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Optimisation of whey permeate anaerobic digestion at different inoculum to substrate ratios and initial pH values under mesophilic conditions

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ABSTRACT

The use of whey permeate as anaerobic digestion (AD) feedstock presents a potential challenge for methane (CH_4) production due to its acidity and high carbon content. This study investigated the optimal parameters of whey permeate AD by incorporating CH_4 yield with CH_4 equivalent of produced volatile fatty acids (VFAs) over volatile solid degradation to determine the mass balance of the system. Batch AD experiments were conducted at various inoculum-substrate ratios (ISRs), initial pH levels, and mesophilic temperatures. Results demonstrated that ISR influences VFA profiles regardless of the variation in temperatures and pH, with the exception at ISR 2. Mass balance analysis showed that the highest yield (1441.42 ± 43.78 $NL_{CH4}kg_{VS}^{-1}$ degraded) was achieved in reactors operated at ISR 2, initial pH of 7.5, at 30°C. The results confirmed a dynamic effect of ISR and temperature throughout whey permeate AD. Finally, response surface methodology was employed to understand operational optimisation based on mass balance data.

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KEYWORDS
Anaerobic digestion; BMP; central composite design; lactose; process optimisation; response surface methodology

1. Introduction

Whey permeate constitutes one of the dairy wastewaters from milk processing. It is generated from membrane filtration of cheese whey during whey protein production (Banaszewska et al. 2014; Slavov 2017). The filtration process retains approximately 80% of the lactose present in milk, transferring it to the resulting whey permeate (Bosco et al. 2018). A comprehensive assessment of the technological and economic feasibility from various whey processing options highlighted that conversion of cheese whey into whey protein leads to a substantial stream of whey permeate that necessitates further treatment due to its significant lactose fraction (Peters 2005). Global whey protein production totalled 2.8 million tons in 2020, with forecasts indicating growth to 3.1 million tons by 2029 (OECD/FAO 2023). This highlights the critical need to develop efficient solutions that

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both sustain the growth of dairy industry, while promoting circular economic practices. Among available wastewater treatment technologies, anaerobic digestion (AD) offers an opportunity for the valorisation of whey permeate by converting its organic matter into bioenergy in the form of methane (CH_4) and valuable volatile fatty acids (VFAs) as its intermediate products. However, despite AD being widely regarded as a cost-effective and reliable technology for wastewater treatment, optimizing its performance for dairy wastewaters remains a challenge (Bella and Rao 2023).

A series of intricate microbiological processes takes place in the AD. These processes involve a diverse array of microorganisms working in sequential stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. These microbial communities are sensitive to environmental conditions, emphasizing the importance of managing various factors in order to maximize AD design and efficiency (Mao et al. 2015). Effective AD operation relies on careful management of the environmental parameters, including inoculum to substrate ratio (ISR), incubation temperature, and pH. The ISR represents the proportion of volatile solids (VS) derived from the inoculum to the VS present in the substrate. A low ISR (indicating a high substrate level) increases the likelihood of acidification and inhibition phenomena as reported by Holliger et al. (2016), especially for easily degradable substrates. Conversely, AD operation with high ISRs reduces the volume of substrate to be processed in the digester and can lead to non-reproducible results, especially when handling heterogeneous substrates (González-Fernández and García-Encina 2009; Raposo et al. 2008). Determining the appropriate balance of ISR is essential in achieving a maximum CH_4 potential and production rate. Empirical studies confirm that the ideal ISR for AD systems is substrate-dependent, with different feedstocks requiring specific ISR (Demichelis et al. 2022; Filer et al. 2019; Flores-Mendoza et al. 2020). For experimental validation, Owen et al. (1979) initially established 1:1 VS substrate-to-inoculum ratio (gVS/gVS) as the fundamental benchmark for AD systems. While their findings offer valuable guidance for ISR determination, substrate-specific ISR variations must be considered. Consequently, a systematic analysis of biochemical methane potential (BMP) protocols by Filer et al. (2019) recommends evaluating at least three distinct ISRs, with particular emphasis on substrate biodegradability.

In addition to ISR, other factors such as temperature and pH play an important role in the AD performance. Temperature significantly influences microbial growth rate, metabolic activities, and population dynamics within the digester. Additionally, temperature also influences the gas transfer rates and the settling characteristics of biological sludges. Anaerobic digesters typically operate within mesophilic (30–38°C) or thermophilic (50–58°C) temperature ranges (Filer et al. 2019). Thermophilic digestion generally exhibits higher degradation rates compared to mesophilic digestion, primarily due to the accelerated biochemical reactions at elevated temperatures. Increasing the operational temperature also offers additional benefits, including increased solids reduction, improved dewatering, and reduction or elimination of pathogenic organisms (Metcalf and Eddy 2014). However, these advantages come with trade-offs, such as increased energy cost, increased odour potential and reduced process stability (Appels et al. 2008). The pH level within the digestate also influences AD kinetics by affecting microbial enzymatic reactions. Low pH levels are associated with VFAs accumulation during acidogenesis, which leads to methanogenesis inhibition, unstable operation or even process failure (Pearse et al. 2018). Conversely, high pH levels can cause inhibitory effects due to the

presence of high free ammonia and ammonium ion concentration (Azkarahman et al. 2021), which can permeate microbial cell membranes and disrupt proton balance (Barber 2016). The optimal pH range for AD is typically maintained between 6.5 and 7.6, as pH levels critically affect microbial enzyme activity, potentially altering their structural conformation and reaction kinetics (Parkin and Owen 1986). Moreover, pH values beyond the recommended range typically result in significantly higher operating costs for AD facilities due to the necessity for pH buffering agents.

Whey permeate contains a significant amount of organic carbon, primarily in the form of lactose (ADPI 2023), making it suitable as the carbon source for CH_4 production via the AD process. In addition, the absence of inhibitory compounds, such as cellulose (Kaldis et al. 2020), further demonstrates the potential of whey permeate to be used as AD feedstock. However, despite its potential, the current practical optimisation of whey permeate AD performance is still rarely investigated, especially when compared to other AD feedstocks (Bella and Rao 2023). Previous studies on the AD of whey permeate primarily focused on the determination and evaluation of the kinetic model (Hwang et al. 1992; Yang and Quo 1991). While these studies provide insight into kinetic parameters of the AD based on experimental data, they do not explicitly address process optimisation for AD. Other studies, such as those by Lee et al. (2008) and Hagen et al. (2014), explored the microbial population dynamics in whey permeate AD. However, AD optimisation through controlled microbial population has been shown to be complex, as demonstrated in the latter study (Hagen et al. 2014), while another study even showed lower conversion rates (Zellner et al. 1987). Alternative strategies, such as co-digestion (Fagbohungbe et al. 2019; Gensollen et al. 2022), offer a potential for whey permeate AD optimisation but face practical challenges due to the variability of co-substrate characteristics (Hagos et al. 2017). Furthermore, direct contact with AD industry operators in the UK revealed persistent difficulties in maintaining stable operations of AD plants utilizing whey permeate. For the first time, this study assessed the performance and mass balance of whey permeate AD at various ISRs, pH levels and temperatures under mesophilic conditions through biochemical methane potential (BMP) tests. The organic matter degradation and VFAs production, as well as CH_4 generation under each condition, were assessed to evaluate overall AD performance. This approach provides insights into potential biorefinery applications, including digestate upcycling, VFAs production, as well as a more justified operational efficiency. In addition, the findings contribute to a more comprehensive understanding of the operational efficiency and optimisation of the AD process for whey permeate.

2. Materials and methods

2.1. Inoculum and substrate

Liquid whey permeate used as a substrate in this study was obtained from acid whey filtration from a creamery plant managed by Future Biogas Ltd., UK. Whey permeate was stored at −20°C and subsequently thawed at room temperature prior to testing. The inoculum used in this study was obtained from a full-scale biogas plant that was previously fed with energy crops (i.e. mainly maize, a little part of rye and beet pulp). Before use in BMP tests, the inoculum was incubated at 30 ± 2°C for a period of 5 days and

sieved to remove part of the non-degraded organic fraction. The incubation and sieving aimed to reduce the endogenous CH_4 production from the inoculum. Both whey permeates and biogas effluent used in this study were provided by Future Biogas. Ltd (Guildford, UK).

Both substrate and inoculum were first dried at 105°C overnight to determine their total solids (TS). Afterwards, each sample was subjected to ignition at 550°C for at least 5 h. The VS was determined by deducting the ash content produced during the ignition and subsequently dividing this difference by the weight of the wet sample. This analysis followed standard methods used for the analysis of water and wastewater (APHA 2017). The pH was measured by using a pH meter equipped with a microelectrode (Mettler Toledo™ SevenEasy™ Plus FP20 pH/mV m). Total organic carbon (TOC) and nitrogen analyses were performed by using a Leco CHN628 instrument (LECO Corp., USA). Lactose, protein and fat content of whey permeate were analysed by using a Lactoscope FTIR Diary Analyser (Delta Instruments, Netherlands).

2.2. Batch digester start-up and experimental design

The biochemical methane potential (BMP) test in this study followed the standard test established by Holliger et al. (2016). The digesters were conducted in a batch mode by using 150 mL bottles, sealed with rubber stoppers and crimped with aluminium caps. Appropriate quantities of inoculum, substrate and deionised water were added to keep the ISR constant at a value of 0.5, 1 and 2 on VS basis (w/w) with the working volume set at 70 mL. The initial pH was adjusted by adding 1 N NaOH until target pH was reached (7, 7.5, and 8). Before each experiment, all bottles were flushed with N_2 gas to remove oxygen and ensure the anaerobic conditions within the digester. During the BMP start-up, we found that the digester with initial pH <7 showed a very low CH_4 production since day 1, thus was excluded from this study. The sealed digesters were then placed in the incubation room at each respective temperature (20, 30, and 37°C). Methane production was measured daily by using the liquid displacement method. In this method, 1 M NaOH solution was used to trap CO_2, enabling CH_4 measurement from the produced biogas. Blanks trials (bottles that only contained inoculum and water) were prepared for all different pH values and temperatures to measure the inoculum's endogenous CH_4 production. The obtained values from the relevant blank trials were subtracted from those acquired from the experimental bottles. The test ended when daily CH_4 production was <1% of the accumulated volume of CH_4 (<BMP1%) for three consecutive days. All experiments took place in triplicate and the BMP is expressed as the volume of dry methane gas under standard conditions (273.15 K and 101.33 kPa) per mass of VS input ($NL_{CH4}kg_{VS}^{-1}$ input). The experimental set up of this study is presented in Table 1.

2.3. Kinetic parameters

The kinetics of AD can be determined by applying various mathematical models on data collected from the biochemical methane potential (BMP) test. In this study, we evaluated nine kinetic models (First-order exponential, Fitzhugh, Cone, BPK, Monomolecular, Logistic, Transference, Richards, and modified Gompertz) against daily CH_4 production

Table 1. Operational parameters of the BMP tests.

Target ISR	Temperature (°C)	Initial pH	Volume (mL)	Total TS (%)	Total VS (%)	C/N ratio	No. of replications
0.5	20	7	70	12.13	10.46	25.00	3
0.5	30	7	70	12.13	10.46	25.00	3
0.5	37	7	70	12.13	10.46	25.00	3
1	20	7	70	10.81	9.08	18.19	3
1	30	7	70	10.81	9.08	18.19	3
1	37	7	70	10.81	9.08	18.19	3
2	20	7	70	9.74	7.96	15.11	3
2	30	7	70	9.74	7.96	15.11	3
2	37	7	70	9.74	7.96	15.11	3
0.5	20	7.5	70	12.13	10.46	25.00	3
0.5	30	7.5	70	12.13	10.46	25.00	3
0.5	37	7.5	70	12.13	10.46	25.00	3
1	20	7.5	70	10.81	9.08	18.19	3
1	30	7.5	70	10.81	9.08	18.19	3
1	37	7.5	70	10.81	9.08	18.19	3
2	20	7.5	70	9.74	7.96	15.11	3
2	30	7.5	70	9.74	7.96	15.11	3
2	37	7.5	70	9.74	7.96	15.11	3
0.5	20	8	70	12.13	10.46	25.00	3
0.5	30	8	70	12.13	10.46	25.00	3
0.5	37	8	70	12.13	10.46	25.00	3
1	20	8	70	10.81	9.08	18.19	3
1	30	8	70	10.81	9.08	18.19	3
1	37	8	70	10.81	9.08	18.19	3
2	20	8	70	9.74	7.96	15.11	3
2	30	8	70	9.74	7.96	15.11	3
2	37	8	70	9.74	7.96	15.11	3
Blank	20	7	70	8.11	6.27	10.78	3
Blank	30	7	70	8.11	6.27	10.78	3
Blank	37	7	70	8.11	6.27	10.78	3
Blank	20	7.5	70	8.11	6.27	10.78	3
Blank	30	7.5	70	8.11	6.27	10.78	3
Blank	37	7.5	70	8.11	6.27	10.78	3
Blank	20	8	70	8.11	6.27	10.78	3
Blank	30	8	70	8.11	6.27	10.78	3
Blank	37	8	70	8.11	6.27	10.78	3

data from the BMP tests. Each result was statistically validated by determining the R^2, Akaike Information Criterion (AIC), and normalised root mean square error (NRMSE) value. The results indicated that the modified Gompertz model provided the best fit to our experimental data. As a result, outcomes from other models were excluded from this study, and only kinetic parameter results obtained from the modified Gompertz model are presented.

Nonlinear regression analyses were applied to the modified Gompertz model, treating it as a deterministic function for kinetic parameters estimation (Zwietering et al. 1990). The mean values were obtained from experimental data of triplicate batch digesters were fitted to the model. Nonlinear regression method was performed with Solver Tool of Microsoft Excel software. The model fit enabled estimation of the lag phase, maximum CH_4 production rate and maximum CH_4 production potential. The modified Gompertz model is presented in THE following equation:

$$Pt = Pexp\left\{-\exp\left[\frac{Rm^e}{P}(\lambda - t) + 1\right]\right\} \tag{1}$$

In the above equation Pt represents the specific CH_4 yield at the given time ($NL_{CH4}kg_{VS}^{-1}$ input), *P* represents the maximum CH_4 yield potential ($NL_{CH4}kg_{VS}^{-1}$ input), *Rm* represents the maximum CH_4 production rate ($NL_{CH4}kg_{VS}^{-1}$ input day^{-1}), λ represents the *x*-axis intercept of the tangent of the cumulative CH_4 yield or the lag phase (day), t is the time point of observation (day), and e is the Euler number. Correlation coefficient (R^2) was calculated to determine model fitness.

2.4. Post-BMP analytical tests

Upon completion of the BMP test, the digestates were subjected to several analyses. The VS degradation was determined by subtracting the post-BMP VS of the blank from the experimental digesters and then dividing the result by the amount of VS input from the substrate. The TOC degradation was determined in a similar manner. Both VS and TOC degradations were expressed as percentage. The VS measurement was carried out by following the American Public Health Association guidelines (APHA 2017), and TOC analysis was performed by using the Leco CHN628 instrument (LECO Corp., USA).

The analysed post-BMP VFAs in the present study were C_2–C_5 acids, i.e., acetic acid (HAc), propionic acid (HPr), iso- and n-butyric acid (HIBu and HBu), iso- and valeric acid (HIVal and HVal) and lactic acid (HLac). Both iso-caproic and caproic acids were excluded from the VFA analysis as both compounds were too small or not detected from all samples. The digestate from the experimental digesters was derivatised following the method of Richardson et al. (1989) and analysed with gas chromatography (Agilent 7890B). The gas chromatography was equipped with HP-5MS capillary column (~30 m × 0.25 mm I.D × 0.25 µm film thickness) and flame ionisation detector (FID) with He as the carrier gas. Injector and detector temperatures were set at 275°C. To validate the derived mass balances, chemical oxygen demand (COD) at the equivalent of each VFA was calculated. Conversion factors of 1.067 g_{COD}/g_{acid} for HAc, 1.514 g_{COD}/g_{acid} for HPr, 1.818 g_{COD}/g_{acid} for HIBu and HBu, 2.039 g_{COD}/g_{acid} for HIVal and HVal, 2.207, and 1.07 g_{COD}/g_{acid} for HLac were used to calculate the COD equivalents of each VFA (Atasoy et al. 2020).

2.5. Mass balance calculations

A mass balance calculation was performed to evaluate the impact of the research treatments on the whey permeate AD efficiency. The mass balance calculations included VS degradation and potential CH_4 yield from VFAs in addition to the cumulative CH_4 production to represent the overall AD processes. The potential CH_4 yield from the VFA was determined by converting the amount of VFAs (g_{COD}) to theoretical CH_4 equivalent (0.35 $L_{CH4}g_{COD}^{-1}$) at the standard temperature and pressure conditions. The mass balance ($NL_{CH4}kg_{VS}^{-1}$ degraded) was calculated as follows (Equation (2)):

$$\text{Mass balance} = \frac{\text{Cumulative } CH_4 + \text{Cumulative } CH_4 \text{ potential from VFA}}{\text{VS degraded}} \quad (2)$$

2.6. Experimental design and statistical model

The statistical analysis conducted was a three-way ANOVA with statistical significance assigned to $p < 0.05$ with the use of SPSS (version 27) software. Fisher's Least Significant Difference test was employed as post-hoc test to determine statistically significant differences between means. In addition, central composite design (CCD) of response surface methodology (RSM) was applied to design the relations between parameters affecting the AD process (ISR and temperature) and mass balance to reflect the AD performance. The initial pH was excluded as an independent variable in the equation following its non-significant effect towards mass balance, as obtained from ANOVA analysis. A quadratic CCD model was employed as the experimental design of RSM. The analysis was performed by using Minitab 21 software. The model function given as follows was utilised to describe the system behaviour (Equation (3)):

$$Y = \beta_o + \beta_i X_i + \beta_j X_j + \beta_{ii} X_i^2 + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 + \beta_{ij} X_i X_j + \in \quad (3)$$

In the above equation, Y represents the predicted response (mass balance), X_i and X_j represent the studied independent variables ISR and temperature respectively, β_0 is the intercept, β_i and β_j are the coefficients associated with the linear effects of X_i and X_j, respectively, β_{ii} and β_{jj} are the coefficients associated with the quadratic effects of X_i and X_j, respectively, β_{ij} is the coefficient associated with the interaction effect between X_i and X_j, and ε represents the error term. The model terms were selected or rejected based on the probability value with a confidence level of 95% ($p < 0.05$). The quality of the fit of the polynomial equation model was expressed through the coefficient of determination (R^2). The visualisation of response surfaces was generated with their respective contour plots based on the effects of the two-factor levels (ISR and temperature). Through the use of both 2D and 3D graphs, the effect of the simultaneous interaction between two factors on the response variable (mass balance) was studied.

3. Results and discussion

3.1. Substrate and inoculum characterisations

The physicochemical analyses of the biogas effluent used as the inoculum in this study (Table 2) adhered to the quality criteria for AD established by Holliger et al. (2016).

Table 2. Physicochemical characteristics of biogas effluent (inoculum) and whey permeate (substrate) of the study.

Parameters	Inoculum	Whey permeate
Total solids (%)	8.11 ± 0.03	15.97 ± 0.45
Volatile solids (%)	6.27 ± 0.01	14.47 ± 037
Ash (%)	1.85 ± 0.02	1.50 ± 0.07
pH	8.09 ± 0.12	5.14 ± 0.01
Total organic carbon (%)[a]	42.12 ± 0.62	41.98 ± 0.08
Nitrogen (%)[b]	3.91 ± 0.01	0.60 ± 0.01
C/N	10.78 ± 0.43	70.35 ± 1.28
Lactose (%)	n/d[2]	11.23 ± 0.02
Protein (%)	n/d[2]	1.35 ± 0.01
Fat (%)	n/d[2]	0.43 ± 0.004

[a]In dry matter.
[b]Not determined.

Whey permeate, as substrate of this study, had volatile to total solids (VS/TS) ratio exceeding 90% and a carbon to nitrogen (C/N) ratio of 70.35. These reflected the abundance of organic matter for AD. Substrates rich in carbon content frequently cause volatile fatty acid (VFA) accumulation in AD systems, whereas nitrogen-rich feedstocks generate high total ammonia nitrogen (TAN) levels. Both VFAs and TAN function as crucial metabolic intermediates, yet at elevated concentrations, they may become process inhibitors (Shi et al. 2013). Thus, the high C/N ratio of the whey permeate underpins its potential as substrate for AD, since its low nitrogen content could reduce the risk of methanogen inhibition due to ammonia (NH_3) accumulation in the digester (Procházka et al. 2012), as long as excessive VFA accumulation is avoided. A high nitrogen content in the substrate promotes the formation of both ammonium ions (NH_4^+) and free NH_3 (Azkarahman et al. 2021), which at elevated concentrations exhibit inhibitory effects on microbial metabolism and cellular proliferation (Chen et al. 2014). This nitrogen-derived inhibition particularly destabilizes the functional balance between acidogenic and methanogenic microbial communities (Zheng et al. 2021). The low nitrogen content of whey permeate is a result of the various processing steps in its production, which include milk coagulation during cheese manufacturing, followed by ultrafiltration of the resulting cheese whey. These processes leave whey permeate as a byproduct with limited nutritional value, predominantly characterised by its high lactose content.

The high C/N ratio, easily degradable carbon content (primarily in the form of lactose), and uniform characteristics of whey permeate highlight its potential for AD. In this study, ISRs 0.5, 1, and 2 corresponded to C/N ratios of 25.00, 18.19, and 15.11, respectively. These C/N ratios are relatively low, with ISR 0.5 being the most suited for AD. The recommended C/N ratio for sustaining bacterial communities in AD processes typically ranges from 20 to 30 (Wang et al. 2012; Meegoda et al. 2018). However, it should be noted that the optimal C/N ratio is not universal and can vary based on the specific inoculum, substrate, and operational configuration of the AD system (Jain et al. 2015). This variability is supported by examples from Yen and Brune (2007), Mata-Alvarez et al. (2014), and Kaldis et al. (2020). Adjusting C/N ratio within the digester can be achieved by modifying the amount of substrate as suggested by Wang et al. (2015). Nonetheless, reducing the ISR to increase C/N ratio in the digester may inadvertently decrease the methanogen population in the digester and subsequently hinder CH_4 production.

3.2. Methane production and kinetics

The daily CH_4 production rate (Figure 1) from the BMP tests in this study exhibited dynamic fluctuations, which reflected successive stages of hydrolysis, acidogenesis, acetogenesis and methanogenesis. This dynamic pattern was evident in the recovery of CH_4 production after a notable decline, followed by another decrease until the end of the BMP test. Methane production started promptly on the first day in all digesters, except for ISR 0.5 under 20°C incubation, regardless of its initial pH levels. The highest daily CH_4 production rate from whey permeate was 101.09 $NL_{CH4}kg_{VS}^{-1}$ input on the first day of digestion at ISR 2, initial pH 7 and 37°C. This value exceeds those found with raw cheese whey (Flores-Mendoza et al. 2020) when used as AD substrate. Furthermore, our observed maximum CH_4 production rate exceeds those documented in previous BMP studies

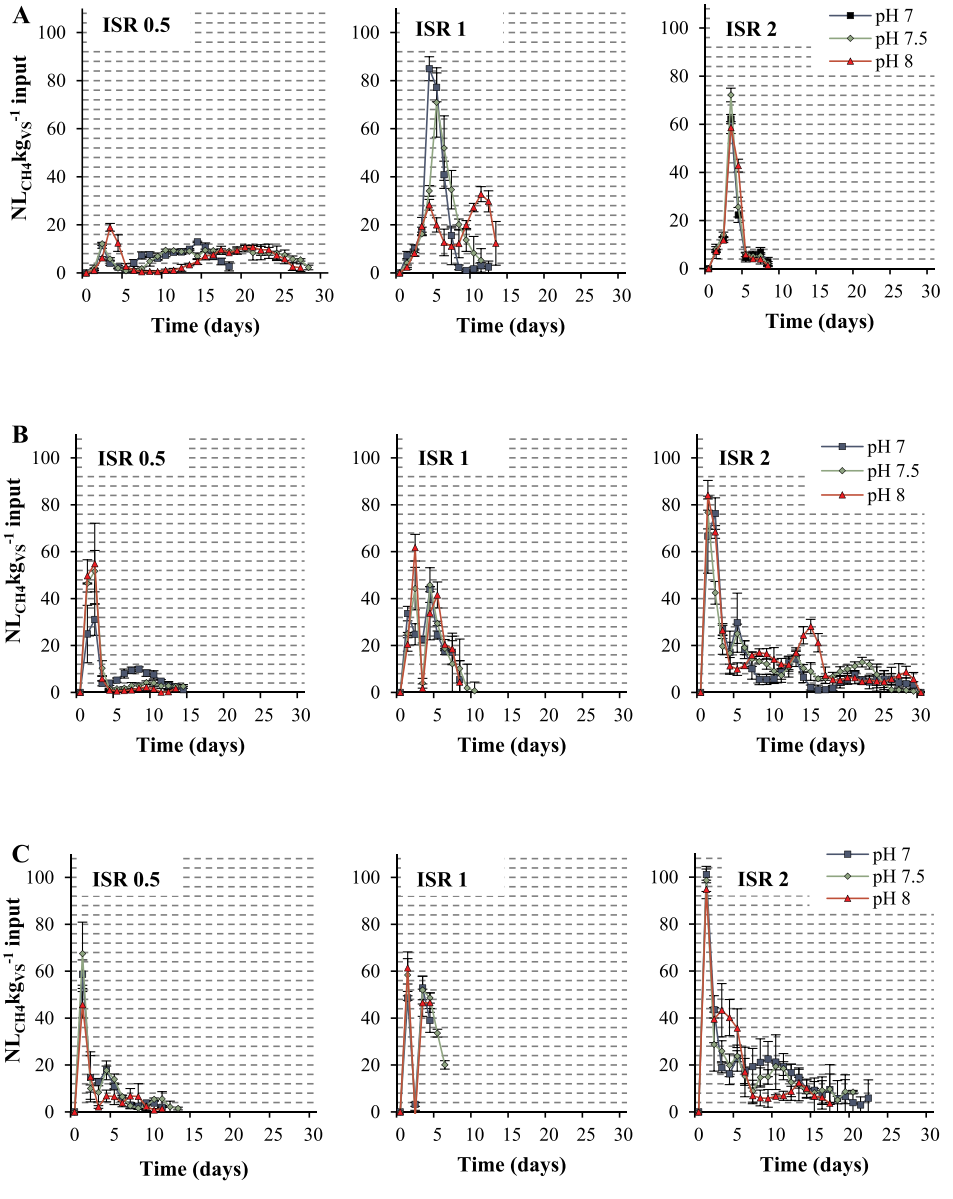

Figure 1. Daily CH_4 production of whey permeate during AD throughout the BMP test at various pH values and ISRs under (A) 20°C; (B) 30°C and (C) 37°C incubation. Error bars indicate standard deviation.

with energy crops and lignocellulosic biomass as the feedstock, such as maize (Raposo et al. 2006), wheat straw (Ferreira et al. 2014), date palm (Mehrez et al. 2022), as well as agro-industrial effluents (Morais et al. 2021). Thus, this confirms the easily degradable characteristics of whey permeate, as peak CH_4 production occurred earlier with higher output.

Additionally, the time required for the daily CH_4 production rate to drop below < BMP1% varied across treatments (Figure 2). This variability was anticipated, given

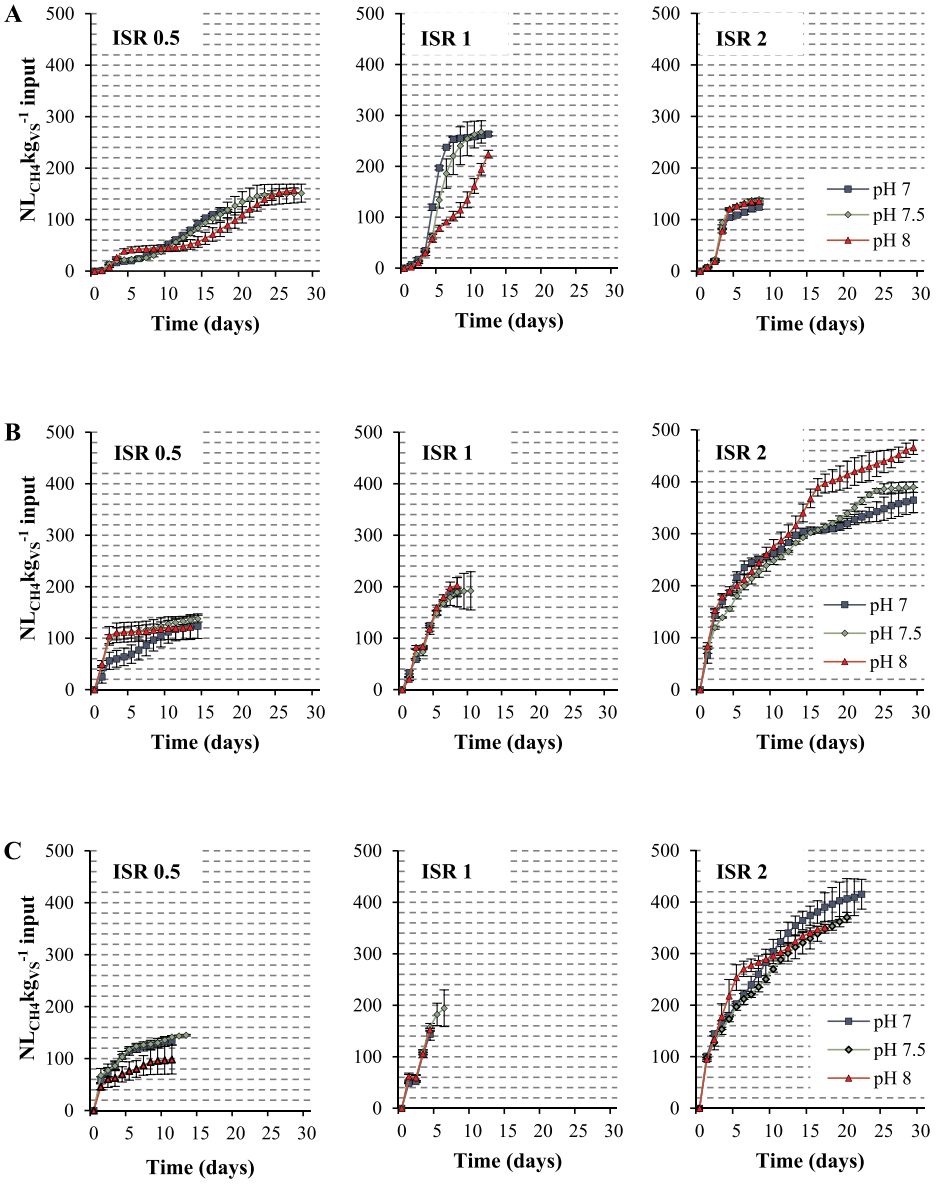

Figure 2. Cumulative CH_4 production during AD of whey permeate throughout the BMP test at various pH values and ISRs under (A) 20°C; (B) 30°C and (C) 37°C incubation. Error bars indicate standard deviation.

that methanogenic activity is influenced by factors such as incubation temperature, ISR and alkalinity as supported by previous studies (Demichelis et al. 2022; Flores-Mendoza et al. 2020). The technical digestion time ($T_{80\text{-}90}$), referring to the time required to attain 80-90% of the maximum CH_4 production from BMP tests can be used to describe the CH_4 production efficiency (Kim and Lee 2005) and estimate the hydraulic retention time (HRT) for continuous anaerobic digestion of the substrate (Kafle and Kim 2013). The $T_{80\text{-}90}$ value of our study (Table 3) has shown to be shorter compared to other

Table 3. Kinetic analysis of whey permeate anaerobic digestion.

Digesters							
ISR	Temp (°C)	pH	*P* CH_4 ($NL_{CH4}kg_{VS}^{-1}$ input)	Rm ($NL_{CH4}kg_{VS}^{-1}$ input.d^{-1})	λ (1/*d*)	R^2	T_{80-90} **(days)**
0.5	20	7	81.40	3.24	13.08	0.99	15–16
		7.5	65.97	3.18	11.65	0.99	19–21
		8	381.78	4.47	45.88	0.97	22–24
	30	7	48.22	4.35	2.80	0.96	9–10
		7.5	45.49	19.64	0.98	0.95	5–10
		8	42.72	30.71	0.91	0.99	2–3
	37	7	47.94	10.23	1.19	0.96	5–7
		7.5	50.89	9.12	1.18	0.94	5–10
		8	34.65	6.43	1.07	0.92	6–8
1	20	7	96.09	34.53	3.72	0.99	6
		7.5	100.22	22.25	4.48	0.99	7–9
		8	167.76	8.59	9.94	0.98	11–12
	30	7	77.11	12.64	2.64	0.99	6–7
		7.5	77.29	12.36	2.71	0.99	6–7
		8	84.24	13.09	2.74	0.98	6–7
	37	7	120.63	15.44	3.45	0.96	4
		7.5	88.11	15.07	2.37	0.97	4–5
		8	144.91	15.79	3.87	0.93	4
2	20	7	43.39	22.52	2.24	0.99	4–6
		7.5	49.23	27.34	2.36	0.99	4–5
		8	49.37	24.08	2.51	0.99	4–5
	30	7	124.48	9.84	2.32	0.94	13–22
		7.5	148.08	6.88	4.31	0.97	17–22
		8	177.30	7.97	4.73	0.97	16–21
	37	7	152.29	10.18	3.57	0.98	12–16
		7.5	138.63	9.06	3.21	0.97	12–16
		8	120.66	17.34	1.89	0.97	8–13

substrates such as apple waste (Kafle and Kim 2013), except for ISR 0.5 under 20°C. In other BMP studies involving other substrates, such as coffee grounds (Dias et al. 2023), solid state anaerobic digested rice straw (Zheng et al. 2022), and bioaugmented corn straw under 20°C incubation (Xu et al. 2022), the T_{80-90} was observed to be longer compared to all treatments in our study. This observation further confirms the easily degradable properties of whey permeate as a suitable substrate for AD, where rapid acidification and methanogenesis processes can take place.

Statistically significant effects on the cumulative CH_4 yield from AD of whey permeate, were observed among different ISRs, temperatures, and initial pH levels ($p < 0.05$). The highest cumulative CH_4 yield (466.29 ± 13.71 $NL_{CH4}kg_{VS}^{-1}$ input) was observed at ISR 2, under 30°C and an initial pH of 8. Research focused on CH_4 production of whey permeate AD is less common when compared to other dairy byproducts, such as cheese whey or other dairy wastewaters (Bella and Rao 2023). Current research provides limited understanding of the ideal operational parameters for AD of whey permeate, especially regarding its efficiency of CH_4 production under practical and reproducible conditions. Recent AD studies of whey permeate employed co-substrates ranging from cattle slurry (Fagbohungbe et al. 2019), to a complex mixture of green beans, cow manure and slaughterhouse sludge (Gensollen et al. 2022). A parallel study where a different source and forms of whey permeate was used, revealed that the maximum CH_4 yield was achieved at similar ISR ranges, but at 37°C and an initial pH of 7.5 (Azkarahman et al. 2025). This highlights that differences in physicochemical composition of a similar type of substrate could also affect its optimum operational

conditions for AD. Moreover, the lower CH_4 yield at ISR 0.5 and higher at ISR 2 observed in this study could be attributed to the accumulation of VFAs in the digester. The evidence supporting this observation can be seen from the total VFAs data obtained post-BMP test (Figure 5), where the highest VFAs accumulation was found at ISR 0.5, with the exception at 20°C. Methanogenesis in AD involves two primary pathways: (1) the acetoclastic pathway, in which acetic acid generated from acetogenesis process dissociates into CH_4 and CO_2 by acetoclastic methanogens; and (2) the hydrogenotrophic pathway, which involves the use of H_2 or formate as an electron donor by hydrogenotrophic methanogen to reduce CO_2, resulting in the production of CH_4 and H_2O (Ferry 2011). Furthermore, previous studies have included specific types of VFAs, such as acetic acid (Zhou et al. 2018) and propionic acid (Demirel and Yenigün 2002; Wang et al. 2006), as indicators to assess the efficiency of acetogenesis and acetoclastic methanogenesis. Our study demonstrated that ISR 2 resulted in significantly lower VFAs accumulation during whey permeate AD. This result indicates a more efficient acetogenesis and methanogenesis process at ISR 2, and ultimately leads to significantly higher CH_4 yield compared to lower ISRs of the study. Interestingly, our study also demonstrated that the highest CH_4 production under 20°C incubation was achieved at ISR of 1. Based on the data of our study, VFAs accumulation under 20°C was not significantly affected by the ISR and the initial pH. This could be due to lower acidogenic and methanogenic activities under this temperature. The findings by Dev et al. (2019) and Rusín et al. (2021) who observed AD under low temperatures have shown similar results.

A high level of VFAs accumulation could inhibit methanogenesis and induce microbial stress due to rapid acidification and subsequent pH reduction (Wang et al. 1999). Consequently, this may lead to process deterioration within the anaerobic digester. The cumulative CH_4 production and VFAs accumulation data in our study suggest that decreasing the incubation temperature allows the digester with reduced level of inoculum to perform a better hydrolysis and methanogenesis rate. However, when the ISR was further reduced to 0.5, an insufficient amount of inoculum hindered the efficiency of the anaerobic digestion process. The total organic carbon (TOC) degradation data (Figure 3) also indicate that the level of inoculum at ISR 0.5 was insufficient to convert the carbon for methanogenesis as compared to higher ISRs. On another BMP study by Raposo et al. (2008), it was also found that ISR 0.5 had lower organic matter removal when compared to higher ISR when sunflower oil cake was used as the substrate. The cumulative CH_4 yields from whey permeate in our study were found to be higher compared to other dairy wastewaters, such as waste milk (Adghim et al. 2020), dairy cattle wastewater (Chou and Su 2019), and even compared to cheese whey (Flores-Mendoza et al. 2020; Pellera and Gidarakos 2016). This discovery presents an improved valorisation approach for milk processing, suggesting that cheese whey should undergo filtration for whey protein production before being subjected to AD in the form of whey permeate. Compared to other substrates (Kaldis et al. 2020), the maximum CH_4 yield in our study was also higher, but lower than swine manure (Sun et al. 2015) and organic fraction of municipal solid waste (Demichelis et al. 2022).

Kinetic analysis revealed the modified Gompertz model's superior fit to our experimental data compared to other observed kinetic models. Thus, only kinetic parameters derived from the modified Gompertz model are presented in this paper. Modified Gompertz model allows for the identification of the lag phase (λ) and the maximum CH_4

production rate (Rm). The model showed high correlation coefficients ($R^2 = 0.92–0.99$; Table 3) to the experimental data. This indicates that the modified Gompertz model adequately described the cumulative CH_4 production. However, the kinetic modelling in our study also showed that even though high R^2 was obtained, the Modified Gompertz model could not accurately calculate the AD kinetic parameters where CH_4 production curve was not sigmoidal. This can be seen on the experimental conditions where R^2 was less than 0.99, such as at ISR 0.5, pH 8, at 20°C or ISR 0.5, pH 8, at 37°C. This finding thus demonstrates that ISR, pH and temperature not only affect the CH_4 production, but also affect the accuracy of the applied kinetic model due to a change in CH_4 production curve. In our study, it was observed that AD of whey permeate at ISR 0.5 under 20°C exhibited the lowest Rm with the highest λ value. This outcome was expected due to the low temperature and limited number of bacteria and archaea performing methanogenesis.

The overall results showed that ISR, temperature and initial pH collectively influence both λ and Rm. For instance, extended λ values were observed at initial pH 7 at ISR 2 and 30°C, however such delay was also observed at initial pH 8 at 37°C with similar ISR. Similarly, variation in Rm values is also observed at different ISR, temperature, and initial pH, with no apparent linear effect from each individual parameter. Theoretically, ISR should not affect cumulative CH_4 yield and only impact the kinetics of CH_4 production. However, experimental data revealed that ISR can influence both cumulative yield and production kinetics. There is compelling evidence indicating that ISR directly affects microbial growth patterns (Raposo et al. 2012). Owamah et al. (2021) found that increased ISRs led to higher λ and lower Rm during the AD of food waste and maize husk. In our study, we found that ISR alone could not account for the observed variations in λ and Rm, as temperature and initial pH also played critical roles in the AD process. Previous studies have highlighted other factors, such as inoculum source and pre-treatments (Cysneiros et al. 2011; Lima et al. 2018), operational conditions (Cysneiros et al. 2012), as well as substrate characteristics (Morais et al. 2021) that can also affect kinetic parameters in AD. However, the impact of initial pH on AD kinetics has rarely been discussed. Our findings suggest that initial pH levels should be considered when determining AD kinetics under a batch system. In practical applications, this insight is particularly relevant for co-digestion processes, where the initial pH may require careful management. Moreover, various mathematical models have been developed to describe AD kinetics, and further comparisons of these models across different substrates would help validate and extend our findings.

3.3. Organic matter degradation

The microbial population in the digester consumes organic matter from the substrate for growth and generates CH_4, along with VFAs and other metabolites during the process (Angelidaki et al. 2018). Volatile solids (VS) and total organic carbon (TOC) are commonly used parameters to assess the organic matter of a substrate (Peces et al. 2014; Wang et al. 2024), with TOC specifically indicating the available organic carbon for biological processes. Through AD, the organic matter of the substrate undergoes sequential stages of hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Thus, organic matter removal of the substrate reflects the AD performance in addition to CH_4

production (Wu et al. 2019). Hydrolysis is the process of breaking H–O bonds through the addition of water. In general, anaerobic microorganisms cannot directly utilize complex organic matter, as it must first be degraded into simpler, soluble compounds (Gujer and Zehnder 1983). A group of bacteria known as hydrolytic bacteria facilitates the breakdown of carbohydrates, proteins, and lipids into simpler compounds such as sugars, amino acids, and long-chain fatty acids. This process occurs through the secretion of extracellular enzymes (Li et al. 2011). During the acidogenesis stage, these products undergo fermentation, leading to the formation of various VFAs, such as formic, propionic, butyric, lactic, and succinic acid, along with ketones and alcohols. Acidogenesis typically progresses at a faster rate than other stages (Zhou et al. 2018). The acids and other compounds produced during the acidogenesis are large molecules and are unsuitable for CH_4 production. Therefore, in acetogenesis, most of these intermediate products are broken down into acetic acid (or its salts), CO_2, and H_2O. Moreover, long-chain VFAs are also converted into acetate or propionate, with H_2 also being generated. During the methanogenesis stage, all intermediate products generated in the previous stages are transformed into CH_4, CO_2, and H_2O (Angelidaki et al. 2018).

In our study, TOC and VS degradation of the substrate from the BMP test were determined to understand the AD efficiency operation. There was statistically significant three-way effect between ISR, temperature, and pH ($p < 0.05$) on TOC and VS degradation of whey permeate. The highest TOC degradation (77.913 ± 1.442%) was found at ISR 2 with an initial pH of 8 under 20°C, while the lowest (23.133 ± 1.427%) was found at ISR 0.5 under the same initial pH level and temperature (Figure 3). Furthermore, post-hoc statistical analysis showed that the TOC degradation at ISR 0.5 was significantly lower than at ISR 1 and 2. This showed that ISR 0.5 was inadequate to perform efficient acidification of the organic carbon on the substrate. In addition, this also confirmed that hydrolysis was the rate-limiting step for AD in this condition. On the contrary, in a study by Demichelis et al. (2022), ISR 0.5 gave the highest TOC removal of organic fraction of municipal solid waste as compared to ISR 1 and 2, which achieved 89.96% of TOC removal when digested with cow agriculture sludge after 10 days of incubation. This contradictory outcome highlights the substrate characteristics during AD. In the case of whey permeate, the hydrolysis process was observed to occur at a faster rate than methanogenesis, leading to the accumulation of VFAs and a reduced TOC degradation at ISR 0.5. The TOC degradations of whey permeate in our study ranged from 23.13 to 77.19%. This value is relatively low when compared to TOC removals reported in other AD studies, such as those by Demichelis et al. (2022) and Brémond et al. (2018), which achieved TOC removals as high as 80–90%. The low TOC degradation observed in our study underpins the potential for further optimisation of AD for whey permeate. Such optimisation should aim to enhance TOC degradation and, consequently, increase CH_4 production.

The high organic load of the dairy wastewaters represents a significant environmental pollutant when improperly disposed of (Bella and Rao 2023). Moreover, the removal of this organic load from dairy wastewaters imposes additional costs to the industry (Arvanitoyannis and Giakoundis 2006). Prior investigations show that the AD process can eliminate over 90% of chemical oxygen demand (COD) from whey permeate (El-Mamouni et al. 1995; Kisielewska et al. 2014). The VS degradation from AD of whey permeate in our study ranged from 36.22 to 81.14% (Figure 4). This level is higher

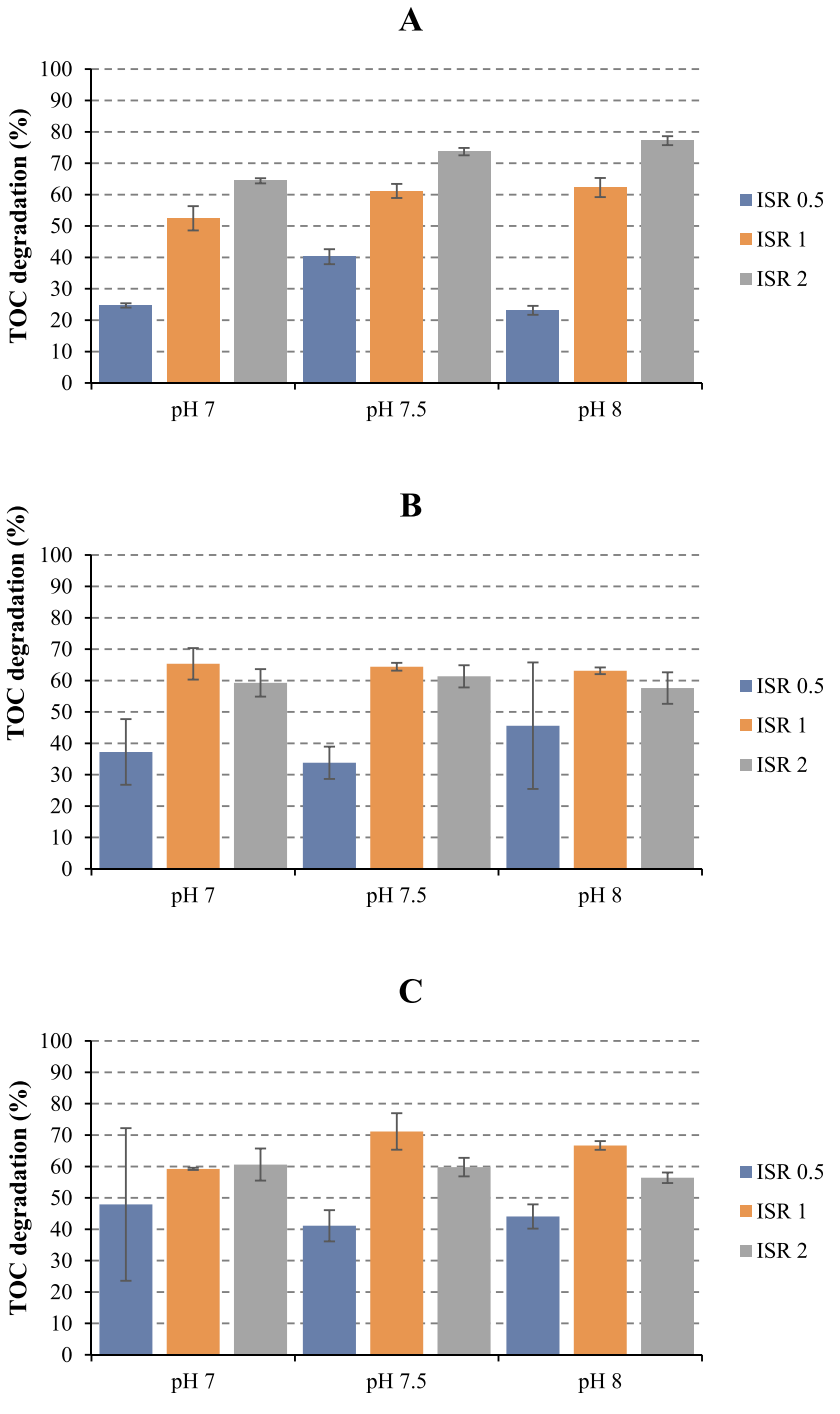

Figure 3. Total organic carbon (TOC) degradation of whey permeate following AD under various pH values and ISRs post-BMP test at (A) 20°C; (B) 30°C and (C) 37°C incubation. Error bars indicate standard deviation.

than the VS degradation observed in the AD of fruit and vegetable wastes (D'Silva et al. 2022), as well as of that food waste inoculated with waste activated sludge (Gaur and Suthar 2017) under batch AD. Moreover, the highest VS degradation of this study closely aligns with reported values where a different form of whey permeate is used as AD feedstock (Azkarahman et al. 2025). The findings from VS degradation in our study add evidence supporting the suitability of AD as a viable method for upcyling whey permeate for bioenergy production. In addition to observing significant VS degradation even under 20°C, our findings indicate that ISR did not show a positive correlation with VS degradation. The extent of VS degradation enables the estimation of CH_4 production from the degraded solids. Excluding any unutilised VS from the calculation could provide a more accurate assessment of AD efficiency.

3.4. Volatile fatty acids profile

The significance of VFAs has recently received increased attention, primarily due to their potential for diverse and valuable applications that extend beyond those of CH_4. VFAs are recognised as green and renewable chemical commodities, offering numerous opportunities across various sectors and feedstocks, including the production of bioplastics and biofuels (Kleerebezem et al. 2015). In our study, the characterisation of VFAs profile aimed not only to gain insight into the AD performance, but also to explore their potential for specific VFA production. Statistically significant ($p < 0.05$) three-way effect between ISR, temperature, and pH was found on the post-BMP VFAs in our study (Figure 5). The highest level VFAs (2.85 ± 0.35 $g_{COD}L^{-1}$) were produced at ISR 0.5 with an initial pH 8 under 37°C. The data show that ISR 2 had lower post-BMP VFAs accumulation compared to other ISRs except under 20°C. This suggests that methanogenesis was more efficient at ISR 2 compared to lower ISRs. Similar findings have also been reported by Raposo et al. (2006) and Demichelis et al. (2022), where lower ISRs resulted in higher VFA accumulation. The VFA concentration would vary significantly as it is affected by several factors including pH, temperature and nutrient availability (Cysneiros et al. 2012; Hawkes et al. 2002). The results of VFA analysis in our study indicate that AD at lower temperatures (20°C) may inhibit methanogenesis, leading to greater VFAs accumulation. Specifically, at ISR 2, VFA levels were higher at 20°C relative to 30 and 37°C.

The VFA analysis in our study demonstrated a substantial influence of the ISR on the dominant VFA compound except at ISR 2. Specifically, at ISR 0.5 and 1, HBu and HPr were the predominant VFAs accumulated across all initial pH levels and incubation temperatures, respectively. At ISR 2, the dominant VFA varied based on the initial pH level and incubation temperature. However, due to the low total VFAs accumulation (0.01–0.26 $g_{COD}L^{-1}$ at 30 and 37°C), the difference was not significant. A notable HBu accumulation was observed at ISR 0.5 at 37°C. This value was lower compared to that postulated by Reddy et al. (Reddy et al. 2018) where anaerobic fermentation of food waste aimed for VFAs production yielded HBu up to 8.9 g/L with bioaugmented culture. Butyric acid is known as a valuable compound for the production of various bio-based products such as bioplastics (Bengtsson et al. 2017; Cao et al. 2011) and biofuels (Maiti et al. 2016), while it is also used in various industries with a worldwide market of approximately 80,000 metric tons per year (Jiang et al. 2018; Wang et al. 2016). The findings in our

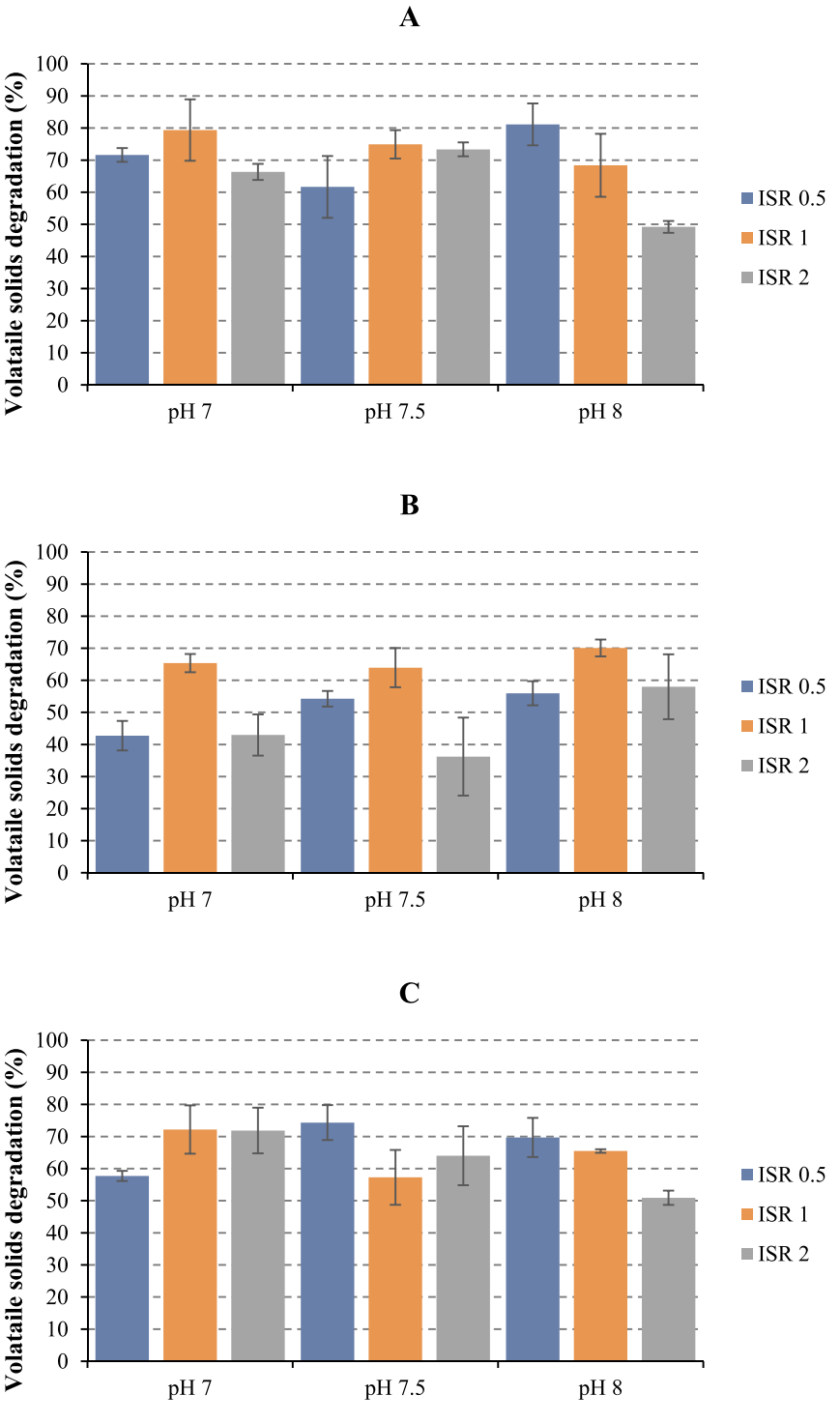

Figure 4. Volatile solids (VS) degradation of whey permeate post-AD BMP test under various pH values and ISRs at (A) 20°C; (B) 30°C and (C) 37°C incubation. Error bars indicate standard deviation.

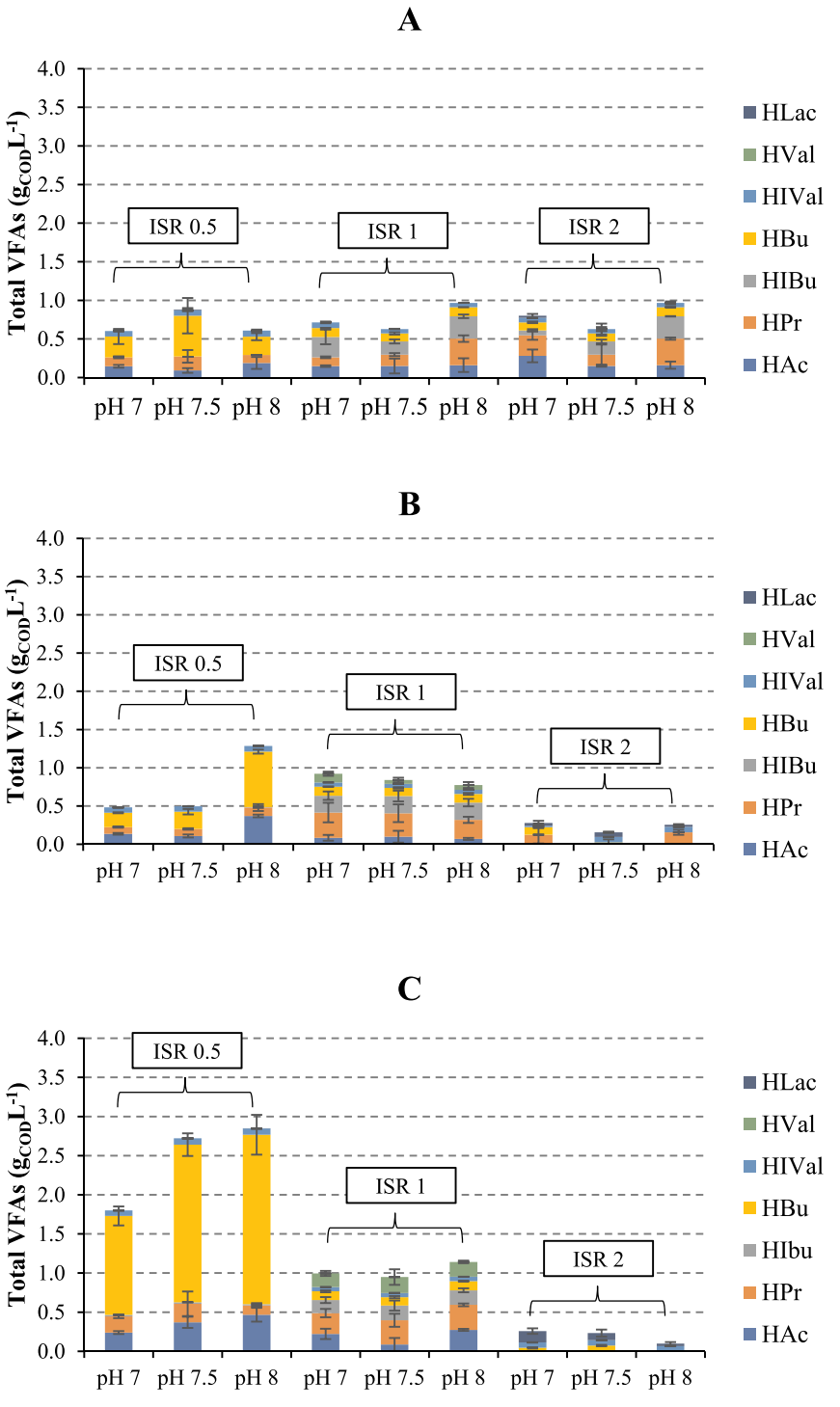

Figure 5. Total volatile fatty acids (VFAs) and their speciation following AD of whey permeate post-BMP test under various pH values and ISRs at (A) 20°C; (B) 30°C and (C) 37°C incubation. Error bars indicate standard deviation.

study thus suggest that potential product diversification could be performed by changing the AD conditions of whey permeate to produce HBu. Additionally, HLa was only detected at ISR 2 under 30 and 37°C incubation, indicating that this condition might not provide an optimal environment for complete conversion of HLa into HAc to be further metabolised by methanogens. These findings agree with those of Bühlmann et al. (2022) who showed that substrate characteristics, inoculum, pH and temperature affect the HLa generation. HAc is commonly the predominant component of the total VFAs, constituting 66% to 80% of the overall VFA composition (Tampio et al. 2019). In our study, a different finding was observed, where HAc was not the dominant VFA component in the digestate. This indicates that methanogenesis by acetoclastic pathway occurred efficiently throughout the research treatments.

Although anaerobic digestion (AD) is conventionally utilised to produce CH_4-rich biogas, VFAs generated during acidogenesis and acetogenesis possess significant value and find applications in various industries (Kleerebezem et al. 2015). Recovering VFAs from AD could enhance the economic feasibility of food waste management and offer an environmentally friendly approach for producing these valuable chemicals (Veluswamy et al. 2021). The VFAs accumulation observed in our study was lower than that of food waste AD (Dhamodharan et al. 2015; Ding et al. 2022) and poultry manure (Rivera et al. 2023), but similar to dairy cattle wastewater (Chou and Su 2019). This low VFAs accumulation indicated an efficient AD processes of whey permeate. Nevertheless, VFAs recovery from whey permeate can be a promising approach for valorisation, especially considering that specific VFA compounds can be recovered in the AD by simply adjusting the ISR as found in our study.

3.5. Operational AD optimisation and validation based on mass balance

The mass balance of whey permeate AD in our study was calculated by including the CH_4 potential yield from the accumulated VFAs along with the cumulative CH_4 yield, with degraded VS as the denominator to identify the optimum AD operation parameters. A statistically significant ($p < 0.05$) effect of ISR and temperature was found on the result of mass balance. The highest mass balance was shown to be achieved at similar conditions observed at cumulative CH_4 yield measurement (ISR 2 with the initial pH of 7.5 under 30°C), that produced 1441.42 ± 43.78 $NL_{CH4}kg_{VS}^{-1}$ degraded (Figure 6). Furthermore, it is also demonstrated that the larger portion of CH_4 potential from VFAs was found at ISR 2 under 20°C and ISR 0.5 under 37°C. These findings revealed incomplete methanogenesis under those conditions and suggest the possibility of recovering VFAs. The explanation for CH_4 as well as VFAs production and organic matter degradation of this study, are discussed in Sections 3.1–3.4. In this section, numerical optimisation derived from mass balance data was performed by employing central composite design (CCD) of response surface methodology (RSM).

Response surface methodology (RSM) is a systematic optimisation approach that can be used to evaluate the effects of AD operational conditions (independent parameters) towards CH_4 yield (dependent parameter), thus enabling the determination of optimal AD operations based on their impact. This methodology combines experimental designs to develop a new dataset by first- or second-order polynomial equations in a systematic test method (Ramaraj and Unpaprom 2019). Moreover, RSM also has distinct

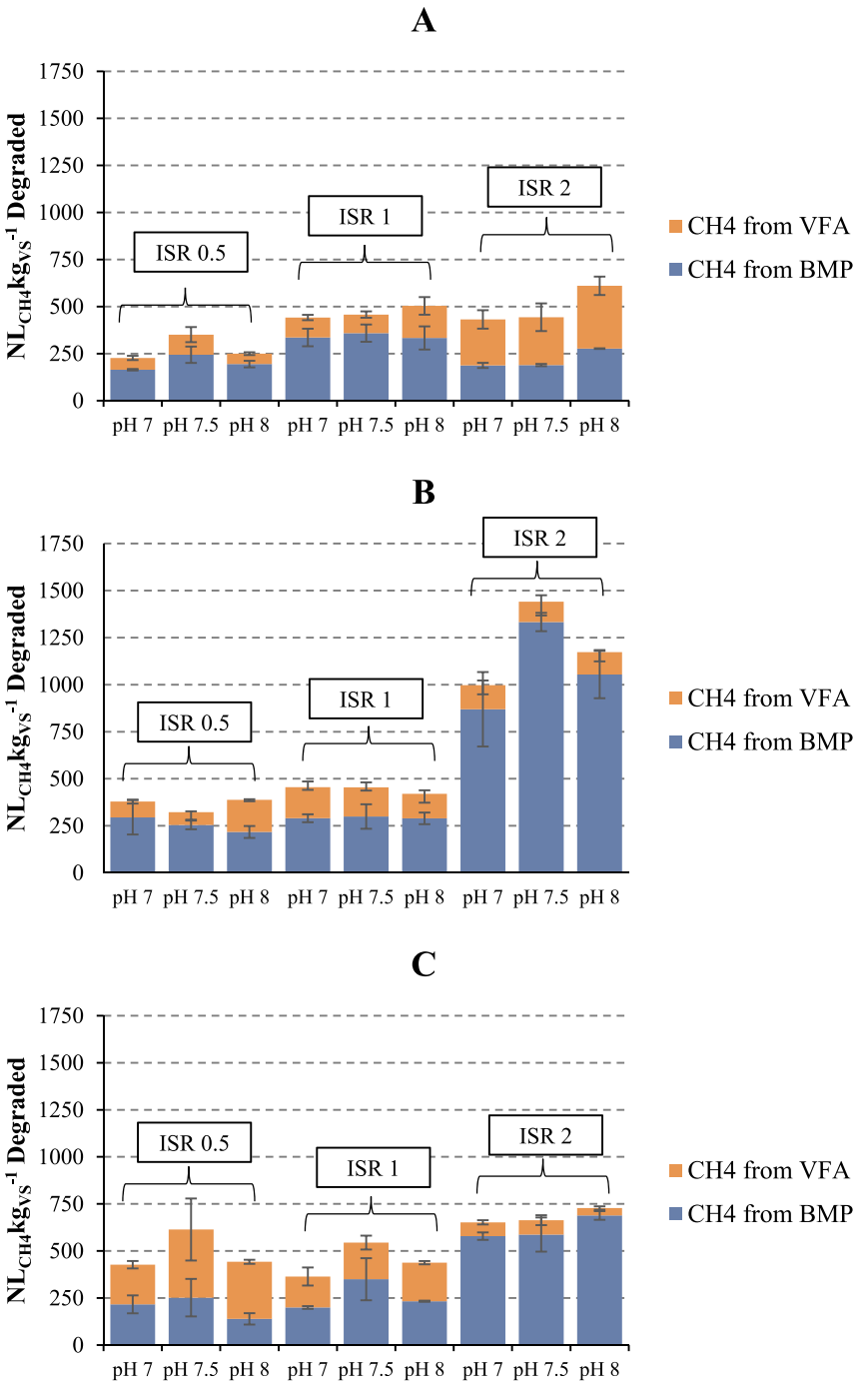

Figure 6. Mass balance of whey permeate following AD of whey permeate post-BMP test under various pH values and ISRs at (A) 20°C; (B) 30°C and (C) 37°C incubation. Error bars indicate standard deviation.

advantages compared to conventional optimisation approaches, particularly in evaluating multifactorial interactions with reduced experimental requirements. The key benefits include precise identification of optimal conditions while assessing their sensitivity to variable fluctuations (Kilickap 2010), quantitative modelling of variable-response relationships with visual projections (Rastegar et al. 2011), and significant reductions in time and resource through a reduced number of experimental trials (Boyacı 2005). Furthermore, central composite design (CCD) is one of commonly used designs of experimental techniques employed in RSM to systematically vary the process parameters and collect data on the target response (Djimtoingar et al. 2022). AD optimisation through CCD of RSM has been used in previous studies with other substrates, such as rice straw (Kainthola et al. 2019; Kainthola et al. 2020), cotton stalk (Zhang et al. 2018), wheat straw (Wang et al. 2013), and sugarcane bagasse (Ghaleb et al. 2020). Since operational AD parameters directly affect methanogenesis, these parameters can be optimised through CCD of RSM for more favourable AD performance (Djimtoingar et al. 2022). The three-way ANOVA of the mass balance in our study revealed that ISR and temperature significantly affect the mass balance of whey permeate AD, while no significant effect was observed from different initial pH levels ($p > 0.05$). As a result, the initial pH level was excluded as independent in the RSM analysis of our study. Most of the RSM analyses for AD only consider CH_4 yield as the response towards its independent factor (Dias et al. 2023; Flores-Mendoza et al. 2020; Yılmaz and Şahan 2020). In our study, mass balance was selected as the response factor, considering that VFAs generation and VS degradation play an important role in AD process. The inclusion of both VFAs and VS degradation to the AD optimisation enables a more justified measurement of the AD performance when compared to the sole CH_4 yield.

The RSM analysis of our study has shown that although temperature has a significant impact, its effect on mass balance is not linear. Both surface and contour plots of the RSM analysis have shown that the saddle point was observed at around 30°C (Figure 7(A,B)). Saleh et al. (2012) employed RSM for CH_4 yield of the co-digestion of palm oil mill effluent (POME) and empty fruit bunch (EFB) by using a Box–Behnken design with four independent variables: temperature, POME volume, inoculum volume and EFB addition. Their results identified temperature as the most statistically significant factor, exhibiting a positive and linear correlation with CH_4 production. A study by Ruiz-Aguilar et al. (2023) employed CCD of RSM analysis from tomato waste AD, and reported a similar effect of temperature towards CH_4 production. In our study, a linear relationship between operational AD temperature and mass balance was not observed. The difference may be arising from the inclusion of VS degradation of the substrate in addition to VFAs accumulation. Saleh et al. (2012) reported that the highest specific biogas production rate and CH_4 percentage for AD of POME were achieved at 43°C and 44°C, respectively. Safari et al. (2018) applied a Box–Behnken design to investigate the effects of temperature, agitation time, total solids, and inoculum on CH_4 yield from co-digested canola residues with cow manure and demonstrated that the maximum CH_4 production occurred at 40.36°C. Moreover, the RSM study on AD of tomato waste plant revealed that the predicted optimal temperature was higher than 43°C (Ruiz-Aguilar et al. 2023). Thus, it can be concluded that optimal temperature within the mesophilic range varies across different substrates. In addition, to different types of substrates, our study suggests that the properties of the substrate also significantly influence the

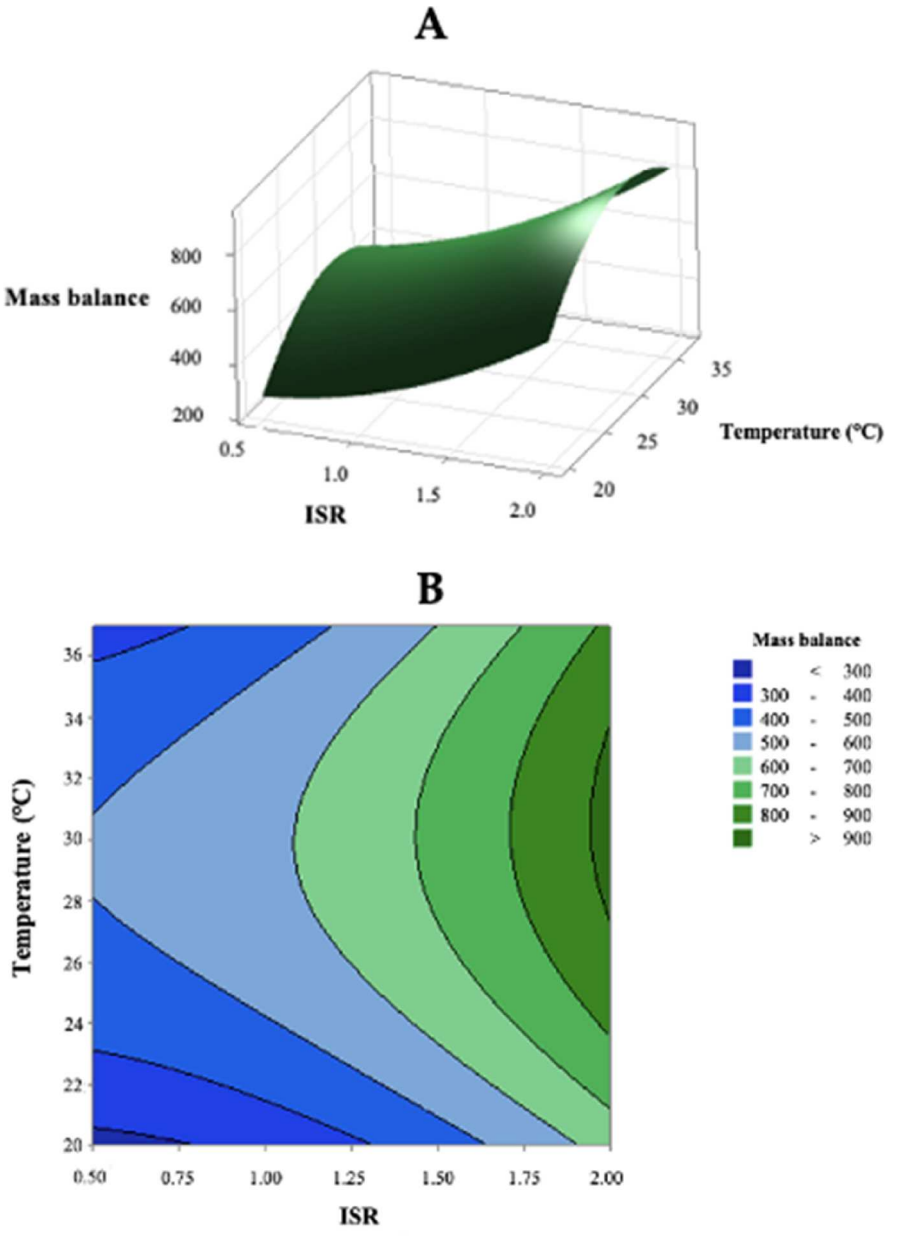

Figure 7. Response surface methodology and central composite design of the mass balance: (A) Surface plot of mass balance by temperature and ISR; and (B) Contour plot of mass balance by temperature and ISR.

effect of the AD parameters. Furthermore, in contrast to the present work, in our parallel study (Azkarahman et al. 2025), where different whey permeate (from different sources) with different physicochemical properties was used as substrate, a linear relationship between temperature and CH_4 yield depending on the ISR was observed.

Aside from temperature, our study also revealed the positive effect of ISR towards mass balance. Higher ISRs consistently improved mass balance, as shown in the surface and contour plot analyses (Figure 7(A and B)). The positive correlation between ISR and AD performance of our study is consistent with other RSM studies that utilize different substrates in an AD system (Ghaleb et al. 2020; Kainthola et al. 2019; Ruiz-Aguilar et al. 2023; Wang et al. 2013; Zhang et al. 2018). However, our study further revealed that the impact of ISR on AD performance may also be influenced by other independent factors of the study. For instance, at an ISR of 1, a higher mass balance value of our study is only observed when incubated at 30°C. Moreover, the results of RSM analysis of our study demonstrated that adjusting the temperature and ISR combination may also be an option to suit the technical operation cost of the biogas plant, especially considering that both ISR and temperature could directly affect the operational cost of the AD plant.

4. Conclusions

The results of this study showed that AD of whey permeate performed better compared to other milk processing wastewaters that have been investigated in other studies. The mass balance analysis revealed that ISR and incubation temperature significantly affect AD performance. In addition, this study has demonstrated, for the first time, that AD of whey permeate can still be performed even at 20°C and ISR 0.5. ISR has been shown to affect the dominant VFA type generated throughout the AD. The optimum condition in this study was found at ISR 2 and initial pH of 7.5 under 30°C, where 1441.42 ± 43.78 $NL_{CH4}kg_{VS}^{-1}$ degraded was produced. Response surface methodology of the mass balance has been presented to describe the optimal combinations of research treatments. Our findings demonstrate the possibility to shift the focus of the whey permeate AD product from CH_4 to VFA by adjusting the ISR. While this study provides valuable insights into AD optimisation of whey permeate, further research is needed to explore the economic assessments in response to varying experimental conditions.

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Author contributions

Conceptualisation and methodology, A.R.A., D.C., A.C., and K-A.G.K.; investigation, A.R.A. and K-A.G.K; writing – original draft preparation, A.R.A; Validation, D.C. and A.C.; Supervision, D.C. and K-A.G.K.; writing – review and editing, K-A.G.K. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author

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