Optimization of the reproduction of *Weissella cibaria* in a fermentation substrate formulated with agroindustrial waste

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**ABSTRACT**

Agroindustrial wastes contain macronutrients and micronutrients essential for the reproduction of lactic acid bacteria. In this research, the reproduction of *Weissella cibaria* was experimentally optimized in a supplemented fermentation substrate (SFS) formulated from pineapple and sacha inchi wastes. Response surface methodology was used to evaluate the influence of the following independent variables: temperature (32–40 °C), pH (5.0–6.0), and stirring speed (SS) (100–150 rpm) on the following dependent variables: viability (Log10 CFU mL−1), biomass production (BWC), lactic acid production (LA), biomass volumetric productivity (VPWC), LA volumetric productivity (VPLA), carbon source consumption (CSC), N2 consumption (N2C), and specific growth rate (µ). The experimental optimization of multiple responses presented a desirability of 76.8%, thus defining the independent variables of the process: temperature = 35.1 °C, pH = 5.0, and SS = 139.3 rpm; and the dependent variables: viability = 10.01 Log10 CFU mL−1, BWC = 2.9 g L−1, LA = 19.4 g mL−1, VPWC = 43.9 g biomass/g CSC, VPWC = 0.49 g L−1 h−1, VPLA = 3.2 g L−1 h−1, CSC = 17.2%, N2C = 63.6% and µ = 0.28 h−1. From these, viability, VPWC, CSC, N2C, and LA presented significant statistical differences, while the independent variable with the least important effect on the process was pH. Under optimal conditions of temperature, pH and SS, SFS favors the reproduction and viability of *W. cibaria*. This provides evidence of a sustainable alternative for the production of probiotics in the context of circular economy.

**Abreviations**

Name Abbreviation
- Biomass
- Colony Forming Units CFU
- Carbon Source Consumption CSC
- Free Amino Nitrogen FAN
- Lactic acid LA
- Lactic Acid Bacteria LAB
- Man Rogosa Sharpe MRS
- Maximum biomass production MBWC
- Nitrogen Consumption N2C
- Reducing sugar concentrations S0 - S
- Regression coefficient - linear model R2

Regression coefficient - logistic equation R2LE
Relative mean error RME
Specific growth Speed µ
Specific growth rate− linear model µL (h−1)
Specific growth rate− logistic equation µL (h−1)
Stirring Speed SS
Supplemented fermentation substrate SFS
Temperature T
Volumetric Productivity of lactic acid VPLA
Volumetric Productivity of *W. cibaria* VPWC
Working volume in the reactor Vf

1. **Introduction**

Agroindustrial wastes are complex and easily decomposed structures; therefore, its accumulation generates environmental problems [1,
In 2017, global waste from the food industry exceeded 1300 million tons [3]. However, these residues are important sources of micro and macronutrients (minerals, vitamins, carbohydrates, lipids, and proteins) [2,4] with great potential for recovery of bioactive compounds through biotechnological processes (hydrolysis, fermentation, extraction, concentration, purification, biocatalysis, among others) [5-7]. In agroindustrial waste, the high concentration of C and N stands out, which determines its potential use as a substrate for fermentation. Elements such as C and N are essential nutrients in the production of organic acids, bioactive peptides, bacteriocins, and probiotic reproduction [6,8-10].

In fermentation processes, commercial substrates represent between 40 and 70% of the costs of the process, and some research highlights the challenges of more profitable and sustainable processes using agroindustrial waste [11]. In addition, the composition of the substrate and the process conditions are factors that favor a greater or lesser production of cellular biomass or other secondary metabolites with wide application in the food industry (organic acids, bacteriocins, exopolysaccharides) [12-14].

Agroindustrial wastes have been evaluated as fermentation substrates mainly for the production of lactic acid (LA) because it is widely used in the food, chemical, medical and pharmaceutical industries [9,15]. In terms of LA productivity, some authors report favorable results when conventional fermentation substrates are replaced by substrates formulated with organic C and N sources. In their review, Ahmad et al. [11] highlighted the use of various organic substrates and food residues for the production of LA, such as cassava bagasse (2.74 g L\(^{-1}\) h\(^{-1}\)), apple pulp (5.41 g L\(^{-1}\) h\(^{-1}\)), and xylose fermented corn liquor (6.15 g L\(^{-1}\) h\(^{-1}\)). For the production of bacterial biomass, the commercial substrate (MRS) has been principally used [16]. This type of substrate favors the metabolic efficiency represented by the specific growth rate, a property that determines the speed of reproduction and increase of cellular biomass in the fermentation kinetics. However, the high cost of commercial substrates reduces the profitability of the biotechnological process [17].

On the other hand, the circular economy is a production and consumption model that involves minimizing the waste generated in production, generating value-added products with said waste [3]. Therefore, the use of agroindustrial waste as fermentation substrates has been relevant in the context of circular economy [15,18,19].

One of the largest waste-generating agrochains is pineapple because most of the primary production is destined to the generation of products such as juices, concentrates, jams, salads, preserves, jellies, cakes, among others [20,21]. The aforementioned process generates production residues that represent 50% of the total weight: crown, epicarp, and cores [21,22]. These wastes are characterized by their high fiber and sugar content, as well as their minerals and to a lesser contain vitamins [23]. Similarly, the sacha inchi seed oil extraction industry generates a cake byproduct that represents between 60 and 75% of the total weight: crown, epicarp, and cores of gold honey variety pineapple (Ananas comosus) (41.33%) [31], the by-product of the sacha inchi oil extraction process (Plaknetilla volubilis) (57.95%), and mineral salts (C\(_2\)H\(_5\)O\(_2\)N\(_2\) = 5.0%, C\(_6\)H\(_5\)O\(_2\)N\(_2\) = 2.0%, K\(_2\)HPO\(_4\) = 2.0% and MgSO\(_4\) = 0.2%), which were obtained from previous research by Micanquer-Carlosama et al. [22].

2. Materials and methods

2.1. Fermentation substrate

A supplemented fermentation substrate (SFS) was formulated from a mixture of powdered residues. The total formulation included epicarps and cores of gold honey variety pineapple (Ananas comosus) (41.33%), the by-product of the sacha inchi oil extraction process (Plaknetilla volubilis) (57.95%), and mineral salts (C\(_2\)H\(_5\)O\(_2\)N\(_2\) = 5.0%, C\(_6\)H\(_5\)O\(_2\)N\(_2\) = 2.0%, K\(_2\)HPO\(_4\) = 2.0% and MgSO\(_4\) = 0.2%), which were obtained from previous research by Micanquer-Carlosama et al. [22].

2.2. Activation and reproduction of W. cibaria in the SFS

A W. cibaria strain from the Strain Bank of the Biotechnology Institute of the National University of Colombia (IBUN Strain and Genes Bank 090 – 03,684; AN: KU132362) was used. The purity of W. cibaria in the strain bank is confirmed by the following tests: ability to assimilate aniline blue, morphology by Gram stain, biochemical confirmation by API 50 CHL kit and effectiveness in antimicrobial activity. The bacteria was activated following the methodology described by Micanquer-Carlosama et al. [22] and its reproduction in the SFS was optimized by evaluating the process conditions (pH, T, and SS). The optimization was performed through discontinuous fermentation in 600 mL batches, using a 1 L capacity reactor (BioFlo® / CelliGen® 115, Germany).

2.3. Fermentation kinetic parameters with W. cibaria

20 mL of fermentation product were taken at 0, 2, 4, 6, 8, 10, 12, and 24 h (0 corresponded to the initial conditions of the substrate). Viability (CFU mL\(^{-1}\)) was measured by plating (36 °C for 48 h) [31] 1 mL of the fermentation product. The remaining volume was centrifuged at 4508 g for 10 min at 4 °C (Penendorf centrifuge 5804 R, Germany), and the supernatant was separated from the precipitate. The supernatant was filtered (0.45 μm cellulose filter) and used to measure: concentration of reducing sugars (g L\(^{-1}\)) by the 3,5-dinitrosalicylic acid methodology (Miller, 1959). The N\(_2\) concentration and LA production (g L\(^{-1}\)) were measured by the method of free amino nitrogen (FAN) (g L\(^{-1}\)) and by reflectometry (Reflectoquant Merck - RQflex Plus 10, Germany), respectively [33]. The precipitate was used to determine biomass production of W. cibaria (B\(_{Wc}\)), which was expressed as g of dry biomass (105 °C, time 5 h) per L of fermented sample (g L\(^{-1}\)). Biomass yield (Y\(_{Bwc/S}\)) (g dry biomass / g CSC), volumetric biomass productivity (VP\(_B\)) (g L\(^{-1}\) h\(^{-1}\)), carbon source consumption (CSC) (%), N\(_2\) source consumption (N\(_2\)C) (%), and volumetric productivity of LA (VP\(_L\)) (g L\(^{-1}\) h\(^{-1}\)) were calculated using Eqs. (2), (3), (4), (5), and (6), respectively.

$$\text{FAN} = \frac{\text{mL NaOH} \times 0.1 N \times (14 \times 1000)}{\text{mL of sample}} \text{ (mg L}^{-1}\text{)}$$

$$Y_{Bwc/S} = \frac{B_{Wc} - B_{w0}}{S_c - S} \text{ (g g}^{-1}\text{)}$$

$$VP_B = \frac{MB_{Wc}}{V_t 	imes t} \text{ (g L}^{-1}\text{h}^{-1}\text{)}$$

$$\text{CSC} = \frac{(S_c - S)}{S_c} \times 100 \%$$
\[ N_{t}C = \frac{(FAN_{t} - FAN)}{FAN_{t}} \times 100(\%)
\]

\[ V_{P,LA} = \frac{LA}{V_{f} + t} \times 100 \ (g L^{-1} h^{-1})
\]

Where, \( B_{W,0} \) and \( B_{W} \) are the dry biomasses, \( S_{0} \) and \( S \) are the reducing sugar concentrations, and \( FAN_{0} \) and \( FAN \) are the free nitrogen concentrations at the initial time and at each sampling time, respectively. \( MB_{W} \): maximum biomass production. \( V_{f} \): working volume in the reactor. \( t \): fermentation time.

2.4. Experimental design and statistical analysis

Response surface methodology was used with a central composite design – centered face \((\alpha = 1)\) (15 experiments), depending on the following independent variables: pH (5.0–6.0), T (32–40 °C), and SS (100–150 rpm); and the dependent variables: viability, \( B_{W,0}, Y_{Wc/S}, VP_{Wc}, CSC, N_{C}, LA \), and \( V_{P,LA} \). The results were analyzed using the analysis of variance (ANOVA) with a significance level of 5% obtained from the Statgraphics software (version XVII. II). The experimental data of the dependent variables are reported as the mean value ± standard deviation. These were obtained from triplicate measurements for each experiment and adjusted to a 2nd order polynomial model (Eq. (7)); where, \( Y \) are the dependent variables, \( \beta_{0} \) is a constant; \( \beta_{1}, \beta_{2}, \beta_{3} \) correspond to the regression coefficients, and \( X_{1} \) and \( X_{2} \) present the independent variables.

\[ Y = \beta_{0} + \sum_{i=1}^{1} \beta_{i} X_{i} + \sum_{i=1}^{1} \beta_{i} X_{i}^{2} + \sum_{i=1}^{1} \beta_{i} X_{i}^{3} \]

An experimental optimization of multiple responses to the fermentation process was carried out. The analysis considered the results of the ANOVA and criteria, weights, and impacts of the dependent variables. These optimum parameters favored the production and viability of \( W. cibaria \) biomass, and the relative mean error (RME) was used to assess the accuracy of the mathematical model.

2.5. Specific growth rate of \( W. cibaria \)

The specific growth rate (\( \mu \) h\(^{-1} \)) was calculated for each of the design experiments, considering the exponential phase (time: 0 → 10 h). This parameter was determined using a model of order 1 (Eq. (8)) (Specific growth rate–linear model \( \mu_{1} \ h^{-1} \)) and the model adjusted by the logistic equation (Eq. (9)) (Specific growth rate–logistic equation \( \mu_{2} \ h^{-1} \)) [17]. These values were compared with the experimental value obtained at the optimal condition.

\[ \ln B_{W} = \mu_{1} + \ln B_{W,0} \]

\[ \frac{dB_{W}}{dt} = \mu_{2} B_{W} \left( 1 - \frac{B_{W}}{MB_{W}} \right) \rightarrow B_{W} = \frac{MB_{W}}{1 + \left( \frac{MB_{W}}{B_{W0}} \right) e^{-\mu_{2} t}} \]

2.6. Morphology of \( W. cibaria \) biomass

The morphological characterization of \( W. cibaria \) biomass was performed by means optical microscopy (Leica ICC50 W, Switzerland) for wet biomass with Gram staining and in Scanning Electron Microscope (JSM-5910LV, JEOL) for dry biomass [34]. The biomass was obtained as a product of the fermentation process with the optimal experimental condition, using a supplemented fermentation substrate (SFS).

3. Results and discussion

3.1. Fermentation kinetic parameters with \( W. cibaria \)

Table 1 shows the dependent variables (viability, \( B_{W,0}, Y_{Wc/S}, VP_{Wc}, CSC, N_{C}, LA \), and \( V_{P,LA} \)) as a function of the independent variables (pH, T, and SS) during the fermentation kinetics of \( W. cibaria \). Table 2 presents the p-values of the dependent variables produced by the response surface methodology. Figs. 1–4 show the surface and volume response graphs of viability, \( B_{W,0}, Y_{Wc/S}, CSC, N_{C} \), and LA respectively with respect to the effects of the independent variables.

The viability of \( W. cibaria \) varied between 9.43 and 10.08 Log\(_{10} \) (CFU mL\(^{-1} \)), and the ANOVA showed significant differences \((p < 0.05)\) with respect to the T and the linear T-SS interaction. A decrease in viability (blue zone) of the order of 0.6 Log\(_{10} \) (CFU mL\(^{-1} \)) is observed in the response volume graph when T increases (mainly between 39 and 40 °C), which is attributed to the effect of heat stress on the bacteria [35].

In general, \( W. cibaria \) presented better viability when the system was operated at low temperatures (32–36 °C), higher SS (130–150 rpm), and pH between 5.0–5.8. In relation to the T-SS interaction, two areas of importance were identified in the response surface graph when the system was operated at higher SS (140–150 rpm); 1) higher viability at low T (32–34 °C); and 2) higher lethality at high T (38–40 °C). This occurred due to the lower or higher thermal stress generated. Zone 1 favored the reproduction of \( W. cibaria \), and this is attributed to mechanical stress caused by agitation and low T. These conditions induce greater metabolic activity of bacterial cells and, consequently, greater resistance to lethality. The above is explained because \( W. cibaria \) is a facultative anaerobic bacterium that adapts to the presence of \( O_{2} \) without altering its reproduction [35,36]. Furthermore, this behavior is affected by the quadratic interaction of the pH of the system; since, it presents curvilinear behaviors that provide both less and greater favorability in different conditions of T and SS.

This is attributed to the fact that LAB are characterized by their easy adaptation to acidic conditions and wide versatility in diverse temperatures (25–50 °C) [13,16]. Similar results were reported by both Lakra et al. [16], using \( Weissella confusa \) MD1 and MD2 (10.34 - 10.39 Log\(_{10} \) CFU mL\(^{-1} \)) with commercial MRS substrate, and by Zannini et al. [37], using \( W. cibaria \) MG1 (1.31 × 10\(^{9} \) UFC mL\(^{-1} \)) with whole quinoa milk substrate. Lower viability was reported by López et al. [29], when using \( W. cibaria \) 3LA (7.50–7.75 Log UFC mL\(^{-1} \)), \( W. confusa \) 1.9 (7.69–8.66 Log UFC mL\(^{-1} \)), \( W. confusa \) L17 (7.33–7.84 Log UFC mL\(^{-1} \)), and \( W. confusa \) Snc40k (7.34–7.94 Log UFC mL\(^{-1} \)) with commercial MRS fermentation as the substrate added with xylan as a source of carbon.

The production of \( B_{W} \) presented average values that fluctuated between 1.99 and 3.47 g L\(^{-1} \). The ANOVA did not show significant differences \((p < 0.05)\) in the production of \( B_{W} \) with respect to the independent variables (T, SS, and pH) or their interactions, showing a homogeneous behavior (green and yellow-green areas) mainly in the ranges between 2.0 and 3.0 g L\(^{-1} \). This situation indicates that \( W. cibaria \) adapted and effectively assimilated the nutrients from the SFS based on pineapple, sacha inchi, and minerals (in the evaluated ranges of the independent variables). The availability of nutrients in SFS favors the cell growth of \( W. cibaria \) [7,8,26] and the generation of secondary metabolites [9]. In addition, the enzymatic hydrolysis process applied to the substrate allows for increasing the content of disaccharides and monosaccharides [22], compounds easily metabolized by LAB during fermentation [15,18]. LAB such as \( W. cibaria \) are nutritionally demanding microorganisms. Therefore, the substrates used for their growth must be a source of the main macronutrients (C and N) and micronutrients (vitamins and minerals), which are essential in fermentation processes [18,27]. The results obtained in the present investigation were superior to those reported by Ma et al. [38], using \( W. paramesentamentos \) JT13 and commercial fermentation substrate (MRS) (1.17 g L\(^{-1} \)), as well as those obtained by Serna et al. [39], when
Table 1
Fermentation kinetic parameters of *W. cibaria* in substrate formulated with agroindustrial wastes (parameters measured at 10 h of fermentation).

| Dependent Variables | Independent Variables | Viability (Log_{10} CFU mL^{-1}) | B_{W0} (g L^{-1}) | Y_{mic/\phi} | VP_{mic} (g L^{-1} h^{-1}) | CSC (%) | N_{4C} (%) | LA (g L^{-1}) | VP_{LA} (g L^{-1} h^{-1}) |
|---------------------|-----------------------|----------------------------------|------------------|-------------|-------------------------|----------|-------------|---------------|-------------------------|
| Run                 | pH                    | T (°C)                           | SS (rpm)         |             |                         |          |             |               |                         |
| 1                   | 5.0                   | 32                               | 100              | 9.49 ± 0.04 | 1.99 ± 0.09             | 66.52 ± 0.33 | 2.20 ± 0.09 | 72.97 ± 0.14 | 14.40 ± 0.24 |
| 2                   | 6.0                   | 32                               | 150              | 9.88 ± 0.01 | 2.10 ± 0.07             | 26.11 ± 0.35 | 9.77 ± 0.16 | 51.35 ± 0.56 | 22.89 ± 0.80 |
| 3                   | 6.0                   | 40                               | 100              | 9.71 ± 0.04 | 2.25 ± 0.05             | 57.50 ± 0.37 | 3.31 ± 0.05 | 61.11 ± 0.56 | 23.49 ± 0.90 |
| 4                   | 5.5                   | 40                               | 125              | 9.54 ± 0.03 | 2.65 ± 0.07             | 27.40 ± 0.44 | 7.28 ± 0.18 | 67.57 ± 0.98 | 20.60 ± 3.43 |
| 5                   | 5.5                   | 36                               | 125              | 9.72 ± 0.05 | 2.80 ± 0.04             | 17.24 ± 0.47 | 14.32 ± 0.18 | 70.27 ± 0.98 | 18.39 ± 3.05 |
| 6                   | 5.0                   | 36                               | 125              | 9.82 ± 0.05 | 3.31 ± 0.04             | 52.90 ± 0.55 | 5.39 ± 0.25 | 66.67 ± 0.40 | 25.40 ± 4.23 |
| 7                   | 5.5                   | 36                               | 125              | 9.99 ± 0.02 | 2.86 ± 0.04             | 14.11 ± 0.48 | 18.53 ± 0.25 | 67.57 ± 0.70 | 24.20 ± 4.03 |
| 8                   | 5.5                   | 36                               | 125              | 10.08 ± 0.02| 2.83 ± 0.05             | 26.87 ± 0.47 | 8.22 ± 0.16 | 68.42 ± 0.46 | 20.30 ± 3.38 |
| 9                   | 5.5                   | 36                               | 150              | 9.90 ± 0.02 | 2.68 ± 0.04             | 6.78 ± 0.45  | 25.37 ± 0.25 | 65.79 ± 0.98 | 19.10 ± 3.18 |
| 10                  | 5.5                   | 36                               | 125              | 10.02 ± 0.2 | 2.91 ± 0.03             | 20.32 ± 0.48 | 15.26 ± 0.25 | 67.57 ± 0.98 | 19.39 ± 3.22 |
| 11                  | 6.0                   | 36                               | 125              | 9.68 ± 0.04 | 3.20 ± 0.04             | 71.38 ± 0.53 | 4.39 ± 0.09 | 70.59 ± 0.62 | 23.40 ± 3.90 |
| 12                  | 5.5                   | 36                               | 100              | 9.87 ± 0.01 | 3.47 ± 0.08             | 16.40 ± 0.58 | 16.73 ± 0.09 | 68.57 ± 0.56 | 44.50 ± 7.42 |
| 13                  | 5.0                   | 40                               | 150              | 9.43 ± 0.04 | 2.69 ± 0.08             | 5.87 ± 0.45  | 38.20 ± 0.34 | 60.53 ± 1.50 | 19.50 ± 3.25 |
| 14                  | 5.5                   | 32                               | 125              | 9.98 ± 0.02 | 3.26 ± 0.05             | 50.43 ± 0.54 | 15.26 ± 0.65 | 68.42 ± 0.66 | 37.50 ± 6.25 |
| 15                  | 5.5                   | 36                               | 125              | 9.99 ± 0.02 | 2.79 ± 0.08             | 11.48 ± 0.46 | 18.40 ± 0.77 | 67.57 ± 0.50 | 20.30 ± 3.38 |

P values of the ANOVA corresponding to the kinetic parameters of fermentation of *W. cibaria* by effects of temperature (T), pH, and stirring speed (SS).

| Dependent variables | Log_{10} CFU mL^{-1} | B_{W0} (g L^{-1}) | Y_{mic/\phi} | VP_{mic} (g L^{-1} h^{-1}) | CSC (%) | N_{4C} (%) | LA (g L^{-1}) |
|---------------------|-----------------------|------------------|-------------|-------------------------|----------|-------------|---------------|
| pH                  | 0.396                 | 0.840            | 0.319       | 0.842                   | 0.845    | 0.209       | 0.834         |
| T                   | 0.026*                | 0.304            | 0.227       | 0.306                   | 0.228    | 0.766       | 0.114         |
| SS                  | 0.617                 | 0.205            | 0.590       | 0.207                   | 0.197    | 0.353       | 0.032*        |
| pH²                 | 0.044*                | 0.803            | 0.008*      | 0.808                   | 0.027*   | 0.216       | 0.268         |
| pH + T              | —                     | 0.170            | 0.136       | 0.171                   | 0.137    | 0.055       | 0.049*        |
| pH + SS             | 0.132                 | 0.176            | 0.698       | 0.177                   | 0.024*   | 0.889       | 0.128         |
| T²                  | 0.053                 | 0.189            | 0.344       | 0.189                   | 0.563    | 0.109       |               |
| T - SS              | 0.043*                | 0.942            | 0.557       | 0.938                   | 0.310    | 0.008*      | 0.494         |
| SS²                 | —                     | 0.358            | 0.043*      | 0.357                   | 0.023*   | 0.046*      | 0.496         |

*Significant for p < 0.05.

Fig. 1. Response surface and volume plots of viability of *W. cibaria* (Log_{10} UFC mL^{-1}) as a function of the independent variables (T, pH, and SS) during the fermentation process.
using W. confusa and a worm meal-based substrate as a source of N$_2$. (1.47 g L$^{-1}$). The Y$_{Wc/S}$ presented significant differences ($p < 0.05$) with respect to the pH-pH and SS-SS interactions, and high variability fluctuating between 5.87 and 66.52g$^{-1}$. The quadratic interactions show the curvature of the response surface graphs, obtaining the highest Y$_{Wc/S}$ at extreme pH (5.0 and 6.0) values and intermediate SS (120 rpm) values (Fig. 2).

CSC presented statistical differences ($p < 0.05$) with the pH-SS, pH-pH and SS-SS interactions, and the CSC fluctuated between 2.2–38.2%. These interactions show a curvature of the response surface graphs, where the lowest CSC consumption was found at pH (5.0–5.2) and SS (100–110 rpm), and the highest consumption was at pH (5.0–5.2) and SS (140–150 rpm) (Fig. 3). The low CSC is attributed to the fact that it takes two routes during the process. In one case, it is a nutrient or energy source for cell construction during the reproduction of W. cibaria [31], and in another, it goes to the production of LA [4,18]. Generally, CSC is progressive during fermentation kinetics [40], and it could even run out at the end of the process. However, the low CSC in this research is attributed, firstly, to the greater depletion of N$_2$ that limits the continuity of cellular reproduction, and, secondly, to the complex molecular structure of SFS. This is rationalized give that pineapple wastes and sacha inchi residues have high fiber and protein contents, respectively [23,26]. Indeed, a greater use of the lignocellulosic material can be achieved through the application of pretreatments (homogenization or thermal and enzymatic hydrolysis) [22].

Serna et al. [41], and Serna et al. [19], evaluated the CSC in W. cibaria and found that the lower or higher CSC depends on the type of substrate used. Therefore, the presence of macro and micronutrients favors its metabolic capacity. On the other hand, various researchers have shown the progressive consumption of the C source in different applications: W. cibaria in commercial MRS substrate [22]; W. cibaria MG1 in maltose and sucrose substrate [37]; B. coagulans on glucose and fructose substrate [42]; L. crustorum W19 and L. sanfranciscensis MR29 on wheat straw hydrolyzate substrate [18], among others.

On the other hand, the T-SS interaction and the quadratic S-S interaction affected the N$_2$C variable. The response surface plot shows a large area where N$_2$C becomes higher (blue area) (70–73%) (Fig. 3). This is consistent with the behavior of the high viability of the microorganism, which varied only by 1 Log unit. These results confirm that the bacterium W. cibaria easily adapts to the evaluated process conditions. It efficiently assimilates and metabolizes the N$_2$ source, which is an essential macronutrient for its cellular structure [5].

The supply of essential nutrients in the fermentation substrate is important for cell reproduction. The cell cycle of bacteria depends on the composition of the substrate, concentration of available nutrients, process conditions, and physical conditions such as pH, T and SS, among others [13,35,36]. The limiting nutrient source of the fermentation process was N$_2$, since this macronutrient was depleted first (73.0%) in (Fig. 3). The low CSC is attributed to the fact that it takes two routes during the process. In one case, it is a nutrient or energy source for cell construction during the reproduction of W. cibaria [31], and in another, it goes to the production of LA [4,18]. Generally, CSC is progressive during fermentation kinetics [40], and it could even run out at the end of the process. However, the low CSC in this research is attributed, firstly, to the greater depletion of N$_2$ that limits the continuity of cellular reproduction, and, secondly, to the complex molecular structure of SFS. This is rationalized give that pineapple wastes and sacha inchi residues have high fiber and protein contents, respectively [23,26]. Indeed, a greater use of the lignocellulosic material can be achieved through the application of pretreatments (homogenization or thermal and enzymatic hydrolysis) [22].
corresponds to a VP$_{LA}$ of 2.4 and 7.42 g L$^{-1}$ h$^{-1}$, respectively.

The response volume graph identifies the process conditions with the highest production of LA: T = 32–36 °C, SS (100–110 rpm), and pH (5.4–6.0) (Fig. 4). This area corresponds to the conditions with the highest production of B$_{WC}$, showing that cell growth is proportional to the production of LA [18]. The above is explained because W. cibaria is a heterofermentative LAB, a characteristic that favors the production of various secondary metabolites (LA, propionate, butyrate, acetate, among others [14]. In the present investigation and under the process conditions evaluated, LA production was favored (44.5 g L$^{-1}$) with mixed culture of W. cibaria WC018 + L. plantarum LP067 using commercial MRS [43] . Other investigations obtained higher concentrations of LA: T (32 °C) compared to pH, SS, and N$_C$, evaluated experimentally. In addition, the theoretical values were predicted by the mathematical and experimental models obtained from 3 replicates at the optimal conditions of the independent variables. The RME was determined in order to validate the results of the mathematical models.

The R$^2$ values showed a good acceptable fit of the mathematical models (R$^2$ ≥ 85%) for the dependent variables log$_{10}$ CPU, Y$_{Bwc/S}$, VP$_{WC}$, CSC, N$_C$, and LA) and their respective R$^2$ values. Table 4 presents the criteria, weights, and impacts established in the experimental optimization of multiple responses during the fermentation process of W. cibaria. In addition, the theoretical values were predicted by the mathematical and experimental models obtained from 3 replicates at the optimal conditions of the independent variables. The RME was determined in order to validate the results of the mathematical models.

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Table 3 presents the regression coefficients of the 2nd order polynomial models of the dependent variables (viability, B$_{WC}$, Y$_{Bwc/S}$, VP$_{WC}$, CSC, N$_C$, and LA) and their respective R$^2$ values. Table 4 presents the criteria, weights, and impacts established in the experimental optimization of multiple responses during the fermentation process of W. cibaria. In addition, the theoretical values were predicted by the mathematical and experimental models obtained from 3 replicates at the optimal conditions of the independent variables. The RME was determined in order to validate the results of the mathematical models.

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3.3. Specific growth rate (μ)

Table 5 presents the experimental and theoretical μ values of W. cibaria obtained from the production kinetics of B$_{WC}$ for each evaluated experiment. In addition, it presents the adjusted R$^2$ coefficients according to the order 1 and logistic models.

The μ values of W. cibaria in the SFS were higher in all cases (0.23 - 0.30 h$^{-1}$) compared to μ$_2$ (0.16 - 0.22 h$^{-1}$). However, the logistic model presented a better regression fit for the estimate of μ for W. cibaria reproduced in the SFS, where the R$_2$ values were 0.99 in all cases. In the model of order 1, the estimate of μ$_1$ fluctuated between 0.85 and 0.95. Similar results of μ have been reported by Serna et al. [39] (0.28 h$^{-1}$) and by Micaquero-Carlosama et al. [22], (0.27 h$^{-1}$). W. cibaria is a rare bacteria species that had a better production and viability of W. cibaria in the SFS.

The behavior of the growth kinetics of W. cibaria obtained under the optimal process conditions was similar to the kinetics obtained when the commercial substrate (MRS) was used [22]. Although, the absence of an adaptation phase was observed (Fig. 5), which indicates the easy and rapid adaptation of W. cibaria to the SFS. This is attributed to the previous adaptation that the bacteria had in the preparation phase for the fermentation inoculum. Furthermore, the optimal process conditions allowed for improved cell production (3.16 ± 0.19 g L$^{-1}$) compared when SFS was used in reference conditions (T = 36 °C, pH = 6, SS = 100 rpm) (2.93 ± 0.03 g L$^{-1}$) [22]. This highest cell growth occurred during the exponential phase (10 h). Next, an asymptotic behavior was identified, and this defined the stationary phase, as well as a B$_{WC}$ corresponding to 3.13 ± 0.05 (g L$^{-1}$). The easy adaptation of W. cibaria in SFS is attributed to the fact that the substrate was specifically formulated to meet the nutritional requirements (macronutrients and micronutrients of W. cibaria).

On the other hand, the evaluation of the μ obtained to the optimal experimental condition and using the order 1 and logistic models, presented the following results: μ$_1$ = 0.24 h$^{-1}$ and μ$_2$ = 0.28 h$^{-1}$, behavior similar to that described for all experiments (μ$_1$ > μ$_2$). Additionally, the assessment of the R$^2$ for the two models reported values of 0.90 and

**Table 3**

| Regression coefficient | log$_{10}$ CPU ml$^{-1}$ | B$_{WC}$ (g L$^{-1}$) | Y$_{Bwc/S}$ | VP$_{WC}$ (g L$^{-1}$ h$^{-1}$) | CSC (%) | N$_C$ (%) | LA (g L$^{-1}$) |
|------------------------|-------------------------|----------------------|-------------|-------------------------------|---------|---------|----------------|
| Constant               | -39.48                  | -114.49              | 576.34      | -19.04                        | -1806.63| 339.77  | -2413.11       |
| pH                     | 8.68                    | 17.43                | 1624.57     | 2.89                          | 571.51  | 0.59    | 549.86         |
| T                      | 0.94                    | 2.97                 | -95.28      | 0.50                          | 15.62   | -10.15  | 41.00          |
| SS                     | 0.16                    | 0.30                 | 7.22        | 0.05                          | -0.35   | -1.51   | 4.20           |
| pH$^2$                 | -0.65                   | -0.25                | 123.41      | -0.04                         | -31.42  | -6.73   | -18.57         |
| pH × T                 | -0.01                   | -0.04                | 0.48        | 0.00                          | -0.10   | -0.14   | -         |
| T$^2$                  | -0.01                   | -0.02                | 0.06        | 0.00                          | 0.07    | -0.04   | -         |
| SS$^2$                 | 0.00                    | 0.00                 | 0.00        | 0.00                          | 0.01    | -0.01   | 0.00           |
| R$^2$                  | 84.94                   | 71.91                | 89.89       | 71.86                         | 93.38   | 95.18   | 69.89          |
15 treatments in the statistical design, which guarantees an acceptable prediction of the model of order 1 and excellent in the logistic model. These values were similar to those achieved for the experiments 5, 7, 8, 10 and 15 are replicates obtained under the same fermentation process conditions. R² values for the experimental and theoretical interpretation of the references to color in this figure, the reader is referred to the web version of this article.

### Table 4

Experimental optimization of multiple responses in the fermentation process with *W. cibaria*.

| Dependent variables                          | Criteria   | Weight | Impact | Theoretical Optimum | Experimental Optimal | RME (%) |
|----------------------------------------------|------------|--------|--------|----------------------|----------------------|---------|
| Viability (Log_{10} CFU mL⁻¹)                | Maximize   | 1.0    | 5      | 10.03                | 10.07 ± 0.05         | 0.39    |
| \( b_w \) (g L⁻¹)                            | Maximize   | 1.0    | 5      | 2.96                 | 3.16 ± 0.19          | 6.33    |
| \( V_{net/s} \)                              | 38.63      | 0.3    | 1      | 45.16                | 25.38 ± 0.11         | 77.93   |
| VPW (g L⁻¹ h⁻¹)                              | Maximize   | 0.9    | 4      | 0.49                 | 0.53 ± 0.03          | 7.55    |
| GSC (%)                                      | Minimize   | 0.6    | 3.0    | 16.95                | 45.60 ± 0.04         | 62.83   |
| NcG (%)                                      | Minimize   | 0.6    | 3.0    | 63.57                | 63.46 ± 1.82         | 0.17    |
| LA (g L⁻¹)                                   | Minimize   | 0.8    | 4.0    | 19.23                | 20.66 ± 1.27         | 6.92    |
| VP_A (g L⁻¹ h⁻¹)                             | Minimize   | 0.8    | 4.0    | 3.20                 | 3.44 ± 0.21          | 6.98    |

Note: Experiments 5, 7, 8, 10 and 15 are replicates obtained under the same fermentation process conditions. \( R^2_{\text{ander}} \): Regression coefficient - linear model and \( R^2_{\text{LE}} \): Regression coefficient - logistic equation.

### Table 5

Results of experimental and theoretical \( \mu \), and the real and adjusted \( R^2 \) coefficients of the growth kinetics of *W. cibaria*.

| Variable | Run | T1  | T2  | T3  | T4  | T5  | T6  | T7  | T8  | T9  | T10 | T11 | T12 | T13 | T14 | T15 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| \( \mu_1 \) (h⁻¹) | 0.23 | 0.23 | 0.25 | 0.26 | 0.28 | 0.29 | 0.27 | 0.27 | 0.27 | 0.27 | 0.30 | 0.29 | 0.27 | 0.27 | 0.28 | 0.26 |
| \( \mu_2 \) (h⁻¹) | 0.16 | 0.16 | 0.18 | 0.19 | 0.21 | 0.22 | 0.20 | 0.20 | 0.20 | 0.21 | 0.22 | 0.22 | 0.20 | 0.21 | 0.20 | 0.20 |
| \( R^2_{\text{order1}} \) | 0.90 | 0.85 | 0.95 | 0.93 | 0.92 | 0.95 | 0.91 | 0.88 | 0.94 | 0.90 | 0.91 | 0.92 | 0.94 | 0.91 | 0.91 | 0.86 |
| \( R^2_{\text{LE}} \) | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |

### 3.4. Morphology of *W. cibaria* biomass

The product of the fermentation process consists of *W. cibaria* biomass and SFS (Fig. 5). Before using the strain, its purity is checked by biochemical tests by API 50 CHL Kit (positive values for Amygdalin, Arbutin, Esculin, Salicin, Cellobiose, Maltose and Sucrose, and Catalase negative). (4) LA production (range 11 to 12 g L⁻¹ during 12 h) and (5) Antimicrobial activity [19,39,41]. Micrograph (A) shows Gram positive stained bacteria and micrograph (B) shows a large amount of agglomerated biomass and in its characteristic bacilli form (Fig. 6).

### 4. Conclusions

The research allowed for experimental optimization of *W. cibaria* reproduction and viability, showing that the temperature and stirring speed independent variables had a greater influence on the fermentation process. The non-extreme conditions of temperature and higher stirring speed allowed for greater use of the substrate and metabolic efficiency of *W. cibaria*, which was reflected in the results obtained in yield and volumetric productivity. It was evidenced that the SFS formulated based on pineapple and sacha inchi wastes provided the macronutrients (C and N₂) and micronutrients (Na, NH₃, K, and Mg) necessary for effective cell reproduction. Therefore, it is inferred that the use of agroindustrial waste as fermentation substrates is an excellent alternative for optimizing probiotic production processes, contributing positively to environmental impacts and, in effect, to the circular economy.

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### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Supplementary materials

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