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Statistical optimization of kojic acid production by a UV-induced mutant strain of *Aspergillus terreus*

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ABSTRACT

The ability of four *Aspergillus* strains for biosynthesis of kojic acid was evaluated among which *Aspergillus terreus* represented the highest level (2.21 g/L) of kojic acid production. Improvement kojic acid production ability of *A. terreus* by random mutagenesis using different exposure time to ultraviolet light (5–40 min) was then performed to obtain a suitable mutant of kojic acid production (designated as $C_{5\text{-}10}$, 7.63 g/L). Thereafter, design of experiment protocol was employed to find medium components (glucose, yeast extract, KH_2PO_4 $(NH_4)_2SO_4$, and pH) influences on kojic acid production by the $C_{5\text{-}10}$ mutant. A 2^{5-1} fractional factorial design augmented to central composite design showed that glucose, yeast extract, and KH_2PO_4 were the most considerable factors within the tested levels ($p < 0.05$). The optimum medium composition for the kojic acid production by the $C_{5\text{-}10}$ mutant was found to be glucose, 98.4 g/L; yeast extract, 1.0 g/L; and KH_2PO_4, 10.3 mM which was theoretically able to produce 120.2 g/L of kojic acid based on the obtained response surface model for medium optimization. Using these medium compositions an experimental maximum Kojic acid production (109.0 ± 10 g/L) was acquired which verified the efficiency of the applied method.

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Introduction

Kojic acid (KA), 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (Fig. 1), was initially obtained from *Aspergillus oryzae* cultivated on steamed rice (named as "koji" in Japanese) in 1907.[1,2] This whitening agent could be crystallized as colorless and prismatic needles and chemically classified as a multifunctional, reactive γ-pyrone with weakly acidic properties.[2,3] KA as a secondary metabolite was usually biosynthesized by several *Aspergillus* and *Penicillium* species as well as some bacterial strains like *Acetobacter* and *Brevibacterium*.[2,4,5] It has several biotechnological applications and widely used in various area including food, medical, and chemical industries.[6–8] In addition, KA as a metal chelating agent with tyrosinase inhibition ability has been extensively applied in cosmetic and pharmaceutical industries.[7,9,10]

Such valuable metabolites production improvement could be attained either by manipulation of the microbial strains or optimization of the fermentation process.[11,12] Random mutation and selection the best mutant possess extensive application for improvement of the industrially valuable strains.[13,14] Simple irradiation by ultraviolet (UV) light or gamma ray might induce a mutant with overproduction ability for a selected metabolite which can increase the efficiency and productivity.[15,16]

Medium optimization and mathematical modeling are among other procedures applied for overproduction of a metabolite in biotechnology.[16–19] Growth of cells and metabolite concentrations are highly influenced by medium compositions and culture conditions.[16,20] It is not easy to investigate all the effective factors and their optimum levels by traditional method which evaluates one factor while keep other factors constant.[20–23] Design of experiments is a group of mathematical and statistical techniques to decrease the total number of experiments, thus aiming for reduction of both costs and time.[18,20,21]

The present study was aimed to develop an *Aspergillus* mutant strain with high ability of KA production using UV irradiation method. Furthermore, the main factors affecting KA biosynthesis by the selected mutant were screened and the related model was evaluated. On the other hand, this research tried to increase KA production by a simple mutation method combined with medium optimization using statistical experimental design.

Materials and methods

Chemicals and fungal strains

Sucrose, glucose, sodium nitrate ($NaNO_3$), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), potassium chloride (KCl), ferrous sulfate ($FeSO_4{\cdot}7H_2O$), ferric chloride ($FeCl_3$), yeast extract, Sabouraud dextrose broth (SDB), potato dextrose broth (PDB), and KA were supplied by Merck Chemicals (Darmstadt, Germany). Four *Aspergillus* strains including, *A. flavus* (PTCC 5004), *A. fumigatus* (PTCC 5009), *A. terreus* (PTCC 5283), *A. niger* (PTCC 5012), and *A. oryzae* (PTCC 5163) were provided by Persian type culture collection (PTCC, Tehran, Iran).

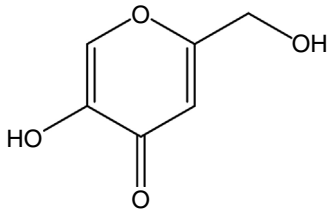

Fig. 1 – The chemical structure of Kojic acid.

Determination of KA concentration

For determination of KA concentration, Bentley's modified method was used based on the complexation of KA with Fe^{3+} ions and subsequent measurement the absorbance of the produced red complex at 500 nm.[24] Briefly, 100 μL from different concentrations of KA (1–32 mg/mL) was transferred to the test tubes containing 900 μL of deionized water and 2 mL of the freshly prepared $FeCl_3$ (0.06 M) in HCl (0.1 M) was added. After mixing the reagents with 12 mL of deionized water, the absorbance of the mixtures was measured at 500 nm using a UV–Vis spectrophotometer (UV-1800, Shimadzu Corporation, Tokyo, Japan). Deionized water was replaced by KA stocks as blank. All of the above-mentioned procedures were repeated three times on three different days and the mean of absorbencies was used for drawing the suitable standard curve.

Screening of KA producers

Into 50 mL of sterile defined medium consisting glucose (30 g/L); $NaNO_3$ (2 g/L); KH_2PO_4 (1 g/L); $MgSO_4{\cdot}7H_2O$ (0.5 g/L); KCl (0.5 g/L); and $FeSO_4{\cdot}7H_2O$ (0.01 g/L) 1 mL of spore stock suspension (1×10^8 spore per mL) of *A. flavus*, *A. fumigatus*, *A. terreus*, *A. niger*, and *A. oryzae* was separately added and each 250-mL inoculated Erlenmeyer flask was then incubated in a shaker incubator (30 °C, 150 rpm) for a period of 30 days. Interval samples (1 mL) were consequently withdrawn aseptically each 24 h. Fungal cells were then harvested by centrifugation (6000 × *g*, 20 min) and the obtained supernatants were used to determine the KA concentrations in the media in comparison with that of the blank (the supernatant obtained from the un-inoculated media) as previously described (see "Determination of KA concentration" section). These experiments were reiterated three times on various days.

UV-induced mutagenesis

The highest KA producer strain was cultivated on potato dextrose agar (PDA) plates for seven days at 30 °C. Then, the produced spores were collected and washed in triplicate with sterile normal saline by sequential centrifugation (7000 × *g*, 10 min). Different test tubes containing the spore suspensions in sterile normal saline solution (OD_{600}, 0.1, 4 mL) were then exposed to UV light in a cabinet containing UV lamp (255 nm) placed at 20 cm distance from the test tube surface. After 5 min, 10 min, 20 min, and 40 min each UV exposed

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