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Mutation and Characterization of an Albino Mutant of *Monascus* sp. Isolated from the Cikapundung River, Bandung

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Monascus **sp. isolated from Cikapundung River, Bandung was mutated using ethyl methanesulfonate (2.5%, 90 min). Previously, this wild type was identified as *Monascus purpureus* ITBCC-HD-F001 employing random amplification polymorphic DNA (RAPD). Stability of the mutant was observed using color consistency and mutant stability (sub-culturing for five generations) tests. Genetic variation of the mutant (*M. purpureus* ITBCC-HD-F002) was confirmed by RAPD. One of the DNA bands of 1150 bp was found in the albino mutant but not in the wild type, so it was considered as a genetic variation resulting from the mutation process. The albino mutant was characterized by comparing the growth curve, biomass production curve, and the monascidin A production curve of both strains i.e. wild type and the albino mutant. Monascidin A production of the mutant was higher than that of the wild type.**

Key words: *Monascus* sp., ethyl methanesulfonate, random amplification polymorphic DNA, albino mutant, monascidin

Monascus purpureus is a rice fermentation fungus used to produce 'angkak'. This fermentation product has been used for a long time as a food colorant, a meat preservative, and a traditional medicine, especially by the people of South China, Japan, and South East Asia. Various secondary metabolites have been isolated from 'angkak', such as pigments, an antihypercholesterolemic agent (monacolin K), and an antibacterial substance (monascidin A) (Blanc *et al*. 1998a; Lakrod *et al*. 2000). Blanc *et al*. (1995) reported that monascidin A was citrinin, a mycotoxin that has carcinogenic, teratogenic, and nephrotoxic properties. The presence of monascidin A in products fermented by *Monascus* raises concerns about the safety of the products.

To obtain fermented products free from monascidin A, Blanc *et al*. (1998b) tested three procedures. Firstly, they conducted a selection among *Monascus* strains to find one that did not produce monascidin A, secondly, they modified fermentation conditions, and thirdly, they degraded monascidin A found in fermentation products. These methods could suppress or eliminate monascidin A, but unfortunately the process also decreased the production of pigments and monacolin K significantly. This could happen because these three secondary metabolites were all synthesized via the polyketide biosynthesis pathway catalyzed by polyketide synthase (PKS). Characterization of enzymatic reactions at the branch point of the three-metabolite biosynthesis pathways is needed to properly develop a strategy to produce pigments and monacolin K free from monascidin A. (Hajjaj *et al*. 1999).

To characterize those enzymatic reactions, a study of PKS enzymes and genes at the molecular level is needed. This kind of study requires a transformation system for *M. purpureus* that uses an albino mutant deficient in monacolin K or citrinin (monascidin A) synthesis as recipients. The use of albino mutant has a higher priority because clone selection for the recipient of pigment biosynthesis gene can be conducted from the colonies that are able to reverse the non-pigment producing capability. (Blanc *et al*. 1998b). This albino mutant is still not available, especially one that is obtained from local Indonesian isolates.

In this research, we carried out the mutation of *Monascus* sp. from local isolates and characterized a resultants albino mutant. These are the initial steps to develop an efficient transformation system for *M. purpureus*.

MATERIALS AND METHODS

***Monascus* sp. Strains.** As the parental strain we used *Monascus* sp. isolated from the Cikapundung River, Bandung. Two other fungal strains were used as standards, i.e. *Monascus purpureus* CECT2955T (Universidad de Valencia, Spanyol) and *Monascus ruber* DSM1561 (Biotechnology Research Center, LIPI, Cibinong). These three fungal strains were grown on yeast extract-malt extract-peptone (YMP) agar (0.3% [w/v] yeast extract, 0.3% [w/v] malt extract, 0.6% [w/v] peptone, 2% [w/v] glucose, 2% [w/v] agar) for 7-10 days at 28 ^oC. The spore suspension was obtained from *Monascus* sp. solid culture on YMP agar, and then the spore concentration was adjusted to: % transmittance (T) = 25% at wavelength (λ) = 660 nm which was equal to 2.25×10^4 spores ml^{-1}.

Identification of *Monascus* sp. Employing RAPD. Identification of *Monascus* sp. was carried out employing RAPD according to the procedure by Campoy *et al*. (2003), and replicated three times. Five milliliters aliquots of each strain *Monascus* sp., *M. purpureus* CECT 2955T and *M. ruber* DSM1561 was inoculated into 45 ml YMP broth medium, and then shaken at 150 rpm at 28 ^oC for 18 h. Fungal mycelium was harvested by centrifugation at 107,141x g at room temperature for three min. After that, fungal DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega). From each strain, 200 ng of DNA was amplified in

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a solution containing 0.5 unit Taq DNA polymerase (Promega), 90 nmoles magnesium chloride (Promega), 8 pmoles primer CRL9 (5'-CAGCCGCCCC-3') or CRL12 (5'-CGCCGCCCG-3') (Proligo-Sigma), 5 nmoles dNTP (Promega) in a 2700 thermocycler (Applied-Bioscience). The PCR program consisted of initial denaturation (4 min, 94 ^oC), followed by 44 cycles each of which consisted of denaturation (40 sec, 94 ^oC), hybridization (60 sec, 34 ^oC), and elongation (120 sec, 72 ^oC), and then terminated by final elongation for 10 min at 72 ^oC. Electrophoresis was conducted on 1% (w/v) agarose gel using IX TAE buffer at 70-80 volt for 90 min.

Mutation of Parental Strain *M. purpureus*. Parental strain *M. purpureus* ITBCC-HD-F001 was mutated using ethyl methanesulfonate (EMS) according to the procedure of Susilowati (1997). A 5 ml aliquot of parental strain suspension was inoculated into 45 ml YMP broth medium, and then shaken at 150 rpm at 28 ^oC for 64 h. Ten milliliters of this liquid culture was then centrifuged at 5357 x g at 4 ^oC for 10 min, and then the mycelium sediment was suspended in 10 ml of 200 mM phosphate buffer pH 7.0. Portions of 1.0 ml mycelium suspension and 100 μl of 2% (w/v) glucose were placed in 25 ml -Erlenmeyer flasks, and then sufficient quantity of EMS and 200 mM phosphate buffer pH 7.0 were added each flask to obtain a variation of EMS concentrations of 0, 1, 2, 3, 4% (v/v). All flasks were shaken at 150 rpm at 28 ^oC for 45 and 60 min. After that, 2 ml of 5% sodium thiosulfate was added. After shaking at 150 rpm at 28 ^oC for 20 min, the fungal suspension was centrifuged at 5357 x g at 4 ^oC for 10 min. The sediment was resuspended in 1 ml of 200 M phosphate buffer pH 7.0 and this suspension was then gradually diluted. A 100 μl aliquot of each dilution was inoculated on YMP agar and incubated for 4-7 days at 28 ^oC. The number of colonies (both red and white) was observed, the percentage of mutant viability and mutation efficiency were determined, and then the death curve was constructed. The mutation process was repeated with EMS concentrations of 0, 1.0, 1.5, 2.0, 2.5, 3.0% (v/v) with 90 min incubation periods.

White colonies from the mutation process were grown again on YMP-G agar (YMP agar with 8% [w/v] glucose) for seven days at 28 ^oC for the color consistency test. The colonies that remained white in color were sub-cultured for five generations on YMP agar for mutant stability testing.

Genetic Variations of *M. purpureus* Albino Mutant. Genetic variation of *M. purpureus* albino mutant was detected employing RAPD with three replications using the same procedure previously used in the identification of the parental strain. *M. purpureus* parental strain was used as the standard.

The Growth of *M. purpureus* Parental Strain and Its Albino Mutant. Five milliliters suspensions of parental strain and albino mutant was each inoculated into 45 ml YMP broth, and then shaken at 150 rpm at 28 ^oC. Culture samples from both strains were taken every six hours until the stationary growth phase was reached. All culture samples were centrifuged at 3571 x g for 10 min to obtain a packed mycelial volume (PMV) (% w/v) and pH of the supernatant of the culture samples. Both data were plotted versus fermentation time (h) on a growth curve.

A 5 ml aliquot of both cultures at optimum age was used as inocula for the production process in 45 ml YMP broth medium. The culture was then shaken at 150 rpm at 28 ^oC. Samples were taken every 24 h, after which all samples were centrifuged at 3571 x g for 10 min. The data of PMV (% w/v) and pH of the supernatant were plotted versus fermentation time (h) on a biomass production curve.

The extraction, identification, and determination of monascidin A content in fermentation samples produced by *M. purpureus* parental strain and albino mutant were conducted employing the procedure of Blanc *et al*. (1995). Ten milliliters aliquots from each strain were extracted three times using methanol. The resultant extraction was filtered, washed two times with isooctane, and then an equal volume of water was added. The extract was acidified using sulfuric acid to give pH of 4.5, then partitioned using methylene chloride. The bottom phase was dried, and then dissolved in methanol. A citrinin standard calibration curve was made at concentration levels of 0, 5, 10, 15, 20, 25, 30, 35, 40 μg/ml using commercial product (Sigma-Aldrich). Identification and determination of monascidin A content were carried out using HPLC (Hewlett-Packard) with a 214 nm UV detector. The mobile phase was methanol:water (1:1), with a 1.5 ml min^{-1} flow rate in a C-18 Hypersil column at 40 ^oC column temperature.

RESULTS

Identification of *Monascus* sp. Identification of *Monascus* sp. employing RAPD using both primers CRL9 and CRL12 resulted in an RAPD band pattern that was identical to that of *M. purpureus* CECT2955T. This RAPD band pattern was different from the band pattern of *M. ruber* DSM1561. Therefore, it was concluded that *Monascus* sp. from Cikapundung River was *M. purpureus*. This was later named *M. purpureus* ITBCC-HD-F001 (Figure 1).

Mutant *M. purpureus* ITBCC-HD-F001. Mutation of *M. purpureus* ITBCC-HD-F001 using 3 and 4% (v/v) EMS with a 60-min incubation time resulted in less than 10% mutant viability and unfortunately under those conditions white colonies could not be obtained from the albino mutant. Later, mutation was conducted using a lower EMS concentration and longer incubation time, i.e. EMS concentrations of 1.0, 1.5, 2.0, 2.5, 3.0% (v/v) for 90 min. Less than 10% mutant viability was obtained at an EMS concentrations of 2.5 and 3.0% (v/v), but only the 2.5% (v/v) EMS concentration could produce white colonies of 1.93×10^3 colonies/ml, with 6.01% mutant viability and 1.08% mutation efficiency. Color consistency and mutant stability tests showed that albino mutant colonies remained white on high-glucose medium (YMP-G) and YMP solid medium over five generations. This was consistent and the stable albino mutant was later named *M. purpureus* ITBCC-HD-F002.

Genetic Variation of *M. purpureus* ITBCC-HD-F002. The determination of genetic variation of *M. purpureus* ITBCC-HD-F002 using RAPD produced almost identical banding pattern compared to that of its parental strain, except for one additional band at 1150 bp produced by primer CRL12. This band is the result from genetic change caused by EMS mutation in the albino mutant's DNA (Figure 2).

The Growth of *M. purpureus* ITBCC-HD-F001 and *M. purpureus* ITBCC-HD-F002. The two growth curves

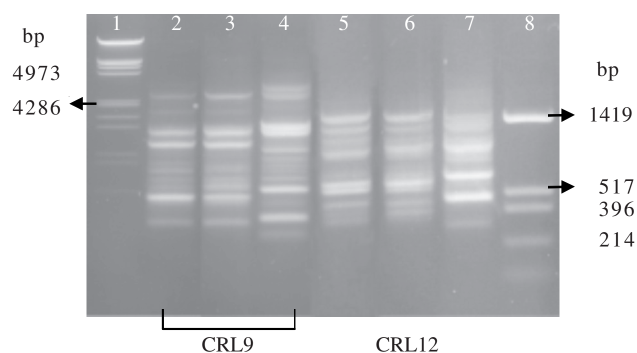

Figure 1 RAPD amplification results of *Monascus* sp. DNA using CRL9 and CRL12 as primers. 1: λ/*Hind*III/*Eco*RI DNA marker, 2 and 5: *M. purpureus* ITBCC-HD-F001, 3 and 6: *M. purpureus* CECT2955T, 4 and 7: *M. ruber* DSM1561, 8: pUC19/*Hinf*I DNA marker.

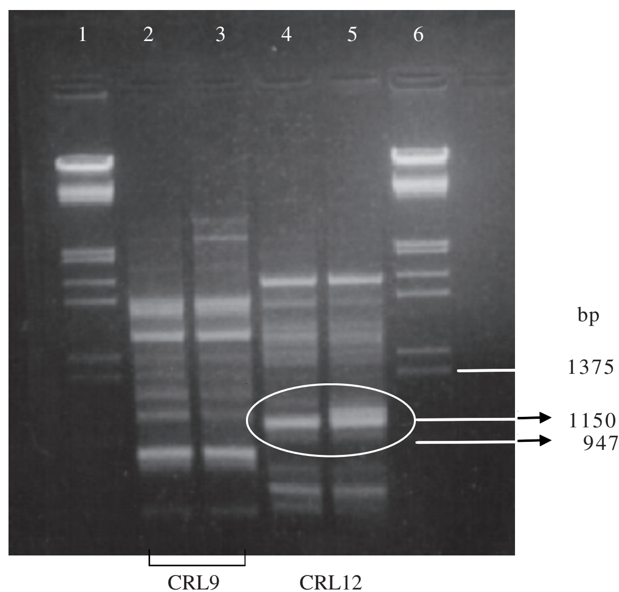

Figure 2 RAPD amplification results of the DNA of *M. purpureus* parental strain and albino mutant. 1 and 6: λ/*Hind*III/*Eco*RI DNA marker, 2 and 4: parental strain (*M. purpureus* ITBCC-HD- F001), 3 and 5: albino mutant (*M.purpureus* ITBCC-HD- F002).

(Figure 3) showed that the growth rate of albino mutant (*M. purpureus* ITBCC-HD-F002) was slower than that of its parental strain (*M. purpureus* ITBCC-HD-F001). The growth curve peak shifted from 66th h in parental strain to 144th h in the albino mutant. The decrease of pH was faster in the parental strain fermentation compared to that of its mutant. The deceleration of growth rate also caused a change in the optimum age of inocula prepared for fermentation, from 64th h in the parental strain to 102nd h in the albino mutant.

The two biomass production curves (Figure 4) showed that biomass production rate for albino mutant was slower than that of its parental strain, therefore production curve peak shifted from 72nd h with 12% (w/v) PMV (parental strain) to 120th h with 11.58% (w/v) PMV (albino mutant). It was also shown that medium pH fluctuated throughout both strains' fermentation processes.

Monascidin A. Identification employing HPLC revealed that both citrinin (monascidin A) standard and fermentation extracts from the parental strain and the albino mutant produced a chromatogram peak with average retention time of 1.7 min. Therefore, it was concluded that monascidin A was found in the fermentation extracts of both strains. Two production curves of monascidin A (Figure 5), indicated that the monascidin A production rate by albino mutant was

Packed mycelial volume (% w/v)

Figure 3 Growth curve of *M. purpureus* parental strain (◇PMV, □ pH) and albino mutant. (△ PMV, ○ pH)

Packed mycelial volume (% w/v)

Figure 4 Biomass production curve of *M. purpureus* parental strain (◇ PMV, □ pH) and albino mutant. (△ PMV, ○ pH)

Monascidin A (μg ml^{-1})

Figure 5 Monascidin A production curve of *M. purpureus* parental strain (◇) and albino mutant (□).

slower than that of its parental strain, resulted in the shifting of production curve peak from 96th h with 19.19 µg ml^{-1} monascidin A content (parental strain) to 144th h with 22.26 µg ml^{-1} monascidin A content (albino mutant).

DISCUSSION

Based on microscopic morphological characteristics on various growth media, *Monascus* sp. can be classified into *M. pilosus, M. purpureus*, and *M. ruber* (Hawksworth and Pitt 1983). It turns out that the identification of *Monascus* is very difficult to do if it is based only on microscopic morphology, so identification needs to be conducted using a molecular biology technique such as RAPD (Lakrod *et al*. 2000). Campoy *et al*. (2003) succeeded in characterizing *Monascus* sp. employing RAPD with 2 decamer (10-bases) primer, namely CRL9 and CRL12. The same procedure succeeded in identifying *Monascus* sp. isolated from Cikapundung River as *M. purpureus*, which was later named *M. purpureus* ITBCC-HD-F001.

An albino mutant was obtained through the mutation process of parental strain *M.purpureus* ITBCC-HD-F001 using 2.5% (v/v) EMS with a 90 min incubation time. This mutant was produced when both mutant viability and mutation efficiency were below 10%, as reported by Susilowati (1997) during the mutagenesis of *Saccharomyces cerevisiae*. Mutation under those conditions resulted in the substitution of base pairing in PKS gene for pigment biosynthesis.

Employing consistency and stability tests, albino mutant colonies were shown to be consistent and stable over five generations. Unstable albino mutants will turn red again on YMP-G medium because a high concentration of glucose can reverse the mutation. The consistent and stable albino mutant from this experiment was later named *M. purpureus* ITBCC-HD-F002.

Analysis of genetic variation caused by mutation was conducted employing RAPD. This analysis produced an additional 1150 bp band. This band was the result of an amplification process by primer CRL12 on one DNA segment of the albino mutant chromosome, which was not present in parental strain DNA. This amplification happened because the an increased homology of this DNA segment to CRL12, which led to base pair substitution caused by EMS.

Characterization of the albino mutant through the comparisons of growth, biomass production, and monascidin A production curves revealed that the growth, biomass production, and monascidin A production rates are slower compared with those of its parental strain. This indicated that EMS mutation decreased the ability of albino mutant to adapt to its growth surroundings. The increase of monascidin A production by the mutant at the peak of the production curve was caused by the accumulation of pigment precursor, which was also the precursor of monascidin A. All of the precursors were shifted to the monascidin A biosynthesis pathway, resulting in an increase of monascidin A production by the mutant.

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