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Increased NADPH concentration obtained by metabolic engineering of the pentose phosphate pathway in *Aspergillus niger*

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Many biosynthetic reactions and bioconversions are limited by low availability of NADPH. With the purpose of increasing the NADPH concentration and/or the flux through the pentose phosphate pathway in *Aspergillus niger*, the genes encoding glucose 6-phosphate dehydrogenase (*gsdA*), 6-phosphogluconate dehydrogenase (*gndA*) and transketolase (*tktA*) were cloned and overexpressed in separate strains. Intracellular NADPH concentration was increased two- to ninefold as a result of 13-fold overproduction of 6-phosphogluconate dehydrogenase. Although overproduction of glucose 6-phosphate dehydrogenase and transketolase changed the concentration of several metabolites it did not result in increased NADPH concentration. To establish the effects of overexpression of the three genes, wild-type and overexpressing strains were characterized in detail in exponential and stationary phase of bioreactor cultures containing minimal media, with glucose as the carbon source and ammonium or nitrate as the nitrogen source and final cell density limiting substrate. Enzymes, intermediary metabolites, polyol pools (intra- and extracellular), organic acids, growth rates and rate constant of induction of acid production in postexponential phase were measured. None of the modified strains had a changed growth rate. Partial least square regressions showed the correlations between NADPH and up to 40 other variables (concentration of enzymes and metabolites) and it was possible to predict the intracellular NADPH concentration from relatively easily obtainable data (the concentration of enzymes, polyols and oxalate). This prediction might be used in screening for high NADPH levels in engineered strains or mutants of other organisms.

The pentose phosphate pathway (PPP) and glycolysis comprise the most central pathways in primary metabolism (Fig. 1). The PPP is believed to be the major source of NADPH required for many biosynthetic and detoxification reactions. The flux through this pathway has been reported to increase at high NADPH requirements, for

Abbreviations
6PG, 6-phosphogluconate dehydrogenase; a, ammonium; ALD, aldolase; ARC, anabolic reduction charge; CRC, catabolic reduction charge; DB, dry biomass; DHAP, dihydroxyacetone phosphate; *e*, exponential growth phase; *E*, extracellular; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate dehydrogenase; GAP, glyceraldehyde 3-phosphate; GLYDH, glycerol dehydrogenase; *I*, intracellular; IAP, induction of acid production; M1PDH, mannitol 1-phosphate dehydrogenase, *n*, nitrate; PGI, phosphoglucose isomerase; PYR, pyruvate; R5P, ribose 5-phosphate; RMSEP, root mean square error of prediction; Ru5P, ribulose 5-phosphate; *s*, stationary phase; S7P, sedoheptulose 7-phosphate, TAL, transaldolase; TKT, transketolase; wt, wild-type; Xu5P, xylulose 5-phosphate; μ_{max}, maximum specific growth rate.

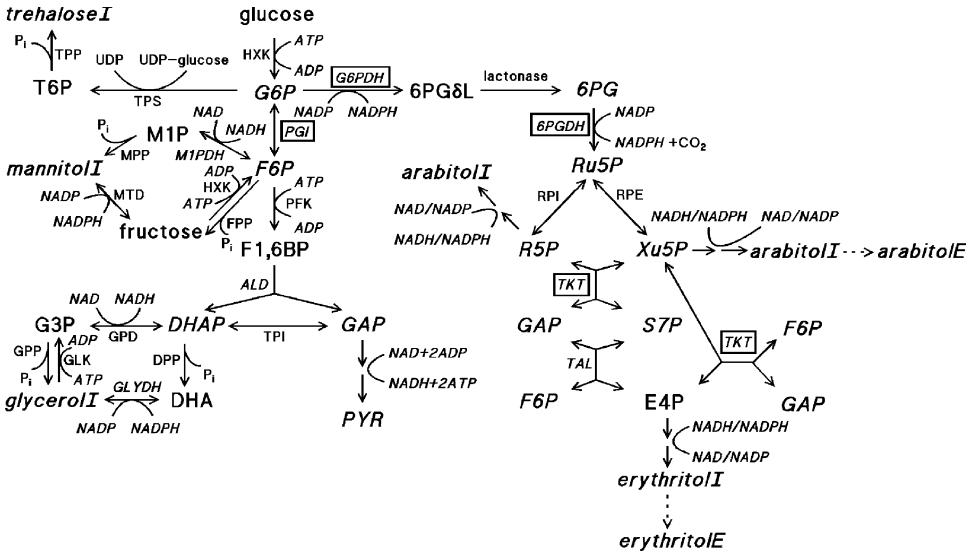

Fig. 1. Glycolysis, pentose phosphate pathway and polyol formation and degradation in Aspergilli. Partly after [50] and [51]. Enzymes in boxes were subjected to metabolic engineering in this study. Metabolites and enzymes in italics were measured in wild-type and engineered strains. Enzymes involved in polyol formation and degradation are probably regulated to prevent potential futile cycles. Two arrows in series mean two or more reactions. E and I indicate extra- and intracellular polyols, respectively. Additional metabolite abbreviations: 6PGδL, 6-phosphoglucono-δ-lactone; DHA, dihydroxyacetone; E4P, erythrose 4-phosphate; F1,6BP, fructose 1,6-bisphosphate; G3P, glycerol 3-phosphate; M1P, mannitol 1-phosphate; T6P, trehalose 6-phosphate. Additional enzyme abbreviations: DPP, dihydroxyacetone phosphate phosphatase; FPP, fructose 6-phosphate phosphatase; GPD, glycerol 3-phosphate dehydrogenase; GPP, glycerol 3-phosphate phosphatase; GLK, glycerol kinase; HXK, hexokinase; MPP, mannitol 1-phosphate phosphatase; MTD, mannitol dehydrogenase; PFK, phosphofructokinase; RPI, ribosephosphate isomerase; RPE, ribulosephosphate 3-epimerase; TPP, trehalose 6-phosphate phosphatase; TPS, trehalose 6-phosphate synthase; TPI, triosephosphate isomerase.

example penicillin formation [1,2], methylenomycin synthesis [3] and reduction of (growth on) nitrate [4,5], and to decrease when the need for NADPH production is decreased [6,7]. In cell-free enzyme systems the NADPH is regenerated enzymatically or electrochemically [8], but whole-cell systems are often the only available, more stable and inexpensive enzyme source [9].

The availability of NADPH in whole-cell systems might be increased by metabolic pathway engineering, e.g. overproduction of enzymes in the PPP or deletion of genes in glycolysis when a hexose is the carbon source. NADPH is produced in two of the steps in the PPP, namely the conversion of glucose 6-phosphate (G6P) to 6-phosphoglucono-δ-lactone (6PGδL), catalysed by glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), and conversion of 6-phosphogluconate (6PG) to ribulose 5-phosphate (Ru5P) catalysed by 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44) (Fig. 1). In the nonoxidative part of the PPP two out of three reactions are catalysed by transketolase (TKT; EC 2.2.1.1). Overproduction of these enzymes might lead to increased flux through the PPP. The level of NADPH has previously been increased in *Escherichia coli* by overproduction of G6PDH or 6PGDH [10] and in *Ralstonia eutropha* by overproduction of G6PDH [11] or by overproduction of 6PGDH or TKT [12].

Glucose 6-phosphate is a branching point to several pathways. It leads to the pentose phosphate pathway, glycolysis and the pathways for biosynthesis of cell wall components. The *Aspergillus niger* gene encoding G6PDH (*gsdA*) has been cloned, but transformation of the fungus with this gene resulted in only low levels of overproduction of G6PDH [13]. The authors suggested that a high level of G6PDH overproduction might result in a low lethal NADP/NADPH ratio in the cell [13]. Therefore, in this study isolation of transformants with a higher overproduction of G6PDH was attempted by a rescue on media giving a high oxidation rate of NADPH to NADP.

In glycolysis the conversion of G6P to fructose 6-phosphate (F6P) is accomplished by phosphoglucose isomerase (PGI). A disruption of the gene encoding for PGI (*pgiA*) is likely to increase the flux through the PPP, as this would force all conversion of G6P to intermediates in glycolysis through the PPP (Fig. 1). Canonaco and coworkers [14] had strong indications that using this strategy in *Escherichia coli* increases the NADPH concentration. We have tried a similar approach and cloned the *pgi* gene (accession number

AJ551177) but we failed to obtain a disruptant, although we analysed more than 120 transformants.

The aim of this study is to increase the availability of NADPH for synthesis or bioconversions by overproduction of three enzymes in the PPP, G6PDH, 6PGDH and TKT. Wild-type and engineered strains were characterized in detail in bioreactor cultures using multivariate data analysis showing that overproduction of 6PGDH resulted in increased NADPH levels.

Results

Cloning of the genes *gndA*, *gsdA* and *tktA*

To be able to manipulate the genes *gndA*, *gsdA* and *tktA* encoding 6PGDH, G6PDH and TKT, respectively, these genes were cloned by screening an *A. niger* N400 genomic library in EMBL4 [15] by hybridization with PCR products obtained by using the PCR oligos in Table 1. Fragments obtained for these three genes were a 5.3 kb *Eco*RI–*Sal*I fragment (AJ551178, *gndA* including 1.1 kb upstream and 2.1 kb downstream of the gene), a 5.0 kb *Sal*I–*Nsi*I fragment (part of S78375 [13], *gsdA* including 1.1 kb upstream and 1.5 kb downstream of the gene) and a 3.8 kb *Eco*RI–*Cla*I fragment (AJ550995, *tktA* including 0.7 kb upstream and 0.5 kb downstream of the gene), which were cloned into pBluescript resulting in the pIM445, pIM440 and pIM448 plasmids, respectively.

The amino acid sequences of 6PGDH and TKT are highly similar to previously published sequences from other organisms. The highest similarities were with 6PGDH from *Aspergillus oryzae* (BAC06328, 94% identity) and the TKT from *Neurospora crassa* (CAC18218, 74% identity), respectively.

Both *gndA* and *tktA* contain an exceptionally long first intron (estimated from alignments with sequences of *gnd* and *tkt* genes of other organisms) of 407 and 267 bp, respectively, which is much longer than generally observed in filamentous fungal genes [16]. Strikingly, this is also the case for the other PPP enzyme-encoding gene *gsdA* [13] cloned so far, but whether this is a general feature of all the PPP genes of *A. niger* still remains to be shown.

Table 2. *A. niger* strains used in this study.

Strain	Trivial name	Genotype[a]	Reference for characterized mutation or strain
NW131	wt	cspA1 goxC17	[33] [34]
NW129		cspA1 goxC17 pyrA6	[39]
NW342	Gnd5	cspA1 goxC17 $[gndA]_5$	This study
NW341	Gnd8	cspA1 goxC17 $[gndA]_8$	This study
NW340	Gnd20	cspA1 goxC17 $[gndA]_{20}$	This study
NW323	Gsd11	cspA1 goxC17 $[gsdA]_{11}$	This study
NW339	Tkt15	cspA1 goxC17 $[tktA]_{15}$	This study

[a] Subscript is copy number estimated by Southern analysis.

Transformations of *A. niger* to obtain overexpression of *gndA*, *gsdA* and *tktA*

With the purpose of overproducing the enzymes 6PGDH, G6PDH and TKT in separate strains, the plasmids pIM445 (*gndA*), pIM440 (*gsdA*) and pIM448 (*tktA*) were used in cotransformations, which resulted among others in the multicopy strains given in Table 2.

After transformation with pIM445 (*gndA*) we isolated 20 transformants of which approximately half overproduced 6PGDH in the range from two- to 13-fold. This is a higher level of overproduction than previously obtained in both *Escherichia coli* [10] and *R. eutropha* [12] which was 1.7 and 3.8 times wild-type activity, respectively. As shown in Fig. 2, the activity did correlate both to the number of copies of the *gndA* gene introduced (up to 20) and to the transcription level. We chose the *gndA* multicopy strain Gnd20 (NW340, Table 2) with 20 introduced copies and a 6PGDH activity of 13 times wt activity for detailed characterization.

Protoplasts transformed with pIM440 (*gsdA*) were plated on minimal media with different carbon and

Table 1. PCR oligos for probes and site-directed mutagenesis.

Oligo	Position[a]	Sequence	Comments
gnd1	1617–1633 (Z46631)	AARATGGTNCAYAAYGG	Degenerate PCR on *A. niger* cDNA
gnd2	1867–1851 (Z46631)	GTCCAYTTNCCNGTNCC	
gsd-1	733–752 (S78375[b])	GCAGCTGGACAGCTTCTGCC	Specific PCR on *A. niger* DNA
gsd-2	1603–1584 (S78375[b])	CGTTCTTGGGCTCAATGGCG	
nctkt1	600–584 (NC4B12-T7[c])	GCCATTGATGCCGTCAA	Specific PCR on *N. crassa* DNA
nctkt4	256–272 (NC4B12-T7[c])	CTGGAAAGCCCTGTTGA	

[a] Position in the accession number given. [b] From [13]. [c] Putative *Neurospora crassa* transketolase EST sequence from http://biology.unm.edu/

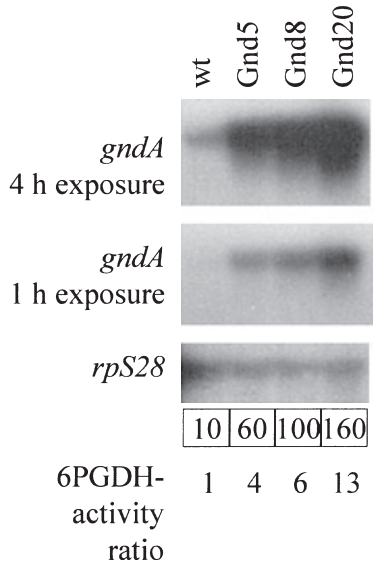

Fig. 2. Transcript analysis of gndA expression in multicopy transformants. The probe was a 0.25 kb PCR product of oligos gnd1 and –2 (Table 1). Both 1 and 4 h exposures to film are shown because of large differences in transcription level. The probe for the loading control was a 0.7 kb *Eco*RI-*Xho*I fragment of ribosomal protein gene *rpS28* [52]. The high intensity on left side of wild-type (wt) band is an artefact due to exposure from a strong neighbouring band. Numbers in boxes indicate the relative levels of *gndA* transcripts corrected for loading differences on basis of the *rpS28* signal. Signals for wt were set at 10. Bottom, 6PGDH-activity relative to wild-type (wt 6PGDH activity = 0.4 U·mg protein^{-1}).

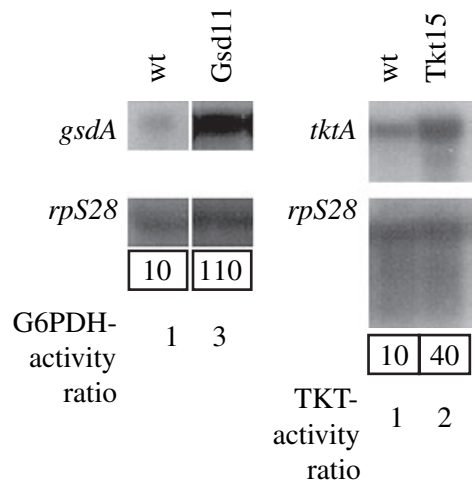

Fig. 3. Transcript analysis of gsdA and tktA expression in multicopy transformants. Probes were a 1.4 kb *Xho*I-*Nco*I fragment of *gdsA* (S78375) and 2.1 kb *Sma*I-*Sph*I of *tktA* (AJ550995), respectively. The probe for the loading control was a 0.7 kb *Eco*RI-*Xho*I fragment of ribosomal protein gene *rpS28* [52]. The numbers in boxes indicate the relative levels of *gsdA* and *tktA* transcripts corrected for loading differences on basis of the *rpS28* signals. Signals for wt were set at 10. Bottom, G6PDH- and TKT-activities relative to wild-type (wt G6PDH activity = 1.0 U·mg protein^{-1}, wt TKT activity = 0.3 U·mg protein^{-1}).

nitrogen sources to obtain different rates of intracellular NADPH oxidation. Approximately half of 30 transformants isolated from each medium (120 in total) overproduced G6PDH. However, the overproduction did not differ significantly between the media and was only up to three times wt activity. This result is in agreement with previous attempts to overproduce G6PDH in *A. niger* [13], *E. coli* [10] and *R. eutropha* [11]. The rescue of transformants on media which led to increased oxidation of NADPH therefore had no influence on the G6PDH overproduction levels obtained. However, whereas van den Broek and coworkers [13] found only up to four introduced copies of the *gsdA* gene, we found up to 40 introduced copies, but the number of introduced copies did not correlate with the degree of overproduction of the enzyme. This was confirmed by transcript analysis (Fig. 3), which showed very high transcription levels compared to wild-type even for strains with few introduced copies. We therefore concluded that the *gsdA* gene product(s) must be subject to post-transcriptional regulation, either at the mRNA or at the enzyme level. We chose *gsdA* multicopy strain Gsd11 (NW323, Table 2) with 11 introduced copies and a G6PDH activity of three times wt activity for detailed characterization.

After transformation of *A. niger* with pIM448 (*tktA*) we isolated 20 transformants of which approximately one third overproduced TKT, but we found only up to two times wt activity. This level of overproduction is comparable to that previously obtained in *R. eutropha* [12], but in *Saccharomyces cerevisiae* [17] and *Corynebacterium glutamicum* [18] overproduction of up to 15 and 30 times wt activity, respectively, was obtained. Southern analysis showed up to 15 introduced copies and no apparent correlation with enzyme activity, but the differences and the accuracies in enzyme activity were too low to exclude this. In contrast to the high transcription level of the *gndA* and *gsdA* multicopy strains, the transcription level of the *tktA* multicopy strains was only slightly higher than wild-type level (Fig. 3), which confirmed the low level of overproduction of only twofold. One reason for this could be that the 0.7 kb promotor of pIM448 is too short to obtain high level transcription. The *tktA* multicopy strain Tkt15 (NW339, Table 2) with 15 extra (introduced) copies and a TKT activity of two times wt activity was chosen for detailed characterization.

Detailed characterization of wild-type and overproducing strains

To determine physiological changes caused by overproduction of G6PDH, TKT or 6PGDH, repeated batch cultures were performed in computer controlled bioreactors with the wild-type, Gsd11, Tkt15 and Gnd20 strains. Macro-morphology profiling (BR Poulsen, AB Sørensen, T Schuleit, GJG Ruijter & J Visser, unpublished results) showed that the cultures were without large pellets containing a substrate diffusion-limited centre and contained about 30% (dry biomass, DB) pellets smaller than 0.3 mm diameter and 70% (dry biomass) free hyphae. This mainly filamentous morphology was obtained only at low pH (here at pH 3). If the pH was increased above 4.5, pellet fraction and size increased resulting in diffusion-limited biomass in the centre of large pellets (> 0.3 mm diameter).

The added titrants in the exponential growth phase were NaOH and HCl in ammonium and nitrate cultures, respectively, and they were added in quantities equivalent to the amount of these nitrogen sources. This can be explained by (a) only small quantities of organic acid were produced during the exponential phase, and (b) release of a proton upon the uptake of an ammonium ion [19] and uptake of a proton upon the uptake of a nitrate ion. The added titrant in the stationary phase of both ammonium and nitrate cultures was NaOH, which was caused by equivalent quantities of organic acid produced [20].

Performing partial least square (PLS) regressions

For future metabolic engineering of strains with the purpose of obtaining increased NADPH levels and for the understanding of the regulation of the NADPH level it is important to know which variables are correlated with NADPH concentration. Because of the relatively high number of variables obtained in this study from analysis of samples from exponential (e) and stationary (s) phases not all correlations are obvious. One statistical tool, which is suitable to find correlations between multiple variables and at the same time to make a regression in order to predict one or more variables, is a Partial least square (PLS) regression. In a PLS regression the most important part of the variation in the X-variables for description of the Y-variables is found as one or more principal components (PCs). Details of algorithms used in PLS regressions are given in Martens and Næs [21], Höskuldsson [22] and Esbensen [23].

We performed PLS regressions to predict the NADPH concentration (Y) from, and find correlations with, the other measured variables (X). The variables G6PDH, 6PGDH, TKT, mannitol 1-phosphate dehydrogenase (M1PDH; EC 1.1.1.17), sedoheptulose 7-phosphate (S7P), dihydroxyacetone phosphate (DHAP), xylulose 5-phosphate (Xu5P), F6P, pyruvate (PYR), ribose 5-phosphate (R5P), glyceraldehyde 3-phosphate (GAP), 6PG, NADP, NADPH, NADH, erythritolI, arabitolI, mannitolI, arabitolE, trehaloseE, oxalate and NADH (E, extracellular; I, intracellular) were skewed and therefore preprocessed by log-transformation. The rest of the variables [Aldolase (ALD; EC 4.1.2.13), transaldolase (TAL; EC 2.2.1.2), PGI, glycerol dehydrogenase (GLYDH; EC 1.1.1.156), G6P, ADP, AMP, NAD, catabolic reduction charge (CRC), glycerolI, trehaloseI, glycerolE, erythritolE and mannitolE] had a skewness between –l and 1 and were not preprocessed. Citrate, DB and maximum specific growth rate (μ_{max}, h^{-1})/induction of acid production (IAP, h^{-1}), were excluded from the regression, because they are very different in the exponential and stationary phases. ATP and energy charge were excluded because for some of the samples from the cultures of overproducing strains the determination of ATP was not reproducible. We found no explanation for this other than that the turnover of ATP is very high. Ru5P was excluded, because of an incomplete dataset for this variable. Anabolic reduction charge (ARC) was excluded because it is calculated partly from the Y-variable (NADPH).

Figure 4 shows a PLS regression with both exponential phase (e) and stationary phase (s) samples from ammonium (a) and nitrate (n) grown cultures after excluding variables with only minor correlation with NADPH [TAL, M1PDH, S7P, R5P, GAP, 6PG, ADP, AMP, CRC, glycerolE, arabitolE, mannitolE and citrate] from the prediction. Two PCs were chosen since this gave a minimum in the Y-variance. Figure 4A shows the scores on the two PCs of the samples (from different strains and different conditions). Figure 4B shows the X-loading weights of the X-variables (measured variables other than NADPH) and the Y-loading of NADPH on the two PCs. As NADPH is in the first quadrant of Fig. 4B (the four quadrants in a system of coordinates are ordered from top right and numbered counterclockwise) all the variables in this quadrant are positively correlated to NADPH in the two PCs. The variables in the third quadrant are all negatively correlated to NADPH in the two PCs. The variables in the fourth quadrant are mainly positively correlated to NADPH, because they are positively correlated in PC1 and negatively correlated in PC2, and much more of the NADPH is explained on PC1 (73%) than on PC2 (12%). For the same reason the variables in the second quadrant are

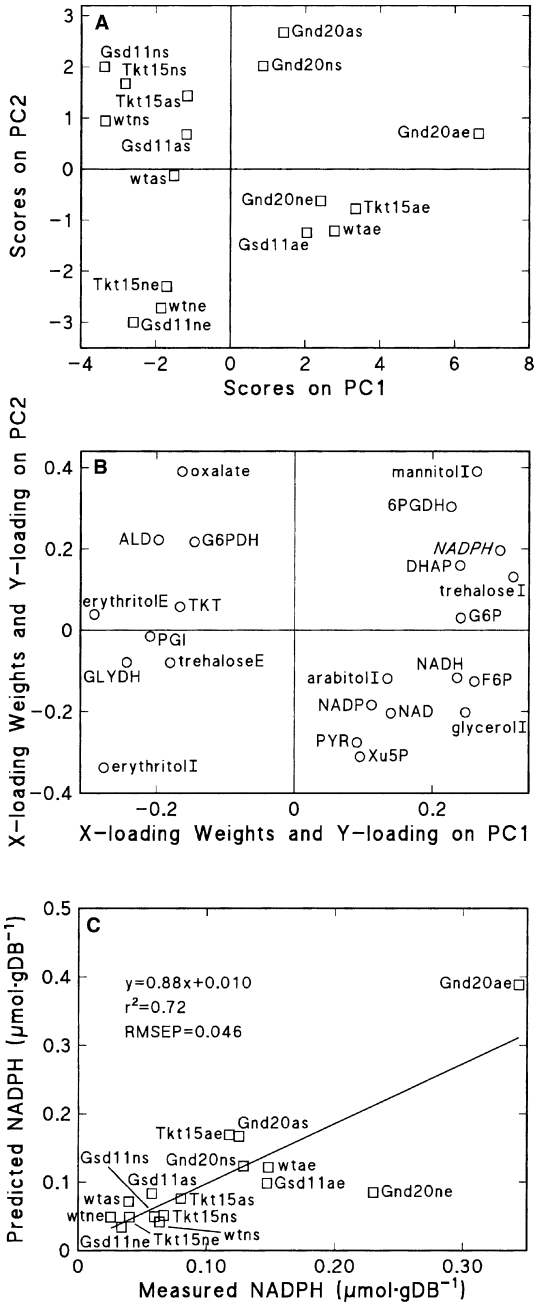

Fig. 4. Prediction of NADPH using a partial least square (PLS) regression with samples from exponential and stationary phase. Variables with less correlation to NADPH were excluded (TAL, M1PDH, S7P, R5P, GAP, 6PG, ADP, AMP, CRC, glycerolE, arabitolE and mannitolE). Gsd11, Tkt15 and Gnd20 are strains overproducing G6PDH, TKT and 6PGDH, respectively. a and n are cultures with ammonium and nitrate, respectively, as final cell density limiting substrate. e and s are exponential and stationary phase, respectively. RMSEP is root mean square of error of prediction. Two PCs were used. X explained, 40% on PC1 and 17% on PC2. Y (NADPH) explained, 73% on PC1 and 12% on PC2. (A) Scores, (B) X-loading weights and Y-loadings, (C) predicted vs. measured NADPH.

mainly negatively correlated to NADPH. Similarly, the samples (Gnd) in the first quadrant of Fig. 4A have a tendency to have a high NADPH level. However, the position of the samples in Fig. 4A is influenced by the level of all variables in these samples. For example, the samples of the strains in the third quadrant of Fig. 4A have a tendency to a high erythritolI level, because this variable is in the third quadrant of Fig. 4B. A total of 85% of NADPH is explained on the basis of two principal components. The coefficient of determination (r^2) is 0.72, which confirms the correlation, although it is not very precise. The root mean square error of prediction, or average relative error in prediction (RMSEP) is 0.046 μmol·g DB^{-1} (Fig. 4C), which corresponds to about 20% of the NADPH concentration in Gnd20 in the exponential phase and is satisfactory considering that the coefficient of variation (CV = standard deviation/average × 100%) of the NADPH determination is about 30%.

Samples from exponential (e) and stationary (s) phase form two separate groups in Fig. 4A. This is expected, as identical conditions such as growth in exponential phase have a tendency to result in the same concentrations of variables. Similarly, the conditions ammonium (a) and nitrate (n) have a tendency to form separate groups. There is a tendency that the variation in nitrogen source is on PC1; nitrate scores low on PC1 and ammonium scores high on PC1. Similarly, there is a tendency that the variation in growth phase is on PC2: exponential phase scores low on PC2 and stationary phase scores high on PC2. In addition, the strain Gnd20 forms a group, although it is relatively scattered, which indicates that this strain differs from the other strains; the main differences being high 6PGDH activity and NADPH concentration.

Without the intermediary metabolites a good correlation was obtained when the samples from the stationary phase are also excluded (Fig. 5). A total of 96% of NADPH is explained by two PCs, the coefficient of determination (r^2) is 0.76 and RMSEP is 0.067 μmol·g DB^{-1}, corresponding to about 30% of the NADPH concentration in Gnd20 in exponential phase and in the range of the CV of the NADPH determination.

Discussion

Obvious tendencies found by characterization of the wild-type

In the exponential phase of the ammonium cultures all of the PPP enzyme activities were lower and the NADPH concentration was higher than during other

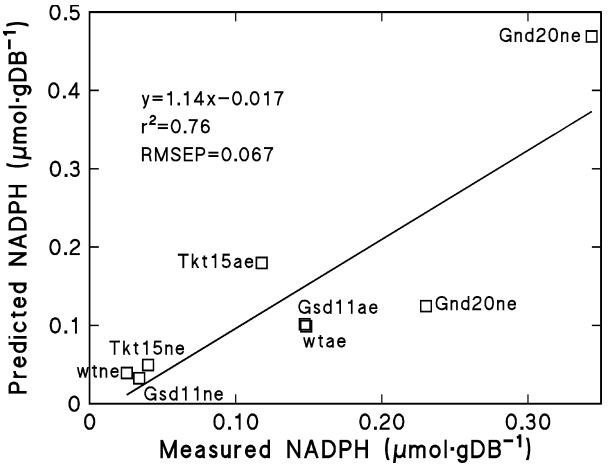

Fig. 5. Prediction of NADPH using a partial least square (PLS) regression with samples from exponential phase only and without values of intermediary metabolites. Variables with less correlation to NADPH were excluded (TAL, M1PDH, arabitolI, glycerolE and arabitolE). Legends as in Fig. 4. Two PCs were used. X explained, 62% on PC1 and 19% on PC2. Y (NADPH) explained, 89% on PC1 and 7% on PC2.

conditions. It is difficult to explain the higher PPP enzyme activities in stationary phase, because in this phase most of the carbon taken up is converted to carbohydrate as storage compounds [20] (about 35%) and to oxalate (about 20%) and only about 6% to polyols. Of the products formed only polyol formation requires NADPH, and similar quantities of polyols were formed in both the exponential and stationary phases.

In the exponential phase of the nitrate cultures high PPP enzyme activities and a low NADPH level were probably caused by a high demand for NADPH for the reduction of nitrate. It is possible that the control mechanism for the high and low PPP enzyme activities in the exponential phase of the nitrate and ammonium cultures, respectively, is the NADPH level as suggested by the results of Witteveen *et al.* [24] and Hankinson [25]. During growth on ammonium NADPH consumption is low compared to growth on nitrate and therefore the concentration of NADPH is high. This leads to the down-regulation of PPP genes. Conversely, during growth on nitrate NADPH consumption is high and therefore the concentration of NADPH is low which makes the up-regulation of PPP genes necessary.

Concentration of intermediary metabolites had a general tendency to be higher in exponential phases than in stationary phases. Whether this is a result of growth or part of the regulation of growth is unknown to us.

Obvious tendencies found by characterization of overproducing strains

The 6PG level was generally increased in the Gsd11 strain and generally decreased in the Gnd20 strain. This is expected, as 6PG is the product and the substrate for the enzyme overproduced in Gsd11 and Gnd20, respectively. Although NADPH is a product of both enzymes it was only increased in the latter strain (under all conditions). Also the concentration of NADP and NAD had a tendency to be increased in Gnd20, which might counterbalance the regulatory effect of the high NADPH concentration. The lack of a significant increase in NADH parallel to the increase in NADPH indicates that there is no significant transhydrogenase activity in *A. niger* under the conditions used here, whereas this has been suggested for citric acid-producing mycelia [26]. Overproduction of 6PGDH resulted in an increase of synthesis of pyridine nucleotide cofactors, as the total pool of these increased. Whether the synthesis is regulated by the NADPH concentration remains speculation. The G6PDH overproducing strain has wild-type levels of NADPH under the conditions applied for detailed characterization, which contradicts the arguments used previously [13] that high and lethal concentrations of NADPH are the reason for only low overproduction of G6PDH found in *A. niger*. However, the reason might be too low an NADP concentration, because the concentration of this metabolite had a tendency to decrease in the G6PDH overproducing strain. Another reason for the lack of high G6PDH overproduction might be that this results in high 6PG inhibiting PGI [27] to a level incompatible with growth. This would imply that the absence of PGI activity is lethal, which would be consistent with our results where we were not able to produce a *pgi* disruptant.

Furthermore, it was found that the Tkt15 strain had a tendency to show a higher level of acid production. The reason for this is unknown, but one suggestion could be that in this strain with increased transketolase activity carbon is more efficiently converted from the oxidative PPP via Ru5P to glycolysis in the form of GAP and F6P, and thereby made available for acid production.

Correlations with NADPH deduced from PLS

From Fig. 4B it is possible to deduce a number of correlations with NADPH concentration. The correlations with enzyme concentrations are interesting, because it is possible to change these by genetic engineering.

Firstly, the 6PGDH correlates with NADPH. Similarly, the little success we had with increasing NADPH by overproduction of G6PDH and TKT is confirmed by a negative correlation between these enzymes and NADPH. Also ALD, PGI and GLYDH are negatively correlated to NADPH. However, this does not necessarily imply that high expression of these enzymes is irrelevant for high NADPH production. For example, it is known that the flux through the PPP is increased during growth on nitrate compared to growth on ammonium [4,5] and we observed a two- to fourfold increase in PPP enzyme levels, but a fivefold decrease in NADPH concentration, probably because NADPH is used for the reduction of nitrate. These data apparently have a stronger influence on the calibration of the PLS regression than the data from the Gnd20 strain, in which the G6PDH activity and NADPH concentration were generally increased. Therefore the correlations shown in Fig. 4B should be interpreted with caution taking into account the knowledge of, for example, the pathways shown in Fig. 1. A PLS regression gives correlations but not the cause of the correlations.

Surprisingly 6PG has no strong (negative) correlation with NADPH although the concentration is decreased three- to sevenfold under most conditions in the Gnd20 strain. This may be caused by a three- to sevenfold increase in 6PG and a slight tendency to an increase in NADPH in the Gsd11 strain.

It is possible that PPP flux is increased in the Gnd20 strain and that a higher G6PDH activity is required for this. Concentrations of polyols and intermediary metabolites had a tendency to be increased in this strain which could be caused by a higher NADPH concentration and precursor production originating from an increased flux through the PPP. It seems likely that the increased NADPH and intermediary metabolite levels caused an increased polyol formation. This is probably the reason for the correlation between NADPH, most intracellular polyols and intermediary metabolites. Despite this, the total pool of polyols was only increased significantly (doubled) in the stationary phase of the nitrate cultures of the TKT and the 6PGDH overproducing strains.

Of the polyols only erythritol is negatively correlated with NADPH and has a tendency to be low in the 6PGDH overproducing strain and under conditions with high NADPH concentrations. Low erythrose 4-phosphate concentration might be the cause, but this cannot be confirmed, as even in the wild-type it is too low to be measured in *A. niger* [27]. Alternatively, the formation of erythritol might use NADH as a cofactor instead of NADPH. However, the cofactor is likely to be NADPH in *A. oryzae* [28], but this has not been investigated in *A. niger*.

The consumption of NADPH upon formation of glycerol by GLYDH (Fig. 1) confirms the negative correlation with this enzyme. This is a very interesting observation as it indicates that a disruption or a down-regulation of the gene encoding for GLYDH might result in higher NADPH concentrations.

Applicability of PLS regression to other metabolic engineered strains or mutants

The PLS regression in Fig. 4 shows quite well how variables are correlated to NADPH. However, this prediction of NADPH concentration requires measurement of concentrations of intermediary metabolites G6P, F6P, DHAP, NAD, NADP, PYR and Xu5P. Because sampling for and extraction of intermediary metabolites is quite tedious it would be a great advantage if these could be left out of the prediction. Also, as NADPH is an intermediary metabolite itself one could argue that if an extraction is necessary for the prediction it has little value, as measurement of NADPH concentration in the extract is relatively little work compared to performing the extraction.

The prediction of NADPH in Fig. 5 is sufficiently precise to be used for screening for a strain with elevated NADPH content. Extractions of enzymes and intracellular polyols are relatively simple and they are stable compounds compared to intermediary metabolites. These extractions could therefore be automatized to screen a large number of strains. In addition, all the polyols can be measured by one injection on HPLC.

The PLS regression in Fig. 5 was calibrated with samples from four strains having different PPP enzyme concentrations and cultivated under two different conditions (exponential phase in ammonium or nitrate containing media), which should make it relatively robust. In addition, the variables in these eight samples were in most cases determined as averages of several independent measurements. However, eight samples is an insufficient number to avoid cross validation of the regression, which means that the same samples are used for calibration and validation of the regression. Therefore, whether this calibration is generally applicable to a wide range of different genetically modified strains still remains to be shown.

In our case, samples from the exponential phase were shown to be the most important; a regression using only the samples from the exponential phase was successful, but a regression using only samples from the stationary phase was not. The logarithm of slope plot [29] was therefore an important tool, because it shows exactly

when a culture grows exponentially. The extra- and intracellular polyol concentrations are important for the regression and it might be applicable to other filamentous fungi, as they usually produce polyols. Enzyme concentrations are also important for the prediction of the NADPH concentration and other compounds than polyols which require reduction of NADPH to NADP upon formation may also be useful.

The main conclusion from this study is that NADPH concentration was successfully increased by overproducing 6PGDH (Figs 4C and 5). This is in contrast with previous studies of *A. niger* where overproduction of citrate synthase [30], phosphofructokinase and pyruvate kinase [31] showed no effect on metabolism other than decreased levels of the activator fructose 2,6-biphosphate and of ATP in the phosphofructokinase overproducing strain [32]. Although many significant differences in enzyme and metabolite levels were observed in the 6PGDH overproducing strain compared to wild-type, overproduction had no significant influence on overall physiology. For example, specific growth rate and spore formation remained unchanged, which is of great advantage when propagating the engineered fungal strains. This indicates that *A. niger* has a relatively robust primary metabolism which is in contrast to results obtained in *E. coli* [10,14] and *R. eutropha* [11,12], where increased NADPH levels as a result of metabolic engineering had a negative effect on growth rate.

The increased NADPH concentration might result in increased biotransformation rates of substrates that require reducing equivalents. However, this still remains to be shown by application of strains overproducing 6PGDH in NADPH-dependent processes. Because overproduction of both G6PDH and TKT also results in significant changes in concentrations of intracellular metabolites it would be very interesting to overproduce all three enzymes or combinations thereof in the same strain.

Materials and methods

Strains and culture conditions

We used *Aspergillus niger* NW 131 (*cspA1 goxC17*) as the wild-type (wt) strain, which is a glucose oxidase negative strain [33] with short conidiophores [34]. All strains used were derived from N400 (CBS 120.49) and are listed in Table 2.

Unless stated otherwise, medium composition, plate cultures and bioreactor cultures were as described previously [29]. Shake flask cultures for preliminary characterization (Southern analysis, transcript analysis and enzyme activity) and screening of isolated transformants contained minimal medium (MM) with 70 mM $NaNO_3$ as the nitrogen source and 1% (w/v) glucose as the carbon source. Bioreactor cultures for detailed characterization of strains contained MM with 21 mM NH_4Cl or $NaNO_3$ as the nitrogen source (final cell density limiting substrate) and 5% (w/v) glucose as the carbon source. Titrants for maintaining pH at 3 were 2 M NaOH and 2 M HCl.

Molecular biology techniques

DNA manipulations were essentially as described by [35]. *E. coli* DH5α was used for propagation of plasmid DNA. Unless stated otherwise the plasmid used was pBluescript (SK+). Preliminary and control DNA sequencing were carried out using a Ready Reaction Dye Deoxy Terminater Cycle Sequencing kit (Perkin Elmer, Wellesley, MA) in an Applied Biosystems automatic DNA sequencer model 310 (ABI Prism 310 Genetic Analyser, Perkin Elmer). *tktA* was sequenced by ligating the gene into pUC19 and using ^{33}P-labelled ddNTPs (Amersham-Pharmacia, Piscataway, NJ) by standard methods [35,36] covering all parts of the sequence at least twice in each direction. *gndA* and *pgiA* were sequenced using the BigDye sequencing kit and an ABI Prism 310 capillary sequencer (Perkin-Elmer). *A. niger* DNA was isolated as described in de Graaff *et al.* [37] and RNA was isolated using TRIZOL (Life Technologies, Gaithersburg, MD).

Transformations of *A. niger* were performed essentially as described by Kusters-van Someren *et al.* [38] using 2×10^7 protoplasts. Overexpressing strains were obtained by cotransformation of the uridine requiring strain [39] NW129 (*cspA1 goxC17 pyrA6*) with 1 μg of the plasmid pGW635 containing the *pyrA* gene and 20 μg of a plasmid containing the gene coding for the enzyme to be overproduced. After transformation the protoplasts were plated on minimal medium, which unless otherwise stated contained 0.95 M sucrose as osmotic stabilizer and carbon source in addition to 70 mM nitrate as nitrogen source. The protoplasts transformed with pIM440 (*gsdA*, described above) were plated on minimal media osmotically stabilized with sorbitol and with different carbon and nitrogen sources to obtain different rates of intracellular NADPH oxidation: 1% (w/v) glucose and 70 mM ammonium, 1% (w/v) glucose and 70 mM nitrate, 1% (v/v) dihydroxyacetone and 70 mM nitrate, and 1% (w/v) L-arabinose and 70 mM nitrate, because NADPH is needed for growth on nitrate, dihydroxyacetone and L-arabinose. Copy number of genes introduced in transformants was estimated by Southern analysis.

Sampling and analysis

Culture filtrate samples were obtained as described before [20]. Mycelium samples were collected by filtration in a fun-

nel with a sintered glass filter. After washing, the mat of mycelium was frozen in liquid nitrogen. Dry biomass (DB) samples were sampled directly into a measuring cylinder and mycelium was washed twice on the sintered glass filter by resuspension in distilled water, frozen in liquid nitrogen, and stored at −20 °C. Samples for measurement of enzymes were washed twice with 50 mM potassium phosphate buffer (pH 7) on the sintered glass filter, frozen in liquid nitrogen, and stored at −70 °C. Samples for measurement of intracellular polyols were not washed since this can cause loss of up to 60% of the intracellular (I) polyols [40]; mycelium was frozen in liquid nitrogen, and stored at −70 °C. Sampling for intermediary metabolites was done directly into a methanol buffer at −40 °C to inactivate metabolism [41], and samples were frozen in liquid nitrogen, and stored at −70 °C.

Biochemicals were from Boehringer Mannheim (Mannheim, Germany), Roche (Basel, Switzerland) or Sigma (St. Louis, MO). Glucose was determined either by glucose test strips (Roche), by HPLC analysis or enzymatically essentially as described by Bergmeyer [42]. Nitrate was detected by nitrate/nitrite test strips (Merck). Glucose, polyols and organic acids were determined by HPLC analysis using a Dionex system (Dionex Corp., Sunnyvale, CA, USA). Extracellular (E) concentrations were determined after centrifuging culture filtrate samples to remove any precipitate after freezing. Intracellular (I) polyols were extracted from mycelium according to Witteveen *et al.* [40]. For glucose and polyols, an anion-exchange CarboPac MA1 column (Dionex) was used. Elution was isocratic at 0.4 $mL \cdot min^{-1}$ with 0.48 M NaOH and amperometric detection. For organic acids an Aminex ion exclusion HPX-87H column (BioRad, Hercules, CA), thermostated at 50 °C was used. Elution was isocratic at 0.5 $mL \cdot min^{-1}$ with 25 mM HCl and detection by refractive index and UV at 210 nm. Extracellular polyols and acids were calculated as concentration measured extracellularly ($mol \cdot L^{-1}$) divided by dry biomass concentration (g $DB \cdot L^{-1}$) to compensate for slightly different times of sampling.

Dry biomass samples were lyophilized and weighed. Frozen mycelium sampled for measurement of enzymes and for isolation of DNA and RNA was precooled in liquid nitrogen and powdered in a precooled Teflon container with a stainless steel ball using a Micro-Dismembrator II (B. Braun, Melsungen, Germany). For measurement of enzymes 0.1–0.4 g powder·mL^{-1} was suspended in extraction buffer containing 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol at 0 °C. The suspension was mixed by pipetting and the enzyme extract was obtained as the supernatant after centrifugation at 40 000 ***g*** for 10 min. Enzyme assays were based on measurement of NAD(P)H and performed at 30 °C using a Cobas Bio autoanalyzer (Roche; absorbance at 340 nm, $\varepsilon = 6.22\ mM^{-1} \cdot cm^{-1}$). ALD, G6PDH, PGI and M1PDH activities were determined as described by Ruijter *et al.* [31]. GLYDH activity was determined as described by de Vries *et al.* [43]. 6PGDH was determined as described by Rippa and Signorini [44] with the modification that EDTA was omitted. TAL activity was determined as described in [45] with the modifications that the buffer was 100 mM Pipes pH 7.6, the concentration of F6P was increased to 3 mM and EDTA was omitted. The specific TAL activity was found by subtraction of the M1PDH activity. TKT activity was determined as described by Bruinenberg [45] with the modifications that the buffer was 50 mM Pipes pH 7.6, the concentration of R5P was doubled to 4 mM and the reaction was started with Xu5P. Protein concentration in enzyme extracts was determined after denaturation and precipitation of protein with sodium deoxycholate and trichloroacetic acid [46] using the BCA method as described by the manufacturer (Sigma).

Extraction and determination of intermediary metabolites were performed as described by Ruijter and Visser [41]. The assays for G6P, F6P, PYR, ADP, AMP, NAD and ATP were also as described by Ruijter and Visser [41]. The assay for 6PG was the same as for G6P, except that G6PDH was exchanged with 6PGDH. The assay for NADP was as described by Klingenberg [47] with the modification that 50 mM triethanolamine (pH 7.6) was used, G6P was 0.5 mM, G6PDH was 1.4 $U \cdot mL^{-1}$ and 2.5 mM $MgCl_2$ was added instead of $MgSO_4$. The assay for NADH and NADPH was as described by Klingenberg [47] with the modifications that 50 mM triethanolamine (pH 7.6) was used, 2-ketoglutarate was 1.25 mM and instead of absorbance the fluorescence was measured ($\lambda_{excitation} = 340$ nm and $\lambda_{emission} = 460$ nm, F4500 Fluorescence Spectrophotometer, Hitachi, Tokyo, Japan) to increase the sensitivity. G6P, F6P and S7P were determined in a modified version of the assay developed by Racker [48] in the presence of 25 mM glycylglycine (pH 7.4), 0.5 mM NADP and 0.2 mM GAP by addition of 0.3 $U \cdot mL^{-1}$ 6PGDH, 0.3 $U \cdot mL^{-1}$ PGI and 0.3 $U \cdot mL^{-1}$ TAL, respectively. DHAP, GAP, R5P and Ru5P were determined in a modified version of the assay from [49]. Our assay was carried out in the presence of 25 mM glycylglycine (pH 7.4), 6 mM $MgCl_2$, 2.4 mM thiamine pyrophosphate, 1 mM NADH and 0.5 mM Xu5P by addition of 0.7 $U \cdot mL^{-1}$ glycerol 3-phosphate dehydrogenase, 40 $U \cdot mL^{-1}$ triosephosphate isomerase, 0.33 $U \cdot mL^{-1}$ TKT and 1 $U \cdot mL^{-1}$ ribosephosphate isomerase, respectively. DHAP, GAP and Xu5P were determined in a similar assay by exchanging 0.5 mM Xu5P with 0.5 mM R5P, whereby Xu5P is measured by the addition of 0.33 $U \cdot mL^{-1}$ TKT and the addition of ribosephosphate isomerase is omitted.

Accumulated titrant added to maintain constant pH was analyzed with natural logarithm of slope plots [29] to ensure correct sampling time points (exponential growth phase, e, and stationary phase, s) and to measure the maximum specific growth rate (μ_{max}, h^{-1}) and the rate constant of induction of acid production (IAP, h^{-1}) in the postexponential phase. Samples from exponential growth phase (e)

were taken 13–20 h after inoculation with spores, which corresponds to 1–8 h before exhaustion of the final cell density limiting substrate (ammonium or nitrate). Samples from stationary phase (s) were taken 11–14 h after exhaustion of the final cell density limiting substrate.

Partial least square (PLS) regressions (see Martens and Næs [21], Höskuldsson [22], Esbensen [23] for a general introduction to PLS regression) were made with the statistical software package for multivariate data analysis UNSCRAMBLER vs. 7.8 (CAMO Process AS, Oslo, Norway). Because the number of samples (16) was relatively small, full cross validation was applied. Skewed (asymmetric) variables with a skewness higher than 1 or lower than −1 were preprocessed by a simple log-transformation (a = log[a]), which reduced the absolute value of the skewness to lower than 1. Variables were centralized (subtraction of mean) and weighted (division with standard deviation) to obtain a mean of zero and a standard deviation of 1 for all variables. Variables with little correlation to the Y-variable (low absolute values of X-loading weights) were excluded from the PLS regression, because they contribute little to the prediction but significantly to the error. Several PLS regressions were performed with different X-variables with low X-loading weights excluded to optimize the correlation and minimize the error.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/EJB/EJB4554/EJB4554sm.htm

Table S1. Table of variables measured in the cultures for detailed characterization of wild-type and overproducing strains.

Figure S1. Residual variance of calibrated X and of validated Y (NADPH) in PLS regression shown in Fig. 4.

Figure S2. U vs. T scores on PC1 and on PC2 in PLS regression shown in Fig. 4.

Figure S3. Scores and X-loading Weights and Y-loadings in PLS regression shown in Fig. 5.

Figure S4. Residual variance of calibrated X and of validated Y (NADPH) in PLS regression shown in Fig. 5.

Figure S5. U vs. T scores on PC1 and on PC2 in PLS regression shown in Fig. 5.