JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

AMEDMENT STABLE KOJIC ACID PRODUCED BY NON-TOXINOGENIC ASPERGILLUS ORYZAE USING FIVE LEVELS CENTRAL COMPOSITE DESIGN OF RESPONSE SURFACE METHODOLOGY

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https://doi.org/10.15414/jmbfs.2683

ARTICLE INFO

Received 24. 2. 2020
Revised 23. 1. 2021
Accepted 1. 2. 2021
Published 1. 6. 2021

Regular article

OPEN ACCESS

ABSTRACT

Kojic acid is a remarkable secondary metabolite of *Aspergillus* with various hot spot applications in the field of medicine, cosmetics, food, agriculture, and chemistry field. However the needs of stable large production with safe cultures still need continuous searching. Microbial kojic acid concentrated highly on *Aspergillus* species especially *Aspergillus flavus* group. Ten isolates of *A. flavus* and *A. oryzae* isolated from various Egyptian sources were producible of KA in range 0.091±0.01 to 66.81± 0.95 g/l. *Aspergillus oryzae* (no. 4) that give maximum production was selected as non-toxinogenic safe isolate for optimizing the production by five levels CCD design of RSM. Maximum value of kojic acid with 108.4% increasing was 139.24 g/L (predicted 135.8 g/L) using glucose (+1; 200 g/l), yeast extract (+1; 6 g/l), KH_2PO_4, (+1; 1.5 g/l) and $MgSO_4.7H_2O$ (+1; 1 g/l) through run (24). Model significance and validity tested by R^2 values of KA was 0.987, DM 0.989 and CS 0.9831 and calculated with Derringer's desirability function as 0.937. Optimized kojic acid showed stability against different range of heat stress from 40°C to 100°C during five continuous hours which may attribute that microbial product usually more stable than synthetic ones by attaching it with other active groups that guaranty more stability under stress conditions. *Aspergillus oryzae* (*Ao*-4) represents promising safe isolate for industrial kojic acid production with highly product stability using this significant valid experimental design.

Keywords: Kojic acid, Kojii, Fungi, Derringer's desirability function, industrial, stability

INTRODUCTION

Kojic acid is a major heterocyclic natural secondary metabolite related to weekly organic acid with molecular formula ($C_6H_6O_4$) with chemical structure 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one **(Brtko *et al.*, 2004)**. It has been used highly in cosmetics products like skin-lightning products for its high inhibitory effect against tyrosinase (alternative of hydroquinone) **(Wang *et al.*, 2014)**, is an iron and copper chelator prevent oxidation, hyperpigmentation, photodamage, and skin wrinkling **(Briganti *et al.*, 2003)**. It's used as skin lotions, soaps, creams, and other products **(Faig *et al.*, 2017)**. Also, it has large pharmaceutical applications such as antibacterial (Gram-negative), antiviral, anti-inflammatory properties, pain relief, anti-aging, antidermatophytic, radical scavenging agent, biocompatibility medical and antiprotozoans **(Prignano *et al.*, 2007; Gonçalez *et al.*, 2015; Syamsul *et al.*, 2017)**. In agriculture it's used as antimicrobial, pesticides and insecticidal agent **(Burnett *et al.*, 2010)**. In Japanese foods (soybean paste, sake, soy sauce, and mirin) include as a preservative food antioxidant agent **(Sheikhshoaie *et al.*, 2017)**.
Kojic acid was produced originally from *Aspergillus oryzae* **(Machida *et al.*, 2005)** in high quantities, however it also produced by *Aspergillus* sp., *Penicillium* sp., *Acetobacter* sp., and *Bacillus* sp. **(Masse *et al.*, 2001; Machida *et al.*, 2005; Pildain *et al.*, 2008; Vasntha *et al.*, 2014).** *Aspergillus* spp. were known to produce large amounts of kojic acid like *A clavatus, A awamorii, A fumigatus, A candidus, A. flavus, A. parasiticus, A. oryzae*, and *A. tamarii* **(Kwak and Rhee, 1992; Lee *et al.*, 2006; Terabayashi *et al.*, 2010; Chang *et al.*, 2011; Prabu *et al.*, 2011; Mahmoud *et al.*, 2020a).** Researcher's didn't ignore the risk of aflatoxin produced by *Aspergillus* strains, **Madihah *et al.* (1996)** showed that aflatoxin synthesis by kojic acid producing isolates like *A. flavus* could inhibited using suitable medium and culture conditions, also both kojic acid and aflatoxin follows different synthesis pathways in *Aspergillus* sp. **(Basappa *et al.*, 1970)**. Several methods were used for KA analysis including column chromatography, voltammetry, mass spectrometry, thin-layer chromatography, high-performance liquid chromatography but the easiest way was spectrophotometry detection as its generate brown reddish color when react with ferric chloride **(Tanigaki *et al.*, 1980; Dobias and Brtko, 1985; Frisvad, 1987; GoTo *et al.*, 1990; Pildain *et al.*, 2008).**
Medium constituents especially the carbon and nitrogen sources are the highest parameters that effects on kojic acid production. Glucose considered the best carbon source used highly for kojic acid production as to the structure similarity with kojic acid. Scientist suggested that, kojic acid formed directly from glucose during the fermentation without any carbon chain cleavage into smaller fragments **(Kitada *et al.*, 1967; Basappa *et al.*, 1970; Chang *et al.*, 2011).**
A classical optimizing method depends on studying one factor each time neglecting the interaction between factors that could increase the production by 100% or more, while statistically optimization used the parameter interactions in convenient runs number saving time and reduce the error possibility by introducing the predicted values **(Xu *et al.*, 2003; Chen *et al.*, 2009).** Experimental designs represent a new way for production optimization which could easily save the experiment time and introduce possible parameter combinations with various KA quantities. Response Surface Methodology is one of the effective experimental designs that explain the quantitative experimental data by simultaneously multivariate equations **(Vohra and Satyanarayana, 2002; Desai, 2008).** It is widely utilize in the bioprocess technology and optimization of numerous types of growth parameters giving the best combination of the parameters with responses prediction **(Grahovac *et al.*, 2014; Kong *et al.*, 2014).**
Aspergillus flavus group, especially *A. flavus* and *A. oryzae*, represents the most significant producing fungal group of kojic acid **(Machida *et al.*, 2005).** However most of studies discussed the effect of fermentation parameters on kojic acid production using one- factor only method and neglect the interaction between these parameters and its effects in increasing the production process especially by using statistical tool like response surface methodology. So, the aim of this study was design to examine the ability of some *A. flavus* and *A. oryzae* isolates to produce KA. Also, studying the interaction process between different medium constituents on the KA production and evaluate the stability properties of the microbial kojic acid from non-toxicogenic *A. oryzae* were aimed.

MATERIAL AND METHODS

Fungal isolation

Ten isolates of *Aspergillus flavus* group (*A. flavus* and *A. oryzae*), were isolated from soil, milk, and spoiled nuts samples using direct and dilution plate methods and incubated at 28±1°C on potato dextrose (PDA) medium containing (g/l): 200, scrubbed and diced potato; 15, dextrose; 20, agar; 1000 distilled water**.** Medium initial was justified to pH 5.6 with 1N HCl and autoclaved for 20 min. at 121ºC. Sterilized medium supplemented with bactericidal agents (chloramphenicol) **(Booth, 1971; Mohamed and Mahmoud, 2018).** Generated fungal isolates were identified according to its growth, macroscopic and microscopic features described by **Raper and Fennell (1965), Domsch *et al.* (1980) and Bennett (2010).** Cultures examined using Olympus CX41, Japan microscope for microscopic properties after staining by lactophenol cotton blue for clear image analysis **(Ibrahim *et al.*, 2020)**. Purified isolated maintained on PDA slants, preserved at 4± 1 °C and sub-cultured every two weeks until using.

Screening kojic acid medium and inoculum preparation

For the harvesting of *Aspergillus* spores the fungal isolates were re-cultured on the same preservation medium (PDA) at 28±1°C aerobically for 3 days **(figure 1(a)).** *Aspergillus* spores were collected off medium by scratching the growth surface and mixed with sterilized 0.1% (v/v) tritonx100 in deionized water, and vortex for 5 min then diluted with the same steps to 3×10^5 spore/ml. Kojic acid screening medium includes (g/l): glucose 100; KH_2PO_4, 1.0; yeast extract, 5.0; $MgSO_4.7H_2O$, 0.5 and 1000 distilled water **(Ariff *et al.*, 1996; Liu *et al.*, 2016; Mahmoud *et al.*, 2019).** Before sterilizing the medium by autoclaving (20 min., 1.5 atmospheric pressure at 121°C) initial pH was set to 3 by 1N HCl. After sterilization, the medium was fortified with membrane sterilized (0.22 mm pore size) chloramphenicol as bacteriostatic agent at 250 mg/ml **(Ibrahim and Mahmoud, 2019)**. Each 100 mL medium inoculated with one ml contains 3×10^5 spore of *Aspergillus* inoculum prepared suspension stock, incubated aerobically at 28±1°C incubator with rotary shaker (150 rpm) for 7days. After that, culture flask filtered on weighed filter paper (Whatman No. 113), washed trice by distilled water and dried in hot air oven at 70 °C for 24 h. to estimated *Aspergillus* dry (DM) mass. Supernatants were collected and centrifuged at 4,000×g for 10 min then clear supernatants were used for quantitative estimation of KA **(figure 1(b))** and the consumed sugars (CS).

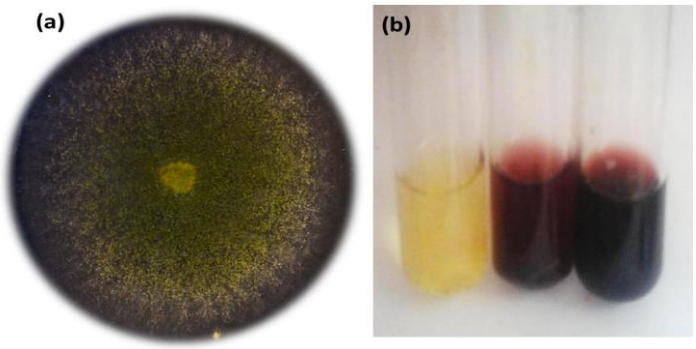


Figure 1 (a) growth of *Aspergillus oryzae* on PDA medium after three days, (b) purple-red color developed after the addition of ferric chloride reagents against kojic acid free samples (yellow).

Selection of the most potent fungal isolate for safety test

Aspergillus oryzae isolate number four was selected as the highest KA producer from the screening experiment and tested for its ability to produce mycotoxins especially aflatoxins as following; the fungus spore suspension was inoculated into liquid PDA medium and incubated for 10 days at 28±1°C in rotary shaker incubator with speed 150 rpm. After ten days culture flask were homogenized with equal volume of chloroform in high speed homogenizer for 5 min., and then filtrated to remove the fungal mycelia. Organic chloroform layer was separated from aqueous layer using separation funnel on sodium sulfate anhydrous and being allowed to evaporate in order to concentrate it to approximately 1 ml **(Scott *et al.*, 1970; Sadhasivam *et al.*, 2017)**. Presence of aflatoxins in crude extract was detected using thin layer chromatography (TLC) as following; silica gel plates (SiO_2, 60 GF254) were injected with 10 µl crude extract leave the spot to dry in cold air flow, placed in solvent chamber contains in chloroform: acetone at a ratio 90:10; as running solvents. After running step, the developed plates were dried in air and examined under shortwave (254 nm) and longwave (356 nm) ultraviolet radiation. When aflatoxin is present, developed blue or green colours bands of aflatoxins will observed **(Scott *et al.*, 1970; El-Kady and Moubasher, 1982; Lin *et al.*, 1998)**.

Maximization of kojic acid production by RSM

For maximizing kojic acid production central composite design (CCD) of response surface methodology (RSM) was utilized. The four production medium constituents including glucose, yeast extract, KH_2PO_4, and $MgSO_4.7H_2O$ were analyzed in five levels contains very low concentration (-2), low concentration (-1), the original constituent concentration (0), and high concentration (+1), higher concentration (+2) as shown in table **(1).** A set of twenty four factorial runs in trice and one run represents the center point (for reproducibility) were performed **(Yan *et al.*, 2014; Nafady *et al.*, 2015; Mahmoud *et al.*, 2020b)**. The variables are following equation **(1)** for the statistical calculation:

$$x_i = X_i - X_0/\delta X \qquad (1)$$

Where x_i represents the dimensionless number of X_i as independent variable; X_i represents the experimental value of the variable; X_0 represents the value of X_i at the center point; δX represents the step change in variable i experimental numbers to a variation of a unit for dimensionless value of variable i. Each variable role, interactions, and statistical analysis to calculate the predicted values is calculated by applying the quadratic equation **(2):**

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^{\,2} + \sum \beta_{ij} x_{ij} \qquad (3)$$

Where Y represents the predicted values, β_0 represents the offset term, β_i represents the linear effect, β_{ii} represents the squared effect, β_{ij} is the interaction effect, x_{ij} represents the independent variables levels. To confirm the selected optimized conditions Derringer's desired methodology was used; trice experiments were conducted under the previous cleared optimized conditions then compared the actual and predicted values for the validity of the models.

Stability of Kojic acid at different temperatures

The stability of kojic acid at different temperatures was determined as following. Fermentation broth of the highest KA in the best optimized medium constituents was centrifuge at 4,000×g for 5 min. to remove any fungal residuals and used. At the beginning of the experiment the crude KA quantity was measured as initial concentrations. Glass tubes containing five ml of the aqueous KA broth were incubated in water bath at 40, 60, 80, and 100°C, samples periodically withdrawn during the incubation period at 0, 1, 2, 3, 4, and 5 h. Changing in KA concentration was measured spectrophotometerically at 500nm using ferrous chloride reagent. The degradation ratio calculated as the ratio between KA at zero time and after the treatment. All treatments were in triplicate and the standard deviations were estimated **(Santos-Edinum *et al.*, 2013).**

Chemical analysis

Spectrophotometer measurements analysis was estimated using a T60 split beam UV spectrophotometer covering the wavelength range of 190–1100 nm. Kojic acid was determined as **Bentley (1957) and (2006)** by ferric chloride (1% in 0.1N HCl) reagent, purple-red color will developed and measured against free blank quantitatively at 500 nm. Kojic acid concentrations were calculated from absorbance using standard curve of pure kojic acid as g/l **(Mahmoud *at el.*, 2020a).** Residual sugars were measured using anthrone-sulfuric acid method as decribed by **Yemm and Willis (1954)** by reacting anthrone-sulfuric acid reagent with the fungal supernatant at 100ºC in water bath for ten minutes. After cooling, the absorbance of developed green color was detected at 620 nm against free sample, then the glucose concentration was calculated from standard glucose curve (g/l).

Statistical analysis

Statistical analysis was done using the statistical software of statistical program Design Expert 7.0.0 (Minneapolis, Stat-Ease Inc., USA). Analyses of experimental data were performed using multiple regression analysis. Linear, quadratic regression coefficients and the interaction that involved in the design model were analysis by analysis of variance (ANOVA). The quality of the regression model was expressed as R^2, runs significance checked by *F*- test with probability ranges ($p \leq 0.05$). To clear the variables relationships and the optimum variables concentrations, response surface (3D) plots and curves were drawn.

RESULTS

Screening for kojic acid production and selection of the most potent fungal isolates

Ten isolates of *Aspergillus flavus* group including four isolates of *A. flavus* and six isolates of *A. oryzae* were isolated from different sources air, soil, milk, and spoiled nuts and tested for kojic acid production on glucose medium **(figure 2).** All isolates were positive to KA production with wide quantities range from 0.091±0.01 g/l (*A. flavus* no.9) to 66.81± 0.95 g/l (*A. oryzae* no. 4). The isolates

also give various mass of growth between 18.13±0.34 g/l (*A. oryzae* no. 3) and 37.3± 0.71 g/l (*A. oryzae* no. 1) with sugar consumption percentage from 27.58±1.45% (*A. flavus* no.9) to 90.175±1.13% (*A. oryzae* no. 4). *Aspergillus oryzae* (*Ao*-4) isolate was selected as the most kojic acid potent isolate giving 66.81± 0.95 g/l with 21.6± 0.65 g/l dry mass and highest sugar consumption percentage 90.175±1.13% comparing to the other isolates. Mycotoxins analysis gives negative results, no aflatoxin bands appeared, which combine the isolate as safe industrial potential isolate. Brief morphology (macroscopic and microscopic) description of the selected isolate was cleared in **figure (3).** *Aspergillus oryzae* grown on Czapek's dextrose agar medium (CzD) and Potato dextrose agar medium (PDA) appeared as yellowish margins shifting to yellow-green towards the colony center when it young (3 days) shifting to greyed-brown when it became old. The developing colony lacks exudates with colorless reverse. Microscopic features includes colorless long conidiophores 15-25 µm, large radiate conidial head with sub-globose vesicle (18-45 µm) without metulae, conidial chains with globose to sub-globose, smooth to rough conidia 4-6 µm diameter.

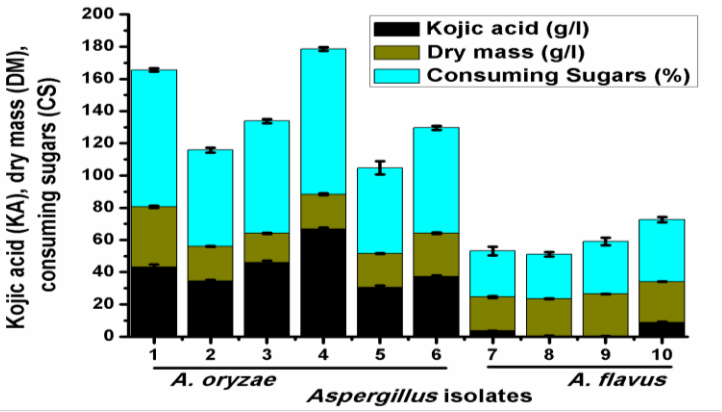


Figure 2 Screening for kojic acid production (g/l), dry mass (g/l) and sugar consumption (%) by ten isolates of *Aspergillus* (numbered 1-10) on kojic acid production medium.

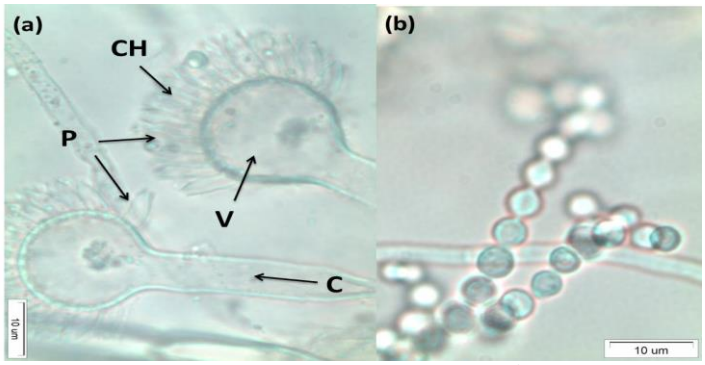


Figure 3 *Aspergillus oryzae* (Ahlburg) Cohn microscopic features, includes (a) conidial head (CH), phialid (P), conidiophore (C), vesicle (V) in the left figure and (b) conidial chains in the write figure, Bars, 10µm.

Maximization of kojic acid production by RSM

For maximizing kojic acid production statistical optimization using CCD design of response surface methodology was performed on medium containing four constituents (glucose, yeast extract, KH_2PO_4, and $MgSO_4.7H_2O$) at five levels (-2, -1, 0, +1, +2) as mentioned in table **(1).** Predicted values of the estimated runs calculated by applying multiple regression analysis using second-order polynomial **equation (2).** Kojic acid predicted values calculated by **equation (3),** dry mass (g/l) by **equation (4)** and sugar consumption (%) by **equation (5)** as following:

Kojic acid (g/l) = 68.14 + 22.29 * A + 15.59 * B + 7.42* C+ 6.5* D+ 10.10* AB + 7.08* AC + 5.44* AD + 4.18* BC + 0.0139* BD + 3.37* CD + (-6.26)* A^2 + (-2.58) * B^2 + (-6.54) * C^2 + (-4.03) * D^2 **(3)**

Dry mass (g/l) = 26.47 + 13.07 * A + (-7.31) * B + 1.71* C+ 0.41* D+ (-3.9)* AB + 0.82* AC + 0.66* AD + (-4.46)* BC + 1.4* BD + 0.06* CD + 0.77* A^2 + 0.44* B^2 + 1.63 * C^2 + (-0.026) * D^2 **(4)**

Sugar consumption (%) = 93.02 + (-11.74)* A + 8.29* B + 6.04* C+ 6.19* D+ 1.05* AB + 2.86* AC + 0.26* AD + (-1.16)* BC + 0.37* BD + 2.33* CD + (-8.57)* A^2 + (-5.04) * B^2 + (-5.7) * C^2 + (-8.02) * D^2 **(5)**

Where A, B, C, D are the coded values of glucose, yeast extract, KH_2PO_4, and $MgSO_4.7H_2O$, respectively. Maximum experimental value of kojic acid was 139.24 g/L, whereas the predicted corresponding value was 135.8 g/L using glucose (+1; 200 g/l), yeast extract (+1; 6 g/l), KH_2PO_4, (+1; 1.5 g/l) and $MgSO_4.7H_2O$ (+1; 1 g/l) through run (24) with 33.47 g/l dry mass and 82.25% sugar consumption**.** Maximum experimental value of dry mass was 63.33 g/l, with corresponding predicted value 62.16 g/L using glucose (+1; 200 g/l), yeast extract (-1; 4 g/l), KH_2PO_4, (+1; 1.5 g/l) and $MgSO_4.7H_2O$ (-1; 0.1 g/l) through run (13)**.** Highest sugar consumption percentage observed in run (2) with experimental value 94.15% and predicted 95.31% using glucose (-1; 50 g/l), yeast extract (+1; 1.5 g/l), KH_2PO_4, (+1; 1.5 g/l) and $MgSO_4.7H_2O$ (+1; 1 g/l) as cleared in **in table (1)**.

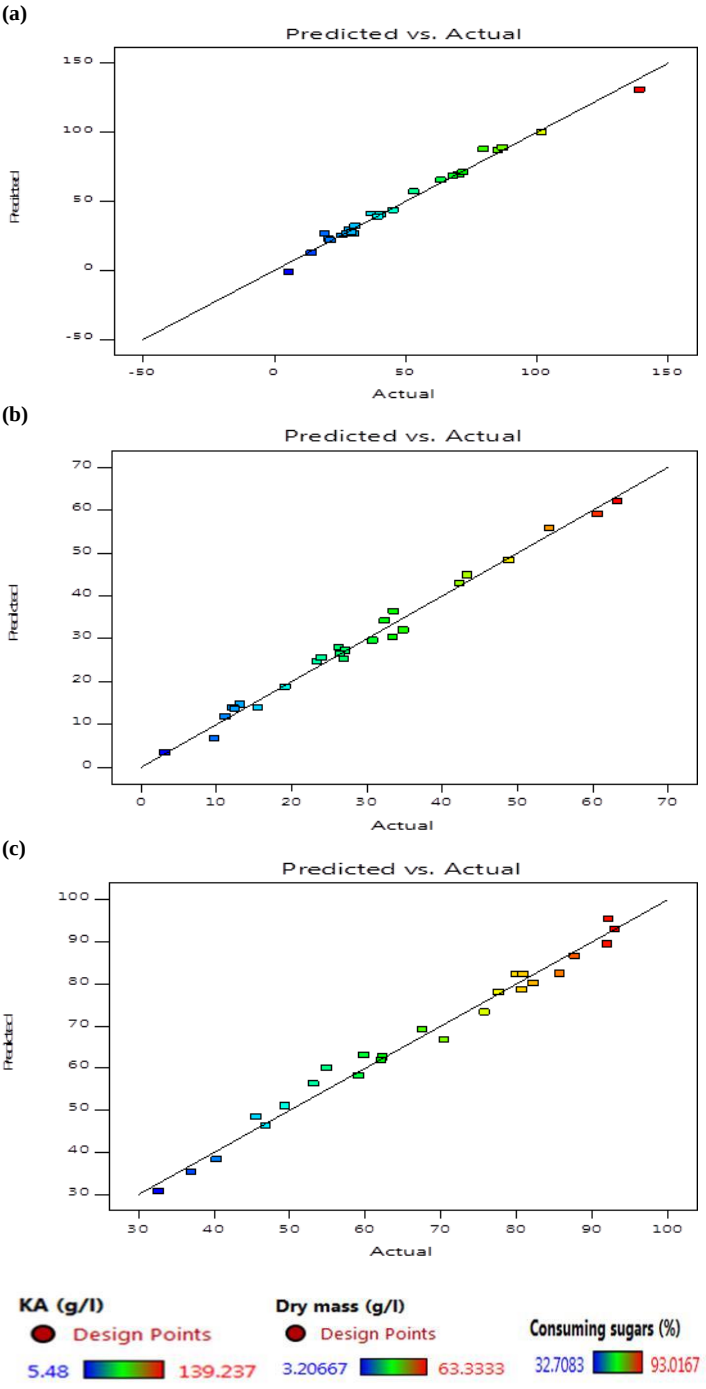


Figure 4 Comparison between the predicted and the actual values of kojic acid production (a), dry mass (b), and consuming sugars (c) by *Aspergillus oryzae* (Ahlburg) Cohn.

The predicted values of KA, DM, and CS of the response surface model were located in close proximity to the experimental ones as shown in **Table 1** and **figures (4a** for KA, **4b** for DM, and **4c** for CS**)** which supported the consideration of RSM optimizing method is sufficient to illustrate and explain data variations and variables actual relationships. These polynomial Eqs **(3, 4, and 5)** was further tested for confirmation the model suitability and significance by an analysis of variance (ANOVA) as shown in **table 2**. The model *F* and *P*-values of kojic acid (54.1; P<0.0001), dry mass (64; P<0.0001), and consuming sugars (41.63; P<0.0001). The test estimated the model failure of represent data

in the experimental values at points by lack of fit significance that preferred non-significant for the model signify. Lack of fit values was no significant relative to the pure error (lack of fit P<0.0001) proved that the model was significant. To evaluate the model goodness and fitting, different evaluated statistical parameters like coefficient (R^2) estimation was calculated, R^2 values of KA was 0.987, DM 0.989 and CS 0.9831 with adjusted R^2 values were 0.9687, 0.9735 and 0.9595 for KA, DM and CS, respectively which indicates that the entire variation was explained by the model.

Table 1 Response surface experimental design with operational variables; glucose (A), yeast extract (B), KH_2PO_4 (C), and $MgSO_4.7H_2O$ (D), actual and predicted responses; kojic acid yield (KA; g/l), dry mass (DM; g/l), and consuming sugars (CS; %).

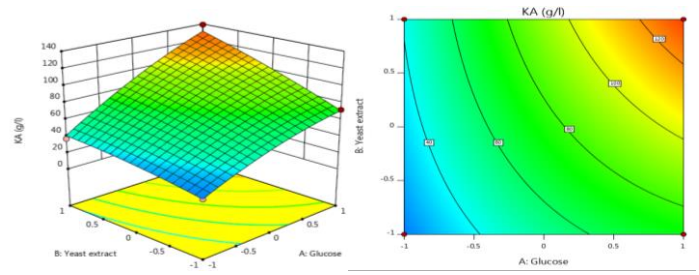
Run Order	A: Glucose	B: Yeast extract	C: KH_2PO_4	D: $MgSO_4.7H_2O$	KA (g/l)		Dry mass (g/l)		Consuming sugars (%)	
					Actual value	Predicted value	Actual value	Predicted value	Actual value	Predicted value
1	-1 (50)	-1 (4)	1 (1.5)	1 (1)	21.42	21.61	23.40	24.70	85.73	82.40
2	-1 (50)	1 (6)	1 (1.5)	1 (1)	36.99	40.97	11.20	11.77	94.15	95.31
3	1 (200)	-1 (4)	-1 (0.5)	1 (1)	45.44	43.65	43.33	44.85	40.35	38.29
4	-1 (50)	1 (6)	-1 (0.5)	1 (1)	25.80	25.20	19.20	18.78	87.70	86.60
5	0 (100)	0 (5)	-2 (0.1)	0 (0.5)	29.56	27.13	30.80	29.57	59.13	58.14
6	0 (100)	0 (5)	2 (2)	0 (0.5)	53.37	56.80	33.60	36.42	80.83	82.29
7	-1(50)	-1 (4)	-1 (0.5)	-1 (0.1)	19.35	27.01	13.20	14.65	62.27	62.62
8	0 (100)	0 (5)	0 (1)	0 (0.5)	68.14	68.14	26.47	26.47	93.02	91.02
9	2 (300)	0 (5)	0 (1)	0 (0.5)	79.69	87.68	54.27	55.68	36.99	35.25
10	0 (100)	-2 (3)	0 (1)	0 (0.5)	27.55	26.64	42.33	42.85	53.19	56.26
11	0 (100)	0 (5)	0 (1)	-2 (0)	39.54	38.83	24.00	25.55	45.58	48.54
12	-1(50)	-1 (4)	-1 (0.5)	1 (1)	20.87	22.56	15.53	13.86	67.53	69.07
13	1 (200)	-1 (4)	1 (1.5)	-1 (0.1)	40.45	40.26	63.33	62.16	46.83	46.22
14	-1(50)	1 (6)	1 (1.5)	-1 (0.1)	30.88	31.89	9.80	6.70	77.70	78.05
15	1 (200)	1 (6)	1 (1.5)	-1 (0.1)	101.89	99.98	26.33	28.00	62.17	61.86
16	1 (200)	-1 (4)	-1 (0.5)	-1 (0.1)	30.56	26.35	48.87	48.29	32.71	30.78
17	-2 (10)	0 (5)	0 (1)	0 (0.5)	5.48	-1.50	3.21	3.38	80.00	82.21
18	0 (100)	0 (5)	0 (1)	2 (1.5)	63.48	65.20	27.13	27.18	75.78	73.30
19	-1(50)	-1 (4)	1 (1.5)	-1 (0.1)	14.23	12.59	27.00	25.24	70.47	66.62
20	1 (200)	1 (6)	-1 (0.5)	1 (1)	85.28	86.71	32.40	34.16	54.96	60.04
21	-1(50)	1 (6)	-1 (0.5)	-1 (0.1)	28.93	29.60	12.27	13.96	80.70	78.67
22	1 (200)	-1 (4)	1 (1.5)	1 (1)	71.92	71.03	60.67	58.97	59.79	63.06
23	1 (200)	1 (6)	-1 (0.5)	-1 (0.1)	70.33	69.36	34.87	31.98	49.42	51.04
24	1 (200)	1 (6)	1 (1.5)	1 (1)	139.24	135.80	33.47	30.43	82.25	80.18
25	0 (100)	2 (7)	0 (1)	0 (0.5)	87.10	89.01	12.53	13.61	92.02	89.42

Table 2 Analysis of variance (ANOVA) for response surface (RSM) quadratic model of kojic acid (KA; g/l), dry mass (DM; g/l) and consuming sugars (CS; %) by *Aspergillus oryzae*.

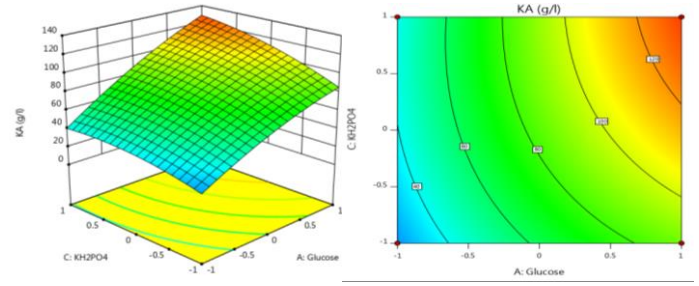
Source	Sum of Squares				Mean Square			*F*-value			*p*-value		
	KA (g/l)	DM (g/l)	CS (%)	df	KA (g/l)	DM (g/l)	CS (%)	KA (g/l)	DM (g/l)	CS (%)	KA (g/l)	DM (g/l)	CS (%)
Model	24200.06	6125.46	8017.15	14	1728.58	437.53	572.65	54.10	64.00	41.63	< 0.0001	< 0.0001	< 0.0001
A-Glucose	11928.75	4102.59	3308.66	1	11928.75	4102.59	3308.66	373.32	600.11	240.50	< 0.0001	< 0.0001	< 0.0001
B-Yeast extract	5833.56	1281.88	1650.28	1	5833.56	1281.88	1650.28	182.57	187.51	119.96	< 0.0001	< 0.0001	< 0.0001
C-KH_2PO_4	1321.20	70.50	874.60	1	1321.20	70.50	874.60	41.35	10.31	63.57	< 0.0001	0.0093	< 0.0001
D-$MgSO_4.7H_2O$	1043.10	4.00	920.15	1	1043.10	4.00	920.15	32.64	0.5853	66.88	0.0002	0.4619	< 0.0001
AB	1633.36	243.88	17.77	1	1633.36	243.88	17.77	51.12	35.67	1.29	< 0.0001	0.0001	0.2823
AC	802.28	10.78	130.88	1	802.28	10.78	130.88	25.11	1.58	9.51	0.0005	0.2378	0.0116
AD	472.77	7.02	1.12	1	472.77	7.02	1.12	14.80	1.03	0.0815	0.0032	0.3347	0.7811
BC	279.41	318.62	21.35	1	279.41	318.62	21.35	8.74	46.61	1.55	0.0144	< 0.0001	0.2413
BD	0.0031	31.55	2.21	1	0.0031	31.55	2.21	0.0001	4.61	0.1605	0.9924	0.0572	0.6971
CD	181.46	0.0625	87.01	1	181.46	0.0625	87.01	5.68	0.0091	6.32	0.0384	0.9257	0.0307
A^2	443.05	6.64	829.72	1	443.05	6.64	829.72	13.87	0.9711	60.31	0.0040	0.3477	< 0.0001
B^2	75.11	2.19	287.35	1	75.11	2.19	287.35	2.35	0.3210	20.89	0.1562	0.5835	0.0010
C^2	483.64	30.10	366.99	1	483.64	30.10	366.99	15.14	4.40	26.68	0.0030	0.0623	0.0004
D^2	183.54	0.0075	727.22	1	183.54	0.0075	727.22	5.74	0.0011	52.86	0.0375	0.9742	< 0.0001

The significance of individual variables and interactions cleared in **table (2)** as the ANOVA results. Individual variables glucose (A), and yeast extract (B) were significant (P<0.0001) in their effects on kojic acid production, dry mass and consuming sugar, while KH_2PO_4 (C) (*P* 0.0093), and $MgSO_4.7H_2O$ (D) (*P* 0.4619) were non-significant for dry mass. Interaction between variables found to be non-significant (P>0.0001) for BC (yeast extract; KH_2PO_4) and BD (yeast extract; $MgSO_4.7H_2O$) in kojic acid production; AC (glucose; KH_2PO_4), AD (glucose; $MgSO_4.7H_2O$), BD (yeast extract; $MgSO_4.7H_2O$), CD (KH_2PO_4; $MgSO_4.7H_2O$) for dry mass and for consuming sugars the interaction between variables was not-significant. Response surface plots and contour plots can be used for the 3D visualization of the interaction between the pair-wise of the four factors selected, when the other two factors are constant as cleared in **figure (5)** explaining the effect of A; glucose; B: yeast extract; C: KH_2PO_4; D: $MgSO_4.7H_2O$ on kojic acid production (a), dry mass (b), and consuming sugars (c) by *Aspergillus oryzae* (Ahlburg) Cohn. The interaction between AB (glucose; yeast extract), AC (glucose; KH_2PO_4), and CD (KH_2PO_4; $MgSO_4.7H_2O$) was drawn for kojic acid production (g/l) **pictures 5(a1, a2, a3)**; AB (glucose; yeast extract), AC (glucose; KH_2PO_4), and BC (yeast extract; KH_2PO_4) for dry mass (g/l) **pictures 5(b1, b2, b3)** and AB (glucose; yeast extract), AC(glucose; KH_2PO_4), and BD (yeast extract; $MgSO_4.7H_2O$) for consuming sugars (%) **pictures 5 (c1, c2, c3)**.

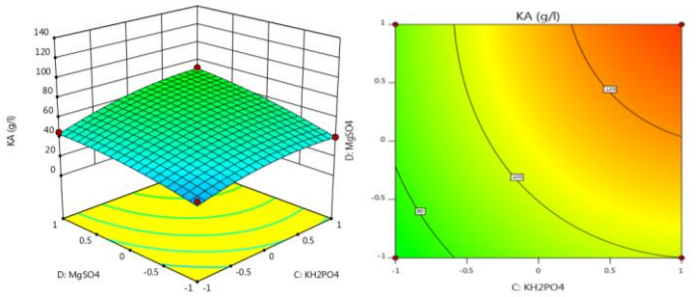
(a1)



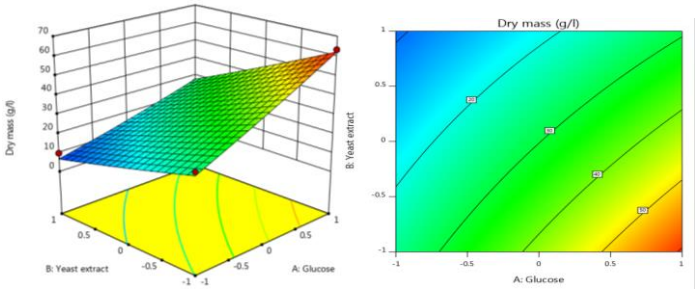
(a2)



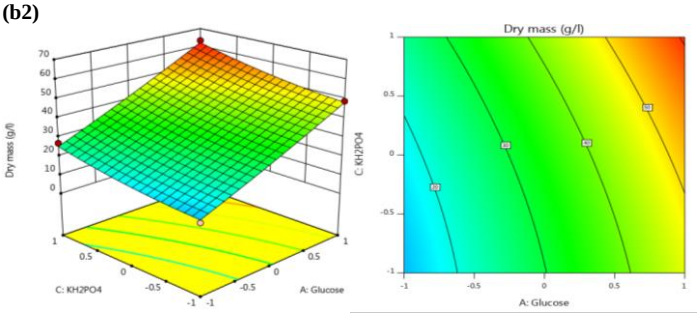
(a3)



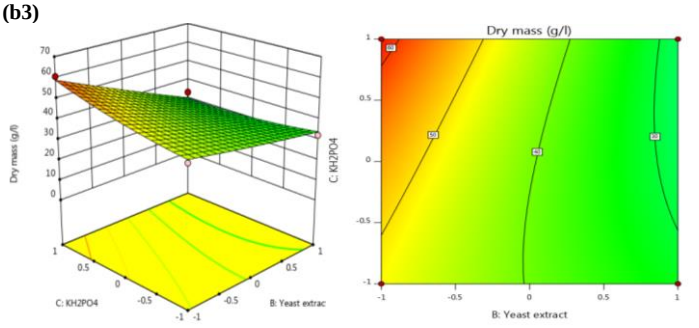
(b1)



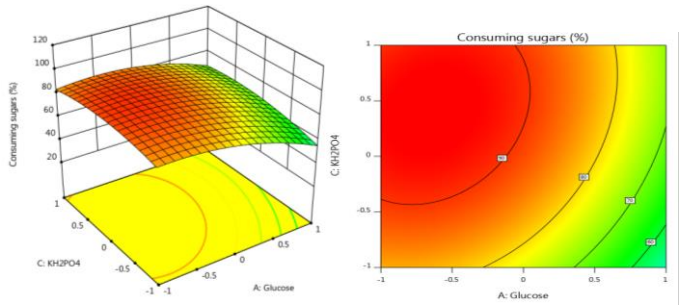
(b2)



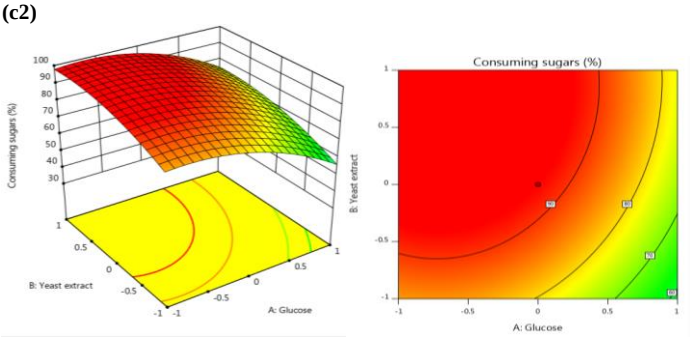
(b3)



(c1)



(c2)



(c3)

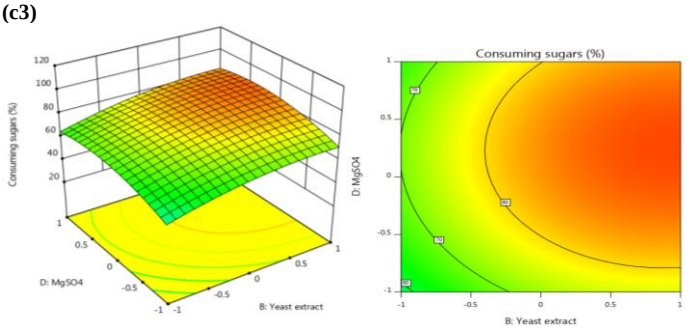


Figure 5 3D plots and contour plots of response surface design explaining the effect of A; glucose; B: yeast extract; C: KH_2PO_4; D: $MgSO_4.7H_2O$ on kojic acid production (a), dry mass (b), and consuming sugars (c) by *Aspergillus oryzae* (Ahlburg) Cohn.

Derringer's desirability function for model validation

Previous results showed the different treatment (25 runs) that has effects on the three responses (KA, DM, and CS), however it was necessary to define the totally most desirable concentrations of the medium constituents. Optima treatments for high kojic acid production were estimated by application the Derringer's desirability function which combines variable levels that jointly maximize the target response. By applying this function methodology, the optimum levels of the various parameters were glucose (1; 200g/l), yeast extract (1; 6 g/l), KH_2PO_4 (1; 1.5 g/l), and $MgSO_4.7H_2O$ (1; 1 g/l), which gives 130.801 g/l predicted kojic acid, 31 g/l dry mass, and 87.22% sugar consumption with overall value of desirability 0.937 **(Figure 6).** For model validation, triplicate experiments performed under the same above-mentioned optima conditions and the mean values confront with predicted values, actual values were in agreement with predicted values with standard error ± 4.2 using desirability functions that indicating the sufficiency of quadratic model developed.

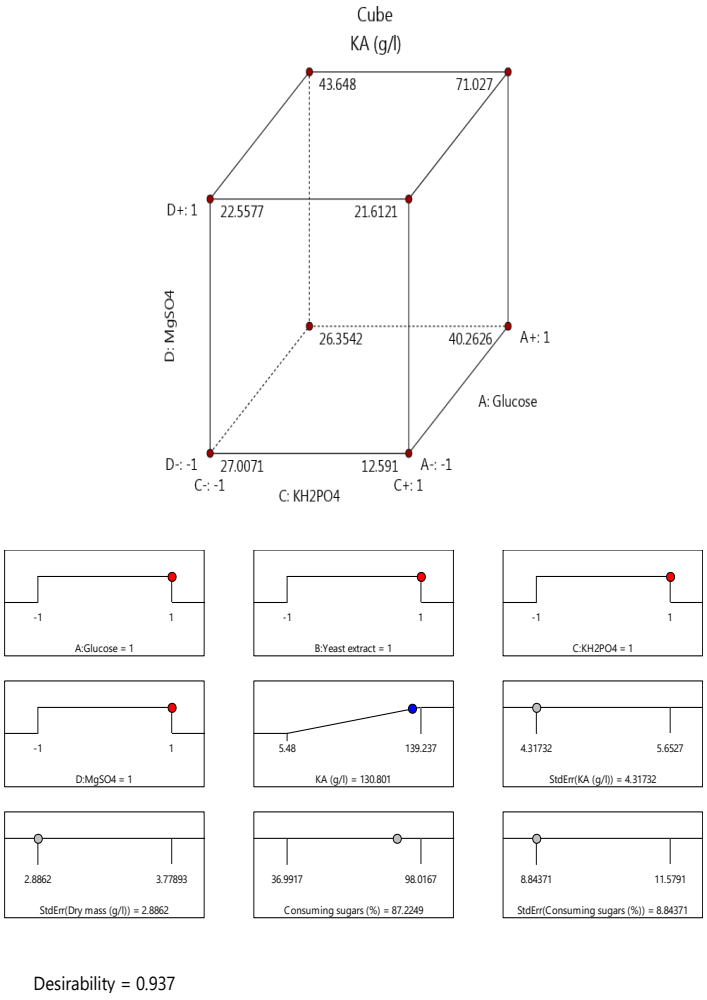


Figure 6 Optimization desirability ramp plot for kojic acid production by *Aspergillus oryzae*

Stability of Kojic acid at different temperatures

Microbial kojic acid stability represents a significant factor that influence on its industrial application, extraction and purification strategies. Therefore, the stability of kojic acid against different heat stress (40°C, 60°C, 80°C, and 100°C) during five hours (0, 1, 2, 3, 4, and 5 h.) has been estimated as shown in **figure (7)**. Overall, kojic acid preserve its stability in the first hour for all tested temperatures (slight changes at 100°C), then start to change with percentage related to each specific temperature. At 40°C, KA preserved its stability during the five hours from 136.36 g/l KA at zero time to 134.45 g/l KA after 5h.; at 60°C, KA stability change slightly during the five hours from 136.36 g/l KA at zero time to 127.05 g/l KA after 5h.; at 80°C, KA stability decreased during the five hours from 136.36 g/l KA at zero time to 104.32 g/l KA after 5h. At 100°C, KA stability decreased especially after two hours from 136.36 g/l KA at zero time to 90.59 g/l KA with 33.6% reduction after 5h.

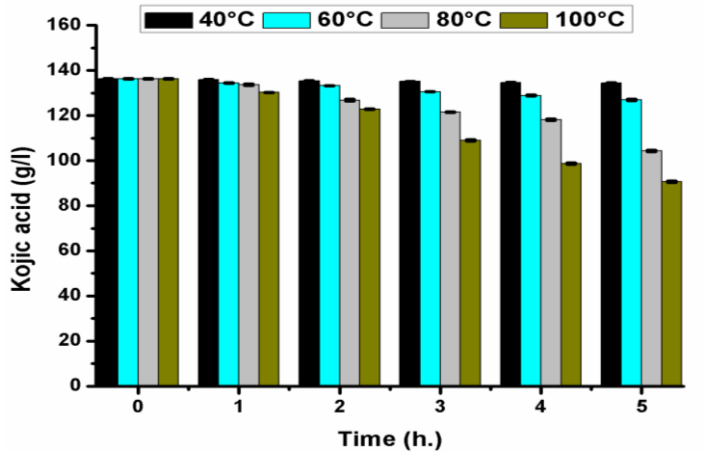


Figure 7 Stability of optimized kojic acid (g/l) produced by *Aspergillus oryzae* under different temperature degrees (40°C, 60°C, 80°C, and 100°C) after 0, 1, 2, 3, 4, and 5 hours.

DISCUSSION

Kojic acid acts as remarkable heterocyclic natural secondary metabolite of *Aspergillus* with various hot spot applications in cosmetics products of skin-lightning cosmetics **(Wang *et al.*, 2014),** prevent hyper-pigmentation and skin wrinkling **(Briganti *et al.*, 2003)**, antibacterial, anti-inflammatory, anti-aging, antidermatophytic, and biocompatibility medical **(Gonçalez *et al.*, 2015; Syamsul *et al.*, 2017)**. Also, it used as pesticides, insecticidal agent **(Burnett *et al.*, 2010),** and food antioxidant agent **(Sheikhshoaie *et al.*, 2017).** Microbial kojic acid concentrated highly on *Aspergillus* species especially *Aspergillus flavus* group. *Aspergillus clavatus*, *A fumigatus*, *A candidus*, *A awamorii*, *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. tamarii* produced kojic acid **(Kwak and Rhee, 1992; Terabayashi *et al.*, 2010; Chang *et al.*, 2011)**.

Ten isolates of *A. flavus* and *A. oryzae* isolated from various Egyptian sources were producible of KA in range 0.091±0.01 to 66.81± 0.95 g/l. In agreement with our screening data; **Manabe *et al.* (1981)** recorded that *A. flavus* could produce 40 g/L KA after optimization, **Kwak and Rhee (1991)** obtained 80 g/l KA from immobilized *A. oryzae* cells, **El-Sharkawy (1995)** obtained 60 g/l KA by *A. flavus* ATCC 9179 using immobilization, **Ogawa *et al.* (1995)** obtained 20g/L KA by *A. oryzae* NRRL 484, **Wakisaka *et al.* (1998)** obtained 24 g/L KA from *A. oryzae*. **Liu *et al.* (2016)** obtained 83.47 g/L KA by *Aspergillus oryzae* after several optimization processes. **Mahmoud *at el.* (2020a)** recorded 26.63 ± 0.04 g/l KA using zinc complexes as stimulator from *Aspergillus flavus.* Using *Aspergillus flavus* strains in the industrial production of kojic acid put producers and researcher's in critical situation regarding the risk of aflatoxin production by this isolates, however some researchers belived that if the isolate has the ability to produce aflatoxin, using a suitable medium and culture conditions for KA production could inhibit the aflatoxin synthesis **(Basappa *et al.*, 1970; Madihah *et al.*, 1996)**. Utilization of safe isolates of *Aspergillus* species for kojic acid production, avoiding us critical issues regarding how to inhibit these toxins. *Aspergillus oryzae* (*Ao*-4) that give maximum production was selected as non-toxinogenic safe isolate for optimizing the production by five levels CCD design of RSM. According to several researchers not all kojic acid producers are toxinogenic, several kojic acid producers are non-aflatoxin synthesizers **(Basappa, 1970; Madihah *et al.*, 1996; Bracarense and Takayashi, 2014).**

Maximum value of kojic acid with 108.4% increasing was 139.24 g/L (predicted 135.8 g/L) using glucose (+1; 200 g/l), yeast extract (+1; 6 g/l), KH_2PO_4, (+1; 1.5 g/l) and $MgSO_4.7H_2O$ (+1; 1 g/l) through run (24) in our study. Model significance and validity tested by R^2 values of KA was 0.987, DM 0.989 and CS 0.9831 and calculated with Derringer's desirability function as 0.937. Glucose represents the most favorable carbon source for kojic acid by *Aspergillus* species **(Rosfarizan and Ariff, 2000).** It's believed that the six carbon ring of glucose represented as a precursor for kojic acid synthesis **(Megalla *et al.*, 1986).**The fungus utilizes glucose molecules initially for its growth, and then later synthesizes kojic acid within the early stationary and decline phase **(Kitada *et al.*, 1967).** High glucose concentration not necessary utilize in the production it converted to much growing mass of the fungus, which cleared adverse relation between growth and production **(Ariff *et al.*, 1996)**. On the other hand, **Futamura *et al.* (2001)** obtained 40g/L of kojic acid by *Aspergillus oryzae* MK-107-39 using corn starch as carbon source. **Wan *et al.* (2005)** produced 41g/L KA using glucose, rice bran, KH_2PO_4, and $MgSO_4$ by *A. oryzae*, and **Hazzaa *et al.* (2013)** produced 25.5 g/L KA using glucose, ammonium nitrate, KCl, and MgSO4 by *A. oryzae*. **Yan *et al.* (2014)** produced 33.1g/L KA by *A.oryzae* M866 using corn stalk, peptone, KH_2PO_4 and $MgSO_4$.

The traditional optimization process was one-factor-at-a-time method which used in most kojic acid production researches, involves test one factor with keeping the other factors constant under the specific conditions **(Alexeeva *et al.*, 2002; Kumar *et al.*, 2003; Patidar *et al.*, 2005; Ahamed *et al.*, 2006).** New statistical optimization methods like response surface methodology introduced the main effects and interaction between variables with lowest experimental numbers, saving much time and draw clear pictures for the interaction **(De Lima *et al.*, 2010; Zohri *et al.*, 2018; Mahmoud *et al.*, 2020b)**. **Coelho *et al.* (2010)** utilize glycerol as carbon source for kojic acid production using CCD design and obtained 18.8 g/l from *A. flavus* NRRL 626. In this study, optimized kojic acid showed stability against different range of heat stress from 40°C to 100°C during five continuous hours which may attribute that microbial product usually are more stable than synthetic ones by attaching it with other active groups that guaranty more stability under stress conditions. This stability give it the advantages to be applied in industrial rang tolerate the different extraction and purification process through the industrial process which will need more research on its stability during all these process. Clearly, RSM designs save much time in optimizing kojic acid production with clarity the factorial interaction with less number. Also, *Aspergillus oryzae Ao*-4 represents a promised industrial potential safe isolate with stable product of kojic acid.

CONCLUSION

Aspergillus oryzae (*Ao*-4) represents promising safe isolate for industrial kojic acid production with highly stable product and significant valid experimental design. Response surface methodology offers saving in time during optimizing

microbial product with clarity the interaction between variables with less number of experiment leading it as one of the most effective and valid way for kojic acid maximization. The selected isolate (*Aspergillus oryzae Ao*-4) is promised industrial potential safe isolate with stable kojic acid.

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