



Hydrolytic enzyme activity in high-rate anaerobic reactors treating municipal wastewater in temperate climates

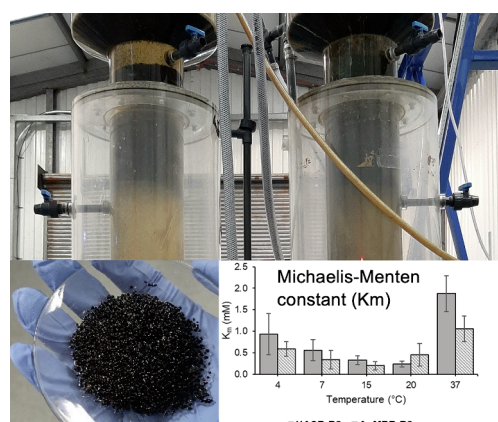
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HIGHLIGHTS

- 2 pilot-reactors treated settled wastewater at <20 °C with different seed-sludge.
- Similar methane production and hydrolysis rates were measured in the UASB and AnMBR.
- Hydrolytic enzymes (β -glucosidase, protease and lipase) were not psychrophilic.
- Hydrolytic enzymes had higher affinity to substrates at the operational temp. <20 °C.
- Effective adaptation of enzymes to <20 °C irrespective of seed-sludge source.

GRAPHICAL ABSTRACT



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ABSTRACT

Particulate matter hydrolysis is the bottleneck in anaerobic treatment of municipal wastewater in temperate climates. Low temperatures theoretically slow enzyme-substrate interactions, hindering utilization kinetics, but this remains poorly understood. β -glucosidase, protease, and lipase activities were evaluated in two pilot-scale upflow anaerobic sludge blanket (UASB) reactors, inoculated with different sludges and later converted to anaerobic membrane bioreactors (AnMBRs). Despite similar methane production and solids hydrolysis rates, significant differences emerged. Specific activity peaked at 37 °C, excluding the predominance of psychrophilic enzymes. Nevertheless, the Michaelis-Menten constant (Km) indicated high enzyme-substrate affinity at the operational temperature of 15–20 °C, notably greater in AnMBRs. It is shown, for the first time, that different seed sludges can equally adapt, as hydrolytic enzymatic affinity to the substrate reached similar values in the two reactors at the operational temperature and identified that membrane ultrafiltration impacted hydrolysis by a favourable enzyme Michaelis-Menten constant.

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1. Introduction

The considerable energy demand and production of excess sludge of aerobic processes have encouraged the implementation of high-rate anaerobic reactors, especially the upflow anaerobic sludge blanket (UASB) reactor, as an alternative for municipal wastewater treatment. Interesting advantages of anaerobic wastewater treatment include energy savings, due to lack of aeration and biogas recovery, lower sludge production and the possibility of recovering nutrients from the effluent (Robles et al., 2021).

Municipal anaerobic wastewater treatment differs from anaerobic digestion in several key aspects. In anaerobic wastewater treatment, the feed wastewater typically contains low solids (<300 mg TSS/L) contrasting with standard anaerobic digestion which involves a feedstock with higher solid concentrations (>3000 mg TSS/L), operates at much higher temperatures (>30 °C) and the hydraulic retention time and a solid retention time are the same (Tchobanoglous et al., 2003). Notably, municipal anaerobic wastewater treatment has been tested in large reactors in temperate climates and at temperatures as low as 10 °C (Table 1) with COD removals >80 %, showing the potential of the technology (Table 1).

Municipal wastewater is characterised by low-strength streams, rich in particulate organic matter, such as carbohydrates, proteins and lipids. Anaerobic degradation is initiated by fermentative bacteria, whose hydrolytic enzymes break down these complex polymeric substrates into soluble molecules. In temperate climates (<20 °C) hydrolysis has been identified as the rate-limiting step, causing accumulation of non- or partially-hydrolysed solids in the reactor (Ribera-Pi et al., 2020). Low temperatures can reduce diffusion rates of the substrates and increase the viscosity of the medium, leading to mass transfer limitations (Dev et al., 2019). These changes result in an increase of the kinetic requirements to overcome the activation energy barrier and a decline of the reactions rates (Dhauaniya et al., 2019).

Psychrophiles are microorganisms inhabiting cold environments with optimal growth at 15 °C (De Maayer et al., 2014) and the remarkable Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 has been widely investigated for its fast growth at a wide temperature range from −2.5 to 25 °C (Mocali et al., 2017). To maintain an appropriate enzymatic rate and sustain essential cellular processes, psychrophiles synthesise psychrophilic enzymes, which have developed survival mechanisms to overcome the low-temperature drawbacks (Margesin et al., 2007). To reduce the activation energy, these enzymes decrease the number of enthalpy-driven interactions that need to be disrupted during activation, leading to an increase in flexibility of the active site of the enzyme, at the expense of activity at higher temperatures. This causes a lower stability of the protein structure and a looser binding of the substrate in psychrophilic enzymes, resulting in a lower substrate affinity compared to that of mesophilic counterparts (Collins & Margesin, 2019).

Due to the higher activity at low temperatures, the use of a psychrophilic inoculum could be a valid solution to sustain hydrolysis in UASB reactors operated in temperate climates. Recent studies have highlighted the efficacy of psychrophilic microorganisms and their hydrolytic enzymes even at temperatures as low as 5 °C, showcasing their remarkable activity to cold environments, with enzymes being

commercially explored such as xylanase, pectinases and esterases, primarily in the food industry (Kumari et al., 2021). However, the availability of this type of seed sludge is limited since it can only be sourced in extreme environments difficult to access. Moreover, temperate climates are susceptible to seasonal temperature variations, which would affect the physical properties of wastewater and could negatively impact the psychrophilic enzymes, due to their structural instability.

Another possibility is to use a mesophilic inoculum and implement different strategies to improve the kinetic rates at low temperature. Hydrolysis is a mass transfer limited process, therefore increasing the contact between the substrate and the sludge would facilitate the increase of hydrolytic efficiencies, reducing the accumulation of solids. Several studies have reported a successful use of a mesophilic inoculum at low temperatures, in particular in anaerobic membrane bioreactors (AnMBRs) (Gouveia et al., 2015; Hejnic et al., 2016; Ribera-Pi et al., 2020; Wang et al., 2019).

AnMBRs are high-rate anaerobic reactors in which a membrane is placed after or inside the biological reactor and allows the complete retention of solids and biomass, increasing the sludge retention time. Ribera-Pi et al., (2020) observed that the rate of solids hydrolysed increased in an AnMBR compared to a UASB reactor and overall AnMBRs were able to improve the organic removal efficiencies of UASB reactors (Ribera-Pi et al., 2020; Wang et al., 2019).

While these studies from literature have shown that the anaerobic treatment of municipal wastewater is feasible in temperate climates and even without a psychrophilic inoculum, the hydrolytic mechanisms and adaptation to low temperatures are still unclear. Only few studies have reported activities of hydrolytic enzymes in anaerobic reactors treating wastewater (Anupama et al., 2008; Berrio-Restrepo et al., 2017; Petropoulos et al., 2018). However, the variation with temperature of enzymatic activity and substrate affinity in high-rate anaerobic reactors for municipal wastewater treatment has not been measured yet. Therefore, in this study, the activity of the sludge employed in two UASB reactors, inoculated with different types of sludge, and then converted in AnMBRs, to promote hydrolysis, has been investigated at different temperatures to advance the current knowledge on the adaptation of anaerobic hydrolytic enzymes to low temperatures. Enzyme activity was tested at different temperatures to determine whether a sustained operation of the reactors at temperatures <20 °C would promote the development of hydrolytic enzymes of psychrophilic nature and substrate affinity was tested at different temperatures to assess the adaptation of the sludge. Moreover, the analysis of sludge samples from UASB reactors and AnMBRs allowed to understand how the presence of an ultrafiltration membrane would affect those parameters.

2. Materials and methods

2.1. Description and operation of the pilot-scale plants

Two 70 L cylindrical UASB reactors were operated in parallel and fed with the same settled wastewater, pumped from the bottom with peristaltic pumps (520Du, Watson Marlow, Falmouth, UK) to achieve a hydraulic retention time (HRT) of 8 h. Each reactor was inoculated with 11 L of sludge derived from a different source. The first reactor, UASB-R1, was seeded with mesophilic granular sludge from food processing

Table 1

Large-scale AnMBR studies from literature treating municipal wastewater in temperate climates. COD: chemical oxygen demand. (*): $L_{CH4}/g_{COD, in}$.

Volume (m ³)	HRT (h)	Temperature (°C)	Influent COD (mg/L)	COD removal (%)	Methane yield ($L_{CH4}/g_{COD, rem}$)	Reference
0.31	7	18 ± 2	892 ± 271	87 ± 1	0.18–0.23	(Gouveia et al., 2015)
0.5	8–10	10–28	1729 ± 914	85–94	0.09–0.14	(Peña et al., 2019)
1.42	11 ± 3	12.7–31.5	610 ± 260	88 ± 7	0.14 ± 0.06*	(Lim et al., 2019)
2.1	24.4 ± 0.4	27 ± 1	510 ± 87	88	0.108 ± 0.018	(Seco et al., 2018)
5	12	25	403.1 ± 28.1	90	0.25	(Kong et al., 2021)
40.8	25–41	10–27	755–1403	87.2 ± 6.1	0.07–0.17*	(Robles et al., 2022)

wastewater treatment (89 % volatile solids (VS) to total solids (TS) and 55 gVS/L), while the second reactor, UASB-R2, was inoculated with sludge coming from municipal anaerobic digestion and pulp and paper processing wastewater treatment (79 % VS/TS, 33 gVS/L) and had been employed for over five years in the same reactor aimed at anaerobic wastewater treatment in the UK, making it already adapted to the studied conditions (Ribera-Pi et al., 2020; Wang et al., 2018a). Three lamella settlers were placed along the height of each reactor and a three-phase separator on top facilitated the collection of effluent and biogas. The effluent was then recirculated using peristaltic pumps (620Du, Watson Marlow, Falmouth, UK) to achieve an upflow velocity of 0.8 m/h.

The two UASB reactors were subsequently converted into AnMBRs with the addition of an external submerged hollow-fibre ultrafiltration membrane tank (25 L). For each AnMBR, the effluent overflowed into the membrane tank and the retentate was recycled back to the UASB reactor. The upflow velocity was kept at 0.8 m/h, while the HRT of both UASB reactors varied between 4–6 h and was constant at 1 h for both membrane tanks. Permeate was collected with a peristaltic pump (520Du, Watson Marlow, Falmouth, UK). AnMBR-R1 (originally UASB-R1) was connected to a mini C-MEM cartridge (SFC Umwelttechnik GmbH, Slazburg, Austria) with high density polyethylene fibres with pore size of 0.02 µm and membrane area of 1.4 m². AnMBR-R2 (originally UASB-R2) was fitted with a Zenon ZW-10 module (GE Water & Process Technologies, Oakville, Canada) with polyvinylidene fluoride (PVDF) fibres with pore size of 0.04 µm and membrane area of 0.93 m². Pressure transducers (PX319-030A5V, Omega, Manchester, UK) and a multichannel data logger (ADC-20, Pico Technology, St Neots, UK) were used to record the transmembrane pressure. AnMBR-R1 was equipped with a permeate tank for backwashing. Nitrogen-enriched air was used for gas sparging (NG6, Noblegen gas generator, Gateshead, UK), which was regulated by a solenoid valve (Type 6014, Burkert, Ingelfingen, Germany) connected to a timer relay (PL2R1, Crouzet, Valence, France). The two membranes were operated in pseudo dead-end filtration mode, alternating filtration with gas sparging and relaxation or backwashing. AnMBR-R1 operation included filtration for 5 min and backwashing with gas sparging for 30 s, as suggested by the supplier, while AnMBR-R2 employed cycles of filtration (9 min) followed by relaxation and gas sparging (1 min), according to the optimal operational conditions identified for this membrane module by Wang et al. (2018b). The use of different membrane modules or filtration cycles is not expected to influence the tests as the purpose of this study is to better understand the adaptation to low temperature of hydrolytic enzymes in UASB reactors and investigate how the activity is affected by the presence of an ultrafiltration membrane.

2.2. Wastewater and biogas analysis

Before the enzymatic tests, the UASB reactors were in operation for over 10 months at temperatures between 6 and 20 °C, while the AnMBRs were running for more than 1 month at around 20 °C, achieving in both cases stable removals. The data here analysed for effluent quality and methane production span 3 months for UASB reactors and 2 months for AnMBRs in steady-state operation, during which the samples for enzymatic analysis were collected. Wastewater samples of influent, UASB effluent and AnMBR effluent were analysed multiple times a week in duplicates. Total suspended solids (TSS), volatile suspended solids (VSS) and biochemical oxygen demand (BOD) were measured according to Standard Methods (APHA, 2012). Total and soluble chemical oxygen demand (COD, sCOD) and sulphate (SO₄) were analysed with cell test kits (Merck KGaA, Darmstadt, Germany). Wastewater samples for sCOD and SO₄ were filtered at 0.45 µm with retention membrane filters (Whatman, Cytiva, Little Chalfont, UK). Temperature and pH were measured on site with a portable Multi Meter (HQ40D, Hach Lange GmbH, Düsseldorf, Germany).

The total methane produced was calculated combining gaseous and

dissolved methane. The production of biogas was measured with gas meters (TG 0.5, Ritter, Bochum, Germany) and the composition was determined with in-line CO₂ and CH₄ sensors (BCP-CO₂ and BCP-CH₄, BlueSens gas sensor GmbH, Herten, Germany). Dissolved methane in the effluent of UASB reactors was measured following the method described by Cookney et al. (2016) and the concentration was calculated according to Equation (1) (Souza et al., 2011):

$$[CH_4]_{diss} = \frac{([\%CH_4]_{gas}/100) \cdot \rho \cdot V_{gas} + (P_T - P_V) \cdot K_H \cdot V_{liq}}{V_{liq}} \quad (1)$$

where, $[CH_4]_{diss}$ is the concentration of dissolved methane (mg/L), $[\%CH_4]_{gas}$ is the concentration of methane in the headspace (%), ρ is the density of methane (651 mg/L at 20 °C and 0.988 atm), V_{gas} is the volume of gas in vial (mL), P_T is the atmospheric pressure (0.988 atm at Cranfield, UK), P_V is the water vapour pressure (0.023 atm at 20 °C), K_H is the Henry's constant for methane (24.9 mg L⁻¹ atm⁻¹ at 20 °C) and V_{liq} is the volume of liquid in vial (mL). For AnMBRs, since the membrane tanks were not sealed and a direct measurement would have not been representative, the dissolved methane was estimated using Henry's Law as described in Equation (2):

$$\frac{C_g}{C_s} = H_u \quad (2)$$

where, C_g is the concentration of methane in the gas phase, C_s is the saturation concentration of methane in the liquid phase and H_u is the dimensionless Henry's Law constant, depending on temperature. Hydrolysis percentages were calculated according to Equation (3) (Elmitwalli et al., 2002):

$$\text{Hydrolysis}(\%) = 100 \cdot \frac{CH_4 \text{ as COD} + sCOD_{out} - sCOD_{in}}{COD_{in} - sCOD_{in}} \quad (3)$$

where, the subscripts "in" and "out" indicate the influent and effluent wastewater, respectively, and 0.25 L_{CH₄}/g_{COD} was used for the conversion between methane and COD. The rate of solids hydrolysed in the reactor was expressed as the load of solids removed per unit of biological reactor volume (70 L), multiplied by the hydrolysis percentage (Ribera-Pi et al., 2020). Statistical tests were performed to evaluate first outliers, then normality (Shapiro-Wilk test). Welch's *t*-test was applied for analysis of the means, using a 95 % confidence limit for wastewater and methane data and a 85 % confidence limit for enzymatic data where not indicated otherwise.

2.3. Microbial community

The microbiome of the sludge was analysed to determine the bacterial differences in UASB reactors and AnMBRs. Sludge samples, one for each reactor (UASB-R1, UASB-R2, AnMBR-R1, AnMBR-R2), used for enzymatic analysis, were kept in the cold room and shipped to an external laboratory for analysis. DNA extraction was performed with Qiagen DNeasy Powersoil Pro Kit, according to kit's instructions. PCR analysis and sequencing were carried out with the Nanopore 16S Barcoding Kit (SQK-RAB204) and the Nanopore MinION Flowcell (R9.4.1), respectively. Despite it was possible to analyse only one sludge sample for each reactor, the use of a larger sample size for microbial analysis would increase the reliability of the results, reducing the chance of random error in sample collection and analysis.

2.4. Enzyme assays

Enzymatic tests were performed on sludge samples from UASB-R1, UASB-R2, AnMBR-R1 and AnMBR-R2, collected from the sampling point at the bottom of the UASB reactors. For each enzyme and protein assay, three sludge samples per reactor, collected over the course of maximum three weeks to limit microbiome and temperature variability,

were analysed in triplicates. Samples were sonicated for 15 min at 30 kHz at room temperature and then centrifuged at 5000 rpm for 10 min. The supernatant was collected and analysed for protein concentration and enzymatic activity.

The protein concentration was determined with the Bradford method (Quick Start™ Bradford Protein Assay, Bio-Rad Laboratories Ltd., Watford, UK), using gamma-globulin as standard (0.125–2 mg/mL) in multiwell microplates. The absorbance of the reaction well at 595 nm, measured with a microplate reader (Infinite M200 Pro, Tecan, Austria), was corrected based on sample and reagent blanks.

To investigate the degradation of the main particulate organic components of municipal wastewater (carbohydrates, lipids and proteins), the enzymes considered in this study were β -glucosidase, lipase and protease, as they are regarded as equally important in the hydrolytic process (Whiteley & Lee, 2006). Colorimetric assays, measured with a microplate reader (Infinite M200 Pro, Tecan, Austria), were used to determine specific activity and kinetics constants for each enzyme.

Specific activity, defined as the concentration of product released in a certain amount of time divided by the protein concentration, was measured after 2 h of incubation using a single substrate concentration and at three different temperatures (4, 20, 37 °C). Samples and substrate controls were used to correct the final values. A first-order kinetics (Michaelis-Menten model) was used to describe the rate of enzymatic hydrolysis in this study (Whiteley & Lee, 2006). The Michaelis-Menten equation was solved using a non-linear regression in order to determine the Michaelis-Menten constant (K_m), which provides an indication of substrate affinity. As for specific activity values, controls were run to assess colour development in sample and substrate blanks. The assays were carried out for β -glucosidase and lipase at different temperatures (4, 7, 15, 20, 37 °C). It was not possible to measure K_m for protease as, when stopping the reaction after 15 min, the colour developed in the sample blank was too similar to the colour of the reaction wells. Table 2 reported a summary of the assays carried out in this study.

The assay to determine β -glucosidase kinetics parameters used 4-nitrophenyl β -D-glucopyranoside (N7006, Sigma-Aldrich, UK) as substrate, in the range 0.5–5 mM, and 4-nitrophenol (241326, Sigma-Aldrich, UK), measured at 405 nm, as standard. Sodium carbonate (1 M) was used as stopping reagent and sodium chloride (0.14 M) as buffer (Kreutz et al., 2016). Sample and substrate were placed with a ratio 1:1 (0.5 mL) in a microcentrifuge tube and incubated at the temperatures of interest. The reaction was stopped after 15 min with 0.5 mL of stopping reagent and the tubes were centrifuged according to Kreutz et al. (2016). The supernatant was transferred to a microplate (250 μ L) to read the absorbance of the released product. The assay to determine specific activity followed the same procedure, stopping the reaction after 2 h and using a concentration of substrate of 2 mM.

Lipase kinetics parameters were determined using 4-nitrophenyl palmitate (N2752, Sigma-Aldrich, UK) as substrate with concentrations ranging from 0.1–0.5 mM. Stock and working solutions were prepared according to Gessesse et al. (2003). The assay was carried out at the different temperatures in a microplate, using 25 μ L of sample and 225 μ L of substrate (1:9), measuring the release of 4-nitrophenol (241326, Sigma-Aldrich, UK) at 405 nm after 20 min. The assay to determine specific activity followed the same procedure, using 0.2 mM as substrate concentration and measuring the absorbance after 2 h.

Protease assay was performed only for the determination of specific activity at different temperatures after a 2-h incubation, according to the methodology developed by Sigma-Aldrich for protease colorimetric detection (PC0100, Sigma-Aldrich, UK) using casein (2 mg/mL) as substrate. Tyrosine, which is a product of the cleavage of casein, was used as standard and its presence was revealed with the Folin and Ciocalteu's reagent at 660 nm, after the addition of 10 % w/w trichloroacetic acid.

3. Results and discussion

3.1. Microbiome characterisation

The bacterial diversity of the two UASB reactors and the two AnMBRs is reported in Fig. 1. While the microbiomes of the two inocula were extremely different, reflecting their different origin (Supplementary Materials), after over 10 months of operation in the same conditions, the two UASB reactors developed similar relative abundances, although more samples would be required to ascertain the replicability of the results (Fig. 1). During the operation of the UASB reactors, the predominant order was *Campylobacteriales* (*Proteobacteria* phylum). The other bacterial orders identified in the samples are involved in the hydrolysis of particulate matter. *Lactobacillales* (*Bacilli* class, *Firmicutes* phylum) can grow anaerobically and produce lactic acid from carbohydrates fermentation (Aguirre & Collins, 1993). *Eubacteriales* or *Clostridiales* are obligate anaerobes belonging to the *Clostridia* class and *Firmicutes* phylum, which have been identified as a dominant class in the microbial community of anaerobic reactors and are able to degrade lipids, proteins and polymeric carbohydrates (Fykse et al., 2016). *Synergistales* (*Synergistetes* phylum) are syntrophic fermentative bacteria that degrade amino acids into volatile fatty acids and develop syntrophic relationships with methanogens, contributing to acidogenesis and acetogenesis (Damodara Kannan et al., 2020).

With the addition of the membrane, the overall abundance of bacteria with hydrolytic abilities, identified in the UASB reactors, increased from 17.3 % to 34.3 % in AnMBR-R1 and from 20.8 % to 73.3 % in AnMBR-R2. Moreover, the presence of other orders, involved in hydrolysis, increased. The proliferation of *Bacteroidales* (*Bacteroidota* phylum), capable of sugar fermentation, suggests an enhancement in volatile fatty acids degradation in AnMBRs (Azman, 2016). However, a codominance of *Bacteroidales* and *Clostridiales*, previously reported in similar operational conditions (Ribera-Pi et al., 2020), was not observed in this study. The combined relative abundance of *Syntrophobacteriales*, *Syntrophales* and *Syntrophorhabdiales*, belonging to the *Deltaproteobacteria* class and *Proteobacteria* phylum, increased in AnMBRs, reaching 9.2 % in AnMBR-R1. These bacteria can degrade short-chain fatty acids, such as acetic, propionic, and butyric acid, to carbon dioxide and hydrogen in syntrophic relationships with methanogens (Nakasaka et al., 2020). However, these orders have been characterised by metabolic flexibility and can grow as sulphate-reducing bacteria or fermentative microorganisms, depending on the environmental conditions (Plugge et al., 2011). Moreover, the fluctuation in their abundance has been correlated to COD:SO₄ ratio, as it was observed that a decrease in COD:SO₄ led to a microbial shift to incomplete-oxidizing sulphate-reducing bacterial species, which could cause volatile fatty acids accumulation

Table 2

Summary of enzymatic tests carried out in this study on sludge samples from pilot-scale UASB reactors and AnMBRs to evaluate specific activity and substrate affinity of β -glucosidase, lipase and protease.

Enzyme	Substrate	Substrate concentration (mM)	Incubation time (min)	Incubation temperature (°C)
β -glucosidase	4-nitrophenyl β -D-glucopyranoside	0.5–5	15	4, 7, 15, 20, 37
	4-nitrophenyl β -D-glucopyranoside	2	120	4, 20, 37
Lipase	4-nitrophenyl palmitate	0.1–0.5	20	4, 7, 15, 20, 37
	4-nitrophenyl palmitate	0.2	120	4, 20, 37
	Casein	2 (mg/mL)	120	4, 20, 37

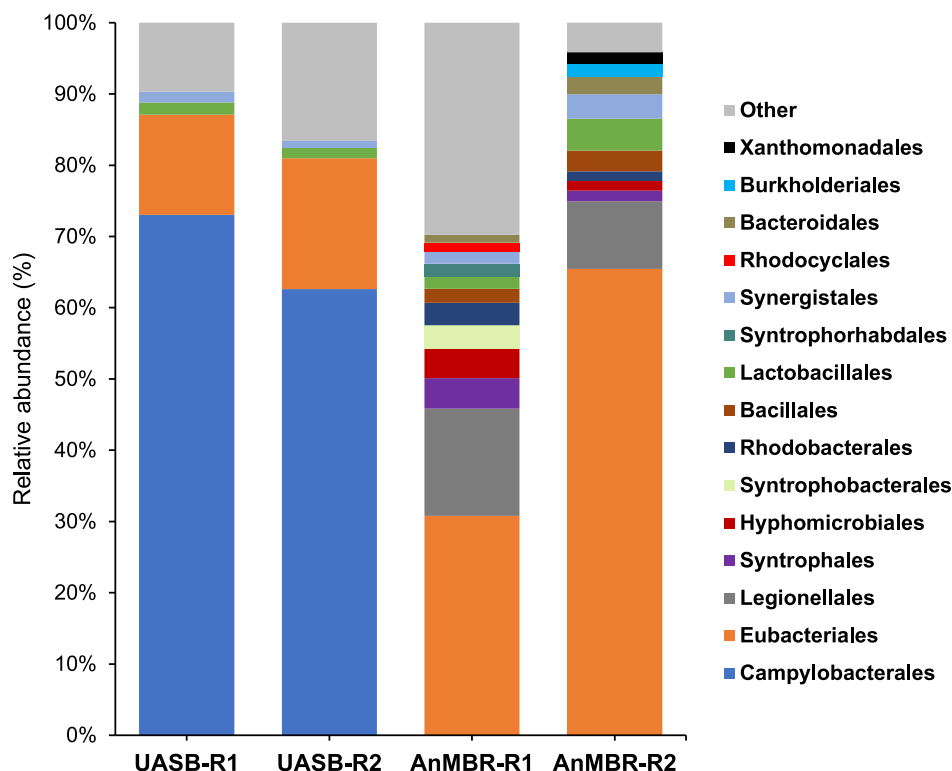


Fig. 1. Bacterial community of sludge samples from pilot-scale UASB reactors and AnMBRs, described by orders (relative abundance cutoff of 1%).

and reduced methane production (Lu et al., 2018).

Regarding archaeal communities, while no data was collected in this study, literature data suggest that the addition of a membrane affects to some extent the relative abundances of methanogens in the sludge. However, the evolution is not univocal. Ozgun et al. (2015) observed that after membrane addition, the genus *Methanosaeta* (acetoclastic methanogens) increased from less than 15 % to 56 % in relative abundance, while *Methanobacterium* (hydrogenotrophic methanogens) decreased by 45 %. Similarly, Petropoulos et al. (2019) reported that

Methanosaeta dominated the archaeal communities in the AnMBR reactor, although particularly on the surface of the membrane, while hydrogenotrophic methanogens were more abundant in the UASB reactor. On the other hand, Smith et al. (2015) found that hydrogenotrophic methanogenesis was the preferred pathway in the biofilm, but not in the suspended biomass, and Ribera-Pi et al. (2020) did not observe a marked shift in the composition of the methanogenic community between a flocculent UASB reactor and AnMBR. Therefore, analysis of the archaeal community would be necessary to investigate

Table 3

Influent and effluent wastewater characterisation, removal efficiencies and methane production of the two UASB reactors and AnMBRs. The star (*) indicates the parameters for which two reactors with the same configuration showed significantly different values. n.a.: not applicable.

		UASB influent	UASB-R1 effluent	UASB-R2 effluent	AnMBR influent	AnMBR-R1 effluent	AnMBR-R2 effluent
Characterisation							
Temperature	°C	16.1 ± 2.8	15.2 ± 3.0	15.3 ± 3.0	20.6 ± 1.0	20.0 ± 1.7	20.0 ± 2.1
COD	mg/L	243 ± 61	99 ± 24*	121 ± 32*	350 ± 104	37 ± 11*	29 ± 2*
sCOD	mg/L	46 ± 11	37 ± 9	35 ± 9	58 ± 8	34 ± 9	26 ± 4
BOD	mg/L	94 ± 26	44 ± 10	53 ± 10	139 ± 40	15 ± 5	16 ± 1
TSS	mg/L	130 ± 30	35 ± 8*	46 ± 10*	207 ± 55	5 ± 3	4 ± 2
VSS	mg/L	110 ± 28	32 ± 7*	42 ± 9*	186 ± 51	4 ± 2	3 ± 2
SO ₄	mg/L	64 ± 8	40 ± 13*	49 ± 11*	51 ± 4	19 ± 7	19 ± 7
COD:SO ₄		3.8	n.a.	n.a.	6.9	n.a.	n.a.
Removals							
COD	%	n.a.	57 ± 12*	49 ± 10*	n.a.	88 ± 4*	92 ± 2*
sCOD	%	n.a.	18 ± 12	23 ± 13	n.a.	43 ± 14	54 ± 4
BOD	%	n.a.	50 ± 12	43 ± 11	n.a.	89 ± 5	87 ± 2
TSS	%	n.a.	74 ± 5*	61 ± 11*	n.a.	98 ± 2	98 ± 1
SO ₄	%	n.a.	36 ± 20*	24 ± 13*	n.a.	70 ± 9	60 ± 15
Methane							
Total CH ₄ production	L/d	n.a.	2.7 ± 1.1	2.3 ± 0.6	n.a.	5.3 ± 1.7	5.7 ± 0.7
CH ₄ production (gas)	L/d	n.a.	0.4 ± 0.3	0.7 ± 0.2	n.a.	1.6 ± 0.8	1.3 ± 0.6
CH ₄ production (dissolved)	L/d	n.a.	2.3 ± 0.9	1.6 ± 0.6	n.a.	3.7 ± 1.0	4.4 ± 0.3
Methane yield	L _{CH4} /g _{CODrem}	n.a.	0.13 ± 0.07	0.14 ± 0.08	n.a.	0.15 ± 0.09	0.16 ± 0.10

the impact of the membrane in this study as no clear conclusions can be drawn from literature. However, for this study, the analysis of the bacterial community is more relevant to the scope compared to the analysis of the *Archaea*.

3.2. Effluent quality, methane production and hydrolysis

While both UASB reactors and AnMBRs were operating in steady-state conditions when the enzymatic tests were carried out, the influent wastewater, which affects the microbiome of the sludge, was statistically different between the two periods (Table 3). In particular, COD and TSS concentrations increased from 243 to 350 mg/L and from 130 to 207 mg/L, respectively, when the AnMBRs were in operation. BOD:COD ratio remained stable at around 0.39 and COD was mainly in particulate form (around 80 %) in both periods (Table 3). However, COD:SO₄ increased from 3.8 to 6.9 during AnMBR operation, which could have triggered the increase of *Deltaproteobacteria*, observed in the microbiome characterisation.

Removals for COD, TSS and SO₄ in UASB-R1 were higher than the ones in UASB-R2, respectively 57 %, 74 % and 36 % against 49 %, 61 % and 24 %. During the AnMBRs operation, the effluent streams were more similar, with the exception of COD removal, which was higher in AnMBR-R2 (92 % against 88 % in AnMBR-R1) (Table 3). Overall, the removal efficiencies achieved in this study in all reactors were in line with literature data for the treatment of municipal wastewater in temperate climates (9–18 °C) with the membrane ultrafiltration after the UASB promoting enhanced COD removal from 50–60 % to 88–92 %, as also described in other studies (Gouveia et al., 2015; Ribera-Pi et al., 2020; Wang et al., 2019). Furthermore, the average total methane production was not significantly different between the two UASB reactors and the two AnMBRs. More data on the long-term performance of UASB reactors and AnMBRs are reported in Supplementary Information.

Recently a life cycle cost (LCC) analysis of an AnMBR demonstration plant, which was operated for three years treating municipal wastewater at ambient temperature, revealed that the capital expenditure (CapEx) was mainly influenced by the cost of the ultrafiltration membranes, the combined heat and power technology for energy recovery and the reactor construction, while the main contributors to the operational expenditure (OpEx) were energy requirements, membrane replacement and discharge fee (Jiménez-Benítez et al., 2023). The maximum total annualised cost (TAC) was €0.19/kg_{CODrem}, with CapEx being the main contributor. To compare, the TAC of conventional activated sludge found in literature varied between €0.14–2.51/kg_{CODrem} (Jiménez-Benítez et al., 2023). To improve the economic sustainability of AnMBRs, the parameters to optimise are: membrane fouling, hydraulic retention time, biogas requirements, for reactor mixing and membrane stirring, and energy recovery efficiency (Jiménez-Benítez et al., 2023).

To better understand how the removal efficiencies translated into hydrolytic efficiencies, hydrolysis and solids hydrolysed were calculated for each reactor (Table 4). The two UASB reactors and the two AnMBRs did not display statistical differences in these parameters. It was observed that UASB-R1 presented marginally higher hydrolysis and rate of solids hydrolysed than UASB-R2, which could be linked to higher COD and TSS removals observed in Table 3, while, during AnMBR operation, AnMBR-R2 was performing slightly better in terms of

hydrolytic efficiencies, in line with COD removals (Table 3). It is well established that improving the hydrolysis stage with enzymes can enhance the anaerobic digestion process. Enzymes have been shown to be effective in pre-treating wastewater, resulting in a substantial improvement in biogas production, increasing it by up to 76 % (Liew et al., 2020).

In AnMBRs, the hydrolysis percentage resulted lower than in UASB reactors, as previously observed by Ribera-Pi et al. (2020), which could seem to contradict the hypothesis that the use of a membrane would promote hydrolysis due to higher solids retention in the reactor. However, when the hydrolysis percentages were applied to the solids removed, the rate of solids hydrolysed clearly showed that the AnMBR allowed for better solids hydrolysis. The results from Table 4 suggest that enzymatic activities of hydrolases in this study would be expected to be different between UASB reactor and AnMBR, but not between the two reactors with the same configuration, but with different seed sludge, since no statistical difference was identified ($p > 0.05$).

3.3. Protein concentration and specific enzymatic activity

The protein concentration of the sludge samples collected for enzymatic tests from UASB reactors and AnMBRs was measured to obtain a first indication of enzymes content in the reactors. UASB-R2 displayed higher average concentration (584 mg/L) compared to UASB-R1 (447 mg/L), while AnMBR-R2 concentration (434 mg/L) was slightly lower than AnMBR-R1 (451 mg/L) (Fig. 2). Overall, neither the addition of the membrane nor the type of sludge did have a marked effect on the protein concentration of sludge, as all the observed series of data were not statistically different from one another ($p > 0.05$).

The protein concentration was used to determine the specific enzymatic activity (SEA) of the enzymes at different temperatures (4, 20, 37 °C) (Fig. 3). In all configurations, the activity of each enzyme increased with temperature, especially when incremented to 37 °C. AnMBRs presented higher activities compared to UASB reactors in all cases, except for β -glucosidase at 20 °C (0.17 ± 0.02 nmol/(mg • min) for UASB-R1 and 0.16 ± 0.08 nmol/(mg • min) for AnMBR-R1 ($p > 0.15$)) (Fig. 3a) and protease at 4 °C (6.84 ± 2.02 nmol/(mg • min) for UASB-R1 and 4.73 ± 1.81 nmol/(mg • min) for AnMBR-R1 ($p > 0.15$)) (Fig. 3e), suggesting that overall the presence of the membrane helped increasing hydrolytic activity. This is in line with the bacterial composition analysis (Fig. 1), which reported an increased abundance of bacteria with hydrolytic abilities with the addition of the membrane, and the improved rates of solids hydrolysed observed in AnMBRs (Table 3). Statistical differences ($p < 0.15$) between UASB reactors and AnMBRs were identified particularly for protease in both setups and all

Table 4

Average of hydrolysis percentages and rates of solids hydrolysed in the UASB reactors and AnMBRs operated during this study.

		UASB-R1	UASB-R2	AnMBR-R1	AnMBR-R2
Hydrolysis	%	28.3 ± 9.5	24.7 ± 9.2	18.2 ± 9.5	21.4 ± 12.0
Rate of solids hydrolysed	g/(L • d)	0.86 ± 0.29	0.82 ± 0.28	1.57 ± 0.35	1.87 ± 0.41

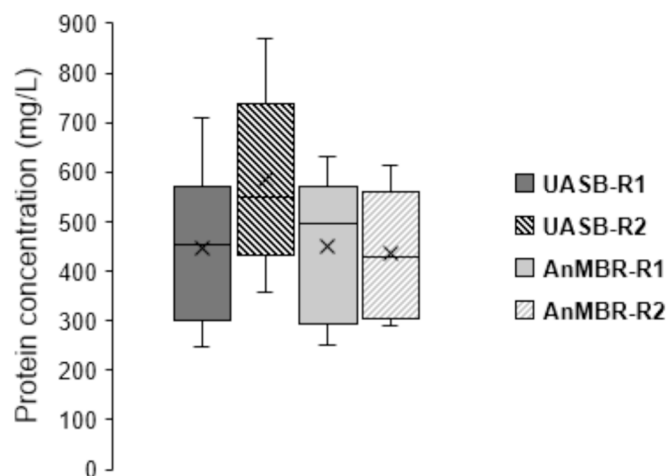


Fig. 2. Protein concentration in the sludge samples from UASB reactors and AnMBRs used for enzymatic assays.

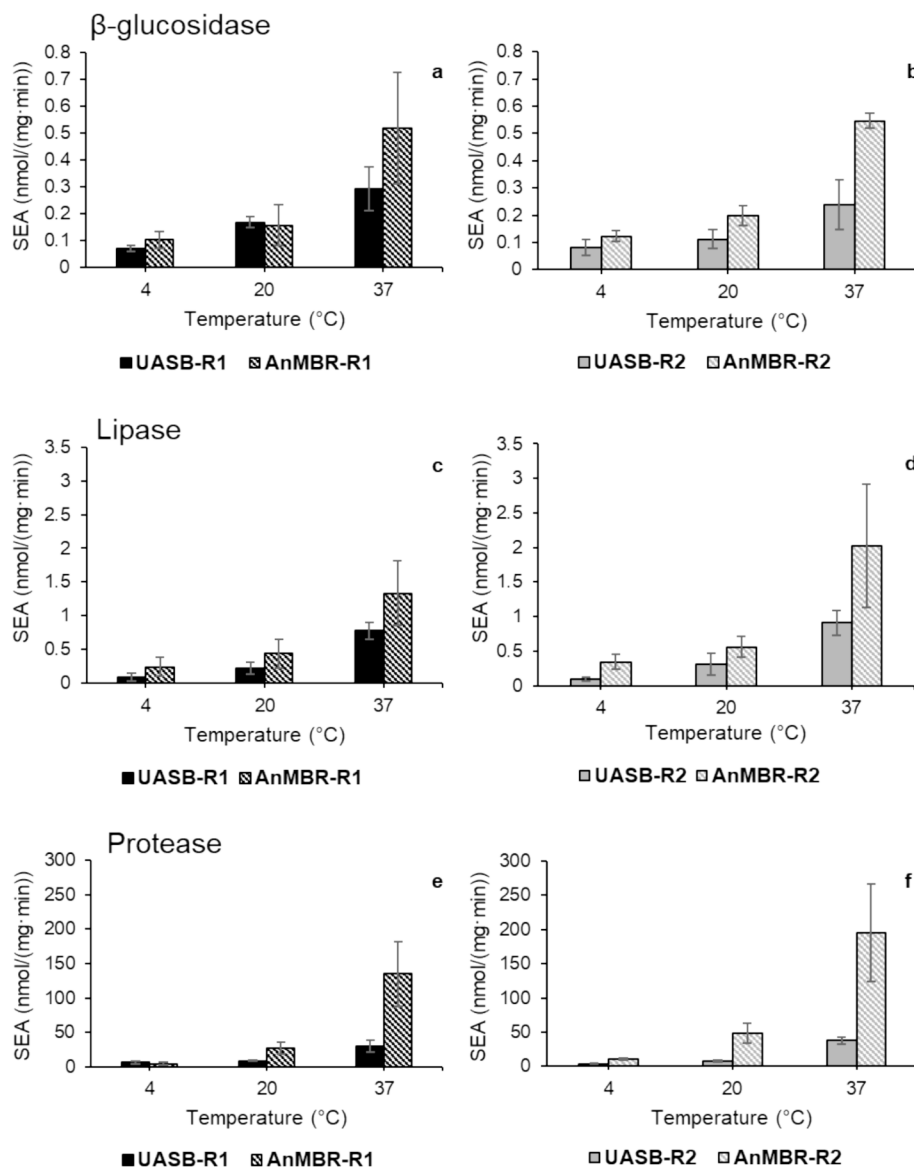


Fig. 3. Specific enzymatic activity of β -glucosidase (3a, 3b), lipase (3c, 3d) and protease (3e, 3f) in the different sludge samples (UASB-R1, UASB-R2, AnMBR-R1 and AnMBR-R2) at 4, 20 and 37 °C.

temperatures, except for UASB/AnMBR-R1 at 4 °C. The highest specific activity observed for β -glucosidase was 0.52 nmol/(mg • min) in AnMBR-R1 and 0.55 nmol/(mg • min) in AnMBR-R2 (Fig. 3a-b). Regarding lipase, specific activity reached 1.33 nmol/(mg • min) in AnMBR-R1 and 2.02 nmol/(mg • min) in AnMBR-R2 (Fig. 3c-d). Ultimately, protease presented the highest values for specific activity, achieving 135 nmol/(mg • min) in AnMBR-R1 and 196 nmol/(mg • min) in AnMBR-R2 (Fig. 3e-f). Between AnMBRs, the specific enzymatic activity of AnMBR-R2 was slightly higher than AnMBR-R1, matching the trend in hydrolysis percentages (Table 4), however, no significant difference was revealed with statistical tests, except for protease at 4 and 20 °C, with AnMBR-R2 performing better. Similarly, the two UASB reactors did not display statistically different enzymatic activity at the same temperature, except for β -glucosidase at 20 °C and protease at 4 °C, with UASB-R1 achieving higher activities in those assays.

Assays at temperatures >37 °C would allow to determine which is the optimal temperature for enzymatic activity of the sludge used in this study, corresponding to the maximum of specific activity observed. The fact that the enzymes displayed higher activities at 37 °C suggests that the operation for over a year at temperatures <20 °C did not cause the

development of psychrophilic microorganisms that secreted psychrophilic enzymes, which present peak activity between 20 and 30 °C (Feller & Gerday, 2003). However, the temperature for maximum enzyme activity could not always match the optimal growth temperature of the microorganism (Brenchley, 1996). In particular, it was reported that the enzymes activity could be less well adapted to cold compared to microbial growth and present higher activity at temperatures beyond the growth range of the bacteria (Huston et al., 2000). Petropoulos et al. (2018) reported that lipase activity in the sludge from batch reactors treating municipal wastewater decreased with temperature, as observed in this study, and adaptation of the sludge at different temperatures (4 and 15 °C) did not have a significant impact when measuring activity at 37 °C. Therefore, to investigate the adaptation of the sludge samples to the operational conditions of this study (<20 °C), the affinity of the sludge, expressed in terms of K_m , the half-saturation constant of the Michaelis-Menten model, was measured for different substrates at different temperatures.

3.4. Enzymes substrate affinity at different temperatures

Results from Fig. 4 revealed that β -glucosidase and lipase exhibited lowest K_m values at around 15–20 °C, corresponding to the average operational temperature of the reactors during the analysed period (Table 3). The trend did not change when UASB reactors were converted in AnMBRs. Overall, the enzymes from AnMBRs showed lower K_m values compared to UASB reactors, except for lipase at 20 °C in UASB/AnMBR-R2 (Fig. 4d). No statistical difference was observed in the two AnMBRs, as well as in the two UASB reactors, with the exception of lipase at 15 and 37 °C ($p < 0.15$). Only a few cases tested presented a significantly lower K_m after the addition of the membrane (β -glucosidase at 7 °C in UASB/AnMBR-R2, lipase at 15 °C in UASB/AnMBR-R1 and lipase at 37 °C in UASB/AnMBR-R2) (Fig. 4b–4c–4d). Lowest K_m values for β -glucosidase and lipase were 0.37 mM in AnMBR-R1 and 0.52 mM in AnMBR-R2, both at 15 °C (Fig. 4a–b), and 0.26 mM in AnMBR-R1 and 0.20 mM in AnMBR-R2, both at 15 °C (Fig. 4c–d), respectively.

The highest K_m values were normally observed at the highest and lowest temperatures assayed, therefore 4–7 °C and 37 °C. This was expected since these temperatures are outside the operational range measured at pilot-scale (13–21 °C). However, in the UASB reactors, before the period here analysed, experienced also temperatures down to 6 °C and, from Fig. 4, lipase in UASB reactors seemed to reflect that, since K_m values were lower at 4 °C than at 37 °C (0.84 mM at 4 °C and 1.21 mM at 37 °C in UASB-R1 and 0.93 mM at 4 °C and 1.87 mM at 37 °C in UASB-R2) (Fig. 4c–d).

It was shown that enzymes display lowest K_m values at the physiological temperature of the producing microorganism (Kulakova et al., 2004). Kulakova et al. (2004) observed that the optimal temperature for a psychrophilic esterase was around 35 °C. However, this is enzyme dependant, commercial pectinases have been shown to be active between 10–60 °C, and despite not being classified as psychrophilic, exhibit effectiveness at lower temperatures, are also finding applications in the food and beverage sectors (Kumari et al., 2021). In this study lowest K_m values were found at 25 °C, which was the optimal growth temperature of the bacteria producing the enzyme. Therefore, the trend in Fig. 4 suggests that the physiological temperature of the hydrolytic bacteria in this study was 15–20 °C, showing sludge adaption to the

operational temperature. In a study that investigated the breakdown of organics in soils, it was demonstrated that as temperature increased from 0 to 40 °C, enzyme activity of hydrolytic enzymes (i.e.: β -glucosidase, cellobiohydrolase and hemicelluloses (xylanase) generally heightened, while the Michaelis-Menten constant underwent alterations, impacting enzyme-substrate interactions and subsequently affecting catalytic efficiency and the two were not factors were not necessarily aligned (Razavi et al., 2015) as shown in this study in UASB reactors.

There is no indication in literature that the assays for enzymatic activity developed for activated sludge are influenced by the presence of oxygen (Coelmont et al., 2023), therefore, the specific activity of anaerobic sludge can be measured in aerated conditions. In this study, the assays were adapted from literature for activated sludge. However, despite the clear trend, the high standard deviations in Fig. 4 and the difficulty in carrying out the short-term enzymatic test for protease suggest that more replicates may be needed to define the K_m values and modifications to the protease assay could be considered to try to reduce the colour development in the sludge sample blank. A different substrate (azocasein) was tested, following the procedure described by Gessesse et al. (2003), with a similar outcome. Possible solutions could include a different procedure for the preparation of the sludge sample (concentration, extraction, purification etc.), the use of a different type of assay (e.g. fluorescence-based assays) or the investigation of a related enzyme, like amino-peptidase.

4. Conclusions

Two pilot-scale UASB reactors, subsequently converted in AnMBRs, were seeded with two different types of mesophilic sludge and their operation, to treat municipal wastewater in temperate climates (<20 °C), was assessed in terms of effluent quality, methane production and hydrolysis percentages. Removals of COD and TSS were around 50 % and 60 % in UASB reactors and 90 % and 98 % in AnMBRs, respectively, and production of methane and hydrolysis percentages were not statistically different between the two UASB reactors and the two AnMBRs.

To better understand the adaptation of the sludge to low

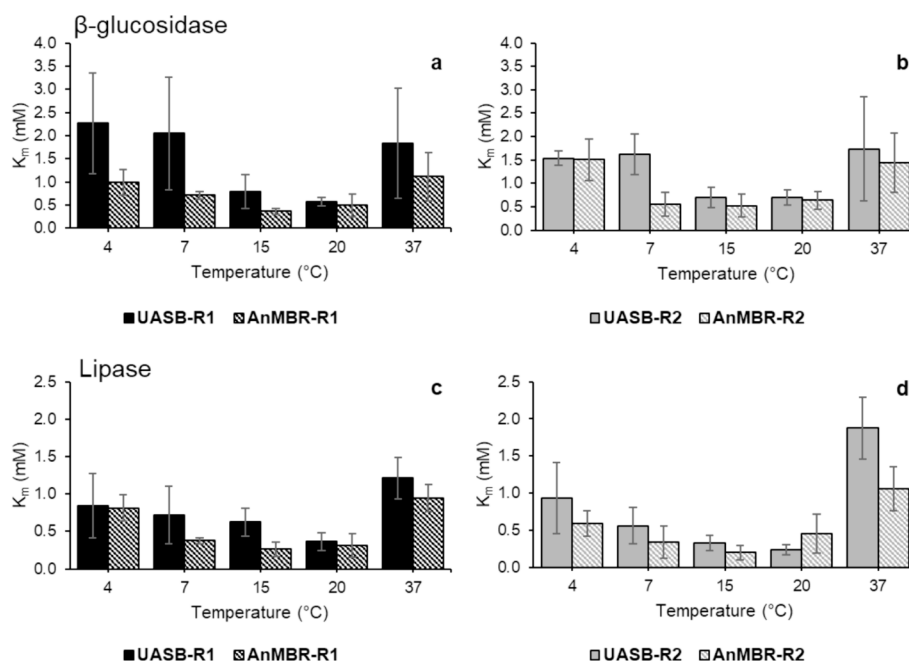


Fig. 4. Michaelis-Menten constant (K_m) for β -glucosidase (4a, 4b) and lipase (4c, 4d) at different temperatures (4, 7, 15, 20, 37 °C), measured in sludge samples from UASB reactors and AnMBRs.

temperature, sludge samples were collected from the two reactors and in the two configurations to measure the activity of hydrolytic enzymes (β -glucosidase, protease and lipase). The specific activity increased with temperature (from 4 °C to 37 °C) for all three enzymes, implying that the operation at low temperatures did not cause the development of psychrophilic enzymes in the mesophilic sludge. However, the Michaelis-Menten constant (K_m) revealed that the enzymes, in all configurations, displayed higher affinity, and therefore lower K_m , at the operational temperature of the reactors (15–20 °C). The hydrolytic enzymes present lower K_m values at the physiological temperature of the producing bacteria. Therefore, the results from this study suggest that both inocula adapted successfully to the low-temperature municipal wastewater treatment, matching the temperature for optimal bacterial growth to the operational one, while the hydrolytic enzymes maintained higher activities at mesophilic temperatures (37 °C).

CRediT authorship contribution statement

Eleonora Paissoni: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bruce Jefferson:** Writing – review & editing. **Ana Soares:** Writing – review & editing, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

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 file made available in the Cranfield University Depository with a DOI

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2024.130975>.

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