Research article

Formulation of a fermentation substrate from pineapple and sacha inchi wastes to grow *Weissella cibaria*

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ABSTRACT

Gold honey variety pineapple wastes and sacha inchi sub-products (SIS) were characterized in their elemental, physical, and chemical form in order to formulate a supplemented fermentation substrate (SFS) for the growth *Weissella cibaria*. The peels and fresh cores of the pineapple (FPP, FPC) were dried and ground (PPP, PPC) and then mixed (MCPP). The following procedures were then undertaken: a physicochemical characterization (moisture, a_w, pH, acidity, and soluble solids) of the SIS, FPP, FPC, PPP, and PPC; a proximal characterization of he FPP, FPC, SIS, and SFS; and an elemental analysis ($C–N_2–H_2–O_2–S$) of the MCPP, SIS, and *W. cibaria*, which allowed the stoichiometric equation to be defined and the SFS to be formulated. We then evaluated the effect that homogenization and heating to boiling point had on the concentration of reducing sugars in the SFS (g L^{-1}). Finally, *W. cibaria's* kinetic fermentation parameters were evaluated in the SFS and in a commercial substrate (control). The results showed FPP and FPC yields of 26.02 ± 0.58 and $14.69 \pm 1.13\%$, respectively; a higher total sugar content in FPC (7.21%) than in FPP (6.65%); a high crude protein content in SIS (56.70%), and a $C:N_2$ ratio of 6.50:1.00. Moreover, the highest concentration of reducing sugars (4.44 ± 0.29 g L^{-1}) in the SFS was obtained with 5 h of hydrolysis under homogenization pre-treatments and heating until boiling. The SFS allowed the adaptation of *W. cibaria*, and there was a biomass production of 2.93 g L^{-1} and a viability of 9.88 log CFU mL^{-1}. The formulation of an unconventional fermentation substrate from -Agro-industrial wastes of pineapple and sacha inchi to produce valuable products (such as lactic acid biomass through fermentation), is an excellent perspective for large-scale application.

1. Introduction

The use of agro-industrial wastes to produce lactic acid (LA) and probiotic biomass has been studied in recent years (Cizeikiene et al., 2018; Rolim et al., 2018). Since agro-industrial and organic wastes are materials rich in lignocellulosic compounds, they can be important sources of micro and macro nutrients as well as direct sources of C and N_2 in fermentation processes.

Processed pineapple (*Ananas comosus*) is one of the world's most widely consumed tropical fruits. The residues generated during the processing of the fresh peel of the pineapple (FPP), Fresh core of the pineapple (FCP), and crown represent between 45 and 65% of the total weight of the fruit; about 76% of this waste is fibre, 99.2% is the insoluble fraction, and 0.8% is the soluble fraction (Selani et al. 2014). These residues have been used in several ways (Difonzo et al. 2018) including

the use of pineapple wastes as substrates to obtain bioactive compounds such as biopolymers (Vega-Castro et al., 2016), polyphenols (Sepúlveda et al., 2018), biohydrogen, biogas, and alcohols such as ethanol or butanol (Khedkar et al., 2017), which have the potential for industrial application. Among the evaluated residues, the pineapple peel is outstanding due to the high concentration of reducing sugars (97 g L^{-1}) obtained through the application of acid hydrolysis as a pre-treatment (Vega-Castro et al. 2016). In addition, pineapple waste has been used to produce cellular protein (Mensah and Twumasi, 2017). Pineapple is part of the bromeliad family, and, in 2017, world production was 27 million tons with a harvested area of 1 million ha. This is mainly concentrated in Asia and America, particularly in Costa Rica, the Philippines, Brazil, China, and Colombia (FAOSTAT, 2018).

Moreover, sacha inchi (*Plukenetia volubilis*) is a native plant to the jungle region between Peru, Bolivia, and Brazil. Although the crop is still

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incipient, world production has increased in recent years, mainly in Peru followed by Thailand and then Colombia: countries that export to Europe (France, Spain, and Germany), South Korea, and the United States (Saengsorn and Jimtaisong, 2017). The main interest in this crop has been the seed to obtain oil, which is characterized by having a high unsaturated fatty acid content: mainly oleic acid, linoleic, and linolenic (Vásquez-Osorio et al., 2017). The oil extraction process generates a sub-product with a high protein content (59%) (Wang et al., 2018), which represents between 60 and 75% of the seed's total weight (Vásquez-Osorio et al., 2017). Currently, these sub-products are not effectively used in production systems (Dai and Huang, 2016) that cause environmental problems due to their easy decomposition (Neethu et al. 2015). The scientific literature does not report the use of this sub-product as a fermentation substrate.

In the bioprocessing industry, there is interest in the use of residual materials due to their easy accessibility and the C and N_2 content, necessary elements in fermentation processes (Neethu et al. 2015); however, the greatest interest is the possibility of reducing the costs associated with the substrate, given that these constitute between 60 and 70% of the total cost of the process (Khedkar et al. 2017).

Moreover, different studies have demonstrated the probiotic ability of *Weissella cibaria*. This lactic acid bacterium survives in low pH environments; resists passage through the small intestine (Garcia and Serna, 2015); has efficient antimicrobial activity against pathogenic microorganisms for plants, animals, and man (*Xanthomonas albilineans, Staphylococcus aureus* and *Streptococcus agalactiae*) (Serna et al., 2010; Serna, Camargo and Rengifo, 2013a; Serna, Mera, and Angulo, 2013b), has inhibitory effect gainst Gram negative microorganisms such as *Escherichia coli* and *Klebsiella pneumoniae* (Serna and Valencia, 2013), and has the ability to adhere to bovine mammary gland epithelium (Serna and Pabon, 2016). These characteristics promote its application in food industry products. The aim of this research was to characterize fresh and powder peel of the pineapple (FPP, PPP) and the fresh and powder cores (FPC, PPC) of gold honey variety pineapple and sacha inchi sub-product (SIS), in elemental, physical, and chemical terms in order to formulate a supplemented fermentation substrate (SFS) to grow *W. cibaria* lactic acid bacteria (LAB).

2. Materials and methods

2.1. Wastes conditioning and yield assessment

FPP and FPC were used from gold honey variety pineapples that have a degree 4 ripeness according to the colour scale for fresh pineapples (Colombian technical standard, CTS 729-1) and a 1.70 ± 0.23 kg average weight. The fruits were purchased at a local supermarket (Valle del Cauca, Colombia). In addition, SIS was used. This was obtained from the process of extracting oil from the seeds, which was provided by Agroincolsa SAS (Putumayo, Colombia).

The following materials were characterized: FPP, FPC, powdered pineapple peel (PPP), powdered pineapple core (PPC), a mixture of PPP and PPC with 1.5:1.0 ratios, respectively, mixture of core and peel powder (MCPP), SIS, and supplemented fermentation substrate (SFS).

The pineapples were washed with pressurized water, sanitized with hypochlorite solution (200 ppm), and then peeled. The percentage yield of FPP, FPC, and crown was calculated using Eq. (1) for which the total weight corresponds to the whole pineapple, including the crown. When calculating the PPP and PPC yield, the total weight corresponds to the addition of both (PPP + PPC).

$$Yield = \frac{Waste\ weight}{Total\ weight} * 100\ (\%) \qquad (1)$$

The powdered pineapple wastes were obtained by freeze-drying FPP and FPC using a freeze-dryer (Labconco, Freezone 6 Plus, USA) operating with a 0.110 mbar vacuum, -80 °C condenser temperature, and 0.03 °C

min^{-1} heating speed until a final temperature of 26 °C was reached. The freeze-dried pineapple wastes (PPP, PPC) and the SIS was independently subjected to a grinding process (IKA A11 knife mill, Brazil) and sieved using a 16, 30, and 60 mesh number. The material was selected with a <250 μm (60 mesh) particle size (Codex Standard 152–1985). In addition, the pineapple wastes ratios for FPP/FPC and PPP/PPC were calculated.

2.2. Sources of C and N_2

MCPP and SIS were used as sources of C and N_2 to formulate a fermentation substrate. This substrate (SFS) was supplemented with $C_2H_3NaO_2$, $C_6H_5O_7*2NH_3$, K_2HPO_4, and $MgSO_4$ (analytical grade reagents, Sigma Aldrich. Germany), and the commercial substrate De Man Rogosa Sharpe (MRS) (1960) was taken as a reference. The SFS was pretreated, hydrolysed, and finally used to reproduce a LAB.

2.3. Lactic acid bacteria

W. cibaria bacterium from the Biotechnology Institute of the National University of Colombia (IBUN 090–03684 strain and gene bank) was used to test the efficiency of SFS.

2.4. Proximal analysis

Proximal analyses were performed on FPP, FPC, SIS, and SFS. The amount of total sugars was determined using the Antrona method with an UV-VIS spectrophotometric (Thermo Scientific - Genesys 10UV, USA) (Miller, 1959); the total protein content was performed with the Kjeldahl method No. 2.062 (AOAC, 1990); neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the gravimetric Van Soest method (AOAC 973.18, 1990); ethereal extract was determined with the Soxhlet methodology (AOAC 920.39, 1990); and mineral content (Ca, Cu, P, Fe, Mg and K) was determined with atomic absorption spectrometry (AOAC 942.05, 1990).

2.5. Physicochemical properties

The pH of FPP, FPC, PPP, PPC, SIS, and MCPP were measured according to CTS 4592 (ICONTEC, 1999); acidity was measured according to CTS 4623 (ICONTEC, 1999); soluble solids ($^{\circ}$Brix) were measured according to CTS 4624 (ICONTEC, 1999); moisture was measured according to AOAC 130.15, (1990); and water activity (a_w) was measured using a dew point hygrometer (25 °C) (AquaLab 4TE).

2.6. Elemental analysis

Elemental analyses (C, N_2, O_2, H_2, S) were performed on MCPP, SIS, *W. cibaria* biomass grown in MRS (*Wc*-MRS), and *Weissella cibaria* in supplemented fermentation substrate (*Wc*-SFS) using an Elemental Analyzer (Exeter CE-440, USA) according to ASTM D5373 (2016). Based on the results, the number of atoms for elements C, H_2, N_2, and O_2 was estimated, and the empirical molecular formulas of MCPP, SIS, *Weissella cibaria* in Man Rogosa Sharpe (*Wc*-MRS), and *Wc*-SFS (Ebbing and Gammon, 2016) were obtained.

2.7. Stoichiometry of the chemical reaction of fermentation

To calculate the coefficients of the stoichiometric equation of *Wc*-MRS production, Eq. (2) was used. This equation corresponds to the general equation of biomass production and LA, where the first and second terms corresponded to the MCPP and SIS wastes, respectively, the third term to *Wc*-MRS, and the fourth term to LA. The terms α, Biomass yield on substrate consumed ($Y_{X/S}$) and Product yield on substrate consumed ($Y_{P/S}$) refer to the coefficients of the balanced equation, and

the x, y, z, f, g, h, i, j, k, l sub-indices correspond to the estimated value of atoms for the elements C, H_2, N_2, and O_2 for each of the compounds. The coefficients were determined for batch lactic fermentation.

$$C_x O_y H_z + \alpha C_f O_g H_h \rightarrow Y_{x/s} C_i H_j O_k N_l + Y_{p/s} C_3 H_6 O_3 \qquad (2)$$
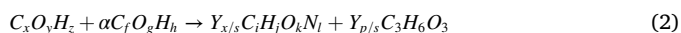
2.8. C:N_2 ratio of MCPP and SIS, and substrate formulation

Molecular weights of MCPP, SIS, and *Wc*-MRS compounds were estimated based on their empirical formulas. The coefficients α, $Y_{X/S}$, and $Y_{P/S}$ from Eq. (2) were determined with a matter balance. The following hypotheses were considered for the balance: closed system, anaerobic, and 100% substrate conversion. Using the molecular weights and coefficients from the balanced chemical equation, we obtained the relationship C:N_2, which was provided by MCPP and SIS. The SFS was formulated for the growth of *W. cibaria* using the C:N_2 relation.

2.9. SFS pre-treatments

The SFS was diluted in distilled water (0.1 kg L^{-1}) and three pre-treatments were applied; Treatment with homogenization and enzymatic hydrolysis (T1): homogenization (15000 rpm, time 5 min) in ulltraturrax (LSK High Shear Mixer, New York); Treatment heating to boiling and enzymatic hydrolysis (T2): heating to boiling (1 min), and Treatment with homogenization, boiling and enzymatic hydrolysis (T3): corresponding to T1+T2. Each treatment was then hydrolysed with the enzyme Cellic®CTec2 (Novozyme, Denmark) (0.01 L kg^{-1} of SFS on a dry basis). The process was carried out at optimal enzyme conditions, 45 °C and 150 rpm with continuous orbital homogenization in a Shaker (VWR-Incubating Orbital Shaker, USA) (Hu et al. 2016). Each treatment was performed in triplicate. The concentration of reducing sugars (g L^{-1}) was determined at the beginning of each pre-treatment and during hydrolysis (every hour for 5 h). We took 10 mL samples; they were then centrifuged at 2862 g for 10 min at 4 °C (Penendorf centrifuge 5804 R, Germany). The reducing sugars were determined in the supernatant using the 3.5-dinitrosalicylic acid method (Miller, 1959) with a UV-VIS spectrophotometer (ThermoScientific - Genesys 10UV, USA). The treatment with the highest concentration of reducing sugars for the reproduction of *W. cibaria* was selected from the statistical analysis.

2.10. Fermentative reproduction of Weissella cibaria

W. cibaria was propagated by discontinuous fermentation in the pre-treated SFS (*Wc*-SFS), and, as a control treatment, *W. cibaria* was grown on MRS substrate (*Wc*-MRS). Batches of 0.6 L were prepared using a 1 L capacity reactor (BioFlo®/CelliGen® 115, Germany) at constant process conditions (36 °C, pH = 6, 100 rpm, 24 h). Substrates were sterilized at 121 °C for 15 min and then incorporated with activated inoculum (10% v/v) following Serna and Rodríguez's (2007 and 2013a) methodology but including some modifications. Adapting the bacteria to the substrates was performed in two generations: in the 1st generation, the activation was carried out in commercial MRS substrate that was supplemented with glucose (0.04 kg L^{-1}), with a 0.005 L working volume; and, in the 2nd generation, the bacteria was adapted in pre-treated SFS with a 0.05 L working volume.

Fermented samples (20mL) were measured for 24 h at various times (0, 2, 4, 6, 8, 10, 12, and 24 h), and the bacteria was counted (colony forming units, CFU mL^{-1}) by plate sowing (36 °C, for 48h). Subsequently, each sample was centrifuged at 2862 g for 10 min at 4 °C, filtered (0.45 μm qualitative filter), and the biomass concentration (g L^{-1}) was determined by dry weight according to the AOAC (934,01. 1990) standard method. The following variables were quantified in the supernatants: LA (g L^{-1}) (through reflectometry, Relectoquant Merck - Roflex Plus 10, Germany), nitrogen source consumption (g L^{-1}) (through determining free amino nitrogen (FAN), using NaOH (0.1 N) with Eq. (3) and the

carbon source consumption (%) with Eq. (4). In fermentation kinetics the maximum specific growth rate (μ) was calculated whit Eq. (5).

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$$FAN \ (mg/L) = \frac{mL \ NaOH \ (0.1N)*1.4*1000}{(mL \ of \ sample)} \qquad (3)$$

$$SC = \frac{(S_o - S_t) * 100}{S_o}\% \qquad (4)$$

$$\mu = \frac{dx}{dt} \ h^{-1} \qquad (5)$$

S_0 and S_t represent the concentrations of reducing sugars at 0 and sampling time, respectively.

2.11. Statistical analysis

In the fermentative process with *W. cibaria* grown in the pre-treated SFS, a completely random unifactorial design was used. Pre-treatment of the substrate was the independent variable, which had four levels: T1, T2, T3, and Control treatment (T0) (control treatment); and the reducing sugars (g L^{-1}) was the dependent variable. Furthermore, a unifactorial design was used in the fermentative reproduction process of *W. cibaria*: the independent variable was the substrate with two levels (SFS and MRS); and the dependent variables were biomass production (g dry cell weigth L^{-1}), viability (CFU mL^{-1}), C source consumption (%), N_2 source consumption (%), and LA production (g L^{-1}). Data in triplicate were analysed by variance analysis (ANOVA). Tukey's mean comparison test was applied with a 95% confidence level using Minitab software (version 18).

3. Results and discussion

3.1. Characterization of pineapple and sacha inchi wastes

3.1.1. Proximal characterization
Table 1 shows the yields of FPP, FPC, PPP, and PPC gold honey variety pineapple wastes and the relationships between the different types of wastes (fresh and powdered). The results show representative values for these different types of wastes: FPP had the highest percentage participation, followed by FPC, and then crown.

It has been reported that during the commercial production of pineapple juice around 20–40% of wastes are generated by FPP and FPC (Khedkar et al. 2017). This percentage is similar to the one obtained in this research and has led to research for which these wastes are used as substrates in fermentative and hydrolytic processes to obtain bioactive compounds (Kaprasob et al. 2017). Moreover, Sepúlveda et al. (2018)

Table 1. Yield percent (R) and the ratio of peel wastes to fresh cores (FPP/FPC) for peel and powder cores (PPP/PPC) that come from the gold honey variety pineapple.

Pineapple wastes	Yield (%)	Ratio
FPP	26.02 ± 0.58	–
FPC	14.69 ± 1.13	–
Fresh Crown	13.68 ± 1.55	–
PPP	60.52 ± 1.26	–
PPC	39.48 ± 1.26	–
FPP/FPC	–	1.76
PPP/PPC	–	1.53

Table 2. Proximal characterization of fresh peels and cores (FPP and FPC) from gold honey variety pineapples, SIS, and SFS.

Component	FPP	FPC	SIS	SFS
Total sugars (% p/p)	6.65	7.21	4.93	30.51
Protein (% p/p)	0.80	0.40	56.70	33.90
NDF (% p/p)	5.40	1.80	14.20	17.00
ADF (% p/p)	2.50	1.00	5.90	9.30
Ethereal extract (% p/p)	–	–	6.56	3.58
Ca (mg/kg)	189.00	57.00	0.42	240.00
Cu (mg/kg)	4.90	4.90	19.00	13.00
P (mg/kg)	264.00	49.00	0.81	850.00
Fe (mg/kg)	8.00	4.90	66.00	48.00
Mg (mg/kg)	81.00	121.00	0.49	270.00
K (mg/kg)	0.20	819.00	0.90	1390.00

reported that 96% of the total pineapple wastes correspond to organic material and 4% to inorganic, which potentiates its use in bioprocesses.

The proximal characterizations of FPP and FPC, SIS, and SFS are shown in Table 2. FPC and FPP had low sugar and protein contents; however, they are considered important contents for the formulation of fermentation substrates because they constitute essential macronutrients as sources of C and N$_2$ (Hu et al. 2016; Neethu et al. 2015). In general, there is no detailed information on the main components of pineapple wastes, so the results shown in our research are relevant to different developments.

Vega-Castro et al. (2016) obtained higher values in sugars with the same variety (27.08%), while Roha et al. (2013) reported similar values in the cayena lisa variety (7.37% with respect to total waste). Regarding the protein content of FPP, Vega-Castro et al. (2016) reported a similar value (0.63%). The NDF and ADF contents were higher for FPP than for FPC due to that, in the cell wall of the peels there are more complex structures, such as cellulases, hemicelluloses, and lignins. These lignocellulosic compounds can be found between 42 and 70% in FPP (Dai and Huang, 2016; Khedkar et al. 2017); therefore, they are considered important sources of carbohydrates that can be made through hydrolytic processes that make them more available (Banerjee et al., 2017). Variations in proximal composition are attributed to factors including variety, soil, primary production, climatic conditions, and state of ripeness (Difonzo et al. 2018).

The contents of P, Ca, K, and Mg in FPP and FPC are part of the microelements necessary for the growth of bacteria and yeasts (Cizeikiene et al. 2018). Ca and Mg favour lignocellulosic enzymatic activity when used as fermentation substrates (Cizeikiene et al. 2018).

The protein content of FPP and FPC was low, while SI had a considerably high protein and lignosellulosic content (Table 2). Therefore, SIS favours SFS since N$_2$ is a necessary element in microbial metabolism during cell growth (Mensah and Twumasi, 2017). Similar protein contents in SIS (59%) have been reported by Wang et al. (2018). Moreover, the fat content of SIS is composed mainly of polyunsaturated fatty acids including oleic, linoleic, and linolenic acids (Vásquez-Osorio et al., 2017), which are acids that favour the growth of lipolytic bacteria (Neethu et al. 2015).

3.1.2. Physicochemical properties

The moisture content, a$_w$, total soluble solids (TSS), pH, and acidity of FPP, FPC, PPP, PPC, SIS, and MCPP are given in Table 3. The high moisture and a$_w$ values in FPP and FPC, are favourable environments for microbial growth; while they were significantly lower and much more microbiologically stable for PPP, PPC, MCPP, and SIS. In other words, they tend to become more conserved with time.

Some research has reported moisture values between 70.9% and 80% for pineapple wastes (Vega-Castro et al. 2016) and an a$_w$ of 0.14 for freeze-dried pineapple pomace (Selani et al. 2014). Meanwhile, SIS reports moisture values between 3.3 and 8.32% (Wang et al.2018) and a$_w$ values of 0.5 (Vásquez-Osorio et al., 2017). An a$_w$ of 0.374 $\pm$ 0.01 was obtained in the SFS, which provides stability in the substrate.

The wastes' TSS are mainly comprised of sugars and a small proportion of acids, salts, and hydrosoluble components, which can serve as minor elements to improve the nutritional conditions of microorganisms used in the fermentation processes.

Acidic pH values of FPP, FPC, PPP, PPC, and MCPP (3.5–3.7) may limit microbial growth in SFS because they reduce their metabolic capacities (Moumita et al. 2017). However, the presence of SIS allowed the final pH of the SFS to be increased and be able to quickly adapt to the culture medium, which improved the fermentation conditions as well as its cellular reproduction (Idris and Suzana, 2006; Rolim et al. 2018). Studies have reported pH values of 4.03 (ground pineapple peel); 4.5 (liquid pineapple extract) (Idris and Suzana, 2006; Difonzo et al. 2018); and 6.4 for SIS (Vásquez-Osorio et al., 2017). Acidity values have been reported between 0.11 and 2.01% (citric acid) (Idris and Suzana, 2006; Selani et al. 2014).

3.1.3. Elemental analysis and empirical formulas of MCPP, SIS, biomass Wc-MRS, and Wc-SFS

Table 4 shows the results of the elemental analysis and the estimation of the empirical formulas and molecular weights of the MCPP, SIS, *W. cibaria* lyophilized biomass reproduced in a commercial substrate (*Wc*-MRS), and *W. cibaria* lyophilized biomass reproduced in the pretreated SFS (*Wc*-SFS).

Elemental analysis showed that there was a high concentration of C in MCPP and SIS, and N$_2$ only in BSI (p $\leq$ 0.05); therefore, using them as fermentation substrates to produce biomass could be an effective alternative in order to substitute commercial substrates given that C and N$_2$ are majority elements required for bacterial cell growth (Mensah and Twumasi, 2017; Cizeikiene et al. 2018). Additionally, SIS is an important source of peptides, essential amino acids, vitamins and minerals (Wang et al. 2018); and the mixture of SIS with MCPP represents a good source of carbohydrates and minerals.

The H$_2$ contents were of the same order of magnitude in all wastes assessed. The higher O$_2$ content in MCPP could be attributed to the higher presence of functional groups (OH and CHO for carbohydrates). However, the difference between the concentrations of H$_2$ and O$_2$ is mainly due to their molecular weights (Ebbing and Gammon, 2016). S was the element that had the lowest concentration, and significant differences (p < 0.05) were exhibited among all wastes. MCPP had a higher concentration, which was mainly due to the mineral content found in pineapple wastes (Table 1).

Table 3. Physico-chemical characterization of fresh peel (PFP, PFC), powder core (PPP, PPC), and a mixture of peel and powdered core (MCPP) for gold honey variety pineapple and SIS.

| Property	FPP	FPC	PPP	PPC	MCPP	SIS
Moisture (%)	85.01 $\pm$ 0.49	88.74 $\pm$ 0.25	4.77 $\pm$ 0.53	5.11 $\pm$ 0.24	6.53 $\pm$ 0.33	6.57 $\pm$ 0,15
a$_w$	0.981 $\pm$ 0.007	0.993 $\pm$ 0.006	0.389 $\pm$ 0.038	0.306 $\pm$ 0.040	0.387 $\pm$ 0.015	0.595 $\pm$ 0.019
TSS (^{O}Brix)	11.40 $\pm$ 0.56	13.73 $\pm$ 0.42	13.53 $\pm$ 0.06	17.73 $\pm$ 0.40	16.07 $\pm$ 0.21	0.40 $\pm$ 0.01
pH	3.64 $\pm$ 0.12	3.69 $\pm$ 0.17	3.64 $\pm$ 0.02	3.52 $\pm$ 0.03	3.58 $\pm$ 0.38	6.42 $\pm$ 0.04
Acidity (%)	0.58 $\pm$ 0.01	0.64 $\pm$ 0.03	3.11 $\pm$ 0.04	2.80 $\pm$ 0.09	3.11 $\pm$ 0.04	0.49 $\pm$ 0.05

Table 4. Elemental analysis, empirical formulas, and molecular weights of the mixture of peels and powder cores (MCPP) for gold honey variety pineapple, SIS, and biomasses that are reproduced in MRS (*Wc*-MRS) and in supplemented fermentation substrate (*Wc*-SFS).

Element	MCPP (%)	SIS (%)	*Wc*-MRS (%)	*Wc*- SFS (%)
C	38.91 ± 0.45^b	43.99 ± 0.52^a	38.62 ± 0.67^b	43.36 ± 2.04^a
N_2	2.03 ± 0.06^d	10.05 ± 0.39^a	9.24 ± 0.16^b	7.27 ± 0.21^c
O_2	51.65 ± 0.90^a	34.16 ± 0.41^b	33.11 ± 0.37^b	32.32 ± 2.82^b
H_2	6.11 ± 0.23^a	6.32 ± 0.35^a	6.66 ± 0.75^a	6.20 ± 0.38^a
S	0.11 ± 0.03^c	0.64 ± 0.05^a	0.26 ± 0.02^b	0.33 ± 0.03^b
Empirical Formula	$CH_{1.87} ON_{0.04}$	$CH_{1.71} O_{0.58} N_{0.20}$	$CH_{2.06} O_{0.64} N_{0.21}$	$CH_{1.70} O_{0.56} N_{0.14}$
Molecular Weight (g mol^{-1})	30.46	25.81	27.25	24.70

Different letters for each row indicate a significant difference ($p \leq 0.05$).

Furthermore, the elemental analyses performed on the microbial biomass obtained in *Wc*-MRS and *Wc*-SFS allowed us to identify that the elemental composition of the lactic acid bacterium (*W. cibaria*) depends on the composition of the substrates used for cell growth. The greatest variations were found in the contents of C, O_2, and N_2. The N_2 content of *Wc*-SFS < *Wc*-MRS was consistent with the resultant matter balance of the proportions of SIS and MCPP used. This is because the N_2 quantified in the SFS is not fully available; it is, instead, immersed in the complex protein structures (Salmerón et al., 2014).

Eq. (6) shows the coefficients of the estimated stoichiometric equation for biomass ($Y_{X/S}$) and LA ($Y_{P/S}$) production of *W. cibaria*. It uses MCPP and SIS as sources of C and N_2. The coefficients were calculated from the empirical formulas.

$$CH_{1,87}ON_{0,04} + \alpha CH_{1,71}O_{0,58}N_{0,20} \rightarrow Y_{X/S}CH_{2,06}O_{0,64}N_{0,21} + Y_{P/S}CH_2O \quad (6)$$
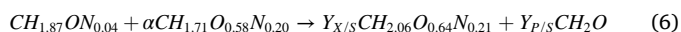
The estimated values of the coefficients from material balances were as follows:

$$\alpha = 1,267; \quad Y_{x/s} = 1,395; \quad Y_{p/s} = 0,872.$$

The elemental analysis and stoichiometric equation were determined. Then, we estimated the relation between the pineapple and sacha inchi wastes that were to be used (MCPP:SIS) in the SFS, based on the total C + N_2 of each waste. Table 5 shows the concentrations of C and N_2 (g $L_{culture\ medium}^{-1}$) per 100g of MCPP and SIS as well as the contents of (C + N_2) and the MPC:SIS ratio (C + N_2).

Since the C:N_2 ratio of fermentation substrates is specific to each microorganism, the high or low ratio of C:N_2 will determine biomass and/or LA production (Zhang et al., 2007). In the case of *W. cibaria*, this ratio was 0.713:1.00, which is because the SIS is richer in (C + N_2) than the MCPP. The literature review did not report results on C:N_2 ratios for the test materials. Ayeleru, Okonta, and Ntuli (2018), reported a high C:N_2 ratio (22.66) in substrates that had mixtures of food wastes for composting applications. For fermentation processes, this ratio is dependent on the type of microorganism and its metabolic needs (Vogel and Torado, 2014).

The final formulation of SFS on the basis of 1 $L_{culture\ medium}$ is shown in Table 6. It is important to emphasize that this formulation is specific for the growth and propagation of *W. cibaria*; however, it could be polyfunctional or extensive to other microorganisms using the same substrate. As such, we recommend performing an elemental analysis of the microorganism and recalculating the MCPP:SIS ratio.

Until now, there have been no formulations of pineapple + sacha inchi substrates that have been developed from elemental analysis. Other studies have evaluated the growth of *W. confusa* bacteria using substrates based on agricultural residues of sugar cane as sources of C (Serna and Rodríguez, 2007) as well as guava and worm seed flours or earthworm flour as sources of N_2 (Serna et al., 2013b, 2013c), but none of these studies reports the results as a function of the C:N_2 relationship.

3.2. Application of SFS as fermentation substrate

3.2.1. Pre-treatments of the SFS

Figure 1 shows the kinetics of concentration for reducing sugars through the effect of SFS pre-treatments (T0, T1, T2, and T3) during the enzymatic hydrolysis process. Reducing sugars had statistically significant differences ($p < 0.05$) with respect to pre-treatment and time factors. T0 (control) showed a fluctuation in the concentration of reducing

Table 6. Formulation of a supplemented fermentation substrate (SFS) based on 1 $L_{culture\ medium}$.

Compound	Quantity (g)
MCPP	41.33
SIS	57.95
$C_2H_3NaO_2$ (Sodium acetate)	5.00
$C_6H_5O_7 \cdot 2NH_3$ (di-ammonium hydrogen citrate)	2.00
K_2HPO_4 (Dipotassium phosphate)	2.00
$MgSO_4$ (Magnesium Sulfate)	0.20
Total	108.48
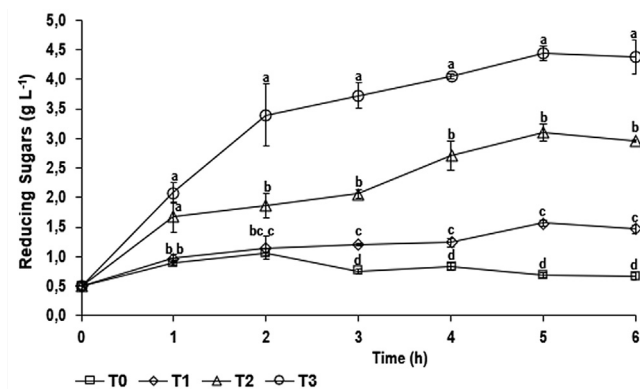
Different letters for each kinetic indicate a significant difference ($p \leq 0.05$)

Figure 1. Kinetics of the concentration for reducing sugars in a supplemented fermentation substrate (SFS) and pre-treated: T0 (control), T1: homogenization (15000 rpm, time 5 min), T2: heating to boiling (1 min), and T3: T1+T2. All the treatments enzymatically hydrolysed.

Table 5. Concentrations and ratios of C and N_2 for 100 g of powdered mixture of peels with their gold honey variety pineapple core (MCPP), SIS, and a mixture of MCPP whit SIS.

Waste	C (gL^{-1})	N_2 (gL^{-1})	C:N_2	(C + N_2)
MCPP	39.42	1.91	20.64	41.33
SIS	46.54	11.41	4.08	57.95
(MCPP + SIS)	85.96	13.32	6.45	
MCPP:SIS (C + N_2)	–	–	–	0.713:1.00

sugars (Figure 1), which could be explained by a possible inhibition of Cellic®CTec2 (Novozyme) during the hydrolysis process, maybe due to the presence of proteolytic enzymes in the MCPP (Sepulveda et al., 2018).

With T1, the reducing sugars experienced a slight increase during hydrolysis time, wich was mainly due to homogenization. This reduced the particle size and produced greater interaction of the enzyme with the substrate (Banerjee et al. 2017). The greatest increase of the concentration of reducing sugars over time was observed in T2 and T3. Significant differences were obtained between the treatments; there was a higher concentration of reducing sugars in T3. The enzyme acts with greater efficiency in T3 due to the combined effect of homogenization and heating until boiling; the process of heating until boiling inhibited the present proteolytic enzymes (Cizeikiene et al. 2018). In general, all treatments showed asymptotic behaviour after 5 h of hydrolysis. Studies have reported that the use of techniques such as delignification and autohydrolysis in pineapple wastes makes it possible to increase the concentration of reducing sugars (Sepúlveda et al. 2018). This is because, when biological materials are used with complex compounds (lignocellulosic structures), pre-treatments are required to improve the availability of the macro and micronutrients contained within them (Hu et al. 2016).

3.2.2. Reproduction kinetics of W. cibaria in two fermentation substrates (Wc-MRS and Wc-SFS)

Figure 2 shows the biomass production kinetics of *W. cibaria* (g dry cell weigth L^{-1}) and LA (g L^{-1}) in a control substrate (*Wc*-MRS) and in *Wc*-SFS. Figure 3 shows the viability kinetics for *W. cibaria* (Log CFU mL^{-1}) in *Wc*-SFS, and *Wc*-MRS.
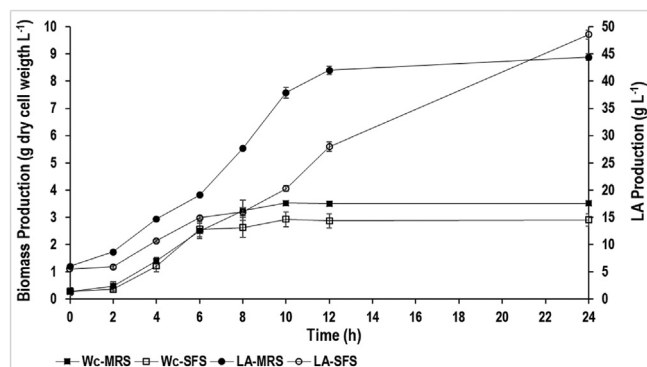

Figure 2. Biomass production kinetics of *W. cibaria* and LA on MRS and SFS substrates. *Wc*-MRS: *W. cibaria* reproduced in control substrate (MRS); *Wc*-SFS: *W. cibaria* reproduced in supplemented fermentation substrate (SFS); LA-MRS: concentration of lactic acid in MRS; LA-SFS: concentration lactic acid in SFS.
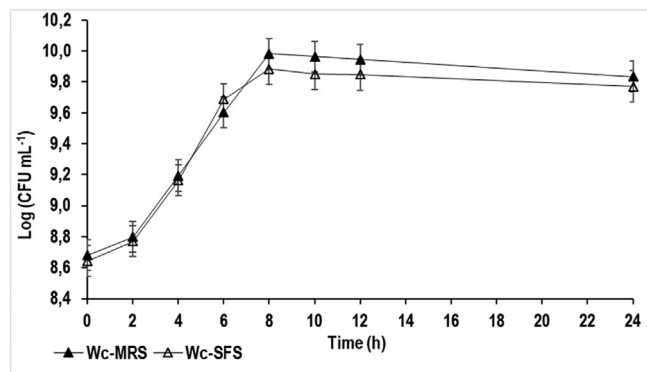

Figure 3. Growing kinetics (viability) for *W. cibaria* on supplemented fermentation substrate (*Wc*-SFS) and control substrate (*Wc*-MRS).

The behaviours of the growth kinetics of *Wc*-SFS and *Wc*-MRS were similar during the adaptation, exponential, and stationary phases. However, ANOVA showed significant differences (p < 0.05) in biomass production with respect to the treatment factor from t = 8. During the exponential phase an increase in biomass production was observed over time, reaching a maximum level at 10 h (*Wc*-SFS: 2.93 ± 0.03 g L^{-1} and *Wc*-MRS: 3.52 ± 0.05 g L^{-1}). Finally, the stationary phase was observed from t = 10 to t= 24. The maximum specific growth rate of *W. cibaria* was similar in both growth substrates: MRS ($0.27 h^{-1}$) and SFS ($0.26 h^{-1}$).

Among the secondary products of fermentation, the content of LA is important for its commercial value. The production the LA in *Wc*-SFS and *Wc*-MRS presented significant differences (p < 0.05) with respect to treatment and time. There was always a higher level of AL-MRS than LA-SFS; however, at 24 h of fermentation with *Wc*-SFS, a higher concentration was reached (48.6 ± 0.04 g L^{-1}) corresponding to 4 g L^{-1} more than what was obtained with the control substrate. Lignocellulosic biomass has generally been considered a biological material with high potential for LA production. The concentration of AL found in our research were higher than for those found by Probst et al. (2015), who reported (30 g L^{-1}) of lactic acid using biological residues fermented by their native microbiota.

In relation to biomass production, our results are promising when compared with other research that uses strains of the same genus. Serna, Rengifo, and Rojas (2013c) obtained 1.47 g L^{-1} of AL with *W. confusa* using worm flour as a source of N_2. Serna et al. (2010) obtained 3.07 g L^{-1} using milk supplemented with yeast extract as the source of N_2 and glucose as the source of C.

The viability kinetics had no significant differences (p < 0.05) with respect to the type of substrate. Time was statistically significant. In both substrates, the maximum viability was reached at 8 h.

International regulations establish that the viability of LABs that are probiotic in character and can be potentially applied in functional foods should be greater than 10^6 CFU g^{-1} (Bosnea et al. 2017). The results show that the biomass of *Wc*-SFS was very viable. Rolim et al. (2018) found a minor viability with *W. cibaria 10M* using a fermentation substrate based on orange juice, sucrose, and malt extract (5.4×10^8 CFU mL^{-1}); this viability was lower than what was found in our research. Rizzello et al. (2019), using LABs reproduced in bean flour, obtained a viability of 8.4 ± 0.4 and 9.5 ± 0.1 Log CFU g^{-1}.

Finally, consumption of the C and N_2 sources for *Wc*-SFS and *Wc*-MRS are shown in Table 7.

Statistically significant differences were obtained in the consumption of C and N_2 between substrates and fermentation time. The consumption rate of the two sources of macronutrients was recorded until *W. cibaria* reached the exponential phase. During the adaptation and growth phases, bacteria reproduced and used the highest nutrient content for cell metabolism (Mensah and Twumasi, 2017), while, during the stationary

Table 7. Consumption of C (%) and N_2 (%) sources during fermentation processes with *W. cibaria* reproduced on MRS substrate (*Wc*-MRS) and on supplemented fermentation substrate (*Wc*-SFS).

Time (h)	Consumption of C (%)		Consumption of N_2 (FAN%)	
	Wc-MRS	*Wc*-SFS	*Wc*-MRS	*Wc*-SFS
0	0	0	0	0
2	10.94 ± 0.03^{aa}	1.69 ± 0.02^{ba}	8.93 ± 0.11^{aa}	3.03 ± 0.06^{ba}
4	16.14 ± 0.01^{ab}	12.30 ± 0.03^{bb}	17.86 ± 0.11^{ab}	12.42 ± 0.07^{bb}
6	27.35 ± 0.09^{ac}	22.83 ± 0.01^{bc}	32.14 ± 0.04^{ac}	$30.\pm 0.06^{bc}$
8	31.73 ± 0.01^{ad}	25.94 ± 0.01^{bd}	42.86 ± 0.04^{ad}	48.18 ± 0.04^{bd}
10	36.65 ± 0.07^{ae}	30.39 ± 0.01^{be}	51.79 ± 0.07^{ae}	60.91 ± 0.04^{be}
12	39.11 ± 0.09^{af}	35.70 ± 0.06^{bf}	60.71 ± 0.03^{af}	$73,03 \pm 0.03^{bf}$
24	44.31 ± 0.07^{ag}	$38,74 \pm 0.01^{bg}$	62.50 ± 0.06^{ag}	73.33 ± 0.03^{bg}

Different letters for each row and different second letters for each column indicate a significant difference (p ≤ 0.05).

phase, macronutrient consumption was reduced since the nutrients are only used for maintenance. There was higher consumption of C and N_2 for *Wc*-SFS with respect to *Wc*-MRS. These differences can be explained by the composition of the substrates. Although MRS is a pure and compositionally enriched substrate, SFS is a substrate with structurally complex compounds and, therefore, simple compounds are not as frequently available (Dai and Huang, 2016).

In general, LABs are nutritionally demanding since, for their reproduction, they need to supply the metabolism's needs using greater sources of C and N_2 during the reproduction stage (exponential phase) (Hu et al. 2016). Any of these elements may be depleted during the fermentation process, which limits cell growth (Vogel and Torado, 2014). This situation depends on the strain of bacteria used and the compositional content of the substrate. Salmerón et al. (2014) obtained different profiles of reducing sugars and FAN in barley, oats, and malt although with behaviours like those obtained in this research (Table 7).

4. Conclusions

The wastes of pineapple and Sacha inchi can be used (for their physicochemical and elemental properties and for their macronutrient and micronutrient content (mainly carbohydrates and proteins)), to formulate a fermentation substrate, which is appropriate for the growth of lactic acid bacteria such as *W. cibaria*. This manuscript is of scientific relevance as the scientific literature has not yet reported studies with a similar approach to what has been presented in this research.

The application of combined pre-treatments (homogenization and heating to boiling), followed by enzymatic hydrolysis in a substrate formulated with pineapple and sacha inchi wastes, increases the availability of reducing sugars and, therefore, improves the efficiency of the use of these wastes as fermentation substrates. Finally, it is recommended that future research evaluates the effectiveness of this substrate according to the fermentation operating conditions: pH, temperature, agitation, and aerobic and anaerobic environments. This is because the composition of the culture medium, the biomass concentration, and the production of metabolites constantly change due to the metabolism of the microorganisms.

Declarations

Author contribution statement

Adriana Micanquer-Carlosama: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Misael Cortés-Rodríguez & Liliana Serna-Cock: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

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