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The Influence of Biochar Augmentation and Digestion Conditions on the Anaerobic Digestion of Water Hyacinth

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Abstract: The augmentation of biochar (BC) during anaerobic digestion (AD) has been identified as a potential strategy for improving the AD of complex feedstocks. This study evaluates the influence of oak wood biochar 450 °C and fermentation conditions during the AD of the invasive aquatic plant, water hyacinth (WH). Factorial 2^2 design of experiments (DOE) allowed the evaluation of the effect of the crucial processing conditions, inoculum-to-substrate ratio (ISR) and biochar load. Further optimisation was performed to identify the best processing conditions for the AD of WH, at an ideal ISR of 1. The contour plots suggested that methane yield is favoured at biochar loads of $\leq$0.5%, whereas the production rate is favoured by increasing biochar loads. However, biochar addition offered no further improvement or significant effect on the digestion of WH. The subsequent AD of WH samples collected from different locations in India and Uganda exhibited variable biochemical methane potential (BMP) yields. BC addition had little effect on BMP performance, and in some cases, it even reduced the BMP. This study concludes that the amendment potential of biochar is influenced by digestion conditions and the substrate, particularly when working with complex substrates.

Keywords: anaerobic digestion; water hyacinth; biochar; design of experiments (DOE); inoculum-to-substrate ratio (ISR)

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1. Introduction

The production and use of biogas from organic waste embody the concept of a circular economy by improving waste management and resource efficiency while reducing greenhouse gas (GHG) emissions [1]. Anaerobic digestion (AD) is designed for the sustainable management of numerous waste materials and the production of biogas (methane and carbon dioxide) and digestate. The biogas is usually destined for heat, steam, and electricity, while the digestate is employed as fertiliser in agriculture [2,3]. The development of biogas into a sustainable energy future fluctuates among countries since it depends on a myriad of factors, including technological development, feedstock availability, prevailing market conditions and policy priorities [1]. Even though AD is a robust and largely established technology, it continues to face challenges. The operational instability of the process represents a major problem for AD as inhibitory compounds present on the organic feedstocks or produced during their hydrolysis detriment the process yields [2,3]. Hence, AD still requires gradual technological changes for increasing performance and economic viability.

Several approaches proposed for improving AD performance include integration with other technologies, and the addition of adsorptive carbonaceous materials, such as biochar (BC), for amendment and/or immobilisation of cells [4]. The advantages of using BC over other materials include low cost, environmental sustainability, the capacity to use a variety of biomass feedstocks for its production, and the capacity to improve the quality of the digestate. BC is obtained from the pyrolysis of biomass under anoxic or limited oxygen conditions and high temperatures. The BC has advantageous physicochemical properties, including large surface area and surface functionality, which can be further tailored to obtain desired characteristics [5,6]. The main impact of BC addition on AD includes a reduction in the lag phase, the promotion of hydrolysis and acidogenesis-acetogenesis, buffering acid stress, stabilisation of methanogenesis, enhancing methane yields and production rate, while promoting syntrophic interactions [7]. BC is also reported to accelerate acetic and butyric acid formation and further VFA degradation, and stimulate the growth of methanogens [8]. These effects have been attributed to the role of BC in facilitating the direct interspecies electron transfer (DIET) process, increasing the electrical conductivity of the sludge, reducing ammonia inhibition, buffering acids, and even the adsorption of CO_2 for improving the quality of biogas [9,10].

To understand the relationship between BC and its effect on AD, previous work conducted in the research group has highlighted the importance of the physicochemical properties of the biochar [11]. Nonetheless, it is equally important to consider the influence of the chosen substrate and its biochemical composition. BC amendment has moderately favoured the AD of glucose [12,13], and food waste [14]. These publications agree on the fact that substrates are quickly transformed into VFAs, whose further consumption towards methane generation is favoured by BC. For complex substrates, BC addition is reported to improve the AD of anaerobic sludge [15], dairy manure [16], and swine manure [17]. Other publications for the AD of anaerobic sludge reported that BC offered no improvement in methane yield, although it increased the production rate [9,10]. Conversely, for the seaweed *Laminaria digitata*, BC addition modestly enhanced methane generation and even exhibited a detrimental effect at increasing BC loads [18]. The variations in AD performance suggest that the BC effect is substrate dependent, although this correlation has not been addressed.

The use of complex substrates in AD is key for developing the biogas industry of a country or a region. To achieve this, several aspects must be taken into consideration, such as feedstock availability, economics, regulatory issues, and national bioenergy production targets. To promote an efficient AD scenario, substrate selection must consider the re-use or recycling of existing and long-term available raw materials. Agricultural, industrial, municipal waste and food crops are considered to represent the highest market maturity and economic efficiency in biogas plants. Nonetheless, agricultural residues require pre-treatments for decomposing the lignin fraction and making the fermentable constituents available, whereas food crops compete with food security and prices [19]. Alternative options that could represent a reliable supply are non-edible plants with rapid growth, such as water hyacinth (WH).

WH (*Eichhornia crassipes*) is an aquatic invasive macrophyte with adaptative phenology growing throughout the tropical zone around the globe. The flexible morphology and capacity to hyper-accumulate nutrients available in water bodies give WH an outstanding adaptation and invasive potential. In natural environments, WH out-competes and negatively affects flora and fauna, hence the importance of its removal and further utilisation for economic viability [20]. WH grows uncontained in water bodies in over 50 countries, principally in Southeast, Central and Western Asia and Central America, and is predicted to expand into higher latitudes as a consequence of temperatures rise due to climate change [21]. Given its biology, the eradication of WH is practically impossible, and due to its high content of water (95%), transport, storage or disposal is very costly. Hence, directing the control of WH towards sustainable utilisation via AD could increase the energetic and ecological development of urban and rural areas. The ability of AD to process wet biomass makes this technology highly suitable to utilise WH for methane generation [22]. Aquatic biomass can thus support energy generation while its removal from water bodies could beneficiate local ecosystems.

The use of WH as a substrate for AD could provide a source of electricity and cooking gas in rural areas. India is one of the countries most affected by WH proliferation, while persistently struggling to fulfil their energy demand, thereby they are supporting the development of bioenergy. The high ash and water content of WH complicates its use in gasification or pyrolysis, hence it has been suggested as a feedstock for biogas production. Employing locally available biomass for off-grid biogas generation could provide cooking and lighting energy to villages while trying to solve the trilemma of preserving energy resources, achieving economic sustainability and preventing environmental degradation [23]. WH exhibits some advantages as a substrate for AD, due to its carbohydrate fraction comprised mainly of cellulose and hemicellulose that could be easily fermented into methane, whereas its protein content could give WH an adequate C/N ratio (~15–30) for AD. Nonetheless, there is limited work on the AD of WH, with moderate biomethane yields found within a range of 114–240 mL CH_4/g [24,25]. Most literature reports employ WH samples from one single location, while this work evaluates WH samples from four locations in two countries across two continents. To enhance the transformation of complex feedstocks such as WH into methane, it is necessary to establish suitable AD conditions. Inoculum-to-substrate ratio (ISR) and BC load influence AD efficiency, hence the necessity to establish optimal conditions [7,26]. The initial activity and general performance of the digester are influenced by the inoculation, while an optimum ISR preserves digester stability, by preventing the accumulation of volatile fatty acids (VFAs), and reducing the need for nutrient media supplementation, thus improving methane production [27]. The BC load is key during BC augmentation since adequate amounts could improve AD performance [15] while higher doses could even exhibit an inhibitory effect [28]. To the best of our knowledge, the augmentation of lignocellulosic-derived BC as an additive during the AD of water hyacinth has not been reported. Furthermore, there are also no publications regarding the influence of augmentation of a true biochar for the AD of WH, or the implementation of design of experiments (DOE) for supporting correlations and optimisation.

The overall aim of this research is to determine the capacity of biochar augmentation to improve the digestion performance of water hyacinth and to establish how fermentation conditions influence the AD of raw WH. To achieve this, a full factorial 2^2 experimental design was implemented for evaluating the process variables ISR and BC load. This is followed by a comparison of the conditions resulting from the DOE on the AD of WH samples from other sources.

2. Materials and Methods

2.1. Substrate, Inoculum, and Biochar

Mesophilic anaerobic sludge was collected from the Esholt wastewater treatment plant in Bradford, United Kingdom. The inoculum was stored at 4 °C, and before use, it was homogenised by filtration through a mesh (1 mm). The total solids (TS) and volatile solids (VS) were determined gravimetrically for adjusting the desired ISR [29]. WH samples collected from different sources in India and Uganda were oven dried, milled, and stored in a solid container at room temperature. The commercial holm oak wood biochar was produced at 450 °C for 24 h in a batch mono retort kiln by a pyrolysis plant operated by Proininso (Málaga, Spain). The details of the reactor system are proprietary.

2.2. Composition of Biochar and Water Hyacinth Substrates

Table 1 describes the sampling site and the chemical composition of the WH substrates and the biochar. Proximate analysis was determined with the thermogravimetric analyser TGA/DSC 1 (Mettler Toledo, GmbH, Greifensee, Schweiz, Switzerland). Approximately 10 mg of the sample was analysed under an N_2 flow (50 mL/min) and a heating rate of 25 °C/min from 25 to 105 °C, held at 105 °C for 10 min, then heated up to 900 °C, and held at 900 °C for 10 min—at that point, the flow was switched from N_2 to air for 15 min to promote complete combustion. Differences in mass loss during the heating stages allowed calculating the percentage of moisture, volatile matter (VM), fixed carbon (FC) and ash.

Table 1. Proximate, ultimate, and biochemical composition of the substrate water hyacinth and the oak wood biochar.

Material	VBU-WH	MM-WH	PV-WH	UG-WH	Biochar
Sampling site	Goyal Para pond, India	Mula Mutha River, India	Pavana River, India	Lake Victoria, Uganda	
VM (%, db)	73.4	76.2	74.0	85.4	21.1
FC (%, db)	10.4	15.4	12.3	<1	67.2
Ash (%, db)	16.0	7.8	13.7	14.6	11.7
C (%)	34.2	36.3	33.0	36.1	65.7
H (%)	4.1	4.6	4.6	3.1	2.7
N (%)	1.8	3.0	3.1	2.5	0.6
O (%)	43.7	48.1	45.1	43.6	19.3
S (%)	0.0	0.2	0.5	0.1	0.0
C/N	17.8	12.3	10.6	14.5	-
Cellulose (%)	32.1	26.4	17.4	25.1	-
Hemicellulose (%)	25.5	16.1	8.3	22.6	-
Lignin (%)	4.7	7.9	11.2	6.8	-
Oils (%)	0.44	-	-	1.0	-
Protein (%)	8.42	-	-	12.4	-
Free sugars * (%)	8.7	-	-	11.8	-
BMP_{Th} (mL CH_4/g VS)	383.4	331.8	351.3	352.6	-

* Determined by difference; - not measured.

Elemental analysis used the automatic CHNOS Thermo Instruments Flash (EA 1112 Series, Thermo Scientific, Waltham, MA, USA). An amount of 2.5–3.0 mg of sample was combusted at 1000 °C, within an atmosphere of helium and a determined amount of oxygen. Certified biomass was used as reference materials (Elemental Microanalysis, Devon, UK) and ran in parallel. The elemental composition was calculated by converting the carbon to CO_2, nitrogen to NO_x, sulphur to SO_2 and hydrogen to H_2O. The values are expressed as the elemental percentage over the total dry weight. Total oxygen (O) was calculated by difference. The physicochemical properties of the biochar were characterised elsewhere [11].

Table 1 shows the biochemical composition of water hyacinth substrates. The neutral detergent fibre (NDFom, or aNDFom using amylase, STM 016), acid detergent fibre (ADFom, STM 017) and acid detergent lignin (ADLom, STM 043) were determined using the Gerhardt Fibrecap system as described by Fettweis and Kühl [30]. The protein content was determined by the Kjeldahl method, using a conversion factor of 4.64 [31].

2.3. Biochemical Methane Potential

The quantification of the biochemical methane potential (BMP) was performed by the Automatic Methane Potential Test System (AMPTS II) (Bioprocess Control, Lund, Sweden). The BMP was calculated according to the equation:

$$BMP = \frac{\text{Volume } CH_4 \text{ from sample } (mL) - \text{Volume } CH_4 \text{ from blank} (mL)}{\text{g VS of substrate fed in digester}} \quad (1)$$

Reactors of 500 mL and a working volume of 400 mL were used for the AD experiments. Digestion conditions consisted of inoculum at 10 g VS/L, substrate at 5–10 g/L, and BC at 0–3 (% w/v). Controls consisted of inoculum and substrate without BC, and a blank containing only the inoculum to subtract residual methane emissions was performed in parallel. The reactors were flushed with nitrogen for promoting anaerobic conditions and afterwards incubated at 37 °C for 30 days. The reactors were automatically stirred every 10 min for 60 s.

2.4. Theoretical Biochemical Methane Potential

The theoretical biochemical methane potential (BMP_{Th}) of the WH substrates was calculated based on Boyle's equation:

$$BMP_{th} = \frac{22400 \times \left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8}\right)}{12c + h + 16o + 14n} \tag{2}$$

where c, h, o and n state the molar fractions of C, H, O and N, respectively [32,33]. This equation assumes a complete substrate transformation into CH_4 and its by-product CO_2, with a 100% efficiency. The BMP_{Th} values are expressed on VS basis by considering only the biodegradable content and thus subtracting the ash fraction from the calculation.

2.5. Kinetic Analysis

From all the available kinetic equations for modelling methane generation, the modified Gompertz model is the most employed. This model offers the best fitting because it can accurately simulate the cumulative generation of methane [34]. Kinetic parameters were estimated as predictor variables by fitting the experimental BMP values to the modified Gompertz model [35]:

$$BMP(t) = BMP_{max} \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{BMP_{max}}(\lambda - t) + 1\right]\right\} \tag{3}$$

where BMP(t) represents the cumulative methane yield at time t (day), and BMP_{max} stands for maximum methane yield, both expressed as mL CH_4/g VS. μ_m states for the methane production rate expressed as mL CH_4/g VS·day, λ represents the lag phase in days, and e is the exponential of 1.

2.6. Anaerobic Biodegradability

The anaerobic biodegradability (BD) of methane depends on the degradability degree of the WH substrate. The BD was calculated based on the BMP_{Th} of the substrate and the final experimental BMP yield (BMP_{Exp}) for each treatment of study [33].

$$BD\ (\%) = \frac{BMP_{Exp}}{BMP_{Th}} \times 100 \tag{4}$$

2.7. Design of Experiments

To determine the effect of biochar addition and ISR during the AD of WH, a full factorial 2^2 DOE was created. The two independent variables of this study were evaluated at two levels, ISR (1–2) and BC load (0–3%) with 3 replicates and 3 centre points, as shown in Table 2. The inoculum concentration was fixed at 10 g VS/L, while the substrate ranged from 5 to 10 g VS/L to achieve the desired ISR.

Table 2. Full factorial 2^2 experimental design used for the anaerobic digestion of water hyacinth.

Reactor No.	Orthogonal Design		Actual Value	
	ISR	BC Load	ISR	BC Load (%)
R1, R2, R3	−1	−1	1	0
R4, R5, R6,	−1	1	1	3
R7, R8, R9	0	0	1.5	1.5
R10, R11, R12	1	−1	2	0
R13, R14, R15	1	1	2	3

2.8. Optimisation

A factorial regression model was employed for analysing the experimental methane production (BMP_{Exp}), and the kinetic variables BMP_{max} and μ_m. Contour plots were produced for evaluating the influence of the mentioned experimental factors on the variables of response [36]. Optimisation with the desirability (D) function was applied at the DOE data to establish the BC load and ISR conditions necessary to maximise the response variables BMP_{Exp}, BMP_{max} and μ_m. Composite D ranges from zero to one, where one is an ideal scenario and zero indicates that at least one response is outside the acceptable limits.

2.9. Anaerobic Digestion of Water Hyacinth Substrates

The AD of MM-WH, PV-WH and UG-WH substrates was performed on the AMPTS system as previously described. The necessary amount of WH substrates was calculated based on their chemical composition to achieve an ISR of 1. Oak wood BC 450 °C was added at 0.5% (*w*/*v*). Controls consisting of inoculum and each WH substrate, and the blank were performed in parallel. All runs were performed by duplicate.

2.10. Volatile Fatty Acids and Alcohols Analysis

Samples from the digesters taken at the end of the AD were centrifuged and syringe filtered (0.2 µm) to quantify the accumulated volatile fatty acids (VFAs) and alcohols. A gas chromatograph (GC) Agilent 7890A, (Agilent Technologies LDA UK, Stockport, Cheshire, UK) containing a DB-FFAP column (30 m × 0.32 mm, film thickness of 0.5 µm) and a flame ionisation detector (FID) was used for the analysis. at 200 °C with nitrogen as make-up gas. The autosampler took 10 µL of the filtered sample and injected it into the GC at a 5:1 split ratio. The inlet port operated at 150 °C and used helium at 10 mL/min as the carrier gas. The column oven used increasing temperatures starting at 60 °C for 4 min, heated then to 140 °C with a ramp of 10 °C/min. The final temperature reached 200 °C with a ramp of 40 °C/min, the column was held at 200 °C for 5 min. The volatile acid standard mix (Merck Life Science UK Limited, Gillingham, Dorset, UK) and alcohols made from high purity individual reagents were analysed in parallel as standards. The GC ChemStation software Rev. B.04.03-SP1 (Agilent Technologies LDA UK Limited, Stockport, Cheshire, UK) was used for data acquisition.

2.11. Statistical Analysis

The DOE factorial design, analysis of response variables, models and optimisation were performed with the Minitab 27 software. The effect of the independent variables ISR and BC load over the response variables was performed by analysis of variance (ANOVA) and linear regression, both at a confidence level of $p < 0.05$. The rest of the statistical analyses, including the Gompertz kinetic parameters calculation, and significant tests via ANOVA and Tukey's post hoc test, were performed with the SPSS Statistics 26 software, all at a $p < 0.05$.

3. Results

3.1. Design of Experiments

3.1.1. Anaerobic Digestion

Figure 1 shows the average BMP curves obtained by each condition within the factorial design for the AD of WH. Methane production started on day one for all the conditions. The final BMP yields were found in a range of 165.3–208.9 mL CH_4/g VS, comprising up to 21% of the difference among the conditions. These values were within the reported range of 113–268 mL CH_4/g for untreated or dried WH (Table 3). BMP yields differ among publications, although they agree on the improved biodegradability achieved by implementing pre-treatments. Moreover, the differences in methane yields are influenced by the location, seasonal variation, biomass maturity, pre-treatment, digestion conditions, and the methodology employed for measuring methane and biogas [37]. The WH was added to the digester as a dried substrate, although it has been stated that digesting WH as received without a drying process or that endured a pre-treatment leads to considerable higher biogas generation than WH dried in an oven [38]. These variations make difficult a quantitative comparison with the literature, and therefore considering the role of some of these factors within equivalent experiments could allow the improvement of such understanding.

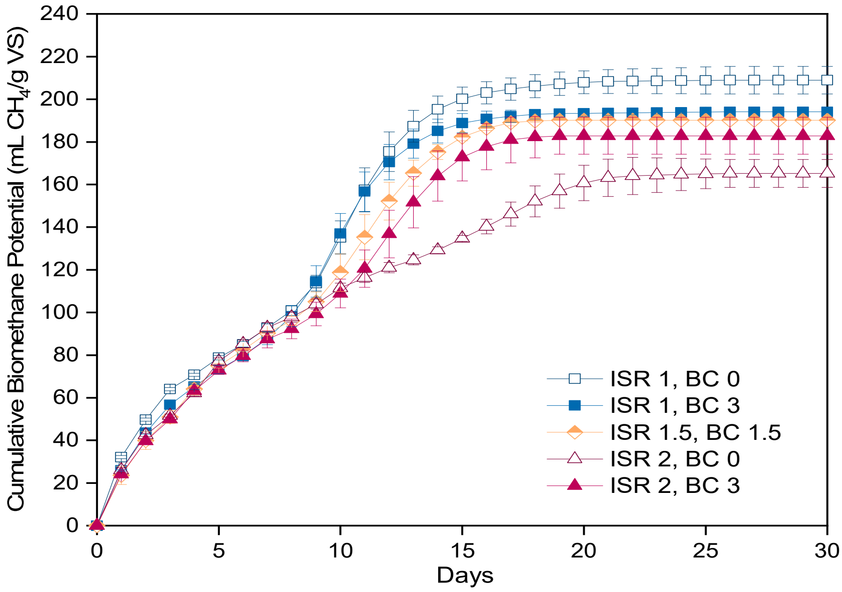

Figure 1. Biomethane production for the full factorial 2^2 experimental design used for the anaerobic digestion of VBU water hyacinth.

The BMP curves indicate that higher BMP yields were obtained at a lower ISR (Figure 1). Accordingly, Bhui et al. [39] evaluated the AD of WH with cow dung at an ISR of 0.25, 0.5 1 and 3. Increasing the ISR reduced biogas generation, with a maximum of 406 mL biogas/g VS (236 mL CH_4/g VS) at an ISR of 0.5. Similarly, Romero de León et al. [40] evaluated the AD of WH and its AcoD with the organic fraction of municipal solid waste (OFMSW) at an ISR of 0.5 and 1 and thermophilic conditions. The AcoD offered a nutrient balance that resulted in considerable higher biogas production, quality, and a more efficient substrate degradation than the mono digestion of WH, although the WH by itself exhibited a balanced C/N ratio of 27. Biogas production for WH (339–350 mL biogas/g VS) and its AcoD with OFMWS (483–486 mL biogas/g VS) was considerably higher at an ISR of 0.5 than 1. Methane yield within the biogas was 65% for both mono and co-digestion of WH, although it slightly reduced for the AD of WH at an ISR of 0.5. The higher yields at an ISR of 0.5 than 1 were attributed to differences in the inoculum composition that could have affected the biodegradability of the substrates. They suggested that the digestion of WH was influenced by the choice of inoculum, ISR and the ISR on a VS basis. Accordingly, De la Rubia et al. [41] evaluated the effect of ISR from various inoculum sources: granular biomass from wastewater reactors treating brewery, granular biomass from sugar beet industries, and a flocculent inoculum municipal sewage sludge digestate. When increasing the ISR, the brewery inoculum enhanced BMP, whereas sugar beet reduced it. Conversely, the digesters fed with sewage sludge were not significantly affected by ISR variations.

Table 3. Summary of literature reports for the anaerobic digestion of water hyacinth.

Substrate	Pre-Treatment	Digester	Inoculum	ISR	Biogas (mL/g VS)	Methane (mL/g VS)	μ_m (mL/g VS·Day)	Ref.
WH	Oven dried	AMPTS 500 mL	AS	1 2		209 165	15 10	This work
WH	Oven dried	AMPTS 500 mL	AS	2		103	11	[37]
WH-HC	HTC 150 °C HTC 200 °C HTC 250 °C					191 185 45	21 46 13	
WH-PW	HTC 150 °C HTC 200 °C HTC 250 °C					213 138 149	44 12 14	
WH slurry (HC- PW)	HTC 150 °C HTC 200 °C HTC 250 °C					202 162 146	32 4 39	
WH	Sundried	Bottle 1000 mL	AS	2	143			[42]
WH	Untreated	SB 125 mL	Compost leachate	0.5 1	350 339	246 268		[40]
WH	Untreated	Bottle 2000 mL	CD	0.5 1 3	406 330 383	235 185 241		[39]
WH	Untreated A (121 °C/ 30 min)	Bottle 5000 mL	CD	1 1		113 150		[43]
WH	Untreated H_2SO_4 5%, 30 m H_2SO_4 5%, 45 m H_2SO_4 5%, 60 m H_2SO_4 5%, 75 m	Bottle 1000 mL	CM digestate	3	183 mL 203 mL 384 mL 424 mL 267 mL	11 33 216 273 85	6.7 mL/d 6.2 mL/d 5.7 mL/d 7.2 mL/d 7.9 mL/d	[44]
WH	Untreated Hot air oven	Bottle 1000 mL	CD	0.5 0.67	1396 mL 1522 mL	143 193	100 mL/d 77 mL/d	[45]
WH *	Oven dried *	SB 200 mL FBR 200 L	Manure	1	292 267	 140	 0.052 d^{-1}	[46]
WH	Oven dried	Bottle 250 mL	Poultry litter	6.25 12.5 18.75 25	360 440 480 410		17 [b] 19 [b] 8 [b] 33 [b]	[47]

HC hydrochar and PW process water obtained from the HTC hydrothermal carbonisation of WH substrate; A autoclaved; SB serum bottle; AS anaerobic sludge; CD cow dung; CM cow manure; FBR flooded bed reactor; * nutrient supplementation; [b] biogas; biogas and methane expressed in mL/g VS, and production rate of methane (μ_m) in mL/g VS·d, unless stated otherwise; empty cells state for not reported values.

Improved AD performance and methane generation at a lower ISR have been previously reported. Barua et al. [45] pre-treated WH by hot air oven, reporting increased solubilisation, and further digested with cow dung at an ISR of 0.4–2. The dried WH reached the highest methane production earlier than the untreated sample, at an ISR of 0.67. They attributed this to the breaking of hydrogen bonds within the lignocellulosic polymers due to the heat, thus facilitating the biodegradability of WH. The untreated WH exhibited a higher BMP yield at an ISR of 0.5, while the hot air oven pre-treated WH reached the highest BMP at an ISR of 0.67. Brown et al. [37] pre-treated WH by hydrothermal carbonisation (HTC), separated the liquid (process water) and solid (hydrochar) by-products, and subsequently digested them separately, mixed them as a slurry (PW + HC), and compared them to the oven-dried WH. The AD of dried WH produced 103 mL CH_4/g VS, whereas the digestion of the HTC products exhibited considerably higher and even double BMP yields. Integrating low-temperature HTC and AD improved the energetic output recovered from WH. The digestion of PW offered the most energetically feasible integration route in comparison to the dried substrate. These observations support the benefits of pre-treating the WH substrates for increasing productivity and energetic recovery.

BC addition favoured BMP performance at an ISR of 2, but the opposite happened at an ISR of 1, where BMP yield was even reduced (Figure 1). Deng et al. [18] assessed the effect of BC on the AD of seaweed wet feedstock. They used similar AD conditions to this work, with a substrate concentration of 5 g VS/L, an ISR of 2 and a BC load of 0.031–1% (w/v). They supported the importance of BC load since the BMP was only enhanced with the addition of BC at 0.031 and 0.062%, whereas higher BC loads led to lower BMP than the control. Further addition of BC inhibited methanogenesis, possibly attributed to substrate sequestration and changes in the diversity of microorganisms. Like this work, they did not observe a buffering effect provided by the BC, although the pH was maintained within an adequate range in both cases.

The potential of biochar amendment during the AD of WH has not been properly assessed, although there is one publication integrating the pyrolysis solid fraction 'BC'. Suthar et al. [48] studied the addition of cow dung BC at loads of 0.5, 1.0 and 1.5%, during the AD of dilute acid-thermal treated WH. BC addition increased biogas production up to 73.5%, with the highest yield at a BC load of 1%. The methane content on the biogas at BC loads of 0.5, 1 and 1.5% increased by 13.2, 19.8 and 9.3%, respectively. However, it is important to remark that cow dung has a large content of organic matter and nutrients, particularly cellulose, fibre, N, K and P. Besides, the pyrolysis of cow dung was performed at a very low temperature (350 °C), which resulted in a BC with extremely low content of fixed carbon (7.4%), and higher volatile matter (30.9%) and ash (58.5%). The carbonisation of biomass is not achieved at low temperatures. At 300–400 °C, the O-alkyl C and carbonyl C structures disappear while the presence of alkyl C structures intensifies. Although it is until 500 °C that the alkyl C structure is further destroyed, resulting in a more aromatic BC [49]. Biomass rich in protein or lipids exhibits a more complex pyrolytic behaviour. Hence, the low degree of carbonisation and an easily biodegradable fraction of the cow dung BC, in addition to the nutrients from the ash fraction, particularly K (63.8 g/kg) and Na (40.5 g/kg) could have been used as substrate and nutrients by the inoculum, which could have contributed to the considerable enhancement of biogas [48]. This can be compared to the moderate enhancement of BMP reported for the addition of woody BCs produced at higher temperatures and thus a higher degree of carbonisation [12,14,50].

The amount of VFAs accumulated in the digestate at the end of the AD experiments was negligible for all experiments (<10 mg/L). The pH was measured at the beginning and the end of the digestion period. The pH of the digesters was not fixed to evaluate the effect of ISR and BC load. Even so, all systems were maintained at a suitable pH initially at 7.6–7.7 and finally at 7.1–7.2. It has been reported that VFAs accumulation continually decreased at increasing ISRs during the AD of WH [39]. Nonetheless, the independent variables ISR and BC load did not affect the accumulation of VFAs and pH of the digester.

3.1.2. Kinetic Parameters

Table 4 shows the values of BMP_{max}, μ_m and λ obtained by fitting experimental BMP for the digestion of VBU-WH into the modified Gompertz model. The BMP_{max} was gradually improved by reducing the ISR. This trend could have been influenced by the greater energy and substrate demand for cellular maintenance and growth exerted by higher inoculum concentrations and in consequence a higher ISR. The trend is less clear for the BC load since at an ISR of 1, higher BC loads improved the BMP_{max}, while the opposite behaviour was observed at an ISR of 2. The lag phase was too short and almost negligible for all systems, showing no apparent trend with the independent variables ISR and BC load. On the other hand, μ_m appeared to be improved by reducing ISR and increasing BC load. The oak wood BC produced at 450 °C and used in these experiments has proved to improve and even double μ_m in previous AD experiments. This BC has a large presence of oxygenated functional groups within redox-active moieties that have been attributed a positive role as electron transfer mediators in DIET interactions [11]. The mediating role of the BC improves the formation of the intermediary organic acids and its further consumption towards methane, which is reflected in the faster production rate. Other publications for the AD of complex substrates have also reported an improvement of μ_m due to BC addition [8,9,51].

Table 4. Average kinetic parameters for the full DOE conditions used for the AD of VBU-WH.

ISR	BC Load (%)	BMP_{Exp} (mL CH_4/g VS)	BMP_{max} (mL CH_4/g VS)	μ_m (mL CH_4/g VS·Day)	λ (Days)	R^2
1	0	208.9	222.8	15.0	0.0	0.974
1	3	194.1	204.4	15.3	0.2	0.978
1.5	1.5	190.2	203.0	13.7	0.2	0.981
2	0	165.3	171.8	10.0	0.0	0.982
2	3	182.9	197.4	12.2	0.0	0.979

ISR inoculum-to-substrate ratio; BC biochar; BMP_{Exp} experimental methane yield; BMP_{max} maximum methane yield; μ_m methane production rate; λ lag phase; R^2 coefficient of determination.

3.1.3. Regression Model Fitting

For analysing the factorial design, the parameters BMP_{Exp}, BMP_{max} and μ_m were designated as the most relevant and were thus selected as the response variables. The acquired regression models were statistically significant ($p < 0.05$) and F-value > F-critical at the 0.05 alpha level as stated in Table 5. The factors and interactions with significant specific coefficients ($p < 0.05$) were maintained within the regression models. Thereby, a satisfactory fitting of the experimental data to the quadratic models was obtained (Table 6). The regression models for BMP_{Exp}, BMP_{max} and μ_m, are outlined in Equations (5)–(7), respectively. The independent variable ISR was significant for all responses, although the negative coefficients suggested that increasing ISR could result in lower kinetic parameters. However, BC load had no significant effect on the responses. Given the significant influence of the ISR*BC interaction over the variables BMP_{Exp} and BMP_{max}, the coefficient BC load was maintained for these regression equations due to the hierarchy principle [36].

$$BMP_{Exp} = 188.3 - 13.7 \times ISR + 0.7 \times BC + 8.1\ ISR \times BC \tag{5}$$

$$BMP_{max} = 199.9 - 14.5 \times ISR + 1.8 \times BC + 11.0 \times ISR \times BC \tag{6}$$

$$\mu_m = 13.2 - 2.0 \times ISR \tag{7}$$

Table 5. Analysis of variance for the factorial regression models for VBU water hyacinth anaerobic digestion.

Analysis of Variance (ANOVA)			
Variable	BMP_{Exp}	BMP_{max}	μ_m
R^2	0.9017	0.8627	0.8243
Adjusted R^2	0.8749	0.8253	0.7763
Prediction R^2	0.7929	0.7166	0.6797
F value	33.64	23.04	17.20
Model p-value	0.000	0.000	0.000

Table 6. Statistical evaluation of the factors and interactions contained in the factorial regression models for VBU water hyacinth anaerobic digestion.

	Coefficient Probability					
	BMP_{Exp}		BMP_{max}		μ_m	
Term	**Coefficient**	***p*-Value**	**Coefficient**	***p*-Value**	**Coefficient**	***p*-Value**
Constant	188.3	0.000	199.9	0.000	13.2	0.000
ISR	−13.7	0.000	−14.5	0.000	−2.0	0.000
BC	0.7	0.667	1.8	0.429	0.6	0.055
ISR*BC	8.1	0.001	11.0	0.001	0.5	0.145

ISR inoculum-to-substrate ratio; BC biochar; BMP_{Exp} experimental methane yield; BMP_{max} maximum methane yield; μ_m methane production rate; linear regression at a confidence level of $p < 0.05$.

Increasing the ISR negatively impacted the response variables, contrary to most reports where higher initial ISR results in faster fermentation and enhanced BMP yields [27,52]. Others state that increasing the ISR within an adequate range offers no difference [53,54]. Finally, other reports state that increasing the ISR reduced BMP yields [39–41]. Holliger et al. [55] advised the use of inoculum with very low endogenous methane production, and low ISRs for the AD of substrates that result in moderate BMP. This advice could be applied to the conditions used for this experiment since the sewage sludge blank exhibited very low endogenous BMP and the AD of water hyacinth offered moderate BMP and BD.

Even though BC addition improved BMP yield and μ_m, especially at an ISR of 2 (Table 4), it was not statistically significant. The coefficient for the interaction of ISR*BC load for the variables BMP_{Exp} and BMP_{max} was considerably high, almost of the order of the individual ISR coefficient. This suggests a correlated effect of both ISR and BC load over BMP yields. Similarly, Cai et al. [14] investigated the effect of BC load and ISR on the AD of food wastes. They observed that BC addition generally improved AD performance, while at an ISR of 2, BC addition had little effect on BMP. By reducing the ISR to 1 and 0.8, BMP performance was drastically improved by BC addition. They also suggested a correlation between the amount of BC and the concentration of inoculum (ISR) for establishing the effectiveness of BC. In addition, they attributed the positive effect of BC to the immobilisation of cells, the promotion of biofilm growth, and the ability to facilitate the DIET process.

3.1.4. Optimisation

Figure 2 shows the contour plots for the graphical interpretation of the variables ISR and BC load over AD performance. All response variables were favoured by ISRs closer to 1. The stretching of the *Y*-axis indicated that maximum BMP_{Exp} and BMP_{max} values can be obtained at BC loads of 0–1.8 and 0–0.5%, respectively, whereas μ_m was favoured at all BC loads and more significantly at the highest loads close to 3%. It can be summarised from these plots that ISR had a major role on the response variables while exhibiting a significant interaction of BC load and the responses BMP_{Exp} and BMP_{max} as stated on the regression models (Table 6). μ_m was mainly benefited by BC addition, particularly at higher loads ~3%. Even though BC addition offered no statistically significant effect on AD performance, the contour plot suggests that BC loads of $\leq$0.5% could favour methane generation. Furthermore, factorial regression optimisation for methane production was carried out using the desirability function by considering the response variables BMP_{EXp}, BMP_{max} and μ_m. The optimum conditions for the AD of WH corresponded to an ISR of 1 and a BC load of 0%. A D-value of 0.88 indicated a satisfactory prediction given that all responses were predicted within acceptable limits.

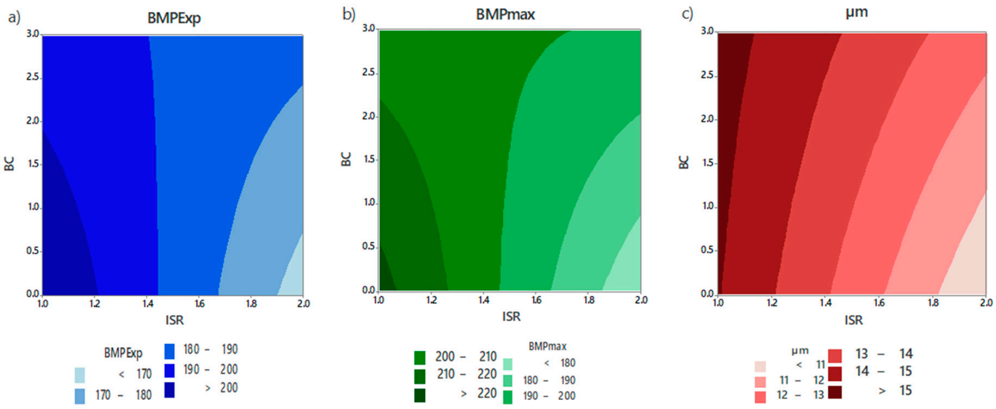

Figure 2. Contour plot for the optimised area of the methane parameters: (**a**) experimental biochemical methane potential (BMP_{Exp}); (**b**) maximum biochemical methane potential (BMP_{max}); (**c**) methane production rate (μ_m).

3.1.5. Effect of Biochar Load

The optimisation with the desirability function established an ISR of 1 and a BC load of 0% as the best digestion conditions. Nonetheless, the contour plots suggested a positive influence of BC loads of ≤0.5% over the BMP and even higher BC loads of ≤3% for μ_m. To corroborate these observations, the further digestion of VBU-WH at an ISR of 1 with the addition of BC at 0, 0.5, 0.75 and 1.0% (*w*/*v*) was performed (Figure 3). The BC load of 0.5% reached slightly higher BMP yields (4%), whereas it considerably improved the production rate. Conversely, the addition of 0.75 and 1% of BC exhibited a detrimental effect on BMP yields. Similarly, Shen et al. [9] added 0.8–1.5% of corn stover BC during the AD of sludge, exhibiting no significant effect on BMP yield, while μ_m was favoured principally at a BC load of 0.8%. Linville et al. [28] reported an analogous behaviour for the AD of food waste amended with fine walnut shell biochar. Small amounts of fine walnut shell biochar FWSB (0.4%) improved AD, while higher doses (0.7%) exhibited an inhibitory effect. Higher BC loads could increase the concentration of potential inhibitory compounds produced during pyrolysis, such as phenols, organic acids from the thermal degradation of the hemicellulose and lignin, polycyclic aromatic hydrocarbons (PAH) resulting from the aromatisation reactions, and pyrazines produced from Maillard reactions [56]. Therefore, it is important to establish a suitable BC load since small doses could facilitate the syntrophic metabolism and AD performance, whereas excessive doses could hinder the digestion of WH.

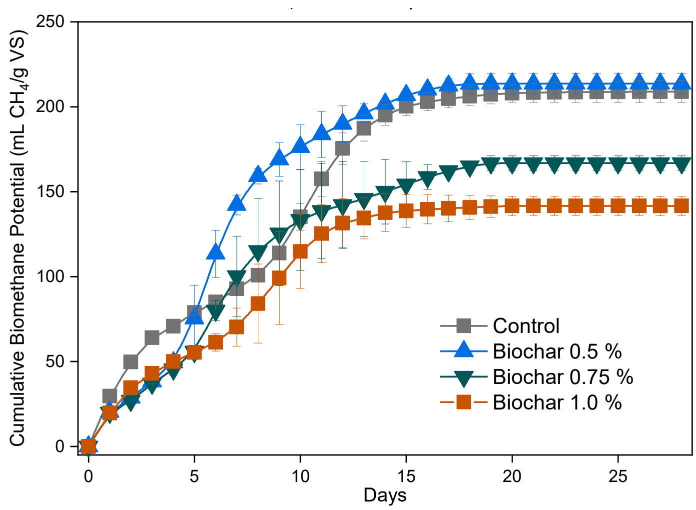

Figure 3. Cumulative biomethane production during anaerobic digestion of VBU-WH augmented with variable concentrations of biochar.

3.2. Anaerobic Digestion of Water Hyacinth Substrates from Different Sources

3.2.1. Biochemical Methane Potential

The further digestion of three additional WH substrates at an ISR of 1 with BC at 0 and 0.5% (w/v) was contrasted to the VBU-WH DOE experiments. All systems exhibited a rapid initial methane generation (Figure 4). The final BMP yields for the controls without BC varied significantly ($p < 0.05$), exhibiting the following order VBU-WH > MM-WH > PV-WH > UG-WH. These differences could be attributed to the chemical and biochemical composition of the WH samples. The highest BMP was achieved by the VBU-WH samples that also exhibited the highest cellulose and hemicellulose content, C/N ratio and BMP_{Th}, and the lowest ash content (Table 1). The cellulosic polymers were easily hydrolysed and converted into methane, whereas increasing the recalcitrant lignin content often restricts the access to the carbohydrates, thus affecting their fermentation [57]. Furthermore, the lowest BMP yields were obtained by digesting the UG-WH. This substrate exhibited an adequate C/N ratio and cellulosic fractions. However, the larger content of protein and oils for UG-WH in comparison to the VBU-WH could have been detrimental to AD. A large protein content would result in more inhibitory ammonia released into the digestate, whereas the slower degradation rate of lipids leads to their accumulation, which is reported to block the mass transfer process for the methanogens [58,59]. The second- and third-best BMP producers were MM-WH and PV-WH, respectively. These two substrates contained less ash and hemicellulose, and more lignin. They also exhibited lower and even imbalanced C/N ratios (10.6–12.3) that could have hindered their digestion. Adequate C/N ratios are found in a range of 15–30, thus lower values often result in a longer retention time, poor methane production or even digester failure [59]. In summary, the best performance for the digestion of VBU-WH in comparison to the others could be attributed to a more accessible, degradable, and balanced composition.

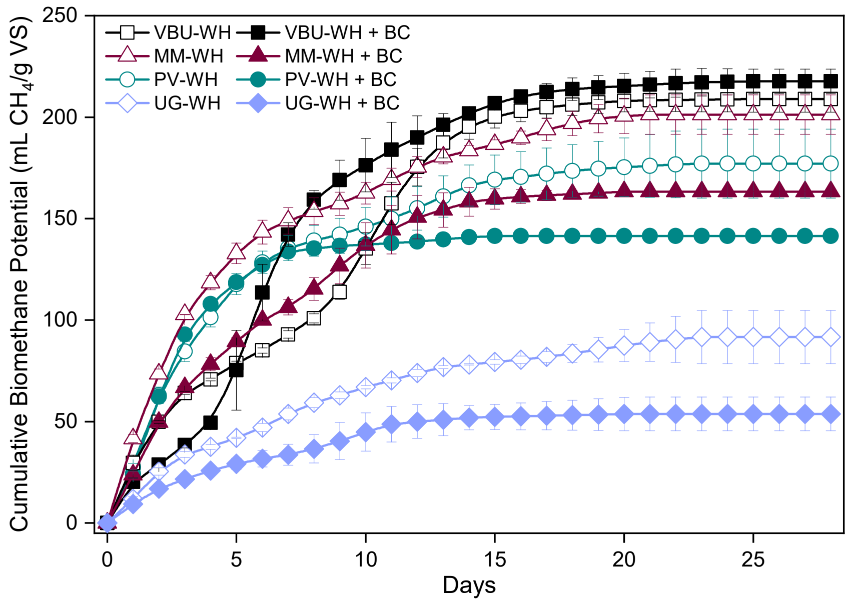

Figure 4. Cumulative biomethane potential during the anaerobic digestion of water hyacinth feedstocks with and without the addition of biochar at 0.5%.

The effect of the oak wood biochar produced at 450 °C over the BMP generated from the four WH substrates differed largely. The addition of 0.5% of BC slightly improved BMP yield for the VBU-WH, whereas the yield of the other three WH substrates was hindered. The addition of this same biochar at a load of 3% has positively improved the digestion of cellulose [11], and the co-digestion of the microalgae *Chlorella vulgaris* and cellulose at low ISR (0.5–0.9). The further optimisation of these co-digestion experiments established an ISR of 2, a BC load of 0.58% and C/N ratio 25 as the most appropriate conditions [26].

Conversely, this study exhibited a mild or even negative impact due to BC addition. This behaviour suggests that the BC effect as an amendment additive in AD is highly influenced by the digested substrate.

3.2.2. Kinetic Parameters

Methane generation started on day one, hence the lag phase was negligible for all systems (Table 7). The addition of biochar 0.5% improved the μ_m for VBU-WH and PV-WH by 1.7- and 1.6-fold the control, respectively, while it reduced the μ_m for MM-WH and UG-WH. The BMP_{max} was generally reduced by BC addition, except for the least producer UG-WH. Nevertheless, BC addition had no significant effect on the kinetic variables ($p > 0.05$).

Table 7. Kinetic parameters calculated with the modified Gompertz model for the anaerobic digestion of water hyacinth feedstocks.

	Experimental		**Gompertz Model**			
	BMP_{exp} (mL CH_4/g VS)	**BD (%)**	**BMP_{max} (mL CH_4/g VS)**	**μ_m (mL CH_4/g VS·Day)**	**λ (Days)**	**R^2**
VBU-WH	208.9	54.5	222.8	15.0	0.0	0.974
VBU-WH + BC 0.5%	217.7	56.8	217.4	24.9	1.5	0.991
VBU-WH + BC 0.75%	173.3	45.2	179.3	17.4	1.0	0.990
VBU-WH + BC 0.1%	141.7	37.0	145.1	13.0	0.4	0.978
MM-WH	201.3	60.7	196.6	20.2	0.0	0.967
MM-WH + BC 0.5%	163.3	49.2	164.6	15.8	0	0.989
PV-WH	177.1	50.4	172.9	19.8	0.0	0.977
PV-WH + BC 0.5%	141.4	40.2	140.5	32.6	0.2	0.995
UG-WH	91.6	26.0	93.4	6.8	0.0	0.987
UG-WH + BC 0.5%	53.7	15.2	54.4	5.0	0	0.983

VBU-WH water hyacinth from Goyal para pond, India; MM-WH water hyacinth from Mula-Mutha river, India; PV-WH water hyacinth from Pavana river, India; UG-WH water hyacinth from Lake Victoria, Uganda; BC biochar; BMP_{Exp} experimental methane yield; BD biodegradability; BMP_{max} maximum methane yield; μ_m methane production rate; λ lag phase; R^2 coefficient of determination.

3.2.3. Biodegradability

The BMP_{Th} yields were calculated based on the chemical composition of the WH substrates (Table 1) by using Boyle's equation and expressed on a dry ash-free (daf) basis to establish the BD of the AD runs. The BMP_{Th} values for VBU-WH, MM-WH, PV-WH and UG-WH were estimated at 383.4, 331.8, 351.3 and 352.6 mL CH_4/g VS, respectively. The BD values ranged from 25.8 to 60.7% (Table 7). The ability to biodegrade a substrate is limited by the complexity, toxicity, and bioavailability of the molecule. Even though MM-WH exhibited the lowest BMP_{Th}, it also showed some of the highest BMP yields, thus the considerably higher BD (59.2–60.7%). BC addition had no statistically significant effect over the BD of WH substrates ($p > 0.05$).

3.2.4. Volatile Fatty Acids and pH

Figure 5a shows the accumulated intermediary alcohols and VFAs measured at the end of the digestion, those mostly found were methanol, acetone, ethanol, and acetic acid. Increasing the BC load resulted in a greater accumulation of VFAs for VBU-WH and MM-WH. These two WH substrates exhibited C/N ratios, 17.8 and 12.3, respectively. This is of relevance since the production and accumulation of VFAs during AD is highly related to the nature of the employed substrate, particularly the C/N ratio [7]. In the case of PV-WH, the addition of BC exhibited a greater acetone and ethanol accumulation, in agreement with the lower BMP yields in comparison to the control. Notwithstanding, the total amount of VFAs was extremely low, below 40 mg/L for all experiments, which could be considered negligible. The pH was measured at the beginning and the end of the digestion period, without pH adjustment. All systems started at a suitable pH of 7.5–7.8 and suffered little variation by the end of the digestion with values ranging from 6.8 to 7.4 (Figure 5b). In summary, the digestion of the different WHs resulted in minimal accumulation of VFAs and pH variation without exhibiting a significant difference due to BC addition.

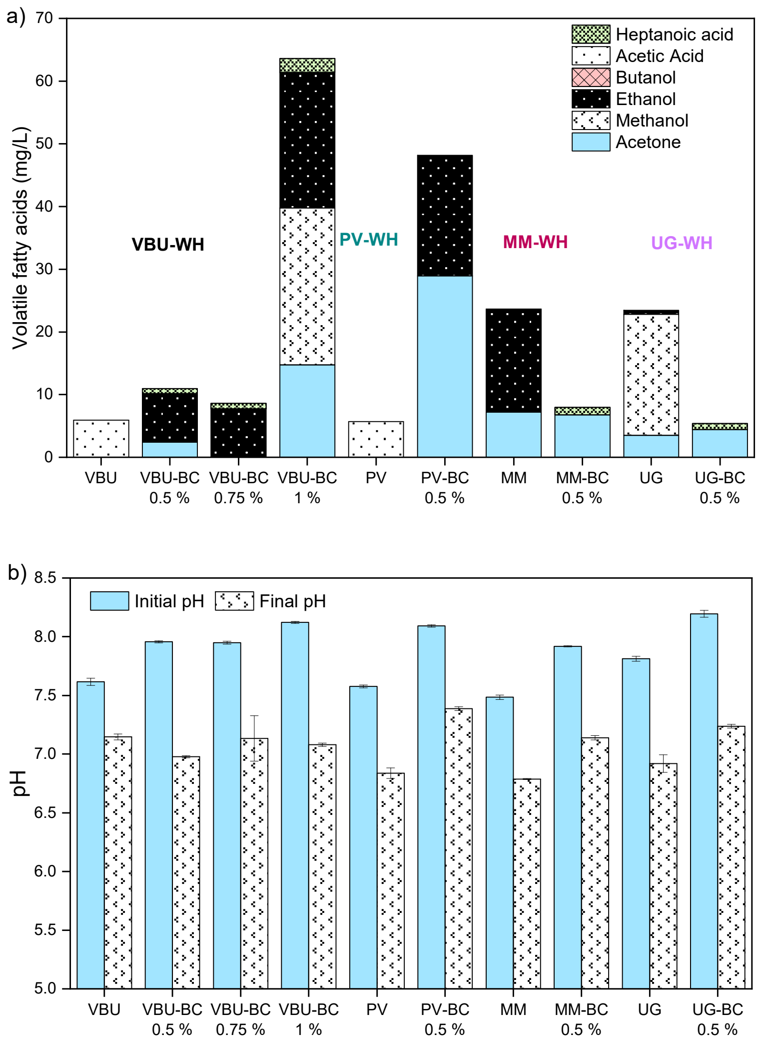

Figure 5. Anaerobic digestion of water hyacinth substrates augmented with biochar: (**a**) VFAs as analysed by GC; (**b**) pH.

Uganda and India are affected by the proliferation of WH while struggling to meet the energy demand. Thus, the integration of thermochemical technologies and AD for the management of WH could provide direct benefits. It would be valuable to evaluate biochar or even charcoal locally produced on the digesters located in rural communities. Additionally, evaluate how the BC influences the quality of the digestate and the microbial communities involved in AD. This approach could improve the understanding of how biochar interacts with the system in real and diverse operation conditions. Moreover, the large-scale AD industry is developed only in a few countries, especially in Europe, hence the sources and availability of inoculum differ among countries. Therefore, it would be interesting to explore inoculums from local and highly available sources in India and Uganda, such as animal dung. This research is ongoing in our laboratories and within an international collaboration, hence this and other outcomes will be reported in future work.

4. Conclusions

The regression models for the AD of WH established the importance of the ISR in the process, suggesting that increasing ISR could result in lower BMP yields and μ_m. The BC load had no significant effect on the production of methane, although it showed a positive interaction with the ISR. Further optimisation stated that an ISR closer to 1 was ideal for the AD of WH, while a BC load of <0.5% could favour μ_m. The AD of WH samples collected from different locations in India and Uganda provided variable BMP yields in agreement with their different biochemical compositions. For these substrates, BC addition had little effect on BMP performance; and in some instances, it even reduced methane generation. Such variations between the WH samples and our previous work suggest that the BC effect is influenced not only by digestion conditions but also by the chosen substrate. Hence, it is necessary to create an understanding of these relationships to establish the best AD conditions for each system of study.

Author Contributions: J.Q.-N.: conceptualisation, investigation, data curation, formal analysis, methodology, project administration, resources, software, validation, visualisation, writing—original draft, and writing—review and editing. A.J.B.: conceptualisation, investigation, project administration, resources, and supervision. L.A.F.: conceptualisation, project administration, and supervision. D.G.B.: data curation and formal analysis. A.B.R.: conceptualisation, funding acquisition, investigation, project administration, resources, supervision, validation, visualisation, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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