STATISTICAL OPTIMIZATION OF CELLULASES PRODUCTION FROM A STREPTOMYCES SP. STRAIN

(Optimización estadística para la producción de celulasas de una cepa de Streptomyces sp.)

Luis Edgardo Ojeda[1,2], Nirza Noguera-Machado[1,3], Luis Pérez-Ybarra[3], Pablo Pizzanni[4], Juan Fernández Molina[5]

[1]Universidad de Carabobo, Facultad de Ciencias de la Salud, Instituto de Investigaciones Biomédicas "Dr. Francisco Javier Triana Alonso". Maracay-Venezuela.
[2]Universidad de Carabobo, Facultad de Ciencias de la Salud, Escuela de Medicina, Departamento de Fisiología y Bioquímica. Maracay-Venezuela.
[3]Universidad de Carabobo, Facultad de Ciencias de la Salud, Escuela de Bioanálisis, Departamento de Ciencias Básicas. Maracay-Venezuela.
[4]Universidad Rómulo Gallegos, Área Agronomía, Instituto para el Desarrollo Sostenible de los Sistemas Agroambientales. Guárico-Venezuela.
[5]Programa Ciencias Del Agro y Del Mar. Vicerrectorado de Infraestructura y Procesos Industriales. UNELLEZ-San Carlos, Venezuela. Email: jjflearnturbo@gmail.com.

Corresponding author: lojeda2@uc.edu.ve

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SUMMARY

The Streptomyces genus is a group of Gram-positive bacteria, which play an important role in the decomposition of organic matter through the production of lignocellulolytic enzymes. The aim of the present work was to evaluate the effect of the culture conditions (pH, temperature and stirring speed) on the growth of a wild strain of *Streptomyces sp.* and the production of cellulases. For this, an experiment was designed with a 33 factorial treatment arrangement with three replications, whose levels were pH: 6.5, 7.5, and 8.5, temperature: 35, 37, and 39°C, and stirring speed: 100, 150, and 200 rpm. The biomass measured was expressed in mg/mL, and the response surface method was used for this variable analysis. Enzymatic activity was evaluated by determining free glucose from cellulose. As optimum values, pH 7.5 and stirring speed of 170 rpm were obtained. There was no temperature effect. The increase in enzyme activity coincided with the biomass increase, with a maximum after 36 hours of incubation. These conditions, as well as the synthesis time, are advantageous from an economic point of view for industrial use.

Keywords: *Streptomyces sp.*, cellulases, response surface, biotechnology

RESUMEN

El género Streptomyces es un conjunto de bacterias Gram positivas, que cumplen un importante rol en la descomposición de la materia orgánica mediante la producción de enzimas lignocelulolíticas. El presente trabajo tuvo como objetivo evaluar el efecto de las condiciones de cultivo (pH, temperatura y velocidad de agitación) sobre el crecimiento de una cepa silvestre de Streptomyces sp. y la producción de celulasas. Para ello, se diseñó un experimento con arreglo factorial de tratamientos 33 con tres repeticiones, cuyos niveles fueron pH: 6,5, 7,5 y 8,5, temperatura: 35, 37 y 39°C, y velocidad de agitación: 100, 150 y 200 rpm. Se midió la biomasa expresada en mg/mL, y se utilizó el método de superficie de respuesta para el análisis de esta variable. La actividad enzimática fue evaluada mediante la determinación de glucosa libre a partir de celulosa. Como valores óptimos se obtuvieron pH de 7,5 y velocidad de agitación de 170 rpm. No hubo efecto de la temperatura. El incremento de la actividad enzimática coincidió con el aumento de la biomasa, con un máximo a las 36 horas de incubación. Estas condiciones, así como el tiempo de síntesis, son ventajosas desde un punto de vista económico para el uso industrial.

Palabras Clave: *Streptomyces sp.*, celulasas, superficie de respuesta, biotecnología

INTRODUCTIÓN

The *Streptomyces* genus is distributed widely on nature and a great number of species have been isolated until now, they differ among each other in physiology, biochemical activity, and morphology (Azimi *et al.*, 2017). From the point of view of pharmacology, this genus is very relevance, because its antimicrobial and antifungal capacity has been discovered from a diverse number of molecules (Azimi *et al.,* 2017; Kibret et al., 2018). Furthermore, many studies have demonstrated its capacity to produce different commercial enzymes of interest, such as laccases (Lisov *et al.,* 2018), α-amylases (Alagu *et al.*, 2020), xylanase (Zhang *et al.,* 2017; Pennacchio *et al.,* 2018), cellulases (Saini y Aggarwal, 2019; Soeka *et al.*, 2019; Correa *et al.*, 2019), proteases (Galal *et al.*, 2020), among others.

Of this enzymes group, the cellulases are of great economic importance due to their various applications in the bioethanol production (Suriya *et al.*, 2016), textile industry, paper manufacturing, detergent sector and in the food industry (Pinheiro *et al.*, 2017). These enzymes catalyzed the cellulose hydrolysis to glucose (Soeka *et al.*, 2019) and are classified as:

exoglucanases (EC 3.2.1.74), endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), y α-glucosidases (EC 3.2.1.21) (Pinheiro *et al.*, 2017).

The cellular growth, is one of the aspect that must be considered in these processes, actually, because the synthesis velocity is proportional to the biomass speed (Ramos-Sánchez *et al.*, 2018). Accordingly, conditions for the ideal bacterial growth must be established. It has been described that in the cases of the cellulases, the temperature plays an important role in their synthesis (Srivastava *et al.*, 2020). However, the Streptomyces genus can be developed in a wide temperature range, that is between 20 and 250C (Rodrígues *et al.*, 2020).

In view of this, the objective of the present investigation was to optimize the conditions for the growth of a wild Streptomyces sp. strain, regarding to pH, temperature, and stirring

speed. Furthermore, the kinetic of cellulases production was evaluated using the optimized conditions.

MATERIALS AND METHODS

Microrganism

A *Streptomyces sp.* strain identified and isolated from the section of Biotecnología Agroindustrial del Instituto de Investigaciones Biomédicas "Francisco Javier Triana Alonso" at the Universidad de Carabobo, Maracay-Venezuela was used. The qualitative cellulolytic activity was confirmed by the Red Congo test and the growth in solid medium using cellulose as a carbon source (Da Vinha *et al.*, 2011).

Optimization conditions for the *Streptomyces sp.* **strain**

A 3^3 factorial arrangement treatments, with three variables or factors, all qualitative was considered: pH, temperature, and stirring speed (rpm), under a complete randomized design with three replications for a total of 81 experimental units. In addition, the biomass production was used as the dependent variable (mg/mL) (Table 1).

For the cultures growth, the medium suggested by Pometto and Crawford (1986), was used, this contains 6.0 Na_2HPO_4 5,3; K_2HPO_4: 1,98: 0.2 $MgSO_4$; 0.2 NaCl and 0.005CaCl2; yeast extract all in g/Lt and 0.125% cellulose and traces of ($ZnSO_4$, $MnCl_2$ y $FeSO_4$) salts. The vials ere sterilized and inoculated with a spores solution ($1{,}8x10^9$ spores/mL de *Streptomyces sp.*) and placed in agitation for 24h. The used culture conditions are shown in the following table:

Table 1. Preliminary conditions for the factorial arrangement showing factors pH (A), temperature (B), and agitation speed (C).

pH	Temperature (°C)	Agitation speed (rpm)
6.5 (-1)	35 (-1)	100 (-1)
7.5 (0)	37 (0)	150 (0)
8.5 (1)	39 (1)	200 (1)

Note: The level of coded factor is in parenthesis.

To verify the biomass production from the results of the factorial experiment, a response surface analysis (RSA) was carried out for the complete quadratic model with doble interactions. the model equation is as follow:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3 + \beta_7 x_1^2 + \beta_8 x_2^2 + \beta_9 x_3^2 + \varepsilon$$

An estimation of response Surface analysis (RSA) was carried out for the complete quadratic model with doble interaction to verify if there existed an optimum value for the biomass production from the results of the factorial experiment, the general model equation is as follow:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3 + \beta_7 x_1^2 + \beta_8 x_2^2 + \beta_9 x_3^2 + \varepsilon$$

Where, y is the response variable, x_1 is the pH, x_2 is the temperature, x_3 is the stirring speed and ε is the random effect non explained by the model.

Once the model was estimated,the effects that didn't contribute to any statistically significant information were discarded and the reduced model that best fitted the biomass production was selected. For statistically significant effect ($p \leq 0,05$) an analysis of variance (ANOVA) was considered.

Finally, the estimated function was verified if it reached a stationary point that can be local extreme, in this case, in such a way that the objective function corresponded to a local maximum. The data was processed using the MNITAB 18.0 statistical software for Windows.

Growth evaluation of Streptomyces sp, using the optimized conditions

A culture medium was prepared using the method suggested by Pometo and Crawford (1986), the medium was sterilized, and a volume of spores solution ($1.8x10^9$ spores/ mL de *Streptomyces sp.*) was inoculated, and the bacteria was placed to grow under the optimized conditions resulted. The culture was evaluated every two hours for a 40h period, the biomass, pH changes, and turbidity were resolved during this time, and the catalytic activity was determined with the supernatant.

Estimation of biomass

The culture sample medium was collected in a tube previously weighed and centrifuged to 800 rpm for 10 min. The supernatant was discarded and the sediment was washed three times with distilled water and placed in a drying oven at 900C for 48h to constant weight and the result expressed in mg/mL (Lan *et al.*, 2016).

Turbidity determination

A 1 mL culture sample was taken under sterility conditions for the evaluated times and the tur bidity was determined by measuring the absorbance of the culture at 600 nm, a BECKMAN DU spectrophotometer was used. The pH was determined using a pH meter.

Cellulolytic activity

A modified methodology suggested by (Sinjaroonsak *et al.*, 2020) was used to produce free glucose from enzymatic hydrolysis of crystalline cellulose. A solution of 0.25% cellulose in 100 mM buffer phosphate (pH = 7) was prepared and sterilized until further use. A mixture (1:1) of cellulose solution with the supernatant obtained from the culture was placed in an incubator at 350C, 150 rpm, and the reaction was stopped by putting the assay tubes in ice until posterior analysis.The modified methodology proposed by Trinder (Glucose Oxidase-Peroxidase) (Noguera-Machado *et al.*, 2019) was performed to determining free glucose. A calibration curve was used, and the absorbance values were converted to glucose (mg/dL).

RESULTS AND DISCUSION

The observed and estimated (predicted) experimental means obtained by the response surface model for the different variable combinations (pH, temperature, and stirring speed) of the factorial 3^3 experiments are illustrated in table 2.

Table 2. Coded factors levels pH (A), temperature (B), stirring speed (C) for the mathematical. Observed and predicted mean values for independent variable (Biomass, mg/mL).

Treatment (A, B, C)	Observed Median	Predicted Median
-1, -1, -1	0.0347	0.0376
-1, -1, 0	0.0423	0.0506
-1, -1, 1	0.0607	0.0495
-1, 0, -1	0.0407	0.0376
-1, 0, 0	0.0467	0.0506
-1, 0, 1	0.0420	0.0495
-1, 1, -1	0.0410	0.0376
-1, 1, 0	0.0633	0.0506
-1, 1, 1	0.0417	0.0495
0, -1, -1	0.0599	0.0552
0, -1, 0	0.0473	0.0683
0, -1, 1	0.0790	0.0672
0, 0, -1	0.0433	0.0552
0, 0, 0	0.0957	0.0683
0, 0, 1	0.0400	0.0672
0, 1, -1	0.0517	0.0552
0, 1, 0	0.0773	0.0683
0, 1, 1	0.0777	0.0672
1, -1, -1	0.0523	0,0332
1, -1, 0	0.0410	0.0462
1, -1, 1	0.0527	0.0451
1, 0, -1	0.0183	0.0332
1, 0, 0	0.0413	0.0462
1, 0, 1	0.0420	0.0451
1, 1, -1	0.0360	0.0332
1, 1, 0	0.403	0.0462
1, 1, 1	0.0497	0.0451

The estimated complete quadratic model with doble interactions showed as an equation:

$$\hat{y} = 0{,}0635 - 0{,}0022x_1 + 0{,}0005x_2 + 0{,}0060x_3 - 0{,}0024x_1x_2 + 0{,}0008x_1x_3 - 0{,}0004x_2x_3 - 0{,}0198x_1^2 + 0{,}0071x_2^2 - 0{,}0071x_3^2$$

For this model, the doble interaction did not present any significant effect (p>0,05), nor the temperature linear and quadratic effects (p>0,05). The exploration region indicated that the variables pH and stirring speed showed a quadratic form with down concave, therefore, it is reasonable that they reach a maximum in such a region, contrary behavior was shown by the temperature variable, consequently, it could reach a minimum in the exploration region, see figure 1, in addition, it was observed that the uppermost biomass averages were gotten with the pH variable, indicating that it would contribute more information to the phenomenon.

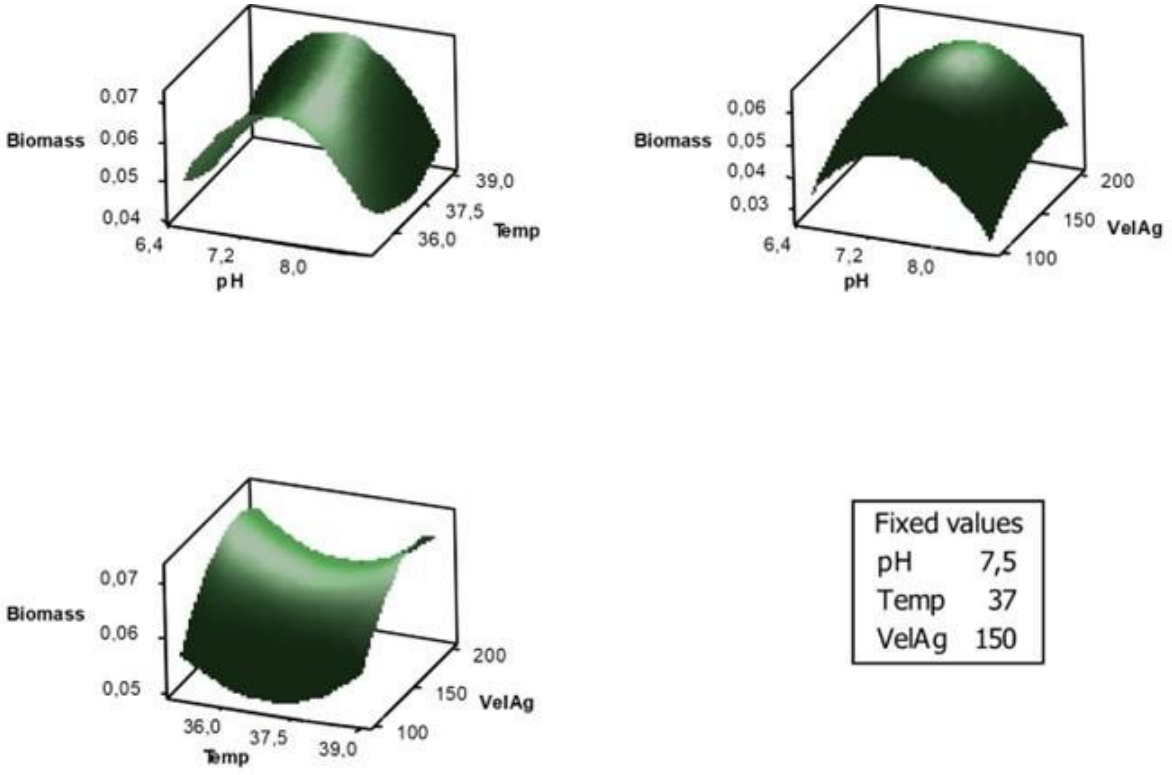

Figure 1. Response Surface graphic images for the response studied variables.

However, as the quadratic and linear effects, in addition to the doble interactions can be discarded, it is possible to estimate a reduced model without interaction, considering the pH and stirring speed variables only, that is,

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_3 + \beta_3 x_1^2 + \beta_4 x_3^2 + \varepsilon.$$

For this new model, the estimated equation was given by:

$$\hat{y} = 0{,}0683 - 0{,}0022x_1 + 0{,}0060x_3 - 0{,}0198x_1^2 - 0{,}0071x_3^2$$

In this model, the effect of the stirring speed (p=0.109) and the pH linear effect (p=0.389) did not cause statistical significance at 5% level, nevertheless, the equation did not show lack of fit (p=0,687), that is, it presented a goodness of fit for the reduced quadratic model suggested.

Further analysis at the stationary point, indicated that the maximum was reached at pH=7.45 and a stirring speed of 170.1 rpm. In practical terms this becomes a pH=7.5 and a stirring speed of 170 rpm. This result indicates that any sustained temperature (35, 37 o 39 °C) can be used along with the optimized stirring speed (170 rpm) and pH of 7.5.

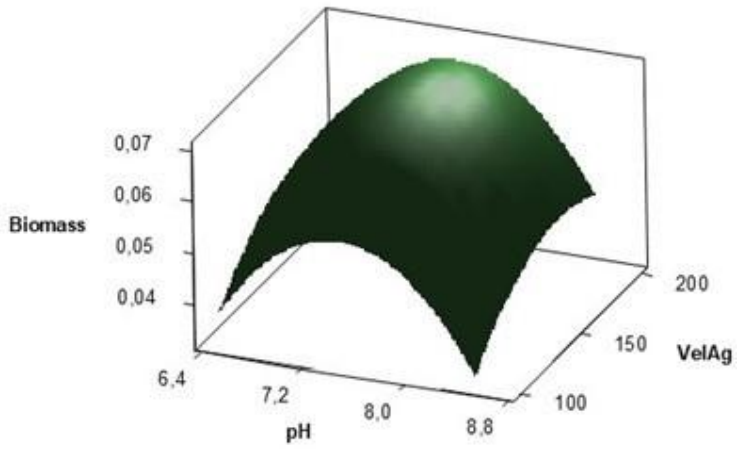

Figure 2. Response Surface graphic image under optimal conditions for the growth of *Streptomyces sp*.

The results obtained after optimization show the microorganisms capacity to work in a wide range of temperature, this property make it ideal to be used technologically on tropical climates, in which the temperatures prevail the same all year round, additionally, it will reduce costs if it would be used in industrial processes because it can support changes in tis variables without losing activity.

Kinematic growth and Cellulolytic activity

As part of these results, it was planned to do an assay with the culture optimal conditions. It was done with the purpose of observing the pH behavior, turbidity, and bacteria biomass production as a function of time, and in this way find out what would be the most convenient time for the microorganism to grow. In addition, the cellulolytic activity of supernatants collected during the biomass production was determined (see fig. 3).

As observed from figure 3, the pH (red line) showed a moderate increase during the culture period, confirming the bacterial growth and the capacity of the salts presented to buffering the change and favoring the raise of this microorganism as accounted for by Pometo and Crawford (1986).

With respect to the production of biomass (blue line) and cellulolytic activity (yellow line), the observed behavior showed a narrow relation between the bacterial growth and the production of extra cellular enzymes with cellulolytic activity. This behavior has been reported by (Lan et al., 2016; Celaya-Herrera *et al*., 2020), for *Streptomyces sp., Streptomyces viridobrunnes* (Da Vinha et al., 2011) y *Streptomyces drozdowiczii* (Grigorevski et al., 2005) strains. The major elimination of cellulases occurred after 30h of culture, in all cases.

During the first 10h non representative changes were observed with respect to the turbidity (black line). The exponential growth is observed from the 26h on, and the culture stopped growing at 32h of incubation, suggesting that the bacteria has reached the stationary phase.

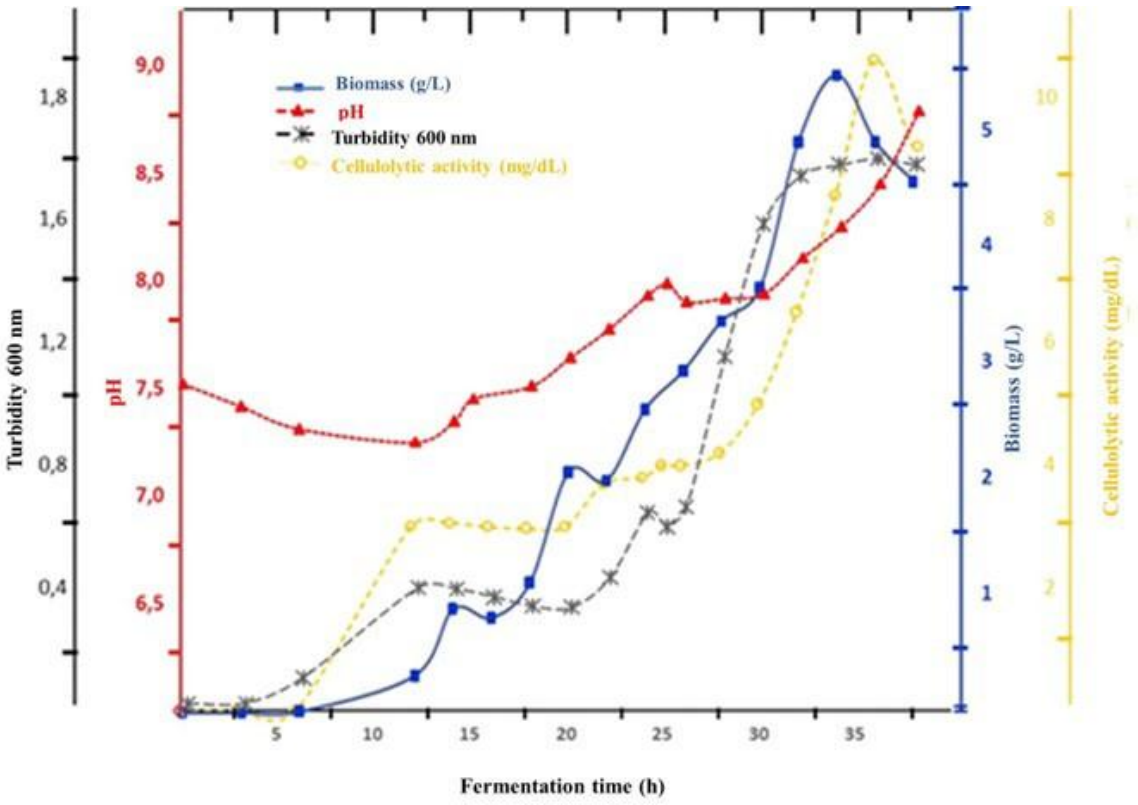

Figure 3. Behavior of variables pH, turbidity, biomass production and cellulolytic activity during the growth of Streptomyces sp. using optimal conditions.

The observed behavior indicates a possible use now to evaluating the microbial performance as time function to be used as time monitoring, because the maximum absorbance values concede with the cellulolytic activity and biomass production maximums. Besides, it is a rapid test that allows evaluation in real time if growth occurs.

The growth of *Streptomyces* presents several advantages in relation to other microorganisms: growth capacity in a wide temperature range, low pathogenicity and sporulated form are some of the characteristics that favor its biotechnological potential use.

CONCLUSIONS

It was statistically demonstrated that the *Streptomyces sp*. strain reached its maximum proliferation at 170 rpm and pH=7.5, independent of the temperature utilized for the culture growth. The uppermost biomass production and major cellulolytic activity was observed at 36h, suggesting that this is the right time for the growth of this bacteria. Since the culture of this bacteria is independent of temperature, suggests that it can be cultivated without a temperature control, which will reduce costs if it is utilized on an industrial level.

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