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Biochemical pathways and kinetic determinants of methane production in the anaerobic digestion process

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ABSTRACT

Anaerobic digestion (AD) is a microbial process in which organic matter is broken down by microbial communities in the absence of oxygen, producing biogas composed mainly of methane and carbon dioxide. The process proceeds through hydrolysis, acidogenesis, acetogenesis and methanogenesis, and methane is the main energy-rich component of the resulting gas. Methane production during AD is controlled by interactions among biochemical pathways, reaction kinetics and microbial activity. Although these aspects are commonly discussed separately, their interdependence largely determines process efficiency and stability. This review presents an integrated analysis of the biochemical and kinetic mechanisms governing methane formation, linking microbial metabolism with substrate conversion dynamics and process performance. Particular emphasis is placed on the relationship between hydrolysis, methanogenesis, methane accumulation kinetics and the factors limiting methane production, including substrate resistance to biodegradation, operating conditions, inhibition and inefficient electron transfer. The review also discusses current approaches for enhancing methane production by increasing substrate availability, supporting microbial cooperation and applying kinetic-based process optimisation. Its distinctive feature is the integration of biochemical pathways, kinetic interpretation and process-limiting factors into one coherent framework for analysing methane production. This approach may support the development of more efficient, stable and predictable anaerobic digestion systems.

Keywords: anaerobic digestion, biochemical methane potential, methane production kinetics, hydrolysis, methanogenesis, kinetic modelling, microbial interactions.

INTRODUCTION

Anaerobic digestion is widely used for organic waste treatment and renewable energy recovery. Its importance has grown with the need to reduce biodegradable waste streams and improve the sustainability of energy systems (Appels et al., 2008; Kumar et al., 2024). Although the process is commonly divided into hydrolysis, acidogenesis, acetogenesis and methanogenesis, these stages form a continuous biochemical network in which the products of one microbial group become substrates for another. Methane production therefore depends on both feedstock composition and the rate and balance of successive biochemical conversions (Pavlostathis and Giraldo-Gomez, 1991; Batstone et al., 2002; Nayeri et al., 2024).

Methane formation is closely linked to substrate degradation and intermediate conversion. Hydrolysis often controls the release of soluble compounds from complex or particulate substrates (Vavilin et al., 2008; Prasad et al., 2024). By contrast, rapid degradation of readily biodegradable matter may intensify acid formation and promote the accumulation of volatile fatty acids. This may lower pH, inhibit methanogenic activity

and disturb process stability (Chen et al., 2008; He et al., 2024). High biodegradability does not therefore guarantee efficient methane production. Stable conversion requires coordination between substrate breakdown, syntrophic oxidation and methanogenic activity.

Operating and environmental conditions further influence this balance. Temperature, pH, organic loading rate, retention time, ammonia concentration, trace element availability and substrate composition affect microbial growth and reaction rates (Demirel and Scherer, 2011; Yenigün and Demirel, 2013). Syntrophic interactions and electron transfer are also important because they connect the bacterial conversion of intermediates with methanogenesis (Liu et al., 2012; Kato et al., 2012; Lovley, 2017). Anaerobic digestion should therefore be viewed as a dynamic microbial system in which biochemical pathways, reaction kinetics and operating conditions shape methane production and process stability.

The aim of this article is to review the biochemical pathways and kinetic determinants of methane production during anaerobic digestion. Particular attention is given to the links between metabolic transformations, reaction rates and the factors that disturb efficient substrate conversion.

BACKGROUND

Current evidence indicates that the final biochemical methane potential (BMP) value alone is not sufficient to assess anaerobic digestion performance. It does not describe degradation dynamics, process stability or the extent to which methane potential is realised in practice (Pilarska et al., 2026). This broader view is reflected in the analysis by Pilarski and Pilarska (2025), who linked kinetic limitations with substrate composition, pretreatment, organic loading, retention time and early-warning indicators. Their analysis showed that kinetic parameters are most useful when they support operating decisions. Wu et al. (2025) found that greater treatment capacity and energy recovery under organic overloading may be accompanied by reduced resilience to disturbance. Alves et al. (2026) likewise observed that adding green waste to food waste affected digestion time and stability, although the gain in final methane yield was limited. Final yield and the course of digestion may therefore lead to different assessments of the same substrate mixture.

Work on kinetic modelling also confirms that no single approach is suitable for every digestion system. Lower et al. (2025) compared four models for the thermophilic digestion of Lemnaceae biomass. As the inoculum had been acclimatised to the same feedstock, no clear lag phase occurred, and the first-order and transference equations gave the best fit. Kusi et al. (2025) examined fibrous materials and slaughterhouse waste, where an adapted hybrid formulation described the more complex degradation pattern more accurately. Tiwari et al. (2025) compared the first-order, modified Gompertz and Chen–Hashimoto models for DIET-enhanced (DIET – direct interspecies electron transfer) digestion of wheat husk. Following calibration and validation, the Chen–Hashimoto model provided the highest predictive accuracy. The suitability of a given equation therefore depends on the substrate, inoculum history and operating conditions. Estimated parameters should describe the system under study rather than be regarded as universal biological constants.

Research on plant-based feedstocks provides a clearer view of how chemical composition shapes methane production profiles. Pilarski et al. (2026a) examined ten batches of maize silage and found that residual organic matter and hemicellulose shortened the lag phase and increased the maximum methane production rate. The acid detergent lignin-to-cellulose ratio influenced the slower part of the conversion curve (Prasad et al., 2024). Greater electrical conductivity and increased Na, Cl and S loads were associated with a later onset of methane formation, whereas the C/N ratio and trace elements involved in methanogenesis were linked with improved stability (Demirel and Scherer, 2011). In ten sugar beet pulp variants, a larger proportion of simple sugars increased the rapidly degradable fraction and shortened the lag phase.By contrast, greater fibre content reduced the rate constant of the slowly degradable fraction and lowered organic matter conversion (Pilarski et al., 2026b). Individual feedstock components may therefore affect different stages of methane release and require different feeding strategies and retention times.

Digestion performance may also improve when conditions favour microbial activity and cooperation. Better biomass retention can support bacterial growth, enzymatic activity and biogas production, while stable pH and alkalinity help maintain process balance (Pilarska et al., 2019; Nayeri et al., 2024). However, an observed

improvement should not be attributed too quickly to a single mechanism. The same caution applies to direct interspecies electron transfer. Changes in methane production and acetate or hydrogen concentrations provide only indirect evidence, whereas reliable confirmation requires complementary electrochemical, microbiological and molecular methods (Lovley, 2017; Khalid et al., 2025). Assessment should therefore include methane production dynamics, kinetic parameters, microbial response and operational stability.

BIOCHEMICAL BASIS OF METHANE PRODUCTION

Methane production in the AD process results from interconnected biochemical reactions carried out by specialised microbial communities rather than by a single metabolic pathway. Understanding these microbial interactions, carbon conversion pathways and methanogenic mechanisms provides the foundation for interpreting the kinetic behaviour, stability and efficiency of anaerobic digestion systems.

Microbial communities and biochemical pathways

Anaerobic digestion is carried out by complex microbial consortia in which different functional groups perform complementary biochemical roles. No single group of microorganisms can convert complex organic matter directly into methane. Instead, methane formation depends on the sequential and partly simultaneous activity of hydrolytic, fermentative, syntrophic and methanogenic populations. In the Anaerobic Digestion Model No. 1, Batstone et al. (2002) described AD as a network of biochemical conversions involving disintegration, hydrolysis, acidogenesis, acetogenesis and methanogenesis. This approach is important because it shows that the process is controlled not by a single reaction but by the balance between several microbial groups and their metabolic products.

The first functional groups involved in AD are hydrolytic and fermentative bacteria. They decompose complex polymers, such as carbohydrates, proteins and lipids, into soluble compounds that can be further metabolised. Appels et al. (2008) emphasised the central role of these initial transformations in the anaerobic degradation of organic matter, especially in systems treating sludge and complex wastes. Hydrolysis is therefore not only a preliminary stage but also a biochemical gateway that determines how much substrate becomes available for subsequent microbial communities. Its efficiency depends on the structure of the feedstock, the accessibility of organic fractions and the activity of extracellular enzymes. This is particularly important for particulate and lignocellulosic materials, for which Vavilin et al. (2008) identified hydrolysis kinetics as a key element controlling anaerobic degradation.

The soluble products formed during hydrolysis are converted by acidogenic bacteria into volatile fatty acids, alcohols, hydrogen and carbon dioxide. These compounds then become substrates for acetogenic and syntrophic bacteria, which transform higher organic acids and alcohols into acetate, hydrogen and carbon dioxide. This part of the process is especially sensitive because many acetogenic reactions are thermodynamically favourable only when hydrogen is maintained at a low concentration. For this reason, the activity of hydrogen-consuming methanogens is directly linked to the efficiency of syntrophic oxidation. Pavlostathis and Giraldo-Gomez (1991) showed that anaerobic treatment kinetics must be understood through the interactions among substrate conversion, microbial growth and environmental conditions rather than through isolated reaction rates. Methanogenic archaea complete the biochemical sequence by converting acetate, hydrogen, carbon dioxide and selected one-carbon compounds into methane. Although methanogens are essential for the final energy recovery stage, they are generally slower-growing and more sensitive to environmental stress than many bacterial groups. Disturbances in pH, ammonia concentration or volatile fatty acid accumulation may therefore affect methanogenesis and destabilise the entire process. Chen et al. (2008) linked many inhibition phenomena in AD to the disruption of microbial activity and intermediate conversion, while Yenigün and Demirel (2013) highlighted ammonia as a particularly important factor affecting methanogenic performance.

Thus, methane production should be interpreted as the result of microbial cooperation rather than as the final step of a simple linear pathway. Recent reviews by Nayeri et al. (2024) and Kumar et al. (2024) confirm that the performance

of AD systems depends on the integration of microbial mechanisms, substrate properties and operating conditions. This perspective is essential for linking biochemical pathways with process kinetics, because changes in microbial balance affect both the rate of organic matter conversion and the stability of methane production.

Carbon and electron flow in anaerobic digestion

Carbon and electron flow are central to methane formation during anaerobic digestion. Organic carbon enters the process mainly in the form of carbohydrates, proteins and lipids. These compounds are first converted into soluble monomers and then into volatile fatty acids, alcohols, acetate, hydrogen and carbon dioxide. In this sequence, carbon is progressively redistributed among dissolved intermediates, microbial biomass and gaseous end products. The final direction of this flow depends on the balance between fermentative bacteria, syntrophic oxidisers and methanogenic archaea (Batstone et al., 2002; Appels et al., 2008).

Electron flow is equally important because methane formation requires the transfer of reducing equivalents generated during substrate degradation. During acidogenesis and acetogenesis, electrons are carried mainly by hydrogen, formate or reduced organic compounds. These carriers must be rapidly consumed to keep syntrophic reactions thermodynamically favourable. If hydrogen or reduced intermediates accumulate, the oxidation of propionate, butyrate and other compounds becomes less efficient, and the whole process may slow down. This explains why anaerobic digestion is not only a carbon conversion process but also a system of controlled electron disposal. Methanogenic archaea close this biochemical network by using acetate or by reducing carbon dioxide with hydrogen-derived electrons. In this way, they remove key intermediates and maintain the conditions required for upstream bacterial metabolism. Acetate is the main direct carbon precursor of methane in many digesters, while hydrogen and carbon dioxide support hydrogenotrophic methanogenesis. The balance between these routes affects both methane yield and process stability. A disturbance in this balance may lead to volatile fatty acid accumulation, pH decline and reduced methanogenic activity (Chen et al., 2008; Nayeri et al., 2024).

Recent studies have expanded this classical view by showing that electrons may also be exchanged directly between microorganisms. In direct interspecies electron transfer, conductive materials or electroactive cells may support electron movement without hydrogen serving as the main intermediate. This mechanism has been linked to improved syntrophic cooperation and faster methane formation in systems containing conductive minerals, activated carbon or electroactive microorganisms such as *Geobacter*, *Methanosarcina* and *Methanosaeta* (Kato et al., 2012; Rotaru et al., 2014; Lovley, 2017). Therefore, carbon conversion and electron transfer should be considered together because both determine how efficiently reducing equivalents are channelled towards methane rather than contributing to the accumulation of unstable intermediates.

Methanogenesis pathways

Methanogenesis is the final biological stage of anaerobic digestion and the process directly responsible for methane formation. It is carried out exclusively by methanogenic archaea, which occupy a unique ecological niche within anaerobic microbial communities. Their activity not only determines methane yield but also maintains the thermodynamic conditions required for upstream microbial reactions. Consequently, methanogenesis represents the key biochemical link between intermediate carbon metabolism and energy recovery in anaerobic digestion (Nayeri et al., 2024).

Three principal metabolic pathways are involved in biological methane formation, namely acetoclastic, hydrogenotrophic and methylotrophic methanogenesis. Their relative contributions depend on substrate composition, reactor configuration and operating conditions. In agricultural and municipal digesters, acetoclastic methanogenesis is generally regarded as the dominant route because acetate is the main intermediate generated during the degradation of carbohydrates, proteins and lipids. Approximately two-thirds of the methane produced under mesophilic conditions originates from acetate, while the remaining fraction is mainly formed through the reduction of carbon dioxide with hydrogen. The balance between these pathways is dynamic and may change in response to environmental stress or variations in substrate characteristics (Kumar et al., 2024).

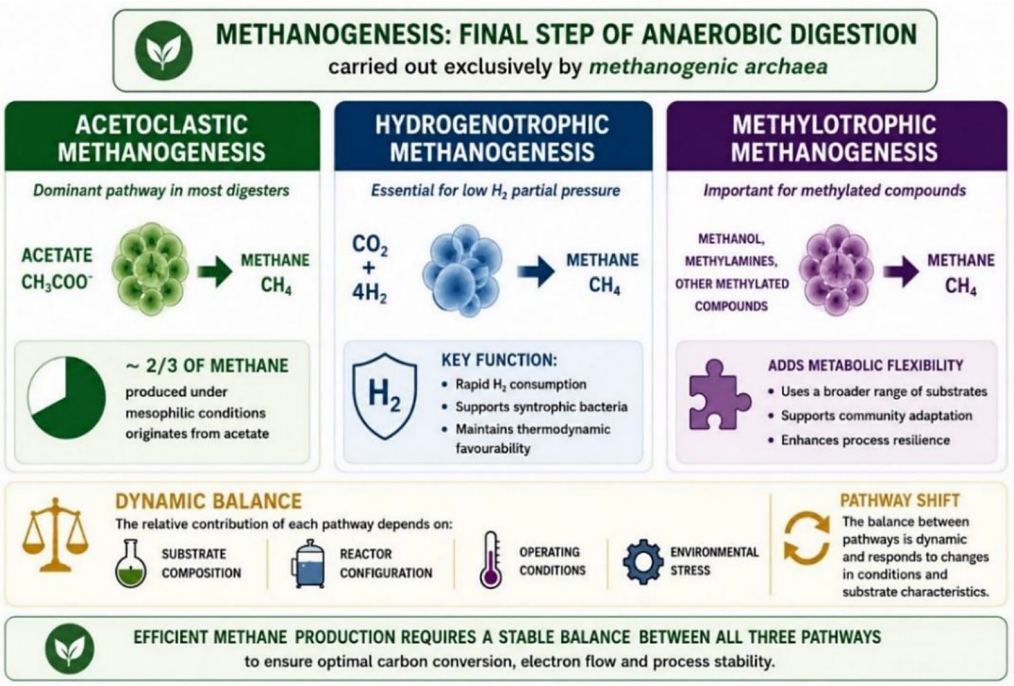

Figure 1. Principal methanogenesis pathways during anaerobic digestion (authors' own elaboration prepared using Canva's AI features)

Hydrogenotrophic methanogenesis plays a particularly important role in maintaining a low hydrogen partial pressure within the digester. Rapid hydrogen consumption enables syntrophic bacteria to oxidise volatile fatty acids and other reduced intermediates that would otherwise accumulate. This close metabolic cooperation is essential because several acetogenic reactions become thermodynamically unfavourable when hydrogen concentrations increase. Consequently, the efficiency of methane formation depends not only on methanogenic activity itself but also on the continuous exchange of metabolic intermediates between different microbial populations (Pavlostathis and Giraldo-Gomez, 1991; Chen et al., 2008). Although methylotrophic methanogenesis usually contributes less to total methane production, it becomes important in digesters treating substrates containing methanol, methylamines or other methylated compounds. This pathway expands the metabolic flexibility of anaerobic microbial communities and enables efficient utilisation of a broader range of organic substrates. Recent studies also indicate that methanogenic pathways are not independent but may shift in response to environmental conditions, microbial adaptation and process stability. Such metabolic flexibility improves the resilience of anaerobic digestion and helps maintain methane production despite fluctuations in substrate composition or operational parameters (Demirel and Scherer, 2011; Nayeri et al., 2024).

Recent advances in microbial ecology further suggest that methane formation should be interpreted as the outcome of coordinated metabolic interactions rather than isolated biochemical reactions. The recognised roles of syntrophic cooperation and direct interspecies electron transfer demonstrate that carbon conversion and electron exchange are closely interconnected within methanogenic communities. Therefore, understanding methanogenesis requires consideration of both metabolic pathways and the microbial interactions that sustain them. This provides a basis for improving methane production and process stability (Kato et al., 2012; Lovley, 2017).

KINETIC BASIS OF METHANE FORMATION

Methane production kinetics determine the rate and progression of anaerobic digestion beyond the final methane yield. This section examines the key kinetic aspects of hydrolysis, methane accumulation and kinetic modelling in anaerobic digestion.

Hydrolysis as a kinetic gateway

Hydrolysis is generally regarded as the principal kinetic gateway of anaerobic digestion because it controls the release of soluble compounds from complex organic matter. Unlike the subsequent microbial stages, which utilise readily available intermediates, hydrolysis determines how rapidly carbon enters the anaerobic conversion pathway. Consequently, the overall rate of methane formation is frequently limited by substrate depolymerisation rather than by methanogenic activity itself. This limitation is particularly evident during the digestion of lignocellulosic biomass, agricultural residues and other particulate substrates, where restricted accessibility of biodegradable fractions slows the entire process. Recent reviews by Kumar et al. (2024) and Nayeri et al. (2024) emphasise that the kinetics of anaerobic digestion are governed by the interactions among substrate properties, microbial activity and operating conditions rather than by a single biochemical reaction.

The rate of hydrolysis depends on both biological activity and substrate characteristics. Hydrolytic microorganisms secrete extracellular enzymes that convert carbohydrates, proteins and lipids into soluble sugars, amino acids and long-chain fatty acids. However, enzyme production alone does not guarantee rapid degradation. Particle size, lignin content, cellulose crystallinity, moisture distribution and surface accessibility strongly influence enzyme–substrate interactions and, consequently, the availability of biodegradable organic matter. Vavilin et al. (2008) demonstrated that hydrolysis kinetics become particularly important when slowly degradable substrates dominate the feedstock, while Prasad et al. (2024) showed that disruption of lignocellulosic structures markedly improves substrate accessibility and accelerates subsequent methane formation.

Figure 2 presents an example of a full-scale agricultural biogas plant equipped with a dedicated acid hydrolysis reactor, in which the initial hydrolysis stage enhances the conversion of complex organic substrates.

Hydrolysis dynamics influence the digestion process. Insufficient hydrolysis limits the supply of soluble intermediates, prolongs the lag phase and reduces methane production rates. In contrast, the rapid release of readily biodegradable compounds may temporarily exceed the conversion capacity of downstream microbial communities, resulting in volatile fatty acid accumulation and transient process instability. Donatelli and Chang (2024) demonstrated that methane production kinetics are closely linked to substrate composition and degradation dynamics, whereas Llanos-Lizcano et al. (2024) showed that the shape of methane production curves reflects the synchronisation between substrate degradation and microbial conversion rather than the final methane yield alone.

Recent evidence suggests that the hydrolysis rate should be considered a controllable engineering parameter rather than an inherent substrate property. Anacleto et al. (2024), in a comprehensive meta-analysis, demonstrated that the response to pretreatment depends strongly on substrate composition, indicating that no single

Figure 2. Full-scale agricultural biogas plant (0.499 MW) with a dedicated acid hydrolysis stage

strategy is universally effective. This finding reinforces the view that optimisation of methane production should begin with improving substrate accessibility and controlling hydrolysis kinetics. Because hydrolysis determines the availability of carbon for all subsequent microbial transformations, it establishes the kinetic framework for the entire anaerobic digestion process.

Dynamics of methane accumulation

Methane accumulation reflects the dynamic progression of anaerobic digestion rather than its final conversion efficiency. Unlike the ultimate methane yield, which represents the biochemical potential of a substrate, the methane accumulation profile integrates the rates of hydrolysis, intermediate conversion and methanogenesis throughout the digestion process. It therefore provides valuable information on substrate biodegradability, microbial adaptation and process performance. Consequently, substrates producing similar methane yields may exhibit markedly different accumulation patterns, indicating differences in conversion dynamics rather than in methane potential alone. Donatelli and Chang (2024) demonstrated that methane production kinetics vary considerably with the chemical composition of food waste fractions, while Llanos-Lizcano et al. (2024) confirmed that kinetic parameters offer important insight into substrate degradation behaviour.

A typical methane accumulation curve consists of three characteristic phases: an initial lag phase, a period of rapid methane production and a final plateau corresponding to substrate depletion. The lag phase reflects the time required for microbial communities to establish efficient metabolic cooperation under the prevailing process conditions. This phase is influenced by inoculum activity, substrate properties, temperature and the inoculum-to-substrate ratio. It is followed by the exponential phase, during which methane production reaches its highest rate, before gradually approaching a plateau as biodegradable organic matter becomes exhausted. The duration and slope of each phase provide insight into the efficiency of substrate conversion and the metabolic response of the microbial community. Rapid methane accumulation generally indicates efficient synchronisation between hydrolysis, acidogenesis and methanogenesis, whereas a prolonged lag phase or a gradual increase in methane production may indicate limited substrate availability or slower microbial adaptation. For this reason, methane accumulation curves are used not only to quantify methane production but also to evaluate the progression and stability of the anaerobic digestion process. The transition between these stages is continuous rather than discrete because microbial activity adjusts continuously to changes in substrate availability.

The shape of the methane accumulation curve is highly sensitive to changes during anaerobic digestion. Limited substrate accessibility or low biodegradability frequently prolong methane production, whereas readily degradable feedstocks generally accelerate methane formation but may also promote the temporary accumulation of intermediates if microbial conversion becomes unbalanced. Recent interlaboratory studies by Hafner et al. (2026) and Casavant et al. (2025) further demonstrated that reliable interpretation of methane accumulation requires standardised experimental conditions, particularly with respect to inoculum quality and test completion criteria. Consequently, methane accumulation curves are recognised not only as a means of reporting methane production but also as indicators of substrate biodegradability, microbial adaptation and overall process performance. Figure 3 presents a conceptual methane accumulation curve highlighting the principal phases and key kinetic parameters of anaerobic digestion.

Modelling methane production kinetics

Kinetic models provide a quantitative framework for describing methane production beyond the final methane yield. By characterising the dynamics of methane accumulation, they enable the estimation of key process parameters, including the maximum methane production rate, lag phase and degradation constant. These parameters facilitate the interpretation of substrate biodegradability and process performance under different operating conditions. They also allow comparisons between substrates that differ in composition, degradation behaviour and methane production dynamics (Pilarski et al., 2026a; Pilarski et al., 2026b). Da Silva et al. (2024) demonstrated that advanced kinetic approaches can describe methane production profiles more accurately than conventional first-order models, particularly for heterogeneous substrates.

Among the available approaches, first-order, modified Gompertz and logistic models remain

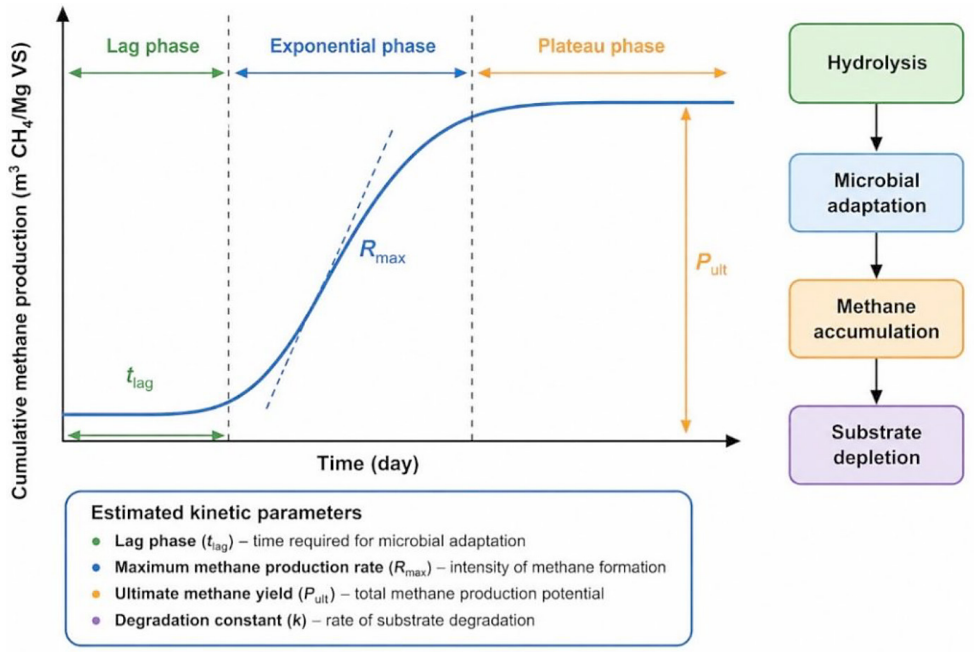

Figure 3. Conceptual methane accumulation curve during anaerobic digestion and its key kinetic parameters (authors' own elaboration prepared using Canva AI)

the most widely applied because they combine relatively simple mathematical formulations with good predictive performance. First-order models are commonly used to describe substrate degradation, whereas Gompertz-type models better capture microbial adaptation and the lag phase preceding intensive methane production (Pilarski et al., 2026a; Pilarski et al., 2026b). Llanos-Lizcano et al. (2024) showed that kinetic modelling facilitates comparison of different organic substrates by identifying differences in degradation rate rather than methane yield alone. Likewise, Catenacci et al. (2022) reported that reliable estimation of first-order model parameters allows earlier prediction of biochemical methane potential, reducing the duration of laboratory experiments.

Beyond the interpretation of laboratory results, kinetic models increasingly support reactor optimisation, process design and scale-up. They are used to evaluate pretreatment efficiency, compare co-digestion scenarios and predict process performance under changing operating conditions. Manthos et al. (2023) demonstrated that kinetic information obtained from BMP tests can improve substrate selection and operational planning in continuous anaerobic digestion systems. In addition, Pilarski et al. (2020) proposed a biochemical methane potential correction coefficient to improve the interpretation of laboratory BMP results and their application to full-scale digesters. Consequently, kinetic modelling has evolved from a descriptive tool into an important component of process evaluation, optimisation and full-scale application in modern anaerobic digestion systems. The principal kinetic models used for methane production analysis are presented in Table 1.

Table 1. Overview of the principal kinetic models used for methane production analysis

Model	Main application	Strengths	Limitations	Reference
First-order	Describes substrate degradation kinetics	Simple, robust and suitable for comparing substrate biodegradability	Does not account for microbial adaptation or lag phase	Catenacci et al. (2022); Llanos-Lizcano et al. (2024)
Modified Gompertz	Describes cumulative methane production kinetics	Accurately estimates lag phase, maximum methane production rate and methane potential; widely used for BMP analysis	Empirical model with limited mechanistic interpretation	Donatelli and Chang (2024); da Silva et al. (2024)
Logistic	Describes cumulative methane production	Simple mathematical formulation and good fit for sigmoidal methane production curves	Less suitable for complex degradation patterns and prolonged lag phases	Llanos-Lizcano et al. (2024); Cabrita and Santos (2023)

FACTORS LIMITING METHANE PRODUCTION

Methane production is determined not only by biochemical pathways and reaction kinetics but also by factors that restrict microbial activity and process efficiency. These limitations arise from substrate properties, operating conditions and microbial interactions, which often act simultaneously. Figure 4 summarises the principal factors limiting methane production and their interrelationships during anaerobic digestion.

Substrate resistance to biodegradation

Substrate biodegradability is one of the principal factors limiting methane production because it determines how efficiently organic matter can be converted into soluble compounds available for microbial metabolism. Organic substrates differ considerably in their susceptibility to biological degradation owing to differences in chemical composition, structural organisation and the accessibility of biodegradable fractions. Easily degradable compounds, including simple carbohydrates, proteins and lipids, are converted rapidly, whereas lignocellulosic biomass, crop residues and other structurally complex materials degrade much more slowly. Consequently, methane production is frequently constrained not by the theoretical methane potential of the substrate but by the limited availability of biodegradable organic matter. He et al. (2024) emphasised that substrate composition remains one of the primary determinants of methane production efficiency, particularly during the anaerobic digestion of heterogeneous organic wastes. Li et al. (2024) also identified substrate biodegradability as a key factor controlling process performance under both laboratory and full-scale conditions.

The resistance of lignocellulosic substrates results primarily from the complex structure of the plant cell wall. Cellulose fibres are embedded within a matrix of hemicellulose and lignin, which restricts enzymatic access and considerably slows hydrolysis. In addition, the crystalline structure of cellulose and the strong interactions between structural polymers reduce the availability of biodegradable carbohydrates despite their relatively high carbon content. As a result, only a fraction of the organic material is readily converted during the initial stages of anaerobic digestion, whereas the remaining fraction requires substantially longer retention times. Prasad et al. (2024) demonstrated that disruption of lignocellulosic structures significantly enhances substrate accessibility and improves methane production, confirming that structural recalcitrance rather than organic content alone often determines process efficiency. Similar conclusions were reached by Anacleto et al. (2024), whose meta-analysis showed that the effectiveness of pretreatment strongly depends on the chemical and structural characteristics of the processed biomass.

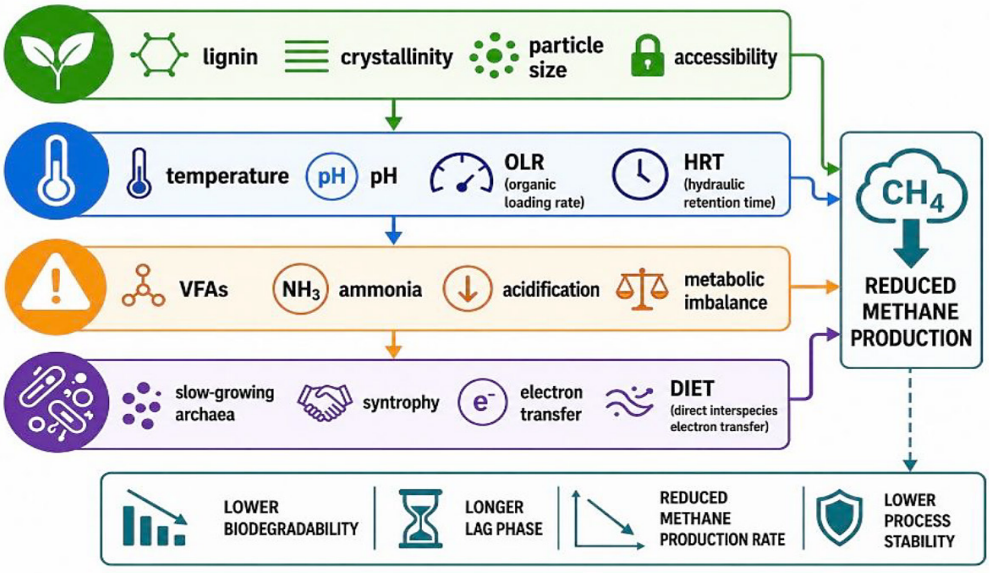

Figure 4. Integrated scheme of the principal factors limiting methane production during anaerobic digestion (authors' own elaboration prepared using Canva AI)

Resistance to biodegradation is influenced not only by chemical composition but also by several physical properties that directly affect microbial access to the substrate. Particle size, porosity, moisture distribution, bulk density and the degree of prior biological or mechanical degradation affect interactions among microorganisms, extracellular enzymes and organic matter. Consequently, substrates with comparable volatile solids content may exhibit markedly different methane production kinetics because the biodegradable fraction is not equally available for microbial conversion. Conventional compositional analyses alone are therefore often insufficient to predict methane production dynamics. Toufexis et al. (2024) indicated that reliable evaluation of biomethane potential requires consideration of substrate quality alongside its physical characteristics, whereas Manthos et al. (2023) demonstrated that substrate-specific degradation behaviour substantially influences the optimisation of continuous anaerobic digestion systems.

From a practical perspective, substrate resistance is not a fixed barrier but a property that can be partly modified through appropriate process design. Mechanical size reduction, thermal and chemical pretreatments, biological conditioning and co-digestion may improve the availability of biodegradable fractions by disrupting structural barriers or balancing feedstock composition. However, treatment efficiency remains substrate-specific and depends on both feedstock characteristics and operating conditions. Li et al. (2024) emphasised that improvement strategies should therefore be selected according to substrate properties rather than routinely applied, while Paranjpe et al. (2023) showed that combining complementary substrates through co-digestion can partly compensate for limited biodegradability and improve overall methane production. These findings indicate that substrate resistance should be regarded as a process characteristic rather than a fixed property, as it influences methane formation kinetics and can be reduced through targeted engineering interventions.

Environmental control of reaction rates

Environmental conditions directly regulate the rate of biochemical reactions throughout anaerobic digestion and strongly influence methane production efficiency. Even readily biodegradable substrates may not be converted effectively if process conditions limit microbial activity or disturb metabolic interactions. Among the main process variables, temperature, pH, organic loading rate and hydraulic retention time exert the greatest influence on reaction kinetics because they affect microbial growth, enzymatic activity and substrate conversion rates. Li et al. (2024) emphasised that stable operating conditions are essential for sustaining methane productivity in mono-digestion and co-digestion systems. Similarly, Lee et al. (2024) demonstrated that operating temperature strongly affects methane production, with mesophilic and thermophilic conditions resulting in different degradation dynamics.

Temperature influences not only reaction rates but also the balance between successive stages of anaerobic digestion. Higher temperatures may accelerate biochemical conversion and shorten degradation time, but they can also increase the sensitivity of microbial communities to process fluctuations. Reaction rates are also influenced by pH, which affects enzyme activity, acid formation and methanogenic metabolism. Deviations from the optimum range reduce methane production by weakening syntrophic cooperation and inhibiting methanogenic archaea. Excessive organic loading or insufficient hydraulic retention time may further disturb conversion dynamics by causing soluble intermediates to be produced more rapidly than they can be transformed. He et al. (2024) indicated that properly adjusted operating parameters are necessary to prevent process deterioration, while Manthos et al. (2023) showed that optimisation of substrate use and operating conditions can improve reactor performance.

Environmental factors rarely act independently. A change in one parameter may modify the effect of another, influencing microbial activity, substrate conversion and methane production at the same time. For example, increasing the organic loading rate without adequate retention time may destabilise microbial metabolism even when temperature and pH remain favourable. Efficient methane production therefore depends on maintaining balanced operating conditions rather than maximising individual parameters. As noted by Toufexis et al. (2024), optimisation of anaerobic digestion should consider the combined effects of environmental conditions, substrate characteristics and microbial activity rather than individual variables alone.

Inhibition and process imbalance

Inhibition in anaerobic digestion usually occurs when the formation of intermediates exceeds the capacity of microorganisms to convert them into methane. The most common signs are volatile fatty acid accumulation, pH decline, reduced methanogenic activity and slower gas production. This imbalance may result from excessive organic loading, rapid degradation of readily biodegradable substrates, ammonia toxicity or abrupt changes in operating conditions. He et al. (2024) indicated that food waste digestion is particularly sensitive to acidification because its organic matter is rapidly degraded, which may intensify acidogenesis.

Process imbalance is closely linked to the different sensitivities of microbial groups. Acidogenic bacteria usually respond more rapidly to substrate availability than methanogenic archaea, which exhibit slower growth and greater sensitivity to environmental disturbances. As a result, acid formation may proceed even when methane formation is already limited. Li et al. (2024) emphasised that stable co-digestion performance requires a balance among substrate degradation, intermediate conversion and methanogenic activity. Similarly, Paranjpe et al. (2023) showed that co-digestion can reduce instability by improving nutrient balance and buffering capacity.

The practical significance of inhibition lies in its early detection rather than in the separate description of individual inhibitors. A decline in methane production rate, a prolonged lag phase or a delayed plateau may indicate that the process is shifting from efficient conversion towards metabolic stress. For this reason, inhibition should be interpreted as a dynamic disturbance of biochemical and kinetic balance, rather than solely as the effect of a single toxic compound. This perspective links process monitoring with methane production kinetics and shows that stable methane production depends on maintaining metabolic balance throughout anaerobic digestion.

Methanogenic limitations and electron transfer

Methane formation represents the final stage of anaerobic digestion and depends on the efficiency of electron transfer between syntrophic bacteria and methanogenic archaea. Unlike the preceding biochemical reactions, methanogenesis proceeds only when reducing equivalents generated during substrate degradation are continuously transferred and consumed. Any disturbance **in** this interaction limits methane production, even when hydrolysis and acidogenesis remain active. Nayeri et al. (2024) emphasised that efficient methanogenesis requires close coordination between microbial populations, while Kumar et al. (2024) identified microbial interactions as one of the principal determinants of process stability and methane productivity.

A major limitation arises from the different physiological characteristics of syntrophic bacteria and methanogenic archaea. Syntrophic microorganisms rapidly oxidise fermentation intermediates, whereas methanogens exhibit slower growth and greater sensitivity to environmental fluctuations. Consequently, methane production depends on maintaining a balance between the formation of intermediates and their subsequent conversion. If electron transfer becomes inefficient, hydrogen and volatile fatty acids accumulate, reducing the thermodynamic feasibility of syntrophic oxidation and progressively slowing methane generation. As discussed by Li et al. (2024), maintaining metabolic cooperation among microbial groups is therefore essential for sustaining stable reactor performance.

Recent studies have shown that DIET may provide a more efficient mechanism of microbial communication than conventional interspecies hydrogen transfer. In DIET, electrons are exchanged directly between microorganisms through electrically conductive pili or conductive materials, reducing energy losses associated with diffusible electron carriers. This mechanism can enhance syntrophic metabolism, accelerate methane formation and improve process stability, particularly under high organic loading conditions. The concept was first demonstrated by Rotaru et al. (2014) and subsequently reviewed by Lovley (2017), while more recent studies continue to identify DIET as an important route for improving methane production efficiency. Conductive materials, including activated carbon, biochar, magnetite and iron-based minerals, facilitate electron transfer by providing conductive pathways that strengthen microbial interactions (Liu et al., 2012; Kato et al., 2012).

The practical significance of electron transfer extends beyond microbial physiology because it directly influences methane production kinetics and reactor stability. Enhanced

electron exchange may shorten the lag phase, accelerate methane accumulation and improve the conversion of fermentation intermediates into methane. Consequently, strategies that stimulate syntrophic cooperation are increasingly recognised as effective approaches to overcoming methanogenic limitations. As noted by Dębowski et al. (2024), optimisation of microbial activity should be combined with improvements in substrate accessibility and operating conditions, whereas Toufexis et al. (2024) highlighted that efficient biomethane production ultimately depends on the integration of biochemical pathways, microbial interactions and process engineering rather than on the optimisation of individual process stages alone.

EMERGING STRATEGIES FOR ENHANCING METHANE PRODUCTION

Improving methane production requires first improving substrate availability for microbial conversion. Mechanical, thermal, chemical and biological pretreatments accelerate hydrolysis by disrupting lignocellulosic structures, increasing the accessible surface area and facilitating enzymatic degradation. However, recent evidence indicates that pretreatment efficiency depends strongly on substrate composition and should therefore be selected according to feedstock characteristics rather than applied routinely. Anacleto et al. (2024) demonstrated that methane yield improvements vary considerably among biomass types, while Prasad et al. (2024) highlighted that appropriate pretreatment substantially enhances the biodegradability of lignocellulosic substrates. Co-digestion provides an additional strategy by balancing nutrient composition, improving the C/N ratio and promoting the use of complementary substrates. According to Li et al. (2024), substrate selection and appropriate feedstock combinations are essential for maintaining stable methane production, whereas Paranjpe et al. (2023) showed that co-digestion improves process resilience while increasing methane yield. These findings indicate that improving substrate availability should be regarded as the foundation for enhancing both methane production kinetics and process efficiency.

Supporting microbial activity is equally important because methane production ultimately depends on the coordinated metabolism of syntrophic bacteria and methanogenic archaea. Current strategies increasingly focus on stimulating microbial cooperation rather than simply increasing microbial abundance. Conductive materials, including biochar, activated carbon and iron-based minerals, promote direct interspecies electron transfer (DIET), strengthen syntrophic interactions and accelerate methane formation by improving electron exchange between microbial populations (Liu et al., 2012; Rotaru et al., 2014). Dębowski et al. (2024) demonstrated that process intensification technologies can significantly improve anaerobic digestion performance in agricultural systems, while Toufexis et al. (2024) emphasised that efficient biomethane production requires the integration of substrate quality, microbial activity and process engineering. Recent reviews by Kumar et al. (2024) and Nayeri et al. (2024) further indicate that maintaining a diverse and metabolically balanced microbial community enhances process stability, accelerates methane production and improves the resilience of anaerobic digesters under changing operating conditions.

Sustained improvement of anaerobic digestion requires effective process monitoring and optimisation. Modern process control combines conventional operating parameters with kinetic indicators describing methane accumulation, substrate degradation and microbial adaptation (Manthos et al., 2023; Hafner et al., 2026). Kinetic models, biochemical methane potential tests and standardised analytical procedures provide early indications of process performance and support optimisation before full-scale implementation. Manthos et al. (2023) demonstrated that kinetic information obtained from BMP tests facilitates substrate selection and operational planning in continuous digesters. Likewise, Hafner et al. (2026) and Casavant et al. (2025) highlighted the importance of standardised BMP testing for obtaining reliable and comparable methane production data, whereas Cabrita and Santos (2023) emphasised the value of BMP assays for substrate evaluation and process optimisation. Consequently, future improvements in methane production are expected to rely increasingly on the integration of substrate engineering, microbial management and data-driven process control supported by reliable monitoring and predictive kinetic analysis.

CONCLUSIONS

This review presented methane production as a biochemical and kinetic process governed by microbial cooperation, substrate conversion, methanogenic pathways and electron transfer. Based on the literature reviewed, the following conclusions were formulated:

1. Methane production should be interpreted as the result of coordinated microbial interactions rather than a simple sequence of independent biochemical stages.
2. Hydrolysis remains a key kinetic gateway because it controls the availability of soluble compounds for subsequent microbial conversion.
3. Methane accumulation curves and kinetic models provide more information than the final methane yield alone, especially regarding the lag phase, degradation rate and process stability.
4. Substrate recalcitrance, environmental conditions, inhibition and inefficient electron transfer are the main factors limiting methane production.
5. Effective enhancement of methane production requires integrated approaches that combine substrate pretreatment, microbial activity support, co-digestion and reliable process monitoring.

Efficient methane production depends on the interplay among biochemical pathways, reaction kinetics and microbial cooperation. Understanding these mechanisms provides the basis for improving the efficiency and stability of anaerobic digestion.

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