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Research Article

Optimization of kojic acid production conditions from cane molasses using Plackett-Burman design

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ABSTRACT

Fungal synthesis of kojic acid has gained more interest in these days as an alternative way to chemical synthetic. The aspect of the microbial fermentation process is to develop a suitable culture medium to obtain the maximum amount of kojic acid using statistical methods. In this study; different selected three isolates of *Aspergillus flavus* (No 1, 2 and 3) were screened for their ability to produced kojic acid and the isolate No 3 was the highest kojic acid producer one. The capability of *A. flavus* No 3 to produce kojic acid was improved using Plackett-Burman design. From ten different agro-industrial wastes cane molasses recorded the highest kojic acid productivity with 2.24 g/l^{-1} day^{-1} and was the most effective parameter plays a crucial role in Plackett-Burman design. Maximum kojic acid production (24.65 g/l) by *A. flavus* (No. 3) obtained under the fermentation conditions: incubation temperature at 25°C, incubation time 9 days, pH 3, inoculum size 0.5%, shaking rate at 150 rpm and medium constituents: Cane molasses 60 g/l, yeast extract 7 g/l, KH_2PO_4 2 g/l, $ZnSO_4 \cdot 7H_2O$ 100 µg/l and $MgSO_4 \cdot 7H_2O$ 1 g/l with regression analysis (R^2) 99.45% and 2.33-fold increase in comparison to the production of the original level (10.6 g/l).

Keywords: Kojic-acid; Agro-industrial wastes; Optimization; Plackett-Burman; *Aspergillus*.

Abbreviations: Czapek's dextrose agar medium (CZD), kojic acid (KA), Consuming sugars (CS), Dry mass (DM).

1. INTRODUCTION

Kojic acid (5-hydroxy-2-hydroxymethyl-l-pyrone) is an organic acid has a weak acidic property crystallizes in form of colorless and prismatic needles [1]. The melting point of kojic acid ranges from 151-154°C. Kojic acid is soluble in water (3.95 g/100 ml at 20°C), ethanol and ethyl acetate. On the contrary, it is less soluble in ether, alcohol ether mixture, chloroform and pyridine [2-4].

Kojic acid is a major secondary metabolite can be produced from carbohydrates by using different carbon and nitrogen sources, also using agriculture wastes under aerobic fermentation strategies. Kojic acid is produced by *Aspergillus* spp.

belonging mainly to the section *flavi*: *Aspergillus flavus* [5-9], *Aspergillus oryzae* [10-14], *Aspergillus oryzae* var *effusus* [15], *Aspergillus tamarii* [16] and *Aspergillus parasiticus* [6, 7, 14, 17-19], as well as *Penicillium* sp. and certain bacteria [14, 20, 21].

Glucose, sucrose, acetate, ethanol, arabinose and xylose have been used as carbon sources for kojic acid production. Glucose is the best carbon source for kojic acid production due to the similarity of its structure to that of kojic acid. It has been suggested that, during the fermentation, kojic acid is formed directly from glucose without any cleavage of the carbon chain into smaller fragments [5, 22, 23]. Utilization of agro-industrial wastes or by-products for the fungal production of useful products has been recommended by many investigations such as cheese whey [24-26], sugar cane molasses [27-30], fruits, vegetables, corn steeps liquor [9, 31].

Kojic acid is a natural antibiotic agent, early as 1934 it was reported that kojic acid inhibited the growth of Gram-negative more strongly than that of Gram- positive bacteria [32]. This property was rediscovered much later and the antibiotic action of culture filtrates of several fungi was shown to be due to the presence of kojic acid and it is used in the medical field as a pain killer and anti-inflammatory drug [33]. In the food industry, KA used as one of the precursors for flavor enhancers [34]. Kojic acid has the ability to prevent the undesirable melanosis (blackening) of agricultural products by inhibiting polyphenol oxidase [35], and used as a skin care product for whitening [4] and as a protective against U.V. light. It has been used for the production of miso, soya sauce and sake in Japan for a long time [36, 37].

Agro-industrial wastes, include wastes generated during the industrial processing of agricultural or animal products or those obtained from agricultural activities in the form of straw, stem, stalk, leaves, husk, shell, peel, lint, seed, pulp, legumes or cereals (rice, wheat, corn, sorghum and barley), bagasse' from sugarcane or sweet sorghum milling, spent coffee grounds, brewer's spent grains, and many others. These wastes are mainly composed of sugars, fibers, proteins, and minerals. The chief constituents of such agro-industrial wastes include cellulose, hemicelluloses and lignin, collectively being called "lignocellulosic materials" [38]. Cheap agro-industrial sources such as wheat bran, soy bean meal, corn steep liquor, sugarcane bagasse', whey, etc. have been used as carbohydrate as well as nitrogen sources in the lieu of synthetic ones [39]. Different types of treatments (physical, chemical, and enzymatic) can be given to these by-products in order to make them easily consumed by microbes [40].

A classical method of optimizing the fermentation conditions and medium constituents depends on single parameter whilst all the other factors are maintained at a fixed level. However, statistical planned experiments effectively explained the interaction of parameters and minimize the error in determining the effect of parameters [41, 42]. The design of experiment reduces the number of experiments and increases process efficiency [43, 44]. Statistically designed experiments are used for optimization strategies such as screening experiments and optimization for targeted response [45]. Plackett-Burman design used which greatly enhance the yield of product, reduces time, cost, process variability and has been successfully used to optimize many bioprocesses [46, 47]. The Plackett-Burman design was developed by Plackett and Burman in 1946. It is two-level fractional design for studying up to k=N-1, where k are variables and N is the number of runs. These designs have complex alias structures, and hence, this design was generally preferred for screening of significant factors [48].

The main objective of this work was to test the ability of different *Aspergillus flavus* isolates to produce kojic acid on both glucose and agro-industrial wastes media, secondly to improve the production by investigating the effect of several variables on the KA production process and recorded the optimum fermentation conditions for the highest KA production using Plackett-Burman design as a statistical approach.

2. MATERIALS AND METHODS

2.1. Microorganisms

Three isolates of *Aspergillus flavus* proved previously as highly kojic acid producers [9] and proved as non-toxigenic producers (data not recorded here) were selected for this study. These isolates were maintained on Czapek's dextrose agar medium (CZD) aerobically and stored at 4±1°C until

using (sub-cultured every 30 day). Prior to the experiments *A. flavus* isolates grown on CZD medium at 28±1°C for 4 days aerobically. Homogeneous spore suspension obtained by scrapping fungal hyphae and suspended it in sterilized distilled water containing 0.01% (v/v) tween 80 until spore suspension 3×10^6 spore/ml and stirred for 30 min. then using it as inoculum.

2.2. Medium and culture conditions.

Modified Czapek's dextrose liquid medium used for kojic acid production [49] containing (g/l): glucose, 100.0; yeast extract, 5; KH_2PO_4, 1.0 and $MgSO_4.7H_2O$, 0.5. These contents were dissolved in 1000 ml distilled water with initial pH adjusted to 3 before autoclaving. After sterilization in an autoclave at 121°C and 1.5 atm pressure for 20 min. chloramphenicol, 250 mg/ml was sterilized separately by membrane filtration, using a membrane of pore size 0.22 mm and added as bacteriostatic agent. Incubation was carried out at 28±1°C on a rotary shaking (150 rpm) for 7 days. All the experiments were carried out independently in triplicates. After the incubation period, mycelium was recovered by filtration through dried and weighed Whatman filter paper (No. 113), washed with distilled water three times and then dried at 70 °C overnight for dry mass (DM) determination. The supernatants were used for quantitative determination of kojic acid (KA) and consuming sugars (CS).

2.3. Screening for kojic acid production on agro-industrial wastes

Ten agro-industrial wastes collected from Agriculture Research Centre and sugar factories were used in this experiment, namely beet molasses, cane molasses, mixed of cane and beet molasses, bagasse, starch water, corn steep liquor, rice straw, *Zea mays* waste, onion waste and peanut waste. The hard wastes were washed to remove dust, separated, dried, ground and sieved through 1-mm mesh screen. All wastes were prepared in concentration 40 g/l. Incubation was carried out at 28±1°C on a rotary shaking (150 rpm) for 7 days. All the experiments were carried out independently in duplicates. The chemical analysis of beet molasses and cane molasses showed in Table 1.

Table 1. Chemical composition of Abo-Qurqas sugarcane and beet molasses, Egypt.

Test	Cane molasses	Beet molasses
Brix	86.50±1.0	82.50±1.0
pH	5.1±0.1	8.1±0.1
Ash %	12.30±0.5	10.50±0.5
Total sugar %	56.0±1.0	57.50±1.0
Non-fermentable sugar %	4.50±0.2	2.00±0.2
Fermentable sugar %	51.50±0.1	55.50±0.1
Reducing sugar %	24.90±0.1	1.82±0.1
Nitrogen %	0.61±0.1	1.3±0.1
Protein %	3.81±0.1	8.12±0.1
Color % brix	22500	16060
CaO %	1.58±0.1	2.00±0.1
P_2O_5 %	0.3±0.01	0.1±0.01
SO_4 g/l	19.0±2.0	5.4±2.0

2.4. Optimization using Plackett-Burman design

Plackett-Burman design was used to screen the fermentation parameters that influenced kojic acid (KA) production [50]. Eleven trails carried out by Plackett-Burman design for screening the fermentation parameters with respect to their main effect and without interaction effects between various constituents of the medium is shown in Table 2. Each independent variable was tested at two levels, high (+1) and low (−1). In each column and row should contain equal number of negative and positive signs. Plackett and Burman design was used to screen and evaluate the important medium components that influence the response. Kojic acid yields are explained by the following polynomial equation:

$$Y=bo + \sum biXi + \sum bijXiXj + Ei \quad (1)$$

Where, Y; the variable dependent response; i; the regression coefficient; X; the independent variable level; b0 is offset term; bij is interaction effect and E; the experimental error. The experimental data were statistically analyzed to determine the significant difference ($p \leq 0.05$) in response under different conditions. The response surface graphs were also plotted using the same software. The quality of fit for the regression model equation was

expressed as R^2. The program Sigma XL (Version 6.12) was used to analyze this experiment.

Table 2. Plackett-Burman design for screening kojic acid production using different variables by *Aspergillus flavus.*

Variable code	Variable	Unit	Level	
			Low (-1)	High (+1)
A	Incubation temperature	C°	25	35
B	Incubation time	D	5	9
C	Fermentation type		Shaking	Static
D	Inoculums size	%	0.5	2
E	Initial pH		3	5
F	Molasses	gl^{-1}	20	60
G	Yeast extract	gl^{-1}	3	7
H	KH_2PO_4	gl^{-1}	0.5	2
J	$ZnSO_4.7H_2O$	μgl^{-1}	0	100
K	Glycine	μgl^{-1}	0	100
L	$MgSO_4.7H_2O$	gl^{-1}	0.1	1

2.5. Analytical analysis

Kojic acid was determined spectrophotometrically using ferric chloride reagent; the developed purple-red color was measured quantitatively against substrate-free blank at 540 nm [2, 20, 51]. The residual sugar was analyzed spectrophotometrically by anthron method using T60 UV with a split beam UV visible spectrophotometer covers a wavelength range of 190-1100 nm [52].

3. RESULTS AND DISSCUSSION

3.1. Kojic acid production by *Aspergillus flavus* isolates

Three isolates of *A. flavus* (No. 1, 2 and 3) were screened for their ability to produced kojic acid on fermented medium. All the isolates grown on the production medium and showed various degrees of dry mass and kojic acid production. A wide variation in KA production on the screening medium ranged from 8.5±0.01 to 10.6±0.01 g/l in submerged cultures and 7.4±0.02 and 8.9±0.01 g/l in static cultures. The highest fungus dry mass and kojic acid producer was *Aspergillus flavus* (No. 3) giving 10.58±0.01 g/l KA (with productivity 1.51 g/l/day) and 6.1±0.53 g/l dry mass so it was selected for the further experiments. Several species of *A. flavus* group were estimated as kA producers such as *A. flavus*, *A. oryzae* and *A. parasiticus* [9, 14, 23, 53-58].

Brief description of kojic acid highly producer *Aspergillus flavus* Link (No. 3); Growth on CZD medium 60 mm in one week, Texture floccose becoming granular, Color bright yellow-green; occasionally yellow-brown, cream reverse. Conidiophores roughened; vesicles globose with radiate or columnar spore production; phialides arising directly or produced on medullae in others; conidia round to elliptical, 3-6 μm; smooth or finely roughened (Fig. 1).

3.3. Screening for kojic acid production on agro-industrial wastes by *Aspergillus flavus* Link

Ten agro-industrial wastes were tested as a carbon source for the growth of *A. flavus* (No. 3) and KA production was illustrated in Fig. 2. *Aspergillus flavus* growth and KA production were largely impressed by the type of waste. The results indicated that cane molasses promoted both fungal growth and kojic acid production (15.71 g/l KA, 20.2 g/l DM) followed respectively by potato waste water (11.4 g/l KA, 17.5 g/l DM), onion wastes (9.49 g/l KA, 9.05 g/l DM) and mixed molasses (9.23 g/l KA, 15.6 DM). It is worthy to mention that bagasse, corn steep liquor, peanut wastes and rice straw contribute low production of kojic acid matching 0.87, 2.37, 3.62 and 3.98 g/l kojic acid, respectively. The current study clearly proved that *A. flavus* could grow well on cane molasses and produced a large amount of kojic acid. Utilization of different carbon sources such as glucose, starch, sucrose, maltose and cellulose by different *Aspergillus* species for kojic acid production were studied by Rosfarizan and Ariff [59]. El-Aasar [19] reported that glucose also has yield the highest kojic acid production by *A. parasiticus* and followed by sucrose and beet molasses. Several quantities of KA

produced by fungi were recorded previously by several researchers such as: Manabe et al. [60] recorded 40 g/l kojic acid by *A. flavus*; El-Sharkawy [57] obtained 60 g/l kojic acid with immobilization technique by *A. flavus* ATCC 9179. Kwak and Rhee [11] produced 80 g/l kojic acid using, also, immobilized cells of *A. oryzae*. Ogawa et al. [61] produced 20g/l KA by *A. oryzae* NRRL 484 using shaking culture. Wakisaka et al. [62] produced 24 g/l KA from the same previous isolate on different medium.

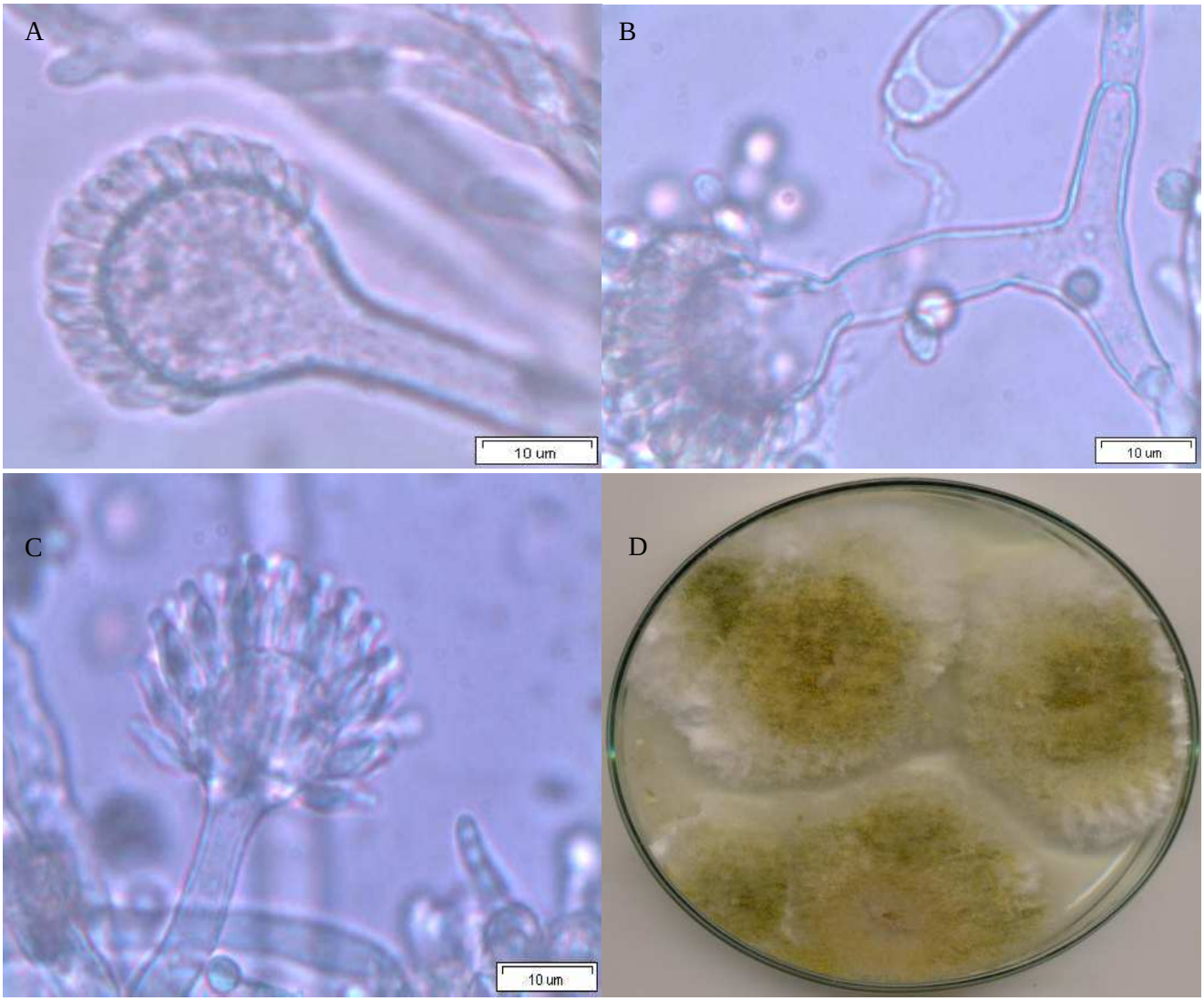

Figure 1. *Aspergillus flavus*, **A, B**: Biserriate phialide (Bi); Conidiophore (Co) and hypha (Hy); **C**: Monoserriate phalide (Mo); Bars, 10 µm; **D**: Fungus growth on Czapek's dextrose agar medium.

3.3. Optimization of KA production using Plackett-Burman design

The highly kojic acid producer (*A. flavus* No.3) was chosen for screening the effects of different parameters on KA production using Plackett-Burman design. Each variable was studied at two levels (-1, 1) as declared in Table 1. Relationship between the response and the screened variables was expressed by the following polynomial equations:

KA (g/l) = (11.98) + (-6.38) * A + (1.35) * B + (-6.36) * C + (-0.54) * D + (-1.67) * E + (7.98) * F + (1.44) * G + (4.31) * H + (-1.06) * J + (-0.388) * K + (4.8) * L. (2)

CS (%) = (8.52) + (-1.89) * A + (-0.96) * B + (-1.05) * C + (-0.48) * D + (1.32) * E + (2.05) * F + (-0.4) * G + (1.32) * H + (1.89) * J + (-0.49) * K +

(0.82) * L. (3)

DM (g/l) = (15.37) + (-0.55) * A + (0.25) * B + (-2.1) * C + (1.74) * D + (1.48) * E + (4.79) * F + (1.77) * G + (0.92) * H + (-0.27) * J + (0.083) * K + (3.47) * L. (4)

The results obtained in Table 3 indicated that there was a wide variation in kojic acid production of 0.82 to 24.65 g/l, consuming sugar 27.33 to 89.87% and dry mass varied between 3.6 and 28.2 g/l indicating the important effect of both medium components and environmental factors on the production of KA. The ANOVA results are shown in Table 4 showed that among the eleven variables, D (inoculums size), K (glycine) in kojic acid production; B (incubation time), J ($ZnSO_4.7H_2O$), K (glycine) in dry mass; G (yeast extract) in consuming sugars were found to be non-significant ($p>0.05$). Among the tested parameters, cane molasses was the most effective parameters plays a crucial role in KA production, dry mass and consuming sugars with 3.99, 4.79 and 7.98 coefficient effect as shown in Pareto-Plot (Fig. 3).

All the predicted values of Plackett-Burman design were located in close proximity to experimental values. This supports the hypothesis that the model Eq. (2, 3, and 4) is sufficient to describe the response of the experimental observations of KA production, dry mass and consuming sugars (Fig. 4). The main effects of different parameters on kojic acid production by *A. flavus* showing effect of two variables (other variables were kept at zero in coded unit indicated in Fig. 5.

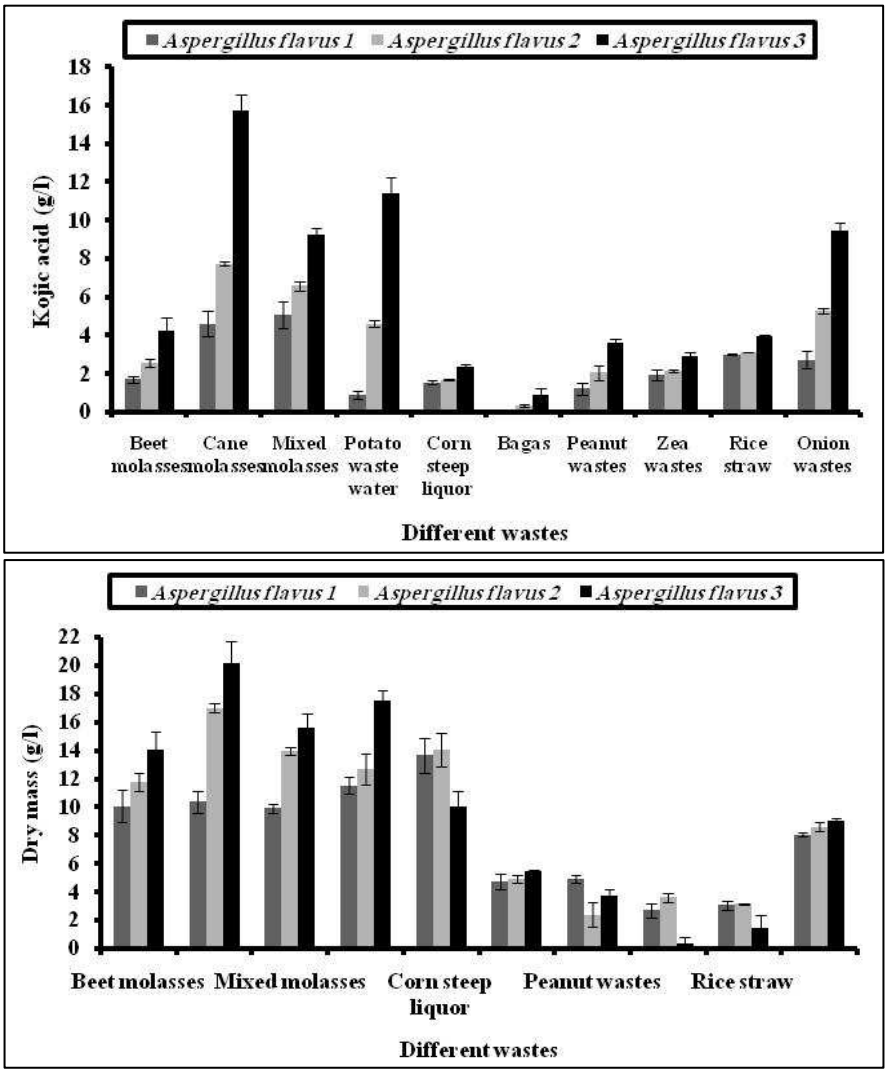

Figure 2. Screening for kojic acid production on different agro-industrial wastes by three species of *Aspergillus*.

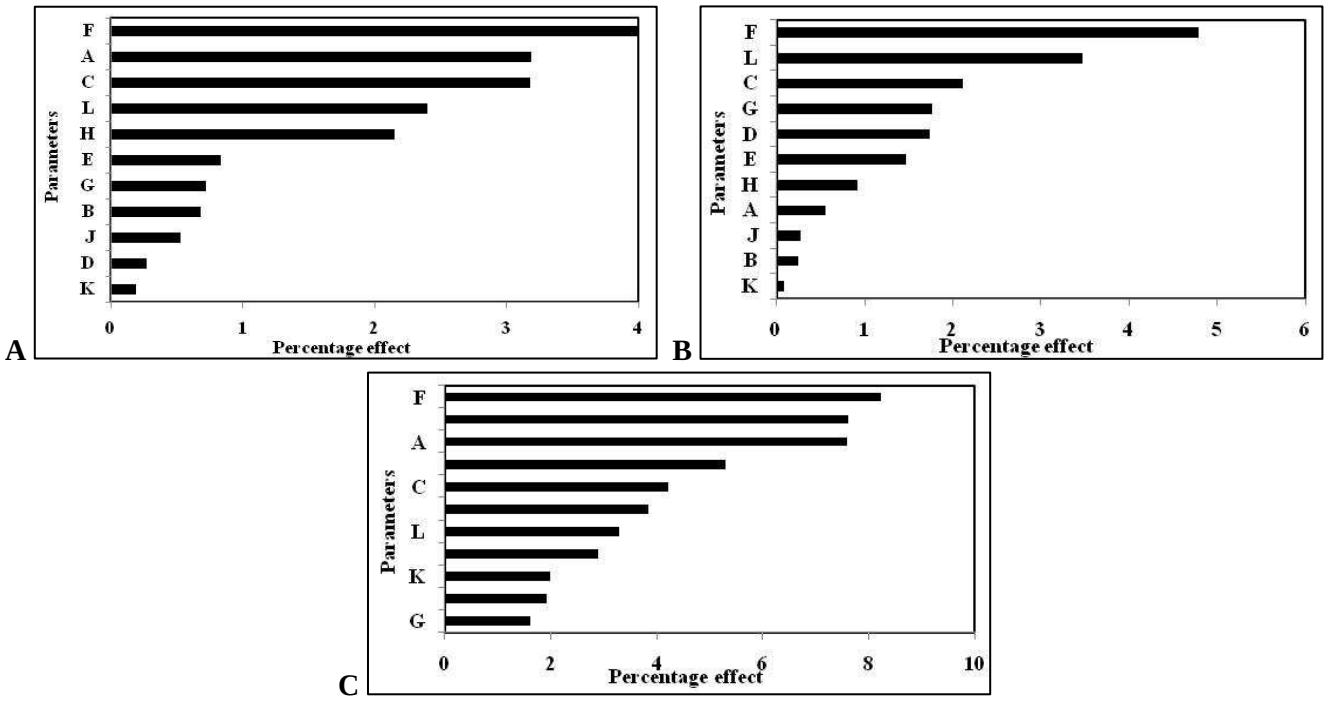

Figure 3. Pareto-Plot for Plackett-Burman parameter determines the effect of each parameter on kojic acid produced by *Aspergillus flavus* (3), A: Kojic acid (g/l); B: Dry mass (g/l) and C: Consumed sugars (%).

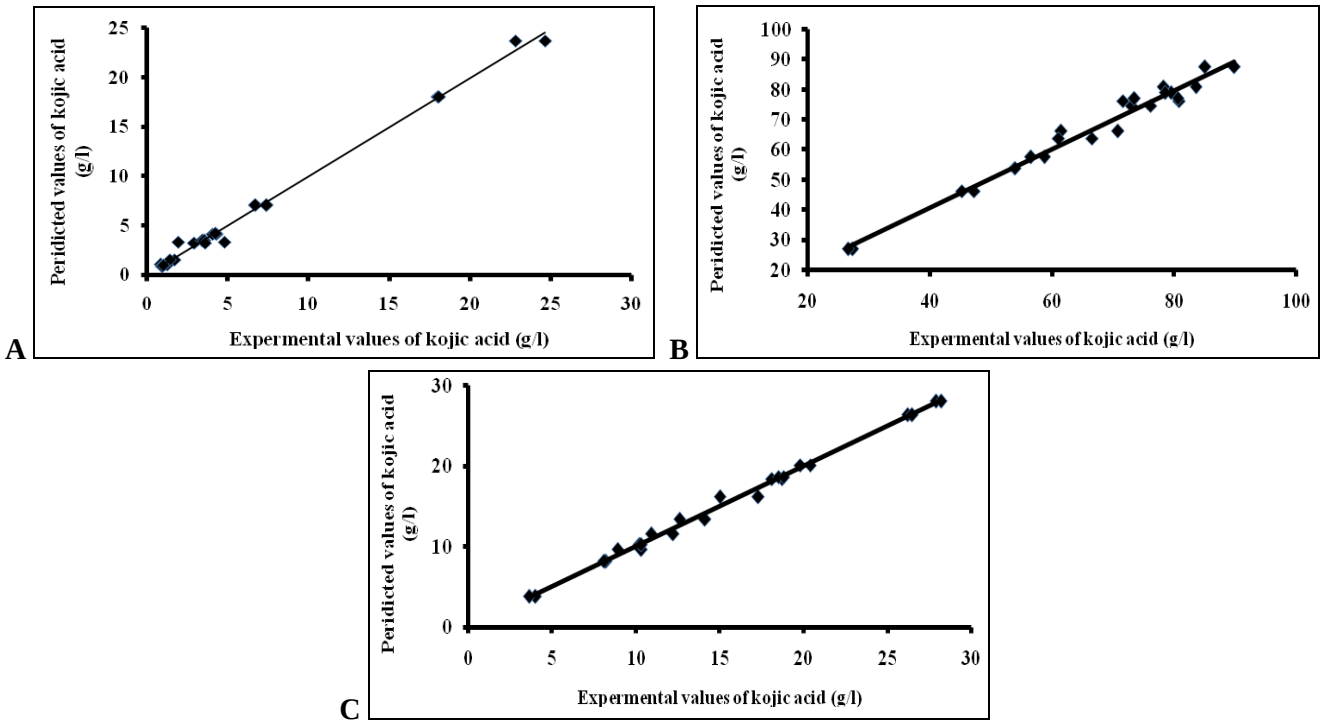

Figure 4. Comparison between kojic acid (g/l) experimental and predicted values of the Plackett-Burman design by *Aspergillus flavus* (3), A: Kojic acid (g/l); B: Dry mass (g/l) and C: Consumed sugars (%).

Three-dimensional response surface curves were generated to study the interaction between each two variables (Fig. 6). The Model F value of KA (value is calculated as ratio of mean square regression and mean square residual due to the real error) was 197.79 ($p<0.05$), DM was 207.74 ($p<0.05$) and CS was 44.09 ($p<0.05$) implies that the model is significant. The R^2 value was 99.45%,

99.48% and 97.59% for KA, DM and CS, respectively indicated that the entire variation was explained by the model. The adjusted R^2 value was 98.95%, 99% and 95.37% for KA, DM and CS, respectively.

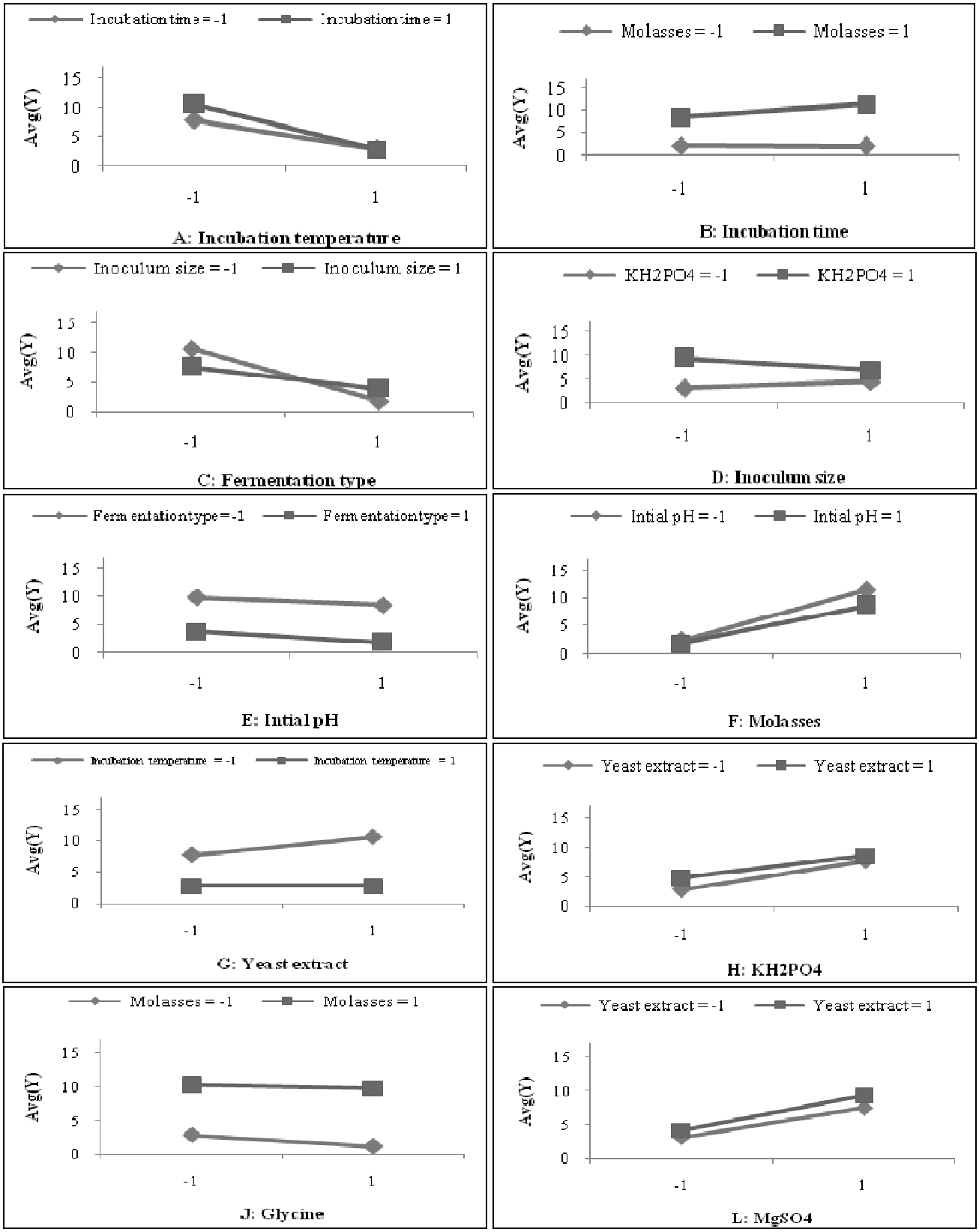

Figure 5. Main effects of different parameters on kojic acid production by *Aspergillus flavus* (3) showing effect of two variables (other variables were kept at zero in coded unit).

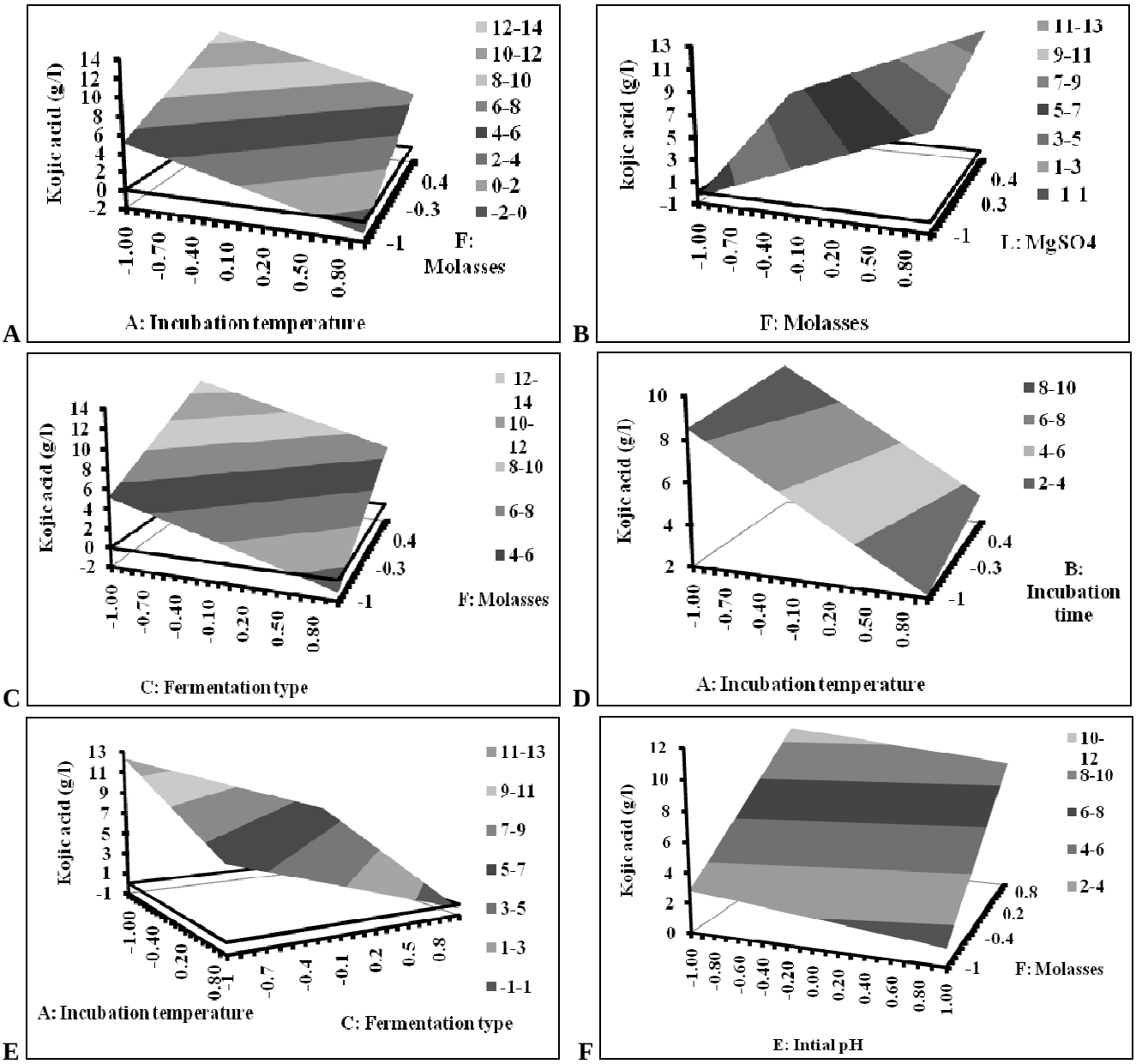

Figure 6. Response surface plots of kojic acid production by *Aspergillus flavus* (3) showing the effect of two variables (other variables were kept at zero in coded unit) : (A) Molasses and Incubation temperature, (B) Molasses and $MgSO_4$, (C) Molasses and fermentation type, (D) Incubation temperature and incubation time, (E) Incubation temperature and Fermentation type, (F) Molasses and initial pH.

Maximum KA production (24.65 g/l) by *A. flavus* obtained under the fermentation conditions: incubation temperature at 25°C, incubation time 9 days, pH 3, inoculums size 0.5%, shaking rate at 150 rpm and medium constituents: Cane molasses 60 g/l, yeast extract 7 g/l, KH_2PO_4 2 g/l, $ZnSO_4.7H_2O$ 100 µg/l and $MgSO4.7H_2O$ 1 g/l. In agreement with our results; Lin et al. [17, 63] showed that the optimal pH values for the production of kojic acid were 4.5, 6.2 and 6.5 by *A. flavus*, *A. parasiticus* and *A. oryzae*. Optimal pH for producing KA 3 obtained by strains of *A. oryzae*, this could be explained by the optimal pH of the KA-producing enzymes is around pH 3.5 [21, 59]. Also, secondary metabolites produced in the late log-stationary phases, in which the cultural medium has already been acidified by various acidic primary metabolites (itaconic acid or citric acid) [64, 65]. Optimum temperature for kojic acid production by fungi in the most of the cases was found to be 25-30°C [63, 66].

Table 3. Plackett-Burman design variables with kojic acid production by *Aspergillus flavus* as response.

Trials	A	B	C	D	E	F	G	H	J	K	L	Kojic acid (g/l)		Dry mass (g/l)		Consumed sugars %	
												Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	-1	-1	1	1	1	-1	1	1	-1	1	-1	1.25	1.04	12.20	11.55	78.25	80.90
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.68	1.54	8.20	8.15	85.06	87.46
3	1	-1	-1	-1	1	1	1	-1	1	1	-1	4.30	4.17	18.10	18.40	45.28	46.28
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	4.24	4.25	4.00	3.80	61.48	66.15
5	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.39	1.54	8.10	8.15	89.87	87.46
6	1	1	-1	1	1	-1	1	-1	-1	-1	1	2.94	3.25	19.80	20.10	79.46	78.98
7	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.82	1.04	10.90	11.55	83.55	80.90
8	-1	1	1	1	-1	1	1	-1	1	-1	-1	6.70	7.06	15.00	16.15	58.76	57.65
9	1	-1	-1	-1	1	1	1	-1	1	1	-1	4.04	4.17	18.70	18.40	47.28	46.28
10	1	1	1	-1	1	1	-1	1	-1	-1	-1	3.44	3.48	12.60	13.35	71.61	76.23
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	22.82	23.74	26.20	26.35	66.60	63.82
12	1	1	1	-1	1	1	-1	1	-1	-1	-1	3.52	3.48	14.10	13.35	80.84	76.23
13	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.99	1.00	10.20	10.25	80.62	77.05
14	-1	-1	-1	1	1	1	-1	1	1	-1	1	18.04	18.06	27.90	28.05	27.33	26.96
15	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.88	0.93	8.90	9.60	53.81	53.89
16	1	1	-1	1	1	-1	1	-1	-1	-1	1	3.57	3.25	20.40	20.10	78.50	78.98
17	-1	1	-1	-1	-1	1	1	1	-1	1	1	24.65	23.74	26.50	26.35	61.04	63.82
18	-1	1	1	1	-1	1	1	-1	1	-1	-1	7.41	7.06	17.30	16.15	56.55	57.65
19	1	-1	1	1	-1	1	-1	-1	-1	1	1	1.92	3.36	18.50	18.65	73.07	74.60
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	4.25	4.25	3.60	3.80	70.81	66.15
21	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.97	0.93	10.30	9.60	53.96	53.89
22	1	-1	1	-1	-1	-1	1	1	1	-1	1	1.01	1.00	10.30	10.25	73.47	77.05
23	-1	-1	-1	1	1	1	-1	1	1	-1	1	18.09	18.06	28.20	28.05	26.58	26.96
24	1	-1	1	1	-1	1	-1	-1	-1	1	1	4.80	3.36	18.80	18.65	76.13	74.60

The sign +1 and −1 represent the two different levels (high and low) of the independent variable under investigation. A: Incubation temperature, B: Incubation time, C: Fermentation type, D: Inoculums size, E: Initial pH, F: Molasses, G: Yeast extract, H: KH_2PO_4, J: $ZnSO_4.7H_2O$, K: Glycine and L: $MgSO_4.7H_2O$.

Table 4. Statistical analysis of Plackett-Burman design of each variable at two levels for kojic acid production by *Aspergillus flavus*.

Variable code	Variable	Coefficient			t value			P value		
		Kojic acid (g/l)	Dry mass (g/l)	Consumed sugars %	Kojic acid (g/l)	Dry mass (g/l)	Consumed sugars %	Kojic acid (g/l)	Dry mass (g/l)	Consumed sugars %
	Constant	5.990	15.367	65.830	40.055	104.901	87.615	0.0000*	0.0000*	0.0000*
A	Incubation temperature	-3.189	-0.550	7.602	-21.325	-3.755	10.118	0.0000*	0.0027*	0.0000*
B	Incubation time	0.677	0.250	3.842	4.526	1.707	5.113	0.0007*	0.1136 ^{N}	0.0003*
C	Fermentation type	-3.179	-2.108	4.223	-21.258	-14.393	5.620	0.0000*	0.0000*	0.0001*
D	Inoculums size	-0.271	1.742	1.928	-1.811	11.890	2.566	0.0952 ^{N}	0.0000*	0.0247*
E	Initial pH	-0.833	1.475	-5.292	-5.574	10.069	-7.043	0.0001*	0.0000*	0.0000*
F	Molasses	3.989	4.792	-8.241	26.675	32.711	-10.968	0.0000*	0.0000*	0.0000*
G	Yeast extract	0.720	1.767	1.617	4.815	12.060	2.151	0.0004*	0.0000*	0.0525 ^{N}
H	KH_2PO_4	2.153	0.917	2.906	14.400	6.258	3.868	0.0000*	0.0000*	0.0022*
J	$ZnSO_4.7H_2O$	-0.529	-0.267	-7.616	-3.541	-1.820	-10.137	0.0041*	0.0937 ^{N}	0.0000*
K	Glycine	-0.194	0.083	1.995	-1.299	0.569	2.655	0.2184 ^{N}	0.5799 ^{N}	0.0210*
L	$MgSO_4.7H_2O$	2.401	3.467	-3.282	16.058	23.665	-4.369	0.0000*	0.0000*	0.0009*

t – student's test, p – corresponding level of significance,* Significant at $p \leq 0.05$, ^{N}, non-significant at $p \geq 0.05$.

4. CONCLUSION

From the outcome of our investigation it is possible to conclude that non-toxigenic *Aspergillus flavus* can be highly recommended in industrial production of kojic acid. Also using statistical method in optimization for improving the production has a great potential for applications and was very effective in our study as the production of KA (24.65 g/l) in this paper increase with 2.33-fold in comparison to the production of original level (10.58 g/l) using Plackett-Burman design.

AUTHORS' CONTRIBUTIONS

A-NAZ and GAEM designed the research plan, drafting and revised. GAEM and RAH carried out the research point by point. All authors helped in collected, re-identifying the fungal strains and approved the manuscript. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

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