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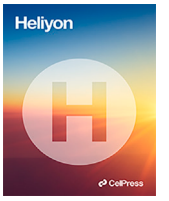

Research article

Electrostatic ethanol fermentation: Experimental study and kinetic-based metabolic modeling

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ABSTRACT

Due to the electrical nature of the cell, it is possible to modulate its behavior through the application of non-lethal external electric fields to improve fermentation processes. In this work, a microbial cell system with a chamber and two electrodes inside and connected to a voltage source was used. One of the electrodes was kept isolated to create an electric field without the flow of current. Cultures with two ethanol-producing microbial strains (*Saccharomyces cerevisiae* and *Zymomonas* mobilis) were conducted in this device. The application of voltages between 0 and 18 V was evaluated to determine the impact of the generated electric field on ethanol production. To analyze the possible effect of the field on the central carbon metabolism in each strain, biochemical-based kinetic models were formulated to describe the experimental fermentation kinetics obtained.

It was found that low applied voltages did not have significant effects on growth rate in either strain, but all voltages evaluated increased substrate consumption and ethanol production rate in *Z. mobilis*, while only 18 V affected these rates in *S. cerevisiae*, indicating that *Z. mobilis* was the most sensitive to the electric field. At the end of the fermentation, significant increases in ethanol yields of 10.7% and 19.5% were detected for *S. cerevisiae* and *Z. mobilis*, respectively. The proposed mathematical models showed that substrate transport through the membrane catalyzed by the phosphotransferase system (PTS) for *Z. mobilis* and hexose transport proteins mechanism and hexokinase (HK) activity for *S. cerevisiae* and the transformation of pyruvate to ethanol, catalyzed by the decarboxylase (PDC) and alcohol dehydrogenase (ADH) enzymes, were the reactions most affected by the application of the external field.

1. Introduction

Global policies addressing the phenomenon of climate change are compelling countries to make changes in the configuration of their energy sources to develop sustainable solutions for energy generation [1]. The transportation sector accounts for 25% of global energy consumption and is responsible for nearly 40% of final greenhouse gas emissions. Furthermore, this sector represents 90% of global oil consumption [2]. In the pursuit of decarbonizing the transportation sector, various strategies have been considered, including electricity, biofuels, and hydrogen [3], where ethanol is the primary biofuel utilized. Currently, between 68 and 73% of ethanol production is used for blending with gasoline in proportions ranging from 15 to 30% ethanol, providing excellent performance

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in internal combustion engines and achieving greenhouse gas emission reductions of up to 13.4% [4–6]. It is projected that ethanol will comprise 55% of the total biofuel production by 2030 [2], highlighting the importance of continuing the development of more efficient production processes.

Ethanol can be produced through the fermentation of biomass feedstocks derived from plant biomass with a high content of starch and sugar (such as potatoes, sugarcane, beets, among others) and agricultural residues (sugarcane bagasse, wheat straw, wood chips, among others), achieving yields between 137-430 L/t for first-generation ethanol and between 30-500 L/t for second-generation ethanol [7]. The microorganism primarily employed in ethanol production through glucose fermentation is the yeast *Saccharomyces cerevisiae*, which has achieved ethanol concentrations in the range of 8-147 g/L and productivities between 0.17-1.38 g/L/h. These values depend on the strain type, the raw material used, and the fermentation operation conditions [8,9]. Other strains of interest for ethanol production include the bacteria *Zymomonas mobilis*, which metabolizes glucose [9–11].

A significant number of studies have been conducted to increase yields and productivities through genetic engineering techniques and process design. For example, employing fed-batch fermentation systems [12], immobilized cell systems [13], high gravity fermentation [14], among others, aims to enhance the ability of new strains to metabolize different types of carbohydrates [15], tolerate the presence of inhibitory compounds [16], and withstand osmotic stress caused by high sugar concentrations [17,18].

Recent advancements in bioelectrochemical systems have not only enabled the production of electric current from organic matter but also increased the production of metabolites such as ethanol, butanol, acetone, among others, using electrofermentation [19,20]. Electrofermentation involves generating a current flow through an electrochemical circuit that induces the modification of intracellular redox potential, promoting the biosynthesis of high-energy reduced compounds in microorganisms [21,22]. Some emerging technologies, such as the use of moderate electric fields (mEF), have also recently shown improvements in fermentation processes [23,24]. This is based on the idea that metabolic processes responding to stress can lead to bioprocess improvements.

A well-established technology involves the application of pulsed electric fields due to their effectiveness in eliminating bacteria and yeast for food preservation. Typically, the electric fields (EF) intensities range from 20 to 80 kV/cm, with voltages between 11 and 60 kV. The durations of these pulses vary from 1 to 300 μs. [25,26]. Studies in *S. cerevisiae* have shown that this type of electric field causes destabilization and perforation of the cell membrane, this condition becomes irreversible, resulting in the disruption of membrane functions and the inactivation of microbial cells as part of the inactivation process [27].

On the other hand, little attention has been paid to the use of moderated electric fields (mEF) (typically 1–1000 V/cm) to promote improvements in fermentative processes. The application of sublethal electric fields to conventional production of biomolecules from fermentations is an emerging technology currently under evaluation [28,29]. The electric field applied to the cellular culture alters the environment associated with fermentation and affects the microbial homeostatic system, considering the natural behavior of the cellular membrane, which can, in turn, impact microbial metabolic pathways [24]. It is considered that the majority of interactions between electromagnetic fields and the cell occur in the membrane.

The application of electric fields with appropriate magnitude and duration causes structural rearrangements of molecules and molecular structures, induced by changes in membrane potential. This modulation affects the behavior of charged molecules, membrane receptors (chemotaxis), and the flow of ions through ion channels [30–32]. However, recent studies have also shown significant effects of mEF on the activity of enzymes such as glucoamylase, pectinase, and peroxidase, among others [33–35]. An electric field can be configured by incorporating isolated electrodes within the fermentation culture, with one connected to the positive pole and the other to the negative pole of a power source. This setup allows to create an energy-free field that acts as a capacitor, within which the microbial culture is contained.

Kinetic models enable capturing the behavior of cellular states over time, providing insights into cellular metabolism and aiding in the identification of physiological changes induced by external factors such as pH, temperature, nutrients, etc. [36–38]. It is possible to associate the metabolic states with parameters introduced in mechanistic kinetic models and thus posit that changes in parameter values may indicate changes in these states [39,40]. The challenge lies in identifying a set of parameters with which the model reproduces experimental conditions and simultaneously aligns with cellular physiological states [38]. Mechanistic kinetic models for large metabolic networks remain uncommon due to the difficulty in adjusting their parameters, whereas the use of simplified networks allows for the formulation of kinetic models that facilitate parameter fitting based on *in vivo* data [37].

Building upon previously discussed aspects, this study aimes to assess the impact of moderate electric fields (mEF) generated by low voltages on the fermentation process of two ethanol-producing microorganisms. Structured and simplified kinetic models were formulated to replicate the experimental results. Subsequently, these models were utilized to elucidate potential effects of mEF on the metabolic reactions involved in the central carbon metabolism of the microorganisms.

2. Materials and methods

2.1. Microorganims and media

S. cerevisiae commercial baking yeast and *Z. mobilis* (ATCC 31821) were maintained at −80 °C for fermentation studies. The strains were activated and subsequently cultured in YPD medium, incubated at 30 °C and 150 rpm for 12 h to obtain the inoculum.

The culture media composition (g/L) for the evaluated strains was as follows: *S. cerevisiae*: glucose (110), $MgSO_4 \cdot 7H_2O$ (1), KH_2PO_4 (2), $(NH_4)_2SO_4$ (3), yeast extract (4), and peptone (3.6); *Z. mobilis*: glucose (60), $MgSO_4 \cdot 7H_2O$ (0.5), $(NH_4)_2SO_4$ (1), and yeast extract (4). The cultures were incubated at 30 °C, 150 rpm, with an initial pH of 6.0 (*S. cerevisiae*) and 5.0 (*Z. mobilis*).

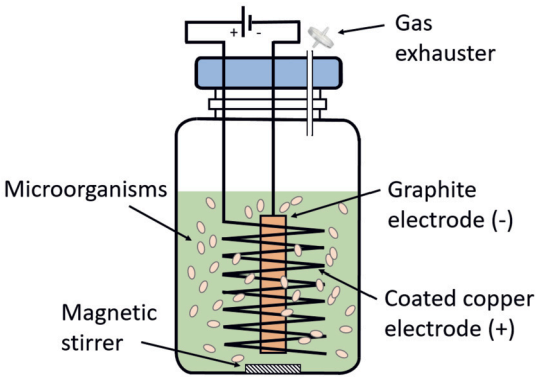

Fig. 1. Microbial Electric Field Device Configuration.

2.2. Microbial cell set up

The electric field fermentation device consisted of a 250 mL Pyrex glass flask with a set of electrodes inside (Fig. 1). The electrodes included a piece of graphite (6 cm long, 2 cm wide, and 0.4 cm thick) and an insulated 14 AWG copper wire, 250 cm in length, coiled relative to the graphite bar (distance between electrodes of 3 cm). The fermentation device was filled with 250 mL of sterilized fermentation medium (120 °C for 15 min), and before inoculation (10% v/v), nitrogen was bubbled for 5 min to create anaerobic conditions. An external power source was used to supply the desired voltage (0, 6, 12, and 18 V), corresponding to an electric field of 0 V/cm, 0.5 V/cm, 1.0 V/cm, and 1.5 V/cm, respectively, which remained constant during the treatment time for each trial. The positive pole was connected to the solenoid (anode), and the negative pole to the graphite electrode (cathode). The cultures were incubated at 30 °C and 150 rpm in triplicate. Periodic samples were extracted to analyze the concentrations of glucose, biomass, and ethanol. The significance of the treatments was determined using ANOVA analysis and the Fisher's LSD (Least Significant Difference) test.

2.3. Analytical methods

Biomass was determined by optical density at 600 nm using a spectrophotometer (Genesys 10S UV-VIS) and then converted to concentration in g/L using a calibration curve. Ethanol analysis was performed on a 7890A series gas chromatograph coupled with an Agilent 5975C mass selective detector (GC–MS, Agilent Technologies Inc., CA, USA). Separation was achieved by using a capillary column (HP-INNOWax, 30 m, 0.25 mm, 0.50 μm). Helium was used as carrier gas with a flow rate of 1 mL min^{-1}. Glucose was analyzed by using an HPLC system (Shimadzu LC-20AT Prominence Tokyo, Japan) fitted with refractive index detector (Shimadzu RID-10A, Tokyo, Japan). Separations were carried out on a Supelcosil LC-NH_2, 25 cm × 4.6 mm, 5 μm column. The mobile phase consisted of mixture of acetonitrile and water (75:25).

The specific growth rate (μ_x) was determined by calculating the slope of the logarithmic growth curve during the exponential phase. The specific rates of substrate consumption (μ_s) and ethanol production (μ_p) were estimated by multiplying the specific growth rate by the corresponding yield on dry biomass during the exponential phase. The overall ethanol yield ($Y_{p/s}$) was calculated using the total glucose consumption and the concentration of ethanol produced.

2.4. Central carbon metabolism

The mathematical models utilized in this study are derived from the metabolic networks depicted in Fig. 2 for *S. cerevisiae* and *Z. mobilis*. These two species are widely recognized as benchmark microorganisms for bioethanol production from glucose [41,42].

The Embden-Meyerhof-Parnas pathway (Fig. 2A) is employed for the yeast *S. cerevisiae*, where glucose is transformed into pyruvate with a net gain of 2 molecules of ATP and a consumption of two molecules of NAD^+ per molecule of glucose. The regeneration of consumed NAD^+ is achieved by transforming pyruvate into ethanol, generating the release of CO_2 [9]. The microorganism *Z. mobilis* catabolizes the sugar substrate through the Entner-Doudoroff pathway (Fig. 2B). In this case, glucose is transformed into pyruvate and glyceraldehyde 3-P through the intermediary 6-phosphogluconate. The glyceraldehyde 3-P is subsequently transformed into pyruvate as well. This metabolic pathway generates a net gain of 1 mol of ATP and consumes one mol of $NADP^+$ and two mols of NAD^+ per mol of glucose. Similarly to the previous case, the regeneration of consumed NAD^+ is achieved by transforming pyruvate into ethanol, generating the release of CO_2 [9].

The kinetics of reactions in the mathematical models used in this study are based on the metabolic networks shown in Fig. 2. It consists of 5 reaction rate equations, which is possible to describe using the enzyme-substrate reaction mechanism, Eq. (1), from which the Michaelis-Menten rate equation (Eq. (2)) is derived [43].

$$E + S \underset{k\_-1}{\overset{k\_1}{\rightleftharpoons}} ES \xrightarrow{k\_2} E + P \tag{1}$$

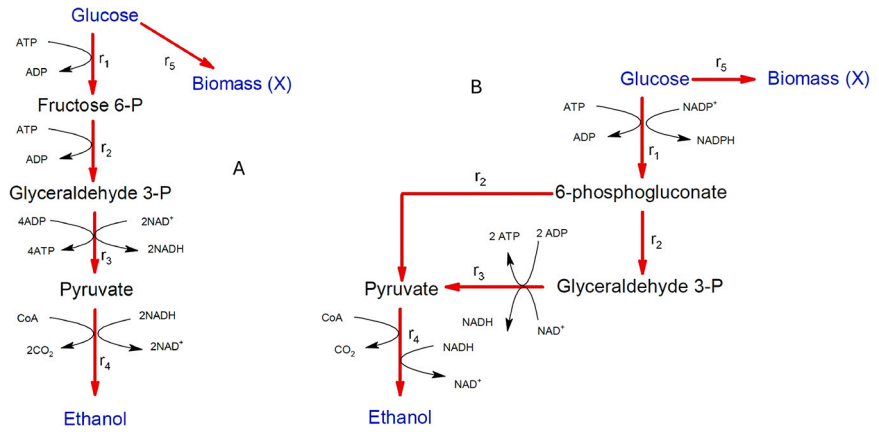

Fig. 2. Central carbon metabolism in anaerobic conditions for ethanol production (A): *S. cerevisiae* and (B) *Z. mobilis*.

$$r_i = r_{i\,max}\,\frac{[S]}{K_M + [S]} \tag{2}$$

Where $[S]$ is the substrate concentration (mM), K_m is the Michaelis-Menten constant (mM), r_i is the reaction rate of component i (h^{-1}), and $r_{i\,max}$ is the maximum reaction rate (h^{-1}.

The kinetic models dealing with the description of reaction velocities in a biological system involve a significant number of variables such as temperature, pH, initial substrate concentration, dissolved oxygen concentration, substrate inhibition, product inhibition, substrate limitation, among others [44].

The kinetic models proposed for the two microorganisms studied are described by Eqs. (3) to (12), where the included variables in mM units are: $[G]$, Glucose concentration; $[F6P]$, Fructose 6-P concentration; $[G3P]$, Glyceraldehyde 3-P concentration, $[Pyr]$, Pyruvate concentration; X, Biomass concentration; $[EtOH]$, Ethanol concentration and $[6PG]$, 6-phosphogluconate concentration.

Fermentation processes of glucose by *S. cerevisiae* are mainly influenced by phenomena such as ethanol inhibition, substrate inhibition, and substrate limitation [44,45]. Ethanol significantly affects glucose transport mechanisms [46,47] and impacts biological processes associated with lipid synthesis in the cellular membrane, reducing water activity in the medium. This results in a decrease in yeast cell growth [46,48]. On the other hand, a high sugar concentration inhibits growth due to excessive osmosis, leading to a low fermentation yield [49,50]. These effects have been incorporated into various proposed models, both structured and unstructured [44,51]. In this work, inhibition effects are included in Eqs. (3) and (7) for *S. cerevisiae*. Fermentation with *Z. mobilis* is primarily affected by substrate inhibition, product inhibition (ethanol), and substrate limitation [52–54]. *Z. mobilis* has a higher tolerance for ethanol than *S. cerevisiae*; however, ethanol inhibits its growth [55,56]. Substrate inhibition on growth has also been reported [57]. Therefore, inhibition effects are included in Eqs. (8) and (12) for *Z. mobilis*.

S. cerevisiae

$$r_1 = V_1\,\frac{[G]}{(K_1 + [G])\left(1 + \dfrac{[EtOH]}{K_{1A}}\right)} \tag{3}$$

$$r_2 = V_2\,\frac{[F6P]}{K_2 + [F6P]} \tag{4}$$

$$r_3 = V_3\,\frac{[G3P]}{K_3 + [G3P]} \tag{5}$$

$$r_4 = V_4\,\frac{[Pyr]}{K_4 + [Pyr]} \tag{6}$$

$$r_5 = V_5\,\frac{[G]}{K_5 + [G]\left(1 + \dfrac{[EtOH]}{K_{5A}}\right)\left(1 + \dfrac{[G]}{K_{5B}}\right)} \tag{7}$$

Z. mobilis

$$r_1 = V_1\,\frac{[G]}{(K_1 + [G])\left(1 + \dfrac{[EtOH]}{K_{1A}}\right)} \tag{8}$$

$$r_2 = V_2\,\frac{[6PG]}{K_2 + [6PG]} \tag{9}$$

$$r_3 = V_3\,\frac{[G3P]}{K_3 + [G3P]} \tag{10}$$

$$r_4 = V_4 \frac{[Pyr]}{K_4 + [Pyr]} \tag{11}$$

$$r_5 = V_5 \frac{[G]}{K_5 + [G]\left(1 + \frac{[EtOH]}{K_{5A}}\right)\left(1 + \frac{[G]}{K_{5B}}\right)} \tag{12}$$

2.5. Mathematical modeling

The modeling approach to describe the process dynamics follows a metabolic-based kinetic approach. The material balance for the metabolite i, Eq. (13), shows that the instantaneous accumulation of component i is due to the net synthesis of the metabolite.

$$\frac{d[i]}{dt} = \sum_{j \in \text{reactions}} \nu_{ij}\, r_j X \qquad i \in \text{metabolites} \tag{13}$$

The synthesis of i is expressed as the product of the stoichiometric coefficient of i in reaction j, ν_{ij}, the reaction rate, r_j, and the biomass concentration, X, over all the reactions in which this metabolite participates. The stoichiometric coefficients, as well as the reaction rates corresponding to the particular metabolism of each strain.

The parameters labeled from V_1 to V_5 in the Eqs. (3) to (12), represent a measure of the enzymatic activity of each enzyme or enzyme pool involved in the reactions depicted in Fig. 2. Parameters K_1 to K_5 represent the Michaelis-Menten constants for each reaction, reflecting the affinity that the enzyme has for the substrate [58]. Additionally, parameters K_{iA} and K_{iB} represent the greater or lesser inhibitory effect that ethanol or substrate has on enzymatic reaction i.

2.5.1. S. cerevisiae

The carbon central metabolism of *S. cerevisiae* described in Section 2.4 shows that material balances, Eqs. (14) to (19), involve the same metabolites and the same number of reaction rates, namely:

$$\frac{d[G]}{dt} = -(r_1 + r_5)X \tag{14}$$

$$\frac{d[F6P]}{dt} = (r_1 - r_2)\, X \tag{15}$$

$$\frac{d[G3P]}{dt} = (r_2 - r_3)\, X \tag{16}$$

$$\frac{d[Pyr]}{dt} = (r_3 - r_4)\, X \tag{17}$$

$$\frac{d[EtOH]}{dt} = r_4\, X \tag{18}$$

$$\frac{dX}{dt} = -r_5\, X \tag{19}$$

Where the specific reaction rates are given by Eqs. (3) to (7).

2.5.2. Z. mobilis

The balances for the carbon central metabolism of *Z. mobilis* follow Eqs. (20) to (25),

$$\frac{d[G]}{dt} = -(r_1 + r_5)X \tag{20}$$

$$\frac{d[6PG]}{dt} = (r_1 - r_2)\, X \tag{21}$$

$$\frac{d[G3P]}{dt} = (r_2 - r_3)\, X \tag{22}$$

$$\frac{d[Pyr]}{dt} = (r_2 + r_3 - r_4)\, X \tag{23}$$

$$\frac{d[EtOH]}{dt} = r_4\, X \tag{24}$$

$$\frac{dX}{dt} = -K_x\, r_5\, X \tag{25}$$

where the reaction rates involved in this model are provided by Eqs. (8) to (12).

2.6. Parameter estimation

The kinetic parameters needed to describe the dynamics of each culture were obtained using the least-squares (LS) method [59]. The mathematical formulation of the LS optimization problem is as follows:

$$\min_{\theta} \frac{1}{2} \sum_{t_k} \sum_{i \in m} ([i]_{pred}(t_k) - [i]_{meas}(t_k))^2$$

$$\text{s.t.} \quad \text{Eqs.(14) to (19) or Eqs.(20) to (25)} \tag{26}$$

where $[i]_{pred}(t_k) - [i]_{meas}(t_k)$ is the model-measurement mismatch at sampling time t_k for each of the m measured metabolites in the experiments (e.g., biomass, ethanol and glucose), and θ represents the set of parameters of the kinetic model. The optimization constraint, Eq. (26), ensures that the parameters fulfill the mass balances for all metabolites in the system.

The optimization and model routines were implemented in MATLAB^{TM} language using *ode15* function to solve the differential equation set and *fmincon* to minimize the least-squares function.

3. Results and discussion

3.1. Experimental study

In this study, the effect of applying a constant electric field on alcoholic fermentation with two different fermenting microorganisms was evaluated. The fields were generated by applying voltages ranging from 0 to 18 V between two insulated electrodes.

In Fig. 3, some significant variables and their behavior under voltage application during *S. cerevisiae* fermentation are presented. As can be seen, the specific growth rate is not affected by the applied voltage. However, a significant effect is observed on the specific glucose consumption rate, specific ethanol production rate, and overall ethanol yield.

The specific glucose consumption rate experienced a 40.8% increase with the application of 18 V compared to the control. The Fisher's Least Significant Difference (LSD) test determined that no significant differences were observed at lower voltages. Meanwhile, the specific ethanol production rate showed an average increase of 47.3% compared to the control with the application of voltages between 6 V and 18 V, with no significant differences observed between them. These specific rates represent the effect of voltage on cultivation dynamics, indicating evidence of significant changes in specific cellular metabolism reactions.

The overall ethanol yields obtained under different applied voltages are also illustrated in Fig. 3. The ANOVA analysis indicated a significant effect ($P < 0.05$) of changing the applied voltage value, and the LSD test showed that the treatment at 18 V is significantly different from treatments at lower voltages and the control. At this voltage, there was a 10.7% increase in the ethanol overall yield compared to the control.

In Fig. 4, the variables of specific growth rate, specific glucose consumption rate, specific ethanol production rate, and overall ethanol yield are presented as a function of applied voltage for *Z. mobilis*. The voltage effect on the specific growth rate was not significant for 12 V and 18 V ($P > 0.05$). However, for 6 V, the effect was nearly significant ($P = 0.048$), with an observed increase of 16% in the specific growth rate.

A significant increase ($P < 0.05$) in specific glucose consumption rate and specific ethanol production rate was observed with the voltage increase for all evaluated voltage values. For the 18 V treatment, the increases were 138% and 142%, respectively. Finally, the overall ethanol yield was also affected by the application of 18 V, showing a 20% increase, while no significant difference was observed with lower voltages.

It has been found that the presence of low and high electric field shows different behaviors regarding cell growth. In general, cell growth is more prominent under low intensity fields, while it decreases with the application of high-intensity fields, inhibiting growth at fields above 25 kV/cm [60,61]. It has been reported for *S. cerevisiae* that low field intensities (under 0.6 kV/cm) do not have a significant impact on growth; however, upon increasing the intensity, cellular growth reaches a maximum point from which the effects of the field begin to be lethal (above 0.8 kV/cm) [62,60].

In this study, the specific growth rate was not affected by the applied electric field. However, the final biomass in both cultures was reduced by approximately 10% (*S. cerevisiae*) and 29% (*Z. mobilis*). This can be explained by considering that cells exposed to an electric field increase their glycolytic flux to produce more energy for maintenance mechanisms due to the stress induced by the electric field [83,82]. This increase in catabolism also leads to higher ethanol production, as ethanol is the final product in the catabolic pathway.

In line with this, increases in the growth of the microalgae *C. vulgaris* have also been reported with field intensities of 2.7 kV/cm [63]. On the other hand, results have been presented for *L. acidophilus*, where the presence of low electric field (1 V/cm) during fermentation does not change the lag time, maximum specific growth rate, and generation time [64]. These results are consistent with those obtained in this work for both *S. cerevisiae* and *Z. mobilis*. On the other hand, a recent study reported that the application of a moderate electric field, generated by voltages between 15 and 30 V, increases the specific growth rate of *S. pastorianus* by 1.7 to 2.7 times compared to the control [29], which contrasts with our results.

Cells have their own electric field generated by their membrane potential (approximately 70-90 mV), which acts as a 'Faraday cage' serving as a shield that prevents weak electric fields from entering the cell [65]. The membrane can be considered non-conductive. When compared to the cytoplasm and the extracellular physiological medium, these have conductivities several orders of magnitude higher than the membrane. This membrane potential generates an intense electric field on the order of 1 kV/cm, below which the effects on cell growth and cellular viability are negligible [66].

The effect of the electric field on *S. cerevisiae* and *Z. mobilis* revealed an increase in the specific substrate consumption rate as the applied field increased. It was observed that *Z. mobilis* was more sensitive to this field, as differences were noted even at the lowest applied voltages, while *S. cerevisiae* was less sensitive to the field, with effects observed only at the highest applied voltage, i.e. 18 V. This higher sensitivity to the electric field is characteristic of Gram-negative bacteria, such as *Z. mobilis*, while Gram-positive bacteria are less sensitive [60].

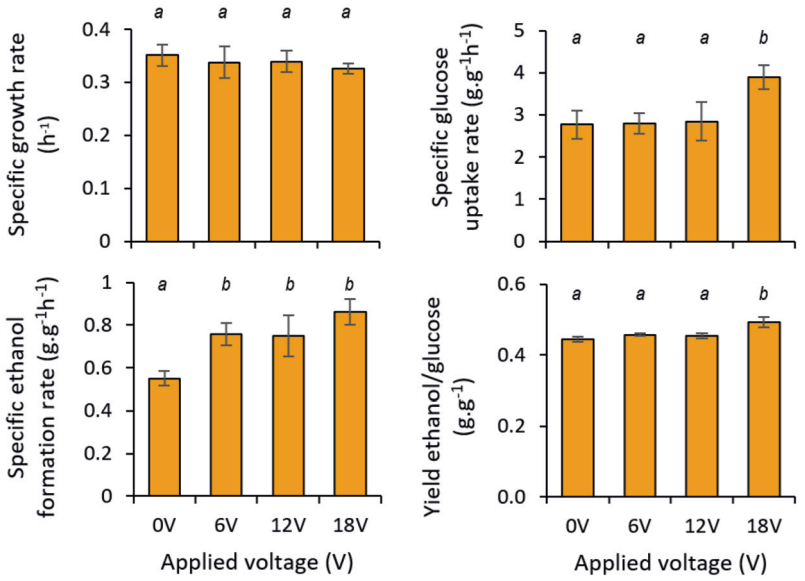

Fig. 3. Experimental kinetic parameters for *S. cerevisiae* fermentations at different applied voltages. Different letters indicate statistical significance ($p < 0.05$). Results are reported as the mean ± standard deviation (n = 3).

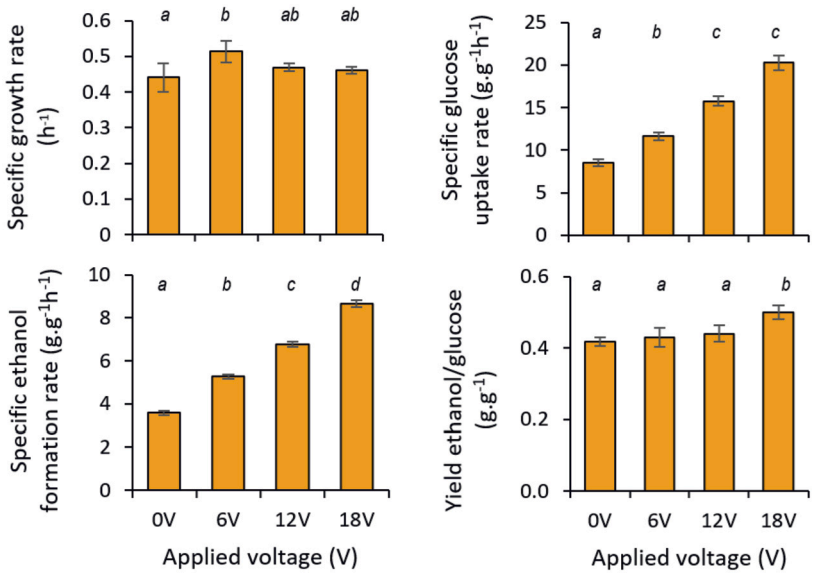

Fig. 4. Experimental kinetic parameters for *Z. mobilis* fermentations at different applied voltages. Different letters indicate statistical significance ($p < 0.05$). Results are reported as the mean ± standard deviation (n = 3).

Transport of sugars represents an important step in sugar metabolism of bacteria and yeast, and is often a limiting step for control of metabolism. Bacteria use the PTS system for sugar transport inside the cell, employing phosphoenolpyruvate as the phosphate group donor, while yeast employ a different mechanism involving the use of hexose transport proteins, these proteins facilitate the passage of sugar across the plasma membrane by facilitated diffusion, which does not require direct ATP expenditure. [67,68]. It has been demonstrated that external electric fields can activate membrane-bound ATPases [69], and although ATPases are not directly used in either of the two transport systems, they are involved in maintaining the proton gradient across the plasma membrane, creating an electrochemical gradient that can be utilized by various transporters to move solutes against their gradient. This could be a possible explanation for the observed effect of the electric field on the specific substrate consumption rate.

Increases in the specific ethanol formation rate and ethanol yield obtained in this work are consistent with the results of other studies. These studies showed that ethanol production with *S. cerevisiae* under an electric field generated by applying voltages between 0 and 15 V increased with higher applied voltages [70]. It has been speculated that the induced electric field by the applied voltage speeds up cellular electron transport to enhance the fermentation rate, thereby generating an increase in ethanol production rate. Other fermentation processes, different from alcoholic fermentation, have also exhibited effects from the application of electric fields.

Table 1
Pearson's correlation coefficients between experimental data and simulated results.

	0 V			18 V		
	Glucose	Biomass	Ethanol	Glucose	Biomass	Ethanol
S. cerevisiae	0.976	0.990	0.987	0.999	0.975	0.982
Z. mobilis	0.992	0.969	0.983	0.999	0.972	0.998

The application of an electric field generated by a voltage of 1.2 V in an anaerobic digestion process significantly increased methane production (by 151%) compared to the treatment at 0 V. It was also proposed that an increase in the electron transfer rate associated with various redox enzymes is possible, as metabolic processes can be modulated by induced electric fields [71]. Furthermore, the effect of a moderate electric field has also enhanced the production of primary metabolites, such as bacteriocin by *Lactobacillus acidophilus*, particularly when the field (1 V/cm) was applied at the early stage of fermentation [64]. These authors suggested that the improvement was due to an increase in transmembrane conductivity and diffusive permeability of nutrients in the presence of an electric field. On the other hand, it was recently reported that the application of fields generated by low voltages between 15 and 30 V reduced ethanol production in *S. pastorianus* [29], which contrasts our study and with the previously presented studies. A transcriptomic analysis conducted in this latter study on *S. pastorianus* indicated that the electric field influences cellular processes and metabolic pathways and is associated with the down regulation of genes related to transport and extracellular regions. This disruption affects cellular trafficking and signaling processes, leading to alterations in intra- and extracellular homeostasis [29]. It is clear from this, that one of the greatest challenges in understanding the observed changes in biological systems resulting from exposure to controlled external factors, such as an electric field, is to be able to relate biological changes to physical mechanisms.

3.2. Kinetic model evaluation

A mathematical model based on the kinetics of central carbon metabolism was proposed to quantitatively verify its consistency with the experimental results and to analyze the effect of the electric field on the main metabolic reactions involved in the model. The fermentation kinetics of the control and 18 V treatments, which showed significant differences in most of the evaluated variables for both strains, were used to adjust the mathematical model. These mechanism-based mathematical models describe systems in terms of identifiable physical processes, with parameters assumed to have fundamental physical significance [72]. Their analysis can be used to better understand the impact of the electric field on fermentation processes.

In Figs. 5 and 6, the experimental data of biomass, glucose and ethanol concentration over time of each fermentation are showed in markers, while the profiles obtained with the proposed mathematical models are also represented by lines. Given that the proposed model was based on reaction rate kinetics for each metabolic reaction, the experimental data were presented in mM units, using an average molecular weight of 26.16 g/gmol for biomass, 180 g/gmol for glucose and 46.1 g/gmol for ethanol, for conversion from the original data in g/L.

Overall, the profiles show good agreement with the experiments, judging by the values of the Pearson's correlation coefficients obtained for each measured experimental variable (Table 1). The correlation coefficients were above 0.975 for *S. cerevisiae* and 0.969 for *Z. mobilis*. These results indicate that the proposed kinetic models are indeed effective in describing the experimental kinetics trends for both microorganisms, and they can be employed to make inferences about the impact of the electric field on cell metabolism.

The parameters identified for the model are shown in Table 2 for the two studied strains at 0 and 18 V. Our analysis relies on identifying the effect of the applied electric field on the metabolic reactions of the network shown in Fig. 2, based on the changes in the parameter values of the proposed kinetic model when voltage is modulated. The parameter V_5, corresponding to the maximum specific growth rate, was not considered as an adjustable parameter and was included as a constant (values taken from Figs. 3 and 4), in the set of parameters to be adjusted for each of the two strains and for each of the voltages evaluated. This was done as a measure to guide the optimization function to adjust a set of parameters that hold physical significance for the system.

The first observation derived from the information presented in Table 2, is about parameters V_1 and V_4 which are related to the specific glucose consumption rate r_1 and specific ethanol production rate r_4 respectively. The increase in these parameters exhibits the same behavior as the experimental rates observed in Figs. 3 and 4. Additionally, it is observed with *S. cerevisiae* that the parameters V_2 and V_3, corresponding to the reactions of glyceraldehyde 3-phosphate formation and pyruvate formation, respectively, also increase in value with the application of the electric field. On the other hand, for *Z. mobilis*, V_2 experiences a decrease while V_3 increases.

For *S. cerevisiae* V_1 changed from 2.77 to 3.90 while for *Z. mobilis* the same parameter changed from 8.52 to 20.3. This result could indicate that the electric field might affect the substrate transport mechanism at the level of the phosphotransferase system (PTS) in *Z. mobilis*, as well as at the level of hexose transport proteins or hexokinase (HK) activity in *S. cerevisiae*. These results correlate with studies where HK enzyme activity increased under electrical stimulation, compared to the absence of this stimulus [73]. Kinetic experimental studies on enzyme hexokinase showed that changes in the reaction rate could be attributed to changes in the maximum reaction rate, suggesting that the increase in reaction rate under electrical stimulation was due to an increase in accessibility to the active site [73]. In other studies, it has also been reported that electrically stimulated *S. cerevisiae* cultures have shown an increased glucose consumption rate [74,75,29]. The transcriptome analysis performed on *S. pastorianus* revealed an increase in mRNA genes corresponding to the transmembrane transport activity when cells are exposed to an electric field generated by a 30 V potential, additionally, among the upregulated genes, those related to the cellular response to oxidative stress were observed [29].

Table 2
Parameters of the adjusted models.

	S. cerevisiae		*Z. mobilis*	
	0V	18V	0V	18V
V1	2.77	3.90	8.52	20.3
V2	0.145	0.497	2337	22.6
V3	0.250	0.387	0.00111	0.484
V4	0.550	0.861	3.58	8.66
V5*	0.351	0.326	0.441	0.460
K1	0.432	1.91	888	858
K1A	9.56	8.20	1637	2348
K2	1.97	82.8	643	0.524
K3	0.0247	0.00869	8265	7887
K4	0.00667	37.2	1.83	0.147
K5	0.454	0.454	4776	4155
K5A	681	454	5885	5286
K5B	1787	1942	263	196
Kx	—	—	36.4	64.4

* Parameter V_5 was not identified. V_i units in (mol·mol^{-1}·h^{-1}) and K_i units in (mM).

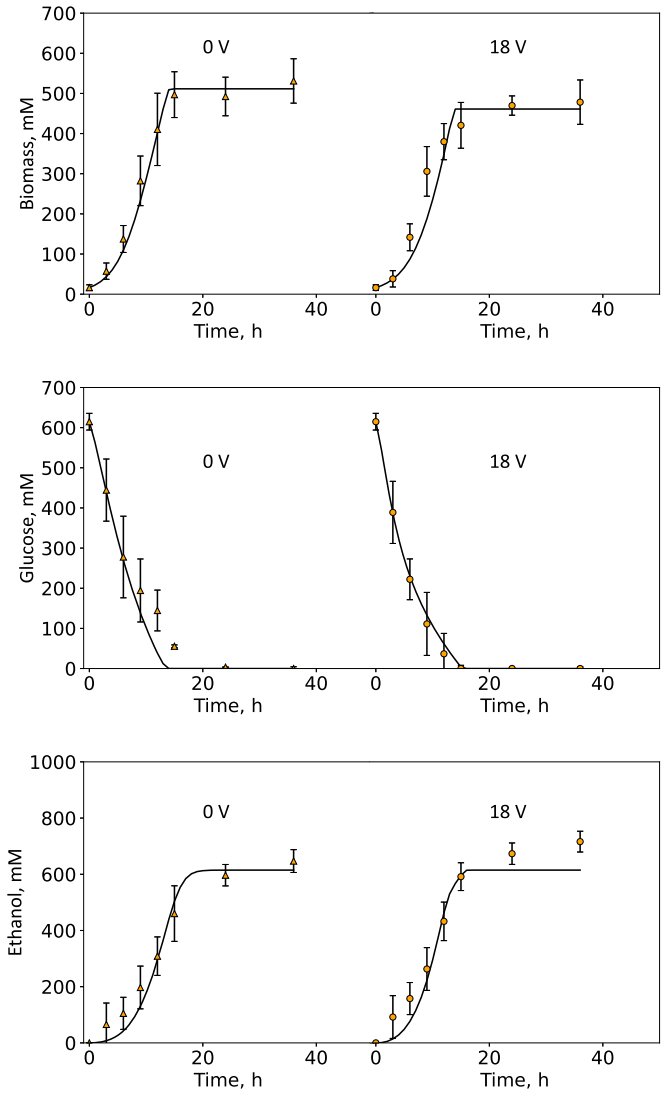

Fig. 5. Experimental kinetic data (markers: △ 0 V; ○ 18 V) and simulation profiles (lines) in the fermentation of *S. cerevisiae*.

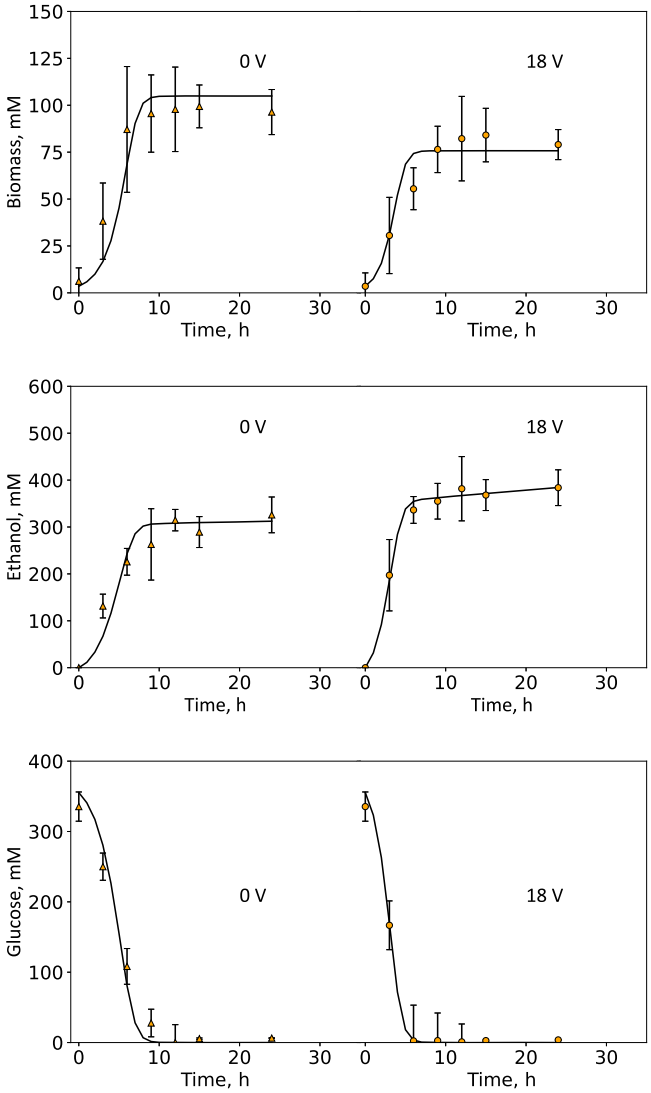

Fig. 6. Experimental kinetic data (markers: △ 0 V; ○ 18 V) and simulation profiles (lines) in the fermentation of *Z. mobilis*.

Another reaction rate affected by the electric field was the specific ethanol production rate r_4, which involves the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). As observed in Figs. 3 and 4, r_4 showed an average increase of 47.3% for *S. cerevisiae*, while the increase was 142% for *Z. mobilis* when a potential of 18 V was applied. The increase in V_4 parameters exhibits the same behavior as the experimental rate r_4. For *S. cerevisiae* V_4 changed from 0.550 to 0.861 while for *Z. mobilis* the same parameter changed from 3.58 to 8.66. Some studies reported that alcohol dehydrogenase and other enzymes can be inductively electrostimulated only when they are bound to membranes, such as in yeast cells. In contrast, under *in vitro* conditions, electric field applications have no significant effect on the original enzyme activity [76,77]. This indicates that the contact of the enzyme with the electric double layer of the membrane could be a prerequisite for the electrostimulation effect to occur [77].

This last aspect is still inconclusive, as recent studies have shown positive effects of applying moderate electric fields to other types of free enzymes such as glucoamylase, laccase, pectinase, lipase [78,35,34,79], while inactivation effects have also been observed, as in the case of peroxidase [33]. This can be explained by the different strategies employed to evaluate the electric fields, which include pulsed electric fields, electrostatic fields with current flow, and electrostatic fields without current flow. It is well known that cell membranes act as capacitors, exhibiting significant electric potential that allows them to modulate a significant number of biological functions such as transport, signaling, mobility, etc. [30–32]. Furthermore, when a substrate is in proximity to the enzyme's active site, strong electrical interactions can occur between the reactants and the polar groups of the enzyme. This highlights that electrostatic interactions is a key factor in the energetics of enzymatic catalysis [80]. These electrical aspects at the cellular and molecular levels are affected to a greater or lesser extent by the presence of external electric fields. Hence, the study of the effect of moderate electric fields on cell behavior and enzymatic catalysis has gained new relevance in contemporary research.

Once the mechanisms by which moderate electric fields act at the cellular and molecular levels are consolidated, their scaling to an industrial level can be considered, either in larger units or through the addition of modular units [81]. For this, design aspects in the configuration of the electrodes (area, spacing, field flow pattern, materials) and aspects such as the geometric configuration, modes of operation, and operating conditions of the bioreactor must be taken into account.

4. Conclusions

In this contribution the effect of applying a constant electric field on the ethanol production by two different microorganisms was studied experimentally and by developing metabolic-based kinetic models.

The experiments showed that the electric field generated at voltages between 6 to 18 V significantly affected the dynamics of the fermentation process, including the substrate consumption rate and ethanol production rate, and increased the final ethanol production, but did not affect the specific growth rate. The overall ethanol yield increased by 11% for *S. cerevisiae* and 20% for *Z. mobilis* when applying a potential of 18 V compared to the controls.

The proposed mathematical models showed good agreement with experimental data confirming that the model structure captures the essential features of the phenomena occurring during the fermentation process both with and without electric fields.

The analysis of the identified model parameters agrees with the experimental findings related to the effect of the electric field on substrate uptake and allows inference about its impact on membrane transport and hexokinase activity. Furthermore, since the model shows an enhanced ethanol production rate with the application of voltage, the effect of the field on alcohol dehydrogenase can also be involved.

Future contributions should address the experimental verification of the effect of the electric field on the activities of enzymes, particularly those involved in central carbon metabolism, or evaluate gene expression through transcriptome analysis. Future works will focus on the optimization of ethanol production under moderated electrical fields.

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CRediT authorship contribution statement

Carlos Alberto García-Mogollón: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Diego F. Mendoza:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Juan Carlos Quintero-Díaz:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting the findings of this study are available within the article.

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