IMPROVEMENT OF KOJIC ACID PRODUCTION by A MUTANT STRAIN OF Aspergillus flavus, N40C10

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Abstract
Improvement of Aspergillus flavus No.193 has been done to obtain a potential mutant which produce kojic acid higher than its parent strain. The mutagenesis was conducted by using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) which combined with UV radiation after protoplast preparation. Screening of mutants were carried out with 1% FeCl₃ in a 96-well microtiter plate. The potential mutants strain were used for fermentation with 100 ml of 10 %w/v YES medium in a 250 ml Erlenmeyer flask and grown at 30°C and 180 rpm. Analysis of kojic acid in the culture fermentation was done by using Spectrophotometry at 502 nm and and quantitatively determined by TLC densitometry at 327 nm. The result showed that flask culture of mutant N40C10 produced a high concentration of kojic acid, which was 8.15 g/L, about twenty times higher than that of the parent strain.

Keywords: Aspergillus flavus, kojic acid, mutagenesis, NTG, UV radiation.

I. Introduction
Kojic acid (5-hidroxy-2-hydroxy methyl-4-pyrene) is a secondary metabolite produced by many species of filamentous fungi, especially by Aspergillus spp. Kojic acid has many biological activities, and widely used in cosmetics as whitening agent.1) It is also used as analgesics in medicine/pharmacy, and as pesticide in agriculture.2,3)

Generally, kojic acid was produced in small quantities by the parent strain, and was isolated firstly by Saito in 1907. Many study have been reported to improve the productivity of kojic acid fermentation using Aspergillus oryzae.1,3,4) Another study reported that Aspergillus flavus was very potential kojic acid producers among other Aspergillus strain, while it also produce aflatoxin as other metabolite.5) This study was carried out to find a hyperproducer mutant of A. flavus through mutagenesis using NTG and UV irradiation after protoplast formation.

II. Materials and Methods
Microorganism and Medium :
A potential isolate of Aspergillus flavus (no.193) of LIPI, Bogor, from our previous study was used for mutagenesis study.6) Stock cultures were maintained in potato dextrose agar (PDA), and preserved at 4°C. Medium for selection of mutant was prepared using 50 g of sucrose, 15 g of rice brand, 1 g of KH2PO4, 0.5 g of MgSO4.7H2O and sterile water to 1000 ml.

Mutagenesis and Screening of Mutants
NTG Mutagenesis
Mutagenesis was carried out according to procedure of Wu et al.1,8) Spores suspension of A. flavus was washed twice with phosphate buffer pH 7, and each time was centrifuged at 3500 rpm for 20 minutes. The spores were then resuspended in sterile water to concentration of 10⁶ spores/ml. Then, 1.0 ml of this suspension was treated with 9.0 ml 0.1% NTG in phosphate buffer, and incubated by shaking at 100 rpm, 28°C for 30 min. Neutralization of mutagen was done by diluting the suspension with 5 % sodium thiosulphate. About 100 µl was pipetted and spread on to PDA dishes and incubated at 28°C for 4 days. Screening of mutants was done in 96-well plates after incubating for 4 d, followed by addition of 1 drop of 1% ferrichloride. The strains which showed deep red-purple were collected. Increase of kojic acid concentration by the improved strains was confirmed by shake flask fermentation. The selected strains were preserved as the sources of further improvement by protoplast preparation and UV irradiation.

Protoplast Preparation
Protoplast were prepared by method of Wu et al.1) with some modifications. The selected strain was growing in 50 ml YEPD medium for 12 h at 28°C 180 rpm. The mycelia were harvested by centrifugation at 5000 rpm for 15 minutes. The precipitated mycelia was washed twice with 0.6 M KCL, and then suspended in 10 ml 1.2% lysing enzyme by shaking at 100 rpm, 28°C for 2 hours. The suspension was filtered through three pieces
of lense paper (substituting for miracloth), and then centrifuged at 500 rpm for 10 min to obtain protoplast. The supernatant was decanted, and protoplast was washed twice with phosphate buffer pH 6 containing 0.6 M KCL. The protoplast was chilled at 4°C, and ready for UV irradiation.

**UV Mutagenesis**

A volume of 100 µl of protoplast suspension was spread on PDA medium containing 0.6 M KCL, and irradiated with UV at 254 nm from the distance of 20 cm for 300 seconds. The irradiated strains were protected from the light, and then incubated at 28°C for 3 d. Similar screening procedure was applied to the mutant strains obtained.

**Kojic acid Fermentation**

For flask fermentation, preculture of an improved strains was prepared with 50 ml YES(yeast extract-sucrose) medium in 100 ml Erlenmeyer flask. Incubation was carried out by shaking at 180 rpm, 30°C for 24 h. Fermentations with different carbon sources and nitrogen sources were done with 100 ml medium and 10%(v/v) inoculum in 250 ml Erlenmeyer flask, and incubated by rotary shaking at 180 rpm, 30°C for 10 d.

**Analytical methods**

Kojic acid was determined by colorimetric method of Bentley, and also quantitatively analysed using TLC-Densitometer (CAMAG III, Switzerland), using silica gel F254 as stationary phase, toluene-ethyl acetate-formic acid(3:6:1) as mobile phase, and UV detector at 327 nm. Biomass was determined by the dry cell weight method. Culture samples were pipetted into a pre-weighed tubes, and centrifuged at 7500 rpm for 15 min. Supernatant was used for kojic acid analysis, and biomass was washed with distilled water, centrifuged, and dried at 105°C to constant weight.

**III. Results and discussion**

**NTG Mutagenesis**

Screening of NTG induced mutants on 96-well plates showed many variants have a deep red-purple colour (Fig.1), which has correlation with high concentration of kojic acid. Twelve potential mutants were collected, few of them were N20A10, N30F10, N40C10, and N50G12.

UV irradiation of protoplast

Variant N30F10 was processed to protoplast formation, and then continued to UV irradiation. The results showed few variants have a deep red–purple colour, more intensive than NTG variants (Fig.2) Direct UV irradiation after NTG treatment without protoplast formation showed no ones have a deep red–purple colour (results not shown).

![Fig.1. Screening results of NTG induced mutants after addition 1% FeCl3. Upper : 40 and 50 minute contact, lower : 20 and 30 minutes contact.](image1)

![Fig.2. Screening results of mutants induced by UV irradiation of protoplast for 300 seconds.](image2)

![Fig. 3. Kojic acid fermentation of some variants compared to their parent strain.](image3)
**Table 1**

Effect of different carbon and nitrogen sources on growth and kojic acid production in shake flasks fermentation by *Aspergillus flavus* N40C10

<table>
<thead>
<tr>
<th>No</th>
<th>Carbon source, (g/l)</th>
<th>Nitrogen source, (g/l)</th>
<th>Biomass (g/l)</th>
<th>Kojic acid (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sukrosa, 100</td>
<td>Yeast extract, 10</td>
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<td>7,595</td>
</tr>
<tr>
<td>2</td>
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<td>7,275</td>
</tr>
<tr>
<td>3</td>
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<td>15.40</td>
<td>6,277</td>
</tr>
<tr>
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<td>Yeast extract, 10</td>
<td>17.85</td>
<td>7,330</td>
</tr>
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<td>5</td>
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<td>Yeast extract, 5 dan CO(NH₂)₂, 0.7</td>
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<td>6,967</td>
</tr>
<tr>
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<td>15.80</td>
<td>5,492</td>
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</tr>
<tr>
<td>9</td>
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<td>Yeast extract, 5 dan (NH₄)₂SO₄, 1.5</td>
<td>15.25</td>
<td>2,143</td>
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<td>1,951</td>
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<tr>
<td>12</td>
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<td>15.25</td>
<td>1,811</td>
</tr>
</tbody>
</table>

*Fermentation was carried out with 100 ml medium in 250 ml Erlenmeyer flask, and incubated by shaking at 180 rpm, 30°C.*

**Kojic acid fermentation**

Comparison of few selected variants grown in flask culture with YES medium showed significant differences in kojic acid concentration (Fig.3). Concentration of biomass and pH of fermentation were also observed during the day of 0, 1, 2, 4, 6, and 8 (data not shown). These kojic acid data showed that visual observation (screening results) was not going along or equivalent with quantitative data. That may be happened if there is other compound instead of kojic acid in fermentation culture which showed similar reaction to kojic acid. Another possibly reason is that UV improved variants were very unstable.

The pHs culture of different variants were relative similar, namely between pH 4.5 – 5.2, but that was a little lower than that of the parent strain, pH 6.1. This description was suitable by the fact that all variants tested grew better than the parent strain.

![Fig.4. Correlation of kojic acid production to cell growth (biomass) and sucrose consumed during 15 d fermentation. Δ, kojic acid; ♦, sucrose; ●, biomass.](image-url)
Variation of nitrogen sources and carbon sources

As the parent strain showed best growth and optimum kojic acid production using yeast extract (5), it could be assumed that mutants strains will have similar properties of growth. To reduce cost, nitrogen source was varied by combining yeast extract with urea and ammonium sulphate. The results of fermentation with different carbon sources showed yeast extract and sucrose were the best nitrogen and carbon source, respectively (see Table 1). There was a significant decrease of biomass by combining yeast extract and urea, while kojic acid concentration showed only a little different to that if yeast extract used. These results recommended us to use combination of yeast extract and urea instead of yeast extract as sole source of nitrogen to increase the efficiency of fermentation.

On the best condition above, kojic acid produced was 8.15 g/l, with yield 0.14 g/g of sucrose consumed. This concentration value is similar to that reported by Wu et al. using yeast extract as nitrogen source (1). Our lower yield was especially influenced by higher growth of variant N40C10, which used much more carbon source for biomass than for kojic acid synthesis.

IV. Conclusion

In conclusion, Variant N40C10 of \textit{A. flavus} mutant was a potential Hyperproducer which needed to develop more over. Its important to used nitrogen source which can reduced biomass or inhibit growth, but do not inhibit kojic acid production.

V. Referensi