Optimization of Cultural Parameters for Cost Effective Production of Kojic Acid by Fungal Species Isolated from Soil

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Novelty of Work

A novel soil isolated fungus Aspergillus flavus FJ537130 was used for kojic acid production. Twelve different cost effective carbon sources were used for the production of kojic acid for the first time and tested for large scale production of value added product like kojic acid.

Introduction

Kojic acid covers a wide range of applications in various fields like food, pharmaceutical, cosmetics, medical, chemical industries etc., It is mainly used as a key ingredient in the preparation of skin care products because it acts as a skin lightening and de-pigmenting agent [1]. It is utilized as a food additive to impede enzymatic browning and used during the preparation of foods like miso, soy sauce, sake etc., [2]. In kojic acid the word ‘koji’ was the name of the starter culture which was widely used in the preparation of oriental fermented food products. Chemically it is called 5-hydroxy-2-hydroxymethyl-γ-pyrone [3]. Numerous fungal strains have been used for the production of kojic acid which includes 19 different Aspergillus species and five Penicillium species and few bacteria like Bacterium xylinoides, Gluconoacetobacteropacus var. mobilis and Gyrinium roseum [4]. Different types of raw materials were used by the earlier researchers to obtain better yields of kojic acid which include various synthetic carbon sources like glucose, sucrose, maltose, xylose, alcohols [5] agro waste by-products, fruit wastes, vegetable wastes, industrial wastes etc., [6]. The biosynthesis of kojic acid through fermentation by Aspergillus often involves the direct conversion of glucose through multistep enzymatic reactions. The following enzymes Glucose-6-phosphate dehydrogenase, Hexokinase and Gluconate dehydrogenase which constitutes a cell bound enzyme system was involved in kojic acid biosynthesis [7,8]. Glucose act as a precursor for kojic acid biosynthesis and production ceases when all the glucose molecules were depleted in the medium [9]. It was identified that, three genes KojR, KojA, KojT which encode transcription factor, kojic acid synthesizing enzyme and a transporter were responsible for kojic acid biosynthesis [10]. Till now it was reported that the better source for the production of kojic acid was glucose. The outstanding applications of kojic acid commercially in various industries and its growing demand world-wide, lead to an extensive research by a number of researchers to find the most applicable and cost-effective method for production of kojic acid. In view of the fermentation economics, any bio production at the industrial level was significantly influenced by the raw material used. The current research provides a cost-effective production of kojic acid in terms of using novel and economic carbon sources to reduce the production cost less than USD 10.00 per kg which serves a market with strong prospects of growth. In Indian market, the cost of kojic acid was USD 3.50 per gram (M/s Himedia laboratories Pvt. Ltd., Visakhapatnam). In the present study the production cost of kojic acid was approximately USD 6.25 with 1 kg of Sago starch substrate and 285 g of kojic acid crystals were obtained. According to Futamura et al. [11] the production cost of kojic acid was USD 9.50 with substrate Corn starch. So the production cost of kojic acid when compared to Futamura et al. [11] was reduced by 35% in the present study. Futamura et al. [11] reported 40 g/l of kojic acid from Corn starch whereas the present study reported 90.8 g/l of kojic acid from Sago starch with Bentley’s method. Hence the production rate of kojic acid in the present study was twice compared to Futamura et al. [11]. From these findings it can be concluded that, the present study was the most reliable and cost-effective method for the production of kojic acid.

Abstract

A novel isolate capable of producing opulent yields of kojic acid with surface fermentation was screened from ten different soil fungi. The organism was confirmed as Aspergillus flavus FJ537130 strain using 18S rRNA based molecular analysis and it was identified as a negative producer of aflatoxin. Though twelve different carbon sources were used, significant yields of kojic acid crystals was noticed with Sago starch. The optimized conditions established were substrate concentration 1000 ml (100 g of starch powder in 1000 ml of H₂O), pH 6.0, Time 28 d, Temperature 28°C, Peptone concentration 4 g/l, KH₂PO₄ concentration 1 g/l, MgSO₄ concentration 0.5 g/l and the yield achieved was 28.5 g/l. The resulted fermentation broth was subjected to solvent extraction followed by gel filtration for the separation of kojic acid and crystallization. The structural characterization of purified kojic acid was confirmed by Proton NMR, FTIR and XRD. The molecular weight and purity of kojic acid was confirmed by LC/MS and HPLC. The isolated kojic acid crystals shows high antimicrobial activity against pathogenic bacteria Staphylococcus aureus and Escherichia coli and maximum zone of inhibition was 9 mm. The inhibitory effect of kojic acid was more on the cell line K562 (Leukemia) when compared to the MDAMB435S (Breast cancer) cell line.
Materials and Methods

The present investigation was planned to conduct a study for the production of kojic acid using soil fungi isolated from paddy soil, peanut soil and garden soil to make the fermentation process economical. Serial dilution technique [12] was employed for isolation. The isolated cultures were maintained and sub cultured in Czapek Dox agar medium at 4°C [13]. Lacto phenol cotton blue staining was used for fungal species identification [14]. The isolated fungal cultures were screened for kojic acid production using Ariff’s glucose medium [15] by employing two different fermentation techniques, surface and sub merged fermentation (100-250 rpm) grown at 28°C for 12 days. Bentley’s colorimetric method was used for the estimation of kojic acid in the fermented broth [16]. Later the higher yielding fungal strain was subjected to optimization process by OFAT strategy at various physicochemical conditions by using production medium designed by Ariff [15] but with replacement of glucose with starch substrates like Sago starch, Cassava, Alocasia macrorrhiza, Ipomea. All the four different starch substrates were subjected to preliminary starch hydrolysis procedure before used for the production [17]. The starch hydrolysis of Sago starch was performed by α-amylase enzyme (purchased from M/s. Coastal Chemical Enterprises Ltd., Visakhapatnam) at a concentration of 9.0 KNU/100 g suspension. The amount of reducing sugars released were estimated using 3,5-Dinitrosalicylic acid method [18]. Liquid substrates like coconut water, paner whey, palmyra sap, fruit based substrates like Musanga calabura, Cashew apple, agro waste by-products like sugarcane bagasse and bran substrates like rice bran and wheat bran. Very few high yielding substrates were selected based on OFAT optimization for statistical optimization using central composite design (CCD) and response surface methodology (RSM) with most significant parameters which influences the production [19-22]. Minitab version 16.0 was used for statistical analysis. To enhance the kojic acid yields from different substrates, the different cultural aspects were used for the increased production of kojic acid. The literature survey had revealed that, different types of enhancers were generally used to increase the yields of kojic acid by the fungal cultures and they include Copper-mononales-nicotinic acid complex and Copper-mononales-riboflavin complex [23], Cycasin or Methyl-azoxymethyl-β-D-glucose [24] Methanol [25]. Addition of Cu (I)–B3 complex to the fermentation medium enhances the yield to 47% by the fungus A. flavus [22]. The complex was utilized by the fungus biochemically in a manner related to that of niacin utilized naturally. According to Bajpai et al. [26] the enzymes glucose dehydrogenase and gluconate dehydrogenase were involved in kojic acid biosynthesis were NAD and NADP dependent produced from nicotinic acid or niacin. When methanol was added to the culture it will reduce the bubble size especially in stirred tank fermenters and increases the aeration rate. This may lead to increase in the production rate of the compound. Methanol at a concentration of 4% v/v, Cu (I)–B2 complex and Cu (I)–B3 complex at a concentration of 0.1 mg/ml were used. The samples were examined for the enhanced production after 24 hours of addition of enhancer. The concentration of kojic acid was determined by Bentley’s colorimetric method and the resulted broth samples were subjected to crystallization and purification process by Sephacryl S-200 gel filtration. The results were expressed in g/l of dry crystals for each and every carbon source. The structure of kojic acid was elucidated by using different biophysical analytical techniques like Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance spectroscopy (1H-NMR) and X-Ray diffraction spectroscopy. High pressure liquid chromatography (HPLC) was used to characterize the purity of the compound [18-21]. The isolated kojic acid crystals were tested for antimicrobial activity against different bacteria and tested for the anti proliferative activity using breast cancer cell lines MDAMB435S and leukemia cell line K562. These cell lines were obtained from National Centre for Cell Science, Pune. They were grown in a minimal essential medium (MEM, GIBCO) supplemented with 4.5 g/l glucose, 2 m ML-glutamine and 5% fetal bovine serum (FBS) growth medium at 37°C in 5% CO2 incubator. The standard MTT assay followed by Mosmann [27] was modified and used to determine the inhibitory effects of test compound kojic acid on cell growth in vitro.

Result and Discussion

Ten different species of soil fungi A. flavus, A. sojae, A. niger, Penicillium chrysogenum, A. terreus, Fusarium solani, A. fumigatus, A. nidulans, Mucor piriformis, A. oryzae were isolated and identified morphologically from three different soil samples paddy soil, peanut soil and garden soil. Out of these, A. flavus and A. sojae were found to be kojic acid producers. The yields obtained were 37.9 g/l by A. flavus and low yield by A. sojae 24.12 g/l. In submerged fermentation technique, A. flavus produced 5.53 g/l of kojic acid at 100 rpm agitation speed and no production was observed with A. sojae. Hence for further studies A. flavus was selected and it was a non-aflatoxin producer [18]. The organism was confirmed as A. flavus FJ537130 strain using 18S rRNA based molecular analysis. It showed 99% maximum identity using BLAST method.

Table 1 represent the kojic acid yields obtained from the 12 carbon sources at optimized process parameters. From the results of OFAT method, more than 50-75% increase in the kojic acid yield was identified after optimization. Maximum production was obtained with M. calabura fruits 86.06 g/l, sago starch 79.47 g/l and palmyra sap 76.31 g/l. The yield obtained with the sago starch 79.47 g/l in the present research was higher than the yield reported by the earlier studies. Potato starch, corn starch and sago starch are used for kojic acid fermentation with the kojic acid producing fungal strain A. flavus S33-2 isolated from morning glory flower. The yield obtained was 1.7 g/l with potato starch, 19.2 g/l with corn starch and 0.3 g/l with sago starch [28]. It was reported that, 40 g/l of kojic acid was obtained with A. oryzae MK-107-39 strain using partially hydrolyzed corn starch supplemented with little amount of corn steep liquor. To enhance the production rate of kojic acid, fermentation was conducted in 8L stirred tank fermenter using gelatinized sago starch by A. flavus reported 16.43 g/l of kojic acid [9]. It was revealed that, maximum kojic acid production 40.67 g/l from potato starch using a potato mutant strain AFUV8 [29]. The fruits of M. calabura produce an opulent yield of kojic acid 86.06 g/l (Bentley’s method) higher than the formerly used fruit based substrates by the earlier researchers. El-kady et al. [6] used various fruit wastes, and reported maximum production 5.9 g/l with oranges, 3.1 g/l with apricot, 4.2 g/l from apple waste. Nurashikin et al. [30] used pine apple waste and reported maximum production 26.3 g/l of kojic acid. The differences in the production may be either due to the culture conditions or due to species differences [31]. The resultant fermented broth was subjected to purification and crystallization. The resultant kojic acid crystals were Sago starch 21.0 g/l, M. calabura 24.3 g/l and palmyra sap 21.94 g/l. Hence, these three substrates were selected for further statistical optimization including CCD and RSM. The resulted statistical optimization with substrate palmyra sap, sago starch and M. calabura fruits had revealed that the experimental values agreed well with the
actual predicted values obtained by regression model which proved the satisfactory validity of the model. The yield obtained as 21.94 g/l by palmyra sap, 21.0 g/l by sago starch and 24.3 g/l by *M. calabura* fruits [19-21]. The other substrates yielded Cassava 17.94 g/l, *Ipomea* 16.0 g/l, *A. macrorhiza* 12.9 g/l, rice bran 9.62 g/l, wheat bran 0.04 g/l, sugarcane bagasse 3.95 g/l, paneer whey 14.2 g/l, coconut water 16.8 g/l and cashew apple 15.9 g/l of kojic acid crystals.

<table>
<thead>
<tr>
<th>'C' Source</th>
<th>Substrate concentration</th>
<th>Peptone</th>
<th>Time</th>
<th>pH</th>
<th>Temperatur</th>
<th>MgSO₄</th>
<th>KH₂PO₄</th>
<th>Final production (g/l)</th>
<th>'p' value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipomea</td>
<td>1000 ml</td>
<td>3 g/l</td>
<td>28 d</td>
<td>5.5</td>
<td>28°C</td>
<td>0.7 g/l</td>
<td>1.5 g/l</td>
<td>53.9 ± 0.2</td>
<td>0.00005</td>
</tr>
<tr>
<td></td>
<td>44.2 ± 0.36</td>
<td>48.9 ± 0.6</td>
<td>29.7 ± 1.12</td>
<td>8.27 ± 0.59</td>
<td>30.4 ± 0.6</td>
<td>7.31 ± 0.42</td>
<td>5.16 ± 0.3</td>
<td>53.9 ± 0.2</td>
<td>0.00005</td>
</tr>
<tr>
<td>Cassava</td>
<td>1000 ml</td>
<td>2 g/l</td>
<td>28 d</td>
<td>5.5</td>
<td>28°C</td>
<td>0.7 g/l</td>
<td>1.5 g/l</td>
<td>65.59 ± 0.5</td>
<td>0.00004</td>
</tr>
<tr>
<td></td>
<td>25.18 ± 0.36</td>
<td>25.9 ± 0.55</td>
<td>27.3 ± 0.55</td>
<td>59.8 ± 0.66</td>
<td>10.15 ± 0.37</td>
<td>6.81 ± 0.25</td>
<td>3.46 ± 0.4</td>
<td>79.47 ± 0.8</td>
<td>0.00004</td>
</tr>
<tr>
<td>Sago</td>
<td>1000 ml</td>
<td>4 g/l</td>
<td>28 d</td>
<td>5.5</td>
<td>28°C</td>
<td>0.5 g/l</td>
<td>1 g/l</td>
<td>40.13 ± 0.9</td>
<td>0.00019</td>
</tr>
<tr>
<td></td>
<td>71.38 ± 0.59</td>
<td>82.5 ± 1.55</td>
<td>66.5 ± 1.81</td>
<td>50.6 ± 1.09</td>
<td>52.6 ± 1.57</td>
<td>12.68 ± 0.87</td>
<td>4.62 ± 0.09</td>
<td>76.31 ± 0.5</td>
<td>0.00001</td>
</tr>
<tr>
<td>Alocasia</td>
<td>1000 ml</td>
<td>2 g/l</td>
<td>21 d</td>
<td>28°C</td>
<td>0.5 g/l</td>
<td>1 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
<td>50.9 ± 2.58</td>
</tr>
<tr>
<td>Palmyra sap</td>
<td>1000 ml</td>
<td>3 g/l</td>
<td>32 d</td>
<td>28°C</td>
<td>0.5 g/l</td>
<td>2 g/l</td>
<td>53.9 ± 0.2</td>
<td>1.5 g/l</td>
<td>79.47 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
<td>57.91 ± 0.68</td>
<td>50.9 ± 2.58</td>
<td>19.3 ± 0.94</td>
<td>4.23 ± 0.2</td>
<td>3.81 ± 0.1</td>
<td>42.7 ± 0.3</td>
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<tr>
<td>Paneer whey</td>
<td>1000 ml</td>
<td>2 g/l</td>
<td>21 d</td>
<td>28°C</td>
<td>0.3 g/l</td>
<td>1 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
<td>50.9 ± 2.58</td>
</tr>
<tr>
<td>Coconut water</td>
<td>1000 ml</td>
<td>4 g/l</td>
<td>16 d</td>
<td>28°C</td>
<td>0.3 g/l</td>
<td>1 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
<td>50.9 ± 2.58</td>
</tr>
<tr>
<td>M.calabura</td>
<td>1000 ml</td>
<td>4 g/l</td>
<td>28 d</td>
<td>28°C</td>
<td>0.7 g/l</td>
<td>2 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
<td>50.9 ± 2.58</td>
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<tr>
<td></td>
<td>65.32 ± 1.45</td>
<td>60.47 ± 1.36</td>
<td>73.6 ± 1.66</td>
<td>28.63 ± 0.36</td>
<td>28.8 ± 2.01</td>
<td>42.5 ± 1.64</td>
<td>31.17 ± 1.77</td>
<td>36.4 ± 1.2</td>
<td>37.4 ± 1.43</td>
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<tr>
<td>Cashew apple</td>
<td>500 ml</td>
<td>4 g/l</td>
<td>16 d</td>
<td>28°C</td>
<td>0.7 g/l</td>
<td>1.5 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
<td>50.9 ± 2.58</td>
</tr>
<tr>
<td>Sugar cane bagasse</td>
<td>30 g/l</td>
<td>4 g/l</td>
<td>21 d</td>
<td>5.5</td>
<td>28°C</td>
<td>0.7 g/l</td>
<td>1.5 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
</tr>
<tr>
<td>Rice bran</td>
<td>10 g/l</td>
<td>4 g/l</td>
<td>21 d</td>
<td>5.5</td>
<td>28°C</td>
<td>0.7 g/l</td>
<td>1.5 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
</tr>
<tr>
<td>Rice bran</td>
<td>10 g/l</td>
<td>4 g/l</td>
<td>16 d</td>
<td>30°C</td>
<td>0.7 g/l</td>
<td>2 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
<td>50.9 ± 2.58</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>20 g/l</td>
<td>4 g/l</td>
<td>16 d</td>
<td>5.5</td>
<td>28°C</td>
<td>0.7 g/l</td>
<td>1.5 g/l</td>
<td>47.21 ± 0.31</td>
<td>78.5 ± 1.65</td>
</tr>
</tbody>
</table>

When the isolated culture was treated with 3 different types of enhancers, the culture showed enhanced production to methanol rather than copper complexes and the yield was increased up to 1-12%. In this report, particularly the production rate was increased in starch substrates followed by bran substrates in response to enhancers than other substrates used. The enhanced yield was showed by the substrates sago starch 28.5 g/l of kojic acid crystals with methanol followed by *M. calabura* fruits 24.71 g/l and palmyra sap 22.83 g/l. Since sago starch released 46.2 g/l of reducing sugars and upon fermentation the sugars were converted to give 28.5 g/l of kojic acid crystals. The ripened fruits of *Muntinga calabura* L. contain 170 g of carbohydrates per 1 kg of fruits which were converted to give 24.71 g/l of kojic acid crystals and on the other hand palmyra sap contain 10.93 g/100 cc of total sugars which were converted to give 22.83 g/l of kojic acid crystals. Elevated production of kojic acid obtained from other substrates cassava 20.3 g/l, *Ipomea* 19.3 g/l, coconut water 17.9 g/l, cashew apple 16.8 g/l, *A. macrorhiza* 12.9 g/l, rice bran 9.62 g/l, wheat bran 0.04 g/l, sugarcane bagasse 3.95 g/l, paneer whey 14.2 g/l, coconut water 16.8 g/l and cashew apple 15.9 g/l of kojic acid crystals.
macrorhiza 16.28 g/l, rice bran 14.9 g/l, paneer whey 14.5 g/l, sugarcane bagasse 4.85 g/l and wheat bran 2.06 g/l. The elution profile of kojic acid from Sephacryl S-200 gel filtration chromatography showed that kojic acid was eluted in fractions 54-56 as a single peak.

The resultant eluted sample was subjected to crystallization and purification and produces a long needle shaped crystals. The FTIR spectrum of test kojic acid sample obtained after purification (Figure 1) showed the peak wave number values for the functional groups similar to that of standard kojic acid.

**Figure 1:** FTIR spectrum of Kojic acid test sample.

The bands appear for functional groups at 3270.8 cm\(^{-1}\), 3179.43 cm\(^{-1}\) (-OH), 2925.17 cm\(^{-1}\), 2854.05 cm\(^{-1}\) (aliphatic-CH), 1660.59 cm\(^{-1}\) (cyclic -C=O), 1611.11 cm\(^{-1}\) (C=C), 1472.61 cm\(^{-1}\) (deformation of -CH\(_2\)), 1074.04 cm\(^{-1}\) (1,4α-disubstituted ring). Whereas the standard sample showed peaks at 3271 cm\(^{-1}\), 3178 cm\(^{-1}\) (-OH), 2926 cm\(^{-1}\), 2866 cm\(^{-1}\) (aliphatic-CH), 1661 cm\(^{-1}\) (cyclic -C=O), 1611.11 cm\(^{-1}\) (C=C), 1474 cm\(^{-1}\) (deformation of -CH\(_2\)), 1076 cm\(^{-1}\) (cyclic C-O-C), 944 cm\(^{-1}\), 866 cm\(^{-1}\) and 778 cm\(^{-1}\) (1, 4 α-disubstituted ring).

The 1H NMR of kojic acid crystals showed (Figure 2) 4 characteristic protonic signals of varying size intensity and few smaller signals. The four peaks obtained at 9.03 (s, 1H), 6.332 (s, 1H), 4.286 (s, 2H), 3.340 (s, OH).

**Figure 2:** Proton NMR of test kojic acid sample.

From the results of HPLC (Figure 5) it was evaluated that, the purity of the compound achieved was 92% where as the standard sample...
Maximum zone of inhibition (9 mm) was observed with the cultures *Staphylococcus aureus* and *Escherichia coli* followed by *B. subtilis* (8 mm) indicates that these organisms were highly sensitive to the antimicrobial compound kojic acid [18]. The compound kojic acid exhibits anti-proliferative activity on the breast cancer cell lines MDA-MB435S and leukemia cell lines K562 (Figure 6 and 7).

The percentage of inhibitory activity for the breast cancer cell line was 22.32% at 100 μg/ml concentration and for the leukemia it was 67.07% at 200 μg/ml concentration.

**Conclusion**

Based on these findings, using of waste carbon sources has proved the auspicious potentiality in exploiting the alternate sources for higher production of kojic acid by *Aspergillus flavus* FJ537130 through surface fermentation. Out of different carbon sources used, elevated yields of kojic acid was noticed with sago starch, *M. calabura* and palmyra sap. Kojic acid crystals showed antimicrobial activity and anti-cancerous activity against pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli* and inhibitory effect was more on the cell line K562 (leukemia) when compared to the MDA-MB435S (breast cancer) cell line. The study in fact helps to scale-up the kojic acid fermentation to large-scale level in order to produce the expensive chemical like kojic acid with low production cost from economical raw materials.

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