British Microbiology Research Journal
7(5): 255-268, 2015, Article no.BMRJ.2015.118
ISSN: 2231-0886

SCIENCEDOMAIN *international*
www.sciencedomain.org

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

Kayitha Bala Durga Devi[1*], Payala Vijayalakshmi[1], Vadlamani Shilpa[1] and Bapatla Veerendra Kumar[1]

[1]Department of Biotechnology, GITAM Institute of Sciences, GITAM University, Visakhapatnam-530045 (A.P.), India.

Authors' contributions

This work was carried out in collaboration between all authors. Author KBDD designed the study, performed the bench work, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors PV and VS managed the statistical analyses of the study and literature searches. Author BVK was the research guide of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2015/15325
Editor(s):
(1) Mehdi Razzaghi-Abyaneh, Department of Mycology, Pasteur Institute of Iran, Iran.
Reviewers:
(1) Ponchang Apollos Wuyep, Plant Science and Biotechnology, University of Jos, Nigeria.
(2) Anonymous, Turkey.
(3) Sang-Han Lee, Department of Food Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea.
(4) Anonymous, Malaysia.
(5) Rajagopalan Prabu, Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia, Department of Bioscience, Christian Eminent Academy, Indore 452001, India.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=992&id=8&aid=8719

Original Research Article

Received 20th November 2014
Accepted 18th March 2015
Published 8th April 2015

ABSTRACT

Two different species of *Aspergillus* are able to produce kojic acid at higher concentrations under static conditions in the present study. Various physicochemical parameters which influence the production of kojic acid are optimized by means of One-factor-at-a-time method. The other objective of this research is to examine the prospects of using novel substrates like Cassava, *Ipomea batatas*, *Alocasia macrorrhiza* tubers and rice bran, wheat bran for the production of value added products like kojic acid. The kojic acid concentration is quantitatively estimated by Bentley's colorimetric method. It is observed that the maximum yield of kojic acid crystals, 22.5 g/L is

**Corresponding author: E-mail: kbaladurgadevi@gmail.com;*

obtained with the substrate Cassava and by the isolate *Aspergillus flavus*. The optimized physicochemical parameters are initial pH - 6.0, Time - 28d, Temperature - 28ºC, Substrate concentration – 100 g/L, Nitrogen source concentration-2 g/L, $MgSO_4.7H_20$-0.5 g/L and KH_2PO_4-2.0 g/L under static conditions. After the fermentation is completed, the fermented broth samples are subjected to crystallization and the isolated kojic acid crystals are examined by Fourier transform infrared spectroscopy and X-ray crystallographic methods. The FTIR spectrum of the sample kojic acid showed bands at 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} (1,4 α -disubstituted ring). The X-ray diffraction spectrum of the sample is similar to standard sample and reveals seven distinctive peaks appeared at 2θ angles 8º, 19.27º, 21.71º, 27.73º, 31.15º, 36.13º, 39.09º. The result confirms the presence of kojic acid. Antimicrobial activity of kojic acid is tested on some Gram positive and Gram negative bacteria. The culture *Escherichia coli* and *Staphylococcus aureus* show equal susceptibility to the antimicrobial compound kojic acid.

Keywords: Kojic acid; fungi; physicochemical conditions; FTIR; X-ray crystallography.

1. INTRODUCTION

Industrial production of organic acids is eventuated by employing various microorganisms using different fermentative techniques. The specific fungi belonging to the genus *Aspergillus* are familiar in excess production of different organic acids like Citric acid, Itaconic acid, Gluconic acid etc. However the organic acids like Malic acid, Gibberellic acid, Kojic acid, Lactic acid etc., are produced in little quantities. These acids are highly advantageous in therapeutics, research and commercial purposes. In the year 1907, Saito isolated the kojic acid from the mycelial mat of *Aspergillus oryzae*. This contaminant is grown in steamed rice. Later the rice was popularly called as Koji and the name Kojic acid was invented in the year 1913 by scientist, Yabuta. He also proposed the kojic acid structure and described it as 5-hydroxy-2-hydroxy methyl-δ-pyranone [1]. Kojic acid is used as a key ingredient in the preparation of skin care products because it acts as a skin lightening and de-pigmenting agent [2]. Kojic acid reduces the melanin synthesis since it represses the catecholase activity of tyrosinase enzyme. It is utilized as a food additive to impede enzymatic browning and used in preparation of foods like miso, soy sauce, sake etc. As kojic acid has a powerful anti-oxidizing activity, it is used widely as preservative and flavour enhancer. Kojic acid also used to treat a disorder called Melasma. So many prospective studies have been developed to produce novel group of kojic acid derivatives which may be used as a potent pharmaceutical agents [2].

Due to its wide application in various industries like cosmetics, the demand for kojic acid has been tremendously increased. Various types of carbon sources like glucose, sucrose, acetate, ethanol, arabinose and xylose have been used for the production of kojic acid. But it is identified that glucose was the most suitable source of carbon for the production of kojic acid because of the structural similarity between glucose and kojic acid. During the synthesis of kojic acid through fermentation, kojic acid is directly produced from glucose without breakage of its carbon skeleton into shorter fragments. It is reported that polysaccharides like starch is the poor source of carbon for kojic acid production [2]. But one study had investigated and found that an isolated fungal strain *A. flavus* from morning glory flower is capable of producing very high yield of kojic acid using corn starch as carbon source. The authors used sago starch and potato starch as carbon sources in their studies [3]. The present research is the first attempt in using cost-effective substrates like Cassava tuber powder, *Ipomea batatas* tuber, *Alocasia macrorrhiza* tuber, Rice bran and Wheat bran for the production of kojic acid. The following enzymes, Glucose-6-phosphate dehydrogenase, Hexokinase and Gluconate dehydrogenase constituting a cell bound enzyme system are involved in the kojic acid biosynthesis. The pathway involves the intermediates, Gluconic acid-δ-Lactone and one of the three compounds Gluconic acid lactone, 3-keto glucose and oxy kojic acid [4]. Owing to the applications of kojic acid in various sectors and its growing demand in the world-wide, an extensive studies has been done by a number of researchers to establish the most pertinent and cost-effective method of kojic acid. Because the economy of any fermentation process at the industrial level was highly effected by the raw

material used, the present study is one such to provide a cost-effective process of kojic acid production using inexpensive carbon sources which have not been commercially exploited.

2. MATERIALS AND METHODS

2.1 Microrganisms

Two different species *Aspergillus flavus* and *Aspergillus sojae* maintained on Czapek Dox agar medium slants at 4ºC were used for the production of kojic acid.

2.2 Screening of Fungal Isolates for Production of Kojic Acid

The seed medium designed by Ariff et al. [5] was used for the preparation of inoculum. The composition of the medium is (g/L) glucose -50, yeast extract -5, KH_2PO_4 -1.0 and $MgSO_4.7H_2O$-0.5. The spore suspension (1×10^7) for inoculation was prepared by adding two drops of 0.1% Tween 80 solution into a test tube containing 10 ml of sterilized distilled water. The spores were carefully scraped from the CZA slants using an inoculation loop and transferred into a test tube. Five ml of spore suspension of each isolated fungal strain was added to 250 ml conical flask containing 50 ml of seed medium. All the flasks were incubated at 28ºC for 12 days. After fermentation, the medium was filtered and the mycelium was severally washed with water and then dried in a hot air oven at 80ºC for 24h. The dry weight of the mycelia mat was determined. The supernatant was subjected to colorimetric method for determination of Kojic acid [6]. Two different fermentation techniques, surface fermentation and submerged fermentation were used for the kojic acid production. Submerged fermentation was conducted in an orbital shaker (M/s. Remi Instruments Ltd.,) at different agitation speeds of 100, 150, 200, 250 rpm. The fungal organisms were later subjected to optimization process at various physicochemical conditions by using the production medium described above [5] replacing glucose with tuber and bran substrates.

2.3 Raw Materials

All the carbon sources were collected from local areas of Visakhapatnam, Andhra Pradesh, India.

2.3.1 Cassava

Cassava was commonly known as *Manihot esculenta* or tapioca root. One hundred grams of Cassava tuber possess an energy value of 670 KJ. It contains protein 1.4 g, fat 0.28 g, carbohydrates 38 g, fibre 1.8 g, sugar 1.7 g, vitamins and minerals. The starch powder was purchased from M/s. Trinethra Supermarket, Visakhapatnam.

2.3.2 *Ipomea*

The dicotyledonous plant, *Ipomea batatas* was commonly called as sweet potato. One hundred grams of raw tuber contains an energy value of 359KJ. Its composition carbohydrates 20.1 g, starch 12.7 g, sugar 4.2 g, dietary fibre 3 g, fat 0.1 g, protein 1.6g and also contains vitamins and minerals. The tubers were washed, peeled, cut, grated and made into powder [7]. The dried powder was stored in containers.

2.3.3 *Alocasia macrorrhiza*

Very large stem tubers of *Alocasia macrorrhiza* were collected from road side. *A. macrorrhiza* L. also called as Gaint Taro. The edible portion of the raw tuber possess an energy value of 293-599 KJ/100 g, water 63-81%, crude protein 0.6-3.3%, fat 0.1-2%, carbohydrate 17-27%, ash 1.1-1.3% and contains little quantities of mineral ions and vitamins. The amount of starch present in the tuber ranges from 16-21% [8]. The tubers were made into starch powder and stored in a sealed container.

2.3.4 Wheat bran

Wheat bran possess an energy value of 1216KJ, protein 16.2 g, fat 5.3 g, carbohydrate 65.1 g, dietary fibre 40.2 g, ash 5.4 g, moisture 8.2g, vitamin E 1.6 mg and vitamin K 83 µg.

2.3.5 Rice bran

Rice bran is one of the by-product obtained through paddy milling process. The nutritional value of rice bran per 100 g is protein 16.5 g, fat 21.3 g, minerals 8.3 g, crude fibre 11.4 g, carbohydrate 49.4 g, starch 24.1 g, free sugar 50 g and also contains minerals and vitamins. The energy value is 359Kcal. Both the rice bran and wheat bran were purchased from Poorna Market in Visakhapatnam.

2.4 Enzymatic Hydrolysis of Various Starches

Among five different economical carbon sources used, fermentation with tuber substrates often involves preliminary starch hydrolysis procedure.

For starch hydrolysis of tuber substrates, initially 100 g of each three powdered tuber samples were weighed and 1L of 0.02M sodium phosphate buffer solution of pH 6.9 was added and the contents were thoroughly mixed. All the beakers were subjected to starch gelatinization procedure by keeping them in boiling water bath shaker RSB-12 (M/s. Remi Instruments Ltd.,) to prevent lump formation for 3 hrs. After that the liquefaction was performed with α-amylase enzyme at a concentration of 9.0KNU/100 g suspension. The contents were incubated at 30ºC. The hydrolysis was performed for 240-300 min. Throughout the incubation time, the samples were withdrawn for every 50min interval and the amount of reducing sugars released were determined using 3,5 Dinitrosalicylic acid method [9]. Finally all the samples were boiled for 5 min to inactivate the enzyme. Each experiment was performed in duplicates and the results were expressed as Standard error means.

2.5 Effect of Physicochemical Factors on Kojic Acid Production

The production medium contain 100 g of carbon source of Wheat bran or Rice bran or 100 ml of starch hydrolysate of Cassava or *Ipomea* or *Alocasia* Peptone 1.0 g/L, KH_2PO_4 - 1.0 g/L, $MgSO_4.7H_2O$ - 0.5 gL. The fermentation was carried out at varying physical conditions Temperature (20ºC-35ºC), pH (4.0-8.0), Time (11d-37d) and chemical conditions like substrate concentration (10 g/L-100 g/L bran substrates, 10 ml-100 ml starch hydrolysate for tuber substrates), Peptone concentration(1-5 g/L), KH_2PO_4 (0.5 g/L-2.5 g/L), $MgSO_4$ concentration(0.1 g/L-0.9 g/L) using one-factor-at-a-time method. Basing on the preliminary screening procedure, it was observed that fungal cultures *A. flavus* and *A. sojae* produce maximum production in surface fermentation rather than submerged fermentation. Hence surface fermentation technique was used to study effect of various physicochemical factors on the production of Kojic acid. When the optimized fermentation conditions were established, the final production was done at flask level and crystallization was performed. The fermented broth samples were filtered and subjected to evaporation in a refrigerator at 5ºC for 24 h. For further extraction of kojic acid, the soluble kojic acid in the fermented broth was extracted using ethyl acetate. Upon evaporation, the extractant yields kojic acid crystals in the form of needles. The crystals were collected and dried at 80ºC for 24 h. For purification of the crystals, repeated crystallization was performed with a mixture of water and acetone. Later the dry weight of the crystals from each sample was determined [10]. After recovery of the pure kojic acid crystals, the purity was tested with Fourier transform infrared spectroscopy (Perkin-Elemer model RX_1) and X-ray crystallography was used to find the structure of kojic acid.

2.6 Statistical Analysis

Each experiment was performed 3 times. The results were analyzed by one-way ANOVA at 95% confidence level and the sample means were analysed by Tukey's test at significance level of $p \leq 0.05$ using STATISTICA 6.0 (Stat-Ease Inc., Tulsa, 130 OK, USA).

2.7 Antimicrobial Activity of Kojic Acid

The agar-slants inoculated with pathogenic bacteria were collected from M/s. Doctor's Diagnostic Centre, Gajuwaka, Visakhapatnam. Individual bacterial suspensions were prepared using a sterile distilled water containing 10^8CFU/ml. By applying spread plate technique, 0.5ml of bacterial inoculum was spread uniformly on a petridish containing Mueller Hinton agar. The isolated kojic acid crystals and standard kojic acid crystals were made into two different suspensions of 0.1 ml with sterile distilled water at a concentration of 250 µg/ml was inoculated into two wells created in the petridish. The plates were incubated at 37ºC for 16-24 hrs. After 24 h, zone of inhibitions surrounding the wells were identified and measured in millimetres [11].

3. RESULTS AND DISCUSSION

3.1 Screening of Kojic Acid Producing Microorganisms

The fungal organisms when tested for the kojic acid production in glucose medium using two different fermentation techniques-surface fermentation and submerged fermentation, the organisms produce high concentrations of kojic acid in surface fermentation technique. The yields obtained were 37.9 g/L by *A. flavus* and 24.12 g/L by *A. sojae*. In submerged fermentation technique, *A. flavus* produce 5.53 g/L of kojic acid at 100 rpm agitation speed and no production was observed with *A. sojae* because the mycelia growth was confined only to the walls of the conical flask and no growth was identified in the centre of the flask. The results are in-line with many earlier findings which reported that surface fermentation is more

effective for kojic acid production than submerged fermentation [12-14].

3.2 Progress of Starch Hydrolysis for Various Starches

Rapid hydrolysis of starch molecules has taken place within the first 120 min of incubation time and then after 120 min, the rate of hydrolysis was decreased till 300 min. It was also observed that starch samples of three different origins used were susceptible to α-amylase action differently and releases reducing sugars at different concentrations (Fig. 1). The concentration of reducing sugars released were 47.1 g/L for Cassava, 42.95 g/L for *Ipomea* and 38.2 g/L for *A. macrorrhiza*. The difference in the susceptibility and mode of enzyme action was based on starch source, enzyme system and botanical origin [15]. The degree of digestibility depends on crystalline polymorphic forms of starch molecules. The starch which exhibit A-type x-ray diffraction spectrum was more susceptible to hydrolysis than with starch of B-type spectra [16].

3.3 Effect of Physicochemical Factors on Kojic Acid Production

The effect of physicochemical parameters on the production of kojic acid was studied with five different raw materials using *A. flavus* and *A. sojae*. Table 1 and Table 3 showed the yields of kojic acid obtained at various process conditions by *A. flavus* and *A. sojae*. Figs. 2-6 represented the error bar plots for most significant factors influence the kojic acid production from the carbon sources with *A. flavus*. From Table 2, it was depicted that *A. flavus* showed significant production ($p<0.05$) with raw material *Ipomea* 53.7 g/L of kojic acid and after filtration followed by crystallization 16 g/L of dry crystals were obtained. Whereas *A. sojae* produced maximum yield 42.2 g/L and upon subsequent crystallization 13.94 g/L of dry crystals were obtained (Table 4). Figs. 7-11 represented the error bar plots for most significant factors influence the kojic acid production from the carbon sources with *A. sojae*. With the substrate *Alocasia*, *A. flavus* produced highest yield of 39.3 g/L with colorimetric method and 12.9 g/L dry crystals after crystallization (Table 2). On the other hand, *A. sojae* showed maximum production 34.4 g/L and 9.8 g/L of dry crystals after crystallization (Table 4). *A. flavus* produced 65.1 g/L concentration of kojic acid and 20.3 g/L of dry crystals with the substrate Cassava (Table 2). The other organism *A. sojae* yielded 79.3 g/L concentration of kojic acid and 22.5 g/L of dry crystals with Cassava (Table 4).

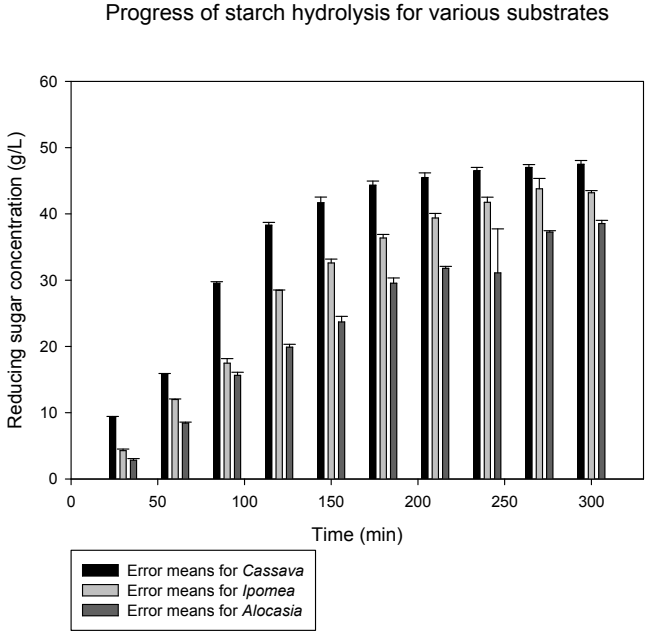


Fig. 1. Progress of starch hydrolysis for various substrates

Table 1. Effect of physico-chemical factors on the production of kojic acid from different carbon sources by *Aspergillus flavus*

Physico-chemical factors	Concentration of kojic acid (g/L)				
Substrate conc. (g/L)	***Ipomea batatas***	***Alocasia macrorrhiza***	**Cassava**	**Wheat bran**	**Rice bran**
10	10.80±1.13	1.32±1.01	2.41±0.51	0.50±0.11	25.50±2.07
20	11.17±2.30	0.36±0.22	4.97±1.10	0.77±0.14	1.68±0.55
30	15.96±2.80	2.44±1.22	7.20±0.96	0.61±0.21	1.68±0.41
40	16.50±3.52	1.86±0.30	7.20±0.82	0.61±0.19	1.42±0.21
50	16.75±3.05	2.50±0.21	7.50±0.16	0.58±0.05	1.45±0.32
60	16.90±2.30	4.80±0.90	7.60±0.62	0.56±0.02	1.38±0.52
70	24.70±5.01	8.31±1.21	7.40±1.21	0.56±0.01	1.29±0.61
80	30.10±4.92	12.56±0.90	7.42±0.92	0.49±0.50	1.21±1.10
90	39.50±3.95	19.30±0.52	19.50±3.96	0.30±0.12	1.20±0.58
100	44.20±0.36	34.73±0.80	25.18±0.36	0.19±0.03	0.88±0.29
pH					
4	3.59±1.12	0.71±0.11	49.01±5.21	-	6.01±1.12
5	6.41±2.15	1.01±0.25	54.41±4.81	0.18±0.02	10.70±1.63
5.5	8.27±0.59	1.93±0.05	59.30±3.92	0.81±0.28	12.01±1.92
6	5.53±1.52	4.52±0.14	59.87±0.66	0.39±0.16	21.41±1.89
7	3.19±0.90	3.64±1.51	49.10±1.92	0.12±0.11	13.20±1.56
8	0.21±0.05	2.40±0.91	45.60±2.82	0.08±0.01	3.31±3.92
Time (d)					
11	0.70±0.01	3.01±0.61	25.61±1.12	0.46±0.01	2.91±0.92
16	2.11±0.56	33.41±5.21	17.11±2.11	0.84±0.29	28.30±1.13
21	13.70±1.21	36.80±1.11	19.40±1.92	0.31±0.12	0.83±0.04
28	29.70±1.12	16.80±3.16	27.30±0.55	0.28±0.16	0.09±0.02
32	18.30±4.32	11.50±2.92	22.51±2.08	0.16±0.12	-
37	4.20±0.92	4.60±0.92	5.71±1.56	-	-
Temp. (ºC)					
20	4.41±1.09	2.93±0.52	0.44±0.11	0.12±0.05	-
25	17.50±2.95	14.11±0.91	8.93±1.92	0.36±0.03	6.30±0.52
28	30.41±0.60	22.41±0.55	10.15±0.37	0.82±0.54	9.71±1.16
30	15.31±4.82	17.90±2.92	9.95±2.26	0.40±0.18	10.96±0.55
35	2.11±0.98	3.24±1.52	3.01±1.32	-	2.19±1.92
Peptone conc. (g/L)					
1	21.66±9.92	1.26±0.58	18.50±3.21	0.29±0.12	4.01±1.12
2	40.80±3.21	3.86±0.14	25.91±0.55	0.42±0.21	9.81±2.09
3	48.91±0.61	1.28±1.12	22.40±4.96	0.58±0.31	13.11±3.82
4	33.31±5.05	2.64±0.98	21.61±3.82	0.64±0.16	15.60±1.83
5	10.40±1.16	0.22±0.15	5.21±1.91	0.59±0.05	12.41±5.82
KH_2PO_4 (g/L)					
0.5	1.19±0.55	2.28±1.15	0.62±0.06	0.13±0.05	3.51±1.62
1	4.65±1.56	3.65±0.41	1.99±0.92	0.32±0.03	8.01±0.98
1.5	5.16±0.31	2.74±0.58	3.08±1.20	0.38±0.02	14.02±3.82
2	5.09±1.65	2.66±1.19	3.46±0.41	0.26±0.12	21.04±1.56
2.5	4.12±0.05	2.64±0.82	1.01±0.82	0.15±0.08	17.50±2.21
$MgSO_4$ (g/L)					
0.1	1.49±0.52	3.26±1.13	1.22±0.91	0.18±0.02	4.10±0.92
0.3	3.81±1.26	3.38±0.63	2.19±1.01	0.21±0.06	11.36±2.81
0.5	4.99±1.05	3.64±0.17	6.81±0.25	0.33±0.03	12.11±1.92
0.7	7.31±0.42	3.14±1.92	4.53±1.25	0.41±0.01	15.86±1.97
0.9	5.51±0.56	3.10±0.69	2.01±0.62	0.40±0.28	9.31±3.21

Table 2. Significant and optimized factors for kojic acid production with *A. flavus*

'C' source	Substrate concentration	Peptone	Time	pH	Temp.	$MgSO_4$	KH_2PO_4
Ipomea	100 ml	3.0 g/L	28d	5.5	28ºC	0.7 g/L	1.5 g/L
'p' value	0.00002	0.00006	0.0004	0.001	0.0001	0.001	0.001
Cassava	100 ml	2.0 g/L	28d	6.0	28ºC	0.5 g/L	2.0 g/L
'p' value	0.00007	0.0001	0.0001	0.00004	0.0004	0.0004	0.004
Alocasia	100 ml	2.0 g/L	21d	6.0	28ºC	0.5 g/L	1.0 g/L
'p' value	0.00017	0.0004	0.0003	0.0003	0.0002	0.0007	0.004
Rice bran	10 g/L	4.0 g/L	16d	6.0	30ºC	0.7 g/L	2.0 g/L
'p' value	0.002	0.004	0.0005	0.002	0.0008	0.005	0.001
Wheat bran	20 g/L	4.0 g/L	16d	5.5	28ºC	0.7 g/L	1.5 g/L
'p' value	0.01	0.02	0.03	0.04	0.12 (NS)	0.0004	0.001

'p' value: Probability value; NS: Non-significant

Table 3. Effect of physico-chemical factors on the production of kojic acid from different carbon sources by *Aspergillus sojae*

Physico-chemical factors	Concentration of kojic acid (g/L)				
Substrate Conc. (g/L)	***Ipomea batatas***	***Alocasia macrorrhiza***	**Cassava**	**Wheat bran**	**Rice bran**
10	3.36±1.02	0.93±0.02	0.53±0.16	2.24±0.42	2.40±0.34
20	5.84±0.92	2.47±0.01	2.17±0.82	3.89±0.26	28.45±0.39
30	7.79±2.08	2.47±0.52	3.60±0.32	3.23±0.46	20.40±0.49
40	7.93±2.92	5.91±1.12	3.61±1.19	3.18±0.54	19.01±0.49
50	10.52±1.93	3.51±1.65	3.95±1.82	3.05±0.60	18.24±0.47
60	10.50±2.86	11.49±3.28	4.40±1.56	3.01±0.60	18.20±0.37
70	15.31±4.01	15.60±2.92	5.38±2.01	2.20±0.15	17.60±0.22
80	22.19±5.12	18.56±4.05	6.21±0.52	2.05±0.42	17.01±0.34
90	26.50±4.26	18.49±3.96	11.97±3.06	2.00±0.50	15.30±0.38
100	29.41±0.36	20.81±0.15	23.60±0.47	0.94±0.22	4.28±0.54
pH					
4	2.08±0.62	0.11±0.03	2.08±1.90	0.03±0.01	4.37±1.12
5	5.14±1.16	0.95±0.16	1.30±0.82	0.29±0.05	8.40±2.92
5.5	7.54±0.45	1.49±0.42	1.60±0.58	0.35±0.02	12.20±2.28
6	5.49±0.95	2.15±1.90	0.68±0.42	2.50±0.49	16.10±3.52
7	3.50±0.26	3.01±0.93	0.91±0.03	1.03±0.26	19.82±0.65
8	0.95±0.52	3.56±0.32	2.42±0.48	0.21±0.01	8.13±1.19
Time (d)					
11	0.79±0.12	2.50±0.12	23.61±0.22	1.91±0.22	3.84±0.92
16	2.36±0.05	10.33±1.15	22.81±0.50	3.91±0.67	24.65±1.06
21	15.75±1.26	16.90±2.32	29.10±0.91	3.05±0.51	24.10±2.31
28	34.82±0.37	30.48±0.35	51.70±0.48	2.06±0.92	4.19±0.54
32	19.90±2.94	24.21±2.96	74.83±0.25	1.90±0.43	1.50±0.02
37	4.30±1.17	2.99±0.75	3.91±0.47	1.01±0.11	0.05±0.01
Temperature (ºC)					
20	4.91±1.14	5.59±1.01	0.31±0.04	-	0.03±0.02
25	13.11±1.72	11.40±2.06	2.02±0.53	1.16±0.03	8.35±0.56
28	18.87±0.15	15.54±0.31	4.21±0.76	3.69±0.57	10.08±1.32
30	9.33±1.98	13.19±2.46	3.93±1.27	3.02±0.54	11.94±0.53
35	1.28±0.54	0.95±0.06	0.63±0.82	1.08±0.04	4.19±1.83
Peptone conc. (g/L)					
1	5.41±0.06	9.81±0.36	30.60±1.57	1.64±0.06	2.24±0.04
2	14.01±1.01	11.31±0.34	32.71±2.82	2.34±0.82	7.01±0.13
3	26.71±0.70	13.60±0.15	22.60±1.00	2.62±0.07	9.91±0.42
4	39.64±0.56	15.20±0.51	19.01±2.06	2.79±0.27	14.34±0.60

Physico-chemical factors	Concentration of kojic acid (g/L)				
Substrate Conc. (g/L)	***Ipomea batatas***	***Alocasia macrorrhiza***	**Cassava**	**Wheat bran**	**Rice bran**
5	3.36±0.36	31.67±0.32	18.21±3.04	2.03±0.18	3.50±0.84
KH_2PO_4 (g/L)					
0.5	1.66±0.47	1.91±0.69	0.51±0.02	2.05±1.01	8.72±1.63
1	3.50±0.78	2.65±1.06	3.83±1.01	2.36±0.97	10.91±2.92
1.5	4.54±0.32	4.27±0.42	7.40±0.71	2.95±0.05	15.10±4.01
2	4.03±1.08	1.53±0.76	5.04±1.97	4.37±1.08	16.22±0.59
2.5	1.44±0.09	0.19±0.05	2.51±0.53	3.03±1.02	7.40±0.36
$MgSO_4$ (g/L)					
0.1	2.29±0.63	0.21±0.02	5.61±0.02	2.01±0.42	2.20±0.62
0.3	5.01±1.42	3.05±0.83	5.94±0.28	2.99±0.56	6.41±1.05
0.5	5.53±0.46	3.68±0.45	7.51±0.43	3.84±0.97	13.40±0.59
0.7	3.10±0.52	2.44±0.54	7.41±1.09	2.80±0.68	13.05±3.92
0.9	0.88±0.12	2.08±0.04	3.82±0.92	2.15±0.71	11.01±2.58

±: Standard deviation

Table 4. Significant and optimized factors for kojic acid production with *A. sojae*

'C' source	**Substrate concentration**	**Peptone**	**Time**	**pH**	**Temp.**	**MgSO4**	**KH_2PO_4**
Ipomea	100 ml	4.0 g/L	28d	5.5	28ºC	0.5 g/L	1.5 g/L
'p' value	0.00005	0.00006	0.00003	0.001	0.00002	0.002	0.001
Cassava	100 ml	2.0 g/L	32d	8.0	28ºC	0.5 g/L	1.5 g/L
'p' value	0.0001	0.0003	0.000004	0.01	0.01	0.001	0.003
Alocasia	100 ml	5.0 g/L	28d	8.0	28ºC	0.5 g/L	1.5 g/L
'p' value	0.00001	0.00003	0.00004	0.002	0.0001	0.005	0.003
Rice bran	20 g/L	4.0 g/L	16d	7.0	30ºC	0.5 g/L	2.0 g/L
'p' value	0.0006	0.0005	0.0006	0.0003	0.0006	0.0006	0.0004
Wheat bran	20 g/L	4.0 g/L	16d	6.0	28ºC	0.5 g/L	2.0 g/L
'p' value	0.001	0.003	0.009	0.012	0.008	0.02	0.01

From the previous research studies on kojic acid production, it came to know that several species of *Aspergillus* produced kojic acid [2]. The demand for kojic acid production has been tremendously increased due to its commercial application and use in various industrial sectors like food, cosmetics etc. As a result, an extensive research is being carried out on the kojic acid production. For better development in the production of kojic acid, an extended research was done in the strain development procedures involving various mutational methods, optimizing media components and environmental factors, utilizing different agro-waste carbon sources [4]. Potato starch, corn starch and sago starch were 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 [3]. 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 [17]. To enhance the production rate of kojic acid, fermentation was conducted in 8L stirred tank fermentor using gelatinized sago starch by *A. flavus* Link 44-1 and reported 16.43 g/L of kojic acid [18]. It was revealed that, maximum kojic acid production 40.67 g/L from potato starch using a potato mutant strain *Aspergillus flavus* UV_8 ($AFUV_8$) [19]. The present research differs mainly in three aspects i.e., utilizing different types of starch substrates *Ipomea*, *Alocasia* and Cassava, fermenting microorganism's *A. flavus* and *A. sojae* isolated and instead of using the starch as partially hydrolyzed starch for fermentation, all the three tuber substrates were completely hydrolyzed by α-amylase and the resulted hydrolysates were used for kojic acid fermentation. In this paper, the maximum kojic acid yield 79.3 g/L was reported with *A. sojae* with the substrate Cassava was higher than the yields obtained by the earlier studies [3,17-19] and lower with the studies of Yan et al. [20] who reported that, the highest percentage of kojic acid 90.8% when the fermentation medium containing glucose and xylose as carbon source

with *A. oryzae* M866 strain. High concentration of kojic acid 83 g/L was obtained with the immobilized cells of *A. oryzae* [19]. A high concentration of kojic acid in the broth causes kojic acid to crystallize in to fine needles [2,21]. Morton et al. [22] reported results in terms of grams of kojic acid crystals. Fifty grams of absolute kojic acid crystals were obtained from 1L culture filtrate. The mother liquor still contains 20-50 g of kojic acid. The fermentation medium contains glucose as carbon source. The yield was higher with the results of the current research and it was found that maximum amount of kojic acid crystals 22.5 g was obtained from 1L Cassava fermented broth.

Figure 2:Effect of Peptone concentration on kojic acid production with the substrate *Ipomea from* A.flavus

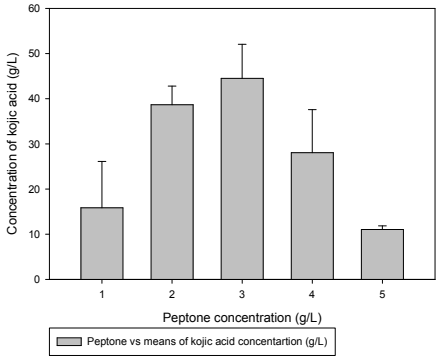
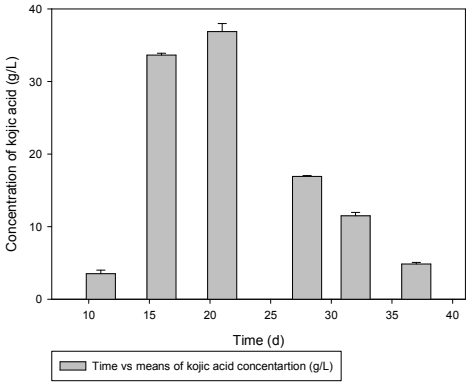


Figure 3: Effect of Time on kojic acid production with the substrate*A.macrorrhiza from* A.flavus



The maximum production of kojic acid 0.74 g/L takes place from wheat bran with the fungus *A. flavus* and subsequent crystallization yielded 0.04 g/L of kojic acid crystals (Table 2) whereas *A. sojae* produces 4.1 g/L concentration of kojic acid and upon further crystallization produced 1.05 g/L of crystals (Table 4). When the rice bran was used as a substrate, *A. flavus* produces 29.4 g/L of kojic acid concentration and 9.62 g/L of crystals (Table 2). The other isolate *A. sojae* produces 30.8 g/L of concentration of kojic acid and upon crystallization yielded 8.2 g/L of kojic acid crystals (Table 4). From the above results it was deduced that *A. sojae* produces maximum yield 30.8 g/L of kojic acid with rice bran and 4.1 g/L of kojic acid with wheat bran. The yield obtained was higher than the yield reported by El-Kady et al. [23]. The authors isolated 278 different types of fungal strains and tested for the kojic acid production. They selected five highly producing fungi grown on various agricultural by-products, industrial by-products etc., and reported 3.0 g/L of kojic acid from wheat bran, 2.8 g/l of kojic acid from rice bran and 21.2 g/L of kojic acid from rice fragment with *A. flavus*. The differences in the production may be either due to the culture conditions or due to species differences [1]. Rice bran and wheat bran were also used as potent nitrogen sources and produced 44.0 g/L and 39.88 g/L of kojic acid respectively [19].

Figure 4: Effect of pH on kojic acid production with the substrate *Cassava from A.flavus*

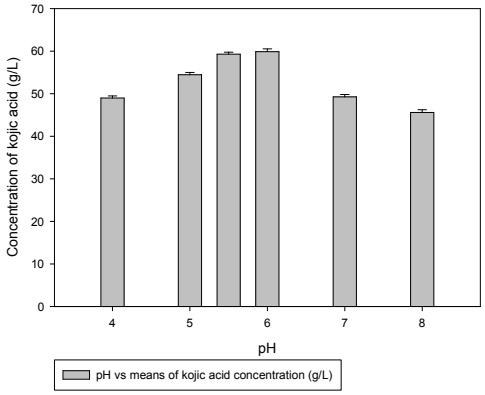
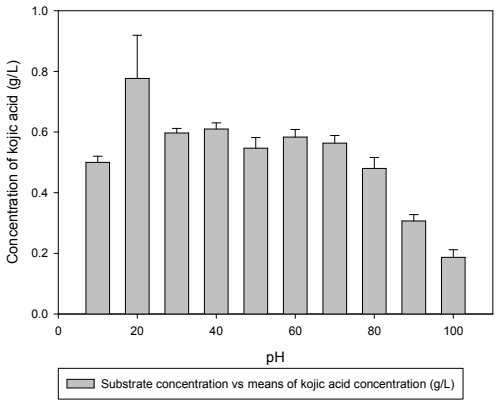


Figure 5: Effect of Substrate concentration on kojic acid production with the substrate Wheat bran from *A.flavus*



The resulted crystals were observed under compound microscope (Fig. 12) and subjected to

further analysis by FTIR and X-ray crystallography. The FTIR spectrum of the sample kojic acid shows the peak wave number values for the functional groups similar to that of standard kojic acid. 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, 4 α -disubstituted ring) (Fig. 13).

The structure was determined with XRD diffractometer (Shimadzu, Tokyo, Japan) with in 2θ angle 70º using Cu Kα radiation. The kojic acid X-ray diffraction spectrum was shown in the (Fig. 14). The spectrum reveals that seven distinctive peaks were appeared at 2θ angles 8º, 19.27º, 21.71º, 27.73º, 31.15º, 36.13º, 39.09º. The highest appeared at 19.27º with a count number 649.92. Similar spectrum was appeared with standard sample.

Figure 6: Effect of Substrate concentration on kojic acid production with the substrate Rice bran from A.flavus

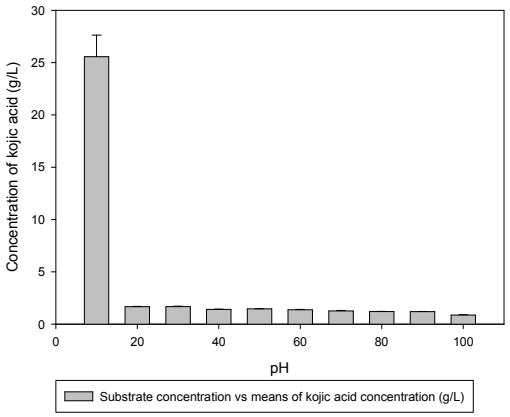


Figure 7: Effect of Peptone concentration on kojic acid production with the substrate *Ipomea* from *A.sojae*

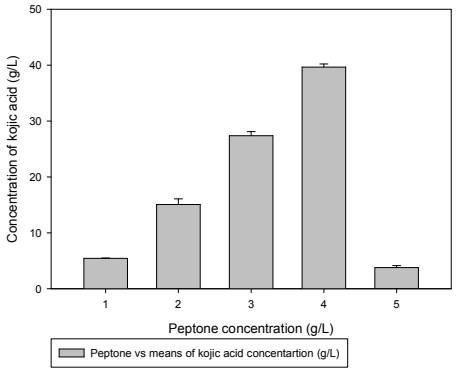


Figure 8: Effect of peptone concentration on kojic acid production with the substrate Alocasia from A.sojae

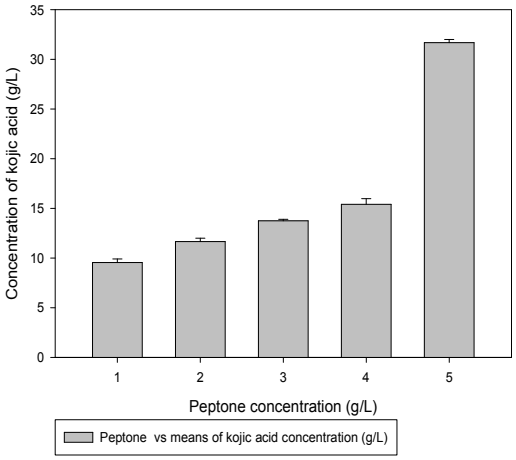


Figure 9: Effect of Time on kojic acid production with the substrate Cassava from A.sojae

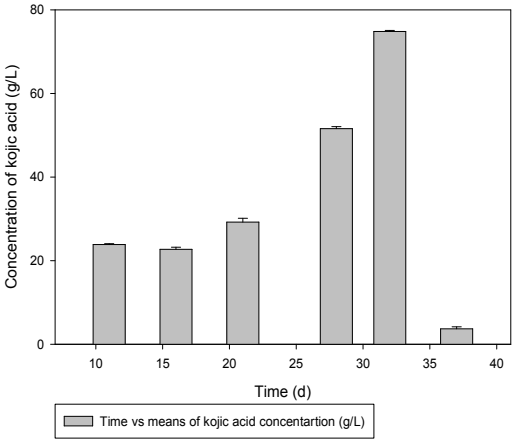


Figure 10: Effect of Substrate concentration on kojic acid production with the substrate Wheat bran from A.sojae

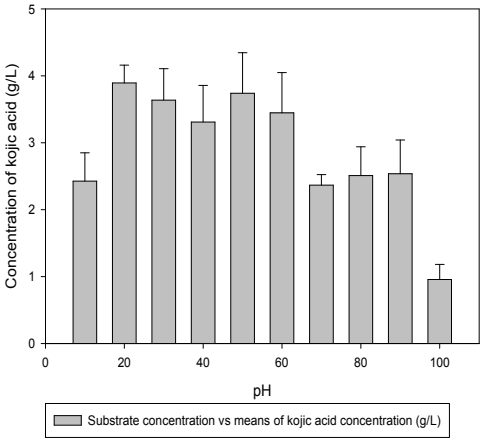
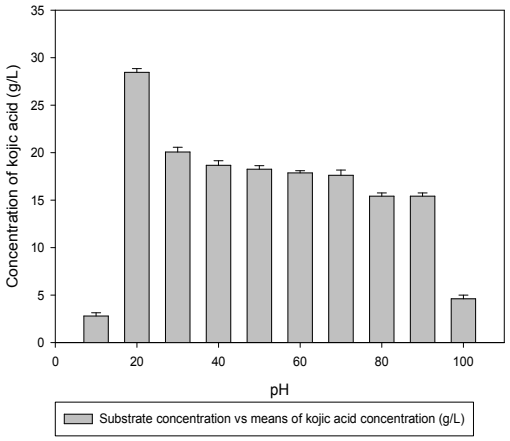


Figure 11: Effect of Substrate concentration on kojic acid production with the substrate Rice bran from A.sojae



3.4 Antimicrobial Activity

Bacteria of distinct genera including *Proteus*, *Staphylococcus, Streptococcus*, *Pseudomonas*, *Bacillus*, *Corynebacterium*, *Clostridium*, *Aerobacter*, *Escherichia*, *Klebsiella*, *Salmonella* etc., were inhibited by kojic acid [20]. The maximum zone of inhibition (12 mm) was observed with the cultures *Staphylococcus aureus* and *Escherichia coli* (Fig. 15) followed by *Bacillus subtilis* (11 mm) indicates that these organisms were highly sensitive to the antimicrobial compound kojic acid. Others showed least sensitivity to kojic acid.

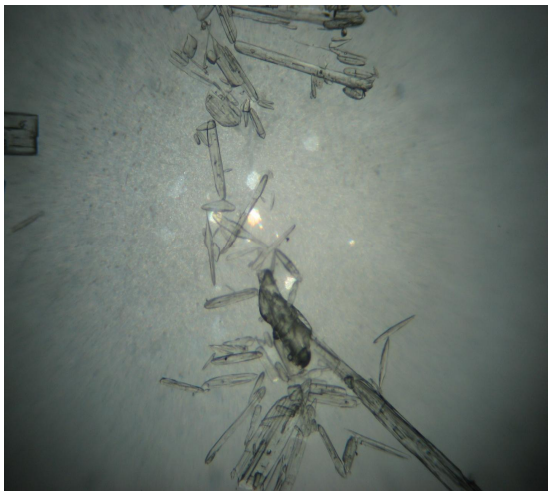


Fig. 12. Kojic acid crystals

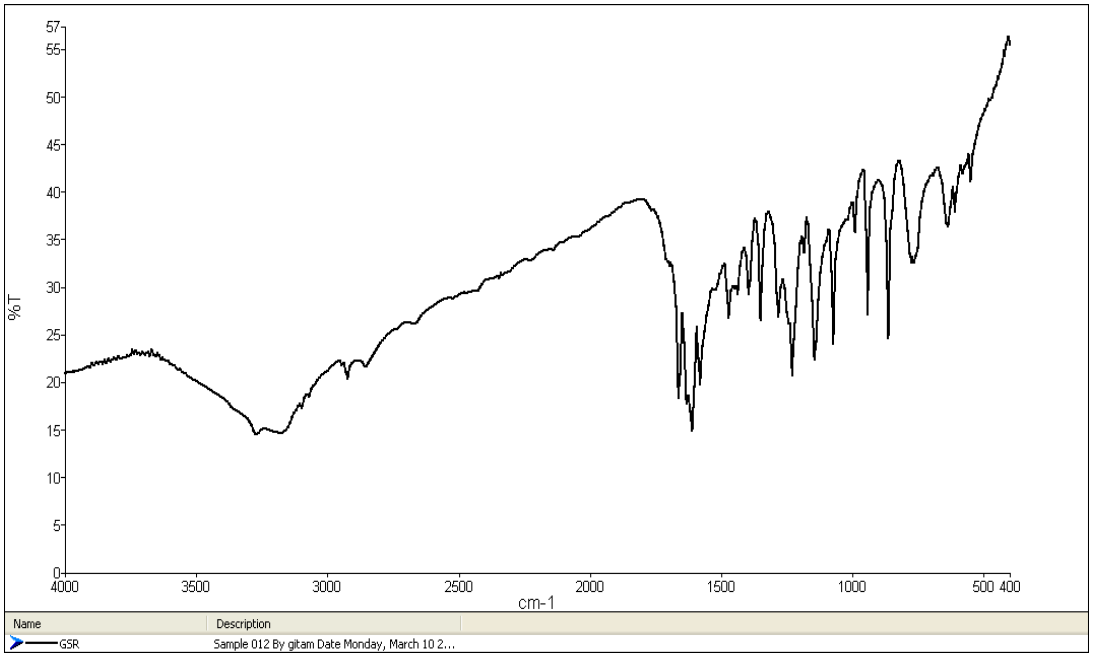


Fig. 13. FTIR spectrum of kojic acid

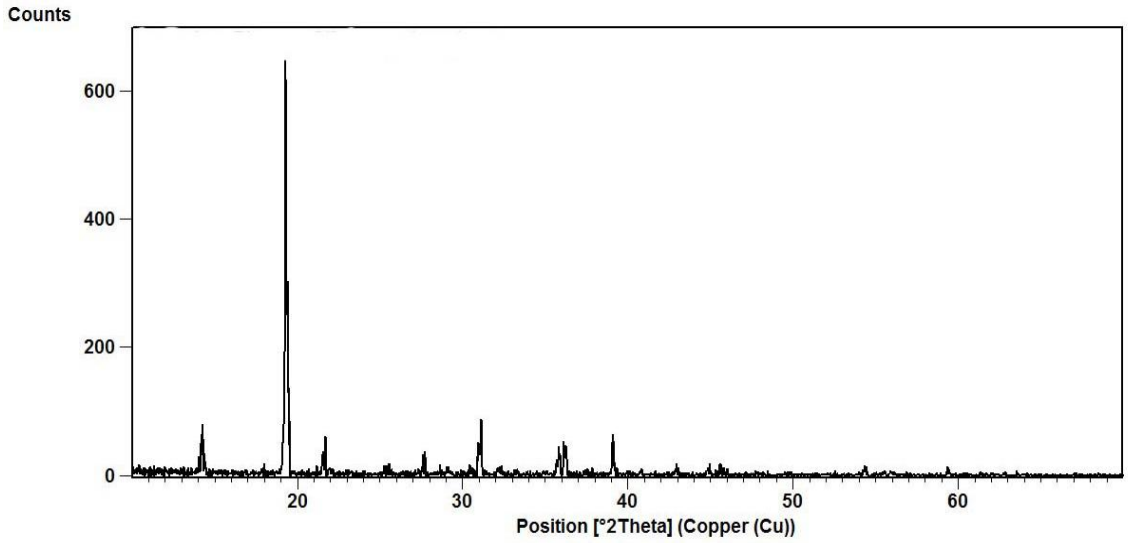


Fig. 14. X-ray crystallography of kojic acid crystal

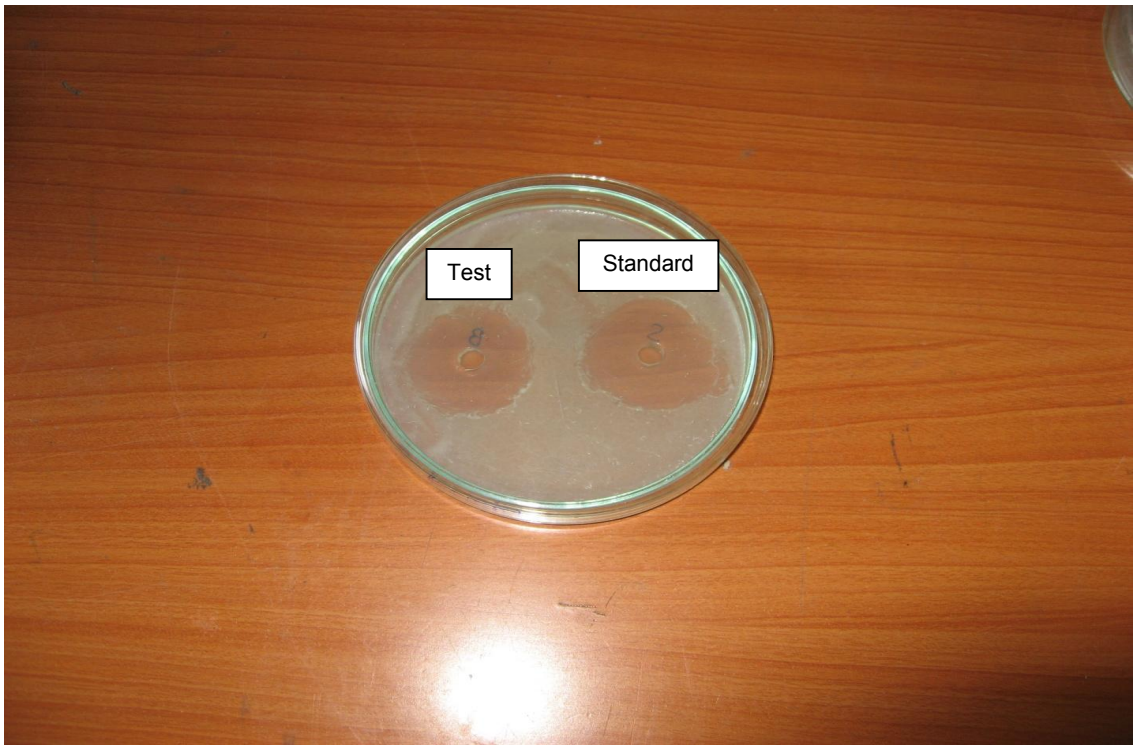


Fig. 15. Zone of inhibition for *Staphylococcus aureus* to kojic acid

4. CONCLUSION

Based on these findings, the tuber and bran substrates have proved the auspicious potentiality in exploiting the alternate sources for higher production of kojic acid by *Aspergillus flavus* through surface fermentation. The study in fact helps to scale-up the kojic acid fermentation to a large-scale fermentor in order to produce the expensive chemical like kojic acid from the economical raw materials.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. El-Aasar SA. Cultural conditions studies on kojic acid production by *Aspergillus parasiticus*. Int J Agric Biol. 2006;8(4):468-473.
2. Rosfarizan M, Mohd Shamzi M, Nurashikin S, Salleh MM, Arbakariya Ariff B. Kojic acid: Applications and development of fermentation process for production. Biotechnol Molecular biology Reviews. 2010;5(2):24-37.
3. Rosfarizan M, Madihah S, Ariff AB. Isolation of a kojic acid producing fungus capable of using starch as a carbon source. Lett Appl Microbiol. 1998;26(1):27-30.
4. Chaudhary J, Pathak AN, Sudarshan L. Production technology and applications of kojic acid, Annual Res Rev Biol. 2014;4(21):3165-3196.
5. Ariff AB, Salleh MS, Gani B, Hassan MA, Rusul G, et al. Aeration and yeast extract requirements for kojic acid production by *Aspergillus flavus* Link. Enzyme Microbiol Technol. 1996;19(7):545-550.
6. Bentley R. Preparation and analysis of kojic acid. Methods Enzymol. 1957;3(1):238-241.
7. Lodha, Nemade. Isolation of Alocasia Indica Linn. Starch and its performance as a disintegrating agent. Int J Pharmaceut Biosci. 2012;3(4):472-477.
8. Foliaki S. In proceedings of taking Taro into the 1990s: A Taro conference, research extension series, Hawaii Institute of Tropical agriculture and human resource, Honolulu. 1990;37-45.

   ISSN:0271-9916 Report No.114.
9. Sadasivam S, Manickam A. Biochemical methods. New Age International publishers Ltd.; 2nd Edition, 2005.
10. Hazza MM, Saad AENM, Hassan HM, Ibrahim EI. High production of kojic acid crystals by isolated Aspergillus oryzae var.effusus NRC14. J Appl sci Res. 2013;9(3):1714-1723.
11. Saleh RM, Kabli SA, Al-Garni SM, Mohamed SA. Screening and production of antibacterial compound from Trichoderma species against human pathogenic bacteria. Afr J Microbiol res. 2011;5(13):1619-1628.
12. Lin MT, Mahajan JR, Dianese JC and Takatsu A. High production of kojic acid crystals by *Aspergillus parasiticus* UNBF A-12 in liquid medium. Appl Environ Microbiol. 1976;32(2):298-299.
13. Wei CI, Huang TS, Fernando SY, Chung KT. Mutagenicity studies of kojic acid. Toxicol Lett. 1991;59(1-3):213-220.
14. Ogawa A, Wakisaka Y, Tanaka T, Sakiyama T, Nakanishi K, Production of kojic acid by membrane-surface liquid culture of *Aspergillus oryzae* NRRL484. J Ferment Bioeng. 1995;80(1):41-45.
15. Franco CML, Ciacco CF, Tavares DQ. Studies on the susceptibility of granular Cassava and corn starches to enzymatic attack. Part 2. Study of the granular structure. Starch. 1988;40(1):29-32.
16. Adejumo AL, Aderibigbe, Owolabi RU. Comparitive studies of starch susceptibilities to α-amylase degradation of different cereal and root crops of Nigeria. Afr J Biotechnol. 2013; 12(29):4663-4669.
17. Futamura T, Ishihara H, Tamura T, Yasutake T, Huang G, Kojima M, Okabe M. Kojic acid production in an airlift bioreactor using partially hydrolyzed raw corn starch. J Biosci Bioeng. 2001;92(4):360-365.
18. Rosfarizan M, Ariff A. Importance of carbon source feeding and pH control strategies for maximum kojic acid production from sago starch by *Aspergillus flavus*. J Biosci Bioeng. 2002;94(2):99-105.
19. Abd El-Aziz B Amany. Improvement of kojic acid production by a mutant strain of *Aspergillus flavus*. J Nat Sci Res. 2013;3(4):31-41.
20. Yan S, Tang H, Wang S, Xu L, Liu H, Guo Y, Yao J. Improvement of kojic acid production in strain *Aspergillus oryzae* B008 mutant strain and its uses in fermentation of concentrated corn stalk hydrolysates. Bioprocess Biosystem Eng. 2014;37(6):1095-1103.
21. Kwak MY, Rhee JS. Control mycelial growth for kojic acid production using Ca-alginate immobilized fungal cells. Appl Microbiol Biotechnol. 1992;36(5):578-583.

22. Morton HE, Kocholaty W, Junowiczkocholaty R, Kelner A. Toxicity and antibiotic activity of kojic acid produced by *Aspergillus luteo*-virescens. J Bacteriol. 1945;50(5):579-584.

23. El-Kady A Ismael, Abdel Naser A Zohri, Shimaa R Hamed. Kojic acid production from agro-industrial byproducts using fungi. Hindawi publishing corporation, Biotechnology Res Int. 2014;1-10.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=992&id=8&aid=8719