A COMPARATIVE STUDY ON THE TRANSFORMATION OF *MONASCUS PURPUREUS* ALBINO'S MUTANT USING TWO DIFFERENT SELECTION MARKERS

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ABSTRACT

A comparative study on the transformation of *Monascus purpureus* albino's mutant was carried out using two different selection markers, phleomycin resistance gene and the nitrate reductase gene, by polyethyleneglycol-mediated protoplast transformation. The first marker is based on the presence of genes conffering resistance to phleomycin (*ble* gene). The other marker, the nitrate reductase gene (*nia*D) from *Aspergillus nidulans*, utilize the conversion of an auxotroph of nitrate reductase from the albino's mutant to prototrophy. The efficiency of transformation using *ble* gene (31-45 transformants/μg plasmid) was higher than the *nia*D gene (2-16 transformants/μg plasmid). Mytotic stability studies demonstrated that all transformants were stabile up to 5 generations. The presence of the *ble* gene and the *nia*D gene in the transformants genome were analysed by PCR. These transformation systems are the first essential step in the cloning of the citrinin (a mycotoxin) biosynthetic genes. They provide the tools to prevent citrinin contamination in the other of *Monascus* metabolites, like azaphilone pigments and monascidin K (an antihypercholesterolemia agent), which used in human food and drugs.

Key words : *Monascus purpureus,* albino's mutant, phleomycin, nitrate reductase, *ble,* *nia*D, transformation.

Introduction

Monascus purpureus is filamentous ascomycetes that form asexual uninucleate spores. They are well known producers of natural pigments which have been widely used for hundreds of years in the Far East as food colorants for rice (angkak), processed meats and wine or in Chinese medicine (Hiroi et al., 1979; Jacobson and Wasileski, 1994). Their relatively high stability with respect to pH and temperature, are interesting features which favour their use as substitutes for synthetic food colorants. Moreover, *M. purpureus* produces statins (such as lovastatin), with 3-hydroxymethylglutaryl-CoA reductase inhibitory properties, used to reduce choresterol levels in blood (Endo, 1985).

As a part of the investigation on this fungal pigments, an antibacterial compound monascidin A, have been isolated from supernatant cultures. But, Blanc et al. (1995) have characterized this antibiotic as citrinin, a theratogenic and nephrotoxic agent. It is essential that the *Monascus* products avoid the occurrence of this mycotoxin.

According to last study, production of citrinin can be avoided by detoxification of the pigments, the use of non producing citrinin strains or fermentative conditions of non production citrinin. But there are no effective control producers for preventing contamination of citrinin, because the three metabolites are produced by *Monascus* through the same polyketides pathways (Blanc et al, 1998). Only little is known about this biochemical pathways in *Monascus*, due to a lack of knowledge of the genes involved. The number of chromosomes, the genome size and the possibility of the transforming this fungi with exogenous DNA are unknown too. This basic information is essential to start any molecular study on the genes involved in pigment, lovastatin or citrinin production by *M. purpureus* (S. Campoy et al., 2003).

Introduction of DNA in different filamentous fungi has been achieved by polyethyleneglycol-mediated protoplast transformation (Cantoral et al., 1987; Punt et al., 1987). Transformants strains can be selected by the use of dominant selectable markers or by the positive screening of auxotrophic mutants for a defined gene. Recently, reports of filamentous fungal transformation using dominant resistance genes, such as hygromycine or phleomycin resistance have appeared (M.J. Daboussi et al., 1989). An alternative approach has been achieved in different filamentous fungi using nitrate reductase deficient mutants (*nia*D) selected through their resistance to chlorate (Maladier et al., 1989).

In this work, we report on a comparative study of the transformation of *M. purpureus* with different exogenous plasmid DNA by protoplast transformation. These vectors are based in presence of genes coffering resistance to phleomycin or based in the complementation of *nia*D mutant. These transformation systems are the first essential step in the cloning of the citrinin biosynthetic genes. Once a gene involved in citrinin biosynthesis is clearly characterized, such gene will be disrupted in order to obtain citrinin negative genetically designed strains. There is an effective control producers for preventing contamination the products of *M. purpureus* with citrinin.

Materials and methods

Strains and culture conditions

M. purpureus ITBCC-HD-F002, a strain non producing pigments (albino) and lovastatin, was obtained by ethyl methanesulphonate (EMS) mutagenesis from the natural isolate, *M. purpureus* ITBCC-HD-F001. This mutant was routinely grown in solid YMP medium (0.3 % yeast extract, 0.3 % malt extract, 0.6 % peptone, 2.0 % glucose and 1.5 % bacto-agar) for 7-10 days at 28°C. Spores from several plates of solid YMP medium were collected using sterile saline. The spores were used to inoculate liquid cultures (in liquid YMP medium).

Plasmids

The plasmids used in this study were :

1. pULJL43 (4.6 kb), an integrative plasmid in *Penicillium chrysogenum* containing the phleomycin resistance gene (*ble*) from *Streptoalloteichus hindustanus* expressed using the *pcb*C promoter and the CYC1 terminator from *Saccharomyces cerevisiae* at the 3' end of the *ble* gene (kindly provided by S. Campoy, Instituto de Biotecnologia de Leon, Spain).
2. pSTA14 (7 kb), an integrative plasmid containing the nitrate reductase gene (*nia*D) from *Aspergillus nidulans* (kindly provided by S.S. Sandhu, Departement of Biological Science, Rani Durgawati University, India).

Determination of phleomycin minimum inhibitory concentration (MIC)

Growth inhibition of *M. purpureus* ITBCC-HD-F002 was tested by plating 10^5 spores on solid YMP medium supplemented with phleomycin in different concentrations, i.e. 0, 0.5, 1.0, 1.5, 2.0, and 2.5 μg/mL The resulting plates were incubated at 28°C for 7-14 days.

Mutagenesis *M. purpureus* ITBCC-HD-F002 with ethyl methanesulphonate (EMS) and chlorate

Liquid YMP medium (50 mL) was inoculated with 10^5 spores/mL of *M. purpureus* ITBCC-HD-F002 and incubated for 62 h at 28°C on rotary shaker at 150 rpm. About 10

mL of cultures was concentrated by centrifugation (10.000 x g, 10 min, at 4°C) and resuspended in 10 mL stabilizing buffer (0.2 M phosphate buffer pH 7.0). One mL of this suspension was incubated in a solution containing 100 µL 2 % glucose, 850 µL stabilizing buffer and 50 µL EMS at 28°C for 90 min on rotary shaker at 150 rpm. The treatment was stopped by the addition of 2 mL 5 % sodium thiosulphate and incubated for 20 min at 28°C on rotary shaker at 150 rpm. The suspension was concentrated by centrifugation (10.000 x g, 10 min, at 4°C) and resuspended in 1 mL stabilizing buffer.

EMS mutants were grown on minimal medium/MM (2.5 % potato dextrose agar, 5% glucose, 0.15 % NaCl, 0.1 % $MgSO_4$ and 0.25 % KH_2PO_4) supplemented by various concentration of $KClO_3$ (0.1, 0.2, 0.3, 0.4 and 0.5 mM) and 5 mM urea. These plates were incubated at 28^{o}C for 7-14 days.

Chlorate-resistant mutants were selected to different classes on the basis of their growth on MM containing one of the four following nitrogen sources : 23 mM $NaNO_3$, 10 mM $NaNO_2$, 5 mM ammonium tartrat and 0,7 mM glutamate). All cultures were maintained at 28°C for 7-14 days. The *nia*D mutants were grown on all nitrogen source, except nitrate.

Protoplast preparation and transformation process

The transformation procedure applied is based on the protocol described by Herzog *et al.* (1996), with several modifications. About 50 mL of liquid YMP medium (pULJL43) was inoculated with 10^5 spores/mL (from *M. purpureus* ITBCC-HD-F002 and for *nia*D mutant, respectively) and incubated for 20 h at 28°C on rotary shaker at 150 rpm. The resulting mycelium was filtered through Whatman No.1 filter paper, washed twice with 0.6 M KCl and resuspended in 20 mL 0.6 M KCl including the lytic enzyme. The following lytic enzymes were tested to obtain protoplasts : (1) lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich) at 5 mg/mL (2) a mixture of lysing enzyme from *T. harzianum* (Sigma-Aldrich) at 5 mg/mL and cellulase (Sigma-Aldrich) at 10 mg/mL (3) a mixture of glucanex (Sigma-Aldrich), cellulase (Sigma-Aldrich), and maserozyme (Sigma-Aldrich) at 10 mg/mL, respectively. The suspension was incubated at 28°C with orbital shaking (100 rpm) and protoplast formation was followed under the microscope for up to 3 h. The protoplasts were filtered through Whatman No.1 filter

paper, concentrated by centrifugation (3,000 g, 20 min), and washed twice with 50 mM $CaCl_2$.

In transformation experiments, 100 µL aliquots of protoplast suspensions (10^7 protoplast/mL) was mixed with 10 µg plasmid DNA followed by the addition of 50 µL PEG solution (60 % PEG 6000, 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$). After 20 min incubation on ice, 1 mL PEG solution was added. Following 15 min further incubation at room temperature, aliquots were poured onto solid YMP medium including 5 µg/mL phleomycin (for pULJL43) or selection medium (MM supplemented with 23 mM $NaNO_3$) (for pSTA14) The resulting plates were incubated at 28°C. The first transformants were visible after 3-7 days. After 7 days (for pULJL43) or 14 days (for pSTA14), the total number of transformants was determined and colonies selected for further studies were transferred to fresh plates. The stability of the mutant was tested in YMP medium including 5 µg/mL phleomycin (for pULJL43) or selection medium (for pSTA14) until fifth generations.

DNA isolation and amplification

DNA was extracted from the transformants by *Wizard Genomic DNA Purification Kit* (Promega). PCR analysis for detection of *ble* gene in putative transformants was performed using primer pair bleF (5'-AAGTTGACCAGTGCCGTTCC-3') and bleR (5-GTCGGTCAGTCCTGCTCCTC-3'), which amplified 373 bp DNA fragment of *ble* gene. PCR amplification included an initial denaturating step of 3 min at 94°C (hot start), followed by 30 cycles of 1 min denaturation (94°C), 1 min annealing (56°C) and 1 min polymerization (72°C).

PCR analysis for detection of *nia*D gene in putative transformants was performed using primer pair niaDF (5'GGAGGGCGAGTGTCAAGT3') and niaDR (5'GCCCCAGTTCCCATTCGTC3'), which amplified 811 bp DNA fragment of *nia*D gene. PCR amplification included an initial denaturating step of 3 min at 94°C, followed by 30 cycles of 1 min denaturation (94°C), 1 min annealing (56°C) and 1.5 min polymerization (72°C).

Results and discussion

The transformation of *M. purpureus* ITBCC-HD-F002 protoplast with pULJL43 plasmid

In order to optimize the formation of protoplasts from *M. purpureus* ITBCC-HD-F002, mycelia treated with lysing enzyme from *T. harzianum* yielded about 2.89 X 10^7 protoplast/mL. With lysing enzyme and cellulase, the protoplasts yield increased to 1.01 X 10^8 protoplasts/mL The best yield (9.8 X 10^8 protoplasts/mL) was reached with glucanex, cellulase and maserozyme. The improved protoplast yield with glucanex and masrozyme suggests that the cell wall structure of *M. purpureus* may have a complex polymer in addition to glucan that is resistant to the *T. harzianum* lytic enzyme.

M. purpureus ITBCC-HD-F002 is very sensitive to phleomycin, being unable to grow in plates supplemented with 1 µg phleomycin/mL. This protoplast are about 2.5 fold more sensitive to phleomycin than the other *M. purpureus* (S. Campoy et al., 2003). This was considered suitable for the selection of resistant colonies in transformation experiments.

Transformation experiments were performed with 10^7 protoplasts and 10 µg of plasmid DNA. The transformation resulted in a good transformation system in which 31-45 transformants /µg plasmid were obtained (Table 1).

Table 1. The Transformation Results of *M. purpureus* ITBCC-HD-F002 Protoplast with pULJL43 Plasmid

No.	Protoplast amount (protoplast/mL)	Plasmid concentrations (µg)	Tansformation frequencies (transformants/µg plasmid)
1	10^7	0	0
2	10^7	10	40
3	10^7	10	31
4	10^7	10	45

The stability of the transformants was tested in solid YMP medium supplemented with 5 µg phleomycin /mL. About 50 transformants pULJL43 of *M. purpureus* ITBCC-HD-F001 were submitted to five rounds of replication and 100 % of them retained the ability to grow in the presence of phleomycin (Figure 1).

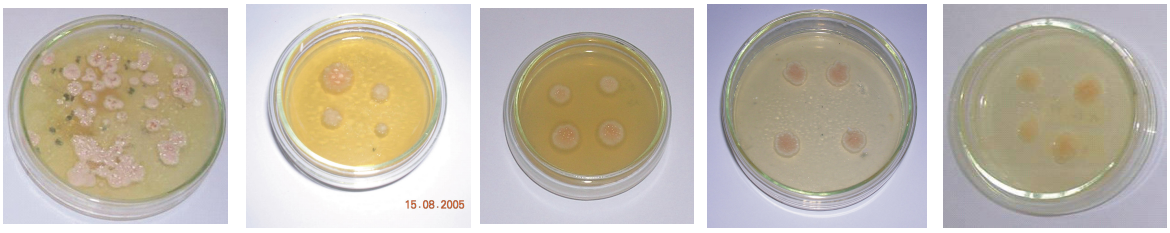


Figure 1. The stability test results of pULJL43 transformants from *M. purpureus* ITBCC-HD-F002 : A. First generations; B. Second generations C. Third generations; D. Fourth generations; E. Fifth generations

PCR analyses targeted at the *ble* gene yielded of the expected size DNA fragment (373 bp) in transformants (Figure 2)

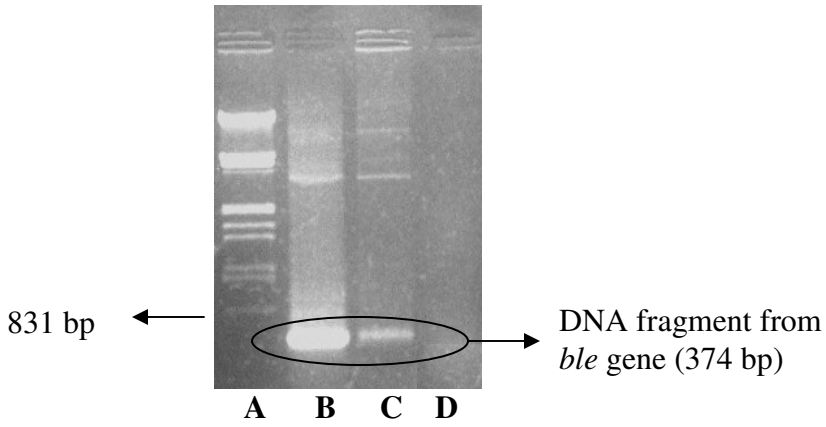


Figure 2. PCR detection of the *ble* gene from pULJL43 transformants *M. purpureus* ITBCC-HD-F002 : A. DNA marker λ/*HindIII*/*EcoR*I; B. DNA of pULJL43 plasmid (positive control); C. DNA of pULJL43 transformants from *M. purpureus* ITBCC-HD-F002; D. DNA of *M. purpureus* ITBCC-HD-F002 (negative control)

The transformation of *nia*D mutant protoplasts from *M. purpureus* ITBCC-HD-F002 with pSTA14 plasmid

Since nitrate reductase deficient mutants can be selected on the basis of their resistance to chlorate, we tested *M. purpureus* for its sensitivity to chlorate. Generally, *nia*D mutants of fungi are selected by spontaneous mutations on appropriate MM supplemented with various concentration of $KClO_3$ and a nitrogen source (Dabousi et al,

1989). But, it has been observed that *nia*D mutants isolated simply by spontaneous mutation on chlorate were not stable (S.S. Sandhu et al, 1991). We developed to isolate stable *nia*D mutants of *M. purpureus* by treating mycelium with EMS and chlorate.

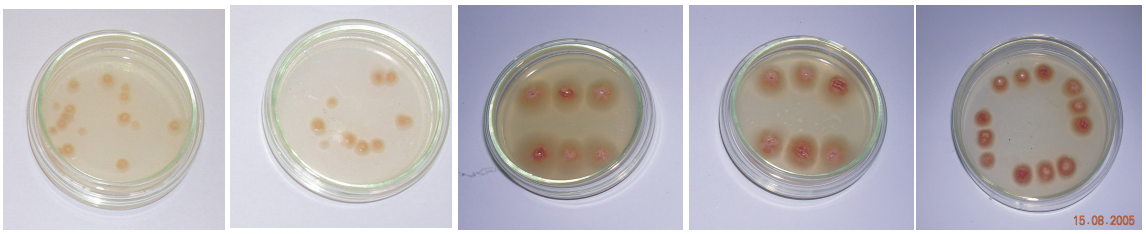
After mutagenesis by EMS (2.5%, 90 min) and 0.4-0.5 mM chlorate, we obtained 11 chlorate resistance mutants. The selected mutants were transferred to MM containing nitrate as the sole nitrogen source. Two were found to be *nii* mutant, while one was *nia*D mutant.

In typical experiment approximately 10^7 protoplast were mixed with 10 µg of plasmids pSTA14. The protoplast were plated on a medium containing nitrate as the sole nitrogen source. The controls, protoplast incubated with aquades, produced no transformant. Growing colonies with aerial mycelium were obtained for 7-14 days after plating : 10 µg of plasmids pSTA14 produced transformation frequencies that ranged from 2-16 transformants/µg DNA (Table 2).

Table 2. The Transformation Results of *nia*D Mutant Protoplast from *M. purpureus* ITBCC-HD-F002 with pSTA14 Plasmid

No.	Protoplast amount (protoplasts/mL)	Plasmid concentrations (µg)	Tansformation frequencies (transformants/µg plasmid)
1	10^7	0	0
2	10^7	10	10
3	10^7	10	16
4	10^7	10	4
5	10^7	10	8
6	10^7	10	4
7	10^7	10	2
8	10^7	10	2

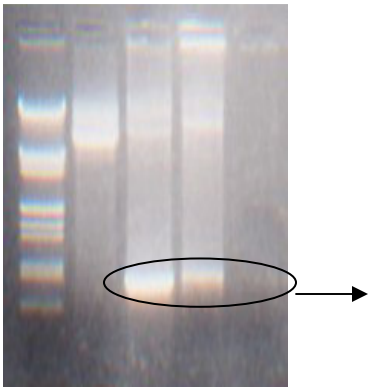
Stable transformants were recovered until fifth generation in selection medium (Figure 3).



A **B** **C** **D** **E**

Figure 3. The stability test results of pSTA14 transformants from *M. purpureus* ITBCC-HD-F002 : A. First generations; B. Second generations;C. Third generations;D. Fourth generations; E. Fifth generations

PCR analyses targeted at the *ni*D gene yielded of the expected size DNA fragment (811 bp) in transformants (Figure 4)



A **B** **C** **D**

Figure 4. PCR detection of the *nia*D gene from pSTA14 transformants from *M. purpureus* ITBCC-HD-F002 : A. DNA marker λ/*HindIII*/*EcoR*I; B. DNA of *nia*D mutant from *M. purpureus* ITBCC-HD-F002 (negative control); C. DNA of pSTA14 plasmid (positive control); D. DNA of pSTA14 transformants from *M. purpureus* ITBCC-HD-F002

A comparative study on the transformation of *M. purpureus* ITBCC-HD-F002 using two different selection markers

Comparison of protoplast transformation using this two different selection markers in *M. purpureus* ITBCC-HD-F002 was made. The efficiency of transformation with genes coffering resistance to phleomycin was higher than the transformation based in the

complementation of *nia*D mutant, which makes the former system ideal to transform *M. purpureus* ITBCC-HD-F002 due its simplicity.

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