World J Microbiol Biotechnol (2011) 27:3019–3023
DOI 10.1007/s11274-011-0782-7

SHORT COMMUNICATION

Production of a heterologous recombinant protein using fragments of the glyceraldehyde-3-phosphate dehydrogenase promoter from *Penicillium camemberti*

Yeison Espinosa · Jovanka Trebotich · Francisco Sepúlveda · Jeisson Cadena · María-José Vargas-Straube · Inmaculada Vaca · Paulina Bull · Gloria Levicán · Renato Chávez

Received: 19 February 2011 / Accepted: 7 May 2011 / Published online: 15 May 2011

Abstract The biotechnological applications of cheese-ripening fungi have been limited by a lack of genetics tools, in particular the identification and characterization of suitable promoters for protein expression. In this study, the suitability of the glyceraldehyde-3-phosphate dehydrogenase (*gpdP*) promoter from *Penicillium camemberti* to drive the production of a recombinant protein was evaluated. The *gpdP* gene and its promoter were isolated using PCR and Genome Walker. The promoter of *gpdP* has two regions with high identity to the regulatory elements *gpd*-box and *ct*-box previously described in *Aspergillus nidulans*. Two fragments of the promoter containing the *gpd*- and *ct*-box or the *ct*-box alone were used to drive the in vivo production of recombinant *β*-galactosidase using *A. nidulans* as host. Our results indicate that larger fragment containing *gpd*-box enhances the production of *β*-galactosidase activity levels respect to *ct*-box alone, and that both boxes are necessary to obtain maximal enzymatic activity production. The smaller fragment (187 nt) containing the *ct*-box alone was able to trigger up to 27% of *β*-galactosidase activity, and to our knowledge this is the smallest fragment from a *gpd* gene used to produce a recombinant protein. Differences were not observed when glycerol, galactose or glucose were used as carbon sources, suggesting that the promoter activity is carbohydrate-independent. This is the first report in which a *Penicillium gpd* promoter is used for recombinant protein production. Our results open the way for the future development of a system for recombinant proteins expression in the biotechnologically important cheese-ripening fungus *P. camemberti*.

Keywords *Penicillium camemberti* · Glyceraldehyde-3-phosphate dehydrogenase promoter · Heterologous protein expression · *β*-galactosidase

Y. Espinosa · J. Trebotich · F. Sepúlveda · J. Cadena · M.-J. Vargas-Straube · G. Levicán · R. Chávez (✉)
Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Usach, Alameda 3363, Correo 33, Casilla 40, Estación Central, Santiago, Chile
e-mail: renato.chavez@usach.cl

I. Vaca
Departamento de Química, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile

P. Bull
Facultad de Ciencias Biológicas, P. Universidad Católica de Chile, Alameda 340, Casilla 114-D, Santiago, Chile

Introduction

The cheese-ripening fungi have worldwide importance to the food industry, because they are responsible for flavor and other organoleptic properties in some kinds of cheese and other foods. In particular, *P. camemberti* is the most important organism colonizing the Camembert and Brie cheeses, among others. *Penicillium camemberti* possesses an enzymatic battery that allows its use as ripening agent. These positive characteristics could be improved using genetic engineering, thus enhancing these important enzymatic activities. However, suitable genetic tools have not been developed for this organism, thus hindering improvement of its positive features. On the other hand, as part of the cheeses, the mycelium of *P. camemberti* has been eaten by people for centuries without harm to human health. Consequently, is considered a generally regarded as safe (GRAS) organism. Since materials produced by GRAS organisms are safe (Hjört 2003), *P. camemberti* could offer important advantages as potential producer of recombinant enzymes to be used as food additives or for pharmaceutical

purposes. Therefore, in order to expand applications of *P. camemberti* for genetic improvement programs or as host for recombinant protein expression, suitable promoters should be identified and characterized.

The glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter is an ideal candidate for recombinant protein expression. As a key enzyme in glycolysis, glyceraldehyde-3-phosphate dehydrogenase protein (GAPDH) can represent up 5% of cellular proteins in higher eukaryotes, which correlates with a high level of *gpd* mRNA (Holland and Holland 1978; Piechaczyk et al.1984). Therefore, fungal *gpd* promoters are highly active, being successfully used for the expression of heterologous genes in several yeasts and fungi (for recent examples see Gonzalez et al. 2010; Sharma and Kuhad 2010). However, and despite their potential applications, little is known about critical parts of *gpd* promoters necessary to express recombinant proteins. The fungal *gpd* promoters usually contain two elements (called "boxes") that could be important for their activity (Punt et al. 1990): the *gpd*-box (a region highly conserved in *Aspergillus gpd* promoters) and the *ct*-box (a proximal C + T rich region). Although in almost all the fungal promoters analyzed only the *ct*-box is found (Kuo et al. 2004; Nitta et al. 2004; Vastag et al. 2004; Fei et al. 2006; De Maeseneire et al. 2008), both boxes have been found in few of them (Punt et al. 1990; Liao et al. 2008). In these scarce cases, different fragment promoters have been used to produce recombinant proteins, concluding that although the proximal *ct*-box alone seems enough to drive the expression of a protein, the maximal expression levels would be reached in the presence of the additional *gpd*-box (Punt et al. 1990, 1992; Liao et al. 2008).

To date, the use of any *Penicillium gpd* promoter for recombinant protein production has not been carried out yet. This would be useful especially in *Penicillium* considered as GRAS organisms, such as the cheese-ripening fungus *P. camemberti*. Therefore, in this work we explore the potential of *P. camemberti gpd* promoter to drive the production of a recombinant protein. Our results open the way for the future use of this promoter for genetic improvement of this organism, or its use for the recombinant proteins production.

Materials and methods

Strains, vectors and primers

P. camemberti strain CECT 2267 (Chávez et al. 2010) was used in this work. All the rest of strains and plasmids have been previously described (Díaz et al. 2008). Primers used are listed in Table 1.

Table 1 Sequences of primers used in this work

Primer name	Sequence
GPDFW	5′-AAGGC (CT) GTCGGCAAGGT-3′
GPDRV	5′-CCACTCGTTGTCGTACCA-3′
GPD-CAJAGPDCT[a]	5′-AGACTCGGATCCCCTATTGGTA GACGGGA-3′
GPD-PROMO-1[a]	5′-AGACTCGGATCCGATTGCGG TTTACTGGA-3′
GPD-CAJACT[a]	5′-AGACTCGGATCCGTCCATTGCGT CAGTCA-3′

[a] *Bam*HI sites are underlined

Amplification and sequencing of *gpdP* gene and promoter from *P. camemberti*

Based on a multiple alignment of several filamentous fungal *gpd* gene sequences, the degenerate primer GPDFW and the specific primer GPDRV were designed. Using these primers for PCR with *P. camemberti* genomic DNA, a specific DNA fragment (380 nt) was obtained, cloned in pGEM-T and sequenced. This fragment was used as scaffold for the amplification of the rest of the upstream and downstream sequences using *P. camemberti* Genome Walker libraries (Navarrete et al. 2009). The sequence of *P. camemberti gpdP* gene described in this work has been deposited in Genbank under the accession number N° HQ423172.

DNA isolation and Southern blot analysis

Genomic DNA from *P. camemberti* was isolated as described (Navarrete et al. 2009) and Southern blot was carried out according to Díaz et al. (2008). Membranes were hybridized with a 565 nt fragment containing the central part of the *gpdP* gene.

Construction of the plasmids for β-galactosidase expression and transformation of *A. nidulans*

The longer fragment containing both the *ct*-box and the *gpd*-box (410 nt) from *P. camemberti gpdP* was obtained by PCR using primers GPD-CAJAGPDCT and GPD-PROMO-1, digested with *Bam*HI and ligated upstream of the *lacZ* reporter gene in plasmid pAN49-1 (Kanemori et al. 1999). Competent *E. coli* were transformed with ligation mix and recombinant plasmids were purified, thus obtaining plasmid pGPD-CT. The shorter fragment containing only the *ct*-box (187 nt) was amplified using primers GPD-CAJACT and GPD-PROMO-1, and treated as above, thus obtaining plasmid pCT. The orientation of the fragments and the absence of undesirable mutations were determined by direct sequencing of the recombinant plasmids.

Aspergillus nidulans A722 strain was transformed with pCT or pGPD-CT according to the protocol previously described (Díaz et al. 2008). For each transformation, around 12 transformants were obtained. Copy number of each reporter plasmid was determined by Southern blot using part of the *lacZ* gene as probe, and the endogenous *gpdA* probe from *A. nidulans*. *A. nidulans* contains only one copy of the *gpdA* gene (Punt et al. 1988), and for this reason this gene was used as a normalization control for Southern blots. The radioactivity of each probe was quantified in a liquid scintillation counter, and equivalent amounts of radioactivity were used for the Southern blot experiments.

Growth of selected transformants in different carbon sources and β-galactosidase assay

1×10^7 spores from selected transformants were grown in flasks containing 100 ml of the minimal medium described before (supplemented with *p*-amino benzoic acid) with 1% glucose, galactose or glycerol for 48 h at 37°C and 200 rpm. After this time, mycelia were harvested and β-galactosidase was measured using the protocol described by Díaz et al. (2008). In brief, extracts of the transformants were added to *o*-nitrophenyl galactopyranoside solution to produce *o*-nitro phenol (oNP), which can be measured by a spectrophotometer at 420 nm. The μmoles of oNP produced were estimated from a calibration curve. The β-galactosidase activity obtained was normalized for protein concentration (determined by the Bradford method) in each measurement.

Results and discussion

Characterization of the *gpdP* gene and promoter from *P. camemberti*

The *gpdP* gene consists of 1,268 nt. Using bioinformatic tools and comparison with others fungal *gpd* genes, it is predicted that this gene contains four introns which are 55, 66, 65, and 65 nt in length. Two putative polyadenylation signals were observed, at 247 and 439 nt downstream from the stop codon. Southern blot analysis suggests the existence of a single genomic copy of *gpdP* gene in *P. camemberti* (data not shown).

The deduced GAPDH protein consists of 338 amino acids, which according to BLAST, presents a high degree of identity to other deduced eukaryotic gliceraldehyde-3-phosphate dehydrogenase, mainly to those from *P. chrysogenum* (94% identity) and several species of the genus *Aspergillus*. By multiple alignment of our deduced protein with 14 fungal gliceraldehyde-3-phosphate dehydrogenase protein sequences obtained from Genbank (data not shown), the catalytic motif ASCTTNCL which is typical in GAPDH proteins, was observed. This motif contains the putative catalytic residue C152. The highly conserved amino acids H179, R297, and R234, putatively involved in catalysis, were also found.

A 429 nt sequence of the *gpdP* promoter was sequenced. The *gpdP* promoter contains a *ct*-box located 97 nt upstream of the starting ATG codon, and a *gpd*-box located 323 nt upstream of the starting codon (Fig. 1a). Pairwise aligment of these boxes with those described in the *A. nidulans gpdA* promoter (Punt et al. 1990) shows a high identity between *ct* (71% identity) and *gpd* (88% identity) boxes (Fig. 1b), suggesting that they could be important regulatory elements for the expression of the *P. camemberti gpdP* gene. Interestingly, both *gpd*- and *ct*-boxes are about 70–80 nt nearer to the coding sequence than the position described for the *A. nidulans gpdA* promoter (Fig. 1b), which suggests that in *P. camemberti* a shorter promoter sequence would be enough to trigger gene expression. This is a valuable advantage for the design of plasmids for heterologous expression of proteins (Liao et al. 2008). Other elements present in the promoter region of the *gpd* gene from *A. nidulans* (e.g., *pgk*-box and *alc*-box) were not found in the promoter of *P. camemberti gpdP* gene.

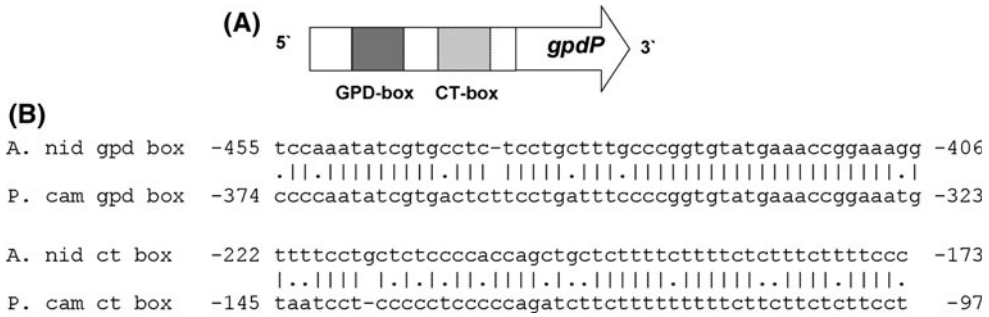

Fig. 1 **a** Schematic representation of the *gpdP* promoter showing the *ct*- and the *gpd*-boxes. **b** Pairwise alignment between *A. nidulans* (A. nid) and *P. camemberti* (P. cam) *gpd* boxes (*upper alignment*) and *ct*-boxes (*lower alignment*). Identities are indicated by *vertical lines*. Mismatches are indicated by *dots*. DNA sequences are numbered with reference to the translation start site as +1. Note that both *gpd*- and *ct*-boxes are about 70–80 nt nearer to the coding sequence than the position described for the *A. nidulans gpdA* promoter

Use of two fragments of *gpdP* promoter to drive the intracellular production of β-galactosidase in *A. nidulans*

To date, *P. camemberti* promoters have not been used to produce heterologous recombinant proteins. Since there is no similar experimental system available in *P. camemberti* for this purpose, we overcame this difficulty using a heterologous approach. As the *gpdP* promoter from *P. camemberti* has a similar structure to the *gpdA* promoter from *A. nidulans* and their main regulatory elements (*ct*- and *gpd*-boxes) have high identity (Fig. 1b), *A. nidulans* was chosen. Other researchers have successfully used *Aspergillus* reporter systems to analyze heterologous genes (Kanemori et al. 1999; Díaz et al. 2008).

To prove that *gpdP* promoter isolated from *P. camemberti* is suitable to drive the production of a recombinant protein, two expression vectors named pGPD-CT (containing the larger 410 bp fragment of *P. camemberti gpd* promoter including the *gpd*- and the *ct*-boxes) and pCT (containing the shorter 187 bp fragment with *ct*-box alone) were constructed and used to transform *A. nidulans*. Some randomly chosen clones from each transformation were assayed by Southern blot and two clones (named GPD-CT-4 and CT-20), which contained one copy of the respective plasmid (data not shown) were used for further experiments. Preliminary assays with the selected clones indicated that they reach maximal values of β-galactosidase activity at around 48 h of culture. Therefore, all assays were carried at this time.

Previous data suggested that the maximal level of expression for recombinant proteins would be reached using *gpd* promoter fragments containing both *ct*- and *gpd*-boxes, although the proximal *ct*-box alone seems enough to drive the expression of a reporter protein (Punt et al. 1990; Liao et al. 2008). Figure 2 shows that the fungus transformed with pGPD-CT produced up 3–6 times (depending the carbon source) more β-galactosidase activity that the observed in fungus transformed with pCT plasmid. Thus, our results indicate that the *gpd*-box enhanced the production of β-galactosidase activity levels respect to *ct*-box alone and that both boxes are necessary to obtain maximal enzymatic activity.

It is interesting to remark that the smaller 187 nt fragment of the *gpdP* promoter is enough to produce a 27% (as average) of the β-galactosidase activity obtained with the full promoter. In yeasts and fungi, the minimal size for promoter activity is different in each case. In *Beauveria bassiana*, expression of β-glucuronidase was achieved using a 726 nt fragment of its *gpd* promoter (Liao et al. 2008). In *Lentinula edones*, a minimal size of 442 nt of its *gpd* promoter was necessary to obtain transformants resistant to hygromycin (Hirano et al. 2000) and in the yeast *Candida bombicola*, 190 nt promoter part was enough to obtain hygromycin-resistant transformants (Van Bogaert et al. 2008). To our knowledge, the fragment (187 nt) containing the *ct*-box used by us is the smallest fragment from a *gpd* gene used to produce a recombinant protein.

Finally, we assayed the production of β-galactosidase activity in these clones using glucose, galactose and glycerol, and no significant statistical differences in β-galactosidase activity were found when these carbon sources are interchanged (Fig. 2). This result was independent of the size of the promoter fragment used for recombinant protein production. The results obtained suggest a carbohydrate-independent expression pattern, being the carbon source indifferent for the protein expression. Usually, microorganisms prefer easily fermentable monosaccharides such as glucose. Other alternative carbon sources as galactose and non-fermentable glycerol are used in absence of glucose and require other enzymatic activities, which imply a hierarchy of regulatory networks (Schüller 2003). So, it

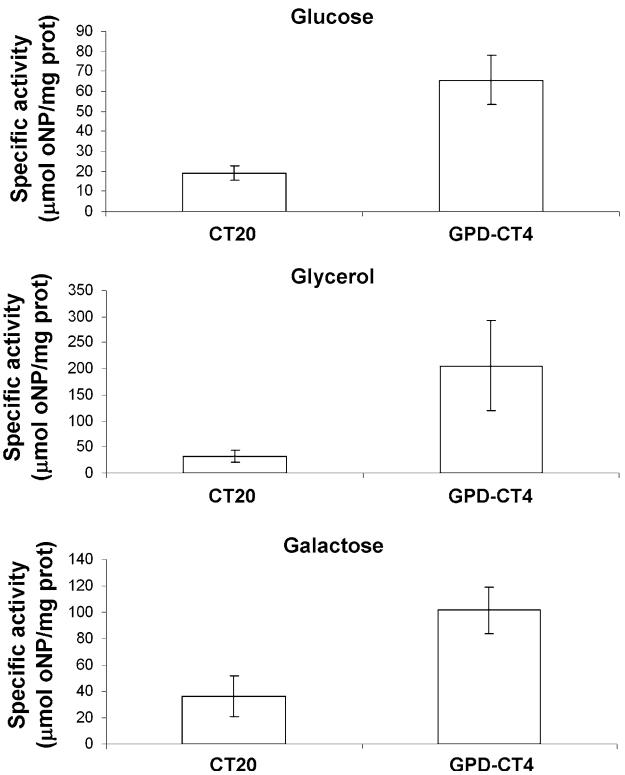

Fig. 2 Production of β-galactosidase activity in clones CT-20 (containing only the *ct*-box) and GPD-CT-4 (containing both *gpd*- and *ct*-boxes) using different carbon sources. Measurements were performed in triplicate for each transformant and carbon source, and standard error is showed by error bars. Mann–Whitney test using Graphpad Prism 4 was used for statistical evaluation ($P < 0.05$). Statistical significant differences were found in β-galactosidase activity when different sizes of promoters are used, but no when carbon sources are interchanged

could be speculated that the absence of some important regulatory element in the promoter (e.g. CreA) produced the lack of differences in β-galactosidase activity with glucose, glycerol, or galactose. The CreA protein is a wide-domain carbon catabolite repressor influencing several metabolic pathways, including some glycolytic genes (van der Veen et al. 1995). We looked for putative binding sites for CreA in the *gpdP* promoter fragments used, and we found six consensus sequences for this regulator (data not shown). Two of these sites were located in the shorter promoter segment, and the remaining four in the rest of the promoter. Thus, and although it should be investigated in more detail, this observation suggests that the lack of differences in β-galactosidase activity using different carbon sources cannot be attributed to the absence of an important regulator such as CreA.

Summarizing, *P. camemberti gpd* promoter was able to drive the production of a recombinant protein (β-galactosidase). Maximal enzymatic activity was obtained with full promoter containing both the *gpd*- and *ct*-box, while a small fragment (187 nt) was enough to produce recombinant protein. Our results open the way for the future development of plasmids for recombinant protein expression in this important industrial organism.

Acknowledgments This work was supported by grants from FONDECYT project 11060003 and DICYT-USACH project 021043 CHR. I. Vaca acknowledges support of Programa Bicentenario de Ciencia y Tecnología program (Chile), project PDA13. We thank Patricia Román and Andrés Cortés for technical assistance, Dr. Claudio Acuña for statistical analysis of data, and Dr. Jaime Eyzaguirre for critical reading of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

References

Chávez R, Roa A, Navarrete K, Trebotich J, Espinosa Y, Vaca I (2010) Evaluation of properties of several cheese-ripening fungi for potential biotechnological applications. Mycoscience 51:84–87

De Maeseneire SL, Dauvrin T, Jonniaux J-L, Van Bogaert INA, Soetaert WK, Vandamme E (2008) Cloning and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene and the use of its promoter for expression in *Myrothecium gramineum*, a novel expression host. FEMS Microbiol Lett 281:140–146

Díaz J, Chávez R, Larrondo LF, Eyzaguirre J, Bull P (2008) Functional analysis of the endoxylanase B (*xynB*) promoter from *Penicillium purpurogenum*. Curr Genet 54:133–141

Fei X, Zhao MW, Li YX (2006) Cloning and sequence analysis of a glyceraldehyde-3-phosphate dehydrogenase gene from *Ganoderma lucidum*. J Microbiol 44:515–522

Gonzalez M, Goddard N, Hicks C, Ovalle R, Rauceo JM, Jue CK, Lipke PN (2010) A screen for deficiencies in GPI-anchorage of wall glycoproteins in yeast. Yeast 27:583–596

Hirano T, Sato T, Yaegashi K, Enei H (2000) Efficient transformation of the edible basidiomycete *Lentinus edodes* with a vector using a glyceraldehyde-3-phosphate dehydrogenase promoter to hygromycin B resistance. Mol Gen Genet 263:1047–1052

Hjört CM (2003) Production of food additives using filamentous fungi. In: Heller KJ (ed) Genetically engineered food. Wiley-VCH Verlag GmbH&Co. KGaA, Weinheim, pp 87–99

Holland MJ, Holland JP (1978) Isolation and identification of yeasts messenger ribonucleic acid coding for enolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase. Biochemistry 17:4900–4907

Kanemori Y, Gomi K, Kitamoto K, Kumagai C, Tamura G (1999) Insertion analysis of putative functional elements in the promoter region of the *Aspergillus oryzae* taka- amylase A gene (*amyB*) using a heterologous *Aspergillus nidulans amdS-lacZ fusion gene system*. Biosci Biotechnol Biochem 63:180–183

Kuo C-Y, Chou S-Y, Huang C-T (2004) Cloning of glyceraldehyde-3-phosphate dehydrogenase gene and use the *gpd* promoter for transformation in *Flammulina velutipes*. Appl Microbiol Biotechnol 65:593–599

Liao XG, Fang WG, Zhang YJ, Fan YH, Wu XW, Zhou Q, Pei Y (2008) Characterization of a highly active promoter, PBbgpd, in *Beauveria bassiana*. Curr Microbiol 57:121–126

Navarrete K, Roa A, Vaca I, Espinosa Y, Navarro C, Chávez R (2009) Molecular characterization of the *niaD* and *pyrG* genes from *Penicillium camemberti*, and their use as transformation markers. Cell Mol Biol Lett 14:692–702

Nitta Y, Miyazaki Y, Nakamura M, Iimura Y, Shishido K, Kajita S, Morohoshi N (2004) Molecular cloning of the promter region of the glyceraldehyde-3-phosphate dehydrogenase gene that contributes to the construction of a new transformation system in *Coriolus versicolor*. Mycoscience 45:131–136

Piechaczyk M, Blanchard JM, Marty L, Dani C, Panabieres F, El Sabouty S, Fort P, Jeanteur P (1984) Post-transcriptional regulation of glyceraldehyde-3-phosphate dehydrogenase gene expression in rat tissues. Nucleic Acids Res 12:6951–6963

Punt PP, Dingemanse MA, Jacobs-Meijsing BJM, Pouwels PH, van den Hondel CAMJJ (1988) Isolation and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*. Gene 69:49–57

Punt PP, Dingemanse MA, Kuyvenhoven A, Soede RDM, Pouwels PH, van den Hondel CAMJJ (1990) Functional elements in the promoter region of the *Aspergillus nidulans gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase. Gene 93:101–109

Punt PP, Kramer C, Kuyvenhoven A, Soede RDM, Pouwels PH, van den Hondel CAMJJ (1992) An upstream activating sequence from the *Aspergillus nidulans gpdA*. Gene 120:67–73

Schüller HJ (2003) Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. Curr Genet 43:139–160

Sharma KK, Kuhad RC (2010) Genetic transformation of lignin degrading fungi facilitated by *Agrobacterium tumefaciens*. BMC Biotechnol 10:67

Van Bogaert INA, De Maeseneire SL, Develter D, Soetaert W, Vandamme EJ (2008) Cloning and characterisation of the glyceraldehyde 3-phosphate dehydrogenase gene of *Candida bombicola* and use of its promoter. J Ind Microbiol Biotechnol 35:1085–1092

van der Veen P, Ruijter GJG, Visser J (1995) An extreme *creA* mutation in *Aspergillus nidulans* has severe effects on D-glucose utilization. Microbiology 141:2301–2306

Vastag M, Kasza Z, Àcs K, Papp T, Schwab H, Vágvölgyi C (2004) Cloning and sequencing of the glyceraldehyde-3-phosphate dehydrogenase gene from the zygomycetes *Rhizomucor miehei*. Antonie van Leeuwenhoek 86:111–119