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Improving penicillin biosynthesis in *Penicillium chrysogenum* by glyoxalase overproduction

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ABSTRACT

Genetic engineering of fungal cell factories mainly focuses on manipulating enzymes of the product pathway or primary metabolism. However, despite the use of strong promoters or strains containing the genes of interest in multiple copies, the desired strongly enhanced enzyme levels are often not obtained.

Here we present a novel strategy to improve penicillin biosynthesis by *Penicillium chrysogenum* by reducing reactive and toxic metabolic by-products, 2-oxoaldehydes. This was achieved by overexpressing the genes encoding glyoxalase I and II, which resulted in a 10% increase in penicillin titers relative to the control strain.

The protein levels of two key enzymes of penicillin biosynthesis, isopenicillin N synthase and isopenicillin N acyltransferase, were increased in the glyoxalase transformants, whereas their transcript levels remained unaltered. These results suggest that directed intracellular reduction of 2-oxoaldehydes prolongs the functional lifetime of these enzymes.

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1. Introduction

β -lactam antibiotics represent a class of important pharmaceuticals of major clinical value. Their economic significance is stressed by the fact that these antibiotics contribute to over 40% of the total antibiotic market (Kresse et al., 2007). The β -lactam antibiotic penicillin (PEN) is industrially produced by the filamentous fungus *Penicillium chrysogenum*. The PEN biosynthetic machinery is compartmentalized in *P. chrysogenum* in the cytosol and microbodies (peroxisomes) (Evers et al., 2004; Müller et al., 1991; Turner, 1992). The process starts with the condensation of three amino acids into the tripeptide α -aminoadipoyl-cysteine-valine (ACV). ACV is subsequently converted by cytosolic isopenicillin N synthase (IPNS) to isopenicillin N (IPN), which contains the characteristic β -lactam backbone. The third enzyme of PEN biosynthesis, isopenicillin N acyltransferase (IAT) is localized in peroxisomes (Müller et al., 1992). IAT catalyzes the incorporation of a novel side chain, using phenylacetyl CoA or phenoxyacetyl CoA as a substrate. The enzyme phenylacetyl CoA

ligase (PCL) is known as the major phenyl acetic acid activating enzyme (Lamas-Maceiras et al., 2006). PCL is also a peroxisomal enzyme, thus stressing the importance of this organelle in efficient PEN production in *P. chrysogenum* (Kiel et al., 2009; Meijer et al., 2010).

Since the isolation of the first PEN producing strain in 1943, random mutagenesis and selection procedures have been successful in generating a strain lineage that shows enhanced PEN production levels. More recently, also genetic engineering was introduced in strain improvement programs. These efforts have primarily focused on increasing the levels of (or introducing new) enzymes in the product pathway and on engineering of primary metabolism.

At present, there is an urgent need for the development of novel strategies because of the limited success of further enhancing the biosynthesis performance of current production strains by random mutagenesis approaches and the exhaustion of targets for metabolic engineering. A new strategy in this regard is to increase the period over which biosynthetic enzymes of secondary metabolism are functional. This was accomplished in the present study by 'boosting' the ability of the fungal cell factory to detoxify reactive products of metabolism. An important example of such a compound is methylglyoxal which is formed as by-product of the triosephosphate isomerase reaction in glycolysis (Thornalley, 2008). Catalyzed by glyoxalase I (GLO1, EC 4.4.1.5, lactoylglutathione lyase) and II (GLO2, EC 3.1.2.6, hydroxacylglutathione hydrolase), 2-oxoaldehydes are converted into their corresponding 2-hydroxy acids in two consecutive steps (Thornalley, 2008). Stressing the importance of the glyoxalase system on fungal

Abbreviations: ACV, α -aminoadipoyl-cysteine-valine; AT, acyltransferase; BSA, bovine serum albumin; GLO1, glyoxalase I; GLO2, glyoxalase II; HPLC, high pressure liquid chromatography; IAT, isopenicillin N acyltransferase; IPNS, isopenicillin N synthase; MG, methylglyoxal; PCL, phenylacetyl CoA ligase; PEN, penicillin; RT, room temperature; SDLGSH, S-D-lactoylglutathione; TCA, trichloroacetic acid; WT, wild type.

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viability, it was shown that overexpression of glyoxalase I and II encoding genes leads to increased survival on media containing glucose (2% concentration) as a sole carbon source in the fungal aging model *Podospora anserina* (Scheckhuber et al., 2010).

Here we demonstrate that overexpression of the corresponding genes (*PcGlo1*, *PcGlo2*) in *P. chrysogenum* results in an increase in PEN production. Double *PcGlo1/PcGlo2* overexpression strains (*PcGLO1/2OEx*) showed an increase of PEN titers up to 10%. These findings correlate with an increased content of IPNS and IAT protein in *PcGLO1/2OEx* strains.

2. Methods

2.1. Strains/cultivation

DS17690 (Harris et al., 2006) was used as a high PEN production strain of *P. chrysogenum*. Sporulation of mycelia was stimulated by growth on R agar (Bartoszewska et al., 2011) at 25 °C for 10–11 days. For production of PEN V, spores were inoculated in 50 ml PEN production medium (Nijland et al., 2010) +0.05% phenoxyacetic acid for 1 h at RT before transfer to shake flasks. Cultures were subsequently incubated in an orbital shaker (200 rpm) for 10 d at 25 °C or 30 °C. For cloning purposes, *Escherichia coli* strain DH5 α was used.

2.2. Construction of *PcGlo1* and *PcGlo2* overexpression plasmids

For the construction of the *PcGlo1* overexpression vector *pPcGlo1Ex1*, the *PcGlo1* cDNA was amplified by PCR from a *P. chrysogenum* cDNA library using oligonucleotides *cGlo1f1* and *cGlo1r1* (Table 1). The PCR product was cut with *HindIII/EcoRV* and cloned into the *HindIII/SmaI* site of vector *pGBRH2* (Kiel et al., 2005) containing the *IPNS* promoter and *AT* terminator sequences from *P. chrysogenum*. The strategy for the construction of the *PcGlo2* overexpression vector *pPcGlo2Ex1* is similar but uses oligonucleotides *cGlo2f1* and *cGlo2r1* (Table 1). Both constructs were verified by sequencing. The glyoxalase I/II overexpression cassettes were isolated from *pPcGlo1Ex1* and *pPcGlo2Ex1* by *NotI* restriction and subsequently purified using the Nucleospin kit (Machery Nagel, Düren, Germany).

2.3. Transformation and selection of *P. chrysogenum*

Protoplasts of *P. chrysogenum* high PEN production strain DS17690 were prepared and subsequently co-transformed with the linear *PcGlo1/PcGlo2* expression cassettes and the *amdS* (acetamidase synthase) selection marker from plasmid *pSUS15* (lab collection) according to a previously published protocol (Cantor et al., 1987). Transformants were selected on plates containing acetamide as the sole nitrogen source and further analyzed by colony PCR (Bartoszewska et al., 2011) using oligonucleotides *cGlo1f1/cGlo1r1* or *cGlo2f1/cGlo2r2* (Table 1). Positive candidates were transferred to R agar to induce sporulation of the mycelium. Spores were streaked out on acetamide plates to receive single

colonies, which were transferred to fresh acetamide medium and tested again using colony PCRs for the presence of the *PcGlo1/PcGlo2* expression cassettes.

2.4. Preparation of crude cell extracts

Protease inhibitor cocktail (final concentrations in the medium: 10 μ M 4-(2-aminoethyl)-benzenesulfonylfluoride, 0.14 μ M E-64, 0.22 μ M pepstatin A and 50 μ M 1,10-phenanthroline in dimethylsulfoxide) (Sigma Aldrich, Zwijndrecht, The Netherlands) was added to the culture. Three ml of the culture was filled into a French Press chamber and exposed to high pressure. Protein concentration of the cell extracts was determined by using a kit from BioRad (Munich, Germany). Trichloroacetic acid (TCA) extracts of whole cells were prepared as described previously (Kiel et al., 2009).

2.5. Determination of glyoxalase I and glyoxalase II activity

Glyoxalase I activity, modified after Basu et al. (1988): assay buffer containing 3 mM methylglyoxal, 1 mM reduced glutathione, 16 mM MgSO_4 and 33.3 mM potassium phosphate (pH 7.0) was freshly prepared and kept at RT for 1 h so that the glyoxalase I substrate, hemithioacetal, could be formed. Upon addition of cell extract, the increase of absorbance at 240 nm (i. e., formation of S-D-lactoylglutathione [SDLGSH]) was measured in a Lambda 35 UV/VIS spectrophotometer (Perkin-Elmer, Waltham, MA, USA) for 3 min. Enzymatic activity was calculated by using the molar coefficient of extinction for the formed product, SDLGSH ($3100 \text{ M}^{-1} \text{ cm}^{-1}$ at 240 nm).

Glyoxalase II activity, modified after Maiti et al. (1997): protein was added to assay buffer (300 μ M SDLGSH in 33.3 mM potassium phosphate [pH 7.0]) and the decrease in absorbance was measured at 240 nm for 3 min.

2.6. qPCR analysis of *IPNS* and *IAT* transcript levels

Quantitative PCRs for the determination of *IPNS* and *IAT* transcript levels were determined according to a previously published protocol (Nijland et al., 2010).

2.7. Western blot analysis

Western blotting was performed by established techniques. After blocking, the blots were incubated with primary antibodies against *IPNS* (1/10,000) or *IAT* (1/5000) (supplied by DSM, Delft, The Netherlands). As loading control, antibodies against translation elongation factor 1 α (eF1 α) from *Hansenula polymorpha* (1/5000) (lab collection) were utilized. Blots were decorated using α -IgG coupled with alkaline phosphatase, 1/10,000 (Sigma Aldrich, Zwijndrecht, The Netherlands).

2.8. HPLC determination of PEN V

Levels of PEN V in spent media were determined by high pressure liquid chromatography (HPLC) using an isocratic flow of acetonitrile (350 g/l), KH_2PO_4 (640 mg/l) and H_3PO_4 (340 mg/l). The peaks were separated on a Platinum EPS 5 μ m C18 column (Grace, Deerfield, IL, USA) at a flow rate of 1 ml/min. The detection wavelength was set to 254 nm.

2.9. Protein identification in Coomassie stained gels

Proteins bands of interest were cut from an SDS-PAGE gel containing separated TCA extracts from the strains of interest and analyzed by

Table 1
Oligonucleotides used in this study.

Name	5'–3' Sequence
<i>cGlo1f1</i>	AAA <u>AAGCTT</u> ATGGCTTCGGATACCTCC
<i>cGlo1r1</i>	AA <u>GATATC</u> CTACCAATCTCCAGTCCG
<i>cGlo2f1</i>	AAA <u>AAGCTT</u> ATGCATATCCAATCAATTCC
<i>cGlo2r1</i>	AA <u>GATATC</u> TTACATCGAATCTTCATCTC
<i>cGlo2r2</i>	CGGCACTGAGAATGACCCC

Recognition sites for restriction enzymes are underlined.

A

PcGLO1 1 mas--dtstyklntmmrvkdprksvefykflglnlvntidmpewkfcnyflaydgp--aslqgarhwtdrnavlelthnygtendpnys
 AfGLO1 1 mat--dtikylnhtmirvkdprksvefykflglnqldfpenkfslyflayngp--qslqgdhrwtdrnavlelthnygtendpnys
 AnGLO1 1 mat--dtskyklntmlrvkdprkslefykylglsgvnqidfpenkfslyflaydgp--qslqgerhftdrngvlelthnhtgeddpnys
 NcGLO1 1 maatttdvgnkykfnhsmirvkdprksvefykflglnslkkslspkafldlyflgydap--gavsganilwdrreglielthnygtessdpnyk
 PaGLO1 1 masttdvknfklnhtmirvkdprksvefykflglnslkkslspkafldlyflgydap--gavshgknlwdrreglielthnygtendpeyk
 SmGLO1 1 maatttdvknkykfnhsmirvkdprksvefykflglnslkkslspkafldlyflgydapggavsganilw-drreglielthnygtessdsyk

PcGLO1 87 vvnngntephrfgghiaaisvndieaackriedagypfqkkltegrmrhifakdpdgywveii-rrededlst---t--tdpgsyrlnhtn
 AfGLO1 87 vvnngntephrfgghiaaisvndieaackriedagypfqkkltegrmrhifakdpdgywveii-rrededlst---a--tdpgtyrlnhtn
 AnGLO1 87 vvnngntepyrghiaaisvndieaackriedagypfqkkltegrmrhifakdpdgywveii-rrededlst---a--tdpgsyrlnhtn
 NcGLO1 89 innngvpehrfgfhtcdisvndlgaacqriedagypfqkkltegrmrhifaldpdgywveii-grkpveetegvke--tdlkyrmnhtn
 PaGLO1 89 innngvpeyrghfhtcdisvndlgaacqriedagypfqkkltegrmrhifaldpdgywveii-grkpveeteg---vretldlgyrmnhtn
 SmGLO1 90 innngvpehrfgfhtcdisvndlgaacqriedagypfqkkltegrmrhifaldpdgywveii-grkpveetse---iketdlkyrmnhtn

PcGLO1 171 lrvkdaeslskfyqesmgmtlvrtienpenkfnlyflgypasnpiekegkngvaeweglleltnygtetekqegpyvnhngntepqgfggh
 AfGLO1 171 lrvksaetslskfyqesmgmtlvrtienkdaafnlyflgypasnpieqegaknpvaeweglleltnygtetekqegkvyhdgnseppqgfggh
 AnGLO1 171 lrvksaetslskfyqesmgmtlvrtienkdaafnlyflgypasnpieqegaknpvaeweglleltnygtetekqegpyvnhngntepqgfggh
 NcGLO1 176 lrvkdgekslskfyqesmgmtlvrtieakeagfnlyflgy-----gdekqntadreglleltnygtetekdenfshyngndqpgqfggh
 PaGLO1 176 lrvkdaerslskfyqesmgmtlvrtieakeagfnlyflgyegeqgavveg--gdtakreglleltnygtetekdenfshyngndqpgqfggh
 SmGLO1 177 lrvkdaekskskfyqesmgmtlvrtieakeagfnlyflgy-----gdekqntadreglleltnygtetekdenfshyngndqpgqfggh

PcGLO1 261 cisvddleaacdrfeslskvnfkrltdgrmhniafilpdpgywievvqnggikrtgdw 318 a.a.
 AfGLO1 261 cvsddlnaacdrfeslnvwnkrltdgrmknvafildpdpgywievqnealkrtgsnw 318 a.a. 83%
 AnGLO1 261 cisvddlnaacdrfeslnvwnkrltdgrmknyvafildpdpgywievqnealkrtgsl 318 a.a. 81%
 NcGLO1 258 cvsndieaacerleglksvwnkrltdgrmknvafildpdpgywievqnerftgqanf 315 a.a. 61%
 PaGLO1 264 cvsdslekaacerfeglgvswkrltdgrmknvafildpdpgywievqnerfadkanf 321 a.a. 62%
 SmGLO1 259 clsvndieaacerleglnvwnkrltdgrmknvafildpdpgywievqnerftgqsnf 316 a.a. 62%

B

PcGLO2 1 mhiqsiptwtgkgnnyaylvtdeptkqsviidpanppevapelpksqiasgkidltaiivnthhhwdhaggngdilkqf--k
 AfGLO2 1 mhvqsiptwtgkgnnyaylvtdeptkesviidpanppevapelpdaqikagkiklsaiivnthhhwdhaggngnmlkhf--g
 AnGLO2 1 mhvqsiptwtgkgnnyaylvtdeptkesviidpanppevapelpksqiasgkidltaiivnthhhwdhaggngnmlkhf--g
 NcGLO2 1 mhiqsiptwtgssdnyaylvvddkskdavidpanppevapvllkaiddgsikltaiivnthhhwdhaggngtklrltalglp
 PaGLO2 1 mhiqsiptwtgssdnyaylvvddkskdavidpanppevapvllkaiddgsikltaiivnthhhwdhaggngtklrltalglp
 SmGLO2 1 mhiqsiptwtgssdnyaylvvddkskdavidpanppevapvllkaiddgsikltaiivnthhhwdhaggngtklrltalglp

PcGLO2 79 g-lqviggkdcasvktphageefkigdrisvkalhtpchtqdsicymqgdg--qravftgdtlfiggcgrffegnake
 AfGLO2 79 k-lpviggrncqsvtqtpahgeetfkigerisvkalhtpchtqdsicymqgdg--ekvvftgdtlfiggcgrffegnake
 AnGLO2 79 q-lsviggrncqsvtqtpahgeetfkigerisvkalhtpchtqdsicymqgdg--dkvvftgdtlfiggcgrffegnake
 NcGLO2 81 n-leiiggrdcckvknvpahgqgfign-iavkalhtpchtqdsicwfmqgdg--dkvvftgdtlfiggcgrffegnake
 PaGLO2 76 sklpiiggrdcavhktphagettigenikvkalhtpchtqdsicwlmeketgdkviftgdtlfiggcgrffegnake
 SmGLO2 81 n-leiiggrdcckvknvpahgqgfign-iavkalhtpchtqdsicwfmqgdg--dkvvftgdtlfiggcgrffegnake

PcGLO2 156 mhkalnetlaalpddtkvypgheytkanvkfciavsqtepiikkqlafadqknqtkgkftigdeklnhvfmrndpeiqrk
 AfGLO2 156 mhkalnetlaalpddtkvypgheytkanvkfciavsqtepiikkqlafadqknqtkgkftigdek-----dpeiqrk
 AnGLO2 156 mhkalnetlaalpddtkvypgheytkanvkfciavsqtepiikkqlafadqknqtkgkftigdeklnhvfmrndpeiqrk
 NcGLO2 157 mhkalnetlaalpddtkvypgheytkanvkfciavsqtepiikkqlafadqknqtkgkftigdeklnhvfmrndpeiqrk
 PaGLO2 156 mhealnvvlagvgddrvypgheytkanvkfciavsqtepiikkqlafadqknqtkgkftigdeklnhvfmrndpeiqrk
 SmGLO2 157 mhkalnetlaalpddtkvypgheytkanvkfciavsqtepiikkqlafadqknqtkgkftigdeklnhvfmrndpeiqrk

PcGLO2 236 tgktdpvdmalremkns- 255 a.a.
 AfGLO2 227 tgktdpvdmalremkns- 246 a.a. 80%
 AnGLO2 236 tgktdpvdmalremkns- 255 a.a. 86%
 NcGLO2 237 tgktdpvaimtklremknsfk 257 a.a. 68%
 PaGLO2 236 tgktdpvaimtklremknsfk 256 a.a. 65%
 SmGLO2 237 tgktdpvaimtklremknsfk 257 a.a. 70%

Fig. 1. Homology analysis between fungal proteins of the glyoxalase system: (A) comparison of the amino acid sequences of glyoxalase I proteins from *P. chrysogenum* (PcGLO1, UniProt accession number B6GZZ1), *Aspergillus fumigatus* (AfGLO1, Q4WN17), *Aspergillus niger* (AnGLO1, NCBI Reference Sequence: XP_001394288.2), *Podospora anserina* (PaGLO1, B2AQW8), *Neurospora crassa* (NcGLO1, Q7S6M0) and *Sordaria macrospora* (SmGLO1, F7VW73), (B) comparison of the amino acid sequences of glyoxalase II proteins from *P. chrysogenum* (PcGLO2, UniProt accession number B6HM01), *Aspergillus fumigatus* (AfGLO2, Q4WVP5), *Aspergillus niger* (AnGLO2, NCBI Reference Sequence: XP_001401257.2), *Podospora anserina* (PaGLO2, B2B554), *Neurospora crassa* (NcGLO2, Q1K7C3) and *Sordaria macrospora* (SmGLO2, F7VWX7). Amino acids that are identical to the *P. chrysogenum* sequences are printed in black. Homologous and non-homologous amino acids are printed in gray and red lettering, respectively. After each sequence the length of the protein and the identity relative to the *P. chrysogenum* GLO1 and GLO2 sequence is given. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mass spectrometry (MALDI-TOF peptide mass fingerprint and MALDI-TOF/TOF peptide sequencing) by Alphalyse A/S, Odense, Denmark.

2.10. Assay to determine methylglyoxal sensitivity

50 ml of PEN V production medium was inoculated with sporulating mycelium grown on 15–20 rice grains for 1 h at room temperature with occasional mixing. 5 µl of a 1/10 dilution in sterile water was spotted onto R agar plates supplemented with

increasing concentrations (0%–0.15%) methylglyoxal (40% stock solution, Sigma Aldrich, Zwijndrecht, The Netherlands). The plates were incubated at 25 °C for six days.

2.11. Detection of methylglyoxal-modified proteins

The levels of methylglyoxal-modified proteins were determined using the OxiSelect™ Methylglyoxal (MG) ELISA kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions.

Methylglyoxal–protein adducts were probed with specific monoclonal antibodies. Subsequently the samples were washed and treated with horseradish peroxidase conjugated secondary antibodies and a substrate solution. Substrate turnover leads to the formation of a product that can be measured in a photometer at a wavelength of 450 nm. These data are compared to a standard curve prepared from methylglyoxal–bovine serum albumin (supplied with the kit).

2.12. Glyoxalase homology analyses

Homology between related proteins was determined using the BLOSUM62 algorithm implemented in 'Clone Manager Suite 6' (Scientific and Educational Software, Cary, NC, USA).

3. Results

3.1. Identification of PcGLO1 and PcGLO2 in the *P. chrysogenum* genome

The amino acid sequences of *P. anserina* GLO1 (UniProt accession number B2AQW8) and GLO2 (UniProt accession number B2B554) were used to search the genomic sequence of *P. chrysogenum* (van den Berg et al., 2008) for the corresponding homologs via the BlastP algorithm (Altschul and Lipman, 1990). For each protein, one homolog, Pc12g09820 (PcGLO1) and Pc21g08590 (PcGLO2) was identified (Fig. 1). The sequences of PcGLO1 and PcGLO2 are strongly conserved relative to their *P. anserina* counterparts, displaying 62% and 65% sequence identity, respectively (Fig. 1). It should be noted that both deposited sequences were corrected regarding the translation start of the proteins. The annotated sequence for Pc12g09820 starts at amino acid position +14 while the annotated sequence for Pc21g08590 starts at amino acid position –23. This correction was possible due to homology analysis including glyoxalase I and II sequences from various filamentous fungi (i.e., *Aspergillus fumigatus*, *Aspergillus niger*, *P. anserina*, *Neurospora crassa* and *Sordaria macrospora*) (Fig. 1).

3.2. Generation of glyoxalase I/II overexpression strains

We analyzed the impact of the glyoxalase system on PEN production by analyzing double (*PcGlo1/PcGlo2*) overexpression

strains. Two independent glyoxalase I/II double overexpression strains (PcGLO1/2OEx) were analyzed for enzyme activities (Table 2). The data revealed that in both strains the levels of glyoxalase I and II activity were strongly enhanced (Table 2) relative to those of the parental strain DS17690 (hereafter designated as control). The highest activities were obtained in strain #7

Table 2

Enzyme activities of PcGLO1 and PcGLO2 in mycelial extracts from control and PcGLO1/2OEx overexpression strains.

	GLO1 [U/mg protein]	GLO2 [U/mg protein]
25 °C		
Control	0.27	0.11
PcGLO1/2OEx #7	13.01	11.83
PcGLO1/2OEx #12	7.74	18.28
30 °C		
Control	0.11	0.08
PcGLO1/2OEx #7	8.06	4.19
PcGLO1/2OEx #12	4.95	5.48

The strains were grown in PEN production media for 10 days at either 25 °C or 30 °C.

Table 3

Determination of PEN V levels in control and PcGLO1/2OEx overexpression strains.

Strain	Growth temperature (°C)	PEN V [g/l]	%	PEN V [mg/g dry weight]	%	Ysp [mg/g]
Control	25	1.13	100	60.1	100	15.07
PcGLO1/2OEx #7	25	1.19*	105	96.0	160	15.87
PcGLO1/2OEx #12	25	1.18*	104	86.8	144	15.73
Control	30	0.86	100	48.9	100	11.47
PcGLO1/2OEx #7	30	0.95**	110	66.0	135	12.67
PcGLO1/2OEx #12	30	0.94**	109	58.8	120	12.53

Ysp: mg PEN per gram carbon source.

* $P < 0.05$ (control strain vs. PcGLO1/GLO2OEx; Student's *t*-test, two-tailed).

** $P < 0.01$ (control strain vs. PcGLO1/GLO2OEx; Student's *t*-test, two-tailed).

Fig. 2. Transformants PcGLO1/2OEx #7 and PcGLO1/2OEx #12 show enhanced resistance to methylglyoxal: suspensions of germinating spores was spotted on agar plates containing increasing concentrations of methylglyoxal. The plates were incubated for six days at 25 °C. PcGLO1/2OEx #7 and PcGLO1/2OEx #12 are able to tolerate higher methylglyoxal levels compared to the control strain (C, DS17690).

Fig. 3. Analysis to reveal differences in protein abundance between the control strain and transformant PcGLO1/2OEx #7: (A) 1D SDS-gel electrophoresis for the detection of differential abundance of proteins in the control and glyoxalase overexpressor PcGLO1/2OEx #7. Positions of proteins present at higher abundance in the overexpression strain are indicated by red arrows. 1: IPNS, 2: PcGLO1 and 3: PcGLO2 (determined by mass spectrometry) and (B) densitometry analysis of the gel lanes indicated in panel C (black: PcGLO1/2OEx #7, red: control). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Enhanced IPNS and IAT levels are present in mycelial extracts of PcGLO1/2OEx strains: (A) detection of IPNS levels by Western blotting in extracts prepared from strains that were grown on PEN production medium for 10 days at either 25 °C or 30 °C and (B) similar analysis for the determination of IAT protein levels (A). In (A) and (B) images from different parts of the same gel have been grouped (indicated by dividing lines).

which showed a 48-fold increased PcGLO1 activity together with a 108-fold increased PcGLO2 activity.

We next determined whether PcGLO1/2OEx strains show increased resistance against externally added methylglyoxal. As shown in Fig. 2, both transformants (PcGLO1/2OEx #7 and PcGLO1/2OEx #12) are able to tolerate enhanced methylglyoxal levels relative to the control strain. For example, the control strain hardly grows on plates supplemented with 0.15% methylglyoxal while both mutants are able to do so (Fig. 2, bottom row). These results indicate that increased levels of enzymatically active glyoxalase I and II are present in both transformants.

Taken together, our data indicated that strains with enhanced glyoxalase I and II activities were obtained.

3.3. PEN levels are enhanced in glyoxalase overproducing strains

Subsequently, PEN production of the glyoxalase I/II double overexpression strains was determined. To this purpose cells were grown for 10 days in PEN V production media at 25 °C. As shown in Table 3, both strains showed a significant increase in PEN production (both in g PEN/l and in g PEN/g dry weight).

1D SDS-PAGE was performed to detect differences in protein patterns between the control and the PcGLO1/2OEx strain #7 (Fig. 3A). Coomassie brilliant blue staining led to the identification of three enhanced protein bands that were present in PcGLO1/2OEx #7 relative to the control. Mass spectrometry revealed that two of these bands represented PcGLO1 and PcGLO2, as expected. Interestingly, however, the third band was identified as IPNS (Fig. 3A, B), a finding that was confirmed in Western blots

Table 4

Determination of relative IPNS and IAT transcript levels in control and PcGLO1/2OEx overexpression strains.

	Control	PcGLO1/2OEx #7
IPNS	207	204
IAT	0.67	0.63

γ -actin transcript levels was used as reference. X fold changes in transcript quantity relative to γ -actin (transcript level set to 1) are given in the table.

decorated with antibodies against IPNS (Fig. 4A). This positive effect on protein abundance was not confined to IPNS alone as also IAT appeared to be present at elevated levels (Fig. 4B).

Cooling costs are an important economic factor in large scale industrial PEN fermentations. Hence, performing *P. chrysogenum* fermentations at higher growth temperatures would be very cost effective. However, it has been demonstrated that IPNS is instable at enhanced growth temperatures (Gidijala et al., 2008). Growth of the control strain at 30 °C is accompanied by a drop in PEN titers of 24% (Table 3), whereas the PEN titers produced by the mutants show a decrease of approximately 20% when the growth temperature was enhanced to 30 °C. Moreover, when normalizing the PEN production to biomass, PEN productivities (g/g dry weight) in the PcGLO1/2OEx strains (66.0 mg/g dry weight [PcGLO1/2OEx #7] and 58.8 mg/g dry weight [PcGLO1/2OEx #12]) were similar to the productivity of the control strain at 25 °C (60.1 mg/g dry weight [control]). Also at 30 °C protein levels of IPNS and IAT are substantially increased in PcGLO1/2OEx mutants (Figs. 3 and 4).

3.4. qPCR analysis of IPNS and IAT transcript levels

Increased protein levels of IPNS and IAT can be related to either elevated gene transcription or improved protein stability. Quantitative PCR (qPCR) using oligonucleotides directed against *IPNS*, *IAT* and the reference gene γ -actin was performed to determine the relative transcription levels of these genes (Table 4). *IPNS* transcript is present in higher amounts in both samples (control strain DS17690 and glyoxalase transformant PcGLO1/2OEx #7) than either *IAT* or γ -actin transcript (Table 4). This finding is consistent with the fact that IPNS but not IAT is readily visible in Coomassie stained gels (Fig. 3A). Between the control and the glyoxalase transformant there are no significant differences in *IPNS* and *IAT* transcript levels (Table 4). These results suggest that not an induction of gene expression but increased stability of IPNS and IAT leads to the elevated levels of these proteins in glyoxalase transformants.

3.5. PcGLO1/2OEx transformants show reduced methylglyoxal-mediated protein modifications

To test our hypothesis that the overexpression of *PcGlo1* and *PcGlo2* leads to decreased levels of intracellular methylglyoxal, we determined the extent of methylglyoxal-mediated protein modifications. As shown in Fig. 5, both transformants show a reduction in methylglyoxal-mediated protein modification relative to the parental strain.

3.6. Analysis of mycelial deterioration

Mycelial autolysis and fragmentation is a known factor that limits the biosynthetic capacity of fungal cell factories (Paul and Thomas, 1996). Overproduction of both glyoxalase I and II increases the replicative lifespan of *P. anserina* (Scheckhuber et al., 2010). Hence, a possible additional reason for the improved performance of the glyoxalase mutants could be related to a delay in mycelial fragmentation/autolysis. However, we observed no differences in this regard when mycelium samples from the control and a transformant were analyzed by light microscopy at different time points (Fig. 6). Therefore, overexpression of glyoxalase-coding genes has no effect on time-dependent mycelium deterioration.

4. Discussion

Here we demonstrate the positive effect of decreasing toxification by 2-oxoaldehydes by boosting the endogenous glyoxalase system of an important fungal cell factory, *P. chrysogenum*.

The reaction of 2-oxoaldehydes with proteins leads to the formation of advanced glycation end-products (AGEs) (Grillo and Colombatto, 2008). Molecular mechanisms capable of repairing proteins that are modified in this way are not known, so the only remaining option is proteolysis (e.g. by the proteasome) and the costly synthesis of new protein.

We show that both at standard and elevated growth temperatures, double glyoxalase I/II overexpression strains of *P. chrysogenum* (PcGLO1/2OEx) are able to produce enhanced levels of PEN relative to the control. This correlates with increased levels/stability of two enzymes of the PEN biosynthetic pathway, namely IPNS and IAT. As IPNS is very susceptible to inactivation by post-translational modifications (Dubus et al., 2000; Perry et al., 1988), enhanced protection against modification by 2-oxoaldehydes results in less functional impairment and subsequent degradation of the protein. The beneficial effect on IAT protein levels in the glyoxalase transformants is most likely a significant factor in the observed increase in PEN production, because IAT activity was recently reported to be a limiting factor in PEN biosynthesis in high-production strains like DS17690 (Nijland et al., 2010).

In a previous study it was shown that overexpression of both genes encoding the two enzymes of the glyoxalase system in *P. anserina* leads to an increased replicative lifespan when strains are grown on media containing elevated amounts of glucose (Scheckhuber et al., 2010). *P. chrysogenum* does not replicatively age but subject to autolysis and fragmentation during cultivation in liquid media. Due to the fact that microscopic analyses regarding the occurrence of autolysis in control and glyoxalase overexpressors revealed no differences it can be ruled out that increased PEN levels in PcGLO1/2OEx are due to increased 'stability' of the mycelium. The factors influencing replicative ageing in *P. anserina* and autolysis in *P. chrysogenum* are probably distinct.

We previously increased PEN production in *P. chrysogenum* by enhancing peroxisome numbers or inhibiting of autophagy. An increase in peroxisome numbers concomitant with increased PEN titers was obtained by overexpression of *PEX11* (Kiel et al., 2005) or *PEX14-17* (Opalinski et al., 2010), which both resulted in an approximately 2-fold increase. Inhibition of autophagy by deletion of *ATG1* resulted in a smaller increase (PEN titers increased to 125% relative to the parental strain) (Bartoszewska et al., 2011), in the same range as our current observations (increase to 110% by *PcGlo1* and *PcGlo2* overexpression). It must be noted that the last

Fig. 5. Transformants PcGLO1/2OEx #7 and #12 show reduced protein modification by methylglyoxal: (A) standard curve using different concentrations of methylglyoxal-modified BSA and (B) determination of methylglyoxal-modified proteins in the WT control strain (DS17690) and the two transformants PcGLO1/2OEx #7 and #12 (unit: ng modified protein/μg total protein). The mean values of seven measurements each are given.

Fig. 6. Analysis of hyphae fragmentation by light microscopy: samples from the control strain (DS17690) and the glyoxalase I/II transformant PcGLO1/2OEx #7 were taken at the indicated time points from cultures (grown in PEN V production media). Mycelial fragmentation starts to occur after seven days of growth. No differences in hyphal fragmentation between the control strain and the transformant are detected.

two studies were performed with the relatively high producing strain, DS17690, whereas the earlier studies were performed with low producing strains (NRRL1951 for *PEX14-17* overexpression and Wisconsin 54-1255 for *PEX11* overexpression). This may explain why a smaller difference is observed in relative PEN titers. In addition, however, different cultivation conditions were used, which makes these studies difficult to compare. The titer of the low producing NRRL1951 strain is in the range of milligrams per liter, whereas current industrial strains produce 30–55 g/l PEN (van den Berg et al., 2008). The parental strain (DS17690) used in our current study produces approximately 1 g/l. Whether an increase in PEN levels can also be achieved by overproduction of *PcGlo1* and *PcGlo2* in current industrial strains is unknown as these strains are not freely available.

5. Conclusions

The *P. chrysogenum* genome contains one gene for glyoxalase I and one gene for glyoxalase II. Co-overexpression of both genes leads to increased levels of isopenicillin N synthase and isopenicillin N acyltransferase protein levels and enhanced PEN production. At elevated fermentation temperatures (30 °C) *PcGLO1/2OEx* strains produce up to 10% higher PEN titers relative to the parental strain.

Genetic engineering strategies to improve fungal cell factories by overexpression of genes involved in the product pathway have only a limited effect if the proteins are unstable. Our current results offer a novel approach for efforts to stabilize such proteins.

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