Research Article

Development of a pyrG Mutant of Aspergillus oryzae Strain S1 as a Host for the Production of Heterologous Proteins

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The ease with which auxotrophic strains and genes that complement them can be manipulated, as well as the stability of auxotrophic selection systems, are amongst the advantages of using auxotrophic markers to produce heterologous proteins. Most auxotrophic markers in Aspergillus oryzae originate from chemical or physical mutagenesis that may yield undesirable mutations along with the mutation of interest. An auxotrophic A. oryzae strain S1 was generated by deleting the orotidine-5′-monophosphate decarboxylase gene (pyrG) by targeted gene replacement. The uridine requirement of the resulting strain GR6 pyrGΔ0 was complemented by plasmids carrying a pyrG gene from either Aspergillus nidulans or A. oryzae. β-Galactosidase expression by strain GR6 pyrGΔ0 transformed with an A. niger plasmid encoding a heterologous β-galactosidase was at least 150 times more than that obtained with the untransformed strain. Targeted gene replacement is thus an efficient way of developing auxotrophic mutants in A. oryzae and the auxotrophic strain GR6 pyrGΔ0 facilitated the production of a heterologous protein in this fungus.

1. Introduction

In Japan, the filamentous fungus Aspergillus oryzae is extensively used in the production of miso (soybean paste), sake (rice wine), and shoyu (soy sauce). Due to its proven safety record in the food industry, A. oryzae has been assigned the status Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration (USFDA) and the World Health Organization (WHO) [1]. A. oryzae is also used for the production of homologous and heterologous proteins and metabolites [2]. The A. oryzae genome consists of eight chromosomes totalling 37 megabases (Mb) predicted to harbour 12,074 protein coding genes [3].

The pyrG gene encodes orotidine-5′-monophosphate (OMP) decarboxylase, an enzyme of the pyrimidine biosynthesis pathway that is important for uridine synthesis. The mutagen 5-fluoroorotic acid (5-FOA) inhibits cell proliferation in most organisms by entering the pyrimidine synthesis pathway where it is converted into the pyrimidine analogue fluoroorotidin monophosphate. Through the subsequent action of ribonucleotide reductase, fluoroorotidin monophosphate is converted intofluorodeoxyuridine, a potent suicidal inhibitor of the enzyme thymidylate synthase, which converts deoxyuridine monophosphate into the essential DNA precursor thymidylate [4]. Resistance to 5-FOA can be achieved by blocking the pyrimidine
pathway through mutational inactivation of orotidine-5'-monophosphate (OMP) decarboxylase which catalyzes the decarboxylation of orotidine monophosphate (OMP) to form uridine monophosphate (UMP). In filamentous fungi, pyrimidine auxotrophs obtained through inactivation of the OMP decarboxylase gene (usually designated \( \text{pyrG} \)) are auxotrophic for uridine and/or uracil and are resistant to 5-FOA [5, 6]. The generation of 5-FOA-resistant \( \text{pyrG} \) mutants has been reported in other fungi including \( \text{A. aculeatus} \), \( \text{A. niger} \), \( \text{Sclerotinia sclerotiorum} \), and \( \text{Trichoderma reesei} \) [5, 7–9]. Most \( \text{pyrG} \) mutants were isolated following either ultraviolet (UV) or chemical mutagenesis. Mutations induced in these ways are often unstable (i.e., they revert to the wild type) and may include multiple, undesirable mutations. In contrast, mutants generated by targeted gene replacement are stable as the targeted gene is removed from the genome. It is, however, more difficult to mutate multiple genes this way [10, 11].

A BLAST search using the \( \text{pyrG} \) sequence of \( \text{A. oryzae} \) S1 against the \( \text{A. oryzae} \) RIB40 genome database at the National Institute of Technology and Evaluation (NITE; http://www.bio.nite.go.jp/dogan) showed that \( \text{pyrG} \) is present as a single copy on chromosome 7 (contig SC011, location 2219667...2220565).

In this study, a gene replacement cassette was constructed consisting of the native \( \text{pyrG} \) locus extending 1.5 to 2.0 kb beyond the upstream and downstream termini of the \( \text{pyrG} \) coding region but lacking the open reading frame of the gene. Transformation of \( \text{A. oryzae} \) strain S1 with the gene replacement cassette generated 5-FOA-resistant uracil and uridine auxotrophs. Putative \( \text{pyrG} \Delta 0 \) mutants were verified by comparing their growth on selective and nonselective media, PCR analyses of the \( \text{pyrG} \) region in the putative mutants and complementation with plasmids containing wild-type homologous and heterologous \( \text{pyrG} \) genes. Suitability of the \( \text{pyrG} \Delta 0 \) mutant as a host for heterologous protein production was assessed using strain GR6 harbouring a plasmid in which the \( \text{pyrG} \) and heterologous \( \beta \)-galactosidase genes were integrated.

2. Materials and Methods

2.1. Fungus, Cultivation, and Plasmids. \( \text{A. oryzae} \) strain S1 was obtained from the Fungal Stock Culture Collection of the Molecular Mycology Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The fungus was maintained on a Potato Dextrose Agar (PDA; Difco, France) at 30°C and subcultured monthly. Fungal cultivation was carried out by inoculating onto a fresh PDA plate. Cultures were incubated for 7 days at 30°C. Plasmid ANEp2 (Figure 1(a)) is a shuttle vector capable of autonomous replication in Aspergilli [12]. In addition to the pUC18 backbone ANEp2 carries the \( \text{A. nidulans} \) \( \text{pyrG} \) gene as a selectable marker, an expression cassette (\( \text{GlaPr-lacA-GlaTt} \)) that expresses an \( \text{A. niger} \) secreted \( \beta \)-galactosidase, and the AMA1 sequence that supports autonomous replication in Aspergilli. ANEp2 was used to functionally test for complementation of uridine auxotrophy and reporter gene expression in an \( \text{A. oryzae} \) \( \text{pyrG} \) mutant. Plasmid pUC\(_{\text{pyrG}}\) Ao containing the \( \text{pyrG} \) of the \( \text{A. oryzae} \) strain S1 was also used for complementation of uridine auxotrophy (Figure 1(b)) [13]. \( \text{Escherichia coli} \) strain DH5α was used for DNA manipulation.

2.2. Media. Polypeptone dextrin (PD) medium containing 1.0% (w/v) polypeptone, 2.0% (w/v) glucose, 0.5% (w/v)
The Scientific World Journal

The construction of pGEM-5 endonuclease mapping and sequencing were used to verify Trichoderma harzianum et al. [14] and Liew [17] as follows:

2.4. Spheroplast Preparation and Transformation.

The upstream region was amplified with the primers 5′-pyrG′-region antisense primer outside the pyrG′ gene replacement cassette

Pyr5′-out

The cassette used to delete the A. oryzae pyrG (Figure 2) was assembled as follows:

2.3. Replacement Cassette Construction. The cassette used to delete the A. oryzae pyrG (Figure 2) was assembled as follows: genomic DNA of A. oryzae strain S1 was used as the PCR template to amplify the 1.6 kb and 2.0 kb regions upstream and downstream of the pyrG coding region, respectively. The upstream region was amplified with the primers 5′-pyr and revint-XhoI while the downstream region was amplified using the primers fwdint-XhoI and 3′-pyr (Table I). Both PCR products were ligated into the vector, pGEM-T Easy (Promega, Fitchburg, WI, USA). Plasmids pGEM-5′-pyr and pGEM-3′-pyr were identified by restriction endonuclease digestion and verified by sequencing. Finally, the two regions flanking the pyrG coding region were assembled into the pyrG replacement cassette by ligating the plasmids pGEM-5′-pyr and pGEM-3′-pyr digested with XhoI and Sall. Restriction endonuclease mapping and sequencing were used to verify the construction of pGEM-5′-3′-pyr.

2.4. Spheroplast Preparation and Transformation. Spheroplasts were produced based on the methods of Ushijima et al. [14] and Liew [17] as follows: A. oryzae conidia (10⁷ conidia/mL) were cultivated in 50 mL PD at 30°C for 20 h. Mycelia (3 g wet weight) were pretreated with DTT-lysis buffer containing 10 mM dithiothreitol (DTT) and 10 mM Tris-HCl for 1 h at 30°C by shaking at 75 rpm prior to incubation with 70 mg/mL lyzing enzyme (Trichoderma harzianum, Sigma-Aldrich Corp. St. Louis, MO, USA) solution containing osmotic buffer (0.8 M MgSO₄ in 0.01 M phosphate buffer) for 4 h at 30°C (agitation—75 rpm). The mixture containing spheroplasts was transferred to a 50 mL centrifuge tube (Greiner Bio-One, UK) and 0.6 M sorbitol was added slowly to the mixture. The mixture was spun at 4,000 rpm, 4°C for 30 min. The interphase layer between the lysis buffer and sorbitol was collected and transferred to a new 50 mL tube with the addition of STC buffer (1.2 M sorbitol, 0.01 M Tris-HCl, 0.01 M CaCl₂). Transformation was carried out according to Storms et al. [12] and Wernars et al. [18] using 1.6 × 10⁸ viable spheroplasts in STC mixed with 10 µg purified PCR product (3.6 kb amplified DNA fragment consisting of the 5′-pyr and 3′-pyr regions of the pyrG gene replacement cassette) and 1.2 M sorbitol as the osmotic stabilizer. Subsequently, 0.5 mL aliquots containing 8 × 10⁶ transformed spheroplasts were mixed into 0.5% CD overlay agar, poured onto 2% CD agar plates supplemented with 0.2% uracil and 20 mM uridine, and incubated at 30°C for 4–6 days.

2.5. Screening and Analysis of Transformants. 5-FOA resistant colonies were tested for auxotrophy by patching on CD medium and CD medium with uracil and uridine. Verification that the 5-FOA-resistant auxotrophs arose via deletion of the pyrG coding region was done by PCR analysis of total DNA using primers Pyr5′-out and Pyr3′-out (Table I) designed to be complementary to the known genomic (obtained from the A. oryzae RIB40 NITE genome database) sequence just beyond the upstream and downstream boundaries of the replacement cassette. The pyrGΔ0 mutants were also tested by complementation with plasmids pUC_pyrGpA and ANEp2 harbouring A. oryzae and A. niger pyrG, respectively.

2.6. β-Galactosidase Expression. Levels of β-Galactosidase activity expressed by A. oryzae strain GR6 pyrGΔ0 and GR6 pyrGΔ0 transformants harbouring ANEp2 were determined using filtered medium from 7-day old static cultures. Static cultures were generated by inoculating 1.0 × 10⁶ conidiospores into Petri dishes containing 25 mL of MMJ medium as described by Käfer [19] using 15% maltose as the carbon source (along with the necessary supplements) [20], at 30°C.
β-Galactosidase assays were performed by adding culture filtrates to a reaction-buffer containing 50 mM sodium acetate (pH 4.1) and 4 mg/mL of orthonitrophenyl-β-D-galactoside (ONPG), and incubating for 10 min at 37°C. Assays were stopped by adding 1 volume of 1 M sodium carbonate and the absorbance was read at 415 nm. β-Galactosidase activity is expressed in units (U) per mL of culture, where one unit is the amount of enzyme needed to hydrolyze 1 μmol of ONPG to orthonitrophenol per min at 37°C. Western blot analyses were carried out using 20 μL samples of concentrated culture filtrate using ultra-filtration spin columns with a molecular weight cut-off of 10 kDa (“Vivaspin,” Sartorius, Göttingen, Germany) and fractionated by 10% SDS-PAGE. Fractionated proteins were transferred to nitrocellulose membranes and visualized using a rabbit anti-lac A antiserum, an anti-rabbit IgG labelled with horseradish peroxidase (Promega) and a chemiluminescent detection kit (Thermo Fisher Scientific, Waltham, MA, USA). Rabbit serum containing polyclonal antibodies directed against A. niger β-galactosidase was generated as follows. A. niger β-galactosidase was expressed and purified by ion exchange chromatography. Purified β-galactosidase (200 μg with complete Freund’s Adjuvant for immunization) was introduced by subcutaneous injections into New Zealand white rabbits. The initial injection was followed by two booster injections (100 μg purified β-galactosidase with Incomplete Freund’s Adjuvant). For serum preparation, the collected blood was stored at 4°C overnight and centrifuged for 30 min at 4,000 rpm. The collected supernatant was stored at −20°C. Enzyme-linked immunosorbent assay (ELISA) was used for the evaluation of antibody positive titer in the rabbit serum.

3. Results

3.1. Deletion of the A. oryzae Strain S1 pyrG. The 3.6 kb pyrGΔ0 gene replacement cassette was amplified using primers 5’pyr and 3’pyr, and plasmid pGEM-5’3’pyr as the template (Figure 4(a)). Generation of a pyrGΔ0 derivative of A. oryzae was performed by transforming strain S1 with the 3.6 kb pyrGΔ0 cassette. Initial attempts to regenerate transformed spheroplasts were performed on CD plates supplemented with uracil, uridine, and 5-FOA. This strategy, which selects for pyrGΔ0 gene replacements immediately after transformation of spheroplasts with the PCR product, is similar to the approach used to isolate pyrG mutants of Trichoderma reesei (irradiated spores are plated out on minimal medium containing uridine and 5-FOA—Long et al. [5]). However, colonies derived from the experiments described here with pyrG failed to yield FOA resistant colonies. Perhaps 5-FOA-resistant pyrGΔ0 mutants were not obtained because multiple nuclei were present in the spheroplasts, and therefore, a single pyrGΔ0 locus could
not prevent production of the suicide inhibitor FdUMP. To address this possibility, the transformation protocol was modified by allowing nuclei in which the pyrG gene was deleted to segregate from wild-type nuclei by plating the transformed spheroplasts (about $8 \times 10^5$ per plate) onto nonselective regeneration plates containing uracil/uridine. Once a lawn of conidia was produced, the conidia were collected, diluted and plated at $8 \times 10^6$ spores per plate on CD selective medium with 5-FOA, uracil, and uridine. Ten randomly selected colonies were chosen from over 100 colonies that formed and purified to homogeneity by two rounds of plating on CD uracil and uridine plates. Conidia prepared using the purified colonies were harvested and plated onto CD plates without uracil and uridine ($10^5$ conidia per plate). Only one of the putative mutants, designated GR6 (Gene Replacement mutant number 6) did not grow and was therefore assumed to be completely stable (Figure 3).

3.2 Verifying that GR6 Harbours a pyrGΔ0 Locus. PCR amplifications with primers Pyr3'-out and Pyr3'-out using the genomic DNA of strain S1 and strain GR6 as the templates, showed that a 0.9 kb deletion was introduced into the pyrG region of strain GR6 (Figure 4(b)), which is consistent with generation of the desired pyrGΔ0 locus by homologous recombination.

Then, the pyrG auxotroph GR6 was transformed with plasmids pUC_pyrGAo and ANEp2. These plasmids contain the wild-type pyrG from A. oryzae (pUC19) and A. nidulans, respectively. Both plasmids were introduced into and restored uracil and uridine prototrophy of GR6 with frequencies of three transformants per μg of plasmid DNA for pUC_pyrGAo and 50 transformants per μg of plasmid DNA for ANEp2. Furthermore, the frequency ratio of pyrG prototrophs obtained when transformation was performed with the pUC_pyrGAo versus the AMA plasmid was about 0.1. This is in accordance with the range of prototrophy frequency ratios obtained with integrating versus autonomously replicating plasmids previously reported with other fungal species [12, 21].

3.3. Protein Expression. Total secreted proteins in culture filtrates of the untransformed strain GR6 pyrGΔ0 and GR6 pyrGΔ0 transformed with ANEp2 were analyzed by SDS-PAGE (Figure 5(a)) and Western blotting (Figure 5(b)). SDS-PAGE showed a very prominent 110 kDa protein, which is about the size expected of the A. niger β-galactosidase (Swiss-Prot accession number P29853) (UnitProtKB; http://www.uniprot.org/uniprot/P29853) [22] in the culture filtrate of ANEp2 GR6 pyrGΔ0 which was absent in the untransformed strain GR6 pyrGΔ0. Western blot analyses confirmed that the major band was the A. niger β-galactosidase. As expected from the above, culture filtrates of the ANEp2 GR6 pyrGΔ0 transformants possessed high levels of β-galactosidase activity (135 U/mL), as compared to the untransformed strain GR6 pyrGΔ0 which expressed β-galactosidase at 0.80 U/mL.

4. Discussion

Replacement of a gene requires homologous recombination of the introduced DNA with the genome: gene replacement is achieved by a precise double crossover event [23]. We first sought these events by selecting for FOA-resistant clones. FOA inhibits the growth of the fungus but permits multiplication of cells deficient in OMP decarboxylase and because of this, are auxotrophic for uridine. Initially, we selected for pyrGΔ0 gene replacements immediately after transfection using a similar plating procedure as has been used to isolate pyrG mutants of T. reesei, A. aculeatus, and Penicillium nalgiovense from irradiated spores [5, 7, 21]. In our hands, this technique failed to yield FOA resistant colonies.
This was probably because multiple nuclei were present in each spheroplast, and therefore a single pyrGΔ0 locus could not prevent production of the suicide inhibitor FdUMP. To test this, the transformation protocol was modified to allow cells containing putative pyrG deletions to segregate from wild-type ones by plating the transformed spheroplasts onto nonselective regeneration plates containing uracil and uridine. Then, the conidia from the nonselective plates were plated-out on CD selective medium containing 5-FOA, uracil and uridine. This led to isolation of the mutant strain, GR6 pyrGΔ0. PCR based tests confirmed that the pyrG region of strain GR6 was deleted.

It was possible to complement the uridine requirement of GR6 pyrGΔ0 by introducing the plasmids pUC_pyrGAo and ANEp2 carrying A. oryzae or A. nidulans pyrG, respectively. This suggests that the AMA1 region of ANEp2, which supports autonomous plasmid replication in other fungi, also supports autonomous replication in A. oryzae. Furthermore, the transformation frequency of ANEp2 was about 10-fold greater than that obtained with the integrative plasmid pUC_pyrGAo. Transformation frequency ratios obtained with pUC_pyrGAo as compared to plasmids harbouring AMA1 were in accordance with those obtained with integrating versus autonomously replicating plasmids [12]. Also, Fierro et al. [21] transformed P. nalgiovense with integrative versus autoreplicative plasmids and found 60-fold higher frequencies for AMA1-based autonomously replicating plasmids compared with integrative plasmids. Autonomously replicating plasmids carrying the AMA1 region from A. nidulans transformed P. nalgiovense very efficiently and these plasmids also showed mitotic stability with low degrees of reorganization.

β-Galactosidase expression showed that the A. oryzae strain GR6 pyrGΔ0 was able to efficiently produce heterologous secreted proteins using the A. niger transcriptional and translational controls introduced via ANEp2. The strain GR6 pyrGΔ0 transformed with ANEp2 containing lacA encoding A. niger β-galactosidase was more than 150 times higher than that obtained with the untransformed strain. The finding of this study is promising and this paves the way for the application of A. oryzae strain GR6 pyrGΔ0 in heterologous protein production given that this strain is amenable to genetic manipulation and that A. oryzae is widely used in biotechnology and the food industry.

5. Conclusion

Mutant strain GR6, a pyrGΔ0 derivative of the A. oryzae strain S1, was constructed using targeted gene replacement. The pyrG auxotrophs were verified by assessing their ability to grow on selective and nonselective media and PCR analysis. Plasmids pUC_pyrGAo and ANEp2, harbouring the pyrG genes from A. oryzae and A. nidulans, respectively, complemented the uracil and uridine requirement of strain GR6. Transformants of the A. oryzae GR6 mutant carrying the heterologous expression vector ANEp2 were able to functionally express a secreted β-galactosidase at high levels.

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References

[1] T. Kobayashi, K. Abe, K. Asai et al., “Genomics of Aspergillus oryzae,” Bioscience, Biotechnology and Biochemistry, vol. 71, no. 3, pp. 646–670, 2007.

[2] O. P. Ward, W. M. Qin, J. Dhanjoon, J. Ye, and A. Singh, “Physiology and biotechnology of Aspergillus,” Advances in Applied Microbiology, vol. 58, pp. 1–75, 2005.

[3] M. Machida, K. Asai, M. Sano et al., “Genome sequencing and analysis of Aspergillus oryzae,” Nature, vol. 438, no. 7071, pp. 1157–1161, 2005.

[4] J. D. Boeke, F. LaCroute, and G. R. Fink, “A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance,” Molecular and General Genetics, vol. 197, no. 2, pp. 345–346, 1984.

[5] H. Long, T.-H. Wang, and Y.-K. Zhang, “Isolation of Trichoderma reesei pyrG negative Mutant by UV Mutagenesis and Its Application in Transformation,” Chemical Research in Chinese Universities, vol. 24, no. 5, pp. 565–569, 2008.

[6] M. Heerikhuisen, C. Van den Hondel, and P. J. Punt, “Aspergillus sojae,” in Production of Recombinant Proteins—Novel Microbial and Eukaryotic Expression Systems, pp. 191–214, Wiley-VCH, Weinheim, Germany, 2005.

[7] S. Kanamasa, K. Yamaoka, T. Kawaguchi, J.-I. Sumitani, and M. Ara, “Transformation of Aspergillus aculeatus using the drug resistance gene of Aspergillus oryzae and the pyrG gene of Aspergillus nidulans,” Bioscience, Biotechnology and Biochemistry, vol. 67, no. 12, pp. 2661–2663, 2003.

[8] R. V. Miller, E. J. Ford, N. J. Zidack, and D. C. Sands, “A pyrimidine auxotroph of Sclerotinia sclerotiorum for use in biological weed control,” Journal of General Microbiology, vol. 135, pp. 2085–2091, 1989.

[9] W. Van Hartingsveldt, I. E. Mattern, and C. M. J. Van Zeijl, “Development of a homologous transformation system for Aspergillus niger based on the pyrG gene,” Molecular and General Genetics, vol. 206, no. 1, pp. 71–75, 1987.

[10] D. Mueller and U. Stahl, “RNA: a powerful tool for recombination and regulated expression of genes,” in Progress in Botany, pp. 31–45, Springer, Berlin, Germany, 2005.

[11] J. Aranda, M. Poza, B. G. Pardo et al., “A rapid and simple method for constructing stable mutants of Acinetobacter baumannii,” BMC Microbiology, vol. 10, article 279, 2010.

[12] R. Storms, Y. Zheng, H. Li, S. Sillaots, A. Martinez-Perez, and A. Tsang, “Plasmid vectors for protein production, gene expression and molecular manipulations in Aspergillus niger,” Plasmid, vol. 53, no. 3, pp. 191–204, 2005.

[13] S. S. L. Oh, J. M. Leong, A. M. A. Murad, N. M. Mahadi, and F. D. Abu Bakar, “Cloning and analysis of pyrG gene encoding orotidine 5-monophosphate decarboxylase of Aspergillus oryzae strain S1,” Sains Malaysiana, vol. 40, no. 4, pp. 331–337, 2011.

[14] S. Ushijima, T. Nakadai, and K. Uchida, “Interspecific electrofusion of protoplasts between Aspergillus oryzae and Aspergillus sojae,” Agricultural and Biological Chemistry, vol. 55, pp. 129–136, 1991.

[15] Y. Iimura, K. Gomi, H. Uzu, and S. Haru, “Transformation of Aspergillus oryzae through plasmid-mediated complementation of the methionine-auxotrophic mutation,” Agricultural and Biological Chemistry, vol. 51, pp. 323–328, 1987.

[16] J. J. Feng, T. Takahashi, M. Machida, and Y. Koyama, “Identification of a basic helix-loop-helix-type transcription regulator gene in Aspergillus oryzae by systematically deleting large chromosomal segments,” Applied and Environmental Microbiology, vol. 75, no. 18, pp. 5943–5951, 2009.

[17] Y. R. Liew, Protoplasting and cell wall regeneration of the filamentous fungi Aspergillus oryzae strain S1 [B.Sc. thesis], Universiti Kebangsaan Malaysia, Malaysia, 2008.

[18] K. Wernars, T. Goosen, B. M. Wennkees, K. Swart, C.A. Van den Honde, and H.W. Van den Broek, “Cotransformation of Aspergillus nidulans: a tool for replacing fungal genes,” Molecular and General Genetics, vol. 209, no. 1, pp. 71–77, 1987.

[19] E. Käfer, “Meiotic and mitotic recombination in Aspergillus and its chromosomal aberrations,” Advances in Genetics, vol. 19, pp. 33–131, 1977.

[20] “Liquid media, plates and overlayers for A. niger,” 2012, http://clone.concordia.ca/aspergillus/protocols/nigmedia.html.

[21] F. Fierro, F. Laich, R. O. García-Rico, and J. F. Martín, “High efficiency transformation of Penicillium nalgiovense with integrative and autonomously replicating plasmids,” International Journal of Food Microbiology, vol. 90, no. 2, pp. 237–248, 2004.

[22] “UnitProtKB,” 2012, http://www.uniprot.org/uniprot/P29853.

[23] V. L. Prabha and N. S. Punekar, “Genetic transformation in Aspergilli: tools of the trade,” Indian Journal of Biochemistry and Biophysics, vol. 41, no. 5, pp. 205–215, 2004.