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Research article

Effect of metals on mesophilic anaerobic digestion of strawberry extrudate in batch mode

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ABSTRACT

According to recent studies, the anaerobic digestion of strawberry extrudate is a promising option with potential in the berry industry biorefinery. However, the lack and/or unbalance of concentrations of metals in some agro-industrial residues could hamper methane production during the anaerobic digestion of these kinds of wastes. In this study, a fractional factorial design was applied to screen the supplementation requirements regarding six metals (Co, Ni, Fe, Cu, Mn, and Zn) for methane production from strawberry extrudate (SE). The logistic model was used to fit the experimental data of methane production-time. It allowed identifying two different stages in the anaerobic process and obtaining the kinetic parameters for each step. Maximum methane production obtained in the first (B_{max}) kinetic stage, the methane production in the second stage (P), and the maximum methane production rates (R_{max}) concluded a statistically significant effect for Ni and Zn. The second set of experiments was carried out with Ni and Zn through a central composite design to study the concentration effect in the anaerobic digestion process of the strawberry extrudate. The parameters P and R_{max} demonstrated a positive interaction between Ni and Zn. Although, B_{max} did not prove a statistically significant effect between Ni and Zn.

1. Introduction

Strawberries and their by-products are consumed worldwide, increasing their production every year. In the last ten years, world strawberry production has increased by more than 30%, reaching around 9 million tons of strawberries in 2020 (FAO, 2022). With the increase in strawberry production every year, it is also observed an increase in the by-products generated with strawberry concentrate, whose manufacture produces a large amount of waste called strawberry extrudate (SE) (Rodríguez-Gutiérrez et al., 2019; Trujillo-Reyes et al., 2019). At present, SE is dumped in landfills. Still, with appropriate valorisation, it would not produce environmental pollution, and instead, economic benefits could be obtained for the strawberry sector (Cubero-Cardoso et al., 2020b). As seen in previous studies, a promising alternative for the valorisation of the strawberry extrudate could be the anaerobic digestion process (Cubero-Cardoso et al., 2020c; Serrano et al., 2013; Trujillo-Reyes et al., 2019).

Anaerobic digestion is a complex biochemical process in which a highly varied consortium of microorganisms, in the absence of oxygen, decomposes complex organic substances into simple compounds, mainly producing methane and carbon dioxide (Sarker et al., 2019; Wyman et al., 2019). Due to the energetic potential of the generated methane, anaerobic digestion is considered an environmentally friendly technology to convert solid organic wastes, such as SE, into renewable energy (Mirmohamadsadeghi et al., 2019; Trujillo-Reyes et al., 2019). Anaerobic digestion has a low environmental impact and generates a small production of an undesirable excess of biomass. In addition, it could allow working with different temperature ranges, high organic loading rates, high efficiency, and the methane generated could be used as an

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Abbreviations used

B_0	Methane Production at the Start-up.
B_{max}	Maximum Cumulative Methane Production
BMP	Biochemical Methane Potential
CODs	Soluble Chemical Oxygen Demand.
CODt	Total Chemical Oxygen Demand.
P	Methane produced from the point B_0 and determined when the production stabilizes
R_{max}	Maximum Methane Production Rate
R^2	R-squared
R^2-adj	R^2-adjusted
SE	Strawberry Extrudate
TS	Total solids
VFA	Volatile Fatty Acids
VS	Volatile solids

energy source (Fermoso et al., 2018; Mancillas-Salas et al., 2012). The addition of an adequate concentration of metal to the anaerobic reactors has been widely proven to be a key operational parameter for the proper behaviour of the anaerobic digestion which can even result in an increase in methane production (Venegas et al., 2021; Wyman et al., 2019). The importance of metal for anaerobic digestion lies in their role as necessary micronutrients of anaerobic microorganisms involved in the synthesis of some key coenzymes related to the methanogenesis process, such as vitamin B12 or coenzyme A, the microbial cell growth, and as both electron acceptor and donor (Choong et al., 2016; Kang and Ahn, 2022). The needs for metals in anaerobic digestion greatly vary depending on their contents in the substrate and the inoculum (Fermoso et al., 2019). Optimizing anaerobic digestion is intended to identify the required metal and the appropriate range for the optimal functioning of these reactors. Different studies on the selective addition of metals to food waste have shown their effects on the improvement of methane production (Assis and Gonçalves, 2022). The addition of an adequate concentration of metal to the anaerobic digestion of fruit waste, such as strawberry, can enhance the microbial activity, improving the methane production rate and, thus, avoiding the accumulation of soluble compounds that can cause the acidification of the process (Ezieke et al., 2022).

The addition of metal in anaerobic digestion at concentrations higher than those requirements of the microorganisms can also be harmful or inhibitory, as has been observed in various studies (Demirel and Scherer, 2011; Juliastuti et al., 2003; Qi et al., 2021). Therefore, it is important to understand the specific requirements of each anaerobic process, and to add only the metals necessary to improve the performance, and at the same time, to avoid the undesirable operating costs derived from an overestimated dosage. According to some authors, cobalt (Co), iron (Fe), and nickel (Ni) are necessary for the methanogenic stage and the stability of the anaerobic process (Fermoso et al., 2009; Jiang et al., 2017; Osuna et al., 2003; Zhang et al., 2003). Patidar and Tare (2006) observed that combinations of certain metals could have synergistic or antagonistic effects on methane production. These authors evaluated the following metals combinations: Fe/Co, Fe/Ni/Zn (Zinc), Ni/Zn/Co, and Zn, concluding that the trials where Fe/Co and Ni/Zn/Co combinations were added led to reach the maximum total methanogenic activity in a study carried out in batch mode. On the other hand, it was also observed that Ni affects Co and Zn uptake in methanogenic microorganisms (Patidar and Tare, 2006).

In recent years, several studies have demonstrated the high potential of the anaerobic digestion of SE, reporting methane production yields in the range of 325–470 mL CH_4/g VS (Cubero-Cardoso et al., 2020c; Serrano et al., 2013; Trujillo-Reyes et al., 2019). However, the long-term operation of anaerobic reactors treating SE showed an easy trend to the volatile fatty acids (VFA) accumulation and, thus, the acidification of the process (Cubero-Cardoso et al., 2020a). These studies were carried out without any metal supplementation, and it is possible that a lack of metal in a proper concentration could compromise the stability of the process.

This work aimed to study the effect of adding six metals (Co, Ni, Fe, Cu, Mn, and Zn) on the maximum methane yield and the maximum methane production rate in anaerobic digestion of SE. To carry out the study, on the one hand, the fractional factorial design has been used to maximize the number of study factors to be evaluated. On the other hand, a logistic model was used to fit the experimental data of methane production-time and to obtain the kinetic parameters of the anaerobic process. A second set of experiments was carried out with simultaneous Ni and Zn addition through a central composite design to study the synergism/antagonism effects of these two metals in the anaerobic digestion process of SE.

2. Materials and methods

2.1. Strawberry extrudate and sludge

The strawberry extrudate used in the assays was provided by the company “HUDISA S.A.“, which is located in Lepe (Huelva, Spain). Strawberry extrudate was kept under freezing conditions (−20 °C) before its use to prevent its fermentation and deterioration.

The anaerobic sludge used as inoculum was taken from a full-scale anaerobic reactor treating brewery wastewater, located in the brewery “HEINEKEN SPAIN, S. A.” (Seville, Spain). The chemical characterization, metal and other element content of SE and anaerobic inoculum are summarized in Tables S1 and S2, respectively.

2.2. Anaerobic digestion experimental procedure

The anaerobic digestion of strawberry extrudate was evaluated by biochemical methane potential (BMP) tests. BMP tests were carried out in 250 mL Erlenmeyer flasks using a working volume of 240 mL. The inoculum/substrate ratio was 2:1 in g of volatile solid (Raposo et al., 2011). In addition to inoculum and substrate, 50 mL of macronutrients solution (concentration in the solution – g L^{-1}: NH_4Cl, 7.0; K_2HPO_4, 6.25; $MgSO_4\,7\,H_2O$, 5.0; $CaCl_2\,2\,H_2O$, 2.5; yeast extract, 1), 10 mL buffer (Na_2CO, 50 g L^{-1}), and distilled water until reaching the final volume of 240 mL were added to each reactor, varying only the metals added. More details about the experimental conditions used and the methane measurement system can be found in Cubero-Cardoso et al. (2020c). The BMP tests were carried out during the time interval required (c.a. 26-day period) to exhaust the methane production. The biodegradability of the substrate was calculated according to the percentage of the initially added substrate that was converted into methane, expressing both values in terms of COD.

2.3. Experimental designs

In this work, two subsequent assays have been carried out to study the influence of metals on the anaerobic digestion of SE. Six metals (Co, Fe, Ni, Cu, Mn, and Zn) were studied to assess whether their supplementation favours the anaerobic digestion of SE in the first assay. The experimental matrix with metal as factors was built using a fractional factorial design (Table 1) with a two-level design and one centre point, resulting in 9 runs. The nine runs evaluated in the screening were done through BMP tests, in triplicate reactors, with different concentrations, according to the matrix formed (Table 1). The concentrations of added metal were based on the concentrations defined as inhibitory of each metal according to the literature (Fermoso et al., 2009, 2019), as well as their concentrations in the inoculum and substrate (Table S2). The low level (−1) was defined as the initial metal concentration, i.e., metal from anaerobic sludge and SE without supplementation. An increment in

Table 1
Matrix of added and total metal concentrations in the BMP tests using Fractional Factorial design.

	Metal (mg L^{-1})					
Run	Added Co	Added Fe	Added Ni	Added Cu	Added Mn	Added Zn
1	0	0	0	2.342	3.308	18.259
2	0.544	0	0	0	0	18.259
3	0	310.078	0	0	3.308	0
4	0.544	310.078	0	2.342	0	0
5	0	0	2.246	2.342	0	0
6	0.544	0	2.246	0	3.308	0
7	0	310.078	2.246	0	0	18.259
8	0.544	310.078	2.246	2.342	3.308	18.259
9	0.272	155.039	1.122	1.171	1.654	9.13
Run	**Total Co**	**Total Fe**	**Total Ni**	**Total Cu**	**Total Mn**	**Total Zn**
1	0.181	103.359	0.748	3.122	4.411	24.345
2	0.725	103.359	0.748	0.78	1.103	24.345
3	0.181	413.437	0.748	0.78	4.411	6.086
4	0.725	413.437	0.748	3.122	1.103	6.086
5	0.181	103.359	2.994	3.122	1.103	6.086
6	0.725	103.359	2.994	0.78	4.411	6.086
7	0.181	413.437	2.994	0.78	1.103	24.345
8	0.725	413.437	2.994	3.122	4.411	24.345
9	0.453	258.398	1.87	1.951	2.757	15.216

metal concentration of 1.5-fold the initial metal concentration was adopted for centre point (0), whereas a metal supplementation of 3-fold the initial concentration for the high level (+1). The software Statistica® (version 13) was used to build the matrix of Fractional Factorial design and perform the ANOVA test. The required concentrations of the elements were achieved by dosing the following chemical reagents: $CoCl_2·6H_2O$, $FeCl_2·4H_2O$, $NiCl_2·6H_2O$, $CuCl_2·2H_2O$, $MnCl_2·4H2O$, and $ZnCl_2$.

In the second assay, the influence of different concentrations of Ni and Zn was evaluated using a central composite design experiment. The central composite design has been effectively used to optimize the process factors in BMP tests and, thus, to enhance the performance of these processes (Mlaik et al., 2022). This method also provides efficient, reliable and accurate results as it allows assessment of several factors with optimum experimental sets offering quantitative results (Paul Choudhury and Kalamdhad, 2021). In the present research, Ni and Zn were selected based on their statistically significant and positive effects on the anaerobic digestion of SE determined in the first assay. The central composite design resulted in 10 runs with replicate, with 2 centre points using software Statistica® (version 13) (Table 2). The centre point of the previous fractional factorial design was adopted as the low level (−1) in the present composite central design for both metal, Ni, and Zn for verifying an optimal region indicated by the significance of the curvature factor in the previous experiment. A metal concentration of 2-fold the initial one was adopted as an increment to calculate the concentration in each level.

2.4. *Kinetic study*

The kinetic parameters for the different BMP tests performed were determined from the experimental data of cumulative methane production and digestion time by applying the Logistic model (Sigmoidal with four parameters). The replicates were fit to the model by using non-linear regression applying the software Statistica® (version 13). The Logistic kinetics model has been applied by authors in BMP tests of different substrates (Donoso-Bravo et al., 2010; Li et al., 2012). In the present study the model was slightly modified to incorporate the initial cumulative methane production at the start/up of the assay (Equation (1)).

$$B = B_0 + P / [1 + exp(4 \cdot R_{max} \cdot (t - \lambda) / (P + 2))] \tag{1}$$

Table 2
Central composite design matrix with added and total Ni and Zn.

	Metal (mg L^{-1})	
Run	Added Ni	Added Zn
1	1.122	9.130
2	1.122	33.474
3	4.114	9.130
4	4.114	33.474
5	0.502	21.302
6	4.734	21.302
7	2.618	4.088
8	2.618	38.516
9	2.618	21.302
10	2.618	21.302
Run	**Total Ni**	**Total Zn**
1	1.870	15.216
2	1.870	39.560
3	4.862	15.216
4	4.862	39.560
5	1.250	27.388
6	5.482	27.388
7	3.366	10.174
8	3.366	44.602
9	3.366	27.388
10	3.366	27.388

*Centre points were run in two sets.

Where B is the cumulative methane production during the assay (mL CH_4 g VS^{-1}), B_0 is the cumulative methane production at the start-up of the assay (mL CH_4 g VS^{-1}), P is the methane produced from the point B_0 and was determined when the production stabilizes (mL CH_4 g VS^{-1}), B_{max} is the maximum cumulative methane production obtained by summing B_0 and P. R_{max} is the maximum methane production rate (mL CH_4 g VS^{-1} d^{-1}), and λ (d) is the lag time. Additionally, R^2, percentage of error (%), and standard error of estimate (σ_{est}) were determined to evaluate the adjustment of the experimental data to the model and the precision of the results. The error (%) was defined as the difference in percentage between the final experimental cumulative methane production and the theoretical value predicted by the model.

In the assay using fractional factorial design, all reactors presented a change in the kinetics. Therefore, the logistic model was applied twice to fit the first and second kinetic stages separately, as shown in Figure S1.

2.5. *Statistical analysis*

The responses considered to evaluate the effect of the factors, i.e., metal assessed, were P, B_{max}, and R_{max}. In the fractional factorial design, the responses were used to fit a linear equation as a faction of the factors. The ANOVA analysis was performed to evaluate if each factor was significant at a confidence level higher than 90%. The responses in the central composite design were used to fit a quadratic model using quadratic and linear terms for the factors Ni, Zn, and the interaction between them. ANOVA was performed, with a confidence level of 90%, depending on the setting, and response surfaces were built. After that, the desirability approach was used to analyse the best condition for all responses simultaneously. In this approach, the values estimated using the model fitted (y_i) are converted into values from 0 to 1 (**Equation (2)**), for which a value equal to 1 represents the highest desired value for the response (H), and 0 represents the value out of the acceptable range (L). Then, global desirability is calculated to find the best condition considering both responses (Equation (3)).

$$d = (y_i - L) / (H - L) \tag{2}$$

$$D = (d_1 \times d_1 \times \ldots \times d_n)^{1/n} \tag{3}$$

2.6. Chemical analyses

The following chemical analyses were used for characterization of the SE, inoculum and the effluents from each BMP test at the end of the process. The concentration of total solids (TS), total volatile solids (VS), total mineral solids, pH, and alkalinity were determined according to the recommendations of the Standard Methods of APHA (APHA, 2017). Total chemical oxygen demand (CODt) was determined using the method described by Raposo et al. (2008). Soluble COD (CODs) was determined by closed digestion and the colorimetric standard method 5220D (APHA, 2017). Individual volatile fatty acids (VFA) (C2–C5) were determined using a Shimadzu gas chromatograph (GC-2010). The GC was equipped with a column of 100% ethylene glycol composition of 0.25 mm × 25 m and a flame ionization detector (FID). The oven temperature was gradually increased from 100 to 170 °C at a rate of 5 °C·min^{-1}. Nitrogen (30 mL min^{-1}), hydrogen (40 mL min^{-1}), and air (399.8 mL min^{-1}) was used as carrier gas at a flow rate of 40.1 mL min^{-1} at 456 kPa. The concentrations of dissolved metals were determined by filtration of the liquid sample with a 0.20 μm filter in an oxygen-free atmosphere measured by ICP-OES (VARIAN 720-ES). Total metals concentrations in solid samples were determined through ICP-OES after aqua regia digestion in a microwave oven (Pinto-Ibieta et al., 2016). Total phenols were determined by Folin-Ciocalteu spectrophotometric method (Singleton and Rossi, 1965) after extraction with methanol/water solution (80:20) for 1 h at 70 °C. Samples preparation included either centrifugation at 400*g* for 5 min and subsequent filtration through 0.45 μm filters (García et al., 2016). Results were expressed as milligrams of gallic acid equivalents per kilogram of the sample. The content of total sugars was determined by the anthrone colorimetric method (Mokrash, 1954) using a spectrophotometer (Biorad iMark Microplate Reader. USA). Samples preparation included either centrifugation at 400*g* for 5 min and subsequent filtration through 0.45 μm filters. Results were expressed as milligrams of glucose equivalents per kilogram of the sample.

3. Results

3.1. Screening of the effect of metals (co, fe, ni, cu, mn, and zn) on strawberry extrudate anaerobic digestion

A high variation in the ultimate methane yield was observed for the different metal supplementation conditions, ranging from 303 ± 1 mL CH_4 g VS^{-1} to 437 ± 27 mL CH_4 g VS^{-1} (Fig. 1 A and B). A slight increase in methane production was observed during the first day of experiments regardless of the supplemented metal (Fig. 1 A and B). Next, from day 1 to day 5, approximately a lag phase was observed in all curves. From day five onwards, continuous exponential growth is observed until approximately day 13, when production becomes constant for all runs. Further production was observed for runs 4 to 9 until it reached stabilization around day 26. These runs presented a similar ultimate methane production, i.e., around 365 ± 40 mL CH_4·gVS^{-1}.

The effluent characterization at the end of the BMP digestion time showed that the pH values were very similar in all the runs, varying in a short range between 7.5 and 8.2 (Table 3). The amount of organic matter (VS and CODs) after the anaerobic digestion processes of the different substrates was in all cases less than 14 g VS kg^{-1} and 1.3 g O_2 L^{-1}, respectively. VFA only represented about 15% of the CODs, reaching concentrations lower than 0.2 g O_2 L^{-1} at all the runs (Table 3). The total phenols determined after the anaerobic digestion processes of the different substrates were, in all cases, less than 215 mg gallic acid eq. L^{-1} (Table 3). Finally, on the contrary, biodegradability based on methane production is in all runs around 50%.

As runs 4–9 demonstrated a change in kinetic pattern and, therefore, two stages clearly differentiated, the experimental data were split to fit equation (3) and obtain the kinetic parameters for the 1st and 2nd steps (Table S3). The different mixes of metals give the difference in methane production found to be significant, as seen in other studies (Choong et al., 2016). The parameters *P*, B_{max}, and R_{max} from the 1st and 2nd kinetic steps were used as responses to evaluate the effect of the factors, i.e., metals, on anaerobic digestion, using SE as substrate. First, all six metal sets as factors on fractional factorial design were used in a linear equation as independent variables to estimate the responses individually. Then, the factors with the highest p-value were removed to decrease the mean squared error and approximate the R^2-adjusted (R^2-adj) to the R-squared (R^2), avoiding the excess of variables misleading the goodness-of-fit.

The response of *P* from 1st step was significantly improved by the Zn (p-value 0.0009) and hindered by Fe (p-value 0.0006) supplementation (Figure S2A). The curvature was also positively significant, meaning that the concentration in the centre point might represent a region with higher values than the extremes for any or both factors (Figure S2A). The Cu positive effect was considered to fit the linear model. Despite it was not significant at 95% of confidence, Cu (p-value 0.1816) contributed to the goodness-of-fit of the linear fitting (R^2: 0.9915; R^2-adj: 0.9830; mean squared error: 11.9334) (Figure S2A). The improvement in P was 8% when the total Zn concentration increased from 6.086 to 24.345 mg L^{-1} (Figure S2B). Regarding the P from the 2nd kinetic step, Ni (p-value

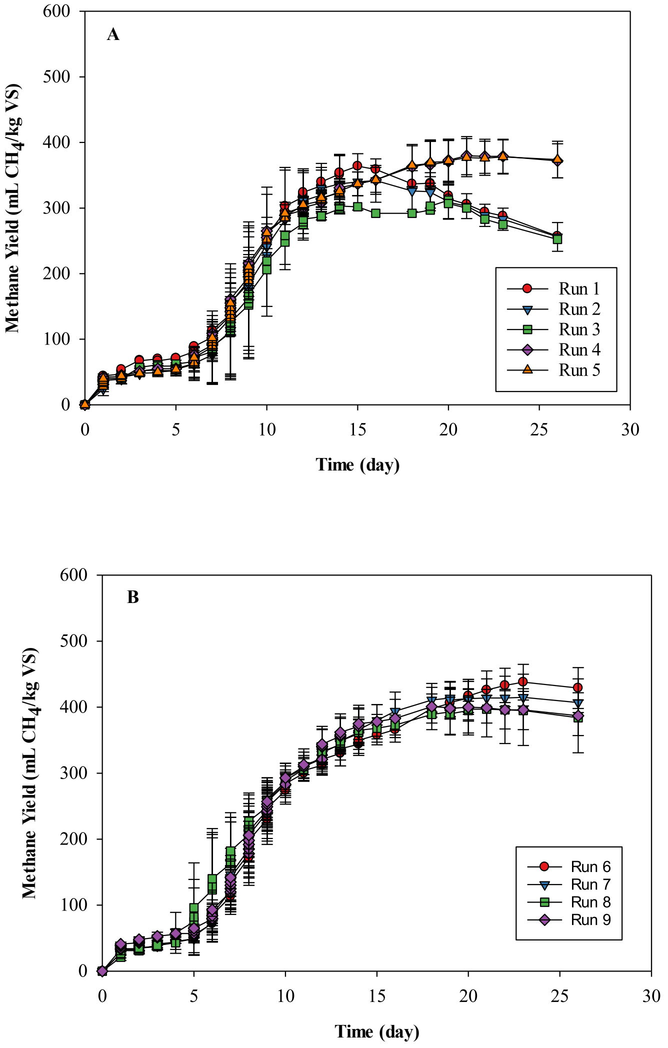

Fig. 1. (A) Methane yield in the BMP tests of SE from 1 to 5 runs (Table 1) with six (Co, Fe, Ni, Cu, Mn, and Zn) metal combinations by an experimental matrix of a Fractional Factorial design with their standard deviations; **(B)** Methane yield in the BMP tests of SE from 6 to 9 runs (Table 1) with six (Co, Fe, Ni, Cu, Mn, and Zn) metals combinations by an experimental matrix of a Fractional Factorial design with their standard deviations.

Table 3
Analytical characterization of anaerobic digestion effluents at the end of the biochemical methane potential tests from SE (mean ± standard deviation values).

Runs	pH	Alkalinity (mg $CaCO_3$ L^{-1})	VS (g VS kg^{-1})	CODs (mg O_2 L^{-1})	VFA (mg O_2 L^{-1})	Total Phenols (mg Gallic acid L^{-1})	Biodegradability (%)
1	7.6 ± 0.1	8332 ± 40	13.6 ± 0.1	1288 ± 8	81 ± 0	210 ± 5	43 ± 1
2	7.6 ± 0.1	7751 ± 402	13.1 ± 0.4	1215 ± 90	90 ± 14	212 ± 1	43 ± 3
3	7.5 ± 0.1	7019 ± 197	11.5 ± 0.3	1027 ± 23	154 ± 0	176 ± 6	42 ± 3
4	8.2 ± 0.1	6885 ± 213	11.7 ± 0.5	1110 ± 113	63 ± 31	203 ± 6	49 ± 4
5	8.2 ± 0.1	7144 ± 211	12.0 ± 0.6	1313 ± 91	80 ± 57	176 ± 12	49 ± 4
6	8.2 ± 0.1	7030 ± 186	11.8 ± 0.3	1217 ± 111	118 ± 77	177 ± 10	55 ± 3
7	8.1 ± 0.1	6353 ± 544	10.4 ± 1.1	983 ± 127	80 ± 27	155 ± 23	55 ± 2
8	8.1 ± 0.1	6482 ± 271	12.2 ± 0.5	1217 ± 220	152 ± 115	158 ± 3	53 ± 2
9	8.0 ± 0.3	7205 ± 274	13.1 ± 0.9	1291 ± 192	149 ± 56	182 ± 26	53 ± 5

0.0740) supplementation showed a significant positive effect on the response at the level of significance of 0.1 (Figure S2C). The factors Co, Zn, and Fe, were considered for the linear model (R^2: 0.7462; R^2-adj: 0.4930; mean squared error: 11.9334), despite they were not statistically significant to the response. The improvement of P was 204% with the addition of Ni (Figure S2D).

The parameter B_{max} from the 1st kinetic stage reflected the P behaviour resulting in a similar statistically significant enhancement by the supplementation of Zn (p-value 0.0333) and a reduction by Fe addition (p-value 0.0092) (Figure S3A), as well as the curvature significance. Ni and Co effects were negative and not significant at a 95% confidence level but contributed to the goodness-of-fit of the linear fitting (R^2: 0.9743; R^2-adj: 0.9315; mean squared error: 51.4974). The supplementation of Zn, i.e., increasing the total concentration in the system from 6.086 to 24.345 mg L^{-1}, enhanced the B_{max} of the 1st kinetic step by 6% (Figure S3B). The global B_{max}, considering the contribution from the 2nd kinetic step, was positively affected by Ni (p-value 0.0208) (Figure S3C). For the linear fitting, the factors curvature, Co, and Fe were important to the goodness-of-fit (R^2: 0.8224; R^2-adj: 0.6449; mean squared error: 765.0171), however, they were not statistically significant. The global B_{max} increased by 21% when the total Ni concentration in the system increased from 0.748 to 2.994 mg L^{-1} (Figure S3D).

Ni (p-value 0.0263) was the only statistically significant metal affecting the R_{max} positively from 1st kinetic step, which received a negative impact of the addition of Mn (p-value 0.0114) (Figure S4A). The linear fitting of the significant factors to the response R_{max} from 1st kinetic step resulted in R^2: 0.7819; R^2-adj:0.7092; and mean squared error: 8.9051. The supplementation of Ni increased the R_{max} by 11% in the 1st kinetic stage (Figures S4B). The R_{max} considering only the 2nd kinetics was also positively affected with Ni addition (p-value 0.0083), allowing it to reach a value 419% higher (Figures S4C and D). The goodness-of-fit of the linear model was R^2: 0.8785; R^2-adj:0. 0.7570; and mean squared error: 13.8468. Fe and Co showed positive and Mn negative effects that contributed to the model but did not achieve the significance level.

3.2. The synergic/antagonistic influence of Ni and Zn on anaerobic digestion of strawberry extrudate

As Ni and Zn presented statistically significant positive effects on the variables and kinetic parameters of anaerobic digestion from SE, these two metals were selected as factors of a BMP assay using the central composite design. It was possible to observe differences in the methane productions among the ten runs, ranging from 438 ± 2 mL CH_4 gVS^{-1} to 561 ± 16 mL CH_4 gVS^{-1} (Fig. 2). Similarly, to the initial set of experiments, at the beginning of the test, all runs had a small increase in methane production during the first 3 days (Fig. 2 A and B). From the first three days, a difference of more than 100 $mLCH_4$ gVS^{-1} can be observed among different runs. Next, from day 3 to day 5, approximately a lag phase was observed in the curves of all runs except for 7, 8, 9, and 10, which follow a growth exponentially. From day 7 onwards, continuous exponential growth was observed, and it lasted until day 25. Runs from 1 to 6 presented a slight change in kinetics on day 14, but runs 7 to 10 presented no change, thus, only one kinetic model was fitted in these assays.

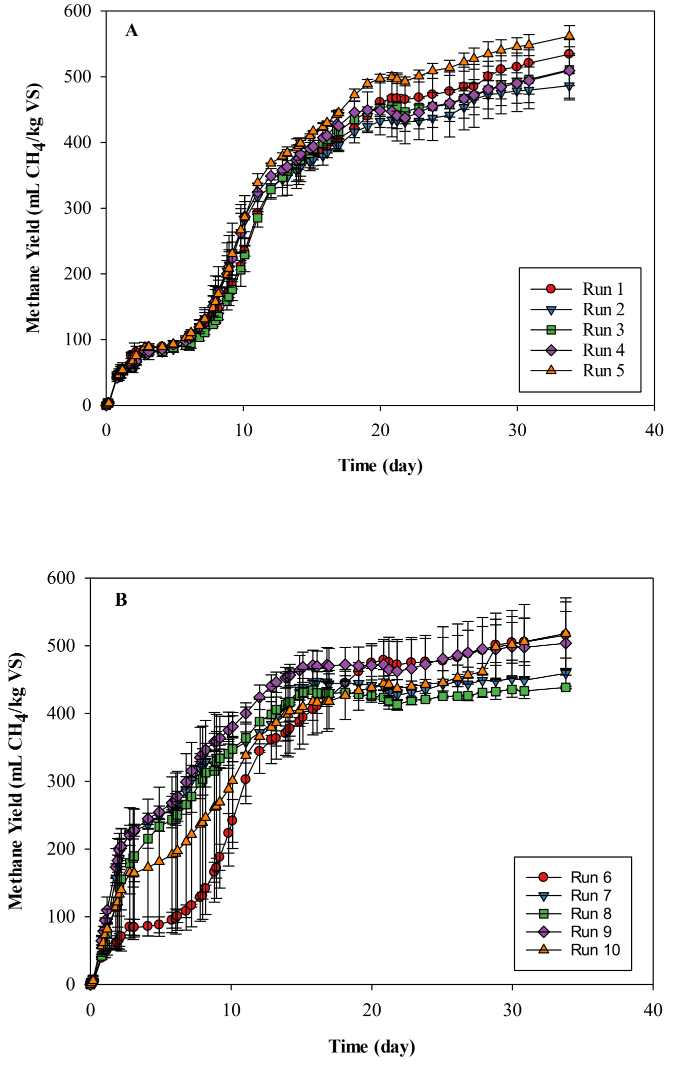

Fig. 2. **(A)** Methane yield in the BMP tests of SE from 1 to 5 runs (Table 2) with Ni and Zn combinations by an experimental matrix of a central composite design with their standard deviations; **(B)** Methane yield in the BMP tests of SE from 6 to 10 runs (Table 2) with Ni and Zn combinations by an experimental matrix of a central composite design with their standard deviations.

The pH in all runs was 7.6, which is in the optimal range for an adequate anaerobic digestion process, except in run 7, for which a pH of 8.2 was observed, these values being similar to the previous BMPs (Table 4). As in the previous BMPs, adequate buffering of anaerobic digestion systems (BMPs) has been described, with alkalinity

Table 4
Analytical characterization of effluents from the anaerobic digestion process of SE at the end of the biochemical methane potential (BMP) tests with their standard deviations.

Runs	pH	Alkalinity (mg $CaCO_3$ L^{-1})	VS (g VS kg^{-1})	CODs (mg O_2/L^{-1})	VFA (mg O_2 L^{-1})	Total Phenols (mg Gallic acid L^{-1})	Biodegradability CH_4 (%)
1	7.6 ± 0.1	7836 ± 80	13.2 ± 0.2	973 ± 84	46 ± 10	117 ± 3	71 ± 1
2	7.6 ± 0.1	7612 ± 66	13.2 ± 0.1	1011 ± 40	46 ± 1	118 ± 1	64 ± 2
3	7.6 ± 0.1	7747 ± 44	13.1 ± 0.1	1056 ± 144	52 ± 4	120 ± 2	66 ± 3
4	7.6 ± 0.1	7281 ± 214	13.5 ± 0.3	1064 ± 165	48 ± 9	120 ± 6	68 ± 3
5	7.6 ± 0.1	7427 ± 413	13.4 ± 0.6	1008 ± 159	62 ± 17	140 ± 17	75 ± 2
6	7.7 ± 0.1	7542 ± 218	13.1 ± 0.1	1264 ± 59	62 ± 32	125 ± 9	69 ± 3
7	8.2 ± 0.8	7144 ± 653	13.3 ± 0.3	1218 ± 719	84 ± 79	108 ± 2	61 ± 3
8	7.5 ± 0.1	6808 ± 47	13.6 ± 0.3	843 ± 230	22 ± 1	104 ± 2	58 ± 1
9[a]	7.5 ± 0.1	7594 ± 5	13.0 ± 0.8	1168 ± 256	25 ± 1	98 ± 11	69 ± 10
10[a]	7.6 ± 0.1	7425 ± 190	13.3 ± 0.1	993 ± 68	24 ± 2	86 ± 1	65 ± 1

[a] Centre points were runs in two sets.

concentrations presenting values in a range of 6000–8000 mg $CaCO_3$ L^{-1}. The concentrations of organic matter (VS and CODs) after the anaerobic digestion processes of the different conditions were similar to the previous BMPs, i.e., less than 13 g VS kg^{-1} and 1.3 g O_2 L^{-1}. Only 7% of CODs corresponded to VFA, not reaching a concentration higher than 0.1 g O_2 L^{-1} in VFA (Table 4). In addition, only acetic acid was observed. The concentrations of total phenols determined after anaerobic digestion processes were low, i.e., less than 140 mg of gallic acid eq. L^{-1}, being less than the reported inhibitory concentration. Finally, the biodegradability based on methane production was around 70%, which increased by 20% compared to the previous BMPs (Table 4).

In the BMPs designed with the central composite design, after day 3 only a slight change occurred in CH_4 production kinetic, thus the raw data were fitted to only one kinetic model (Table 3). The higher range concentration of Ni and Zn used in this BMP indeed stimulated the CH_4 production resulting in higher B_{max} than those obtained in the previous BMPs (Table S4). However, the overall rate R_{max} represented by one kinetics showed smaller values for the higher range concentration of Ni and Zn.

The kinetic parameters P, R_{max}, and B_{max} were used to analyse the effect of Ni and Zn and their interactions, as well as to fit a quadratic model. Considering P in the quadratic model, the quadratic factor Ni was positive and statistically significant (p-value 0.0277), the interaction Ni and Zn were positive, with p-value equal to 0.1698, and Zn impacted the response linearly, with p-value of 0.2929. The obtained model (R^2: 0.9404; R^2-adj:0.9107; lack of fit: 0.2852, and mean squared error: 47.7264) presented a minimum region for P at Ni concentrations around 2.5–4.5 mg L^{-1}. The addition of Zn at Ni concentration below 2.5 mg L^{-1} showed an antagonist effect, however, at Ni concentrations higher than 4.5 mg L^{-1} the effect of both metals was synergic (Fig. 3B).

R_{max} can be estimated by a quadratic model using only Ni concentrations, being the quadratic term statistically significant at 0.1 level of significance (p-value 0.0969), whereas the linear term was not significant (p-value 0.4401) (Fig. 3C). The model fitted (R^2: 0.7507; R^2-adj:0.6794; lack of fit: 0.4688, and mean squared error: 10.6722) showed a minimum region for R_{max} at a similar Ni concentration as occurred for P.

Conversely, for B_{max}, the quadratic factors Ni and Zn were statistically significant at 0.1 level of significance, respectively, with p-value equal to 0.0564 and 0.0963, and interaction between Ni and Zn was not significant (p-value 0.1698), but was considered in the model (Fig. 3E). For B_{max}, the model fitted showed a region of maximum response for total Ni concentrations lower than 2.5 mg L^{-1} and Zn concentrations lower than 20 mg L^{-1}, with synergistic interaction between the metals, however, the model presented low goodness-of-fit (R^2: 0.4956; R^2-adj:0.2434; lack of fit: 0.1398, and mean squared error: 47.7264).

The desirability functions were used to determine the Ni and Zn concentrations that improve P and Rmax simultaneously (Derringer and Suich, 1980). Bmax was not included due to its low goodness-of-fit. The desirable values of P and Rmax were defined as, respectively, 500 mL CH_4 gVS^{-1} and 60 mL CH_4 gVS^{-1} d^{-1}. The maximum global desirability obtained was 0.69 when concentrations of Ni and Zn of 5.48 and 10.17 mg L^{-1} were supplemented, respectively (Fig. 4).

4. Discussion

The experimental trend of methane production in both BMP tests, with the same substrate from SE, was similar to that described by Cubero-Cardoso et al. (2020c). Furthermore, similar methane production and biodegradability were obtained for both cases, and all anaerobic reactors were stable, maintaining appropriate pH and alkalinity values. In most cases, both BMP tests maintained the recommended pH range for an adequate methanogenic activity, i.e., 7.3–7.8 (Wheatley, 1990) (Tables 3 and 4). The lack of acidification throughout the tests, despite the acidic character of the substrates, i.e., pH 3.7 (Table 1), indicated a proper buffering of the anaerobic systems, which presented alkalinity concentration values in a range of 6000–8000 mg CaCO3 L^{-1} (Table 1).

In the kinetic study of both assays, two different exponential phases can be observed, obtaining two differentiated kinetics. The change in the kinetics of microbial metabolism is not an uncommon phenomenon and could be explained by the complexity of the substrate (Kiyuna et al., 2017). The degradation of the complex substrate can result in the initial consumption of compounds more easily available to the microorganisms, and after their exhaustion, a change to degradation of more recalcitrant ones or by-products formed in the system takes place (Kiyuna et al., 2017).

Different studies on the selective addition of metal to food waste, as it can be in the SE, have shown effects on the improvement of methane production (Assis and Gonçalves, 2022). In this sense, the mesophilic anaerobic digestion of food residues and the effects of metals (Fe, Co + Fe, and Co + Ni + Fe) were recently studied (Kang and Ahn, 2022). Supplementation with 0–5 mg Co/L, 0–10 mg Ni/L, or 0–200 mg Fe/L can significantly improve methane productivity in mesophilic digesters. Adding micronutrients increased biogas production, but the excessive addition of metals hampered production. Supplementation with Fe + Co or Fe + Co + Ni increased biogas production more than Fe alone. Within design limits, he following concentrations of three metals were optimal in this process [Co] = 0.33 mg L^{-1}, [Ni] = 0.43 mg L^{-1}, and [Fe] = 5.35 mg L^{-1} (Kang and Ahn, 2022). In this line, Zhang et al. (2015) also demonstrated the effects of adding metallic elements on the anaerobic digestion of food waste. They found that metals supplemented at moderate concentrations greatly improved methane yield. However, excessive Fe and Ni supplementation exhibited obvious toxicity to methanogens. However, Fe + Co + Mo + Ni supplementation obtained the highest methane yield of 504 mL g VS^{-1} and the highest increase of 35.5% compared to the reactor without metal supplementation (372 mL g VS^{-1}) (Zhang et al., 2015).

In the present research, the concentrations of added metals were based on their concentrations in the inoculum and substrate (Table S2)

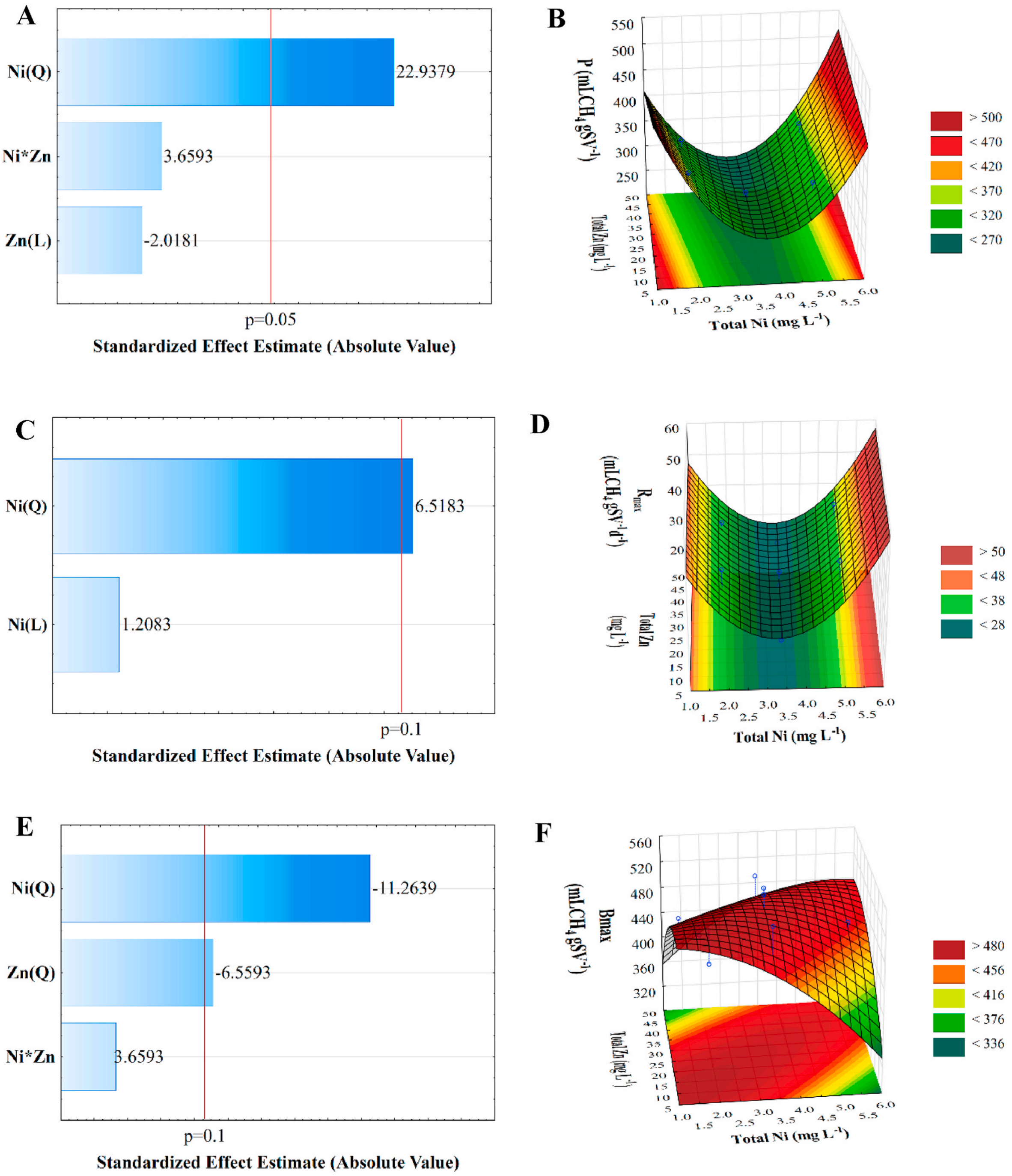

Fig. 3. Assessment of the effects of Ni and Zn on kinetic parameters from the anaerobic digestion of SE using central composite design. For P: (A) Pareto chart and (B) Surface response; *Rmax*: (C) Pareto chart and (D): Surface response; and for global B_{max} (E) Pareto chart and (F) Surface response.

and they were lower than the toxic concentrations of each metal found in the literature (Fermoso et al., 2009, 2019). Four elements had significant effects on the anaerobic digestion process of the SE, i.e., Fe, Mn, Zn, and Ni. Fe is usually reported as a stimulatory metal due to its presence in several metalloenzymes important for anaerobic digestion (Fermoso et al., 2009). However, the current work demonstrated that for an initial Fe concentration of 103.359 mg L^{-1} in the system, the increase of 3-fold this value caused a negative effect on anaerobic digestion. Fe is a well-studied metal due to its involvement in forming biogas from acetic acid, and it has a negative influence when there is a high concentration of Fe in the reactor (Wang et al., 2020). A similar negative effect of Mn was verified and reported by Braga et al. (2017) when evaluating the specific methanogenic activity using blackwater as substrate, increasing the Mn concentration from 0.89 to 1.44 mg L^{-1}. In the present study, the Mn negative effect was checked by supplementing the initial concentration, 1.103 mg L^{-1}, with 3.308 mg L^{-1}. This result indicates that an increase in the Mn concentration in the system might hinder the process, i.e., whether a continuous operation results in Mn accumulation on the sludge or due to the fluctuation of metal concentration in the inlet. Chan et al. (2019) found a stimulatory effect of Zn supplementation on the anaerobic digestion of a mixture of food waste and domestic wastewater. These authors added Zn in a range from 50 to 100 mg L^{-1} to a UASB reactor operated in intermittent feed mode, and Zn demonstrated to play an important role in the short chain fatty acids removal, consequently increasing the methane yield and methane production rate. As can be seen, the Zn concentration values reported by

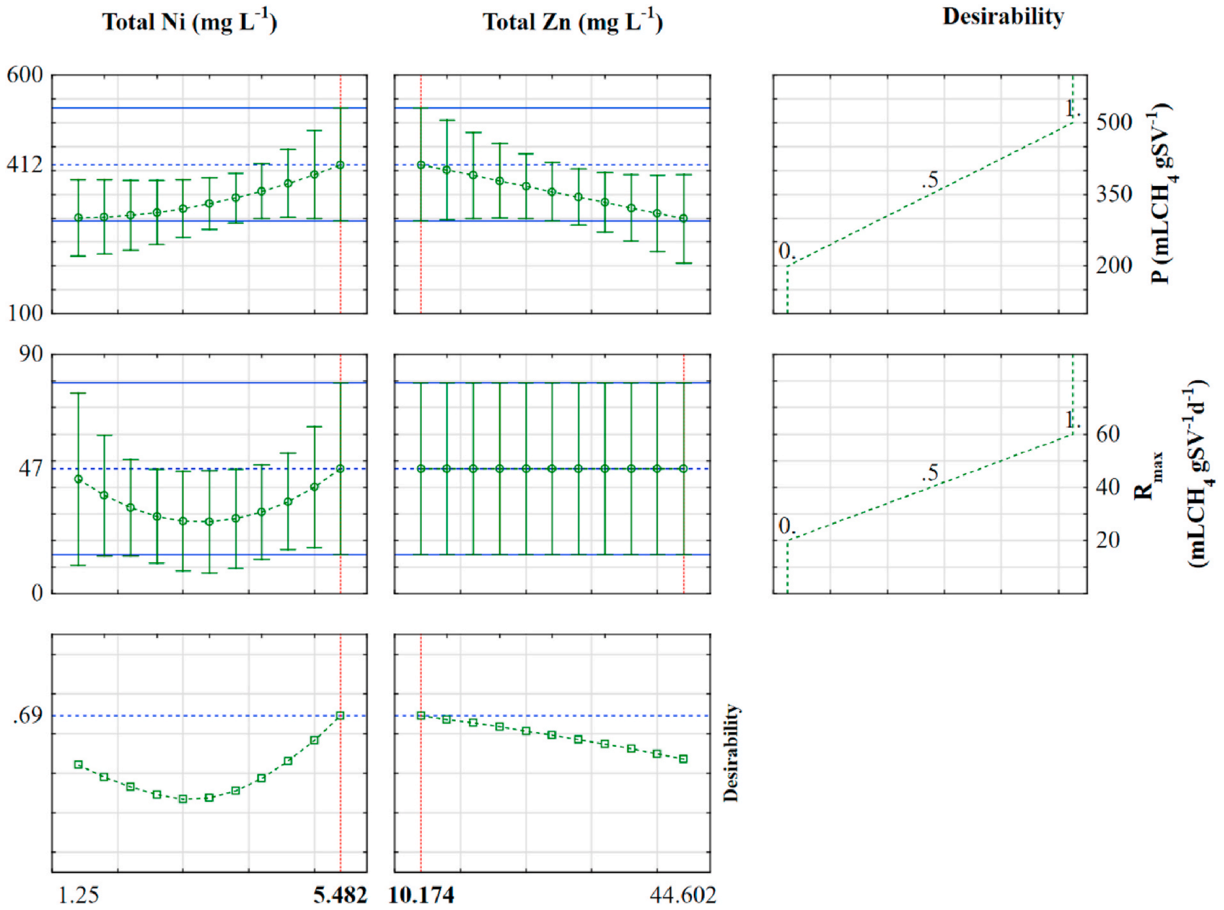

Fig. 4. Profiles for predicted values and desirability considering the variables *P* and R_{max}.

Chan et al. (2019) were much lower than those added in the present work. Ni is also an essential metal for anaerobic digestion and participates in all process steps. Along with Fe, Ni is present in hydrogenases, important in hydrolytic and acidogenesis steps (Mulrooney and Hausinger, 2003). Ni is also the metal core of F430, which is the co-factor of methyl CoM reductase and involved in the final stage of methanogenesis and common to all pathways (acetoclastic, hydrogenotrophic, and methylotrophic); and it composes the acetyl Co-A synthase and CO-dehydrogenase (Can et al., 2014). Considering the initial metal concentrations in the system, 0.748 mg L^{-1}, the Ni supplementation is important to the whole process, allowing the development of the 2nd kinetics. It is worth noticing that a total Ni concentration higher than 1.25 and lower than 4.64 results in global desirability lower than 0.45. The metal concentrations in continuous systems can vary during the reactor operation, either due to the metal inlet with the substrate and accumulation in the sludge or due to metal loss with solids particles from sludge or along with effluent (Roussel et al., 2019). Thus, metal monitoring in the system is essential to avoid inhibition processes due to excess or lack of metal.

Accordingly, the present work results indicate that the CH_4 production from more available compounds (1st Kinetics) in the SE might require a higher concentration of Zn than its available content in the system. In addition, Ni can stimulate the degradation of recalcitrant or secondary compounds, enhancing the global CH_4 production in the system. For the process to be more economically and environmentally sustainable, co-digestion with co-substrates rich in metals, in this case, Zn and Ni, or the addition of waste products from combustion processes have been proposed (Ezieke et al., 2022; SilvadaBraga et al., 2021). In this sense, the addition of mature leachate to food waste provided ammonium to avoid acidification and metals (mainly Fe and Ni) for microbial growth and activity to improve the anaerobic digestion of food waste, and the efficiencies of all stages of the anaerobic digestion process of this waste (Liu et al., 2022). In this case, the predominant methanogenic genera were shifted to adapt the changing condition, thus stabilizing the system.

5. Conclusions

The addition of six metals (Co, Ni, Fe, Cu, Mn, and Zn) to the anaerobic digestion of strawberry extrudate (SE) in batch mode resulted in methane productions ranging from 303 ± 1 mL CH_4 g VS^{-1} to 437 ± 27 mL CH_4 g VS^{-1} and around 50% of biodegradability. The logistic kinetic model was applied to fit the experimental data of methane production-time and it allowed identifying two different stages in the anaerobic process of SE and obtaining the kinetic parameters for each step. The parameters *P*, B_{max}, and R_{max} showed a statistically significant effect for Ni and Zn. Co and Cu did not significantly affect methane production within the tested range, while Mn and Fe negatively affected the anaerobic digestion of strawberry extrudate. The second BMP test related to anaerobic digestion from this substrate with the addition of Ni and Zn resulted in methane productions in the range from 438 ± 2 mL CH_4 g VS^{-1} to 561 ± 16 mL CH_4 g VS^{-1} with 70% of biodegradability. The parameters *P* and R_{max} demonstrated a positive interaction between Ni and Zn. However, B_{max} did not demonstrate a statistically significant effect between Ni and Zn.

Author contributions

Conceptualization, **J.C.C.**, **A.F.M.B.**, **A.T.R.**, **A.S.**, and **F.G.F.**; methodology, **A.F.M.B.**, **A.T.R.**, **G.A.S.**, and **J.C.C.**; formal analysis, **A.**

F.M.B., and **A.S.**; resources, **F.G.F.**, and **R.B.**; writing original draft preparation, **J.C.C.** and **A.F.M.B.**; writing review and editing, **A.S., R.B.** and **F.G.F.**; supervision, **F.G.F.**; funding acquisition, **R.B.**, and **F.G.F.** All authors have read and agreed to the published version of the manuscript.

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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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2022.116783.

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