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Influence of Fermentation Conditions on Humic Acids Production by *Trichoderma viride* Using an Oil Palm Empty Fruit Bunch as the Substrate

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***Abstract*—**Humic acids (HA) were produced by a *Trichoderma viride* strain under submerged fermentation in a medium based on the oil palm empty fruit bunch (EFB) and the main variables of the process were optimized by using response surface methodology. A temperature of 40°C and concentrations of 50g/L EFB, 5.7g/L potato peptone and 0.11g/L $(NH_4)_2SO_4$ were the optimum levels of the variables that maximize the HA production, within the physicochemical and biological limits of the process. The optimized conditions led to an experimental HA concentration of 428.4±17.5 mg/L, which validated the prediction from the statistical model of 412.0mg/L. This optimization increased about 7–fold the HA production previously reported in the literature. Additionally, the time profiles of HA production and fungal growth confirmed our previous findings that HA production preferably occurs during fungal sporulation. The present study demonstrated that *T. viride* successfully produced HA via the submerged fermentation of EFB and the process parameters were successfully optimized using a statistics-based response surface model. To the best of our knowledge, the present work is the first report on the optimization of HA production from EFB by a biotechnological process, whose feasibility was only pointed out in previous works.

***Keywords*—**Empty fruit bunch, humic acids, submerged fermentation, *Trichoderma viride*.

I. Introduction

HUMIC Acids (HA), part of the organic matter in soil, are the soil fraction that is most resistant to microbial degradation. They are complex polymeric organic acids with a wide range of molecular weights, and they can be heterogeneous mixtures of a variety of organic compounds, including aromatic, aliphatic, phenolic, and quinolic functional groups [1]. In addition, HA's size, chemical composition, structure, and functional groups may vary greatly, depending on its origin and the age of the material [2]. They are one of most active fractions of organic matter and affect a variety of chemical, physical, and biological reactions. From an agricultural point of view, they improve the absorption of nutrients by both plants and soil microorganisms, positively affect the dynamic of nitrogen and phosphorus in soil, stimulate plant respiration and photosynthesis, and favor the formation of soil aggregates, among other benefits [3]. Moreover, they have deservedly received growing attention from the field of biomedicine, primarily due to their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities [4], which are of great importance for pharmaceutical and biomedical applications. Traditionally, HA is extracted from lignite, brown coals and humified organic materials [5]. However, harvesting humic acids from such non-renewable carbon resources can be expensive and environmentally/ecologically unsustainable. Moreover, the extraction of peat leads to the destruction of peatlands, which is associated environmental problems [6]. It is thus desirable to use more ecologically sustainable precursors/feedstocks for HA and develop cheaper and cleaner methods for the extraction of this valuable product.

For economic reasons, industrial fermentations involve complex, almost undefinable substrates that are often the by-products of other industries. Empty fruit bunch (EFB), a cellulosic material source containing 43.8% cellulose, 35.0% hemicelluloses, and 16.4% lignin [7], is a strong candidate for use as a fermentation substrate. EFB is the product of oil palm processing, waste that has not been completely utilized, and it is produced in large quantities: 23% of the fresh fruit bunch [8] used in oil palm production remains as EFB. The global production of oil palm has doubled over the past ten years and is expected to double again in the next decade [9]. Thus, there is a growing interest in making EFB useful, reducing the large volume of waste and its ensuing environmental problems [10].

Trichoderma species, recognized as lignocellulose degrading agents, are frequently found in soil as well as on substrates like wood, bark, and other fungi, demonstrating their high opportunistic potential and their adaptability to various ecological conditions [11]. This characteristic allows the genus to be used in many and varied biotechnological applications. Moreover, the conidial mass of *Trichoderma* is a proficient propagule and tolerates downstream processing [11]. Because *Trichoderma* fermentation is a highly aerobic process [13], submerged fermentation, in which the organism is grown in a vigorously aerated and agitated liquid medium [14], is widely used. Furthermore, liquid cultures are homogenous, making them easier to control, maintain, and monitor [15], so this fermentative process has been employed extensively in industries for large-scale production [16].

The reports on HA production by biotechnological route are still scarce in the literature. The first work focused in the biotechnological process for HA production was performed by our group, since an initial work on these acids production by

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solid state fermentation of EFB using *T. viride* aimed only their characterization. Furthermore, in our previous work, has been shown the superiority of EFB for HA production by submerged fermentation with *T. viride*, compared to other cellulosic substrates [17].

In the present study, an investigation was carried out to determine the optimum medium composition and fermentation conditions for HA production. Temperature, pH and EFB, peptone, K_2HPO_4 and $(NH_4)_2SO_4$ concentrations were the variables studied for their individual and interactive effects. For this purpose, a Plackett&Burman (PB) [18] design was used to screen for the significant parameters, followed by the application of a Central Composite Rotatable Design (CCRD) to determine the response surface. The optimized conditions pointed out from the statistical model were validated by experimental assays in triplicate. Finally, the time profile of HA production was obtained and related to the fungal growth phase.

II. Material and Methods

A. Microorganisms and Inoculum

For storage, the *T. viride* strain culture was grown on potato dextrose agar plates at 24°C for 10 days. After sporulation, the spores were resuspended in a sterile 20% glycerol solution, which was stored in 1.2-mLcryotubes at -70°C.

The inoculum was prepared in 500mL Erlenmeyer flasks, each of which contained 300mL of culture media with 30g/L oats and 5g/L peptone, prepared as described in our previous article [19]. The culture flask was inoculated with 1mL of the spore suspension (1.26×10^7 spores) and incubated at 24°C and 150rpm for 120 hours to produce a large number of spores.

B. Submerged Fermentation

EFB (provided by Oil Palm S/A - Agro-industrial OPALMA, Bahia, Brazil) was milled to achieve a standardized particle size of between 125 and 500μm (115 and 32 Tyler series mesh, respectively). The composition of the EFB particles was determined by a CNH elemental analyzer (Perkin Elmer Series II 2400, Maryland, USA). The mass percentages were 48.0±0.7% carbon, 2.6±0.1% nitrogen and 6.1±0.2% hydrogen. These results are the means of triplicates with their average deviations. Potato peptone was purchased from Fluka Analytical (France); $(NH_4)_2SO_4$and K_2HPO_4 were purchased from Ecibra (Brazil).

The fermentation cultures were housed in 500mL Erlenmeyer flasks that contained 270mL of culture media inoculated with 30mL of inoculum (3.52×10^6 spores/mL), prepared as described in section 2.1. The culture flasks were incubated at 150rpm. Over the course of 120 hours of cultivation, samples were withdrawn every 24 hours for analysis.

C. Statistical Analysis

1) Plackett&Burman Design:

This design was used to screen for the selected variables that significantly affected the HA production. Six variables were screened: pH, temperature (°C), EFB (g/L) as a carbon source, potato peptone (g/L) as an organic nitrogen source, and K_2HPO_4 (g/L) and $(NH_4)_2SO_4$ (g/L) as inorganic nitrogen sources (Table I). Each factor of this statistical analysis was examined at three levels—low (−), high (+) and central (0)—to evaluate both the linear and curvature effects of the variables. The samples that were withdrawn every 24 hours throughout the cultivation were processed to determine their HA concentrations, and the values obtained after 120 hours of fermentation were used as the response variables. Table II shows the PB experiment design, with 16 trials that varied each of the six factors, and the resulting HA amounts produced. This statistical design does not consider the interactions between the selected variables and follows a linear approach to screen the factors [18]. The effects of the variables are summarized in Table III, as obtained by Statistica software, version 8.0 (Statsoft, Oklohoma, USA). The variables that were considered to have a significant effect on the response (p-value<0.1) were then optimized by CCRD, a response surface methodology.

TABLE I
The Lower (-1), Higher (+1) and Central (0) Levels of the Six Variables Screened by the Pb Design

Variables	Units	Experimental values		
		-1	0	+1
EFB	g/L	10.0	20.0	30.0
Peptone	g/L	1.00	3.85	6.70
pH	-	4.0	6.0	8.0
Temperature	°C	25.0	30.0	35.0
K_2HPO_4	g/L	0.28	1.54	2.80
$(NH_4)_2SO_4$	g/L	0.24	0.77	1.30

TABLE II
THE EXPERIMENTAL PB DESIGN USED TO SCREEN SIX VARIABLES, WITH THEIR REAL AND CODE VALUES (IN PARENTHESES) AND THE OBSERVED VALUES OF THE HA PRODUCTION RESPONSE

Trial	Experimental values						HA (mg/L)[a]
	EFB (g/L)	Peptone (g/L)	pH	Temperature (°C)	K_2HPO_4 (g/L)	$(NH_4)_2SO_4$ (g/L)	Observed
1	30.0 (+1)	1.00 (-1)	8.0 (+1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	12.6
2	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	0.28 (-1)	0.24 (-1)	85.2
3	10.0 (-1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	0.24 (-1)	48.9
4	30.0 (+1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	38.3
5	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	107.0
6	30.0 (+1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	44.3
7	10.0 (-1)	6.70 (+1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	46.9
8	10.0 (-1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	46.9
9	10.0 (-1)	1.00 (-1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	1.30 (+1)	10.3
10	30.0 (+1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	3.3
11	10.0 (-1)	6.70 (+1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	1.30 (+1)	47.6
12	10.0 (-1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	20.2
13	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	58.8
14	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	58.1
15	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	57.5
16	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	60.8

[a]HA concentrations were obtained after 120 hours of fermentation.

TABLE III
A SUMMARY OF THE ESTIMATED EFFECTS OF THE PB-TESTED VARIABLES

Factor	Estimated effect	Standard error	t(8)	*p*-value
Mean/Interc.	42.6	3.1	13.6	<0.0001
Curvature	32.3	12.5	2.6	0.0323
EFB	11.6	6.3	1.9	0.0998
Peptone	41.4	6.3	6.6	0.0002
pH	-6.0	6.3	-1.0	0.3671
Temperature	26.3	6.3	4.2	0.0030
K_2HPO_4	1.7	6.3	0.3	0.7984
$(NH_4)_2SO_4$	-21.7	6.3	-3.5	0.0085

2) Central Composite Rotatable Design:

After screening the significant variables, a CCRD was used to optimize four variables: the temperature (°C) and the levels of EFB (g/L), potato peptone (g/L), and $(NH_4)_2SO_4$ (g/L). Each variable was studied at five coded levels (−2, −1, 0, +1, +2), and the model included 28 runs with four replicates at the center value, as shown in Table IV. A multiple regression analysis of the model and the construction of response surface graphs were performed by Statistica, version 8.0 (Statsoft, Oklohoma, USA). The quality of the regression equation was determined by the coefficient of determination (R^2), and its significance was judged by an F test. The fitted second order polynomial equation was explained in the form of three-dimensional graphs to show the relationship between the response and the experimental variables. The point optimization method was used to optimize the maximum response of each variable. The samples withdrawn every 24 hours throughout the cultivation were processed to determine their HA concentrations, and the values obtained after 120 hours of fermentation were used as the response variables.

3) Statistical Model Validation:

To validate the model obtained, experiments using the optimized variables were conducted in triplicate for 120 hours. The mean values were used to confirm the results of the CCRD analysis. The values of the independent variables of the model that maximize the HA production within the ranges studied (-2 to +2), as well as the predicted HA concentration using these values, were obtained by Solver Microsoft Office Excel program.

Moreover, while performing the validation assays, the samples withdrawn every 24 hours throughout the cultivation were processed to determine their HA and cellular protein concentrations. Graphs were constructed to represent the behavior of the parameters during the optimized fermentation process.

TABLE IV
THE EXPERIMENTAL CCRD DESIGN OF THE MEDIA NUTRIENTS AND PHYSICAL PARAMETERS, WITH BOTH REAL AND CODED VALUES (IN PARENTHESES) AND THE OBSERVED VALUES OF THE HA PRODUCTION RESPONSE

Trial	Experimental values				HA (mg/L)[a]	HA (mg/L)	Relative deviation (%)
	EFB (g/L)	Peptone (g/L)	Temperature (°C)	$(NH_4)_2SO_4$ (g/L)	Observed	Predicted	
1	20.0 (-1)	2.00 (-1)	25.0 (-1)	0.44 (-1)	45.4	89.1	49.0
2	20.0 (-1)	2.00 (-1)	25.0 (-1)	1.10 (+1)	51.1	67.2	24.0
3	40.0 (+1)	2.00 (-1)	25.0 (-1)	0.44 (-1)	89.8	49.8	-80.3
4	40.0 (+1)	2.00 (-1)	25.0 (-1)	1.10 (+1)	16.9	27.9	39.3
5	20.0 (-1)	5.70 (+1)	25.0 (-1)	0.44 (-1)	70.3	69.8	-0.7
6	20.0 (-1)	5.70 (+1)	25.0 (-1)	1.10 (+1)	79.0	47.9	-65.1
7	40.0 (+1)	5.70 (+1)	25.0 (-1)	0.44 (-1)	44.9	83.6	46.3
8	40.0 (+1)	5.70 (+1)	25.0 (-1)	1.10 (+1)	27.4	61.7	55.6
9	20.0 (-1)	2.00 (-1)	35.0 (+1)	0.44 (-1)	112.3	79.4	-41.5
10	20.0 (-1)	2.00 (-1)	35.0 (+1)	1.10 (+1)	75.4	57.5	-31.1
11	40.0 (+1)	2.00 (-1)	35.0 (+1)	0.44 (-1)	87.5	127.5	31.4
12	40.0 (+1)	2.00 (-1)	35.0 (+1)	1.10 (+1)	91.7	105.6	13.1
13	20.0 (-1)	5.70 (+1)	35.0 (+1)	0.44 (-1)	157.2	145.6	-7.9
14	20.0 (-1)	5.70 (+1)	35.0 (+1)	1.10 (+1)	61.0	123.7	50.7
15	40.0 (+1)	5.70 (+1)	35.0 (+1)	0.44 (-1)	239.9	246.9	2.8
16	40.0 (+1)	5.70 (+1)	35.0 (+1)	1.10 (+1)	269.6	225.0	-19.8
17	30.0 (0)	3.85 (0)	20.0 (-2)	0.77 (0)	46.3	40.2	-15.1
18	30.0 (0)	3.85 (0)	40.0 (+2)	0.77 (0)	172.2	193.8	11.2
19	30.0 (0)	0.15 (-2)	30.0 (0)	0.77 (0)	6.8	1.0	-600.7
20	30.0 (0)	7.55 (+2)	30.0 (0)	0.77 (0)	117.4	101.0	-16.2
21	10.0 (-2)	3.85 (0)	30.0 (0)	0.77 (0)	74.8	86.0	13.1
22	50.0 (+2)	3.85 (0)	30.0 (0)	0.77 (0)	152.7	148.0	-3.2
23	30.0 (0)	3.85 (0)	30.0 (0)	0.11 (-2)	142.3	138.9	-2.4
24	30.0 (0)	3.85 (0)	30.0 (0)	1.43 (+2)	98.5	95.1	-3.6
25	30.0 (0)	3.85 (0)	30.0 (0)	0.77 (0)	137.5	117.0	-17.5
26	30.0 (0)	3.85 (0)	30.0 (0)	0.77 (0)	136.3	117.0	-16.5
27	30.0 (0)	3.85 (0)	30.0 (0)	0.77 (0)	139.4	117.0	-19.2
28	30.0 (0)	3.85 (0)	30.0 (0)	0.77 (0)	136.8	117.0	-16.9

[a]HA concentrations were obtained after 120 hours of fermentation.

D.HA Quantification

The following methods were adapted from Badiset al. [20]. The samples were centrifuged at 10,000g for 15min (Rotina 380 R Centrifuge, Hettich Zentrifugen, Tuttlingen, Germany), and the supernatant fractions were filtered using the Microfilter syringe system (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fractions were diluted 5-fold with a 0.5M NaOH solution at pH 4.5±0.01, and the absorbances at 350nm were measured. Standard curves were obtained from the absorbances at 350nm of known concentrations of commercial HA (Sigma-Aldrich, United Kingdom) in a 0.5M NaOH solution at pH 4.5±0.01.

E. Protein Quantification

An indirect estimation of the fungal biomass was made by determining the protein concentration. Protein quantification was performed according to the method adapted by Callow and Ju[21] to quantify only the cellular proteins. Culture samples (3.0mL) were collected and centrifuged at 10,000g for 10min to obtain pellets. The supernatants were collected for further processing. The pellets were re-suspended and washed twice with de-ionized water. After each wash step, the biomass was centrifuged and the water discarded. To release the intracellular proteins, the pellets were suspended in 3.0mL of 1 N sodium hydroxide and heated at 100°C for 10min. After cooling, the digested samples were centrifuged at 10,000g for 10min to remove the cell debris and other solids; the supernatants were then collected and their protein concentrations were determined via the bicinchoninic acid assay [22], using a commercial kit (BCA Protein Assay, Thermo Scientific, USA). Standard curves were constructed using bovine serum albumin.

III. RESULTS AND DISCUSSION

Selection of Critical Media Components and Fermentation Conditions:

Six variables were screened by PB, as shown in Table I. The temperature and the concentrations of EFB, peptone and $(NH_4)_2SO_4$ were the four of those screened variables that were considered to have a statistically significant effect on the HA production by presenting a p-value<0.1 (Table III). Among these four variables, only the $(NH_4)_2SO_4$ concentration had a negative effect on HA production. The peptone concentration had the greatest positive effect on HA production, followed by temperature and EFB concentration. These four variables were selected for further optimization by CCRD to maximize the HA production. K_2HPO_4 concentration and pH had no significant effect on HA production at the confidence level studied (90%). According to Bhattiprolu [23], *T. viride* is able

to grow under wide range of pH (4.0 to 9.0), which may explain the fact that this factor has not been statistically significant. Regarding to the K_2HPO_4 concentration, the average chemical formula of HA elucidated by Schnitzer and Khan [24] is $C_{187}H_{186}O_{89}N_9S_2$, which do not contain phosphorus and potassium, explaining the absence of significant effect of this factor.

Media components and fermentation conditions optimization: Following the variable screening, a CCRD optimization including 28 experiments was performed to determine the optimal levels of the four significant factors (the temperature and the EFB, peptone and $(NH_4)_2SO_4$ concentrations) that affected HA production. The real and coded values of each variable used in the trials, as well as the resulting HA concentration obtained after 120 hours of fermentation, are shown in Table IV. The values of the parameters considered not statistically significant by the PB (the pH and K_2HPO_4 concentration) were fixed at the central level (code 0) shown in Table I.

According to the regression analysis of the CCRD (Table IV), the model terms temperature (Q), EFB (Q), $(NH_4)_2SO_4$ (L), $(NH_4)_2SO_4$ (Q), temperature (L) by $(NH_4)_2SO_4$, peptone (L) by EFB (L), peptone (L) by $(NH_4)_2SO_4$ (L) and EFB (L) by $(NH_4)_2SO_4$ (L) were not significant ($p>0.10$). However, the terms $(NH_4)_2SO_4$ (L) and peptone (L) by EFB (L) were ultimately included in the model because their *p*-values were very close to 0.10.

TABLE V
A SUMMARY OF THE REGRESSION COEFFICIENTS FOR THE CCRD

Factor	Regression coefficients	Standard error	t(13)	*p*-value
Mean/Interc.	137.5	18.0	7.6	<0.0001
Temperature (L)	38.4	7.3	5.2	0.0002
Temperature (Q)	-8.1	7.3	-1.1	0.2879
Peptone (L)	25.0	7.3	3.4	0.0047
Peptone (Q)	-19.9	7.3	-2.7	0.0178
EFB (L)	15.5	7.3	2.1	0.0549
EFB (Q)	-7.0	7.3	-1.0	0.3573
$(NH_4)_2SO_4$ (L)	-10.9	7.3	-1.5	0.1598
$(NH_4)_2SO_4$ (Q)	-5.3	7.3	-0.7	0.4793
Temperature (L) by Peptone (L)	21.4	9.0	2.4	0.0333
Temperature (L) by EFB (L)	21.9	9.0	2.4	0.0303
Temperature (L) by $(NH_4)_2SO_4$ (L)	-1.5	9.0	-0.2	0.8732
Peptone (L) by EFB (L)	13.3	9.0	1.5	0.1635
Peptone (L) by $(NH_4)_2SO_4$ (L)	1.5	9.0	0.2	0.8671
EFB (L) by $(NH_4)_2SO_4$ (L)	3.9	9.0	0.4	0.6717

Explained variance (R^2) = 83.8%

The linear effect of the temperature variable was the most significant, with a *p*-value < 0.0002, which can be explained by the fact that enzyme activities as well as regulation and transport systems are in generally affected enormously by the temperature in microbial systems [25]. The linear effect of EFB concentration, as well as of temperature, was positive, once this component was the main carbon source for HA production by *T. viride* in the studied media. Regarding the nitrogen sources, both peptone (L) and $(NH_4)_2SO_4$ (L) concentration had significant effect on HA production although the first had a positive effect and the second a negative effect. Juwon and Emmanuel [26] tested in their work the influence of nitrogen sources in growth and enzymes production by *T. viride* strain, where they observed that organic nitrogen substrates, like peptone, supported better biomass yield and enzyme activity of the fungus as compared to the inorganic nitrogen substrates tested.

The interactions temperature (L) by peptone (L), temperature (L) by EFB (L) and peptone (L) by EFB (L) had positive coefficients, increasing HA production, whereas the $(NH_4)_2SO_4$ (L) and Peptone (Q) terms had negative coefficients, decreasing HA production.

A nine-variable quadratic polynomial regression model (1) was used to predict the HA production as a function of the four process parameters, including the temperature (T, ◦C), peptone concentration (P, g/L), EFB concentration (EFB, g/L) and $(NH_4)_2SO_4$ concentration ($(NH_4)_2SO_4$, g/L). The equation was constructed from the statistically significant terms and includes $(NH_4)_2SO_4$ (L) and peptone (L) by EFB (L). The values for the independent variables in (1) are the coded values.

$$HA\ (mg/L) = 117.02 + 38.40*T + 25.01*P - 16.50*P^2 + 15.49*EFB - 10.95*(NH_4)_2SO_4 + 21.40*T*P + 21.86*T*EFB + 13.28*P*EFB \quad (1)$$

The statistical significance of the model was checked by an F test (ANOVA), and the results are shown in Table VI. The F test value (10.27) for the regression was highly significant (5.1 times higher than the critical value of 2.02), and the percent of variation in the model was suitable (R^2=81.2%). Taking into account the inherent variability of bioprocesses, the model could be considered predictive and was therefore used to generate a contour plot and a response surface (Fig. 1) for HA production.

TABLE VI
ANOVA RESULTS FOR THE PROPOSED MODE

Source of variance	Sum of squares	Degrees of freedom	Mean squares	*F*test[a]
Regression	84,304.38	8	10,538.05	10.27
Residual	19,501.66	19	1,026.40	
Total	103,806.04	27		

Explained variance (R^2) = 81.2%; [a]$F_{0.10;\ 8;\ 19}$ ($F_{tabulated}$) = 2.02

Equation (1) was used to plot 3-D response surfaces and their corresponding 2-D contours in Statistica 8.0 to show how the HA production would be affected by different levels of the four process variables. The surface and contour plots are shown in Fig. 1. The response surfaces can be used to explain how two process parameters interact with each other when the other two parameters are fixed at their central levels. Each 3-D response surface curve has a corresponding 2-D contour curve, representing an infinite number of points for two independent process parameters. In the contour curves, the color level represents the different responses; the darker area demonstrates the conditions that lead to a higher HA production. Figs. 1 (f) and (l) show that there are no significant interactions between the $(NH_4)_2SO_4$ concentration and either the temperature or the EFB concentration, when the other two process parameters are fixed at their central levels. Figs. 1 (b), (d) and (f) show that the highest temperature level (+2) is required to achieve a high HA production. The same analysis can be performed for the EFB concentration, and according to Figs. 1 (d), (h) and (l), a high HA production is achieved when 50g/L EFB is used. On the other hand, the best $(NH_4)_2SO_4$ concentration level is -2, both economically and for HA production. No increase is seen when the peptone concentration is raised from 5.70 g/L (+1) to 7.55 g/L (+2).

Statistical Model Validation:

According to Solver Microsoft Excel program, values that optimize the production of HA within the levels studied are Temperature, EFB and Peptone concentration level +2 and $(NH_4)_2SO_4$ level -2. The analysis of the 2D contour curves shown in Fig. 1 allows similar conclusions, which demonstrate that the highest levels (+2) of temperature and EFB and the lowest level (-2) of $(NH_4)_2SO_4$ lead to the highest HA production. However, according to the Figs. 1 (b), (h) and (j), both the +1 and +2 levels of peptone lead to a higher HA production and no increase is seen when the peptone concentration is raised from 5.70g/L (+1) to 7.55g/L (+2), justifying its use at the +1 level to reduce the fermentation cost.

Although the spectrum of HA production (Fig. 1) indicates that levels above those studied in this work for temperature and EFB concentration may lead to higher yields of HA, physicochemical and biological constraints don't allow to adopt these levels. The use of temperatures above 40°C is not secure to maintain cell viability, as well as higher EFB concentrations compromise the suspension of substrate, not adding beneficial effects on the fungal growth.

Therefore, the +2 levels for the EFB concentration and temperature, -2 for the $(NH_4)_2SO_4$ concentration and +1 for the peptone concentration, which correspond to the real values of 40°C, 50g/L EFB, 5.7g/L peptone and 0.11g/L$(NH_4)_2SO_4$, were selected as the optimal conditions for HA production. Experiments were carried out in triplicate with the predicted conditions and concentrations to validate the model. The similarity between the predicted response of HA production (412.0mg/L) and the experimental value (428.4±17.5mg/L) proves the validity of the model. Moreover, the relative deviations in the Table IV trials that had similar levels to those used in the model validation (trials 18, 20, 22 and 23) were low, with a high degree of similarity between the predicted and experimental values, further validating the model.

Comparing the amount of HA produced after optimization with that produced in our previous work [17], which is, as far we know, the first submerged fermentation process proposed for HA production; a 7.3-fold increase was achieved. This is a high value when compared to the other bioprocess optimization methods using *Trichoderma* seen in literature. Singhania et al. [27] used process optimization to improve the cellulase production by *T. reesei* RUT C30 under solid-state fermentation, resulting in a 6.2-fold increase in production. El-Sayed [28] increased by 2.2-fold the production of L-glutaminase by *T. koningii*, using wheat bran as a substrate. Alam et al. [29] reached an approximately 1.5-fold increase in cellulase activity by statistically optimizing the process conditions in a liquid state bioconversion using *T. harzianum*.

The time profiles of HA production and cellular proteins concentration is represented in Fig. 2. According to this figure it is possible to observe that the concentration of HA increases throughout the fermentation time due to its accumulation in the medium. HA are the fractions of the organic matter that is most resistant to microbial degradation [1], which may explain the observed accumulation. This confirms that the HA concentration after 120 hours of fermentation was the appropriate variable to choose as the response in the statistical analysis (both PB and CCRD).

The concentration of cellular proteins was used as an indirect estimate of biomass. Christias et al. [30] determined in their work the amount of protein present in the biomasses of five different genera of fungi, obtaining a range of 30% to 40%. By applying these results to the present study, it is estimated that the *T. viride* biomass began at approximately 4.7-6.2g/L, relative to the biomass from the inoculum, and reaches 34.6-46.1g/L, a population increase of nearly 7.4-fold.

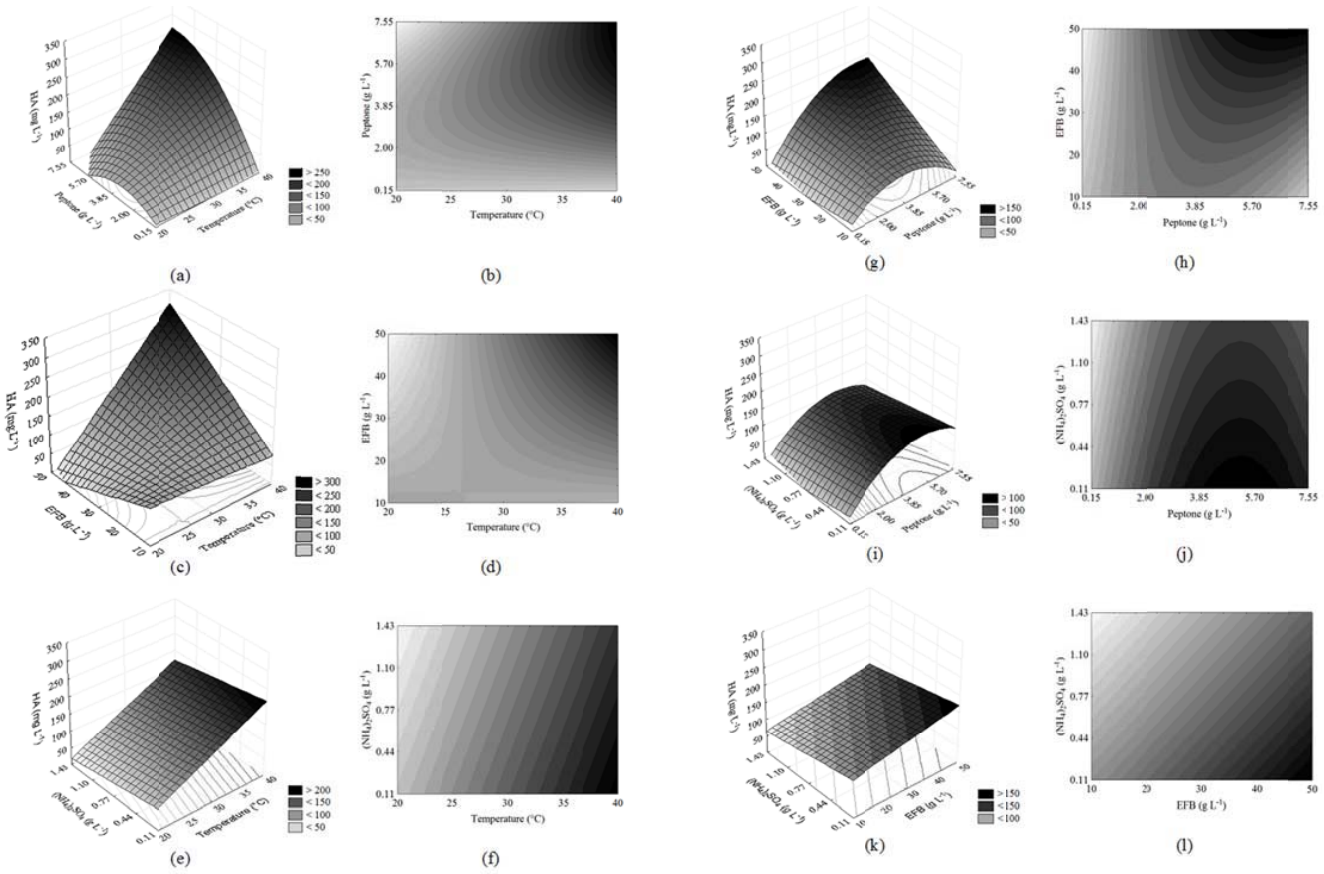

Fig. 1 Response surface plots for HA production showing the interactive effects of: (a) the temperature and the peptone concentration, keeping the EFB and $(NH_4)_2SO_4$ concentrations at central values (3D); (b) the temperature and the peptone concentration, keeping the EFB and $(NH_4)_2SO_4$ concentrations at central values (2D); (c) the temperature and the EFB concentration, keeping the peptone and $(NH_4)_2SO_4$ concentrations at central values (3D); (d) the temperature and the EFB concentration, keeping the peptone and $(NH_4)_2SO_4$ concentrations at central values (2D); (e) the temperature and the $(NH_4)_2SO_4$ concentration, keeping the peptone and EFB concentrations at central values (3D); (f) the temperature and the $(NH_4)_2SO_4$ concentration, keeping the peptone and EFB concentrations at central values (2D); (g) the peptone andEFB concentrations, keeping the temperature and the $(NH_4)_2SO_4$ concentration at central values (3D); (h) the peptone and EFB concentrations, keeping the temperature and the $(NH_4)_2SO_4$ concentration at central values (2D); (i) the peptone and $(NH_4)_2SO_4$ concentrations, keeping the temperature and the EFB concentration at central values (3D); (j) the peptone and $(NH_4)_2SO_4$ concentrations, keeping the temperature and the EFB concentration at central values (2D); (k) the EFB and $(NH_4)_2SO_4$ concentrations, keeping the temperature and the peptone concentration at central values (3D); (l) the EFB and $(NH_4)_2SO_4$ concentrations, keeping the temperature and the peptone concentration at central values (2D)

Fig. 2 indicates fast growth of the fungus between 0 and 48 hours of fermentation and mild growth between 48 and 120 hours, with a distinct change in the growth rate between these two ranges. In our previous work [17], where the proposed process for HA production had not yet been optimized, the greatest *T. viride* growth rate was also observed between 0 and 48 hours, showing a decrease in this rate between 48 and 120 hours, where the growth was almost absent. Comparing the growth rates of the two works, the fact there is still growth between 48 and 120 hours of fermentation in the optimized medium, which was absent in this period in our previous work, is mainly due to the increased availability of cellulosic substrate in the optimized medium (50 g/L of EFB) than in the previous work (20 g/L of EFB).

Regarding the HA production in the optimized culture media, Fig. 2 shows that there is an substantial increase after 96 hours of fermentation, being produced during 24 hours (between 96 and 120 hours of fermentation) the equivalent to 1.8-fold which was produced in the first 96 hours (between 0 and 96 hours). Moreover, according to Fig. 2, it is possible to conclude that HA production occurs mainly when the *T. viride* growth tends to cease, in other words, when the mycelial form, typical of fungal growth, is being transformed into spores. Even as in our previous work[17], where image analysis of the fermentation samples were also made, this statement demonstrates that HA production by *T. viride* by SF of EFB occurs preferably during the fungal sporulation. Siddiqui et al. [31] confirmed through their experiments the fungicidal activity of HA isolated from empty fruit bunch of oil palm compost, which indicates that the similarity between the time duration of sporulation and HA production might be a niche preservation strategy of the *T. viride*.

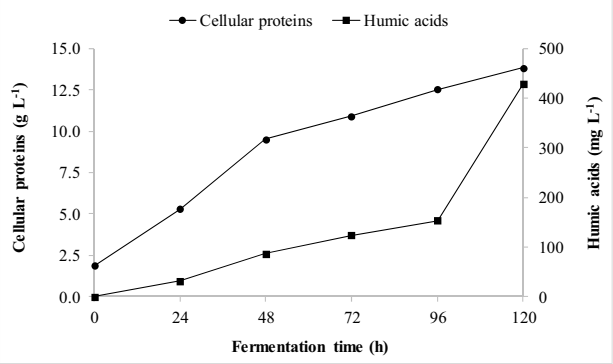

Fig. 2 The cellular proteins and HA concentration over time during submerged fermentations using the *T. viride* strain and the parameters optimized by CCRD

IV. Conclusions

The present study demonstrated that *T. viride* successfully produced HA via the submerged fermentation of EFB. HA production levels were influenced by the temperature as well as the EFB, peptone and $(NH_4)_2SO_4$ concentrations, and these parameters were successfully optimized using a statistics-based response surface model. The screening and optimization methodologies described here represent valuable tools for the development of cost-effective industrial fermentation media, particularly because the carbon source is an underutilized residue, adding value to the procedure. The profile of HA production and fungal growth obtained by the validation assays throughout the fermentation duration indicates that HA production preferably occurs during fungal sporulation and that cultivation studies beyond 120 hours can yield even more of these acids. Furthermore, the production of HA in a controlled bioreactor as well as its purification would aid the characterization of this process.

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