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Improved electroporation-mediated non-integrative transformation of *Thermomyces lanuginosus*

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ABSTRACT

The development of an efficient fungal expression system for recombinant proteins requires an improved transformation system for the host organism. We report a facile, efficient and highly reproducible electroporation-mediated transformation system for *Thermomyces lanuginosus* with a transformation efficiency of 1.27×10^3 transformants/μg DNA. Conidia of *T. lanuginosus* were stably transformed to hygromycin B resistance using the pBC-hygro plasmid construct. Optimal electroporation conditions for maximum transformation of 10^8 conidia ml^{-1} in 1.2 M sorbitol buffer (15 mM DTT, 5% DMSO) were a field strength of 5.5 kV/cm for 10 ms and a DNA concentration of 0.5 μg μl^{-1}. Transformants were recovered in prewarmed potato dextrose broth supplemented with 1.2 M sorbitol for 1–2 h at 50 °C. The presence of the hygromycin B phosphotransferase (*hph*) gene and non-integrative transformation was confirmed by PCR, Southern hybridization analysis and plasmid recovery. Transformants exhibited altered phenotype with reduced pigmentation and transformants were found to be mitotically stable after 15 sequential transfers on nonselective media without selective pressure.

1. Introduction

Because of their biological niche, thermophilic fungi have been widely exploited particularly in the production of industrial enzymes (Maheshwari et al., 2000; Singh et al., 2003; Li et al., 2005). *Thermomyces lanuginosus*, a thermophilic fungus is one of the best naturally-occurring overproducers of endoxylanase. It produces high levels of cellulose-free xylanase belonging to family 11 of glycoside hydrolases. *T. lanuginosus* also produces a range of other secreted degradative enzymes such as α-amylase, glucoamylase (Nguyen et al., 2002), pectinase (Puchart et al., 1999), phytase (Berka et al., 1998), protease (Hasnain et al., 1992) and lipase (Berg et al., 1998). These enzymes have been studied to a limited extent and are reported to be thermostable catalysts. Genes encoding other proteins such as kinesins, actin, phytase and protein kinase have been cloned and characterized (Wildeman, 1988; Munholland et al., 1990; Berka et al., 1998; Sakowicz et al., 1999).

Development of a transformation system for filamentous fungi is necessary for the cloning and mutagenesis of genes (Nevalainen, 2001; Meyer et al., 2003). It could also prove to be valuable in basic research for genetic manipulations suitable for commercial exploitation. Filamentous fungi have been transformed by standard procedures like lithium acetate transformation (Barratt et al., 1965; Gomi et al., 1987; Koukaki et al., 1989; Kiuchi et al., 1991; Yanai et al., 1990; Manczinger et al., 1997), $CaCl_2$- and PEG-mediated transformation (Harashima et al., 1984), electroporation (Richey et al., 1989; Chakraborty et al., 1991; Sánchez and Aguirre, 1996), microprojectile bombardment (Barreto et al., 1997; Téo et al., 2002) and *Agrobacterium*-mediated transformation (De Groot et al., 1998).

Previously, Chadha et al. (2000) reported on integrative transformation of *T. lanuginosus* by electroporation with a transformation frequency of 4.4 transformants/μg DNA. *Humicola grisea* var. *thermoidea* was reported to be transformed to hygromycin resistance by lithium acetate and electroporation to obtain 32 and 25 transformants, respectively (Dantas-Barbosa et al., 1998). In this report, we describe an improved electrotransformation protocol for *T. lanuginosus* with an enhanced transformation efficiency. The experiments outlined in this study constitute the first report on the optimization of conditions for electroporation-mediated, non-integrative transformation of *T. lanuginosus*.

2. Materials and methods

2.1. Strains and plasmids

T. lanuginosus DSM 5826 obtained from the German Type Culture Collection was maintained as mycelia in 30% glycerol at −80 °C and on Potato Dextrose Agar (PDA, Difco, Detroit, MI) plates. The plasmid vector pBC-hygro (6.8 kb) was a gift from Dr. P. Silar (Centre de Génétique Moléculaire du CNRS, France). It is derived from the pBluescript SK+ vector (Stratagene Cloning Systems Inc., La Jolla, CA, USA) carrying the hygromycin B resistance cassette included in the

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AvaI–SphI restriction fragment from plasmid pMOcosX. In this vector the *hph* coding sequence is under the control of the *Neurospora crassa cpc-1* promoter and the *Aspergillus nidulans trpC* terminator.

2.2. Media, chemicals and culture conditions

For DNA preparation and transformation experiments, *T. lanuginosus* was grown in Emerson YpSs medium (Cooney and Emerson, 1964) containing per litre: 4 g yeast extract; 1.5 g soluble starch; 1 g KH_2PO_4 and 0.5 g $MgSO_4.7H_2O$ (pH 7). The cultivation was carried out in 100 ml of medium in 250 ml Erlenmeyer flasks for 4 days at 50 °C, 150 rpm. Transformants were stabilized on minimal selective medium (Barratt et al., 1965) containing per litre: 20 g xylose; 1.5 g KH_2PO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g KCl; 4.4 g $ZnSO_4.7H_2O$; 1.0 g $MnCl_2.4H_2O$; 0.32 g $CoCl_2.6H_2O$; 0.32 g $CuSO_4.5H_2O$; 1.47 g $CaCl_2.2H_2O$; 1.0 g $FeSO_4.7H_2O$ and 20 g agar. The plasmid was propagated in *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, MD, USA) grown in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, pH 7.2) supplemented with chloramphenicol (100 μg ml^{-1}). The enzymes for DNA extraction were purchased from Roche Diagnostics (Mannheim, Germany) and the QIAmp tissue kit (Qiagen Inc., Santa Clarita, CA, USA) or Magic Miniprep kit (Promega, Madison, WI, USA) were used for the genomic DNA and plasmid isolations. To develop a dominant selection system in *T. lanuginosus*, the drug sensitivity of the wild-type strain was determined by plating different densities of spores on PDA amended with 25, 50, 100, 200 and 500 μg ml^{-1} of hygromycin B (Sigma Chemical Co., St. Louis, MO, USA). *T. lanuginosus* failed to grow at hygromycin B concentrations of 50 μg ml^{-1} and greater.

2.3. Optimization of electroporation conditions

Transformations were performed on germinating conidia. The conidia scraped from a 7-day culture grown on PDA were used to inoculate 100 ml YpSs medium in 250 ml flasks and incubated at 50 °C, 200 rpm for 4 h. After germination, the conidia in the culture fluid were filtered through sterile miracloth (Calbiochem, San Diego, CA, USA). The conidia were washed twice with 25 ml of ice cold sterile water, then once with 25 ml of 1.2 M sorbitol and collected by centrifugation (5 min, 4000 ×g, 4 °C). The conidia were resuspended in 1 ml of 1.2 M sorbitol to which varying concentrations of dithiothreitol (0–25 mM — Gácser et al., 2005) and dimethyl sulphoxide (0–10% — Hill, 1989) were added in combination or alone and incubated at 37 °C with occasional inversions for 10 min (DMSO) or 15 min (DTT alone or in combination). Aliquots of 80 μl of germinating conidia (ca 10^8 ml^{-1}) were mixed with 0.5 μl (1 μg μl^{-1}) of plasmid DNA, transferred into a prechilled microfuge tube and subjected to electroporation in a prechilled electroporation cuvette with an electrode gap of 0.2 cm using a Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA). The mixture of conidia and DNA was electroporated by a single exponential pulse ranging between 3 and 6.5 kV/cm (25 μF capacitance, 200 Ω resistance) and pulse durations between 2 and 20 ms. Each set of experiments included controls in which transforming DNA or pulse was omitted. The effect of buffer ionic strength on the transformation efficiency was investigated by electroporating the germinating conidia and DNA in three different buffers: buffer I (1.2 M sorbitol, pH 7.2), buffer II (270 mM sucrose, 10 mM Tris–HCl, pH 7.5) and buffer III (270 mM sucrose, 1 mM lithium acetate, 10 mM Tris–HCl, pH 7.5). After pulsing, the effect of the recovery medium on transformation efficiency was determined by transferring the electroporated conidia to 1 ml of prewarmed (50 °C) medium I (PD broth supplemented with 1.2 M sorbitol) or medium II (1.2 M sorbitol) or medium III (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). Aliquots from the recovery medium were mixed into 7.5 ml of prewarmed top agar (1%), supplemented with 50 μg ml^{-1} of hygromycin and overlaid on PD agar plates. Putative transformants were scored after incubation at 50 °C for 5–10 days. The putative transformants were carefully excised and transferred to fresh PDA plates. All experiments were performed in duplicate and the results were averaged, with a variation of transformation efficiency of ±5%.

2.4. Plasmid stability studies

Selected transformants of *T. lanuginosus* were allowed to sporulate and single spore isolates were used to obtain cultures for further analysis. The mitotic stability of the primary transformants was assessed by culturing 60 randomly selected transformants on nonselective medium (PDA without hygromycin). Following fifteen cycles of cultivation on nonselective medium, the colonies which appeared between 5 and 7 days on nonselective medium were subsequently transferred to selective medium (amended with 50 μg/ml of hygromycin). Only those transformants that grew equally well on PDA and selective medium supplemented with hygromycin were picked and subjected to further stabilization by successive transfers on minimal selective medium amended with higher titres of hygromycin (200 μg ml^{-1}). PDA plates were inoculated with a mycelia plug of 0.3 cm diameter and incubated at 50 °C for 14 days. Daily observations of colour and shape of colonies were recorded.

2.5. Molecular analysis of transformants

Total DNA from transformants and the wild-type strain was isolated according to the method of Chow and Käfer (1993), with some modifications. The spores from the wild type strain of *T. lanuginosus* and the presumptive transformants were grown in 100 ml YpSs medium overnight at 50 °C and 200 rpm. Approximately 100 mg of the mycelium was harvested, ground in liquid nitrogen and resuspended in 900 μl of 5× TE buffer (pH 8.0) followed by 90 μl 10% sodium dodecyl sulphate (Sambrook et al., 1989). Protein and cell debris were precipitated by the addition of 100 μl of 5 M potassium acetate. The supernatant was precipitated with 70% ethanol, centrifuged for 30 s and the pellet was air dried, resuspended in 50 μl TE buffer (pH 8.0). The presence of the hygromycin resistance gene was confirmed by PCR analysis of total DNA isolated from the untransformed parental strain and the hygromycin-positive strains using hygromycin specific primers (forward: 5′CGTCTGTCGAGAAGTTTC3′, reverse: 5′GTCAGGACATTGTTGGAG3′) designed to amplify a 557 base pair internal region of the *hph* gene. PCR reactions were performed using AccuPower PCR premix (Bioneer Corp, Alameda, CA, USA). The PCR reaction mixture of 100 μl contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTPs, 30 pmol of each primer and 1.5 units of Taq DNA polymerase. The cycling regimen in a Geneamp 2700 PCR system (Applied Biosystems, Foster City, CA, USA) included an initial denaturation cycle at 95 °C for 2 min followed by 35 cycles at 95 °C of 30 s each, 30 s annealing at 55 °C and 1 min elongation at 72 °C followed by a 7 min final elongation step at 72 °C. The PCR products were resolved by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. The pBC-hygro plasmid DNA and DNA from untransformed *T. lanuginosus* were used as positive and negative controls, respectively, in all PCR reactions.

2.6. Southern hybridization analysis

Ten micrograms of total DNA isolated from the parental strain and PCR-positive transformants (Chow and Käfer, 1993) was subjected to Southern hybridization analysis. Uncut or restriction endonuclease digested samples of DNA, size fractionated on a 0.8% agarose gel, were blotted on positively-charged nylon membranes using standard techniques (Sambrook et al., 1989). The blots were hybridized with the labelled 557 bp sequence spanning the *hph* gene. Probe labelling,

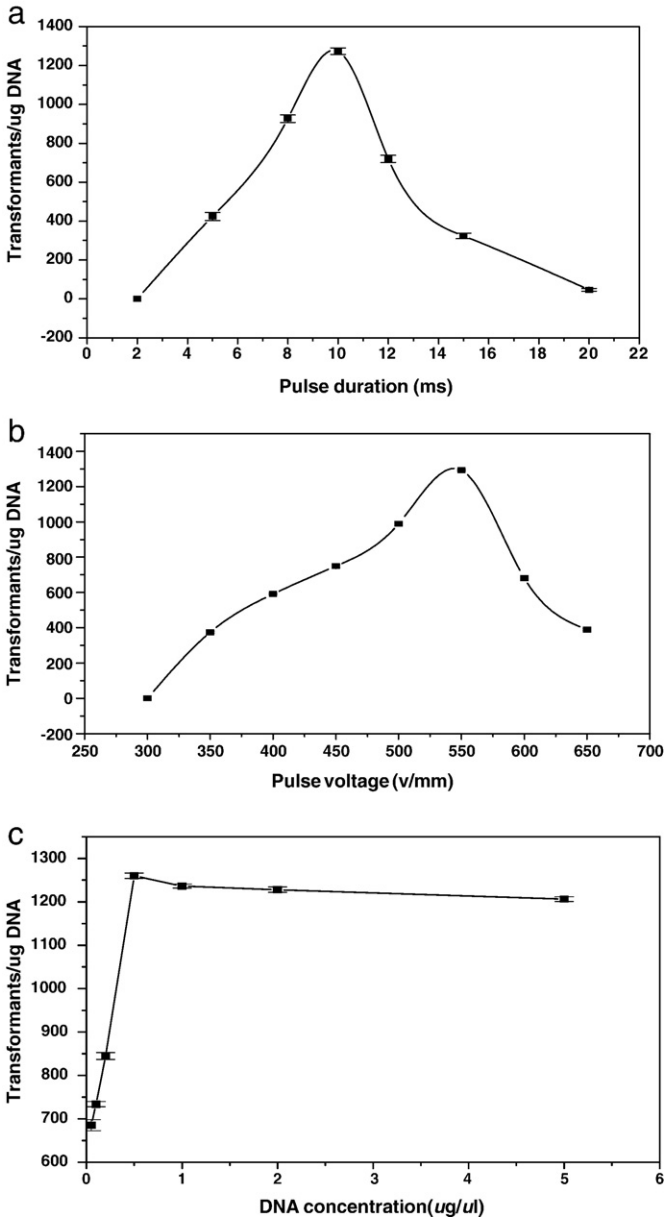

Fig. 1. Optimization of electroporation parameters for transformation of *Thermomyces lanuginosus*. The effect of a) pulse voltage b) pulse duration and c) DNA concentration on transformation efficiency are shown. Optimal conditions: field strength — 5.5 kV/cm, pulse duration — 10 ms, concentration of cells — ca 1×10^8/ml, DNA concentration — 0.05 µg, electroporation buffer — 1.2 M sorbitol supplemented with 15 mM DTT and 10% DMSO. Results are an average of three replicate experiments with a SD of ±5%.

hybridization and detection were carried out using the Amersham ECL Direct Nucleic Acid Labeling and Detection System (Amersham Pharmacia, Little Chalfont, UK).

2.7. Plasmid recovery and restriction digestion

In order to ascertain whether the transformants contain free plasmid DNA, "minipreps" of plasmid DNA from transformants and the wild-type strain were isolated according to the method of Paietta and Marzluf (1985), with some modifications. The spores from the wild type strain of *T. lanuginosus* and the presumptive transformants were grown in 100 ml YpSs medium overnight at 50 °C and 200 rpm. Approximately 100 mg of the mycelium was harvested, frozen overnight at −70 °C and then lyophilized. The lyophilized mycelium was ground and resuspended in 500 µl of lysis buffer (100 mM Tris–HCl [pH 8.0], 50 mM EDTA, 100 mM NaCl, 10 mM 2-mercaptoethanol, 1% SDS), vortexed and the mixture was incubated at 65 °C for 10 min. Protein and cell debris were precipitated by the addition of 100 µl 5 M potassium acetate. The supernatant was precipitated with 70% ethanol, centrifuged for 30 s and the pellet was air dried and resuspended in 50 µl TE buffer (pH 8.0). The DNA recovered from the transformants was restricted with EcoRI and HindIII. Restriction fragments were analyzed on horizontal slab gels containing 0.8% agarose in TAE buffer at 100 V.

3. Results and discussion

3.1. Determination of optimal conditions for transformation efficiency

The optimal field strength and pulse duration are critical parameters in determining the transformation efficiency. Initial transformants were obtained at a field strength of 3.5 kV/cm and no transformants were obtained at a field strength of 3 kV/cm (Fig. 1b). This showed the dependence of transformation efficiency on electric field strength and the requirement of a minimal field intensity. The optimal field strength was found to be 5.5 kV/cm. No transformants

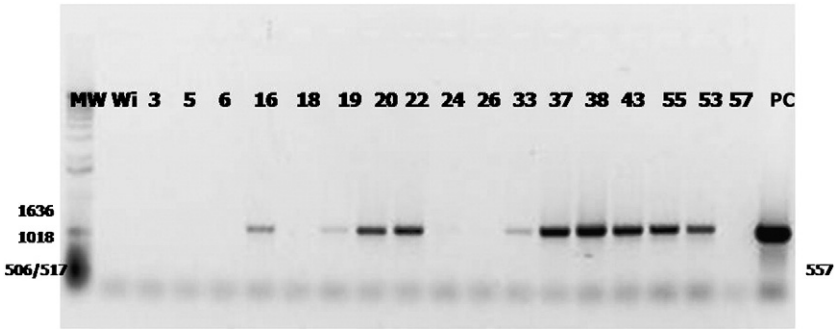

Fig. 2. Amplification of the *hph* gene fragment in *Thermomyces lanuginosus* transformants by PCR Lane 1: MW: molecular weight size standards (Invitrogen 1 kb DNA ladder), Lane 2: Wi: wild type, Lane 3–17: transformants, Lane 18: PC: plasmid (pBC-hygro) control.

were obtained at a pulse duration of 2 ms and the optimum pulse duration was 10 ms (Fig. 1a). Further increase in pulse length resulted in a decrease in the number of transformants.

When the field strength and pulse duration were maintained at optimized settings and the DNA concentration varied from 0.05 μg μl^{-1} to 2 μg μl^{-1}, the number of transformants increased linearly up to 0.5 μg μl^{-1} and then remained constant as the DNA concentration was further increased. For 10^8 conidia/ml, a DNA concentration of 0.5 μg μl^{-1} gave maximum number of transformants (Fig. 1c).

The effect of pretreatment with thiol compounds on transformation efficiency was examined by using DTT at varying concentrations from 0–20 mM in combination with DMSO or alone. Pretreatment with thiol compounds like DTT is reported to weaken disulphide bridges and thereby increase the porosity of the cells which facilitates the uptake of DNA (Gácser et al., 2005). DMSO was reported to increase the transformation efficiency of lithium acetate-mediated transformation of intact yeast cells (Hill, 1989). It was attributed to the changes in the lipid arrangement bringing structural changes in the membrane (Soni et al., 1993; Melkonyan et al., 1996). We found that the transformation efficiency increased linearly with an increase in concentration of DTT up to 15 mM and for 30 min. Further increases in the concentration of DTT to over 20 mM and longer incubation times did not improve transformation efficiency. Similarly, pretreatment with 7.5% DMSO for 30 min also yielded efficiency comparable to that of the 15 mM DTT concentration. The combined effect of 15 mM DTT and 5% DMSO increased the number of transformants (1.29×10^2/μg DNA) which could be due to the additive effect of both the substances in increasing the membrane permeability to DNA uptake.

The buffer composition affected transformation efficiency. The transformation efficiency was further increased to 1.22×10^3 transformants/μg DNA using buffer I (1.2 M sorbitol) compared to buffer II and buffer III which gave 9.4×10^2 and 6.1×10^2 transformants/μg DNA, respectively. This could be due to the high ionic strength of buffer II and buffer III which resulted in arcing that subsequently affected the survival of conidia. This finding supported the use of 1.2 M sorbitol as an electroporation buffer, affording continuous osmotic support for the electrically compromised conidia (Van den Hoff et al., 1990). Optimized electroporation conditions adopted for all the experiments included the use of this buffer in combination with the other optimal parameters.

Transformation efficiency was improved (1.27×10^3 transformants/μg DNA) when the population of pulsed conidia were allowed to recover in medium I (prewarmed PD broth supplemented with 1.2 M sorbitol) for 1–2 h at 50 °C compared to the other two recovery media. Medium II and medium III did not result in a significant increase in transformation efficiency. These results are in good agreement with research showing that an incubation step is necessary to allow the expression of the antibiotic resistance gene and the osmotic stabilization is desirable to increase the effectiveness of transformation (Becker and Guarente, 1991).

3.2. PCR analysis of transformants

PCR analysis of the transformants on agarose gels yielded the expected 557 bp *hph* gene fragment (Fig. 2). Of 17 transformants analyzed, 10 were positive for the presence of *hph* gene after PCR reactions. There was no amplification in the water control and fungal DNA from untransformed wild-type fungus, suggesting that the PCR reactions did not yield false positives (Fig. 2). Apparently, the transformants 3, 5, 6, 18, 24, 26 and 57, lost the plasmid during the stabilization process. Dantas-Barbosa et al. (1998) have reported that the transformants resulted from electroporative transformation of *H. grisea* var. *thermoidea* could be analyzed for detection of the *hph* gene by PCR analysis only.

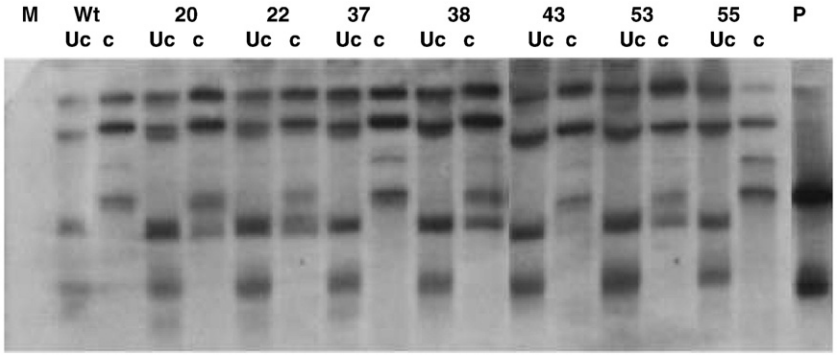

Fig. 3. Southern hybridization analysis of total DNA from *Thermomyces lanuginosus* transformants. Genomic DNA was digested with HindIII, run on a 0.7% gel and transferred to a nylon membrane before probing with a 557 bp *hph*-gene fragment. Lane 1: M — molecular weight size standard (Invitrogen 1 kb DNA ladder), Lane 2–3 Wt: wild type (uncut and cut), Lane 4–17: transformants (uncut and cut), Lane 18: PC — plasmid (pBC-hygro).

3.3. Southern analysis of transformants and plasmid recovery

Transformants were found to be stable under hygromycin selection for a period of over 6 months which is suggestive of stable rather than transient transformation. PCR amplification with specific primers verified the presence of plasmid DNA in the transformants. Southern blot analysis of undigested and HindIII digested total DNA of transformants also demonstrated the presence of plasmid DNA but, in addition, verified that intact supercoiled plasmids were present in undigested samples. These were converted to linear DNA molecules after digestion (Fig. 3). The episomal replication and extrachromosomal maintenance was confirmed by recovery of plasmid DNA from the hygromycin-resistant transformants. The restriction pattern of the plasmid DNA rescued from *E. coli* was the same as that of the plasmid vector (data not shown). Previous analyses by Southern hybridization after electroporation of *T. lanuginosus* had verified the insertion of plasmid DNA (Chadha et al., 2000) using an integrative vector but the transformation efficiency reported was low. Contrarily, we have transformed *T. lanuginosus* using pBC-hygro and optimized electroporation conditions to obtain significantly higher frequencies which are 1000-fold higher than reported by Dantas-Barbosa et al. (1998) and Chadha et al. (2000).

In genetic manipulation of filamentous fungi, the autonomously replicating plasmids are reported to be generally more effective than the integrative plasmids for efficient gene replacement or expression of a heterologous gene to analyze its function because of the high transformation efficiency and the ectopic integration of transforming DNA into the genome can be avoided (Ruiz-Díez, 2002; Fierro et al., 2004). Our results on mitotic stability and transformation efficiency is in agreement with that reported for *Penicillium nalgiovense* (Fierro et al., 2004) and *Penicillium chrysogenum* (Fierro et al., 1996). There were reports on transforming *Monascus purpureus* using an autonomously replicating vector to obtain aureobasidin-resistant transformants at an efficiency of 17 transformants/μg DNA (Shimizu et al., 2006) and long term maintenance of episomal plasmids (Aleksenko and Clutterbuck., 1995). It is not certain that their stable maintenance is as a result of a specific segregation mechanism or a very efficient replication origin which generates a sufficiently high copy number for the plasmid. The latter would appear to be the more likely explanation.

3.4. Morphological characterization of transformants

Morphological characterization and sporulation assays of transformants confirmed that a few transformants differed from the wild-type strain with less pigmentation. A few transformants exhibited a distinctive phenotype with conidia varying from dark grey to albino. Transformants 20, 22, and 43 produced albino conidia with less pigmentation compared to the dark grey conidia and the intense wine red pigmentation of the wild-strain. The reason for this is unknown. The phenotype and the colour of the conidia of the rest of the transformants were similar to the parental strain but pigmentation varied, ranging from tea-yellow to light wine red. All the transformants grew consistently on PDA or selective medium containing hygromycin. The grey colour in xylanase preparations from wild-type *T. lanuginosus* limits the use of the enzyme in biobleaching of pulp whereas albino transformants with reduced or no pigmentation will have potential industrial application in the bleaching of paper and pulp (Gutiérrez et al., 2001; Christopher et al., 2005). We are currently characterizing the albino transformants and also investigating the possibility of using the xylanase from the albino transformants in biobleaching of pulp and paper. We intend using the newly-developed transformation system of *T. lanuginosus* to introduce genetically modified xylanases having increased thermal and pH stability into this organism as well as examining the potential for expression of heterologous proteins in this fungus.

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References

Aleksenko, A., Clutterbuck, A.J., 1995. Recombinational stability of replicating plasmids in *Aspergillus nidulans*. Curr. Genet. 28, 87–93.
Barratt, R.W., Johnson, G.B., Ogata, W.N., 1965. Wild-type and mutant stocks of *Aspergillus nidulans*. Genetics 52, 233–246.
Barreto, C.C., Alves, L.C., Aragao, F.G., Rech, E., Schrank, A., Vainstein, M.H., 1997. High frequency gene transfer by microprojectile bombardment of intact conidia from the entomopathogenic fungus *Paecilomyces fumosoroseus*. FEMS Microbiol. Lett. 156, 95–99.
Becker, D.M., Guarente, L., 1991. High Efficiency Transformation of Yeast by Electroporation. . Methods Enzymol, vol. 194. Academic Press, New York, pp. 182–187.
Berg, O.G., Cajal, Y., Butterfoss, G.L., Grey, R.L., Alsina, M.A., Yu, B., Jain, M.K., 1998. Interfacial activation of triglyceride lipase from *Thermomyces* (*Humicola*) *lanuginosa*: kinetic parameters and a basis for control of the lid. Biochemistry. 37, 6615–6627.
Berka, R.M., Rey, M.W., Brown, K.M., Byun, T., Klotz, A.V., 1998. Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*. Appl. Environ. Microbiol. 64, 4423–4427.
Chadha, B.S., Kaur, R., Saini, H.S., Singh, S., 2000. Characterization of hygromycin — resistant transformants of thermophilic fungus *Thermomyces lanuginosus*. World J. Microbiol. Biotechnol. 16, 303–306.
Chakraborty, B.N., Patterson, N.A., Kapoor, M., 1991. An electroporation-based system for high-efficiency transformation of germinated conidia of filamentous fungi. Can. J. Microbiol. 37, 858–863.
Chow, T.Y.-K., Käfer, E., 1993. A rapid method for isolation of total. nucleic acids from *Aspergillus nidulans*. Fungal Genetics Newsletter 40, 25–27.
Christopher, L., Bissoon, S., Singh, S., Szendefy, J., Szakacs, G., 2005. Bleach-enhancing abilities of *Thermomyces lanuginosus* xylanases produced by solid state fermentation. Process Biochem. 40, 3230–3235.
Cooney, D.G., Emerson, R., 1964. *Humicola insolens* and *Humicola grisea* var. *thermoidea*. Thermophilic Fungi. W. H. Freeman, San Francisco, pp. 77–79.
Dantas-Barbosa, E.F., Araújo, L.M.P., Moraes, M.H., Vainstein Azevedo, M.O., 1998. Genetic transformation of germinated conidia of the thermophilic fungus *Humicola grisea* var. *thermoidea* to hygromycin B resistance. FEMS Microbiol. Lett. 169, 185–190.
De Groot, M.J.A., Bundock, P., Hooykaas, P.J.J., Beijersbergen, A.G.M., 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat. Biotechnol. 16, 839–842.
Fierro, F., Kosalková, K., Gutiérrez, S., Martin, J.F., 1996. Autonomously replicating plasmids carrying the *AMA1* region in *Penicillium chrysogenum*. Current Genet. 29, 482–489.
Fierro, F., Laich, F., Ramón, O., Garcia-Rico, Martin, J.F., 2004. High efficiency transformation of *Penicillium nalgiovense* with integrative and autonomously replicating plasmids. Int. J. Food Microbiol. 90, 237–248.
Gácser, A., Salomon, S., Schäfer, W., 2005. Direct transformation of a clinical isolate of *Candida parapsilosis* using a dominant selection marker. FEMS Microbiol. Lett. 245, 117–121.
Gomi, K., Limura, Y., Hara, S., 1987. Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. Agric. Biol. Chem. 51, 2549–2555.
Gutiérrez, A., del Rio, J.C., Jesús Martinez, M., Martínez, M.T., 2001. The biotechnological control of pitch in paper pulp manufacturing. Trends Biotechnol. 19, 340–348.
Harashima, S., Takai, A., Oshima, Y., 1984. Transformation of protoplasted yeast cells is directly associated with cell fusion. Mol. Cell. Biol. 4, 771–778.
Hasnain, S., Adeli, K., Storer, A.C., 1992. Purification and characterization of an extracellular thiol-containing serine proteinase from *Thermomyces lanuginosus*. Biochem. Cell. Biol. 70, 117–122.
Hill, D.E., 1989. Integrative transformation of yeast using electroporation. Nucl. Acids Res. 17, 8011.
Kiuchi, N., Naruse, A., Yamamoto, H., Sekiguchi, J., 1991. Transformation of *Penicillium urticae* with plasmids containing the hygromycin B resistance gene. Agric. Biol. Chem. 55, 3053–3057.
Koukaki, M., Giannoutsou, E., Karagouni, A., Diallinas, G., 1989. A novel improved method for *Aspergillus nidulans* transformation. J. Microbiol. Methods 55, 687–695.
Li, X.T., Jiang, Z.Q., Li, L.T., Yang, S.Q., Feng, W.Y., Fan, J.Y., Kusakabe, I., 2005. Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. Bioresour. Technol. 96, 1370–1379.
Maheshwari, R., Bharadwaj, G., Bhat, M.K., 2000. Thermophilic fungi: their physiology and enzymes. Microbiol. Mol. Biol. Rev. 64, 461–488.
Manczinger, L., Komonyi, O., Antal, Z., Ferenczy, Z., 1997. A method for high-frequency transformation of *Trichoderma viride*. J. Microbiol. Methods 29, 207–210.
Melkonyan, H., Sorg, C., Klempt, M., 1996. Electroporation efficiency in mammalian cells is increased by dimethyl sulfoxide (DMSO). Nucl. Acids Res. 24, 4356–4357.
Meyer, V., Mueller, D., Strowig, T., Stahl, U., 2003. Comparison of different transformation methods for *Aspergillus giganteus*. Curr. Genet. 43, 371–377.

Munholland, J.M., Kelly, J.K., Wideman, A.G., 1990. DNA sequences required for yeast actin gene transcription do not include conserved CCAAT motifs. Nucl. Acids Res. 18, 6061–6068.

Nevalainen, H., 2001. Strain improvement in filamentous fungi — an overview. In: Khachatourians, G.-G., Arora, D.K. (Eds.), Applied Mycology and Biotechnology. . Agriculture and Food Production, vol. 1. Elsevier Science, Netherlands, pp. 289–304.

Nguyen, Q.D., Rezessy Szabó, J.M., Claeyssens, M., Stals, I., Hoschke, A., 2002. Purification and characterisation of amylolytic enzymes from fungus *Thermomyces lanuginosus* strain 34626. Enzyme Microb. Technol. 31, 345–352.

Paietta, J., Marzluf, G.A., 1985. Plasmid recovery from transformants and the isolation of chromosomal DNA segments improving plasmid replication in *Neurospora crassa*. Curr. Genet. 9, 383–388.

Puchart, V., Katapodis, P., Biely, P., Kremnicky, L., Christakopoulos, P., Vrsanska, M., Kekos, D., Macris, B.J., Bhat, M.K., 1999. Production of xylanases, mannanases, and pectinases by the thermophilic fungus *Thermomyces lanuginosus*. Enzyme Microb. Technol. 24, 355–361.

Richey, M.G., Marek, E.T., Schardl, C.L., Smith, D.A., 1989. Transformation of filamentous fungi with plasmid DNA by electroporation. Phytopathology 79, 844–849.

Ruiz-Díez, B., 2002. Strategies for the transformation of filamentous fungi. J. Appl. Microbiol. 92, 189–195.

Sakowicz, R., Farlow, S., Goldstein, L.S.B., 1999. Cloning and expression of kinesins from the thermophilic fungus *Thermomyces lanuginosus*. Prot. Sci. 8, 2705–2710.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a laboratory Manual, 2nd Ed. Cold Spring harbor Laboratory, Cold Spring Harbor, New York, USA.

Sánchez, O., Aguirre, J., 1996. Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. Fungal Genet. Newslett. 43, 48–51.

Shimizu, T., Kinoshita, H., Nihira, T., 2006. Development of transformation system in *Monascus purpureus* using an autonomous replication vector with aureobasidin A resistance gene. Biotechnol. Lett. 28, 115–120.

Singh, S., Madlala, A.M., Prior, B.A., 2003. *Thermomyces lanuginosus*: properties of strains and their hemicellulases. FEMS Microbiol. Rev. 27, 3–16.

Soni, R., Carmichael, Murray, J.A.H., 1993. Parameters affecting lithium acetate mediated transformation of *Saccharomyces cerevisiae* and development of a rapid and simplified procedure. Curr. Genet. 24, 455–459.

Téo, S.J., Bergquist, P.L., Nevalainen, H., 2002. Biolistic transformation of *Trichoderma reesei* using the BioRad seven barrels Hepta Adaptor system. J. Microbiol. Methods 51, 393–399.

Van den Hoff, M.J., Labruyere, W.T., Moorman, A.F., Lamers, W.H., 1990. The osmolarity of the electroporation medium affects the transient expression of genes. Nucl. Acids Res. 18, 6464.

Wildeman, A.G., 1988. Putative ancestral actin gene present in a thermophilic eukaryote: novel combination of intron positions. Nucl. Acids Res. 16, 2553–2564.

Yanai, K., Horiuchi, H., Takagi, M., Yano, K., 1990. Preparation of protoplasts of *Rhizopus niveus* and their transformation with plasmid DNA. Agric. Biol. Chem. 54, 2689–2696.