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4 **Influence of growth kinetics of microalgal cultures on biogas**
5 **production**
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Abstract

The microalgal cultures growth curve shows different phases involving changes in the physical-chemical characteristics of the microalgae. The aim of this study was to determine if the physico-chemical differences involve different biogas productions during the different stages of growth kinetics of *C. sorokiniana*. The microalgae growth kinetics was evaluated in batch cultures. Three phases were differentiated on the growth curve: P1, beginning of the exponential growth; P2, end of the exponential growth, and P3, steady state phase. The physico-chemical characterization, the biochemical methane potential, the biogas production rates by the Gompertz model, and the energy balance for each one of the three phases of the growth curve were evaluated. Significant differences were established in the four parameters evaluated. The highest biogas production and maximum production rate was obtained at the end of exponential growth P2, with 322 mL CH₄/g VS and 17.6mL CH₄/g. VS/d, respectively. Regarding the energy balances, the methane potential from the biomass harvested in P2 and P3 phases contributed with 16% and 14% of the harvesting requirements, respectively.

Key words: anaerobic digestion, biogas, growth phases, growth kinetics, microalgae.

1. Introduction

The microalgae anaerobic digestion is often proposed to overcome the high energy requirements of a microalgal biorefinery [1,2]. The biomethanization of microalgae for biogas production depends, among other parameters, on the physico-chemical composition of the microalgae [3]. Lipids have the greatest theoretical biomethane potential of the main microalgae components, corresponding to 1.014 L CH₄/g VS. However, they are the slowest to hydrolyse, followed by proteins with 0.851 L CH₄/g VS and carbohydrates with 0.415 L CH₄/g VS [3].

It has been described that microalgae presents growth kinetics in which it is possible to distinguish at least five phases: i) the lag phase, in which growth is delayed due to the presence of non-viable cells or physiological adjustments to adapt to the new medium conditions; ii) the exponential phase, where cells grow and divide as an exponential function of time, with non-limiting light intensity and nutrients; iii) the linear phase, in which the light becomes limited due to the increase in microalgae quantity, decreasing the growth rate; iv) the steady state phase, where there is nutrient limitation, determining when growth stops, and v) the death phase, where microalgae cell concentration declines rapidly due to a depletion of nutrients and pH disturbance [4,5]. In each of these phases, the physico-chemical composition of the microalgae changes. For example, in the steady state phase, in which the nutrients are exhausted, storage carbon products such as starch and lipids begin to accumulate [4,6]. Currently, there are no studies linking the biomethane potential with the different microalgal growth phases.

The aim of this study was to determine if there are significant differences in biogas production at different phases of the growth kinetics of the *Chlorella sorokiniana* microalgae in batch cultures. *Chlorella sorokiniana* is morphologically very simple but is diverse in physic-chemical characteristics. Its hemicellulotic cell wall is the main reason for the rigidity of the cells. The cell wall composition, as well as the whole microalgae

composition, present different characteristics at the different phases of the growth kinetics [7]. The methane produced was compared with the energy requirements of the microalgal harvesting process.

2. Material and Methods

2.1. Batch cultivation of the *C. sorokiniana* microalgae

The *C. sorokiniana* microalgae was donated by the University of Huelva, Spain. This microalgae was previously isolated from a sludge anaerobic digester effluent belonging to a wastewater treatment plant in Spain, and was subsequently identified using molecular analysis.

For microalgal growth, a Sueoka culture medium was used, which is composed of 0.72 [g/L] KPO_4H_2 , 1.44 [g/L] $\text{K}_2\text{PO}_4\text{H}$, 0.061 [g/L] $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002 [g/L] $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 [g/L] NH_4Cl , 0.95 [g/L] KNO_3 , 5[ml/L] hunter traces (2.28 [g] H_3BO_3 , 4.4 [g] $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$, 1.02 [g] $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 1.0 [g] $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.32 [g] $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.32 [g] $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.22 [g] $\text{Mo}_7\text{O}_{24}(\text{NH}_4)_6 \cdot 4\text{H}_2\text{O}$). This culture medium was chosen because it contains the components required for non-limiting growth conditions. The culture conditions used were: temperature of $21 \pm 2^\circ\text{C}$, artificial lighting source F24-39W with $I = 127.60 \mu\text{mol}$ of photons $/(\text{m}^2 \times \text{s})$, a 24-h light photoperiod, and an atmospheric air aeration of 1.3-1.5 L/min.

The growth kinetics of the *C. sorokiniana* cultures was determined by measuring the biomass concentration over time by optical density at 600nm. The dry weight estimation was determined based on the Volatile Solids (g VS/L) of the microalgal culture [8]. Subsequently, the dry weight concentration was related to the optical density at 600nm (spectrophotometer Jenway 6715 UV/VIS), obtaining a linear relationship between both variables (Figure 1). This linear relationship was evaluated and confirmed for each studied phase. The culture biomass concentration was expressed in g of VS per L of microalgal culture.

Figure 1

Once the growth kinetics was described, three growth phases were differentiated: P1, beginning of exponential growth; P2, end of exponential growth, and P3, steady state phase of the culture. After reaching these phases, the microalgal cultures were harvested by centrifugation with a Hermle Z 400 centrifuge (Labortechnik GmbH, P: 480 W).

2.2. Characterization of microalgal cultures

The characterization of cultures into the three different growth phases was based on the measurement of their physico-chemical composition, composed by protein content, lipids, carbohydrates, and recalcitrant material. For these analyses, samples from P1, P2, and P3 were lyophilized at a pressure of 10-15 mTorr and a temperature of -53°C using the ILSHIN Mod. FD5518 freeze dryer.

Protein determination was performed using the Kjeldahl method through the measurement of total organic nitrogen [9,10]. Lipid identification was conducted using the Soxhlet method, [11]. Carbohydrate determination was performed using the Dubois method [12]. Finally, recalcitrant material determination, measured as the insoluble fiber content of the sample, was determined using acid digestion followed by alkaline digestion to remove proteins, sugars, and lipids until the residue was considered as recalcitrant material [11].

2.3. Biomethane potential and CH₄ productivity

Biogas production generated by cultures of *C. sorokiniana* in phases P1, P2, and P3 was evaluated using the biochemical methane potential test (BMP). BMP is a conventional test to evaluate the anaerobic biodegradability of any substrate [13].

The anaerobic inoculum used in the BMP tests was obtained from a lab-scale anaerobic reactor that treats sewage sludge. BMP tests were performed in 100 mL flasks under

mesophilic conditions (37°C). In all cases, the substrate to inoculum ratio was maintained at 0.5 g. VS_{substrate}/g VS_{inoculum}. The biogas produced was passed through a NaOH solution to capture CO₂; the remaining gas was assumed to be methane. The test ended once biogas production ceased. Each experiment was carried out in triplicate.

The quantification of accumulated methane production was measured in time (mL CH₄ accumulated/g. VS.) and normalized to mL CH₄/g VS.

Subsequently, biogas productivity was determined with the modified Gompertz model [14], using the production values obtained from the BMP test, whose equation can be expressed as (Equation 1):

$$B = P \times \exp\left(-\exp\left(\frac{Rm \times e}{P}(\lambda - t) + 1\right)\right) \quad (1)$$

Where *B* represents the accumulated volume of biogas production at time *t* (d), *P* is the maximum biogas production potential (mL CH₄/ g VS), *Rm* is the maximum production rate (mL CH₄/g VS/d), *λ* is the duration of the lag phase (h) and *t* is the incubation time (d).

2.4. Analytical methodologies

All analyses were performed in triplicate, estimating mean values and their standard deviations. Both the microalgal biomass as well as the inoculum were characterized, estimating the following parameters: total solid (TS) concentration, and volatile solids (VS) concentration, according to standard methods [11]. pH was analysed by means of a pH meter (HI/111 Hanna Instrument).

2.5. Energy balance

Energy balance was based on the difference between the energy obtained as CH₄ in each phase and the energy required for microalgae harvesting, since this is one of the steps that requires most of the energy [15]. Energy balance for each of the phases was

calculated considering an energy input (E_i) that corresponded to the specific energy used for microalgae harvesting in each one of the three phases and an energy output (E_o), considering the biogas production in each of the three phases. E_i and E_o were calculated using Equations 2 and 3 [16,17]

$$E_i = \frac{P \times t}{V \times TS \times \left(\frac{VS}{TS}\right)} \quad (2)$$

Where E_i is the input energy (KJ/g VS); P is the power applied in centrifugation (480 W); t is the centrifugation time needed to reach around 9 g TS/L after centrifugation (s); V is the volume of the culture media (L); TS is the total solid concentration before centrifugation (g TS/L) and VS is the total solid concentration before centrifugation (g VS/L).

$$E_o = \frac{\Delta P \times \xi}{10^6} \quad (3)$$

Where E_o is the output energy (kJ/g VS); ΔP is the net methane production mL CH₄/g VS, and ξ is the specific heat of the methane (35600 KJ/m³ CH₄).

The energy balance is expressed as the ratio between energy input and energy output. Positive balance values or values greater than 1 indicate net energy production.

2.6. Statistical analysis

The evaluation whether there were differences between the three growth phases: P1, P2, and P3 was done in relation to the following parameters: biomethane potential, biogas production, physico-chemical composition, and energy balance with the ANOVA parametric test [18], considering a significance level of α 0.05 using the Statistica 13 software (version 13.0, StatSoft Inc., Tulsa, USA). Each time that the variance analysis was used, compliance with the assumptions of normality and homoscedasticity was verified as well as the absence of correlation between means and variances. When these assumptions were not met, a corrective transformation of the data was performed. Post-

hoc Bonferroni correction was applied [18] for comparisons between each of the phases (P1-P2, P1-P3, P2-P3).

3. Results and discussion

3.1. Growth phases and culture characterization

The microalgal cultures achieved a biomass concentration of 1.9 g VS/L in a period of 15 days, exhibiting a typical growth curve for microalgal batch cultures (Figure 2).

Figure 2

Phase P1 (beginning of growth phase) was achieved on the fourth day of culture. In this period, microalgae concentration reached 0.6 g VS/L (0.94 g VS/g TS). P2 (end of growth phase) was achieved on the eleventh day of culture, recording a microalgae concentration of 1.6 g VS/L (0.94 g VS/g TS). Finally, phase P3 (steady state of the culture) was achieved on the fifteenth day with a microalgae concentration of 1.9 g VS/L (0.95 g VS/g TS) (Figure 2).

Table 1 shows the characterization of microalgal biomass in the different growth phases. Similar protein concentration values were recorded in phases P1 and P2 with 45.5% and 45.9%, respectively. Meanwhile, a strong decline was recorded in phase P3 with 27.4%. Although no significant differences were observed between P1 and P2, they were observed between P3 and the other two phases. Mata et al. [5], in an analysis of biofuel production from microalgae, claimed that microalgal cultures generally present a high protein concentration during their growth phases. The same authors claimed that the protein content decreases in the steady state; hence increasing glycogen and carbohydrate concentrations. In our study, a significant increase in carbohydrates was recorded in the steady state phase (P3) (Table 1).

Table 1

Lee et al. [4] refer to the accumulation of nutrients such as starch and lipids during the steady state phase (P3) as a survival strategy for microalgae in the face of nutrient limitations. Decrease of protein concentration in P3 might be associated with the increase in carbohydrates in the same phase, as well as the slight increase in recalcitrant material (Table 1).

The amount of recalcitrant material allows us to estimate the non-degradable portion of the microalgae substrate [6]. An increase in recalcitrant material was observed in P3 with 7.1% in comparison to the values of P1 with 4.03% and P2 with 4.7%. Probably, this increase is associated to the gradual increase in cell wall thickness throughout the different phases. Yamamoto et al. [19] studied cell wall synthesis and formation of some microalgal species of the Chlorellaceae family using electron transmission microscopy. These authors indicated that daughter cell wall synthesis and formation for *Chlorella sorokiniana* begins in an early stage, and thickness gradually increases throughout their cell growth phase. Therefore, the thickening of the cell wall is more likely to happen during the steady state phase of the culture (P3), and thus, the cell wall of microalgae in this growth state is more difficult to hydrolyse. Similar lipid concentration values were recorded for the three growth phases; P2 exhibited the highest percentage of lipids (26.2%), followed by P3 with 24.5%, and P1 with 23.2%. No significant differences were recorded between the phases regarding lipid concentration.

3.2. Influence of microalgae growth kinetics on biogas potential and productivity

Table 2 shows CH₄ production potential and biodegradability (%) for each of the growth phases.

Table 2

The values of CH₄ produced varied between 242 mL CH₄/g VS for P3, 304 mL CH₄/g VS for P1, and the maximum was recorded in P2 with 322 mL CH₄/g VS (Figure 3, Table 2).

Figure 3

Different biogas yields from microalgae have been reported, varying in a wide range from 50 to 800 mL CH₄/g VS. The wide range of biogas production potentials and percentages may be determined by different factors, including the physico-chemical composition of the different microalgae species used.

Biodegradability percentages were estimated based on the theoretical methane production of protein, lipid and carbohydrates against the recorded methane production values (Table 2). The highest biodegradability percentage was achieved in P2 with 67.3%. P3 recorded the lowest biodegradability percentage with 37.1%. It is possible to relate the low biodegradability percentage of P3 with cell wall structure in this growth curve state. As previously discussed, the cell wall is likely to be much thicker in the steady state phase; for this reason, it would be more difficult to hydrolyse than in previous phases. In this sense, the degree of biodegradability of microalgal cell walls may be determined by at least two factors: a) thickness and recalcitrant compounds of the cell wall, as the cell wall is the first defence mechanism regarding a modulated cellular response to hydrolytic enzymes produced by bacteria [3] and/or b) due to the very nature of the hydrolytic enzymes released by the anaerobic inoculum, which may be deficient in amount or effectiveness in the degradation of macro-compounds of the microalgal cell wall [20].

The kinetic parameters obtained from the modified Gompertz equation (Equation 1) were also evaluated (Table 2). For P2, the highest CH₄ productivity rate was recorded with an R_m of 17.6 mL CH₄/g VS/d, while P3 recorded the lowest productivity with an R_m of 11.1 mL CH₄/g VS/d. It is possible to find a wide variation of biogas productivities from microalgae cultures in the literature. Lee et al. [21] estimated a R_m of 8 mL CH₄/g VS/d. VS for biogas productivity from *Hydrodictyon reticulatum*, while Chantrasakdakul et al. [22] reported a R_m of 30 mL CH₄/g VS/d for *Chlorella vulgaris*. This variation in parameter values may be explained by the diverse physico-chemical compositions of the microalgae, which depend on the culture method and species used, as previously described. In this context, Pavlostathis & Giraldo [6] mention that substrate conversion rates depend on the type of predominant microorganism, which also depends on the substrate type and origin of the inoculum.

Regarding substrate conversion, it is possible to point out that, in general, proteins are hydrolysed much slower than carbohydrates, while the biodegradation of soluble carbohydrates is fast and almost complete [6]. In the case of lipids, it is reported that, in general, their hydrolysis is even slower than carbohydrates and proteins [3]. In addition, Rinzema et al. [28] report that total lipid conversion is limited both by the conversion of long-chain fatty acids as well as by physical dissolution processes and the mass transfer of these acids. It is interesting that P3, with the highest carbohydrate concentration and the lowest proteins concentration out of the three phases (Table 1), resulted in the lowest methane yield and lowest methane production rate (Table 2). Therefore, in this study, it is possible to associate the low biogas production and productivity of P3 with the state of development of the cell wall [19].

3.3. Evaluation of the energy cost in the process

Energy balances were estimated for phases P1, P2, and P3 of the *C. sorokiniana* cultures according to Equation 2 and Equation 3 (Table 3).

Table 3

The energy needed for harvesting was in all cases much higher than the energy produced by methane production. We must consider that the harvesting process used in this study can be easily optimized in a full-scale application; a much lower energy requirement can be assumed in a full-scale application. The energy output base on methane production considered 100% conversion form methane to energy, however the efficiency conversion will decrease this energy output value. Nevertheless, the ratio between E_i and E_o gives a good indication about the contribution of the energy produced by anaerobic digestion to the harvesting, the most energy-demanding process.

The energy produced with the P2 biomass will contribute with 14% of the harvesting process, being only 5% for the P1 phase. Despite the fact that CH_4 production values are similar for P1 and P2, the energy requirements to harvest P1 were much greater than for P2. This is due to the lower concentration of microalgal biomass in P1 compared to P2. This lower concentration entails greater centrifugation time, and therefore, greater energy consumption [24]. Additionally, the possible smaller size of cells with less cell wall formation would contribute to an even greater calculated energy consumption [19].

It is interesting to notice how the contribution made by the P3 and P2 methane potential to the harvesting process were very similar (Table 3), even when P3 showed a much lower methane productivity and biodegradability (Table 2). Nevertheless, the time required to reach this P3 growth phase is necessarily more elevated than for P2. For this reason, the operating energy required to reach this phase would be higher than the required energy to reach the P2 phase. However, as previously mentioned, the energy balance estimated

here only considers energy for the harvesting of each of the growth phases, since growth operation costs are considered much less in comparison to those for harvesting [15].

4. Conclusions

The studied *C. sorokiniana* presented significant differences in relation to its physico-chemical composition in the different growth phases studied. Similarly, there were differences in biogas production and productivity from microalgal cultures in the different growth phases. The results provided by the present study can be considered to implement a strategy for the optimization of biogas production into a *C. sorokiniana* biorefinery or other microalgae-like biorefinery.

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Figure captions

Fig. 1. Calibration curve for *Chlorella sorokiniana* culture concentration was carried out in triplicate. The dry weight concentration was related to optical density at 600 nm, obtaining a linear relationship between both variables. Mean values $\pm$ SD.

Fig. 2. Growth kinetics of *Chlorella sorokiniana* in a 5-L batch culture. P1, beginning growth phase. P2, end of growth phase. P3, stationary state of the culture. The growth kinetics was carried out in triplicate. Mean values $\pm$ SD.

Fig. 3. Biomethane potential test (BMP) for phases P1, P2, and P3 of cultures of *C. sorokiniana*. Each BMP was carried out in triplicate for each phase. Mean values $\pm$ SD.

Table 1. Characterization of microalgal biomass in different growth phases. Each characterization was carried out in triplicate.

Characterization	P1	P2	P3
Proteins (%)	45.90 ^a	45.50 ^a	27.40 ^b
Lipids (%)	23.20	26.20	24.50
Carbohydrates (%)	23.90 ^a	23.70 ^a	44.10 ^b
Recalcitrant material (%)	4.03 ^a	4.70 ^b	7.10 ^c
pH	6.90	6.80	6.90

^{a, b, c} significant differences, where $p \leq 0.05$. Post-hoc Bonferroni test.

Table 2. Biochemical methane potential test (BMP) and CH₄ Productivity for phases P1, P2 and P3 of the *C. sorokiniana* cultures. Each BMP was carried out in triplicate. Mean values ± SD.

BMP	P1	P2	P3
Biodegradability (%)	41.9±1 ^a	42.8±1 ^b	36.4±4 ^c
Biomethane potential (mL CH ₄ accumulated/g. VS)	304±5 ^a	322±7 ^a	242 ± 28 ^b
Gompertz			
λ(h)	12±5 ^a	24±1 ^a	12 ± 0.5
<i>R_m</i> (mL CH ₄ /g. VS/d)	17.5 ± 0.5 ^a	17.6 ± 0.3 ^a	11.1 ± 1.5 ^b
<i>P</i> (mL CH ₄ /g. VS)	309±6 ^a	321±7 ^a	247 ± 28 ^b
R ²	0.877	0.901	0.905

^{a, b, c} significant differences, where $p \leq 0.05$. Post-hoc Bonferroni test.

Table 3. Energy balance evaluation for each phase of the cultures of *C. sorokiniana* based on recorded CH₄ production (*E_i*) and energy consumed in the harvesting process (*E_o*).

Phases	CH ₄ production (mL CH ₄ /g VS)	Energy input (<i>E_i</i> = kJ g TS)	Energy output (<i>E_o</i> = kJ g TS)	<i>E_o/E_i</i> (%)
P1	304	240	10.82	5
P2	322	72	11.46	16
P3	242	60.63	8.62	14

Figure 1

Figure 2

Figure 3