High Production of Kojic Acid Crystals by Isolated Aspergillus oryzae var.effusus NRC14

1M.M. Hazzaa, 2Abd EL-Naby M. Saad, 3Helmy M. Hassan and 2Eman I. Ibrahim

1Botany Department, 2Faculty of Science, Benha University and 3Microbial Chemistry Department, National Research Center.

ABSTRACT

Among 20 fungal strains grown on the solid glucose salts medium, 10 Aspergillus spp. have a strong red purple color in plates and the appearance of red color was taken as the production of kojic acid. The fungi in the plates showing the highest color intensities were selected by visual observation followed by a second selection using 250 ml Erlenmeyer flasks fermentation. Of the ten Aspergillus strains grown statically or shaken on a fermentation medium (pH, 4.0) for 18 days at 28°C, A. oryzae var.effusus NRC14, A. flavus NRC13, A. tamarii NRC18 and A. parasiticus were found to be the most highly active organisms for kojic acid production, whereas in two cases, (static and shaken cultures), kojic acid produced by A. oryzae var.effusus NRC14 was higher than that produced by A. flavus NRC13, A. tamarii NRC18 and A. parasiticus. On the other hand, a static culture of A. oryzae var.effusus NRC14 produced kojic acid higher than a shaken culture did (42.0, 25.5g kojic acid/L) at 150 rpm. In general, A. oryzae var.effusus NRC14, A. flavus NRC13, A. tamarii NRC18 and A. parasiticus produced 0.626, 0.602, 0.379, 0.305, 0.447, 0.288, 0.262 and 0.088 g kojic acid/g glucose consumed. Aflatoxins, B1, B2, G1 and G2 were observed in culture filtrates of A. flavus NRC13 and A. parasiticus whereas no aflatoxins were detected in A. oryzae var.effusus NRC14 culture filtrate. A. oryzae var.effusus NRC 14 biomass produced from the first grown on a glucose salts medium can be resuspended in a glucose salts medium (original medium) or in citrate phosphate buffer, pH4.0, buffer supplemented with 10% glucose or 10% glucose and 0.112% NH4NO3 produced kojic acid lower than growth medium one, while the biomass and glucose consumed in both original medium and buffer supplemented with glucose and nitrogen were increased compared with the growth medium one. No kojic acid was formed if buffer used for resuspension lacked glucose. The produced kojic acid was extracted in the form of needles and it identified according to its melting point, UV absorption spectrum and infrared spectrum.

Key words: Fungi, Biomass, Resuspension, Kojic acid, Aflatoxins, A. oryzae var.effusus NRC 14, A. flavus NRC 13, A. tamarii, A. parasiticus.

Introduction

Kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrene) was originally isolated in Japan by Saito in 1907 from mycelia of Aspergillus oryzae grown on steamed rice. Kojic acid is produced by Aspergillus spp. belonging mainly to the section flavi (flavus– oryzae – tamarii). Among them A. flavus (Basappa et al., 1970; Ariff et al., 1996; Saad et al.,1996; Chang et al.,2011 ; Prabu et al.,2011), A. oryzae (Kwak and Rhee 1991, 1992; Takamizawa et al., 1996; Wan et al., 2005;Terabayashi et al., 2010), A. oryzae var.effusus (Kumda and Asao ,1996; Lee et al., 2006), A. tamarii (Gould, 1938) and A. parasiticus (Lin et al., 1976; Nandan and Polasa, 1985; Coupland and Niehaus, 1987; Saad et al., 1996; EL-Aasar, 2006; Chang et al.,2011). Kojic acid can be produced either by using aerobic batch fermentation (Kitada et al., 1971; Madihah et al., 1996) or resuspended cell material in buffered solution containing only glucose (Bajpai et al., 1981,1982; Ariff et al., 1997) were reported to have the ability to produce large amount of kojic acid. Isolates of A. flavus and A. parasiticus vary in their ability to produce aflatoxins. Diener and Davis (1966) reported that 40 % of A. flavus isolates collected throughout the world didn’t produce aflatoxins. Relatively few studies have compared aflatoxin- producing and non-producing isolates of Aspergillus (Gupta et al., 1970; Schmidt et al., 1977; Saad et al., 1996). MuraKami and Suzuki (1970) found that certain morphological and cultural traits were commonly exhibited by toxin producers. Similarly, Parrish et al., (1966) reported that kojic acid production was a common trait of aflatoxin producers of A. flavus and A. parasiticus. However, kojic acid was also produced by some non-toxicogenic strains. Gupta et al., (1970) found that the toxigenic strain formed less kojic acid than did the non-toxicogenic strain in a glucose– salts medium. Heathcote et al., (1965) have suggested that kojic acid is a precursor of aflatoxins.

Corresponding Author: Abd EL-Naby M.Saad, Microbial Chemistry Depart, National ResearchCenter (NRC), Cairo, Egypt.
E-mail: saad_abdelnaby@yahoo.com
Various types of compounds, such as glucose, sucrose, acetate, ethanol, arabinose and xylose (Kitada et al., 1967; Basappa et al., 1970) have been used as carbon sources for kojic acid production. It is well known that glucose is the best carbon source for kojic acid production due to the similarity of its structure to that of kojic acid. 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 (Kitada et al., 1967; Megalla et al., 1986). Kojic acid crystallizes in form of colorless, prismatic needles that sublime in vacuum without any changes. Meanwhile, the melting point of kojic acid ranges from 151°C -154°C (Ohyama and Mishima, 1990). Kojic acid is soluble in water (3.95g/100ml. at 20°C), ethanol and ethyl acetate. On the contrary, it is less soluble in ether, alcohol ether mixture, chloroform and pyridine (Bentley, 1957; Wilson, 1971). Kojic acid has a maximum peak of ultraviolet absorption spectra at 280-284nm (Choi et al., 2002; Watanabe – Akanuma et al., 2007).

The market for kojic acid has been developed for some 40 years since 1955, where Charles Pfizer and Company, USA, announced the first attempt to manufacture this organic acid. In short, the interest in kojic acid is increasing enormously with a growing presence in industries related to its applications, especially in cosmetic and health care industries (Brkko et al., 2004; Bentley, 2006). It is primarily functions as the basic material for the production of skin whitening creams, skin protective lotions, whitening soaps and tooth care products. Kojic acid has the ability to act as the ultraviolet protector whereby, it suppresses hyper-pigmentation in human skins by restraining the formation of melanin through the inhibition of tyrosinase formation, the enzyme that is responsible for skin pigmentation (Ohyama And Mishima, 1990; Noh et al., 2009; Lajis et al., 2012), an antioxidant, a bacterio static, a metal chelating agent, food as a preservative, as an antioxidant for fat and oils, in the preparation of derivative esters, in adhesives, in chelate - forming resins and as a plant growth - regulating agent to increase production (Cabanes et al., 1994; Chemos Group, 2000; Jarchem industries, 2000).

The purpose of this study was to isolate of some fungi capable of producing kojic acid crystals and recycling of a selected fungal biomass which has a high kojic acid crystals production for increasing the amount of kojic acid.

**Material and Methods**

Mold culture:

Throughout this study twenty fungal strains were examined for kojic acid production, eight of them were already known *Aspergillus flavus* ATCC 9643™ was obtained from American Type Culture Collection,10801 university Blvd. Manassas, VA 20110, USA; *Aspergillus flavus* EMCC274, *Aspergillus flavus* EMCC275, *Aspergillus oryzae* EMCC163 and *Rhizopus oryzae* EMCC611 were obtained from Egyptian Microbiology Culture Collection, Microbiology Resources Centre (Cairo Mircen), Faculty of Agriculture, Ain Shams University, Egypt; *Aspergillus parasiticus* and *Aspergillus niger* were obtained from the Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt and *Aspergillus niger* NRRL595 was obtained from ARS culture collection (NRRL), Peoria, Illinios, USA ; and twelve isolates were collected from different habitats (air, 2 isolates; marine water, 2 isolates; soil, 8 isolates).

The isolated fungi were identified by The Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt based on hyphal morphology and colony characters using an image analysis system.

**Culture conditions:**

The fungal strains were maintained on potato dextrose agar (PDA) slants at 4°C and sub-cultured at intervals from 15-30 days.

**Culture method:**

Spores scraped from each slant culture incubated at 28°C for 6 days on the PDA medium were diluted with sterile d.w for inoculation. The spores solution was inoculated into Petri dishes each containing 20 ml of PDA medium, pH, 4. The cultures were incubated at 28°C for 6 days.

**Isolation of some fungi capable of producing kojic acid crystals:**

Circular mycelia plug (4mm in diameter) growing on PDA dishes were replicated to a pair of Petri dishes containing 20 ml of the production medium which consisted of (g/l): glucose, 100; NH₄NO₃, 1.125; MgSO₄·7H₂O, 0.5; KCl, 0.1; H₃PO₄, 0.063 ml and agar, 20.(May et al., 1931).

The medium was adjusted to pH 4 with 1N HCl prior to sterilization. The Petri dishes were incubated at 28°C for 12 days. After cultivation period, ferrous chloride solution as a colorant was added. The strains showing a strong red purple color were picked and stocked for a second selection procedure using a 250 ml...
Erlenmeyer flasks. The cultures were incubated static or shaken (150rpm) at 28°C for 18 days. All cultures were run in duplicates. The medium was decanted, the mycelium washed several times with distilled water and dried in the oven at 80°C for 24h. The supernatant was utilized for the determination of kojic acid, glucose consumed and its final pH value. For successful production of kojic acid even on a commercial scale, the biomass have a high kojic acid crystals production were resuspended in the above mention liquid medium or in a simple fermentation medium containing phosphate buffer, buffer supplemented with 10% glucose only or supplemented with 10% glucose and 0.112% NH₄NO₃.

Isolation of kojic acid:

The culture broth was filtered through two layers of cheesecloth into another flask and maintained under refrigeration at 5°C. After one night of storage, the precipitated crystals were separated by filtration. The crystals were collected, dried at 80 °C for 24 h. and weighed (Lin et al., 1976). For further kojic acid extraction, the filtrate was then extracted with ethyl acetate and kojic acid crystals were recovered by evaporation and weighed (Barnard and Challenger, 1949). They were combined and purified by repeated crystallization from a mixture of acetone and water (Lin et al., 1976). The crystalline product was identified by its melting point (m.p), UV absorption spectra and infrared spectrum.

Detection of aflatoxins in the culture filtrate:

Aflatoxins were extracted with chloroform, purified by repeated precipitation by n-hexan and dissolution into chloroform (Murakami, 1971) and then subjected to HPLC Model 1525(USA).

Determination of kojic acid and glucose:

Kojic acid was determined by the method of Bentley (1957). Glucose was determined by DNS method (Chaplin and Kennedy, 1986).

Results and Discussion

Identification of the isolated fungi:


Isolation of a high level kojic acid producer strains:

Among 20 fungal strains grown on the solid glucose salts medium, 10 *Aspergillus* spp. (No.11-20) have a strong red purple color in plates and the appearance of red color was taken as the production of kojic acid (Fig.1A&B). These results agreed with the results of Murakami (1971), Futamura et al., (2001).

![Fig. 1](image_url)
The fungi in the plates showing the highest color intensities were selected by visual observation followed by a second selection using a 250 ml Erlenmeyer flask fermentation. Of the ten Aspergillus strains grown statically or shaken on a fermentation medium (pH4.0) for 18 days at 28°C, A. oryzae var. effusus NRC 14 was higher than that produced by A. flavus NRC 13, A. tamarii NRC 18 and A. parasiticus (42.0, 41.0, 22.0, 17.7, 25.5, 17.3, 15.2 and 5.6 g/L, respectively, Tables 1&2), while initial glucose was reduced from 100 g/L to 33.0, 32.0, 42.0, 43.0, 36.0 and 38.0 g/L, respectively. Kojic acid yields of 42.0%, 41.0%, 22.0%, 17.7%, 25.5%, 17.3%, 15.2% and 5.6%, respectively and glucose utilization values of 67%, 68%, 58%, 57%, 60%, 58% and 64% respectively, (Table 1&2). On the other hand, a static culture of A. oryzae var.effusus NRC 14 produced kojic acid higher than a shaken culture did (42.0, 25.5 g kojic acid/L) at 150 rpm. In general, A. oryzae var.effusus NRC14, A. flavus NRC 13, A. tamarii NRC 18 and A.parasiticus produced 0.626, 0.602, 0.379, 0.305, 0.447, 0.288, 0.262 and 0.088 g kojic acid /g glucose consumed, respectively. In addition to kojic acid production, the biomass dry weight values were 6.4, 7.4, 7.0, 6.4, 10.6, 7.0, 7.9 and 7.5 g/L, respectively (Tables 1&2).

Table 1: Production of Kojic acid via surface fermentation by 10 Aspergillus spp.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Final pH</th>
<th>Kojic acid (g/L)</th>
<th>Kojic acid (yield%)</th>
<th>Residual glucose (g/L)</th>
<th>Consumed glucose (g/L)</th>
<th>g kojic acid / g glucose consumed</th>
<th>Biomass dry weight (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. oryzae var.effusus NRC 14</td>
<td>3.51</td>
<td>25.5</td>
<td>25.5</td>
<td>43</td>
<td>57</td>
<td>0.447</td>
<td>10.6</td>
</tr>
<tr>
<td>A. flavus NRC 13</td>
<td>2.62</td>
<td>17.3</td>
<td>17.3</td>
<td>40</td>
<td>60</td>
<td>0.298</td>
<td>7.0</td>
</tr>
<tr>
<td>A. flavus NRC 18</td>
<td>3.34</td>
<td>15.2</td>
<td>15.2</td>
<td>42</td>
<td>58</td>
<td>0.262</td>
<td>7.9</td>
</tr>
<tr>
<td>A. parasiticus</td>
<td>2.45</td>
<td>5.6</td>
<td>5.6</td>
<td>36</td>
<td>64</td>
<td>0.088</td>
<td>7.5</td>
</tr>
<tr>
<td>A. oryzae var.effusus NRC 14</td>
<td>2.38</td>
<td>0.3</td>
<td>0.3</td>
<td>35.5</td>
<td>64.5</td>
<td>0.005</td>
<td>5.5</td>
</tr>
<tr>
<td>A. tamarii NRC 18</td>
<td>4.33</td>
<td>12.6</td>
<td>12.6</td>
<td>37</td>
<td>63</td>
<td>0.200</td>
<td>16.4</td>
</tr>
<tr>
<td>A. parasiticus</td>
<td>4.42</td>
<td>0.1</td>
<td>0.1</td>
<td>34</td>
<td>66</td>
<td>0.022</td>
<td>11.1</td>
</tr>
<tr>
<td>A. flavus EMCC 274</td>
<td>3.00</td>
<td>0.1</td>
<td>0.1</td>
<td>42</td>
<td>58</td>
<td>0.002</td>
<td>6.7</td>
</tr>
<tr>
<td>A. flavus EMCC 275</td>
<td>3.33</td>
<td>12.3</td>
<td>12.3</td>
<td>73</td>
<td>3.5</td>
<td>0.168</td>
<td>11.5</td>
</tr>
<tr>
<td>A. oryzae var.effusus NRC 14</td>
<td>3.09</td>
<td>4.0</td>
<td>4.0</td>
<td>23</td>
<td>77</td>
<td>0.052</td>
<td>16.4</td>
</tr>
</tbody>
</table>

Kojic acid yield (%), expresses kojic acid formed of initial glucose in medium.

The above results show that A. oryzae var.effusus NRC 14 produced more kojic acid than A. flavus NRC 13, A. tamarii NRC 18 and A. parasiticus when grown on static or shaken cultures, whereas the biomass dry weight produced by A.oryzae var.effusus NRC 14 was more than A. flavus NRC 13, A. tamarii NRC 18 and A. parasiticus when grown on shaken culture (Tables 1&2).

Kojic acid is produced by Aspergillus spp. belonging mainly to the section flavi (flavus-oryzae-tamarii), among them, A. flavus (Basappa et al., 1970; Ariff et al., 1996). A. oryzae (Kwak and Rhee, 1992, Tamizawa et al., 1996) was reported to have the ability to produce large amounts of kojic acid. A very high yield (that is, 0.453 g kojic acid / g glucose) was obtained in the fermentation using A. flavus (Ariff et al., 1997, Rosfarizan and Ariff, 2007). The mutation of A. oryzae ATCC 22788 via NTG treatment and UV irradiation, followed by protoplast found to improve kojic acid production (41 g/L) of about 100 times been reported that kojic acid can be easily conjugated (Demain, 1973). The highest yield of kojic acid (0.6 g kojic acid / g glucose) was obtained in the fermentation using glucose as a carbon source by various kojic acid producing strains (Kitada et al., 1967). Thus, glucose is not only used as a carbon source for biomass built-up, but it is also used as a precursor for kojic acid synthesis (Arnstein and Bentley, 1956; Kitada and Fukimbara, 1971).

The results obtained indicated that static cultures of two strains (A. oryzae var.effusus NRC 14 and A. flavus NRC 13) gave more kojic acid than the shaken ones at 150 rpm. These results agreed with the results of Wei et al., (1991), who found that A. candidus ATCC44054 grown without agitation produced more kojic acid in the
modified Czapek-Dox liquid medium than cultures shaken at 150 rpm. Saad et al., (1996), found that a static culture of A. parasiticus produced more kojic acid than a shaken culture one at 150 rpm. Also, Ogawa et al., (1995), found that the amount of kojic acid increased more than that obtained by means of the culture in the shaken flasks.

The results mentioned above indicated that the maximum kojic acid production by A. oryzae var. effusus NRC14 and A. flavus NRC13 was achieved when the initial pH of production medium which containing glucose as a carbon source and ammonium nitrate as the nitrogen source was 4.0. Most studies conducted on the effects of culture pH towards the growth and production of kojic acid was based on the initial culture pH (Lin et al., 1976; Clevstrom and Ljunggren, 1985). The maximum kojic acid production was achieved at pH 3.08 when ammonium nitrate was used as the nitrogen source (Kitada et al., 1967).

**Characterization and Examination of A. oryzae var. effusus NRC14 and A. flavus NRC 13:**

Characterization and Examination of two strains producing high amounts of kojic acid are based on such morphological characteristics as the diameter, color, and texture of the colony, the size and texture of conidia, and the conidiophores structure. The data are illustrated in Tables (3 & 4) and Figs. (2&3).

### Table 3: Characterization and Examination of A. oryzae var. effusus NRC14.

<table>
<thead>
<tr>
<th>Character</th>
<th>Examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture exam:</td>
<td></td>
</tr>
<tr>
<td>Growth characteristics</td>
<td>Colonies on Czapek agar at 25 °C attaining a diameter of 3.0-4.5 cm within 7 days gives greenish yellow colonies.</td>
</tr>
<tr>
<td>Microscopic exam:</td>
<td></td>
</tr>
<tr>
<td>Vesicle diam.</td>
<td>Sub-globes 18.9 μm in diameter.</td>
</tr>
<tr>
<td>Sterigmata</td>
<td>Uniseriate 9.2X3.0 μm</td>
</tr>
<tr>
<td>Conidiophores diam.</td>
<td>10.8 μm in diameter.</td>
</tr>
<tr>
<td>Conidia</td>
<td>Globose, smooth, 6.5 μm in diameter.</td>
</tr>
</tbody>
</table>

### Table 4: Characterization and Examination of A. flavus NRC14.

<table>
<thead>
<tr>
<th>Character</th>
<th>Examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture exam:</td>
<td></td>
</tr>
<tr>
<td>Growth characteristics</td>
<td>Colonies on Czapek agar at 25 °C growing rapidly yellowish green colonies with yellow to pale brown reverse.</td>
</tr>
<tr>
<td>Microscopic exam:</td>
<td></td>
</tr>
<tr>
<td>Vesicle diam.</td>
<td>Sub-globes 25.9 μm in diameter.</td>
</tr>
<tr>
<td>Sterigmata</td>
<td>Uniseriate 10X4.2 μm</td>
</tr>
<tr>
<td>Conidiophores diam.</td>
<td>10.2 μm in diameter.</td>
</tr>
<tr>
<td>Conidia</td>
<td>Globose, smooth, 4.4 μm in diameter.</td>
</tr>
</tbody>
</table>

**Fig. 2:** Aspergillus oryzae var. effusus.

**Fig. 3:** Aspergillus flavus.
Aflatoxins, B₁, B₂, G₁, and G₂ were observed in culture filtrates of *A. flavus* NRC13 and *A. parasiticus*, whereas no aflatoxins were detected in *A. oryzae var. effusus* NRC14 culture filtrate (Fig. 4a, b, c).

These results agreed with the results of Wogan *et. al.*, (1966), who reported that *A. flavus* is known invade agricultural products and produce a group of toxic metabolites known as aflatoxins. Also, Basappa *et. al.*, (1970) found that resting *A. flavus* synthesized more aflatoxin in addition to kojic acid on a medium with glucose than they did with any other substrate tested. Saad *et. al.*, (1996) suggested that aflatoxins B₁,B₂,G₁ and G₂ were observed in the culture filtrate of *A. parasiticus* NRRRL6111, whereas Lee *et. al.*, (2006) reported that *A. oryzae var. effusus* isolated from Zea mays, USA synthesized kojic acid and has no record of producing aflatoxins. Thus, the isolated *A. oryzae var. effusus* NRC14 was selected for the subsequent experiment.

*Aspergillus oryzae var. effusus* NRC14 biomass recycling for successful of kojic acid production:

It is apparent that *A. oryzae var. effusus* NRC14 can be first grown in a glucose salts medium (original medium) until develops the ability to synthesize kojic acid, and then it can be resuspended into a simple fermentation medium for successful production of kojic acid even on a commercial scale. On the basis of the data present in Table (5), it can be calculated that an intact mycelium resuspended in a glucose salts medium or in citrate phosphate buffer, buffer supplemented with 10% glucose only or buffer supplemented with 10% glucose and 0.112% NH₄NO₃ yield 0.346, 0.0, 0.123 and 0.401 g kojic acid/g glucose consumed, respectively, as compared with 0.626 g kojic acid/g glucose consumed obtained during the first growth in the production medium, in addition to increase the intact mycelia and glucose consumption in both original medium and buffer supplemented with glucose and nitrogen (10.6g, 11.0g wet weight), (9.25, 7.98 g glucose/100 ml), respectively.

The data show that resuspension of the intact mycelium does not impair its ability to synthesize kojic acid in glucose salts medium or buffer supplemented with glucose and nitrogen. The data presented in Table (5) indicate the need for external substrates such as carbon and nitrogen sources for kojic acid production and biomass built up. These results was similar with the results of Bajpai *et. al.*, (1982). Who suggested that, limitation of nitrogen source is required to suppress the growth during resuspended cell system, so that, more glucose can be converted to kojic acid. Also, Kwak and Rhee (1992) found that the rate of kojic acid production and glucose consumption by immobilized cell cultures, increased proportionally with the increase in the nitrogen content.

For kojic acid recovery, *Aspergillus oryzae var. effusus*NRC14 culture broth was mainted under refrigeration (5°C), after one night of storage we observed that many yellowish-brown needles crystals were present in the flasks (Fig.5), for further kojic acid extraction, the soluble kojic acid in the broth was extracted by using ethyl acetate, evaporation of the ethyl acetate extract yields crystalline kojic acid in the form of needles.

<table>
<thead>
<tr>
<th>Resuspension media</th>
<th>Final pH</th>
<th>Wet weight of biomass before resuspension</th>
<th>Wet weight of biomass after resuspension</th>
<th>Kojic acid (g/100ml)</th>
<th>Consumed glucose (g/100ml)</th>
<th>g Kojic acid / g glucose consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose salts (original)</td>
<td>4.40</td>
<td>5.05</td>
<td>10.6</td>
<td>3.20</td>
<td>9.25</td>
<td>0.346</td>
</tr>
<tr>
<td>Citrate phosphate buffer</td>
<td>4.00</td>
<td>4.36</td>
<td>5.5</td>
<td>0.00</td>
<td>0.00</td>
<td>0.000</td>
</tr>
<tr>
<td>Citrate phosphate buffer +10%glucose</td>
<td>4.30</td>
<td>4.60</td>
<td>5.2</td>
<td>0.17</td>
<td>1.38</td>
<td>0.123</td>
</tr>
<tr>
<td>Citrate phosphate buffer +10%glucose +0.112%NH₄NO₃</td>
<td>4.50</td>
<td>5.46</td>
<td>11</td>
<td>3.20</td>
<td>7.98</td>
<td>0.401</td>
</tr>
</tbody>
</table>
The crystals were collected, dried at 80 °C for 24 h. An average of 39.0 g of crystals/L was obtained. They were combined and purified by repeated crystallization from a mixture of water and acetone, yielding colorless long needles. The extracted crystals gave wine-red color with ferric chloride solution and the appearance of red color was taken as kojic acid (Fig.6). These results agreed exactly with the results found by Lin et al., (1976).

**Fig. 5:** Showed the precipitated kojic acid crystals of the form of needles after one night of storage at 5 °C.

**Fig. 6:** Showed the extracted crystals gave a wine red color with ferric chloride reagent.

**Identification of extracted kojic acid:**

A purity of the crystalline kojic acid was determined by HPLC (Shimadzu Class-VP V5.03) and it was 99%. The purified crystals was identified by its melting point (m.p), UV absorption spectrum and infrared spectrum. The m.p (in °C ) has given as 152-153 °C , the same result was observed in case of pure kojic acid obtained from Riedel- De Haen AG Seelze - Hannover and it agreed with Barnard and Challenger (1949), who found that m.p of kojic acid crystals in the form of colorless, prismatic needles was 152-153 °C. The infrared spectra of extracted acid and its standard mentioned above are given in (Fig.7).

**Fig. 7:** Infrared spectra of (a) standard kojic acid, (b) extracted kojic acid by cooling at 5 °C, and (c) extracted kojic acid by ethyl acetate.
It is evident that the extracted kojic acid has identical spectra to those of the standard kojic acid. The pyrone-carbonyl frequency of kojic acid was 1651 cm\(^{-1}\), the carbonyl group of the 5-acetate group was at 1760 cm\(^{-1}\). The infrared spectrum of kojic acid showed double-bound vibration at 1577 cm\(^{-1}\) and 1605 cm\(^{-1}\) (Kuhn, 1950). Also, the extracted kojic acid has identical peak of UV absorption spectrum to that of the standard kojic acid; the maximum peak was 282 nm (Fig. 8).

These results were agreed with the results of Choi \textit{et al.}, (2002) and Watanabe-Akanuma \textit{et al.}, (2007) who suggested that kojic acid has a maximum peak of ultraviolet absorption spectra at 280-284 nm.

\textbf{Fig. 8:} UV absorption spectra of (a) standard kojic acid, (b) extracted kojic acid by cooling at 5 °C, and (c) extracted kojic acid by ethyl acetate.

\textbf{References}


