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Production of Kojic Acid by *Aspergillus niger* M4 with Different Concentrations of Yeast Extract as a Nitrogen Source

Tomasa Quiterio-Gutiérrez [1], Susana González-Morales [2], José Antonio González-Fuentes [3], Adalberto Benavides-Mendoza [3], Fabián Fernández-Luqueño [4], Julia Medrano-Macías [3] and Armando Robledo-Olivo [5,*]

1 Postgraduate Program in Protected Agriculture, Antonio Narro Autonomous Agrarian University, Saltillo 25315, Coahuila, Mexico
2 CONACyT-Department of Horticulture, Antonio Narro Autonomous Agrarian University, Saltillo 25315, Coahuila, Mexico; qfb_sgm@hotmail.com
3 Department of Horticulture, Antonio Narro Autonomous Agrarian University, Saltillo 25315, Coahuila, Mexico
4 Sustainability of Natural Resources and Energy Programs, CINVESTAV-Saltillo, Ramos Arizpe 25900, Coahuila, Mexico
5 Fermentations and Biomolecules Laboratory, Food Science and Technology Department, Antonio Narro Autonomous Agrarian University, Saltillo 25315, Coahuila, Mexico
* Correspondence: armando.robledo@uaaan.edu.mx; Tel.: +52-(844)-411-0209

Abstract: In agro-industrial processes, microorganisms that are not pathogenic and that generate molecules are generally recognized as safe (GRAS). The *Aspergillus niger* fungus has different industrial applications, being used to produce citric acid and 166 other secondary metabolites. The objective of this research was to optimize a culture medium to induce the production of kojic acid (KA) by the *Aspergillus niger* M4 strain in a liquid fermentation process. Four fermentative kinetics were developed in flasks, using different levels of yeast extract in (1) 0.05 g/L, (2) 0.10 g/L, (3) 2.5 g/L, and (4) 2.5 g/L + Zinc sulfate. The culture medium conditions influenced the formation and speed of biomass and the synthesis and yield of KA. The optimum production points were from 72 h and 96 h with 0.552 g/L and 0.510 g/L of KA using 2.5 g/L of yeast extract and with a pH of 5.5. The *Aspergillus niger* M4 strain had the ability to produce kojic acid, which was induced by the concentration of the nitrogen source.

Keywords: kojic acid; *Aspergillus niger*; liquid fermentation; kinetic modeling

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1. Introduction

Aspergillus is a genus of ascomycete fungi of the *Aspergillaceae* family that contains a large number of species and is widely distributed in natural environments [1]. *Aspergillus niger* is a fungus of great industrial economic importance due to its enhanced adaptive metabolic capacity for carbohydrate hydrolysis and the production of organic acids and proteins; thus, it is used commercially to produce citric acid [2,3]. Approximately 166 secondary metabolites derived from *A. niger* have been detected, which can be grouped into five different types: pyranone (α-pyrones, γ-pyrones, and naphtho-γ-pyrones), alkaloid, cyclopentapeptide, polyketide, and sterol [4,5]. Within the γ-pyrones, there is kojic acid (KA), a metabolite of great industrial importance due to its weak-acid characteristics, which allow it to have several industrial applications, such in the fields of cosmetics, food, chemicals, pharmaceuticals, agriculture, and medicine [6]. Some uses of KA in the food industry include antifungals, antioxidants, preservatives, and food additives to prevent the enzymatic discoloration of vegetables, crabs, and shrimps [7,8]. In cosmetics, it has been used as a skin-lightening agent [9,10] as it is a copper chelator and inhibits the activity of tyrosine containing copper ions at the active site, forming a KA-Cu-Tyrosine complex,

which reduces enzyme activity in human cells by reducing melanin content [6,8]. KA (5-hydroxy−2-hydroxymethyl-γ-pyrone) is a secondary metabolite produced by a wide range of fungi of the genus *Aspergillus*, such as *A. terreus* [11], *A. parasiticus*, and *A. flavus* [12]; fungi of the genus *Penicillium*; and certain bacteria [13]. A prominent strain is *Aspergillus oryzae* [14], called 'koji-kin' in Japanese, which is the origin of the common name of this organic acid [15]. A mutant of *A. niger* (FGSC A1279) has also been detected as a producer of KA [16]. The industrial importance of the *A. niger* strain lies in its adaptability to different carbon sources and culture medium conditions, allowing biomass waste substrates to be used for low-cost bioprocesses. The metabolism of this micro-organism can be modified by changing the composition of the medium and the mode of fermentation [5], which improves the production of secondary metabolites by optimizing fermentation processes [11]. Different methods can be used, such as experimental designs, mathematical methods, and kinetic models, for the optimization of fermentative processes [17]. Some of the conditions of the culture medium are pH, temperature, carbon, and nitrogen source, as well as incubation periods. For the production of KA, different sources of carbon (glucose, xylose, sucrose, starch, maltose, lactose, or fructose) and nitrogen (NH_4Cl, $(NH_4)_2S_2O_8$, $(NH_4)_2NO_3$, yeast extract, or peptone) have been used with the *Aspergillus flavus* strain [17]. Alternatively, when using inorganic sources of nitrogen, nitrate ions can inhibit enzymes that are involved in KA biosynthesis by reducing their production [18]. Yeast extract (YE) as an organic source at concentrations between 1% and 5% significantly increases the synthesis and production of KA by using *A. terreus* and *A. sojae* [11,19]. High concentrations of the carbon source and low availability of the nitrogen source are key features to enhance KA production [20]. The unused carbon source that remains in the culture medium can be converted into KA by the enzymatic activity that is found attached to the outer membrane of non-growing mycelial cells [20,21]. This has given place to the favorable use of YE as an organic nitrogen source compared to as an inorganic source. On the other hand, Badar et al. [22] evaluated the impact of pH variation on KA production using two *Aspergillus* species: *A. flavus* with a pH of 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 producing 8, 10, 6, 4, and 3 g/L KA, respectively, with the best performance being at a pH of 4.5 for 20 days, and *A. oryzae* with a pH of 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 producing 5, 3, 2, 1.6, 1.1, and 0.5 g/L KA, respectively, with the best performance being at a pH of 3.5 for 20 days. Promsang et al. [23] argued that to produce KA with a different strain of *A. oryzae*, a pH between 3.0 and 5.5 is required. Other studies reported that during fermentation with *A. flavus* after 7 days of incubation at 30 °C, a maximum production of KA was reached at a pH of 4.0 [24]. For the isolation of *A. flavus* and *A. oryzae* for KA production, the culture medium composition consisted of glucose, YE, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, with an initial pH of 3.0 and heat stress above 40 °C [25]. Some authors have added $ZnSO_4 \cdot 7H_2O$ into fermentative processes [23,26] to act as a modulator of cellular amino acid metabolism and redox balance, exerting an antioxidant effect and inhibiting the presence of acetic acid [27]. Although some authors have previously proposed the use of $ZnCl_2$, for the production of KA with the *A. parasiticus* strain, Zn^{2+} concentrations (10 μM) did not affect KA production [28]. Although culture media with the use of $ZnSO_4 \cdot 7H_2O$ have already been made for the production of KA, the authors did not evaluate the influence of the nutrients; they only focused on the different sources of nitrogen. From the above information, one can hypothesize that the conditions of the culture medium during the liquid fermentation of the fungus *Aspergillus niger* M4 may be modified to generate an induction in the synthesis of KA. In the present investigation, the *A. niger* M4 strain was subjected to liquid fermentation, using diverse levels of yeast extract as a nitrogen source, the addition of zinc sulfate as a nutrient, and pH adjustments of the medium at different times for kojic acid obtention.

2. Materials and Methods

2.1. Microorganism

In the present study, the *Aspergillus niger* M4 (code KY825168.1) strain was used (Property of the Fermentations and Biomolecules Laboratory of the Food Science and

Technology Department at the Antonio Narro Autonomous Agrarian University). The strain was preserved at −20 °C in a medium containing skim milk (10% *w*/*v*) and glycerol (5% *w*/*v*). The reactivation of the fungus was conducted in Petri dishes that contained potato dextrose agar (PDA, Bioxon México) and that were incubated for 7 days at 30 °C. For the preparation of the inoculum, the spores were collected using a sterilized solution of Tween 80 (Hycel, Mexico) at 1%, and the spores were counted in a Neubauer chamber.

2.2. Fermentation Conditions

For the fermentation kinetics, a basic medium composed of 100 g/L of glucose as a carbon source, 1 g/L of KH_2PO_4, and 0.5 g/L of $MgSO_4 \cdot 7H_2O$ was used. Diverse levels of yeast extract (YE) were used as a nitrogen source, which were 0.05 g/L, 0.10 g/L, and 2.5 g/L, and an additional medium containing 2.5 g/L of YE + 0.1 g/L of $ZnSO_4 \cdot 7H_2O$ (YE + Zn). The pH of the initial medium was 5.5 for each media with YE variations. The fermentations were conducted in Erlenmeyer flasks, and 20 mL of medium was added with inoculation of 1×10^6 spores per mL of *A. niger* M4. The flasks were incubated at 30 °C and continuously shaken at 125 RPM (INNOVA 44 Orbital Incubator), taking samples at various times for the analytical determinations. For each YE level, a completely randomized design was developed, where each flask was considered as an experimental sample reactor, and intervals of 24 h were considered as the experimental time, which was conducted in triplicate for a total of 78 reactors at times of up to 552 h. The complementary pH test for 3.5 and 5.5 pH values was carried out in the same manner described above, placing 36 reactors at a time of 120 h, monitoring them every 24 h. A pH evaluation was conducted as it has been reported that the pH of a medium determines the growth rate and fermentation processes of KA-producing microorganisms [24]. For subsequent analyses, the liquid content of the flasks after filtration was used, considering it as raw fermentation extract (RFE).

2.3. Analytical Determinations

To obtain the biomass, each reactor was taken at its respective fermentation time, obtaining the RFE by double filtration (Whatman N°42) using a Kitasato flask and a vacuum pump. The mycelium obtained in the first filter was dried in an oven and kept for 24 h at 80 °C to determine the biomass by weight difference. The filtered RFE was stored at −20 °C for further analysis. All samples were analyzed in triplicate and the averages obtained were reported.

The glucose content was quantified by the Miller method [29], placing 1 mL of RFE and 1 mL of DNS reagent into glass tubes. The tubes were boiled in a water bath for 5 min; then, the reaction was stopped with an ice bath for 5 min. A total of 5 mL of distilled water was added, shaken, and left to stand for 5 min at room temperature, then read using a UV-Vis spectrophotometer (Thermo Fisher Scientific, model G10S, Waltham, MA, USA). A calibration curve was made with dextrose (Jalmek) at 1000 mg/L.

KA quantification was performed using both the spectrophotometric technique and HPLC (high-performance liquid chromatography). For spectrophotometer quantification, the method described by Bentley [30] was used, which involved adding 1 mL of previously filtered RFE and 2 mL of 1% $FeCl_3$ prepared with HCl (0.1 N) and diluting the mixture with 5 mL of distilled water. Readings were taken at 505 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific, model G10S, Waltham, MA, USA). The calibration curve was prepared using standard-grade kojic acid (Sigma-Aldrich) at 1000 mg/L. The data analyzed were expressed in grams of KA per liter of fermentation (g/L).

To determine the KA content by HPLC, the method described by Promsang et al. [23] was used, with some modifications. The RFE obtained was filtered again through 13 mm nylon membranes with a 0.45 µm pore. The filtered samples were degassed before being injected into the high-performance liquid chromatography (HPLC) equipment. To analyze the kojic acid, a Luna C18(2) separation column (5 µm; 150 × 4.6 mm) and UV Detector at 270 nm were used. Mobile phase (A) consisted of 94.9% HPLC grade water, 5% acetonitrile,

and 0.1% formic acid; mobile phase (B) consisted of 94.9% acetonitrile, 5% HPLC grade water, and 0.1% formic acid. The proportion of solvents A:B was 40:60, with a flow rate of 1 mL/min and a temperature of 22 °C. The injection volume was 100 µL, with a retention time of 12 min per sample. The calibration curve was performed with HPLC-grade KA at 75 mg/mL.

To measure the antioxidant capacity in the fermentations with a different pH, two radical scavenging methods, DPPH and ABTS, were used. The first method was that described by Brand-Williams et al. [31], with some modifications. A 0.1 mM mixture of radical DPPH (2,2-Diphenyl−1-(2,4,6-trinitrophenyl)hydrazin−1-yl) was prepared, diluting it with 80% methanol. The absorbance of the solution was adjusted to 0.700 ± 0.02 at 520 nm using a fully automatic microplate reader (BIOBASE-EL 10A, Jinan, SHG, China). For microplate quantification, 6 µL of RFE and 234 µL of DPPH (0.1 mM) were added to each well, allowed to settle for 30 min, and then reading was performed at 520 nm.

The second method (ABTS) was the spectrophotometric method of Re et al. [32], with some modifications. The ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline−6-sulfonic acid)) radical was prepared at 7 mM, with 2.45 mM of potassium persulfate at a concentration of 1:1 v/v. The mixture was settled for 16 h at 30 °C. The absorbance of the solution was adjusted with 20% ethanol to 0.700 ± 0.02 at 750 nm using a fully automatic microplate reader (BIOBASE-EL 10A, Jinan, SHG, China). For quantification, 5 µL of RFE and 240 µL of ABTS were added to microplate wells, with a stabilization time of 6 min, before being read at 750 nm. Both radical capture methods were expressed as percentage inhibition, using the following formula:

$$\text{Inhibition } [\%] = \left[\mathbf{1} - \frac{\boldsymbol{A_{sample}}}{\boldsymbol{A_{control}}}\right] \times \mathbf{100} \quad (1)$$

where $\boldsymbol{A_{sample}}$ is the absorbance of the sample and $\boldsymbol{A_{control}}$ is the blank absorbance for the ABTS with 20% ethanol.

2.4. Calculations of Parameters Associated with the Fermentative Process

The Verhulst logistic growth, Pirt, and Luedeking-Piret models were used to estimate the theoretical values of the biomass, substrate, and product coefficients, respectively, as described by Robledo et al. [33].

The growth rate of the microorganism was measured by estimating the number of cells ($\boldsymbol{X}$) over time ($\boldsymbol{t}$), considering the carrying capacity of the system ($\boldsymbol{X_{max}}$) and the maximum rate of cell growth ($\boldsymbol{\mu_{max}}$):

$$\frac{\boldsymbol{dX}}{\boldsymbol{dt}} = \boldsymbol{\mu_{max}} \cdot \boldsymbol{X}\left(\frac{\boldsymbol{X_{max}} - \boldsymbol{X}}{\boldsymbol{X_{max}}}\right) \quad (2)$$

with the solved form

$$\boldsymbol{X_{(t)}} = \frac{\boldsymbol{X_{max}}\boldsymbol{X_0}}{(\boldsymbol{X_{max}} - \boldsymbol{X_0})\boldsymbol{e}^{-\boldsymbol{\mu_{max}}\boldsymbol{t}} + \boldsymbol{X_0}} \quad (3)$$

where $\boldsymbol{X_0}$ are the cells present in the medium when $\boldsymbol{t}$ = 0. For values of $\boldsymbol{\mu_{max}} > 0$, the resulting growth curve has a sigmoidal shape and is asymptotic to the carrying capacity ($\boldsymbol{X_{max}}$). The higher $\boldsymbol{\mu_{max}}$ is, the faster the curve reaches the load capacity ($\boldsymbol{X_{max}}$).

For the production of biomass, it is required to consume substrate ($\boldsymbol{S}$) for the synthesis of new cells ($\boldsymbol{S_G}$) and to maintain existing cells ($\boldsymbol{S_M}$). Substrate consumption will always be related to time and the biomass in the culture medium, and can be represented by the following expression:

$$-\frac{\boldsymbol{dS}}{\boldsymbol{dt}} = \frac{\boldsymbol{1}}{\boldsymbol{Y_{X/S}}} \cdot \frac{\boldsymbol{dX}}{\boldsymbol{dt}} + \boldsymbol{m} \cdot \boldsymbol{X} \quad (4)$$

where $\boldsymbol{m}$ is the cell maintenance coefficient and $\boldsymbol{Y_{X/S}}$ is the maximum growth yield coefficient when $\Delta \boldsymbol{S_M}$ = 0.

$$\boldsymbol{Y_{X/S}} = \frac{\boldsymbol{X_2} - \boldsymbol{X_1}}{(\boldsymbol{S_1} - \boldsymbol{S_2})_{\boldsymbol{G}} + (\boldsymbol{S_1} - \boldsymbol{S_2})_{\boldsymbol{M}}} \quad (5)$$

$$Y_{X/S} = \frac{X_2 - X_1}{(S_1 - S_2)_G} \quad (6)$$

where X_1 is the biomass initial condition and X_2 is the biomass maximum value; S_1 is the substrate initial condition and S_2 is the substrate concentration maximum value. The solution of Equation (4) can be obtained as a function of X as follows:

$$S_{(t)} = S_0 - \frac{X - X_0}{Y_{X/S}} - \frac{m \cdot X_{max}}{\mu_{max}} \cdot \ln\left[\frac{X_{max} - X_0}{X_{max} - X}\right] \quad (7)$$

where S_0 is the initial condition for substrate level S.

The kinetics of product (P) formation was modeled using the equation as follows:

$$\frac{dP}{dt} = Y_{P/X} \cdot \frac{dX}{dt} + k \cdot X \quad (8)$$

where $Y_{P/X}$ is the product yield in terms of biomass (units of product per unit of biomass) and k is the secondary coefficient of product formation or destruction.

$$Y_{P/X} = \frac{P_2 - P_1}{X_1 - X_2} \quad (9)$$

where P_1 is the product initial concentration and P_2 is the maximum product concentration; X_1 is the biomass initial condition and X_2 is the biomass concentration at the product maximum value. It is possible to solve Equation (8) as a function of biomass.

$$P_{(t)} = P_0 + Y_{P/X} \cdot (X - X_0) + \frac{k \cdot X_{max}}{\mu_{max}} \cdot \ln\left[\frac{X_{max} - X_0}{X_{max} - X}\right] \quad (10)$$

Substrate consumption (q_S) and product formation (q_P) rates are linked to transformation yields and growth rates, respectively.

$$q_P = \mu_{max} \cdot Y_{P/X} \quad (11)$$

$$q_S = \frac{\mu_{max}}{Y_{X/S}} + m \quad (12)$$

2.5. Statistical Analysis

The results were represented as the mean of the data ± standard deviation and performed for three replicates per sample. The data were processed with the statistical software Minitab (version 17.1.0). All data presented a normal distribution and an analysis of variance (ANOVA), and a Tukey comparison test of means ($p < 0.05$) was performed. For all the adjustments of the experimental data to the models, the Excel Solver tool was used, seeking to minimize the squared errors between the experimental values and the model. As confidence indicators of the models, the mean square error (*MSE*) and R^2 values were used.

3. Results

The adaptability of *A. niger* for the production of industrial metabolites is based on its growth environment. The components present in the fermentative medium that go from the concentration into the source of nitrogen, carbon, and minerals have an influence on the production of KA [24]. Four different media were evaluated in the production of KA; the response to the formation of biomass, reduction of sugars, and production of KA were also evaluated. The biomass formation of the different levels of kojic acid production is shown in Figure 1. The concentrations of 0.05 g/L and 0.10 g/L of YE presented an exponential phase between the first and third days, increasing from 1.44 g X/g glucose to

2.11 g X/g glucose for 0.05 g/L of YE; for 0.10 g/L of YE, the values on the first and third days were 1.47 g X/g glucose and 2.12 g X/g glucose. With an addition of 2.5 g/L of YE, they increased from 2.18 g X/g glucose to 6.04 g X/g glucose between the first and fourth days. For conditions with 2.5 g/L of YE + Zn, the increase was from 1.26 g X/g glucose to 5.13 g X/g glucose in the same lapse of 4 days. After the fourth day of fermentation for the four conditions evaluated, the growth process reached a stationary phase. The maximum growth occurred after 12 days of fermentation under the conditions of 2.5 g/L of YE, increasing the biomass by 6.4 times compared to zero time.

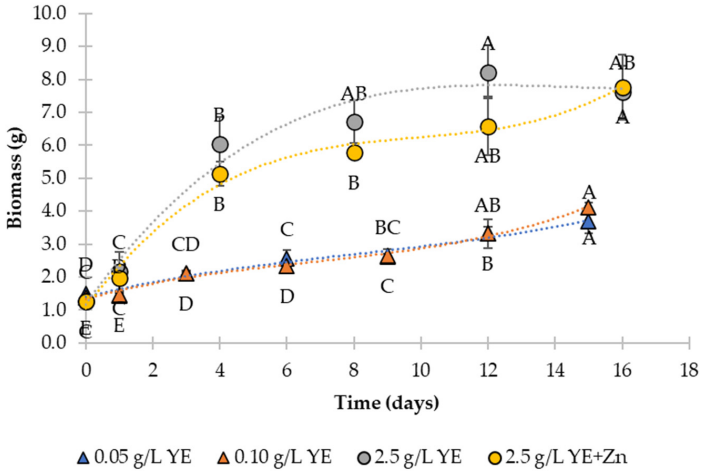

Figure 1. Biomass formation of *Aspergillus niger* M4 using different concentrations of yeast extract as the sole nitrogen source. The dotted lines represent a polynomial trend line of the 3rd order. Different uppercase letters indicate statistically significant differences between the same concentrations of yeast extract (Tukey $p < 0.05$).

Sugars, such as glucose, are essential for the production of secondary metabolites [34]. The glucose content is shown in Figure 2. During the fermentation of 0.05 g/L and 0.10 g/L of YE, in the first few hours, glucose was reduced by 66% and 60%, respectively, while for 2.5 g/L of YE and 2.5 g/L of YE + Zn, glucose was reduced by 19% and 12%, respectively, for the same period of time. The highest glucose consumption of 89.4% was reached with 2.5 g/L of YE + Zn on the 12th day of fermentation.

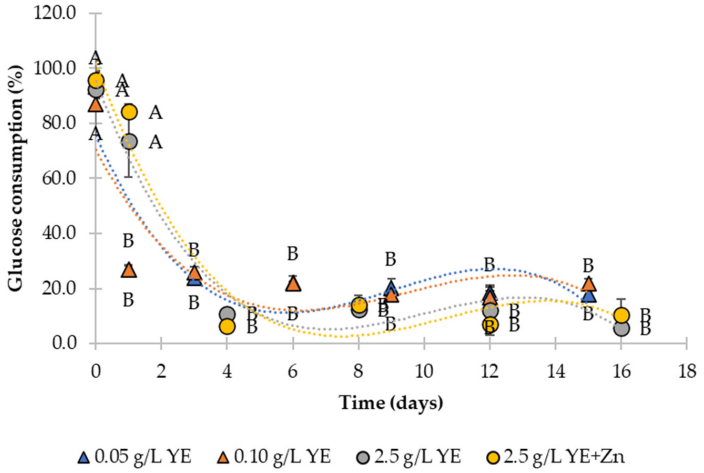

Figure 2. Glucose consumption by *Aspergillus niger* M4 using different concentrations of yeast extract as the sole nitrogen source. The dotted lines represent a polynomial trend line of the 3rd order. Different uppercase letters indicate statistically significant differences between the same concentrations of yeast extract (Tukey $p < 0.05$).

The production of the *Aspergillus niger* M4 fungus to synthesize kojic acid is shown in Figure 3. KA production started from the third day of fermentation for all YE concentrations evaluated. For the concentration of 0.05 g/L of YE, a maximum yield of 0.150 g KA/L was obtained after 15 days. On the other hand, the concentration of 0.1 g/L of YE had a maximum yield of 0.071 g KA/L after 6 days of fermentation. For the concentrations of 2.5 g/L of YE and 2.5 g/L of YE + Zn, maximum yields of 0.523 g KA/L and 0.240 g KA/L were obtained, respectively, both at 4 days of fermentation.

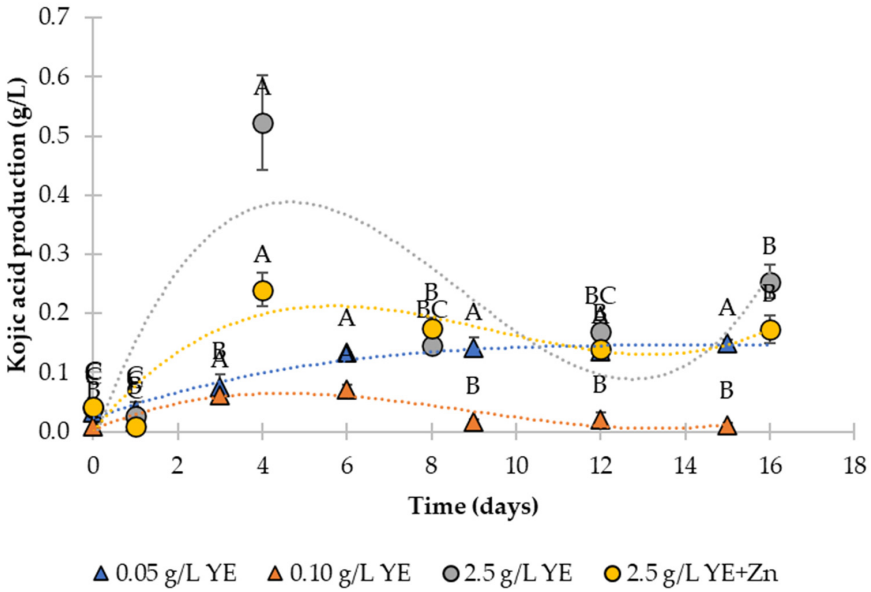

Figure 3. Kojic acid production in liquid medium by *Aspergillus niger* M4 using different concentrations of yeast extract as the sole nitrogen source. The dotted lines represent a polynomial trend line of the 3rd order. Different uppercase letters indicate statistically significant differences between the same concentrations of yeast extract (Tukey $p < 0.05$).

Table 1 was generated with the kinetic parameters from the evaluations with all concentrations of yeast extracts. The highest parameters of maximum growth velocity (μ_{max}), biomass-substrate yield ($Y_{X/S}$), product-to-biomass yield ($Y_{P/X}$), substrate consumption rate (q_S), and product formation rate (q_P) were reached for the concentration of 2.5 g/L YE. The values of $Y_{P/X}$ and q_P for 0.05 g/L YE and of q_P for 0.10 g/L YE presented insignificant values in the range of 10^{-7}–10^{-9}. For all of the YE concentrations, maintenance coefficient values (m) and product formation (k) are shown.

Table 1. Kinetic parameters of fermentation using different concentrations of yeast extract as the sole nitrogen source. The values were calculated from the respective models mentioned above.

Parameter	0.05 g/L YE	0.10 g/L YE	2.5 g/L YE	2.5 g/L YE + Zn
μ_{max} (h^{-1})	0.006 ± 0.001 c	0.004 ± 0.001 c	0.035 ± 0.001 a	0.024 ± 0.002 b
$Y_{X/S}$ (g X/g S)	0.019 ± 0.001 c	0.020 ± 0.003 c	0.082 ± 0.001 a	0.052 ± 0.004 b
m (g X/g S h)	0.000 ± 0 b	0.000 ± 0 b	0.000 ± 0 a	0.000 ± 0 b
$Y_{P/X}$ (g KA/g X)	0.000 ± 0 b	0.007 ± 0.006 b	0.032 ± 0.004 a	0.029 ± 0.001 a
k (g KA/g X h)	0.000 ± 0 a	0.000 ± 0 a	0.000 ± 0 a	0.000 ± 0 a
q_S (g glu/g X h)	0.296 ± 0.062 ab	0.199 ± 0.051 b	0.430 ± 0.001 ab	0.460 ± 0.084 a
q_P (g KA/g X h)	0.0000 ± 0 c	0.0000 ± 0 c	0.0011 ± 0.0001 a	0.0007 ± 0.0001 b

Values are presented as mean (± standard deviation, $n = 3$). Different lowercase letters in the same row indicate statistically significant differences ($p < 0.05$).

For the standardization of the induction of KA, a kinetic of 120 h was submitted with the use of 2.5 g/L of YE as a nitrogen source (Figure 4). The optimal points of production were at 72 h and 96 h, with 0.522 g/L and 0.510 g/L of KA produced, respectively. The results of this study showed that, in terms of general production, fermentation with

2.5 g/L of yeast extract in the culture medium was favorable for KA production and that fermentation times were shorter, indicating that the metabolite was best obtained under the aforementioned conditions.

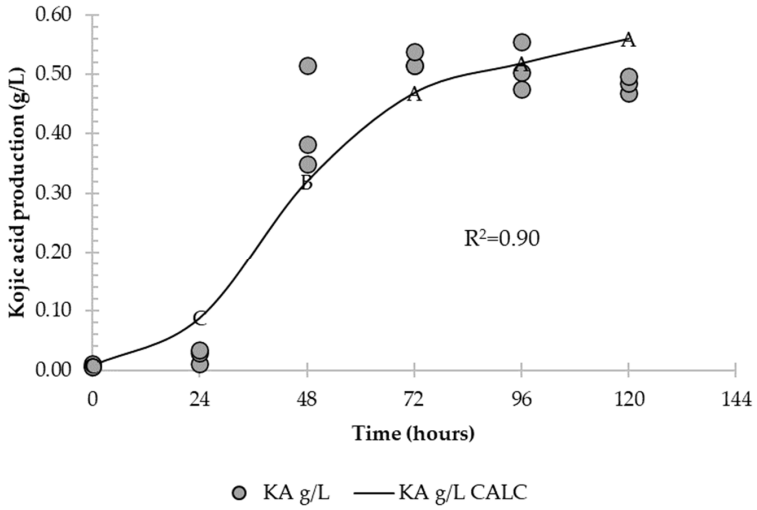

Figure 4. Production of kojic acid by *Aspergillus niger* M4 in liquid medium fermentation at 120 h using 2.5 g/L of YE as the sole nitrogen source. The solid line represents the values calculated using Equation (10) [R^2 = 0.9473; *MSE* = 0.0083]. Different uppercase letters indicate statistically significant differences (Tukey $p < 0.05$).

3.1. Effect of pH on Kinetic Parameters

To investigate the variability of KA production when using the conditioned culture medium, the effect of the pH of the growth medium was evaluated, being at pH 3.5 and pH 5.5 at a time of 120 h. The biomass growth kinetics process for 120 h is shown in Figure 5. For the time 72 h with a pH of 3.5, there was an increase in biomass of 1.067 g/L, while with a pH of 5.5 at times of 72 h and 96 h, the increase was 1.500 g/L and 1.622 g/L, respectively. The experimental data were adjusted for Equation (3). A pH of 5.5 was more favorable during the biomass growth phase; therefore, the addition of acid to adjust the pH to 3.5 of the culture medium was not effective for the fungal activity, affecting the production of KA.

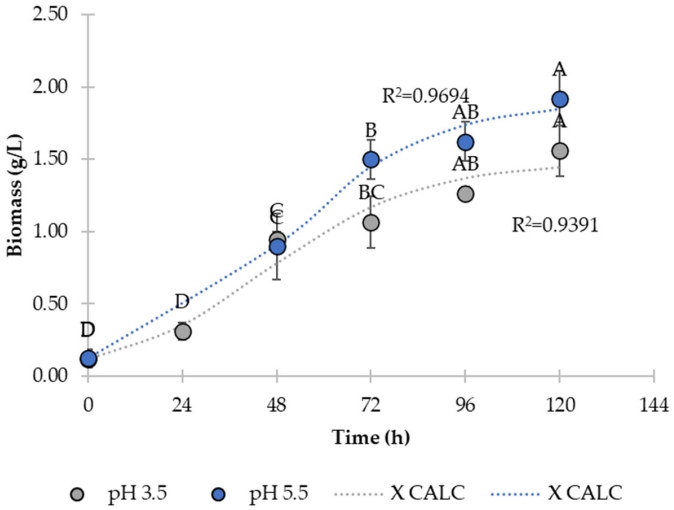

Figure 5. Biomass formation at 120 h using 2.5 g/L of yeast extract as the sole nitrogen source. The dotted line represents the values calculated using Equation (3) [pH 3.5: R^2 = 0.9391; *MSE* = 0.0168; pH 5.5: R^2 = 0.9694; *MSE* = 0.0138]. Different uppercase letters indicate statistically significant differences (Tukey $p < 0.05$).

3.2. Substrate

The consumption of glucose thereby reducing sugar can be observed in Figure 6. In conditions of a pH of 3.5, the decrease occurred after 48 h, while at a pH of 5.5, the decrease occurred after 72 h. The maximum glucose consumption was 68.2% at a pH of 3.5 and 32.3% at a pH of 5.5, both at 120 h of the fermentation process. The glucose present in the culture medium was mostly accumulated under conditions of a pH of 5.5, while a pH of 3.5 influenced the reduction of sugars; however, the pH adjustment interfered with the production of KA (Figure 6).

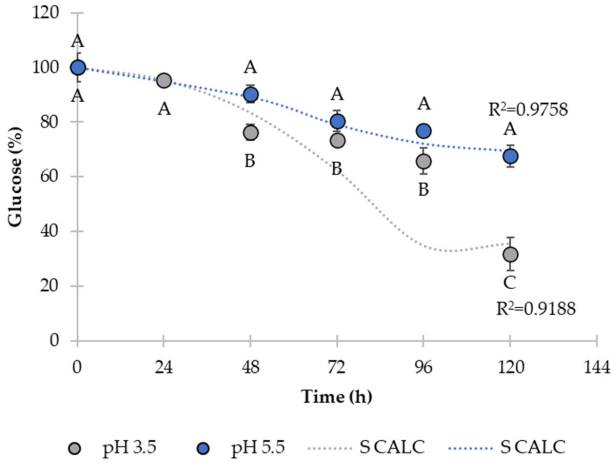

Figure 6. Substrate at 120 h using 2.5 g/L of yeast extract as the sole nitrogen source. The dotted line represents the values calculated using Equation (7) [pH 3.5: R^2 = 0.9188; *MSE* = 11.1924; pH 5.5: R^2 = 0.9758; *MSE* = 1.9768]. Different uppercase letters indicate statistically significant differences (Tukey $p < 0.05$).

3.3. Product

Regarding product formation (Figure 7), at a pH of 3.5, the presence of KA was obtained at 48 h, producing 0.252 g/L, and this value remained stable until near the end of the process. On the other hand, with a pH of 5.5, the product was generated at 48 h with a production of 0.265 g/L, with an increasing tendency until the end of the process, reaching a maximum value of 0.436 g/L.

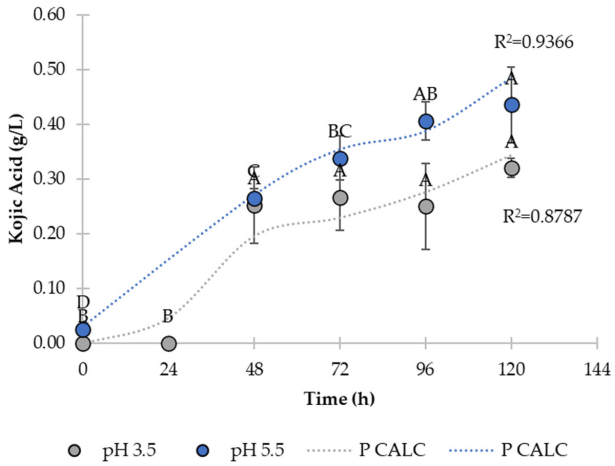

Figure 7. Production of KA over 120 h using 2.5 g/L of yeast extract as the sole nitrogen source. The dotted line represents the values calculated using Equation (10) [pH 3.5: R^2 = 0.8787; *MSE* = 0.0022; pH 5.5: R^2 = 0.9366; *MSE* = 0.0025]. Different uppercase letters indicate statistically significant differences (Tukey $p < 0.05$).

The RFE measured by HPLC can be observed in Figure 8. With a pH of 3.5, 0.396 g/L of KA was produced at 72 h; however, KA production decreased during the remaining fermentation time at 96 h and 120 h. With a pH of 5.5, after 96 h, 0.491 g/L of KA was produced, which was above the highest value obtained with a pH of 3.5; the highest value obtained with a pH of 5.5 was 0.931 g/L of KA at 120 h. To create optimal conditions to produce KA by means of *Aspergillus niger*, the pH of the initial medium should be pH 5.5 since adjusting the pH to 3.5 may make the medium too acidic for the fungus.

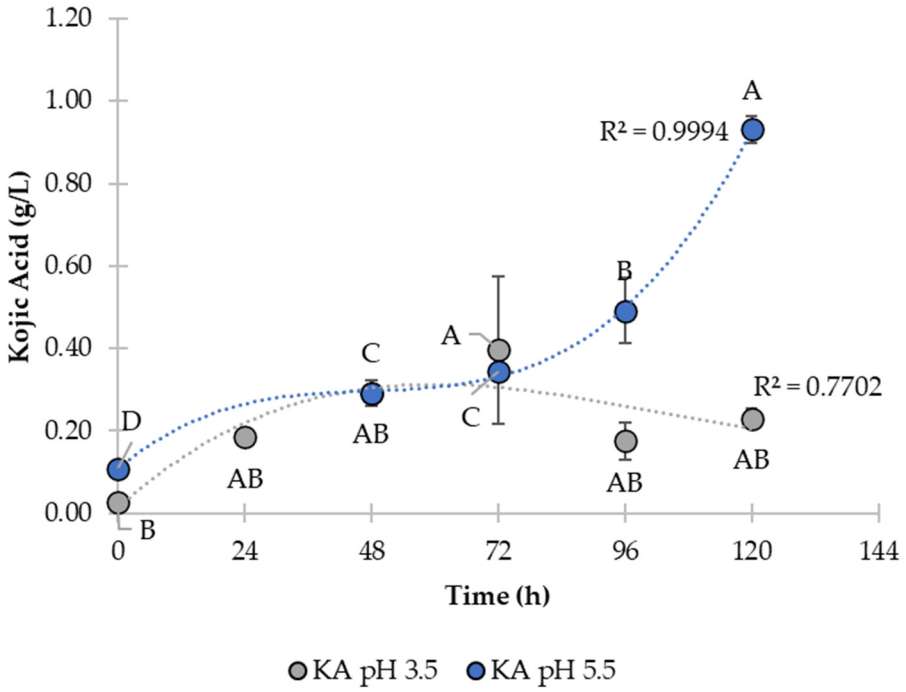

Figure 8. Kojic acid measurement by HPLC over 120 h of fermentation using 2.5 g/L of yeast extract as the sole nitrogen source. Dotted lines are the 3rd order polynomic tendency line [pH 3.5: $y = 2 \times 10^{-6}x^3 - 0.0002x^2 + 0.011x + 0.10733$; pH 5.5: $y = 2 \times 10^{-6}x^3 - 0.0002x^2 + 0.011x + 0.1071$]. Different uppercase letters indicate statistically significant differences (Tukey $p < 0.05$).

All modeled parameters are summarized in Table 2, where it can be observed that medium conditions with a pH of 5.5 favored biomass production and yielded better KA production. Outstanding results were obtained concerning growth speed (μ_{max}), whereby conditions with a pH of 5.5 were the fastest in terms of fungal growth and biomass product yield ($Y_{P/X}$), presenting the highest number of grams of KA per gram of glucose.

Table 2. Kinetic parameters of fermentation using two values of pH in the culture medium and only 2.5 g/L of yeast extract as the sole nitrogen source. The values were calculated from the respective models mentioned above.

Parameters (Units)	pH 3.5	pH 5.5
μ_{max} (h^{-1})	0.049 ± 0.001	0.065 ± 0.004
$Y_{X/S}$ (g X/g S)	0.345 ± 0.010	0.173 ± 0.068
m (g X/g S h)	0.135 ± 0.015 *	0.108 ± 0.016 *
$Y_{P/X}$ (g KA/g X)	0.231 ± 0.005	0.246 ± 0.005
k (g KA/g X h)	0	0
q_S (g glucose/g X h^{-1})	0.276 ± 0.017	0.347 ± 0.005
q_P (g KA/g X h^{-1})	0.015 ± 0.003 *	0.016 ± 0.001 *

Values are presented as mean (±standard deviation, $n = 3$). Asterisks in the same row indicate NO statistically significant differences ($p < 0.05$).

3.4. Antioxidant Capacity by DPPH and ABTS

Assays based on electron transfer measure the ability of an antioxidant to reduce an oxidant, which is represented by the color change that occurs when it is reduced and correlates to the antioxidant concentration in the sample (ABTS or DPPH). Although the reducing capacity of a sample is not directly related to its radical scavenging capacity, it is still an important parameter of antioxidants [35]. On the other hand, the inhibition percentage of both assays (ABTS and DPPH) is expressed using effective concentration values (EC_{50}), which are reported as the number of antioxidants necessary to reduce the initial concentration of sequestering radicals by 50% [36]. When measuring the antioxidant capacity of DPPH and ABTS, all values were below the EC_{50} (Table 3). However, the highest values were reached when the KA accumulation was highest. From the perspective of analysis, the purification of the KA metabolite could be carried out to avoid interferences in the analysis of the antioxidant capacity.

Table 3. Percentage inhibition of DPPH and ABTS in two fermentation processes at pH 3.5 and pH 5.5.

Time (h)	pH 3.5		pH 5.5	
	DPPH (% Inhibition)	ABTS (% Inhibition)	DPPH (% Inhibition)	ABTS (% Inhibition)
0	1.89 ± 1.32 b	0.062 ± 0.91 b	8.42 ± 1.07 c	12.88 ± 1.25 b
24	15.64 ± 095 a	9.92 ± 1.03 a	nd	nd
48	17.25 ± 1.38 a	14.36 ± 3.99 a	14.37 ± 0.82 b	18.04 ± 2.54 a
72	16.37 ± 2.64 a	13.71 ± 1.84 a	17.12 ± 7.41 ab	15.45 ± 1.18 ab
96	18.44 ± 0.27 a	11.68 ± 3.96 a	16.59 ± 6.11 ab	16.18 ± 0.99 a
120	18.41 ± 0.51 a	14.48 ± 2.28 a	18.73 ± 4.93 a	17.56 ± 1.53 a
CV	8.76	23.98	12.64	9.96

Values are presented as mean (± standard deviation, $n = 3$). Different lowercase letters in the same column indicate statistically significant differences ($p < 0.05$).

4. Discussion

Endophytic fungi are recognized as a source of bioactive secondary metabolites with various potential applications in medicine, agriculture, and the pharmaceutical industry [37]. Fermentative processes use fungi to convert substrates into different products [38]. Kojic acid is an important biomolecule that is produced by fermentative biological processes [39]. Some organic nitrogen sources, such as peptone and yeast extract [40], and some inorganic sources [11,41], act as precursors in the production of kojic acid. The growth medium and conditions for the production of metabolites influence the formation of biomass, carbon source consumption, and KA synthesis. Kojic acid is commonly produced mainly by species such as *A. oryzae* and *A. flavus* [30]; however, studies of these species are currently mainly based on molecular modulation [42]. Other *Aspergillus* species, such as *A. tamarii*, *A. parasiticus*, *A. terrus*, and *A. sojae*, and some of the *Penicillium* genus [6], have secondary metabolites with multiple applications, initially in cosmetics, and later in food, medicine, pharmaceutical products, and agriculture [34]. KA is produced under different fermentative processes, with some physical parameters regarding, for example, agitation and aeration, static, incubation period, optimal temperatures, and adequate pH. The culture conditions are characteristic for each producing microorganism; in addition, the culture medium must contain a source of nitrogen, carbon, and mineral nutrients suitable for KA synthesis [19,31–33,43].

In the present investigation, seven fermentative processes using an *Aspergillus niger* strain were developed, with different levels of yeast extract as an organic nitrogen source, and two pH levels. KA could be produced by modifying the culture conditions, such as the carbon and nitrogen sources, incubation temperature, and pH of the medium [44]. When using 0.05 g/L of yeast extract, the formation of biomass started at 72 h, increasing gradually until 360 h; on the other hand, the reducing sugars followed an inverse trend, decreasing

over time. Some investigations report that the fungal growth of *Aspergillus sojae* started after 24 h and at a maximum at 96 h, with a dry weight of 3.67 g/L, and that kojic acid production was achieved 24 h after the cell growth reached the stationary phase [19]. In another study, the production of KA and the fungal biomass of *Aspergillus oryzae* increased as fermentation progressed, reaching a maximum biomass value at day 12 and reducing glucose up to 42% coincidentally with KA production [41]. Saraphanchotiwitthaya and Sripalakit [45] observed a consumption of more than 90% glucose on day 12 by the *Aspergillus oryzae* strain, and KA production began to increase from day 6.

Regarding our study, the use of 0.10 g/L of YE followed the same formation of biomass and sugar consumption as observed by Saraphanchotiwitthaya and Sripalakit [45]; however, KA production showed a peak on day 6 (144 h) and, subsequently, there was a decrease in the metabolite. When there were high concentrations of YE, more than 90% of the glucose was consumed for cell development during the growth phase, leaving less than 10% for the conversion of KA during the production phase [17]. This is a possible reason for the lower KA production. The decrease in KA after day 6 may have been due to an accumulation of other organic acids or unknown substances that inhibited the production of KA [17]. According to other investigations, decreases in the KA value can be generated by different factors, such as nitrogen source concentrations, carbon concentrations, temperature, pH, and incubation period, among others [43]. Each one of these factors will be different for each producer microorganism, even with the *A. oryzae* producer strain itself, where production times can be extended up to 20 days with temperatures of 40 °C, generating amounts of KA of 0.2 g/L [22], although, KA production of up to 42 g/L has been reported [46]. Other authors have reported favorable production in ranges of 25 °C to 30 °C [47]. For example, Rasmey and Abdel-Kareem [43] reported that with temperature peaks above 30 °C, there was a decrease in KA from 68 g/L to 0.5 g/L of KA with *A. oryzae*, and above temperatures of 45 °C, production reached 0 g/L.

The fermentation process with 2.5 g/L of YE showed the best yields for biomass production and KA synthesis, with optimum values being observed at 96 h. The same behavior concerning KA values was observed with the fermentation process with 2.5 g/L of YE + Zn, but in smaller amounts compared to the process with 2.5 g/L of YE. This effect may have been because the $ZnSO_4 \cdot 7H_2O$ did not act as a modulator of cellular amino acid metabolism and redox balance, reducing the antioxidant effect and promoting the presence of acetic acid [27]. Modifying the YE and glucose levels could maximize KA production; in addition, there was an interaction between YE, glucose, and KH_2PO_4 [11] that may have represented a significant effect on KA production. It has been reported that concentrations of phosphorus (KH_2PO_4) in a culture medium for the production of KA could generate variability in productivity. By increasing concentrations of phosphorus (KH_2PO_4 to 1.5 g/L) in a medium with glucose, the production of KA was affected [43], while other authors found that, with 10.3 mM of KH_2PO_4, a considerable interaction was observed between glucose and phosphate, with an optimal level of yeast extract [11]. This comparison was made in order to understand that with the addition of $ZnSO_4$, there was productivity, but it was lower, and that the use of KH_2PO_4 did not interfere with the use of 2.5 g/L of YE. There are several ways to add zinc to a culture medium, but adding Zn^{2+} (10 µM) during fermentation did not affect KA synthesis; however, when salts such as NaCl were added to the culture medium, it inhibited KA production [28]. This may indicate that the salts provided by $ZnSO_4$ decreased production but did not inhibit it. In studies performed by Ariff et al. [48] with the *Aspergillus flavus* strain, the maximum concentration of KA achieved was at the end of the fermentation process at 564 h, with a yield of 0.325 g KA/g glucose and a total production of 0.0576 g KA/L·h. The production of KA was improved with the use of 2.5 g/L of YE, at a smaller scale in the fermentative time. With other producing strains, such as *A. terrus*, as observed by Shakibaie et al. [11], the use of 1 g/L of yeast extract produced a maximum value of KA (2.21 g/L), and concentrations of 5 g/L also induced the production of KA with the *A. flavus* strain, with a production of 25.8 g/L [49], although

this was not comparable to the production of KA by *A. niger*, where the conditioning of the source of nitrogen was appropriated to achieve production.

As the KA production times in YE screening fermentation were below 144 h, the standardized conditions were implemented until 120 h, with 2.5 g/L of YE yielding the best result. In the first 24 h, the biomass increased until 120 h, while the greatest sugar consumption occurred between 72 h and 96 h, the same as the optimal times for KA production. With this standardization, fermentation times were shortened, optimizing production. El-Kady et al. [50] optimized the nutritional conditions for the *Aspergillus flavus* strain to maximize the production of KA using 100 g/L of glucose, 5 g/L of YE, and 1.5 g/L of KH_2PO_4, as sources of carbon, nitrogen, and phosphorus, respectively. The production of KA varied from 7 to 10 days depending on the type of producing strain, such as *A. fumigatus* (0.37 g KA/L), *A. ustus* (0.29 g KA/L), *A. flavus* (0.28 g/L), and *A. terreus* (2.21 g/L) [11]. Synthesis by *Aspergillus* spp. depended on the growth medium and incubation conditions, which both had a strong influence on the production of KA [24]. Among the above-mentioned compounds, the principal compound that affected the induction or repression of KA production was the YE. In fermentations with 5 g/L of YE, an increase in the concentration of KA can be obtained; however, at concentrations of 10 g/L of YE, KA production is lower [17].

Regarding the equations mentioned above, all YE levels that were evaluated revealed important kinetic parameters that facilitate the optimal KA production process. Both 2.5 g/L of YE and 2.5 g/L of YE + Zn treatments were the best conditions to obtain a maximum growth rate, which was likely due to the YE concentration (which was higher than that for the other two treatments), which thereby permits the usage of nitrogen molecular forms to perform cell formation during the fermentation process. The best product biomass yield was also achieved with 2.5 g/L of YE and 2.5 g/L of YE + Zn treatments, showing that biomass production and growth rate are linked to KA synthesis, and the concentration of YE induces metabolite production. The use of $ZnCl_2$ did not affect micellar growth for the *A. parasiticus* strain, presenting a maximum value of KA production at 72 h (10.5 mmol KA/g dry weight), demonstrating that there were no negative effects with the use of $ZnCl_2$. However, under the presence of salts such as NaCl, there was an inhibition of the production of KA [28].

The biomass did not present any variation with the addition of zinc; however, KA production was lower when adding a zinc source. The presence of this molecule in its sulfated form may have generated an inhibitive effect, decreasing the synthesis yield.

The biomass product yield was considered to determine whether *Aspergillus niger* M4 is a strain that can be used for the synthesis and production of KA. The medium used was adjusted to a pH of 3.5 and 5.5 following the optimal conditions observed in previous experiments (120 h using 2.5 g/L of YE). To describe the fermentation behavior of KA production by *Aspergillus* spp. at various pH levels, the proposed models based on logistic and Luedeking-Piret equations were considered adequate [49]. In this research, the biomass (fungal growth), substrate (reducing sugars), and product (kojic acid production) parameters were considered for the simulation of KA production during fermentation processes. Ariff et al. [48], suggested that the proposed models based on logistic equations are sufficient to describe fungal growth, glucose consumption, and kojic acid production. With respect to the fermentation process with a pH of 3.5 or of 5.5, it was found that a pH of 3.5 had a negative impact on production, with 72 h being the optimal point of KA production, thereby affecting the concentration of KA. It should be emphasized that *A. niger* is a producer of other organic acids, which could generate interference by having a moderately acidic pH. In a study by Rosfarizan et al. [12], as the pH decreased, in addition to glucose reduction, KA was accumulated, but this was accompanied by other organic acids such as oxalic, succinic, lactic, and citric acids.

When the culture medium had an initial pH of 5.5, there was an increase in KA production after 72 h, which remained constant until 120 h. Chib et al. [19] found that during their fermentative process to produce KA, the pH of the medium increased from 5 to

6.2 at 96 h, and did not interfere with the production of KA when using *A. sojae*, resulting in a production of 12.2 g of KA/L at 28 °C and 120 h. Badar et al. [22] reported that the yield of KA by the *A. oryzae* strain was affected when high agitation and pH conditions were applied, obtaining on day 20 concentrations of 5.0, 3.0, 2.0, 1.6, 1.1, and 0.5 g of KA/L with a pH of 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5, respectively. On the other hand, Bala et al. [51] revealed that in 28 days at 29 °C under the culture medium optimized, a pH of 6.0 was required to produce a maximum yield of 60 g of KA/L with the *A. flavus* strain. Even *A. niger* required an initial pH of 6.5 to produce other compounds such as citric acid, which reduced the pH to 1.5, facilitating the formation of other compounds [52]. Therefore, most fungi show extensive growth and sporulation in a weakly acidic to neutral environment, giving them the opportunity to produce a greater number of organic acids [24]. KA production was slightly affected by a pH of 3.5, producing half of the KA. According to reports, the pH of a fermentative media is essential and favors the production of KA. For example, production levels improved with a pH of 5.0 [24,53], while other authors found that a pH of 6.0 was favorable for the production of KA [51]. In other cases, a pH of 5.0, which increased to 6.2 during fermentation, did not influence the production of KA [19]. However, for *A. niger*, it is essential to start a fermentative stage with a weakly acidic to neutral pH to achieve the synthesis of KA. Thus, it is required that the initial pH of the culture media must be optimized so that it can be adapted to the microorganism, substrate, and production method [54]. From what has thus far been reported, there are some species that produce KA, among which *A. oryzae* and *A. flavus* stand out, followed by *A. parasiticus*, *A. sojae*, *A. terrus*, and *A. fumigatus*. Some authors have chosen to use different means to improve productivity, replacing some carbon sources with cheap substrates, such as corn, straw, or grape waste, to improve the efficiency of KA production. In addition, KA production has been studied via mutagenesis and other technologies that allow for the detection of groups of genes that stimulate KA synthesis. On the other hand, *A. niger* is a fungus, which is widely used in the food industry as a producer of organic acids and is commonly used commercially for the production of citric acid, for which approaches have been supported only with the aforementioned producer strains. In this experiment, we decided to use a medium similar to those reported with different concentrations of the nitrogen source to induce the synthesis of KA; however, the productivity was lower compared to that achieved with other strains. Chang et al. [42] identified a group of genes of *A. flavus* that modulate the synthesis of KA; within their research was *A. niger*, which contains a single group of KA genes, being a kojR homolog (AN09g05060), which encodes a 63% amino acid sequence identity for the *A. oryzae* KojR proteins [55]. It was also observed that the kojR gene of *A. flavus* was involved in the production of KA [42]. Thus, the induction of kojic acid by *A. niger* likely requires the interference of other physical or chemical methods. These could be different temperatures, exposure to UV radiation, agitation, or the use of other sources of carbon and nitrogen that potentiate production and reduce costs.

The use of a quantification method previously described by HPLC helped to verify that KA was produced using the *A. niger* M4 strain. With a pH of 3.5, 0.3963 g of KA/L was obtained at 72 h, while with a pH of 5.5, 0.9310 g of KA/L was obtained at 120 h. The variation in terms of KA quantification between the spectrophotometric method and HPLC may lie in interferences. When measuring coloration, only the KA that had reacted with $FeCl_3$ could be measured, while in HPLC, the molecule was detected in the retention times of the standard, giving a greater certainty of the presence of KA in the samples, according to the area and retention times. Nevertheless, the colorimetric method could be used as a quick and reliable technique for KA measurement. The data presented by Promsang et al. [23] using HPLC achieved KA production of 1.5 g of KA/L in a retention time of 4.24 min using *A. oryzae*. The *A. niger* fungus is distinguished for being a good producer of organic acids and different enzymes such as amylases, invertase, pectinases, phytases, and proteinases [56]. Although, *A. niger* has not been reported as a strain that produces KA. Recently, in a study conducted by Li et al. [16], who constructed seven histone deacetylase-deficient strains of *A. niger* to investigate the metabolic profiles of secondary

metabolic pathways according to metabolomic analysis, KA production increased in the HosA mutant, suggesting that HosA histone deacetylases play an important role in the biosynthesis of secondary metabolites in *A. niger* FGSC A1279.

Although the production of kojic acid in this investigation was quantitatively low, future investigations could be conducted where the fungus is subjected to stress due to temperature, UV radiation, agitation, and immobilization, in addition to using other kinds of carbon sources, such as industrial waste or other sources of nitrogen, in order to potentiate production and lower costs.

5. Conclusions

The *Aspergillus niger* M4 strain was able to grow in a liquid medium with different yeast extract levels. The maximum growth rate in the fermentation conditions was related to the yeast extract concentration. The addition of zinc sulfate during the fermentation process did not increase the growth rate or the product biomass yield.

Kojic acid synthesis can be induced by the *A. niger* M4 strain, varying the yeast extract concentration up to 2.5 g/L. The pH of the culture medium slightly affected kojic acid production, but pH values close to neutral could increase the metabolite synthesis and fungal growth speed.

Further investigations could be conducted to seek cheaper carbon sources, such as agroindustrial or agricultural wastes, in the form of vegetal biomass.

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