Research Article

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

Durgadevi KB[*], Vijayalakshmi P, Shilpa V and Kumar BV

Department of Biotechnology, GITAM Institute of Sciences, GITAM University, Visakhapatnam, Andhra Pradesh, India

[*]**Corresponding author:** Durgadevi KB, Research Scholar, Department of Biotechnology, GITAM Institute of Sciences, GITAM University, Visakhapatnam, Andhra Pradesh, India, Tel: +919866182496; Fax: +918912790399; E-mail: kbaladurgadevi@gmail.com

Abstract

A novel isolate capable of producing opulent yields of kojic acid with surface fermentation was screened from ten different isolated soil fungi. The organism was confirmed as *Aspergillus flavus* FJ537130 strain using 18SrRNA 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 H_2O), pH 6.0, Time 28 d, Temperature 28°C, Peptone concentration 4 g/l, KH_2PO_4 concentration 1 g/l, $MgSO_4$ 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.

Keywords: *Aspergillus flavus* FJ537130; One factor at a time method; Response surface methodology; Structural characterization; Anti proliferation activity; Fermentation

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 *Pencillium* species and few bacteria like *Bacterium xylinoides*, *Glucono-acetobacteropacus 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.

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 straining 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 identified using 18SrRNA based molecular analysis and further 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-Dinitrosalicyclic acid method [18]. Liquid substrates like coconut water, paneer whey, palmyra sap, fruit based substrates like *Muntingia 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 twelve different sources, three different enhancers were tested 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-monovales-nicotinic acid complex and Copper-monovales-riboflavin complex [23], Cycasin or Methyl-azoxymethyl-β-D-glucose [24] Methanol [25]. Addition of Cu (I)-B$_3$ 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)–B$_2$ complex and Cu (I)–B$_3$ 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 (^{1}H-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% CO_2 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. terrus, 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 18SrRNA 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* Link 44-1 and 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. macrorrhiza* 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.

'C' Source	Substrate concentration	Peptone	Time	pH	Temperature	MgSO$_4$	KH$_2$PO$_4$	Final production (g/l)	'p' value
Ipomea	1000 ml	3 g/l	28 d	5.5	28°C	0.7 g/l	1.5 g/l	53.9 ± 0.2	0.000005
	44.2 ± 0.36	48.9 ± 0.6	29.7 ± 1.12	8.27 ± 0.59	30.4 ± 0.6	7.31 ± 0.42	5.16 ± 0.3		
Cassava	1000 ml	2 g/l	28 d		28°C	0.5 g/l	2 g/l	65.59 ± 0.5	0.00002
	25.18 ± 0.36	25.9 ± 0.55	27.3 ± 0.55	59.8 ± 0.66	10.15 ± 0.37	6.81 ± 0.25	3.46 ± 0.4		
Sago	1000 ml	4 g/l	28 d		28°C	0.5 g/l	1 g/l	79.47 ± 0.8	0.00004
	71.38 ± 0.59	82.5 ± 1.55	66.5 ± 1.81	50.6 ± 1.09	52.6 ± 1.57	12.68 ± 0.87	4.62 ± 0.09		
Alocasia	1000 ml	2 g/l	21 d		28°C	0.5 g/l	1 g/l	40.13 ± 0.9	0.00019
	34.73 ± 0.8	3.86 ± 0.14	36.8 ± 1.1	4.52 ± 0.14	22.4 ± 0.55	3.64 ± 0.17	3.65 ± 0.4		
Palmyra sap	1000 ml	3g/l	32 d		28°C	0.5 g/l	2 g/l	76.31 ± 0.5	0.00001
	47.21 ± 0.31	78.5 ± 1.65	75.91 ± 0.68	50.9 ± 2.58	6.46 ± 0.15	5.59 ± 0.11	3.84 ± 0.19		
Paneer whey	1000 ml	2 g/l	21 d		28°C	0.3 g/l	1 g/l	42.7 ± 0.3	0.00002
	6.84 ± 0.06	3.53 ± 0.07	42.2 ± 1.21	3.84 ± 0.1	19.3 ± 0.94	4.23 ± 0.2	3.81 ± 0.1		
Coconut water	1000 ml	4 g/l	16 d		28°C	0.3 g/l	1 g/l	48.13 ± 1.1	0.0001
	30.8 ± 1.42	46.7 ± 1.5	46.1 ± 1.75	36.4 ± 1.2	15.2 ± 1.81	37.4 ± 1.43	3.76 ± 0.16		
M.calabura	1000 ml	4 g/l	28 d		28°C	0.7 g/l	2 g/l	86.06 ± 0.9	0.00003
	65.32 ± 1.45	60.47 ± 1.36	73.6 ± 1.66	28.63 ± 0.36	28.8 ± 2.01	42.5 ± 1.64	31.17 ± 1.77		
Cashew apple	500 ml	4 g/l	16 d		28°C	0.7 g/l	1.5 g/l	46.3 ± 1.41	0.0003
	16.59 ± 1.77	40.65 ± 1.03	42.67 ± 1.91	19.2 ± 0.67	34.1 ± 5.07	20.4 ± 2.13	21.5 ± 2.1		
Sugar cane baggase	30 g/l	4 g/l	21d	5.5	28°C	0.7 g/l	1.5 g/l	17.8 ± 0.7	0.0005
	19.6 ± 1.67	8.56 ± 1.67	1.32 ± 0.22	12.69 ± 2.1	15.52 ± 0.97	9.5 ± 0.86	6.72 ± 1.15		
Rice bran	10 g/l	4 g/l	21 d	5.5	28°C	0.7 g/l	1.5 g/l	17.8 ± 0.7	0.0005
	25.5 ± 2.07	15.6 ± 1.83	1.32 ± 0.22	12.69 ± 2.1	15.52 ± 0.97	9.5 ± 0.86	6.72 ± 1.15		
Rice bran	10 g/l	4 g/l	16 d		30°C	0.7 g/l	2 g/l	30.7 ± 1.2	0.0005
	25.5 ± 2.07	15.6 ± 1.83	28.3 ± 1.13	21.41 ± 1.89	10.96 ± 0.55	15.86 ± 1.97	21.04 ± 1.56		
Wheat bran	20 g/l	4 g/l	16 d	5.5	28°C	0.7 g/l	1.5 g/l	0.95 ± 0.19	0.01
	0.77 ± 0.14	0.64 ± 0.16	0.84 ± 0.29	0.803 ± 0.28	0.82 ± 0.54	0.4 ± 0.01	0.38 ± 0.02		

Table 1: Kojic acid concentrations at optimized cultural parameters.

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 substrate 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 16.87 g/l, *A.*

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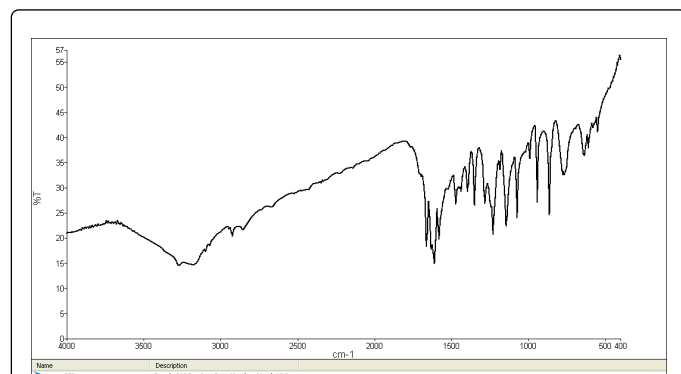
Figure 1: FTIR spectrum of Kojic acid test sample.

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} (cyclic C-O-C), 943.58 cm^{-1}, 863.66 cm^{-1} and 775.65 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 ^{1}H 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).
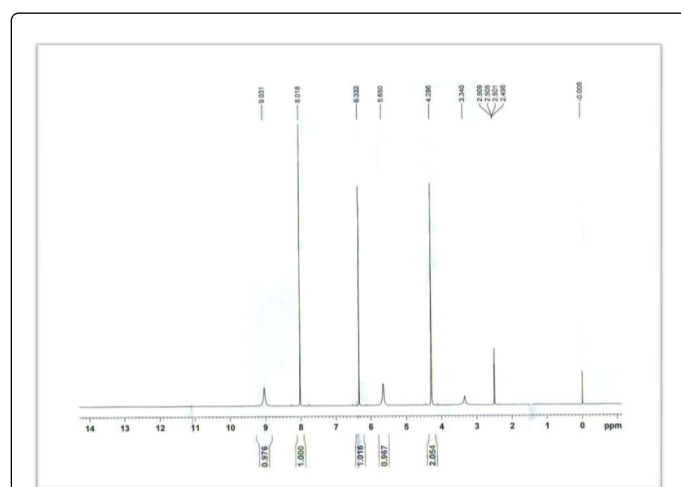

Figure 2: Proton NMR of test kojic acid sample.

The result was similar to standard sample. Peaks appeared at 9.03 (s, 1H), 6.24 (s, 1H), 4.16 (s, 2H), 3.65 (s, OH). The X-ray diffraction spectrum of test kojic acid sample showed seven characteristic peaks at 2θ angles of 9°, 22°, 26°, 31°, 33°,38° and 42° (Figure 3).
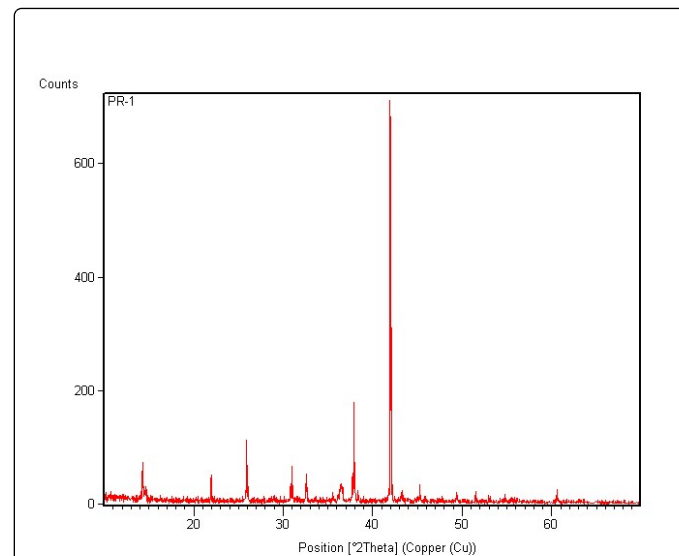

Figure 3: X-ray diffractogram of test kojic acid sample.

The X-ray diffraction spectrum of the standard kojic acid sample showed seven distinctive peaks were appeared at 2θ angles 9°, 22°, 26°, 31°, 38°, 42°. The resultant mass spectra of kojic acid crystal showed that the molecular ion peak was located at 142.07 (Figure 4), which confirmed the presence of kojic acid because the molecular weight was similar to standard kojic acid (kojic acid mass spectrum, Mass bank record JP003237).
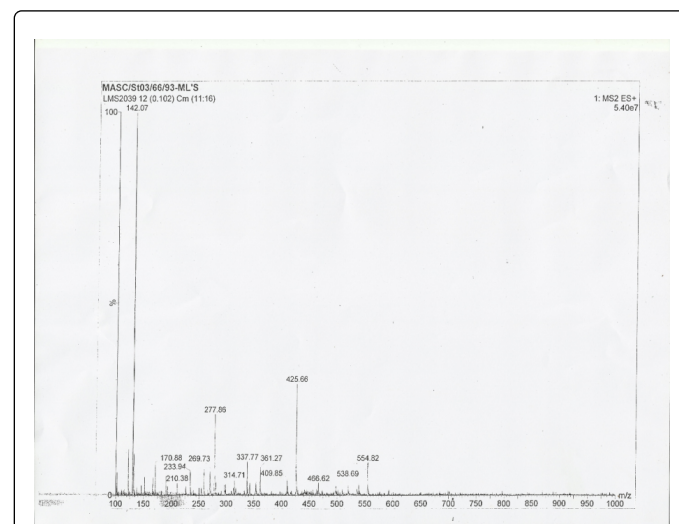

Figure 4: Mass spectrum of kojic acid.

From the results of HPLC (Figure 5) it was evaluated that, the purity of the compound achieved was 92% where as the standard sample

shows 97.2% purity. The retention time of the test compound was 6.901 min whereas for standard it was 6.621.
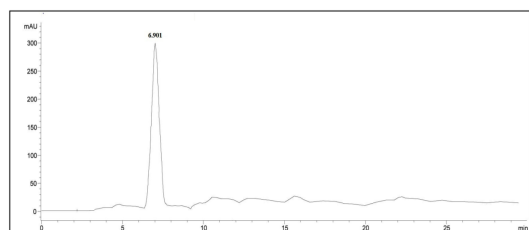

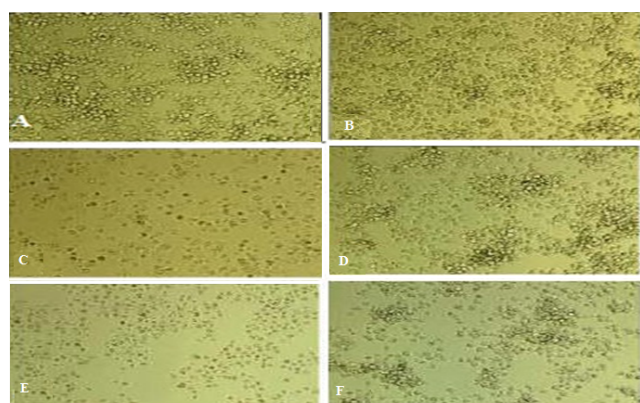**Figure 5:** Elution profile of test kojic acid sample by HPLC.

Figure 6: Activity of kojic acid on MDA-MB435S (breast cancer) cell lines before and after addition. A - Control; B - 12.5 µg/ml; C - 25 µg/ml; D - 50 µg/ml; E - 100 µg/ml; F - 200 µg/ml.
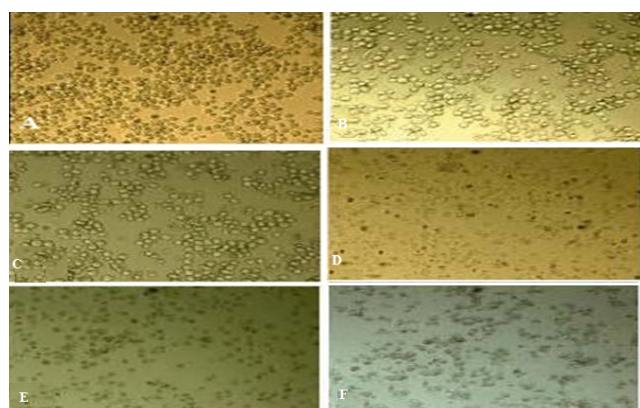

Figure 7: Activity of kojic acid on K562 (Leukemia) cell lines before and after addition. A - Control; B - 12.5 IU/ml; C - 25 IU/ml; D - 50 IU/ml; E - 100 IU/ml; F - 200 IU/ml.

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 MDAMB435S 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 MDAMB435S (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.

Acknowledgement

References

1. Ahn KS, Moon KY, Lee J, Kim YS (2003) Downregulation of NF-kappaB activation in human keratinocytes by melanogenic inhibitors. J Dermatol Sci 31: 193-201.

2. Wood BJB (1998) Microbiology of Fermented Food (2ndedn.) Springer, London.

3. Yabuta T (1924) The constitution of kojic acid, a d-pyrone derivative formed by *Aspergillusflavus* from carbohydrates. J Chem Soc 125: 575-587.

4. Arnstein HR, Bentley R (1953) The biosynthesis of kojic acid. II. The occurrence of aldolase and triosephosphate isomerase in *Aspergillus* species and their relationship to kojic acid biosynthesis. Biochem J 54: 508-516.

5. Barnard D, Challenger F (1949) The formation of kojic acid from ethyl alcohol by A. oryzae and the action of this mould on some carbohydrate derivatives. J chem Soc: 110-117.

6. El-Kady IA, Zohri AN, Hamed SR (2014) Kojic Acid production from agro-industrial by-products using fungi. Biotechnol Res Int 2014: 642385.

7. Arnstein HR, Bentley R (1953) The biosynthesis of kojic acid. I. Production from (1-14C) and (3:4-14C2) glucose and (2-14C)-1:3-dihydroxyacetone. Biochem J 54: 493-508.

8. Basappa SC, Sreenivasamurthy V, Parpia HA (1970) Aflatoxin and kojic acid production by resting cells of *Aspergillus flavus* Link. J Gen Microbiol 61: 81-86.

9. Rosfarizan M, Ariff A (2002) Importance of carbon source feeding and pH control strategies for maximum kojic acid production from sago starch by *Aspergillusflavus*. Journal of Bioscience and Bioengineering 94: 99-105.

10. Terabayashi Y, Sano M, Yamane N, Marui J, Tamano K, et al. (2010) Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*. Fungal Genet Biol 47: 953-961.

11. Futamura T, Ishihara H, Tamura T, Yasutake T, Huang G, et al. (2001) Kojic acid production in an airlift bioreactor using partially hydrolyzed raw corn starch. J Biosci Bioeng 92: 360-365.

12. Dubey RC, Maheshwari DK (2003) Practical microbiology (2ndedn.) S Chand Ltd, India.

13. Pitt JL, Hocking AD (2009) Fungi and Food spoilage (3rdedn.) Springer Link Publications, New York.

14. Mackie, McCartney (1996) Practical medical microbiology (14thedn.) Churchill Livingstone, London.

15. Ariff AB, Salleh MS, Gani B, Hassan MA, Rusul G, et al. (1996) Aeration and yeast extract requirements for kojic acid production by *Aspergillusflavus* Link. Enzyme and Microbial Technology l19: 545-550.

16. Bentley R (1957) Preparation and analysis of kojic acid. Methods in Enzymology 3: 238-249.

17. Padmaja G, Moorthy SN, Nambisan B, Babu L, Sundaresan S, et al. (2001) Analytical Metrologies for Tropical Tubercrops. Central tubercrops research institute, India, pp. 34-38.

18. Miller GL (1959) Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31: 426-429.

19. Devi BDK, Vijayalakshmi P, Shilpa V, Kumar VB (2015) Optimization of cultural parameters for cost effective production of kojic acid by fungal species isolated from soil. British Microbiology Research Journal 7: 255-268.

20. Devi BDK, Vijayalakshmi P, Kumar VB (2014) Response surface methodology for the optimization of kojic acid production by *Aspergillus flavus* using Palmyra sap as a carbon source. European Journal of Biotechnology and Bioscience 2: 52-57.

21. Devi BDK, Vijayalakshmi P, Kumar VB, Prasad VSSLT (2014) Statistical optimization of kojic acid production through response surface methodology by *Aspergillus flavus* using Sago starch hydrolysate as a carbon source. Asian Journal of Applied Science and Engineering 3: 53-60.

22. Devi BDK, Vijayalakshmi P, Shilpa V, Prasad VSSLT, Kumar VB (2015) Response surface methodology for the optimization of kojic acid production by *Aspergillus flavus* using Muntingiacalaburafruits as a carbon source. Indian Journal of Science and Technology 8: 556-561.

23. Megalla SE, Nassar AY, Gohar MA (1987) The role of copper(I)-nicotinic acid complex on kojic acid biosynthesis by Aspergillus flavus. J Basic Microbiol 27: 29-33.

24. Tadera K, Yagi F, Kobayashi (1985) Effects of cycasin on kojic acid-producing molds. Agric Biol Chem 49: 203-205.

25. Madihah MS, Ariff AB, Hassan MA, Rusul G, Karim MIA (1996) Enhanced kojic acid production by *Aspergillus flavus* Link in Growth medium containing methanol. ASEAN Food J 11: 158-162.

26. Bajpai P, Agrawala PK, Vishwanathan L (1981) Enzymes relevant to kojic acid biosynthesis in *Aspergillus flavus*. J Gen Microbiol 127: 131-136.

27. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.

28. Rosfarizan M, Madihah S, Ariff AB (1998) Isolation of a kojic acid producing fungus capable of using starch as a carbon source. Letters in Applied Microbiology 26: 27-30.

29. Abd-El-Aziz (2013) Improvement of kojic acid production by a mutant strain of *Aspergillus flavus*. Journal of Natural sciences Research 3: 31-41.

30. Nurashikin S, Rusley EZ, Husaini A (2013) Solid-state bio conversion of pineapple residues into kojic acid by *Aspergillus flavus*: A prospective study. World academy of Science, Engineering and Technology 7: 147-147.

31. El-Aasar SA (2006) Cultural conditions studies on kojic acid production by *Aspergillus parasiticus*. Int J Agric Biol 8: 468-473.