidulans*

Wild-type *A. nidulans* grew rather well on lactose. In a submerged AMM2 medium containing ammonium-phosphate as a nitrogen source, 15 g/l lactose was consumed within approximately 70 hours time. Similarly, it took three days for the fungus to sporulate on plates. It was therefore concluded that *A. nidulans* is able to utilize lactose as a carbon source.

While no extracellular or cell-wall bound beta-galactosidase activity was detected in any stage of the cultivation, intracellular activity was present throughout. *In silico* studies using the beta-galactosidase sequence of the yeast *Kluyveromyces lactis* obtained from Entrez-database (www.ncbi.nlm.nih.gov), BLAST search was carried out into one of the free *N. crassa* databases (www-genome.wi.mit.edu). Two homologue sequences detected were BLAST-ed into the Cereon database of *A. nidulans* (microbial.cereon.com). Two homologues were found again, that were BLAST-ed into a non-species dependent Entrez database. The first of the two sequences (1309 bp) is identical to the *K. lactis – N. crassa* beta-galactosidase (e-106), while the other is 1.554 bp long and highly similar to the beta-galactosidase sequence of certain bacteria (*Thermotoga neapolitana*, *Caldicellulosiruptor lactoaceticus*, *Arthrobacter sp*. [e-112; e-141; e-130, respectively]). They contain none of the known signal sequences thereby indirectly confirming our conclusion on the localization of beta-galactosidase activity in *A. nidulans*.

**III. Formation of the beta-galactosidase activity in *A. nidulans***

*A. nidulans* beta-galactosidase activity was not detected on glucose or glycerol, but was clearly present in mycelia grown on lactose or D-galactose. Beta-galactosidase activity on lactose peaked during the phase of fast growth and rapid carbon substrate consumption and declined thereafter. Essentially similar results were detected under pH-controlled (pH 6.5) and non-controlled conditions (during which the pH decreased to 3.5). Interestingly, the beta-galactosidase activity of mycelia growing on lactose was lower than that during growth on D-galactose and could be raised further by the addition of D-galactose, suggesting that during growth on lactose enzyme induction is not maximal.

Addition of glucose to lactose-grown cultures resulted in an almost complete disappearance of beta-galactosidase activity within a few hours. Beta-

galactosidase activity began to increase again when about two-thirds of the glucose had been consumed. Uptake of lactose, however, was also suspended as long as glucose was present in the medium, and was reinitiated after the exhaustion of glucose. The rapid disappearance of beta-galactosidase activity in the presence of glucose indicated rapid degradation and a short half-life.

The above results demonstrate that glucose causes an immediate decrease of beta-galactosidase activity, but they do not distinguish whether this is due to inducer exclusion (e.g. by competition with or inhibition of lactose transport by D-glucose) or to true carbon catabolite repression. In order to test whether the carbon catabolite repressor CreA is involved in this phenomenon, we made use of the *A. nidulans* mutant strain CreA.4, in which the *creA* locus had been completely deleted (Shroff et al. 1997). When the glucose-pulse experiment was repeated with this strain, both lactose consumption and beta-galactosidase activity remained unaffected by glucose. Thus, the effect of glucose on lactose uptake and beta-galactosidase activity is due to CreA-dependent carbon catabolite repression. Furthermore, the presence of 2-dezoxy-glucose did not interfere with the lactose uptake in the *creA* mutant strain.

To investigate the effect of CreA on beta-galactosidase formation in more detail, the *A. nidulans* mutant strain CreAΔ4 was grown on glucose and glycerol, respectively, and the formation of beta-galactosidase measured. In contrast to the wild-type strain, in which no beta-galactosidase was measurable under these conditions, the mutant strain formed a beta-galactosidase activity that equals 17% of the maximal levels observed in the wild-type strain on lactose. This indicates that constitutive beta-galactosidase formation is subject to CreA-dependent carbon catabolite regulation, but that carbon catabolite derepression is not sufficient to attain high levels of beta-galactosidase activity, and it is induction that accounts for the major part of *A. nidulans* beta-galactosidase formation. Simultaneous addition of D-glucose and D-galactose reduced the beta-galactosidase activity by about 30 % but did not affect the

uptake of D-galactose, thus indicating a partial interference of D-glucose with the induction process as well. This effect also depended on CreA, as D-galactose induction of beta-galactosidase was completely unaffected by glucose in the *A. nidulans* mutant strain CreA.

The data show that the effect of glucose occurs on at least at three levels, which were all dependent on the carbon catabolite repressor CreA: (1) a blockade of constitutive beta-galactosidase formation; (2) a partial interference with induction, such that in a CreA-negative background 150 % of the wild-type levels of induction were obtained; and (3) a repression of lactose uptake by glucose. Interference of CreA-dependent carbon catabolite regulation with gene expression at multiple regulatory levels is not unknown in *Aspergilli*, and such "double-lock mechanisms" have been reported, e.g. for *alcA* regulation in *A. nidulans* and xylanase biosynthesis in *A. niger*. However, interference of CreA with inducer-transporting permeases (such as observed here with the lactose permease) has not yet been shown. Glucose inactivation of lactose transport in *K. marxianus* has also been reported, but it is not known whether it depends on carbon catabolite repression.

The formation of beta-galactosidase activity in some other fungi (*Aspergillus niger*, *Penicillium canescens*) has been reported to be also induced by L-arabinose and other hemicellulose monomers. This was also found to be the case with *A. nidulans*. L-arabinose (but not D-arabinose) as well as D-xylose induced beta-galactosidase formation, with L-arabinose leading to levels even higher than those obtained with D-galactose. As in the case of D-galactose, significantly higher activities were observed in the *creA*-mutant strain. The reason for beta-galactosidase induction by L-arabinose and D-xylose is unknown, but may be related to the concomitant occurrence of D-galactose, L-arabinose and D-xylose in natural polysaccharide carbon sources for *A. nidulans*.

At suboptimal concentrations of D-galactose addition of L-arabinose further induced beta-galactosidase activity, and *vice versa*. However, maximal induction by either of the monosaccharide inducers was not further increased by addition of the other inducer.

In order to identify which stage of D-galactose metabolism is essential for beta-galactosidase formation, *A. nidulans* mutants defective in galactokinase and galactose-1-phosphate uridylyl transferase activity were used. The galactokinase mutant still formed beta-galactosidase activity upon addition of D-galactose. The maximal activities were similar to those of the wild-type strain, but required much lower D-galactose concentrations. Similar results were obtained in the wild-type strain with D-fucose, a non-metabolizable D-galactose analogue. These data indicate that D-galactose metabolism via galactose kinase is fully dispensable for beta-galactosidase induction by D-galactose.

Mutants defective in galactose-1-phosphate uridylyl transferase activity, however, behaved differently: in these strains, a constitutive level of beta-galactosidase activity which was approximately twice as high as that in the *creA* mutant strain was already formed on glucose or glycerol as carbon sources. Most interestingly, this activity could not be further induced by D-galactose but was fully inducible by L-arabinose. The time course of the formation of beta-galactosidase activity displayed a strictly growth-associated pattern in in the galactose-1-phosphate uridylyl transferase mutants, while growth of these mutants was comparable to that of the wild-type, thus ruling out that the beta-galactosidase formation could be the result of differences in the growth rate.

The results described above could be interpreted in terms of regulation of beta-galactosidase by the uridyltransferase or by the accumulation of galactose-1-phosphate. In order to discriminate rigorously between these two possibilities, a *galD*/*galE* double mutant (*A. nidulans* EFES1) was created to determine whether its beta-galactosidase induction resembled that of *galD* or *galE*. Beta-galactosidase activity in this strain could not be raised by D-galactose above the

constitutive level, while its inducibility by L-arabinose was maintained. Moreover, the time course of the formation of beta-galactosidase activity on glycerol was almost identical to that of the transferase mutant. The results are thus the same as those obtained with the *galD* single mutant, indicating that the observed effect is due to a regulatory role of the uridyltransferase enzyme rather than a rise in galactose-1-phosphate concentration in *A. nidulans*.

This effect is similar, although not identical, to the effect of the corresponding *GAL7* mutation in *K. lactis* (Cardinali et al. 1997), and therefore the induction of â-galactosidase in *K. lactis* and *A. nidulans* may occur by similar mechanisms. In yeasts, galactose induction proceeds by galactose binding to Gal3p (or Gal1p in *K. lactis*), which interacts with Gal80p and prevents it from inhibiting the transcriptional activator Gal4p. Mutations in *K. lactis GAL1* (encoding the D-galactose kinase) which impair kinase activity do not eliminate the ability of Gal1p to induce beta-galactosidase. Thus, assuming that the *A. nidulans* galactokinase mutant still forms the respective, albeit inactive polypeptide, the mechanism of induction as revealed in *K. lactis* may also account for beta-galactosidase induction in *A. nidulans*. However, if so, then the constitutive induction of beta-galactosidase formation in these mutant strains requires a different interpretation. In *K. lactis*, this finding has been suggested to be due to the internal accumulation of low D-galactose concentrations during idiophase. If this explanation also applies to *A. nidulans*, it would be difficult to understand why the low level of constitutive formation is not further inducible by D-galactose. Also, in *A. nidulans*, the constitutive formation of beta-galactosidase occurs throughout the entire growth phase and not only during idiophase as in yeast. Thus in *A. nidulans*, it appears more likely that galactose-1-phosphate uridylyl transferase acts as a repressor of beta-galactosidase biosynthesis and that the inducer D-galactose is mainly needed to antagonize this repression.

Whatever the mechanism of D-galactose induction is, the fact that beta-galactosidase activity in galactose-1-phosphate uridylyl transferase mutant strains is still fully inducible by L-arabinose indicates that signaling by these two monosaccharides involves two different pathways, of which that of L-arabinose is not impaired in the transferase mutant. On the other hand, the fact that a mixture of both monosaccharides cannot induce beta-galactosidase activity over the level achieved by either sugar alone suggests that both ultimately stimulate the same transcriptional activator. No such findings have been reported for *K. lactis*. Thus, the components governing the molecular mechanisms of beta-galactosidase induction in *A. nidulans* and *K. lactis* may not be homologous.

## IV. REDUCTIVE GALACTOSE CATABOLISM IN *A. NIDULANS*

An important achievement of ours was the demonstration that in contrast to the literature (Roberts 1970), *A. nidulans* mutants in galactokinase (*galE*) can still grow on D-galactose. A detailed comparison of the conditions used by the former authors and us revealed that they used nitrate as a nitrogen source, whereas we used ammonium phosphate. Consequently, we tested whether the nitrogen source would be responsible for the apparent contradiction between the results. The nitrogen source indeed strongly affects the ability of the *galE* mutant to grow on D-galactose: whereas the *galE* mutant exhibited a strongly reduced growth on D-galactose with nitrate as a nitrogen source, it grew well with ammonium as a nitrogen source, independently of the anion of the ammonium salt (sulphate, chloride, phosphate). The pH of the medium was controlled at 6.5, thus excluding the possibility that the effect of the nitrogen source is due to the different behaviour of the pH in nitrate and ammonium supplemented cultures. Control experiments with D-glucose, D-fructose and glycerol as carbon sources, respectively, indicated that the nitrogen source had no general influence on growth or carbon uptake by the *galE* mutant.

In case the reductive D-galactose shunt is NADPH-dependent, the phenomenon could be explained by the requirement for NADPH, which may not be available during growth on nitrate because of the requirement and high affinity for NADPH of the nitrate and nitrite reductases.

In order to learn whether the alternative pathway of D-galactose catabolism can fully compensate for the loss of galactokinase in *galE*, the growth of the *A. nidulans* wild-type and the *galE* mutant were compared. Biomass formation of the wild-type strain on D-galactose as a sole carbon source was a great deal faster, resulting in an approximately doubled growth rate compared to the *galE* mutant. A significant difference was also apparent in the biomass yield ($Y_{x/s}$) of the wild-type and the mutant for D-galactose: whereas the wild-type strain displayed a rather constant $Y_{x/s}$ value throughout its phase of D-galactose utilization, the *galE* mutant – while starting with the same value – gradually declined its $Y_{x/s}$ to 60 % until the end of D-galactose utilization, thus suggesting the accumulation of a byproduct.

Since the identification of this byproduct may provide us with a hint hint towards the nature of the alternative pathway operating in the *galE* mutant, we analyzed the culture fluid and the biomass for accumulation of a compound not present in the nutrient medium. This analysis revealed the presence of very high concentrations (over 400 mM) of intracellular galactitol. Galactitol transiently accumulates in the *A. nidulans galE* mutant, and thus is apparently further catabolized. Interestingly, a much lower but nevertheless clearly measurable concentration of galactitol also accumulated in mycelia of the wild-type strain when growing on D-galactose or lactose, but not on any other carbon sources tested (L-arabinose, D-xylose, acetate, D-fructose, D-glucose, glycerol). It rose in the exponential phase of the cultivation, and increased until most of the carbon source had been consumed, after which galactitol was also rapidly utilized. Thus, the reaction forming galactitol and the enzymes catalyzing its

further metabolism - similarly to the *galE* mutant - are apparently present in the wild-type strain as well.

Dialysed cell-free extracts of *A. nidulans* catalyzed the strictly NADPH-dependent reduction of D-galactose into galactitol, and the $NADP^+$-dependent oxidation of galactitol into D-galactose.

In order to identify the enzyme which catalyzes the degradation of galactitol, we first used dialysed cell-free extracts of the wild-type and the *galE* mutant strains to test for the presence of such an enzyme. Both strains possess a galactitol dehydrogenase activity which requires $NAD^+$ but not $NADP^+$ as a cofactor. Since it was reported that an L-arabitol dehydrogenase mutant of *A. nidulans* (G094; *=araA1*) was unable to grow on galactitol, we also tested the galactitol dehydrogenase activity in this mutant strain  It indeed lacked the enzyme activity.

The reaction product of L-arabitol dehydrogenase with L-arabitol is L-xylulose, but the product of its reaction with galactitol is unknown. In order to identify this endproduct, and thus identifying the subsequent intermediate of the alternative pathway, the products from the *in vitro* enzymatic conversion of galactitol by cell-free extracts of the *A. nidulans* wild-type strain, as well as the *galE* and *araA1* mutants were purified by HPLC and subjected to NMR analysis. This analysis identified the product of the reaction as sorbose, and further polarimetric analysis specified it as L-sorbose both in the wild-type and *galE* strains. On the other hand, no end-product was detected in the *araA1* mutant even after a prolonged time of incubation. In the reverse direction, L-sorbose could be converted into galactitol in the presence of NADH (but not NADPH) by cell-free extracts of the wild-type and the *galE* strains, but – most importantly – not by the cell-free extract of the L-arabitol dehydrogenase mutant.

Knowing the chemical nature of the respective compound, we investigated whether it might accumulate in the mycelia to any extent when

grown on galactitol as a sole carbon source. In fact, both the wild-type as well as the *galE* mutant strain, but not the *araA1* mutant, accumulated a very low (20-60 μM), but nevertheless detectable amount of L-sorbose, which was the only hexose found.

Having identified L-sorbose as an intermediate in the alternative D-galactose catabolic pathway, we were interested in its further metabolism. Elorza and Arst (1971) provided evidence for L-sorbose catabolism via D-sorbitol to D-fructose, which could then be phosphorylated by hexokinase. In order to prove that hexokinase is responsible for L-sorbose metabolism and thus is involved in the alternative D-galactose degrading pathway in *A. nidulans*, a *galE / frA1* double mutant was constructed. This mutant is unable to grow on D-galactose. Further, both the *galE / fraA1* and the *frA1* mutant were unable to grow on either galactitol or L-sorbose.

Having identified hexokinase as an essential enzyme for L-sorbose catabolism, there would still be another possibility of its involvement, namely the direct phosphorylation of L-sorbose. To learn if L-sorbose could indeed be phosphorylated by *A. nidulans*, we tested the ability of dialysed cell-free extracts of *A. nidulans* wild-type, *galE* and *araA1* strains, to phosphorylate it. These cell-free extracts indeed phosphorylated L-sorbose in an ATP-dependent manner (Fig. 2). The product of the reaction was identified as a phosphate ester of L-sorbose by NMR and by demonstrating that treatment of the phosphorylated product by alkaline phosphatase resulted in the disappearance of L-sorbose phosphate and the re-formation of L-sorbose.

To prove that the phosphorylation of L-sorbose is indeed due to hexokinase activity, we repeated these experiments with the *A. nidulans frA1* mutant which is impaired in hexokinase activity. Indeed, cell-free extracts of this mutant failed to form L-sorbose phosphate under the conditions which worked with the wild-type, *galE* and *araA1* strains. This is consistent with the

broad specificity of hexokinase towards various aldoses and ketoses (Puri et al. 1988).

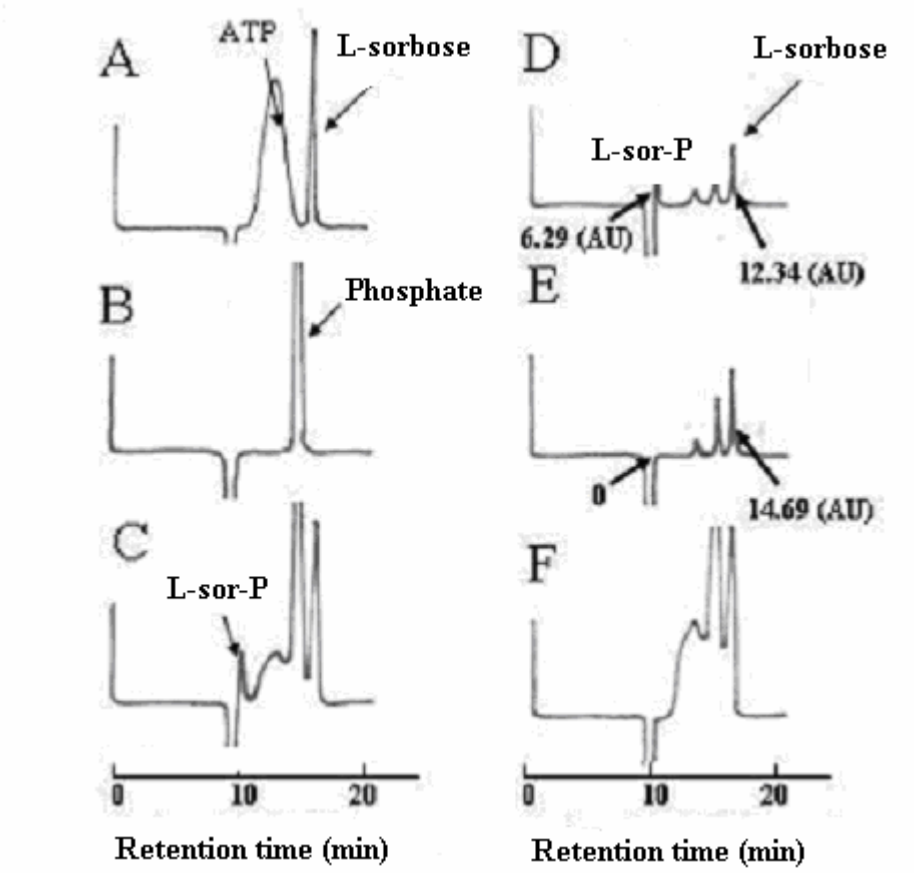


**Figure 2.** HPLC profiles. **A.** ATP and L-sorbose. **B.** Cell-free extract. **C.** Hexokinase reaction profile with L-sorbose using cell-free extracts from the *A. nidulans galE* mutant following 48 h of incubation. **D**. Alkaline phosphatase treatment of the L-sorbose phosphate containing mixture at 0 h. **E.** Alkaline phosphatase treatment of the L-sorbose phosphate containing mixture at 3 h. **F.** Hexokinase reaction profile with L-sorbose using cell-free extracts from the *A. nidulans frA1* mutant following 48 h of incubation. AU: arbitrary unit

An interesting feature of this novel pathway (Fig. 3.) is that it does not involve pathway-specific enzymes, but makes use of proteins which have already been shown to be involved in other pathways. Hence, the oxidation of galactitol occurs by L-arabitol dehydrogenase, which has been characterized to

be involved in the catabolism of L-arabinose, while the phosphorylation of either L-sorbose or D-fructose occurs by hexokinase.

The existence of this novel pathway was also confirmed in another filamentous *Ascomycetes* fungus, *Trichoderma reesei* (Seiboth et al. 2004).

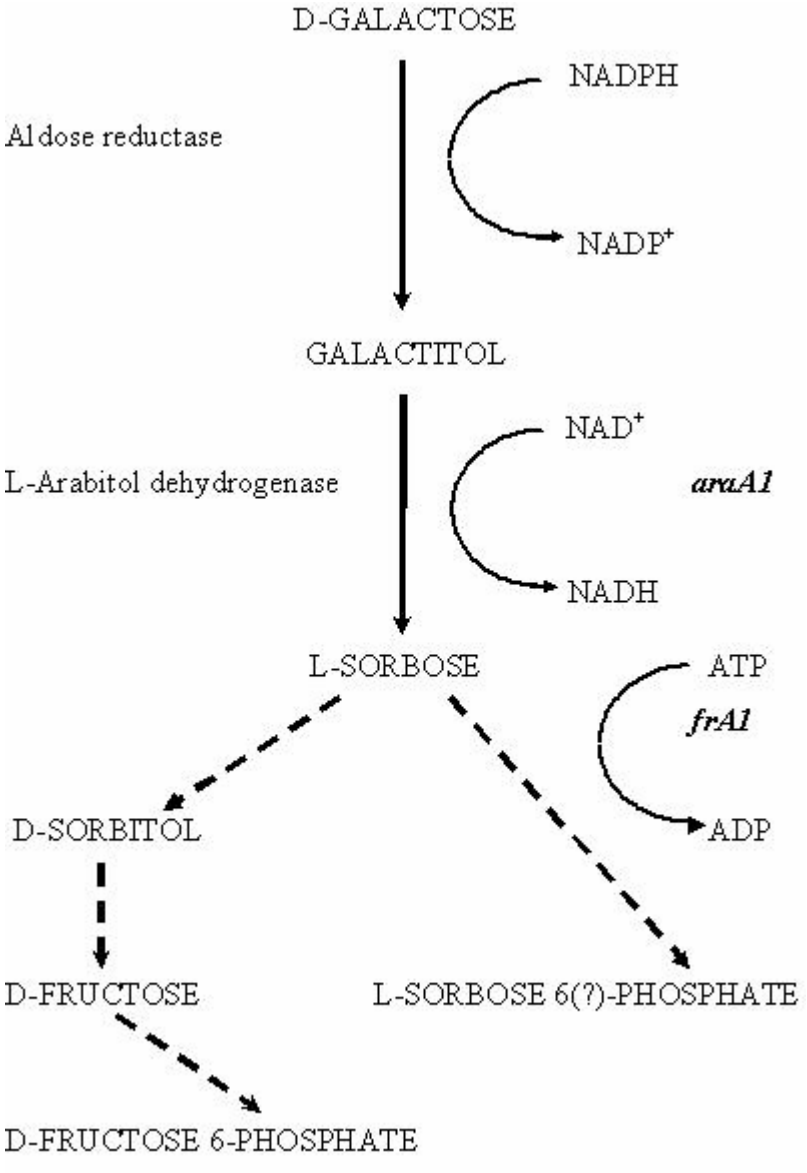


**Figure 3.** Schematic representation of the reductive D-galactose degrading pathway. Broken lines indicate alternative catabolic pathways.

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## THESISES

1. Lactose uptake of *Aspergillus nidulans* is subject to CreA-dependent glucose regulation.
2. Formation of the β-galactosidase activity in *Aspergillus nidulans* is subject to multi-level, CreA-dependent glucose regulation.
3. Formation of the β-galactosidase activity of *Aspergillus nidulans* is induced by D-galactose and L-arabinose in unidentical ways.
4. *Aspergillus nidulans* possesses a galactokinase-independent D-galactose degrading pathway which contains galactitol and L-sorbose as intermediates, and L-arabinitol-4-dehydrogenase and hexokinase as essential enzymes.
5. L-sorbose is an *in vitro* substrate for *Aspergillus nidulans* hexokinase, while the fungus is able to utilize L-sorbose as a sole carbon source.
6. We developed a novel, HPLC-based ion exchange chromatography method that determines fungal galactokinase activity within 5 % error. It relies on measuring the amount of galactose-1-phosphate formed during the reaction of D-galactose + ATP → D-galactose-1-P + ADP. The method has been successfully tested in *Aspergillus nidulans* and *Trichoderma reesei*.

# PUBLICATION LIST

## PUBLICATIONS RELEVANT IN THE THESIS

1) **Fekete E**, Karaffa L, Sándor E, Seiboth B, Biró S, Szentirmai A, Kubicek CP (2002): Regulation of formation of the intracellular β-galactosidase activity of *Aspergillus nidulans*. Archives of Microbiology 179: 7-14. **Impact factor: 2.156**

2) **Fekete E**, Karaffa L, Sándor E, Bányai I, Seiboth B, Gyémánt Gy, Sepsi A, Szentirmai A, Kubicek CP (2004): The alternative D-galactose degrading pathway of *Aspergillus nidulans* proceeds via L-sorbose. Archives of Microbiology 181: 35-44. **Impact factor: 2.156**

3) Seiboth B, Hartl L, Pail M, **Fekete E**, Karaffa L, Kubicek CP (2004): The galactokinase of *Hypocrea jecorina* is essential for cellulase induction by lactose but dispensable for growth on D-galactose. *Molecular Microbiology* 51: 1015-1025 **Impact factor: 6.398**

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1) Karaffa L, Sándor E, Fekete E, Szentirmai A (2001): The biochemistry of citric acid accumulation by *Aspergillus niger*. *Acta Microbiologica et Immunologica Hungarica* 48: 429-441.

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## INTERNATIONAL, PEER REVIEWED ABSTRACTS, PROCEEDINGS

**1)** Sándor E, Karaffa L, Pócsi I, Fekete E, Kozma J, Szentirmai A (1997): Is there any causal relationship between fragmentation and cephalosporin C production of *Acremonium chrysogenum*? *Acta Microbiologica et Immunologica Hungarica* 44: 414.

**2)** Sándor E, Fekete E, Karaffa L, Szentirmai A, Pócsi I (1999): Effect of sulphur-containing amino acids on the glutathione metabolism and cephalosporin C production of *Acremonium chrysogenum*. *Acta Microbiologica et Immunologica Hungarica* 46: 347-348.

**3)** Karaffa L, Fekete E, Sándor E, Sepsi A, Seiboth B, Szentirmai A, Kubicek CP (2002): Carbon catabolite repression in the regulation of β-galactosidase activity in *Aspergillus nidulans*. *Acta Microbiologica et Immunologica Hungarica* 49: 261-265.

**4)** Fekete E, Sándor E, Sepsi A, Szentirmai A, Kubicek CP, Karaffa L (2002): Analysis of the phenotype of an *Aspergillus nidulans* mutant deficient in galactose-1-phosphate-uridylyl transferase activity. *Acta Microbiologica et Immunologica Hungarica* 49: 396.

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## LECTURES, POSTERS

**1)** Fekete E, Sándor E, Biró S, Kozma J, Szentirmai A, Karaffa L: Az *Aspergillus nidulans* laktóz anyagcseréjének néhány sajátsága (IX. Fermentációs Kollokvium, Debrecen, 2000).

**2)** Fekete E., Karaffa L, Sepsi A, Sándor E, Biró S, Szentirmai A, Kubicek CP: A karbon katabolit represszió szerepe az *Aspergillus nidulans* β-galaktozidáz enzimének képződésében (az 50 éves Magyar Mikrobiológiai Társaság jubileumi nagygyűlése, Balatonfüred, 2001).

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**6)** Karaffa L, Sándor E, Fekete E, Szentirmai A, Pócsi I: Az oxigén és oxigén szabadgyökök szerepe az *Acremonium chrysogenum* cephalosporin C termelésében (II. Magyar Mikológai Konferencia, Szeged, 2002).

**7)** Balogh G, Sándor E, Fekete E, Juhász A, Seress P, Bakondi I, Oláh A, Pethő Cs, Karaffa L, Szentirmai A: Characterization of a fermentation by the lovastatin producer *Aspergillus terreus* (II. Magyar Mikológai Konferencia, Szeged, 2002).

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**10)** Fekete E, Sándor E, Szentirmai A, Kubicek CP, Karaffa L: A galaktóz lebontás alternatív útvonala *Aspergillus nidulans*-ban (A Magyar Mikrobiológiai Társaság Nagygyűlése, Balatonfüred, 2002).

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**13)** Serester OK, Fekete E, Kónya J, Nagy N, Szentirmai A, Karaffa L: A laktóz permeáz vizsgálata *Aspergillus nidulans*-ban (A Magyar Mikrobiológiai Társaság Nagygyűlése, Balatonfüred, 2002).

**14)** Fekete E, Sándor E, Szentirmai A, Kubicek CP, Karaffa L: Regulation of formation of the intracellular β-galactosidase activity in *Aspergillus nidulans* (1st FEMS Congress of European Microbiologists, Ljubljana, Szlovénia, 2003).

**15)** Karaffa L, Fekete E, Sándor E, Szentirmai A, Kubicek CP: Reductive pathway of galactose catabolism in *Aspergillus nidulans* (1st FEMS Congress of European Microbiologists, Ljubljana, Szlovénia, 2003).

**16)** Sándor E, Novák E, Serester O, Fekete E, Szentirmai A, Karaffa L: Regulation of the alternative oxidase activity and gene expression by the nitrogen source in *Aspergillus nidulans* (A Magyar Mikrobiológiai Társaság XIV. nemzetközi nagygyűlése, Balatonfüred, 2003).

**17)** Fekete E, Sándor E, Serester O, Novák E, Szentirmai A, Kubicek CP, Karaffa L: β-galactosidase genes in *Aspergillus nidulans* (A Magyar Mikrobiológiai Társaság XIV. nemzetközi nagygyűlése, Balatonfüred, 2003).