(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 January 2014 (03.01.2014)

(10) International Publication Number
WO 2014/003555 A1

(51) International Patent Classification:
C12P 37/00 (2006.01) *C12P 1/02* (2006.01)

(21) International Application Number:
PCT/NL2013/050457

(22) International Filing Date:
26 June 2013 (26.06.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

12173925.4	27 June 2012 (27.06.2012)	EP
61/671,848	16 July 2012 (16.07.2012)	US

(71) Applicant: **RIJKSUNIVERSITEIT GRONINGEN** [NL/NL]; Broerstraat 5, NL-9712 CP Groningen (NL).

(72) Inventors: **SCHECKHUBER, Christian Quintus**; c/o Groningen Biomolecular sciences and Biotechnology, Institute, Department of Molecular Cell Biology, Nijenborgh 7, NL-9747 AG Groningen (NL). **VAN DER KLEI, Ida Johanna**; c/o Groningen Biomolecular sciences and Biotechnology, Institute, Department of Molecular Cell Biology, Nijenborgh 7, NL-9747 AG Groningen (NL).

(74) Agent: **JANSEN, C.M.**; Johan de Wittlaan 7, NL-2517 JR Den Haag (NL).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: IMPROVED PENICILLIN PRODUCTION

(57) Abstract: The invention relates to improved means and methods for producing a compound of interest in fungal host cells. Provided is a method for producing at least one secondary metabolite in a fungal host cell, comprising culturing a fungal host cell capable of producing said metabolite under conditions allowing for production of said metabolite, wherein the host cell has been modified to display elevated levels of glyoxalase I and glyoxalase II activity.

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IMPROVED PENICILLIN PRODUCTION

The invention relates to improved means and methods for producing a compound of interest in fungal host cells.

Fungal cell factories are of major importance for the production of various valuable compounds like biofuels, enzymes and pharmaceutical products (e. g. insulin, hepatitis B antigen) that are indispensable in our daily lives. An eloquent example of this is the β-lactam antibiotic penicillin (PEN) that is massively used for therapy of infectious diseases. This secondary metabolite is industrially produced by the filamentous fungus *Penicillium chrysogenum*. The PEN biosynthetic machinery is compartmentalized in *P. chrysogenum* in the cytosol and microbodies (peroxisomes) (Müller et al., 1991; Turner, 1992; Evers et al., 2004). The process starts with the condensation of three amino acids into the tripeptide α-aminoadipoyl-cysteinyl-valine (ACV). ACV is subsequently converted by cytosolic isopenicillin N synthase (IPNS) into isopenicillin N (IPN), which contains the characteristic β-lactam backbone. The third enzyme of PEN biosynthesis, isopenicillin N acyl transferase (IAT) is located to 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). Also PCL is a peroxisomal enzyme, thus stressing the importance of this organelle in efficient PEN production in *P. chrysogenum* (Kiel et al., 2009).

Since the isolation of the first PEN producing strain in 1943, random mutagenesis and selection procedures have been successful in generating next generations of strains that show enhanced PEN production levels. More recently also genetic engineering was introduced in strain improvement programs. This was not only performed to enhance PEN yields, but also to produce new products (i.e. cephalosporins). These efforts have primarily focused on increasing the levels of (or introducing new) enzymes in the product pathway, on engineering of primary metabolism and on increasing peroxisome numbers to increase PEN production (Thykaer and Nielsen, 2003; Meijer et al. 2010).

At present, however, there is an urgent need for the development of novel strategies because of the limited success of further enhancing the productivity of current production strains by random mutagenesis approaches and the exhaustion of targets for metabolic engineering.

In this regard, the present inventors followed a new approach with the aim to increase the time period over which biosynthetic enzymes of secondary metabolism are functional. Furthermore, they aimed to reduce the costs of industrial fermentation. Metabolic activity of cellular factories and continuous stirring of the growth medium leads to substantial heat increases. Constant external cooling, e.g. by an external cooling jacket, is typically required to avoid cell death of the microorganisms and loss of valuable biomass. Cooling is a critical cost factor for industrial fermentations.

It was surprisingly found that overexpression of genes encoding the two components of the glyoxalase system, glyoxalase I (GLO1) and glyoxalase II (GLO2), in a fungal host cell has a positive influence on the production of a secondary metabolite. More specifically, double overexpression strains (PcGLO1/2OEx) of *P. chrysogenum* but not single overexpression strains (PcGLO1OEx, PcGLO2OEx) were able to produce up to 63% more PEN than the control when growth parameters are set to 10 day incubation time and 25°C cultivation temperature. As is shown herein below, it was observed that the use of glyoxalase I/II double overexpression strains in biotechnological applications not only provides a viable strategy for increasing the production of valuable compounds but also reduces the need for cooling of the production medium, thereby reducing operating costs. More specifically, PEN production in the double transformants was significantly increased (up to 37%) at 30°C compared to the control strain grown at the same temperature, which is well above the standard operating temperature of 25°C. The results are not limited to PEN biosynthesis in *P. chrysogenum* but are readily transferred to many other applications that rely on fungal performance. For example, *P. chrysogenum* is also used as a host for other important compounds e.g. other antibiotics.

Based on the present findings, the performance of other fungal cell factories/pathways in industrial large-scale fermentations can be significantly improved. This is highly attractive for fungal production of compounds that are constantly in high demand like organic acids, polysaccharides, vitamins, alkaloids, enzymes and pharmaceuticals.

Accordingly, the invention provides a method for producing at least one secondary metabolite in a fungal host cell, comprising culturing a fungal host cell capable of producing said metabolite under conditions allowing for production of said metabolite, wherein the host cell has been genetically modified to display elevated levels of glyoxalase I (EC 4.4.1.5, lactoylglutathione lyase) and glyoxalase II (EC 3.1.2.6, hydroxacylglutathione hydrolase) activity, and wherein culturing may be performed with reduced or even without external cooling of the fermenter.

The glyoxalase system is a set of enzymes that carry out the detoxification of methylglyoxal and several other reactive aldehydes that are produced as a normal part of metabolism. This system has been studied in both bacteria and eukaryotes. For example, methylglyoxal reacts non-enzymatically with glutathione. The resulting hemithioacetal is a substrate for glyoxalase I which catalyzes the formation of S-D-lactoylglutathione. The S-D-lactoylglutathione is then hydrolyzed to D-lactate and glutathione by glyoxalase II. Glutathione functions as an anchor of the substrates in the active sites of the two enzymes. Glyoxalase I and glyoxalase II are not structurally related.

Effects of genetic modifications in the glyoxalase system on various biological aspects have been studied before. For example, Singla-Pareek *et al.* (2003) report the effect of overexpression of either *GLO1* or *GLO2* or both on the tolerance of tobacco plants to NaCl. They conclude that simultaneous overexpression of both enzymes results in the highest tolerance compared to the single mutants. Overexpression of glyoxalase I and II encoding genes in the filamentous fungus *Podospora anserina,* which is a model system for studying ageing, was shown to lead to increased survival on growth medium containing glucose (2% concentration) as a carbon source (Scheckhuber et al., 2010). *P. chrysogenum* does not age replicatively but it autolyses during cultivation in liquid medium. Microscopic analyses regarding the occurrence of autolysis in control and glyoxalase overexpressors according to the invention revealed no differences. It can therefore be ruled out that increased PEN levels in *PcGlo1/PcGlo2* overexpressors are due to increased 'stability' of the mycelium.

WO2012/015949 discloses the production of carbon-based products by employing engineered host cells which overexpress genes encoding enzymes for futile cycle pathways. *GLO1* and *GLO2* are mentioned among a large list of candidate genes. WO2012/015949 is silent about thermal sensitivity of host cells, let alone that it teaches or suggests the advantageous effects of combined *GLO1/GLO2* overexpression

Thus, the use of glyoxalase I/II double overexpression strains in biotechnological applications according to the present invention as a strategy to increase the production of valuable compounds with reduced or no external cooling has not been disclosed or suggested in the art.

As used herein, the expression "modified to display elevated levels of glyoxalase I and II activity" is meant to indicate that the enzyme activity levels are increased as compared to the fungal host cell that has not been genetically modified. Activity of glyoxalase I and glyoxylase II can be determined by assays known in the art. Suitable assays are exemplified herein below. Preferably, the enzyme activity is elevated at least 10-fold, more preferably at least 15-fold, e.g. 17-, 18-, 19, 20-fold or even higher as compared to the unmodified host cell. The absolute enzyme activity required will depend on various factors, e.g. the host cell used, the culturing conditions and the metabolite of interest. For example, good results were obtain in a *P. chrysogenum* double mutant displaying a GLO1 activity of at least 4 µmol/min/mg protein and a GLO2 activity of at least 10 µmol/min/mg protein. In one embodiment, the fungal host cell is modified to display a GLO1 activity of at least 10 µmol/min/mg protein and a GLO2 activity of at least 15 µmol/min/mg protein.

The expression "reduced external cooling" is meant to indicate that less cooling is performed than what is typically needed for a fungal cell factory wherein *GLO1* and *GLO2* are not expressed. For example, it refers to a culturing temperature which is at least 2, 3, 4 or 5°C higher than what is used for a host cell not showing increased levels of GLO1 and GLO2. The cooling level/operating temperature will depend on various factors. For example, for *P. chrysogenum* the operating temperature is suitably set at above 25°C, like 27°C, 28°C, 29°C, 30°C or even higher. In one embodiment, the invention provides a method for producing at least one secondary metabolite in a fungal host cell, comprising culturing a fungal host cell capable of producing said metabolite under conditions allowing for production of said metabolite and wherein the culturing temperature is in the range of 26-32°C, preferably 26-30°C, like 27-30°C or 26-29°C, wherein the host cell has been modified to display elevated levels of glyoxalase I (GLO1) and glyoxalase II (GLO2) activity.

Preferably, the host cell overexpresses the genes encoding glyoxalase I and II activity. In one embodiment, the genes encoding for glyoxalase I and/or II activity are derived from a filamentous fungus. The genes encoding glyoxalase I and II activity may but do not have to be derived from the same microorganism.

See Fig. 1 for the amino acid sequences of glyoxalase I proteins derived 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). Panel B shows a comparison of the amino acid sequences of glyoxalase II proteins derived from *P. chrysogenum* (PcGLO2, UniProt accession number B6HM01), *Aspergillus fumigatus* (AfGLO1, Q4WVP5), *Aspergillus niger* (AnGLO2, NCBI Reference Sequence: XP_001401257.2), *Podospora anserina* (PaGLO2, B2B554), *Neurospora crassa* (NcGLO2, Q1K7C3) and *Sordaria macrospora* (SmGLO2, F7VWX7).

The invention is suitably practised using an enzyme comprising a sequence shown in Figure 1, a fragment or functional homolog thereof. In a preferred embodiment, the genes encoding for glyoxalase I and/or II activity are derived from *P. chrysogenum*. Other preferred enzymes are those derived from *Aspergillus fumigatus* or from *Aspergillus niger*.

The terms "enzyme activity " and "enzymatic activity" are used interchangeably and refer to the ability of an enzyme to catalyse a specific chemical reaction, for example the isomerization of the hemithioacetal to S -2-hydroxyacylglutathione derivatives for GLO1 enzyme activity and the hydrolysis of S-lactoylglutathione to glutathione and lactate for GLO2.

As used herein, the expression "functional homolog" refers to a variant enzyme with a different amino acid sequence while retaining the desired catalytic activity. For example, yet to be identified glyoxalase I and II enzymes from other organisms may be used. As another example, the homolog may have one or more conservative amino acid substitutions wherein an amino acid is replaced with another amino acid of similar chemical structure such that it has no effect on protein function.

It is well known in the art that a polypeptide can be modified by substitution, insertion, deletion and/or addition of one or more amino-acids while retaining its enzymatic activity. For example, substitutions of one amino acid at a given position by a chemically equivalent amino-acid that do not affect the functional properties of a protein are common. For the purposes of the present invention, substitutions are defined as exchanges within one of the following groups:

1) Small aliphatic, non-polar or slightly polar residues : Ala, Ser, Thr, Pro, Gly
2) Polar, negatively charged residues and their amides : Asp, Asn, Glu, Gln
3) Polar, positively charged residues : His, Arg, Lys
4) Large aliphatic, non-polar residues : Met, Leu, Ile, Val, Cys
5) Large aromatic residues : Phe, Tyr, Trp.

Thus, changes which result in substitution of one negatively charged residue for another (such as glutamic acid for aspartic acid) or one positively charged residue for another (such as lysine for arginine) can be expected to produce a functionally equivalent product.

The positions where the amino acids are modified and the number of amino-acids subject to modification in the amino acid sequence are not particularly limited. The man skilled in the art is able to recognize the modifications that can be introduced without affecting the activity of the protein. For example, modifications in the N- or C-terminal portion of a protein would not be expected to alter the activity of a protein.

The term "comprising the sequence" means that the amino acid sequence is not strictly limited to a sequence shown in Figure 1 but may contain additional amino acids. The term "a fragment of" means that the sequence of the enzyme may include less amino acid than what is shown in Figure 1 but still enough amino acids to confer glyoxalase I or II activity.

In one embodiment, the enzymes show at least 60%, preferably at least 75%, more preferably at least 80% or 90% sequence identity with a sequence shown in Figure 1, provided that the enzyme activity is maintained.

Preferably, the homolog contains one or more stretches of at least two conserved amino acid sequences (see residues indicated with an asterisk). More preferably, the homolog contains at least 60%, preferably at least 75%, more preferably at least 80% or at least 90% of the conserved residues.

Very useful genes include those encoding *PcGlo1* or *PcGlo2,* and homologs thereof showing at least 60%, preferably at least 80%, more preferably at least 90% sequence identity, and having the desired enzymatic activity. Overexpression of the desired enzymes can be achieved by methods known in the art. The genes can be introduced in the host cell using appropriate expression vectors. One vector may contain one or both of the genes.

Also provided is the use of the combination of the genes encoding for glyoxalase I and II activity to enhance the production of at least one secondary metabolite in a fungal host cell.

Given the strong conservation of the components of the glyoxalase system, the person skilled in the art will appreciate that the concept of the invention is suitably applied to any fungal cell factors currently used in the field of biotechnology, such as *Aspergillus niger*, *S. cerevisiae* (baker's yeast), *Hansenula polymorpha* and *Kluyveromyces lactis*. In a specific aspect, the fungal host cell is a species of the genus Penicillium, preferably selected from the group consisting *P. chrysogenum* or of the genus Acremonium, preferably selected from the group containing *Acremonium chrysogenum*.

Transformation and selection of the transformed fungal host cell can be done according to established techniques, e.g. by the formation of protoplasts and a suitable expression vector. Suitable procedures for transformation of fungal host cells are described in the art and include procedures for transformation of filamentous fungal host cells using *Agrobacterium tumefaciens*. Other methods like electroporation, described for *Neurospora crassa,* may also be applied.

Fungal cells are preferably transformed using co-transformation, i.e. along with the glyoxalase genes of interest also a selectable marker gene is transformed. This can be either physically linked to the gene of interest (i.e. on a plasmid) or on a separate fragment. Following transformation, transformants are screened for the presence of this selection marker gene and subsequently analyzed for the presence of the gene(s) of interest. A selectable marker is a product, which provides resistance against a biocide or virus, resistance to heavy metals, prototrophy to auxotrophs and the like. Useful selectable markers include, but are not limited to, amdS (acetamidase), argB (ornithinecarbamoyltransferase), bar (phosphinothricinacetyltransferase), hygB (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase), sC or sutB (sulfate adenyltransferase), trpC (anthranilate synthase), ble (phleomycin resistance protein), as well as equivalents thereof.

Very good transformation efficiencies can be obtained using a mass ratio between DNA fragment containing gene of interest and DNA fragment containing the marker of around 20 to 1. It is also preferred to use a strong and constitutive promoter for successful overexpression of the gene(s) of interest. For example, for overexpression in *P. chrysogenum* the gene can be placed under the control of the *P. chrysogenum* IPNS promoter.

The vector may be an autonomously replicating vector, i.e. a vector, which exists as an extra chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, an extra chromosomal element, a mini chromosome, or an artificial chromosome. An autonomously maintained cloning vector for a filamentous fungus may comprise the AMA1 –sequence. Alternatively, the vector may be one which, when introduced into the cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The integrative cloning vector may integrate at random or at a predetermined target locus in the chromosomes of the host cell. In a preferred embodiment of the invention, the integrative cloning vector comprises a DNA fragment, which is homologous to a DNA sequence in a predetermined target locus in the genome of host cell for targeting the integration of the cloning vector to this predetermined locus. Preferred target loci in this context can be loci that are not part of a functional gene (i.e. intergenic regions or pseudogenes); loci that are not essential for the fermentation process (i.e. the *niaD* gene of *Penicillium chrysogenum*, encoding nitrate reductase); loci that give rise to high expression (i.e. as described in EP 357127). In order to promote targeted integration, the cloning vector is preferably linearized prior to transformation of the host cell. Linearization is preferably performed such that at least one but preferably either end of the cloning vector is flanked by sequences homologous to the target locus. The length of the homologous sequences flanking the target locus is at least 30 bp, preferably at least 0.1 kb, more preferably at least 0.2 kb, still more preferably at least 0.5 kb, even more preferably at least 1 kb, most preferably at least 2 kb. Individual colonies can be verified for integration of the glyoxalase cassettes into the genome by routine methods, preferably by colony PCR.

The host cell may be provided with homologous or heterologous genes to enhance GLO2/2 activity. In other words, the level of the endogenous enzymes may be increased and/or genes encoding enzymes from other organism(s) may be introduced. In one embodiment, the fungal host cell overexpresses its endogenous genes encoding glyoxalase I and II activity. For example, the host cell is *P. chrysogenum* overexpressing the *PcGlo1*and *PcGlo2* genes or homologs thereof. In another embodiment, the fungal host cell overexpresses genes encoding glyoxalase I and II activity that are derived from a different origin. Although fungal sources are preferred, glyoxalase genes from other sources (e.g. plants) may also be used due to the high level of conservation.

A method of the invention is advantageously used to enhance the production of any secondary metabolite of interest. In one embodiment, the secondary metabolite is an enzyme, biofuel, organic acid, polysaccharide, vitamin, alkaloid or pharmaceutical product. Exemplary pharmaceutical products are insulin, hepatitis B antigen and antibiotics. Preferred secondary metabolites include β-lactam antibiotics, in particular penicillin. The term "penicillin" is often used generically to refer to benzylpenicillin (penicillin G), procaine benzylpenicillin (procaine penicillin), benzathine benzylpenicillin (benzathine penicillin), and phenoxymethylpenicillin (penicillin V). In one specific aspect, the invention provides a method for producing phenoxymethylpenicillin in a fungal host cell, comprising culturing a fungal host cell capable of producing phenoxymethylpenicillin under conditions allowing for production of phenoxymethylpenicillin, wherein the host cell has been modified to display elevated levels of glyoxalase I (GLO1) and glyoxalase II (GLO2) activity.

In addition to the genetic modification to increase the glyoxalase enzymes, the host cell may be genetically engineered in at least one pathway involved in the production and/or breakdown of said at least one secondary metabolite.

Also provided is the use of the combination of genes encoding for glyoxalase I and II activity to reduce the thermal sensitivity of a fungal host cell, in particular a host cell for use in the production of at least one secondary metabolite of interest. The genes may be derived from a fungus, in particular genes encoding an enzyme shown in Fig. 1, or a homolog thereof. Genes derived from *P. chrysogenum*, are preferred.

A still further aspect relates to a recombinant fungal host cell overexpressing the genes encoding for glyoxalase I and II activity derived from *P. chrysogenum* or homologs thereof, for instance those shown in Figure 1. As is clear from the above, such host cell has unexpected properties and is advantageously used as fungal cell factory for the production of various valuable compounds like biofuels, enzymes and pharmaceutical products. Exemplary host cells of the invention overexpressing *Glo1* and *Glo2* include species of the genus *Penicillium, Aspergillus niger, S. cerevisiae, H. polymorpha, K. lactis.*

LEGEND TO THE FIGURES

Figure 1. Homology analysis between fungal proteins of the glyoxalase system using ClustalW2. Panel **A** Comparison of the amino acid sequences of glyoxalase I proteins from *P. chrysogenum* (PcGLO1, UniProt accession number B6GZZ1, corrected as described in the results section), *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). Panel **B** Comparison of the amino acid sequences of glyoxalase II proteins from *P. chrysogenum* (PcGLO2, UniProt accession number B6HM01, corrected as described in the results section), *Aspergillus fumigatus* (AfGLO1, Q4WVP5), *Aspergillus niger* (AnGLO2, NCBI Reference Sequence: XP_001401257.2), *Podospora anserina* (PaGLO2, B2B554), *Neurospora crassa* (NcGLO2, Q1K7C3) and *Sordaria macrospora* (SmGLO2, F7VWX7). After each sequence the length of the protein and the identity relative to the *P. chrysogenum* GLO1 and GLO2 sequence is given. "*" residues in that column are identical in all sequences in the alignment; ":" conserved substitutions have been observed; "." means that semi-conserved substitutions are observed.

Figure 2. Determination of IPNS abundance in mycelial extracts from wild-type (WT) and overexpression strains (PcGLO1/2OEx). The strains were grown in PEN production medium for 10d at either 25°C or 30°C. Protein extracts were subjected the Western blot analysis to determine IPNS expression. As a loading control, membranes were decorated with antibodies against EF1α (translation elongation factor 1α). Shown is a quantitative analysis of the Western blots shown in A using the gel analyzer plugin from ImageJ. Panel A: growth at 25°C. Panel B: growth at 30°C.

Figure 3. Determination of IAT abundance in mycelial extracts from wild-type (WT) and overexpression strains (PcGLO1/2OEx). The strains were grown in PEN production medium for 10d at either 25°C or 30°C. Protein extracts were subjected the Western blot analysis to determine IAT expression. As a loading control, membranes were decorated with antibodies against EF1α (translation elongation factor 1α). Shown is a quantitative analysis of Western blots using the gel analyzer plugin from ImageJ. Panel A: growth at 25°C. Panel B: growth at 30°C.

Figure 4. Transformants PcGLO1/2OEx #7 and PcGLO1/2OEx #12 show enhanced resistance to methylglyoxal Different dilutions of a suspension of germinating spores were spotted on agar plates containing increasing concentrations of methylglyoxal. The plates were incubated for 6 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).

Figure 5. Transformants PcGLO1/2OEx #7 and #12 show reduced protein modification by methylglyoxal **(A)** Standard curve using different concentrations of methylglyoxal-modified BSA. **(B)** Determination of methylglyoxal-modified proteins in the control strain (DS 17690) and the two transformants PcGLO1/2OEx #7 and #12. The mean values of seven measurements each are given.

EXPERIMENTAL SECTION

Material and methods

Strains / cultivation

DS 17690 (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 (250 ml size). Cultures were subsequently incubated in an orbital shaker (200 rpm) for 10d at 25°C or 30°C, respectively. For cloning purposes, *E. coli* strain DH5α was used.

Construction of PcGlo1 and PcGlo2 overexpression plasmids

For the construction of the *PcGlo1* overexpression vector p*PcGlo1*Ex1, the *PcGlo1* cDNA was amplified by PCR from a *P. chrysogenum* cDNA library using oligonucleotides cGlo1f1 and cGlo1r1 (see Table 1). The PCR product was cut with *Hin*dIII/*Eco*RV and cloned into the *Hin*dIII/*Sma*I site of vector pGBRH2 (Kiel et al., 2005) containing the *IPNS* promoter and *AT* terminator sequences from *P. chysogenum*. This enables a high level of constitutive expression of *PcGlo1*.

The strategy for the construction of the *PcGlo2* overexpression vector p*PcGlo2*Ex1 is similar but uses oligonucleotides cGlo2f1 and cGlo2r1. Both constructs were verified by sequencing. The glyoxalase I / II overexpression cassettes were isolated from p*PcGlo1*Ex1 and p*PcGlo2*Ex1 by *Not*I restriction and subsequently purified using the Nucleospin kit (Machery Nagel, Düren, Germany).

Name	5'-3' sequence
cGlo1f1	AAA AAGCTT ATGGCTTCCGATACCTCC
cGlo1r1	AA GATATC CTACCAATCTCCAGTCCG
cGlo2f1	AAA AAGCTT ATGCATATCCAATCAATTCC
cGlo2r1	AA GATATC TTACATCGAATTCTTCATCTC
cGlo2r2	CGGCACTGAGAATGACCCC

Table 1. Oligonucleotides used. Recognition sites for restriction enzymes are underlined.

Transformation and selection of P. chrysogenum

Protoplasts of *P. chrysogenum* high PEN production strain DS17690 were prepared and subsequently cotransformed with the linear *PcGlo1* / *PcGlo2* expression cassettes and the *amdS* (acetamidase synthase) selection marker from plasmid pSUS15 (lab collection) accoriding to a previously published protocol (Cantoral et al., 1987). Transformants were selected on plates containing acetamide as the sole nitrogen source.

Verification of P. chrysogenum *transformants by colony PCR*

Individual colonies were cultivated on selection medium containing sucrose. Small pieces of mycelium were picked and crushed in a centrifuge tube containing 15 µl MilliQ water. After addition of 50 µl MilliQ water the samples were mixed by vortexing and kept on ice to prevent degradation of genomic DNA. 1 µl of the mixture was used as a template in centrifuge tubes containing oligonucleotides cGlo1f1 / cGlo1r1 or cGlo2f1 / cGlo2r2 (0.5 µM each), dNTPs (0.2 mM), Phire polymerase (0.4 µl) (Finnzymes, Vantaa, Finland) and 1x

Phire reaction buffer (Finnzymes, Vantaa, Finland) in a total volume of 20 µl. Parameters of PCR programs were: 98°C, 4 min (1x); 98°C 5 sec, 63°C 5 sec, 72°C 10 sec (35x); 72°C 1 min (cGlo1f1 / cGlo1r1) and 98°C, 4 min (1x); 98°C 5 sec, 62°C 5 sec, 72°C 10 sec (35x); 72°C 1 min (cGlo2f1 / cGlo2r2), respectively. C-1000 (BioRad, Munich, Germany) was used as thermocycler for conducting PCR reactions.

From each transformation twelve colonies were picked and transferred to an acetamide plate. Colony PCRs were subsequently performed to verify the integration of the glyoxalase cassette(s) into the genome (data not shown). Oligonucleotides were designed to flank regions that include an intron so it was possible to distinguish endogenous copies (containing the intron) from integrated copies (not containing the intron because cDNA sequences were used). Positive candidates were transferred to R agar to induce sporulation of the mycelium. This step is necessary to remove possible background contamination. Spores were streaked out on acetamide plates to receive single colonies. Some of these were transferred to fresh acetamide medium and tested again using colony PCRs (data not shown). In total, from each transformation (PcGLO1OEx, PcGLO2OEx and PcGLO1/2OEx) several independent transformants were positively identified.

Preparation of cell extracts via French Press and determination of protein concentration

Before cell extracts were prepared, 50 µl 1/10 diluted protease inhibitor cocktail (100 mM AEBSF, 1.4 mM E-64, 2.2 mM pepstatin A and 500 mM 1,10-phenanthroline in dimethylsulfoxide) (Sigma Aldrich, Zwijndrecht, The Netherlands) was added to the culture. After mixing 3 ml of the culture was filled into a French Press chamber (small version of the chamber, SLM Aminco [Urbana, IL, USA]). The contents of the chamber were exposed to high pressure (1000 units on the gauge of the French Press (SLM Aminco [Urbana, IL, USA])) before the cell extracts were collected in a pre-cooled tube which was immediately kept on ice. Protein concentration of the cell extracts was determined by using a kit from BioRad (Munich, Germany) which is based on the Bradford method (Bradford, 1976).

Assays for the determination of glyoxalase I and glyoxalase II activity

Glyoxalse 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. 30 µg of protein was added to 1 ml assay buffer in a quartz cuvette (108.002 QS, Hellma, Müllheim, Germany) and quickly mixed. 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 three minutes. Enzymatic activity was calculated by using the molar coefficient of extinction for the formed product, SDLGSH (3100 M^{-1} cm^{-1} at 240 nm). Glyoxalase II activity (modified after Maiti et al., 1997): 15 µg of protein was added to 1 ml assay buffer (300 µM SDLGSH in 33.3 mM potassium phosphate [pH 7.0]) and quickly mixed. The measurement was performed identically to the glyoxalase I activity assay, with the exception that the decrease of absorbance (degradation of SDLGSH) was determined.

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 hour 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.

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 that is supplied with the kit.

Western blot analysis

Protein samples (30 µg) were incubated at 100°C for 5 min in SDS (sodium dodecyl sulphate) sample buffer (0.1 M Tris/HCl [pH 6.8], 4% SDS, 20% glycerol, 10% ß-mercaptoethanol, 0.002% bromophenolblue). The samples were electrophoretically separated by using 12.5% polyacryalamide separation gels. After separation, proteins were transferred to a nitrocellulose membrane by using a custom-built semi dry blotting system (43.2 mA/gel for 45 min to 1 h). Proteins on the membranes were visualized by briefly staining in Ponceau S solution (0.2% Ponceau S / 3% trichloroacetic acid) to verify correct transfer. After washing (in demineralized water) the transfer membrane was incubated in TBST (Tris buffered saline, 0.05% Tween-20) + 1% skimmed milk powder for 30 min at RT for blocking unspecific binding sites on the membrane. Subsequent incubation in primary antibody solution was performed at RT for 30 min. Primary (rabbit) antibodies against IPNS (1/10000) or IAT (1/5000) were provided by DSM, Delft, The Netherlands. As loading control, (rabbit) antibodies against translation elongation factor 1α (eF1α) from *Hansenula polymorpha* (1/5000) (lab collection) were utilized. After washing in TBST (3x, 10 min each) membranes were incubated in secondary antibody solution (α-IgG [rabbit] coupled with alkaline phosphatase, 1/10000 [Sigma Aldrich, Zwijndrecht, The Netherlands]) for 30 min at RT. Membranes were washed again (3x, 10 min each) before incubation in 1/50 diluted NBT/BCIP substrate solution (Roche, Mannheim, Germany) for 1 h to visualize antibody binding to target proteins. The membranes were photographed using a Geldoc imaging system (BioRad, Munich, Germany).

HPLC determination of PEN V

Levels of PEN V in the production medium were determined by high-pressure liquid chromatography using an isocratic flow of acetonitrile (350 g/liter), KH_2PO_4 (640 mg/liter) and H_3PO_4 (340 mg/liter). The peaks were separated on a Platinum EPS 5-µm C18 column (Grace, Deerfield, IL, USA) at a flow rate of 1 ml/min. The detection wavelength was set to 254 nm (Harris et al., 2006).

Glyoxalase homology analyses

Homology between related proteins was determined using ClustalW2.

Results

Identification of PcGLO1 *and* PcGLO2 *in the* P. chrysogenum *sequence*

The published amino acid sequences of *Podospora 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. The sequences of PcGLO1 and PcGLO2 to their *P. anserina* counterparts are strongly conserved, displaying 62% and 65% sequence identity, respectively (Fig. 1). It should be noted that both sequences had to be corrected regarding the correct translation start of the proteins. The annotated sequence for Pc12g09820 starts too late at amino acid position +14 while the annotated sequence for Pc21g08590 starts too early 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*, *Podospora anserina, Neurospora crassa* and *Sordaria macrospora*) (Fig. 1).

Construction of overexpression plasmids and transformation

After the identification of *PcGlo1* and *PcGlo2* in the genome of *P. chrysogenum* we set out to characterize the impact of its glyoxalase system on PEN production by creating and analyzing various transgenic glyoxalase strains (i. e., single *PcGlo1* overexpression, single *PcGlo2* overexpression and double (*PcGlo1/PcGlo2*) overexpression). Plasmids for modulation of the glyoxalase system in *P. chrysogenum* were constructed, verified by restriction analyses and sequencing of the insertion fragment and subsequently co-transformed (together with an amdS [acetamide synthase] cassette) into protoplasts prepared from the high PEN producing *P. chrysogenum* strain DS17690. Transformants were selected on medium containing acetamide as the sole nitrogen source.

Verification of glyoxalase transformants

To show that the PcGLO1/2OEx transformants indeed contain elevated levels of glyoxalase I and/or II activity, spectrophotometric assays on mycelial extracts isolated from strains grown at either 25°C or 30°C in PEN production medium were performed. Results are shown in Table 2. In all transformants, glyoxalase activity was strongly improved compared to the untransformed DS17690 strain after 10d (PcGLO1/2OEx, Tab. 2, PcGLO1OEx and PcGLO2OEx not shown). At 25°C, PcGLO1/2OEx 2 shows 55x increased PcGLO1 activity and 254x increased PcGLO2 activity, for example. Glyoxalase activities decrease at 30°C but they are still highly increased in the transgenic strains (PcGLO1: up to 86x; PcGLO2: up to 113x [Tab. 2]). Taken together, these data show that the aim to generate strains which contain boosted glyoxalase I (and II) activity was successfully achieved.

Table 2. Enzymatic activities of PcGLO1 and PcGLO2 in mycelial extracts from WT and PcGLO1/2OEx overexpression mutants. The strains were grown in PEN production medium for 10d at either 25°C or 30°C.

	GLO1 [µmol/min/mg protein]	**GLO2 [µmol/min/mg protein]**
25°C		
WT	0.27	0.11
PcGLO1/2OEx 2	14.84	27.99
PcGLO1/2OEx 5	6.99	19.78
PcGLO1/2OEx 6	4.84	20.00
PcGLO1/2OEx 7	13.01	11.83
PcGLO1/2OEx 8	10.75	17.20
PcGLO1/2OEx 9	6.77	1.94
PcGLO1/2OEx 12	7.74	18.28
30°C		
WT	0.11	0.08
PcGLO1/2OEx 2	9.46	9.03
PcGLO1/2OEx 7	8.06	4.19
PcGLO1/2OEx 12	4.95	5.48

Determination of PEN levels

To address the question whether overexpression of glyoxalase genes has an influence on PEN production transformants and the control strain DS17690 were grown in PEN V production medium for 10d at either 25°C (standard temperature) or 30°C (elevated temperature). PEN V determination in filter-sterilized culture supernatants was determined by HPLC. Results are shown in Table 3. Most glyoxalase I/II double overexpression mutants are able to produce more PEN V than the control (increases of up to 63% [PcGLO1/2OEx 7]). Also at 30°C most PcGLO1/2OEx mutants show increases in PEN V production (up to 37% [PcGLO1/2OEx 7]). At this temperature, PcGLO1/2OEx strains are able to produce a PEN V amount that is comparable to a WT culture grown at standard temperature. In contrast to the glyoxalase I/II double overexpression mutants, single *PcGlo1* or *PcGlo2* overexpressors produce similar amounts of PEN V to the control (data not shown).

Table 3. Determination of PEN V levels in WT and PcGLO1/2OEx overexpression mutants after 10 day culturing in production medium.

Strain	**Growth temperature**	**PEN V [g/l]**	**Dry weight [mg]**	**Rel PEN Titre (% of control)**
WT	25°C	1.13	940	100
PcGLO1/2OEx 2	25°C	1.13	630	149
PcGLO1/2OEx 5	25°C	1.17	820	119
PcGLO1/2OEx 6	25°C	1.15	630	152
PcGLO1/2OEx 7	25°C	1.19	610	163
PcGLO1/2OEx 8	25°C	1.08	650	138
PcGLO1/2OEx 12	25°C	1.18	670	147
WT	30°C	0.86	877	100
PcGLO1/2OEx 5	30°C	1.30	1170	113
PcGLO1/2OEx 7	30°C	0.95	710	137
PcGLO1/2OEx 12	30°C	0.94	800	120

Abundance of IPNS and IAT in glyoxalase transformants

IPNS is a key enzyme involved in PEN biosynthesis. It is also highly susceptible to inactivation/damage with a concomitant loss of catalytic activity (Perry et al., 1988; Dubus et al., 2000). To test whether the increased PEN

levels in PcGLO1/2OEx strains are due to increased IPNS levels an immunodetection analysis was performed. All tested glyoxalase I/II mutants contain elevated levels (up to 3x) of IPNS at either 25°C (Fig. 2A) or 30°C (Fig. 2B). These results show that overexpression of both glyoxalase genes correlates with increased synthesis/stability of IPNS.
Similar to IPNS, peroxisomal IAT is present in increased levels in PcGLO1/2OEx mutants grown at 25°C in production medium (Fig. 3A). At 30°C, two of the three PcGLO1/2OEx mutants display strongly increased IAT levels whereas one is similar to the wild-type (Fig. 3B). Taken together, there is a tendency in PcGLO1/2OEx mutants to contain higher levels of biosynthetic enzymes of PEN.

Resistance to methylglyoxal

We determined whether PcGLO1/2OEx strains show increased resistance against externally added methylglyoxal. As shown in Fig. 1, 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. 4, bottom row). These results indicate that increased levels of enzymatically active glyoxalase I and II are present in both transformants.

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.

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Claims

1. A method for producing at least one secondary metabolite in a fungal host cell, comprising culturing a fungal host cell capable of producing said metabolite under conditions allowing for production of said metabolite, wherein the host cell has been modified to display elevated levels of glyoxalase I (GLO1) and glyoxalase II (GLO2) activity and wherein said culturing is performed with reduced or without external cooling.

2. Method according to claim 1, wherein said host cell overexpresses the genes encoding GLO1 and GLO2 activity.

3. Method according to claim 2, wherein the genes encoding for GLO1 and/or GLO2 activity are derived from a filamentous fungus, preferably from *P. chrysogenum*.

4. Method according to claim 2 or 3, wherein the genes encode an enzyme comprising an amino acid sequence shown in Figure 1, a homolog showing at least 60%, preferably at least 80%, more preferably at least 90% sequence identity with said amino acid sequence, or a fragment thereof, provided that the encoded enzymes have GLO1 and GLO2 activity, respectively.

5. Method according to any one of the preceding claims, wherein the fungal host cell is a species of the genus Penicillium or of the genus Acremonium, preferably selected from the group consisting of *P. chrysogenum, Acremonium chrysogenum, Aspergillus niger, S. cerevisiae, H. polymorpha,* and *K. lactis.*

6. Method according to claim 5, wherein the fungal host cell is *P. chrysogenum* or *Acremonium chrysogenum.*

7. Method according to any one of the preceding claims, wherein the secondary metabolite is an enzyme, biofuel, organic acid, polysaccharide, vitamin, alkaloid or pharmaceutical product.

8. Method according to claim 7, wherein the secondary metabolite is a β-lactam antibiotic, preferably penicillin.

9. Method according to any one of the preceding claims, wherein the host cell is genetically engineered in at least one pathway involved in the production and/or breakdown of said at least one secondary metabolite.

10. Use of the combination of the genes encoding for glyoxalase I and II activity to enhance the production of at least one secondary metabolite in a fungal host cell.

11. Use of the combination of the genes encoding for glyoxalase I and II activity to reduce the thermal sensitivity of a fungal host cell, preferably a host cell for use in the production of at least one secondary metabolite.

12. Use according to claim 10 or 11, wherein the genes are derived from *P. chrysogenum.*

13. A fungal host cell overexpressing the genes encoding for glyoxalase I and II activity derived from *P. chrysogenum.*

14. A host cell according to claim 13, wherein the host cell is a species of the genus Penicillium or of the genus Acremonium, preferably selected from the group consisting of *P. chrysogenum, Acremonium chrysogenum, Aspergillus niger, S. cerevisiae, H. polymorpha,* and *K. lactis.*

Figure 1

A

```
PcGLO1          --MASDTSTYKLNHTMMRVKDPKRSVEFYKFLGLNLVNTIDMPEWKFCNYFLAYDGPA-S 57
AfGLO1          --MATDTIKYKLNHTMIRVKDPKRSVEFYKFLGLNQIQQLDFPENKFSLYFLAYNGPQ-S 57
AnGLO1          --MATDTSTYKLNHTMLRVKDPKRSVEFYKFLGLNQVQQLDFPDNKFSLYFLAYNGPQ-S 57
NcGLO1          MAATTDVQNYKFNHSMIRVKDPKESVKFYEFLGMSLLKKLSFPEAKFDLYFLGYDAPG-A 59
PaGLO1          MASTTDVKNFKLNHTMIRVKDPKESVKFYNHLGLSLLKKLSFPDAKFDLYFLGYDAPG-A 59
SmGLO1          MAATTDVKNYKFNHSMIRVKDPKESVKFYEFLGMSLLKKLSFPEAKFDLYFLGYDAPGGA 60
                   ::*. .:*:**:*:******.**:**:.**:. :: :.:*: **  ***.*:.*  :

PcGLO1          LQGARHWTDRNAVLELTHNYGTENDPNYSVVNGNTEPHRGFGHIAISVDNIEAACKRIED 117
AfGLO1          LQGDRHWTDRNAVLELTHNYGTENDPNYTVANGNTEPHRGFGHIAISVDNIEAACKRLED 117
AnGLO1          LQGDRHWTDRNAVLELTHNYGTENDPNYSITNGNTEPHRGYGHIAISVDNIEAACQRLED 117
NcGLO1          VSAGANLWDREGLIELTHNYGTESDPNYKINNGNVEPHRGFGHTCISVDNLQAACQRLED 119
PaGLO1          VSHGKNLWDREGLIELTHNYGTENDPEYKINNGNVEPYRGFGHTCISVDNIQAACQRIED 119
SmGLO1          VSAGANLWDREGLIELTHNYGTESDDSYKINNGNVEPHRGFGHTCISVDNLQAACQRLED 120
                :.   :  **:.::*********.* .*.: ***.**:**:** .*****::***:*:**

PcGLO1          AGYPFQKKLTEGRMRHIAFAKDPDGYWVEIIRRA---DEDLSTTTDPGSYRLNHTMLRVK 174
AfGLO1          AGYQFQKKLTEGRMKHIAFVKDPDGYWVEIIRRH---DQDVGTATDPGTYRLNHTMLRVK 174
AnGLO1          AGYAFQKKLTEGRMRHIAFVKDPDGYWVEIIKRR---DEAMSTSTDPATYRLNHTMLRVK 174
NcGLO1          AGYKFQKKLSDGRMKHIAFALDPDGYWVEIIGRKPVEETEGVKETDLKTYRMNHTMLRVK 179
PaGLO1          AGYKFQKKLTDGKMRHIAFALDPDGYWVEIIGQRPVEETEGVRETDLGTYRMNHTMIRVK 179
SmGLO1          AGYRFQKKLSDGRMKHIAFALDPDGYWVEIIGRKPVEETSEIKETDLKTYRMNHTMLRVK 180
                *** *****::*:*:****. ********** :    :      **  :**:****:***

PcGLO1          DAEASLKFYQESMGMTLVRTIENPENKFNLYFLGYPASNPEIKEGAKNGVAEWEGLLELT 234
AfGLO1          SAETSLKFYQEVMGMTLVRTIENKDAAFNLYFLGYPASNPTAQEGAKNPVAEWEGLLELT 234
AnGLO1          DAEASLKYYQEVLGMTLVRTIENKEAAFNLYFLGYPASNPQATEGANNPVAEWEGLLELT 234
NcGLO1          DGEKSLKFYQEVMGMKLVRTHEAKEAGFNLYFLGYGDEK--------QNTADREGLLELT 231
PaGLO1          DAERSLEFYREVMGMKLFRTHEAKEAGFNLYFLGYEGEQGAVVE--GGDTAKREGLLELT 237
SmGLO1          DAEKSLKFYQEVLGMKLVRTHEAKEAGFNLYFLGYGDEK--------QNTADREGLLELT 232
                ..* **::*:* :**.*.** *  :  ********  .:          .*. *******

PcGLO1          WNYGTEKQEGPVYHNGNTEPQGFGHICISVDDLEAACDRFESLKVNFKKRLTDGRMHNIA 294
AfGLO1          WNYGTEKQEGKVYHDGNSEPQGFGHICVSVDDLNAACDRFESLNVNWKKRLTDGRMKNVA 294
AnGLO1          WNYGTEKQEGKVYHNGNDEPQGFGHICVSVDDLNAACERFESLKVNWKKRLTDGRMKNVA 294
NcGLO1          WNYGTEKDENFSYHNGNDQPQGFGHICVSVDNIEAACERLEGLKVNWKKRLTDGRMKNVA 291
PaGLO1          WNYGTEKEEGFSYHDGNKGPQGFGHICVSVDSLEKACERFEGLGVSWKKRLTDGRMKNVA 297
SmGLO1          WNYGTEKDENFSYHNGNDQPQGFGHICLSVDNIEAACERLEGLNVNWKKRLTDGRMKNVA 292
                *******:*.  **:**  ********:***.:: **:*:*.* *.:*********:*:*

PcGLO1          FILDPDGYWIEVVQNQGIKRTGDW 318
AfGLO1          FILDPDGYWIEVIQNEALKRTSNW 318 83%
AnGLO1          FLLDPDGYWIEVIQNEALKRTSNW 318 81%
NcGLO1          FVLDPDNYWIELVQNERFTGQANF 315 62%
PaGLO1          FVLDPDGYWVEIVQNERFADKANF 321 63%
SmGLO1          FVLDPDGYWIELVQNERFTGQSNF 316 63%
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B

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PcGLO2          MHIQSIPMWTGKGNNYAYLVTDEPTKQSVIIDPANPPEVAPELKSQIASGKIDLTAIVNT 60
AfGLO2          MHVQSIPIWTGKGNNYAYLVTDEPTKESVIIDPANPPEVAPELDAQIKAGKIKLSAIVNT 60
AnGLO2          MHVQSIPMWTGKGNNYAYLVTDEPTKESVIIDPANPPEVAPTLKSQIDAGKIKLTSIVNT 60
NcGLO2          MHIKSIPMWTGSSDNYAYLVVDDKSKDAVIIDPAHPEEVAPVLKKAIDDGSIKLTAIVNT 60
PaGLO2          MHIRSIPMWG---DNYAYLVVDDKSKDAVIIDPANPPEVLPVLEEAIANKEINLTAIVNT 57
SmGLO2          MHIKSIPMWTGSSDNYAYLVVDDKSKDAVIIDPANPEEVAPVLKKAIDDGSIKLTAIVNT 60
                **::***:*    :******.*: :*::******:* ** * *.  *   .*.*::****

PcGLO2          HHHWDHAGGNGDILKQF--KGLQVIGGKDCASVTKTPAHGEEFKIGDRISVKALHTPCHT 118
AfGLO2          HHHWDHAGGNNEMLKHF--GKLPVIGGRNCQSVTQTPAHGETFKIGERISVKALHTPCHT 118
AnGLO2          HHHWDHAGGNNEILKTF--GQLSVIGGKNCQSVTKTPAHGEVFKIGERISVKALHTPCHT 118
NcGLO2          HHHWDHAGGNTKLRTALGLPNLEIIGGKDCEKVNKVPAHGQGFNIGN-IAVKALHTPCHT 119
PaGLO2          HHHRDHAGGNEALLSKL-PSKLPIIGGRDCAHVTKTPAHNETFTIGENIKVKALHTPCHT 116
SmGLO2          HHHWDHAGGNTKLRTALGLPNLEIIGGRDCEKVNKTPAHGEGFNIGS-IAVKALHTPCHT 119
                *** ******  : . :    * :***::*  *.:.***.: *.**. * **********

PcGLO2          QDSICYYMQDGD--QRAVFTGDTLFIGGCGRFFEGNAKEMHKALNETLAALPDDTKVYPG 176
AfGLO2          QDSICYYMQDGD--EKVVFTGDTLFIAGCGRFFEGNAQEMHKALNETLASLPDDTRVYPG 176
AnGLO2          QDSICYFMQDGD--DKVVFTGDTLFIGGCGRFFEGTAPEMHKALNETLAALPDDTKVYPG 176
NcGLO2          QDSICWFMQDGD--DKVVFTGDTLFHGGCGRFFEGNGAEMHKALNETLASLPDETRVFPG 177
PaGLO2          QDSICYLMEDKETGDKVIFTGDTLFIGGCGRFFEGTGEEMHEALNVVLAGVGDDVRVYPG 176
SmGLO2          QDSICWFMEDGD--DKVVFTGDTLFHGGCGRFFEGNGAEMHKALNETLAALPDETRVFPG 177
                *****: *:* :  ::.:******* .********.. ***:*** .**.: *:.:*:**

PcGLO2          HEYTKANVKFCIAVSQTEPIKKLQAFADQNKQTQGKFTIGDEKLHNVFMRVNDPEIQKVT 236
AfGLO2          HEYTRSNVKFCLTVSQSEPIKKLEAYANQHQQTQGKFTIGDEK---------DPEIQKKT 227
AnGLO2          HEYTKGNVKFCLAVSQSEPIKKLEAFANANQETQGKFTIGDEKLHNVFMRVNDPEIQKKT 236
NcGLO2          HEYTKSNAKFCMSVLQSEAVKALQAFAEKHRETQGKFTIGDEKKHNVFMRPHDPEIQKAT 237
PaGLO2          HEYTKSNVKFGVSVLQSEAVRALEAFADDCKETQGKFTIGDEKQHNVFMRPQDPAIQKAT 236
SmGLO2          HEYTKSNVKFCMSVLQSEAVKALSAFADKNQETQGKFTIGDEKKHNVFMRPQDPVIQKAT 237
                ****:.*.** ::* *:*.:: *.*:*:  ::***********         ** *** *

PcGLO2          GKTDPVDVMAALREMKNSM- 255
AfGLO2          GKTDPVEVMAALREMKNAM- 246 80%
AnGLO2          GKTDPVEVMAALREMKNNM- 255 86%
NcGLO2          GETDPVAIMTKLREMKNNFK 257 68%
PaGLO2          GETDPIAIMTKLREMKNNFK 256 65%
SmGLO2          GETDPVAIMTKLREMKNSFK 257 70%
                *:***: :*: ****** :
```

Figure 2

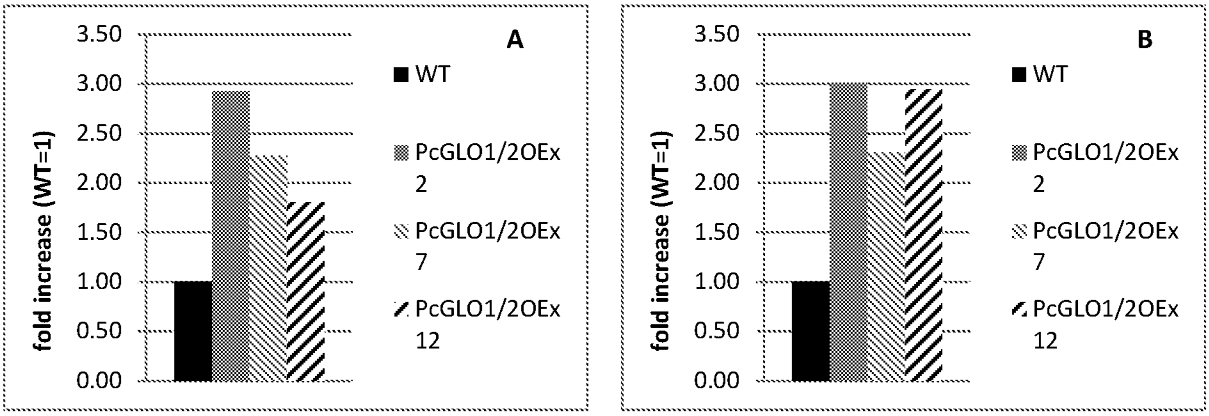


Figure 3

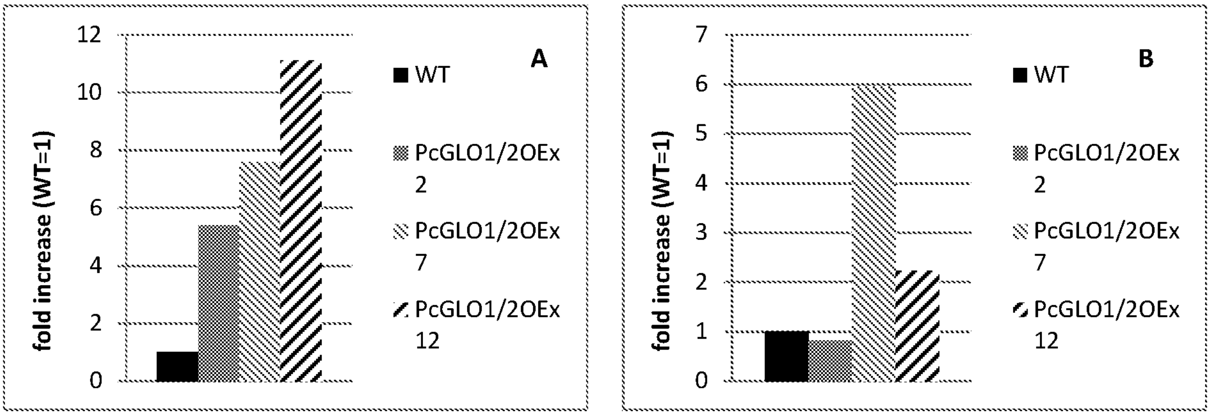


Figure 4

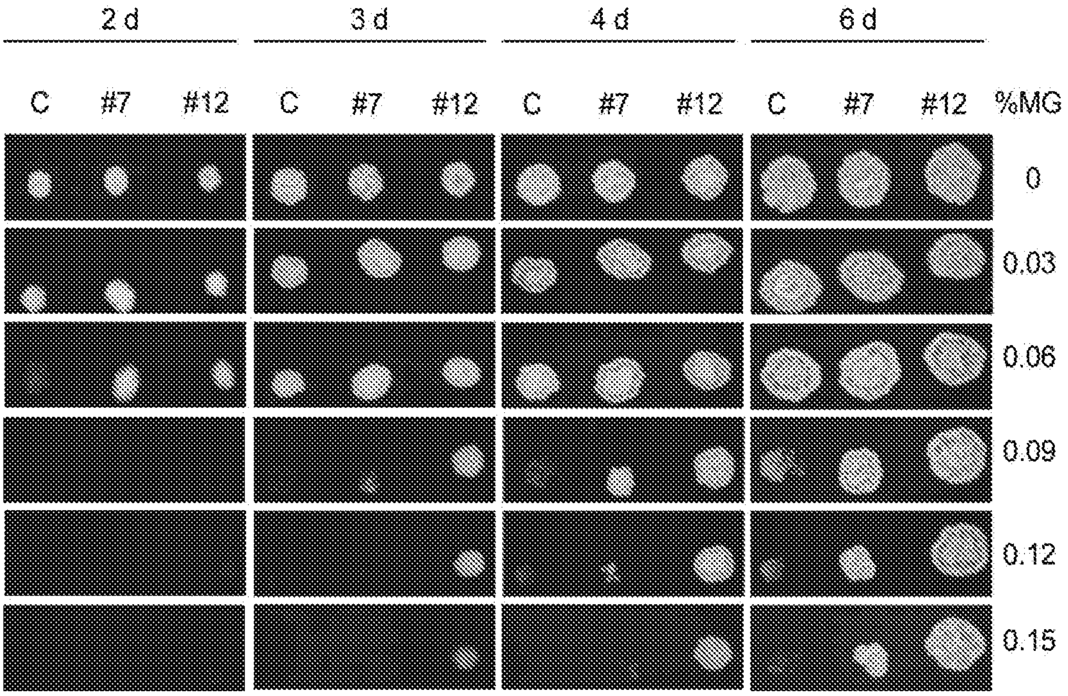
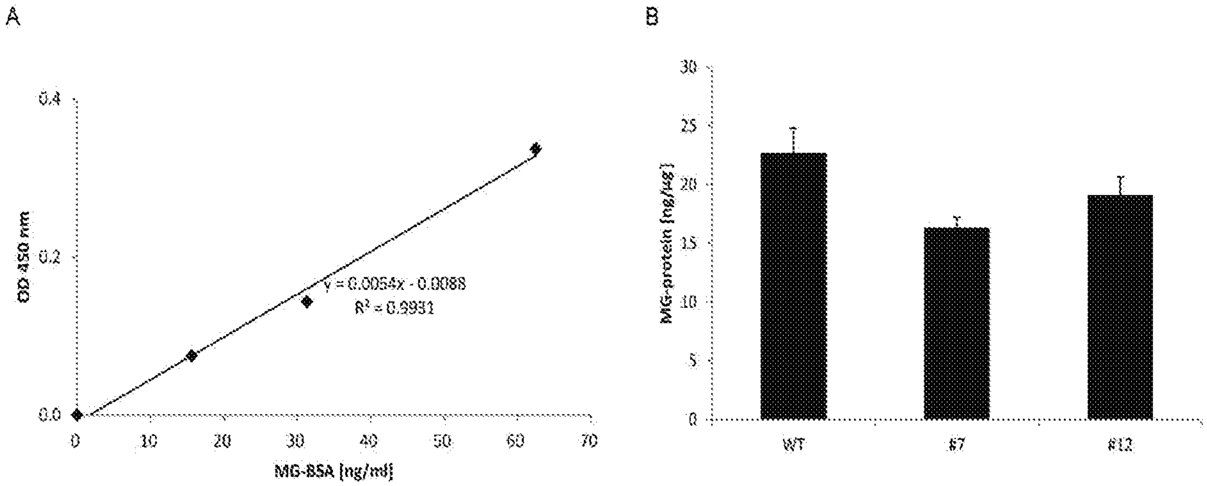


Figure 5


A
0.4
0.2
0.0
OD 450 nm
y = 0.0054x - 0.0088
R² = 0.9931
0
10
20
30
40
50
60
70
MG-BSA [ng/ml]
B
30
25
20
15
10
5
0
MG-protein [ng/µg]
WT
#7
#12

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2013/050457

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P37/00 C12P1/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHECKHUBER CHRISTIAN Q ET AL: "Modulation of the glyoxalase system in the aging model Podospora anserina: effects on growth and lifespan", AGING-US, vol. 2, no. 12, December 2010 (2010-12), pages 969-980, XP002690452,	1,2,4,9, 12-14
Y	the whole document	3,5-9,11

X	WO 2012/015949 A2 (JOULE UNLTD TECHNOLOGIES INC [US]; SKRALY FRANK ANTHONY [US]; REPPAS N) 2 February 2012 (2012-02-02)	10,12-14
Y	pages 1-20; claim 66	3,5-9,11

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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search 8 October 2013	Date of mailing of the international search report 17/10/2013
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Schneider, Patrick

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WATANABE T ET AL: "Selection of stress-tolerant yeasts for simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash to ethanol", BIORESOURCE TECHNOLOGY, ELSEVIER BV, GB, vol. 101, no. 24, 1 December 2010 (2010-12-01), pages 9710-9714, XP027246719, ISSN: 0960-8524 [retrieved on 2010-07-24] page 9710 - page 9711 page 9713 -----	1-14
A	GREWAL H S ET AL: "Fungal production of citric acid", BIOTECHNOLOGY ADVANCES, ELSEVIER PUBLISHING, BARKING, GB, vol. 13, no. 2, 1 January 1995 (1995-01-01), pages 209-234, XP004045669, ISSN: 0734-9750, DOI: 10.1016/0734-9750(95)00002-8 page 220 -----	1-14
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/NL2013/050457

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