TRANSFORMATION OF pULJL43 PLASMID INTO *MONASCUS PURPUREUS* ALBINO MUTANT

Marlia Singgih Wibowo[1)], Tutus Gusdinar[1)], Haryanto Dhanutirto[1)], Tiana Milanda[1)] , Dudi Hardianto [2)]

[1)] **School of Pharmacy, Institut Teknologi Bandung, Jalan Ganesa 10 Bandung, Indonesia**

[2)] **BPP-Teknologi, Serpong, Indonesia**

ABSTRACT

Monascus purpureus, a red angkak mould, produces various secondary metabolites, such as pigments, monacolin K, and citrinin. The pigments are used as food and cosmetic colorants, Monacolin K has anti hypercholesterolemia activity , whereas its citrinin, eventhough has antimicrobial activity against gram positive bacteria, unfortunately showed nephrotoxic properties. Many researches have been done in investigating the way to eliminate this toxic metabolite. Since citrinin's precursor is as same as its pigments, their biosynthesis can be manipulated by genetic engineering method, however it needs comprehensive information about genetic transformation because there are still very limited information on *Monascus* transformations.

This research aimed to study the method of inserting pULJL43 plasmid containing *ble* gene into genome of *M. purpureus* KM2 (albino mutant), to obtain stable transformants. This was a preliminary work, as part of our major research for further genetic engineering application to eliminate citrinin.

The wild type of the fungus was firstly mutated to be albino mutant. The research was initiated by the determination of minimum inhibitory concentration (MIC) of phleomycin from the albino's. The plasmid pULJL43 from *E. coli* was isolated, analysed its purity by electrophoresis and the concentration of the plasmid was determined by spectrophotometric method. Then, the protoplasts of albino mutant were produced. The plasmid was transform to this protoplast by protoplast-polyethylene glycol (PEG) method. Plasmid-containing transformants were produced and then grown on phleomycin-containing YMP solid medium. The transformation efficiency (transformant colonies per μg pULJL43 plasmid) was determined, and the transformants stability was observed for 5 generations. The 5th generation stability was observed too in ten times phleomycin MIC concentration in YMP solid medium. Detection insertion gene (*ble* gene) was characterized by PCR. Electrophoresis of PCR product of transformant's DNA and pULJL43 plasmid as positive control, showed no difference.

The research results showed that the transformation of pULJL43 plasmid into protoplast of *Monascus* albino mutant was succeeded and it was proved by PCR. The minimum inhibitory concentration of phleomycin for albino mutant was 1 μg/mL, whereas the transformants were grown on YMP solid medium containing 5 μg/mL phleomycin, and 31 to 45 transformants were yielded per μg plasmid. Stable transformants were proved stable for 5 generations and the 5th generation still grown in YMP solid medium containing 10 μg/mL phleomycin,.

Keywords : *Monascus purpureus*, transformation, pULJL43 plasmid, *ble* gene, protoplast, albino mutant

Introduction

Monascus purpureus, a red angkak mould, produces various secondary metabolites, such as pigments, monacolin K, and citrinin. The pigments are traditionally used as food and cosmetic colorants, the Monacolin K has anti hypercholesterolemia activity , whereas its citrinin, eventhough has antimicrobial activity against gram positive bacteria, unfortunately showed nephrotoxic properties (1,2). Many researches have been done in investigating the way to eliminate this toxic metabolite. Since citrinin's precursor is as same as its pigments, their biosynthesis can be manipulated by genetic engineering method (3,4), however it needs comprehensive information about genetic transformation because there are still very limited information on *Monascus* transformations. Data on transformations are essential to start any molecular study on the genes involved in pigment and other metabolites production by *Monascus*.

One of many vectors used in genetic transformations is bacterial or fungal plasmid. In this research work, plasmid pULJL43 is used to study the transformation of *M. purpureus*. The plasmid is integrative plasmid from *Penicillium chrysogenum* which contains resistant gene marker for antibiotic pleomycin (*ble* gene). The size of this plasmid is 4.6 kb and categorized as R plasmid (3)

To study the transformation, the wild type of *M. purpureus* should be mutated into albino mutant, and further the protoplast of this mutant was prepared to transform the plasmid.

This research aimed to study the method of inserting pULJL43 plasmid containing *ble* gene into protoplast of *M. purpureus* KM2 (albino mutant), to obtain stable transformants. This was a preliminary work, as part of our major research for further genetic engineering application to eliminate citrinin.

Materials and Method

Strain of Microorganism and Plasmid

M. purpureus KM2, from the collection of Bioprocess Laboratory, School of Pharmacy ITB, Indonesia, an albino strain of *M. purpureus* which was obtained by mutagenesis using chemical mutagen, EMS (Ethyl Methanesulphonate) 2.5% v/v for 90 minutes.

Plasmid pULJL43 is an integrative plasmid from *Penicillium chrysogenum* which contains phleomycin resistant gene (*ble*) from *Streptoalloteichus hindustanus* expressed using the pcbC promoter and CYC1 terminator from *Saccharomyces cerevisiae* at the 3' end from *ble* gene. The size of this plasmid is 4.6 kb and categorized as R plasmid (3)

Material

The media used in this study were : YMP medium (yeast extract 0.3%, malt extract 0.3%, pepton 0.6%, glucose 2.0%, agar 2.0%), LB medium (trypton 1%, sodium chloride 1%, yeast extract 0.5% agar 2.0%),

Sodium chloride (Merck), tryptone (Difco), phleomycin (Sigma), glucanex (Sigma), cellulase (Sigma), maserozyme (Sigma), tris chloride (Merck), calcium chloride (Merck), poliethilenglycol 6000 (Merck), glucose (Merck), Whatman filter paper No.1, agarose (Promega), ethidium bromide, magnesium chloride, *Taq* DNA polymerase, deoksinucleotide triphosphate (dNTP), marker *Hind*III/*Eco*RI, primer *foward* 5' AAGTTGACCAGTGCCGTTCC 3', primer *reverse* 5' GTCGGTCAGTCCTGCTCCTC 3', *QIAprep Spin Miniprep Kit,* TAE buffer 50X, loading buffer and etanol 70%.

Determination of MIC of phleomycin

M. purpureus KM2 grown on YMP solid medium at 28^0C for 7days. The mycelia were harvested and grounded in sterile water until homogeneous, and the transmitant was measured to get T = 25% at λ = 660 nm (2,25 X 10^4 CFU). The culture was then transffered to YMP solid medium containing phleomycin with various concentrations from 0; 0.5; ; 1.0; 1.5; 2.0; 2.5 μg/mL. The fungus growth was observed to obtain the culture which was still grown on concentration of phleomycin introduced. The observation was until 10 days.

Isolation of the plasmid

The research was initiated by isolating the plasmid pULJL43 from *E. coli* using *QIAprep Spin Miniprep* Kit. The plasmid was analysed its purity by electrophoresis and the concentration of the plasmid was determined by spectrophotometric method (3).

Protoplast preparation

The protoplast was prepared in 50 mL of YMP liquid medium, incubated for 20 hours at room temperature, shaked 200 rpm on orbital shaker. The mycelia were harvested using Whatman filter paper No. 1 and washed with sterile water. The mycelia were suspended in 20 mL of lytic enzyme mixture (glucanex, cellulase, and maserozyme 10 mg/mL each). The suspensión was incubated for 3 hours at room temperature with shaked 100 rpm on orbital shaker. The protoplast suspension was obtained and filtered through a sterile Whatman filter paper No. 1 and the filtrate was centrifuged at 3.000 rpm for 20 minutes. The pellets were washed with 50 mM of calcium chloride. The amount of protoplasts were determined using haemocytometer.

Transformation by protoplast-PEG method

Into 9,8 x 10^7 of protoplasts of *M. purpureus* KM2 in 100 μl of 50 mM calcium chloride, 10 μg pULJL43 plasmid and 50 μl of polyethylenglycol solution (PEG 6000 60%; tris chloride buffer 10 mM pH 7.5; calcium chloride 50 mM) were added, and the suspension was soaked in ice cubes for 20 minutes. One ml of PEG solution was added and the suspension was incubated at room temperature for 15 minutes.

The transformants were then plated on YMP solid medium containing 5 μg/mL phleomycin and incubated at room temperature. The experiment was observed within 10 days, and the amount of transformants grown were counted started on day 5. As negative control, sterile water was used instead of the plasmid. The transformants were then tested for stability by replating the transformants 5 times (5 generation). The 5^{th} generation stability was tested too by growing this transformant on solid medium containing 2.5, 5, 7.5 and 10 μg/mL phleomycin

Detection insertion gene by PCR

The transformation result was analysed by PCR method (9). The primers used were 20 bp each (primer *foward* 5' AAGTTGACCAGTGCCGTTCC 3', primer *reverse* 5' GTCGGTCAGTCCTGCTCCTC 3') and obtained by DNAStar program. The PCR was performed using 15,8 μl of aquabidest, 2,5 μl of *Taq* buffer solution, 0,5 μl of dNTP, 0,5 μl of primer forward, 0,5 μl of primer reverse, 0,2 μl of *Taq* DNA polymerase and 5 μl DNA from the transformants.

As positive control, 10 μl of plasmid pULJL43 was used, and the DNA from *M. purpureus* KM2 was used as negative control. PrePCR was done at 94°C for 3 minutes. PCR was performed for 30 cycles of 94°C (1 min) for denaturation, 56°C (1 min) for

hybridization, and 72°C (1 min) for polymerization. PostPCR was 72°C for 10 minutes. The PCR product was detected using electrophoresis with ethidium bromide and uv detector.

Results and Discussion

Introduction of DNA into filamentous fungi using protoplast transformation has been reported by several researchers (3,5,8,9). In this work, before transformation was carried out, *M. purpureus* was mutated from high-producing pigments strain become albino (non-producing pigments) strain. The strain was named *Monascus purpureus* KM2. This mutant was very sensitive to phleomycin. The mutant proved was being unable to grow in agar plates supplemented by phleomycin 1,0 µg/ml. After the mutation, the protoplast was made because the cells have to be converted from non-competent cells to become a competent cells.

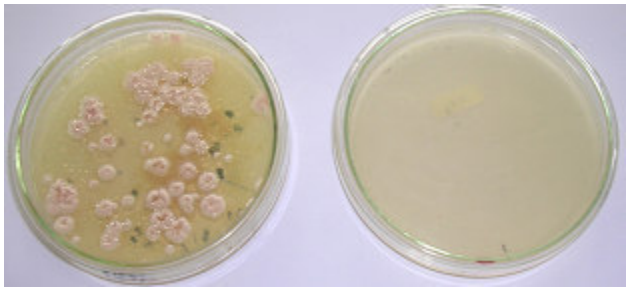
pULJL43 plasmid is a well-known plasmid from *P.chrysogenum* and it contains the phleomycin resistence gene (*ble*) as a marker for selecting the transformants.

The concentration of plasmid isolated from *E.coli* was 17.5 µg per 50 µL (0.350 µg/µL). This amount was used for transformation process which is minimum 10 µg of plasmid required for good transformation process(5).

For protoplast production, three enzymes were used : glucanex, cellulose and maserozyme. The protoplast obtained were 9.8 x 10^8 protoplasts/ml. This numbers were sufficient for transformation process. According to Woloshuk (9) this minimum amount of protoplast was 10^7 protoplasts/ml.

Since the KM2 is very sensitive to phleomycin and unable to grow on medium with 1 µg/mL phleomycin, the transformants will be very easy to be selected if the growth media supplemented by phleomycin. The transformants selected in this work were able to grow in YMP medium containing 5 µg/ml phleomycin.

Figure 1. showed the transformants obtained after 10 days of incubation.



(A) **(B)**

Figure 1. Transformant from *M. purpureus* KM2 in YMP solid medium containing phleomycin 5 µg/mL after 10 days of incubation (A) with plasmid pULJL43 and (B) without plasmid (negative control).

Number of transformants obtained was 31 to 45 transformants per µg pULJL43 plasmid, they were considered stable, the diameter of colonies were between 1,00 to 1,40 cm. It was considered as good transformation result, compared to Campoy, *et.al.*,2003 who resulted 40-50 transformans using similar method (3).

The transformation efficiency (transformants colonies per μg pULJL43 plasmid) was determined and these plasmid-containing transformants produced were also tested for their stability by replicating on phleomycin-containing YMP solid medium. The transformants stability was observed for 5 generations and the 5th generation was still grow on 10 μg/mL phleomycin.

Insertion of ble gene into transformant genome was proved by PCR. As shown in Figure 2, the electrophoregram of transformants and pULJL43 plasmid as positive control, showed a identical bands on 374 bp, while the negative control *M. purpureus* KM2 showed no band at all.

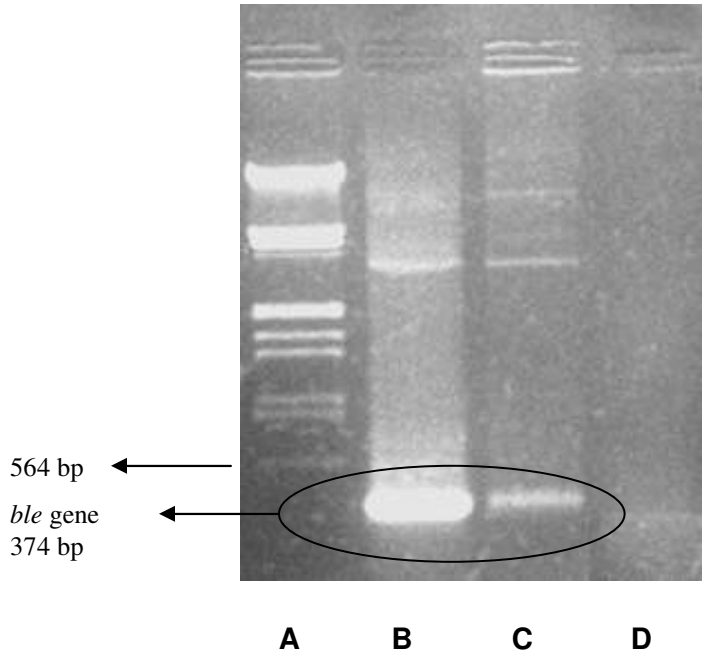


Figure 2. Electrophoregram of PCR product (A) Marker DNAλ *Eco*RI-*Hind*III, (B) plasmid pULJL43 (control positive), (C) transformant, and (D) DNA from *M. purpureus* KM2 (control negative)

The PCR result showed that transformation was succeed. The similar bands were shown on lane B (plasmid) and C (transformants) whereas no band appeared on lane D (negative control). These bands indicating that pULJL43 was inserted in *Monascus* genome.

Conclusion

The transformation of pULJL43 plasmid into protoplast of *M. purpureus* KM2 was succeeded and insertion of *ble* gene to this fungus geneome was confirmed by PCR. The minimum inhibitory concentration of phleomycin for albino mutant was 1 µg/ml whereas the transformants were grown on YMP medium containing 5 µg/ml phleomycin, and 31 to 45 transformants were yielded per µg plasmid. Stable transformants were proved stable for 5 generations and the 5^{th} generation was still grow on 10 µg/mL phleomycin.

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