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Article

Biofuels from Micro-Organisms: Thermodynamic Considerations on the Role of Electrochemical Potential on Micro-Organisms Growth

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Abstract: Biofuels from micro-organisms represents a possible response to the carbon dioxide mitigation. One open problem is to improve their productivity, in terms of biofuels production. To do so, an improvement of the present model of growth and production is required. However, this implies an understanding of the growth spontaneous conditions of the bacteria. In this paper, a thermodynamic approach is developed in order to highlight the fundamental role of the electrochemical potential in bacteria proliferation. Temperature effect on the biosystem behaviour has been pointed out. The results link together the electrochemical potential, the membrane electric potential, the pH gradient through the membrane, and the temperature, with the result of improving the thermodynamic approaches, usually introduced in this topic of research.

Keywords: biofuels; biomethane; nonequilibrium thermodynamics; bacteria; energy engineering; biodiesel



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1. Introduction

In 2017, UN IPCC Conference in Marrakesh suggested containing the increase in temperature up to 2 °C, in order to prevent the overcoming of the thermal threshold for a possible disastrous climate change [1] due to the use of fossil fuels as main source of energy [2]. In the last decade a growing interest in biofuels from bacteria has emerged, due to their sustainable characteristics [3–17].

However, at present, the third generation of biofuels is not yet competitive, in comparison to fossil fuels, because they present high costs of production and extraction, in relation to the amount of fuel obtainable per unite volume of culture [18].

In order to improve their competitiveness, the control of bacteria growth is required. This topic has been studied in the second half of the XX century, starting with Monod [19,20], who developed a whole theory on bacteria growth, by introducing a hyperbolic relationship, which links the amount of limiting resources in the environment to the growth rates of microorganisms. This first result has been improved by coupling it to other models able to fit the experimental data [21–24]. In 1956, the concept of continuous culture has been introduced with the aim to obtain biological cultures able to operate continuously for a long time [24,25]. This approach showed, in 1958, that one of the fundamental requirement of life is the maintenance of concentration or osmotic gradients [26]. Then, the Black Cox model was introduced in order to obtain the relationships among energy, biomass and yield [24], by pointing out that the growth of bacteria, on a single carbon compound as sole energy source, uses a constant amount of energy, per electron, to produce dry weight [27]. Mayberry et al. pointed out the role of the electrons (as electric charge) in the bacteria growth, in relation to the Oxygen fluxes [28]. This thermochemical approach led to confirm the Thornton’s rule [29]. However, the thermodynamic approach has been highlighted to be too dependent by the approximation of enthalpy and Gibbs free energy, with the consequence to require a new development.

In this context, biofuel production by microorganisms must be analysed in order to optimise their production process [30–32], and it can be carried out from an improvement of the thermodynamic approach to biophysical processes. We have recently developed a thermodynamic approach to mutualism [4,33], in order to improve the biodiesel production from bacteria and algae [34–40], in the context of a new general approach to sustainability [41]. So, we suggest an explanation to the behaviour of the bacteria in relation to a new nonequilibrium thermodynamic approach to bacteria membrane. Indeed, bacterial electrical signalling has experimentally been proven to be able to regulate a great variety of physiological processes, mediated by membrane electric potential [42–45], such as proliferation [46].

In accordance with the previous biological and biochemical results, the inflow of nutrients and outflow of waste products occur through the membrane, with a related restoration of ionic gradients, changed by the transport systems. There is a great number of theoretical and computational studies on the membrane mechanics, but these models cannot obtain a full description of the membrane dynamical and chemical behaviour [47]. There is a continuous interaction between bacteria and their environment; indeed, all bacteria are able to modify their environment by secretion of enzymes, toxins, or pheromones and by macromolecules on their external membrane surfaces. This interaction is the key-point of our scientific interest: we wish to point out the conditions of bacteria environment control, useful to improve the production of biofuels by micro-organisms.

In this paper, we develop the analysis of the role of the membrane potential in the behaviour of the bacteria, in relation to their ability to produce biofuels, in order to suggest an approach to improve their productivity, starting from a *natural* behaviour of these living biosystems. Last, we suggest a possible improvement of this behaviour, by introducing the cooperative interaction between different species (mutualism).

2. Materials and Methods

The aim of this paper is to introduce, into the thermodynamic analysis of the biofuels production by bacteria, a nonequilibrium thermodynamic approach to membrane heat and mass transport, recently developed in relation to life [48] and applied to cancer [49–52] and glaucoma [53], with results in accordance with the experimental results in literature, both in relation to cancer [54–80] and to glaucoma [81–89].

The nonequilibrium thermodynamic approach has been obtained by introducing the Onsager general phenomenological relations, previously related only to membrane electric potential, and, here, generalised by introducing the electrochemical potential [48,49,90–93]:

$$\begin{cases} \mathbf{J}_e = -L_{11} \frac{\nabla \mu_e}{T} - L_{12} \frac{\nabla T}{T^2} \\ \mathbf{J}_Q = -L_{21} \frac{\nabla \mu_e}{T} - L_{22} \frac{\nabla T}{T^2} \end{cases} \quad (1)$$

where \mathbf{J}_e is the net current density [A m^{-2}], if the effect of some different species f ions are considered or simply the effect of one species if only one ion species is considered, \mathbf{J}_Q denotes the heat flux [W m^{-2}], $\mu_e = \mu + Ze\phi$ [92–94] is the electrochemical potential [J mol^{-1}], with μ the chemical potential [J mol^{-1}], ze the electric charge [A s mol^{-1}], and ϕ the membrane potential [V], T is the living cell temperature and L_{ij} represent the phenomenological coefficients, such that [94] $L_{12}(\mathbf{B}) = L_{21}(-\mathbf{B})$ (Onsager-Casimir relation [95]), and $L_{11} \geq 0$ and $L_{22} \geq 0$, and [94] $L_{11}L_{22} - L_{12}L_{21} > 0$.

The result consists in modelling the life cycle of a living cell by introducing two related processes [48,49]:

- A continuous energy generation (metabolism), due to the ion fluxes: the ions and metabolites fluxes can be described by imposing $\mathbf{J}_e \neq \mathbf{0}$ and $\mathbf{J}_Q = \mathbf{0}$;
- A continuous heat fluxes from the cell to its environment: The heat exchange towards the environment can be described by imposing $\mathbf{J}_e = \mathbf{0}$ and $\mathbf{J}_Q \neq \mathbf{0}$.

In this way, we can split the life cycle into two thermodynamic processes, as usually done in thermodynamics for any complex process [91,96].

If the ions and metabolites fluxes occur, $\mathbf{J}_e \neq \mathbf{0}$ and $\mathbf{J}_Q = \mathbf{0}$, so, by taking into account the Equations (1), it is possible to obtain [48,90,91]

$$\frac{d\mu_e}{dT} = -\frac{L_{21}}{L_{11}} \frac{1}{T} \tag{2}$$

with a related heat flux [90,91]:

$$\frac{du}{dt} = -\nabla \cdot \mathbf{J}_Q \tag{3}$$

where u is the internal energy density [J m^{-3}].

Living bacteria exchange heat power towards their environment by convective transmission and thermal infrared emission, so, as a consequence of the First Law of Thermodynamics, it follows [51]

$$\frac{du}{dt} dV = \delta\dot{Q} = -\alpha (T - T_0) dA - \epsilon_{irr} \sigma_{SB} (T^4 - T_0^4) dA \tag{4}$$

where $\epsilon_{irr} \approx 0.97$ is the emissivity factor [97–99], $\sigma_{SB} = 5.67 \times 10^{-8} \text{ W m}^{-2}\text{K}^{-4}$ is the Stefan–Boltzmann constant, α is the coefficient of convection, A area of the external surface of the cell membrane, V is the cell volume, T is the mean temperature of the external surface of the bacteria membrane, and T_0 is the temperature of their environment.

So, considering the Equations (3) and (4), together with the Divergence Theorem [100], the heat power exchanged:

$$\dot{Q} = \int_A \mathbf{J}_Q \cdot \hat{\mathbf{n}} dA \approx \alpha (T - T_0) A + \epsilon_{irr} \sigma_{SB} (T^4 - T_0^4) A \tag{5}$$

However, considering Equation (1), and that $\epsilon_{irr} \sigma_{SB} (T^4 - T_0^4) \ll \alpha (T - T_0)$, and the second hypothesis of our approach ($\mathbf{J}_e = \mathbf{0}$, $\mathbf{J}_Q \neq \mathbf{0}$), we can obtain [48]:

$$\frac{d\mu_e}{d\ell} = \frac{T J_Q}{\left(L_{22} \frac{L_{11}}{L_{12}} - L_{21} \right)} = -\frac{\alpha T (T - T_0)}{\left(L_{22} \frac{L_{11}}{L_{12}} - L_{21} \right)} \tag{6}$$

where ℓ is the length of bacteria membrane and $|\nabla\mu_e| \approx d\mu_e/d\ell$. This last relation represents a link between the bacteria membrane electric potential and the temperature of the bacteria but to their heat exchange, too. Now, Equation (6) allow us to obtain:

$$\alpha T (T - T_0) = -\left(L_{22} \frac{L_{11}}{L_{12}} - L_{21} \right) \frac{\partial\mu_e}{\partial\ell} \tag{7}$$

Considering that [93]:

$$\left(L_{22} - L_{21} \frac{L_{12}}{L_{11}} \right) = K_J T^2 \tag{8}$$

where K_J is the Thomson coefficient [93], it is possible to obtain that:

$$\frac{\partial\mu_e}{\partial\ell} = \frac{\partial\mu_e}{\partial T} \frac{\alpha}{K_J} (T_{surf} - T_0) \tag{9}$$

which, considering that $\mu_e = \mu + ze\phi$, allows us to obtain:

$$\frac{\partial\mu}{\partial\ell} = -ze \frac{d\phi}{d\ell} + \frac{\partial\mu_e}{\partial T} \frac{\alpha}{K_J} (T_{surf} - T_0) \tag{10}$$

We can point out that the first fundamental quantity in the equation is the chemical potential, which is defined as [91]:

$$\mu_i = \left(\frac{\partial G}{\partial n_i} \right)_{T,p,n_{k \neq i}} \approx \frac{G}{n_i} = g \tag{11}$$

where G is the Gibbs energy, g is the Gibbs molar specific energy, n is the number of moles, and p is the pressure. Moreover, the Gibbs energy is related to the membrane electric potential by the Nernst equation [101]:

$$\Delta g = F \Delta \phi - 2.3R T_0 \Delta \text{pH} \tag{12}$$

where $F = 96485 \text{ A s mol}^{-1}$ is the Faraday constant and $R = 8.314 \text{ J mol}^{-1} \text{ K}^{-1}$ is the universal constant of ideal gas. Now, introducing the Equation (12) into the Equation (10), we can obtain:

$$\frac{\partial \mu_e}{\partial T} = \frac{K_J}{\alpha} \frac{F + ze}{T_{surf} - T_0} \frac{d\phi}{d\ell} - \frac{K_J}{\alpha} \frac{2.3R T_0}{T_{surf} - T_0} \frac{d\text{pH}}{d\ell} \tag{13}$$

As a consequence of the previous processes, a density entropy rate due to irreversibility (dissipation function [90]) is generated [102]:

$$\sigma = -\frac{1}{T_0} \sum_{i=1}^N \mathbf{J}_i \cdot \nabla \mu_i = \sum_{i=1}^N J_i \frac{z_i e}{T_0} \frac{d\phi}{d\ell} + J_u \frac{\alpha}{K_J} \left(1 - \frac{T_{surf}}{T_0} \right) \sum_{i=1}^N \frac{\partial \mu_{e,i}}{\partial T} \tag{14}$$

where T_0 is the environmental temperature, $\sum_{i=1}^N \mu_i \mathbf{J}_i$ is the contribution of the inflows and outflows, and μ is the chemical potential. Considering that $\sigma \geq 0$, we can find the condition of control for the previous relations:

$$\begin{aligned} \sum_{i=1}^N J_i \frac{z_i e}{T_0} \frac{d\phi_i}{d\ell} &\geq -J_u \frac{\alpha}{K_J} \left(1 - \frac{T_{surf}}{T_0} \right) \sum_{i=1}^N \frac{\partial \mu_{e,i}}{\partial T} \Rightarrow \\ &\Rightarrow \sum_{i=1}^N J_i z_i e \nabla \phi_i \geq J_u \frac{\alpha}{K_J} (T_{surf} - T_0) \sum_{i=1}^N \frac{\partial \mu_{e,i}}{\partial T} \end{aligned} \tag{15}$$

3. Results

The fundamental result of this paper is Equation (10). Indeed, this equation represents a link among the chemical potential gradient and the electric potential through the membrane and the thermophysical properties of the membrane and the environmental fluid, related to the temperature of the micro-organisms membrane external surface. Indeed, the electrochemical potential variation with temperature in Equation (13) shows a dependence on the pH gradient. This result is in accordance with the behaviour of the analysis of the lipid membranes, related to chemical stimuli; indeed, lipid membranes are extremely responsive to chemical stimuli, with particular regards to pH gradients [47], as experimentally proven by using synthetic membranes [47,103]. In this context, it is possible to change the pH gradient by introducing the interaction between different species, as verified experimentally, for example, for mutualism between *Chlorella vulgaris* and *Spirulina platensis* [104].

Proteins play a fundamental role in ion transport. Proteins in the cytosolic can be modified in their functions by phosphorylation or dephosphorylation. An ion actively crosses the membrane against its electrochemical potential, whereby the necessary energy is derived either from the hydrolysis of ATP, or from the movement of a cotransported, or coupled ion along its electrochemical gradient. In this context, the role played by the H^+ -ATPase is fundamental, because it moves positive charges into the cell, while it generates membrane voltage and a pH gradient [105–107]. Protein phosphorylation is an important

cellular regulatory mechanism, because many enzymes and receptors [51,108,109] are activated or deactivated by phosphorylation [110–113].

Moreover, the second fundamental quantity in Equation (10) is the coefficient of convection α [114], directly related to the characteristic length $\langle R \rangle = V/A$. This is a geometrical quantity, and it is related to the shape of the micro-organism. The geometrical characteristic of a system plays a fundamental role in the heat exchange. Recently, it has been shown its importance in the bacteria biological behaviour [115,116], pointing out also a fundamental role in the biological behaviour of the bacteria.

The last fundamental quantity for the behaviour of the micro-organisms is related to the control of the environmental temperature. It is possible to point out that hyperpolarisation is generated in relation to the temperature difference between micro-organisms and their environment, showing the fundamental role of the environmental temperature, and in accordance with the experimental data [46,117–119]: the biomass concentrations results for ethanol $3.43 \pm 0.08 \text{ g L}^{-1}$ and acetate $0.93 \pm 0.12 \text{ g L}^{-1}$ *Clostridium carboxidivorans* at 37 °C [120], higher than at 25 °C, (ethanol $1.58 \pm 0.03 \text{ g L}^{-1}$ and acetate $0.61 \pm 0.15 \text{ g L}^{-1}$ [120]).

In order to obtain biofuels from micro-organisms, all the above mentioned quantities result fundamental and, one of the main issues is how to increase their production, by modifying the micro-organisms behaviour. In relation to our result (Relation (15)), we can point out that a possible way to improve the production of biofuels by micro-organisms can be obtained by increasing the ion and metabolite fluxes, in relation to the heat flux. Indeed, in nature, it is possible to observe that mutual interactions between different species can improve or modify the micro-organisms metabolism and their metabolites fluxes [121]. As a matter of fact, in nature, microbial consortia are widely diffuse, where living organisms create a community [122–125]. Thus, the coexistence of more species in the same environment can lead to a phenomenon called symbiosis [126,127]. The interaction between different species can bring to different effects for the two symbionts, that can be positive or negative [128]: mutualism, cooperation, commensalism, predation, parasitism, amensalism, and competition. In this context, a possible improvement in biofuels production from micro-organisms can be obtained by exploiting the capability of different species to positively interact among them. The main communication/interaction path, among the symbionts, is just the mutual exchange of metabolites [129–136].

In some biotechnological applications, the creation of artificial consortia among different species of micro-organisms through cocultures, has been positively applied, obtaining an improvement for their relative end-products production [137–141].

4. Discussion and Conclusions

Thermodynamics can improve the comprehension of the microorganisms growth and, consequently, it can allow us to improve their productivity of chemical compounds useful to biofuels production. The thermodynamic black box model has been proved to be a good tool for the evaluation of the microbial growth. In particular, it is useful to define the conditions of optimal growth, in relation to the interaction between the microorganisms and their environment. However, this interaction is based on fluxes through the microorganisms membrane. Our results improve this approach, by analysing both the thermal and the electric fluxes. Indeed, the comprehension of the effects of the exogenous stimuli represents a fundamental improvement in the understanding of the bacterial electrophysiology [46,142,143].

So, biofuel and bioplastic production can be related to the proliferation, which is controlled just by membrane electric potential, driven by ATP synthesis [118,144–146]. The fundamental role of the membrane electric potential has been experimentally pointed out, by the analysis of the energy used by *Escherichia coli* to maintain its membrane electric potential; indeed, it results in around half of its total energy consumption [147].

Our results suggest explanation to some experimental evidence; indeed, an exogenous electrical stimulus has been shown to shape the proliferative capacity of bacteria, by inducing hyperpolarization in the cells [46].

Moreover, within the temperature range of life for the micro-organisms, higher temperature shortens the lag period and stimulates cell growth, in accordance with the microbial metabolism [148–152]. In our relation, the metabolism is taken into account by the surface temperature, while the environmental conditioning is expressed by the environmental temperature.

We highlight that a variation of the micro-organisms behaviour can be induced by the symbiosis between two different species. Indeed, in order to improve the formation of the useful high-value biomolecules, for biofuels production from micro-organisms, an interesting approach is to exploit the *natural* positive mutual interactions between different species. This could lead to a variation in their metabolites exchanges across their membranes and to a related change of the pH gradient [104]. For example, the cocultivation of two different species can bring to an enhancement of the lipid biomolecules inside the cells [153], which are the useful molecules employed for the biodiesel production [154]. Several studies have investigated the cocultivation of different micro-organisms, with the aim of improving their lipid concentration:

- In Ref. [155], the species *Chlorella pyrenoidosa* and *Rhodospiridium toruloides* have been cocultivated, obtaining $4.60 \text{ g}_{\text{lipid}} \text{ L}^{-1}$ (compared respectively to $3.00 \text{ g}_{\text{lipid}} \text{ L}^{-1}$ and $3.40 \text{ g}_{\text{lipid}} \text{ L}^{-1}$, respectively for each single species);
- In Ref. [156], the species *Spirulina platensis* and *Rhodotorula glutinis* have been cocultivated, obtaining $0.467 \text{ g}_{\text{lipid}} \text{ L}^{-1}$ (compared respectively to $0.013 \text{ g}_{\text{lipid}} \text{ L}^{-1}$ and $0.135 \text{ g}_{\text{lipid}} \text{ L}^{-1}$ for the single species);
- In Ref. [157], the species *Chlorella sp.* and *Toluraspore* have been cocultivated, obtaining $2.42 \text{ g}_{\text{lipid}} \text{ L}^{-1}$ (compared respectively to $0.052 \text{ g}_{\text{lipid}} \text{ L}^{-1}$ and $1.141 \text{ g}_{\text{lipid}} \text{ L}^{-1}$ for the single species);
- In Ref. [158], the species *Chlorella sp.* and *Monoraphidium sp.* have been cocultivated, obtaining an improved lipid productivity of $29.52 \pm 1.13 \text{ mg}_{\text{lipid}} \text{ L}^{-1} \text{ d}^{-1}$ (compared respectively to $17.99 \pm 3.39 \text{ mg}_{\text{lipid}} \text{ L}^{-1} \text{ d}^{-1}$ and $17.70 \pm 1.19 \text{ mg}_{\text{lipid}} \text{ L}^{-1} \text{ d}^{-1}$ for the single species).

These examples represent some application of the mutualism to produce biodiesel; other promising consortia involve the production of biohydrogen [159], biomethane [160], and bioethanol [161,162]. Thus, the establishment of consortia, such as the microalgae-bacteria ones, can be a powerful tool to improve microalgal biomass production and to enhance the production of the high-value compounds useful to the biofuels production [163]. These processes involve a large amount of exchanged metabolites, molecular signals, and transporters, which induce a variation in the fluxes exchanged by the micro-organisms themselves, on their membrane potential and on the existing pH gradient.

Our results are in agreement with the recent open problems highlighted in the new frontiers in microbiological researches for industrial use of bacteria [142]. Indeed, *Bacillus subtilis* is an example of industrialised bacterium to hydrolyze polypeptides through its secreted proteases and to convert amino acids into advanced biofuels and ammonia fertilizer [164]. Just in relation to *Bacillus subtilis*, some experiments of stimulation of the membrane have been developed by using an electric stimulus of $60 \text{ mV } \mu\text{m}^{-1}$ AC 0.1 kHz for 2.5 s [46]: a hyperpolarization response has been shown, concluding that electrical stimulation causes the efflux of K^+ cations. The authors pointed out that *Bacillus subtilis* reacts to external stimuli by maintaining the resting-state membrane potential, but to do so it consumes a constant amount of ATP in order to keep the intracellular K^+ level. Moreover, the opening of voltage-gated K^+ channels, with the related hyperpolarization due to K^+ efflux, has an effect on the proliferative capacity of the cell [46]. These experimental results represent a further proof of our thermophysical results and considerations. Indeed, cell membranes contain enzymes complexes which further the oxidative phosphorylation

along which electrical potential of H⁺ ions, and chemical potential of reduced transporters (i.e., NADH, etc.), play a fundamental role in ATP production. Last, we can highlight that mutual interactions among different species can represent an interesting approach to improving the production of biofuels and must be considered an important topic of investigation for future development in optimisation of biofuels production.

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