Production of Kojic Acid by *Aspergillus candidus* in Three Culture Media

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**ABSTRACT**

*Aspergillus candidus* ATCC 44054 grown without agitation produced more kojic acid in the modified Czapek-Dox liquid medium than cultures shaken at 100 rpm. Of the three culture media tested, yeast extract-sucrose medium permitted more kojic acid production by the fungus than modified Czapek-Dox liquid medium or Tadera medium. Maximal kojic acid (57-59 mg/ml) was produced in the yeast extract-sucrose medium on days 9-12. No aflatoxin by the fungus was detected.

Kojic acid (5-hydroxy-2-hydroxymethyl-r-pyrone), a fungal metabolite produced by many species of *Aspergillus* and *Penicillium* (10), has recently been shown to inhibit polyphenoloxidase (PPO) activity of mushroom, potato, apple, and crustaceans including white shrimp, grass prawn, and Florida spiny lobster (3,11). A Japanese product containing kojic acid, ascorbic acid, and citric acid has been patented as medium A in this study. The medium of Tadera et al. (12) consisting of 5% glucose, 0.6% peptone, 0.1% KH₂PO₄, 0.1% MgSO₄·7H₂O, 0.001% CaCl₂, and 0.001% FeCl₃ was designated as medium B. Yeast extract/sucrose medium (6) containing 2% yeast extract and 20% sucrose was medium YES.

**Effect of shaking on kojic acid production**

One hundred 50-ml Erlenmeyer flasks containing 15 ml of medium A were used to evaluate agitation on time-related changes in kojic acid production, medium pH, and fungal cell mass (dry weight). The flasks were wrapped with perfection Dubl-Chem-Faced milk filters (Schwartz Mag. Co., Two Rivers, WI) and Whatman filter paper #4 (Whatman International Ltd., Maidstone, England) to allow free passage of air. After the medium was autoclaved at 121°C for 15 min, 0.1 ml of a spore suspension (1.4 x 10⁷ spores/ml) was inoculated into each flask. Fifty flasks were incubated without agitation in the dark at room temperature (25 ± 2°C) and the other 50 at 25°C in a Gyrotory Water Bath Shaker (New Brunswick Scientific Co., New Brunswick, NJ) at a shaking speed of 100 rpm.

Three flasks from each group were removed every 3 d. The flask contents were filtered through a 0.45-µm filter (GA6; Gelman Sciences Inc., Ann Arbor, MI). The pH of the filtrates was measured using a pH meter. The mycelial mass was scraped onto a preweighed aluminum pan, dried in an oven for 2 d at 80°C, and then reweighed to determine dry weight fungal mass. The entire experiment was repeated once and the data points in each experiment represented the average values of three flasks.

**Kojic acid production in three liquid culture media under stationary conditions**

Fifty 125-ml Erlenmeyer flasks containing 25 ml liquid medium (A, B, or YES) were used to determine time-related changes in kojic acid production, medium pH, and fungal cell mass. The flasks were prepared and the media inoculated with the spore suspension as previously described. Following stationary incubation of the cultures at room temperature (25 ± 2°C) in the dark, three flasks were removed every 3 d and samples handled as previously described. The experiment was performed twice. Again, the data points represented the averages of three flasks.

**Quantitation of kojic acid in culture media**

Kojic acid produced in culture media was identified from its melting point, UV-Vis spectra in water and methanol, and the R₆ value and color reaction with a reagent containing FeCl₃ on paper chromatography (5,9,10). Only one spot with R₆ value correspond-
Detection of aflatoxin

At each sampling interval, 5-ml aliquot of the media was extracted three times with an equal volume of chloroform-methanol (9:1, v/v) in a separate funnel. After the solvent extracts were pooled and the solvent removed by rotary evaporation, the residue was dissolved in 500 μl benzene-acetonitrile (98:2, v/v). Samples were spotted along with aflatoxin B standard (Sigma) on analytical thin-layer chromatographic (TLC) plates (Fisher Rediplate, 250-μm silica gel) and developed with a chloroform-acetone-isopropanol (CAI; 85:15:2.5, v/v/v) solvent system. The plates were examined for aflatoxin fluorescence under long-wavelength ultraviolet light in a Chromato-Vue Cabinet (Model CC-20G, Ultra-Violet Products Inc., San Gabriel, CA). Visual detectability of aflatoxin B on TLC plates was as low as 25 ng.

Inhibitory effect of culture media on mushroom tyrosinase activity

The method of Saruno et al. (11) was adopted to determine if the culture media containing kojic acid inhibited mushroom tyrosinase activity. A 900-μl aliquot of the media (kojic acid adjusted to 80 μg/ml with the respective medium or distilled water) or standard kojic acid at 80 μg/ml was added to a cuvette containing 1.1 ml of mushroom tyrosinase (9.1, v/v) in a separate funnel. After the solvent extracts were pooled and the solvent removed by rotary evaporation, the residue was dissolved in 500 μl benzene-acetonitrile (98:2, v/v). Samples were spotted alongside aflatoxin B standard (Sigma) on analytical thin-layer chromatographic (TLC) plates (Fisher Rediplate, 250-μm silica gel) and developed with a chloroform-acetone-isopropanol (CAI; 85:15:2.5, v/v/v) solvent system. The plates were examined for aflatoxin fluorescence under long-wavelength ultraviolet light in a Chromato-Vue Cabinet (Model CC-20G, Ultra-Violet Products Inc., San Gabriel, CA). Visual detectability of aflatoxin B on TLC plates was as low as 25 ng.

RESULTS AND DISCUSSION

Preliminary studies showed that the only approach to accurately quantitate kojic acid in medium A, B, or YES applying the method of Bentley (2) was to use the dose-related absorbance standard curve, prepared by dissolving kojic acid in the respective medium at 0.05 to 0.75 mg/ml. The use of the dose-related absorbance standard curve derived from aqueous kojic acid solutions failed to provide accurate quantitation for spiked kojic acid in various medium systems (data not shown). The medium composition apparently affected the absorbance readings of kojic acid in these media.

Surface culture methods have been applied in most prior kojic acid fermentation studies. (1,2,7,10,12). Agitation of A. candidus during cultivation did not enhance kojic acid production; stationary cultures produced more kojic acid than those shaken at 100 rpm (Fig. 1). Maximum production of kojic acid occurred after 12-15 d of cultivation for stationary cultures, and after 18-24 d for those agitated. More mycelial mass (at least 2-fold more) was produced by stationary cultures than shaken. As with the other fungal secondary metabolites, most production of kojic acid occurred after the accelerated increase in fungal mass (Fig. 1).

A comparison of the three media A, B, and YES revealed that medium YES was superior in supporting kojic acid production by A. candidus (Fig. 2). Maximal production at approximately 60 mg/ml occurred on days 9-12 of cultivation in YES, which was about three times more than in medium B. Medium YES has been reported to be the most favorable for kojic acid production; Bajpai et al. (1) showed that kojic acid at 81.5 mg/ml was produced by A. flavus in this medium. Maximal kojic acid production at about 40 mg/ml occurred on days 12-15 in medium A. Medium A was better than medium B in supporting kojic acid production by A. candidus.

Kojic acid disappeared almost completely from YES and medium B after 27 d of cultivation. Although kojic acid was still present in medium A at this time, the compound disappeared eventually on prolonged cultivation (2). The old fungal mycelia may play a role in causing the disappearance of kojic acid in these media.

The mycelial mass collected from YES on day 12 or 15 weighed about three times more than those from medium A or B (Fig. 2). All three media had a decrease in pH values following fungal growth. As time proceeded, the pH increased above initial values (Fig. 2).

Unlike many strains of A. flavus and A. parasiticus which produce aflatoxin, A. candidus did not produce aflatoxin in any of the three media throughout the entire cultivation period. The chloroform-methanol extracts of the culture media failed to show any fluorescence under UV light; TLC analysis of the solvent extracts on silica plates also failed to show any fluorescent spots corresponding to aflatoxin standard.

The culture media had a dose-related inhibitory effect on mushroom tyrosinase (data not shown) similar to aqueous kojic acid solution (Fig. 3). A kojic acid solution at 80 μg/ml inhibited approximately 85% of the enzyme activity. The culture medium could thus be used directly for industrial application after removal of the fungal mycelia and dilution with water to a working concentration of 150 μg/ml.

The results show that YES is a suitable medium for kojic acid production by A. candidus. The compound showed an inhibitory effect on mushroom tyrosinase. Since kojic acid production can be affected by several factors, it is worthwhile to explore such possibilities to enhance its production. Tadera et al. (12) demonstrated that the addition of cycasin in culture medium increased kojic acid production by A. oryzae, A. flavus, or A. tamarii. Formation of kojic acid occurs most readily in highly acidic substrates (approximately pH 2-3) containing about 10% glucose or xylose and high levels of phosphate, but lacking nitrogen (4). Therefore, modification of the YES medium following these principles may enhance kojic acid production by A. candidus.

REFERENCES
Figure 1. A comparison of shaking versus stationary culture condition on time-related changes of (a) medium pH, (b) mycelial weight, and (c) kojic acid production by A. candidus grown in medium A.

Figure 2. Time-related changes of (a) medium pH, (b) mycelial weight, and (c) kojic acid production due to A. candidus growth in media A, B, and YES. The cultures were maintained at stationary position throughout the cultivation period.

Figure 3. Dose-related inhibitory effect of aqueous kojic acid at 20-150 μg on mushroom tyrosinase using DL-DOPA as the substrate.