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“Estudio de la influencia del campo eléctrico en los metabolismos de la digestión anaerobia durante el tratamiento de un agua residual doméstica”

TESIS

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Universo, ¿qué más es posible?

“Nuestro miedo más profundo no es ser inadecuado, nuestro miedo más grande es nuestro poder incommensurable, es nuestra luz, no nuestra oscuridad lo que nos aterra. Optar por la mezquindad no sirve al mundo, no hay lucidez en encogerse para que los demás no se sientan inseguros junto a ti, nuestro destino es brillar como los niños, no es el de unos cuantos, es el de todos, y conforme dejamos que nuestra luz propia alumbre, inconscientemente permitimos lo mismo en los demás y al liberarnos de nuestro propio miedo, nuestra presencia automáticamente libera a otros”

-Marianne Williamson

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Summary

The growing need for efficient wastewater treatment has driven research to increase anaerobic digestion performance, particularly at low temperatures, where metabolic activity decreases. This study examines using a low magnitude controlled electric field to modify the anaerobic digestion (AD) process. This intervention enables efficient operation at temperatures below 35°C, improving the efficiency of biogas production.

Anaerobic granular sludge was first stabilized at room temperature (23 °C) and subsequently utilized to study each phase of the anaerobic digestion process (i.e., hydrolysis, acidogenesis, acetogenesis and methanogenesis) in batch culture. Each stage was supplied with a specific carbon source, such as sucrose, glucose, a mixture of volatile fatty acids (VFAs), and acetate, respectively, to enable precise characterization. The experimental units consisted of a two-electrode RuO₂/Ti (ruthenium oxide-coated titanium) cell, which was operated with short reaction times to avoid biofilm formation, thus isolating the effect of electrochemically generated oxygen (O₂) on anaerobic digestion. Three electric field magnitudes were tested: 1.21, 1.45, and 1.64 V/cm. The results showed that the application of the electric field increased dissolved oxygen levels in the medium, influencing each stage of the anaerobic digestion process. Optimal performance was achieved at 1.21 V/cm, with a dissolved oxygen production rate of 0.0753 mg O₂/L·min. This oxygen was rapidly consumed by facultative bacteria, as evidenced by a dissolved oxygen consumption rate of 0.484 ± 0.04 mg O₂/L·min. Notable improvements were observed across all stages of digestion, demonstrating the positive impact of the electric field on the overall process. Hydrolysis efficiency improved by 52%, driven by a 53% increase in extracellular enzyme activity observed after three hours of electric field application. The presence of O₂ influenced the acidogenesis and acetogenesis stages, shifting metabolism toward oxidation. This resulted in increased carbon dioxide (CO₂) production and reduced acetate formation. A significant observation was the 34% increase in volatile fatty acid (VFA) consumption during acetogenesis under the influence of the electric field. This improvement enhanced syntrophic interactions with acidogenesis, effectively addressing challenges often posed by VFA imbalances.

Methane (CH₄) production showed a significant increase, with methanogenic activity reaching 0.89 ± 0.02 g COD-CH₄/g VSS·d, marking a 39% improvement compared to the control. Remarkably, this level of methanogenic activity at room temperature closely matched the performance typically observed at 35 °C in conventional reactors.

To assess the effects of temperature, anaerobic digestion was evaluated at 10, 18, and 35 °C. Corn starch, serving as the carbon source, was provided at a concentration of 0.8 g chemical oxygen demand (COD)/L, with an electric field of 1.21 V/cm applied. The results demonstrated that at 18 °C, COD removal efficiency reached $89 \pm 1\%$, a performance level typically achieved at 35 °C in the control setup. Biogas analysis revealed a compositional shift under the influence of the applied electric field, with the introduction of approximately 2% hydrogen (H₂), indicating the role of electrochemically generated H₂ in enhancing CH₄ production. Further testing at 18 °C using a carbon-free mineral medium and an electric field of 1.21 V/cm showed a 2.6-fold increase in biogas production (95% CH₄, 5% H₂) compared to the control, highlighting the electric field's effect on hydrogenotrophic activity.

Hydrogenotrophic activity was further examined by scaling the process using two bench-scale up-flow anaerobic sludge blanket (UASB) reactors. One reactor (RC) served as the control without electrodes, while the experimental reactor (ER) incorporated electrodes within the sludge blanket zone to evaluate the effect of the electric field on anaerobic digestion. Both reactors, with an operating volume of 2 liters and a hydraulic retention time (HRT) of 12 hours, were fed municipal wastewater supplemented to 2 ± 0.1 g COD/L. In the ER, an electric field of 1.28 V/cm was applied using RuO₂/Ti titanium mesh electrodes strategically placed to optimize electron transfer with the biomass.

The results from the scaled-up system revealed an 86% increase in biogas production in the experimental reactor (ER), with a biogas composition of 68% CH₄, 21% CO₂, and 11% H₂, compared to 78% CH₄ and 22% CO₂ in the control. Additionally, the biogas from the ER exhibited a 23% higher calorific value, and CH₄ yield nearly doubled. Morphological analysis of the granular sludge exposed to the electric field showed a 17% reduction in sedimentation velocity and a 44% increase in sludge volume index (SVI). Scanning electron microscopy (SEM) images revealed a more compact structure and a greater presence of filamentous microorganisms. The extracellular

polymeric substances (EPS) content in the sludge increased by 52%, and hydrogenotrophic methanogenesis emerged as the dominant pathway, contributing to 65% of CH₄ production, with the remaining 35% attributed to the acetoclastic pathway.

These findings highlight the potential of low-magnitude electric fields to enhance anaerobic digestion, particularly at low temperatures, by significantly improving both COD removal efficiency and methane yield. This approach offers a promising pathway toward more sustainable and efficient wastewater treatment across a range of environmental conditions.

Resumen

La creciente necesidad de tratamientos eficientes para las aguas residuales ha impulsado investigaciones destinadas a incrementar el rendimiento de la digestión anaerobia, especialmente a bajas temperaturas, donde la actividad metabólica suele disminuir. Este estudio analiza el uso de un campo eléctrico controlado de baja magnitud como una modificación al proceso de digestión anaerobia (DA). Esta intervención permite operar de manera eficiente a temperaturas inferiores a 35°C, mejorando la eficiencia de la producción de biogás.

Inicialmente, un lodo granular anaeróbico se estabilizó a temperatura ambiente (23 °C) en un reactor UASB y posteriormente se utilizó para analizar cada etapa de la digestión anaerobia (hidrólisis, acidogénesis, acetogénesis y metanogénesis) en cultivos discontinuos. Cada etapa de la DA se alimentó con una fuente de carbono específica: sacarosa, glucosa, una mezcla de ácidos grasos volátiles (AGVs) y acetato, respectivamente; con la finalidad de tener una caracterización que represente cada etapa. Las unidades experimentales consistieron en una celda de dos electrodos de RuO₂/Ti (titanio recubierto de óxido de rutenio), que se operaron con tiempos de reacción cortos para evitar la formación de la biopelícula y aislar el efecto del O₂ generado electroquímicamente en la digestión anaerobia. Se probaron tres magnitudes de campo eléctrico: 1.21, 1.45 y 1.64 V/cm. Los resultados experimentales revelaron que el campo eléctrico aumentó el oxígeno disuelto en el medio, lo que modificó cada etapa de la digestión anaeróbica. Los resultados óptimos se obtuvieron a 1.21 V/cm, lo que resultó en una tasa de producción de oxígeno disuelto de 0.0753 mg O₂/L·min, que fue consumida por la presencia de bacterias facultativas a una tasa de consumo de oxígeno disuelto de 0.484 ± 0.04 mg O₂/L·min. Se observaron mejoras significativas en todas las etapas de

la digestión anaeróbica. Por ejemplo, la eficiencia de la hidrólisis aumentó en un 52%, lo que se atribuyó a un aumento del 53% en la actividad enzimática extracelular después de tres horas de aplicación del campo eléctrico. La influencia del O₂ se extendió a las etapas de la acidogénesis y acetogénesis, lo que activó el metabolismo oxidativo con un aumento en la producción de CO₂ y una disminución en la formación de acetato. Un hallazgo notable que se observó fue el aumento del 34% en el consumo de AGVs durante la acetogénesis bajo la influencia del campo eléctrico, lo que mejoró la interacción sintrófica con la acidogénesis, que a menudo es la etapa desafiante por los desbalances que genera la presencia de los AGVs. La producción de metano (CH₄) también experimentó un aumento sustancial, con una actividad metanogénica de 0.89 ± 0.02 g DQO-CH₄/g SSV·d, lo que representa un aumento de 39% respecto al control. Sorpresivamente, la actividad metanogénica obtenida a temperatura ambiente estuvo muy cerca de los niveles que normalmente se alcanzan a 35 °C en reactores convencionales.

Por otro lado, para analizar con mayor profundidad los efectos de la temperatura, la DA se estudió a 10, 18 y 35 °C, con el empleo de almidón de maíz como fuente de carbono (0.8 g DQO/L) y un campo eléctrico de 1.21 V/cm. Los resultados mostraron que, a 18 °C, la eficiencia de remoción de la DQO fue de $89 \pm 1\%$, un porcentaje de eficiencia que se alcanzó a 35 °C en el estudio control sin campo eléctrico. El análisis del biogás reveló un cambio en su composición química cuando se aplicó el campo eléctrico, con un 2% de H₂, lo que sugiere que el H₂ generado electroquímicamente participó en la producción de CH₄. Adicionalmente, se realizó un cultivo discontinuo para verificar si el lodo anaerobio tenía la capacidad metabólica para metanizar utilizando el H₂ generado electroquímicamente. El estudio se realizó a 18 °C, 1.21 V/cm y un medio mineral libre de fuente de carbono. Los resultados revelaron una composición química del biogás de 95% CH₄ y 5% H₂ junto con un aumento en la producción de biogás de 2.6 veces en comparación con el control. Esto evidenció el impacto del campo eléctrico en la actividad hidrogenotrófica.

Finalmente, el proceso se escaló utilizando dos reactores tipo UASB a nivel laboratorio. El reactor sin electrodos (RC) se empleó como control, mientras que el reactor experimental (RE) contenía los electrodos en la zona de lodos para evaluar la influencia del campo eléctrico en la digestión anaeróbica. Ambos reactores tuvieron un volumen de operación de 2 litros, se operaron a un tiempo de retención hidráulica (TRH) de 12 horas, y se alimentaron con aguas residuales

municipales suplementadas con 2 ± 0.1 g DQO/L. Al RE se le aplicó un campo eléctrico de 1.28 V/cm a través de electrodos de malla de titanio RuO_2/Ti , los cuales fueron colocados estratégicamente en la zona de lodos para optimizar la transferencia de electrones. En el RE se observó un incremento de 86% en la producción de biogás y una composición en el biogás de 68% CH_4 , 21% CO_2 y 11% H_2 , en comparación con el 78% CH_4 y el 22% CO_2 del control. El biogás del RE presentó un poder calorífico de un 23% más alto con respecto al control, y la producción de CH_4 casi se duplicó. Los cambios morfológicos en el lodo granular expuesto al campo eléctrico mostraron una disminución del 17% en la velocidad de sedimentación y un aumento del 44% en el índice volumétrico del lodo (IVL). Las micrografías revelaron una estructura más compacta y una presencia significativa de microorganismos filamentosos. El contenido total de sustancias exopoliméricas extracelulares (SPE) en el lodo aumentó en un 52%, y la metanogénesis hidrogenotrófica se convirtió en la vía dominante, contribuyendo con un 65% en la producción de CH_4 , en contraste con el 35% generado mediante la vía acetoclástica.

Estos hallazgos destacan el prometedor potencial de los campos eléctricos de baja magnitud para optimizar la digestión anaeróbica, especialmente en condiciones de baja temperatura. La tecnología demostró su capacidad para incrementar significativamente la eficiencia en la remoción de la DQO y la producción de metano, superando limitaciones tradicionales del proceso. Como resultado, esta innovación biotecnológica representa una solución viable y sostenible para mejorar el tratamiento de aguas residuales, ofreciendo un rendimiento eficiente en una amplia variedad de condiciones ambientales.

Introduction

The increasing challenges posed by rapid urbanization, industrial expansion, and the corresponding surge in wastewater generation have made wastewater treatment a critical global issue. Conventional treatment methods, especially aerobic processes, require substantial energy input and produce significant sludge, bringing economic and environmental concerns. These limitations have sparked a search for more efficient, sustainable, and cost-effective treatment technologies.

Anaerobic digestion (AD) has emerged as a promising alternative. Unlike aerobic methods, AD reduces energy costs and recycles valuable resources, such as biogas, a renewable energy source. Through this biochemical process, anaerobic microorganisms break down complex organic compounds into simpler substances, primarily methane (CH_4) and carbon dioxide (CO_2), producing biogas that can be harnessed for energy. The benefits of AD are clear: it effectively removes chemical oxygen demand (COD) from wastewater, reduces sludge production by up to 80%, and offers a potential source of renewable energy that could meet approximately 20% of global natural gas demand.

However, AD has its challenges. Its optimal performance is temperature-dependent, with an ideal range between 30°C and 45°C. This limits its applicability in colder climates and hinders its use in ambient temperature conditions. Moreover, although the process is efficient, it often requires longer retention times and may underperform under certain environmental conditions.

Recent advancements have introduced electrochemical methods to enhance AD performance, significantly improving COD removal and methane production. By applying a low-magnitude electric field, electrochemical systems activate electroactive microorganisms, accelerating the conversion of organic matter to methane. These systems, consisting of an anode, cathode, aqueous conductor, and power source, represent an exciting frontier in wastewater treatment. However, key questions remain: How do these electric fields impact microbial metabolism, and to what extent do they enhance the kinetics of the process? Furthermore, the application of electrochemical systems has mostly been limited to temperatures above 35°C, leaving a gap in understanding their behavior in colder environments.

One of the least explored areas in this field is the effect of electrochemical reactions on suspended granules in anaerobic systems and the role of water electrolysis. Water electrolysis generates H_2 and O_2 , which could potentially be integrated into anaerobic biochemical pathways, influencing microbial metabolism and boosting methane production.

This research aims to fill this gap by investigating how controlled electric fields influence anaerobic metabolism at different process stages. By understanding the dynamic effects of electrochemical reactions, this study seeks to establish strategies for improving COD removal and methane production in up-flow anaerobic sludge blanket (UASB) reactors, particularly under ambient temperature conditions. This approach could provide a more efficient alternative for domestic wastewater treatment, overcoming the typical temperature limitations of traditional AD processes.

The structure of this thesis is as follows:

Chapter I: Review the fundamentals and background on applying electric fields in anaerobic digestion.

Chapter II: Exploration of the influence of electric fields on anaerobic metabolism and kinetics during synthetic wastewater treatment.

Chapter III: Identification of key stages for improving anaerobic process performance.

Chapter IV: Comparison of the effects of different temperatures on wastewater treatment.

Chapter V: Application of operating parameter selection in a UASB reactor for domestic wastewater treatment and its impact on anaerobic sludge.

Chapter I: How does a low magnitude electric field influence anaerobic digestion in wastewater treatment? A review

1 Introduction

Anaerobic digestion (AD) is a complex oxidation-reduction biochemical process performed by archaea and bacteria to biotransform complex carbonaceous organic matter into simple molecules such as CH_4 , CO_2 , H_2S , and traces of other gases; this mixture is called biogas. This technology is widely applied to produce energy as biogas from solid waste and wastewater. The production of biogas in 2018 was around 35 million tons of oil equivalent, representing a fraction of the estimated global potential; full utilization of the potential could cover about 20% of the current world demand for natural gas (International Energy Agency & International Environmental Agency, 2020). At the same time, internationally, the anaerobic process is more relevant to treating wastewater with low biomass production than aerobic technologies due to lower operating costs (Anderson Ken et al., 2003).

Notwithstanding the long history of research and accomplishments, AD still faces critical technical challenges, mainly in the first three stages of AD (Ye et al., 2018), causing imbalanced stoichiometries for the following reaction steps (Park et al., 2018). For instance, at a high organic loading rate (OLR), acidogenesis produces excessive volatile fatty acids (VFAs), inhibiting methanogenesis, with low methane yields alongside long stabilization periods (Chen et al., 2008; Wang et al., 2019). Temperature, one of the most relevant operational requirements, is difficult to control in cold climates. For example, temperatures below 30 °C hinder the transport of substrate within cells and influence the metabolic activities of microorganisms, affecting the gas transfer rate, sludge settling, the physicochemical properties of the wastewater, and the capacity of the reactors (Khanal, 2009). Due to the shortcomings of AD, countless advances have been made to address this issue, such as operating high-temperature reactors, use of conductive materials, pretreatments, and co-digestion, among others (Ariunbaatar et al., 2014; Park et al., 2018; Schievano et al., 2012; Zhang et al., 2019). However, while progress has been made, none of these proposals has resolved the problems; thus, more research is needed (Li et al., 2011).

In recent years, electrochemistry has enhanced AD performance, improving chemical oxygen demand (COD) removal and biogas yield (Clauwaert et al., 2008). In this sense, compared to conventional AD, electro-bioremediation has improved the performance of anaerobic reactors, highlighting high COD removal efficiencies, short stabilization times, and speed up the methane

production due to the participation of microorganisms electrochemically activated (Park et al., 2020).

Despite the advances, certain drawbacks such as electrochemical losses, mass transfer troubles, increases in internal resistance, uncertain metabolic effects, and lack of a methodology clear for the selection of a suitable current strength remain a challenge (Beegle & Borole, 2018; Logan & Rabaey, 2012; Wang et al., 2017). This review addresses the need to review the basics of the biochemistry of AD and the low-magnitude electric field (LMEF) in anaerobic reactors during wastewater treatment to inspire new research ideas.

2 Fundamental aspects of anaerobic digestion

Anaerobic digestion is a syntrophic biological process performed by bacteria and archaea to biotransform complex carbonaceous organic matter into simple molecules such as CH₄, CO₂, and traces of H₂S. This mixture is called biogas, with a calorific value of 50 - 55 MJ /kg. When the wastewater contains more than 2000 mg COD/L (Rajendran et al., 2020), the methane produced is enough to be used in engines or turbines to generate electricity, in boilers to produce heat, purify to introduce it into the networks of natural gas, or used as a base for the synthesis of methanol (Abdelsalam & Samer, 2019).

AD involves four main biological stages (Figure 2.1). Hydrolysis represents the initial stage, where complex organic molecules (e.g., polysaccharides, lipids, and proteins) are hydrolyzed into soluble compounds that can be transferred through the cell and used as energy and carbon sources for the following stage. These are carried out by extracellular enzymatic activity produced by *Bacteroides*, *Clostridium*, *Butyrivibrio*, *Eubacterium*, and *Bifidobacterium* (Corrales et al., 2015; Meegoda et al., 2018).

In the acidogenesis stage, facultative bacteria, such as *Streptococci*, *Enterobacteria*, *Clostridium*, and *Lactobacillus*, transform the monomers previously synthesized into acetic, propionic, and butyric acids, known as AGVs. Simultaneously, CO₂ and H₂ are produced.

The H₂ concentration influences the product type; at low H₂ concentrations, CO₂, H₂, and acetate would be the end products. At elevated H₂ concentrations, propionate, ethanol, and lactate would be the end products (Novaes, 1986). In acetogenesis, the products from the acidogenesis

process cannot be directly metabolized to methane; they need to be biotransformed into suitable substrates. As a result, VFAs with long carbon chains and alcohols are converted to acetate, H_2 , and CO_2 . Bacteria involved in this stage are H_2 producers (e.g., *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, and *Proteobacteria*) and acetate producers (e.g., *Clostridium aceticum* and *Acetobacterium woodii*).

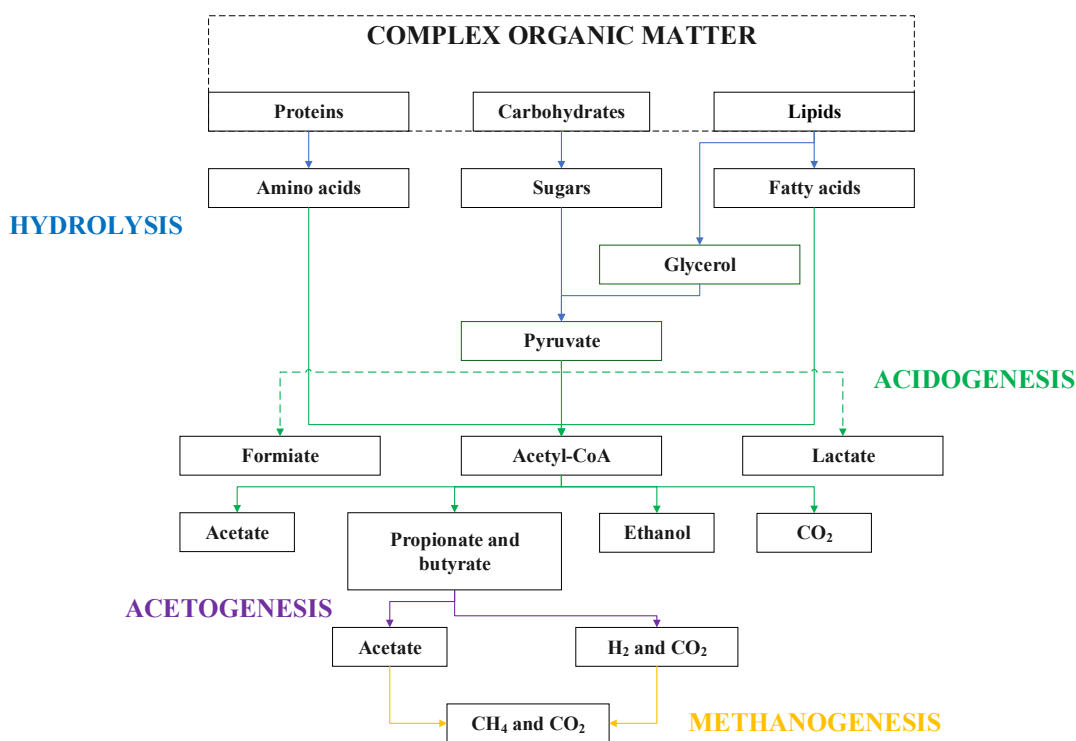


Figure 2.1 Simplified biochemical pathway of anaerobic digestion

In the last step of AD, acetate, H_2 , and CO_2 are transformed to CH_4 and CO_2 by *Acetoclastic methanogenesis* (e.g., *Methanosaeta* and *Methanosarcina*) and *Hydrogenotrophic methanogenesis* (e.g., *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, *Methanospirillum*, and *Methanothermobacter*) (Kim & Whitman, 2014). The former consumes acetate to produce 70% methane from acetic acid decarboxylation, and the latter consumes H_2 and CO_2 to make the remaining 30% methane. Likewise, methane production via methanol, formate, and methylamines has also been reported (Enzmann et al., 2018). A genus of bacteria called reducing sulfate can increase at high sulfate concentrations. These bacteria use organic matter as an energy source to reduce sulfate to H_2S , which usually happens when the stoichiometric

COD/Sulfate ratio is less than 8 (Tabla-Vázquez et al., 2020). The sulfate-reducing bacteria are inhibitors of methanogenic activity due to substrate competition.

AD is complex due to the number of microorganisms involved in biochemical pathways and the enormous variables that could modify their metabolisms, such as macronutrients (i.e., N, P, and S), temperature, pH, ORP, and inhibitors. Fernández & Seghezzo (2015) mentioned an optimal ratio of BOD₅:N:P of 100:0.5:0.1 for the balanced growth of the anaerobic microorganism, Murphy & Thamsiriroj (2013) mentioned two ratios for water treatment: a ratio of COD: N:P of 350:7:1 is required for high-loading organic rates (> 0.5 kg COD/kg VSS-d), whereas 1,000:7:1 for low-loading organic rates (<0.5 kg COD/kg VSS-d).

Micronutrients or trace elements are essential to complete biochemical pathways, and they must be present in wastewater or externally supplemented. Liu et al. (2011) observed that Fe addition plays a substantial role in specific metabolic pathways, resulting in better granulation. Ni and Co addition, in concentrations of 100 nM and 50 nM, respectively, enhanced the acetic acid conversion (Anukam et al., 2019). Abdelsalam & Samer (2019) reported an increase in methanogens microorganisms in Fe, Co, and Ni presence. Zandvoort et al. (2006) We also observed an increase of methanogen microorganisms by adding Fe, Ni, Co, Cu, Zn, and Mo traces.

Temperature influences the metabolic behavior of the AD in different ways: methane production, COD degradation rate, saturation constant (K_s), maximum specific growth rate (μ_{max}), enzymatic reaction rate, and diffusion rate from the liquid to the cell (Khanal, 2009). Most of these coefficients improve with increasing temperature, but eventually, they decrease as the temperature starts to inactivate the enzymes (Jacobo López et al., 2019). Jr et al. (2011) observed that the low temperature hinders the substrate transport into the cells, negatively affects the gas transfer rate, and decreases the solids settling characteristics. Elmitwalli et al. (2001) observed that at 13 °C, the hydrolysis rate and the degradation of organic matter decreased notably in domestic wastewater treatment. Even so, they obtained a COD removal efficiency of 74%. Jacobo López et al. (2019) achieved 0.8 L biogas/d at a temperature of 18 °C, observing an experimental growth of 0.11 kg VSS/kg COD. Donoso-Bravo et al. (2009) observed that acidogenesis was the step more affected by temperature. Temperature plays a vital role in AD. In addition, it usually influences the capacity of the reactors; for temperatures of 10 - 15 °C they can only treat loads of

1-5 Kg COD/m³·d, while for temperatures of 20 - 30 °C it is recommended 5 -15 Kg COD/m³·d. Methanogenic archaea are microorganisms mesophilic with an optimal temperature of around 35 °C. However, for each degree centigrade that falls regarding the optimal value, the anaerobic activity decreases by 11% (Khanal, 2009). Low temperatures do not mean AD is impossible; however, it impacts the methanogenic activity, decreasing the methane production yield (Cecconet et al., 2022).

The low pH modifies the enzyme's charges (e.g., in carboxyl and amine groups), substrate ionization, and denatures enzymes. Ortega (2006) showed that the optimal values for each stage of AD are hydrolysis-acidogenesis = 7.2-7.4, acetogenesis = 7.0-7.2, and methanogenesis = 6.5-7.5. Nonetheless, Fernández and Seghezzo (2015) mentioned that operating at acidic pH (i.e., 3.5-5.5) in well-acclimatized reactors is possible. However, low pH modifies the enzyme's charges (e.g., in carboxyl and amine groups), substrate ionization, and denatures enzymes. In the influent, the alkalinity of 2500 mg CO₃²⁻/L is necessary to buffer the pH, ensuring the stability of the AD. Proteins and sulfate in high concentrations can provide alkalinity without adding chemicals like carbonates (Khanal, 2009). The acidification of the liquid medium is often caused by VFA accumulation or excessive generation of CO₂. This issue might be controlled by diminishing the COD loading rate in such a way that accumulated VFAs are degraded faster than produced; this will help to remove excess VFAs by facultative bacteria (Sung Jae Lee, 2008).

Oxidation-reduction potential (ORP) indicates the oxidizing or reducing environmental conditions inside the wastewater. Methane formation or growth of methanogenic bacteria does not occur until the ORP is less than -300 mV. The ORP value must be kept between -150 mV and -350 mV for adequate growth of obligate anaerobes. Low ORP values prevent oxidizing sources such as oxygen, sulfates, nitrates, or nitrites (Ortega, 2006). Those oxidizing agents affect mainly acetogenic and methanogenic bacteria. Some authors reported optimal values for each AD stage. Sung Jae Lee (2008) mentioned that ORP around -300 mV characterizes hydrolysis. Vongvichiankul et al. (2017) indicated that the ORP value for the acidogenesis stage is -284 ± 32.71 mV, while the ORP value for the methanogenesis phase is -335.63 ± 28.97 mV.

Ammonium is contained in the influent or produced by hydrolysis of organic compounds like proteins or amino acids during AD. Different microorganisms can be inhibited at ammonium

concentrations from 100-3000 mg/L; toxicity by ammonia gas might be avoided by controlling the neutral pH in the reactor. H₂ at a high concentration can cause positive values in the Gibbs free energy ($G^0 > 0$), and, therefore, it blocks the acetogenesis stage, inhibiting the degrading bacteria of propionic acid, accumulating it. In an anaerobic system well operated, the content of VFAs in the discharge is 50-250 mg acetic acid/L. When the symbiosis between acidogens and methanogens is broken, the VFAs break down. The acid's dissociated form cannot permeate through the cell membrane, but the undissociated form does, causing toxic effects (Serrano-Meza et al., 2020).

Feeding into the reactor oxygen in small amounts is fine since the microorganisms that initiate degradation remove it. However, a higher oxygen input inhibits the process but recovers without difficulty when oxygen is removed, either by stripping or by the action of facultative microorganisms (Fernández & Seghezzo, 2015). Heavy metals are toxic to methanogenic bacteria in the following order: Ni > Cu > Pb > Cr > Zn. However, the presence of H₂S removes these metals in the form of insoluble sulfides. Even so, the concentration should not exceed 0.5 g H₂S/L to avoid inhibition troubles.

3 Effects of the electric field at the cellular level

Low magnitude electric field (LMEF) has been successfully used in medicine, biotechnology, food processing (Kovačić et al., 2021), and recently in wastewater treatment improvement. The electric field could influence cell development, maintenance, and control; LMEF can particularly enhance the cell membrane's mass transport rates. This phenomenon is called electroporation, and its mechanism is not yet well understood. Still, it is not necessarily the same as electroporation (Li et al., 2013), which use high-voltage electric shocks to introduce DNA into cells (Potter & Heller, 2010). Electroporation allows the extraction of carbohydrates, lipids, and proteins, among other biomolecules, without losing cellular integrity (Eing et al., 2013). Naturally, substrate diffusion in the cell is carried out by the plasma membrane, which is chemically made up of lipids (i.e., phospholipids, cholesterol, and glycolipids). These lipids have a hydrophilic and hydrophobic section. The polar area is dense, and the non-polar part is more elongated. Due to the non-covalent bonds, the chemical interaction between these lipids is weak. Different proteins are embedded within the lipid bilayer, acting as channels and pumps

to transport the molecules across the membrane (Kotnik et al., 2012). In fact, without these proteins, the membrane would be impenetrable.

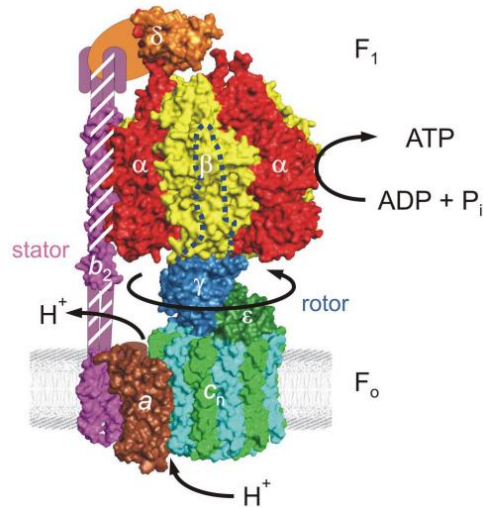


Figure 3.1 The structure and geometry of ATP synthase. F₀ structure is the lipid bilayer. F₁ structure is the transmembrane section (Reprinted with permission from Elsevier from Ref. Weber J, 2007)

ATPases can also be found in the cell membrane, such as ATP synthase (ATPsyn). ATPsyn is a protein providing energy to carry out biochemical pathways (Weidemüller & Hauser, 2008). In the geometry of ATPsyn, there are two main chemical structures, F₀ and F₁ (Figure 3.1). The F₀ structure converts a transmembrane H⁺ gradient into a rotary pair or reverses it to function as an ion pump. The F₁ structure synthesizes ATP, although it can also hydrolyze it, releasing energy to act as a motor. (Oster & Wang, 2000). ATP synthesizes proteins and nucleic acids and produces molecule transport, cell movement, and cell division (Uzman et al., 2000). ATP bridges catabolism and anabolism due to the energy from catabolism (i.e., ATP) essential for microbial growth. In biochemical pathways, regulatory enzymes are involved in cell development and survival that would be sensitive to ATP levels; for example, these enzymes are activated by AMP and by high levels of ATP (Bonora et al., 2012).

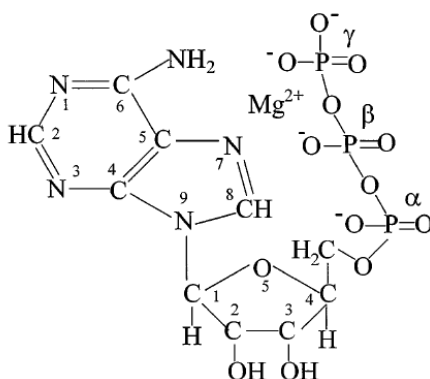


Figure 3.2 Chemical structure of ATP-Mg²⁺ (Clarke et al., 1996)

Therefore, ATP plays an essential role in cells, so it seems paradoxical that high levels of ATP can be unfavorable (Pontes et al., 2015). For example, anabolic processes consume 70% of stored ATP, being the most expensive route (Gaal et al., 1997). ATP is activated by Mg²⁺, as shown in Figure 3.2 (Pecoraro et al., 1984). Magnesium plays a crucial role in protein synthesis (i.e., in maintaining the structure of ribosomes) and translation initiation. For instance, McCarthy (1962) showed that when Mg²⁺ was absent from *E. coli* culture, a loss of ribosomes was observed, and a lack of microbial growth concluded that microbial growth depends on ribosomes.

However, when Mg²⁺ was re-spiked into the culture medium, it led to the regeneration of ribosomes and the re-initiation of microbial growth. For example, a single ribosome can chelate more than 170 Mg²⁺ ions (Schuwirth et al., 2005) and a bacterium can contain up to 70,000 ribosomes (V. Shen & Bremer, 1977). Therefore, high ATP production would chelate Mg²⁺ ions, limiting biochemical processes like cell division.

The electric field has been reported to influence enzymatic activity and cell growth. Weidemüller & Hauser (2008) found that when an electric field is applied, Na⁺/K⁺-ATPase is selectively activated. Zrimec et al. (2002) found that LMEF (< 50 V/cm) increased ATP production in *E. coli*. Valle et al. (2007) observed that when a current of 40 mA was applied in *Bacteria B. cereus*, the ATP content and enzyme activities were stimulated. Velasco-Alvarez et al. (2011) observed in *Aspergillus niger* exposed to 6 mA for 24 h, a stimulation of catabolism, with low biomass production. While Liu et al. (2017) observed in a denitrifying bacterium that the ATP content and cell membrane permeability increased significantly at 200 to 250 mA/m².

These findings suggest that membrane permeabilization can occur even when applied to low-magnitude electric fields. Tsong (1991) suggested that permeabilization can occur in protein channels and the lipid bilayer, producing an osmotic imbalance. Changes in sodium and potassium concentrations near the membranes cause cellular stress (Schoenbach et al., 1997). This phenomenon is believed to be due to the dielectric breakdown theory, where the cell membrane undergoes selective damage (de Vito et al., 2008). as bilayer destabilization (Needham & Hochmuth, 1989). Free diffusion through the membrane allows low-weight molecules to be transported (Teissie et al., 2005).

The potential difference inside and outside the cell is due to a natural potential difference regulated by metabolic activity or induced by an external electric field (Teissie et al., 2005). The natural potential difference varies between -20 and -200 mV, but it is up to the type of cell membrane (Alberts et al., 2018). In this way, an LMEF could be applied to a microbial culture to permeabilize the cell membrane reversibly. Thus, the uptake of substrates and the export of metabolic products can be improved, redirecting metabolic fluxes by the law of mass action. Therefore, metabolic processes could be made more efficient by an LMEF application.

Therefore, if the external electric field generates transmembrane potentials equal to or greater than those generated by the cell, it will modify the ion concentration gradient, as shown in Figure 3.3, where E represents the cell's natural transmembrane potential and E_c , a critical transmembrane potential (Balint et al., 2013). This is reversible due to the low current magnitude; the cell membrane will return to its initial conditions when the electric field is off (Sersa et al., 2008).

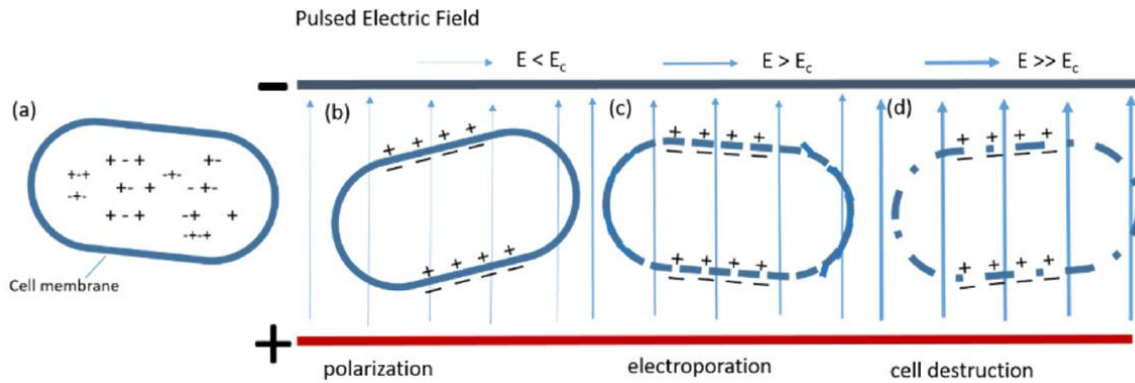


Figure 3.3 Mechanism of cell membrane permeabilization by LMEF. a) charge distribution before the LMEF, b) cell polarization, c) cell permeabilization (reversible), d) cell lysis (irreversible) (Capodaglio, 2021)

4 Electric enhancement of anaerobic digestion

Bioelectrochemical systems (BES) for methane production have been an accidental finding that resulted in a promising green technology (Van Eerten-Jansen et al., 2011). Initially, their main objective was the generation of bioelectricity using microbial fuel cells (MFC) or the removed contaminants using microbial electrolysis cells (MEC) (Capodaglio et al., 2015). BES has been combined with anaerobic digestion to improve methane yield under mesophilic conditions (Feng et al., 2015). Two routes can drive methane production (Figure 4.1), either directly where biomass takes charges from the electrode (Eq. 1) through outer membrane cytochromes of electroactive microorganisms (EM) and transfer them to methanogenic archaea that use charge to reduce CO_2 to CH_4 , or indirectly when H_2 is electrochemically produced (Eq. 2) and it is used together with other intermediate products such as acetate and CO_2 to produce CH_4 (Eq. 3).



$$E = -0.244 \text{ V vs NHE}$$



$$E = -0.41 \text{ V vs NHE}$$



Where NHE represents Normal Hydrogen Electrode

The BES are composed mainly of four components: i) anode, ii) cathode, iii) separator, and iv) electronic conductor device, where conductor can act as source or load of electric current. BES are classified into MFC and MEC, mainly; MFC generate electricity from organic matter degradation by microorganisms, while MEC require a supply of electricity to carry out the biochemical reactions for value-added product formation. In MEC, the charges generated by the biological processes are transported from the anode into the cathode with CO_2 reduction (Jeremiasse et al., 2010). Cathode allows the attachment of electrochemically active microorganisms involved in methane production (Figure 4.1). As the third component, the separator allows the movement of cations like Na^+ , K^+ , and H^+ from the anode to the cathode to keep the solution electroneutral (Amrut Pawar et al., 2020). Finally, the electricity source is needed to drive thermodynamically the reaction. For instance, a voltage supply as low as 0.2-0.8 V is enough for the bioelectrochemical reactions to generate CH_4 (Buitrón et al., 2018).

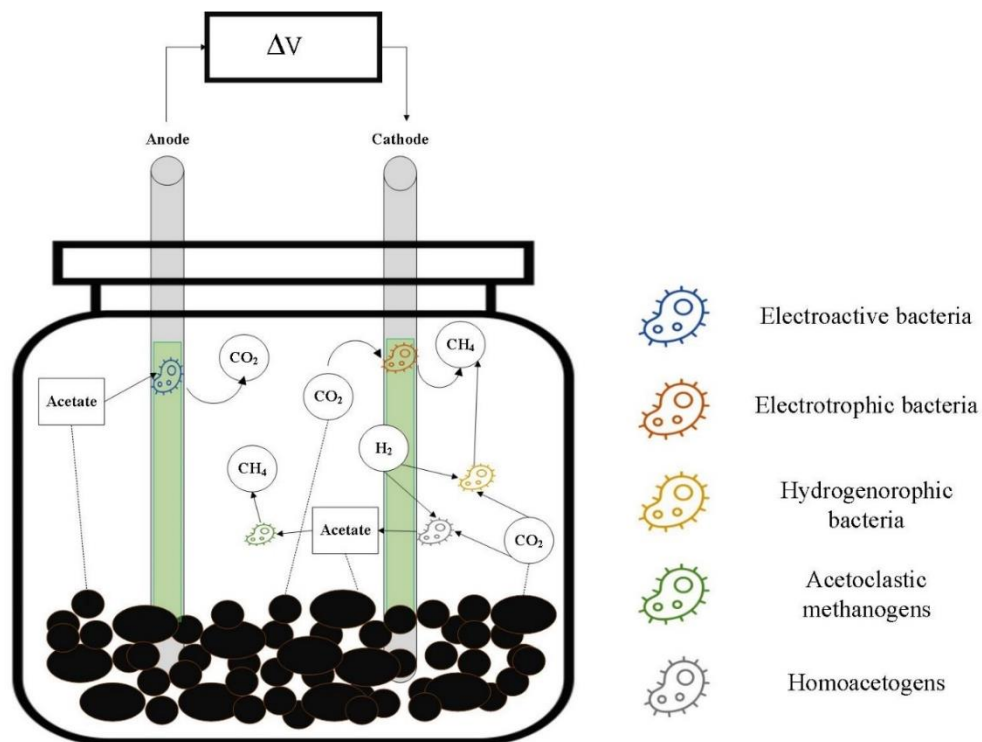


Figure 4.1 Syntrophic mechanism of conventional AD bacteria and bacteria growing in electric field promoted biofilm for methane production, in a microbial electrolysis cell (MEC). V represents the potential difference between electrodes. Modified from Pawar et al.

Electroactive microorganisms (EM) can grow both at the anode and at the cathode, being capable of transporting charge between electronic conductor and microorganism. *Geobacter* species are the most dominant bacteria under electrochemical conditions (Commault et al., 2013). Call et al. (2009) observed that *Geobacter sulfurreducens* predominated at 72% as producing H₂. *Geobacter* bacteria can use an extensive list of organic compounds as substrates, harness electrons coming from the COD oxidation, and transfer them to an electrode (electronic conductor) or other chemical species (Z. Yu et al., 2018).

Table 4.1 summarizes various works focused on improving methane production in wastewater by applying a LMEF. For example, Tartakovsky et al. (2011) created microaerobic conditions due to the water electrolysis, producing O₂ and H₂. The oxygen production did not decrease the acetoclastic methanogenic activity; however, the H₂ production promoted the hydrogenotrophic methanogenic activity, increasing the global methane yield by 10-25% compared to a conventional anaerobic reactor. The authors also showed that microaerobic conditions diminished the content of H₂S in the biogas and improved the stability of the anaerobic process. In addition, the oxygen presence might proliferate the development of aerobic methanotrophic bacteria, which can oxidize methane to CO₂, decreasing the global methane yield. Otherwise, H₂ is indirectly used by hydrogenotrophic archaea to produce methane, but in some cases, there is also an accumulation of H₂, inhibiting microbial activity and decreasing the biogas quality (Y. Chen et al., 2016).

The characteristic of the surface on electrodes seems to be the key to improving the anaerobic digestion process since the material can favor the formation of biofilms due to its porosity. For example, electrode materials can be modified, improving biocompatibility and electron transfer (Tian et al., 2019). De Vrieze et al. (2018) showed that the biofilm around the electrodes represented 0.22 % of the total biomass. However, this amount was sufficient to stabilize the AD with a predominance of *Methanosaeta acetoclastic* bacteria. Other authors report the predominance of *Geobacter* bacteria in the anode (Figure 4.1); these bacteria take charge from the electrode to use in the redox metabolic reactions of acetate to produce H₃O⁺ and CO₂; the suspended biomass metabolism produces the acetate. On the other hand, electrotrophic methanogens attached to the cathode accept the charge to perform metabolic reduction of CO₂ to CH₄. H₂ plus CO₂ are also biotransformed to CH₄ by hydrogenotrophic microorganisms, such

Methanobacterium (Koch et al., 2015), while acetoclastic methanogens are found outside the electrodes (i.e., in the suspended biomass) and participate in the conversion of acetate to CH₄. Tian et al. (2019) found that H₂ consuming methanogens dominated the bioelectrochemical system. Bandara et al. (2011) also suggested that H₂ consuming methanogens dominate at low temperatures.

Table 4.1 Strategies to improve anaerobic digestion through the electric field

Author	Type of organic matter	Electrode material	Operating conditions	Methane production	Microbial diversity	Effects on AD
Tartakovskiy et al., (2011)	Synthetic wastewater	Titanium mesh anode and stainless-steel cathode	T 35°C pH 6.8-7 TRH 6-12 h Potential 2.8-3.5 V	0.46 L CH ₄ /g COD	Not reported	Increment by 10-25% and reactor stability.
De Vrieze et al. (2014)	Molasses	Carbon felt and Ag / AgCl reference electrode	T 34 °C pH 7,67 TRS 20 días Potential 0.5-1V	577 ± 11 mL CH ₄ /L-d	Methanosaetaceae, Methanomicrobiale, Methanosarcinaceae, Actinomycetales, Lactobacillales, Clostridiales and Sphaerochaetales	Stable methane production, immediate recovery of reactor failures, a methanogenic biofilm development.
Koch et al. (2015)	Synthetic wastewater	Graphite rod electrodes and Ag / AgCl reference electrode	T 37 °C pH 7.7-7.8 Potential 0.2, +0.2 V	216 (± 29) mL de CH ₄ /g-COD	<i>Firmicutes</i> , <i>Bacteroidetes</i> , <i>Proteobacteria</i> and <i>Synergistetes</i> (sludge) <i>Geobacter</i> (anode) and <i>Methanobacterium</i> (cathode)	The methane production yield remained constant, with an increase of 27%.
Zhen, (2017)	Synthetic methanolic wastewater	SAE1080 Helical Cathode and Iron Rod Anode	T 35°C TRH 48 h Potential 0.4-0.6 V	1504,7 ± 92,2 mL CH ₄ /L-d	Not reported	The stabilization system increases methane production by 10% and increments in the EPS production.
Tian et al. (2019)	Synthetic wastewater	Carbon felt cathode and modified anode (Gr and MnO ₂ NPs)	T 35°C TRH 48 h Potential 0.4-0.6 V	14.3±1.4 mLCH ₄ /d	<i>Methanoregula</i> , <i>Methanothrix</i> , <i>Methanospirillum</i> , <i>Methanomassiliicoccus</i> , and <i>Methanobacterium</i>	Increment of methane by 25% and EPS production.
Sun et al. (2020)	Synthetic wastewater	Titanium Mesh Electrodes	T 35 °C pH 7.2 TRH 24 h Potential 0.05-0.2 V	280.83 mL CH ₄ /g COD	<i>Methanothrix</i> , <i>Methanomassiliicoccus</i> , <i>Methanoregula</i> , <i>Methanolinea</i> <i>Smithella</i> and <i>Geobacter</i> .	Increment in methane production by 15% and COD removal by 16%. It promoted microbial diversity.
Guo et al. (2022)	Synthetic wastewater	Carbon felt	T 37 °C pH 8 Potential 0.5-1.2 V	477.1 mL CH ₄ /g TVS	<i>Firmicutes</i> , <i>Chloroflexi</i> , <i>Bacteroidete</i> , <i>Proteobacteria</i> , <i>Methanoculleus</i> , <i>Methanobacterium</i> and <i>Methanomassiliicoccus</i>	Stable biofilms formed on the electrode surfaces, facilitating more efficient electron transfer.

By scanning electron microscopy, Guo et al. (2022) show that an increment in the specific surface area favored binding and biofilm formation. For example, the chemical modification of electrodes showed a better electron transfer rate under the same applied voltage, which accelerated the charge transfer between the electrodes and the biofilm (Xie et al., 2012; Yuan & He, 2015). These findings suggest that biofilm provides stability to the anaerobic system since the microorganisms are attached to the electrodes and consume VFAs, acetate, and H₂. It is important to note that the biofilm requires a stabilization time of at least 24 days for the increase in biogas production and COD removal to be considerable. The increase in CH₄ production is the effect of the greatest interest, for instance, Koch et al. (2015) found that the combination of AD and electrochemical technology improved methane production by 27%. Zhen et al. (2017) studied the coupling of microbial electrolysis in a UASB reactor for methanolic biodegradation, where methane production increased by 10%. Tian et al. (2019) developed a BES with graphene/polypyrrole and MnO₂ nanoparticles/PPy-modified electrodes, observing a 22.8-39.0% methane enhancement for UASB reactors operated at low temperatures. Sun et al. (2020) applied a potential of 0.10 V in a UASB reactor observing a methane production of 16% higher than the control.

The electric field also affects the stability and physiology of the granules, although it seems to depend mainly on the properties of the material of the electrodes. For instance, Zhen et al. (2017) suggest that the external energy supplied promoted the structural integrity of the granules since the Fe^{2+/3+} released during anodic corrosion stabilized the extracellular polymeric substances (EPS). The chemical composition of exopolymers revealed that the proportion of proteins was high, producing a water-repellent layer on the surface that gave it better stability and integrity (Tian et al., 2019). The EPS increment is thought due to the electric field stimulating the catabolism of microorganisms by increasing the total content of ATP; it is worth mentioning that exopolymers require high energy for their production (Velasco-Alvarez et al., 2011).

On the other hand, the magnitude of the applied potential is relevant. Sun et al. (2020) studied the influence of different voltages on methane yield, where 0.10 V was the best performer. Chen et al. (2016) reported an increase in methane yield at a voltage less than 0.6 V. H₂ was not observed in this study, indicating that the CH₄ enhancement was not related to the water electrolysis or

methanogenic hydrogenotrophic activity; this observation agrees with the literature because the electrolysis of water requires at least 0.83 V (Chen et al., 2016). Some authors mention the benefits of the by-products (H_2 and O_2) derived from it. For example, O_2 can enhance the microaeration hydrolysis step by having high aerobic and anoxic activity rates compared to the anaerobic process. However, there may be a decrease in methanogenic biomass due to competition with aerobic microorganisms. Despite this, methane production at lower voltages where electrolysis does not occur (<0.8 V) achieves better yields (Zhen et al., 2017). A voltage of 0.6 V has been reported to favor the hydrogenotrophic stage, and a voltage of 0.3 V favors acetoclastic activity; this also allows the desulfurization of the biogas since it has a predominance of methanogens. It has been shown that applying a voltage of 0.5 V in the reactors produces a drastic decrease in the production of methane, but they immediately improve using the values mentioned above.

In addition to applying voltages lower than 0.8 V, ORP values close to -200 to -400 mV can be obtained, improving the reducing conditions due to methanogenic activity. It is expected that by controlling extracellular ORP, intracellular ORP can be controlled biochemically through the balance of reduced/oxidized NAD ($NADH/NAD^+$); thus, regulating enzymatic synthesis (Jiang et al., 2021). Finally, the electric field is expected to induce changes in DNA, protein synthesis, and membrane permeability, thus accelerating cell growth or methane production. However, no evidence exists to support these effects, so research has systematically reviewed different strategies to enhance methane production in recent years. Finally, future research must be undertaken to elucidate the actual impact on metabolism in this context.

5 Conclusions

The electric field influences two crucial aspects at the cellular level due to the difference in electrochemical potential inside and outside the cell, which could accelerate anaerobic metabolism and, on the other hand, microbial diversification with the growth of electroactive microorganisms. However, existing studies do not elucidate how the electric field can be used in a clear way to achieve specific changes in the biochemical pathways of anaerobic digestion to improve methane production since there is no methodology with a solid basis for its effect. Finally, the coupling of electrochemistry could be very useful in solving the problems faced by

AD; however, this review has revealed that there are still exposed aspects to be explored at the cellular, biochemical, and electrochemical levels that require interdisciplinary work.

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Chapter II: Influence of water electrolysis on each stage of anaerobic digestion: A kinetic and metabolic analysis

1 Introduction

Anaerobic digestion (AD) consists of biochemical reactions in which complex organic matter is transformed, under the synergistic effects of several strict and facultative anaerobic microorganisms, into simple chemically stabilized compounds, mainly methane (CH_4) and carbon dioxide (CO_2) (Khanal, 2009). It is a cost-effective and widely applied biotechnology for wastewater treatment, with a high chemical oxygen demand (COD) elimination efficiency of up to 80% at 30-35 °C (Von Sperling & de Lemos Chernicharo, 2015). However, it is a complex biotechnological process that has drawbacks such as long stabilization times (e.g., 2 to 3 months), high sensitivity to various toxic substances (e.g., ammonium), and the need to control temperature (e.g., 25 to 44°C) to achieve acceptable methane production performance (Y. Chen et al., 2008; Mao et al., 2015). It is well known that 30-35°C is the ideal temperature for the best anaerobic digestion performance. However, controlling high temperatures in zones of cold climates in wastewater treatment plants is economically unfeasible. So, developing technologies to operate anaerobic digestion at low temperatures and maintain high performance is a real challenge. Therefore, several studies have addressed these drawbacks. Some of the main strategies are co-digestion, two-stage processes, effluent pretreatment, and one of the most recent is the coupling of an electrochemical system, which has managed to increase COD removal by up to 20% with a CH_4 production of 10-27% compared to a conventional anaerobic reactor (Amrut Pawar et al., 2020; Koch et al., 2015; C. Wang et al., 2019).

Bioelectrochemical systems, particularly microbial electrolysis cells (MECs), require an electric field applied to conduct biochemical reactions to form value-added products (Pawar et al., 2020). The components of most electrochemical systems are i) an anode, ii) a cathode, iii) an aqueous medium as an ionic conductor, and iv) a source of electric current (Capodaglio et al., 2015).

Currently, most research on this type of system has reported the formation of biofilms after 24 days of operation in which the electric field is constantly applied. In these biofilms, bacteria capable of transferring charge (electroactive) develop, which participate as redox mediators in the oxidation-reduction reactions of AD (De Vrieze et al., 2018; Koch et al., 2015). Another essential

aspect that is less studied is the effect of electrochemical reactions (Ec 1 and 2) in these systems and how they influence the granules suspended in the liquid medium.



$$E = -0.244 \text{ V vs NHE}$$



$$E = -0.41 \text{ V vs NHE}$$



Where NHE represents Normal Hydrogen Electrode

Consequently, the intrinsic production of H_2 and O_2 by the electrolysis of water should be one of the variables to consider since AD syntrophy allows microorganisms to couple the O_2 and H_2 produced through electrochemical reactions in their biochemical pathways and, therefore, could modify AD metabolism. For example, Tartakovsky et al. (2011) Observed an improvement in the AD by applying an electric field (potential of 2.8–3.5 V) for water electrolysis at 35 °C; they attributed the higher yield in CH_4 production to the electrochemical production of O_2 that favored the hydrolysis stage and the diminution of H_2S . On the other hand, the authors observed that CH_4 production increased due to H_2 consumption by hydrogenotrophic archaea. Koch et al. (2015) imposed an electric field (cell potential - 0.2, + 0.2 V) using graphite electrodes at 37 °C, improving methane production by 27 %. Tian et al. (2019) Evaluated an electric field (cell potential of 0.4-0.6 V) on the AD in a continuous reactor using a carbon-felt cathode at 35 °C, observing a methane increment of 25% with an increment of exopolymeric substances. Sun et al. (2020) we evaluated an anaerobic reactor at HRT of 24 h, with titanium mesh electrodes at 35 °C, observing an increment of methane production by 15% with changes in the microbial diversity.

Based on the observations mentioned above, it is necessary to develop studies that support the production rate of O_2 and H_2 , their direct effect on each stage of AD at room temperature, and the modification of kinetics or metabolism to better understand bioelectrochemical systems. Therefore, this work aims to evaluate the influence of the production of these gases by water electrolysis in each AD stage by carrying out batch cultures using granular sludge previously stabilized at room

temperature through the rates of specific substrate consumption (i.e., sucrose, glucose, propionate and butyrate, and acetate).

2 Materials and methods

2.1 Stabilization of an anaerobic sludge at room temperature

The anaerobic sludge used for this study was obtained from the IITCA (Inter-American Institute of Water Technology and Sciences) wastewater treatment plant in Atlacomulco, Mexico. This sludge was stabilized in a bench-scale Upflow Anaerobic Sludge Blanket (UASB) bioreactor with an operating volume of 2 L (Figure 2.1). The reactor was fed at 1.38 mL/min with a peristaltic pump (Marlow 114 DV) with a volumetric organic load of 1.5 Kg COD/m³·day at an HRT 24. The initial pH was 7, and the reactor was operated at 23 ± 3 °C. It was fed with the following chemical medium: (in g/L), sucrose (1.5), NH₄Cl (0.85), K₂HPO₄ (0.234), KH₂PO₄ (0.136), MgCl·6H₂O (0.084), NaHCO₃ (2), and yeast extract (0.34). A trace element solution (0.5 mL/L) was added, and the composition of the trace solution (in g/L) was EDTA (0.5), ZnSO₄·7H₂O (0.4), CoCl₂·6H₂O (0.25), MnCl₂ (0.6), CuSO₄·5H₂O (0.25), Na₂MoO₄·2H₂O (0.2), NiCl₂·6H₂O (0.2), MgCl₂ (0.5), FeSO₄ (0.1), and CaCl₂·2H₂O (0.1).

2.2 Characterization of the bioelectrochemical system for batch cultures

Electrochemical characterization was performed in glass electrochemical reactors (two-electrode cells) with 130 mL of liquid and 10 mL of head space. Initially, three materials were evaluated for use as anodes and cathodes: graphite rods, stainless steel rods, and Ti plates coated with RuO₂ (RuO₂/Ti).

The characterization involved applying different electric current intensities (1-20 mA) at 5 mA intervals, verifying a constant cell potential for each current intensity. Cell potential vs. current intensity was reported to find an operating interval in which a resistive behavior was observed. A potentiostat was used to apply the electric current. Additionally, the pH was measured. The material was selected according to its susceptibility to corrosion, biocompatibility with the medium, and resistive behavior.

Once the material was selected, a voltammogram from -1 to 1.7 V with a scan potential of 10 mV/s was performed (in a three-cell electrode) by using mercury/mercurous sulfate/saturated sulfate (SSE) and a carbon cylinder as reference and counter electrodes, respectively, to corroborate that the culture medium was not chemically modified. The voltammogram was carried out with a mineral medium containing sucrose and acetate.

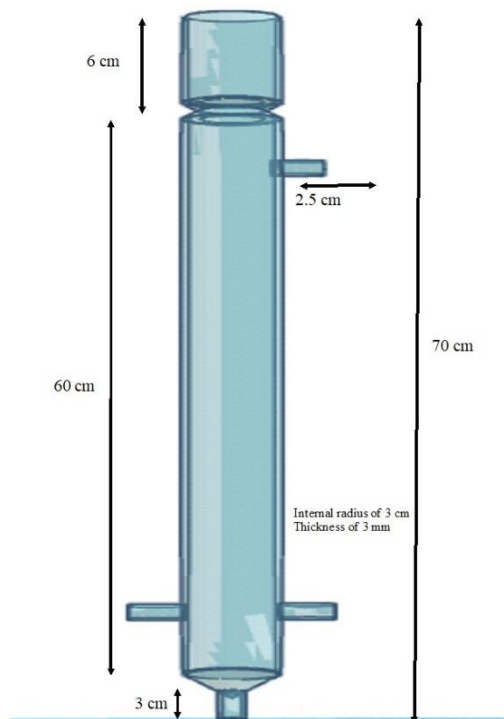


Figure 2.1 Diagram of the bench-scale UASB reactor used to stabilize anaerobic sludge at room temperature.

An amount of 53.46 g of volatile suspended solid (VSS/L) of anaerobic sludge (occupying 33% of the liquid volume) was used as inoculum for UASB. To assess the stability of the anaerobic sludge, parameters such as COD removal efficiency, alkalinity (α index), and biogas production were continuously monitored. Fresh effluent samples were collected, centrifuged at 4000 rpm for 5 minutes, and filtered through a 2.5 μ m filter to determine the soluble COD concentration. Alkalinity was measured by acidifying 100 mL of effluent samples with a 0.1 N H_2SO_4 solution by titration to pH values of 5.75 and 4.3. The volume of acid used to reach each pH was recorded, and

the α index was calculated according to Jenkins et al. (1983). Biogas production was monitored using continuous flow measurement chambers.

A physicochemical characterization of the anaerobic granular sludge was performed during the pseudo-steady state. This included determining specific methanogenic activity (SMA), sludge volumetric index (SVI), sedimentation velocity, VSS, granulometric analysis.

SMA tests were conducted in 100 mL serological bottles inoculated with 2 g VSS/L, using glucose and acetate as carbon sources. The initial pH was adjusted to 7, and the assays were maintained at 30°C. Methane production was quantified using inverted bottles filled with a 1 N NaOH solution to capture and measure the volume of CH₄ produced.

The SVI and sedimentation velocity were determined using a 20 mL sample of concentrated sludge. The sample was washed thrice with a phosphate buffer solution before being transferred to a 100 mL graduated cylinder. The volume was then brought to 100 mL with distilled water. After gently mixing, the sludge was allowed to settle for 30 minutes. The settled sludge volume was recorded every 15 seconds during the first 2 minutes, every minute until 15 minutes, and every 5 minutes after that. Calculations were performed using the following equations.

$$SVI = \frac{\text{volume of sludge settled in 30 min (mL)}}{TSS \left(\frac{g}{L}\right) * \text{initial volume on graduated cylinder (L)}} \quad (\text{Eq. 4})$$

$$SV = \frac{\text{rate of sedimentation} \left(\frac{m}{h}\right)}{\text{area of graduate cylinder (m}^2\text{)}} \quad (\text{Eq. 5})$$

The total, fixed, and volatile solids in the granular sludge were determined using a 40 mL sample of concentrated and homogenized sludge, which was processed according to the standard gravimetric method (FAPHA/AWWA/WEF, 2012). A 250 mL sludge sample was homogenized for granulometry analysis, and the SSV was determined in advance. A 30-mesh sieve (0.6 cm) was placed in a basin with enough water to cover the mesh without exceeding its edge. The sludge sample (250 mL) was then poured into the sieve, submerged, and gently agitated to separate the solids. The granular sludge retained on the sieve was transferred to a 1 L volumetric flask, and VSS₁ was measured.

Subsequently, the process was repeated using a 40-mesh sieve (0.4 cm) with the water from the first flask to obtain VSS2. The remaining water containing the smaller flocs was collected and analyzed to determine VSS3. The corresponding calculations were carried out using the following specific equations.

$$\text{Mass of 0.6 cm granule (M0.6)} = \text{VSS1} * 1L \quad (\text{Eq. 6})$$

$$\text{Mass of 0.4 cm granule (M0.4)} = \text{VSS2} * 1L \quad (\text{Eq. 7})$$

$$\text{Mass of flocc (Mf)} = \text{VSS3} * 1L \quad (\text{Eq.8})$$

$$\text{Total mass} = \text{M0.6} + \text{M0.4} + \text{Mf} \quad (\text{Eq. 9})$$

2.3 Characterization of the anaerobic digestion stages assisted by an electric field

The anaerobic sludge was washed three times with a phosphate buffer solution to eliminate the remaining sucrose, and it was kept fasting for 96 h. All batch cultures were inoculated with 25 mL of sludge previously acclimatized in the UASB, equivalent to 2 g VSS/L as inoculum. Once inoculated into the batch cultures, they were bubbled with N₂ for 3 min to ensure an oxygen-free environment. Initially, batch cultures (without an electric field) were carried out as control assays of each AD stage. Subsequently, the effect of the electric field on each stage was evaluated.

In the batch culture of hydrolysis, the initial substrate concentration was 0.9 g/L sucrose. Sucrose consumption and glucose production were monitored over time. Acidogenesis was performed with 1.0 g/L glucose, where glucose consumption and VFA production profiles were registered. In acetogenesis, 1.0 g/L propionic and butyric acids were added, and VFA consumption and acetate production were monitored. Finally, 1.5 g/L acetic acid was added in the methanogenesis stage and acetate consumption, and the SMA was calculated. Reaction rates were standardized in mol of carbon substrate or product/h (mol C/L-h). Additionally, pH and ORP were monitored.

2.4 Calculation of SMA

The specific methanogenic activity of the sludge is defined as the amount of substrate converted to methane per unit mass of sludge per unit time. It is expressed as g COD-CH₄/g VSS-d. Once the volume of methane production in milliliters (V_{CH₄}) was obtained, it was converted to grams of

COD-CH₄ using a stoichiometric conversion factor. This factor states that four grams of COD are required for every gram of CH₄ produced (Eq. 10).

$$g\text{COD} - \text{CH}_4 = V_{\text{CH}_4} * \frac{1 \text{ g CH}_4}{4 \text{ g COD}} * \text{density of CH}_4 \quad (\text{Eq. 10})$$

A graph was then constructed showing the cumulative volume of methane in g COD-CH₄ versus the time expressed hours. A period was established during which approximately 50% of the expected methane was generated from the added substrate. The slope of the regression line for this segment represents the maximum methane production rate (R_{CH₄}), expressed in grams of COD-CH₄ per hour (g COD-CH₄/h). Once the value of R_{CH₄} was determined, the SMA was calculated using the following equation:

$$SMA = \frac{R_{\text{CH}_4} * 24}{M_{\text{VSS}} * V_S} \quad (\text{Eq.11})$$

Where:

R_{CH₄}= maximum methane production rate (g COD-CH₄/h).

24 = Conversion factor from hours to days

M_{VSS}= Concentration of sludge used in the experiment (g VSS/L)

V_S= Volume of sludge used in the experiment (L)

2.5 Determination of electrochemically O₂ production and facultative activity

In batch cultures, dissolved oxygen (DO) concentration was quantified in the presence and absence of an electric field; this consisted of applying the three selected electric field magnitudes (1.21, 1.45, and 1.64 V/cm). Abiotic (without sludge) and biotic (with 2 g VSS/L of sludge) experiments were performed with constant stirring at 100 rpm for five hours. The reactors were bubbled with molecular nitrogen for 3 min to ensure an oxygen-free environment before applying the electric field.

The sludge's O₂ consumption capacity was determined in independent experiments, in which air was supplied by bubbling until a pseudo-stationary state was reached; once a constant concentration was reached, aeration was stopped, and the DO consumption profile was monitored.

To determine the O₂ consumption capacity of the facultative microorganisms, consumption kinetics were established and adjusted to a Monod-type model. The experimental data of decrement

rates in DO concentration were calculated and graphed as a function of DO concentration. Then they were fitted to obtain the consumption kinetic parameters using the following equation:

$$Q_{O_2} = \frac{Q_{O_2M} C_{O_2}}{K_A + C_{O_2}} \quad (\text{Eq. 12})$$

Where:

Q_{O_2} : rate of oxygen consumption (mg/L-min)

Q_{O_2M} : maximum rate of oxygen consumption (mg/L-min)

C_{O_2} : dissolved oxygen concentration in the medium (mg/L)

K_A : affinity coefficient (mg/L)

Nonlinear regression was used to estimate the parameters through a Marquardt-Levenberg algorithm. Origin Pro Software was used to fit the model.

These experiments were carried out in biotic and abiotic media with continuous stirring at 100 rpm. The medium used for both experiments was described in section 2.1, with sucrose as a carbon source. The DO was quantified with the sensor based on the phenomenon of photoluminescence (WTW™ FDO 925, Germany), with a detection range of 0.00 to 20 mg/L, connected to a multiparameter meter (WTW™ Multi 3034, Germany).

2.6 Determination of enzymatic activity in batch cultures

Enzymatic activity was evaluated to identify when it increased in the presence of the electric field. Batch cultures were conducted in glass electrochemical reactors (two-electrode cells) with 130 mL of liquid and 10 mL of headspace. The electrodes were constructed in titanium plates electrodes coated with ruthenium oxide (RuO₂/Ti), whose dimensions were 7.5 cm long, 0.6 cm wide, and 0.1 cm thick; the submerged area of the electrodes was 9.16 cm², and the separation between electrodes was 2 cm. A high-precision power supply (BK PRECISIONS Model 9120A, Taiwan) was used to apply the electric field (1.21 V/cm).

These cultures were inoculated with 2 g VSS/L, previously acclimated in the UASB reactor. The experimental units were bubbled with N₂ for 3 minutes to ensure an oxygen-free environment. Initially, batch cultures without an electric field served as a control. Following this, the effect of the electric field on enzymatic activity was evaluated. The initial pH was 7, and the assays were

operated at 23 ± 3 °C. It was fed with the following mineral medium: (in g/L), sucrose (1.5), NH_4Cl (0.85), K_2HPO_4 (0.234), KH_2PO_4 (0.136), $\text{MgCl} \cdot 6\text{H}_2\text{O}$ (0.084), NaHCO_3 (2), and yeast extract (0.34). A trace element solution (0.5 mL/L) was added, and the composition of the trace solution (in g/L) was EDTA (0.5), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.25), MnCl_2 (0.6), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.2), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2), MgCl_2 (0.5), FeSO_4 (0.1), and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1). Samples of the liquid phase were taken every five hours to evaluate the enzymatic activity.

An acetate buffer solution at pH 5 and a 1% (w/v) sucrose solution were prepared to measure enzymatic activity. These were mixed in 10 mL test tubes, using 0.35 mL of acetate buffer and 0.5 mL of sucrose solution, and preincubated at 30 °C for 5 minutes to bring the mixture to the optimal temperature for the reaction. A volume of 0.15 mL of the sample was added, and the tubes were incubated for 30 minutes. After this time, 2 mL of DNS reagent was added to stop the reaction, and the reducing sugar assay was completed.

2.7 Analytical methods

The closed reflux method quantified the COD. VSS, SVI, sedimentation velocity, and granulometric were quantified using the standard methods (APHA/AWWA/WEF, 2012) (APHA/AWWA/WEF, 2012). The measurement of the alkalinity ratio due to VFAs to total alkalinity was performed according to the α -index (Jenkins et al., 1983).

pH was measured with a multiparameter (Orion Star™ A215, Indonesia), and ORP was measured with a selective electrode (Cole-Parmer EW-27009-30) connected to a pH/ORP controller meter (Eutech Instruments 800, Singapore).

In batch cultures, total and reducing sugars were quantified by phenol–sulfuric acid (Dubois et al., 1956) and DNS (Miller, 1959) techniques, respectively. Volatile fatty acids were quantified in a gas chromatograph (Agilent Technologies 7820A, Shanghai) with a flame ignition detector (FID) and an Agilent J&W DB-FFAP column, N_2 was used as a carrier gas, a heating ramp from 80 °C to 200 °C was applied, with increments of 25 °C/min. The calibration curve used acetic, propionic, butyric, and valeric acid standards ranging from 10 to 1000 ppm. The biogas composition was determined using helium as the carrier gas with a gas chromatograph (Gow-Mac 580 GC, U.S. A) measured using a Carboxphere 80/100 column with helium as the carrier gas, under these

conditions: column temperature = 40°C, injector temperature = 90°C, detector temperature = 90°C, and a detector current of 120 mA. The calibration curve was prepared using CO₂ and CH₄ standards.

The enzymatic activity of invertase was evaluated since it catalyzes the hydrolysis of sucrose, releasing reducing sugars according to the method described by (Martínez-Trujillo et al., 2020). Reducing sugars were quantified by DNS (Miller, 1959).

2.8 Reaction kinetics

The Gompertz model was a valuable tool for describing the AD stages' kinetics. Nonlinear regression was used to estimate the kinetic parameters through a Marquardt-Levenberg algorithm. The Gompertz model allows starting from a primary function to represent the process of consumption and production of substrates in anaerobic digestion, giving a biological meaning to the following mathematical equation:

$$y = ae^{-be^{-kt}} \quad (\text{Eq. 13})$$

y and t are variables, while a , b , and k are model parameters.

Where:

y : concentration of the product or substrate at a given time (mg/L)

t : reaction time

k : reaction constant (h⁻¹)

a : maximum production or consumption (mg/L)

b : necessary time to reach the maximum consumption rate

e : Euler number ($e = 2.71828...$)

When fitting the kinetic model to experimental data, the Origin Pro Software calculates the kinetic parameters of the Gompertz Model k , a , and b (González-Blanco et al., 2012).

2.9 Statistical analysis

A single-factor analysis of variance (ANOVA) was performed to compare the experimental results obtained in the presence and absence of an electric field. The significance value (α) was 5% using the data analysis add-in of Microsoft Excel software. The initial hypothesis was rejected by comparing the value of F and the critical value of F . A Tukey–Kramer post hoc test was performed.

The analysis was initiated by determining the mean absolute difference between each group based on the means obtained in the ANOVA as follows: Control (C) vs. 1.21 V/cm, C vs. 1.45 V/cm, C vs. 1.64 V/cm, 1.21 V/cm vs. 1.45 V/cm, 1.64 V/cm vs. 1.45 V/cm, and 1.21 V/cm vs. 1.64 V/cm. Subsequently, the critical value of Q is determined using the following equation:

$$Q_{critical\ value} = Q \sqrt{\frac{s^2}{n}} \quad (\text{Eq. 14})$$

where:

- Q = Value of Studentized Range Distribution (Q)
- s^2 grouped = Pooled variance in all groups
- n = Sample size for a given group

To find the value of Q in the table, the degrees of freedom are necessary, and they are calculated as $n-K$, where K represents the number of groups. Finally, the mean absolute difference between each group is compared with the critical value of Q . If the mean absolute difference is more significant than Q 's, then the difference between the group means is significant.

3 Results and discussion

3.1 Stabilization of an anaerobic sludge at room temperature

In a UASB bioreactor, the pseudo-steady state was reached on day 19 (Figure 3.1 a), with a soluble COD removal efficiency of 91.74 ± 3.82 %. This value is similar to that reported by Jacobo López et al. (2019), in which a UASB reactor was operated at 21 °C with wastewater from the chocolate industry, obtaining a COD removal efficiency of 90%. When an anaerobic reactor reaches a steady state, it can achieve COD removal efficiencies up to 80 to 90% despite working at lower than optimal temperatures, which will be proportional to the applied HRT (Rizvi et al., 2015; Víctor Vacca et al., 2008).

The sludge used as an inoculum in the present investigation had a VSS of 81.77 g/L, a settled g rate of 10.2 m/h, a VSI of 11.14 mL sludge/g TS, and a density of 0.97 g/mL. During reactor operation, no significant loss of biomass was observed, attributed to the fact that the density of the granule allowed good sedimentation. According to the granulometry, the anaerobic sludge

presented a composition of 3% floc, 27% granules with a diameter greater than 0.6 mm, and 70% granules with a diameter greater than 0.4 mm. Then, in combination with the HRT used in the present study (i.e., one day), it allowed a longer contact time between organic matter and anaerobic microorganisms. Other factors, such as the characteristics of the inoculum and the type of substrate to be threatened, might affect the removal efficiency, which has already been reported.

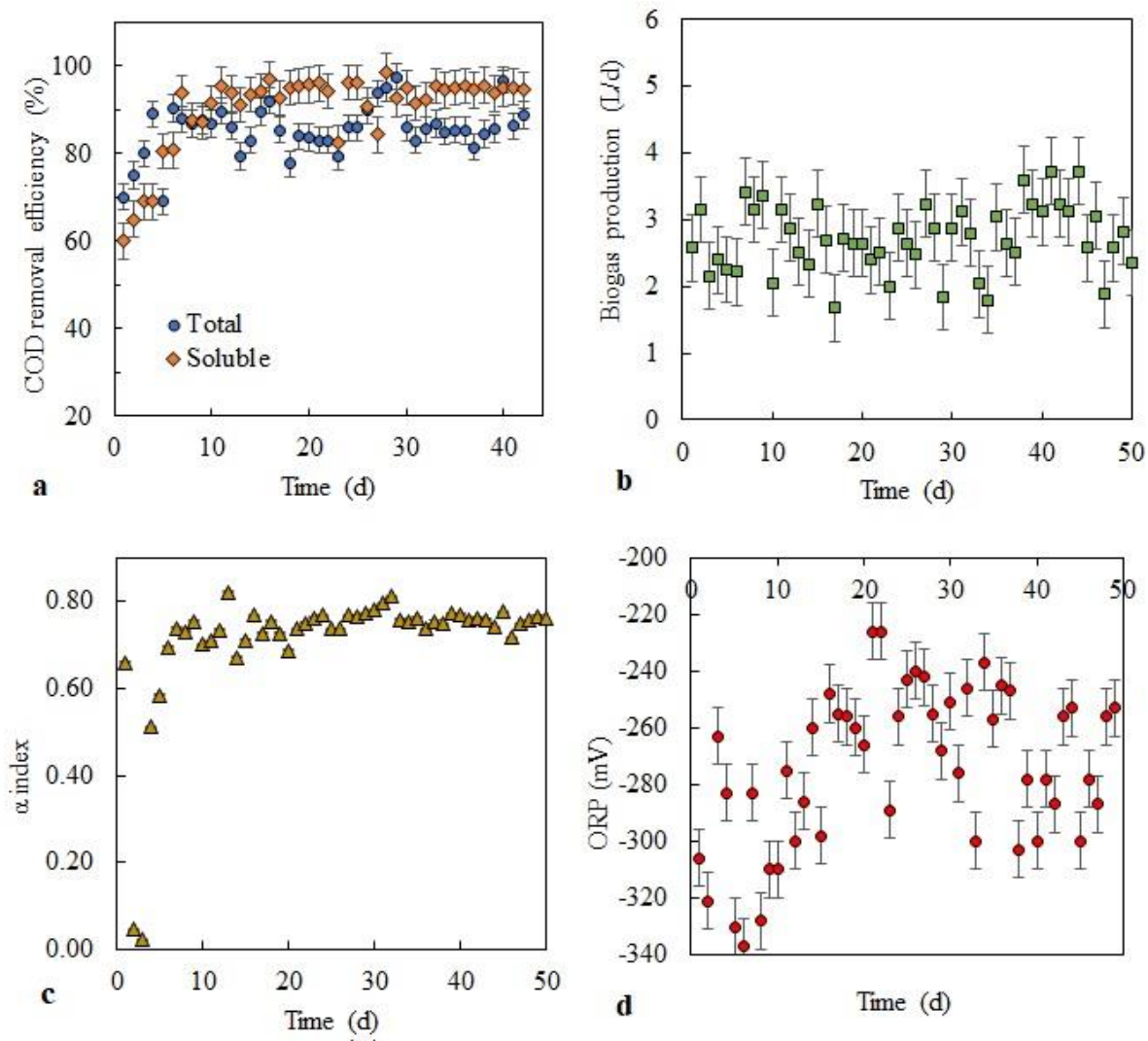


Figure 3.1 Stabilization profiles of the UASB reactor without temperature control, a) COD removal efficiency, b) biogas production, c) α index, d) ORP.

The SMA of anaerobic sludge was 0.12 g CH₄ -COD/ g VSS d at room temperature. The biogas production profile showed a wide variability from 2.05 to 4.95 L/d (Figure 3.1b) due to leakage at the reactor outlet connection. The average production was 1.88 ± 0.3 L/d, and the biogas composition was 80% CH₄ and 20 % CO₂. Based on these data, the methane yield was calculated according to the equation described by Chernicharo. (2007), in which a biomass growth yield (Y_{obs}) considered was 0.17 kg COD granules/kg COD applied, given in the experimental work 406 ± 15 mL CH₄/g COD applied or 338.55 mL CH₄/g COD at standard conditions (1 atm and 0° K). The α index (Figure 3.1c) in the first days of operation was below 0.2, a typical value for the start-up stage. Stability was reached on day 26 with an index value of 0.73 and remained stable with a coefficient of variation of 4.7%. This value means the system has more bicarbonate alkalinity than VFAs, as Jenkins et al. (1983) described. On the other hand, the ORP profile (Figure 3.1d) ranged between -248 and -337 mV, which agrees with those reported for AD (Ortega, 2006) since these values indicate reducing conditions in the culture medium, which mainly favor methanogenic activity. Therefore, it was considered that the inoculum acclimatized satisfactorily at room temperature (23 ± 3 °C).

3.2 Characterization of the bioelectrochemical system

According to the characterization, graphite and stainless-steel electrodes create variations in pH (i.e., > 0.5 units), which could indicate that there are secondary reactions beyond those related to water electrolysis. In contrast, the RuO₂/Ti electrodes keep it constant (i.e., 7). RuO₂/Ti was selected for the coupled low-magnitude electric field on AD because it offers excellent stability, higher corrosion resistance in acidic or alkaline media, lower overpotential for O₂ and H₂ generation, longer service life, and less likelihood of biofilm formation compared to carbon material (Ken & Sinha, 2021). This electrode allowed analysis of the influence of water electrolysis reactions on the AD process associated with microorganisms in the granules without a biofilm on the electrode's surface.

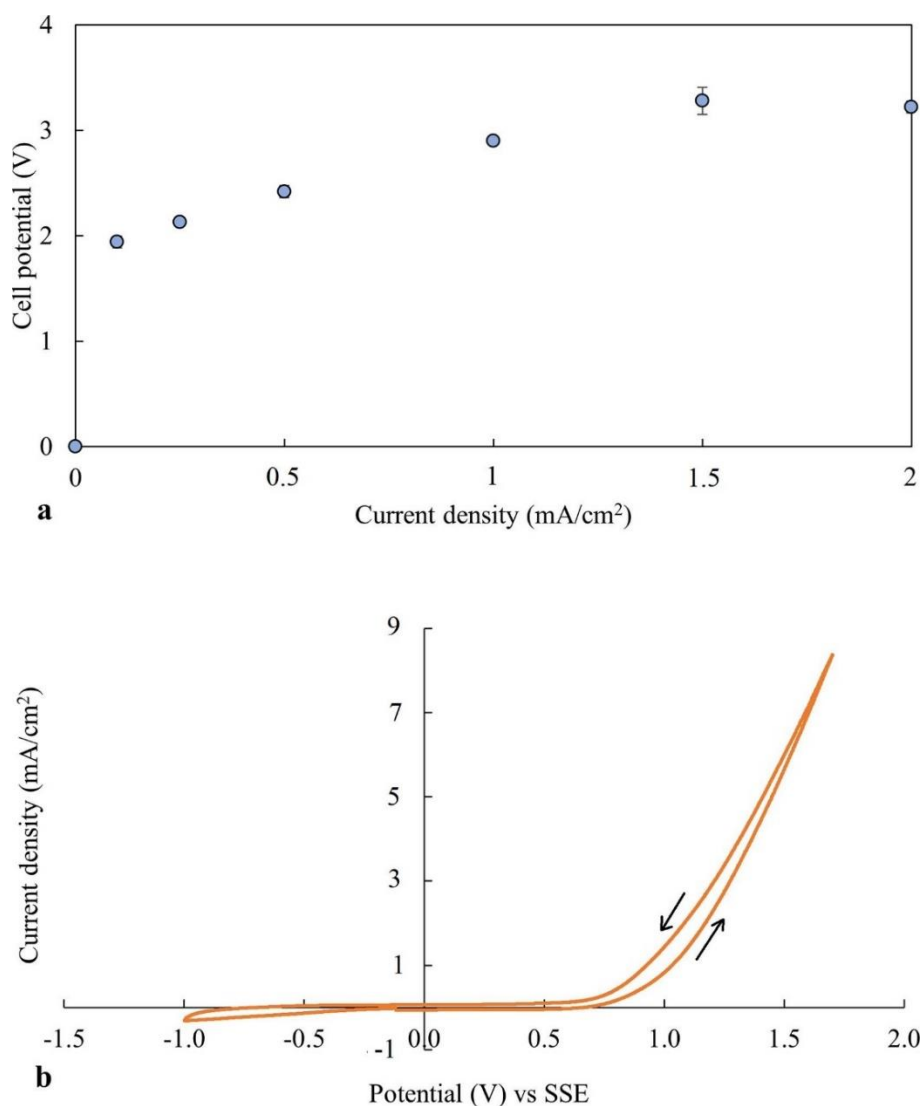


Figure 3.2 Electrochemical characterization of the mineral medium. a) Variation in cell potential (sampled when the potential reaches a stationary state) with imposing different current densities in an abiotic system through Ti/RuO₂ electrodes. b) Voltammogram ($v = 10$ mV/s) obtained for the Ti/RuO₂ electrode in mineral medium in the presence of acetate.

Cell potential (sampled when the potential reaches a stationary state) versus applied current intensity curves were constructed to determine a current intensity interval in which the media behaves like an electrical resistance (Figure 3.2 a). Electrical resistance behavior allows the operation to ensure that the electrochemical reactions on the surface of the electrodes will not alter the chemical composition of the medium. The resistive behavior was observed by applying a

current density within an interval of 0.54 to 1.63 mA/cm², so the electric field magnitudes applied to the cell were 1.21, 1.45, and 1.64 V/cm. The magnitude of the field was reported according to the cell potential obtained for each current intensity (5, 10, and 15 mA) between the separation of the electrodes (2 cm) to obtain the units of V/cm.

The voltammogram showed neither reduction nor oxidation of any chemical component (e.g., sucrose) in the culture medium; only water electrolysis reactions occurred at the anodic and cathodic potential barriers (Figure 3.2 b).

3.3 Influence of electric field on AD metabolism

During the evaluation of the hydrolysis stage, the consumption profiles of nonreducing sugars in the control study were completely consumed at five hours, while the studies with the imposition of the electric field (1.21, 1.45, and 1.64 V/cm) reached it at approximately four hours (Figure 3.3). The effect of the electric field was reflected in the hydrolysis efficiencies, which, when compared at 3 hours, the control had a hydrolysis efficiency of $68 \pm 1.42\%$; in contrast, for the batch cultures with applied electric fields of 1.21, 1.45, and 1.64 V/cm, the hydrolysis efficiencies obtained were $91 \pm 0.28\%$, $83 \pm 0.20\%$, and $93 \pm 1.18\%$, respectively.

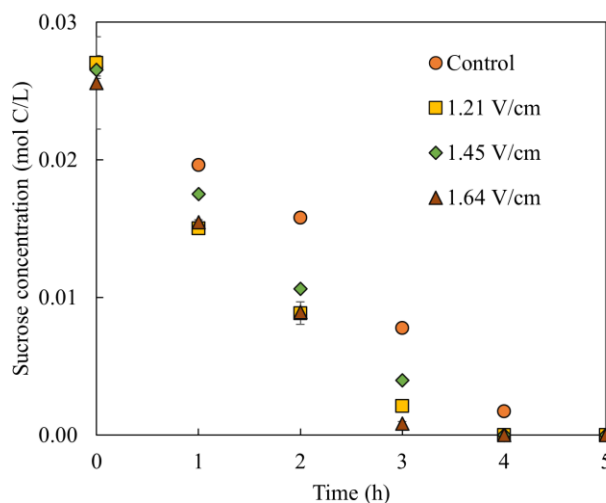


Figure 3.3. Comparison of sucrose consumption profiles in the hydrolysis stage at 23 °C by batch cultures in the absence of the electric field (Control) and the presence of different electric fields (marked as: 1.21, 1.42, and 1.64 V/cm). *Sucrose content is expressed as mol C/L, corresponding to the compound's total carbon concentration.

Some authors suggest that microaerobic conditions generated by water electrolysis reactions could favor the proliferation of facultative bacteria with better hydrolytic performance (Tartakovsky et al., 2011). These facultative bacteria can shield strict microorganisms within the diffusion barrier, preventing full oxygen penetration. High diffusion resistance was created, leaving near-zero dissolved oxygen concentrations at the liquid-biofilm interface (C. F. Shen & Guio, 1996).

The findings of Zhang et al. (2021) further support this explanation, stating that increased extracellular enzyme production under microaerobic conditions enhances the hydrolysis rate of organic matter. A parameter that could show the presence of O₂ was the ORP since it presents less negative values in the presence of the electric field (Figure 3.4 a), and this behavior was proportional to the increase in the magnitude of the electric field. The profiles stabilized from hour two and remained constant until hour five. According to Banerjee (2012), oxygen in anaerobic digestion (AD) stimulates extracellular ORP levels, disrupting the intracellular ORP balance and redirecting metabolic pathways. This shift modifies the microbial community, promoting facultative activity (Wu et al., 2021).

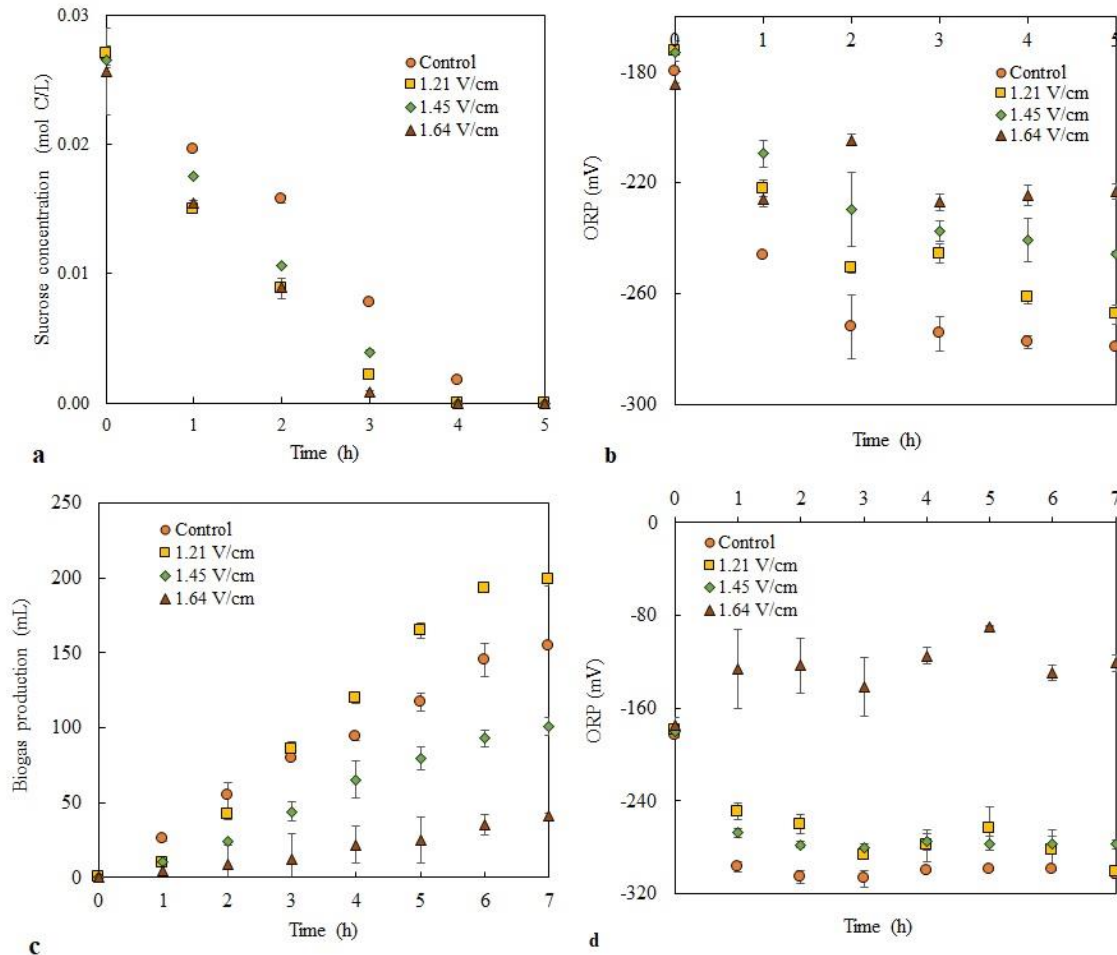


Figure 3.4 Comparison of the profiles of ORP in the four stages of anaerobic digestion at 23 °C by batch cultures in the absence of the electric field (Control) and the presence of different electric fields (marked as: 1.21, 1.42, and 1.64 V/cm). hydrolysis, b) acidogenesis, c) acetogenesis, and d) methanogenesis.

Oxygen's influence extends to the stages of acidogenesis and acetogenesis. Acidogenic bacteria, with a facultative metabolism, could utilize the O_2 produced by water electrolysis (Gerritse et al., 1990), thus oxidizing VFA to CO_2 instead of fermenting them. This phenomenon helps explain the results observed in the acidogenesis stage. The acetate and propionate production profiles (Figure 3.5 a-b) show that the maximum production is reached at three hours for all experiments; however, the acetate concentration produced in the presence of the different electric fields was lower than control, obtaining the lowest concentration at 1.21 V/cm. Despite these differences, production

yields (Table 3.1) did not significantly change. Similar results were obtained by Tao et al. (2020), who observed a 46% decrease in acetate production when 0.4 V/cm was applied.

Acidogenic activity is also susceptible to environmental factors such as H_2 concentration, pH, and temperature. These parameters regulate the type of fermentation that takes place and, therefore, the products obtained, as was observed by Lu et al. (2020), who found that butyrate was the main product under acidic conditions (i.e., pH = 5.0). In contrast, acetate was the dominant product under alkaline conditions (i.e., pH between 7 and 11). Ye et al. (2018) reported that pH 6.0 was optimal for producing VFA, in which butyrate and acetate concentrations predominated, whereas pH 8.0 increased the propionate concentration. Temperature also affects the composition of VFA; for instance, acetate and propionate are the leading products at mesophilic temperatures, whereas, under thermophilic conditions, butyrate is the main product (Zhou et al., 2018). Therefore, the operating conditions in the present work favored acetate and propionate production since the pH was 7.36 ± 0.05 and the temperature was 23.3 ± 0.2 °C. Butyrate was not detected.

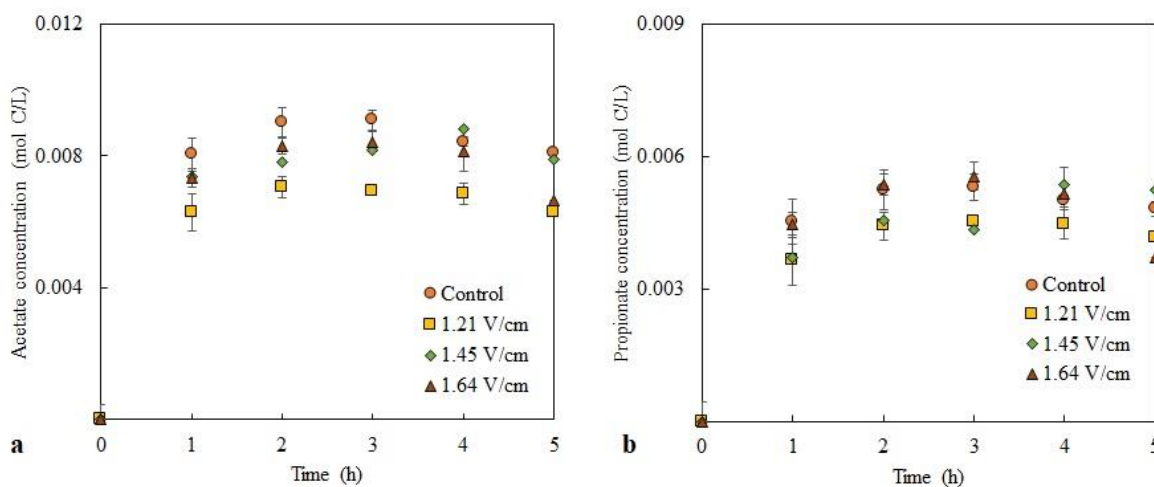


Figure 3.5 Comparison of production profiles of chemical species in the acidogenic phase at 23 °C by batch cultures in the absence of the electric field (Control) and the presence of different electric fields (marked as: 1.21, 1.42, and 1.64 V/cm). a) acetate production, and b) propionate production. * Acetate and propionic content are expressed as mol C/L, corresponding to the compound's total carbon concentration.

In the acetogenesis stage, VFA consumption profiles showed increased concentration during the first two hours, even without the electric field, which could be attributed to residual sugars in the granules (Figure 3.5 a). When applying 1.21 V/cm, VFA consumption rate decreased 69%, while

1.45 and 1.64 V/cm led to increases of 20% and 38%, respectively. Despite these variations, acetate production did not significantly change across the electric field magnitudes (Figure 3.6 b).

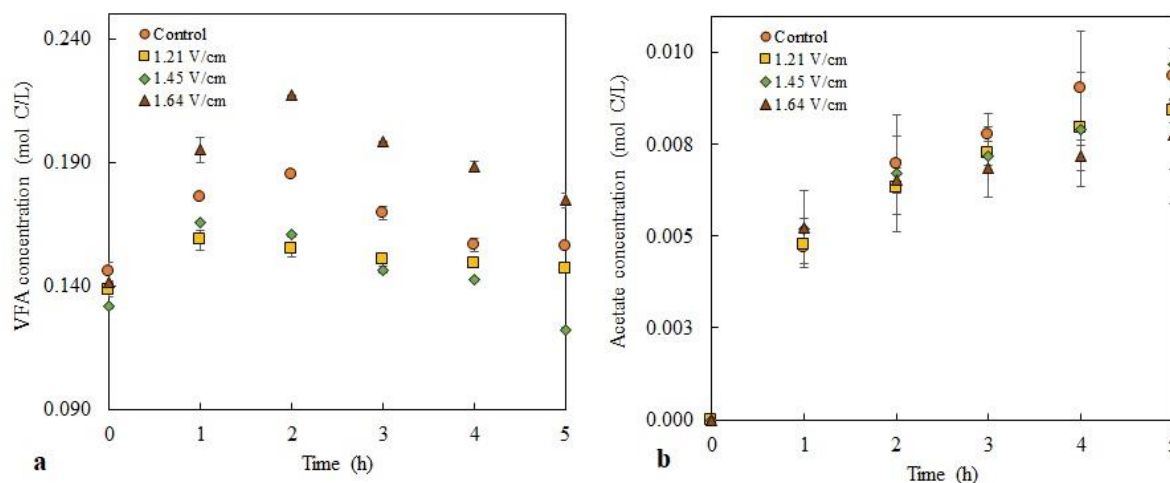


Figure 3.6 Comparison of VFA consumption (butyrate and propionate) at 23 °C by batch cultures in the absence of the electric field (Control) and the presence of different electric fields (marked as: 1.21, 1.42, and 1.64 V/cm). * Acetate and VFA content are expressed as mol C/L, corresponding to the compound's total carbon concentration.

Compared to the control, acetate production yields, relative to VFA consumption, decreased 18% and 44% with 1.45 and 1.64 V/cm, respectively. Conversely, a 47% increase was observed with 1.21 V/cm. The acetogenesis stage is slow and is taken by strictly anaerobic bacteria (Ariesyady et al., 2007). It is worth mentioning that substrate consumption in this stage depends on the concentration of H_2 ; in addition, propionate and butyrate are the most critical syntrophic intermediates, and their consumption is considered the limiting stage due to thermodynamic constraints (ΔG° propionate = +76.1 and ΔG° butyrate = +48.1 kJ/mol) (Amani et al., 2011). Therefore, the role of bacteria consuming H_2 is essential; for example, homoacetogenic bacteria are strictly anaerobic microorganisms that catalyze acetate formation from H_2 and CO_2 . In general, four molecules of H_2 and two molecules of CO_2 are required to complete the process ($\Delta G^{\circ'} = -105$ kJ/mol), so thermodynamically, the homoacetogenic pathway is more favorable for obtaining acetate (Drake et al., 2008). The fermentative stages (acidogenesis and acetogenesis) did not show significant changes in the ORP values (Figure 3.4 b-c). This could indicate that the electric field's influence is insignificant in these stages.

Finally, during the methanogenesis stage, the three tests performed with an electric field showed an adaptation stage at one hour with a biogas production of 12.5 ± 2.5 mL (Figure 3.7 b). In contrast, the control showed a higher biogas production in the first hour of 32.5 mL $\pm$. This was attributed to methanogens being the most susceptible to oxygen because they cannot synthesize the superoxide dismutase enzyme that aerobic organisms use to neutralize reactive oxygen species (i.e., O_2^{2-} , O_2^- , and OH^-). However, in the presence of O_2 to overcome oxidative stress, some methanogenic species may have intrinsic tolerance to oxygen exposure as they adapt to environmental conditions and can even produce superoxide dismutase, which could explain how these experiments showed this adaptive stage (Magdalena et al., 2022). On the other hand, the applied electric field increased CO_2 production (Figure 3.7 a). As mentioned above, the presence of oxygen promoted VFA oxidation.

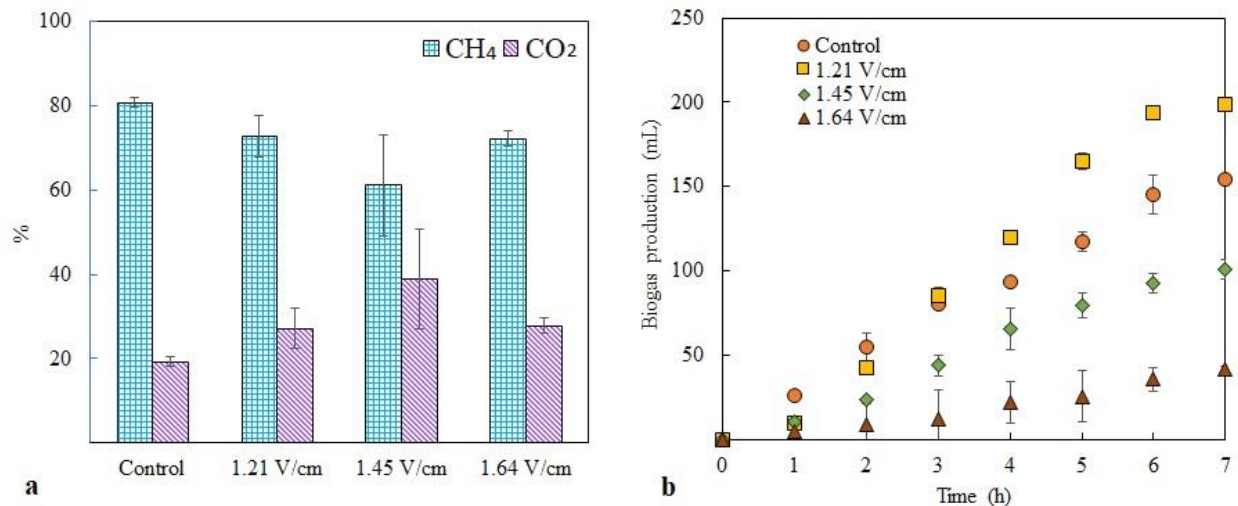


Figure 3.7 Production results of the methanogenesis stage at 23 °C by batch cultures in the absence of the electric field (Control) and the presence of different electric fields (marked as: 1.21, 1.42, and 1.64 V/cm). a) composition of biogas obtained, b) comparison of methane production of methane.

The ORP did not show significant changes concerning the control when applying the electric fields of 1.21 V/cm and 1.45 V/cm. However, when applying 1.64 V/cm, the ORP was less negative than the control's (Figure 3.4 d). The same behavior has been reported for microaerobic conditions in anaerobic treatments (H. Liu et al., 2017). Nghiem et al. (2014) reported that micro aeration (0.14 mL/s) in an anaerobic digester changed the ORP to less negative values, going from -485 mV to -270 mV. Specific methanogenic activity (SMA) served as a response parameter to evaluate the

effect of the electric field on methanogenesis. An increase of 39% was observed when applying the electric field of 1.21 V/cm, while when applying 1.45 and 1.64 V/cm, a decrease of 31% and 79% was observed concerning the control.

Table 3.1 Production yields were obtained for each anaerobic digestion stage in the presence and absence of the electric field.

Stage	Electric field (V/cm)	Y Glucose (mol glucosa-C/mol sucrose-C consumed)	
Hydrolysis	0 (control)	0.13 ± 0.008 ^a	
	1.21	0.14 ± 0.002 ^a	
	1.45	0.11 ± 0.002 ^a	
	1.64	0.15 ± 0.021 ^a	
Acidogenesis		Y Acetate (mol acetate-C/mol glucose-C consumed)	Y Propionate (mol propionate-C/mol glucose-C consumed)
	0 (control)	0.63 ± 0.015 ^a	0.37 ± 0.006 ^a
	1.21	0.55 ± 0.025 ^a	0.32 ± 0.010 ^a
	1.45	0.61 ± 0.010 ^a	0.36 ± 0.010 ^a
	1.64	0.53 ± 0.023 ^a	0.35 ± 0.049 ^a
Acetogenesis		Y Acetate (mol acetate-C/mol VFA-C consumed)	
	0 (control)	0.66 ± 0.096 ^a	
	1.21	0.97 ± 0.183 ^a	
	1.45	0.48 ± 0.180 ^a	
	1.64	0.37 ± 0.011 ^a	
Methanogenesis		Y Methane (mol CH ₄ -C/mol acetate-C consumed)	
	0 (control)	0.32 ± 0.020 ^a	
	1.21	0.31 ± 0.005 ^a	
	1.45	0.19 ± 0.015 ^b	
	1.64	0.12 ± 0.020 ^b	

*The difference between letters indicates that they are significantly different.

In this context, oxygen could significantly impact AD if facultative bacteria are present. An alternative study was conducted to support this proposal, in which three magnitudes of electric field were applied in abiotic experiments. These experiments resulted in similar oxygen saturation concentrations (Figure 3.8 a-c). However, it was observed that the dissolved oxygen (DO) production rate increased proportionally with the magnitude of the electric field, registering values of 0.075, 0.082, and 0.094 mg O₂/L-min for electric fields of 1.21, 1.45, and 1.64 V/cm, respectively.

In contrast, the biotic experiments showed different behavior. The microorganisms consumed almost entirely the DO. The lowest DO concentration (i.e., 0.04 ± 0.01 mg/L), was obtained when applying a field of 1.21 V/cm (Figure 3.8 a). However, as the electric field magnitude increased to

1.45 and 1.64 V/cm, DO accumulation was observed in the aqueous stage starting at minute 200. This could indicate that the microorganism's ability to consume oxygen is overwhelmed under these conditions, likely due to the increased oxygen production by the electric field.

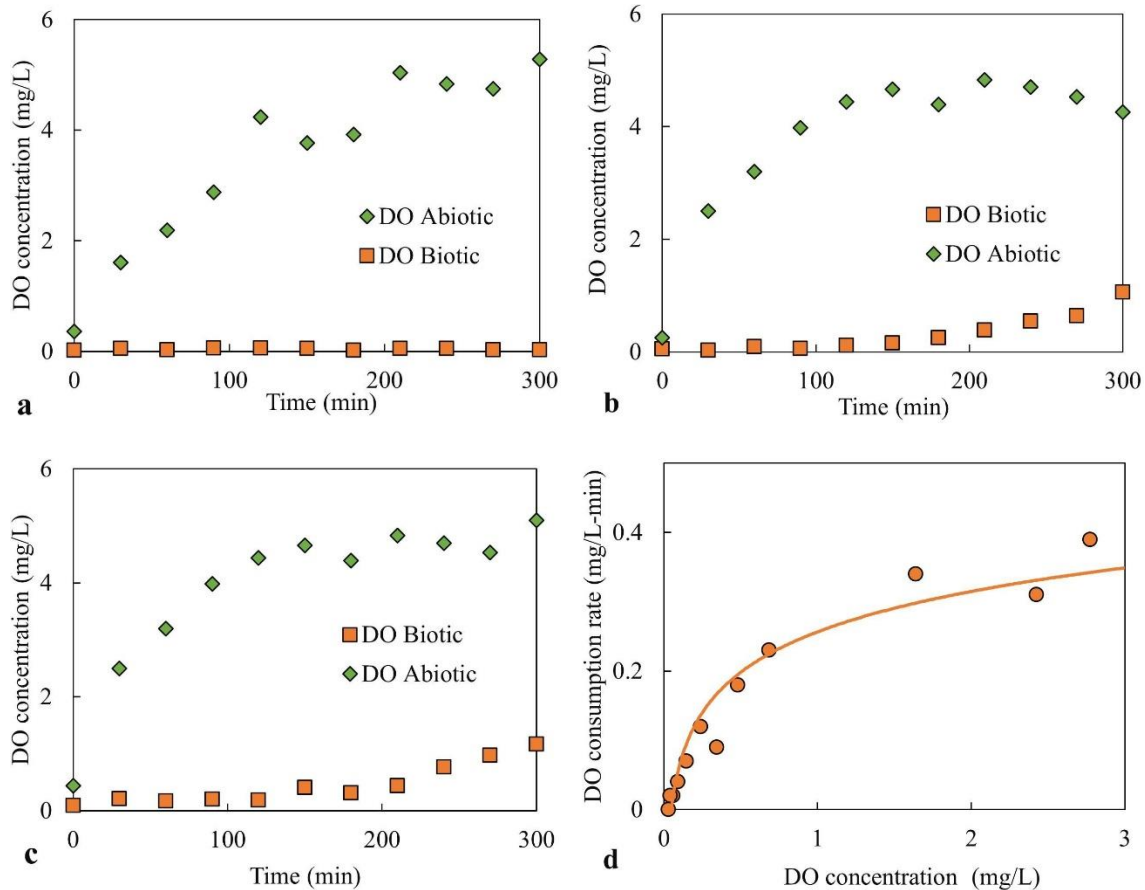


Figure 3.8 Comparison of dissolved oxygen (DO) concentration in the hydrolysis stage at 23 °C by batch cultures in the absence of the electric field (Control) and the presence of different electric fields (marked as: 1.21, 1.42, and 1.64 V/cm) d) kinetic analysis of oxygen consumption, based on the Monod-type model (Continuous line), obtained in the biotic system (previously saturated with O₂) in the absence of an electric field.

To better understand this phenomenon, anaerobic sludge's maximum oxygen consumption rate was calculated under controlled conditions, where the aqueous stage was saturated with oxygen without applying an electric field. The maximum consumption rate obtained was 0.484 ± 0.04 mg O₂/L-min. This reference rate suggests that microorganisms have a high oxygen consumption capacity. The difference in DO consumption rates observed between the biotic and abiotic

experiments indicates that the oxygen generated electrolytically at higher electric field magnitudes was enough for oxygen saturation water. On the other hand, Figures 3.8. b and c show that reached O_2 concentration was higher than 1 mg/L, which can switch the metabolic from fermentative to oxidative due to the maximum oxygen consumption capacity can be observed (Fig. 3.8 d), diminishing the methanogenic activity.

Based on these results, an analysis of invertase enzymatic activity was conducted to determine whether an electric field could enhance the excretion of extracellular enzymes. The experiment, detailed in section 2.5, utilized sucrose as a carbon source with an applied electric field of 1.21 V/cm, which had previously demonstrated optimal performance (Figure 3.9).

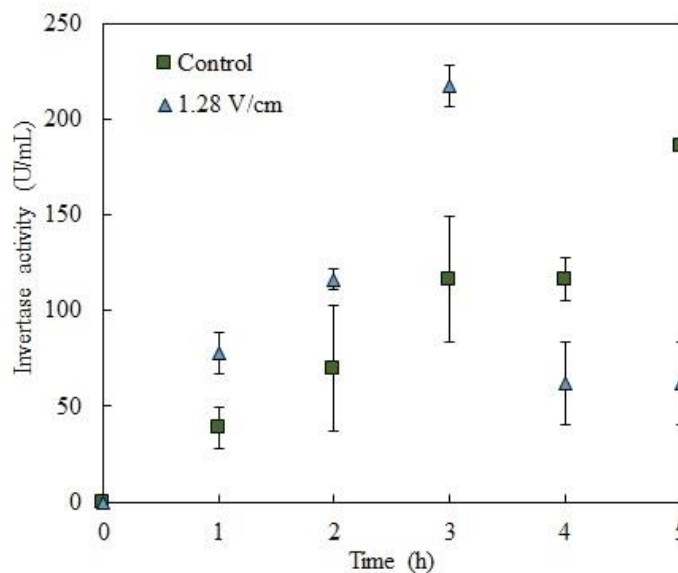


Figure 3.9 Comparison of invertase activity in the absence of electric field (Control) and presence of electric field (1.28 V/cm) at 23 °C.

The findings revealed a significant increase in invertase activity under the influence of the electric field. At the three-hour mark, the enzyme activity reached 217 ± 11 U/mL, while the control only reached 116 ± 33 U/mL. However, after this peak, the activity in the electric field treatment

declined sharply, whereas the control saw a gradual increase, reaching its highest value at hour five.

These results strongly suggest that the electric field stimulates facultative bacteria to excrete higher quantities of extracellular enzymes during the initial stage of anaerobic digestion (AD). This enhancement in enzyme activity may accelerate the overall degradation process in AD by promoting more efficient substrate breakdown in the early phases.

3.4 Influence of the electric field on the kinetics of AD

For the kinetic analysis, global reaction rates were assumed as first order, such as any poblational growth model, this analysis revealed that the hydrolysis stage increased the reaction kinetic constant during sucrose consumption: 52, 42, and 72% for electric field magnitudes of 1.21, 1.45, and 1.64 V/cm, respectively, compared to the control (Table 3.2). These results indicated that the characteristic time of the hydrolysis process decreases with increasing electric field, which could improve the overall AD performance.

Table 3.2 Influence of the magnitude of the electric field on the kinetic parameters of AD phases.

Phases	Electric field (V/cm)	k (h ⁻¹)	Characteristic time (h)
Hydrolysis	0 (control)	0.63 ± 0.00 ^a	1.59 ^a
	1.21	0.97 ± 0.02 ^b	1.03 ^b
	1.45	0.90 ± 0.00 ^c	1.12 ^c
	1.64	1.08 ± 0.01 ^d	0.93 ^d
Acidogenesis	0 (control)	1.19 ± 0.05 ^a	0.84 ^a
	1.21	0.77 ± 0.01 ^b	1.29 ^b
	1.45	0.84 ± 0.02 ^b	1.19 ^b
	1.64	1.26 ± 0.01 ^a	0.80 ^a
Acetogenesis	0 (control)	1.17 ± 0.12 ^a	0.86 ^a
	1.21	1.76 ± 0.14 ^a	0.57 ^a
	1.45	1.53 ± 0.02 ^a	0.65 ^a
	1.64	2.69 ± 0.95 ^a	0.42 ^a
Methanogenesis	0 (control)	0.40 ± 0.01 ^a	2.53 ^a
	1.21	0.55 ± 0.02 ^a	1.83 ^a
	1.45	0.50 ± 0.10 ^a	2.08 ^a
	1.64	0.46 ± 0.04 ^a	2.21 ^a

*The difference between letters indicates that they are significantly different.

During the fermentative stages, acidogenesis showed a decrease in the reaction rate constant for glucose consumption compared to the control when electric fields of 1.21 and 1.45 V/cm were applied, with reductions of 35% and 29%, respectively. This was reflected in the characteristic time; for example, with 1.21 V/cm, there was a 54% reduction compared to the control, which could prevent acidification of the system due to the accumulation of volatile fatty acids (VFA). The acetogenesis stage showed a 34% increase under the same electric field, indicating faster VFA consumption, leading to acetate production for CH₄ generation. This could improve the syntrophy between these two stages, which often exhibit an imbalance between VFA production and consumption.

The characteristic times revealed that methanogenesis remains the slowest stage. However, with the application of a 1.21 V/cm electric field, the time for this stage decreased by 28%, accelerating CH₄ production. In summary, the electric field influences the reaction kinetics of each stage, reducing reaction times and stabilizing the overall process.

4 Conclusions

Anaerobic digestion is typically performed at a minimum temperature of 35 °C, which poses an operational disadvantage for its applicability in cold geographical regions. This study demonstrated that water electrolysis reactions induced by a low-magnitude electric field, assessed at 23 °C, significantly improved the kinetics and metabolic behavior of anaerobic digestion, resulting in a 39% enhancement in methanogenic activity.

The application of an appropriate electric field serves as a valuable tool for modifying the metabolic processes involved in anaerobic digestion. Firstly, micro-aerobic conditions generated by water electrolysis enhanced the hydrolysis rate of sucrose at all tested electric field magnitudes, thereby improving the organic matter removal from wastewater. Additionally, an improved syntrophy between the production and consumption of volatile fatty acids (VFAs) was observed due to alterations in the kinetics of the acidogenesis and acetogenesis stages.

Notably, the methanogenic stage was positively stimulated at the lowest electric field magnitude (1.21 V/cm), with increased acetate consumption leading to enhanced methane production. These

novel findings present a promising alternative for the technological development of wastewater treatment, employing a controlled low-magnitude electric field to optimize desired metabolic products without requiring temperature control. Furthermore, this approach demands shorter hydraulic retention times compared to conventional technologies, allowing for the design of more compact and efficient wastewater treatment plants by integrating anaerobic digestion with low-magnitude electric fields.

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Chapter III: Influence of water electrolysis on hydrolysis and methanogenesis stages of anaerobic digestion at room temperature: Kinetic and metabolic analysis

1 Introduction

Anaerobic digestion (AD) represents a cost-effective and widely applied biotechnology to treat high-COD wastewater. It is a complex biotechnological process requiring high temperatures (e.g., 35-44°C) to achieve high methane yields (Mao et al., 2015). Controlling high temperatures in cold climate zones in wastewater treatment plants is economically unfeasible. Therefore, developing technologies to operate AD at low temperatures while maintaining high yields is a real challenge, and this is the main goal of the present work.

Currently, there are some strategies to enhance CH₄ yield, but one of the most recent is the coupling of electrochemical systems, improving COD removal and CH₄ production. For instance, 0.05 to 3.5 V applied on AD promotes electroactive biofilms on the electrode surface, allowing attached bacteria to transfer charges, turning them into redox mediators, thus increasing CH₄ yield (De Vrieze et al., 2018). Koch et al. (2015) imposed a cell potential of 0.2 V, using graphite electrodes at 37 °C, improving CH₄ yield by 27 %. Sun et al. (2020) evaluated an anaerobic reactor at HRT of 24 h, with titanium mesh electrodes (0.05–0.2 V) at 35 °C, observing an increment of methane production by 15% with shifts in the microbial diversity. The effect of electrochemical reactions in these systems has been studied little, specifically in the granules suspended in the liquid medium. Consequently, the intrinsic production of H₂ and O₂ by the electrolysis of water should be one of the variables to consider since AD syntrophy allows microorganisms to couple the O₂ and H₂ produced through electrochemical reactions in their biochemical pathways and, therefore, could regulate AD metabolism. For example, Tartakovsky et al. (2011) observed an AD improvement by applying a cell potential of 2.8–3.5 V for water electrolysis at 35 °C; the authors attributed the higher CH₄ yield to the electrochemical production of O₂ that favored the hydrolytic activity.

Based on the observations mentioned above, it is necessary to develop studies that support the production rate of O₂ and H₂ and their direct effect on each AD stage, mainly in the hydrolysis and methanogenesis, considered the limiting steps. Therefore, this work aims to evaluate the influence of oxygen production by water electrolysis in hydrolysis and methanogenesis activities using only granular sludge previously stabilized at room temperature in batch cultures.

2 Materials and methods

2.1 Inoculum and culture medium

An anaerobic sludge from a candy-manufacturing wastewater treatment plant was stabilized in a laboratory-scale Upflow Anaerobic Sludge Blanket (UASB) reactor with an operating volume of 2 L. It was fed with the following chemical medium: (in g/L), sucrose (1.5), NH_4Cl (0.85), K_2HPO_4 (0.234), KH_2PO_4 (0.136), $\text{MgCl}\cdot 6\text{H}_2\text{O}$ (0.084), NaHCO_3 (2), and yeast extract (0.34). A trace element solution (0.5 mL/L) was added, with the following composition (in g/L), EDTA (0.5), $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ (0.4), $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ (0.25), MnCl_2 (0.6), $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ (0.25), $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ (0.2), $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ (0.2), MgCl_2 (0.5), FeSO_4 (0.1), and $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ (0.1).

2.2 Characterization of the bioelectrochemical system for batch cultures

Electrochemical characterization was performed in glass electrochemical reactors (two-electrode cells) with 130 mL of liquid and 10 mL of head space. The electrodes were constructed in Ti plates coated with RuO_2 (RuO_2/Ti), whose dimensions were 7.5 cm long, 0.6 cm wide, and 0.1 cm thick; the submerged area of the electrodes was 9.16 cm^2 , and the separation between electrodes was 2 cm. The electric field is associated with the cell potential where two Ti/RuO_2 electrodes are immersed in the granules and culture medium. A low electric current is imposed, and the cell potential developed is monitored with the imposition time. The electric field vs. current density curve has been used to find an operating interval in which a resistive behavior was observed to ensure no chemical alterations in the culture chemical composition, Figure Aa (see chapter II). A high-precision power supply (BK PRECISIONS Model 9120A, Taiwan) was used to apply electric current. A voltammetric study was conducted using a mineral medium containing sucrose and acetate. A voltammogram from -1 to 1.7 V with a scan potential of 10 mV/s was performed (in a three-cell electrode) using mercury/mercurous sulfate/saturated sulfate (SSE) and a carbon cylinder as reference and counter electrodes, respectively.

2.3 Characterization of the anaerobic digestion stages applying an electric field

Batch cultures were inoculated with 2 g VSS/L previously acclimatized in the UASB. Experimental units were bubbled with N_2 for 3 minutes to ensure an oxygen-free environment. Initially, batch cultures (without an electric field) were carried out as a control assay. Subsequently, the effect of the electric field on hydrolysis and methanogenesis was evaluated by applying three

magnitudes (1.21, 1.45, and 1.64 V/cm). In the hydrolysis culture, sucrose was used as a carbon source at 0.9 g/L, where sucrose consumption and glucose production were monitored over time. In the methanogenic culture, 1.5 g/L acetic acid was added; acetate consumption and methane production were plotted, and the specific methanogenic activity (SMA) was determined. Origin Pro Software was used to calculate the kinetic parameters using the Gompertz Model. A single-factor analysis of variance (ANOVA) was performed to compare the experimental results obtained in the presence and absence of an electric field. The significance value (α) was 5% using the data analysis add-in of Microsoft Excel software.

2.4 Determination of O₂ production and facultative activity

In batch cultures, the dissolved oxygen (DO) concentration was quantified in the presence and absence of an electric field; this consisted of applying the three selected electric field magnitudes. Abiotic (without sludge) and biotic (with 2 g VSS/L of sludge) experiments were performed with constant stirring at 100 rpm for five hours. The O₂ consumption capacity of the sludge was determined in independent experiments in which air was supplied by bubbling until a pseudo-stationary state was reached; once a constant concentration was reached, aeration was stopped, and the DO consumption profile was monitored. The consumption kinetics were established and adjusted to a Monod-type mode to determine the O₂ consumption capacity of the facultative microorganisms.

2.5 Analytical methods

pH was measured with a multiparameter (Orion Star™ A215, Indonesia). ORP was measured with a selective electrode (Cole-Parmer EW-27009-30) connected to a pH/ORP controller meter (Eutech Instruments 800, Singapore). Phenol-sulfuric acid and DNS techniques were used to quantify total and reducing sugars, respectively. Volatile fatty acids were quantified in a gas chromatograph (Agilent Technologies 7820A, Shanghai) with a flame ignition detector (FID), using N₂ as a carrier gas, with a heating ramp from 80 °C to 200 °C, with increments of 25 °C/min, with an Agilent J&W DB-FFAP column. The biogas composition was determined using helium as the carrier gas with a gas chromatograph (Gow-Mac 580 GC, U.S. A) with a thermal conductivity detector (TCD). The operating conditions were as follows: $T_{\text{column}} = 40\text{ }^{\circ}\text{C}$, $T_{\text{injector}} =$

90 °C, $T_{\text{detector}} = 90\text{ °C}$, detector current: 120 mA. The DO was quantified with the sensor based on the phenomenon of photoluminescence (WTW™ FDO 925, Germany).

3 Results and discussion

3.1 Characterization of the bioelectrochemical system

Cell potential versus applied current density curves were constructed to determine a current density interval in which the media behaves like an electrical resistance. Electrical resistance behavior allows the operation to ensure that the electrochemical reactions on the surface of the electrodes will not alter the chemical composition of the medium (Gómez-Flores et al., 2020). The resistive behavior was found by applying an interval of 0.54 to 1.63 mA/cm²; the electric field magnitudes applied to the cell were 1.21, 1.45 and 1.64 V/cm.). The voltammogram obtained in a mineral medium containing sucrose and acetate showed neither reduction nor oxidation of any chemical component in the culture medium; only water electrolysis reactions occurred at the anodic and cathodic potential barriers.

3.2 Effect of the electric field on the hydrolysis stage

The consumption profile of sucrose in the control study was consumed at five hours, while the studies with the imposition of 1.21, 1.45, and 1.64 V/cm reached it at approximately 4 hours (Figure 3.1a). The electric field effect was reflected in the hydrolysis efficiencies, which when compared at 3 hours, the control had a hydrolysis efficiency of $68 \pm 1.42\%$. In contrast, in the applied electric fields of 1.21, 1.45, and 1.64 V/cm, the hydrolysis efficiencies obtained were $91 \pm 0.28\%$, $83 \pm 0.20\%$, and $93 \pm 1.18\%$, respectively.

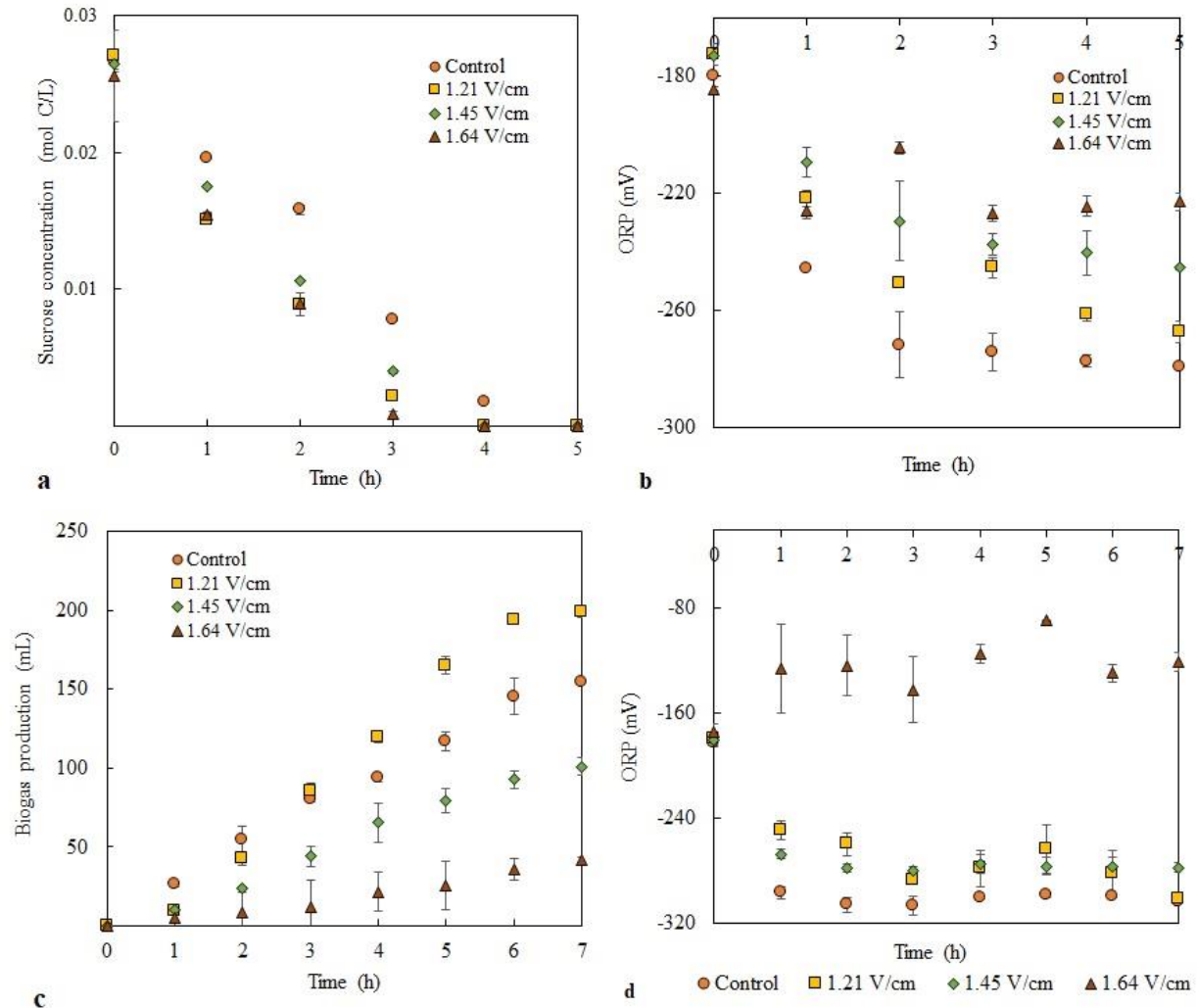


Figure 3.1 Comparison of hydrolysis and methanogenic profiles and ORP behavior at 23 °C in the absence of the electric field (C) and the presence of different electric fields (marked in the figure: 1.21, 1.42, and 1.64 V/cm). a) Sucrose consumption, b) ORP during the hydrolysis, c) biogas production, and d) ORP during methanogenesis.

The kinetic analysis revealed that the reaction constant increased compared to the control by 52, 42, and 72% for electric field magnitudes of 1.21, 1.45, and 1.64 V/cm, respectively (Table 3.1). These results indicated that the characteristic time of the hydrolysis process decreases with increases in the electric field, which could improve the AD's overall performance. Tartakovsky et al. (2011) suggested that microaerobic conditions by water electrolysis might favor the proliferation of facultative bacteria with better hydrolytic activities due to aerobic routes. On the

other hand, Zhang et al. (2021) indicated that microaerobic conditions might increase extracellular enzyme production, speeding up the organic matter hydrolysis.

Table 3.1 Influence of the magnitude of the electric field on the kinetic parameters of AD phases

Stages	Electric field (V/cm)	k (h ⁻¹)	Characteristic time (h)
Hydrolysis	0	0.63 ± 0.00 ^a	1.59 ^a
	1.21	0.97 ± 0.02 ^b	1.03 ^b
	1.45	0.90 ± 0.00 ^c	1.12 ^c
	1.64	1.08 ± 0.01 ^d	0.93 ^d
Methanogenesis	0	0.40 ± 0.01 ^a	2.53 ^a
	1.21	0.55 ± 0.02 ^a	1.83 ^a
	1.45	0.50 ± 0.10 ^a	2.08 ^a
	1.64	0.46 ± 0.04 ^a	2.21 ^a

*The difference between letters indicates that they are significantly different.

In abiotic experiments, three electric field magnitudes were applied to support the previous proposals, reaching similar oxygen saturation concentrations (Figure 3.2 a). However, the production rate increased proportionally to the magnitude of the electric field: 0.0753, 0.0822, and 0.0943 mg O₂/L-min for 1.21, 1.45, and 1.64 V/cm, respectively (Figure 3.2 a-c). In biotic experiments, DO is consumed almost entirely, in which the lowest concentration of DO was obtained at 1.21 V/cm (Figure 3.2 a). The data obtained in the biotic system (previously saturated with O₂) in the absence of the electric field (Figure 3.2 d) showed a maximum oxygen consumption rate of 0.484 ± 0.049 mg O₂/L-min with an affinity constant of 0.877 ± 0.222 mg/L. Based on the above, the oxygen consumption rates were estimated at 0.016 ± 0.002, 0.123 ± 0.020, and 0.154 ± 0.012 mg O₂/L-min for the applied electric fields of 1.21, 1.45, and 1.64 V/cm, respectively. These results show how, from 200 minutes, the DO begins to accumulate in the aqueous stage with 1.45 and 1.64 V/cm since the capability of microorganisms to consume O₂ is overwhelmed (Figure 2b-c). At the same time, when 1.21 V/cm was applied, facultative bacteria maintained a microaerobic environment with a concentration of 0.04 ± 0.01 mg/L (Figure 3.2 a). Therefore, it can be concluded that the electric field decreases the characteristic time for the hydrolysis stage, which is attributed to the fact that the electrochemically generated oxygen favors the metabolism of facultative bacteria.

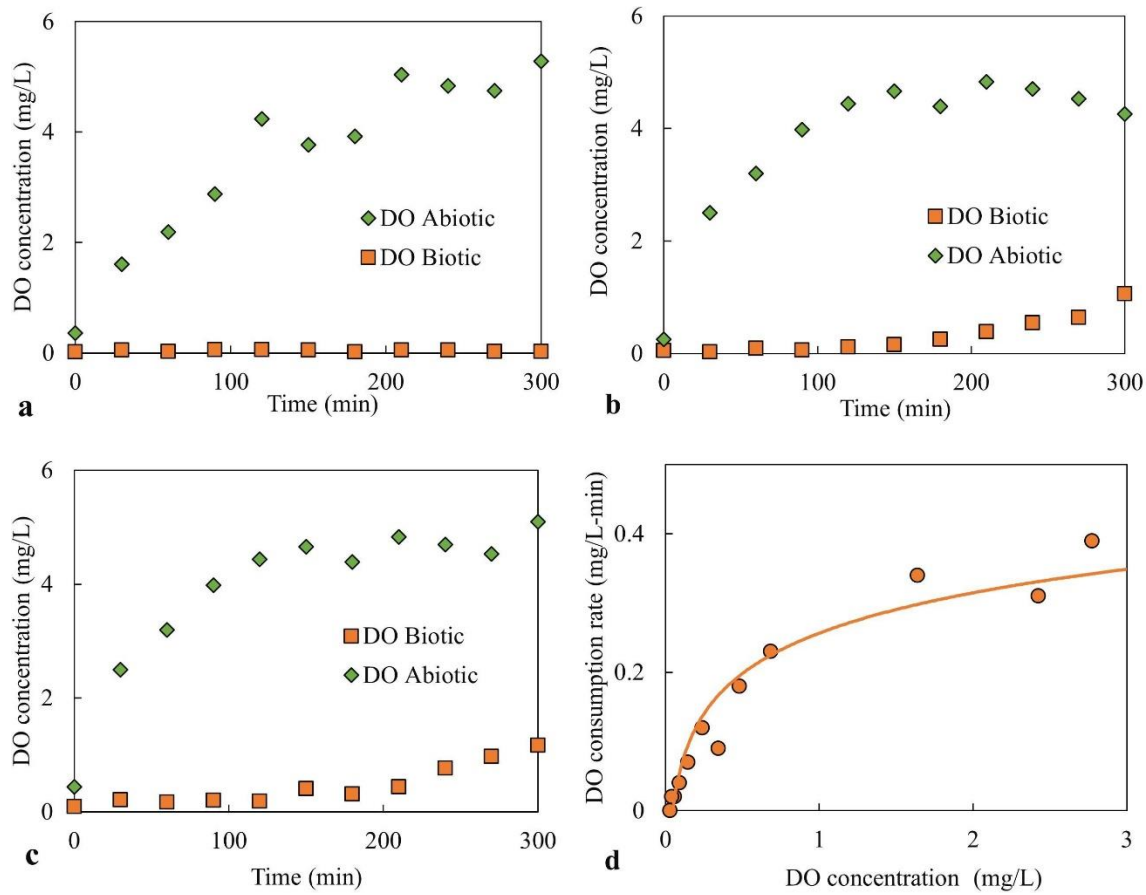


Figure 3.2 Comparison of dissolved oxygen (DO) concentration in the hydrolysis stage at 23 °C by batch cultures in the absence of the electric field (Control) and the presence of different electric fields (marked as: 1.21, 1.42, and 1.64 V/cm) d) kinetic analysis of oxygen consumption, based on the Monod-type model (Continuous line), obtained in the biotic system (previously saturated with O₂) in the absence of an electric field.

On the other hand, in all batch cultures, pH values were within the range of 7.2 to 7.7, an acceptable value for anaerobic digestion. The ORP in the hydrolysis stage displayed less negative values in the presence of the electric field, and this behavior was proportional to the increase in electric field magnitude (Figure 3.1 b). The profiles were stabilized from hour two and remained constant until hour five; the control presented a value of -272 ± 11.3 mV, while the electric field presented values of -251 ± 0.7 , -229 ± 3.5 and -226 ± 2.8 mV for electric field magnitudes of 1.21, 1.45 and 1.64 V/cm, respectively.

3.3 Effect of the electric field on the methanogenesis stage

Batch cultures fed with acetic acid to evaluate methanogenic activity showed that the three tests performed with an electric field present an adaptation stage at one hour with a biogas production of 12.5 ± 2.5 mL (Figure 3.1 c). In contrast, the control showed a higher biogas production in the first hour of $32.5 \text{ mL} \pm 2.1$. Methanogens are the most susceptible to oxygen because they cannot synthesize the superoxide dismutase enzyme that aerobic organisms use to neutralize reactive oxygen species. However, in the presence of O_2 to overcome oxidative stress, some methanogenic species may have intrinsic tolerance to oxygen exposure as they adapt to environmental conditions and can even produce superoxide dismutase, which could explain how these experiments showed this adaptive stage (Magdalena et al., 2022).

On the other hand, the control presented an SMA value of 0.64 ± 0.003 g COD- CH_4 /g VSS-d; this agrees with data reported in the literature (Gonzalez-Tineo et al., 2020). At the same time, assays in the presence of an electric field were 0.89 ± 0.02 , 0.44 ± 0.05 , and 0.13 ± 0.001 g COD- CH_4 /g VSS-d for magnitudes of electric fields of 1.21, 1.45, and 1.64 V/cm, respectively; the batch cultures performed at 1.21 V/cm presented an increase of 39% compared to the control.

Most known methanogenic archaea are mesophilic and have an optimum temperature of 35 °C, diminishing the SMA when temperature is dropped. For example, Kayranli & Ugurlu (2012) using acetate obtained an SMA of 0.74 g COD/g VSS-d at 25 °C, 0.70 g COD/g VSS-d at 15 °C, and 0.68 g COD/g VSS-d at 10 °C, in comparison with the research of Soto et al. (1993), who observed an SMA between 1.078 and 0.975 g COD/g VSS-d at 35 °C. In the present study, the SMA exceeds the value reported at 25 °C, meaning that the electric field enhances this biological process, competing successfully with conventional AD at 35 °C.

Finally, 1.21 and 1.45 V/cm of electric fields did not affect the reducing conditions since the ORP was like the culture control, maintaining values of -277.33 ± 2.1 to -302.5 ± 2.8 mV from hours two to five (Figure 3.1 d). However, at 1.64 V/cm, the ORP was less negative regarding the control, with an average value of -120.08 ± 7.2 from hours two to seven. Nghiem et al. (2014) reported that micro aeration in an anaerobic digester changed the ORP to less negative values, from -485 mV to -270 mV.

4 Conclusions

By conducting water electrolysis caused by a low-magnitude electric field at 23 °C, the outcome of anaerobic digestion was strongly impacted. The micro-aerobic conditions enhanced the hydrolysis rate of sucrose at any electric field, thus improving organic matter removal. The microbial methanogenic activity was positively stimulated at the lowest electric field since acetate consumption was increased with a concomitantly enhanced methane production. These novel findings open an alternative to applying significant development using a controlled low-magnitude electric field to redirect the desired products without temperature control.

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Chapter IV: Enhancing anaerobic digestion metabolism at low temperatures through an electric field application

1 Introduction

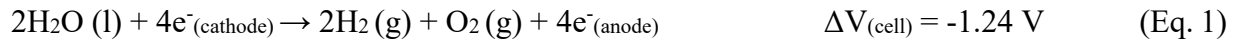
In recent years, anaerobic digestion (AD) has gained attention as a sustainable solution for wastewater treatment, offering multiple benefits over conventional methods. AD emerges as a beacon of hope in wastewater treatment since it efficiently eliminates high organic loads (i.e., 1 to 3 kg COD/m³) and produces 70-80% less sludge than aerobic processes. It significantly decreases energy and operating costs since oxygen is not required. Moreover, it paves the way for the recovery of valuable resources, such as biogas, a blend of 70 % methane (CH₄) and 30 % carbon dioxide (CO₂), which can serve as a renewable energy source (Qadri et al., 2021).

The efficiency of anaerobic digestion is highly sensitive to environmental conditions, with temperature being a particularly crucial parameter. Temperature influences microbial activity and regulates metabolic reaction rates within AD, directly affecting biogas yield and composition. Optimal AD performance is typically achieved within a temperature range of 30-40°C; below this range, microbial activity diminishes due to lower kinetic energy and slows molecular mobility and collision rates. This decline impacts the hydrolysis rate and methane production, potentially leading to system instability (Nie et al., 2021).

When applied to domestic wastewater treatment, AD faces limitations at ambient temperatures, requiring extended hydraulic retention times (HRT) to achieve high COD removal efficiencies. (Y. Chen et al., 2008). Nevertheless, maintaining optimal temperatures in anaerobic reactors is often unfeasible due to the significant costs associated with the heating system. Within this framework, efforts to enhance anaerobic digestion performance have spurred the exploration of diverse strategies, such as co-digestion, substrate pretreatment, two-stage processing, and the application of low-intensity electric fields (Mendoza-Tinoco et al., 2023).

These strategies have been instrumental in increasing biogas production and improving the degradation of complex organic matter. However, the absence of research proposing a strategy to overcome the low-temperature limitation in these processes underscores the need for further exploration in this field. Recent advancements in anaerobic digestion research reveal promising techniques to enhance methane production, even under suboptimal temperature conditions. For instance, a recent study by Mendoza-Tinoco et al. (2024) on the impact of a low-intensity electric

field during the critical stages of anaerobic digestion -hydrolysis and methanogenesis- demonstrates that CH₄ production yields at ambient temperature (23 °C) can challenge those typically achieved at 35 °C. The study showed that water chemical electrolysis, which generates oxygen (O₂) and hydrogen (H₂) (Eq. 1), can modify anaerobic metabolism by enhancing hydrolysis in the presence of O₂ and providing an alternative pathway for CH₄ production. In this pathway, H₂ combines with CO₂ via hydrogenotrophic methanogens to produce CH₄ (Eq. 2) (Amrut Pawar et al., 2020).



Other studies have indicated that electric fields can enhance the performance of anaerobic reactors operating at lower temperatures than typical mesophilic conditions (30 - 37 °C). For example, Yu et al. (2019) reported that, during the treatment of swine manure waste at 25°C with an applied electric field of 0.7 V, similar yields were achieved as in a conventional reactor at 35°C, with hydrogenotrophic methanogens found in higher abundance. Liu et al. (2011) using granular activated carbon electrodes, applying a cell potential of 0.090 V versus an Ag/AgCl_(s) reference electrode at 10 °C, and using acetate as a carbon source increased CH₄ production 6.6-fold compared to the control.

Despite these findings, the influence of H₂ on the quality of the generated biogas and methanogenic metabolism, and especially whether it is possible to increase the methane yield at temperatures below 25 °C, has not been explored. Therefore, this study aims to investigate the effects of applying an electric field on COD removal and biogas production in batch cultures, focusing mainly on the interactions between temperature, methanogenic metabolism, and electrochemical processes.

2 Materials and methods

2.1 Inoculum and growth medium

The anaerobic sludge used in this study was sourced from a wastewater treatment plant at a candy manufacturing facility. The sludge was stabilized in a bench-scale Upflow Anaerobic Sludge Blanket (UASB) bioreactor with a working volume of 2 liters. The reactor was fed with the following chemical medium (in g/L): corn starch (1.5), NH_4Cl (0.85), K_2HPO_4 (0.234), KH_2PO_4 (0.136), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.084), NaHCO_3 (2), and yeast extract (0.34). A trace element solution (0.5 mL/L) was added, and the composition of the trace solution (in g/L) was EDTA tetrasodium salt (0.5), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.25), MnCl_2 (0.6), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.2), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2), MgCl_2 (0.5), FeSO_4 (0.1), and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1).

The UASB reactor was operated until it reached the stationary phase, defined as the point at which the COD removal efficiency exhibited a variation coefficient lower than 15%. Once this state was achieved, the anaerobic sludge was used to evaluate the batch cultures.

2.2 Evaluation of the effect of electric field on performance at different operating temperatures

Batch cultures were conducted in glass electrochemical reactors (two-electrode cells), using titanium plates coated with ruthenium oxide (RuO_2/Ti) as both the anode and cathode, whose dimensions were 7.5 cm long, 0.6 cm wide, and 0.1 cm thick; the submerged area of the electrodes was 9.16 cm^2 , and the separation between electrodes was 2 cm. The operating volume was 130 mL of liquid and 10 mL of headspace, inoculated with 35 mL of sludge previously acclimated in the UASB, equivalent to 2 g VSS/L. Anaerobic sludge was washed three times with phosphate buffer to remove any remaining carbon source. It was fed with the mineral medium described in section 1.1 and used corn starch at a concentration of 0.8 g COD/L as a carbon source, resulting in a 0.4 g COD/g VSS ratio. Once inoculated into the batch cultures, the reactors were bubbled with N_2 for 3 minutes to ensure an oxygen-free environment.

Three temperatures were evaluated (i.e., 10, 18, and 35 °C) to compare the average temperature of wastewater with the optimal temperature for anaerobic digestion. This selection allows for analyzing how process efficiency varies at temperatures below, near, and within the optimal range,

applying an electric field of 1.21 V/cm. According to results from a previous study, this field magnitude can modify anaerobic metabolism without inhibiting it (Mendoza-Tinoco et al., 2024). A high-precision power supply (BK PRECISIONS Model 9130A, Taiwan) was used to apply an electric current. A control assay in the absence of the electric field was included for each experiment, and a blank was performed to rule out biogas production due to any residual corn starch in the anaerobic sludge.

Soluble COD consumption, biogas production, and composition were monitored over time. Fresh effluent samples were collected every hour, centrifuged at 4000 rpm for 5 minutes, and then filtered using a 2.5 μm filter to determine the soluble COD. The volume of CH_4 produced was quantified using inverted bottles containing a 1 N NaOH solution. The CO_2 from the biogas is converted to carbonate and absorbed into the solution so that only the CH_4 gas passes through the solution and the volume equivalent is expelled through the bottle; the volume is proportional to the CH_4 gas that enters the bottle and is collected in a graduated cylinder. A neutral saline solution was used for total gas quantification.

2.3 Analytical methods

In batch cultures, the chemical oxygen demand (COD) was quantified using the closed reflux method, as described in the standard methods. (APHA/AWWA/WEF, 2012), with a spectrophotometer (Thermo Scientific GENESYS 20, USA). The biogas composition was determined using a gas chromatograph (Gow-Mac 580 GC, USA) equipped with a thermal conductivity detector (TCD). For the determination of H_2 , an Alltech Silica gel column 60/80 was used, with nitrogen as the carrier gas, under the following operating conditions: column temperature = 90°C , injector temperature = 110°C , detector temperature = 100°C , and detector current = 80 mA. To determine CH_4 and CO_2 , a Carbosphere 80/100 column was used with helium as the carrier gas under the following operating conditions: column temperature = 40°C , injector temperature = 90°C , detector temperature = 90°C , and detector current = 120 mA. The calibration curve was prepared using CO_2 , CH_4 , and H_2 standards.

2.4 Determination of specific methanogenic activity (SMA) and methane production yield

The specific methanogenic activity of the sludge is defined as the amount of substrate converted to methane per unit mass of sludge per unit time. It is expressed as g COD- CH_4 /g VSS-d. Once the

volume of methane production in milliliters (V_{CH_4}) was obtained, it was converted to grams of COD- CH_4 using a stoichiometric conversion factor. This factor states that four grams of COD are required for every gram of CH_4 produced (Eq. 3).

$$gCOD - CH_4 = V_{CH_4} * \frac{1 g CH_4}{4 g COD} * \text{density of } CH_4 \quad (\text{Eq. 3})$$

A graph was then constructed showing the cumulative volume of methane in g COD- CH_4 versus the time expressed hours. A period was established during which approximately 50% of the expected methane was generated from the added substrate. The slope of the regression line for this segment represents the maximum methane production rate (R_{CH_4}), expressed in grams of COD- CH_4 per hour (g COD- CH_4 /h). Once the value of R_{CH_4} was determined, the SMA was calculated using the following equation:

$$SMA = \frac{R_{CH_4} * 24}{M_{VSS} * V_S} \quad (\text{Eq.4})$$

Where:

R_{CH_4} = maximum methane production rate (g COD- CH_4 /h).

24 = Conversion factor from hours to days

M_{VSS} = Concentration of sludge used in the experiment (g VSS/L)

V_S = Volume of sludge used in the experiment (L)

The yield of production of CH_4 , is used to estimate the energy potential of wastewater, i.e., the CH_4 production per g of COD removed and was calculated using the following equation:

$$Y = \frac{V_{CH_4}}{Q_{COD} * V_{reactor}} \quad (\text{Eq. 5})$$

Where:

Y = Yield of CH_4 production (ml CH_4 /g COD removed)

V_{CH_4} = Accumulated CH_4 (mL)

Q_{COD} = COD removed (g)

$V_{reactor}$ = Liquid volume (L)

The data obtained were normalized using the ideal gas equation (Eq. 6). By assuming a pressure of 1 atm and a temperature of 0 °C (273.15 K), the volumes of methane produced were adjusted to

reflect what they would be under these standard conditions. This normalization allows the results to be comparable with those of other studies in the literature.

$$V_2 = \frac{P_1}{P_2} * \frac{T_1}{T_2} * V_1 \quad (\text{Eq. 6})$$

Where:

V_2 = Normalized yield (ml CH₄/g COD removed)

V_1 = Experimental yield (ml CH₄/g COD removed)

P_1 =Atmospheric pressure of Mexico City (0.769 atm)

P_2 = Standard pressure (1 atm)

T_1 = Experimental temperatures (°K)

T_2 = Standard temperature (273.15 °K)

2.5 Statistical analysis

A single-factor analysis of variance (ANOVA) was performed to compare the experimental results obtained in the presence and absence of an electric field. The significance value (α) was 5% using the data analysis add-in of Microsoft Excel software. The initial hypothesis was rejected by comparing the value of F and the critical value of F. A Tukey–Kramer post hoc test was performed. The analysis began by determining the mean absolute difference between each group based on the means obtained in the ANOVA. Subsequently, the critical value of Q is determined using the following equation:

$$Q_{critical\ value} = Q \sqrt{\frac{s^2}{n}} \quad (\text{Eq. 7})$$

where:

- Q = Value of Studentized Range Distribution (Q)
- s^2 grouped = Pooled variance in all groups
- n = Sample size for a given group

To find the value of Q in the table, the degrees of freedom are necessary, and they are calculated as $n-K$, where K represents the number of groups. Finally, the mean absolute difference between

each group is compared with the critical value of Q . If the mean absolute difference is more significant than Q 's, then the difference between the group means is significant.

3 Results and discussion

The anaerobic digestion process was initially analyzed in terms of COD removal efficiency and COD consumption rate under varying temperatures in the presence of a fixed electric field. The results revealed that applying an electric field led to improvements in COD removal efficiency of 6%, 19%, and 10% at temperatures of 10 °C, 18 °C, and 35 °C, respectively, compared to the controls (Table 3.1). Notably, at 18 °C with the electric field, the COD removal efficiency reached $89 \pm 1\%$. In contrast, achieving this same efficiency in the control setup required a temperature of 35°C, underscoring the significant influence of the electric field on the anaerobic process. On the other hand, the kinetic profile was also affected, with more notable improvements being observed at 18 °C and 35 °C. At these temperatures, the application of the electric field enhanced the volumetric COD consumption rate by 1.67-fold and 1.14-fold, respectively, compared to the controls. These initial studies provided clear evidence of the significant impact of the electric field in enhancing anaerobic digestion, even at low temperatures.

Table 3.1. Comparison of the soluble COD consumption rate in the absence (control) and presence of an electric field (1.21 V/cm) at temperatures of 10, 18, and 35°C.

Temperature (°C)	COD removal efficiency (%)		COD consumption rate (mg/L-h)	
	Control	1.21 V/cm	Control	1.21 V/cm
10	57 ± 0.15^a	63 ± 7^a	188.35 ± 12^a	177.8 ± 29^a
18	70 ± 3^a	89 ± 1^b	170.5 ± 39^a	275 ± 18^b
35	89 ± 2^b	94 ± 4^b	341.5 ± 28^b	391.5 ± 21^b

*The difference between letters indicates that they are significantly different.

A clear correlation is observed between temperature, COD removal efficiency, and biogas production (Table 3.1; Figure 3.1). In the control cultures, the enhancement of anaerobic digestion with increasing temperature is well-documented, for example, below 35°C, the metabolic flexibility of anaerobic bacteria allows them to adapt through modifications in DNA, RNA, cell membranes, and protein types (Nie et al., 2021). However, despite this adaptability, a prolonged hydraulic retention time (HRT) is necessary to achieve high COD removal efficiency and compensate for the lowered metabolic activity (Chen et al., 2008).

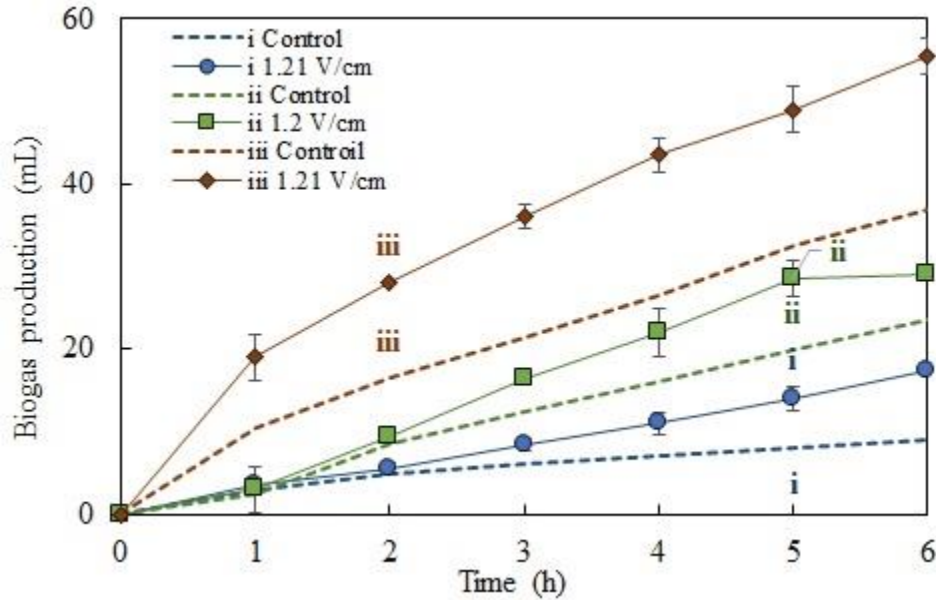
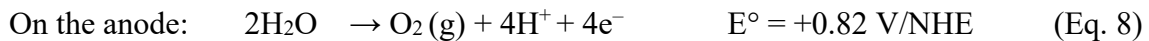


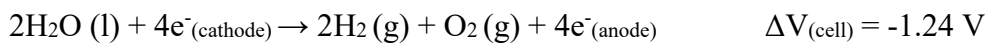
Figure 3.1 Biogas production profiles, in the absence (control, discontinuous lines) and presence of the electric field, continuous lines) (1.21 V/cm) at temperatures (i) 10, (ii) 18, and (iii) 35 °C.

In terms of biogas composition, all control cultures predominantly produced CH_4 and CO_2 at average percentages of $79 \pm 1.5\%$ and $20.6 \pm 1.5\%$, respectively. However, when an electric field was applied at different temperatures, the biogas composition shifted, resulting in the emergence of a third component, H_2 , at approximately 2% (Figure 3.2). This evidence rules out the possibility that anaerobic microorganisms can fully adapt to low temperatures within short culture times. Therefore, the observed improvement in anaerobic digestion can be attributed primarily to the influence of the electric field since it causes the electrolysis of water, producing O_2 by oxidizing the water at the anode (Eq. 8) and H_2 by reducing water at the cathode (Eq. 9).



Where NHE represents Normal Hydrogen Electrode

The cell reaction is given as follows:



(Eq.10)

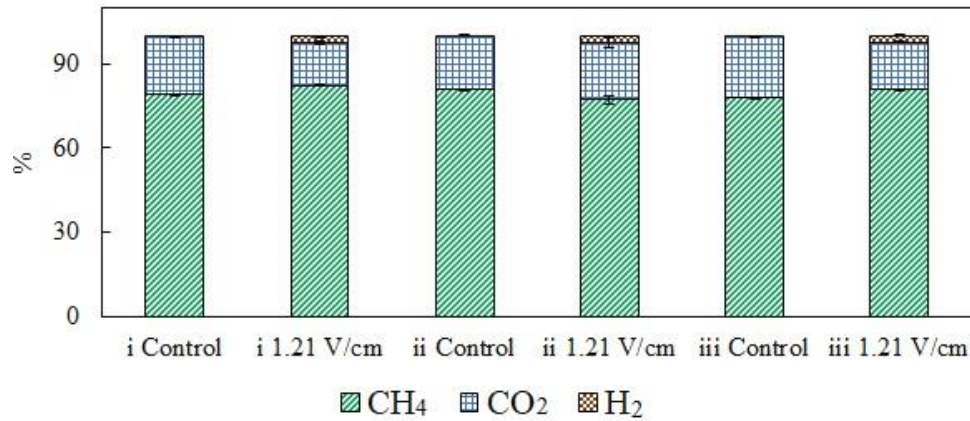


Figure 3.2. Comparison of the biogas composition (%) produced in the absence of the electric field (Control) and the presence of the electric field (1.21 V/cm) at temperatures of (i) 10, (ii) 18, and (iii) 35 °C

The O₂ and H₂ produced electrochemically are the key in this biological behavior, since these products might influence the metabolism of anaerobic digestion in different ways. On one hand, facultative anaerobic bacteria may consume O₂, thereby enhancing the secretion of hydrolytic enzymes that facilitate the degradation of organic matter. (Mendoza-Tinoco et al., 2024; Zhang et al., 2021). On the other hand, the observed increase in biogas production could be influenced by H₂ generated electrochemically, which may have been utilized through hydrogenotrophic activity or supported microbial community proliferation. The latter is highly plausible, as hydrogenotrophic methanogenesis has been shown to exhibit doubling times as short as 2 hours (Richards et al., 2016). Future studies focusing on characterizing microbial structure and abundance could provide valuable insights to support this hypothesis.

The hydrogenotrophic activity in anaerobic sludge is also further supported by the findings of Nozhevnikova et al. (2007), who demonstrated that CH₄ production increases at low temperatures (< 25°C) when substrates like acetate and H₂ are provided. It is very clear that substrate addition enhances hydrogenotrophic activity, suggesting that the limitation of metabolic activity depends on H₂ availability. For example, the solubility of H₂ at 20 °C and 1 atm is approximately 1.6 mg/L, which inherently limits hydrogenotrophic activity. However, electrochemically produced H₂, encapsulated in fine microbubbles, significantly increases the surface area, enhancing the transfer of H₂ from the gas phase to the liquid phase (Fu et al., 2022).. This improved transfer boosts its

availability to hydrogenotrophic methanogens, thereby supporting their metabolic activity more effectively.

The combined effect of low temperatures and an applied electric field impacted methanogenic activity. In the batch cultures conducted at 10 °C, 18 °C and 35 °C under the application of an electric field, the specific methanogenic activity significantly improved compared to the control (Figure 3.3a). This improvement in the AD was also evident in the methane production (Figure 3.3b), where clearly demonstrates how the application of an electric field enhanced the methane yield at all evaluated temperatures. It suggested the utilization of an additional energy source to enhance methane generation. This observation further supports the results indicating that H₂ production via electrochemical reactions serves as the secondary energy source, as previously discussed. These results are highly promising, as they indicate the ability to develop advanced biotechnologies capable of functioning effectively in climates below 20°C while ensuring high COD removal efficiencies and significant methane production yields.

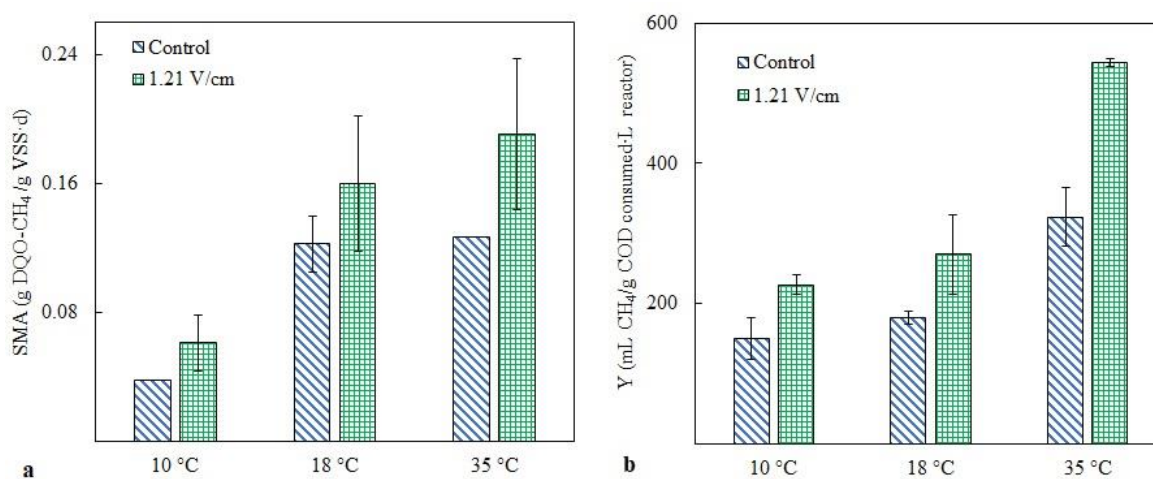


Figure 3.3. Comparison of a) SMA and b) methane production yield in the absence (control) and presence of the electric field (1.21 V/cm) at temperatures of 10, 18, 26, and 35 °C.

In this context, to verify if the anaerobic sludge has the capability to use the H₂ produced electrochemically, an independent experiment was performed at 18°C, where 1.21 V/cm was applied using a mineral medium without adding a carbon source. This experiment aimed to quantify the biogas production resulting from the hydrogenotrophic pathway, based on the idea that the electrochemically produced H₂ could be used to produce CH₄ using only the CO₂ provided

by the added sodium bicarbonate. Parallel to these studies, a control experiment was conducted using the same medium without applying an electric field. The results revealed that biogas production was minimal in the control study, likely due to the residual organic matter (Figure 3.4). In contrast, the batch culture with the application of an electric field demonstrated a 2.6-fold increase in biogas production (blend of 95% CH₄ and 5% H₂) compared to the control. These results provide clear evidence that the anaerobic consortium exhibited hydrogenotrophic metabolic activity, enabling methane production through the consumption of H₂ and CO₂.

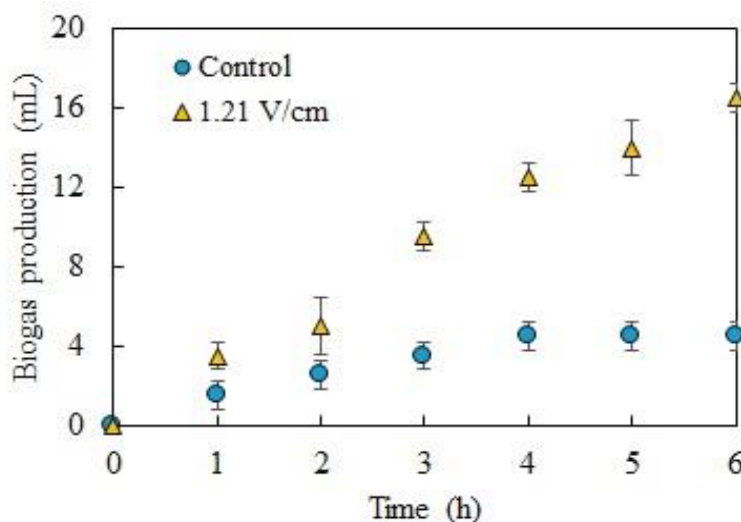


Figure 3.4. Biogas production for an anaerobic sludge in a mineral medium without adding a carbon source at 18 °C, in the absence (control) and applying a low-magnitude electric field (1.21 V/cm).

A stoichiometric analysis was conducted to verify whether the CH₄ produced corresponded to the H₂ generated electrochemically. It was calculated that 0.0020 moles of H₂ would be required to produce the 0.00052 moles of CH₄ obtained under the influence of the electric field. However, based on Faraday's first law, the applied electric field could generate only 0.00056 moles of H₂. This stoichiometric discrepancy indicates that the observed increase in methane yield cannot be solely explained by H₂ production from water electrolysis. Instead, it suggests that the electric field may enhance biological H₂ production. Further investigation is required to elucidate the underlying reaction mechanisms.

The findings of this research demonstrate that applying a low-magnitude electric field can positively alter anaerobic metabolism at low temperatures, enhancing COD removal and methane production. This approach enables low-temperature processes to achieve yields comparable to those of conventional, temperature-controlled systems, positioning it as a competitive and potentially more sustainable alternative for wastewater treatment and biogas production in cold climates.

4 Conclusions

This study demonstrated that applying an electric field of 1.21 V/cm significantly enhanced COD removal efficiency in batch cultures at 18 °C. Additionally, the electric field influenced the composition and production of biogas, resulting in the detection of 2% H₂, increasing the biogas's calorific value. The findings suggest that water electrolysis, producing both O₂ and H₂, was vital in improving methane production yields. It achieved comparable results to those observed in batch cultures at 35°C without the electric field. Specifically, O₂ contributed to the degradation of organic matter, while H₂ supported methane production through the hydrogenotrophic pathway.

Although the electrochemical generation of H₂ at lower temperatures was not the sole factor responsible for the increased methane yields, it created reducing conditions that enhanced biological H₂ production. This interaction underscores the potential of integrating electrochemical processes to optimize biogas production, particularly in low-temperature environments.

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Chapter V: Enhanced Hythane production in a UASB reactor coupled with an electrochemical system for domestic wastewater treatment at room temperature

1 Introduction

Anaerobic digestion is a promising technology for wastewater treatment, in which complex organic matter is degraded and transformed into value-added products such as biogas and biofertilizers (Mainardis et al., 2020). Upflow anaerobic sludge blanket reactor (UASB) is one of the most widely used systems in the world for wastewater treatment, as it promotes the development of granular sludge, which allows the retention of highly active biomass, with excellent sedimentation capabilities facilitating the separation of sludge and effluent. The UASB configuration promotes efficient mixing of biomass and wastewater, allowing the microbial consortium in the granular sludge to remove pollutants, while the generated biogas aids in the mixing process (Chong et al., 2012).

The advanced wastewater treatment systems aim not only to improve contaminant removal efficiency but also to generate valuable by-products like biogas. Among these, Hythane, a mixture of hydrogen (H_2) and methane (CH_4) (10%-30% H_2 and 70%-90% CH_4) has gained attention due to its environmental benefits. By integrating technologies that optimize its production, it can not only take advantage of wastewater to produce energy but also significantly contribute to decrease harmful emissions, such as carbon dioxide (CO_2) and nitrogen oxides (NO_x), aligning with global sustainability goals (Lay et al., 2020).

Several research groups have focused on utilizing anaerobic digestion metabolisms to enhance H_2 production in this scientific field. For instance, the two-stage anaerobic digestion model provides the option for rate maximization by applying different operating conditions for hydrolysis/acidogenesis and methanogenesis. In the first reactor, the fermentative stages are carried out without strict temperature conditions, while in the second reactor, temperatures above 35 °C are applied to enhance methanogenic activity. This approach allows H_2 and CH_4 production from wastewater, achieving significantly higher energy yield than the single-stage UASB reactor (Rajendran et al., 2020). Intanoo et al. (2016) developed a two-stage AD process using UASB reactor to produce hydrogen and methane from wastewater. Results showed a hydrogen production yield of 39.83 l H_2 /kg chemical oxygen demand (COD) removal at a COD loading rate of 25 kg/m³ d, increasing more than 80% methane production compared to the 50-75% production rate from a single-stage anaerobic bioreactor. While the research achievements in methane production in two

stages clearly show a lot of promise, an economic feasibility assessment must be performed to compare the additional cost of a two-stage arrangement (Mao et al., 2015).

Due to the importance of Hythane, researchers have explored different strategies to increase H_2 production, including applying low-magnitude electric fields (LMEF) to stimulate microbial activity and boost hydrogen generation. LMEF has been shown to increase CH_4 production under mesophilic conditions. For example, Zhen et al. (2017) enhanced the biodegradation of methanolic wastewater at 35 °C by coupling a microbial electrolysis cell with a UASB reactor, achieving a 10% higher CH_4 yield compared to the UASB control. Similarly, Tartakovsky et al. (2011) reported that applying an LMEF in a UASB reactor at 35 °C led to a 10-25% increase in global methane yield compared to the control. This latest study highlights that water electrolysis was a key factor in increasing CH_4 production. In this address, Mendoza-Tinoco et al. (2024) suggested that electrochemically produced O_2 might have created microaerobic conditions enhancing organic matter degradation at low temperature.

On the other hand, H_2 production can intervene in different stages of anaerobic digestion, influencing the production of volatile fatty acids (VFAs), especially the generation of acetate through the homoacetogenic pathway and the production of CH_4 through the hydrogenotrophic pathway (Anderson Ken et al., 2003). In addition, some authors suggest that electrochemically generated H_2 could favor the development of hydrogenotrophic microorganisms (Koch et al., 2015).

The present study aims to scale up the process to laboratory level, imposing an LMEF in a UASB reactor operated at room temperature to treat domestic wastewater and obtain Hythane. In addition, it seeks to determine possible modifications in the physiology of the granular sludge.

2 Materials and methods

2.1 Reactor configuration, inoculum, and culture medium

Two bench-scale UASB reactors were utilized as experimental units. One reactor (RC) was not equipped with electrodes and served as the control. The second reactor (RE) had electrodes installed in the sludge blanket zone to evaluate the influence of the electric field on anaerobic digestion. Both reactors had an operational volume of two liters, with the following dimensions: an internal radius of 0.03 m, a wall thickness of 0.003 m, and a height of 0.645 m.

The anaerobic sludge utilized in this study was obtained from a wastewater treatment facility associated with a candy manufacturing plant. At the onset of the experiment, the sludge exhibited a volatile suspended solids (VSS) concentration of 56.7 g/L.

The reactors were fed at a rate of 2.5 mL/min using a peristaltic pump (Marlow 114 DV), with a hydraulic retention time (HRT) of 12 hours and a volumetric organic loading rate of 3.6 kg of chemical oxygen demand concentration (COD) per cubic meter per day. The initial pH was set at 7, and both reactors were maintained at 25 ± 3 °C throughout the operation. Anaerobic sludge with a concentration of 38.61 g/L of volatile suspended solids was used as inoculation in each UASB reactor, occupying one third of the liquid volume.

The biological reactors were fed municipal wastewater from the Cerro de la Estrella treatment plant in Iztapalapa, CDMX. The physicochemical characteristics of the wastewater are outlined in Table 1.1. The wastewater was supplemented with carbohydrates to achieve a COD concentration of 2 ± 0.1 g/L. Two carbon sources were utilized: sucrose, which was employed to stabilize the anaerobic sludge, and corn starch, which was introduced during the application of the electric field to assess its impact on the biological process. Additionally, 2 g/L of sodium bicarbonate was added to enhance the system's alkalinity, ensuring optimal conditions for microbial activity and process stability.

Table 1.1 Physicochemical characterization of domestic wastewater from the Cerro de la Estrella treatment plant.

Parameter	Value
COD Total (mg/L)	281 ± 67
COD Soluble (mg/L)	161 ± 14
pH	7.6 ± 0.2
Salinity (IOT scale)	0.4 ± 00
Conductivity (μS/cm)	962 ± 44
Specific resistance (Ω • cm)	1043 ± 46
TDS (g/L)	982± 60

To assess the stability of the anaerobic sludge, parameters such as COD removal efficiency, alkalinity (α index), and biogas production were continuously monitored. Fresh effluent samples were collected, centrifuged at 4000 rpm for 5 minutes, and filtered through a 2.5 μm filter to determine the soluble COD concentration. Alkalinity was measured by acidifying 100 mL of effluent samples with a 0.1 N H₂SO₄ solution by titration to pH values of 5.75 and 4.3. The volume of acid used to reach each pH was recorded, and the α index was calculated according to Jenkins et al. (1983). Biogas production was monitored using continuous flow measurement chambers.

2.2 Electrochemical system

The electrochemical system consisted of four titanium mesh electrodes coated with ruthenium oxide (RuO₂/Ti), each with an active surface area of 95.62 cm². These electrodes were strategically positioned within the sludge blanket zone to ensure direct contact with the biomass, thereby enhancing electron transfer. The system was powered by a high-precision power supply unit (BK PRECISION Model 9120A, Taiwan), which regulated the applied current with exceptional accuracy (Figure 2.1).

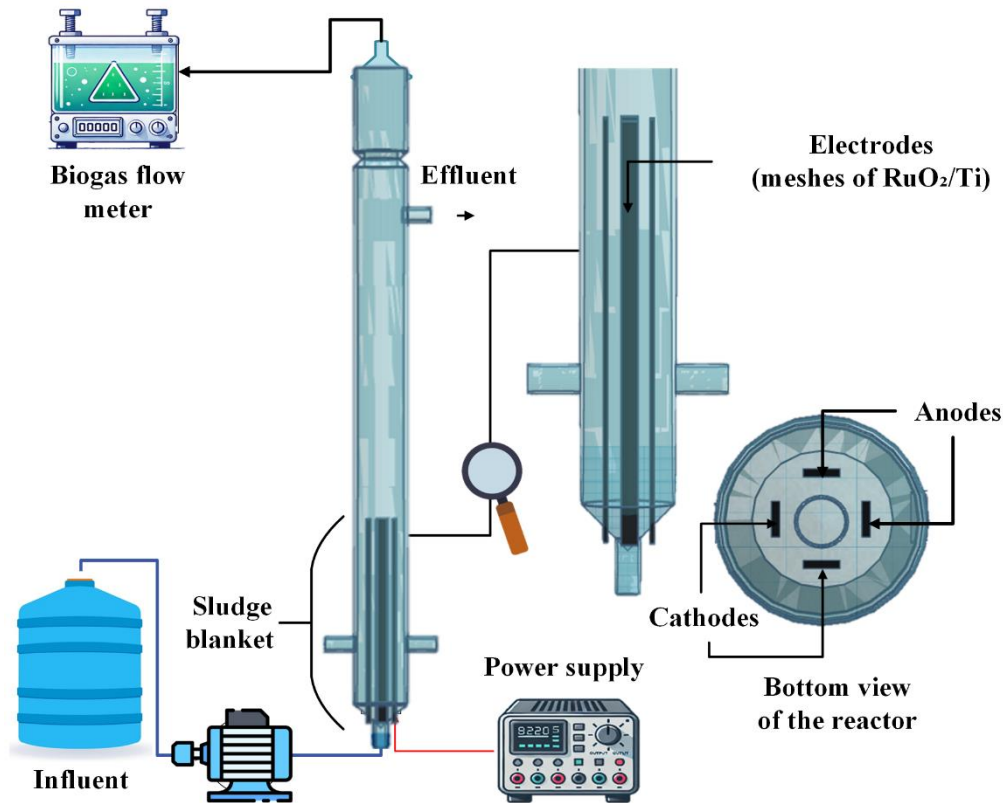


Figure 2.1 Schematic diagram of UASB reactor configured with four titanium mesh electrodes coated with ruthenium oxide.

The applied electric current density was selected based on a previous study conducted with batch cultures, which demonstrated optimal performance at 0.54 mA/cm^2 , this generated an electric field strength of 1.28 V/cm (Mendoza-Tinoco et al., 2024). To scale this current density for the present system, the current density used in batch cultures (0.54 mA/cm^2) and the current surface area of the electrodes were considered, resulting in an applied current of 50 mA . The magnitude of the field was determined by dividing the cell potential (2.56 V) for the applied current density by the distance between the electrodes (2 cm), yielding the final value in V/cm .

2.3 Physicochemical characterization of anaerobic granular sludge

A comprehensive physicochemical characterization of the anaerobic granular sludge was performed during the pseudo-steady state. This included the determination of specific methanogenic activity (SMA), sludge volumetric index (SVI), sedimentation velocity, VSS, exopolymetric substances (EPS), granulometric analysis, and scanning electron microscopy (SEM). SMA tests were conducted in 100 mL serological bottles inoculated with 2 g VSS/L , using glucose

and acetate as carbon sources. The initial pH was adjusted to 7, and the assays were maintained at 30°C. Methane production was quantified using inverted bottles filled with a 1 N NaOH solution to capture and measure the volume of CH₄ produced.

The SVI and sedimentation velocity were determined using a 35 mL sample of concentrated sludge. The sample was washed thrice with a phosphate buffer solution before being transferred to a 100 mL graduated cylinder. The volume was then brought to 100 mL with distilled water. After gently mixing, the sludge was allowed to settle for 30 minutes. The settled sludge volume was recorded every 15 seconds during the first 2 minutes, every minute until 15 minutes, and every 5 minutes after that. Calculations were performed using the following equations.

$$SVI = \frac{\text{volume of sludge settled in 30 min (mL)}}{TSS \left(\frac{g}{L} \right) * \text{initial volume on graduated cylinder (L)}} \quad (\text{Eq. 1})$$

$$SV = \frac{\text{rate of sedimentation} \left(\frac{m}{h} \right)}{\text{area of graduate cylinder (m}^2\text{)}} \quad (\text{Eq. 2})$$

The total, fixed, and volatile solids in the granular sludge were determined using a 40 mL sample of concentrated and homogenized sludge, which was processed according to the standard gravimetric method (FAPHA/AWWA/WEF, 2012). A 250 mL sludge sample was homogenized for granulometry analysis, and the SSV was determined in advance. A 30-mesh sieve (0.6 cm) was placed in a basin with enough water to cover the mesh without exceeding its edge. The sludge sample (250 mL) was then poured into the sieve, submerged, and gently agitated to separate the solids. The granular sludge retained on the sieve was transferred to a 1 L volumetric flask, and VSS1 was measured.

Subsequently, the process was repeated using a 40-mesh sieve (0.4 cm) with the water from the first flask to obtain VSS2. The remaining water containing the smaller flocs was collected and analyzed to determine VSS3. The corresponding calculations were carried out using the following specific equations.

$$\text{Mass of 0.6 cm granule (M0.6)} = VSS1 * 1L \quad (\text{Eq. 3})$$

$$\text{Mass of 0.4 cm granule (M0.4)} = VSS2 * 1L \quad (\text{Eq. 4})$$

$$\text{Mass of floc (Mf)} = VSS3 * 1L \quad (\text{Eq. 5})$$

$$Total\ mass = M_{0.6} + M_{0.4} + M_f \quad (\text{Eq. 6})$$

To extract EPS from granular sludge homogenized, 2 mL samples by duplicate were placed in Falcon tubes and filled with distilled water to a final volume of 10 mL. The samples were then centrifuged at 3000 rpm for 25 minutes. After centrifugation, the supernatant was carefully separated and filtered through 1.2 μm filters. The remaining pellet was resuspended in distilled water to restore the initial volume and homogenized using a shaker at 5000 rpm. The samples were heated in a water bath at 80°C for 10 minutes. Once cooled, the supernatant was filtered again to complete the extraction process.

2.4 Analytical methods

Chemical oxygen demand was quantified using the closed reflux method, whereas VSS, SVI, sedimentation velocity and granulometric were measured—according to standard methods (APHA/AWWA/WEF, 2012). The ratio of volatile fatty acids alkalinity to total alkalinity was assessed using the α index, as described by Jenkins et al. (1983). Salinity, conductivity, total dissolved solids, and specific resistance were measured with a TATRACON 925 electrode (Germany), connected to a WTW™ Multi 3034 multiparameter meter (Germany). pH levels were determined with an Orion Star™ A215 multiparameter device (Indonesia).

The biogas composition was analyzed using a gas chromatograph (Gow-Mac 580 GC, USA) with a thermal conductivity detector (TCD). For-H₂ analysis, an Alltech Silica gel column (60/80) was used with nitrogen as carrier gas, under the following conditions: column temperature = 90 °C, injector temperature = 110 °C, detector temperature = 100 °C, and a detector current: 80 mA. Methane and carbon dioxide were measured using a Carbosphere 80/100 column with helium as the carrier gas, under these conditions: column temperature = 40°C, injector temperature = 90°C, detector temperature = 90°C, and a detector current of 120 mA. Calibration curves were prepared using standard CO₂, CH₄, and H₂ gases.

The content of EPS was determined in two fractions: EPS bound to the granular structure and soluble EPS, following the method outlined by Le-Le-Clech et al. (2006). EPS concentrations were measured based on protein and carbohydrate content using the Lowry et al. (1951) and Dubois et al. (1956) methods, respectively. Additionally, the granular sludge was analyzed using a scanning

electron microscope (JEOL JSM-5900 LV, Japan), with sample preparation conducted as described by Murtey & Ramasamy (2021).

2.5 Calculation of specific methanogenic activity (SMA) and biochemical methane potential (BMP)

The specific methanogenic activity of the sludge is defined as the amount of substrate converted to methane per unit mass of sludge per unit time. It is expressed as g COD-CH₄/g VSS-d. Once the volume of methane production in milliliters (V_{CH₄}) was obtained, it was converted to grams of COD-CH₄ using a stoichiometric conversion factor. This factor states that four grams of COD are required for every gram of CH₄ produced (Eq. 7).

$$g\text{COD} - \text{CH}_4 = V_{\text{CH}_4} * \frac{1 \text{ g CH}_4}{4 \text{ g COD}} * \text{density of CH}_4 \quad (\text{Eq. 7})$$

A graph was then constructed showing the cumulative volume of methane in g COD-CH₄ versus the time expressed hours. A period was established during which approximately 50% of the expected methane was generated from the added substrate. The slope of the regression line for this segment represents the maximum methane production rate (R_{CH₄}), expressed in grams of COD-CH₄ per hour (g COD-CH₄/h). Once the value of R_{CH₄} was determined, the SMA was calculated using the following equation:

$$SMA = \frac{R_{\text{CH}_4} * 24}{M_{\text{VSS}} * V_S} \quad (\text{Eq.8})$$

Where:

R_{CH₄}= maximum methane production rate (g COD-CH₄/h).

24 = Conversion factor from hours to days

M_{VSS}= Concentration of sludge used in the experiment (g VSS/L)

V_S= Volume of sludge used in the experiment (L)

The biochemical methane potential (BMP) is used to estimate the energy potential of wastewater, i.e., the CH₄ production per g of COD removed and was calculated using the following equation:

$$BMP = \frac{Q_{\text{CH}_4} * \text{HRT} * 1000}{Q_{\text{COD}} * V_R} \quad (\text{Eq. 9})$$

Where:

Q_{CH_4} = Methane production rate (L/d)

HRT= Hydraulic Retention Time (d)

1000= Conversion factor from liter to milliliters

Q_{COD} = COD removed (g/L)

V_R = Reactor volume (L)

The data obtained were normalized using the ideal gas equation, considering a 1 atm and zero °C pressure.

2.6 Statistical analysis

A t-student analysis for two independent samples was performed using the data analysis add-in of Microsoft Excel software to compare the experimental results obtained in the presence and absence of an electric field. The significance level (α) was 5 %, and the hypothesized mean difference was zero. The difference between means is statistically significant if the p-value is less than the significance value.

3 Results and Discussion

3.1 Stabilization of anaerobic sludge in the absence of electric field

To better understand the operational phases and performance of the reactors, initially the study was divided into two distinct stages, each characterized by different wastewater compositions and supplementation. The main goal was to evaluate the reactors' response to varying carbon sources, as well as to monitor the transition from one steady state to another. Sludge stabilization was carried out in two stages to minimize the potential stress associated with changes in feed composition. A sudden shift in substrate characteristics could disrupt the biological process, leading to imbalances in degradation and possible inhibition of microbial activity (Khanal, 2009). The conditions and key observations during these stages are outlined below.

In the first stage, which spanned from day 1 to day 11, municipal wastewater supplemented with sucrose was used. During this stage, both reactors reached a pseudo-steady state on day 6. In the second stage, starting on day 12, both reactors were fed with domestic wastewater supplemented with corn starch, reaching a pseudo-steady state on day 20 (Figure 3.1).

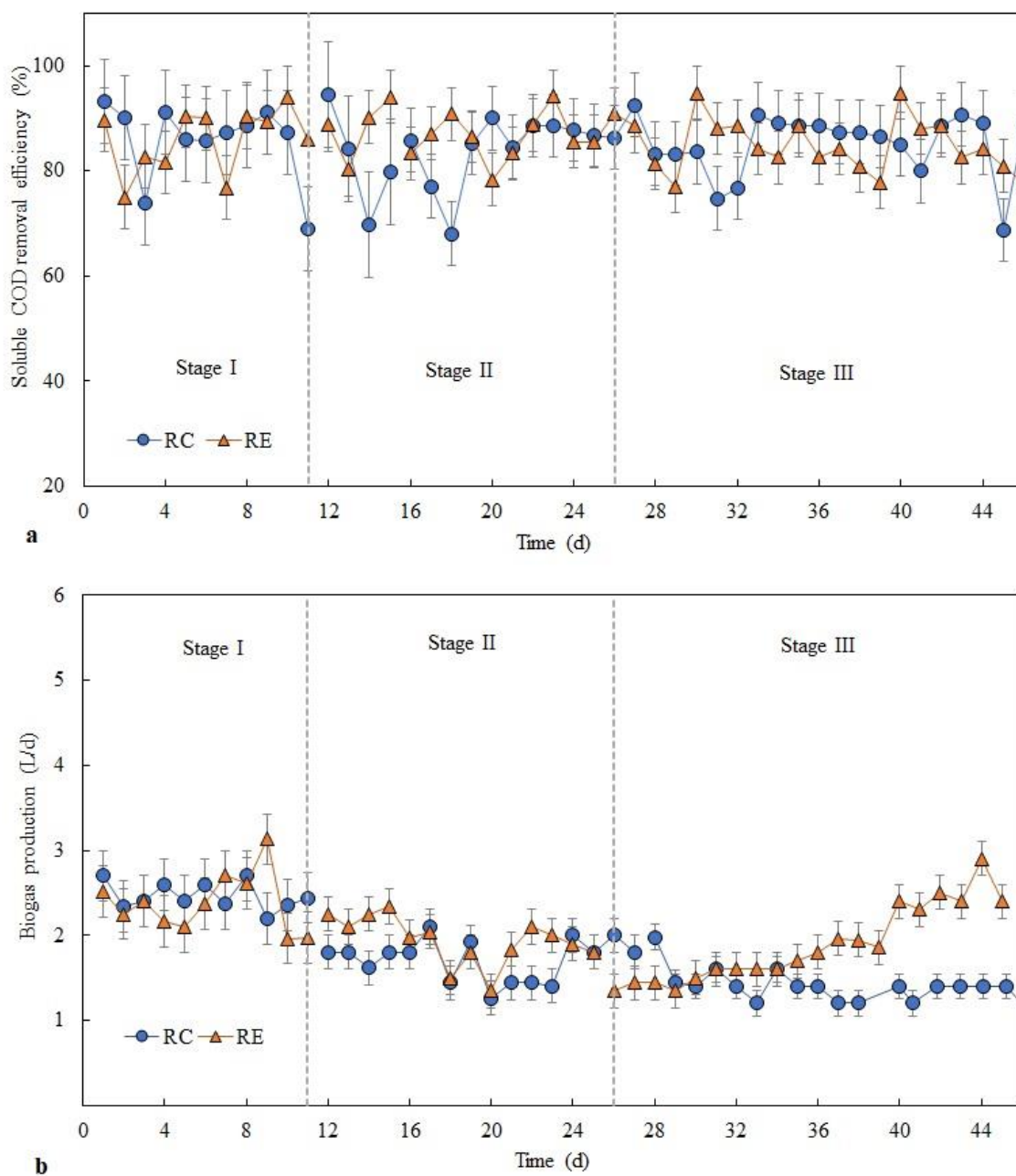


Figure 3.1 Comparison of stabilization profiles of the control reactor (RC) and the reactor with RuO₂/Ti electrodes (RE). The temperature was controlled at 25 ± 3 °C in both reactors. a) Soluble COD removal efficiency, b) Biogas production. Stage I: feeding with domestic wastewater supplemented with sucrose, stage II: feeding with domestic wastewater supplemented with corn starch, stage III: application of the electric field feeding a domestic wastewater supplemented with corn starch.

The transition from sucrose to corn starch as a substrate led to a decrease in removal efficiency of 3-7% and a notable 30% decrease in biogas production. This decline can be attributed to the structural differences between the two substrates. Sucrose, a disaccharide, is a simple sugar rapidly metabolized by microorganisms, resulting in higher and more immediate biogas yields. In contrast, corn starch is a polysaccharide, requiring an additional enzymatic hydrolysis step to break it down into simpler sugars that microorganisms can metabolize. This additional step slows the overall process, leading to decrease biogas production. Moreover, incomplete corn starch degradation can lead to the accumulation of VFAs, which can disrupt the microbial community by inhibiting methanogenic microorganisms. This imbalance hampers the efficiency of the anaerobic digestion process, further contributing to the observed decrease in biogas yield.

To further assess the impact of the experimental conditions, the reactors' performance was analyzed during stages I and II, focusing on the role of the electrodes in the sludge blanket. The following analysis examines whether their presence influenced key operational parameters, including biogas production, before applying the electric field. Experimental results clearly showed that the electrodes in the sludge blanket did not significantly influence biogas production or other key parameters during stages I and II, as summarized in Table 2.1.

Table 3.1 Comparison of the response variables in the pseudo-steady state

Parameter	Stage I (1-11 d)		Stage II (12-24 d)		Stage III (25-45 d)	
	R _C	R _E	R _C	R _E	R _C	R _E
Total COD efficiency removal (%)	84 ± 5	83 ± 8	76 ± 4	76 ± 5	88 ± 5	88 ± 4
Soluble COD removal efficiency (%)	86 ± 8	86 ± 6	75 ± 5	76 ± 5	84 ± 8	86 ± 5
α index	0.77 ± 0.01	0.72 ± 0.04	0.73 ± 0.01	0.71 ± 0.01	0.69 ± 0.05	0.69 ± 0.01
Biogas production (L/d)	2.5 ± 0.2	2.4 ± 0.3	1.7 ± 0.3	1.9 ± 0.3	1.34 ± 0.14	2.5 ± 0.2

* Stage I: feeding domestic wastewater supplemented with sucrose, stage II: feeding domestic wastewater supplemented with corn starch, and stage III: applying the electric field to feed domestic wastewater supplemented with corn starch.

3.2 Influence of the electric field on the performance of a UASB reactor.

To better understand the electric field's impact on reactor performance, key parameters such as COD removal efficiency, biogas production, and sludge characteristics were closely monitored during the application phase. The following results highlight the changes observed and their potential causes.

In the initial days of electric field application (days 26-29), a decrease in COD removal efficiency (10%) and biogas production (46%) was observed. Despite these declines, the COD consumption profile remained consistent with the control reactor. However, a notable increase in flocculent sludge was detected in the reactor with the electric field, leading to higher soluble COD concentrations in the effluent, as confirmed by granulometric analysis (please refer to the following section for further details). This rise in soluble COD can be attributed to sludge disintegration, likely caused by the stress associated with gas bubble formation during water electrolysis in the reactor. The production of gas bubbles also contributed to the fluidization of the granular sludge.

Biogas production gradually increased after the electric field adaptation period, reaching a pseudo-steady state by day 39. At this point, biogas production showed an 86 % increase compared to the control (Figure 3.1 b). This significant rise suggests that, following the initial adaptation phase and changes in reactor dynamics, the electric field positively influenced overall process efficiency.

In addition to the increase in biogas production, a notable change in biogas composition was observed. The control reactor produced a gas mixture of 78 % CH₄ and 22 % CO₂, while the reactor exposed to the electric field yielded 68 % CH₄, 21 % CO₂, and 11 % H₂. Based on this composition, the biogas might be classified as Hythane (Cavinato et al., 2011). This gas mixture is known to diminish CO₂ equivalent and NO_x emissions while enhancing the fuel's heating value (Mutaf et al., 2023). The energetic calculation of biogas shows that the calorific value of the biogas produced in the control reactor was 43 MJ/kg. In contrast, the biogas generated under the influence of the electric field reached 53 MJ/kg, representing a 23% increase in energy content. Finally, the CH₄ yield increased approximately twofold compared to the control (Figure 3.2).

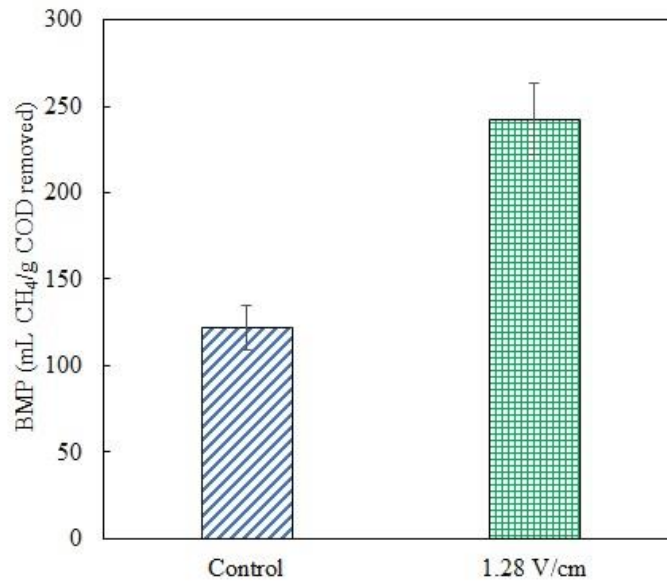


Figure 3.2 Comparison of methane production yield in the absence of an electric field (Control) and the presence of an electric field (1.28 V/cm).

Several hypotheses have been proposed regarding the enhancement of biogas production due to the by-products of electrolysis, such as oxygen and hydrogen. These gases are thought to influence various metabolic pathways in anaerobic digestion, potentially boosting microbial activity and optimizing biogas yields. Oxygen can interfere with the strictly anaerobic environment, potentially affecting the activity of methanogenic microorganisms. At the same time, hydrogen might serve as an additional electron donor, potentially enhancing specific pathways of biogas production, such as hydrogenotrophic methanogenesis.

These interactions could lead to shifts in the microbial community and changes in process efficiency. For example, in a previous study, an electric field of 1.21 V/cm was applied for 6 hours in batch cultures using synthetic wastewater. This resulted in a notable increase in hydrolytic enzyme activity at the 3-hour mark (data in preparation for publication). This effect is attributed to oxygen production via water electrolysis, creating micro-aeration conditions that may promote the growth of facultative microorganisms (H. Chen et al., 2020). These conditions allow the coexistence of facultative and strict anaerobic microorganisms due to the low solubility of oxygen in water and the rapid oxygen consumption by facultative microorganisms. Additionally, the

structure of microbial aggregates protects methanogens from oxygen stress by limiting oxygen diffusion. Furthermore, some methanogens exhibit an intrinsic tolerance to oxidative stress (Botheju et al., 2010). It is important to note that using electrochemical reactions to supply oxygen has been relatively underexplored. However, it may offer one of the most precise methods for regulating oxygen concentration. Oxygen generated through electrolysis produces smaller bubbles, which increases the surface area for gas exchange and enhances oxygen utilization efficiency. Additionally, the airflow rate generated by electrolysis is significantly lower than that of traditional air blowers, leading to decrease heat loss and minimal gas volatilization (Fu et al., 2022).

On the other hand, H_2 generated through water electrolysis can be utilized by hydrogenotrophic methanogenesis pathways, where CO_2 is reduced by H_2 to produce CH_4 . Research has shown that injecting H_2 into anaerobic reactors can convert over 40% of the CO_2 present into CH_4 , thus significantly decreasing the CO_2 concentration in the biogas (Luo & Angelidaki, 2013). Moreover, the introduction of H_2 promotes the growth of hydrogenotrophic microorganisms. For instance, Bassani et al. (2015) proposed a two-stage reactor system to improve biogas quality by coupling the CO_2 in the biogas with external H_2 , which is subsequently converted into CH_4 through hydrogenotrophic methanogenesis. This system, tested under mesophilic conditions, resulted in biogas with an average methane content of 89% following H_2 supplementation. Adding H_2 significantly increased the relative abundance of hydrogenotrophic archaea, with *Methanoculleus* emerging as the dominant genus. The observed shift from acetoclastic to hydrogenotrophic methanogenesis indicates a clear preference for the hydrogenotrophic pathway in elevated H_2 levels.

Moreover, systems coupled with electrochemical processes have shown an increase in the hydrogenotrophic pathway. In a study by Tao et al. (2020) the impact of a low-voltage electric field on hydrogen production from the anaerobic digestion of waste-activated sludge at 35 °C was investigated by applying an electric field of 0.40 V/cm. The results showed a 14% increase in hydrogen production, attributed to enhanced activity of functional enzymes such as NADH, acetate kinase, and butyrate kinase, which play critical roles in the fermentation process where most H_2 is generated. The study also highlighted that the electric field might influence membrane permeability

(related to the higher electric field), as evidenced by an 83% increase in protein concentration in the liquid phase.

Other studies have explored the influence of the electric field on hydrogen-producing enzymes. Pyruvate can be reduced to biohydrogen by two fundamental hydrogenases, namely, [FeFe]-hydrogenase and [NiFe]-hydrogenase (Saravanan et al., 2021). The [FeFe]-hydrogenase plays a crucial role in strict anaerobes by removing excess H^+ equivalents, while nitrogenase primarily reduces N_2 to NH_3 , but in the absence of N_2 , it can also reduce protons to molecular hydrogen (Bedoya et al., 2008). During water electrolysis, the electric field generates an excess of H^+ , which these enzymes utilize to produce H_2 . Marshall et al. (2017) analyzed a mixed acetate-producing microbial community in a microbial electrolysis system and observed high levels of transcripts for soluble hydrogenases, particularly near the electrode surface.

In this context, the electric field can enhance the biological production of H_2 , significantly increasing the yield of a single-stage process conducted at room temperature. This approach streamlines Hythane production-by eliminating the need for strict temperature control, typically required in conventional processes.

3.3 Influence of the electric field on the physiology of anaerobic granular sludge.

Once a pseudo-steady state was achieved, physicochemical characterization of the sludge was performed on day 45 (Table 2.2). An increase in the flocs was observed, confirmed through granulometry. The application of the electric field promoted the growth of the granules, followed by their subsequent breakdown. This was evidenced by a 17 % diminution in sedimentation and a 44 % increase in the SVI. However, there were no significant differences in the concentration of volatile suspended solids.

Table 3.2 Comparison of physicochemical characterization of anaerobic sludge in the absence of electric field (R_C) and presence of electric field (R_E).

Parameter	R_C	R_E
Sedimentation rate (m/h)	3.4 ± 0.2	2.8 ± 1
SVI (mL of sludge/g TS)	11.5 ± 2.7	16.6 ± 2.8
VSS (g/L)	46 ± 2.6	49 ± 8.3
Fraction of granular sludge >0.6 cm	61%*	71%
Fraction of granular sludge >0.4 cm	30%*	11%
Fraction of floc sludge	9%*	17%

Statistical analysis was performed by comparing the values from the control reactor versus the reactor with an electric field; * indicates a significant difference between the pairs.

This phenomenon can be described by the selection pressure theory defined by Hulshoff Pol et al. (1983), which details how hydraulic and gas loading rates affect sludge retention with varying settling characteristics. Under conditions of high selection pressure, lighter, dispersed sludge is washed out, while heavier components are retained within the reactor. This process favors the formation of sludge nuclei composed of voluminous aggregates that gradually become denser as bacterial growth occurs both on their surface and internally. Once these aggregates reach a critical size, they fragment, creating new nuclei for further growth.

In contrast, the upward flow of liquid and the internal gas production do not influence the size distribution of the granules. Instead of modifying the size of the aggregates, these shear forces cause the wear of the smallest particles (Ali et al., 2021).

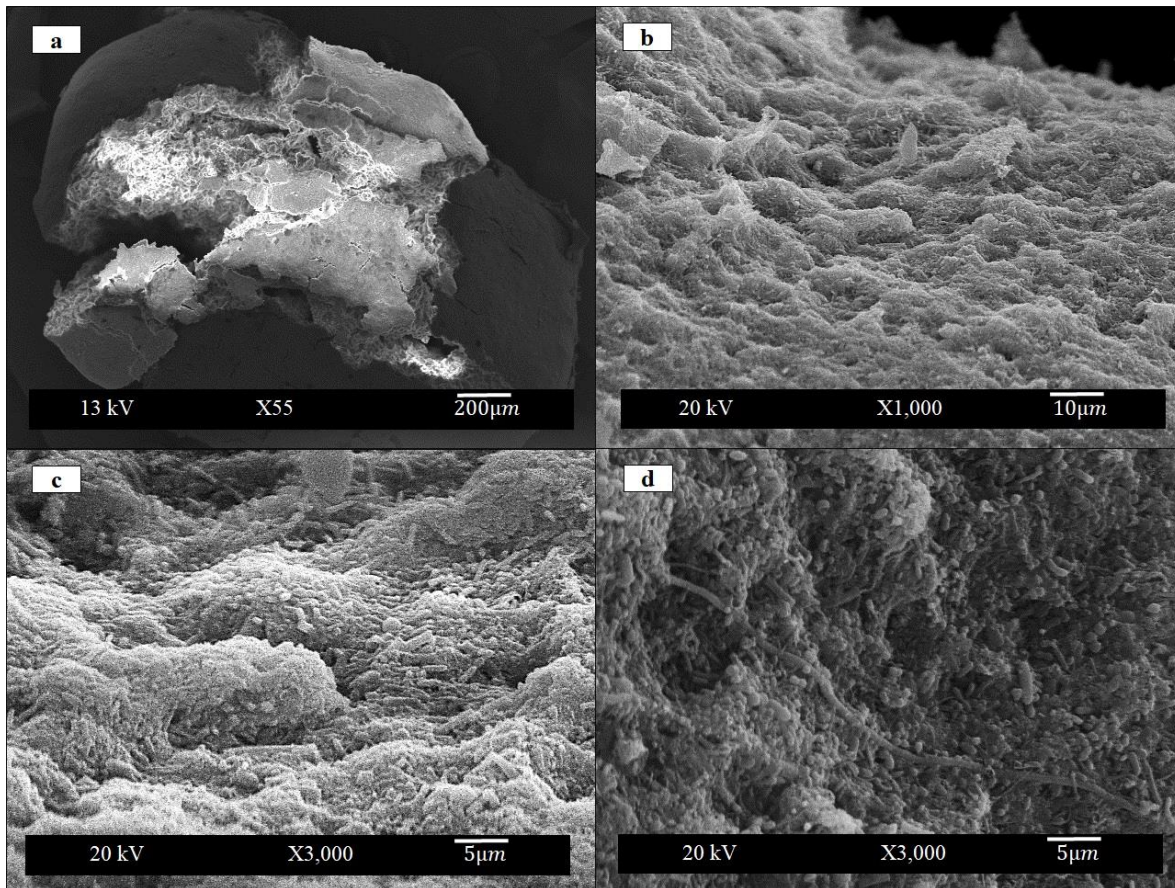


Figure 3.3 Scanning electron micrographs (SEM) of granular sludge without the electric field. a) panoramic view of the granule, b) view of the granule surface, c) close-up of the granule surface, and d) inside the granule.

To better understand the impact of the electric field on the composition and production of extracellular polymeric substances, a comparative analysis was conducted between the control sludge and the sludge exposed to the electric field. The following results highlight the variations in EPS composition and the significant increase observed in total EPS under electric field conditions.

The EPS in the anaerobic sludge showed a composition of 90 % protein and 10 % carbohydrate in the control. In comparison, the proportion of the sludge exposed to the electric field was 88 % protein and 12 % carbohydrate. Although there is no significant difference in the proportion of proteins and carbohydrates, a 52 % increase in total EPS was observed in the sludge exposed to the

electric field concerning the control. Other studies have also reported this behavior. For example, Tian et al. (2019), using graphene/polypyrrole-modified electrodes and MnO₂/PPy nanoparticles, observed that applying a cell potential of 0.4 V (cell potential) at 12 °C resulted in a two-fold increase in EPS in suspended sludge samples. Zhen et al. (2017), using SAE1080 Helical Cathode and Iron Rod Anode applying a cell potential of 2 V at 35 °C, reported an increase of 22% in the ratio of proteins.

This phenomenon might also explain the 10 % increase in large granules (diameter > 0.6 cm) observed in the presence of the electric field. EPS plays a crucial role in microbial aggregation by serving as an adhesive that binds individual bacterial cells, thereby facilitating the initiation of the granulation process. The primary EPS producers are acidogenic microorganisms, such as *Clostridium*, which, in collaboration-with methanogenic archaea, contribute to the formation of primary granule structures (Hulshoff Pol et al., 1983). Additionally, the imposition of an electric field may alter the charge distribution between the solid and liquid phases, leading to changes in the surface tension of the granular sludge (Ermakov, 2023). This alteration could trigger an adaptive response, wherein anaerobic bacteria increase EPS production as a protective mechanism under stress conditions.

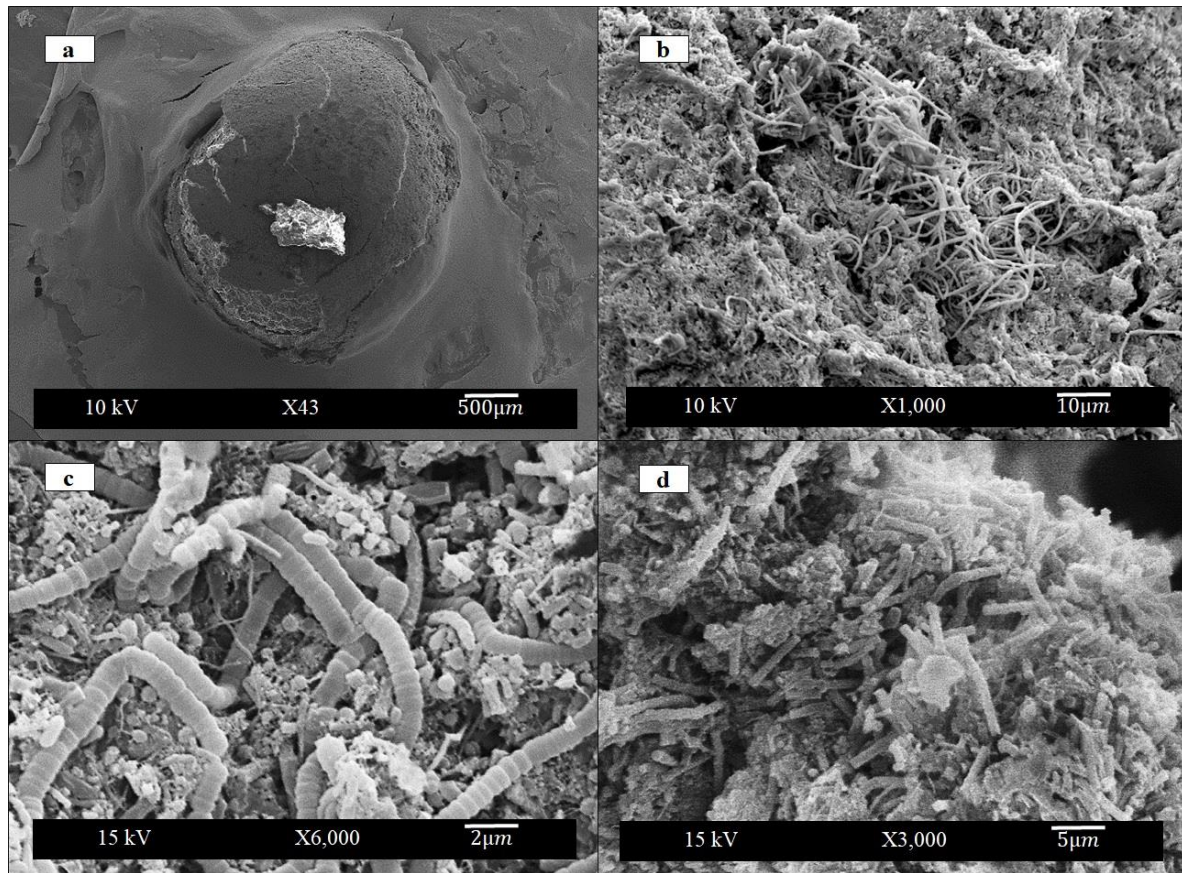


Figure 3.4 Scanning electron micrographs (SEM) of granular sludge exposed to a low-magnitude electric field. a) Panoramic view of the granule, b) View of the granule surface, c) Close-up of the granule surface, and d) View of the granule inside.

SEM micrographs of the control granular sludge show a compact and uniform structure (Figure 3.3a). The granule surface shows bacillus-like, coccobacillus-like, and filamentous microorganisms (Figure 3.3b-c), while the interior shows a more significant number of short filamentous and more elongated microorganisms (Figure 3.3d). In contrast, SEM micrographs of the granular sludge exposed to the electric field reveal an even more compact structure (Figure 3.4a), with an increase of elongated filamentous microorganisms on the granule surface (Figure 3.4b). In addition, clustered cell structures in the form of filaments that are not present in the control sludge (Figure 3.4c) are observed, and there is an increase in the number of filamentous microorganisms within it (Figure 3.4d).

The morphology of the elongated filaments closely resembles that of *Methanosaeta* species, which are archaea with a thread-like structure that plays a crucial role in stabilizing the granular

structure of anaerobic sludge (Show et al., 2020). The observed coccobacilli and bacilli display characteristics like those of bacteria of the genus *Clostridium*, which are typically present in the early stages of anaerobic digestion. The short filaments observed could also correspond to hydrogenotrophic archaea (Owusu-Agyeman et al., 2019), further supporting their role in methane production through hydrogen utilization.

These findings suggest that exposing granular sludge to an electric field stimulates the growth of filamentous microorganisms and significantly enhances the granular structure. The increase in EPS, combined with filamentous growth, appears to be a critical factor in optimizing the anaerobic digestion process. However, further research is necessary to identify the microbial communities involved accurately and to elucidate the underlying mechanisms by which the electric field exerts its effects.

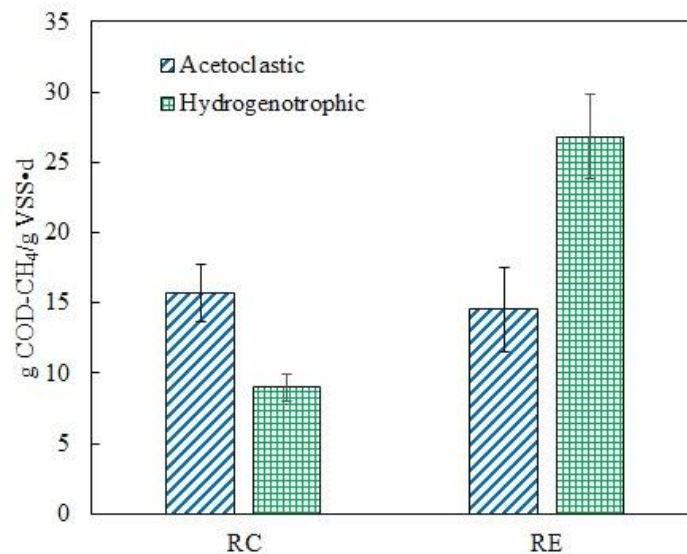


Figure 3.5 Comparison of specific activity methanogenic of anaerobic granular sludge in the absence of an electric field (RC) and the presence of an electric field (RE) at 30° C.

Finally, evaluating the specific methanogenic activity revealed a significant shift in the anaerobic metabolism. The sludge exposed to the electric field showed a marked increase in hydrogenotrophic activity, accounting for 65% of total methane production, while 35% was generated through the acetoclastic pathway (figure 3.5). This shift is notable, as the literature

typically reports that 70% of methane is produced via the acetoclastic route and only 30% through the hydrogenotrophic pathway under mesophilic conditions. Methane production from acetate is generally more favorable, as the hydrogenotrophic route is less efficient due to the low solubility of molecular hydrogen (Anderson Ken et al., 2003). This is further reflected in the control sludge, where methane production occurred primarily via acetoclastic methanogenesis (64%), with 36% generated through the hydrogenotrophic route.

4 Conclusions

The application of a 1.28 V/cm electric field in a UASB reactor operating at room temperature has proven to be a promising strategy for producing Hythane from domestic wastewater treatment in a single stage process, achieving COD removal efficiencies of 86%. This study introduces an innovative approach to anaerobic sludge acclimation-eliminating the need for complex temperature control systems.

A key finding of this research is that water electrolysis can enhance the hydrolysis of complex organic matter, like corn starch, under microaerobic conditions. This not only boosts hydrogen production, but also significantly increases methane generation by modifying anaerobic pathways and enhancing hydrogenotrophic activity. As a result, reactor performance doubled compared to the control, with biogas production reaching 2.5 L/day and a composition of 68% CH₄, 21% CO₂, and 11% H₂. The enhanced production also raised the fuel's calorific value by 23% compared to the control.

Furthermore, the electric field improved the granular sludge structure, promoting the excretion of EPS and stimulating the growth of filamentous microorganisms, which further contributed to the system's stability and efficiency.

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General conclusions

This study demonstrates that the application of a low-magnitude electric field can significantly enhance anaerobic digestion through multiple mechanisms. Notably, the process was optimized even at low temperatures, with a field strength of 1.21 V/cm significantly improving COD removal and methane production at 18 °C—reaching levels comparable to conventional mesophilic systems operating at 35 °C. Process efficiency improved across key stages: hydrolysis showed a 52% increase driven by a 53% rise in extracellular enzyme activity after three hours of electric field exposure; acetogenesis benefited from a 34% increase in volatile fatty acid (VFA) consumption, supporting syntrophic interactions and mitigating VFA accumulation; and methanogenesis saw a 39% rise in activity, reaching 0.89 ± 0.02 g COD-CH₄/g VSS·d. Electrochemically generated oxygen played a significant role during acidogenesis and acetogenesis, enhancing CO₂ production and decreasing acetate accumulation, with facultative bacteria consuming oxygen at a rate of 0.484 ± 0.04 mg O₂/L·min. Biogas production also improved substantially: under controlled laboratory conditions (18 °C, no organic carbon), it increased 2.6-fold with a composition of 95% CH₄ and 5% H₂, while in the scaled-up reactor system, production increased by 86%, yielding biogas with 68% CH₄, 21% CO₂, and 11% H₂, and a 23% higher calorific value compared to the control. Additionally, the sludge structure and composition were affected, with a 52% increase in extracellular polymeric substances (EPS), a 17% reduction in sedimentation velocity, and a 44% increase in sludge volume index (SVI); SEM analysis revealed a more compact matrix and increased filamentous microorganism presence. Metabolically, hydrogenotrophic methanogenesis became the dominant pathway, accounting for 65% of CH₄ production, while the acetoclastic route contributed 35%. Overall, these findings suggest that low-magnitude electric field technology offers a promising, sustainable strategy to enhance anaerobic wastewater treatment performance under a variety of environmental conditions, particularly at low temperatures.



Casa abierta al tiempo

UNIVERSIDAD AUTÓNOMA METROPOLITANA

ACTA DE DISERTACIÓN PÚBLICA

No. 00293

Matrícula: 2203802280

Estudio de la influencia del campo eléctrico en los metabolismos de la digestión anaerobia durante el tratamiento de un agua residual doméstica.

En la Ciudad de México, se presentaron a las 12:00 horas del día 27 del mes de marzo del año 2025 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DR. VICTOR SANCHEZ VAZQUEZ
DRA. GEHOVANA GONZALEZ BLANCO
DR. ARTURO CADENA RAMIREZ
DR. ULISES DURAN HINOJOSA

Bajo la Presidencia del primero y con carácter de Secretario el último, se reunieron a la presentación de la Disertación Pública cuya denominación aparece al margen, para la obtención del grado de:

DOCTORA EN BIOTECNOLOGÍA

DE: TANIA PAOLA MENDOZA TINOCO

y de acuerdo con el artículo 78 fracción IV del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

Aprobar

Acto continuo, el presidente del jurado comunicó a la interesada el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.



TANIA PAOLA MENDOZA TINOCO
ALUMNA

REVISÓ

MTRA. ROSALIA SERRANO DE LA PAZ
DIRECTORA DE SISTEMAS ESCOLARES

DIRECTOR DE LA DIVISIÓN DE CBS

DR. JOSE LUIS GOMEZ OLIVARES

PRESIDENTE

DR. VICTOR SANCHEZ VAZQUEZ

VOCAL

DRA. GEHOVANA GONZALEZ BLANCO

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DR. ARTURO CADENA RAMIREZ

SECRETARIO

DR. ULISES DURAN HINOJOSA