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

Ghada Abd-Elmonsef Mahmoud *, Abdel-Naser A. Zohri

Address(es):
Botany and Microbiology Department, Faculty of Science, Assiut University, Assiut 71516, Egypt.

*Corresponding author: ghadamoukabel@aun.edu.eg 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

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), K2HPO4 (+1; 1.5 g/l) and MgSO4•7H2O (+1; 1 g/l) through run (24). Model significance and validity tested by K2 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 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 (C6H6O3) with chemical structure 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (Britko et al., 2004). It has been used highly in cosmetics products like skin-lightening 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, antiatheromatophylic, radical scavenging agent, biocompatibility medical and antiproteozans (Pigriniano et al., 2007; Gonzalez 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 marin) include as a preservative food antioxidant agent (Sheikhshoie et al., 2017). Kojic acid was produced originally from Aspergillus oryzae (Machida et al., 2005) in high quantities, however it also produced by Aspergillus spp., Penicillium sp., Acetobacter sp., and Bacillus sp. (Masse et al., 2001; Machida et al., 2005; Pildain et al., 2008; Vasantha et al., 2014). Aspergillus spp. were known to produce large amounts of kojic acid like A clavatus, A awamori, 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, Madilhab 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 Britko, 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-toxinogenic 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 121°C. 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 1a). 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 × 10^3 spore/mL. Kojic acid screening medium includes (g/l): glucose 100; KH2PO4, 1.0; yeast extract, 5.0; MgSO4.7H2O, 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 × 10^3 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 1b) 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°C in a 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 (SiO2, 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, KH2PO4, and MgSO4.7H2O 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:

\[ x_i = X_i - \bar{X}_i / \delta X_i \]  

Where \( x_i \) represents the dimensionless number of \( X_i \), as independent variable; \( X_i \) represents the experimental value of the variable; \( \bar{X}_i \) represents the value of \( X_i \) at the center point; \( \delta \) 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):

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ijk} X_i X_j X_k \]  

Where Y represents the predicted values, \( \beta_0 \) represents the offset term, \( \beta_i \) represents the linear effect, \( \beta_{ij} \) represents the squared effect, \( \beta_{ij} \) is the interaction effect, \( \beta_{ijk} \) 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 centrifuged 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 spectrophotometrically at 500nm using ferrous chloride reagent. The degradation ratio calculated (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 was 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 et al., 2020a). Residual sugars were measured using anthrone-sulfuric acid method as described by Yemm and Willis (1954) by reacting anthrone-sulfuric acid reagent with the fungal supernatant at 100°C 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 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 90.17±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 90.17±1.13% comparing to the other isolates. Mycotoxins analysis gives negative results, no aflatoxins 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. Morphoscopic features includes colorless long conidiophores 15-25 μm, large radiate conidal 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, KH2PO4, and MgSO4.7H2O) 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) * A2 + (-2.58) * B2 + (+6.54) * C2 + (+4.03) * D2 +**

**Dry mass (g/l) = 26.47 + 13.07 * A + (-7.31) * B + 1.71 * C + 0.41 * D + (-3.90) * AB + 0.82 * AC + 0.66 * AD + (-4.46) * BC + 1.44 * BD + 0.06 * CD + 0.77 * A2 + 0.44 * B2 + 1.63 * C2 + (0.026) * D2 +**

**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) * A2 + (-5.04) * B2 + (-5.7) * C2 + (-8.02) * D2 +**

Where A, B, C, D are the coded values of glucose, yeast extract, KH2PO4, and MgSO4.7H2O, 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), KH2PO4 (+1; 1.5 g/l) and MgSO4.7H2O (+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 63.35 g/l, with corresponding predicted value 62.16 g/l using glucose (+1; 200 g/l), yeast extract (+1; 4 g/l), KH2PO4 (+1; 1.5 g/l) and MgSO4.7H2O (-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), KH2PO4, (+1; 1.5 g/l) and MgSO4.7H2O (+1; 1 g/l) as cleared 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.
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 koji acid production, dry mass and consuming sugar, while KH₂PO₄ (C) (P 0.0093), and MgSO₄·7H₂O (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; KH₂PO₄) and BD (yeast extract; MgSO₄·7H₂O) in koji acid production; AC (glucose; KH₂PO₄); AD (glucose; MgSO₄·7H₂O); BD (yeast extract; MgSO₄·7H₂O); CD (KH₂PO₄; MgSO₄·7H₂O) 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; KH₂PO₄; D; MgSO₄·7H₂O on koji acid production (a), dry mass (b), and consuming sugars (c) by Aspergillus oryzae (Ahlibourg) Cohn. The interaction between AB (glucose; yeast extract), AC (glucose; KH₂PO₄), and CD (KH₂PO₄; MgSO₄·7H₂O) was drawn for koji acid production (g) pictures 5(a1, a2, a3); AB (glucose; yeast extract), AC (glucose; KH₂PO₄), and BC (yeast extract; KH₂PO₄) for dry mass (g) pictures 5(b1, b2, b3) and AB (glucose; yeast extract), AC(glucose), CD(KH₂PO₄), and BD (yeast extract; MgSO₄·7H₂O) for consuming sugars (%) pictures 5(c1, c2, c3). (a1)
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$_2$PO$_4$ (1; 1.5 g/l), and MgSO$_4$·7H$_2$O (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.
Kojic acid acts as remarkable heterocyclic natural secondary metabolite of Aspergillus with various hot spot applications in cosmetics products of skin-lightening cosmetics (Wang et al., 2014), prevent hyper-pigmentation and skin wrinkling (Briganti et al., 2003), antibacterial, anti-inflammatory, anti-aging, antidermatophytic, and biocompatibility medical (González et al., 2015; Syamsul et al., 2017). Also, it used as pesticides, insecticidal agent (Burnett et al., 2010), and food antioxidant agent (Sheikhshoaei et al., 2017). Microbial kojic acid concentration 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 KA at zero time. 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 20 g/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 et al. (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 believed 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; Madilhah 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-toxigenic safe isolate for optimizing the production by five levels CCD design of RSM. According to several researchers not all kojic acid producers are toxigenic, several kojic acid producers are non-aflatoxin synthesizers (Basappa, 1970; Madilhah 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), KH2PO4 (+1; 1.5 g/l) and MgSO4·7H2O (+1; 1 g/l) through run (24) in our study. Model significance and calculated tested by R2 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, KH2PO4, and MgSO4 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 33.1 g/L KA by A. oryzae M866 using corn stalk, peptone, KH2PO4, and MgSO4.

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; Patidhar 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 guarantee 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
microwave 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–1) is promised industrial potential safe isolate with stable kojic acid.

REFERENCES

Ahmad, M.Z., Panda, B.P., Javed, S., Ali, M. (2006). Production of mevastatin by solid-state fermentation using wheat bran as substrate. Res J Microbiol 1, 443–447. https://doi.org/10.1080/14750750600557747
Alexeeva, Y.V., Ivanova, E.P., Bakemina, I.Y., Zyagintseva, T.N., Mikhailov, V.V. (2002) Optimization of glycosidases production by Pseudomonas aeruginosa issachenkonii KMM 3549T. Lett Appl Microbiol. 35, 343–346. https://doi.org/10.1046/j.1472–7286.2002.01189.x
Ariff, A., Hafiz, M.G., Ghim, B., Ong, C.L., Mamat, D.G., Calixto, G.M.F., Corrêa, M.A., Chorilli, M. (2015). Structural characterization and in vitro antioxidant activity of kojic dipalmitate loaded w/o/w multiple emulsions intended for skin disorders. Biomed Res Int. Article ID 304591, https://doi.org/10.1155/2015/304591
GoTo, T., Matsui, M., Kito, T., (1990). Analysis of Aspergillus mycotoxins by gas chromatography using fused silica capillary column. Mycotoxin 31, 43–47. https://doi.org/10.2520/miyco.1990.47.193
Graovac, J., Graovac, M., Dodic, J., Bajić, B., Balaz, J. (2014). Optimization of cultivation medium for enhanced production of antifungal metabolites by Streptomyces hygroscopicos. Crop Protection J 65,143–152. https://doi.org/10.1016/j.cropro.2014.07.020
Hazzaa, M.M., Saad, A.M., Hassan, H.M., Ibrahim, E. (2013). High Production of Kojic acid crystals by isolated Aspergillus oryzae var. effuses NRC1. J Appl Microbiol 9(3), 1714-1723.
Ibrahim, A.B.M., Mahmoud, G.A.E. (2019). Nonstoichiometric Mesoporous Cu1.90 S Nanoparticles Hydrotastically Prepared from a Copper Anthranilato Complex Inhibit Cellulosolysis of Fungal Genie. J Inorg Organomet Polym 29 (4), 1280–1287. https://doi.org/10.1007/s10900-019-01091-6
Ibrahim, A.B.M., Zidan, A.S.A., Aly, A.A.M., Mosbah, H.K., Mahmoud, G.A.E. (2020). Mesoporous cadmium sulfide nanoparticles derived from a new cadmium antilanthanum complex: Characterization and induction of morphological abnormalities in pathogenic fungi. Appl Organometal Chem. e5391. https://doi.org/10.1002/aoc.5391
Kitada, M., Ueyama, H., Fukimbara, T. (1967). Studies on kojic acid fermentation (I) Cultural condition in submerged culture. J Ferment Technol 45,1101-1107. https://doi.org/10.1271/bbb1968.45.1101
Kong, Y., Zou, P., Liao, Q., J., Song, L., Yu, Z., (2014). Medium optimization for the production of anti-cytoxanoder substances by Streptomyces sp. HIC-D1 using response surface methodology. Environ Sci Pollut Res 21,5983–5990. https://doi.org/10.1007/s11356-014-2525-2
Kumar, D., Jain, V.K., Shanker, G., Srivastava, A. (2003). Utilisation of fruits waste for citric acid production by solid state fermentation. Process Biochem 38, 1725–1729. https://doi.org/10.1016/S0032-9592(02)00253-4
Kwak, M.Y., Ryee, J.S. (1992). Control mycelial growth for kojic acid production using Ca-alginate immobilized fungal cells. Applied Microbiol Bio tech 36,578–583. https://doi.org/10.1007/BF00183222
Kwak, M.Y., Ryee, J.S. (1991). Cultivation characteristics of immobilized Aspergillus oryzae For kojic acid production. Biotechnol Bioengin 39, 903-906. https://doi.org/10.1002/bit.260390904
Lee, C.Z., Liou, G.Y., Yuan, G.F. (2006). Comparison of the aflR gene sequences of strains in Aspergillus section Flavi. J Microbiol 152,161-170. https://doi.org/10.1016/j.micres.2005.10.020
Long, W., Wang, Y., Chen, J. (1998). Thin-layer chromatography of mycotoxins and other comparison with other chromatographic methods. J Chromatography A 815(1), 3-20. https://doi.org/10.1016/S0021–8673(97)00793-2
Liu, J.M., Yub, T.C., Lin, S.P., Hsu, R.J., Hsu, K.D. and Cheng, K.C. (2016). Evaluation of kojic acid production in a repeated-batch PCS biofilm reactor. J Biotechnol 218, 41–48. https://doi.org/10.1016/j.jbiotec.2015.11.023
Machida, M., Asai, K., Sano, T., Tanaka, T., Kumagai, T., Kusumoto, A., I., Arima, T., Akita, O. and Futamura, T., Ishaara, H., Tamura, T., Yatsutake, T., Huang, G., Kojima, M., Okabe, M. (2001). Kojic acid production in an airlift bioreactor using partially hydrolyzed raw corn starch. J Biosci Bioengng 92, 360-365. https://doi.org/10.1263/bjb.92.360
Mahmoud G. A.E., Soltan, H.A.H., Abdel-Aleem, W.M. and Osman, S.A.M. (2020). Safe natural bio-pigment production by Monascus purpureus using mixed carbon sources with cytotoxicity examination on root tips of Allium cepa L. J Food Sci Technol. https://doi.org/10.1155/2020-04758-y.
Manabe, M.T., Goto, K., Tanaka, M.S. (1981). The capabilities of Aspergillus flavus group to produce aflatoxins and kojic acid. Report National Food Res Inst 38, 115–120.

Masse, M.O., Duvalllet, V., Borremans, M. and Goeyens, L. (2001) Identification and qualitative analysis of kojic acid and arbutin in skin-whitening cosmetics. Int J Cosmet Sci 23 (4), 219–232. https://doi.org/10.1046/j.1467-2494.2001.00074.x

Megalla, S.E., Bennett, G.A., Ellis, J.J. and Shotell, O.I. (1986) Production of deoxynivalenol and zearalenone by isolates of Fusarium graminearum SCHW. J Bacteriol 167, 415–419. https://doi.org/10.1128/ib.167.2.415-419.86

Mohamed, M.H. and Mahmoud, G.A-E. (2018) Microbial giberellins impact of on Zea mays (L.) plants under different levels of water salinity. Egypt J Soil Sci 58(3), 373–82. https://doi.org/10.12680/ejs.2018.58.3.373

Nafady, N.A., Bagy, M.M.K., Abd-Alla, M.H., Morsy, F.M. and Mahmoud, G.A-E. (2018) Improvement of medium components for high riboflavin production by Aspergillus terreus using response surface methodology. Rendiconti Lincei 26(3), 335–344. https://doi.org/10.1007/s11241-015-0449-7

Ogawa, A., Wakisaka, Y., Tanaka, T., Sakiyama, T. and Nakaniishi, K. (1995) Production of kojic acid by membrane-surface liquid culture of Aspergillus oryzae NRRL484. J Ferment Bioeng 80(1), 41–45. https://doi.org/10.1016/S0922-338X(95)89174-3

Patidar, P., Banerjee, D., Barnerjee, T. and Patil, S. (2005) Chitinase production from different parameters in their stabili- Improving of red colorants production in submerged culture and the effect of... https://doi.org/10.1007/s11274-014-9592-8

Prabu, R., Rosfarizan, M., Shah, U.K.M. and Ariff, A.B. (2011) Improvement of... https://doi.org/10.1007/s12210-009-00074-x

Prignano, F., Ortonne, G., Buggiani, G. and Lotti, T. (2007) Therapeutical approaches in melasma. Dermatologic Clinics 25(3), 337–342. https://doi.org/10.1016/j.detc.2007.04.006

Raper, K.B., and Fennell, D.I. (1965) The Genus Aspergillus. Williams and Wilkins Co. Baltimore.; IX + 686.

Rosfarizan, M. and Ariff, A.B. (2000) Kinetics of kojic acid fermentation by Aspergillus flavus using different types and concentrations of carbon and nitrogen sources. J Indust Microbiol Biotechnol 25(1), 20–24. https://doi.org/10.1007/s83j.10.339

Sadhasivam, S., Britzi, M., Zakin, V., Kostyukovsky, M., Trostanetsky, A., Quinn, E. and Sionov, I. (2017) Rapid Detection and Identification of Mycotoxigenic Fungi and Mycotoxins in Stored Wheat Grain. Toxins 9, 302. https://doi.org/10.3390/toxins9100302

Santos-Edunam, V.C., Roberto, E.C., Teixeira, M.F.S. and Pessoa, A.J. (2013) Improved of red colorants production in submerged culture and the effect of different parameters in their stability. Biotechnol Prog 29, 778–7785. https://doi.org/10.1002/bptp.1720

Scott, P., Lawrence, J., and Van Walbeek, W. (1970) Detection of mycotoxins by thin-layer chromatography: application to screening of fungal extracts. Appl Microbiol 20(5), 839. PMID: 5485087

Sheikhshohaei, M., Sheikhshohaei, I. and Ranjbar, M. (2017) Analysis of kojic acid in food samples uses an amplified electrochemical sensor employing V-O nanoparticle and room temperature ionic liquid. J Mol Liq 231, 597–601. https://doi.org/10.1016/j.molliq.2017.02.039

Syamsul, K., Mat, R.S., Mohd, F.F.H., Mohd, N.H. and Mohamad, R.M. (2017) Evaluation of tyrosinase activity and radical scavenging activity of Kojic acid and Kojic acid monooxalate. Adv Sci Lett 23 (5), 4742–4744. https://doi.org/10.1166/asl.2017.8881

Tanigaki, H., Obata, H. and Tokuyama, T. (1980) The determination of kojic acid using the stopped-flow method. Bull Chem Soc Jpn 53 (11), 3195–3197. https://doi.org/10.1246/bcsj.53.3195

Terabayshi, Y., Sano, M., Yamane, N., Marui, J., Tamano, K., Sagara, J., Dohmoto, M., Oda, K., Ohshima, E., Tachibana, K., Higa, Y., Ohashi, S., Koike, H. and Masuda M. (2010) Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from Aspergillus oryzae. Fungal Genetics Biol 47, 953–961. https://doi.org/10.1016/j.fgb.2010.08.014

Vasitha K.Y., Muruges, C.S. and Sattur, A.P. (2014) A tyrosinase inhibitor from Aspergillus niger. J Food Sci Technol Mys 51, 2877–2880. https://doi.org/10.1007/s00223-014-1395-6

Vohra, A. and Satyanarayana, T. (2002) Statistical optimization of the submerged cultures by Response Surface Methodology to enhance phytase production by Pichia anomala. Process Biochem 37, 999–1004. https://doi.org/10.1016/S0032-9592(01)00308-X

Wakisaka, Y., Segawa, T., Imamura, K., Sakiyama, T. and Nakanishi, K. (1998) Development of a cylindrical apparatus for membrane-surface liquid culture and production of kojic acid using Aspergillus oryzae NRRL484. J Ferment Bioeng 85(5), 488–494. https://doi.org/10.1016/S0922-338X(98)80067-6

Wan, H.M., Chen, C.C., Giridhar, R. and Chang, T.S. (2005) Repeated –batch production of kojic acid retention fermenter using Aspergillus oryzae M5B9. J Ind Microbiol Biotechnol 32, 227–233. https://doi.org/10.1007/s10295-005-0230-5

Wang, K., Li, P.F., Han, C.G., Du, L., Liu, C., Hu, M., Lian, S.J. and Liu, Y.X. (2014) Protective effects of kojic acid on the periphery blood and survival of beagle dogs after exposure to a lethal dose of gamma radiation. Radiat Res 182 (6), 666–673. https://doi.org/10.1667/RR13823.1

Xu, C., Kim, S., Hwang, H. and Choi, J. (2005) Optimization of submerged culture conditions for mycelial growth and exobiopolymer production by Paecilomyces teruiakes C240. Process Biochem 38(7), 1025–1030. https://doi.org/10.1016/j.procbio.2003.09.007

Yan, S., Tang, H., Wang, S., Xu, L., Liu, H., Guo, Y. and Yao, J. (2014) Improvement of kojic acid production in strain Aspergillus oryzae B008 mutant strain and its uses in fermentation of concentrated corn stalk hydrolysates. Biosprocess Biosyst Em 37(6), 1095-1103. https://doi.org/10.1007/s12210-013-1081-5

Yemm, E.W. and Willis, A.J. (1954) The estimation of carbohydrates in plant extracts by anthrone. Biochem J 57(3), 508–514 PMID: 13181867

Zohr, A.A., Mahmoud, G.A-E., Sadik, N.H., Hanafy, R.A. (2018) Optimization of kojic acid production conditions from cane molasses using Plackett-Burman design. Eur J Biolog Res 8(2), 56–69. https://doi.org/10.5281/zenodo.1211157