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Technical feasibility and modeling of enzymatic pre-treatments of organic fraction of municipal solid waste to improve anaerobic digestion

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ABSTRACT

The study explored the combined effects of enzymatic pre-treatment and anaerobic digestion (AD) on the organic fraction of municipal solid wastes (OFMSW) through experimental and multicriteria decision-making approaches. Five enzymes (UPP2, MPCS, USC4, USE2, and A. niger) and their dosages were studied. AD parameters included two inoculum origins (waste active sludge - WAS - and cow-agricultural sludge - CAS), the substrate: inoculum (SI) ratio, and inoculum incubation time (INOC). Desirability functions were used to optimize the multiple experimental responses simultaneously by converting each of them into values from 0 (unacceptable) to 1 (completely acceptable) and then combining these into a global desirability (D). D highlighted that higher enzyme dosages, INOC, and SI, improved AD performances, with optimal DOSE (at the highest level adopted for each enzyme) and INOC (5–10 d). AD tests with the five enzymes increased CH_4 production by 10–13%v/v compared to untreated OFMSW. For UPP2 and MPCS, increasing DOSE boosted the biogas production, while increasing INOC enhanced the CH_4 content. MPCS reached the highest efficiency (478. 43 NL CH_4/kg $_{VS}$ with CAS, SI = 2:1, INOC = 10 d), followed by UPP2. Furthermore, higher INOC reduced A. niger doses, increasing CH_4 production by 9%v/v compared to literature, with 5–10 d INOC (452.86 NL s/kg $_{VS}$ with WAS, SI = 2:1).

1. Introduction

The European Green Deal aims to achieve carbon neutrality by 2050, recognizing the contribution of energy production and consumption, which accounts for about 70% of greenhouse gas (GHG) emissions. Fossil fuels satisfy 88% of Europe's energy needs, and consumption will increase by 50% from 2018 to 2050 (Raja Ram and Nikhil, 2022). The reliance on fossil fuels leads to an increase in GHG concentrations, presenting a significant environmental challenge (Atelge et al., 2020). To address this issue, the transition to renewable energy sources becomes necessary. Biogas, a renewable biofuel primarily composed of methane and carbon dioxide, has a calorific value of about 20 MJ/kg and can be used for electricity, heat, and steam generation (Khanh Nguyen et al., 2021). Anaerobic digestion (AD) of the organic fraction of municipal solid wastes (OFMSW) is a proven technology for biogas production (Li et al., 2018). However, the complex composition of OFMSW, containing carbohydrates, proteins, lipids, vitamins, and minerals that vary by location and season, can lead to incomplete biodegradation (Pramanik et al., 2019). Pre-treatments, categorized as chemical, physical, biological, or combinations thereof, have been explored to improve biodegradability. These pre-treatments break down substrates, enhance microbial interactions, and strengthen hydrolysis rates and nutrient accessibility (Jain et al., 2015). The choice of the pre-treatment depends on the feedstock composition and the energetic financial requirement. In literature, chemical and physical pre-treatments are the most investigated. However, the pros and cons must be analyzed by considering the application of pre-treatments at pilot and full scale. Chemical pre-treatments face equipment corrosion and high costs. Physical pre-treatments demand high energy and may produce inhibitors. Enzymatic pre-treatments are scalable and energy-efficient but expensive. Further research is needed to optimize dosages and applications for these treatments (Uthirakrishnan et al., 2022). Among pre-treatment strategies, enzymatic pre-treatment stands out as it can improve microbial activity in subsequent biological treatments, preventing lipid and fat accumulation in the substrate (Gnaoui

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Nomenclature

AD	anaerobic digestion
A.niger	Aspergillus niger Cellulase
CAS	cow agricultural sludge
COD	chemical oxygen demand
DoE	Design of Experiment
DOSE	dose of enzyme in the model
DRsCOD	disintegration rate based on soluble chemical oxygen demand
ESI	energy sustainable index
INOC	inoculum incubation time
OFMSW	organic fraction municipal solid waste
SI: substrate	inoculum ratio
WAS	waste active sludge
MPCS	MethaPract® CS
TS	total solids
UPP2	UltraPract® P2
USC4	UltraSweep® C4
USE2	UltraSweep® E2
VS	volatile solids

et al. 2022). Previous studies have shown that adding exogenous enzymes improves AD performance in terms of biogas production, and the digestate being utilized as fertilizer (Zhao et al., 2019). Enzymes can be added directly to the AD or adopted in a pre-treatment step. The effectiveness of enzyme addition depends on various factors, including enzyme type, dose, pre-treatment time, substrate type, temperature, and pH. It is necessary to determine when the addition of enzymes (e.g. cellulase, hemicellulase, and protease) to the AD can improve the digestion kinetics and the biogas yield of OFMSW.

To the best of the authors' knowledge, limited attention has been given to the enzymatic pre-treatment of real OFMSW (Fdez et al., 2011; Zhang et al., 2018), and the available scientific studies focused on enzymatic pre-treatments or AD separately without considering their combined effects.

Worth of note are the investigations of the biodegradation of lignocellulosic biomasses (Navarro et al., 2020), biodegradation of lignocellulosic biomasses with lignin-modifying enzymes and carbohydrases (Brémond et al., 2018), and enzymatic saccharification of fat milk substrates (Domingues et al., 2015) before AD.

There are few studies concerning the combined effect of pre-treatments and AD. In detail, for physical pre-treatments, the study by Demichelis et al. (2023) considered the interactions of physical pre-treatment and AD of real OFMSW by considering the response surface. Concerning the chemical pre-treatments, the study of Mahmoodi-Eshkaftaki and Rahmanian-Koushkaki (2021) determined the optimum process for AD of wild tree wastes under alkaline pre-treatments in biogas plants by adopting the desirability analysis (Mahmoodi-eshkaftaki and Mahmoudi, 2021). Furthermore, the same authors (2020) integrated mathematical models and desirability functions to determine the optimal experimental conditions for the co-digestion of grapefruit waste and manure under NaOH and H_2O_2 treatments (Mahmoodi-eshkaftaki and Rahmanian-koushkaki, 2020).

The present study aims to investigate the combined effects of enzymatic pre-treatment and AD on the OFMSW through a multicriteria decision-making approach, employing desirability functions, and simultaneously optimizing multiple experimental responses concerning enzymes and AD process.

Five enzymes, including four commercial mixes and one pure enzyme, were studied for dose and pre-treatment time to assess their applications and enhance their performance. Commercial enzymes were already employed in AD industrial plants with promising results, and the non-commercial enzyme was previously only investigated at the laboratory scale achieving interesting results (Mlaik et al., 2019a). AD was then investigated considering substrate: inoculum ratio, inoculum origin, and inoculum incubation time. Design of Experiment (DoE) and response surface methodology were employed to identify optimal laboratory-scale conditions, considering biogas quality, production performance, and energetic sustainability simultaneously through desirability functions.

The novelty of the study is optimizing the interaction between enzymatic pre-treatment and AD, bridging the gap through the combined experimental and modeling activities. The impact of this study is the enhancement of bio-energy production from bio-residue by promoting renewable energy uses in alignment with the European Green Deal targets.

Table 1
Physical and chemical properties of OFMSW and inocula in terms of total solids (TS), volatile solids (VS), elemental analysis (CHNSO), Total Oxygen Demand (TOC), Chemical Oxygen Demand (COD), and soluble Chemical Oxygen Demand (sCOD).

	OFMSW		WAS		CAS	
	Mean	dev. St	Mean	dev. St	Mean	dev. St
TS (%)	6.91	0.63	5.15	0.14	5.80	0.10
VS (%)	97.12	0.51	70.7	1.1	70.3	1.0
pH (−)	5.50	0.21	7.13	0.16	7.77	0.10
C (%TS)	48.12	0.54	35.44	0.54	40.62	0.68
H (%TS)	6.23	0.76	3.000	0.070	3.020	0.020
N (%TS)	3.10	0.31	4.53	0.14	7.91	0.10
S (%)	0.21	0.10	–	–	0.090	0.010
C/N (−)	15.5	1.4	7.91	0.11	5.11	0.15
TOC (g/kg)	249.1E+02	1.1E+02	9.50	0.10	12.02	0.21
COD (mg/kg)	1.140E+05	7.2E+02	2.50E+03	1.1E+02	1.000E+04	5.1E+02
sCOD (mg/kg)	8.860E+04	5.6E+02	–	–	–	–
Proteins (%TS)[a]	19.38	0.70	–	–	–	–
Lipids (%TS)[b]	21.9	9.8	–	–	–	–
Carbohydrates (%TS)[b]	48.3	8.7	–	–	–	–
Hemicellulose (% TS)[b]	6.2	1.0	–	–	–	–
Cellulose (%TS)[b]	12.3	1.6	–	–	–	–
Lignin (%TS)[b]	5.22	0.90	–	–	–	–

[a] Protein content was calculated according to (Mariotti et al., 2019) with the standard (for foodstuff) conversion factor equal to 6.25.
[b] Data provided by the company plant.

2. Materials and methods

2.1. Substrate and inoculum

The organic fraction of municipal solid waste (OFMSW) deriving from a separate collection of urban waste was provided by San Carlo S.p. A (Fossano, Italy). Two inocula were tested based on the results of the previous study (Demichelis et al., 2022): the first was a mesophilic digestate of wastewater-activated sludge (WAS), according to (Kumar Biswal et al., 2020), provided by SMAT (Torino, Italy); the second was a mesophilic digestate of cow-agriculture sludge (CAS), based on (Gu et al., 2020), supplied by "Cascina La Speranza" (Fossano, Cuneo, Italy). Table 1 summarizes the OFMSW and inocula properties. All the experiments were performed with the same lot of OFMSW and inocula, to limit its effect on the process variability.

2.2. Enzymatic hydrolysis

Enzymatic pre-treatment was carried out before AD to assess the efficiency of the enzymatic hydrolysis on OFMSW without the addition of the inoculum. The study of Romano et al. (2009) demonstrated that the enzymatic pre-treatment before AD was more effective than during AD with inoculum. This depends on the potential inhibitory effect of the microbial components in the inoculum on the enzyme activity.

The initial enzyme dose was determined based on literature recommendations for non-commercial enzyme and technical reports for commercial ones. Subsequently, doses were systematically decreased until the point at which no significant increase in soluble Chemical Oxygen Demand (sCOD) was detected.

The hydrolysis reactions were carried out in Pyrex glass bottles (Duran, Germany) of 500 mL containing 250 g, based on total solid (TS), of untreated OFMSW. The pH of OFMSW was adjusted at the optimum pH for each enzyme with the addition of NaOH 0.1 M, followed by the addition of the required amount of enzyme, mixing with a magnetic stirrer at a constant speed of 200 rpm and controlling the temperature by a thermocouple. Reactions were performed at the optimal temperature indicated for each enzyme, under atmospheric pressure.

No suggestions were provided by the literature and report for the hydrolysis time; hence the enzymatic hydrolysis was performed for the following hydrolysis times: 0, 15, 30, 45, 60, 120, and 180 min, after which no significant increase of sCOD (p-level <0.05), could be appreciated. To evaluate the hydrolysis rate, a sample of 2 g was taken from the reactor through a Teflon pipe by a peristaltic pump, weighed, and transferred to a 50 mL polypropylene falcon tube at intervals of 15 min for the first hour and then every hour, up to 180 min of total treatment (for a total of 6 samples for each enzyme). At the end of the reaction, the samples of hydrolyzed OFMSW were heated at 90 °C for 10 min to denature the enzyme.

The hydrolysis degree was calculated by considering the concentration of sCOD formed at a given pre-treatment time through Eq. (1), according to (Bougrier et al., 2005).

$$DR_{sCOD}(\%) = \frac{SCOD_1}{SCOD_0} \bullet 100 \quad (1)$$

where $SCOD_0$ and $SCOD_1$ are the sCOD before and after pre-treatment, respectively.

Effective hydrolysis was considered only when the disintegration rate $DR_{sCOD} \geq 20\%$, according to the study of Myszograj et al. (2014); Myszograj (2014) on thermal pre-treatments, which are currently considered the most promising and the benchmark for performance.

The commercial enzymes belong to the Biopract enzyme (produced by ABT) and they are currently employed in AD pilot-industrial scales (GmbH, 2022). The optimal temperature and pH conditions were taken by (GmbH, 2022).

UltraPract® P2 (UPP2) is a mix of cellulases, hemicellulases, pectinases, and proteases, which was designed for working in biogas plants on vegetable biomasses. UPP2 exhibits a peak activity with a dose of 1 mL/100 g TS in the pH range 7.0–7.5 with an optimum at 35 °C. The preparation also contains the enzymatic acceleration factor (AC) which should improve its disintegration efficacy. In the present study, UPP2 was studied at the doses: 0.125, 0.250, 0.500, 1.00, and 1.50 mL/100 g TS, to optimize its application from 0 to 180 min at 35 °C.

MethaPract® CS (MPCS) is a mix of cellulases, hemicellulases, and pectinases, which has been recommended for plants fed with vegetable biomasses like corn silage, winter cereals, and grass silage. MPCS exhibits a peak activity with a dose of 1 mL/100 g TS at pH 7.5 with an optimum at 35 °C. In the present study, MPCS was studied at the doses: 0.125, 0.250, 0.500, 1.00, and 1.50 mL/100 g TS, to optimize its application from 0 to 180 min at 35 °C.

UltraSweep® C4 (USC4) is a mix of cellulase, hemicellulase, and pectinases, as MPCS, plus surfactant proteins. USC4 exhibits a peak activity at a dose of 2 mL/100 g TS at pH 7.5 with an optimum at 30 °C. In the present study, USC4 was studied at the doses: of 0.25, 0.50, 1.00, 2.00 mL, and 2.50 mL/100 g TS to optimize its application from 0 to 180 min at 25 °C.

UltraSweep® E2 (USE2) is an innovative product containing surfactant proteins and a mix of cellulases, hemicelluloses, pectinases, and proteases, hence it could be considered a modified version of UPP2 with the addition of surfactant proteins. USE2 exhibits a peak activity at a 2 mL/100 g TS dose at pH 8.5 with an optimum at 25 °C. In the present study, USE2 was studied at the following doses: 0.25, 0.50, 1.00, 2.00 mL, and 2.50 mL/100 g TS to optimize its application from 0 to 180 min at 25 °C.

Aspergillus niger (commercial name A. niger Cellulase) from Pale Yellow Crystals (Sigma-Aldrich, CAS9012-54-8) contains cellulases, β-glucosidases, and hemicellulase for degrading cellulose to fermentable sugars. In the present study, for the inoculation, it was considered that 1 unit of A. niger produces 1.0 μmol of glucose from cellulose, at pH 4.5 and 35 °C after 2 h of incubation at 100 rpm, in agreement with (Mlaik et al., 2019b). The same authors recommended A. niger with an activity of 6 IU/mL of β-glucosidase to obtain an increase in the biogas and methane yields. In the present study, it corresponded to a dose equal to 2 mL/100 g TS; however, its dose was studied here at the levels: 0.25, 0.50, 1.00, 1.50, and 2.00 mL/100 g TS, to optimize its application from 0 to 180 min at 50 °C and 200 rpm. Cellulase activity was determined at 40 °C using carboxymethyl cellulose as a substrate. The reactive mixture contains 0.5 mL of 1% (w/v) substrate in 0.1 M citrate buffer (pH 4.8) and 0.5 mL of culture supernatant. The mixture was incubated at 40 °C for 30 min. One unit of endoglucanase activity was expressed as the amount of enzyme required to release 1 μmol of reducing sugars per mL, using glucose as a standard curve.

2.3. Anaerobic digestion

The AD was performed on enzymatically pre-treated OFMSW achieving $DR \geq 20$ %. The AD of not pre-treated OFMSW was performed as a control. The AD was performed in 1.00 L Pyrex glass bottles (Duran, Germany) with 80 % working volume at 37 °C, operating in batch mode with OFMSW at 6 % of total solids. The AD setup is reported in (Demichelis et al., 2022).

Two inoculum origins were tested (WAS and CAS) and the inocula were separately cultivated under anaerobic conditions at 37 °C in 2 L Pyrex glass bottles (Duran, Germany), for three different periods (0, 5, and 10 d) and then inoculated in the pre-treated OFMSW considering the S: I ratio ranging from 1:2 to 2:1 based on volatile solids (Demichelis et al., 2022). Moreover, AD tests on inocula (according to origin and incubation time) were performed to measure its contribution. The cumulative biogas and methane productions were calculated by subtracting the contribution of the inoculum (according to origin and incubation time). AD tests were stopped when the daily biogas production was below 1 % of the total volume of biogas produced up to that time

(Demichelis et al., 2018).

The biogas collected in a 2 L gas bag was quantitatively measured by water displacement and qualitatively analyzed with an SRA Micro-GC.

2.4. Analytical

The OFMSW and the two inocula were physically and chemically characterized. The total (TS) and volatile (VS) solids content were detected according to UNI EN 15216:2021 and elemental analysis (CHNSO) was performed with the Elemental Macro Cube system (Vario, Germany).

The soluble compounds released from the OFMSW were measured as soluble chemical oxygen demand (sCOD) with LC 514 (Hach). The removed volatile solids (VS removed %) at the end of AD were evaluated through Eq. (2) according to (Li et al., 2018).

$$VS\ removed\ (\%) = 1 - \frac{VS\ output \bullet (1 - VS\ input)}{VS\ input \bullet (1 - VS\ output)} \quad (2)$$

where VS input and VS output are the percentage of volatile solids in the OFMSW before and after AD, respectively.

2.5. Kinetic study

The kinetics of AD was evaluated considering the biogas volumetric rate (Eq. (3)) according to (Zhou et al., 2017).

$$V\ biogas\ rate\ \left(\frac{L}{L \bullet d}\right) = \frac{Biogas\ (L)}{Volume\ of\ reactor\ (L) \bullet time\ (d)} \quad (3)$$

2.6. Energy sustainability

The energy balance was modeled as in a previous study (Demichelis et al., 2023), assuming the ideal gas law, the thermodynamic equilibrium, steady-state conditions, and atmospheric air composition. The thermal load (Q_s) of the system and the energy sustainable index (ESI) were calculated according to (Mehr et al., 2017; Kovalovszki et al., 2020), respectively. Q_s included the thermal load for the enzymatic pre-treatment and AD and the heat lost from the AD. The energy produced was calculated considering that $CH_4 = 7.2$ kWh/m^3 (Rillo et al., 2020). The detailed description of energy analysis calculation is provided in Supplementary Information Appendix A.

2.7. Design of experiments and modelling

2.7.1. Design of Experiments

The effects of the enzymatic pre-treatment and the AD phase on the performances of the process were evaluated by DoE (Box et al., 2005). Five factors were studied, two qualitative and three quantitative.

- The type of enzyme (qualitative factor). Five different enzymes were evaluated: UPP2, MPCS, USC4, USE2, A. niger.
- The type of inoculum (qualitative factor): WAS and CAS.
- The dose of enzyme (DOSE – quantitative factor). Three levels were considered for each enzyme: 0.25, 0.50, and 1.00 mL/100 g TS for UPP2 and MPCS; 0.50, 1.00, and 2.00 mL/100 g TS for USC4 and USE2; 0.50, 1.00, and 1.50 mL/100 g TS for A. niger.
- The ratio SI (quantitative factor) is considered at three levels: 1:2, 1:1, and 2:1.
- The time of incubation of the inoculum (INOC - quantitative factor), considered at three levels: 0, 5, and 10 d.

The three quantitative factors were studied by a central composite design, consisting of 15 experiments (a full factorial design providing $2^3 = 8$ experiments, plus a star design providing (2*3) + 1 = 7 experiments) to which one more replication of the center of the experimental domain was added to evaluate the experimental variability, giving a total of 16 experiments. The 16 experiments are reported in Table B1 of Appendix B. The two qualitative factors were studied replicating the experimental design provided in Table B1 for each inoculum origin and for each enzyme separately.

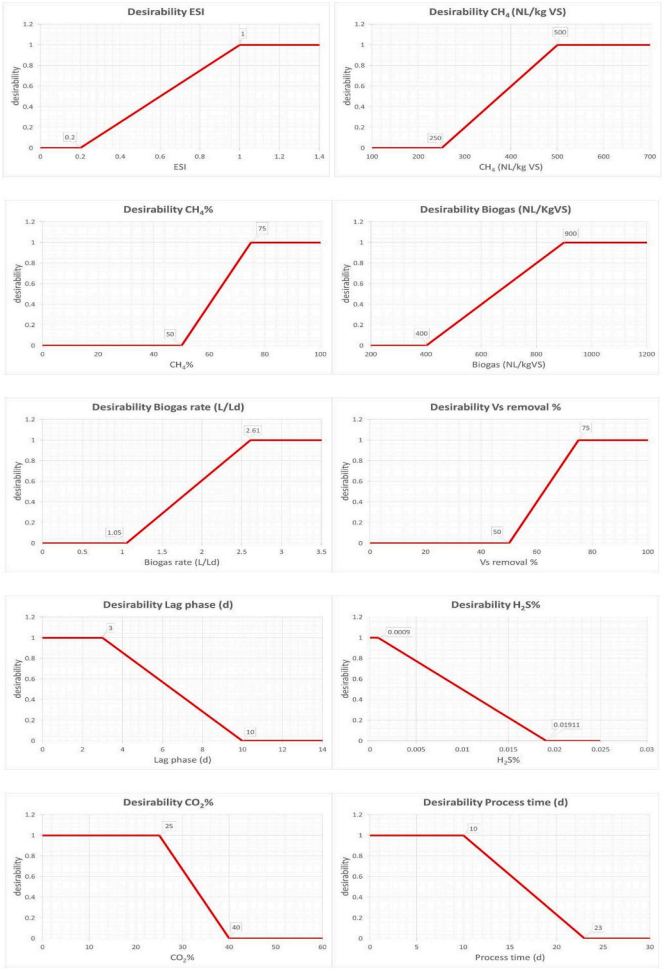

Fig. 1. Desirability functions adopted for each experimental response. Each parameter is reported on the x-axis and each single desirability is reported on the y-axis. For each response, the two x-value thresholds T_1 and T_2 used for the calculation of the linear function are indicated in the corresponding graphic. ESI: $T_1 = 0.2$, $T_2 = 1$; CH_4 (NL/kg $_{VS}$): $T_1 = 250$, $T_2 = 500$; CH_4 %: $T_1 = 50$, $T_2 = 75$; Biogas (NL/kg $_{VS}$): $T_1 = 400$, $T_2 = 900$; Biogas rate (L/Ld): $T_1 = 1.05$, $T_2 = 2.61$; Vs removal %: $T_1 = 50$, $T_2 = 75$; Lag phase (d): $T_1 = 3$, $T_2 = 10$; H_2S %: $T_1 = 0.0009$, $T_2 = 0.01911$; CO_2 %: $T_1 = 25$, $T_2 = 40$; Process Time (d): $T_1 = 10$, $T_2 = 23$.

2.7.2. Desirability functions and calculation of regression models

The impact of several factors on process performance was investigated through DoE, considering parameters related to three process aspects: i) ESI; ii) biogas composition, including CH_4 (%), H_2S (%), CO_2 (%); iii) process efficiency, encompassing biogas production (NL/kg $_{VS}$), lag phase (d), process time (d), biogas rate (L/L•d), and VS removal (%).

Multicriteria decision-making approach, employing desirability functions, simultaneously optimized multiple experimental responses.

The desirability functions (Box, 2005) were applied. This method transforms the value obtained for each experimental response into a desirability value ranging from 0 (completely unacceptable) to 1 (completely acceptable). The single desirability values are combined in a global desirability D, through Eq. (4):

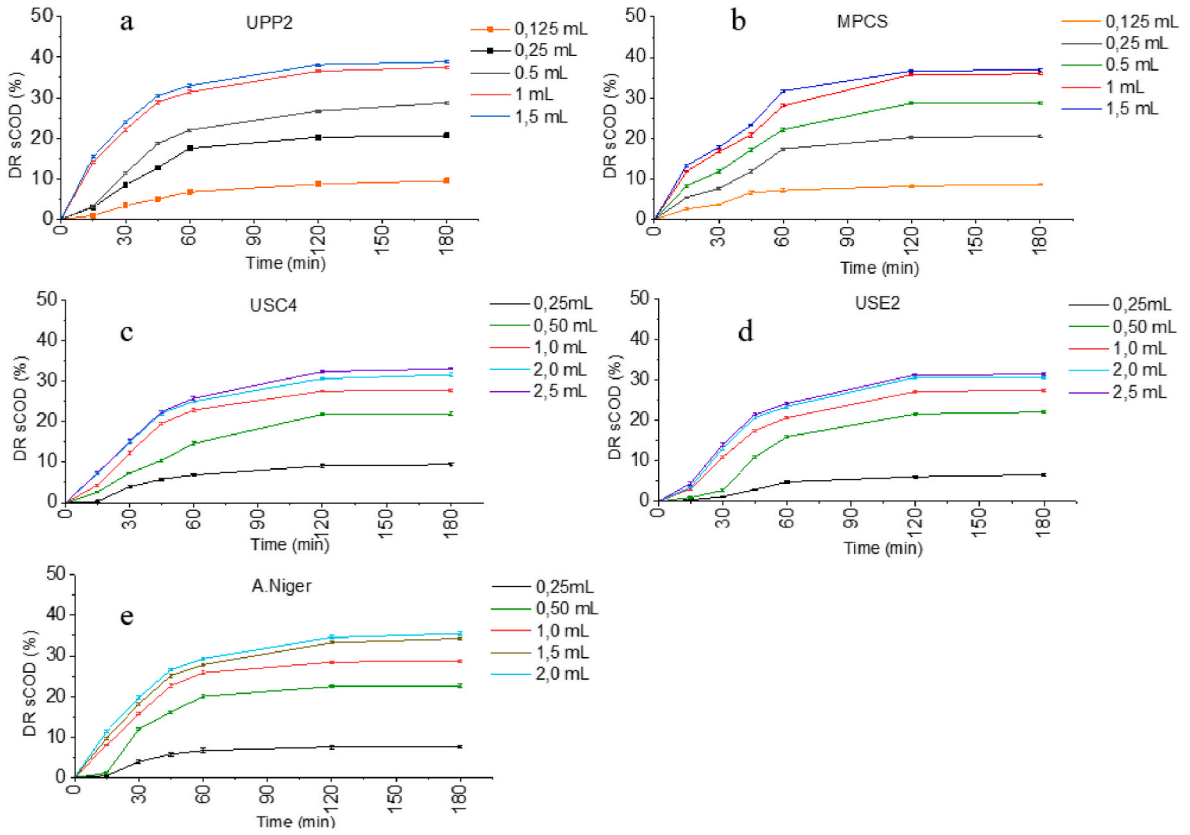

Fig. 2. Disintegration rate (DR) of enzymatic hydrolysis with the five enzymes tested expresses in terms of sCOD. (a) with enzyme UPP2, (b) with enzyme MPCS, (c) with enzyme USC4, (d) with enzyme USE2 and (e) with enzyme A. niger.

$$D_i = \sum_{j=1}^{n} \sqrt[\nu_j]{d_{1,i}^{\nu_1} \bullet d_{2,i}^{\nu_2} \bullet \ldots \bullet d_{n,i}^{\nu_n}} \quad (4)$$

Where D_i is the global desirability for the i-th experiment; n is the number of experimental responses to be combined; $d_{j,\,i}$ is the desirability of the j-th experimental response for the i-th experiment; ν_j is the weight of the j-th experimental response. All the weights were considered equal to 1.

The global desirability (Eq. (4)) corresponds to the geometric average of the single desirability: if just one single value reaches 0, the overall desirability will be null. This behavior has two drawbacks: 1) if the global desirability is 0, it is not possible to verify why the experiment is considered unacceptable: this could be due to several experimental parameters together or just one of them, with the others giving good results; 2) where the global desirability is 0, it is difficult to model D as a function of the studied factors, thus hampering optimization. Fig. 1 represents the single desirability functions adopted for each experimental response. The functions can be divided into two groups. The first group comprises ESI, CH_4 (NL/kg $_{VS}$), CH_4 (%), biogas (NL/kg $_{VS}$), biogas rate (NL/L•d), and VS removal (%). Their desirability $d_i(x)$ is null below threshold T_1, increases linearly between thresholds T_1 and T_2, and is 1 beyond threshold T_2; it is therefore calculated according to Eq. (5), where x is the value of the parameter (ESI, CH_4, etc.) for which the desirability must be calculated:

$$d_i(x) = \begin{cases} 0 \text{ if } x < T_1 \\ \dfrac{T_2 x - T_1}{T_2 - T_1} \text{ if } T_1 < x < T_2 \\ 1 \text{ if } x > T_2 \end{cases} \quad (5)$$

The second group involves H_2S (%), CO_2 (%), lag phase (d), and process time (d). Their desirability $d_i(x)$ is 1 below threshold T_1, decreases linearly between thresholds T_1 and T_2, and is null beyond threshold T_2; it is calculated according to Eq. (6), where x is the value of the parameter (H_2S, CO_2 etc.) for which the desirability must be calculated:

$$d_i(x) = \begin{cases} 1 \text{ if } x < T_1 \\ \dfrac{T_2 - x}{T_2 - T_1} \text{ if } T_1 < x < T_2 \\ 0 \text{ if } x > T_2 \end{cases} \quad (6)$$

Two thresholds for each parameter were chosen to ensure that no 0 value is assigned to any experimental response, facilitating a more straightforward modelling of the global desirability (D). The values of T_1 and T_2 for all the parameters are reported in Fig. 1. The experiments conducted in the DoE build the models, describing each experimental response based on the studied factors, their two-way and three-way interactions, and quadratic effects.

The response surface methodology identified, for each experimental response separately, the best experimental conditions through a grid search algorithm exploring the models in the domain scaled in the range [−1,1] for each factor with a step of 0.1 for each factor included in the model. For each node of the grid, each calculated experimental response was turned into the corresponding value of desirability, and the global desirability D was calculated. It was possible to draw response surfaces in the explored domain showing the trend of the global desirability varying two factors at a time, to evaluate the effect played by each factor on the global desirability and the identification of the experimental conditions providing the highest D values, as the best compromise for all the experimental responses contemporarily.

3. Results

3.1. Enzymatic hydrolysis

The performances of enzymes were evaluated considering hydrolysis time and dosage, the last is a crucial aspect since the addition of enzymes represents an economic cost (Berrocal et al., 2021).

All five enzymes reached the highest DR_{sCOD} (%) at the dosage typically used at industrial plants and in the laboratory, but the optimal treatment time resulted at 120 min. Prolonged treatment did not result in a significant increase (p-level <0.05), in sCOD (Fig. 2). The minimum enzyme dosage was omitted from the AD tests due to achieving a DR_{sCOD} <20%. Whereas intermediate and maximum enzyme dosages underwent evaluation in the AD tests, since $DR_{sCOD} \geq 20\%$.

The selected enzyme dosages used for the DoE for AD test were: 0.25, 0.50, and 1.00 mL/100 gTS for UPP2 and MPCS, 0.5, 1.00, and 2.00 mL/100 g TS for USC4 and USE2, and 0.5, 1.00, and 1.50 mL/100 g TS for A. niger.

Based on DR_{sCOD} after 120 min of treatment at their optimal dosage, the ranking of the enzyme's performance was: 36.59% for UPP2 (1 mL/100 g TS), 35.80% for MPCS (1 mL/100 g TS), 33.30% for A. niger (1.50 mL/100 g TS), 30.63% for USC4 (2 mL/100 g TS), and 30.62% for USE2 (2 mL/100 g TS). The DR_{SCOD} value for A. niger, with an enzyme activity of 6 IU/mL, was 33.30%, which was comparable to the result observed by Mlaik et al. (2019a), which was around 34%.

All the tested enzymes demonstrated activity towards cellulose and hemicellulose, which are the primary components of OFMSW (López-Gómez et al., 2019). All these enzymes can treat vegetable and fibrous biomasses, but UPP2 outperformed the others in terms of DR_{sCOD} and organic matter solubilization due to its comprehensive mix of enzymes, including cellulases, hemicellulases, pectinases, and proteases. This combination allows it to potentially degrade all the components of OFMSW. Cellulases break down β-1,4-linkages in the cellulose polymer, releasing readily digestible sugar units like glucose and other monomers (Mendes et al., 2006).

The unique feature of UPP2 compared to the other tested enzymes is the presence of proteases, which can break peptide bonds and release amino acids suitable for fermentation (Silva et al., 2019) promoting the formation of new protein products from OFMSW. Moreover, the effect of UPP2 in preventing the formation of superficial limiting layers was experimentally evident, while the presence of surfactant proteins in USE4 and USE2 had a negligible impact on OFMSW.

3.2. Evaluation of single response models

The experimental responses can be gathered in two groups: i) technical parameters, including biogas and CH_4 productions, CO_2 and H_2S contents, and VS removal; and ii) kinetic parameters, including the biogas rate, lag phase, and process time. These models were built separately for each enzyme and each inoculum source (WAS and CAS). The single response models and the response surfaces are reported in Appendix C of Supplementary Material. Here, only the most important single models are discussed. The models for ESI are not reported since the R^2 values were <0.8 and cannot be used for a deep evaluation of the effects played by the investigated factors. This R^2 value could be due to a low entity of the effects if compared to the experimental error.

3.2.1. Technical parameters: biogas production

The biogas production models identified two groups based on trends in Fig. C1-C2 and Tables C1 and C2: UPP2, MPCS, and A. niger on one side, and USC4 and USE2 on the other. All the models had $R^2 > 0.97$ and included similar parameters with WAS and CAS, indicating no differences due to the inoculum origin.

For UPP2, MPCS, and A. niger (Fig. C1 a, c, h), the interaction between INOC and DOSE demonstrated that by increasing the DOSE, the biogas production increased, especially at high INOC values. The representation of INOC *vs* SI (Fig. C1 b, d, i), proved that when SI was low or high, an increase of INOC improved the biogas production, particularly at high SI.

It is worth noting that, increasing SI at low INOC, the biogas production slightly decreased since a high SI promotes inhibition, which can be counterbalanced by an inoculum with a methanogenic and acclimatized microbial population (Zhang et al., 2019).

Only for A. niger, the interaction between DOSE and SI (Fig. C1 g) proved that the increase of DOSE improved the biogas production at low and high SI with a quadratic effect, but the increase in biogas was more pronounced for higher DOSE and high SI values (SI = 2:1). This trend can be due to the capability of A. niger to depolymerize cellulose and hemicellulose, making OFMSW more easily digestible (Khoufi et al., 2011), even with low amount of the inoculum.

In this study, when AD was conducted with the maximum A. niger dosage (1.5 mg/100 g TS) and inoculum non-incubate (0 d), at the lowest substrate inoculum ratio (SI) of 1:2, the biogas production of 679–689 NL/kg $_{VS}$ was achieved. This result is comparable to the 672 NL/kg $_{VS}$ reported by Mlaik et al. (2019b)' study (Mlaik et al., 2019b), which used A. niger with an activity level of 6 IUL/mL), equivalent to 1.5 mL/100 g TS.

Whereas, when AD was performed under the same conditions but with the incubated inoculum (5–10 d) the biogas production reached 743–744 NL/kg $_{VS}$, proving the key role of the incubation of the inoculum, in agreement with (Zhang et al., 2019).

For USC4 and USE2 (Table C1 and C2), the interaction between INOC and SI (Fig. C1, e, and f) indicated that the biogas production increased by increasing INOC, while SI showed a less pronounced effect. INOC exhibited a quadratic effect, reaching the highest biogas production when INOC was incubated (5–10 d). The composition of USC4 USE2 is similar to that of MPCS, and UPP2, respectively, but in USC4 and USE2, there are surfactant proteins. AD, without incubation of the inoculum (T0), at low DOSE (0.25–0.5 mL/100 g TS), with USC4 and USE2, reached about 90–94 $\%_{v/v}$ of the biogas production obtained with MPCS and UPP2. This indicates that the use of surfactants to homogenize a heterogeneous feedstock, such as OFMSW, with a non-incubated inoculum, can inhibit the microbial population of the inoculum (Chen et al., 2008). Similar considerations can be derived for CAS (Fig. S2).

3.2.2. Technical parameters: CH_4 production and content

The models of the CH_4 production for USC4, USE2, and A. niger achieved $R^2 > 0.96$, while for UPP2 and MPCS the R^2 was 0.8 with CAS and 0.85 with WAS (Tables C1 and C2).

For UPP2 and MPCS with CAS, the models were simple since high CH_4 production was observed when both DOSE and INOC were at high levels. Hence, the addition of UPP2 and MPCS increased CH_4 production.

For UPP2, MPCS, and USC4 (Fig. C3 a, b, d) for WAS, the interaction between INOC and DOSE showed a similar trend: at high and low DOSE, an increase of INOC increased the CH_4 production. The quadratic effect of DOSE was evident, with the highest CH_4 production occurring at intermediate DOSE levels at both high and low INOC.

Considering WAS, the CH_4 production for UPP2, MPCS, and USC4 was mostly improved by incubating the inoculum (high INOC), rather than acting on the addition or the DOSE of the enzyme. These results agreed with the study of (Li et al., 2013), where the incubation of WAS promoted the acclimation of the microorganisms allowing a higher biodegradation efficiency rather than CAS.

For USE2, USC4, and A. niger, with WAS and CAS, the representation of the interaction between INOC and SI (Fig. C3 c, e, g, and C4), proved that increasing INOC at high and low SI levels, the CH_4 production increased, with a pronounced effect at high SI. However, increasing SI at low INOC, inhibition occurred.

This result confirmed the importance of working with an incubated inoculum (INOC = 5–10 d) which allows operation with a high SI (1:1 or 2:1) without promoting inhibition due to the high concentration of the

acclimatized methanogenic population that can biodegrade the organic matter, as already demonstrated in (Demichelis et al., 2022).

All the AD performed with the five investigated enzymes increased the CH_4 production and content by about 10–13% compared to AD carried out on the untreated OFMSW, according to (Mutschlechner et al., 2015). Considering both CH_4 and biogas productions, for UPP2 and MPCS the increase of the DOSE enhanced the biogas production while increasing INOC enhanced the CH_4 content.

The increase in CH_4 production depends not only on the enzyme and its DOSE but also on the substrate composition. According to (Speda et al., 2017), enzymes are most effective on substrates primarily composed of complex compounds and nutrients, which they hydrolyze into simpler forms. Therefore, both the enzyme and the substrate structure should be considered to enhance the CH_4 production and content.

3.2.3. Kinetic parameters: process time

The models of all five enzymes reached $R^2 > 0.99$ for both origins (Tables C1 and C2). These models exhibited differences depending on the origin of the inoculum.

Considering WAS, the models only contained INOC and its quadratic effect (Figure C6) because an increase in INOC consistently decreased the processing time. Consequently, the best conditions were achieved with high INOC values notwithstanding the values of SI and DOSE. Considering CAS, (Tables C1 C2, and Figure C7) the increase in INOC always reduced the processing time since a high INOC increased the methanogenic population (Zhang et al., 2019)which could faster degrade the OFMSW, producing CH_4. Whereas, at low INOC, an increase in DOSE prolonged the processing time. This shift occurred because a high enzyme dose promotes the degradation of organic substances, which could inhibit the process if the microorganisms in the inoculum are insufficiently available to break down these substances.

Considering CAS, an increase in INOC always caused a significant decrease in the processing time at high and low SI (Fig. C7 c, f, i, l, o). When both SI and INOC were high the processing time was reduced by 5 % compared to the worst condition achieved with low INOC, high SI, and low DOSE. It is noteworthy that SI can be high only when INOC is high, according to (Zeng et al., 2010), or when DOSE is low, as experimentally proven in the present study. Pre-treatment with cellulases and proteases did not significantly reduce the lag phase, but after the lag phase, the biogas production increased compared to not enzymatically treated AD (Romano et al., 2009). The lag phase, and consequently the processing time, are significantly reduced by the action of the incubated inoculum (INOC) as already stated in (Demichelis et al., 2022).

3.2.4. Kinetic parameters: biogas rate

All the models showed $R^2 > 0.99$ (Table C1 and C2). For all the enzymes, the models built with WAS (Fig. C8) were simpler than the ones with CAS (Fig. C9).

Considering WAS, for UPP2, MPCS, and A. niger, the interaction between SI and DOSE exhibited a similar trend (Figure C8 a, b, c): at low SI, the increase of the DOSE did not influence the biogas rate, because the process had a low organic loading rate, according to (Atelge et al., 2020).

Considering WAS, for USC4 and USE2, the interaction between INOC and SI was significant (Fig. C8 d, e): at both high and low SI, an increase in INOC strongly increased the biogas rate, according to (Zhang et al., 2020).

Considering CAS (Fig. C9), the response surfaces were similar for all five enzymes. For the interaction between SI and DOSE (Fig. C9 a, d, g, j, m), at low SI, an increase of the DOSE promoted an increase in the biogas rate, while no significant effect (the biogas rate was always high) was observed when DOSE was increased at high SI values. At a low DOSE, the increase of SI enhanced the biogas rate, while this effect was not observed at high DOSE. The interaction between INOC and DOSE (Fig. C9 b, e, h, k, n) showed a different behavior: at low INOC, an

Table 2
models obtained for the Global Desirability (D) for all the enzymes and separately for WAS and CAS origins. For each model, the R^2 value is reported; for each factor included in the models, the coefficient (Coeff), its standard error (S.E.C.), and the corresponding p-level are reported. The values reported in bold correspond to significant effects (p-level <0.05).

		UPP2: R^2 0.9705			**MPCS: R^2 = 0.9784**			**USC4 R^2 = 0.9659**			**USE2: R^2 = 0.9834**			**A_Niger: R^2 = 0.9890**		
WAS		Coeff	S.E.C.	p-level	Coeff	S.E.C.	p-level	Coeff	S: E.C.	p-level	Coeff	S.E.C.	p-level	Coeff	S.E.C	p-level
	Intercept	**0.710**	**0.012**	**0.0000**	**0.709**	**0.012**	**0.0000**	**0.619**	**0.016**	**0.0000**	**0.6339**	**0.0098**	**0.0000**	**0.6469**	**0.0078**	**0.0000**
	DOSE	**0.0373**	**0.0092**	**0.0020**	**0.0394**	**0.0082**	**0.0004**	**0.028**	**0.010**	**0.0195**	**0.0354**	**0.0074**	**0.0004**	**0.0397**	**0.0059**	**0.0000**
	SI	0.0189	0.0092	0.0655	–	–	–	**0.187**	**0.010**	**0.0000**	–	–	–	**0.0165**	**0.0058**	**0.0165**
	INOC	**0.1718**	**0.0094**	**0.0000**	**0.1853**	**0.0082**	**0.0000**	–	–	–	**0.1954**	**0.0075**	**0.0000**	**0.1807**	**0.0059**	**0.0000**
	$INOC^2$	**−0.046**	**0.015**	**0.0133**	–	–	–	–	–	–	**−0.033**	**0.012**	**0.0202**	–	–	–
	SI*INOC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	$DOSE^2$	–	–	–	−0.052	0.015	0.0053	−0.041	0.019	0.0532	–	–	–	−0.0333	0.0097	0.0057
		UPP2: $R^2$0.9040			**MPCS: R^2 = 0.9462**			**USC4 R^2 = 0.9663**			**USE2: R^2 = 0.9583**			**A_Niger: R^2 = 0.9457**		
CAS		Coeff	S.E.C.	p-level	Coeff	S.E.C.	p-level	Coeff	S.E.C.	p-level	Coeff	S.E.C.	p-level	Coeff	S.E.C.	p-level
	Intercept	**0.706**	**0.011**	**0.0000**	**0.697**	**0.010**	**0.0000**	**0.6327**	**0.0080**	**0.0000**	**0.6177**	**0.0085**	**0.0000**	**0.6512**	**0.0084**	**0.0000**
	DOSE	–	–	–	0.024	0.012	0.0682	0.0203	0.0098	0.0616	**0.023**	**0.010**	**0.0429**	**0.031**	**0.011**	**0.0124**
	SI	–	–	–	–	–	–							–	–	–
	INOC	**0.162**	**0.014**	**0.0000**	**0.175**	**0.012**	**0.0000**	**0.185**	**0.010**	**0.0000**	**0.182**	**0.011**	**0.0000**	**0.156**	**0.011**	**0.0000**
	$INOC^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	SI*INOC	–	–	–	**0.033**	**0.014**	**0.0300**	**0.028**	**0.011**	**0.0268**	–	–	–	–	–	–
	$DOSE^2$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

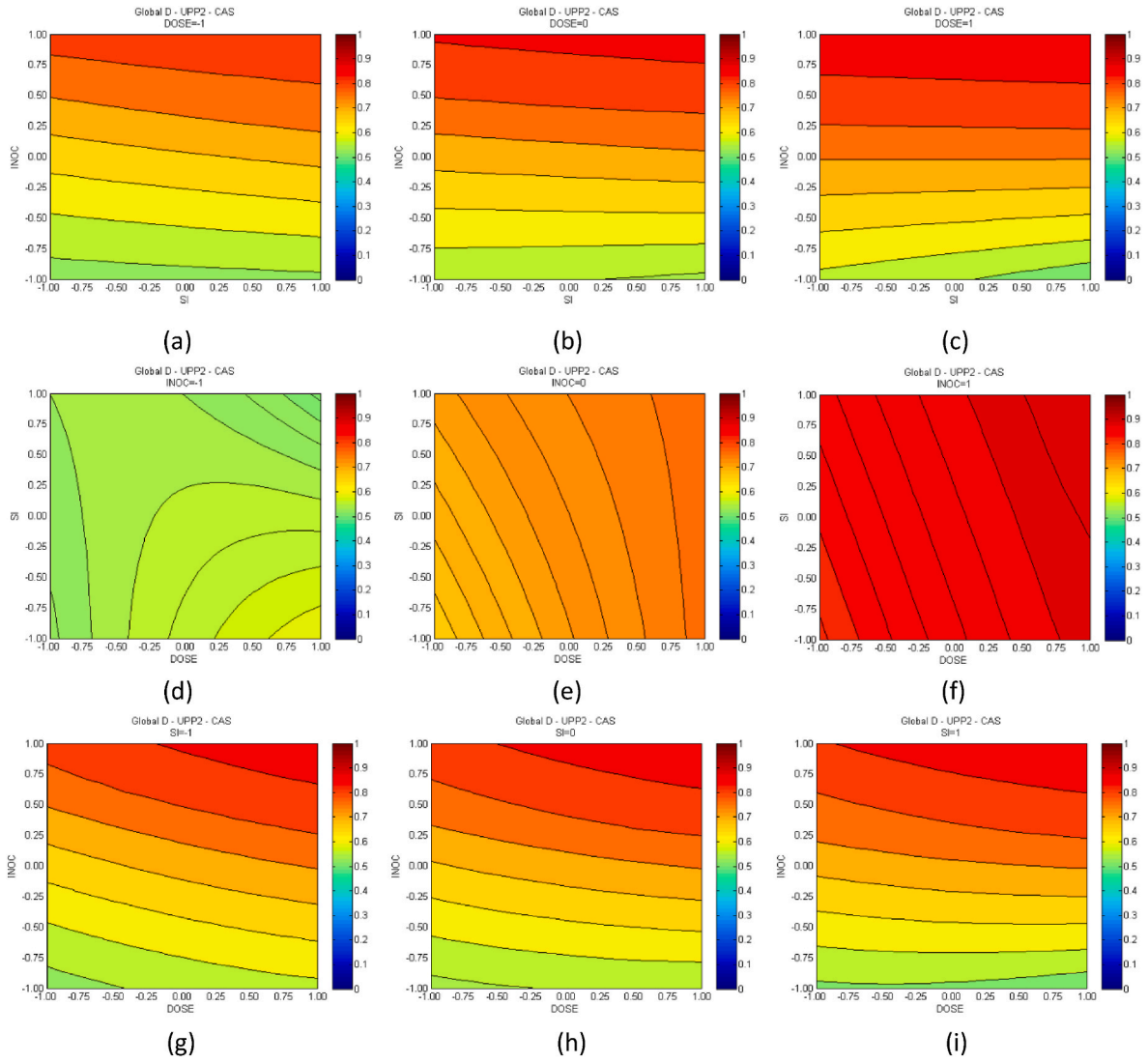

Fig. 3. Contour plots of the response surfaces for the global desirability for UPP2 with CAS; two factors are reported at a time, with the third one at the minimum (−1), intermediate (0), or maximum (+1) level: INOC vs SI with DOSE at −1 (a), 0 (b) and +1 (c); SI vs DOSE with INOC at −1 (d), 0 (e) and +1 (f); INOC vs DOSE with SI at −1 (g), 0 (h) and +1 (i). The x and y axes report, for each factor, the range-scaled values in the [-1,1] interval.

increase in DOSE did not affect the biogas rate (in general, low), while it caused a slight biogas rate increase when INOC was high. At both high and low DOSE, an increase in INOC deeply increased the biogas rate but the effect was higher at high DOSE. Similar considerations can be made for the interaction between INOC and SI (Fig. C9 c, f, l, o). For all the tested enzymes, the biogas rate was primarily influenced by the incubated inoculum (INOC = 5–10 d), and the DOSE was a booster. The biogas rate was not strictly affected by the DOSE as proved in the study of Romano et al. (2009); Romano et al. (2009).

3.2.5. *Kinetic parameters: volatile solids removal*

The models for VS removal showed $R^2 > 0.92$ for UPP2, MPCS, USC4 and USE2, and $R^2 > 0.87$ for A. niger (Table C1 and C2). For UPP2 and MPCS, the models with WAS and CAS were similar, while they were different for USC4 and USE2. Considering the response surfaces with WAS and CAS (Fig. C10-C11), for UPP2 and MPCS, the interaction between INOC and DOSE (Figures C10-C11, a and c) exhibited a similar behavior. At low INOC, the VS removal reached a maximum at intermediate DOSE, while at high INOC, the increase in DOSE improved VS removal, reaching its maximum at medium and high DOSE. The response surfaces with WAS for all the five enzymes demonstrated that the interaction between INOC and SI was significant (Fig. C10, b, d, e, f, g): at high INOC, an increase in SI improved the VS removal, while at low INOC, the variation of SI had less impact and VS removal was low according to (Demichelis et al., 2022). At both high and low SI, an increase in INOC enhanced the VS removal but this improvement was more pronounced at high SI values. For A. niger with CAS, high DOSE and INOC improved VS removal, while the SI was not significant and could be set at the most favorable value (SI = 2:1).

3.3. *Global desirability evaluation*

Table 2 reports the coefficients included in the models calculated for the global desirability D for all the enzymes and for CAS and WAS origins independently. The D values obtained were used to build surface responses representing two factors at a time. Figs. 3 and 4 depict the contour plots of the surface responses obtained for UPP2 and MPCS using inoculum CAS, as representative examples, since they obtained the two most different results. In detail, response surfaces like those reported for UPP2 (Fig. 3) were obtained for all the enzymes both with CAS and WAS, except for MPCS with CAS (Fig. 4).

Considering UPP2 (and all other enzymes except MPCS), INOC, and SI (Fig. 3a–c), at both high and low INOC values, an increase of SI did not significantly affect D (it was low at low INOC and high when INOC was

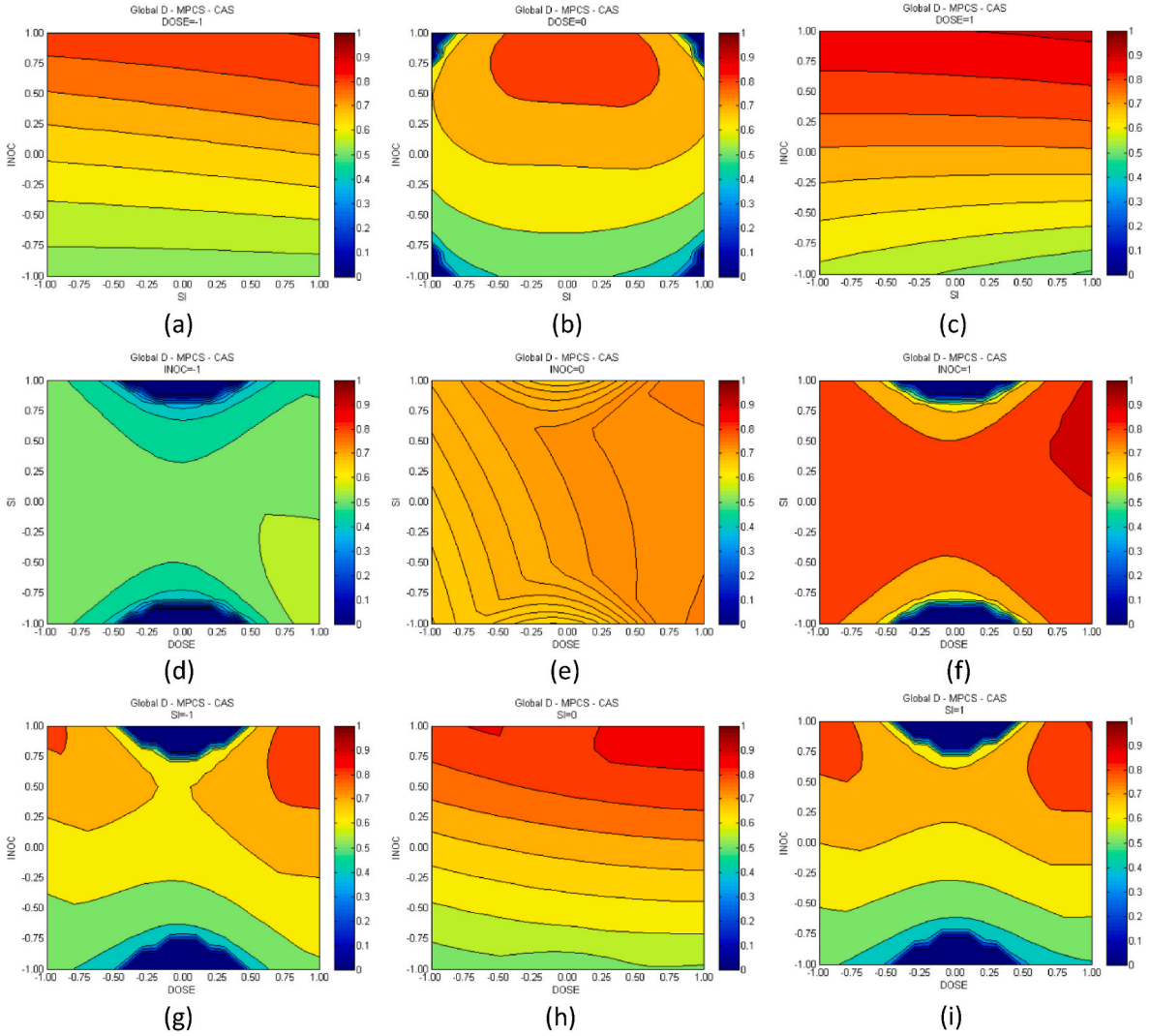

Fig. 4. Contour plot of the response surfaces for the global desirability for MPCS with CAS; two factors are reported at a time, with the third one at the minimum (−1), intermediate (0), or maximum (+1) level: INOC vs SI with DOSE at −1 (a), 0 (b) and +1 (c); SI vs DOSE with INOC at −1 (d), 0 (e) and +1 (f); INOC vs DOSE with SI at −1 (g), 0 (h) and +1 (i). The x and y axes report, for each factor, the range-scaled values in the [-1,1] interval.

high). Whereas at both high and low SI values, an increase of INOC significantly increased D which almost stabilized at INOC values > 0.9. A significant effect of DOSE can be identified since the surface was shifted at higher D values increasing DOSE from −1 to 1. Fig. 3d–f shows the trend of D varying SI and DOSE. When SI was high, an increase of DOSE slightly decreased D, while the effect was opposite at low SI values. No significant effect of SI at high or low DOSE can be detected, while the effect of INOC was evident, with the surface that was significantly shifted at higher D increasing INOC from −1 to 1.

The trend of D varying INOC and DOSE (Fig. 3g–i) proved that at both high and low INOC values, an increase of DOSE slightly increased D. However, D was always high at high INOC and low at low INOC. At both high and low DOSE, increasing INOC significantly increased D which almost stabilizes at INOC values > 0.9; nevertheless, this behavior was more significant at high DOSE values. The effect of SI was not significant, showing the surface that slightly shifts at higher D values increasing SI.

The trends reported in Fig. 3 are shared by all the enzymes with both CAS and WAS, except MPCS with CAS. However, the different enzymes and the two origins can be compared from a quantitative point of view by considering the D values. UPP2 and MPCS showed the best results, with CAS and WAS, with D ≥ 0.9, while the other enzymes (USC4, USE2, A.niger) showed surfaces shifted at lower values, with D < 0.87. In the case of USE2 and USC4, the lower D values can be ascribed to the possible side effects of surfactants in the AD process, as reported in (Chen et al., 2008).

For MPCS with CAS (Fig. 4), considering the response surface of D as a function of INOC and SI (Fig. 4a–c), different trends can be identified: at high and low DOSE, at both high and low INOC, increasing SI, D slightly increased or was stable. At intermediate DOSE levels (Fig. 4b), D is maximum at INOC in the range [0.5,0.8] and SI [−0.5; 0], with the extremes of the domain showing the worst results.

Fig. 4 d-f shows D as a function of SI and DOSE: at both high and low SI values, DOSE had a quadratic effect with a minimum at intermediate DOSE levels. This effect was less evident at intermediate SI values (1:1). This means that when SI was low (1:2) or high (2:1), DOSE should not be kept at intermediate levels. When DOSE was high or low, increasing SI did not affect D which slightly increases or remains stable. The effect of INOC was visible, hence when INOC increased the surface significantly shifted at higher D values.

The interaction between INOC and DOSE (Fig. 4 g-i), showed the quadratic effect of DOSE, for high and low SI values: when INOC was at the extremes (high or low), DOSE should not be kept at intermediate levels, where it showed a minimum. The effect of INOC was evident because an increase in INOC always increased D values.

Table 3 reports the best experimental conditions identified by the

Table 3
Best operative conditions identified by the grid search algorithm for the models built for the Global Desirability (D) and the models based on single parameters providing adequate models ($R^2 > 0.8$) for all the enzymes and with both CAS and WAS origins. "/" corresponds to a parameter that can be kept at any level; "-" is used instead when no adequate model could be calculated (no model at all or $R^2 < 0.8$).

		WAS				CAS			
	Model	**DOSE (mL/100 g TS)**	**S:I**	**INOC (d)**	**Ybest**	**DOSE (mL/100 g TS)**	**S:I**	**INOC (d)**	**Ybest**
UPP2	**D**	1 acceptable up to 0.96	2:1 acceptable up to 1.03:1	10	0.893	1 acceptable up to 0.89	2:1 acceptable up to 0.8:1	10	0.909
	Biogas (NL/kg $_{VS}$)	0.89	2:1	10	734.87 NL/kg Vs	0.89	2:1	10	735.59 NL/kg Vs
	CH_4 (NL/kg $_{VS}$)	0.78	/	10	506.46 NL/kg Vs	1	/	10	504 NL/kg Vs
	CH_4%	–	–	–	–	–	–	–	–
	CO_2%	–	–	–	–	–	–	–	–
	Process Time (d)	/	/	10	10 d	0.25 or 1	2:1 or 1:2	10	9.97 d
	Biogas rate (L/Ld)	0.93	2:1	10	3.83 L/Ld	1	2:1	10	3.87 L/Ld
	Vs removal%	0.89	2:1	10	70.96%	0.89	2:1	10	72.35%
MPCS	**D**	1 acceptable up to 0.96	2:1 acceptable up to 1.78:1	10	0.893	1 acceptable up to 0.96	2:1 or 1:2	10	0.930
	Biogas (NL/kg $_{VS}$)	0.89	2:1	10	720.17 NL/kg Vs	0.89	2:1	10	734.04 NL/kg Vs
	CH_4 (NL/kg $_{VS}$)	0.78	/	10	490.18 NL/kg Vs	1	/	10	500.4 NL/kg Vs
	CH_4%	-	-	-	-	-	-	-	-
	CO_2%	-	-	-	-	0.25 or 1	1:1	5	2.1%
	Process Time (d)	/	/	10	10 d	0.25 or 1	2:1 or 1:2	10	9.97 d
	Biogas rate (L/Ld)	1	2:1	10	3.76 L/Ld	1	2:1	10	3.86 L/Ld
	Vs removal%	0.89	2:1	10	70.32%	0.89	2:1	10	70.57%
USC4	**D**	2 acceptable up to 1.93	2:1 acceptable up to 1.78:1	10	0.836	2 acceptable up to 1.93	2:1 acceptable up to 1.78:1	10	0.867
	Biogas (NL/kg $_{VS}$)	2	2:1	10	739.2 NL/kg Vs	2	2:1	10	739.8 NL/kg Vs
	CH_4 (NL/kg $_{VS}$)	1.78	2:1	10	453.39 NL/kg Vs	2	2:1	10	473.2 NL/kg Vs
	CH_4%	-	-	-	-	2	2:1	10	63.87%
	CO_2%	-	-	-	-	-	-	-	-
	Process Time (d)	/	/	10	10 d	0.5 or 2	2:1 or 1:2	10	9.97 d
	Biogas rate (L/Ld)	2	2:1	10	3.84 L/Ld	2	2:1	10	3.88 L/Ld
	Vs removal%	2	2:1	10	67.91%	2	/	10	67.34 %
USE2	**D**	2	2:1 acceptable up to 1:1	10	0.856	1 acceptable up to 1.93	1:2 acceptable up to 1.78:1	10	0.865
	Biogas (NL/kg $_{VS}$)	2	2:1	10	737.7 NL/kg Vs	2	2:1	10	737.3 NL/kg Vs
	CH_4 (NL/kg $_{VS}$)	2	2:1	10	466.7 NL/kg Vs	0.8	2:1	10	470.83 NL/kg Vs
	CH_4%	2	2:1	10	62.68%	2	2:1	10	64.51%
	CO_2%	-	-	-	-	-	-	-	-
	Process Time (d)	/	/	10	10 d	0.5 or 2	2:1 or 1:2	10	9.97 d
	Biogas rate (L/Ld)	2	2:1	10	3.83 L/Ld	2	2:1	10	3.87 L/Ld
	Vs removal%	2	2:1	10	67.42%	2	/	10	66.72%
A_Niger	**D**	1.5 acceptable up to 1.45	2:1 acceptable up to 1.93:1	10	0.850	1.5	2:1	10	0.885
	Biogas (NL/kg $_{VS}$)	1.5	2:1	10	742.53 NL/kg Vs	1.5	2:1	10	743.29 NL/kg Vs
	CH_4 (NL/kg $_{VS}$)	1.5	2:1	10	479.7 NL/kg Vs	1.5	2:1	10	492.3 NL/kg Vs
	CH_4%	1.5	2:1	10	64.73%	1.5	2:1	10	66.66%
	CO_2%	-	-	-	-	0.5 or 1.5	1:1	5	23.13%
	Process Time (d)	/	/	10	10d	1.5	1:2	10	10.03d
	Biogas rate (L/Ld)	1.5	2:1	10	3.86 L/Ld	1.5	2:1	10	3.9L/Ld
	Vs removal%	1.25	2:1	10	68.32%	1.5	/	10	67.58%

grid search algorithm for the models built for the Global Desirability (D) and the single parameters. For both D and the single responses, with both WAS and CAS, INOC needs to be set at the highest level, proving that the incubation of the inoculum is essential to improve the performances of AD (Zhang et al., 2019).

Concerning the global desirability D, DOSE should be always kept at the highest value, to provide the best overall performances; however, not significantly different results can also be obtained when DOSE was considered in the upper part of the range (>0.96 mL/100 g TS for UPP2 and MPCS; >1.93 mL/100 g TS for USC4; >1.45 mL/100 g TS for A. Niger). This trend was confirmed for all the enzymes with both CAS and WAS with few exceptions needing the use of the maximum DOSE level (USE2 with WAS and A. niger with CAS). For UPP2 with CAS, the range can be further widened up to 0.89 mL/100 g TS.

If the single response models are considered, DOSE should be kept at the highest level for USC4, USE2, and A. niger. While for UPP2 and MPCS, the DOSE can also be reduced at medium/high levels (concerning the ranges here adopted) to improve the biogas and CH_4 production and VS removal. It is worth mentioning that, for A. niger, the optimal DOSE is lower than that indicated in the literature.

SI showed the highest variations: when single response models are considered, SI should be kept at the highest value (2:1) for all enzymes and both with CAS and WAS, apart from some parameters (process time, some models for VS removal with CAS and some model for CH_4 with WAS). When the single responses are considered contemporarily in the global desirability D, the range of SI values providing good results can be widened up to 1.78:1 (for MPCS with WAS, USC4 with CAS and WAS, USE2 with CAS), 1:1 for USE2 with WAS), 1.03:1 or 0.8:1 (for UPP2 with WAS and CAS respectively).

4. Conclusions

The study optimized the interaction between the enzymatic pre-treatment and the anaerobic digestion (AD), exploring dosages and pre-treatment times for five enzymes (UPP2, MPCS, USC4, USE2, and A. niger). and for AD inoculum sources (waste active sludge - WAS - and cow-agricultural sludge - CAS), substrate inoculum (SI) ratio, and inoculum incubation time (INOC). Global desirability highlighted the best pre-treatment and AD conditions, providing the best compromise among all the experimental responses. The best conditions correspond to higher enzyme dosages, SI, and INOC. MPCS exhibited the highest efficiency, followed by UPP2. CAS-based processes outperformed WAS by degrading organic matter more effectively. The findings could be validated at larger scales to demonstrate their effectiveness and then implemented in industrial ones to increase energy yields and contribute to producing renewable energy.

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CRediT authorship contribution statement

Francesca Demichelis: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elisa Robotti:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fabio Alessandro Deorsola:** Supervision, Methodology. **Simone Cerruti:** Methodology, Data curation. **Emilio Marengo:** Supervision, Data curation. **Tonia Tommasi:** Supervision. **Debora Fino:** Supervision, Methodology.

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

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2024.143760.

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