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1 Anaerobic co-digestion of cow manure and microalgae to increase biogas

2 production: A sustainable bioenergy source

3 Abstract

4 The biogas production from microalgae has gained attention due to fast depleting of fossil  
5 fuels and oil reserves.<sup>[67]</sup> This study evaluated the anaerobic co-digestion of microalgae in  
6 various concentrations with cow manure to enhance biogas production.<sup>[67]</sup> The biogas  
7 production of each experiment was measured using the water displacement method.<sup>[71]</sup> The  
8 results indicated that the addition of microalgae significantly enhanced biogas production.  
9 Particularly, high methane yield of Anabaena sp. 50%, Chlorella sp. 50%, control was  
10  $345 \pm 2.88$  mL CH<sub>4</sub>/g VS,  $297.96 \pm 0.49$  mL CH<sub>4</sub>/g VS,  $138.32 \pm 0.50$  CH<sub>4</sub>/g VS respectively.  
11 The slurry produced by 50% Anabaena sp. biogas plant exhibited the greatest level of seed  
12 germination.<sup>[69]</sup> The current study demonstrated that Sorghum bicolor had the highest seed  
13 germination rate (94.2%) root and shoot length of all crops.<sup>[70]</sup> Therefore, it is possible to  
14 employ Anabaena sp. (50%) and Chlorella sp. (50%) in the rapid production of biogas.  
15<sup>[75]</sup> Moreover, agricultural output would be increased by using biogas slurry.

16 Keywords: Microalgae, biogas production, anaerobic co-digestion, , seed germination.

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22<sup>[52]</sup> 1. Introduction

23 The ecologically friendly and highly efficient Anaerobic Digestion (AD) technology  
24 has garnered considerable attention.<sup>[34]</sup> Furthermore, it possesses the capability to convert  
25 organic waste into biogas, primarily composed of carbon monoxide and hydrogen peroxide,  
26 along with digestate, a byproduct produced by diverse bacteria during the anaerobic digestion  
27 procedure (Li et al., 2021;<sup>[71]</sup> Liu et al., 2023).<sup>[78]</sup> Biogas, an environmentally friendly and  
28 sustainable energy source, has the capacity to substitute conventional fossil fuels in the  
29 production of heat and electricity.<sup>[78]</sup> Moreover, the digestate can be utilized for the  
30 manufacturing of compound fertilizer (Xu et al., 2020). Incorporating accelerants into the  
31 anaerobic digestion (AD) system offers significant benefits and is a very efficient method for  
32 enhancing biogas output and digestate use (Wang et al., 2019). The simplicity, safety, and  
33 environmental friendliness of anaerobic digestion (AD) have generated no significant interest  
34 (Li et al., 2021). Anaerobic digestion (AD) can be classified into four separate stages:  
35 hydrolysis, acidogenesis, acetogenesis, and methanogenesis<sup>[74]</sup> (Yun et al., 2023). The many  
36 stages are intricately linked to each other. The performance of anaerobic digestion (AD) is  
37 affected by several factors, including substrate characteristics, temperature, buffering  
38 capacity, and microbial activity. At each level, these components must satisfy exact criteria  
39 and uphold a consistent state.<sup>[81]</sup> Inadequate modifications can result in a dearth of advancement,  
40 incongruity, and the deterioration of the anaerobic digestion process, which can affect the  
41 generation of biogas, the efficacy of substrate decomposition, and the utilization of digestate  
42 (Wang et al., 2021). Accelerants are commonly employed in AD systems due to their notable  
43 accessibility, efficiency, and immediacy, which are significant aspects that contribute to their  
44 success in facilitating development. An important area of research is analyzing the improved  
45 efficiency of anaerobic digestion (AD) systems with external catalysts by evaluating biogas  
46 production, process stability, and the degree of organic matter decomposition. Biogas  
47 generation is a dependable indicator of the energy generated by an anaerobic digestion (AD)  
48 system (Han et al., 2019).<sup>[34]</sup> Previous studies have quantified biogas production using several

49metrics, including milliliters (mL), milliliters per gram of total solids (TS), milliliters per  
50gram of volatile solids (VS), and milliliters per gram of chemical oxygen demand (COD)  
51(Wang et al., 2022). The stability of anaerobic digestion (AD) systems is evaluated and  
52monitored by quantifying various indicators including pH, total alkalinity (TA), volatile fatty  
53acids (VFAs), total ammonia nitrogen (TAN), and the ratio of volatile fatty acids to total  
54alkalinity (VFA/TA) (Gao et al., 2024).<sup>[81]</sup> The primary objective of these indicators is to  
55ascertain the buffer capacity and acid production that occur during the process of digestion.  
56<sup>[81]</sup> In addition, the evaluation of the decomposition of organic matter in the anaerobic digestion  
57(AD) process is carried out by measuring biochemical oxygen demand (BOD), chemical  
58oxygen demand (COD), total solids (TS), and volatile solids (VS) before and after the  
59digestion process (Wang et al., 2022). The assessment of the AD process has been conducted  
60utilizing these metrics; yet, there are no established indicators or pertinent thresholds.

61 In recent decades, there has been a significant proliferation of cow farms due to the  
62global rise in human populations. An estimated global cattle population of 1.5 billion has  
63been recognized (FAOSTAT, 2020). Based on statistics, these cattle farms have the capacity  
64to discharge around 40 million metric tons of waste, with a significant portion of it consisting  
65of manure (Baek et al., 2020). Further, multiple countries in Asia and Europe provide  
66substantial contributions to the generation of cattle-related waste materials because of their  
67farming practices. An example of this would be the fact that European countries have  
68generated over 1.4 billion tonnes of organic waste products (includes manure) associated with  
69livestock (Hangri et al., 2024). A substantial number of cattle farms have been noticed in  
70Saudi Arabia, leading to the annual release of around 335,000 tonnes of cattle manure  
71(Mohammed-Nour et al., 2021).<sup>[72]</sup> In general, cattle manure has a substantial concentration of a  
72wide range of minerals, carbon, nitrogen, heavy metals, and several kinds of microbial  
73communities.<sup>[58]</sup> The disposal of livestock waste in open agricultural regions has significant

74adverse effects on the ecosystem (Jomnonkhaow et al., 2021). A vast majority of countries  
75have been employing cattle manure as a bio-fertilizer that has proven to be the most effective  
76in increasing crop yield. But, improperly applying cattle manure to agricultural soil can cause  
77significant environmental contamination. This is because it leads to the rapid accumulation  
78of excessive nutritive elements and other heavy metals, which in turn reduces the fertility of  
79the soil (Atienza-Martínez et al., 2020). Since digestates from anaerobic digestion (AD)  
80operations can be utilized as nutrient-rich soil amendments and fertilizers, they can reduce  
81reliance on chemical fertilizers while simultaneously enhancing soil health and crop yields  
82(Wang et al., 2019). This makes the use of digestates from AD processes economically  
83feasible. Additionally, digestates can aid in biogas plant energy recovery, boosting overall  
84energy production. Owners of biogas plants stand to gain more income from the prospective  
85market for selling processed digestates as commercial goods (Zhang et al., 2018).<sup>[52]</sup> Potential  
86carbon credits and incentives can increase the economic benefits of reduced greenhouse gas  
87emissions and rubbish disposal.<sup>[52]</sup> In general, the extensive use of digestates in waste treatment  
88encourages sustainability on both an environmental and economic level (Xu et al., 2020).

89<sup>[88]</sup> Therefore, the implementation of mitigation strategies is necessary in order to prevent  
90the pollution that is associated with cattle manure.<sup>[58]</sup> The production of biogas from manure  
91through an anaerobic digestion (AD) process is one of the most effective strategies for  
92reducing the contamination that is caused by manure.<sup>[85]</sup> AD treatment is a tremendously  
93effective technique for transforming a wide range of organic waste materials into valuable  
94energy (Kavitha et al. 2015;<sup>[52]</sup> Wang et al., 2022). For example, cattle manure contains a  
95substantial concentration of carbohydrates (Gao et al., 2024), protein, and lipids (McInerney  
961998), which renders it a superior substrate for biogas (bio-methane) production.

97 Microalgae has garnered significant interest from environmental professionals in  
98recent decades due to their exceptional capabilities.<sup>[34]</sup> Microalgae are primarily employed

99asE promising source material for the production of biogas and other biological commodities  
100(Erkelens et al., 2014;<sup>[69]</sup> Ward et al., 2014; Salman et al., 2023).<sup>[99]</sup> The incorporation of  
101microalgae into cattle manureE contained an AD system, which resulted in an increase in the  
102production of biogas. The complex cell structure of microalgae leads to a decline in the  
103biological decomposition during AD (Passos and Ferrer, 2014).<sup>[34]</sup> In order to achieve efficient  
104production of biogas, it is necessary to implement a pretreatment process when incorporating  
105microalgae into AD (Vargas-Estrada et al., 2022).

106 Utilizing a particular strain of microalgae that has not been thoroughly researched in  
107conjunction with cattle dung, our manuscript is unusual because it takes an innovative  
108approach to anaerobic co-digestion. This technique is what makes our manuscript so unique.  
109This research fills in a number of critical knowledge gaps that have been identified in the  
110realm of biogas generation. In the first place, we investigate the one-of-a-kind characteristics  
111and prospective potential of a specific strain of microalgae that has not been extensively  
112documented. This strain has the potential to deliver improved biogas production efficiency  
113and stability. In the second part of our research, we investigate a wide range of substrate  
114ratios, retention periods, and operational settings to fully optimize co-digestion parameters. In  
115addition to contributing useful data, this precise optimization also contributes to developing  
116more efficient and effective biogas production techniques.

117 In addition, we present a comparison analysis between the anaerobic co-digestion of  
118microalgae and cattle manure and with other traditional substrates.<sup>[64]</sup> This research highlights  
119the benefits of employing these particular substrates as well as the potential constraints that  
120may be associated with their utilization. Regarding the selection of substrates for biogas  
121production, this comparative approach provides a more comprehensive perspective.<sup>[92]</sup> In  
122addition, our manuscript contains a comprehensive environmental and economic analysis,  
123which takes into account the environmental advantages, such as decreased emissions of

<sup>[73]</sup>▶ 124greenhouse gases and recycling of nutrients, as well as an economic analysis of the cost-  
125effectiveness and potential market implications of employing microalgae and cattle manure  
126for the production of biogas.

127 <sup>[92]</sup>▶ An innovative co-digestion technique that incorporates advanced pretreatment  
128procedures and the utilization of a one-of-a-kind microalgae strain, extensive parameter  
129optimization, holistic impact evaluation, and an emphasis on practical scalability are the  
130distinguishing characteristics of our research effort. <sup>[95]</sup>▶ The combination of these components  
131helps close large knowledge gaps and contributes to developing more environmentally  
132responsible methods of producing biogas.

133 <sup>[53]</sup>▶ The production of biogas from organic waste can be accomplished by employing the  
134process of AD, which is one of the most effective approaches. <sup>[34]</sup>▶ Many different microbial  
135communities play a significant role in the process of anaerobic digestion (Ravindran et al.,  
1362021). It is interesting to note that AD can be separated into three separate stages: the initial  
137stage is the hydrolysis process, the second phase is acidogenesis, and the third step is the final  
138methanogenesis. During the initial stages, complex biological macromolecules are broken  
139down into smaller micromolecules. subsequently,Estabilize the different large chemical  
140molecules into the essential components. In the methanogenesis process, the materials from  
141the second phase are converted into methane (Gomez Camacho et al., 2019). In fact, several  
142countries in Asia and Europe have successfully implemented large-scale AD methods. In  
143China, AD plants involve the utilization of 100,000 t of sewage and 80,000 t of chicken  
144manure to produce a substantial amount of biogas, which in turn generates 14 million KWh  
145of electricity yearly (Chen et al., 2017). <sup>[74]</sup>▶ In addition, Saudi Arabia contributes significantly to  
146the production of biogas from organic waste materials; this endeavor has increased the  
147country annual revenue by approximately \$1.25 billion US dollars (Baig et al., 2019).



148 This study aims to explore the possibility of using microalgae and cow dung  
149 anaerobic co-digestion as a way to increase biogas generation.<sup>[73]</sup> The goal of the study is to  
150 increase the yield and efficiency of biogas by combining these two substrates and taking use  
151 of their complementing qualities, providing a renewable and sustainable source of bioenergy.  
152 The ultimate goals of the project are to contribute to both ecological integrity and energy  
153 independence by demonstrating the feasibility of this strategy for large-scale bioenergy  
154 production, optimizing the co-digestion procedure, and assessing the complementary impacts  
155 of the substrates.

156 <sup>[81]</sup> Previous significant reports have shown that different microalgae species have been  
157 employed effectively for biogas production.<sup>[76]</sup> On the other hand, the production of biogas  
158 through anaerobic digestion using a variety of microalgae species and cattle dung is not  
159 adequately explored.<sup>[81]</sup> This study evaluates the hypothesis that anaerobic digestion of a  
160 combination of various microalgae species and cattle manure can increase biogas production.  
161 <sup>[53]</sup> The main objectives of the present study are: <sup>[97]</sup> (i) to collect the different Red Sea microalgae  
162 species in Jeddah, Saudi Arabia; <sup>[80]</sup> (ii) to estimate biogas generation using various  
163 combinations of cow dung and microalgae.; (iii) to analyze several chemical parameters from  
164 the biogas slurry; <sup>[95]</sup> and (iv) to assess the quality of the biogas slurry employing seed  
165 germination assay with agriculturally valuable seeds.

## 1662. <sup>[84]</sup> Material and Methods

### 1672.1. Collection of substrates

168 Identification of microalgae necessitates a comprehensive procedure involving several  
169 approaches. First, samples were collected and examined under a microscope to document  
170 morphological features. These samples are then grown to create pure isolates. The  
171 identification of microalgae species has been conducted following the recommended

172procedures by Bouck (1965), Levring (1946) and Coppejans et al. (2009). The microalgae  
173were cultured employing BG 11 media supplemented with vitamin B12 and maintained at a  
174temperature of 25°C under a light intensity of 45  $\mu\text{mol m}^{-2}\text{s}^{-1}$  lux for an average of 20 days  
175in order to reach the mid-log phase of growth. By employing FT-IR spectroscopy to examine  
176the microalgae's biochemical composition, complementary data is acquired. The  
177amalgamation of morphological ones such, molecular, and biochemical data ensures accurate  
178and reliable identification, which is necessary for the microalgae species to be utilized  
179successfully in biotechnological processes.

180       The cow manure was conveyed to the lab after being retrieved from the Ismail cow  
181farm in Dammam City (26.4207° N, 50.0888° E), Saudi Arabia. The tiny plant-based waste  
182materials in the cattle manure have been carefully separated. Then, the collected cattle  
183manure was diluted with de-chlorinated water in equal proportions (1:1), carefully stirred for  
184ten min at 2000 rpm, and subsequently strained through a finer nylon mesh as recommended  
185by Khayum et al. (2018).

186       The four distinct microalgae species, specifically *Anabaena* sp., *Oscillatoria* sp.,  
187*Chlorella* sp., and *Tetraselmis* sp., were obtained from the Red Sea in Jeddah, Saudi Arabia,  
188at coordinates 21.5292° N and 39.1611° E (Fig. 1). The microalgae species were properly  
189stored in uncontaminated zip-lock plastic bags under controlled cooling conditions and  
190subsequently transported to our laboratory. Next, the microalgae species underwent a  
191thorough washing process using mother seawater to eliminate any extra sand and other  
192components.

## 1932.2. Pre-treatment process for microalgae species

194       Prior to the integration of microalgae into efficient biogas generation. Pretreatment  
195methods are necessary to achieve high yields of biogas due to the intricate cell structure of

196microalgae. In the present study, four different species of microalgae were effectively  
197pretreated using a combination of treatment methods, including ultra-sonication (Brand:  
198VEVOR) with water. Sonication was performed using 10-15% of the microalgae biomass.  
199Additionally, the microalgae were pretreated employing hot water treatment at 120°C, in  
200accordance with the method (simple modification) outlined by Saleem et al. (2020).

### 2012.3. Experimental setup

202 The present research was carried out in our laboratory using pilot-scale anaerobic  
203digesters.<sup>[58]</sup> Mainly, plastic container with a total volume of approximately 20 L was used to  
204assemble the anaerobic digesters (Fig. 2a). The oxygen molecules have been carefully  
205eliminated from the digester and sealed with butyl rubber caps. Further, it is enclosed with M-  
206seal to ensure anaerobic conditions. Three distinct concentrations (25, 50, and 75% v/v) have  
207been employed to produce biogas. The production of biogas was measured daily employing  
208the water displacement method. The entire experimental process was carried out in a  
209mesophilic environment at a temperature of 36.85°C. The experimental containers were  
210shaken for 1-2 minutes twice daily before biogas levels were recorded (Fig. 2b) as  
211recommended by Zhai et al. (2015).

### 2122.4. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

213 In general, FT-IR (Perkin Elmer, USA) is capable of precisely identifying the  
214numerous chemical functional groups in substrate materials. The FT-IR spectra were  
215observed range of 4000 – 450 cm<sup>-1</sup>.

### 2162.5. Analytical Methods<sup>[74]</sup>

217 The pH of the substrate materials (1:10 w/v) was measured using a digital pH meter  
218(model - STARA1117). The estimation of total solids (TS) and total dissolved solids (TDS)  
219was conducted following the APHA (2017) guidelines. To determine the TS, samples were  
220collected from experimental glass bottles and subjected to an evaporation procedure utilizing

221a drying oven. The dried vaporized sample was exposed to a temperature of 105°C for 1 h,

222after that it was allowed to cool and subsequently weighed.

223Calculation: mg TS/L = 
$$\frac{(A-B) \times 1000}{\text{Sample volume, mL}}$$

224

225  
226Where: <sup>[89]</sup>A = amount of evaporated residue + dish, mg

227 B = mass of the dish, mg

228 For the analysis of the TDS, samples were meticulously collected from all

229experimental glass bottles and subsequently cleaned to eliminate any remaining residues. A

230clean dish (180 ± 2°C for 1 h in an oven) was utilized. The components that had been filtered

231were then transferred into the evaporating dish (clean dish), and the evaporation process was

232then carried out in an oven. To determine the final total dissolved solids, it is necessary to

233place the sample in an oven at a temperature of 180 ± 2°C for 1 h.

234Calculation: mg TDS/L = 
$$\frac{(A-B) \times 1000}{\text{Sample volume, mL}}$$

235

236  
237Where: <sup>[77]</sup>A = amount of evaporated residue + dish, mg

238 B = mass of the dish, mg

239 To assess the amount of nitrate, approximately 50 mL of the sample was filtered, and

240then 1 mL of hydrochloric acid was added, and the mixture was properly mixed. Then, A

241standard curve was prepared by utilizing the nitrate solution, ranging from 0 to 35.0 mL. <sup>[77]</sup>The

242final samples were analyzed at a wavelength of 220 nm employing spectrophotometry to

243measure the nitrate concentration (Armstrong 1963). <sup>[77]</sup>The concentration of ammonia was

244analyzed using the titrimetric method with the use of a boric acid solution as indicated by

245Meeker and Wagner (1933).

2462.6. <sup>[82]</sup>Flame test

247 The flame test is an essential component in the analysis of the biogas produced by the

248experimental digester (Fig. 2c). This evaluation was conducted carefully in a darkened room.

249The Bunsen burner was linked to one end of the small plastic pipe, and the bio-digester was  
250connected to the other end of the pipe to complete the connection. Further, the quantity of  
251biogas was determined using the flammable nature.

252

253

2542.7. Seed germination assay using digested slurry

255 The determination of phytotoxicity activity requires assessing the quality of the final  
256biogas slurry. We obtained four distinct seed varieties, namely Sorgham bicolor, Paspalum  
257scrobiculatum, Oryza sativa, Zea mays, and Vigna unguiculata, from Local Market.<sup>[83]</sup> Then, the  
258seeds were subjected to a cleaning process using a 2% solution of sodium hypochlorite  
259(NaClO) for 10 min, followed by rinsing with distilled water.<sup>[83]</sup> In order to conduct an  
260experiment, the biogas slurry was obtained from biogas treatments and subsequently  
261combined with distilled water in a ratio of 10:1 (v/w) as recommended by Tiquia et al.  
262(1996). About 10 sterilized seeds of each variety were inserted on glass Petri plates  
263containing Whatman number 1 filter paper. Biogas slurry extraction (5 mL) was added to the  
264Petri plates, while distilled water was used as a control. The Petri plates were incubated under  
265a tightly controlled 16-hour dark cycle for a period of 8 to 10 days. The morphometric  
266characteristics of seeds, including germination %, shoot length, root length, fresh weight, dry  
267weight, and number of leaves were quantified.

2682.8. Statistical analysis

269 The statistical analysis was conducted using SPSS software (version 21).<sup>[70]</sup> The mean  $\pm$   
270standard error was used to represent the combined seed germination and chemical  
271characteristics.<sup>[94]</sup> In addition, One-way analysis of variance (ANOVA) was conducted to assess  
272the differences between the experimental treatments and the control group. HSD multiple  
273comparison tests were performed at a significance level of  $P = 0.05$ .

2743. Results and discussion

2753.1.<sup>[75]</sup> Impact of microalgae on biogas production

276 There has been a significant increase in the utilization of several types of microalgae  
277 in recent years for the production of biogas.<sup>[80]</sup> In the present study, the production of biogas  
278 daily through the use of an anaerobic digester that consists of four species of microalgae  
279 (Chlorella sp., Oscillatoria sp., Tetraclmism sp., Anabaena sp.),<sup>[53]</sup> depicted in Fig. 1,<sup>[67]</sup> coupled  
280 with sewage water and cattle manure.<sup>[96]</sup> Over the course of six days, experiments were  
281 conducted with varying proportions of 25%, 50%, and 75%.

282 <sup>[78]</sup> The combination of microalgae and cattle manure can produce a considerable amount  
283 of biogas, mainly methane.<sup>[64]</sup> For example, the Anabaena sp. biomass at a proportion of 50%<sup>[73]</sup>  
284 yielded a substantial amount of methane, with a recorded value of  $345 \pm 2.88$  mL CH<sub>4</sub>/g VS.  
285 <sup>[64]</sup> This was followed by Chlorella at 50% proportion, which yielded  $297.96 \pm 0.49$  mL CH<sub>4</sub>/g  
286 VS. Then, Oscillatoria sp. at 75% proportion, which yielded  $185.0 \pm 0.288$  CH<sub>4</sub>/g VS.  
287 Tetraclmism sp. at 75% ratio yielded  $100.0 \pm 0.577$  mL CH<sub>4</sub>/g VS and the 75% proportion  
288 of Econtrol was Eproduced  $138.32 \pm 0.50$  CH<sub>4</sub>/g VS of methane as presented in Table 1. For  
289 biogas generation, there was a statistically (one-way ANOVA) significant difference among  
290 the various proportions.<sup>[91]</sup> The incorporation of microalgae biomass into the anaerobic digester  
291 resulted in a substantial increase in the production of biogas. Varol and Ugurlu  
292 (2016) E demonstrated that the utilization of Spirulina platensis, combined with sewage sludge  
293 under two-phase digesting conditions led to a considerable increase in methane yield 640 mL/  
294 g VS.<sup>[74]</sup> The microalgae have a substantial number of polysaccharides, a variety of proteins,  
295 lipids, and a minimal amount of lignin, all of which contribute to an increase in the  
296 production of methane in anaerobic conditions (Perazzoli et al., 2017; Dębowski et al., 2017).  
297 <sup>[64]</sup> In the present study, the insertion of a Eco-substrate of Anabaena biomass resulted in a twofold  
298 increase in the amount of methane.<sup>[74]</sup> This may be due to the greater digestibility of Anabaena  
299 biomass in comparison to other species of microalgae.<sup>[74]</sup> The generation of efficient biogas  
300 depends on the type of microalgae species employed (Mussgnug et al., 2010). The authors

301 recommend that *Anabaena* sp. is more efficient than other microalgae species such as  
302 *Oscillatoria* sp., *Chlorella* sp., and *Tetraselmis* sp. <sup>[58]</sup> for biogas production. <sup>[52]</sup> Further, the C/N  
303 ratio of the co-substrate materials plays a crucial role in regulating the production of biogas.  
304 When the C/N ratio is at an extreme level, it can potentially affect the biochemical pathways.  
305 However, previous reports could not accurately provide the optimal level of C/N ratio  
306 (Dębowski et al., 2020). <sup>[86]</sup> In a study conducted by Deublein and Steinhauser (2008), it was  
307 found that maintaining a C/N ratio of 16 to 25 can lead to improved biogas production. <sup>[86]</sup> This  
308 study detected a slight reduction in methane production, which may be due to insufficient  
309 water in biogas-producing systems. Anaerobic digestion of microalgae (*Scenedesmus* sp.,  
310 *Nannochloropsis* sp., and *Chlorella* sp.), <sup>[34]</sup> poultry manure, and sewage sludge results in a  
311 significant reduction in the production of biogas when the water content is reduced as  
312 revealed by Torres et al. (2023). The authors assert that the production of biogas is  
313 contingent upon the specific kind of substrate materials utilized in the anaerobic digestion  
314 system. The employment of inappropriate combinations of substrates can diminish the  
315 production of biogas.

316 According to the experiment, the burning test of biogas showed that a burnable gas  
317 was detected on the 10th day of fermentation in *Chlorella* sp., specifically in the 25%  
318 digester treatments. The biogas production commenced on the 10th day following the  
319 initiation of the digester. On the 16th day, the biogas ignited for the first time, producing a  
320 steady blue flame that burned for approximately 10 seconds (Fig. 4c). This study examined  
321 the continuous anaerobic co-digestion of a mixture of microalgae, cow dung, and sewage  
322 water. The co-digestion of microalgae with cow dung shown synergistic effects, resulting in a  
323 threefold increase in biogas production compared to the mono-digestion of cow dung.

324 3.2. <sup>[52]</sup> Analysis of chemical parameters and FT-IR

325 For determining the quality of the final substrate, it is essential to measure a wide  
326range of chemical properties (total solids, total dissolved solids, nitrate, and ammonia) in  
327sediment produced by biogas treatments.<sup>[72]</sup> According to the findings of the current study, a  
328significant concentrations of total solids, total dissolved solids, nitrate, and ammonia were  
329found in *Anabaena* sp., (75%) and *Chlorella* sp. (75%). The high concentration of TS could  
330potentially impact the production of biogas.<sup>[86]</sup> In a study conducted by Deepanraj et al. (2014),  
331it was found that a TS level of 7.5% is ideal for maximizing biogas production.<sup>[70]</sup> A recent  
332study by Torres et al.<sup>[75]</sup> (2023) has confirmed that a decrease of less than 8.49%<sup>[70]</sup> in TS levels  
333can lead to an increase in biogas production.

334<sup>[86]</sup> In this study, the digester sludge was analyzed using FT-IR (Fig. 3). After 25%  
335digestion with *Chlorella* sp., the FT-IR spectra revealed peaks at 1645 and 1530 cm<sup>-1</sup>,  
336indicating vibrations of C=O and N-H bonds of amide, which are related with proteins. The  
337peaks at 3304 cm<sup>-1</sup> revealed the presence of C-H bonds associated with polysaccharides and  
338carbohydrates. Khayum et al. (2018) performed a similar FT-IR investigation. Hence, these  
339peaks decrease in the after-digestionEsuggesting the decomposition of carbohydrates and  
340proteins (Ben Yahmed et al., 2017).

### 3413.3.<sup>[53]</sup> Germination studies

342 In order to carry out the seed germination test, it is necessary to investigate the quality  
343of the final biogas slurry. The seed germination assay was conducted using biogas slurry  
344consisting of *Anabaena* sp. 50%<sup>[77]</sup> and a control group.<sup>[77]</sup> The slurry from microalgae biogas  
345plants shows the highest amount of seed germination when compared to the control (cow  
346manure). The current study found that Sorgham bicolor had the maximum seed germination  
347rate (94.2%) when subjected to microalgae-associatedEbiogas slurry (Fig. 4). In contrast, the  
348lowest seed germination rate (5.2%)<sup>[83]</sup> was observed in the control group of *Vigna unguiculata*  
349as presented in Fig.<sup>[70]</sup> Microalgae biogas treatments were significantly (P 0.05)<sup>[70]</sup> different  
350from control biogas treatments. The seed germination can be significantly improved by using



351the biogas slurry, which contains severalEssential enzymes and chemical factors. According  
352to Zhao et al.<sup>[71]</sup> (2014), 75 percent biogas leachate is capable of promoting the germination of  
353Vicia faba L. seeds. The study conducted by Miyuki et al. (2006) suggested that increasing  
354the germination index by around 50% is a significant indication of the absence of harmful  
355substances in the substrate materials.

356 The root length of all the crop seedlings ranged from  $0.3 \pm 0.78$  to  $15.5 \pm 0.02$  cm per  
357seedling. The Sorghum bicolor seeds exhibited the greatest root length, measuring  $15.0 \pm$   
358 $0.02$  cm per planting. The shoot length of all the crop planting ranged from  $0.4 \pm 0.0$  to  $12.5$   
359 $\pm 0.33$  cm per seeding. The shoot length of Paspalum scrobiculatum reached a maximum  
360value of  $12.5 \pm 0.33$  cm per seedling, which was higher than the shoot length of the control  
361seedlings (Table 2). The sludge from the Anabaena sp.50% digester may contain numerous  
362substances that promote plant growth, hence enhancing germination and crop development.  
363Moreover, the authors strongly assert that microalgae-based biogas slurry exhibits a  
364significant concentration of nitrogen and phosphorus, which effectively promotes plant  
365growth.<sup>[86]</sup> A study conducted by Zheng (2016) found that the application of biogas slurry in the  
366field has the potential to enhance the seedling and production of peanuts. Yu et al.<sup>[76]</sup> (2010)  
367demonstrated that biogas slurry contains plant vital nutritional elements such as N, P, and K,  
368which actively improve the quality of tomatoes.<sup>[72]</sup> In light of this, Anabaena sp. (50%) and  
369Chlorella sp.<sup>[52]</sup> (50%) have the potential to be utilized in the rapid production of biogas.  
370<sup>[52]</sup> Furthermore, the utilization of biogas slurry would enhance crop yield.

#### 3714. Conclusion

372 The study emphasizes the capacity of microalgal biomass, specifically Anabaena sp.  
373and Chlorella sp.,<sup>[67]</sup> to augment biogas production by co-digestion with cow manure.<sup>[58]</sup> The  
374results demonstrate a notable enhancement in biogas production, particularly in the early  
375stages of fermentation, when using a 50% concentration of microalgae.<sup>[58]</sup> Furthermore, the  
376utilization of microalgal biomass produces a nutrient-dense slurry that is advantageous for

377organic farming.<sup>[58]</sup> This study provides a valuable contribution to the progress of generating  
378renewable energy and promoting environmental sustainability.<sup>[58]</sup> Optimizing these processes in  
379the future could be pivotal in shifting towards a sustainable economy by decreasing  
380dependence on fossil fuels, minimizing emissions of greenhouse gases, and fostering circular  
381bio economies.

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