AGROBACTERIUM TUMEFACIENS-MEDIATED GENETIC TRANSFORMATION OF *MONASCUS PURPUREUS* ALBINO MUTANT

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ABSTRACT

Monascus purpureus ITBCC-HD-F002, an EMS-induced albino strain, was transformed into hygromycin B resistance using the hygromycin B phosphotransferase (*hph*) of *Escherichia coli* as the selective trait, governed by *gdp* promoter of *Aspergillus nidulans* in pUR5750 plasmid or CaMV35S promoter in pCAMBIA1304 plasmid. These plasmids were transformed into protoplasts and spores from this fungus mediated by *A. tumefaciens* LBA1100 and *A. tumefaciens* AGL1. The transformation efficiency achieved with pUR5750 mediated by *A. tumefaciens* LBA11 was 346 ± 4.00 transformants/10^7 protoplast, with pUR5750 mediated by *A. tumefaciens* AGL1 was 635.7 ± 13.32 transformants/10^7 spores and with pCAMBIA1304 mediated by *A. tumefaciens* AGL1 was 650.3 ± 10.02 transformants/10^7 spores. This result indicating the highly virulent strain *A. tumefaciens* AGL1 was found to be more efficient in DNA transfer than *A. tumefaciens* LBA1100 and the CaMV35S promoter from pCAMBIA1304 worked well in this fungus. The majority of transformants were mitotically stable up to five generations (92-96 %). The presence of the *hph* genes were detected by PCR. In four randomly chosen transformants, single-copy integration of the marker gene at different chromosomal site were proven by Southern Blot analysis. It favoured single-copy integration in genome, which facilitate genomic study using this method and as a tool for insertional mutagenesis for a mycotoxin gene disruption.. The additional marker in pCAMBIA1304 might serve for physiological study on another metabolite-targeting in this fungus, like natural pigments, antihypercholesterolemia, inflammatory and antitumour agents.

Key words : *Monascus purpureus*, genetic transformation system, *hph, Agrobacterium tumefaciens*

Introduction

Monascus purpureus is a red fungus that produces various secondary metabolites, such as pigments, monacolin K, and citrinin. The pigments are traditionally used as food and cosmetic colorants, whereas monacolin K shows an antihypercholesterolemic activity. Citrinin shows an antimicrobial activity against Gram positive bacteria, but it has carcinogenic, teratogenic and nephrotoxic properties (Blanc *et al*., 1998; Lakrod *et al*., 2000). Many researches have been done to eliminate this toxic metabolite from *Monascus* products. Since citrinin precursor is similar to its pigments, their biosynthesis might be manipulated by the genetic engineering method. However, this method requires comprehensive information about genetic transformation in *M. purpureus* (Blanc *et al*., 1998a; Hajjaj *et al*., 1999). The optimal conditions found for efficient transformation of certain *Monascus* could not be applied to another *Monascus* strain (Campoy *et al*., 2003).

This research was aimed to develop an efficient genetic transformation system that can be used to eventually identify genes involved in *M. purpureus* pigments production. To identify these genes, the transformation must be applied in a nonproducing pigment mutant, called albino mutant, as a host cell obtained from *M. purpureus* local strain. The systems employing *hph* gene in pUR5750 and pCAMBIA1304, conferred resistance transformants cell to hygromycin B. These markers were transformed into the host cells by *Agrobacterium tumefaciens* mediated DNA transfer.

The wild-type *M. purpureus* ITBCC-HD-F001 isolated from Cikapundung river-Bandung was firstly confirmed as *M. purpureus* using Random Amplification Polymorphic DNA (RAPD) method. The fungus was subjected to mutation to obtain an albino mutant by using a mutagenic agent ethyl methanesulfonate (EMS). Stability of the mutant was assayed by *rho-petite* method, color consistency and mutant stability tests. Genetic changes of the mutant was analyzed by the RAPD. The characteristics of the albino were identified, including growth curve, biomass production curve and citrinin production curve. The minimum inhibitory concentration of hygromycin B for albino mutant were determined.

The pUR5750 and pCAMBIA1304 transformed into the albino mutant protoplasts and spores mediated by *A. tumefaciens* LBA1100 and *A. tumefaciens* AGL1. The

presence of the *hph* gene in the transformants genome were detected by Polymerase Chain Reactions (PCR) method and Southern Blot analysis. A comparative study was carried out to obtain the most efficient genetic transformation system for *M. purpureus* ITBCC-HD-F001.

Materials and methods

Strains and culture conditions

M. purpureus ITBCC-HD-F001 (School of Pharmacy ITB, Indonesia) was a high pigment producer strain isolated from Cikapundung-river Bandung. As reference strains, *M. purpureus* CECT2955T (Universidad de Valencia, Spain) and *M. ruber* DSM1561 (Pusat Penelitian Bioteknologi LIPI Cibinong, Indonesia) were used. All fungal strains were maintained on YMP agar slant (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. The spores were collected using sterile aquadest and were used to inoculate liquid cultures in YMP broth.

To achieve *Agrobacterium*-mediated DNA transfer experiments, *A. tumefaciens* LBA1100 (Instituto de Biotechnologia de Leon, Spain) and the highly virulent strain *A. tumefaciens* AGL1 (Pusat Penelitian Bioteknologi LIPI Cibinong, Indonesia) were used. *Agrobacterium* strains were grown in LB agar or LB broth (sodium chloride 0.8%, tryptone 1%, yeast extract 0.5%, pH 7.0) supplemented with kanamycin (100 µg/mL).

Plasmids

The plasmids used in this research were :

1. pUR5750 (15 kb), an *E. coli-Agrobacterium* binary vector containing *E. coli hph* gene inserted between the *gdp* promoter and the *trp*C terminator sequence from *A. nidulans* flanked by the left and right borders of T-DNA (kindly provided by S. Campoy, Instituto de Biotechnologia de Leon, Spain).
2. pCAMBIA1304 (12.3 kb), an *E. coli-Agrobacterium* binary vector containing *E. coli hph* gene inserted between the CaMV35S promoter and CaMV35S *polyA signal* flanked by the left and right borders of T-DNA (pCAMBIA Vectors, Australia).

Confirmation of the fungus as M. purpureus by RAPD method

The confirmation procedure applied is based on the protocol described by Campoy *et al.* (2003) for *M. purpureus* IBCC1. Liquid cultures of *M. purpureus* ITBCC-HD-F001 and its reference strains were incubated at 28°C on a rotary shaker at 200 rpm for 20 hours. Mycelial masses were harvested by centrifugation at 12,000 rpm for 10 min. Total DNA from these strains were extracted using *Wizard Genomic DNA Purification Kit* (Promega). DNA concentration was determined by spectrophotometric method. RAPD reactions were performed using 0.5 µL 20 pmol/µL CRL9 (5'-CAGCCGCCCC-3') or 0.5 µL 20 pmol/µL CRL12 (5'-CGCCGCCCG-3'), 3.6 µL 25 mM magnesium chloride, 0.25 µL 20 mM dNTP, 0.2 µL 50 U/µL *Taq* DNA polymerase, 2.5 µL *Taq* polymerase buffer 10 x and aquabidest until 25 µL total volume. The PCR program was as follows : an initial denaturating step of 4 min at 94°C, followed by 44 cycles of 40 s denaturation at 94°C, 1 min annealing at 34°C and 2 min polymerization at 72°C. RAPD products were electrophoretically separated in 1.5 % agarose in TAE 1X.

Mutation of M. purpureus ITBCC-HD-F001 by ethyl methanesulfonate (EMS)

The mutation procedure applied is based on the protocol described by Susilowati *et al.* (1997) for *Saccharomyces cerevisiae.* Liquid cultures of *M. purpureus* ITBCC-HD-F001 were incubated at 28°C on a rotary shaker at 200 rpm for 20 hours. About 10 mL of cultures was concentrated by centrifugation at 12,000 rpm for 10 min at 4°C and resuspended in 10 mL 0.2 M phosphate buffer pH 7.0. One mL of this suspension was incubated in a solution containing 100 µL 2 % glucose and various volume of 0.2 M phosphate buffer pH 7.0 and EMS to get 0, 1.5, 2.0, 2.5, and 3.0 % v/v EMS concentration. The mixtures were incubated at 28°C on a rotary shaker at 150 rpm for 90 min. The treatment was stopped by the addition of 2 mL 5 % sodium thiosulphate and incubated at 28°C on a rotary shaker at 150 rpm for 20 min. The suspensions were concentrated by centrifugation at 12,000 rpm for 10 min at 4°C and resuspended in 1 mL 0.2 M phosphate buffer pH 7.0. The suspension was then transferred to YMP agar and incubated for 7-14 days at 28°C. Red and white colonies were counted to determine mutant viability and mutant efficiency.

Stability of the mutant was assayed by *rho-petite* method, color consistency and mutant stability tests. The white colonies were transferred to YMP-Gli agar (YMP agar with 3 % v/v glycerine) for rho-petite test. The stable white colonies from YMP-Gli agar were transferred to YMP-G agar (YMP agar with 8 % glucose) for color consistency test.

Further, the stable white colonies were subcultured on YMP agar until five generations for mutant stability test.

Genetics analysis of albino mutant by RAPD method

Genetic changes of the albino mutant was analyzed using RAPD by the same procedure with confirmation of the wild-type. The wild-type *M. purpureus* ITBCC-HD-F001 was used as a reference strain.

Characterization of albino mutant

The mutant characterization applied is based on the protocol described by Blanc *et al*. (1995) for *M. purpureus* NRRL1406 and Kareri *et al.* (2004) for *M. purpureus* ITBCC-HD-F001. The characteristics of the albino included growth curve, biomass production curve and citrinin production curve. Series of 500-mL shake flasks with 95 mL YMP broth were inoculated with 5 mL of spore suspension from the albino mutant and its parent strain. The cultures were maintained at 28°C on a rotary shaker at 200 rpm until the death phase was reached. About 10 mL sample of the cultures were picked up every 6 hours. Packed Mycelia Volume (PMV) % v/v of the cultures was obtained by centrifuged the samples at 4,000 rpm for 10 min. From the supernatant, pH was measured by pHmeter. The PMV and pH data's were plotted to a growth curve. The optimum inoculum was determined and growth rate of the cultures was calculated.

Series of 500-mL shake flasks with 95 mL YMP broth were inoculated with 5 mL of the optimum inoculums from the albino mutant and its parent strain. The cultures were maintained at 28°C on a rotary shaker at 200 rpm until the death phase was reached. About 10 mL sample of the cultures was picked up every 24 hours. The PMV and pH data's were plotted to a biomass production curve. The biomass production rate of the cultures was calculated.

The samples from the cultures were extracted by acetonitrile until three times. The filtered extract was twice defatted with isooctane. After adding an equal volume of water and acidification to pH 4.5 with sulphuric acid (50 : 50 v/v), the extract was partitioned with chloroform. The lower phase was evaporated to dryness, it was then dissolved in methanol and analyzed by HPLC. The concentration of citrinin were plotted

to citrinin production curve. The specific citrinin production rate of the cultures was calculated from the rate of increase in concentration of citrinin in logarithmic phase.

Determination of MIC hygromycin B

The albino mutant was grown on YMP agar containing hygromycin B (0, 2, 4, 6, 8 and 10 μg/mL) at 28^0C for 7-14 days. The fungus growth was observed to obtain the culture which was still grown on certain concentration of hygromycin B.

Transformation pUR5750 and pCAMBIA1304 to the albino mutant protoplast and spores by A. tumefaciens mediated DNA transfer

The transformation procedure applied is based on the protocol described by Sun *et al*. (2002), Campoy *et al.* (2003) and Sugui *et al.* (2003). *A. tumefaciens* LBA1100 was grown on 48 hours in LB agar containing 100 μg/mL kanamycin. The spores were collected using sterile aquadest and were used to inoculate 10 mL LB broth containing 100 μg/mL kanamycin. Liquid cultures were incubated at 28°C on a rotary shaker at 200 rpm for 12 hours. A part of the cultures were inoculated into 10 mL induction medium (LB broth supplemented 200 μM acetosyringone) until an optical density at 600 nm of 0.2 was reached. They were incubated on a rotary shaker at 200 rpm for 5-6 hours or until an optical density at 600 nm of 0.8 (approximately 10^8 cells) was reached.

Spore of the albino strain were obtained by growing this fungus strain on YMP agar for 12-14 days at 28°C, followed by collecting spores using sterile aquadest. The total number of spores was determined by hemacytometer. A part of spores suspension was used to preparing the protoplasts as previously described. The total number of protoplasts was determined by hemacytometer.

Co-cultivation between *A. tumefaciens* and albino mutant were performed as follows : 100 μL containing approximately 10^7 spores were mixed with 100 μL of the *A. tumefaciens* culture and plated onto sterile nylon membrane (Roche-Applied Science) or cellulose membrane (Whatman No. 1 filter paper). These membranes were then incubated at 22-25°C or 26-28°C for 72 hours on induction medium (YMP agar containing 200 μM acetosyringone) for induction of the *vir* genes. Following incubation, the membranes were transferred to selection medium for hygromycin B resistant mutant

(YMP agar containing 50 µg/mL higromycin B and 200 µM cefotaxim to inhibit the growth of *A. tumefaciens*). Further, the pUR5750 and pCAMBIA1304 was transformed into the albino mutant spores mediated by *A. tumefaciens* AGL1.

About 50 transformants were tested for stability by replating the transformants 4 times (4 generations). The fifth generation stability was tested by growing this transformant on selection medium (YMP agar containing 200 µg/mL hygromycin B) and non selection medium (YMP agar).

PCR analysis for detection of the *hph* gene in putative transformants was performed using primer pair hphF (5' TTCGATGTAGGAGGGCGTGGAT 3') and hphDR (5' CGCGTCTGCT GCTCCATACAAG 3'), a primer combination obtained by DNAStar program. PCR amplification included an initial denaturating step of 4 min at 95^{o}C, followed by 35 cycles of 45 s denaturation (94^{o}C), 1 min annealing (60^{o}C) and 1.5 min polymerization (72^{o}C).

Southern Blot analysis

Southern Blot analysis was carried out as described by Sambrook *et al*. (1989) with 5 µg DNA in each sample. DNA probes (pCAMBIA1304/*Hind*III) were labeled by random primer method using *DIG High Prime DNA Labeling and Detection Starter Kit I* (Roche Applied-Science), following the instructions of the manufacturer.

Results and Discussion

Confirmation of the fungus as M. purpureus by RAPD

The wild-type *M. purpureus* ITBCC-HD-F001 isolated from Cikapundung river-Bandung was confirmed as *M. purpureus* using Random Amplification Polymorphic DNA (RAPD) method. Identical bands were observed in *M. purpureus* ITBCC-HD-F001 and *M. purpureus* CECT2955T (Figure 1.), but these bands were different from those of *M. ruber* DSM1561. These results indicated that the strain used in this work was *M. purpureus*.

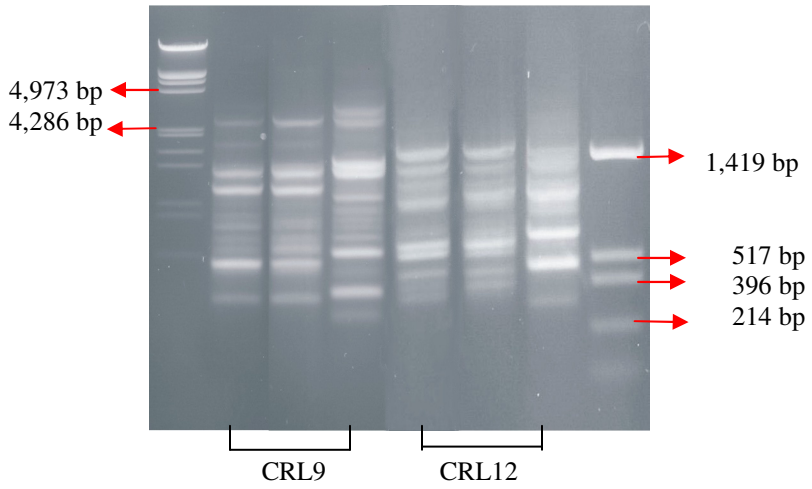

Figure 1. RAPD amplification of *Monascus* spesies DNA using CRL9 and CRL12 (1) DNA marker λ/*Hind*III /*Eco*RI (2, 5) *M. purpureus* ITBCC-HD-F001 (3, 6) *M. purpureus* CECT2955T (4, 7) *M. ruber* DSM1561 (8) DNA marker pUC19/*Hinf*I

Mutation of M. purpureus ITBCC-HD-F001 by EMS

The fungus was subjected to mutation to obtain an albino mutant by using various concentrations of EMS (1.0, 1.5, 2.0, 2.5, and 3.0 % v/v) for 90 minutes. The death curve of *M. purpureus* ITBCC-HD-F001 was presented in Figure 2.

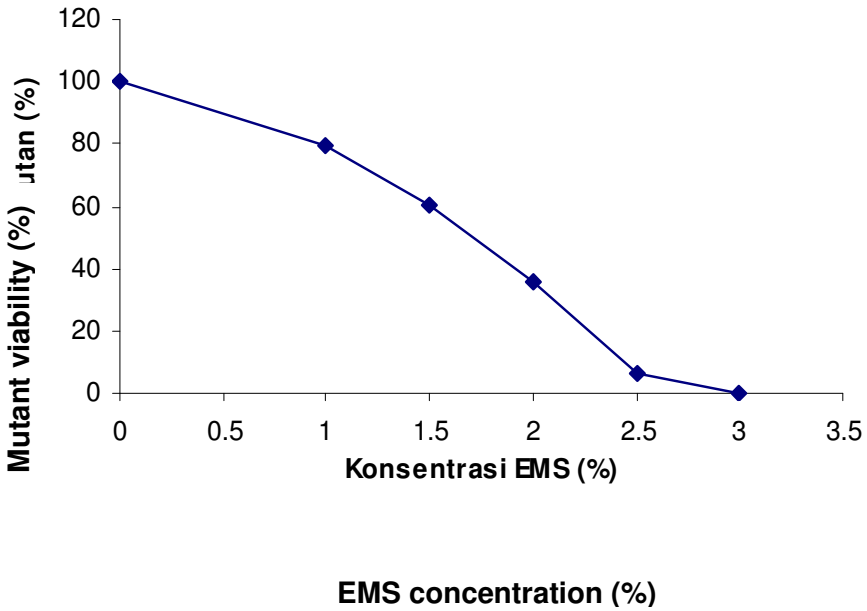

Figure 2. The death curve of *M. purpureus* ITBCC-HD-F001 that mutated by various concentration of EMS for 90 minutes

About 1.93 x 10^3 white colonies were obtained using 2.5 % v/v EMS with 6.01% mutant viability and 1.08 % mutation efficiency. From these results, we know that an albino mutant can be obtained from *M. purpureus* ITBCC-HD-F001 using 2.5 % v/v EMS for 90 minutes.

The stability of the mutant was analyzed by *rho petite* method, color consistency and mutant stability tests. From the results of these tests, we know that the mutant was mutated on choromosomes, consistent in color and stable for five generations. This mutant called *M. purpureus* ITBCC-HD-F002 or albino mutant.

Genetics analysis of albino mutant by RAPD

DNAs from the albino mutant and its parent strains were compared by RAPD (Figure 3.). One of the RAPD's bands of this mutant at 1,150 bp was considered as a genetic change caused by EMS treatment.

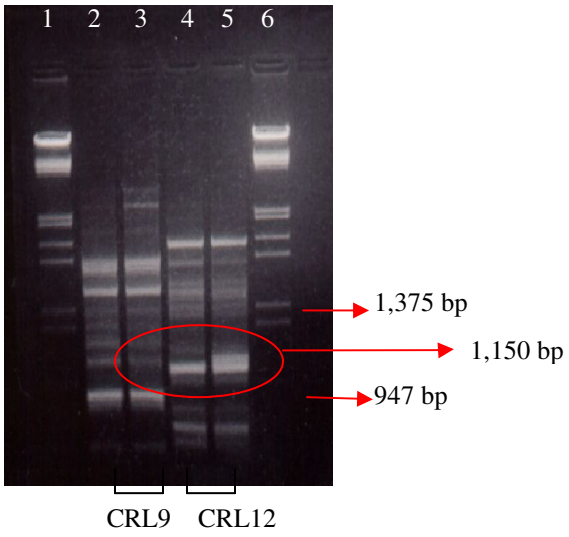

Figure 3. RAPD amplification of *Monascus* spesies DNA using CRL9 and CRL12
(1) DNA marker λ/*Hind*III/*Eco*RI (2, 4) *M. purpureus* ITBCC-HD-F001
(3, 5) *M. purpureus* ITBCC-HD-F002 (6) DNA marker pUC19/*Hinf*I

Characterization of albino mutant

Growth of albino mutant was compared to its parent strain. The growth curve from the wild-type and the albino mutant were presented in Figure 4.

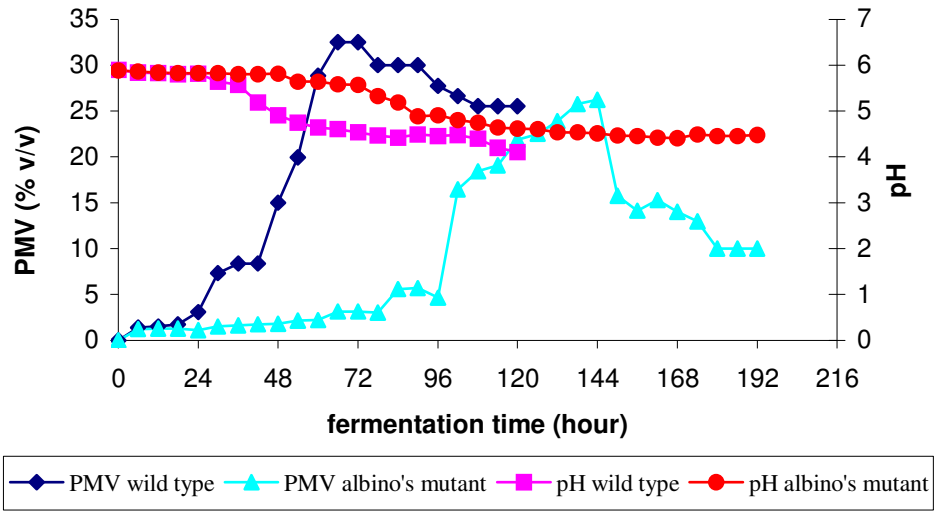

Figure 4. The growth curve from albino mutant and the wild-type

The growth rate of albino mutant (0.182 % v/v per hour) was significantly slower than its parent strain (0.492 % v/v per hour) under the same culture conditions. The peak of this curve shifted from 66 hours (for the wild type) to 144 hours (for the mutant). pH in the broth from the wild-type decreased more rapidly at an early stage of the fermentation. Optimum inoculums that used for fermentation production was removed from 64 hours (for the wild type) to 102 hours (for the mutant).

The biomass production curve from the wild-type and albino mutant were presented in Figure 5.

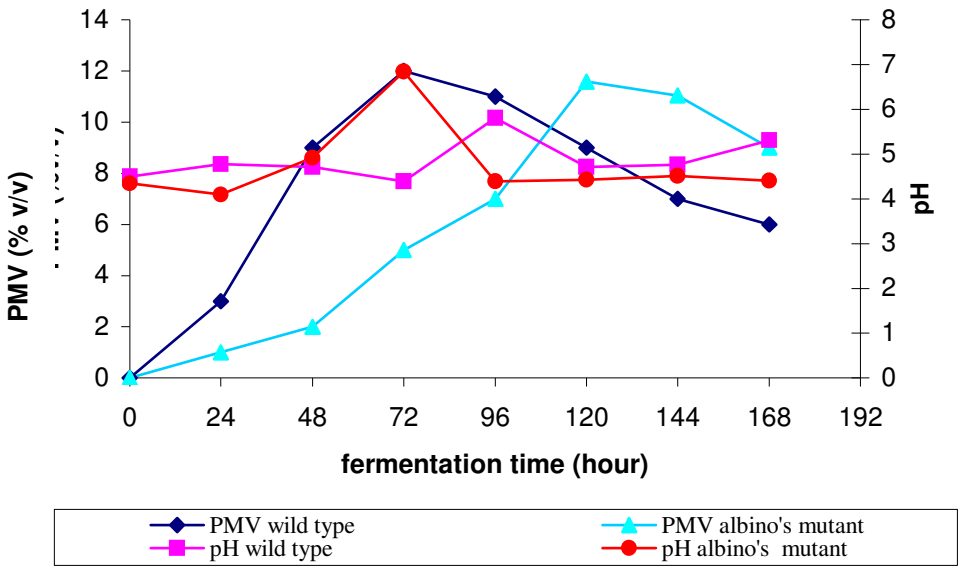

Figure 5. Biomass production curve from the wild-type and the albino mutant

The biomass production rate from the albino mutant (0.097 % v/v per hour) was significantly slower than its parent strain (0.167 % v/v per hour) under the same culture conditions. The maximum biomass production from the mutant (11.58 % v/v) was observed 120 hours after the initiation of the fermentation, whereas the wild type (12 % v/v) was observed at 72 hours. pH from both of the strains fluctuated during the fermentation process.

The citrinin production curve from the wild-type and albino mutant were presented in Figure 6. The citrinin production rate from the albino mutant (0.632 µg/mL per hour) was significantly faster than its parent strain (0.200 µg/mL per hour) under the same culture conditions. The maximum biomass production from the mutant (60.71 µg/mL) and from the wild-type (19.19 µg/mL) was observed 96 hours after the initiation of the fermentation. That is possible because the pigments precursor is similar to citrinin. The mutation of the genes involved in pigments production caused precursor accumulation, which was used to produce citrinin.

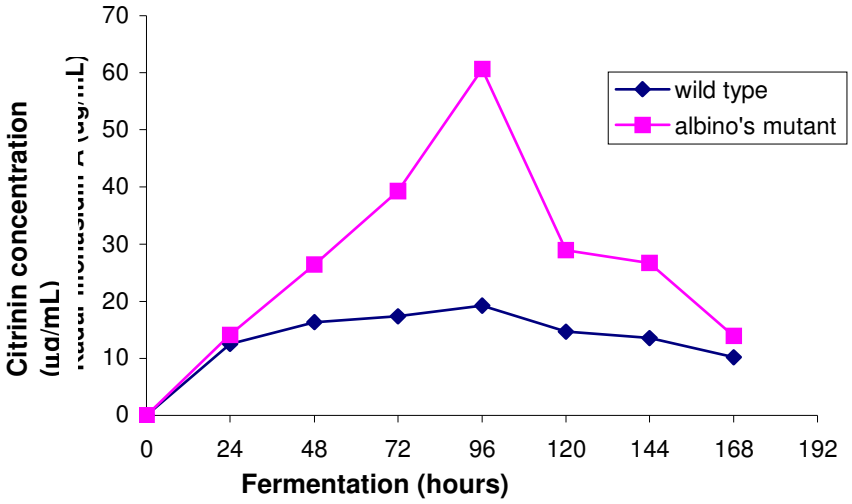

Figure 6. Citrinin production curve from the wild-type and the albino mutant

Determination of MIC hygromycin B

M. purpureus ITBCC-HD-F002 is very sensitive to hygromycin B, being unable to grow in plates supplemented with 4 µg/mL hygromycin B. This strain is more sensitive to these antibiotics than the other *M. purpureus* strain (Campoy *et al*., 2003). This was considered suitable for the selection of resistant colonies in transformation experiments. The transformants were selected for their ability to grown in the presence of 50 µg/mL hygromycin B.

Transformation pUR5750 and pCAMBIA1304 to the albino mutant protoplasts and spores by A. tumefaciens mediated DNA transfer

The pUR5750 was transformed into the albino mutant protoplasts and spores mediated by *A. tumefaciens* LBA1100. Protoplasts and *Agrobacterium* were cocultivated at a ratio of 1:10 (protoplast to bacteria), because this condition resulted in high frequencies of transformants in *Aspergillus fumigatus* (Sugui *et al*., 2005). Two different membranes (cellulose/Whatman No.1 paper filter and nylon membrane) were tested as substrate for cocultivation of *M. purpureus* and *Agrobacterium* cell, because the best results for *A. fumigatus* were obtained with these membranes (Sugui *et al*., 2005). The protoplasts and *Agrobacterium* were cocultivated at 22-25°C or 26-28°C, because these

temperature range applied frequently in several transformation systems for filamentous fungus (Bundock *et al*., 1995; Chen *et al*., 2000; Malonek and Meinhardt, 2001; Sun *et al*., 2002; Sugui *et al*., 2005; Pardo *et al.*, 2005).

The transformation process resulted in 346 ± 400 hygromycin resistant transformants per 10^7 protoplasts. The maximum of transformation efficiency was 350 transformants per 10^7 protoplasts. This transformant only appeared with protoplast as host cell, probably because the bacterial strain used is a slightly virulent bacteria. The transformation results indicate that the nylon membrane is not as efficient as cellulose membrane. The reason for this difference is unclear. It is possible that the chemical properties of this membrane may affect the distributon of protoplasts and *Agrobacterium* cells or inhibit their interaction (Sugui *et al*., 2005). Beside that, the transformants only appear from cocultivation of the cells at 22-25°C. Although 28°C is optimal growth for *A. tumefaciens*, this temperature was not appropriate for T-DNA transfer. It has been proposed that the T-DNA transfer machinery is greatly affected by temperature (Sun *et al*., 2002).

Further, the pUR5750 was transformed into the albino mutant spores mediated by *A. tumefaciens* AGL1. In this experiment the highly virulent strain *A. tumefaciens* AGL1 was found to be more efficient in DNA transfer than *A. tumefaciens* LBA1100. The best yield was 635.7 ± 13.32 hygromycin B resistant transformants per 10^7 spores. The maximum of transformation efficiency was 647 transformants per spores. Additionally, differences in the transformation efficiency depending on the particular promoter expressing the *hph* gene (Campoy *et al*., 2003). To prove this statement, the pCAMBIA1304 was transformed into the albino mutant spores mediated by *A. tumefaciens* AGL1. The best yield was 650.3 ± 10.02 hygromycin B resistant per 10^7 spores. The maximum of transformation efficiency was 660 transformants per 10^7 spores. This result indicating that the CaMV35S promoter functions in this fungus better than *gdp* promoter from pUR5750.

To test the genetic stability of *A. tumefaciens*-mediated transformants, 50 hygromycin B resistent transformant from different transformation systems were allowed to sporulate in selection medium containing 50 µg/mL hygromycin B. After going through four sporulation rounds, 92-96 % of the colonies remainded hygromycin B

resistant. For all the tested transformants from fifth generations, no difference in growth on selection medium (even with 200 µg/mL hygromycin B) and non selection medium was observed. The growth of putative transformants was unaffected, indicating the efficient expression of *hph* gene.

The presence of *hph* gene in the transformants was analysed by PCR and it yielded the expected size DNA fragment (600 bp) appeared in transformants (Figure 7).

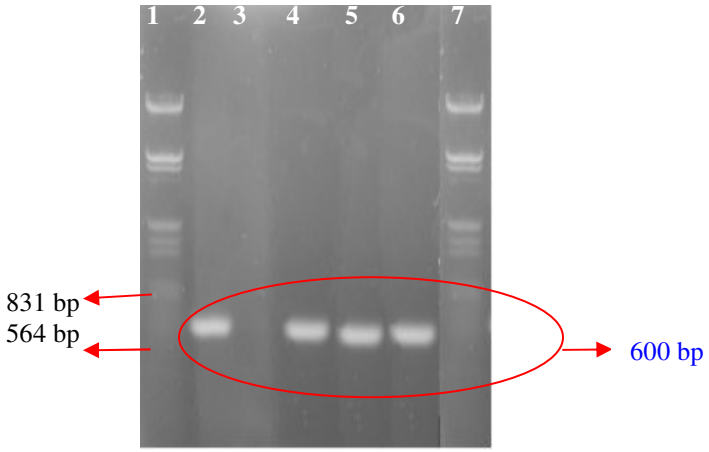

Figure 7. PCR detection of *hph* gene from randomly chosen hygromycin B resistant transformants (1, 7) DNA marker λ/*Hind*III/*Eco*RI (2) pUR5750 as positive control (3) DNA from albino mutant as negative control (4) DNA from hygromycin B resistant transformant (pUR5750/LBA100) (5) DNA from hygromycin B resistant transformant (pUR5750/AGL1) (6) DNA from hygromycin B resistant transformant (pCAMBIA1304/AGL1)

The DNA from these transformants was isolated, digested with *Hind*III and hybridized with a 12.3–kb pCAMBIA1304/HindIII DNA probe containing the *hph* gene. Of the transformants, 100 % showed a single hybridization band.

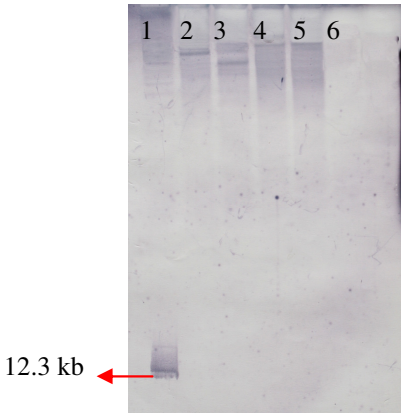

Figure 8. Southern Blot analysis of genomic DNA from randomly chosen hygromycin B resistant transformants. Hybridization was performed using a pCAMBIA1304/*Hind*III as probe (1) pCAMBIA1304/*Hind*III as positive control (2) DNA from hygromycin B resistant transformant/*Hind*III (pUR5750/LBA1100) (3) DNA from hygromycin B resistant transformants/*Hind*III (pUR5750/AGL1) (4-5) DNA from hygromycin B resistant transformant/*Hind*III (pCAMBIA1304 /AGL1) (6) DNA from albino mutant/*Hind*III as a negative control

A comparison of protoplast formation and *Agrobacterium*-mediated DNA transfer in *Monascus* was made. The efficiency of *Agrobacterium*-mediated DNA transformation in *Monascus* was higher than the optimized system for protoplast formation. From three systems which used A*grobacterium* mediated DNA transfer method, pCAMBIA1304/*hph*/AGL1 system resulted in higher transformation efficiency than the other systems. pCAMBIA1304 has seven restriction sites for DNA insertion more than four restriction sites in pUR5750. Beside that, this plasmid has additional selection marker (*mgfp*5:*gus*A:His fusion), which may serve for physiological studies on metabolite targeting in this fungus. These fact make pCAMBIA1304/*hph*/AGL1 system as a former system ideal to transform *Monascus.*

From all genetic transformation system applied in this research it was showed that the most efficient genetic transformation system for M. purpureus ITBCC-HD-F001 is the system employing *hph* gene marker in pCAMBIA1304, transformed into albino mutant by *A. tumefaciens* AGL1.

Conclusions

1. *M. purpureus ITBCC-HD-F002 was an albino mutant that mutated in chromosome, consistant and stable. This mutant has one additional RAPD band that was considered as a genetic change caused by EMS. This mutagenic agent caused decreasing of growth and biomass production rate, but increased citrinin production rate.*
2. *The albino mutant is very sensitive to hygromycin B, whereas a resistance gene from these antibiotics (hph genes) are sufficiently expressed in this mutant for selection of transformants.*
3. *pUR5750 and pCAMBIA1304 can transformed into the albino mutant protoplasts and spores mediated by A. tumefaciens LBA1100 or AGL1. The highly virulent strain of A. tumefaciens was found to be more efficient in DNA transfer than sligthly virulent strain. Additionally, differences in the transformation efficiency depending on the particular promoter expressing the hph gene.*
4. *The most efficient genetic transformation system for M. purpureus ITBCC-HD-F001 is the system employing hph gene marker in pCAMBIA1304, which transformed into albino mutant by A. tumefaciens AGL1.*

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