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OPTIMIZATION OF KOJIC ACID PRODUCTION BY *ASPERGILLUS* COCULTURE AND IT'S APPLICATION AS ANTI-BROWNING AGENT

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Kojic acid has applications in many fields so it is important to increase the amount of kojic acid. In this study production of kojic acid increased using coculture of Aspergillus oryzae ASU44 (OL314732) and A. flavus ASU45 (OL314748) than single culture, after optimizing by Box-Behnken statistical design, the production enhanced to 114.28 g/l using Glucose (150, g/l) (A), Yeast extract (5 g/l) (B), KH_2PO_4 (3 g/l) (C), $MgSO_4.7H_2O$ (0.5 g/l) (D) and pH (3) (E). The design was effective and applicable with coefficient (R^2) 99.1% and adjusted R^2 value 98.1%. Ethyl acetate was the best extraction solvent with ratio 1:1 and the crystals of kojic acid appeared in needle shape after evaporation of ethyl acetate using rotatory evaporator. The crystalized kojic acid has the ability to inhibit the fungal pigment of A. niger ASU311. By increasing the KA concentration, the black color decreased, especially at 10 µg/ml comparing with the control samples. On the other hand, crystalized KA preserved the color of apple juice from the conversion to brown color and the activity increased by increasing the concentration until 50 µg/ml for 6 hrs comparing with the control. Application of kojic acid produced naturally using Aspergillus coculture considered safe and environmentally friendly for increasing the freshness of the fruit juice.

Keywords: *Co-culture. Fungi. Experimental design. Decolorization*

INTRODUCTION

Kojic acid name was derived from "Koji", a fungus or starter inoculum used in oriental food fermenters, many years ago in Japan. This crystalline substance was firstly isolated by Saito (1907)[1], from the mycelia of *A. oryzae* grown on steamed rice. The chemical name of kojic acid is 5-hydroxy-2- hydroxymethyl-ɣ-pyrone[2].Kojic acid is present in colorless prismatic needle form. It has melting point ranging from 151 - 154°C[3], while the boiling point of this substance is 401.67°C at 760 mmHg. The molecular weight of Kojic acid is 142.1[4&5]and its maximum peak of UV Absorption Spectra is at 280-284 nanometer[6]. KA is highly soluble in water, ethanol, ethyl acetate, acetone and ethyl ether and slightly soluble in Ether, Chloroform and Pyridine etc. It is reactive at every position on the ring and number of products which have values in industrial chemistry, such as metal chelates, pyridines, ethers, azodyes, mannich base, and the products of cyanoethylation can be formed from kojic acid[5,7]. It is weakly acidic and combine with metals like Na, Zn, Cu, Ni, Ca & Cd and forms salts[8]. Kojic acid is an organic acid produced biologically as secondary metabolite during the aerobic fermentation of different foods by various types of fungi. kojic acid produced mainly by some species of *Aspergillus* such as: *A. flavus, A. oryzae, A. tamarii and A. parasiticus* as well as *Penicillium* species and certain bacteria[9&10]. Kojic acid can be produced in large amounts by using various carbon and nitrogen sources. Till now among other carbon sources, glucose is the best carbon source for kojic acid production.

Co-culture fermentations may result in increased yield, improving control of product

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qualities and the possibility of utilizing cheaper substrates. Co-cultivation of different microorganisms may also help to identify and develop new biotechnological substances. Because sterile cultivation enables an easy way of controlling microbial growth and product formation, most of the products in industrial biotechnology today are formed using processes involving a single microbial strain. On the other hand, there are many instances where the utilization of co-cultures appears to be advantageous over a single micro-organism because of the potential for synergistic utilization of the metabolic pathways of all involved strains in a co-culture situation[11]. Most bio-transformations in nature take place by the combination of metabolic pathways from different micro-organisms. Fungal co-cultivation has emerged as a powerful strategy for the induction of new bioactive fungal metabolites[12]. For examples, Banerjee *et al*. (2005)[13] described the advantages of tannase production by a co-culture of *Rhizopus oryzae* and *Aspergillus foetidus*. Increase the production of cellulases by a co-culture of two moulds *Aspergillus ellipticus* and *Aspergillus fumigatus* was described by Gupte and Madamwar (1997)[14]. Also, the co-cultivation of *Aspergillus niger* and *Trichoderma reesei* increased cellulase production significantly[15].

Numerous studies associated with the production of kojic acid have generally used *Aspergillus* as a sole culture. However, there was a study used a combination culture of *A. flavus* NSH9 and *A. flavus* Link 44-1 for kojic acid fermentation. Spencer *et al*. (2012)[16] stated that kojic acid produced by mixed cultures of *Aspergillus* molds showed higher yield value than was produced by the sole use of *Aspergillus.* Suryadi and Sukarna (2018)[17] produced kojic acid using combination cultures of *A. oryzae* and *A. tamarii* and founded that the yield value of kojic acid using mixed cultures was higher than that when using a sole culture.

Kojic acid has many economic applications in different eras, such as in the field of medical, it has been reported as having anti-bacterial and anti-fungal potentials. While in case of chemical industries it has been also used for the synthesis of azo-dyes and biodegradable compounds[7&18]. Furthermore, in the field of food industries, it has been used as an anti-browning agent along with the combination of vitamin C[4]. It is used during the food preparation like soy sauce, miso, sake etc as a food additive to inhibit enzymatic browning[19]. It is also used for preservation of canned foods as an anti-browning agent. Fungi that produce kojic acid were used commonly in traditional staple Japanese fermented foods production such as shochu (a distilled liquor); amazake (a sweet beverage) and mirin (a sweet alcoholic seasoning). Most of the healthy foods that are consumed in japan contain kojic acid[20]. kojic acid has anti-oxidizing activity so it is used as flavor enhancer and natural preservative[21]. It prevents fruits from browning like apples after cut[22]. It acts as precursor for flavor enhancers like ethyl maltol and maltol[23]. Comenic acid is derived from kojic acid where it is intermediate in synthesis of maltol that is used as flavor enhancer in food products and constituent in perfumes etc[24]. According to global marketing report the use of kojic acid in cosmetic is increasing day by day. KA is used as primary constituent in skin products as it obstructs the synthesis of pigment, responsible for skin blackening[25]. Kojic acid inhibits the formation of melanin in human skin by inhibition tyrosinase enzyme activity which responsible for the synthesis of melanin[26&27], where it chelates the copper ion which play role in the activation of tyrosinase[28]. Because it acts as skin lightening and de-pigmenting agent, kojic acid was used in preparation of skin care products[29]. It is used in the manufacture of beauty products like sun screens, gels, bubble bath products, bath salts, creams, baby lotions etc., to enhance the skin complexion[25]. Beard and Walton (1969)[30] founded that kojic acid could utilize as insecticides and pesticides agent. Kojic acid is active against several common bacterial strains at dilutions of 1:1,000 to 1:2,000[31].

MATERIALS AND METHODS

Microorganisms

Two highly kojic acid producers were selected from 25 fungal isolates by Kamal-Eldin *et al* (2022)[32]. These two isolates were genetically identified and examined for aflatoxin production and recorded as non-aflatoxin producers (*A. oryzae* ASU44 (OL314732)&*A. flavus* ASU45 (OL314748))[33].

Optimization Using Box-Behnken Statistical Design

Kojic acid production by the single culture from *A. oryzae* ASU44 and *A. flavus* ASU45 were optimized using Box-Behnken Statistical Design to 46.53 and 81.59 g/l respectively in the same laboratory[33].

Antagonistic effect

The antagonistic effect was performed for the co-culture of *A. oryzae* ASU44 and *A. flavus* ASU45 using Czapek's dextrose agar plates. Sterilized medium was poured on plates containing one ml of *A. oryzae* ASU44 spores suspension, mixed well then left to solidify. Then, *A. flavus* ASU45 spores were inoculated in the center of plates. The plates were incubated at 28± 1°C static for five days and observed for clear zone generation**.**

Enhancing kojic Acid Production by Co-culture of *A. oryzae* ASU44 (OL314732) and *A. flavus* ASU45 (OL314748) Using Box-Behnken Statistical Design

Box–Behnken experimental design was used for enhancing kojic acid production *via* studying the interactions between different parameters, the main effects, and quadratic effects of the evaluated variables. Five parameters, 3-levels randomized statistical design with forty-one runs were conducted exploring. Glucose (g/l) (A), yeast extract (g/l) (B), KH_2PO_4 (g/l) (C), $MgSO_4.7H_2O$ (g/l) (D) and pH (E) were stated by three levels low, medium, and high concentrations (-1, 0, +1). The non-linear quadratic statistical model was generated by the quadratic equation (1) as cleared by Liu *et al*. (2013)[34] and Yan *et al*. (2014)[35].

$$Y= \beta_0+ \sum\beta_i x_i +\sum\beta_{ii} x_i^{\,2}+ \sum\beta_{ij}x_{ij} \qquad \textbf{(1)}$$

Y stands for the predicted values of kojic acid, dry mass and residual sugar of tested strain, β_0 is the intercept, β_i is the linear impact, β_{ii} is the squared impact, β_{ij} is the interaction impact, x_i, x_{ij} denotes the independent levels of variables. Response surface plots, curves of actual and expected values, and statistical analysis of all data were used to clarify the variables' linkages. For the validity of the created models, Derringer's required equations were set. Data were analyzed using Design Expert 7.0.0 statistical software from the United States, which included quadratic regression and variable interaction analysis using one-way ANOVA with a P value of 0.05.

Extraction and Crystallization of Kojic Acid

The co-culture of *A. oryzae* ASU44 and *A. flavus* ASU45 were grown on the optimum modified Czapek's glucose broth medium at 28± 2°C for ten days. Kojic acid extracted from the filtrate using the following solvents; ethyl acetate, chloroform, methanol, ethanol, acetone and diethyl ether, individually. Three different ratios used from each solvent (1:1, 2:1 & 1:2; filtrate: solvent) and kojic acid measured using spectrophotometer at wavelength 540 nm according to Sanjotha *et al*. (2019)[10] using ferric chloride ($FeCl_3$) as detecting agent to choose the best extract solvent and its extraction ratio.

Crystallization of the extracted kojic acid was achieved using the best extraction solution (ethyl acetate) as follow: the filtrate stored for one day at 5°C, then evaporated using rotatory evaporator 70°C at 120 rpm, the remaining filtrate after evaporation mixed with the same volume of ethyl acetate (1:1, filtrate: solvent), then the layer of ethyl acetate separated using separation funnel, leaved to evaporate and form crystals[36].

Kojic acid activity in fungal de-pigmentation

Crystalized kojic acid was tested for their ability to de-pigment the black color of *Aspergillus niger* ASU311. *A. niger* inoculated in conical flasks containing YME media include (g/l) (glucose, 10; peptone, 5; yeast extract, 3 and malt extract, 3)[37]. Different concentrations of kojic acid dissolved in distilled water (1:10 µg/ml**)** were added to the flasks, individually. One of these conical flasks was a control without kojic acid. After 7 days of incubation, the content of the flasks filtrated using filter paper. Then 0.5 g of fungal mass (from each kojic acid concentration) added to the tube containing 2.5 g ethyl alcohol then the tubes after 48 hrs were centrifuged 6000 rpm for 10 minutes. The absorbance spectra of the pigment solution were screened at 350–700 nm, then tubes were measured using spectrophotometer at 411nm[38].

Kojic acid activity as anti-browning agent in food industry

Crystalized kojic acid was tested for their ability as anti-browning of apple juice. Apples were peeled and juiced with an ordinary domestic food processor (100 g from apples in 500 ml distilled water). Samples of the juice were poured into tubes, one tube was without kojic acid and the others containing different concentrations of kojic acid dissolved in distilled water (10, 20, 30, 40 & 50 µg/ml). The tubes kept at room temperatures (30°C) for 1, 2, 3, 4, 5, and 6 hrs. The tubes were centrifuged 6000 rpm for ten min. then measured at 421nm using spectrophotometer[39].

RESULTS AND DISCUSSION

Results

Antagonistic effect

There wasn't antagonistic effect between the two fungal strains.

Enhancing kojic Acid Production by Co-culture of *A. oryzae* ASU44 (OL314732) and *A. flavus* ASU45 (OL314748) Using Box-Behnken Statistical Design

The interaction effects, main effects, and quadratic effects of the studied variables on the synthesis of kojic acid by co-culture of *Aspergillus oryzae* ASU44 (OL314732) and *A. flavus* ASU45 (OL314748) in glucose fermentation medium were optimized and evaluated using a Box–Behnken statistical experimental design. Five-parameter (Glucose (100, 150, 200 g/l) (A), Yeast extract (1, 5, 10 g/l) (B), KH_2PO_4 (0, 1, 3 g/l) (C), $MgSO_4.7H_2O$ (0, 0.5, 2 g/l) (D) and pH (3, 5, 7) (E), three-level (-1, 0, +1) statistical design with 41-runs was established as mentioned in Table (1). The predicted values of the completed runs were determined using the equation (1) for second-order polynomial; the predicted values of kojic acid (g/l), was calculated by equation (2) as following:

$$\text{Kojic acid (g/l)} = 63.26+(9.84)\,A +(4.52)\,B + (-0.9161)\,C + (-1.27)\,D +(-24.50)\,E + (7.60)\,AB + (0.13)\,AC + (1.99)\,AD + (-2.03)\,AE + (-0.61)\,BC + (0.3967)\,BD + (0.08)\,BE + (-0.4133)\,CD + (-12.13)\,CE + (-2.34)\,DE + (1.27)\,A^2 + (1.31)\,B^2 + (-1.55)\,C^2 + (4.13)D^2 + (2.17)\,E^2 \quad \textbf{(2)}$$

Maximum experimental values of kojic acid production was 114.28 g/l; whereas the corresponding predicted values was 111.84 g/l obtained in run number (23) using Glucose (150, g/l) (A), Yeast extract (5 g/l) (B), KH_2PO_4 (3 g/l) (C), $MgSO_4.7H_2O$ (0.5 g/l) (D) and pH (3) (E), however the lowest kojic acid production cleared was 15.10 g/l; whereas the corresponding predicted values was 17.94 g/l obtained in run number (7) using Glucose (150, g/l) (A), Yeast extract (1 g/l) (B), KH_2PO_4 (1 g/l) (C), $MgSO_4.7H_2O$ (0.5 g/l) (D) and pH (7) (E).

The predicted values of kojic acid production was found to be very near to the response model experimental data, indicating that the established model is accurate as cleared in **Table (1)** and **Fig. 1**. The statistical model suitability, accuracy, and significance of the quadric polynomial equations were further examined using one-way analysis of variance to corroborate the statistical model suitability, accuracy, and significance (ANOVA) as established in **Table (2)**. The *F* and *P*- values of the tested model of kojic acid production (g/l) were *F;* 103.35 and *P*; <0.0001 indicating that the parameter yielded significant results (Probability ≤0.05). The statistical parameter coefficient (R^2) was also determined to evaluate the model goodness, accuracy, and fitting; R^2 values of kojic acid production (g/l) was 0.991and adjusted R^2 value 0.981 which indicated that the whole variations were explained highly by the statistical model.

Table 1: Box-Behnken design with five variables; Glucose (g/l) (A), yeast extract (g/l) (B), KH_2PO_4 (g/l) (C), $MgSO_4 \cdot 7H_2O$ (g/l) (D) and pH (E) with actual and/or predicted responses of kojic

acid (g/l) (KA) by co-culture of *Aspergillus oryzae* ASU44 (OL314732) and *Aspergillus flavus* ASU45 (OL314748).

Trials	Glucose (g/l)	Yeast extract (g/l)	KH_2PO_4 (g/l)	$MgSO_4 \cdot 7H_2O$ (g/l)	pH (E)	Actual values of KA (g/l)	Predicted values of KA (g/l)
1	1	-1	0	0	0	40.53	42.10
2	0	1	0	0	1	42.88	43.23
3	1	0	0	0	1	66.70	66.22
4	0	1	1	0	0	63.55	64.94
5	0	-1	0	1	0	28.34	29.60
6	0	0	-1	1	0	58.93	60.73
7	0	-1	0	0	1	15.10	17.94
8	0	-1	1	0	0	33.86	31.01
9	1	1	0	0	0	68.45	66.32
10	-1	0	0	0	-1	92.21	95.53
11	0	0	-1	-1	0	82.63	80.75
12	0	1	0	-1	0	41.56	41.18
13	0	0	1	0	1	58.44	59.81
14	0	0	-1	0	-1	113.74	110.63
15	-1	1	0	0	0	35.35	31.45
16	0	-1	0	0	-1	81.30	83.19
17	-1	0	0	-1	0	44.10	49.62
18	1	0	0	-1	0	82.25	83.54
19	0	1	0	1	0	40.98	44.18
20	1	0	1	0	0	108.62	107.47
21	0	0	0	-1	-1	110.64	108.70
22	1	0	-1	0	0	62.59	64.27
23	0	0	1	0	-1	114.28	111.84
24	0	0	0	1	-1	94.44	92.32
25	-1	0	-1	0	0	88.71	89.62
26	0	1	-1	0	0	39.81	41.87
27	0	0	0	-1	1	47.11	45.88
28	-1	0	0	1	0	62.88	61.31
29	0	-1	0	-1	0	40.03	37.70
30	0	0	0	1	1	58.58	57.16
31	0	0	1	1	0	71.74	76.37
32	-1	0	0	0	1	51.37	49.23
33	0	0	1	-1	0	60.49	61.44
34	-1	-1	0	0	0	37.82	37.61
35	1	0	0	0	-1	112.92	117.90
36	-1	0	1	0	0	44.67	42.76
37	0	-1	-1	0	0	59.91	57.74
38	0	0	0	0	0	63.26	63.26
39	0	0	-1	0	1	63.99	64.69
40	1	0	0	1	0	72.54	66.75
41	0	1	0	0	-1	76.55	75.96

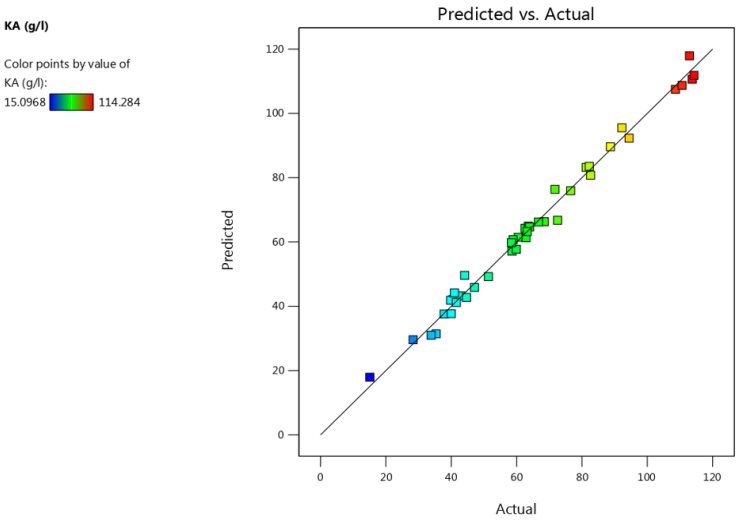


Fig. 1: Comparison between the actual and predicted values of kojic acid production (g/l) by co-culture of *Aspergillus oryzae* ASU44 (OL314732) and *Aspergillus flavus* ASU45 (OL314748).

Table 2: ANOVA results for Box-Behnken quadratic model of kojic acid (g/l) by co-culture of *Aspergillus oryzae* ASU44 (OL314732) and *Aspergillus flavus* ASU45 (OL314748) .

Source	**Sum of Squares**	**Mean Square**	***F*-value**	***P-value***
Model	25543.7	1277.19	103.35	< 0.0001
A-Glucose (g/l)	1550.07	1550.07	125.43	< 0.0001
B-Yeast extract (g/l)	326.33	326.33	26.41	< 0.0001
C-KH2PO4 (g/l)	13.43	13.43	1.09	0.3097
D-MgSO4.7H2O (g/l)	25.96	25.96	2.10	0.1627
E-pH	9600.52	9600.52	776.87	< 0.0001
AB	230.84	230.84	18.68	0.0003
AC	2027.90	2027.90	164.10	< 0.0001
AD	202.74	202.74	16.41	0.0006
AE	7.24	7.24	0.5857	0.4530
BC	619.85	619.85	50.16	< 0.0001
BD	30.86	30.86	2.50	0.1298
BE	264.53	264.53	21.41	0.0002
CD	305.46	305.46	24.72	< 0.0001
CE	9.27	9.27	0.7504	0.3966
DE	191.42	191.42	15.49	0.0008
A^2	52.64	52.64	4.26	0.0523
B^2	1640.78	1640.78	132.77	< 0.0001
C^2	231.40	231.40	18.72	0.0003
D^2	13.44	13.44	1.09	0.3095
E^2	681.44	681.44	55.14	< 0.0001

Response surface plots and the interaction plots draw for the 3D visualization of the cleared interaction between pair-wise of the two factors when the other factor constant as showed in **Fig. (2)** explaining the effect of AB (Glucose (g/l) * Yeast extract (g/l)), AC (Glucose (g/l) * KH_2PO_4 (g/l)), BC (Yeast extract (g/l) * KH_2PO_4 (g/l)) and BD (Yeast extract (g/l) * $MgSO_4.7H_2O$ (g/l)) on kojic acid production (g/l) by coculture of *Aspergillus oryzae* ASU44 (OL314732) and *Aspergillus flavus* ASU45 (OL314748).

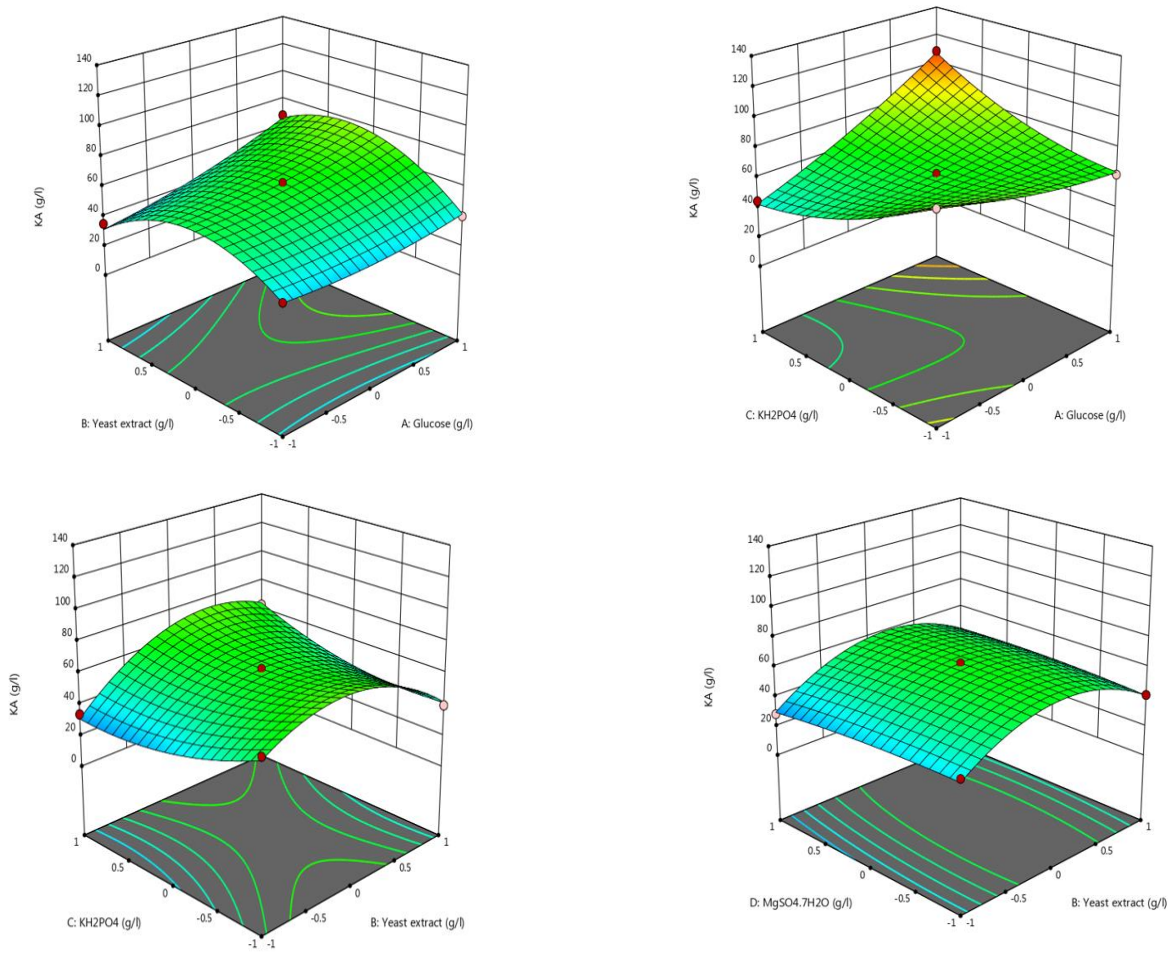


Fig.2: 3D surface plots of Box–Behnken statistical experimental design explaining the interactions effects of AB (Glucose (g/l) * Yeast extract (g/l)), AC (Glucose (g/l) * KH_2PO_4 (g/l)), BC (Yeast extract (g/l) * KH_2PO_4 (g/l)) and BD (Yeast extract (g/l) * $MgSO_4.7H_2O$ (g/l)) on kojic acid (g/l) production by co-culture of *Aspergillus oryzae* ASU44 (OL314732) and *Aspergillus flavus* ASU45 (OL314748).

The significance and effects of each individual variable and/ or the interactions were set in Table (2) as one-way ANOVA results. Individual variables of Glucose (A), Yeast extract (B), KH_2PO_4 (C), $MgSO_4.7H_2O$ (D) and pH (E) have significant effects on dry mass, while among the five variables C (KH_2PO_4) and D ($MgSO_4.7H_2O$) found to be non-significant for kojic acid production but C (KH_2PO_4) found to be non-significant for residual sugar. The interaction between different variables AB (Glucose (g/l) * Yeast extract (g/l)), AC (Glucose (g/l) * KH_2PO_4 (g/l)), AD (Glucose (g/l) * $MgSO_4.7H_2O$ (g/l)), BC (Yeast extract (g/l) * KH_2PO_4 (g/l)), BE (Yeast extract (g/l) * pH), CD (KH_2PO_4 (g/l)* $MgSO_4.7H_2O$ (g/l)), and DE ($MgSO_4.7H_2O$ (g/l) * pH) were found to be significant for kojic acid production (g/l), while the interaction between AE (Glucose (g/l) * pH), BD (Yeast extract (g/l) * $MgSO_4.7H_2O$ (g/l)) and CE (KH_2PO_4 (g/l) * pH) were non-significant for kojic acid production.

Derringer's desirability function was used to calculate an optimal variable concentration for high kojic acid production (g/l). By applying the function, the obtained optimum levels were Glucose (150, g/l) (A), Yeast extract (5 g/l) (B), KH_2PO_4 (3 g/l) (C), $MgSO_4.7H_2O$ (0.5 g/l) (D) and pH (3) (E) were found to be the best levels after using the function, which gives desirability equal to 1.000 for kojic acid production, as cleared at **Fig. (3).**

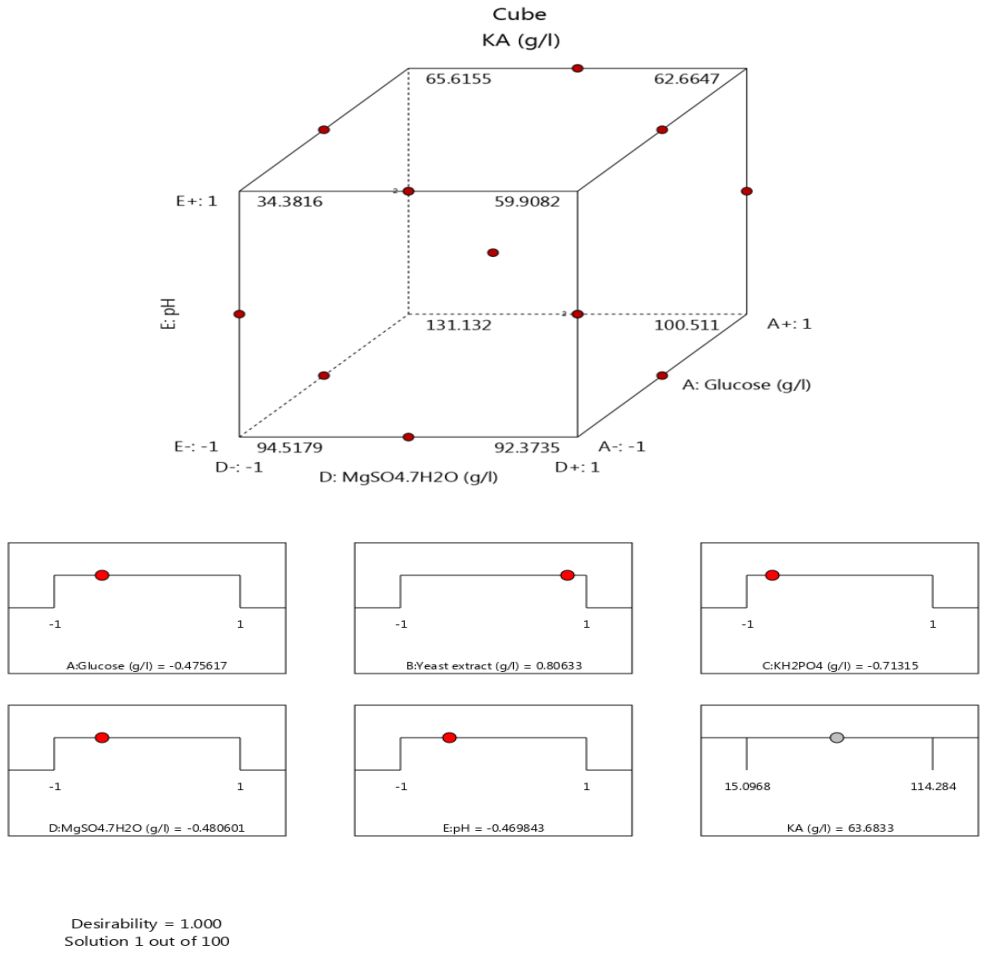


Fig. 3: The statistical optimization desirability ramp plot for kojic acid (g/l) production by co-culture of *Aspergillus oryzae* ASU44 (OL314732) and *Aspergillus flavus* ASU45 (OL314748).

Extraction and Crystallization of Kojic Acid

To choose the best extraction solvent and its extraction ratio, six extraction solvents were used: ethyl acetate, chloroform, methanol, ethanol, acetone and diethyl ether with three different ratios (1:1, 2:1 & 1:2 filtrate: solvent). It was cleared that ethyl acetate and acetone were the best extraction solvents, 1:1 and 1:2 were the best ratios for the extraction. Highest extracted quantity of kojic acid was 96.98±0.24 g/l using ethyl acetate (1:1, filtrate: solvent) as cleared in **Table (3)** & **Fig. (4)**. After evaporation, crystals are formed in needle shape as cleared in **Fig. 5 (a-c)**.

Table 3: Extraction of kojic acid (g/l) produced by co-culture of *A. oryzae* ASU44 (OL314732) and *A. flavus* ASU45 (OL314748). Six separated solvents were tested: ethyl acetate, chloroform, methanol, ethanol, acetone and diethyl ether with three different ratios (1:1, 2:1 & 1:2 filtrate: solvent).

Solvents	Ratios (filtrate: solvent)		
	1:1	2:1	1:2
Ethanol	71.74±0.96	72.28±5.86	78.62±1.9
Methanol	75.62±4.34	62.08±1.38	81.22±2.44
Diethyl ether	83.42±3.08	35.96±0.74	28.68±1.78
Ethyl acetate	96.98±0.24	58.14±4.18	95.62±0.74
Acetone	90.64±2.54	73.26±5.54	91.7±0.9
Chloroform	61.84±6.74	24.32±0.9	41.1±2.22

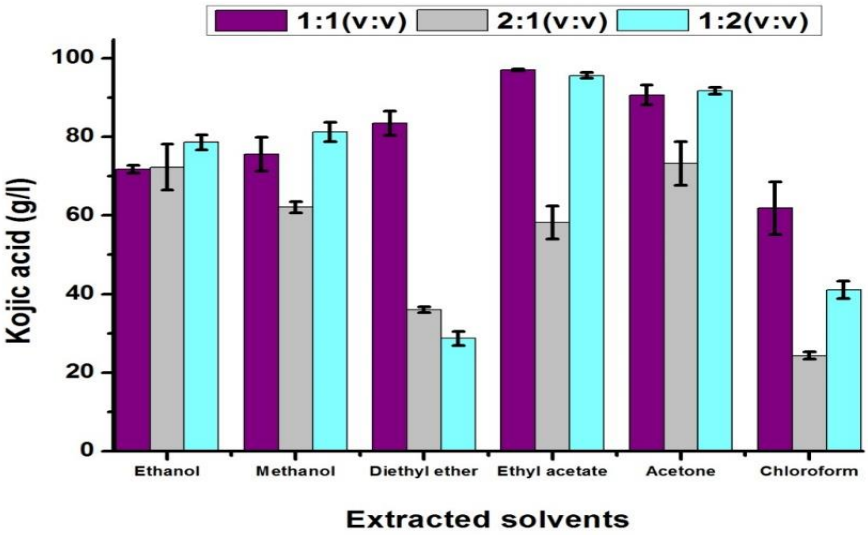
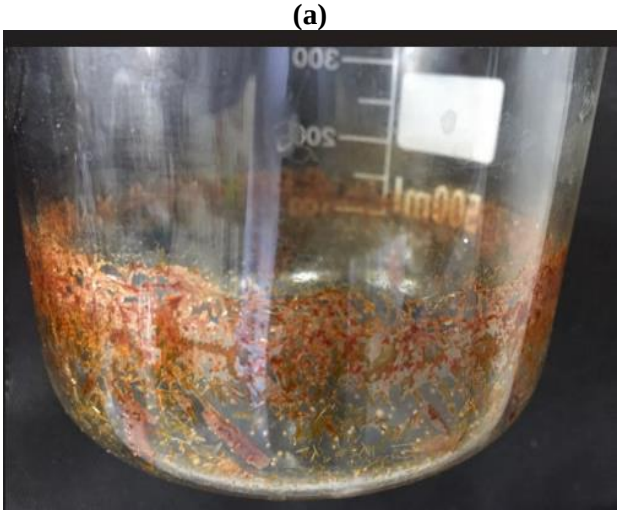
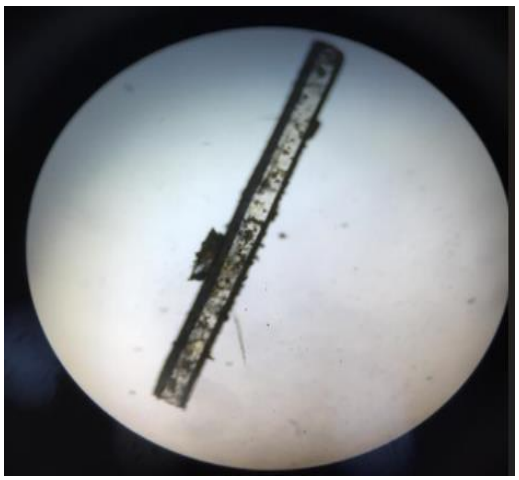


Fig. 4: Extraction of kojic acid (g/l) produced by co-culture of *A. oryzae* ASU44 (OL314732) and *A. flavus* ASU45 (OL314748). Six separated solvents were tested: ethyl acetate, chloroform, methanol, ethanol, acetone and diethyl ether with three different ratios (1:1, 2:1 & 1:2 filtrate: solvent).

(a)



(b)



(c)

Fig. 5: Kojic acid crystals shape visual (a) and under light microscope (b& c).

Kojic acid activity in fungal de-pigmentation

The effects of different kojic acid concentrations (1-10 µg/ml) were cleared in **Fig.s (6& 7).** The suitable wavelength for measuring black pigment of *Aspergillus niger* ASU311 was 411 nm **(Fig., 6)**. Inhibition of fungal pigment formation increased by increasing the concentration of kojic acid compared with the control samples. UV absorbance at concentration from 1 to 4 µg/ml; there wasn't clear inhibition, however high concentrations 7 (1.997), 8 (1.895), 9 (1.607) and 10 (1.459) µg/ml showed good inhibitions in the UV absorbance compared with the control samples (2.736 µg/ ml) as cleared in **Fig.s (6 & 7)**.

Kojic acid activity as anti-browning agent in food industry

Kojic acid activity increased by increasing the concentration until 50 µg/ml, the most effective concentration, where the kojic acid preserved the color of apples from the conversion to brown color as cleared in **Fig.s (8).** Maximum KA activity at 50 µg/ml was 1.48, 1.41, 1.37, 1.35, 1.46, and 1.47 after 1, 2, 3, 4, 5, and 6 hrs, respectively comparing with control samples 1.82, 1.84, 1.83, 1.81, 1.81, and 1.82 after 1, 2, 3, 4, 5, and 6 hrs, respectively.

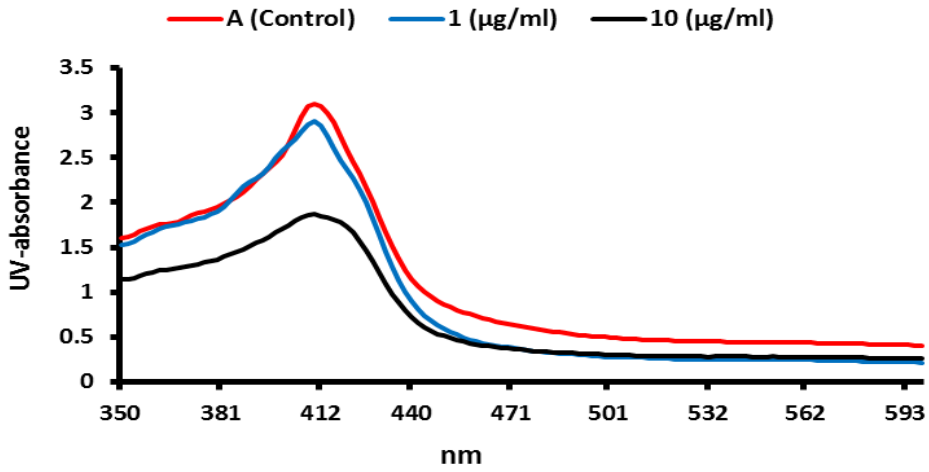


Fig. 6: UV- spectrum for black pigment of *Aspergillus niger* ASU311.

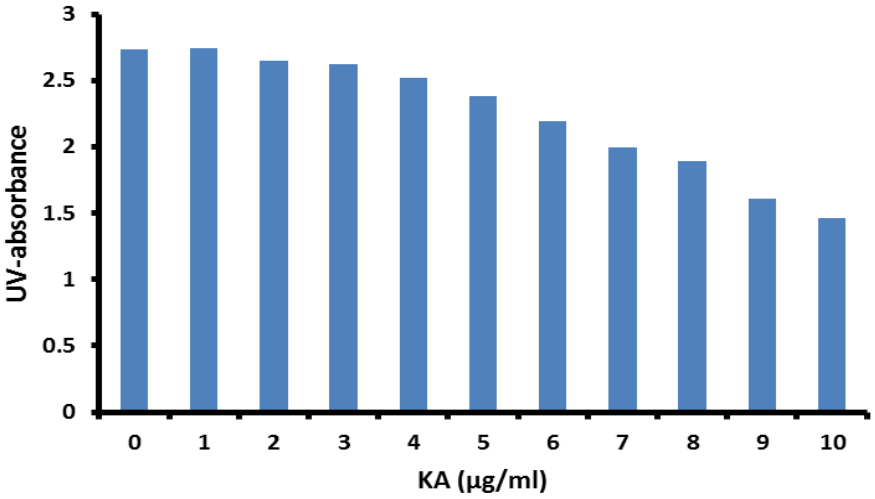


Fig. 7: Effect of different concentrations of kojic acid (0-10 µg/ml) on the black pigment production of *Aspergillus niger* ASU311.

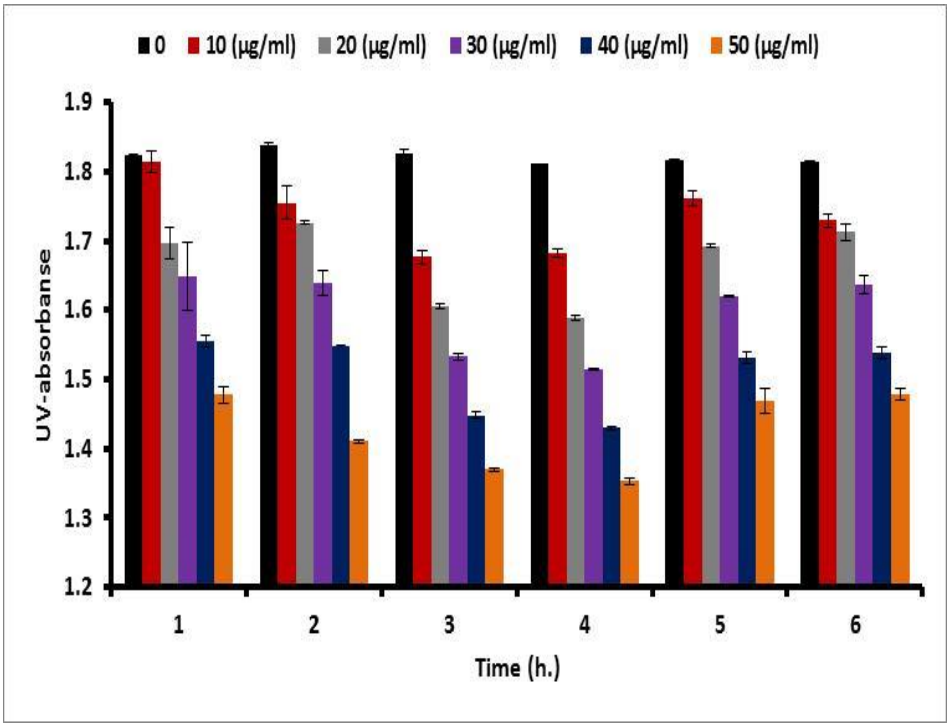


Fig. 8 : Effect of different concentrations of kojic acid (0-50 µg/ml) on fresh apple juice colour during 6 hrs of preservation in room temperature 30° C ±1.

Discussion

Co-culture fermentations may result in increased yield, improving control of product qualities and the possibility of utilizing cheaper substrates. In our study after optimizing kojic acid production by co-culture of *Aspergillus oryzae* ASU44 (OL314732) and *A. flavus* ASU45 (OL314748) using Box-Behnken statistical design (41 runs), kojic acid production enhanced to 114.28 g/l using fermentation medium with 150 g/l glucose, 5 g/l yeast extract, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$ at pH (3). Suryadi and Sukarna (2018)[17] found that Kojic acid production by co-cultures of *A. oryzae* and *A. tamarii* yielded 0.1396 gg^{-1} and the yield value of kojic acid was higher than that when using a sole culture. Numerous studies associated with the production of kojic acid have generally used *Aspergillus* as a sole culture. However, there was a study used a combination culture of *A. flavus* NSH9 and *A. flavus* Link 44-1 for kojic acid fermentation[16]. They reported that kojic acid produced by the mixture of the two *A. flavus* was higher yield than those produced by the sole use of each strains.

Extraction of kojic acid from the fermented medium was performed using six extraction solvents named: ethyl acetate, chloroform, methanol, ethanol, acetone and diethyl ether with three different ratios (1:1, 2:1 & 1:2 filtrate: solvent) of each. The highest extracted quantity of kojic acid was achieved using ethyl acetate (1:1, filtrate: solvent). In agreement with our study, Sanjotha *et al*. (2019)[10] used ethyl acetate, chloroform and methanol (1:1 ratio) for extraction kojic acid and acquired the maximum extracted quantity of kojic acid using ethyl acetate. Also, Chaves *et al*. (2012)[40]; Hazzaa *et al*. (2013)[9] and Ola *et al*. (2019)[41] used ethyl acetate for extraction of kojic acid as effective extraction solution.

Kojic acid was recorded as inhibitor for the formation of melanin by inhibition tyrosinase activity which responsible for the synthesis of melanin[26,27] So, examination of the effect of kojic acid on the pigment formation by *Aspergillus niger* was achieved in this study. The result revealed that kojic acid had the ability to inhibit the fungal pigment formation. The inhibition effect was increased by increasing the kojic acid concentration. Where there wasn't clear inhibition using concentrations from 1 to 4 µg/ml but high concentrations from 7 to 10 µg/ml showed good inhibitions comparing with the control

samples. Chee and Lee (2003)[42] proved that kojic acid significantly reduced the melanization of *Cryptococcus neoformans*. Berthelot *et al.* (2020)[43] showed that kojic acid also significantly reduced the black pigmentation of *Cadophora* sp. and *Phialophora mustea* at concentration 50 µg/ml.

Crystalized kojic acid was effective as anti-browning agent by using concentrations from 10 to 50 µg/ml, where 50 µg/ml was the most effective concentration that preserved the color of apple juice from conversion to brown color for 6 hrs comparing with the control. In this respect, Son *et al*. (2001)[22] founded that the minimum concentration for an effective anti-browning activity of kojic acid on apple slices was 500 µg/ml. A previous study revealed that kojic acid inhibited enzymatic browning in apple slices, and its inhibitory effects were stronger than those of caffeic, ferulic, chlorogenic, coumaric, cinnamic, and gallic acids[44].

Conclusion

It concluded that production of kojic acid by coculture of A. *oryzae* ASU44 (OL314732) and *A. flavus* ASU45 (OL314748) was higher than that produced by single culture. The crystallized kojic acid was effective in fungal depigmentation of *A.niger* ASU311. This study clearly appeared that kojic acid produced by fungi can used effectively for decoloring of black fungi as well as delaying the oxidation of the fresh juice. So, we can recommend using of kojic acid produced by fungi at exact concentration for decoloring of black fungi and as anti-browning agent in fresh juice.

REFERENCES

1. K. Saito, "über die saurebildung bei *Aspergillus oryzae*", ***J Botany,*** 21,7-1(1907).
2. M. Saeedi, M. Eslamifar and K. Khezri,"Kojic acid applications in cosmetic and pharmaceutical preparations", ***Biomed Pharmacother,*** 110,582- 593 (2019).
3. G. A-E. Mahmoud, A.B.M. Ibrahim, P. Mayer, Zn(II) and Cd(II) thiosemicarbazones for stimulation/inhibition of kojic acid biosynthesis from Aspergillus flavus and the fungal defense behavior against the metal complexes' excesses", ***J Biol Inorg Chem,*** 25,797–809 (2020).
4. M. T. de Caldas Felipe, R. do Nascimento Barbosa, J. D. P. Bezerra and C. M. de Souza-Motta, "Production of kojic acid by *Aspergillus* species: Trends and applications", ***Fungal Biol. Rev.,*** 45, 100313 (2023).
5. R. Mohamad, M. S. Mohamed, N. Suhaili, M. M. Salleh and A. B. Ariff, "Kojic acid: Applications and development of fermentation process for production", ***Microbiol Mol Biol Rev,*** 5(2), 24-37 (2010).
6. M. Watanabe-Akanuma, Y. Inaba and T. Ohta, "Mutagenicity of UV-irradiated Maltol in *Salmonella typhimurium*", ***Mutagenesis***, 22(1), 43-47 (2007).
7. G. A-E. Mahmoud, and A. Zohri, "Amedment stable kojic acid production by non-toxicogenic *Aspergillus oryzae* using five levels central composite design of response surface methodology", ***J Microbiol Biotech Food Sci,*** 10(6), e2683 (2021).
8. W. Crueger and A. Crueger, "A Text book of Industrial Microbiology", 2nded. ***Sinauer Associates, Sunderland, MA*** 01375(1984).
9. M. M. Hazzaa, A. M. Saad, H. M. Hassan and E. Ibrahim, "High production of kojic acid crystals by isolated *Aspergillus oryzae* var. *effusus* NRC14", ***J Appl Sci Res,*** 9(3), 1714-1723 (2013).
10. G. Sanjotha, C. T. Shivasharana and S. I. Manawadi, "An in vitro approach for evaluating antimicrobial activity and production of kojic acid by *Aspergillus flavus* isolated from karwar Region", ***J Pure Appl Microbiol,*** 13(4), 2261-2273 (2019).
11. J. Bader, E. Mast-Gerlach, M. K. Popović, R. Bajpai and U. Stahl "Relevance of Microbial coculture fermentations in biotechnology", ***J Appl Microbiol,*** 109(2), 371-387 (2010).
12. S. Bertrand, N. Bohni, S. Schnee, O. Schumpp‹ K. Gindro and J. L. Wolfender, "Metabolite induction via microorganism co-culture: a potential way to enhance

chemical diversity for drug discovery", ***Biotechnol Adv,*** 32(6), 1180-1204 (2014).
13. R. Banerjee, G. Mukherjee and K.C. Patra, "Microbial transformation of tannin-rich substrate to gallic acid through coculture method", ***Bioresour Technol,*** 96(8), 949–953 (2005).
14. A. Gupte and D. Madamwar, "Production of cellulolytic enzymes by co-culturing of *Aspergillus ellipticus* and *Aspergillus fumigatus* grown on bagasse under solid state fermentation", ***Appl Biochem Biotechnol,*** 62, 267–274 (1997).
15. F. Mittermeier, N. Hafner, K. XypoliaVasila and D. Weuster-Botz, "Co-Cultivation of *Aspergillus niger* and *Trichoderma reesei* Enables Efficient Production of Enzymes for the Hydrolysis of Wheat Bran", ***Chem Ing Tech,*** 95(4), 565-575 (2023).
16. A. Spencer, N. Suhaili, K. Bujang and A. Hussaini, "Comparative Study of Kojic Acid Production from Sago Hampas Using Different Strains of Aspergillus flavus via Solid State Fermentation", 2nd ed. ASEAN Sago Symposium. Kota Samarahan, Sarawak, Malaysia: UNIMAS, 29-31 (2012).
17. H. Suryadi and D. K. P. Sukarna, "Kojic acid production using mixed cultures of *Aspergillus oryzae* and *Aspergillus tamarii*", ***Int J Appl Pharm,*** 10, 279- 284 (2018).
18. S. Chib, V. L. Jamwal, V. Kumar, S. G. Gandhi and S. Saran, "Fungal production of kojic acid and its industrial applications", ***Appl Microbiol Biotechnol,*** 107(7-8), 2111-2130 (2023).
19. F. Bashir, K. Sultana, M. Khalid, and H. Rabia, "Kojic acid: a comprehensive review", ***Asian J Allied Health Sci,*** 6(1), 13-21 (2021).
20. Y. Niwa and H. Akamatsu, "Kojic acid scavenges free radicals while potentiating leukocyte functions including free radical generatio", ***Inflammation,*** 15(4), 303-315 (1991).
21. F. C. Wehner, P. G. Thiel, S. J. Van Rensburg, and I. P. Demasius, "Mutagenicity to Salmonella typhimurium of some *Aspergillus and Penicillium* mycotoxins", ***Mutat Res Genet Toxicol Environ Mutagen,*** 58(2-3), 193-203 (1978).
22. S.M. Son, K.D. Moon and C.Y. LLee, "Inhibitory effects of various anti-browning agents on apple slices", ***Food Chem,*** 73, 23- 30 (2001).
23. J. Szklarzewicz, P. Zabierowski, P. Kurpiewsks, K. Mikuriya, M. and D. Yoshioka, "Synthesis, crystal structures and spectroscopic studies of Mo (IV) complexes synthesized in reactions with kojic acid, maltol and ethyl maltol", ***Polyhedron,*** 37(1), 35 – 41 (2012).
24. J. H. McNeill, V. G. Yuen, H. R. Hoveyda and C. Orvig, "Bis (maltolato) Oxovanadium (IV) is a potent insulin mimic" ***J. Med. Chem.,*** 35(8), 1489-1491 (1992).
25. M.O. Masse, V. Duvallet, M. Borremans, and L. Goeyens, "Identification and quantitative analysis of kojic acid and arbutine in skin-whitening cosmetics", ***J Cosmet Sci,*** 23, 219-232 (2001).
26. J. M. Noh, S. Y. Kwak, H. S. Seo, J. H. Seo, B. G. Kim and Y. S. Lee, "Kojic acid–amino acid conjugates as tyrosinase inhibitors" ***Bioorganic Med. Chem. Lett.,*** 19(19), 5586- 5589 (2009).
27. A. F. B. Lajis, M. Hamid and A. B. Ariff, "Depigmenting effect of kojic acid esters in hyperpigmented B16F1 melanoma cells", ***J Biomed Biotech,*** 33, 1-9 (2012).
28. L. Saghaie, M. Pourfarzam, A. Fassihi and B. Sartippour, "Synthesis and Tyrosinase inhibitory properties of some novel derivatives of kojic acid", ***Int J Pharm Sci Res,*** 8(4), 233 (2013).
29. K. S. Ahn, K. Y. Moon and Y. S. Kim, "Kojic down-regulation of NF—KB activation in human keratinocytes by melanogenic inhibitors", ***J Dermatol Sci,*** 31(3), 193 - 201(2003).
30. R. L. Beard and G. S. Walton, "Kojic acid as an insecticidal mycotoxin" ***J Invertebr Pathol,*** 14(1), 53-59 (1969).
31. H. E. Morton, W. Kocholaty, R. Junowicz-Kocholaty and A. Kelner, "Toxicity and antibiotic activity of kojic acid produced by *Aspergillus luteo-virescens*", ***J Bacteriol,*** 50(5), 579-584 (1945).

32. N. A. Kamal-Eldin, A. A. Zohri, G.A-E. Mahmoud, "Kojic acid production by rhizospheric fungi recovered from Egyptian plants", ***Int Multidiscip Res J,***51(1), 89–102 (2022).
33. N.A.M.M. Kamal- Eldin, "Some applications of kojic acid produced by fungi", ***M Sc Thesis Microbiology Department, Faculty of Science, Assiut University***, (2022).
34. H. Liu, K. Wang, W. Schlindwein and M. LI, "Using the Box–Behnken experimental design to optimize operating parameters in pulsed spray fluidized bed granulation ", ***Int J Pharm,*** 448(2), 329-338 (2013).
35. S. Yan, H. Tang, S. Wang, L. Xu, H. Liu, Y. Guo and J. Yao, "Improvement of kojic acid production in strain *Aspergillus oryzae* B008 mutant strain and its uses in fermentation of concentrated corn stalk hydrolysates", ***Bioprocess Biosyst Eng,*** 37(6),1095-1103 (2014).
36. H. M. Hassan, A. M. Saad, M. M. Hazzaa and E. I. Ibrahim, "Optimization study for the production of kojic acid crystals by *Aspergillus oryzae var. effusus* NRC 14 isolate", ***Int J Curr Microbial Appl Sci,*** 3(10), 133-142 (2014).
37. B.M. Bitty and N. Nair, "Isolation and Cellulase production of *Aspergillus niger*", ***J Pest Sci***,1(2), 74-78 (2014).
38. M. A. Toma, K. N. H. Nazir, M. M. Mahmud, P. Mishra, M. K. Ali, A. Kabir, M.A.H. Shahid, M.P. Siddiqui and M. A. Alim, "Isolation and identification of natural colorant producing soil-borne *Aspergillus niger* from Bangladesh and extraction of the pigment", ***Foods,*** 10(6), 1280-1286 (2021).
39. H. Özoğlu and A. Bayındırlı," Inhibition of enzymic browning in cloudy apple juice with selected anti-browning agents", ***Food Cont,*** 13(4-5), 213-221 (2002).
40. F. C. Chaves, T. J. Gianfagna, M. Aneja, F. Posada, S. W. Peterson and F. E. Vega "*Aspergillus oryzae* NRRL 35191 from coffee, a non-toxigenic endophyte with the ability to synthesize kojic acid", ***Mycol Prog,*** 11(1), 263-267 (2012).
41. A. R. Ola, G. Metboki, C. S. Lay, Y. Sugi, P. D. Rozari, D. Darmakusuma and E. H. Hakim, "Single production of kojic acid by *Aspergillus flavus* and the revision of flufuran ", ***Molecules,*** 24(22), 4200-4215 (2019).
42. H. Y. Chee and E. H. Lee, "Fungistatic activity of kojic acid against human pathogenic fungi and inhibition of melanin-production in Cryptococcus neoformans" ***Mycobiology,*** 31(4), 248-250 (2003).
43. C. Berthelot, A. Zegeye, D. A. Gaber, M. Chalot, P. Franken, G. M. Kovács, and D. Blaudez, "Unravelling the role of melanin in Cd and Zn tolerance and accumulation of three dark septate endophytic species", ***Microorganisms,*** 8(4), 537- 553 (2020).
44. K. M. Moon, E. B. Kwon, B. Lee and C. Y. Kim, "Recent trends in controlling the enzymatic browning of fruit and vegetable products",***Molecules,*** 25(12), 2754-2763(2020).

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تحسين إنتاج حامض الكوجيك المتبلور من فطرة الأسبرجيللس وتطبيقة كعامل مضاد للتلون

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حامض الكوجيك له تطبيقات في العديد من المجالات لذلك من المهم زيادة كمية حامض الكوجيك. في هذه الدراسة تم زيادة إنتاج حامض الكوجيك باستخدام مزرعة مساعدة من فطري A. oryzae ASU44 (OL314732) و A. flavus ASU45 (OL314748) ، وقد تم تحسين إنتاجية الحامض من خلال التصميم الإحصائي Box-Behnken ، تم تحسين الإنتاج إلى ١١٤,٢٨ جم / لتر باستخدام الجلوكوز (١٥٠ ، جم / لتر) (أ) ، مستخلص الخميرة (٥ جم / لتر) (ب) ، KH2PO4 (3 جم / لتر) (MgSO4.7H2O (0.5 ،C) جم / لتر) (D) ودرجة الحموضة (٣) (هـ). كان التصميم فعالاً وقابلاً للتطبيق مع معامل (R2 99.1٪) وقيمة R2 المعدلة ٩٨,١٪. كان أسيتات الإيثيل أفضل مذيب استخلاص بنسبة ١: ١ وظهرت بلورات حامض الكوجيك على شكل إبر بعد تبخر أسيتات الإيثيل باستخدام المبخر الدوراني. حامض الكوجيك المتبلور لديه القدرة على تثبيط الصبغة الفطرية لـ Aspergillus niger ASU311. مع زيادة تركيز KA ، انخفض اللون الأسود ، خاصة عند ١٠ ميكروغرام / مل مقارنة بعينات التحكم. من ناحية أخرى ، حافظت KA المتبلورة على لون عصير التفاح من التحول إلى اللون البني وزاد النشاط بزيادة التركيز حتى ٥٠ ميكروغرام / مل لمدة ٦ ساعات مقارنة مع المجموعة الضابطة. ويعد تطبيق حامض الكوجيك المنتج من الاسبرجيللس طريقة صديقة للبيئة للحفاظ علي جودة العصائر الطازجة.