POLITECNICO DI TORINO

Master's Degree In Chemical and Sustainable Processes Engineering

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Master's Degree Thesis

Effect of pretreatment and co-digestion on the biogas production of microalgae and chicory roots

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Abstract

Anaerobic digestion is a process that is attracting growing interest from the scientific community as a pathway for sustainable energy production. Microalgae, in particular, are a focus of research due to their ability to grow rapidly on non-arable land (thus avoiding competition with food production) and their high biogas yield compared to lignocellulosic biomass. A major challenge in the anaerobic digestion of microalgae is the biodegradation of the cell wall and the solubilization of macromolecules.

This study aims to deepen the understanding of how pretreatments applied to raw microalgae, targeting cell wall degradation, impact methane production by anaerobic microorganisms, as well as to examine the effects of co-digestion with chicory roots to explore potential synergistic effects.

The tests evaluated ultrasound and thermal pretreatments at varying intensities, along with anaerobic co-digestion of different mixtures containing various ratios of raw microalgae and chicory roots.

The results indicate that ultrasound pretreatment of *Spirulina* microalgae reduces methane production, while thermal pretreatment accelerates the production kinetics in the early stages but does not increase the final yield compared to untreated microalgae. Co-digestion with equal proportions of the biomasses, or with a higher proportion of microalgae, achieves greater methane production than would be expected from the digestion of each substrate individually.

Summary

Introduzione

La digestione anaerobica è una tecnica fondamentale nel campo dell'energia rinnovabile, nell'ottica di ridurre l'uso di combustibili fossili trasformando i rifiuti organici in risorse energetiche sostenibili. La digestione anaerobica utilizza microrganismi in un ambiente privo di ossigeno per degradare materiali organici e produrre biogas, composto principalmente da metano (CH_4) e anidride carbonica (CO_2), il quale può essere sfruttato per la produzione continua di energia. Questo processo è considerato neutro dal punto di vista delle emissioni di CO_2, poiché il gas rilasciato durante la produzione è compensato dal CO_2 assorbito dai materiali organici nel loro ciclo di vita.

Processo di digestione anaerobica

La digestione anaerobica si articola in quattro fasi principali:

- Idrolisi: in questa fase iniziale, i composti polimerici complessi, come carboidrati, proteine e lipidi, vengono convertiti in molecole più semplici e solubili. Ad esempio, i carboidrati si trasformano in zuccheri, le proteine in amminoacidi e i lipidi in acidi grassi e alcoli.

- Acidogenesi: i composti solubili formati in idrolisi vengono ulteriormente trasformati in acidi grassi volatili, CO_2, H_2 e altri composti organici. L'acido acetico, in particolare, rappresenta un prodotto fondamentale poiché viene trasferito direttamente all'ultima fase del processo.

- Acetogenesi: gli acidi grassi volatili prodotti nella fase precedente vengono convertiti in acido acetico, H_2 e CO_2. Alcune reazioni chiave in questa fase includono la trasformazione dell'etanolo e di altri composti organici in prodotti utilizzabili nella fase finale di metanogenesi.

- Metanogenesi: è la fase finale del processo, dove l'acido acetico, l'idrogeno e il CO_2 vengono trasformati in metano. Circa il 66% del metano proviene dalla

decarbossilazione dell'acetato, mentre il resto deriva dalla riduzione del CO_2 con idrogeno.

Queste fasi coinvolgono diversi consorzi batterici specializzati e richiedono condizioni ambientali specifiche per ottimizzare la produzione di biogas.

Parametri chiave della digestione anaerobica

Per massimizzare l'efficienza della digestione anaerobica, è fondamentale monitorare alcuni parametri essenziali:

- Temperatura: i microrganismi coinvolti nella digestione anaerobica sono classificati in base alla temperatura ottimale per il loro sviluppo. I batteri mesofili lavorano tra 20 e 45°C, mentre quelli termofili tra 45 e 60°C. Sebbene i batteri termofili possano produrre biogas più rapidamente, il processo risulta più stabile e con un'effluente di migliore qualità usando batteri mesofili.

- pH: un pH neutro (6,5-7,5) è ideale per mantenere la stabilità del processo. Durante la digestione, l'accumulo di acidi grassi volatili può ridurre il pH, inibendo i batteri metanogeni. Per questo, si utilizzano tamponi basici per bilanciare l'acidità.

- Rapporto C/N: un corretto rapporto tra carbonio e azoto (C/N) è essenziale per la nutrizione dei batteri. Idealmente, dovrebbe oscillare tra 16 e 30. Se è troppo alto, la produzione di biogas cala a causa della scarsità di azoto; se è troppo basso, si formano ammoniaca e altri prodotti tossici che ostacolano la crescita batterica.

- Carico Organico (OLR): rappresenta la quantità di materiale organico introdotto giornalmente per unità di volume del reattore. Un carico eccessivo accelera l'idrolisi e l'acidogenesi, ma può portare all'accumulo di acidi grassi, abbassando il pH e destabilizzando il processo.

- Tempo di Ritenzione: rappresenta il tempo medio di permanenza del substrato nel reattore. Un tempo di ritenzione adeguato assicura che i substrati vengano completamente convertiti in biogas.

- Agitazione: essenziale per mantenere la concentrazione uniforme di nutrienti e prevenire la sedimentazione dei solidi, l'agitazione può essere ottenuta tramite agitatori meccanici o pompe centrifughe.

Utilizzo delle microalghe nella digestione anaerobica

Le microalghe rappresentano un'interessante alternativa per la digestione anaerobica, con vantaggi unici rispetto alle biomasse tradizionali:

- Producono più metano rispetto ad altre biomasse.
- Crescono rapidamente e assorbono efficacemente CO_2 e nutrienti dall'ambiente.
- Possono prosperare in acque reflue e salmastre, riducendo l'uso di terreni coltivabili e non competendo con la produzione alimentare.
- Possiedono una bassa percentuale di lignina, una sostanza difficile da degradare nella digestione anaerobica.

Il contenuto di proteine nelle microalghe, però, aumenta la quantità di azoto, riducendo il rapporto C/N e rendendo meno favorevole la digestione anaerobica. Inoltre, possono assorbire metalli pesanti dalle acque reflue, che in concentrazioni elevate risultano tossici per i microrganismi anaerobici.

Pretrattamenti delle microalghe

Per migliorare la digeribilità delle microalghe, vengono utilizzati vari metodi di pretrattamento:

- Ultrasuoni: una tecnica che utilizza onde sonore ad alta frequenza per provocare il fenomeno della cavitazione, causando la rottura delle pareti cellulari delle microalghe. È un trattamento rapido e con bassa tossicità.
- Trattamento termico: questo pretrattamento sfrutta il calore per aumentare la solubilità delle molecole organiche, facilitando l'idrolisi. Le temperature usate vanno da 100 a 300°C a pressioni elevate, ma un calore eccessivo può produrre composti resistenti alla degradazione.

Studi mostrano che il pretrattamento ad ultrasuoni e termico può migliorare la produzione di metano, ma i risultati variano molto a seconda della specie di microalga utilizzata e delle condizioni di trattamento.

Co-digestione con altre biomasse

La co-digestione delle microalghe con biomasse ricche di carbonio, come rifiuti agricoli o letame, migliora il rapporto C/N, stabilizza il processo e aumenta la produzione di biogas. La co-digestione offre ulteriori vantaggi, tra cui:

- Incremento della capacità di carico di biomassa.
- Diluizione di inibitori, come sali o polifenoli.
- Bilanciamento dei nutrienti e maggiore stabilità del processo.

Studi dimostrano che la co-digestione può migliorare la produzione di biogas fino al 260%, a seconda delle specie di microalghe e del substrato aggiunto. Ad esempio, la co-digestione di microalghe con letame di pollo o paglia di grano ha portato a incrementi significativi della resa di metano.

Materiali e Metodi

Obiettivi dello studio

Lo studio mira a:

- Ottenere risultati preliminari sull'effetto della co-digestione di microalghe e radici d'indivia nella produzione di metano.
- Analizzare l'impatto di pretrattamenti idrotermici e ultrasonici sulla produzione di metano dalle microalghe.

Per raggiungere questi obiettivi, sono stati condotti tre test di BMP (Biochemical Methane Potential). Le condizioni di pretrattamento valutate includono:

- Ultrasuoni a 400W per durate di 5, 10 e 15 minuti.
- Trattamenti idrotermici a 120°C e 140°C per 15 minuti.

La co-digestione è stata eseguita utilizzando diverse proporzioni di microalghe e radici d'indivia (25%-75%, 50%-50%, e 75%-25%).

Analisi e strumenti

AMPTS (Automatic Methane Potential Test System)
I test BMP sono stati condotti con l'AMPTS II, che permette di misurare il volume di metano prodotto durante la degradazione anaerobica della biomassa. L'AMPTS è composto da tre unità:

- UNITÀ A: bagno d'acqua termostatico per incubazione dei campioni a 37°C.
- UNITÀ B: assorbimento del gas, in cui una soluzione alcalina trattiene CO_2 e H_2S, lasciando passare solo il CH_4.
- UNITÀ C: misura del volume di gas tramite un dispositivo a flusso d'acqua.

Analisi elementare CHNS/O
Con il dispositivo Flash 2000, sono state misurate le quantità di carbonio, idrogeno, azoto, zolfo e ossigeno. Questo permette di determinare la composizione chimica della biomassa e di calcolare la produzione teorica di metano.

Procedure sperimentali

Dopo aver effettuato le misurazioni di massa secca e massa volatile delle varie biomasse, incluso l'inoculo, sono stati eseguiti tre test BMP:

- *AMPTS Compiègne*: valutazione delle microalghe trattate con ultrasuoni e idrotermici. Le microalghe sono state diluite per ottimizzare l'applicazione degli ultrasuoni.
- *AMPTS 1 Beauvais*: valutazione delle radici d'indivia e microalghe senza trattamento e con ultrasuoni a 400W per 15 minuti.
- *AMPTS 2 Beauvais*: valutazione della co-digestione di microalghe e indivia in diverse proporzioni (25%-75%, 50%-50%, 75%-25%).

In tutti i test, è stato scelto un rapporto inoculo/substrato pari a 3. Le bottiglie, rappresentanti ciascuna un mini-reattore, sono state riempite con 250 mL di inoculo, la massa di substrato necessaria a raggiungere il rapporto di proporzione desiderato, e acqua fino a raggiungere un volume totale di 350 mL. Tutti i test sono stati effettuati in duplicato o triplicato, insieme a un controllo positivo in cui si effettua la digestione anaerobica della cellulosa, e un controllo negativo con inoculo solo, necessari per validare l'affidabilità dell'esperimento.

Trattamento dei Dati

I dati sono stati elaborati per rimuovere il contributo del metano prodotto dall'inoculo e normalizzati per il contenuto di massa volatile. I valori relativi a test con lo stesso substrato sono stati mediati ed è stata calcolata la deviazione standard. Il modello cinetico di Gompertz è stato utilizzato per descrivere la produzione di metano. Il modello viene descritto dall'equazione

$$V = V_{max} \cdot exp\left(-exp\left(\frac{R_{max} \cdot exp(1) \cdot (\text{Lag phase} - t)}{V_{max}} + 1\right)\right) \tag{1}$$

dove si possono evidenziare la fase di adattamento microbico (lag phase), la fase esponenziale e quella stazionaria. V_{max} rappresenta la produzione massimale di metano, mentre R_{max} è la velocità massima di crescita.

Analisi finale

L'ultimo step dell'analisi dei dati è l'utilizzo dei risultati ottenuti dal test CHNS/O per calcolare la produzione teorica di metano di una determinata biomassa. Per fare ciò, si

parte dall'equazione generica che rappresenta una reazione di metanazione:

$$\begin{aligned} C_nH_aO_bN_cS_d + \left(n - \frac{a}{4} - \frac{b}{2} + 3\frac{c}{4} + \frac{d}{2}\right) H_2O \rightarrow \\ \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + 3\frac{c}{8} + \frac{d}{4}\right) CO_2 \\ + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - 3\frac{c}{8} - \frac{d}{4}\right) CH_4 \\ + cNH_3 + dH_2S \end{aligned} \tag{2}$$

Da questa reazione, si deriva l'equazione di Buswell per il calcolo della produzione teorica di metano:

$$\text{Produzione teorica} = \frac{n}{2} + \frac{a}{8} - \frac{b}{4} - 3\frac{c}{8} - \frac{d}{4} \ \left[\text{mol/mol}_{biomassa}\right] \tag{3}$$

Dunque, a partire dalle percentuali in massa di carbonio, idrogeno, azoto, zolfo e ossigeno presenti in ciascuna biomassa utilizzata nei diversi test, sono stati effettuati calcoli per determinare i coefficienti stechiometrici della formula chimica del substrato. Successivamente, sono state stimate le produzioni teoriche di metano, che sono state confrontate con i risultati ottenuti sperimentalmente.

Risultati e discussione

AMPTS Compiègne

L'esperimento condotto a Compiègne ha misurato la produzione di metano dalle microalghe sottoposte a diversi pretrattamenti (ultrasuoni e idrotermici). Come mostrato nella figura 1, i pretrattamenti con ultrasuoni hanno ridotto la produzione di metano, probabilmente a causa dell'accumulo di acidi grassi volatili (VFAs), che inibiscono i microorganismi responsabili della metanogenesi. Al contrario, il trattamento idrotermico ha migliorato le cinetiche iniziali di produzione senza tuttavia influire sul volume totale di metano prodotto.

Dati principali:

- Le microalghe senza pretrattamento hanno raggiunto 299,3 $\pm$ 4.1 $\text{NmL}_{CH_4}/\text{g}_{MV}$.
- I trattamenti ultrasonici hanno ridotto il metano del 24%-30%, mentre il trattamento idrotermico ha aumentato la produzione del 2% rispetto al campione non trattato.

Modello di Gompertz
Per caratterizzare le cinetiche di produzione di metano, è stato usato il modello di Gompertz, che ha mostrato un buon adattamento ai dati sperimentali.

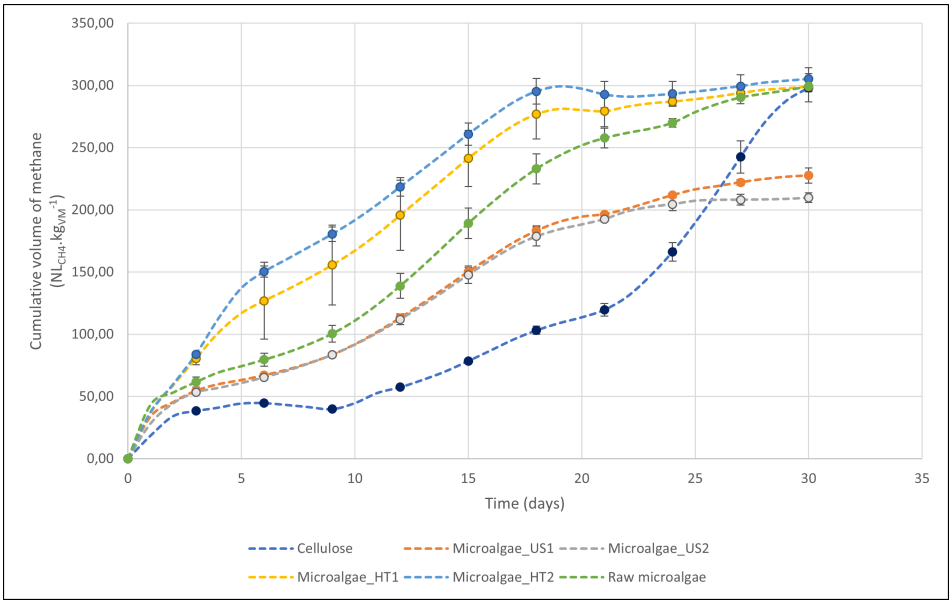

Figure 1: Risultati trattati AMPTS Compiègne

Table 1: Identificazione con una cinetica di tipo Gompertz

	Microalghe US1	Microalghe US2	Microalghe HT1	Microalghe HT2	Microalghe brute
V_{max}	269.55 ± 14.35	241.30 ± 1.56	312.80 ± 10.75	309.0 ± 9.76	357.67 ± 10.51
Lag phase	-0.7 ± 0.11	-0.53 ± 0.14	-0.9 ± 0.15	-0.94 ± 0.10	0.1 ± 0.27
R_{max}	9.65 ± 0.35	9.76 ± 0.48	17.13 ± 3.15	19.75 ± 0.83	12.73 ± 0.56
MAPE (%)	6.84 ± 0.59%	8.62 ± 1.46%	4.84 ± 0.66%	4.37 ± 0.03%	7.71 ± 0.18%
R^2	0.99 ± 0.00	0.98 ± 0.00	0.99 ± 0.00	0.98 ± 0.00	0.99 ± 0.00

Il parametro R_{max} ha confermato che le microalghe trattate termicamente mostrano una velocità di produzione di metano più elevata, mentre i campioni trattati con ultrasuoni hanno registrato una produzione inferiore, come visibile dalla figura 2, dove i punti sono i valori sperimentali, mentre le curve rappresentano il modello teorico.

Analisi elementare ed efficienza

I risultati dell'analisi elementare (CHNS/O) hanno permesso di calcolare le produzioni teoriche di metano e confrontarle con i dati sperimentali. Le microalghe trattate con ultrasuoni hanno avuto l'efficienza più bassa, mentre quelle trattate termicamente hanno mostrato un'efficienza più alta (fino al 60%). I risultati ottenuti sono in tabella 2.

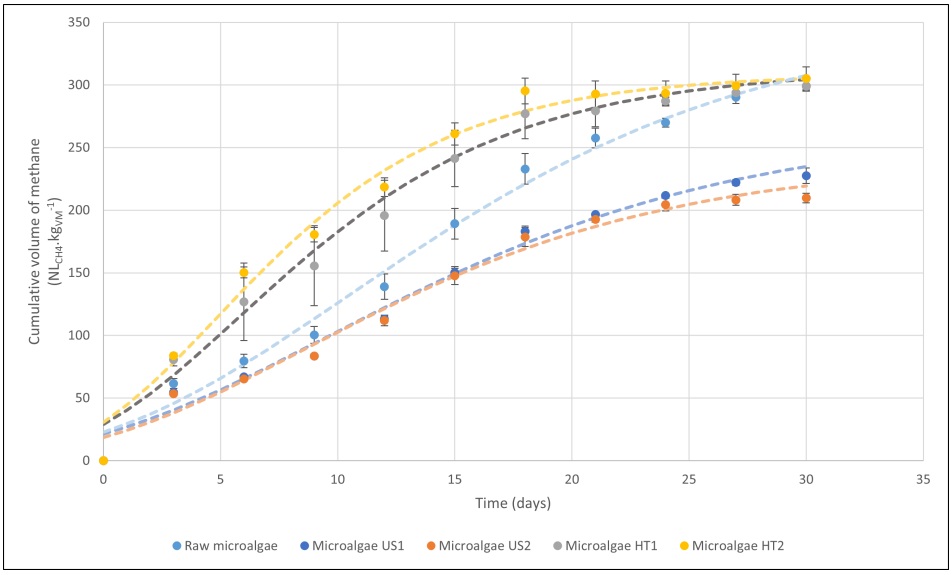

Figure 2: Approssimazione grafica della cinetica di Gompertz - Compiègne

Table 2: Produzioni teoriche

Substrato	Produzione teorica [NmL_{CH_4}/g_{MV}]	Produzione effettiva [NmL_{CH_4}/g_{MV}]	Efficienza [%]
Microalghe brute	549.9 ± 56.2	299.3 ± 4.1	54.4
Microalghe US1	537.8 ± 23.3	227.6 ± 6.2	42.3
Microalghe US2	538.9 ± 35.1	209.7 ± 3.9	38.9
Microalghe HT1	494.6 ± 24.1	299.1 ± 4.2	60.5
Microalghe HT2	509.0 ± 26.5	305.2 ± 9.2	60.0

AMPTS 1 Beauvais

Questo esperimento ha coinvolto microalghe, indivia e microalghe trattate con ultrasuoni (400W per 15 minuti). L'indivia ha mostrato un'inibizione iniziale della produzione di metano, dovuta a zuccheri e polifenoli che riducono il pH e creano condizioni sfavorevoli per i microorganismi. Anche in questo caso, le microalghe trattate con ultrasuoni hanno prodotto circa il 28% in meno di metano rispetto al campione non trattato.

Dati principali:

- Produzione media di metano: microalghe ($315{,}8 \pm 6.8$ NmL_{CH_4}/g_{MV}), microalghe con ultrasuoni ($227{,}7 \pm 21.3$ NmL_{CH_4}/g_{MV}), radici d'indivia ($302{,}6 \pm 12.9$ NmL_{CH_4}/g_{MV}).

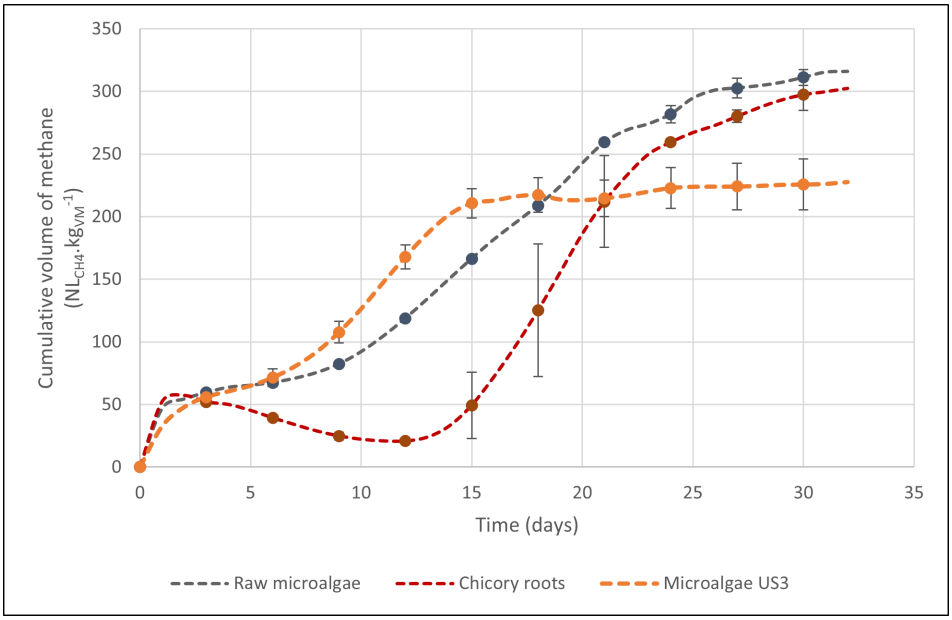

Figure 3: Risultati trattati AMPTS 1 Beauvais

Modello di Gompertz

Il modello di Gompertz ha mostrato difficoltà a rappresentare l'inibizione iniziale dell'indivia, evidenziando limiti nell'adattarsi a substrati che presentano inibizioni temporanee, come visibile dalla figura 4.

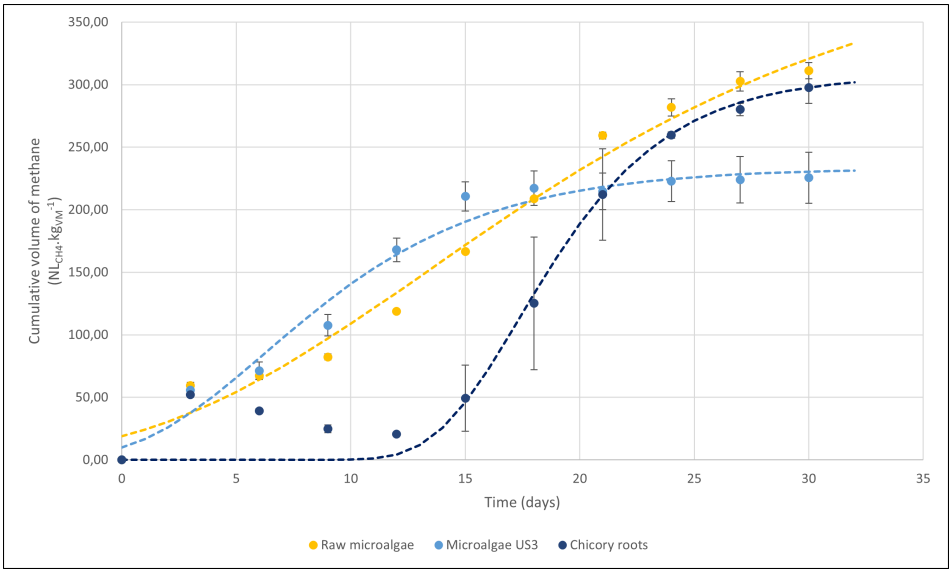

Figure 4: Approssimazione grafica della cinetica di Gompertz - Beauvais 1

Analisi elementare ed efficienza
L'analisi ha mostrato un'efficienza relativamente alta (74.4%) per l'indivia rispetto alle microalghe, suggerendo che la co-digestione d'indivia e microalghe potrebbe aumentare la produzione di metano.

Table 3: Produzioni teoriche

Substrato	Produzione teorica [NmL_{CH_4}/g_{MV}]	Produzione effettiva [NmL_{CH_4}/g_{MV}]	Efficienza [%]
Radici d'indivia	406.6 ± 75.9	302.6 ± 18	74.4

AMPTS 2 Beauvais

In questo esperimento, sono state testate combinazioni di microalghe e radici d'indivia in varie proporzioni (25%-75%, 50%-50%, 75%-25%). I risultati hanno evidenziato che le miscele con indivia al 50% e 25% hanno superato in produzione sia le microalghe sia l'indivia utilizzate singolarmente, suggerendo un effetto sinergico. Al contrario, la miscela con il 75% d'indivia ha sofferto dell'inibizione iniziale (figura 5).

Dati principali:

- La miscela 25% microalghe - 75% radici d'indivia ha riscontrato l'inibizione da parte delle radici d'indivia, con una produzione di 291.1 ± 15.1 NmL_{CH_4}/g_{MV}.
- La miscela 50%-50% ha ottenuto una produzione di 339.6 ± 0.1 NmL_{CH_4}/g_{MV}.
- La miscela 75% microalghe - 25% radici d'indivia ha dato la produzione media più elevata, pari a 342.0 ± 15.7 NmL_{CH_4}/g_{MV}.

Modello di Gompertz
Anche in questo caso, il modello di Gompertz non ha rappresentato con precisione la produzione di metano, poiché l'inibizione dell'indivia ha alterato la curva.

Analisi elementare ed efficienza
I dati ottenuti dall'analisi CHNS/O sono stati utilizzati per trovare le produzioni teoriche. Questi sono stati confrontati con i valori di produzione prevista basandosi su quella ottenuta dai substrati presi individualmente, oltre che a quelli sperimentali.

Table 4: Produzione sperimentale dei substrati presi singolarmente

Microalghe	Radici d'indivia
315.83 ± 8.92	302.63 ± 17.96

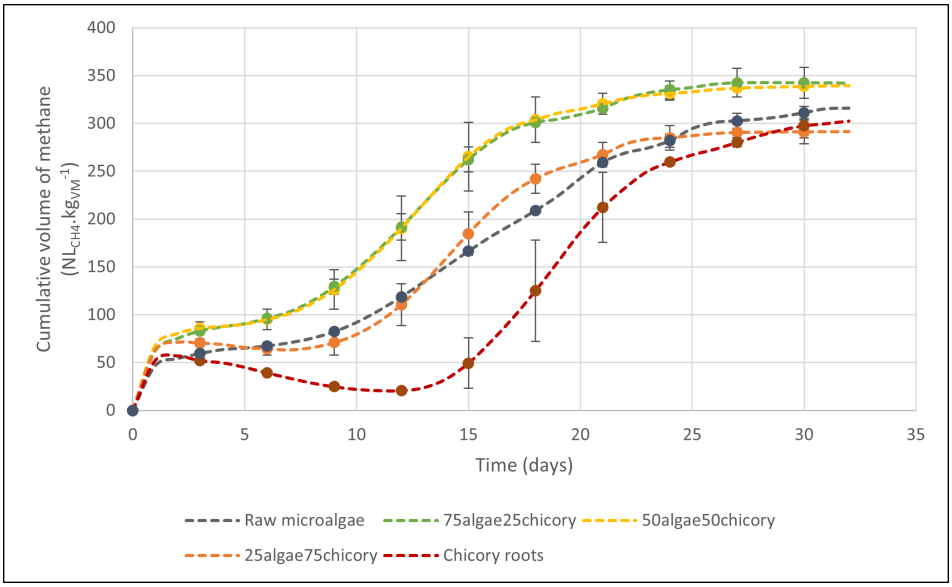

Figure 5: Risultati trattati AMPTS 2 Beauvais

Table 5: Produzione teorica dei substrati presi singolarmente

Microalghe	Radici d'indivia
549.89 $\pm$ 56.20	406.57 $\pm$ 75.91

Table 6: Produzioni teoriche e previste

Mix μalgae - radici d'indivia	Produzione teorica	Produzione prevista	Produzione effettiva	Miglioramento	% della produzione teorica
25% - 75%	442.40	305.93	291.12	-4.84%	66.80%
50% - 50%	478.23	309.23	339.62	+9.83%	71.02%
75% - 25%	514.06	312.53	341.99	+9.43%	66.53%

I risultati hanno mostrato che le miscele con il 50% o 75% di microalghe hanno una produzione maggiore di quanto previsto, suggerendo che la co-digestione potrebbe essere ottimizzata ulteriormente.

Conclusioni

Questo studio ha dimostrato che la digestione anaerobica delle microalghe può contribuire alla transizione energetica, nonostante i limiti legati alla bassa degradabilità della parete cellulare e al basso rapporto C/N. I pretrattamenti hanno effetti diversi: mentre gli ultrasuoni riducono il potenziale di metano per le microalghe *Spirulina*, il trattamento

termico migliora le cinetiche senza aumentare la produzione totale. La co-digestione con indivia, ricca di carbonio, si è rivelata promettente per miscele con microalghe al 50% o 75%.
Possibili sviluppi futuri includono:

- L'uso di specie di microalghe alternative o l'applicazione di pretrattamenti chimici o biologici.
- La co-digestione delle radici d'indivia con microalghe pretrattate termicamente per ottimizzare l'efficienza.

Acknowledgements

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Chapter 1

Introduction

Anaerobic digestion is a biological process in which anaerobic microorganisms break down organic materials in the absence of oxygen. The result of this digestion is the production of biogas, which is mainly composed of 50-70% methane (CH_4) and 30-40% carbon dioxide (CO_2), that can be employed in continuous power generation.
Biogas can be considered an excellent tool in the transition from fossil fuel-based energy to renewable, clean, and sustainable energy. Additionally, anaerobic digestion offers a new alternative for the management and disposal of municipal, industrial, or agricultural organic waste: waste becomes a resource once again, capable of serving a new purpose. Biogas produced through anaerobic digestion can be considered a clean energy source because it is derived from biomass rather than fossil fuels. The CO_2 released during its production is equivalent to the amount absorbed by the biomass throughout its life cycle. As a result, the net emissions are effectively zero.

1.1 Anaerobic digestion process

The process involves a series of complex reactions in which various polymeric substances, such as carbohydrates, proteins, and lipids, are broken down by anaerobic microorganisms, ultimately resulting in the formation of single-carbon molecules like CO_2 and CH_4. Those reactions can be divided in four different stages:

- **Hydrolysis**: in this stage, complex polymeric molecules are hydrolyzed into simple soluble molecules. In particular, proteins are converted into soluble peptides and amino acids, carbohydrates into soluble sugars and lipids into fatty acids and alcohols [1]. The reaction that take place is [2]:

$$(C_6H_{10}O_5)n + nH_2O = n(C_6H_{12}O_6) \tag{1.1}$$

- **Acidogenesis**: at this stage, the soluble compounds formed in the previous phase are converted into volatile fatty acids, carbon dioxide, hydrogen, and other organic

compounds. The most important of these acids is acetic acid (CH_3COOH), which is directly transferred to the final stage of anaerobic digestion, while the others undergo further transformations in the following stage. The various reactions that can occur are [2]:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + CO_2 \tag{1.2}$$
$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \tag{1.3}$$
$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \tag{1.4}$$
$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \tag{1.5}$$
$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \tag{1.6}$$

- **Acetogenesis**: here, the volatile fatty acids with more than two carbon atoms produced during the acidogenesis are converted into acetic acid, hydrogen and carbon dioxide. The reactions happening in the third stage are [2]:

$$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2 \tag{1.7}$$
$$2CH_3CH_2OH + 2CO_2 \rightarrow CH_4 + 2CH_3COOH \tag{1.8}$$
$$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2 \tag{1.9}$$
$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2 \tag{1.10}$$
$$CH_3CHOHCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 2H_2 \tag{1.11}$$

- **Methanogenesis**: in the final stage, acetic acid, hydrogen and carbon dioxide are converted into the biogas. In particular, 66% of methane comes from acetic acids with the acetate decarboxylation and the remaining 34% is formed from carbon dioxide reduction. The final reactions are [2]:

$$CH_3COOH \rightarrow CH_4 + CO_2 \tag{1.12}$$
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \tag{1.13}$$

Acidogenesis and acetogenesis are called as acid formation stage, while methanogenesis is considered the methane formation stage. For each stage, different bacteria consortia are involved in the process. [3]

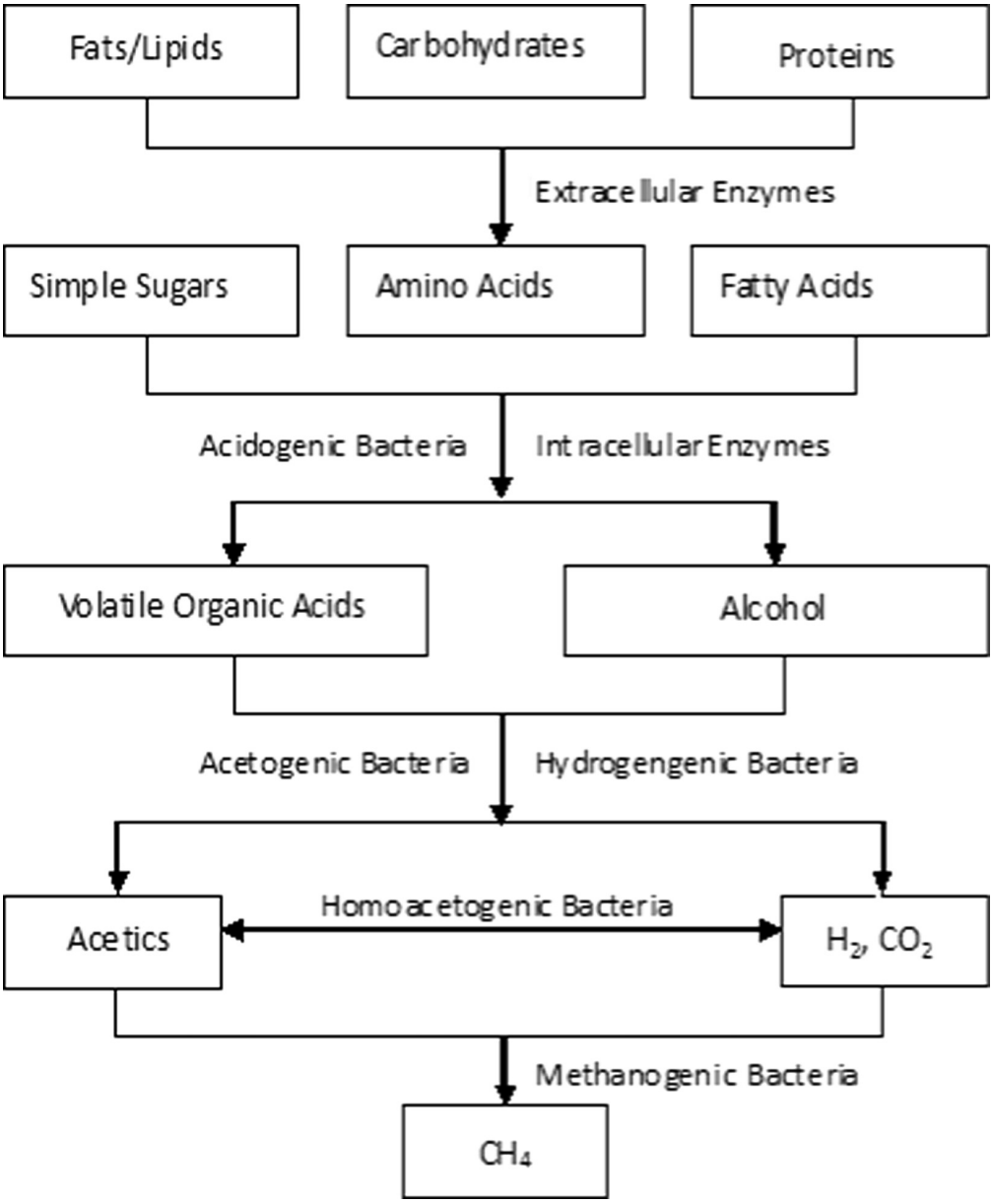


Figure 1.1: Anaerobic digestion process [4]

1.2 Parameters influencing the process

To optimize the performance of anaerobic digestion, certain parameters must be carefully controlled:

- **Temperature**: microorganisms involved in anaerobic digestion are classified according to the temperature range in which they function: psychrophilic (<20°C),

mesophilic (20-45°C), and thermophilic (45-60°C). In general, psychrophilic microorganisms are not used in anaerobic digestion, as the process is not viable at temperatures below 10°C [3]. Higher temperatures accelerate the kinetics of biogas production, making the thermophilic regime more efficient than the mesophilic one in terms of biogas yield. However, during thermophilic anaerobic digestion, acidification can occur, inhibiting biogas production. Other problems include increased energy consumption, greater sensitivity to temperature fluctuations, and the potential formation of toxic byproducts at higher temperatures. On the other hand, mesophilic bacteria exhibit a higher quality effluent and higher stability. Thus, an effective solution to optimize the process could involve conducting the initial stages under thermophilic conditions to capitalize on the enhanced kinetics, while performing methanogenesis under mesophilic conditions to achieve a higher-quality final product. However, this approach necessitates a two-phase, discontinuous process, which may not always be feasible to implement [4].

- **pH**: the optimal pH for the anaerobic digestion process should be maintained between 6.5 and 7.5, indicating a neutral pH level [5]. However, the pH does not remain constant during the process because volatile fatty acids are produced more rapidly than methane, resulting in a decline in the system's pH, which inhibits the activity of methanogens. Consequently, it is necessary to add basic buffering solutions to prevent acidification and maintain system stability [6].

- **C/N ratio**: the C/N ratio reflects the nutrient supply for microorganisms, with carbon-rich compounds (like carbohydrates) and nitrogen (in the form of proteins or ammonium nitrates) serving as their main sources of nourishment [3]. Bacteria consume carbon about 30 times faster than nitrogen, so a high C/N ratio is necessary to meet their nutritional needs [7]. Therefore, in order to optimize the process, a C/N ratio between 16 and 30 would be ideal [3][2]. If the C/N ratio is too high, biogas production decreases because methanogenic bacteria consume nitrogen rapidly for their protein requirements, leaving insufficient nitrogen to react with carbon. On the other hand, if the C/N ratio is too low, excess nitrogen is released as ammonia, causing the pH to become alkaline, which inhibits bacterial activity and slows down the process [2].

- **Organic Loading Rate (OLR)**: in continuous anaerobic digestion, this factor represents the daily input of volatile matter per unit of reactor volume. Intuitively, increasing the Organic Loading Rate (OLR) should boost biogas production. However, this can destabilize the system, as a high OLR accelerates hydrolysis and acidogenesis without a corresponding increase in methanogenesis. As a result, volatile fatty acids (VFA) accumulate, lowering the pH and creating an environment unfavorable for anaerobic digestion [4]. The optimal OLR range is between 0.5 and 2 kg of total volatile solids per unit volume per day [8].

- **Retention time**: in continuous anaerobic digestion, this parameter represents the average retention time. There are two types of retention times: Hydraulic Retention Time (HRT) and Solid Retention Time (SRT) [4]. HRT refers to the duration the substrate stays inside the reactor, and it is calculated using the following formula [9]:

$$HRT = \frac{V}{Q} \tag{1.14}$$

  SRT represents the time that suspended solids, including the microorganisms responsible for anaerobic digestion, remain in the reactor. At a specific temperature, the quantity of substrate that microorganisms can consume is limited. As a result, to digest a certain amount of substrate, an appropriate number of microorganisms must be introduced. The relationship between the amount of substrate and the quantity of microorganisms is referred to as the food-to-microorganism ratio (F/M). A lower F/M ratio results in a greater percentage of the substrate being converted into biogas [2]. The retention time depends on the Organic Loading Rate (OLR), the system temperature, and the substrate composition [4].

- **Mixing/agitation**: mixing is crucial for ensuring the homogeneity and stability of the process. It helps maintain uniform concentrations of all system components, consistent temperature, and overall equilibrium of key parameters throughout the system. It is also useful to prevent sedimentation of the solid [10]. Mixing can be achieved in two ways: by using mechanical stirrers or by recirculating the digester's fluid with centrifugal pumps [11].

1.3 Microalgae

Microalgae are a group of unicellular or simple multicellular microorganisms that are becoming an increasingly viable option for anaerobic digestion due to several advantageous properties [12].

1.3.1 Advantages

Microalgae possess unique characteristics compared to traditional biomasses used so far:

- They produce higher amounts of methane compared to other biomass sources [12].
- They have rapid growth rates with a doubling time < 24h [13].
- They have the ability to better capture atmospheric CO_2 and nutrients than terrestrial plants [14].
- They can thrive in wastewater and seawater, reducing the need for arable land use, without competing with food supply chain [15].

- Very low or negligible percentage of lignin: the presence of lignin, which is abundant in lignocellulosic biomasses, is an inhibiting factor in anaerobic digestion, as it is very difficult to degrade during the hydrolysis process and is toxic to the microorganisms involved in the process [16].

These characteristics give microalgae significant potential as a biomass source.

1.3.2 Cells composition

The microalgae cells have a composition, based on volatile solids (VS), divided as follows [17]:

- 7-69% carbohydrates
- 15-84% proteins
- 1-64% lipids

Additionally, using the Buswell equation

$$\begin{aligned} C_nH_aO_bN_cS_d + \left(n - \frac{a}{4} - \frac{b}{2} + 3\frac{c}{4} + \frac{d}{2}\right) H_2O \rightarrow \\ \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + 3\frac{c}{8} + \frac{d}{4}\right) CO_2 \\ + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - 3\frac{c}{8} - \frac{d}{4}\right) CH_4 \\ + cNH_3 + dH_2S \end{aligned} \tag{1.15}$$

$$\text{Theoretical production} = \frac{n}{2} + \frac{a}{8} - \frac{b}{4} - 3\frac{c}{8} - \frac{d}{4} \ \left[\text{mL}_{CH_4}/\text{g}_{VS}\right] \tag{1.16}$$

the theoretical biodegradability of the components can be calculated [18]:

- Carbohydrates: 415 $\text{mL}_{CH_4}/\text{g}_{VS}$
- Proteins: 496 $\text{mL}_{CH_4}/\text{g}_{VS}$
- Lipids: 1014 $\text{mL}_{CH_4}/\text{g}_{VS}$

The high protein content in microalgae indicates a significant amount of nitrogen, resulting in a low C/N ratio, which is unfavorable for anaerobic digestion. A table showing some examples of composition and C/N ratio for various microalgae species can be seen in table 1.1:

Table 1.1: Composition of different microalgae types [18]

Algal species	Experiments	Composition	Conditions	References
Chlorella vulgaris	Batch	Proteins (35.94% VS) Carbohydrates (20.01% VS) C/N (6.43)	19°C for 25 d	[19]
Chlorella sp	Batch	Proteins (43.43% TS) Carbohydrates (45.15% TS) Lipids (3.42% TS) C/N (4.86)	35°C for 35 d	[20]
C. pyrenoidosa	Batch	Proteins (40.92% TS) Carbohydrates (25.30% TS) Lipids (13.65% TS) C/N (8.27)	36°C for 30 d	[18]
C. minutissima	Batch	Proteins (43.78% TS) Carbohydrates (14.59% TS) Lipids (16.32% TS) C/N (5.89)	36°C for 30 d	[18]

This condition implies that the hydrolysis and anaerobic digestion of microalgae produce ammonia, which can exist in free form (NH_3) or ionic form (NH_4^+) [18]. Non-ionized ammonia can penetrate the cell membrane of microorganisms, affecting their osmotic pressure and thus being even more toxic than the ionized form [21]. Concentration limits can be defined corresponding to specific effects in different experiments:

- A concentration of NH_3-N of 1756-4968 mg/L causes the inhibition of the methanogens responsible for the degradation of the algal biomass [22].
- NH_4^+ concentration below 200 mg/L promotes the growth of anaerobic microorganisms and supports their metabolism [18].
- NH_3 concentration of 560-568 mg/L causes the inhibition of methanogenesis efficiency by 50% at a pH of 7.6 [23].
- A concentration of 785 gNH_4-N/m^3 causes the inhibition of the process for a temperature of 20°C [24].

1.3.3 Cultivation

One major advantage of microalgae is that they can be cultivated near existing industrial plants, eliminating the need for fertile land and avoiding competition with land used for food production. The culture systems can be classified as open or closed systems. The first type is opened to the atmosphere, so it's able to exploit natural resources like atmospheric light; closed systems are also known as photobioreactors: they have higher

initial investment costs, but they enable a controlled environment that promotes the optimal growth of microalgae. The nutrients essential for microalgae growth are primarily macronutrients (carbon, nitrogen, and phosphorus) generally following a C:N:P ratio of around 106:16:1. This ratio can be adjusted depending on the culture medium to optimize growth conditions. Among the nitrogenous compounds beneficial for microalgae growth are nitrites (NO_2^-) and nitrates (NO_3^-), inorganic compounds that are often abundant in wastewater. Likewise, microalgae can absorb phosphorus from wastewater to synthesize essential organic molecules, such as DNA, RNA, ATP, and phospholipid membranes [25].
A drawback of cultivation in wastewater is the high efficiency of microalgae in adsorbing and bioaccumulating heavy metals [26]. Cu, Ni, Zn, and Cd are beneficial for anaerobic digestion within certain limits [27], but above certain concentrations, they produce inhibitory effects on the activity of the microbial consortium. The presence of micropollutants in wastewater above a certain concentration also has an inhibitory effect on anaerobic digestion [18].

1.3.4 Harvesting

There are several types of microalgae harvesting methods, which differ in terms of energy consumption, efficiency, and potential use of chemicals, and they can be summarized in figure 1.2.

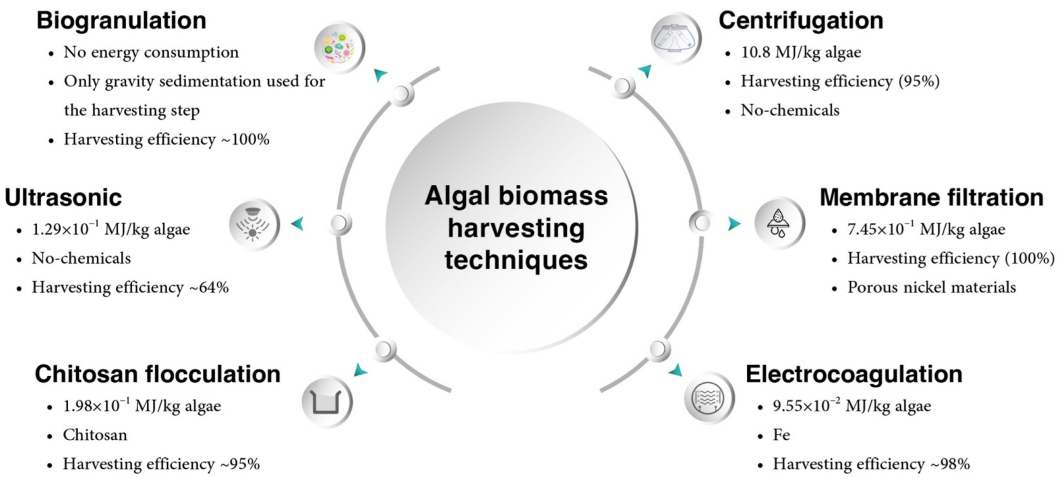


Figure 1.2: Harvesting techniques for microalgal biomass [18]

The choice of appropriate method is depended on microalgae specie, cell density and culture condition. The choice for proper harvesting procedure should be adjusted to the desired product quality. For the low value products gravity sedimentation, sedimentation enhanced by flocculation or settling ponds might be used. For high value products,

continuously operating centrifugation should be used [28]. Sodium hydroxide (NaOH) can be used for harvesting via flocculation [29]. This substance interacts with the carbohydrates and proteins of microalgae, altering their structure. While a minimal concentration of sodium is beneficial for the activity of methanogens, beyond a certain threshold, an inhibitory effect occurs [30].

1.4 Pretreatments

A key factor in determining the quality of microalgae is the digestibility of its cell wall, which is influenced by its composition of cellulose, hemicellulose, and other hard-to-degrade biopolymers. To address this challenge, pretreatment methods, whether physical, chemical, or biological, can be employed [31] [32]. The types of pretreatment commonly applied today include [28]:

- Mechanical: ultrasounds, high pressure homogenization, size reduction and sonication
- Thermal hydrolysis: heat, microwave
- Biological: enzymes
- Chemical: oxidation, alkali treatments, addition of acids, ionic liquids

1.4.1 Ultrasound

Ultrasound is an increasingly used mechanical pretreatment due to its high efficiency, low toxicity, and rapid application speed [33]. Ultrasound are sound waves with frequencies higher than the upper limit of human hearing range, from 20 kHz to 10 MHz [34]. Ultrasonic cell disruption operates through the process of cavitation: energy is transferred into a liquid solution, causing alternating cycles of compression and rarefaction that produce pressure fluctuations. During rarefaction, when the pressure drops below the vapor pressure, bubbles form and grow over successive cycles. As these bubbles enlarge to a point where the ultrasonic energy can no longer contain the vapor inside, they collapse violently during compression, releasing substantial energy. This energy from cavitation is what ultimately causes the rupture of microalgae cell walls [35] [36].

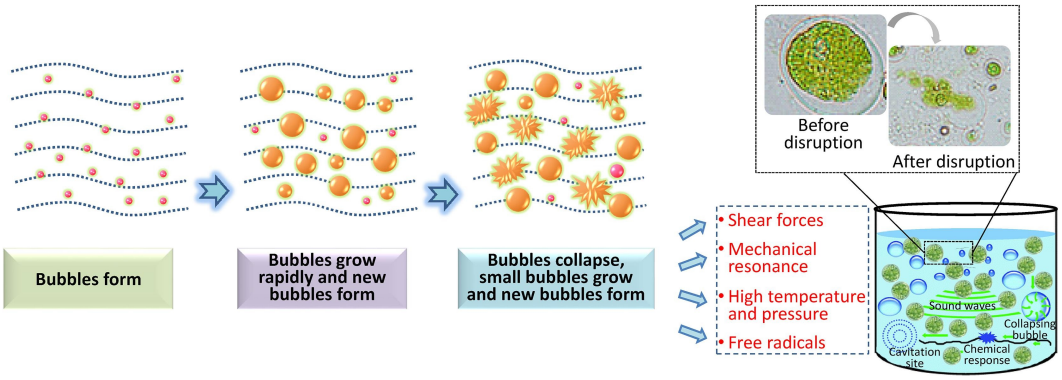
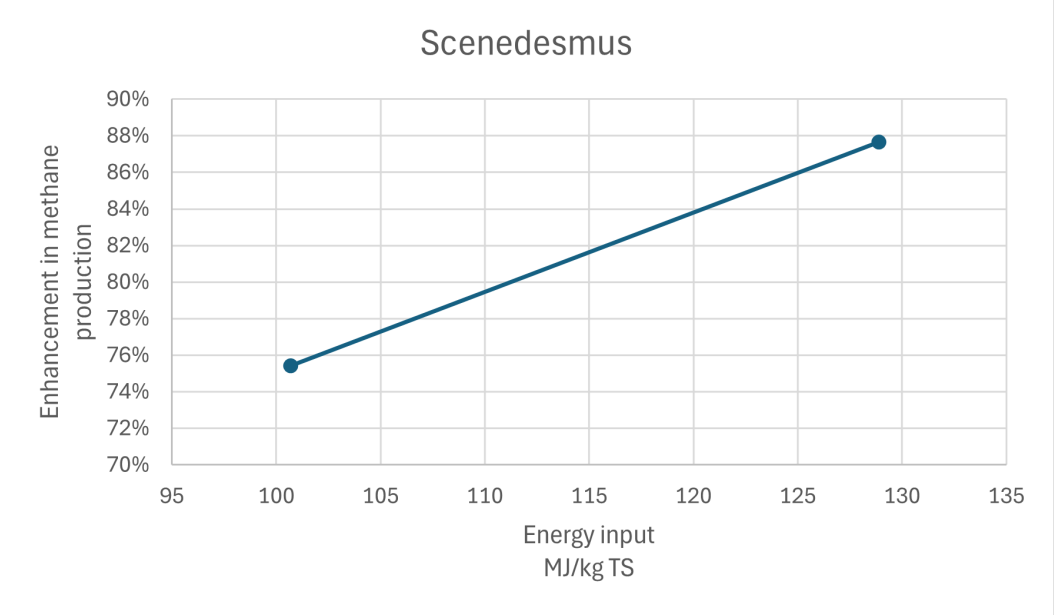
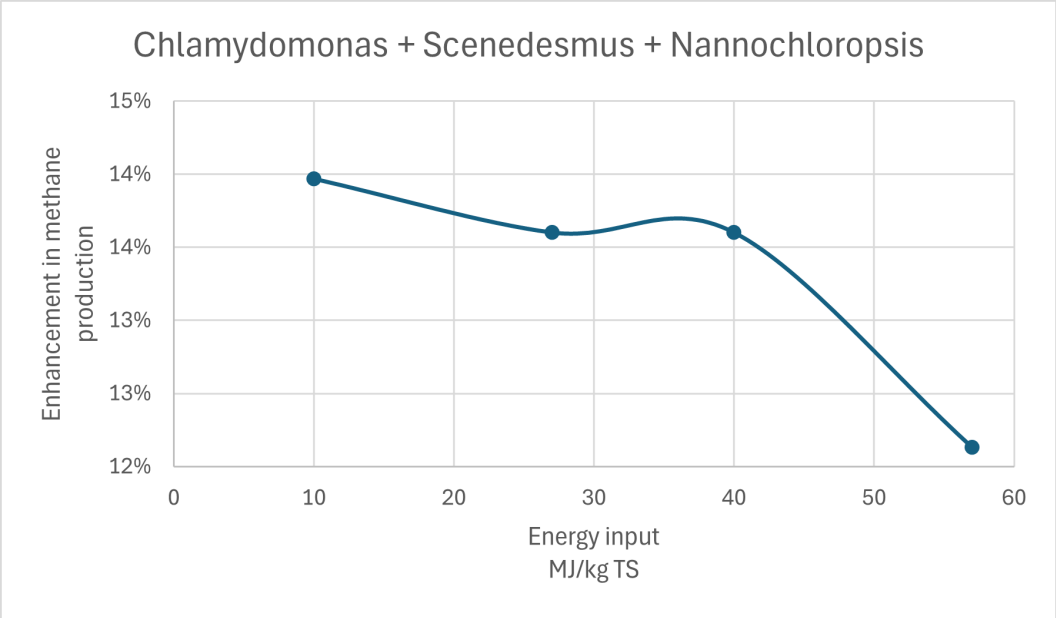
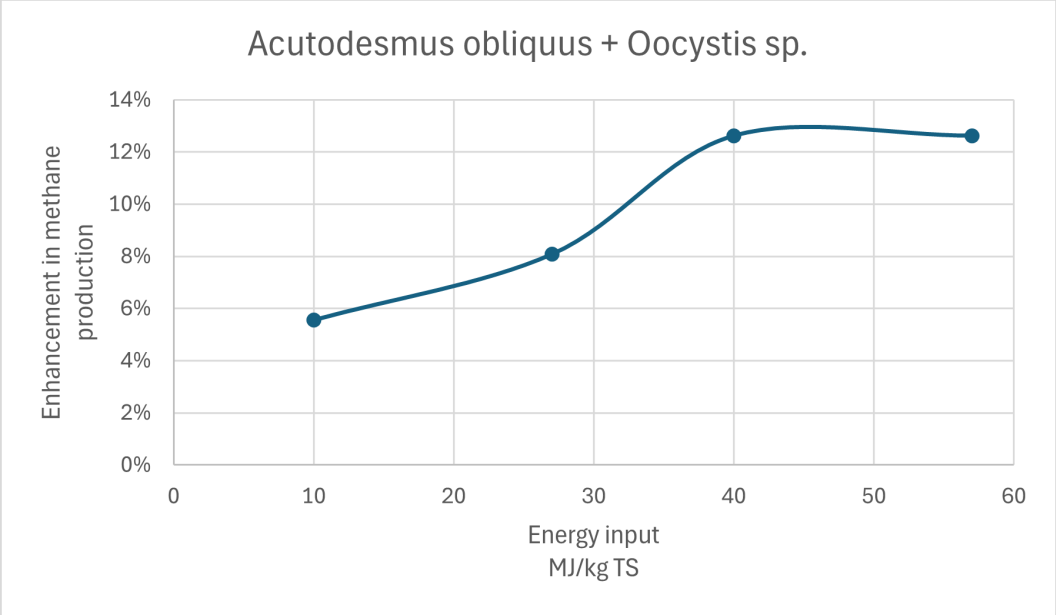


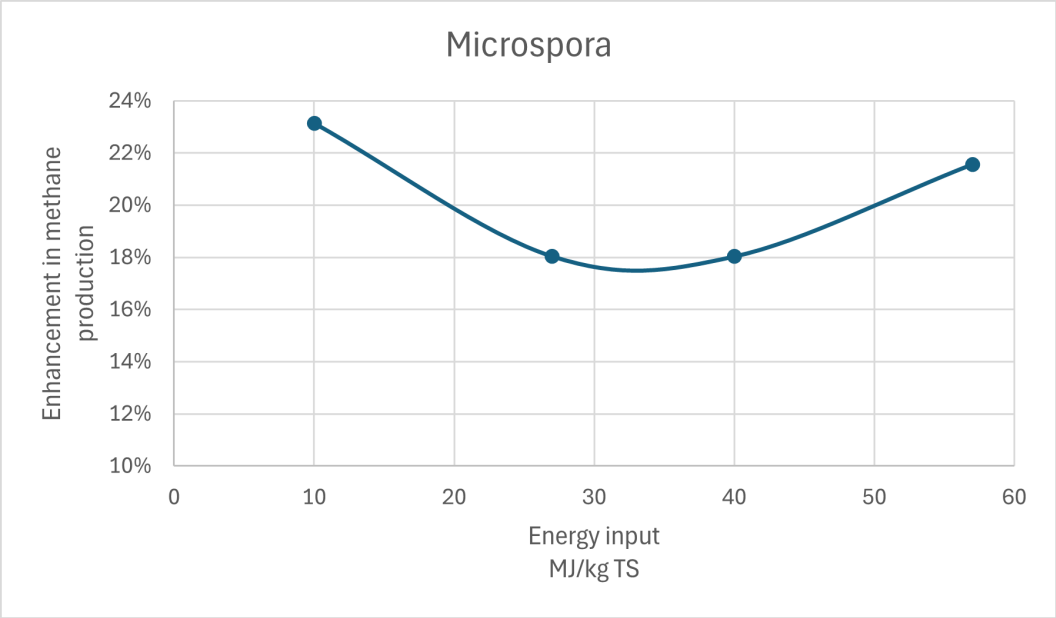
Figure 1.3: Ultrasonic mechanisms towards cell disruption [36]

The table 1.2 provides a summary of key findings from the literature on the anaerobic digestion of microalgae treated with ultrasonic pretreatment. For the mixtures of different species of microalgae, the percentages are refered to the dry masses.
As shown, the results for different biomass types are not directly comparable due to their strong dependence on the unique characteristics of each species. Consequently, results for the same microalgae species were compared across different input energy levels. The graphs were produced after a thorough analysis of the articles listed in the table, with the energy units standardized to MJ/kg TS, and they show the relationship between methane production improvement due to pretreatment application and the pretreatment intensity.




Chlamydomonas + Scenedesmus + Nannochloropsis
Enhancement in methane production
15%
14%
14%
13%
13%
12%
0
10
20
30
40
50
60
Energy input
MJ/kg TS

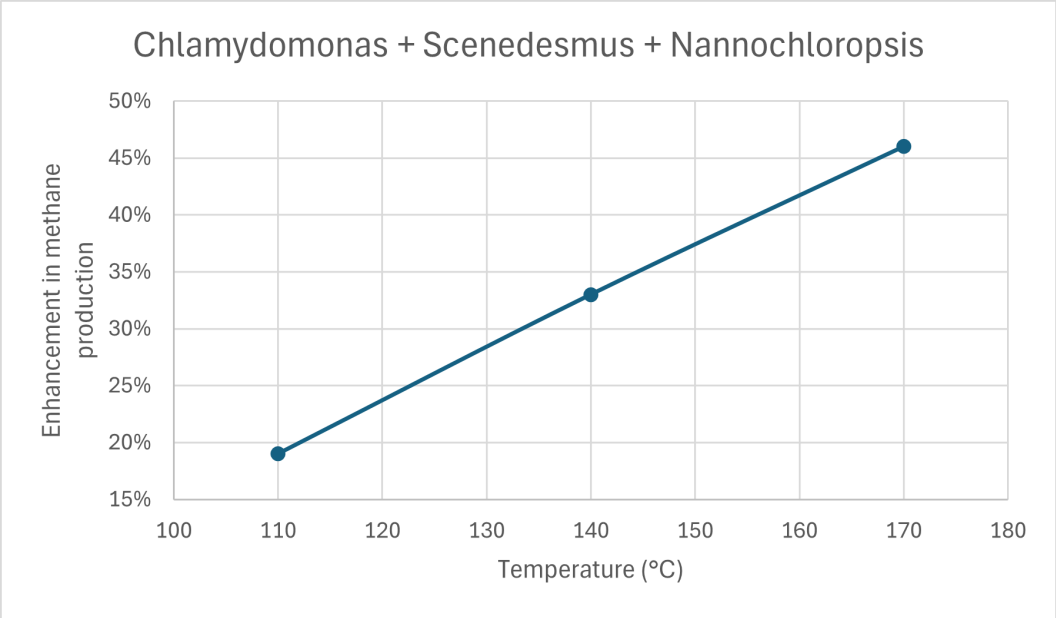

Acutodesmus obliquus + Oocystis sp.
Enhancement in methane production
14%
12%
10%
8%
6%
4%
2%
0%
0
10
20
30
40
50
60
Energy input
MJ/kg TS



The figures demonstrate that ultrasonic pretreatment does not consistently enhance methane production, nor does it follow a predictable linear or monotonic trend. This indicates that the impact of pretreatment on a specific microalgae species cannot be reliably predicted without first conducting experimental trials.

1.4.2 Thermal pretreatment

Thermal pretreatments increase the solubilization of specific organic fractions and improve the hydrolysis of the polymeric molecules that form the cell wall. Heat breaks the hydrogen bonds within the crystalline structures of cellulose and hemicellulose. These pretreatments are classified into two types: those conducted below 100°C at atmospheric pressure and those conducted above 100°C (up to 300°C) under higher pressure. Pretreatments using an aqueous solvent at temperatures above 100°C are known as hydrothermal [42] [43]. Typically, a temperature between 100°C and 140°C is applied with pressures ranging from 1 to 2 bar. Higher temperatures may trigger the Maillard reaction, leading to the formation of recalcitrant compounds [44]. Some results from the literature on the effects of hydrothermal pretreatment are summarized in table 1.3. Also in this case, the percentages of the mixtures are referred to the dry masses.
In this case as well, it can be observed that increasing pretreatment intensity does not always lead to higher methane production. Specifically, for the species *Microspora*, as the biomass is subjected to higher temperatures, the improvement in methane production diminishes. That means that, also for thermal pretreatment, experimental tests are necessary in order to predict the result obtained after a certain pretreatment.


Chlamydomonas + Scenedesmus + Nannochloropsis
Enhancement in methane production
50%
45%
40%
35%
30%
25%
20%
15%
100
110
120
130
140
150
160
170
180
Temperature (°C)

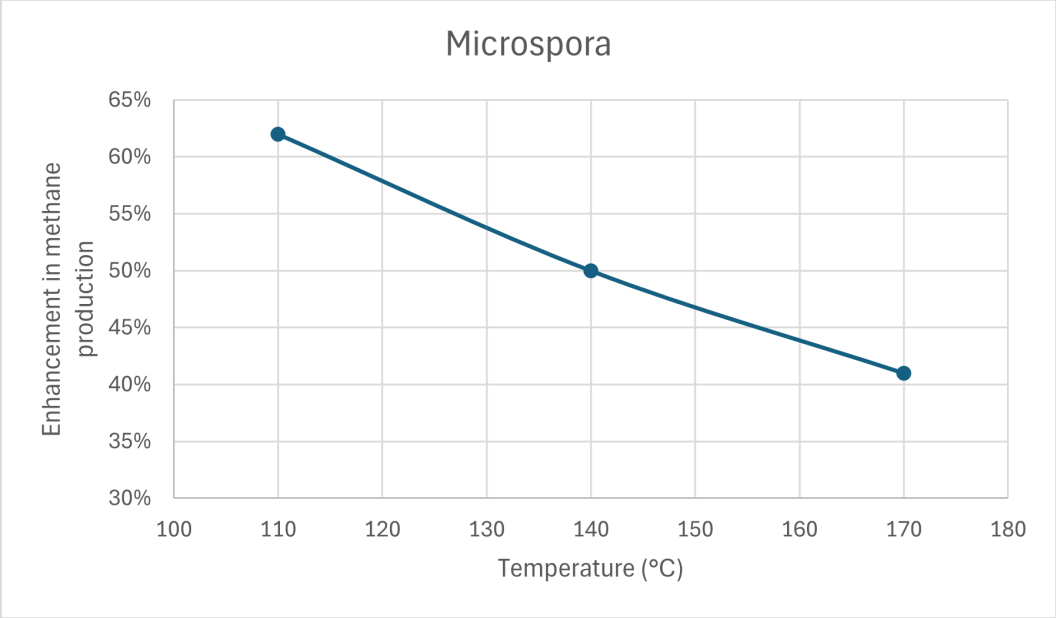

Microspora
Enhancement in methane production
65%
60%
55%
50%
45%
40%
35%
30%
100
110
120
130
140
150
160
170
180
Temperature (°C)

Table 1.2: Findings in literature on anaerobic digestion of microalgae after ultrasonic pretreatment

Strain	Reactor type conditions	Operating conditions	Biogas/methane production before pretreatment	Biogas/methane production after pretreatment	Enhancement	Ref
Spirulina maxima	60 days (35°C, semi-continuous)	10 min - Polytron Generator	0.19 L CH_4/g VS	0.17 L CH_4/ g VS	-11.8%	[37]
Scenedesmus sp.	34 days (35°C, batch)	130 MJ/kg - 30 min	0.0818 L CH_4/g COD	0.1535 L CH_4/g COD	+88%	[38]
H. reticulatum (filamentous algae)	45 days (35°C, batch)	50-5000 J/mL	0.170 L CH_4/g VS	0.310-0.350 L CH_4/g VS	+82.3-105.9%	[39]
Chlorella vulgaris	25 days (35°C, batch)	200 J/mL	0.230 L CH_4/g VS	0.440 L CH_4/g VS	+91%	[40]
40% Chlamydomonas 20% Scenedesmus 40% Nannochloropsis	60 days (35°C, batch)	10 MJ/kg TS	0.272 L CH_4/g VS	0.310 L CH_4/g VS	+14%	[41]
40% Chlamydomonas 20% Scenedesmus 40% Nannochloropsis	60 days (35°C, batch)	27 MJ/kg TS	0.272 L CH_4/g VS	0.309 L CH_4/g VS	+14%	[41]
40% Chlamydomonas 20% Scenedesmus 40% Nannochloropsis	60 days (35°C, batch)	40 MJ/kg TS	0.272 L CH_4/g VS	0.309 L CH_4/g VS	+14%	[41]
40% Chlamydomonas 20% Scenedesmus 40% Nannochloropsis	60 days (35°C, batch)	57 MJ/kg TS	0.272 L CH_4/g VS	0.305 L CH_4/g VS	+12%	[41]
58% Acutodesmus obliquus 36% Oocystis sp. 1% Phormidium 5% Nitzschia sp.	60 days (35°C, batch)	10 MJ/kg TS	0.198 L CH_4/g VS	0.209 L CH_4/g VS	+6%	[41]
58% Acutodesmus obliquus 36% Oocystis sp. 1% Phormidium 5% Nitzschia sp.	60 days (35°C, batch)	27 MJ/kg TS	0.198 L CH_4/g VS	0.214 L CH_4/g VS	+8%	[41]
58% Acutodesmus obliquus 36% Oocystis sp. 1% Phormidium 5% Nitzschia sp.	60 days (35°C, batch)	40 MJ/kg TS	0.198 L CH_4/g VS	0.223 L CH_4/g VS	+13%	[41]
58% Acutodesmus obliquus 36% Oocystis sp. 1% Phormidium 5% Nitzschia sp.	60 days (35°C, batch)	57 MJ/kg TS	0.198 L CH_4/g VS	0.223 L CH_4/g VS	+13%	[41]
Microspora	60 days (35°C, batch)	10 MJ/kg TS	0.255 L CH_4/g VS	0.314 L CH_4/g VS	+23%	[41]
Microspora	60 days (35°C, batch)	27 MJ/kg TS	0.255 L CH_4/g VS	0.301 L CH_4/g VS	+18%	[41]
Microspora	60 days (35°C, batch)	40 MJ/kg TS	0.255 L CH_4/g VS	0.301 L CH_4/g VS	+18%	[41]
Microspora	60 days (35°C, batch)	57 MJ/kg TS	0.255 L CH_4/g VS	0.31 L CH_4/g VS	+22%	[41]

Table 1.3: Findings in literature on anaerobic digestion of microalgae after hydrothermal pretreatment

Strain	Reactor type conditions	Operating conditions	Biogas/methane production before pretreatment	Biogas/methane production after pretreatment	Enhancement	Ref
40% Chlamydomonas 20% Scenedesmus 40% Nannochloropsis	60 days (35°C, batch)	110°C, 1 bar, 15 min	0.272 L CH_4/g VS	0.323 L CH_4/g VS	+19%	[41]
40% Chlamydomonas 20% Scenedesmus 40% Nannochloropsis	60 days (35°C, batch)	140°C, 1.2 bar, 15 min	0.272 L CH_4/g VS	0.362 L CH_4/g VS	+33%	[41]
40% Chlamydomonas 20% Scenedesmus 40% Nannochloropsis	60 days (35°C, batch)	170°C, 6.4 bar, 15 min	0.272 L CH_4/g VS	0.398 L CH_4/g VS	+46%	[41]
58% Acutodesmus obliquus 36% Oocystis sp. 1% Phormidium 5% Nitzschia sp.	60 days (35°C, batch)	110°C, 1 bar, 15 min	0.198 L CH_4/g VS	0.219 L CH_4/g VS	+11%	[41]
58% Acutodesmus obliquus 36% Oocystis sp. 1% Phormidium 5% Nitzschia sp.	60 days (35°C, batch)	140°C, 1.2 bar, 15 min	0.198 L CH_4/g VS	0.26 L CH_4/g VS	+31%	[41]
58% Acutodesmus obliquus 36% Oocystis sp. 1% Phormidium 5% Nitzschia sp.	60 days (35°C, batch)	170°C, 6.4 bar, 15 min	0.198 L CH_4/g VS	0.307 L CH_4/g VS	+55%	[41]
Microspora	60 days (35°C, batch)	110°C, 1 bar, 15 min	0.255 L CH_4/g VS	0.413 L CH_4/g VS	+62%	[41]
Microspora	60 days (35°C, batch)	140°C, 1.2 bar, 15 min	0.255 L CH_4/g VS	0.382 L CH_4/g VS	+50%	[41]
Microspora	60 days (35°C, batch)	170°C, 6.4 bar, 15 min	0.255 L CH_4/g VS	0.359 L CH_4/g VS	+41%	[41]
70% Chlorella sp. 30% Scenedesmus sp.	23 days (37°C, batch)	120°C, 30 min	0.336 L CH_4/g VS	0.405 L CH_4/g VS	+20%	[45]
Nannochloropsis salina	40 days (38°C, batch)	100°C, 8h	0.347 L biogas/g VS	0.549 L biogas/g VS	+58%	[46]

1.5 Co-digestion

Due to the high protein content in microalgae, which leads to a low C/N ratio, co-digestion with carbon-rich biomass can effectively raise the net C/N ratio, enhancing the efficiency of anaerobic digestion [47]. Co-digestion of microalgae not only achieves a favorable C/N ratio but also boosts biogas production by enhancing process stability, increasing biomass loading capacity, diluting inhibitors (such as salts, polyphenols, and sulfated polysaccharides), providing buffer capacity to the digestate, balancing nutrients, and creating net synergistic effects [48]. Improvements in biogas or methane production can vary between 4% and 260% [12], depending on the microalgae species and co-digestion system used, as highlighted in several studies from the literature in table 1.4.

Table 1.4: Findings in literature on co-digestion of microalgae and other biomasses

Co-digestion system	Substrates load in co-digestion	Improvement by co-digestion	Ref
Chlorella sp. and chicken manure	Mass ratio of chicken manure to Chlorella 8:2	Methane yield improved by 22.88%	[49]
Mixed microalgae and wheat straws	50% of microalgae + 50% of wheat straw	Methane yield improved by 77%	[50]
Scenedesmus sp. and pig manure	50% VS of *Scenedesmus sp.* + 50% VS of pig manure	Methane yield improved by 50%	[51]
Arthrospira platensis and seaweed	15% VS of *A. platensis* + 85% of seaweeds	No significant change in methane yield	[47]
Lipid extracted *Chlorella sp.* and lipid-rich fat	50% of *Chlorella* biomass + 50% of fat	Methane yield increased by 260%	[52]

Chapter 2

Materials and methods

The study presents two main objectives:

- To obtain preliminary results on the effect of co-digestion between microalgae and chicory roots on methane production.
- To study the effect of pretreatments (hydrothermal and ultrasonic) on methane production from microalgae.

To achieve this, three different BMP tests were conducted to evaluate all the analyzed conditions. The considered pretreatments are:

- Ultrasound: 400W for 5 min (120 kJ)
- Ultrasound: 400W for 10 min (240 kJ)
- Ultrasound: 400W for 15 min (360 kJ)
- Hydrothermal: 120°C for 15 min
- Hydrothermal: 140°C for 15 min

Co-digestion was performed in combination with chicory roots in different proportions:

- 25% VS microalgae - 75% VS chicory roots
- 50% VS microalgae - 50% VS chicory roots
- 75% VS microalgae - 25% VS chicory roots

Additionally, an elementary analysis test was conducted to evaluate the elemental composition of the biomasses used and thus the stoichiometric methane production that would be obtained under ideal conditions. This was useful to measure the degradability of the biomasses in relation to each type of treatment.

2.1 AMPTS

The BMP test (Biochemical Methane Potential), or Methane Potential Test, allows determining the maximum biogas production resulting from the degradation of a biomass sample by anaerobic bacteria. The biogas production rate is determined by measuring the volume of produced gas over time. In order to evaluate the BMP, we use the Automatic Methane Potential Test System II (AMPTS II, Bioprocess Control, Lund, Sweden): it follows the same measuring principles as conventional instruments for gas volume measurements, which make the analysis comparable with standard methods. The analysis and data recording are automatic during the incubating period.

Figure 2.1: AMPTS III [53]

It is composed of different units:

- UNIT A - *sample incubation unit*: in this unit, 15 vessels contain small amounts of the biomass with suitable microbial inoculum and are incubated at the desired temperature (mesophilic conditions) in a thermostatic water bath. The temperature was maintained at a constant value of 37.0°C.

- UNIT B - *Gas-absorption unit*: the gas produced in each bottle passes through an individual vial containing a solution that can absorb certain fractions of the gas. In this case, an alkaline solution (NaOH, 3 mol/L) is used in order to retain acid fractions like CO_2 and H_2S, so that only CH_4 (and other remaining traces like H_2) will pas through the gas monitoring unit. Thymolphthalein (2',2"-Dimethyl-5,5"-di-iso-propylphenolphthalein, $C_{28}H_{30}O_4$, CAS 125-20-2, ACS reagent, dye content 95%) is used as pH indicator to each vial to control the acid binding capacity of the solution.

- UNIT C - *Gas volume measuring device*: the volume of gas released is measured using a wet gas flow measuring device with a multi-flow cell arrangement (15 cells). It works according to the principle of liquid displacement and buoyancy and

monitor ultra-low gas flows. An integrated embedded data acquisition system is used to record, display and analyse the results.

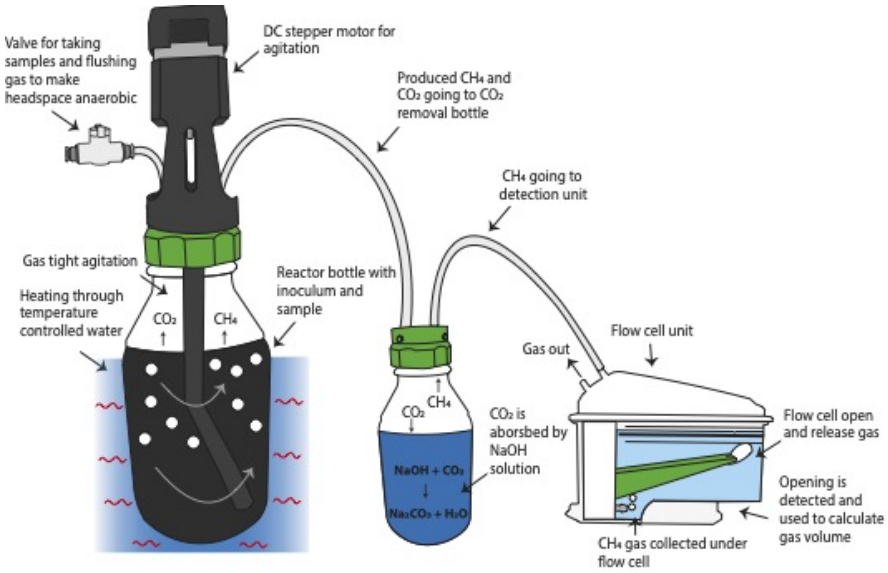


Figure 2.2: Operating diagram [53]

2.2 CHNS/O

The CHNS/O elemental analysis is used to determine the quantities of carbon, hydrogen, nitrogen and sulfur (CHNS) or oxygen (O) present in a solid or liquid sample. To do this, the Flash 2000 machine (ThermoFisher, Massachussets, USA) was used, consisting of one column for the CHNS test and another for measuring the percentage of oxygen.

2.2.1 CHNS analysis

The sample (of a mass between 0.5 and 1.5 mg) is put in a tin container (Ø 5 mm x H 8 mm, 157 μ L) with Vanadium Pentoxide (V_2O_5) as catalizer and is injected into a combustion tube in a furnace heated to 950°C under excess oxygen.

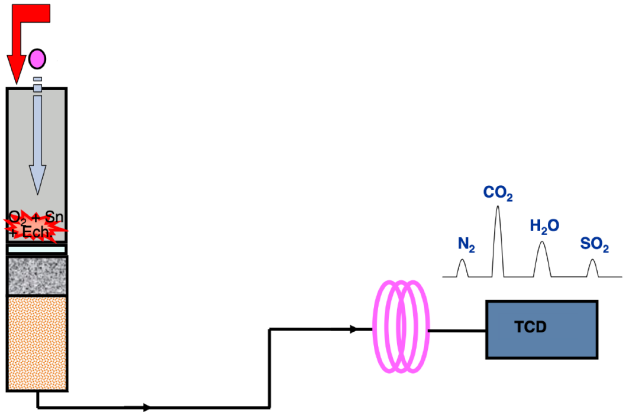


Figure 2.3: Diagram of the principle of CHNS analysis

The sample is injected when there's enough oxygen concentration and goes into the oven under a helium flow and undergoes a flash combustion. Then there is additional catalytic oxidation, reduction of NO_x to N_2 and SO_3 to SO_2, and fixation of excess O_2. After the catalytic conversion, the gas flow is composed of N_2, CO_2, H_2O and SO_2. The gases are then separated on a gas chromatographic (GC) elution column, detected by a Thermal Conductivity Detector (TCD), then quantified by integration of the corresponding peaks.

2.2.2 Oxygen analysis

The sample (of mass between 0.5 and 1.5 mg), put in a silver container (Ø 4 mm x H 6 mm, 75 μL), undergoes catalytic pyrolysis in the absence of oxygen in a tube heated to 1065°C. The oxygenated compounds are converted to CO, and other compounds to N_2, H_2 and CH_4.

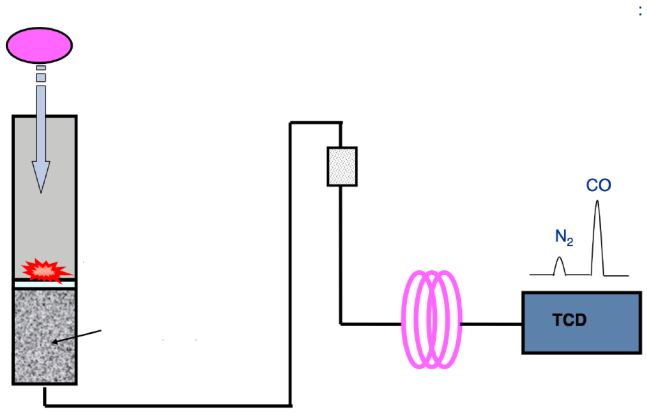


Figure 2.4: Diagram of the principle of oxygen analysis

The gases are separated on a gas chromatographic (GC) elution column and CO is quantified with the same method as before.
The machine is controlled via the "EAGER Xperience for Flash" software, which allows the selection of the operating mode depending on whether the CHNS test or the oxygen test needs to be performed.

For CHNS analysis, the samples are placed in tin containers with a small amount of vanadium pentoxide, while for the oxygen test, silver containers are used without the addition of any catalyst.

Figure 2.5: CHNSO machine

2.3 Experimental procedure

To carry out the experiment, 3 BMP test were conducted. For the first one, an AMPTS III machine were used at UTC (Compiègne), while for the other two tests, two AMPTS II machines were used at the UniLaSalle Polytechnic Institute facility (Beauvais). Each AMPTS machine has 15 bottles, so a total of 45 bottles were available for the test. This setup allowed each condition to be tested in duplicate or even triplicate, helping to avoid measurement issues and providing an uncertainty value. The utilized inoculum is from the Chemin du roi in Saint Crépin Ibouvillers (60), processing silages, beet co-products, animal waste, and it provides all the anaerobic microorganisms necessary for methanation. It was filtered through a sieve with a mesh size of 2.5 mm. The chosen microalgae species is Spirulina, it was purchased from Spiru'force (Hyeres, France) and it has been stored by freezing at -18°C.

2.3.1 Dry mass and volatile mass measurement

The first step before planning experimental activities is to measure the dry mass (DM) and volatile mass (VM) of each substrate, including the inoculum. This is necessary because each substrate has a different chemical composition and moisture content. For instance, organic compounds such as carbohydrates, proteins, and lipids tend to volatilize at high temperatures, whereas mineral substances remain. This enables optimization of the process and allows for more accurate prediction of its behavior. To measure the dry mass, the samples were dried overnight in an oven at 105°C. For the volatile mass, the samples were placed in a furnace at 550°C for 2 hours. The volatile mass is calculated as the difference between the dry mass and the mass remaining after heating in the furnace, so it represents a percentage of the dry mass. The tests were conducted in triplicates. For the inoculum, the value of VM was taken from previous tests made on the same sample, so a value of standard deviation is not available. The measurement for the microalgae solution was conducted on a single sample, so no uncertainty value is available for it. The values of dry mass and volatile mass obtained are represented in table 2.1.

	Dry mass	Volatile mass
Inoculum	9.5% $\pm$ 0.1%	66.0% $\pm$ 0.4
Raw microalgae Compiègne	28.2% $\pm$ 0.7%	91.1% $\pm$ 2.2
Raw microalgae Beauvais	21.3 $\pm$ 0.1%	91.6 $\pm$ 0.1%
Chicory roots	11.3 $\pm$ 0.5%	88.1 $\pm$ 5.4%
Microalgae solution	12.4%	91.6%

Table 2.1: Dry masses and volatile masses

The raw microalgae used in the Compiègne and Beauvais tests were purchased together.

However, based on visual observations during the experiment, it was decided to repeat the dry mass and volatile mass tests, which resulted in different values.

2.3.2 AMPTS Compiègne

In this test, all the different conditions for the microalgae treatment were analyzed:

- Raw microalgae
- Ultrasound: 400W for 5 minutes (120 kJ)
- Ultrasound: 400W for 10 minutes (240 kJ)
- Hydrothermal: 120°C for 15 minutes
- Hydrothermal: 140°C for 15 minutes

Prior to pretreatment, it was necessary to prepare an aqueous solution of microalgae. The microalgae, stored by freezing, were thawed and added to water until achieving a solution with a 12.4% dry mass concentration.
For ultrasound treatment, Dr. Hielscher Ultrashall prozessor with a frequency of 20 kHz was used. During the pretreatment, an increase in temperature was observed, but no control or measurement of the reached temperature was performed. An initial dilution was required to ensure effective processing. A highly concentrated solution hinders proper ultrasound propagation and uniform pretreatment throughout the solution volume. Hence, 100 mL of water was added to reduce the solid mass concentration. Subsequently, vacuum evaporation was employed to restore the desired initial concentration by evaporating the previously added 100 mL of water. The treatment was conducted under continuous power flow.
For the hydrothermal treatment, a high-temperature, high-pressure mechanically stirred reactor of the Paar type (Illinois, USA) was utilized. To reach 120°C, the oven was initially turned off at 60°C, after which the temperature continued to rise until reaching the setpoint temperature. Instead, for reaching 140°C, the oven was turned off at 80°C.
In addition to the BMP of the substrate, blank assays (background methane production from the inoculum) and positive controls with cellulose are conducted (theoretical BMP =414 NL_{CH_4}/kg_{VS}). The BMP of the substrate and the positive control are determined by subtracting the methane production of the blanks from the total methane production of the substrate/positive control assays [54].
For the preparation of the mini-reactors, specific conditions were chosen:

- A total volume of 350 mL was selected to ensure 150 mL of headspace; a volume of 400 mL was avoided since, from previous experience, there's the risk of foaming formation that contaminates NaOH units

- An inoculum/substrate ratio=3

An inoculum/substrate ratio of 3 means that the volatile solid mass of the inoculum must be three times that of the substrate. The choice of the inoculum/substrate ratio comes from some considerations: a high concentration of solids in the substrate complicates mixing and can lead to the accumulation of fatty acids, which in turn lowers the pH and creates an unfavorable environment for microorganisms. Additionally, the buildup of ammoniacal nitrogen from microalgae contributes to toxicity, further inhibiting microbial activity. On the other hand, larger digester volumes and higher process heating costs may be necessary in case of too low substrate concentration, in order to avoid the wash-out of the biomass. In conclusion, an intermediate value of 3 was chosen. [55]
After adding an equal volume of 250 mL of inoculum to each bottle and the respective masses of substrate to maintain the inoculum/substrate ratio, water was added to achieve a total volume of 350 mL. The same total volume was applied for all the conditions tested.
So, after calculating all the masses to be added, the bottles were prepared with the quantities specified in the table 2.2.

2.3.3 AMPTS 1 Beauvais

This test was conducted to assess the BMP of chicory roots and raw microalgae, along with an additional pre-treatment condition: ultrasound at 400W for 15 minutes. During the experiment, visual observation revealed physical properties of the microalgae aqueous solution that differed from those of the solution used in the tests conducted in Compiègne, such as reduced adhesion to the walls and lower viscosity. This led to the hypothesis of a lower DM and VM content compared to the previous sample. New measurements of these parameters were therefore conducted to obtain the actual inoculum/substrate ratio used. The result of these new tests, reported in the table 2.1, shows an effective inoculum/substrate ratio closer to 4. In this case as well, cellulose was used as a positive control, and the inoculum alone served as the negative control.

2.3.4 AMPTS 2 Beauvais

In this latest test, the BMP of mixtures of microalgae and chicory roots were tested in various proportions, alongside the positive and negative controls. The masses of the substrates to be added were calculated based on their dry masses and volatile masses. The selection of the co-digestion ratios to be examined was made in the same way as in article [56]. For example, for the mix containing 25% microalgae and 75% chicory roots, given the desired amount of total volatile substrate mass, the formulas to calculate the masses to be added are as follows:

$$m_{\mu\text{algae}_{25\%}} = 0.25 \cdot \frac{VM_{\text{desired}}}{DM_{\mu\text{algae}} \cdot VM_{\mu\text{algae}}} \tag{2.1}$$

$$m_{\text{chicory}_{75\%}} = 0.75 \cdot \frac{VM_{\text{desired}}}{DM_{\text{chicory}} \cdot VM_{\text{chicory}}} \tag{2.2}$$

Therefore, the composition of the bottles for this test is summarized in the table 2.4. The masses of microalgae and chicory roots for the different mixes are summarized in the table 2.5.

2.4 Data treatment

The Gas Endeavour software calculates the biogas production rate by measuring the volume of gas produced over time. The results can be exported as an Excel file, containing the normalized volume (NmL) values of methane produced in each mini-reactor. Data collection can be halted once the values stabilize and no longer show significant changes. The stabilization condition, where x_i means the cumulative biomethane production on day i, is

$$x_{i+1} - x_i < \frac{1}{100} \cdot x_i \tag{2.3}$$

The raw results are difficult to interpret and evaluate qualitatively, thus requiring further processing.
The first step in data processing is to subtract the inoculum's contribution (negative control) from the methane production. This is done by calculating the daily average volume of methane produced by the two inoculum-only bottles and subtracting this average from the production values of all other bottles.
Subsequently, the values are normalized relative to the volatile organic matter content in their respective bottles. Each production value is divided by the corresponding VM value.
The final step is to average the trials with the same substrate and calculate the standard deviation thanks to the corresponding Excel function, thus providing a measure of uncertainty. With these values, it is possible to calculate the coefficient of variation (CV), which is defined as:

$$CV = \frac{\text{Standard deviation}}{\text{Average value}} \cdot 100 \tag{2.4}$$

At this point, the final values of mean, standard deviation and coefficient of variation have been obtained.

2.4.1 Gompertz model

The kinetic model of methane production from the analyzed substrates is described using the Gompertz model due to its ability to effectively capture the microbial growth dynamics, including the lag phase at the start of the process [57]. This initial lag phase reflects the period during which microorganisms adapt to the environmental conditions, prior to entering the exponential growth phase, where microbial activity and methane production increase rapidly. Eventually, the system reaches a stationary phase as substrate availability decreases, leading the Gompertz curve to plateau at its maximum value. Additionally, the Gompertz model is relatively simple to apply, requiring only a few parameters, and offers greater flexibility compared to other models like the first-order kinetic or logistic models. While these alternatives may describe the exponential growth phase well, they are less effective in capturing the transitions from the lag phase to exponential growth and from exponential growth to the stationary phase.

$$V = V_{max} \cdot exp\left(-exp\left(\frac{R_{max} \cdot exp(1) \cdot (\text{Lag phase} - t)}{V_{max}} + 1\right)\right) \tag{2.5}$$

In the equation, the parameters represent:

- V is the methane production at time t [g/L]
- V_{max} is the maximum methane production [g/L]
- R_{max} is the maximum growth rate [h^{-1}]
- *Lag phase* is the initial period of adaptation before exponential growth begins [h]
- t is the time at which the methane production V is measured [h]

The quality of the approximation by the Gompertz model is represented by the parameters:

- Coefficient of determination R^2: it indicates how well the Gompertz model approximates the observed data points. R^2 ranges from 0 to 1, where 1 indicates a perfect fit.
- MAPE (Mean Absolute Percentage Error): represents the average percentage of absolute errors between predicted values and actual observed values, expressed as a percentage. It is a measure of relative accuracy, indicating the average percentage deviation of predictions from actual values. A lower MAPE indicates better predictive accuracy of the model, as it reflects a lower average percentage error between predictions and observed data.

The model can be analyzed using a dedicated Scilab script that, using the volumetric methane production values over time (in days) as input, applies the Gompertz function to determine the key parameters of the model.

2.4.2 Elemental analysis

The final stage of data analysis is the elemental analysis, carried out using the CHNS/O test. The output from the Flash 2000 instrument is an Excel file that reports, for each sample analyzed, the mass composition of nitrogen, carbon, hydrogen, sulfur, oxygen, and proteins. Moreover, the software automatically calculates the mean and standard deviation for measurements taken from different samples of the same substance. According to stoichiometry, the methanation reaction for a generic organic component is

$$\begin{aligned} C_nH_aO_bN_cS_d + \left(n - \frac{a}{4} - \frac{b}{2} + 3\frac{c}{4} + \frac{d}{2}\right) H_2O \rightarrow \\ \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + 3\frac{c}{8} + \frac{d}{4}\right) CO_2 \\ + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - 3\frac{c}{8} - \frac{d}{4}\right) CH_4 \\ + cNH_3 + dH_2S \end{aligned} \tag{2.6}$$

Thus, based on the stoichiometric coefficients of the various components within the starting biomass, it is possible to calculate the theoretical methane production that would be obtained under ideal conditions of anaerobic digestion and, therefore, the efficiency of the reaction in our specific case. To determine the theoretical production of the reaction based on the mass composition detected by the machine, the following steps are required:

- divide all mass compositions by the molar mass of the respective component in order to convert them into a molar composition;
- divide the molar composition values by the smallest among them to find the stoichiometric coefficients;
- calculate the theoretical methane yield using the Buswell's equation:

$$\text{Theoretical production} = \frac{n}{2} + \frac{a}{8} - \frac{b}{4} - 3\frac{c}{8} - \frac{d}{4} \ \ [\text{mol/mol}_{biomass}] \tag{2.7}$$

- divide by the molar mass of the biomass and multiply by the molar volume to obtain the same unit of measurement as the experimental yield [$\text{NmL}_{CH_4}/\text{g}_{DM}$].

A calculation of the propagation of standard deviation was also conducted to derive the standard deviation associated with the theoretical production, based on the values from the individual measurements. To achieve this, the rules of error propagation for addition/subtraction and multiplication/division were taken into account:

- Z = X + Y or Z = X - Y

$$\sigma_z = \sqrt{\sigma_x^2 + \sigma_y^2} \tag{2.8}$$

- $Z = X \cdot Y$ or $Z = \frac{X}{Y}$

$$\frac{\sigma_z}{Z} = \sqrt{\left(\frac{\sigma_x}{X}\right)^2 + \left(\frac{\sigma_y}{y}\right)^2} \tag{2.9}$$

Here, σ_z is the standard deviation of Z, while σ_x and σ_y are, respectively, the standard deviation of X and Y.

Table 2.2: AMPTS Compiègne

AMPTS Compiègne		*Inoculum*					*Substrate*					$M_{ino} + M_{sub}$ (g)	M_{water} (g)	M_{total} (g)
N°	Title	%DM	%VM	Gross mass of inoculum (g)	Added dry mass (g)	Added volatile mass (g)	%DM	%VM	Gross mass of substrate (g)	Added dry mass (g)	Added volatile mass (g)			
1	Positive control - cellulose	9.5%	66.0%	253.8	24.1	15.9	100.0%	100.0%	5.2	5.2	5.2	259.0	95.0	354.0
2	Positive control - cellulose	9.5%	66.0%	250.7	23.8	15.7	100.0%	100.0%	5.2	5.2	5.2	255.9	96.6	352.5
3	Negative control - inoculum alone	9.5%	66.0%	251.5	23.9	15.8						251.5	101.2	352.7
4	Negative control - inoculum alone	9.5%	66.0%	250.0	23.7	15.7						250.0	104.2	354.2
5	Microalgae US-1	9.5%	66.0%	250.1	23.7	15.7	12.4%	91.6%	46.5	5.8	5.3	296.7	53.9	350.6
6	Microalgae US-1	9.5%	66.0%	250.9	23.8	15.7	12.4%	91.6%	46.5	5.8	5.3	297.4	53.6	351.0
7	Microalgae US-2	9.5%	66.0%	250.7	23.8	15.7	12.4%	91.6%	46.6	5.8	5.3	297.3	53.7	351.0
8	Microalgae US-2	9.5%	66.0%	250.4	23.8	15.7	12.4%	91.6%	46.4	5.8	5.3	296.8	53.7	350.5
9	Microalgae HT-1	9.5%	66.0%	250.2	23.7	15.7	12.4%	91.6%	46.7	5.8	5.3	296.9	53.6	350.5
10	Microalgae HT-1	9.5%	66.0%	250.5	23.8	15.7	12.4%	91.6%	46.5	5.8	5.3	297.0	53.6	350.6
11	Microalgae HT-2	9.5%	66.0%	250.2	23.7	15.7	12.4%	91.6%	47.1	5.8	5.4	297.4	53.7	351.0
12	Microalgae HT-2	9.5%	66.0%	250.2	23.7	15.7	12.4%	91.6%	47.0	5.8	5.3	297.3	53.8	351.0
13	Raw microalgae	9.5%	66.0%	250.1	23.7	15.7	28.2%	91.1%	20.5	5.8	5.3	270.6	79.7	350.3
14	Raw microalgae	9.5%	66.0%	250.3	23.8	15.7	28.2%	91.1%	20.2	5.7	5.2	270.5	79.7	350.2
15	Raw microalgae	9.5%	66.0%	250.2	23.7	15.7	28.2%	91.1%	20.6	5.8	5.3	270.8	79.9	350.6

Table 2.3: AMPTS 1 Beauvais

AMPTS 1 Beauvais		*Inoculum*					*Substrate*					$M_{ino} + M_{sub}$ (g)	M_{water} (g)	M_{total} (g)
N°	Title	%DM	%VM	Gross mass of inoculum (g)	Added dry mass (g)	Added volatile mass (g)	%DM	%VM	Gross mass of substrate (g)	Added dry mass (g)	Added volatile mass (g)			
1	Positive control - cellulose	9.5%	66.0%	250.9	23.8	15.7	100.0%	100.0%	5.2	5.2	5.2	256.1	94.9	351.0
2	Positive control - cellulose	9.5%	66.0%	249.8	23.7	15.6	100.0%	100.0%	5.2	5.2	5.2	255.0	94.9	349.9
3	Positive control - cellulose	9.5%	66.0%	250.0	23.7	15.7	100.0%	100.0%	5.2	5.2	5.2	255.2	94.8	350.0
4	Negative control - inoculum alone	9.5%	66.0%	250.5	23.8	15.7						250.5	100.0	350.5
5	Negative control - inoculum alone	9.5%	66.0%	249.8	23.7	15.6						249.8	100.0	349.8
6	Negative control - inoculum alone	9.5%	66.0%	250.2	23.7	15.7						250.2	100.0	350.2
7	Raw microalgae	9.5%	66.0%	250.4	23.8	15.7	21.3%	91.6%	20.3	4.3	4.0	270.7	79.7	350.4
8	Raw microalgae	9.5%	66.0%	250.0	23.7	15.7	21.3%	91.6%	20.4	4.4	4.0	270.4	79.7	350.1
9	Raw microalgae	9.5%	66.0%	250.1	23.7	15.7	21.3%	91.6%	20.5	4.4	4.0	270.6	79.8	350.4
10	Microalgae US-3	9.5%	66.0%	249.9	23.7	15.7	13.0%	91.6%	45.3	5.9	5.4	295.2	55.9	351.1
11	Microalgae US-3	9.5%	66.0%	250.2	23.7	15.7	13.0%	91.6%	45.6	5.9	5.4	295.8	55.8	351.6
12	Microalgae US-3	9.5%	66.0%	250.0	23.7	15.7	13.0%	91.6%	44.4	5.7	5.3	294.4	55.8	350.2
13	chicory roots	9.5%	66.0%	250.2	23.7	15.7	11.3%	88.1%	38.8	4.4	3.9	289.0	61.2	350.2
14	chicory roots	9.5%	66.0%	250.7	23.8	15.7	11.3%	88.1%	38.8	4.4	3.9	289.5	61.2	350.7
15	chicory roots	9.5%	66.0%	250.0	23.7	15.7	11.3%	88.1%	38.8	4.4	3.9	288.8	61.2	350.1

Table 2.4: AMPTS 2 Beauvais

AMPTS 2 Beauvais		*Inoculum*					*Substrate*					$M_{ino} + M_{sub}$ (g)	M_{water} (g)	M_{total} (g)
N°	Title	%DM	%VM	Gross mass of inoculum (g)	Added dry mass (g)	Added volatile mass (g)	%DM	%VM	Gross mass of substrate (g)	Added dry mass (g)	Added volatile mass (g)			
1	Control positive - cellulose	9.5%	66.0%	251.5	23.9	15.8	100.0%	100.0%	5.2	5.2	5.2	256.7	94.8	351.5
2	Positive control - cellulose	9.5%	66.0%	249.0	23.6	15.6	100.0%	100.0%	5.2	5.2	5.2	254.2	94.8	349.0
3	Positive control - cellulose	9.5%	66.0%	250.0	23.7	15.7	100.0%	100.0%	5.2	5.2	5.2	255.2	94.8	350.0
4	Negative control - inoculum alone	9.5%	66.0%	250.8	23.8	15.7			0.0			250.8	100.0	350.8
5	Negative control - inoculum alone	9.5%	66.0%	250.0	23.7	15.7			0.0			250.0	100.0	350.0
6	Negative control - inoculum alone	9.5%	66.0%	250.0	23.7	15.7			0.0			250.0	100.0	350.0
7	Microalgae 25% + Roots 75%	9.5%	66.0%	250.0	23.7	15.7			34.2	4.4	3.9	284.2	65.8	350.0
8	Microalgae 25% + Roots 75%	9.5%	66.0%	250.0	23.7	15.7			34.3	4.4	3.9	284.3	65.8	350.1
9	Microalgae 25% + Roots 75%	9.5%	66.0%	250.0	23.7	15.7			34.3	4.4	3.9	284.3	65.8	350.1
10	Microalgae 50% + Roots 50%	9.5%	66.0%	250.2	23.7	15.7			29.6	4.4	3.9	279.8	70.4	350.2
11	Microalgae 50% + Roots 50%	9.5%	66.0%	250.2	23.7	15.7			29.4	4.4	3.9	279.6	70.4	350.0
12	Microalgae 50% + Roots 50%	9.5%	66.0%	250.1	23.7	15.7			29.7	4.4	3.9	279.8	70.4	350.2
13	Microalgae 75% + Roots 25%	9.5%	66.0%	250.2	23.7	15.7			24.9	4.3	3.9	275.1	75.1	350.2
14	Microalgae 75% + Roots 25%	9.5%	66.0%	250.4	23.8	15.7			25.0	4.4	3.9	275.4	75.1	350.5
15	Microalgae 75% + Roots 25%	9.5%	66.0%	250.3	23.8	15.7			24.9	4.3	3.9	275.2	75.1	350.3

Table 2.5: Masses of substrates

	N°	Raw microalgae (g)	chicory roots (g)
25% - 75%	7	5.1	29.1
	8	5.2	29.1
	9	5.2	29.1
50% - 50%	10	10.2	19.4
	11	10.2	19.2
	12	10.3	19.4
75% - 25%	13	15.2	9.7
	14	15.3	9.7
	15	15.2	9.7

Chapter 3

Results and discussion

3.1 AMPTS Compiègne

A first evaluation can be done with the figure 3.1, which represents the raw results coming from the Gas Endeavour software.

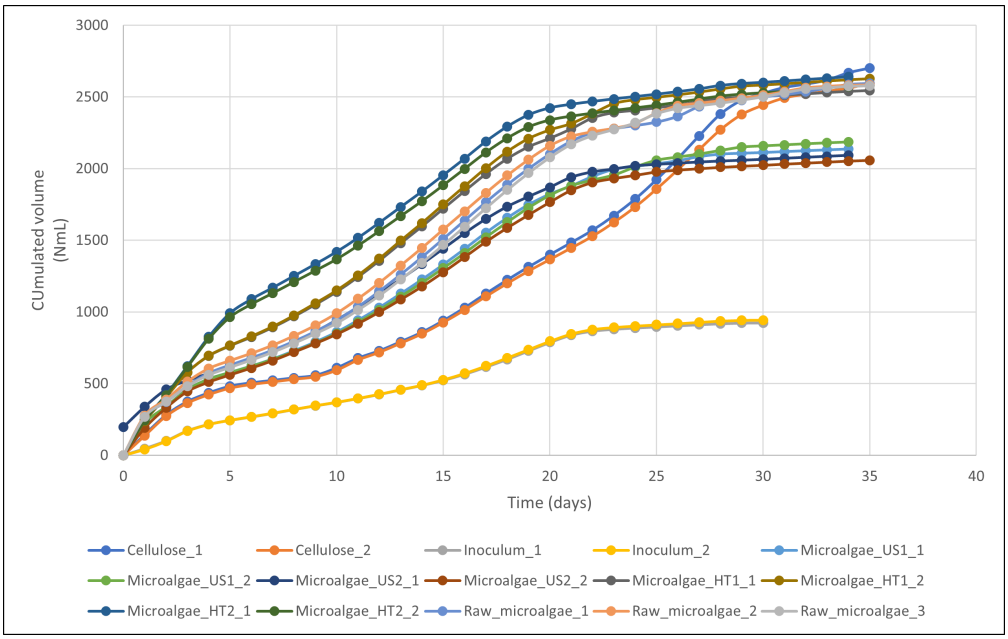


Figure 3.1: Raw results AMPTS Compiègne

The graph is difficult to interpret, so the data processing outlined in the "*Materials and Methods*" section is required to obtain more meaningful results that allow for drawing clear conclusions.

The final experimental values of mean [NmL_{CH_4}/g_{VM}], standard deviation and coefficient of variation have been obtained and are presented in the table 3.1.

Table 3.1: Final experimental values of mean, standard deviation and CV

	Cellulose	Microalgae US1	Microalgae US2	Microalgae HT1	Microalgae HT2	Raw microalgae
Mean	298.2	227.6	209.7	299.1	305.2	299.3
Std. Dev.	11.3	6.2	3.9	4.2	9.2	4.1
CV	3.8%	2.7%	1.9%	1.4%	3.0%	1.4%

The figure 3.2 shows the curves representing the cumulated volume of methane normalized to the amount of organic matter for each substrate, along with error bars indicating the standard deviation.

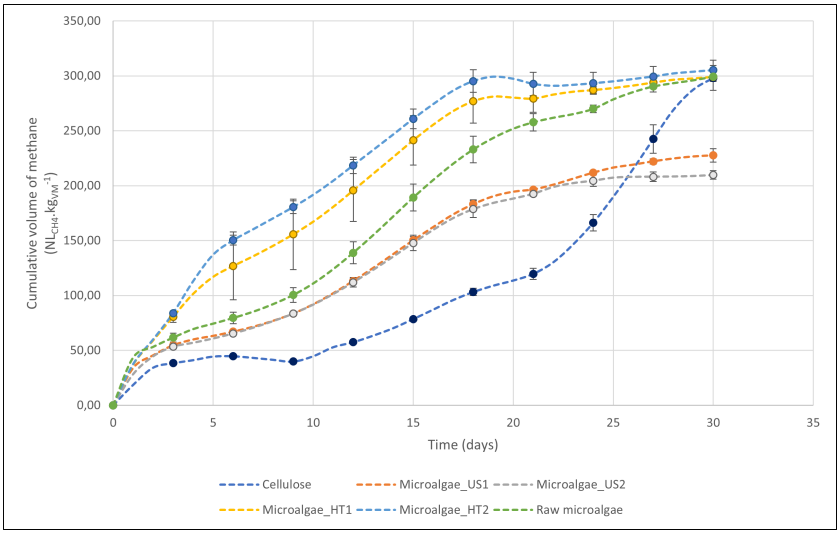


Figure 3.2: Treated results AMPTS Compiègne

The curve representing methane production from cellulose shows a peculiar trend in the initial days due to agitation problems. Around the fifth day, the cap of the mini-reactor was opened for manual agitation. After that, production continued normally.
The graph indicates a negative impact of ultrasonic pretreatment on methane production from microalgae, resulting in a lower BMP compared to untreated microalgae. This result is consistent with what has been reported in the literature for the same treatment on the same species of microalgae, where the result is explained by an increase in the production of VFAs due to ultrasound pretreatment, which accumulate and cause inhibition of the microorganisms responsible for methane production. [37].
In contrast, microalgae that underwent hydrothermal pretreatment show improved production kinetics during the initial BMP analysis period. However, the final methane

production is unchanged compared to untreated microalgae. This means that hydrothermal treatment increases the solubilization rate of the substances contained within the biomass, but for this particular species, it does not increase the total amount of solubilized components.

Table 3.2: Results AMPTS Compiègne

Pretreatment	**BMP (NL_{CH_4} / kg_{VM})**	**Deviation (%)**
Without pretreatment	299.3 ± 4.1	
US1 (400W, 5 min)	227.6 ± 6.2	-24%
US2 (400W, 10 min)	209.7 ± 3.9	-30%
HT1 (120°C, 15 min)	299.1 ± 4.2	-0.07%
HT2 (140°C, 15 min)	305.2 ± 9.2	+2%

3.1.1 Gompertz model

The results obtained are those represented in the table 3.3.

Table 3.3: Identification with a Gompertz kinetics

	Microalgae US1	Microalgae US2	Microalgae HT1	Microalgae HT2	Raw microalgae
V_{max}	269.55 ± 14.35	241.30 ± 1.56	312.80 ± 10.75	309.0 ± 9.76	357.67 ± 10.51
Lag phase	-0.7 ± 0.11	-0.53 ± 0.14	-0.9 ± 0.15	-0.94 ± 0.10	0.1 ± 0.27
R_{max}	9.65 ± 0.35	9.76 ± 0.48	17.13 ± 3.15	19.75 ± 0.83	12.73 ± 0.56
MAPE (%)	6.84 ± 0.59%	8.62 ± 1.46%	4.84 ± 0.66%	4.37 ± 0.03%	7.71 ± 0.18%
R^2	0.99 ± 0.00	0.98 ± 0.00	0.99 ± 0.00	0.98 ± 0.00	0.99 ± 0.00

The result of the graphical approximation is represented in figure 3.3.

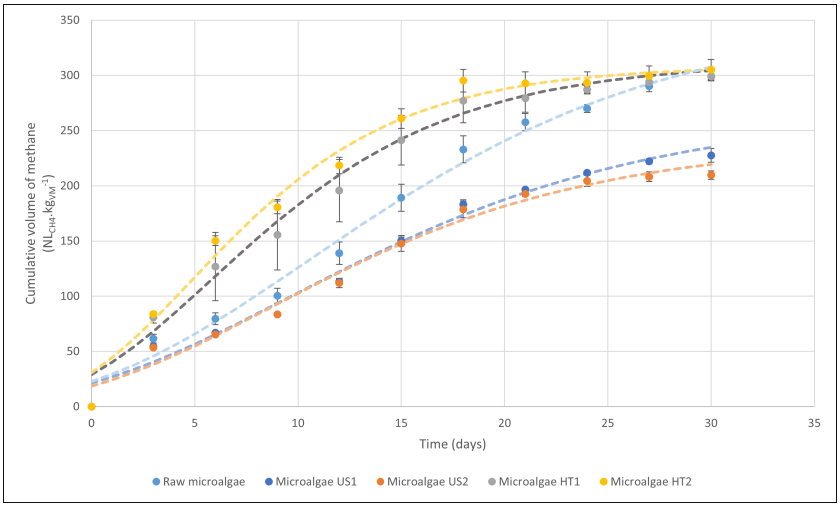


Figure 3.3: Graphical approximation Gompertz kinetics - Compiègne

The continuous lines represent the points found by the model, while the dots represent experimentally obtained values. For clarity, not all points are graphically represented; instead, one point is shown every three days. The vertical lines passing through the experimental points represent the standard deviation values resulting from duplicate or triplicate tests, calculated using Excel functions.
The graph demonstrates a good fit of the Gompertz model to the kinetics of methane production, as evidenced by the low MAPE values and an R^2 value close to 1. The approximation for raw microalgae exhibits the largest error, as indicated by a higher V_{max} value, which was not observed in other cases. Conversely, the increase in kinetics for microalgae subjected to hydrothermal pretreatment is confirmed by the R_{max} parameter.

3.1.2 Elemental analysis

Elemental analysis tests were conducted for all the samples considered. We consider the general formula of the reaction 2.6.
For all the samples, the following stoichiometric coefficients were found: From the

Table 3.4: Stoichiometric coefficients AMPTS Compiègne

	Raw microalgae	Microalgae US1	Microalgae US2	Microalgae HT1	Microalgae HT2
n	4.6 ± 0.2	4.6 ± 0.1	4.6 ± 0.2	4.5 ± 0.0	4.5 ± 0.2
a	7.6 ± 0.5	7.6 ± 0.2	7.7 ± 0.4	7.4 ± 0.8	7.3 ± 0.3
b	1.5 ± 0.4	1.6 ± 0.2	1.6 ± 0.2	1.9 ± 0.1	1.7 ± 0.1
c	1 ± 0.1	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.1
d	0	0	0	0	0

stoichiometric coefficient values, the theoretical yields for the various substrates are obtained. These values can be compared with the experimental results to determine the anaerobic digestion efficiencies.

Table 3.5: Theoretic productions

Substrate	Theoretic production [NmL_{CH_4}/g_{VM}]	Actual production [NmL_{CH_4}/g_{VM}]	Efficiency [%]
Raw microalgae	549.9 ± 56.2	299.3 ± 4.1	54.4
Microalgae US1	537.8 ± 23.3	227.6 ± 6.2	42.3
Microalgae US2	538.9 ± 35.1	209.7 ± 3.9	38.9
Microalgae HT1	494.6 ± 24.1	299.1 ± 4.2	60.5
Microalgae HT2	509.0 ± 26.5	305.2 ± 9.2	60.0

As shown in the table 3.5, the substrate treated with ultrasound exhibits the lowest efficiency. This aligns with the lower methane production compared to untreated microalgae, which could be due to the delayed availability of biomass components. On the other hand, the microalgae subjected to thermal treatment show the highest efficiency, suggesting that the biomass components are immediately more accessible to the microbial consortium. This result is consistent with the production kinetics graph 3.1, which highlights a faster production rate during the first few days for the thermally treated sample.

3.2 AMPTS 1 Beauvais

For the analysis of AMPTS 1 in Beauvais, the data processing followed the same steps as previously described. The substrates analyzed in this test included:

- Raw microalgae
- Microalgae after ultrasound pretreatment at 400W for 15 min
- Chicory roots

In addition to these, the positive control with cellulose and the negative control with inoculum only were also analyzed. Below are the results reported in the table 3.6: mean production [NmL_{CH_4}/g_{VM}], standard deviation, and coefficient of variation.

Table 3.6: Final values of mean, standard deviation and CV

	Cellulose	Raw microalgae	Microalgae US3	Chicory roots
Mean	325.1	315.8	227.7	302.6
Std. Dev.	2.8	6.8	21.3	12.9
CV	0.9%	2.1%	9.3%	4.3%

The trends of cumulative methane volume normalized to volatile solids for each substrate are depicted in figure 3.4.

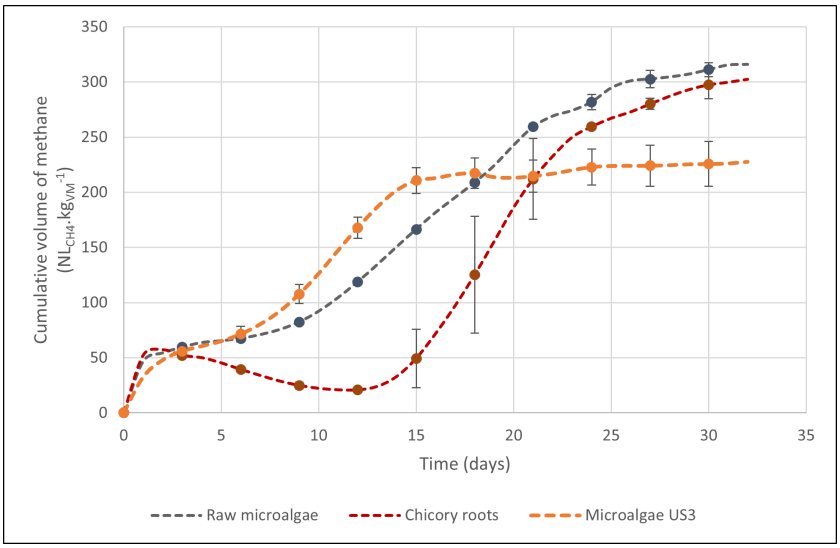


Figure 3.4: Treated results AMPTS 1 Beauvais

Regarding microalgae, a higher production is generally observed compared to the test conducted in Compiègne, where different percentages for DM and VM were also found. However, in this test as well, the biomass treated with ultrasound shows a 28% lower methane production compared to the untreated sample. The most significant result is observed with chicory roots, which exhibit a distinctive trend in the curve: in the first few days, there is a clear inhibition of methane production. This is likely due to the presence of sugars, which cause a drop in pH within the reactor, and polyphenols, which have antimicrobial activity [58], creating unfavorable conditions for the microorganisms responsible for anaerobic digestion, thereby inhibiting their activity. High standard deviation values were observed for certain data points related to the chicory roots. This is because the experiment was conducted in triplicate, and one of the three reactors exhibited significantly more pronounced and prolonged inhibition than the other two. Consequently, the average production value was reduced, and the uncertainty increased, as shown in the table 3.7.

Table 3.7: Cumulated volume of methane [NmL_{CH_4}/g_{VM}]

Time (days)	Reactor 1	Reactor 2	Reactor 3
10	23	20	23
11	21	19	23
12	19	20	24
13	18	25	29
14	18	38	42
15	23	60	66
16	33	89	94
17	50	118	123
18	75	150	151
19	105	182	180
20	139	208	211
21	176	227	234

3.2.1 Gompertz model

The analysis using the Gompertz model provided the results in table 3.8.
These results are represented in figure 3.5.

Table 3.8: Identification with a Gompertz kinetics

	Raw Microalgae	Microalgae US3	Chicory roots
V_{max}	410.67 ± 18.74	233.53 ± 19.47	307.53 ± 13.86
Lag phase	1.47 ± 0.25	0.83 ± 0.30	13.67 ± 1.71
R_{max}	12.70 ± 0.25	15.71 ± 0.43	30.69 ± 2.68
MAPE (%)	10.38 ± 0.47%	9.39 ± 1.17%	30.97 ± 5.34%
R^2	0.98 ± 0.00	0.98 ± 0.01	0.96 ± 0.00

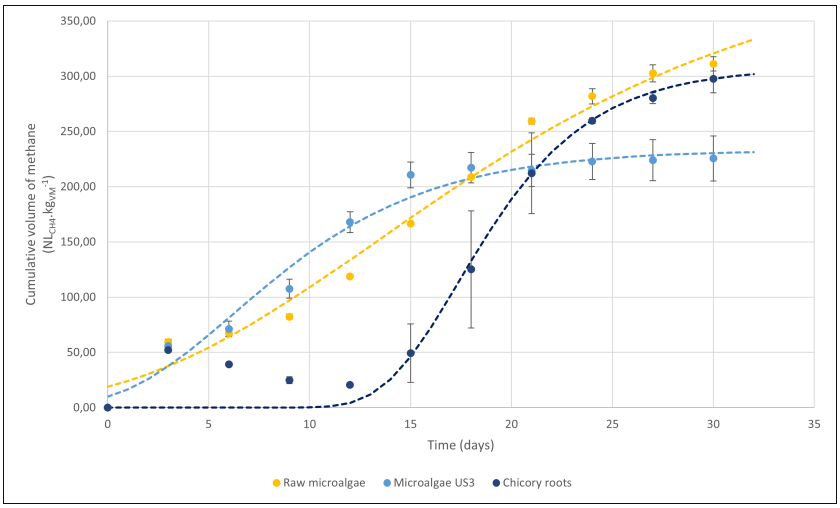


Figure 3.5: Graphical approximation Gompertz kinetics - Beauvais 1

Unlike the case of the AMPTS in Compiègne, the approximation using the Gompertz model is less accurate here, particularly for chicory roots, which exhibit a MAPE of 30.97%. This discrepancy is also clear from the graphical representation: up to day 12, there is a substantial difference between the experimental data and the values predicted by the model. The Gompertz model fails to account for the inhibition observed during the initial phase of anaerobic digestion, which is addressed by considering the lag phase.

3.2.2 Elemental analysis

The test on microalgae subjected to ultrasound pretreatment (400W, 15 min) was not performed, so the only meaningful result is the one concerning chicory roots. With these values, the theoretical production and, consequently, the efficiency can be calculated.
A very high efficiency value was obtained compared to those found for microalgae. This led to the idea of considering the co-digestion of microalgae and chicory roots for

Table 3.9: Stoichiometric coefficients AMPTS 1 Beauvais

	Chicory roots
n	26.6 ± 1.2
a	27.2 ± 8.2
b	18.1 ± 4.8
c	1 ± 0.1
d	0

Table 3.10: Theoretic productions

Substrate	Theoretic production [NmL_{CH_4}/g_{VM}]	Actual production [NmL_{CH_4}/g_{VM}]	Efficiency [%]
Chicory roots	406.6 ± 75.9	302.6 ± 18	74.4

anaerobic digestion.

3.3 AMPTS 2 Beauvais

In this test, various co-digestion conditions between chicory roots and raw microalgae were tested, along with the positive control (cellulose) and the negative control (inoculum only). Specifically, the tested proportions are:

- 25% raw microalgae - 75% chicory roots
- 50% raw microalgae - 50% chicory roots
- 75% raw microalgae - 25% chicory roots

The results of mean production [NmL_{CH_4}/g_{VM}], standard deviation and coefficient of variation for this test are in table 3.11. The values for the 50% microalgae - 50% chicory

Table 3.11: Final values of mean, standard deviation and CV

	Cellulose	25% microalgae 75% chicory roots	50% microalgae 50% chicory roots	75% microalgae 25% chicory roots
Mean	295.1	291.1	339.6	342.0
Std. Dev.	15.1	12.2	0.1	15.7
CV	0.9%	4.2%	0.0%	4.6%

roots test are based on only two reactors instead of three, as one of the tests failed to produce results in the final days of the experiment. To provide a more comprehensive overview, the graph 3.6 depicting the results also includes the curves for raw microalgae and chicory roots, obtained from the AMPTS 1 Beauvais test.

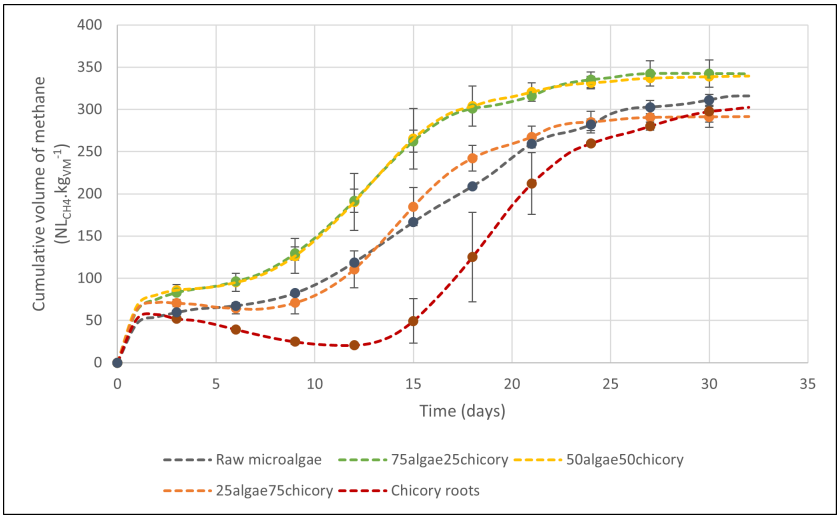


Figure 3.6: Treated results AMPTS 2 Beauvais

The results show that the 50% microalgae - 50% chicory roots mix and the 75% microalgae - 25% chicory roots mix achieve higher methane production compared to both raw microalgae and chicory roots individually. This suggests that co-digestion created a synergistic effect between the two biomasses, leading to better performance than expected from a simple proportional combination. In contrast, the 25% microalgae - 75% chicory roots mix is more impacted by the initial inhibition phase of the chicory roots, resulting in lower methane production than either biomass on its own.

3.3.1 Gompertz model

The Gompertz model provided the following results 3.12.

Table 3.12: Identification with a Gompertz kinetics

	25% μalgae 75% chicory roots	50% μalgae 50% chicory roots	75% μalgae 25% chicory roots
V_{max}	347.07 ± 18.86	373.75 ± 15.77	342.50 ± 80.47
Lag phase	1.90 ± 0.92	0.82 ± 0.61	1.36 ± 0.05
R_{max}	14.10 ± 0.45	17.99 ± 2.56	14.89 ± 2.74
MAPE (%)	$18.75 \pm 3.22\%$	$11.16 \pm 0.51\%$	$12.60 \pm 3.62\%$
R^2	0.95 ± 0.01	0.97 ± 0.00	0.96 ± 0.02

The MAPE values are significant, indicating that the approximation is not entirely reliable. This aligns with the results from the AMPTS 1 Beauvais test, where the Gompertz model did not accurately capture the methane production trend for chicory roots due to the inhibition period. While this effect can be mitigated (especially in the 50% microalgae - 50% chicory roots and 75% microalgae - 25% chicory roots cases) the inhibition phase still has a notable impact on the overall curve. The figure 3.7 clearly shows that the experimental data points deviate significantly from the curve predicted by the Gompertz model. Moreover, while the model's curves do not reach a plateau, in reality, the experiment was stopped once a steady state was achieved, with methane production remaining nearly constant. These discrepancies confirm that the model fails to accurately represent the co-digestion process between microalgae and endive roots.

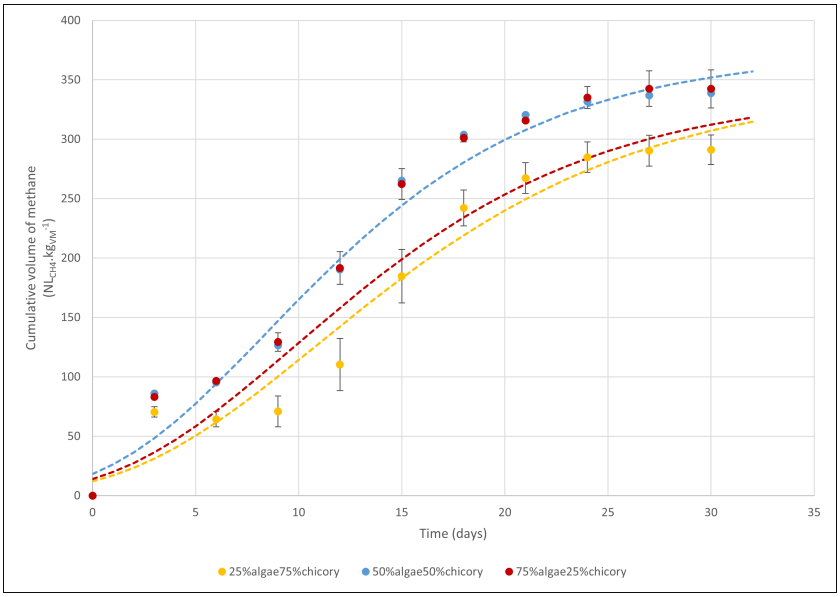


Figure 3.7: Graphical approximation Gompertz kinetics

3.3.2 Elemental analysis

No specific CHNS/O tests were performed for this experiment, but the values from previous analyses can be used to calculate the theoretical production for the various co-digestion ratios. These theoretical results can then be compared with the predicted output based on the individual substrates, as well as with the experimental results. This approach allows for assessing the enhancement due to the synergy between the substrates and the efficiency relative to the theoretical production. The results obtained are summarized in the table 3.15. All the values are in [NmL_{CH_4}/g_{VM}].

Table 3.13: Experimental production of the singles substrates

Microalgae	Chicory roots
315.83 ± 8.92	302.63 ± 17.96

Table 3.14: Theoretic production of the singles substrates

Microalgae	Chicory roots
549.89 ± 56.20	406.57 ± 75.91

As shown in the tables, the mix with a majority of chicory roots produces less than expected based on the experimental results of the individual substrates. This suggests

Table 3.15: Theoretic and predicted productions

Mix μalgae - chicory roots	Theoretic production	Predicted production	Actual production	Enhancement	% of theoretic production
25% - 75%	442.40	305.93	291.12	-4.84%	66.80%
50% - 50%	478.23	309.23	339.62	+9.83%	71.02%
75% - 25%	514.06	312.53	341.99	+9.43%	66.53%

that inhibition from the chicory roots has a significant impact, reducing the overall performance of the mix. In contrast, the 50% microalgae - 50% chicory roots mix and the 75% microalgae - 25% chicory roots mix demonstrate strong synergy, achieving higher production levels than predicted. This finding opens up opportunities for further research in co-digestion, particularly with microalgae subjected to thermal pretreatment, which has already been shown to enhance kinetics in the initial phase of anaerobic digestion.

Chapter 4

Conclusion

In conclusion, anaerobic digestion of microalgae shows considerable promise in advancing the energy transition toward greater sustainability. However, it still faces challenges that open avenues for further research, such as the low degradability of the cell wall and the low C/N ratio, both factors that can inhibit anaerobic digestion. Pretreatments are essential for enhancing biodegradability and solubilizing cell nutrients, yet their effectiveness varies significantly across microalgae species, necessitating experimental validation for each case.

For instance, in this study, ultrasonic pretreatment reduced the methane potential of *Spirulina*, while thermal pretreatment accelerated digestion kinetics in the initial days without increasing overall methane production. In contrast, other studies often report positive effects from both pretreatments. Future directions could include exploring alternative microalgae species or experimenting with different pretreatment types, such as biological or chemical methods, to optimize methane yield and overall process efficiency.

Co-digestion with chicory roots, a carbon-rich biomass, produced promising results for the 50% microalgae - 50% chicory roots and 75% microalgae - 25% chicory roots mixtures, where a synergistic effect between the two biomasses led to an increase in methane production beyond what was expected from the individual biomasses alone. However, the anaerobic digestion of the 25% microalgae - 75% chicory roots mixture resulted in lower methane production than anticipated, likely due to inhibition caused by the chicory roots during the initial days of the experiment. A potential future approach could involve co-digesting chicory roots with microalgae that have undergone prior thermal pretreatment.

Bibliography

[1] Nijaguna. «Biogas Technology». In: *New Age International (P) Limited Publishers* (2012) (cit. on p. 1).

[2] Abbasi et al. «Anaerobic digestion for global warming control and energy generation - An overview». In: *Renewable and Sustainable Energy Reviews* (2012) (cit. on pp. 1, 2, 4, 5).

[3] Deepanraj et al. «Biogas Generation through Anaerobic Digestion Process - An Overview». In: *Research Journal of Chemistry and Environment* (2014) (cit. on pp. 2, 4).

[4] Chunlan et al. «Review on research achievements of biogas from anaerobic digestion». In: *Renewable and Sustainable Energy Reviews* (2015) (cit. on pp. 3–5).

[5] Deublein et al. «Biogas from waste and renewable resources». In: *An introduction* (2008) (cit. on p. 4).

[6] Khanal. «Anaerobic biotechnology for bioenergy production principles and applications». In: *Wiley-Blackwell* (2008) (cit. on p. 4).

[7] Chen et al. «Inhibition of anaerobic digestion process A review». In: *Bioresource Technology* (2008) (cit. on p. 4).

[8] Khoiyangbam et al. «Biogas Technology Towards Sustainable Development». In: *TERI Press* (2011) (cit. on p. 4).

[9] Ekama et al. «Organic material removal». In: *Biol Wastewater Treat: Princ* (2008) (cit. on p. 5).

[10] Karim et al. «Anaerobic digestion of animal waste: Effect of mixing». In: *Bioresource Technology* (2005) (cit. on p. 5).

[11] Nayono S.E. «Anaerobic digestion of organic solid waste for energy production». In: *KIT scientific publishing* (2009) (cit. on p. 5).

[12] Hossain et al. «Biogas from microalgae: Technologies, challenges and opportunities». In: *Renewable and Sustainable Energy Reviews* (2020) (cit. on pp. 5, 16).

[13] Kumar et al. «Recent developments on biofuels production from microalgae and macroalgae». In: *Renewable and Sustainable Energy Reviews* (2016) (cit. on p. 5).

[14] Zabed et al. «Anaerobic digestion of microalgae biomass for methane production». In: *Microalgae biotechnology for development of biofuel and wastewater treatment* (2019) (cit. on p. 5).

[15] Williams P. «Biofuel: microalgae cut the social and ecological costs». In: *Nature* (2007) (cit. on p. 5).

[16] Passos et al. «Anaerobic co-digestion of coffee husks and microalgal biomass after thermal hydrolisis». In: *Bioresource Technology* (2018) (cit. on p. 6).

[17] Ganesh Saratale et al. «A critical review on anaerobic digestion of microalgae and macroalgae and co-digestion of biomass for enhanced methane generation». In: *Bioresource Technology* (2018) (cit. on p. 6).

[18] Tawfik et al. «Sustainable microalgal biomass valorization to bioenergy: Key challenges and future perspectives». In: *Chemosphere* (2022) (cit. on pp. 6–8).

[19] Zhang et al. «Anaerobic co-digestion of microalgae Chlorella vulgaris and potato processing waste: effect of mixing ratio, waste type and substrate to inoculum ratio». In: *Biochemical Engineering* (2019) (cit. on p. 7).

[20] Li et al. «Anaerobic co-digestion of chicken manure and microalgae Chlorella sp.: methane potential, microbial diversity and synergistic impact evaluation». In: *Waste Management* (2017) (cit. on p. 7).

[21] Chen et al. «Inhibition of anaerobic digestion process: a review». In: *Bioresource Technology* (2008) (cit. on p. 7).

[22] Park and Li. «Evaluation of methane production and macronutrient degradation in the anaerobic co-digestion of algae biomass residue and lipid waste». In: *Bioresource Technology* (2012) (cit. on p. 7).

[23] Appels et al. «Principles and potential of the anaerobic digestion of waste-activated sludge». In: *Progress in Energy and Combustion Science* (2008) (cit. on p. 7).

[24] Sukias and Craggs. «Digestion of wastewater pond microalgae and potential inhibition by alum and ammoniacal-N». In: *Water Science Technology* (2011) (cit. on p. 7).

[25] Zuccaro et al. «Chapter 2 - Microalgae Cultivation Systems». In: *Microalgae Cultivation for Biofuels Production* (2020) (cit. on p. 8).

[26] Kropat et al. «Copper economy in Chlamydomonas: prioritized allocation and reallocation of copper to respiration vs. photosynthesis». In: *Proc. Natl. Acad. Sci. U. S. A.* (2015) (cit. on p. 8).

[27] Cai et al. «Effects of adding trace elements on rice straw anaerobic mono-digestion: focus on changes in microbial communities using high-throughput sequencing». In: *Bioresource Technology* (2017) (cit. on p. 8).

[28] Jankowska et al. «Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion». In: *Renewable and Sustainable Energy Reviews* (2017) (cit. on p. 9).

[29] Wu et al. «Evaluation of several flocculants for flocculating microalgae». In: *Bioresource Technology* (2015) (cit. on p. 9).

[30] Jiang et al. «Ammonia inhibition and toxicity in anaerobic digestion: a critical review». In: *Water Process Engineering* (2019) (cit. on p. 9).

[31] Mussgnung et al. «Microalgae as substrates for fermentative biogas production in a combined biorefinery concept». In: *Journal of Biotechnology* (2010) (cit. on p. 9).

[32] Passos et al. «Biogas production from microalgae grown in wastewater: effect of microwave pretreatment». In: *Applied energy* (2013) (cit. on p. 9).

[33] Sivaramakrishnan et al. «Microalgae as feedstock for biodiesel production under ultrasound treatment - A review». In: *Bioresource Technology* (2018) (cit. on p. 9).

[34] Wang et al. «Modeling Bubble dynamics and radical kinetics in ultrasound induced microalgae cell disruption». In: *Ultrasonics sonochemistry* (2016) (cit. on p. 9).

[35] Wang et al. «Algal cell lysis by bacteria: A review and comparison to conventional methods». In: *Algal Res.* (2020) (cit. on p. 9).

[36] Liu et al. «Ultrasound for microalgal cell disruption and product extraction: A review». In: *Ultrasonics Sonochemistry* (2022) (cit. on pp. 9, 10).

[37] Samson et al. «Influence of mechanical and thermochemical pretreatments on anaerobic digestion of Spirulina maxima algal biomass». In: *Biotechnology Letters Vol 5* (1983) (cit. on pp. 14, 34).

[38] González-Fernández et al. «Comparison of ultrasound and thermal pretreatment of *Scenedesmus* biomass on methane production». In: *Bioresource Technology* (2012) (cit. on p. 14).

[39] Lee et al. «Ultrasound pretreatment for enhanced biogas production of waste algal biomass the 1st IWWG-ARB symposium». In: *Hokkaido University* (2013) (cit. on p. 14).

[40] Park et al. «Anaerobic digestion of microalgal biomass with ultrasonic disintegration». In: *International Biodeterioration Biodegradation* (2013) (cit. on p. 14).

[41] Alzate et al. «Biochemical methane potential of microalgae: Influence of substrate to inoculum ratio, biomass concentration and pretreatment». In: *Bioresource Technology* (2012) (cit. on pp. 14, 15).

[42] Rodriguez et al. «Pre-treatment techniques used for anaerobic digestion of algae». In: *Fuel processing technology* (2015) (cit. on p. 12).

[43] Nuchdang et al. «Hydrothermal post-treatment of digestate to maximize the methane yield from the anaerobic digestion of microalgae». In: *Waste management* (2018) (cit. on p. 12).

[44] Passos et al. «Influence of hydrothermal pretreatment on microalgal biomass anaerobic digestion and bioenergy production». In: *ScienceDirect* (2015) (cit. on p. 12).

[45] Cho et al. «Evaluation of thermal, ultrasonic and alkali pretreatments on mixed-microalgal biomass to enhance anaerobic methane production». In: *Bioresource Technology* (2013) (cit. on p. 15).

[46] Schwede et al. «Influence of different cell disruption techniques on mono digestion of algal biomass». In: *Bioenergy Technology* (2011) (cit. on p. 15).

[47] Herrmann et al. «Optimized biogas production from microalgae through co-digestion with carbon-rich co-substrates». In: *Bioresource Technology* (2016) (cit. on p. 16).

[48] Saratale et al. «A critical review of anaerobic digestion of microalgae and macroalgae and co-digestion of biomass for enhanced methane generation». In: *Bioresource Technology* (2018) (cit. on p. 16).

[49] Li et al. «Co-digestion of chicken manure and microalgae Chlorella 1067 grown in the recycled digestate: Nutrients reuse and biogas enhancement». In: *Waste Management* (2017) (cit. on p. 16).

[50] Solé-Bundó et al. «Anaerobic co-digestion of microalgal biomass and wheat straw with and without thermo-alkaline pretreatment». In: *Bioresource Technology* (2017) (cit. on p. 16).

[51] Astals et al. «Anaerobic co-digestion of pig manure and algae: impact of intracellular algal products recovery and co-digestion performance». In: *Bioresource Technology* (2015) (cit. on p. 16).

[52] Park et al. «Evaluation of methane production and macronutrient degradation in the anaerobic co-digestion of algae biomass residue and lipid waste». In: *Bioresource Technology* (2012) (cit. on p. 16).

[53] *BPC Instruments*. `https://bpcinstruments.com/bpc_products/ampts3/`. 2024 (cit. on pp. 18, 19).

[54] Holliger et al. «Toward a standardization of biomethane potential tests». In: *Water Science Technology* (2016) (cit. on p. 23).

[55] Alzate et al. «Biochemical methane potential of microalgae: Influence of substrate to inoculum ratio, biomass concentration and pretreatment». In: *Bioresource Technology* (2012) (cit. on p. 24).

[56] Zhang et al. «Methane yield enhancement of mesophilic and thermophilic anaerobic codigestion of algal biomass and food waste using algal biochar: Semi-continuous operation and microbial community analysis». In: *Bioresource Technology* (2020) (cit. on p. 24).

[57] Liu et al. «Overview of Numerical Simulation of Solid-State Anaerobic Digestion Considering Hydrodynamic Behaviors, Phenomena of Transfer, Biochemical Kinetics and Statistical Approaches». In: *Energies* (2023) (cit. on p. 26).

[58] Othman et al. «Antimicrobial Activity of Polyphenols and Alkaloids in Middle Eastern Plants». In: *Frontiers* (2019) (cit. on p. 39).