@LIMENTECH CIENCIA Y TECNOLOGÍA ALIMENTARIA
ISSN Impreso 1692-7125/ ISSN Electrónico 2711-3035. Volumen 22 No. 1, P. 282 - 294 año 2024
Facultad de Ingenierías y Arquitectura
Universidad de Pamplona

# Optimization of Acetic Acid Yield by *Gluconobacter oxydans* Using a Box-Behnken Design

# Optimización Del Rendimiento De Ácido Acético Por *Gluconobacter oxydans* Utilizando El Diseño De Box-Behnken.

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**Recibido: mayo 13 de 2024; Aprobado: junio 30 de 2024**

## ABSTRACT

This study aims to optimize acetic acid (AA) production by *Gluconobacter oxydans* using a culture medium modified with dairy wastewater, applying a Box-Behnken design. The independent variables evaluated were glucose concentration, temperature (°C), and incubation time (hours). The resulting regression model demonstrated that temperature and incubation time were statistically significant factors influencing AA yield. Optimal conditions for maximum AA production were determined to be 33.484°C, 5.030% (v/w) glucose concentration, and 70.565 hours of incubation, yielding a predicted AA concentration of 4.763 g/100 mL. The

relationships among these variables were modeled with a precision exceeding 91.58%, underscoring the robustness and reliability of the predictions. This optimized approach provides a sustainable method for AA production while repurposing dairy wastewater, contributing to a circular economy framework.

**Keywords:** acetic acid bacteria, dairy wastewater, response surface methodology.

## RESUMEN

Este estudio se enfoca en el proceso de optimización empleando un diseño de Box Behnken para la producción de ácido acético (AA) por *Gluconobacter oxydans* en un medio de cultivo modificado con aguas residuales lácteas. Las variables independientes estudiadas fueron: concentración de glucosa, temperatura (°C) y tiempo de incubación (hr). El modelo de regresión desarrollado indica que la temperatura y el tiempo de incubación fueron determinantes estadísticos para el rendimiento de AA, y las condiciones óptimas para la máxima producción de AA fueron una temperatura de 33.484°C, una concentración de glucosa de 5.030 (v/p), y un tiempo de incubación de 70.565 horas, lo que resultó en un rendimiento predicho de 4.763 (g/100mL). Las relaciones entre estas variables fueron descritas y predichas con precisión, superando el 91.58%. Este método optimizado puede ser utilizado para obtener AA de manera sostenible y limpia, utilizando aguas residuales lácteas en un marco de economía circular

**Palabras clave:** aguas residuales lácteas, Bacterias del ácido acético, Metodología de superficie de respuesta

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@LIMENTECH CIENCIA Y TECNOLOGÍA ALIMENTARIA
ISSN Impreso 1692-7125/ ISSN Electrónico 2711-3035. Volumen 22 No. 1, P. 282 - 294 año 2024
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## INTRODUCTION

Acetic acid (AA) is widely used as a food preservative, solvent, and a key component in various commercial chemicals. Its primary application is in the production of vinyl acetate monomer via oxidative synthesis, which is crucial for the creation of emulsion polymers, resins, and other intermediates used in coatings, textiles, wires, and acrylic fibers (Pal and Navak, 2016). Approximately 15% of global AA production is derived from biological processes, while around 78% is synthesized chemically through methanol carbonylation (de Roos and de Vuyst, 2018). However, chemical synthesis methods for AA present several challenges, including environmental toxicity due to chemical use and the high cost of raw materials compared to biological alternatives (Kalck et al., 2020).

Given these limitations, there is growing interest in exploring biological methods as sustainable alternatives for AA production. Gluconobacter strains have gained attention over the past two decades due to their remarkable ability to partially oxidize sugars and alcohols. These microorganisms, particularly *Gluconobacter oxydans*, act as biocatalysts and have shown potential in increasing the production of oxidized products, such as AA (Es-Sbata et al., 2022).

*Gluconobacter oxyd*ans is an obligate aerobic, mesophilic, Gram-negative bacterium belonging to the acetic acid bacteria genus, typically found in sugar- and alcohol-rich environments (Dai et al., 2022; Mamlouk and Gullo, 2013).

The utilization of alternative substrates, such as organic waste, byproducts from the food industry, and waste from fruit, meat, and dairy processing plants, offers a promising approach for AA production. However, this strategy remains in the early stages of development, with substantial areas yet to be thoroughly investigated. Agro-industrial waste, rich in nutrients, holds considerable potential for producing high-value products like AA (Wang et al., 2022).

Fermentation processes present several advantages, such as the ability to utilize food waste and other organic byproducts, which can be metabolized by microorganisms to produce AA and non-toxic residues. The rapid growth of the dairy industry has led to the generation of significant byproducts, with whey being one of the most prominent. Whey, also known as the serum phase of milk, retains 55% of milk's nutrients and is obtained through processes like fat removal

(skimming) and protein precipitation (casein separation). Despite its nutrient-rich composition, whey is often discarded as wastewater (Zhao et al., 2023).

Improving AA yield through biological processes requires the optimization of various nutritional and physiological factors. As noted by Fasolo et al. (2020), employing experimental design is essential for optimizing biological processes. One of the key objectives of experimental design is optimization, as it enables the prediction of responses across all possible combinations of independent variables and identifies the optimal combination of factors.

Response Surface Methodology (RSM) is a widely used experimental design approach that addresses multi-variable problems by correlating the response with one or more studied factors. Through graphs and polynomials, RSM illustrates functional relationships and determines optimal experimental conditions (Ding et al., 2015). Within RSM, the Box-Behnken Design (BBD) is commonly used, as it involves three-factor levels and avoids extreme combinations of variables. The variation in predicted responses at specific points depends on their distance from the design's midpoint (Fasolo et al., 2020).

In light of the above, this study aimed to optimize the production conditions of acetic acid by *Gluconobacter oxydans* grown in a culture medium modified with dairy wastewater, using a Box-Behnken design.

## MATERIALS AND METHODS

### Cultivation Conditions and Acetic Acid Estimation

The *Gluconobacter oxydans* culture was provided by the Microbiology Laboratory of the University of Cartagena, Colombia. The bacteria were inoculated into Glucose Yeast Carbonate (GYC) broth media to activate the microorganism. For fermentation, a modified culture medium was designed with 12% dairy wastewater, sterilized by autoclave treatment (121°C, 15 lb/15 min). Incubation was carried out for 100 hours with agitation at 120 rpm and submitted to aeration (1 L $h^{-1}$ $L^{-1}$) in an 8 L Frings acetate, with the temperature and glucose content adjusted according to the experimental design before incubation. Acetic acid production was estimated using

the acid-base titration method described by Sharafi et al. (2010).

**Box-Behnken design (BBD) of response surface methodology (RSM).** The study focused on three independent variables with three levels: glucose concentration (2-8 w/v), temperature (25 – 40°C) and incubation time (36 – 94 hr). The design matrix consisted of 15 experimental units, including three replicates of the central point. RSM and the design BBD were employed to analyze these variables. The optimization process and mathematical correlation were performed using the Minitab 17.0 statistical software package.

The second-order polynomial model (equation 1) was used to determine the relationships between independent variables and output (acetic acid yield) (Table 1). The coefficient of determination ($R^2$), in addition to the adjusted $R^2$, was used for statistical assessment to quantify the accuracy and confirm the dependability of the polynomial model equation

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X$$

where Y is the predicted response (acetic acid yield), $\beta_0$ is a model constant, and $X_1$, $X_2$ and $X_3$ are the independent variables corresponding to glucose concentration, temperature (°C) and incubation time (hr) respectively. The terms $\beta_1$, $\beta_2$ and $\beta_3$ represent linear regression coefficients; $\beta_{11}$, $\beta_{22}$ and $\beta_{33}$ are the coefficients of the quadratic terms; and $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ are the coefficients of the interactions.

**Statistical analysis.** An analysis of variance (ANOVA) was performed to assess the significance of the obtained model. A p-value less than 0.05 was considered statistically significant. Contour plots were used to analyze the interactions between two variables.

## RESULTS AND DISCUSSION

**Culture condition for acetic acid (AA) production.** The average AA production ranged between 0.97 and 4.72 g/100 mL, which is similar to those reported by Upadhyay *et al.* (2023), who found AA values between 0.6 and 2.52 g/100 mL for AA-producing bacteria growing in GYC medium. These microorganisms that produce AA can typically be isolated from various environments, including alcoholic beverages,

vinegar, fruits, juices, soil, and water. These microorganisms can utilize glucose, ethanol, lactate, and glycerol as a source of energy to produce AA (Sharafi *et al.,* 2010). In the present study, the production of AA was evaluated using a substrate consisting of a 12% modified culture medium sterilized by autoclave treatment with wastewater from a dairy plant. This opens up the possibility of reducing the production costs of AA at an industrial level.

In Table 1, the BBD design used for the production of acetic acid is presented, where the factors were glucose ($X_1$), temperature ($X_2$), and incubation time ($X_3$). The combinations used in the design show that the highest production of AA (4.72 g/100mL) occurred at 32.5°C with an incubation time of 65 hours and 5% glucose. These findings are similar to those reported by Upadhyay *et al.* (2023), who isolated AA-bacteria from fruit and cow dung and found a maximum AA yield of 4.88 g/100 mL from the bacterial strain isolated from apple waste.

The lowest concentration 0.97 g/100 mL was obtained at 25°C, with an incubation time of 36 hours and 5% glucose. Notably, glucose does not appear to significantly impact AA production, suggesting that the dairy wastewater used to modify the culture medium contains carbon that is utilized for acetic acid production. Typically, AA production is carried out by converting carbohydrates into ethanol and its subsequent oxidation to acid.

**Table 1.** The design of the experiment of the factors dependent on the AA yield.

| Glucose | Temperature (°C) | Incubation time (horas) | Acetic acid yield (g/100 ml) | Predicted value RSM |
|---|---|---|---|---|
| 2 | 25.0 | 65 | 1.42 | 1.61 |
| 5 | 32.5 | 65 | 4.64 | 4.68 |
| 5 | 25.0 | 36 | 0.97 | 1.01 |
| 5 | 40.0 | 94 | 2.94 | 2.89 |
| 8 | 32.5 | 36 | 1.32 | 1.11 |
| 2 | 32.5 | 94 | 2.05 | 2.25 |
| 8 | 40.0 | 65 | 2.55 | 2.35 |
| 5 | 40.0 | 36 | 1.03 | 1.42 |
| 8 | 25.0 | 65 | 1.52 | 1.67 |
| 5 | 25.0 | 94 | 2.36 | 1.96 |
| 2 | 32.5 | 36 | 1.33 | 1.08 |
| 2 | 40.0 | 65 | 2.44 | 2.28 |
| 5 | 32.5 | 65 | 4.72 | 4.68 |
| 8 | 32.5 | 94 | 2.12 | 2.36 |
| 5 | 32.5 | 65 | 4.68 | 4.68 |

The experimental values obtained for the AA yield were subjected to response surface regression analysis. Table 2 presents the results of the model's ANOVA. The regression model was statistically significant ($P < 0.05$), and there was no significance in the lack of fit ($P = 0.685 > 0.05$). Hence, the model properly describes and fits the AA yield. The determination coefficient ($R^2$) for the prediction model obtained from the regression analysis for the response variable was 97.30%, with an adjusted $R^2$ of 92.44% and a predictive $R^2$ of 91.58%.

**Table 2.** ANOVA for the quadratic model of the AA yield.

| Source | Adjusted sum of squares | Adjusted mean squares | *F- value* | *P- value* |
|---|---|---|---|---|
| Model | 23.43 | 2.60 | 20.01 | 0.002 |
| Lineal | 3.81 | 1.27256 | 9.78 | 0.016 |
| Glucose ($X_1$) | 0.0091 | 0.00911 | 0.07 | 0.802 |
| Temperature ($X_2$) | 0.9045 | 0.90451 | 6.95 | 0.046 |
| Incubation time ($X_3$) | 2.9041 | 2.90405 | 22.32 | 0.005 |
| Quadratic | 19.5465 | 6.51549 | 50.07 | 0.000 |
| Glucose*Glucose ($X_1$*$X_1$) | 7.3277 | 7.32767 | 56.32 | 0.001 |
| Temperature *Temperature ($X_2$*$X_2$) | 6.1325 | 6.13247 | 47.13 | 0.001 |
| Incubation time*Incubation Time($X_3$*$X_3$) | 9.0577 | 9.05774 | 69.61 | 0.000 |
| Interaction | 0.0692 | 0.02307 | 0.18 | 0.907 |
| Glucose*Temperature | 0.0000 | 0.00003 | 0.00 | 0.989 |
| Glucose *Incubation Time | 0.0016 | 0.00160 | 0.01 | 0.916 |
| Temperature*Incubation Time | 0.0676 | 0.06760 | 0.52 | 0.503 |
| Lack of fit | 0.2472 | 0.24501 | 0.53 | 0.685 |

The coefficient terms can be interpreted as a proportion of the data variability explained by the model. Adjusted $R^2$ incorporates the effect of the number of factors present and is therefore useful for evaluating the impact of increasing or decreasing the number of terms in the model, and the predictive $R^2$ provides an indication of the predictive capacity of the regression model (Montgomery, 2010).

These findings suggest that the obtained predictive model explains 91.58% of the total variation, indicating a high level of accuracy and reliability of the predictive values, as they closely match the experimental values. This implies that the model can effectively forecast

the AA yield. The subsequent model elucidates the experimental data:

$$Y = 4.68 + 0.0341X_1 + 0{,}3362X_2 + 0.6037X_3 - 0.1409X_1^2 - 1.2891X_2^2 - 1.5662X_3^2 + 0.0032X_1X_2 + 0.0203X_1X_3 + 0.130X_2X_3$$

Where Y is acetic acid yield, $X_1$ is Glucose, $X_2$ is Temperature, and $X_3$ is Incubation Time.

Table 3 shows the values determined for each of the regression coefficients. Incubation time and temperature have significant linear effects on the AA yield, as they correspond to a P-value less than 0.05. In linear terms, the coefficient with the greatest weight, determined by its absolute value, is $X_3$ (incubation time), which has a positive effect. The $X_3$ coefficient is followed by $X_2$ (temperature).

The interaction coefficient ($X_1$*$X_2$, $X_1$*$X_3$, and $X_2$*$X_3$) showed low values. An interaction coefficient with a positive sign represents a synergistic effect, whereas a negative sign indicates an antagonistic interaction between the factors involved (Gibbins et al., 2012). $X_1$, as well as its interactions with $X_2$ and $X_3$, were not significant, as their P-values were greater than 0.05. The negative T-value represents the adverse effect of the variable on AA yield. For example, table 3 shows the adverse effects of the quadratic terms $X_1$, $X_2$, and $X_3$.

The coefficient's sign indicates whether the effect is positive or negative, while its magnitude reflects the strength of the effect. This study establishes that the yield of AA depends primarily on the incubation duration, followed by temperature.

In contrast, Upadhyay et al. (2023) observed a stronger reliance on glucose concentration and incubation period in AA-producing bacteria cultured in GYC broth media. This difference is due to the presence of carbon in the dairy wastewater used. Therefore, in AA production using this substrate, it is not necessary to add glucose as a carbon source.

**Table 3.** Regression coefficients of second-order polynomials for the acetic acid yield

| Term | *Coef.* | *SE Coef.* | *T-value* | *P-value* |
|---|---|---|---|---|
| Constant | 4.68 | 0.208 | 22.47 | 0.000 |
| Glucose ($X_1$) | 0.034 | 0.128 | 0.26 | 0.802 |
| Temperature ($X_2$) | 0.336 | 0.128 | 2.64 | 0.046 |
| Incubation time ($X_3$) | 0.603 | 0.128 | 4.72 | 0.005 |
| Glucose*Glucose | -1.409 | 0.188 | -7.50 | 0.001 |
| Temperature*Temperature | -1.289 | 0.188 | -6.87 | 0.001 |
| Incubation Time*Incubation Time | -1.566 | 0.188 | -8.34 | 0.000 |
| Glucose*Temperature | 0.003 | 0.180 | 0.01 | 0.989 |
| Glucose*Incubation Time | 0.020 | 0.180 | 0.11 | 0.916 |
| Temperature*Incubation Time | 0.130 | 0.180 | 0.72 | 0.503 |

**Optimization of cultivation conditions for acetic acid production**

In previous studies (Upadhyay *et al.,* 2023), the incubation period, ethanol, and glucose concentration were investigated as factors in producing AA by producing bacteria. The present study decided to expand the range of previously studied factors by including temperature as a variable and eliminating ethanol concentration. The production of AA was studied with the assistance of contour plots.

*G. oxydans* primarily show the dependency of AA yield on incubation time and temperature. Figure 1 displays the contour plots of *G. oxydans*, illustrating the interactions between independent variables for AA yield. Typically, *Gluconobacter spp*. Utilize the pentose phosphate pathway for acetic acid production (Gullo *et al.,* 2006). The results of the regression of the response surface data (BBD design) are shown in Table 1. The significance of a correlation coefficient is increased when the T-value is large and the associated P-value is small ($p<0.05$) (Seraman *et al.,* 2010).

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ISSN Impreso 1692-7125/ ISSN Electrónico 2711-3035. Volumen 22 No. 1, P. 282 - 294 año 2024
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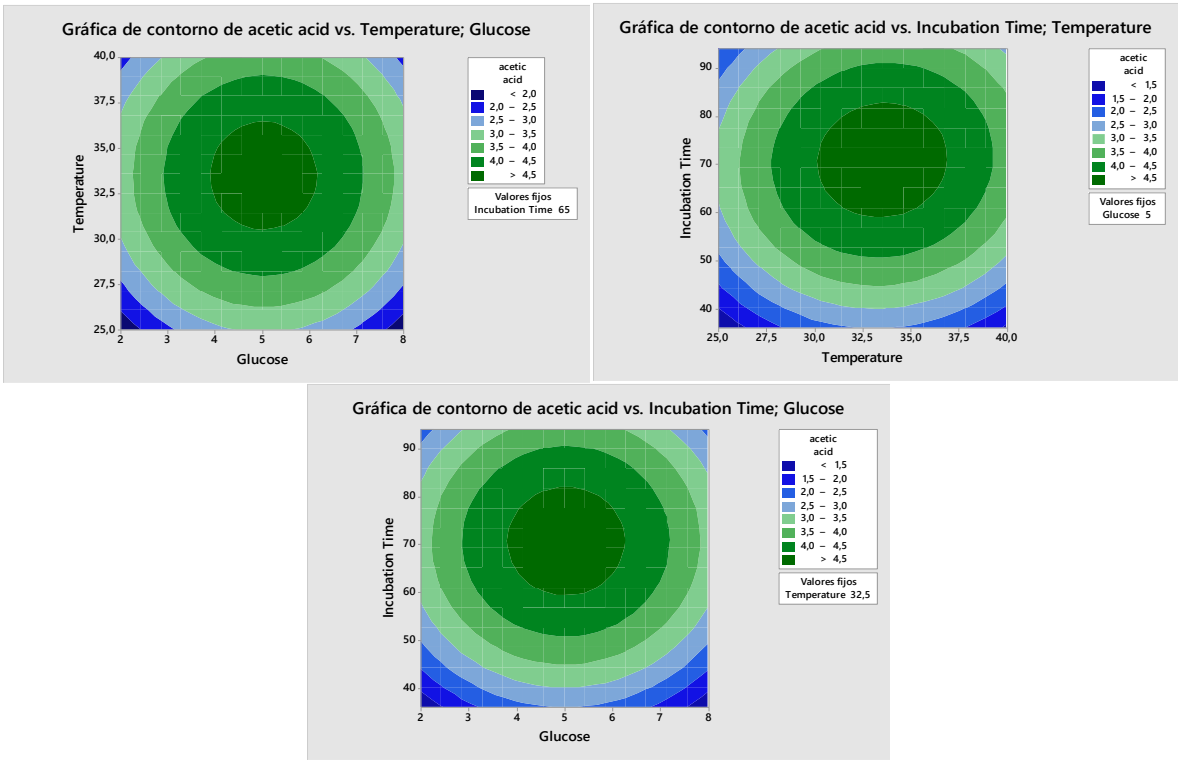


**Figure. 1**. Contour plots of the acetic acid yield showing the interactions of independent variables

The yield conditions of AA were optimized to maximize production using the 'Response Optimizer' function in Minitab® statistical software version 17. The critical values determined by the analysis of the independent variables by the surface model were glucose of 5.030 % (w/v), temperature of 33.484 °C, and incubation time of 70.565 hr. The predicted AA yield under these conditions was 4.763 g/100 mL (Figure 2).

The AA yield was performed experimentally under optimal conditions to verify the mathematical model obtained. The production of AA was 4.82 ± 1.72 g/mL, which coincides with that predicted by the model of 4.76 g/mL, indicating that the model was suitable for describing the AA production.

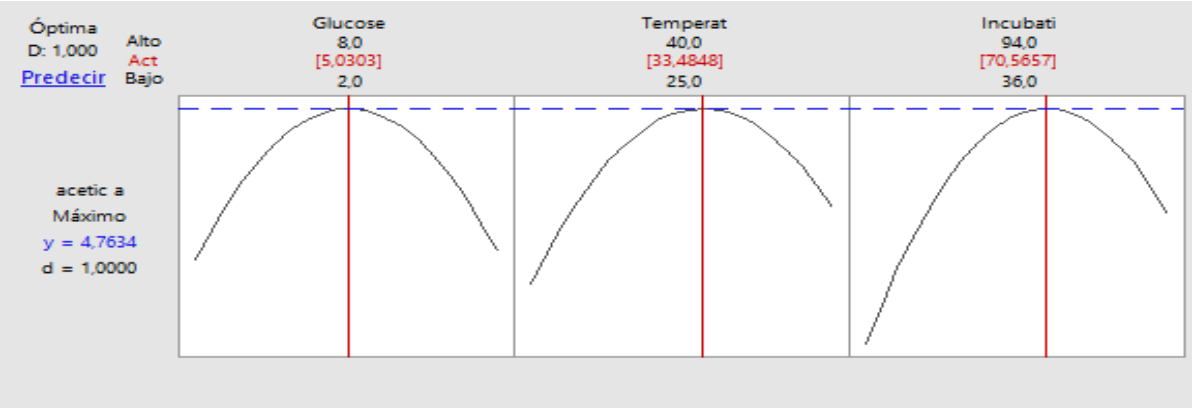


**Figure 2.** Optimization of acetic acid production by *Gluconobacter spp*.

## CONCLUSIONS

Box-Behnken designs represent valuable statistical methodologies for optimizing acetic acid production by *Gluconobacter oxydans.* Temperature and incubation time are critical factors in achieving maximum acetic acid yield. Through experimental investigation, it was determined that the optimal conditions for maximizing yield were a temperature of 33.484°C, a glucose concentration of 5.030 (v/w), and an incubation time of 70.565 hours, resulting in a predicted yield of 4.763 g/100 mL. Employing a carefully constructed second-order polynomial model, the relationships between these variables were accurately described and predicted with a precision exceeding 91.58%. This optimized methodology holds significant promise for industrial-scale production, facilitating the procurement of acetic acid for various industrial applications.

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