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Original Article

Goat manure extracts as a surrogate medium for culturing *Chlorella sorokiniana*

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Abstract

Chlorella sp. has garnered attention as a sustainable feedstock to produce bioactive compounds with potential applications in energy, pharmaceuticals, and agricultural sectors. The commercial viability and sector expansion require a streamlined cultivation process. The chemical nutrient media utilized remain a bottleneck and contribute appreciably to the downstream costs. The study aimed to develop a cheaper and environmentally friendly technique for culturing *Chlorella sorokiniana* UTEX 1230 using goat manure waste. The nitrified air-dried goat manure was aerobically fermented with efficient microbes to augment the nutrient bioavailability. The biomass concentration, biomass productivity, and specific growth rate of *C. sorokiniana* UTEX 1230 were significantly higher in GME10% compared to commercial M8 medium. Moreover, the doubling time was significantly lower for GME10% medium compared to the commercial M8. Carbohydrates were found to be higher in commercial M8, whereas protein content was higher in GME10%. The carbohydrate and protein content showed a clear association with nitrogen concentration in the media, which confirms the role of nitrogen in the synthesis of carbohydrates and proteins in microalgae. These findings provide an insight into the possibility of using animal wastes such as goat manure as a surrogate culturing medium for *C. sorokiniana*. The GME medium is environmentally friendly, as it enhances sustainability by recycling nutrients and reducing the nutrient input costs associated with microalgae biotechnology.

Keywords: Goat manure extract, Nitrogen, Microalgae, Carbohydrates, Protein

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Introduction

The "Green Economy" aims to transition high-energy and chemical input industries into sustainable transformation and efficient resource management (Sutherland et al., 2021). Microalgae remain at the forefront of sustainable technologies due to numerous uses such as medicinal (Khavari et al., 2021), agriculture (Zhang et al., 2024), food (Kusmayadi et al., 2021), and energy (Alishah Aratboni et al., 2019). *Chlorella sorokiniana* is receiving increasing attention due to its high concentration of protein, lipids, carbohydrates, essential elements such as nitrogen and phosphorus, and essential fatty acids such as omega-3 and omega-6 (Lizzul et al., 2018; Tsamesidis et al., 2024). *Chlorella sorokiniana* has been used as an additive in fish feeds to enhance growth performance (Liu et al., 2024) and in energy industries for biofuel production (Faried et al., 2023). Moreover, *Chlorella* spp. were used as bio-stimulants to enhance plant growth (El-Naggar et al., 2020; Popa et al., 2025). The exogenous application of crude extract was found to stimulate seed germination, development, and yields in agriculture (Tian et al., 2022).

Projections indicate that the microalgae industry valuation will exceed 4.6 billion USD by 2027. The commercial application of the functional bio-compounds present within the biomass remains focal to this industry's growth. Moreover, the composition of the growth medium determines the synthesis of these compounds (Abdel-Raouf et al., 2012). According to Patil et al. (2008), large-scale production facilities utilize chemical media to maximize growth and production. However, providing nutrients on such a scale presents an economic challenge that affects the downstream bio-compound processes (Rahardini et al., 2018). The nutrient cost associated with the production of a ton of microalgae biomass in an open pond cultivation system equates to $103, with culturing mediums contributing 10-30% of the total production cost (Yu et al., 2015; Ullmann and Grimm, 2021). Reducing culturing costs by recycling the culture media remains ineffective and not environmentally friendly (Manninen et al., 2016; Chakraborty et al., 2023), and it also presents the risk of pathological infection (Manninen et al., 2016). Moreover, the use of nutrient-rich wastewater and organic fertilizers remains unsustainable due to limited nutrient availability (Clarens et al., 2010; Blair et al., 2014; Fabris et al., 2020).

Therefore, developing cost-effective nutrient strategies is vital to optimizing the economics and sustainability of the cultivation process. Goat manure was found to contain high nutrient levels compared to dairy, chicken, and swine manure (Zhang et al., 2013). According to Sopandi et al. (2020), goat manure provides a feedstock comprising a balanced nutrient profile that fulfils the nutrient requirements for microalgae. The integration of goat manure extract into the medium has the potential to reduce the operating cost of microalgae biotechnology and mitigate pollution of the bio-constituents. The present study aims to explore the possible use of goat manure to develop a culturing medium for the growth of *C. sorokiniana.* It was hypothesised that goat manure-based culturing medium could be a good substitute for the synthetic modified medium for growing *C. sorokiniana*.

Material and Methods

Study design

The study investigated the potential of goat manure extract (GME) to substitute the commercial medium (M8) for growing *C. sorokiniana*. Four treatments were explored for this study. The M8 medium was used as a control (T1), whereas the GME was divided into three treatments, i.e., GME1% (T2), GME5% (T3), and GME10% (T4). A sample size of 10 was used for each treatment.

Experimental material

Chemicals were purchased from Lichro Chemicals and Laboratory Supplies (KwaZulu-Natal, South Africa) and were of analytical grade. Grass-fed goat manure obtained from Eston Goat Farm (-29.87, 30.50, KwaZulu-Natal) was stored for 30 days in open storage. The efficient microbe solution was purchased from Efficient Microbes (South Africa) (Registration Number: B4336) and comprised *Bacillus subtilis, Bifidobacterium animalis, Bi. bifidum, Bi. longum, Lactobacillus acidophilus, L. buchneri, L. bulgaricus, L. casei, L. delbrueckii, L. fermentum, L. plantarum, Lactococcus diacetylactis, Lactococcus lactis, Rhodopseudomonas palustris, R. sphaeroides, Saccharomyces cerevisiae*, and *Streptococcus thermophilus.* The M8 medium was comprised of potassium nitrate (0.75 g/l), potassium dihydrogen phosphate (0.185 g/l), monosodium phosphate (0.065 g/l), Calcium chloride dihydrate (0.003 g/l), ferrous

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sulfate heptahydrate (0.03 g/l), magnesium sulfate heptahydrate (0.1 g/l), and ferric sodium EDTA (0.01 g/l).

Formulation of culture medium

The goat manure (50 g) (Figure 1a) was treated with 100 mL of 2% nitric acid (HNO_3), stirred for 1 hour, and incubated at room temperature for 24 hours (Muhammad Syahren and Wong, 2008). The nitrated goat manure was vacuum-filtered (Rocker 300-LF 31 Vacuum filtration system), washed twice with distilled water, and oven dried for 24 hours at 30°C. The nitrified goat manure (1 g/l) was inoculated with an efficient microbe (EM) (5 mL) and glucose (125 mg). The mixture was placed on an orbital shaker (Labotech OrbiShake Platform Shaker) (120rpm) and incubated at room temperature (24°C ± 1) for 25 days to create a bacterial seed culture. The seed culture was added to 6 litres of distilled water. The goat manure (0.25 g/l) was added daily to the mixture for 30 days. The culture was incubated at room temperature with pH maintained at 7.5 for 50 days to allow for mineralization of nutrients from the substrate. The goat manure extract (GME) was filtered through a muslin cloth to remove large debris and vacuum filtered on a Whatman No.1 filter paper (Figure 1b). The digestion for nutrient analysis was performed following Ngigi and Muraguri (2019), using 5% HNO_3 and 32% hydrogen peroxide (H_2O_2). The analysis was carried out using inductively coupled plasma optical emissions spectrophotometry (ICP-OES) (Thermo Jarrell Ash IRIS Advantage ICP-OES) (Santos et al., 2012; Mowa et al., 2017). Nitrogen content was assessed as per Dumas's method.

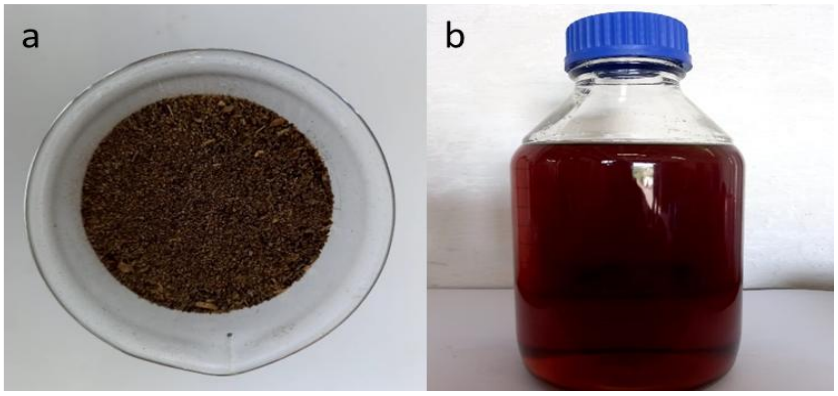

Figure-1. An alternative culturing medium for microalgae derived from goat manure. a. illustrates air-dried goat manure exposed to nitration treatment. b. The medium extract obtained from aerobic fermentation of nitrified goat manure with an efficient microbe solution.

Table-1. Nutrient profile of goat manure culture medium after nitration and aerobic fermentation.

Nutrients	Concentrations (g/l)
N	23.2
P	20.6
K	10.9
Mg	0.1
Ca	1.1
S	0.7
Mn	0.003
Fe	0.575
Zn	0.001
Cu	0.001

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Culturing conditions

Chlorella sorokiniana UTEX 1230, obtained from the School of Life Science (University of Kwa-Zulu-Natal, Westville), had a density of 8.36 x 10^6 cells/ml and a biomass weight of 1.19 g L^{-1}. Microalgae cells (50 mL) were cultured in 500 mL of the culture medium (Figure 2). A modified M8 culture medium was utilized as a control for the experiment (T1). The goat manure extract as a culture medium was evaluated in a series of ratios, i.e., T2: GME1%; T3: GME5%; T4: GME10%. Culture vessels were illuminated with a photoperiod of 16:8 (80 µE m^{-2} s^{-1}; Osram, Germany). Temperature and pH were maintained at 28 ± 1.00°C and 6.8, respectively. The culturing duration was 14 days, with each treatment triplicated.

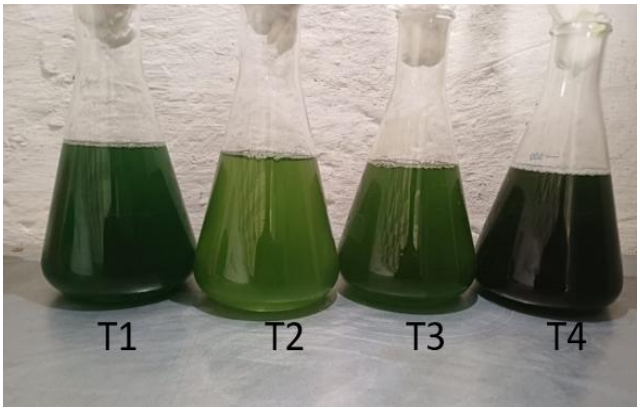

Figure-2. The growth of *Chlorella sorokiniana* UTEX 1230 in different culturing media. T1: Control (M8 medium); T2: GME1%; T3: GME5%; T4: GME10%.

Growth kinetics

Cell density was measured by spectrophotometry ($O.D_{680}$) (Shimadzu UV spectrophotometry [UV-1800], Japan). Cells were harvested at the end of the culturing period by centrifuging 50 ml of the culture solution at 3220 g for 10 minutes at 4°C. Cells were washed twice and placed on a pre-weighed Whatman No.1 filter. Total carbohydrate and protein content were calculated following the phenol-sulfuric method and nitrogen-protein conversion factor (Safi et al., 2013; Tamboli et al., 2020). The biomass productivity (BP) was calculated according to Kumaran et al. (2016), using equation 1.

$$P = \frac{\ln(X2 - X1)}{CultivationTime} \quad (1)$$

where, P is the productivity in g/l Day^{-1}, X2 is the final and X1 is the initial biomass concentration in g/l.

The specific growth rate (SGR) of *Chlorella sorokiniana* UTEX 1230 in the fertilizer medium was calculated according to Wu et al. (2001), using equation 2.

$$\mu = \frac{X2 - X1}{T2 - T1} \quad (2)$$

where µ is the specific growth rate per day, X2 is the final, and X1 is the initial biomass concentration in g/l, and T2 is the final and T1 is the initial culturing time. The doubling time (DT) was calculated following Ranjith et al. (2013), using equation 3.

$$T_2 = \frac{\ln(2)}{\mu} \quad (3)$$

Statistical analysis

Statistical analyses were carried out using R version 4.3.1. The regression analysis of cell density increase with days was carried out using the 'gls' function in the 'nlme' package (Bates et al., 2015), whereas slope comparison was performed using the 'lstrends' function in the 'lsmeans' package (Lenth and Lenth, 2018). Analysis of variance (ANOVA) was used to compare the biomass, biomass productivity, specific growth rate, and doubling time between treatments. The distribution of residuals and homoscedasticity of variance were evaluated using the Shapiro-Wilk and Levene's test, respectively. The post hoc tests were carried out for significant results using the TukeyHSD function. The results were considered significant at $p<0.05$.

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Results

Cell density

The cell density showed an increasing trend from day 1 to day 14, with the slopes showing a significant difference across treatments ($F = 1.87$, $p < 0.001$) (Figure 2). The T2 slope was significantly lower compared to T1 ($p < 0.001$), T3 ($p<0.001$), and T4 ($p<0.001$), whereas the T3 slope was significantly lower compared to T1 ($p<0.001$) and T4 ($p<0.001$) (Figure 2). Nevertheless, the T1 and T4 slopes showed no significant difference ($p = 0.1635$) (Figure 3). The slope generally followed a descending trend: T4 > T3 > T2 > T1 (Figure 3).

Biomass concentration and productivity

The results for biomass concentration (BC) and BP are presented in Figure 4. A similar trend was observed for biomass concentration and productivity (Figure 4). The BC showed a significant difference between treatments ($F = 146$, $p<0.05$), with GME1% showing lower concentration, followed by GME5%, M8, and GME10%, respectively (Figure 4). The BC for GME10% was significantly higher than that of M8 ($p<0.05$) (Figure 4). A similar trend was observed for BP, with a higher productivity being observed for GME10% compared to the M8 ($p<0.05$) (Figure 4).

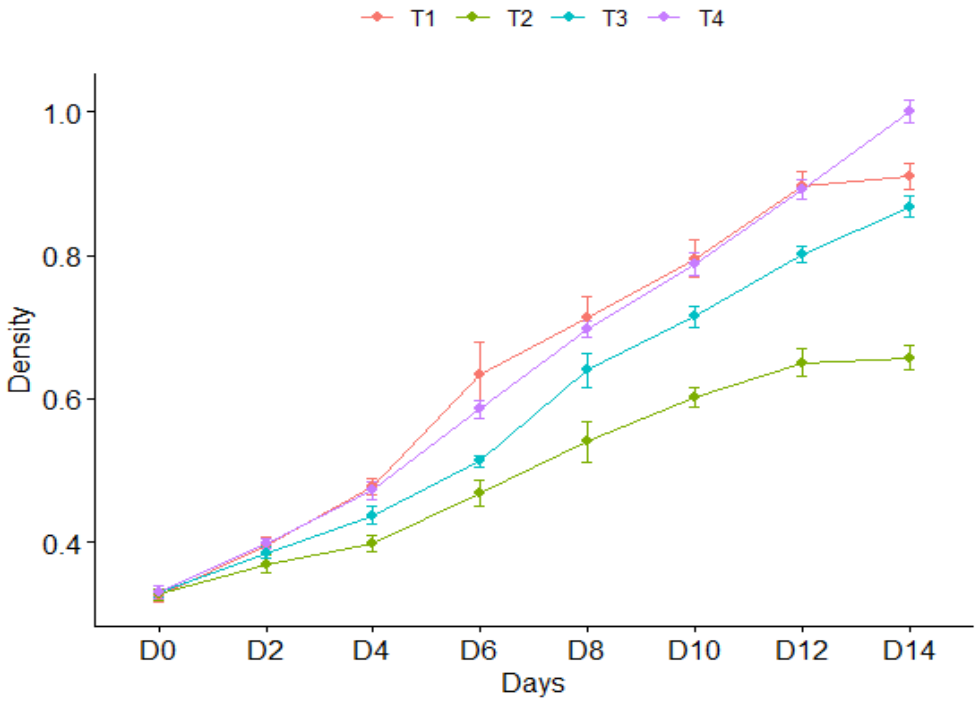

Figure-3. Daily variation of cell density of *Chlorella sorokiniana* in four treatments. T1 (M8), T2 (GME1%), T3 (GME5%), T4 (GME10%).

Specific growth rate and doubling-time

Results for SGR and DT are also presented in Figure 4. There was a significant difference in SGR between treatments ($F = 198.40$, $p<0.05$). The lower SGR was observed for GME1%, followed by GME5%, M8 and GME10%, respectively (Figure 4). The SGR for GME10% was significantly higher than that of the M8 medium ($p<0.05$). In contrast, the doubling time was significantly shorter for GME10%, followed by the M8, GME5%, and GME1%, respectively ($F = 247.30$, $p<0.05$).

Carbohydrate and protein concentration

The carbohydrate and protein composition of the microalgal biomass is presented in Figure 5. Carbohydrates were significantly higher for microalgae cultured in commercial M8 growth medium, followed by GE10%, GME5%, and GME1%, respectively ($F = 105$, $p<0.05$). However,

carbohydrates from the GME1% and GME5% showed no significant difference ($p>0.05$). Nevertheless, the protein content from the GME10% was significantly higher, followed by GME5%, M8 and GME1%, respectively ($F = 118.20$, $p<0.05$). No significant difference was observed for protein content between the M8 and GME5% media ($p>0.05$) (Figure 5).

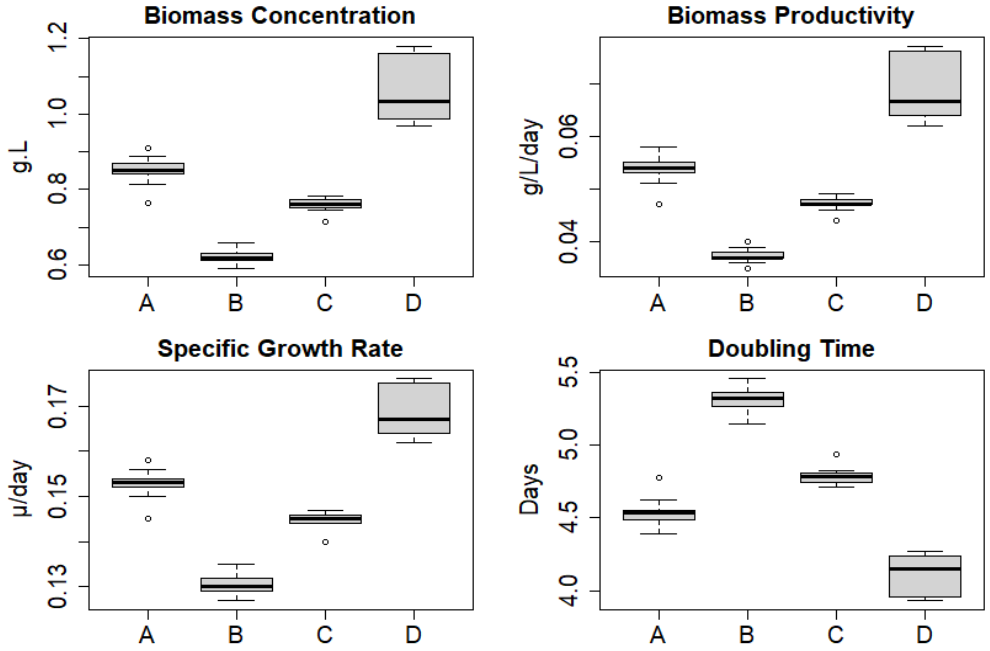

Figure-4. The biomass concentrations, biomass productivity, specific growth rate and doubling time of *Chlorella sorokiniana* UTEX 1230 cultured in different media. A: Control (M8 medium); B: GME1%; C: GME5%; D: GME10%.

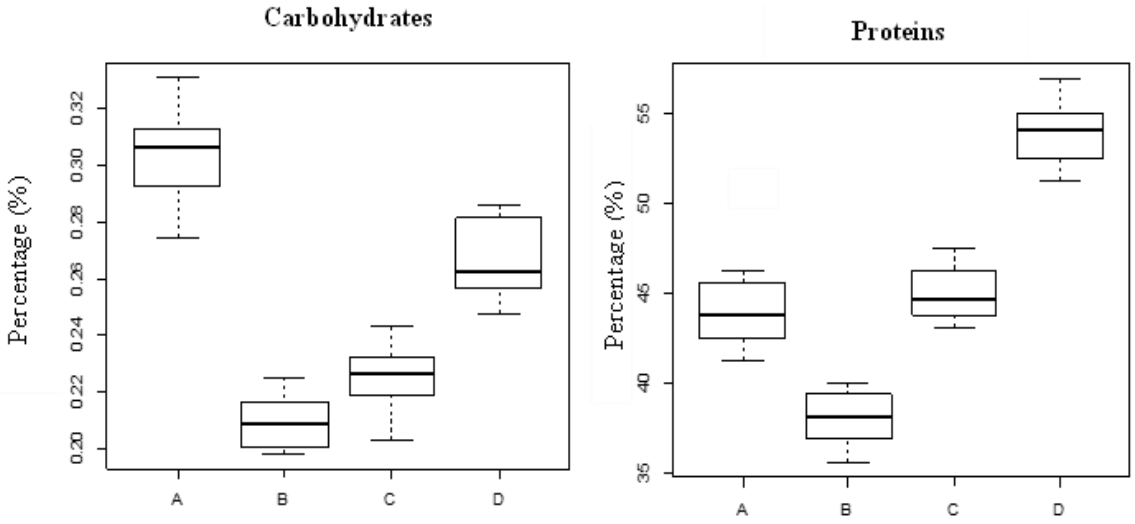

Figure-5. The carbohydrate and protein concentration of *Chlorella sorokiniana* UTEX 1230 cultured in different media. A: Control (M8 medium); B: GME1%; C: GME5%; D: GME10%.

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Cost-Benefit analysis

The M8 culturing medium resulted in R1.04 per gram of biomass compared to the 10%GME, which yielded R0.084 per gram of biomass. The input cost of the M8 medium was R0,87/L, yielding a biomass of 0.849g/L, whereas the 10%GME cost was R0.08/L and resulted in a biomass of 1.063g/L (Table 2). When benchmarked against the M8, the 10%GME was approximately 92% cheaper while generating 25% more biomass. The 10%GME resulted in higher biomass productivity and reduced cost for the medium.

Table-2. A comparative cost of productivity for the M8 and 10%GME.

Production cost	M8	GME (10%)
Medium Cost/l (ZAR)	R8.79	R0.89
Culturing Medium Cost/l (ZAR)	R0.87	R0.08
Biomass Cost/g (ZAR)	R1.04	R0.084

Discussion

Fortification of culture media has shown to receive increasing attention as it enhances mass production of microalgae (de Carvalho et al., 2019). Moreover, the use of livestock wastes seems to provide a promising solution to the expensive culturing cost (Zhu and Hiltunen, 2016). In the present study, cell density increased from day 1 to day 14, with increasing GME concentrations increasing the production rate. The increase in cell production rate in GME10% was found to be comparable to that of the M8 medium, which affirms the potential of GME in enhancing the yield of microalgae. The trend observed in GME was comparable to that observed by Wang et al. (2010) on 10%, 20% and 25% dairy manure extract media. However, the production rate observed in the present study was higher than that reported by Wang et al. (2010) for dairy manure extract. Blanco-Vieites et al. (2024) reported an increasing trend with increasing cow manure extract, with a higher production rate observed at a 50% ratio. Nevertheless, Shen et al. (2008) reported a decrease in cell density with increasing livestock wastes, whereas Han et al. (2017) observed a decrease in cell density with an increase in the ratio of chicken manure. In essence, not all livestock manure can enhance the yield of microalgae, but the goat manure remains a promising solution to enhance the yield of *C. sorokiniana*.

The goat manure is rich in nutrients compared to other ruminants (Zhu et al., 2020; Washaya and Washaya, 2023); therefore, its effect on algal growth may not be mysterious. In the present study, the biomass concentration and productivity increased as the GME ratio increased. The biomass concentration and productivity observed in GME10% were comparable to those observed from M8 medium, with the input cost being lower on the former. Kumaran et al. (2016) reported similar biomass concentration and productivity on *C. vulgaris* cultured in GME10% and GME20%. Sopandi et al. (2020) reported a significantly higher biomass concentration and productivity in a 25% goat manure extract-based medium for *Spirulina platensis* compared to the present *C. sorokiniana* in GME10%. Moreover, the biomass concentration observed for 10% GME was significantly higher compared to that reported by Machado et al. (2020) for organic Bioscape Humix (Atlanlusi, Leiria, Portugal) and synthetic media, whereas Bai et al. (2012) reported biomass concentrations comparable to those reported in the present study in 10% GME. According to Yaakob et al. (2021) and Diaz et al. (2023), the availability of nutrients such as nitrogen and phosphorus in a culturing medium is crucial for the growth of microalgae. Sharma et al. (2020) reported high nitrogen and phosphorus concentrations in goat manure extract compared to cow manure.

Nitrogen is vital for protein and chlorophyll biosynthesis, correlating to higher biomass productivity and cell division, whereas phosphorus contributes to energy molecules within the cell, required for cellular processes (Kumari et al., 2021). Toumi and Politaeva (2021) reported that higher concentrations of nitrate-nitrogen significantly increased the density and biomass growth of *Chlorella.* In the present study, the combination of nitration and aerobic fermentation likely increased the nitrogen content and nutrient bioavailability of the GME10%, which resulted in higher productivity compared to M8. This is supported by Sunaryo et al. (2018), where fermentation of goat manure was found

to increase the nitrogen content. Nevertheless, excess nitrogen and phosphorus in media may become problematic during discharge (Tyagi et al., 2022). Based on the trend observed in the present study, it is likely that an increase in GME concentration in culture media can further enhance the productivity. However, further studies are recommended to explore GME concentrations beyond 10% and the possibility of recycling the media to reduce excess nitrogen and phosphorus.

The SGR for GME10% was higher than that of the amended commercial M8 medium, with the doubling time significantly reduced for GME10% compared to M8 medium. However, GME5% and GME1% showed SGR below the M8 medium, suggesting that the two treatments did not do well compared to M8 medium, and the doubling time was also higher compared to M8 medium. The SGR observed in GME5% was comparable to that observed by Sharma et al. (2020) on *C. homosphaera* and *Scenedesmus obliquus* exposed to GME6% and that reported by Kumaran et al. (2016) on *C. Vulgaris* exposed to 16.6 g L^{-1} goat manure. Moreover, GME was found to give a better yield compared to pig, chicken, cow, and grass wastes on *Chlorella* sp. (Agwa et al., 2012; Chakraborty et al., 2023). Sopandi et al. (2020) reported an increasing specific growth rate of *Spirulina platensis* with increasing GME concentration. Moreover, Sánchez-Zurano et al. (2024) reported lower SGR in 10% milk whey medium in *Chlorella sp.* compared to GME10% observed in the present study. The 10% cow urine medium also showed the *Chlorella* growth rate lower than that observed in the present study on the GME10% (Suresh et al., 2019).

Nevertheless, Kobayashi et al. (2013) found different microalgae species exhibiting different growth rates in anaerobic digested effluent from cattle manure, but with enhanced protein production. Chakraborty et al. (2023) emphasised that organic wastes are rich in nutrients and have the potential to produce higher biomass concentration, productivity, and specific growth rate, and reduce doubling time compared to synthetic media for different *Chlorella* species. It is, therefore, evident that the integration of organics in a growth medium has the potential to produce similar results to those of synthetic media or even better. Moreover, the goat manure-based medium is environmentally friendly with no adverse effect on the aquatic environment (Ma and Jian, 2023).

Carbohydrate and protein content in microalgae may be driven by the dynamics of nitrogen. Low nitrogen concentration was found to enhance the conversion of protein to carbohydrate reserves (Simsek and Cetin, 2019; Kaur et al., 2022). In the present study, the commercial M8 medium produced a higher total carbohydrate content compared to all GME media. The trend of organic-waste-based medium increasing carbohydrate content while decreasing the protein content was also observed by Sharma et al. (2020) in *Chlorella* species. According to Kaur et al. (2022) and Dragone et al. (2011), nitrogen deficiency results in the reduction of cell growth rate and an increase in carbohydrates and starch in microalgae. Sopandi et al. (2020) reported high carbohydrates in culture media with low nitrogen. Nitration, which increased the nitrogen content in GMEs, has had an effect on carbohydrate accumulation in *C. sorokiniana* in the present study.

Contrasting the carbohydrate content, the protein content showed an increasing trend with increasing GME concentrations, with GME10% exhibiting concentrations higher than M8 medium. These results are congruent with Kaur et al. (2022), where high nitrogen concentration in the media enhanced protein synthesis, with low concentration enhancing carbohydrate content. The protein content observed in the present study was comparable to that observed by Kobayashi et al. (2013) from synthetic culture medium, but lower than that from cattle manure extracts. However, the use of ammonium-rich cattle manure culture medium enhanced protein synthesis in *C. sorokiniana* (Ziganshina et al., 2022). Moreover, Sánchez-Zurano et al. (2024) explored milk whey culture media and reported a protein content trend similar to that observed in the GMEs. de Medeiros et al. (2020) reported varying carbohydrate and protein contents in microalgae cultured in different organic media. Organic manure has the potential to substitute for the synthetic ones and leave no residuals that can threaten the environment. In the present study, nitration seems to have had a significant effect on the quality of the GMEs, through increasing nitrogen, which could still be problematic to discharge if the concentration is high.

Nitrogen content influences protein synthesis, with *Chlorella* rapidly absorbing NH_4^+ and incorporating it into amino acids compared to NO_3^- (Simsek and Cetin, 2019). Safafar et al. (2016) reported that in nitrogen-rich media, the fixed carbon is incorporated into the protein synthesis mechanism (Safafar et al., 2016). Additionally, Michelon et al. (2016) reported that protein synthesis is reduced in low nitrogen levels;

concurrently, carbohydrate synthesis is augmented. The observed carbohydrates and protein trend suggests that in commercial media like the M8, rapid nitrogen depletion stimulated carbohydrate production, whereas nitrogen-rich GMEs stimulated protein accumulation in *C. sorokiniana.*

Despite goat manure extracts showing potential to become a surrogate for the synthetic M8 medium for culturing *C. sorokiniana*, mismanagement of goat manure may lead to emission of nitrous oxide and methane (Orangun et al., 2021). Moreover, harmful pathogens such as *Escherichia coli* and *Salmonella* may become a threat to public health if they remain in poorly fermented extracts (Washaya and Washaya, 2023). Fermentation may detoxify contaminants in goat manure (Kingsley Amechi et al., 2018); therefore, utmost attention should be paid to fermentation to ensure good and environmentally friendly extracts. Nevertheless, Washaya and Washaya (2023) indicated that goat manure contributes negligible concentrations of contaminants such as Cu, Ni, and Zn to the environment. The measurement of possible toxicants and pathogens was not within the scope of this work, which limits our argument. Other limitations include not profiling of potentially toxic elements and compounds, and the study was conducted in a controlled environment on a bench-top scale condition, which may hinder predictive modelling of large-scale experimentation. However, more needs to be explored to understand the quantity that may be used without public health implications. Moreover, manure for goat feeding on diverse diets should also be investigated to determine whether diet really matters in the selection criteria.

Conclusion

The study developed a cost-effective and environmentally friendly protocol for culturing *C. sorokiniana* using goat manure extract. Given that goat manure releases heat and can burn plant roots as it breaks down in the soil (Kumaran et al., 2016), different GME percentages were explored. The study discovered that *C. sorokiniana* UTEX1230 cultured in a 10% goat manure extract produced a considerable biomass concentration, biomass productivity, and specific growth rate, which was comparable to those produced by a highly expensive modified M8 culturing medium. Moreover, the doubling time was also significantly reduced compared to the modified M8 culturing medium. Additionally, GME10% significantly enhanced protein synthesis, while the modified M8 medium produced carbohydrate content similar to that produced by GME10%. Nitration of GMEs was the key driver of the quality of this media. The goat manure extract comprises a nutrient profile that enhances *C. sorokiniana* UTEX 1230 growth, and it could be used as a substitute for the expensive modified M8 culturing medium. However, future studies may determine the residual nutrients and other potentially toxic compounds in the spent medium to ensure that what is discharged into the environment is not detrimental, including to public health. Moreover, the exploration of the possibility for recycling spent medium should be considered to reduce residual nutrients before discharge into the environment.

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Conflict of Interest: None.
Source of Funding: None.

Contribution of Authors

Rampersad R.R, Naidoo Y & Lebepe J: Conceptualized the study and designed research methodology.
Rampersad R.R: Performed experiments, collected data and manuscript write-up.
Rampersad RR & Lebepe J: Visualized and analyzed the data and reviewed the literature.
Naidoo Y & Lebepe J: Reviewed and edited the manuscript and supervised the work.

All authors read and approved final draft of the manuscript.

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