

Cultural Conditions Studies on Kojic Acid Production by *Aspergillus parasiticus*

S.A. EL-AASAR

Botany Department, Faculty of Science, Zagazig University, Egypt
E-mail: salahaasar@yahoo.com

ABSTRACT

Five local *Aspergillus* species were screened for kojic acid production statically on four proposed kojic acid producing synthetic medium. *A. parasiticus* was found to be the highly active organism for acid production. The study implicated the optimization of different culture conditions of fermentation medium; initial pH, incubation temperature, carbon source concentration (glucose, sucrose & sugar beet molasses), nitrogen source concentration (yeast extract, peptone, ammonium sulphate, amm. nitrate & their combinations) and changes of kojic acid yield in static and rotary shaking culture. The highest level of kojic acid (34.38 g L⁻¹) was obtained by *A. parasiticus* using fermentation medium of 6% glucose, 1% yeast extract with initial pH 5 and incubated at 28°C for 10 days under rotary shaking culture (220 rpm). Antimicrobial activity of kojic acid was compared to some antibiotics against three Gram – ve, three Gram + ve bacteria and two strains of *Candida*. In conclusion, enhanced production of kojic acid was successfully achieved by optimizing the fermentation conditions for growth and productivity of *A. parasiticus*.

Key Words: Kojic acid; *Aspergillus* spp; Optimization; Antimicrobial activity

INTRODUCTION

Kojic acid was originally isolated in Japan by Saito in 1907 from mycelia of *Aspergillus oryzae* grown on steamed rice. This rice is called “koji” in Japanese, and this name was given to that organic compound by Yabuta in 1913. Later in 1924, Yabuta proposed the structure of kojic acid and introduced that fungal metabolite as 5-hydroxy-2-hydroxymethyl-4-pyranone (Brtko *et al.*, 2001). Kojic acid has several industrial applications. Most important properties of kojic acid are antifungal and antineoplastic activities (Aytemir *et al.*, 2003). It is widely used in medicine as anti-inflammatory drug and pain killer (Kayahara *et al.*, 1990), precursor for flavour enhancers (Le Blanc & Akers, 1989), antibrowning agent in the food industry (Chen *et al.*, 1991) and capability of chelating metals (Wiley *et al.*, 1942). Kojic acid is used in cosmetic industry for its excellent whitening or UV-protective effects (Nakagawa *et al.*, 1995; Masse *et al.*, 2001). This acid can be produced from various carbohydrate sources under an aerobic condition by a variety of microorganisms especially *Aspergillus* spp. Although work on optimization of medium composition and cultural conditions such as pH, temperature, oxygen requirement and mode of fermentor operations, which stimulates production of kojic acid is not yet quantified (Futamura *et al.*, 2001a; Lin, 2001; Gad, 2003). Kojic acid has a polyfunctional heterocyclic, an oxygen containing skeleton with several important reaction centers enabling addition reaction, oxidation and reduction, alkylation and acylation, substitution nucleophilic reaction, a ring opening of the molecule, substitution electrophilic reactions (Brtko *et al.*, 2001). A great variety of kojic acid

derivatives are known for its non-problematic biodegradation with any un-desirable effects on higher organism, thereby representing an attractive polyfunctional skeleton for the development of biologically active compounds via derivatization for the production of antiinflammatory preparations, bronchodilators, local anaesthetics, fungicides, insecticides and/or pesticides. In this study, optimal conditions for kojic acid production have been reported.

MATERIALS AND METHODS

Microorganisms and media. *Aspergillus flavus*, *A. glaucus*, *A. oryzae*, *A. parasiticus* and *A. tamarii*, isolated from soil were tested for kojic acid production. The seed medium proposed by Ariff *et al.* (1996) was used in all experiments for inoculum preparation. The medium consisted of (g L⁻¹); glucose, 50; yeast extract, 5; KH₂PO₄, 1.0 and MgSO₄.7H₂O, 0.5. For inoculum preparation, 5 mL of spore suspension (approximately 10⁶ mL⁻¹) was inoculated into 50 mL medium in a 250 mL Erlenmyer flask. The flasks were incubated at 30°C for 12 days. All experiments were performed at least in duplicate.

Analytical methods. Culture media were decanted, the mycelium was washed several time with distilled water and oven dried (80°C for 24 h) to get mycelial dry weight. The supernatant was used for kojic acid and glucose estimation according to colorimetric method of Bentley (1957) and Dubois *et al.* (1956), respectively.

RESULTS AND DISCUSSION

Many researchers proposed the production of kojic

acid by cultivating different *Aspergillus* spp on kojic acid producing synthetic media. Most of these media contained glucose or sucrose as the sole carbon source; yeast extract or peptone as the sole nitrogen source in addition to magnesium sulphate and potassium phosphate. Futamura *et al.* (2001a) suggested seventy six types of media to enhance the production rate of kojic acid by *A. oryzae* (Mk107-39). Based on the correlation between the kojic acid production rate and product yield, two media were selected as suitable media for kojic acid production. Therefore; in this paper, we were proposed four media for the kojic acid production by the selected *Aspergillus* spp. Medium (M)- 1 (sucrose, 40 g L⁻¹ & peptone, 10 g L⁻¹); M- 2 (glucose, 40 g L⁻¹ & peptone, 10 g L⁻¹); M- 3 (sucrose, 40 g L⁻¹ & yeast extract, 10 g L⁻¹) and M- 4 (glucose, 40 g L⁻¹ & yeast extract, 10 g L⁻¹). Besides, all of these media contained 1.0 g L⁻¹ KH₂PO₄ and 0.5 g L⁻¹ MgSO₄.7H₂O.

Many researchers reported that static fermentation conditions are more effective in kojic acid production than culture in the shake flasks (Lin *et al.*, 1976; Wei *et al.*, 1991; Ogawa *et al.*, 1995; Abd-El-Naby *et al.*, 1996; Ariff *et al.*, 1996; Lin, 2001; Aytemir & Erol, 2003; Gad, 2003). The screened *Aspergillus* spp were grown statically for 12 d on all proposed media for kojic acid production. It was found that M- 4 was the best in kojic acid production followed by M- 3, M- 2 and M- 1 (Fig. 1). Moreover; *A. parasiticus* followed by *A. flavus* were found to be the highly active for kojic acid production. Results conform to many early findings on the production of kojic acid by fermentation as secondary metabolite using *Aspergillus* spp. *A. effuses*, *A. glaucus*, *A. oryzae*, *A. flavus*, *A. gillus gymnosardae*, *A. awamori*, *A. clavatus*, *A. fumigatus*, *A. giganteus*, *A. albus*, *A. candidus*, *A. nidulans*, *A. parasiticus*, several spp of *Penicillium* and *Acetobacter* (Lin *et al.*, 1976; Megalla *et al.*, 1985; Coupland & Niehaus, 1987; Futamura *et al.*, 2001a; Gad, 2003). These differences in production of kojic acid and mycelial mat may be ascribed to either the culture conditions or to species differences.

The effect of pH on the production of kojic acid by *A. parasiticus* was studied by adjusting the pH of the fermentation medium to the desired values with 0.1 N NaOH or 0.1 N HCl, whereas the reported optimal pH for kojic acid production appears to be 5.0 (Fig. 2). The production began to decrease at pH 5.5. The decrease in kojic acid production at high pH values was accompanied by the decrease in mycelial dry weight. This comes in harmony with data obtained by Abd-El-Naby *et al.* (1996), who reported that pH 5.0 was favorable for kojic acid production by a toxigenic strain of *A. parasiticus*. Filamentous fungi, generally, are characteristically tolerant to acidic pH and most of them have an optimum pH between 5 and 6 for cellular growth and several metabolic activities (Rosfarizan *et al.*, 2000). High pH may lead to the growth of microorganism or inhibition of enzymes activities responsible for the biosynthesis of kojic acid. Lin *et al.* (1976) showed two optimal pH values for the production of

Fig. 1. Screening of *Aspergillus* strains for kojic acid production using the proposed fermentation media

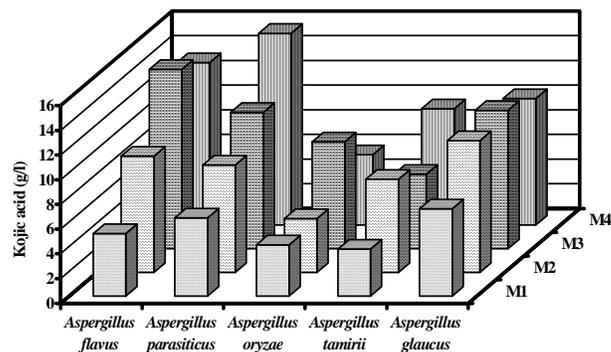
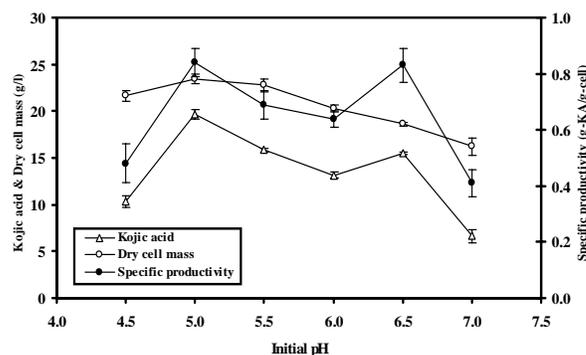


Fig. 2. Relative tolerance of initial pH of the fermentation medium kojic acid production by *A. parasiticus*



kojic acid 4.5 and 6.2 by *A. parasiticus*. Lin *et al.* (2001) reported that pH optima for kojic acid production were 4.5 and 6.5 for *A. flavus* and *A. oryzae* respectively. Enzymes, being proteins, contain ionizable groups; consequently, the pH of the culture medium affects their structure and function (Lekha & Lonsane, 1997). Gad (2003) found that pH 6 seemed to be most favorable for kojic acid production by *A. parasiticus*.

Microbial fermentation is governed by the temperature, but the optimum for synthesis of particular compounds may differ for optimum growth. It is inferred from the data that *A. parasiticus* was able to grow considerable well within a temperature range from 25°C to 32°C (Fig. 3). The optimal incubation temperature for kojic acid production was recorded at 28°C. Generally the optimum temperature for kojic acid production by fungi in most of the cases was found to be 25 - 30°C (Kuwak & Rhee, 1992; Futamura *et al.*, 2001a; Lin, 2001; Gad, 2003).

Upon testing the influence of carbon compounds on the production of extracellular kojic acid as well as growth rate of the tested organism, different carbon sources were used (Fig. 4). These results show that 6% glucose followed by 3% sucrose and 6% beet molasses (supplied by the Delta Sugar Company Egypt) induced maximum kojic acid production in addition to biomass yield. Above a critical

Fig. 3. Relative tolerance of temperature for kojic acid production by *A. parasiticus*

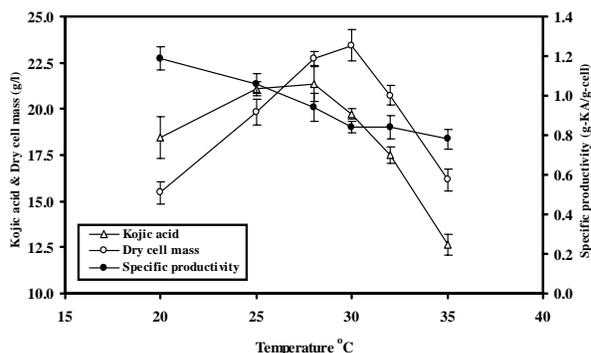
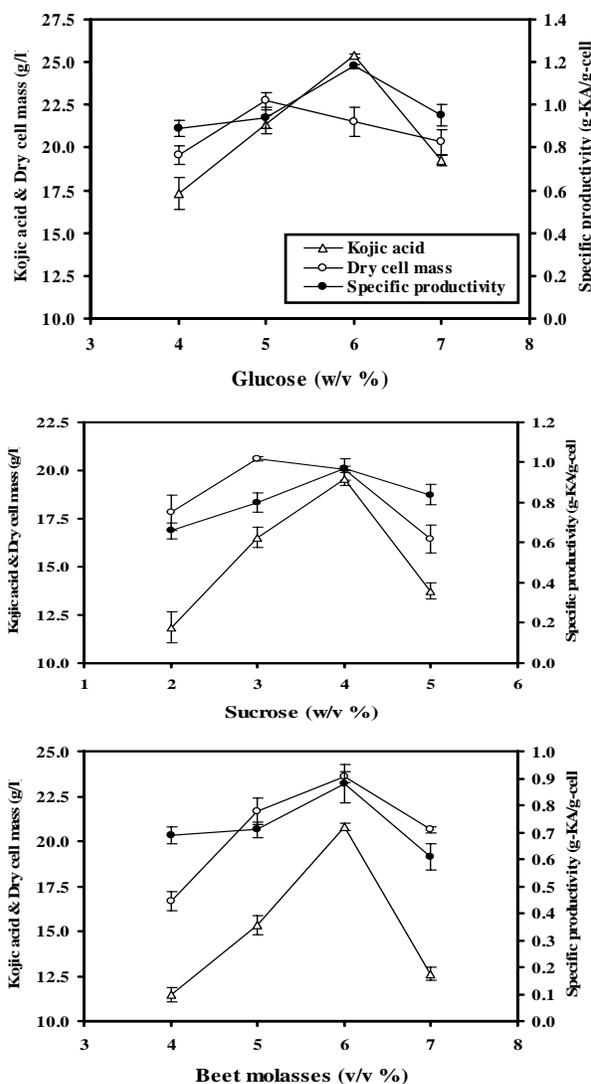


Fig. 4. Kojic acid production with different sugar concentration by *A. parasiticus*



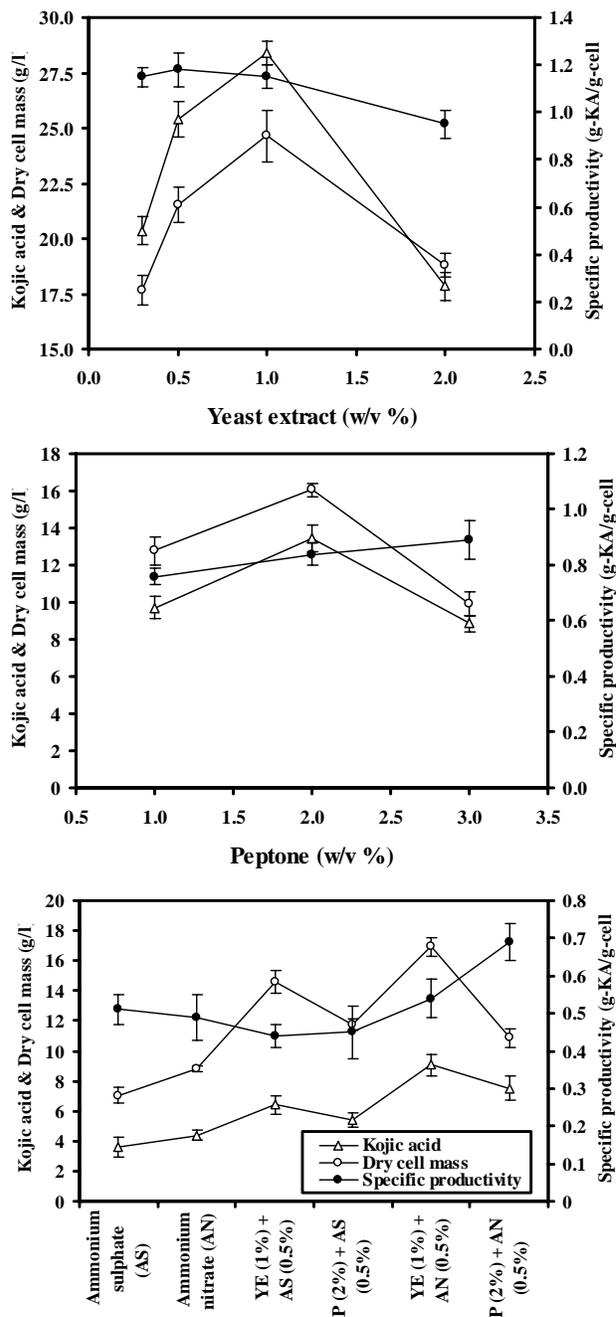
substrate concentration, a decreased water activity and onset of plasmolysis combine to cause a decrease in the rates of

fermentation (Roukas, 1993). Increasing initial sugar concentration resulted in a significant increase in residual sugar, which may be due to inability of the microorganisms to metabolize high levels of sugar. The decreased sugar utilization encountered with the highest concentration probably was due to osmosis effect. It is well known that glucose is the best carbon source for kojic acid production due to structural similarity of both. It has been suggested that during the fermentation kojic acid is formed directly from glucose without any cleavage of the carbon chain into smaller fragments. Moreover, the presence of glucose has a six carbon ring, which acts as precursor for kojic acid synthesis. Kojic acid synthesis only started after growth reached stationary phase and stopped when glucose in the medium was depleted (Kitada *et al.*, 1967; Megalla *et al.*, 1985; Rosfarizan *et al.*, 1998; Futamura *et al.*, 2001a, b). Some molasses sucrose during sugar processing is hydrolyzed into reducing sugar glucose and fructose in beet molasses 22% (w/w) reducing sugar. Gad (2003) studied the effect of beet molasses concentration on kojic acid production by *A. parasiticus* and attained its maximal value at 20% (w/v).

The choice of nitrogen source for the fermentation medium can be an important factor influencing the subsequent fermentation. In most fermentation processes, the concentration of the nitrogen and/or carbon source in the medium is increased to secure high productivity, thereby increasing the viscosity of the culture broth, which leads to a decrease in the oxygen transfer rate in the fermentation, resulting in a low yield of the product. Therefore, when selecting an optimum production medium, it is very important to select a medium yielding low cell concentration (Futamura *et al.*, 2001a). Nitrogen source appears to be one of the most effective factors for the kojic acid production. Yeast extract and peptone nitrogen sources favored and promoted kojic acid production compared with ammonium sulphate and ammonium nitrate. These results showed that the use of 1% yeast extract resulted in the highest kojic acid production (28.41 g L⁻¹) compared with the other nitrogen sources (Fig. 5). On the other hand, the use of ammonium sulphate and ammonium nitrate resulted in small amounts of production. This may suggest a metabolic activation in using the other tested nitrogen sources for kojic acid production. Most likely, this was due to the lack of some essential growth factors in such sources. However, this did not mean that yeast extract had better quality nitrogen, but probably contain higher levels of other essential components required for growth and fermentation, such as vitamins and oligoelements that's why it is usually used in microbiological media (Parrish *et al.*, 1996; Ariff *et al.*, 1996; Bazaraa & Al-Dagal 1999; Futamura *et al.*, 2001b; Lin, 2001; Gad, 2003).

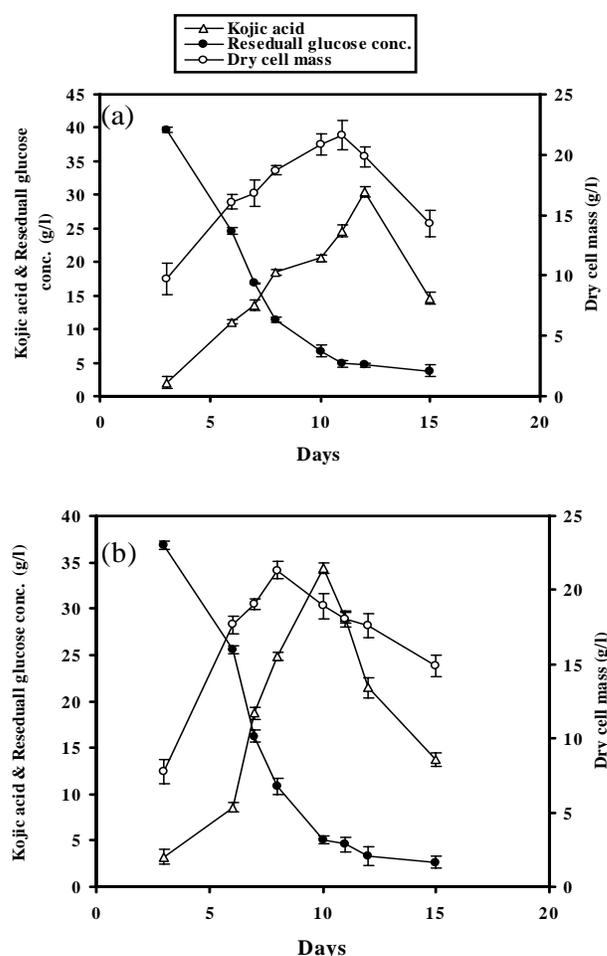
Course of kojic acid fermentation by static and shaking culture were conducted to study changes in kojic acid, residual glucose concentration and dry cell weight (Fig. 6). In both cultivation methods, the pH of the

Fig. 5. Effect of nitrogen sources on kojic acid productivity of *A. parasiticus*



fermentation solutions was decreased to around 3 after 4 - 5 days of cultivation (data not shown). The glucose concentration gradually decreased with concomitant increase in the kojic acid concentration, where most of the glucose supply was exhausted after about 9 days. The growth rate of mycelia reached their maximum 21.63 and 21.32 g L⁻¹ at 11 and 8 days of cultivation in static and shaking cultures then gradually decreased, respectively. The kojic acid concentration reached about 30.45 and 34.38 g L⁻¹

Fig. 6. Changes of kojic acid, residual glucose concentration and dry cell weight of *A. parasiticus* in static (a) and shaking culture (b)



at 12 and 10 days of cultivation and gradually decreased in static and shaking cultures, respectively. The decrease in the kojic acid concentration after reaching a maximum was pronounced in shaking than in the static culture. Such behavior of kojic acid fermentation is usually observed (Ogawa *et al.*, 1995; Ariff *et al.*, 1996; Futamura *et al.*, 2001a; Gad, 2003).

The increasing numbers of pathogen bacteria and fungi that are resistant to the commonly used therapeutic agent is a major worldwide health problem. For this reasons the search for new antimicrobial agents with novel modes of action represents a major target in chemotherapy (Aytemir & Erol, 2003). Therefore, antibacterial and antifungal activities of the synthesized kojic acid produced by *A. parasiticus* were compared to some antibiotics tested. Minimal inhibitory concentrations values (MICs) were presented in the Table I. Three Gram negative (*Pseudomonas aeruginosa*; *E. coli* & *Proteus vulgaris*) and three Gram positive (*Staphylococcus aureus*; *Streptococcus pneumoniae* & *Bacillus subtilis*) bacteria were used as

Table I. Antibacterial and antifungal activities of the synthesized kojic acid by *A. parasiticus* compared to various antibiotics tested (MIC in µg/mL)

Tested organisms	Kojic Acid	Cefraz- idime	Nitrofur- antoin	Fluon- azole
Gram -ve bacteria				
<i>Pseudomonas aeruginosa</i>	188	17	80	
<i>E. coli</i>	178	14	72	
<i>Proteus vulgaris</i>	205	17	65	
Gram +ve bacteria				
<i>Staphylococcus aureus</i>	285	28	85	
<i>Streptococcus pneumoniae</i>	176	32	82	
<i>Bacillus subtilis</i>	244	34	105	
Fungi				
<i>Candida albicans</i>	168			8
<i>Candida krusei</i>	166			56

quality control strains. For testing antifungal activities, *Candida albicans* and *C. krusei* were tested. In this study, MICs values differed according to the bacterial and fungal isolates. Generally MIC of ceftazidime was higher than nitrofurantoin. The results revealed that Ceftazidime (MIC: 28:34 µg mL⁻¹) was found to be potent against Gram positive bacteria and most potent (MIC: 14 - 17 ug mL⁻¹) against Gram negative bacteria. Whilst nitrofurantoin had moderate antibacterial activity (MIC: 80 - 105 ug mL⁻¹) against both Gram negative and Gram positive bacteria. On the other hand, MICs of kojic acid against the bacterial isolates were 176 - 285 µg mL⁻¹ and 166 - 168 ug mL⁻¹ against *Candida krusei* and *C. albicans*. These results were in agreement with Aytemir & Erol (2003) in their study compared the antimicrobial activity of kojic acid and twelve of its intermediate compounds they concluded that MICs of kojic acid ranged between 128 and 256 ug mL⁻¹ against various bacteria and fungi. It is well known that kojic acid and its derivatives are reported to possess pharmacological effects such as herbicidal, pesticidal and insecticidal (Dowd, 1988 & 1991; Blaney & green, 1989; Sadek *et al.*, 1996; Burdock *et al.*, 2001); antimicrobial (Uher *et al.*, 1990 & 1993; Marwaha *et al.*, 1994; Nakagawa *et al.*, 1995; Burdock *et al.*, 2001; Aytemir & Erol, 2003); as well as being a skin whitening agents and antiacne (Cabanes *et al.*, 1994; Cotellessa *et al.*, 1999; Lim, 1999; Burdock *et al.*, 2001). Thus, the search could be extended in future by investigating kojic acid and its derivatives for their antimicrobial activities. In conclusion, enhancement of productivity leading to a reduction in the production cost of kojic acid by *A. parasiticus* and the ascertained biological activity of kojic acid was successfully achieved.

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