Hydrogen production by the hyperthermophilic bacterium *Thermotoga maritima* Part II: modeling and experimental approaches for hydrogen production

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**Abstract**

**Background:** *Thermotoga maritima* is a hyperthermophilic bacterium known to produce hydrogen from a large variety of substrates. The aim of the present study is to propose a mathematical model incorporating kinematics of growth, consumption of substrates, product formations, and inhibition by hydrogen in order to predict hydrogen production depending on defined culture conditions.

**Results:** Our mathematical model, incorporating data concerning growth, substrates, and products, was developed to predict hydrogen production from batch fermentations of the hyperthermophilic bacterium, *T. maritima*. It includes the inhibition by hydrogen and the liquid-to-gas mass transfer of H₂, CO₂, and H₂S. Most kinetic parameters of the model were obtained from batch experiments without any fitting. The mathematical model is adequate for glucose, yeast extract, and thiosulfate concentrations ranging from 2.5 to 20 mmol/L, 0.2–0.5 g/L, or 0.01–0.06 mmol/L, respectively, corresponding to one of these compounds being the growth-limiting factor of *T. maritima*. When glucose, yeast extract, and thiosulfate concentrations are all higher than these ranges, the model overestimates all the variables. In the window of the model validity, predictions of the model show that the combination of both variables (increase in limiting factor concentration and in inlet gas stream) leads up to a twofold increase of the maximum H₂-specific productivity with the lowest inhibition.

**Conclusions:** A mathematical model predicting H₂ production in *T. maritima* was successfully designed and confirmed in this study. However, it shows the limit of validity of such mathematical models. Their limit of applicability must take into account the range of validity in which the parameters were established.

**Keywords:** *Thermotoga maritima*, Hyperthermophile, Hydrogen, Modeling, Inhibition

**Background**

Because of the increasing demand for energy, due to economic and population rapid growths and because of the damaging effect of the fossil energies on the environment (global warming), governments are focusing on alternative energy sources for fuels. In response to this dual problem (depletion and pollution), the development of a new, more environmentally friendly, and healthy energy is necessary.

Presently, the development of biofuels from renewable plant biomass is a way to reduce fossil fuel consumption. Among the potential biofuels, hydrogen appears as one of the energy sources for the future. Indeed, hydrogen is highly reactive, with high energy density (122 MJ/kg, compared to 50.1 MJ/kg for methane, 29.7 MJ/kg for ethanol, and 47.3 MJ/kg for gasoline) and is directly convertible into electricity with high efficiency (>80%). In addition, it is a low-carbon fuel which combustion...
produces only water, making it an excellent candidate in terms of environmental impact. Overall, the use of hydrogen shows a 10% growth per year, leading to represent 8–10% of total energy in 2025.

Currently, hydrogen production is closely dependent on fossil fuels (natural gas and hydrocarbons). However, new approaches for hydrogen production, such as biophotolysis, photofermentation, and dark fermentation, offer less costly technological solutions in terms of energy balance and are friendlier to the environment. Among these techniques, dark fermentation is of great interest because it allows the biodegradation of complex residues using a broad spectrum of microorganisms and enzymes. In addition, it is performed with abundant, inexpensive, renewable, and biodegradable agricultural waste [1].

To be economically viable, one of the main challenges of dark fermentation is to achieve both high hydrogen productivity and yield. Hydrogen is produced by both mesophile and (hyper) thermophile anaerobic bacteria. In general, the latter ones show slightly lower hydrogen production rates but higher yields. The elevated temperature (70–110 °C) has several advantages by reducing (1) the hydrogen solubility which is known to be a strong inhibitor of growth [2–4], (2) the variety in fermentation by-products [5] and (3) the sensitivity to contamination by H₂-consumer and pathogen bacteria present in the waste. Moreover, the high temperature promotes the enzymatic hydrolysis of a wide range of carbohydrates (starch, cellulose, hemicelluloses...) [6–9]. Among the hyperthermophilic anaerobic hydrogen-producing bacteria (>80 °C), *Thermotoga* were one of the most studied [6, 10–12]. Several *Thermotoga* sp. (*neapolitana, maritima, elfii, petrophila, naphthophila,...*) metabolized glucose with high hydrogen yields of 3–4 mol/mol [6, 12–16]. Maximal hydrogen productivity of some *Thermotoga* strains was reported between 2.7 and 12.4 mmol/L h [6, 14, 16, 17]. Higher hydrogen productivity requires determining the conditions influencing the growth and the metabolism of the *Thermotogales*. Among them, optimal concentrations of glucose, yeast, sulfur, dissolved hydrogen, carbon dioxide, etc., have to be established [6, 16, 17]. Most *Thermotoga* species have been reported to reduce elemental sulfur to H₂S [18, 19]. Huber et al. [20] proposed that the addition of elemental sulfur stimulates the growth of *T. maritima* on glucose by reducing the inhibitory effect of hydrogen. Schroder et al. [6] supported this hypothesis by showing that, on glucose, sulfur reduction to H₂S stimulated *T. maritima* growth. On the contrary, Boileau et al. [part I, 21] showed that the addition of small amounts of thiourea, as well as some other sulfur sources, allowed a significant increase of *T. maritima* growth and its hydrogen production. These authors confirmed that the sulfur compound was not used in a detoxification process but rather was assimilated by *T. maritima* leading to an increase in biomass, and therefore in the amount of hydrogen produced.

To improve the understanding of the biological and physical mechanisms that govern the hydrogen production by dark fermentation, mathematical modeling can be an appropriate tool. The aim of the present study is to propose a mathematical model incorporating kinetics of growth, consumption of substrates, product formations, and inhibition by hydrogen in order to predict hydrogen production depending on defined culture conditions.

To the best of our knowledge, the most comprehensive kinetic model predicting the hydrogen production by a thermophilic bacterium was proposed by Ljunggren et al. [3]. This kinetic model takes into account the microbial growth of the extreme thermophilic *Caldicellulosiruptor saccharolyticus*, its substrate consumption and product formations and, the liquid-to-gas mass transfer. This model predicted high oversaturation of hydrogen in the liquid (12–34 times the equilibrium concentration) comparable to the experimentally obtained values. The authors have shown that the dissolved hydrogen concentration was a function of the stripping rate and the hydrogen productivity. In the present study, a mathematical model was developed to predict hydrogen production from batch fermentations of the hyperthermophilic bacterium, *T. maritima*. This model incorporates the kinetics of growth, consumptions of substrates (glucose, yeast, and thiosulfate), and product formations (H₂, CO₂, H₂S, acetate, and lactate). It includes the inhibition by hydrogen and the liquid-to-gas mass transfer of H₂, CO₂, and H₂S. Most kinetic parameters of the model were obtained from batch experiments without any fitting. The limits of its applicability were clearly established.

**Methods**

**Strain and culture medium**

*Thermotoga maritima* strain MSB8 (DSMZ 3109) was cultivated as previously described [part I, 21]. Basal medium containing, per liter: NH₄Cl 0.5 g, K₂HPO₄ 0.3 g, 0.3 g, CaCl₂ 0.1 g, KCl 0.1 g, NaCl 20 g, MgCl₂ 0.2 g, yeast extract 1.0 g, and glucose 20 mmol/L was used. Batch trace mineral element solution (10 mL) was added [part I, 21]. The inoculum was obtained from three bottles of 100 mL each, containing 50 mL of liquid culture.

**Experimental system, operating conditions, and analytical methods**

Experimental system, operating conditions, and analytical methods were specified [part I, 21]. *T. maritima* was batch cultivated in a similar 2-L double-jacket glass bioreactor (FairMenTec, France) with a 1.5-L working volume. The temperature was maintained constant at
80 ± 1 °C and pH was controlled at 7 ± 0.1 by the addition of sodium hydroxide (NaOH 0.5 mmol/L). The inlet gas stream of N₂ was controlled via a mass-flow meter (Bronkhorst, range 0–500 SCCM), and the one composed of the mixture of N₂ and H₂ was prepared using two mass-flow meters (Bronkhorst, range 0–500 SCCM and 0–100 SCCM, The Netherlands). The stirring was set to 350 rpm. The online measurements of CO₂, H₂, and H₂S concentrations, bioreactor liquid volume, and NaOH consumption are described [part I, 21].

For each experiment, three successive batches were carried out. The first batch was always considered as an adaptation batch, the following two being in general well reproducible.

OD (Optical Density), hydrogen, glucose, acetate, lactate, and hydrogen sulfide concentrations were determined as previously described [part I, 21]. Cell dry weight was calculated from OD data using the same relation of 1 OD unit = 330 mg/L.

Thiosulfate concentration was quantified by ion chromatography (761 Compact IC Metrohm, Metrohm, Villebon-sur-Yvette, France) equipped with a Metrosep Anion Supp1 column (Metrohm).

**Mathematical model**

The model developed in this study incorporates the kinetics of growth of *T. maritima*, glucose, yeast extract, and thiosulfate consumptions and product, such as hydrogen (H₂), carbon dioxide (CO₂), hydrogen sulfide (H₂S), acetate, and lactate formations. It takes into account the transfer of H₂, CO₂, and H₂S, as well as the chemical equilibrium between CO₂ and bicarbonates (HCO₃⁻) and between H₂S and hydrosulfide ions (HS⁻).

For the operational parameters of this study, the main end products, resulting from the anaerobic fermentation of glucose by *T. maritima*, are acetate, lactate, H₂, CO₂, biomass, and EPS (Extracellular polysaccharides) [22]. In this case, the following biochemical reaction occurring during the fermentation is:

\[
\text{C}_6\text{H}_{12}\text{O}_6 + k \text{ H}_2\text{O} \rightarrow a \text{ C}_2\text{H}_4\text{O}_2 + b \text{ C}_3\text{H}_6\text{O}_3 + 2a\text{H}_2 + a \text{ CO}_2 + c \text{ Biomass} + d \text{EPS}
\]  

(1)

The value of the stoichiometric parameters \(a, b, c,\) and \(d\) in the Eq. 1 are unknown. The parameter \(a\) was estimated using a methodology described in the Determination of kinetic and mass transfer parameters section. Glucose fermentation in *T. maritima*, under controlled physicochemical conditions, shows that about 5 and 22% of the consumed glucose are converted into biomass and EPS, respectively [22]. These two values of percentages were used to evaluate \(c\) and \(d\) parameters. The stoichiometric parameter \(b\) relates to the lactate production and was determined by the difference between the amount of carbon from the consumed glucose and the amount of carbon found in all the end products: acetate, carbon dioxide, biomass, and EPS.

At low concentration, thiosulfate is a sulfured nutrient for *T. maritima* growth used for the synthesis of cellular materials [part I, 21]. Thus, this sulfur is incorporated to the biomass according to the elemental composition of *T. maritima* (CH₁₆O₀₆N₀₂S₀₀₅) determined by Rinker and Kelly [23].

The low amount of remaining sulfur fuel to the production of hydrogen sulfide as follows:

\[
\text{S}_2\text{O}_3^{2-} + 4\text{H}_2 \rightarrow 2\text{H}_2\text{S} + \text{H}_2\text{O} + 2\text{OH}^-
\]  

(2)

**Growth kinetics, substrate, and nutrient consumptions**

The specific growth rate (\(\mu\)) is given by Monod kinetics. It depends on the glucose (Glu), yeast extract (Yeast), thiosulfate (Thio), dissolved-H₂ ([H₂]) concentrations and the equivalent concentrations of glucose (\(\epsilon_1\)) and thiosulfate (\(\epsilon_2\)) in the yeast extract. Although *T. maritima* can grow (slightly) without yeast extract [part I, 21], we consider that yeast extract is essential for the growth as a first approximation. The mass balance for *T. maritima* growth is described by the following expression:

\[
\frac{dX}{dt} = (\mu - \mu_d)X
\]  

(3)

\[
\mu = \mu_{max}\left(\frac{\text{Glu} + \epsilon_1}{(\text{Glu} + \epsilon_1) + K_{s\text{glu}}}\right)\left(\frac{\text{Yeast}}{\text{Yeast} + K_{s\text{yest}}}\right)\left(\frac{\text{Thio} + \epsilon_2}{(\text{Thio} + \epsilon_2) + K_{s\text{thio}}}\right)\left(1 - \left(\frac{[\text{H}_2]}{[\text{H}_2]_{crit}}\right)^N\right)
\]  

(4)

where \(X\) and \(\mu_{max}\) are the cell mass concentration and the maximum specific growth, respectively. \(\mu_d\) is the cell death rate. \([\text{H}_2]_{crit}\) is the critical dissolved-H₂ concentration for which inhibition is 100%. \(N\) is the exponential parameter describing the level of inhibition. \(K_{s\text{glu}}, K_{s\text{yest}}, K_{s\text{thio}}\) are the saturation constants of glucose, yeast extract, and thiosulfate.

The mass balance of glucose (Eq. 5), yeast extract (Eq. 6), and thiosulfate (Eq. 7) can be written as follows:

\[
\frac{d(\text{Glu} + \epsilon_1)}{dt} = -\left(\frac{\mu}{Y_{\text{X/GLU}}} + m_{\text{GLU}}\right)X
\]  

(5)

\[
\frac{d\text{Yeast}}{dt} = -\frac{\mu X}{Y_{\text{X/YEAST}}}
\]  

(6)

\[
\frac{d(\text{Thio} + \epsilon_2)}{dt} = -\frac{\mu X}{Y_{\text{X/THIO}}}
\]  

(7)

with : \(Y_{\text{X/THIO}} = Y_{\text{X/THIO}}^X + Y_{\text{X/THIO}}^{H_2S}\)
Y_{X/GLU}, Y_{X/YEAST} and Y_{X/THIO} are the yields of biomass on glucose, yeast extract, and thiosulfate, respectively. m_{GLU} is the maintenance coefficient [ratio between the consumption rate of glucose and maximum biomass determined at the end of the growth when the specific growth rate (μ) is close to 0]. Y_{X/THIO} is the sum of two yields: the equivalent sulfur from thiosulfate incorporated into the biomass (Y_{X/GLU}^S) and the equivalent sulfur from thiosulfate released as H_2S (Y_{X/THIO}^S).

Product formation in the liquid phase
The products formed during the fermentation are acetate, lactate, hydrogen, carbon dioxide, and hydrogen sulfide. The product formation rates are expressed using the following equations:

\[
\frac{d\text{Act}}{dt} = Y_{\text{ACT/GLU}} \left( \frac{\mu}{Y_{X/GLU}} + m_{\text{GLU}} \right) X
\]

and Eq. 10 for the determination of lactate (obtained from the Eq. 1):

\[
\frac{d\text{Lact}}{dt} = \frac{d(\text{Glul} + \varepsilon_1)}{dt} (6Y_{\text{ACT/GLU}} - 1.62) - 2 \frac{d\text{Act}}{dt} - \frac{d[\text{CO}_2]}{dt}
\]

\[
\frac{d[H_2]}{dt} = -K_{\text{H2}} ([H_2] - [H_2]^*) + Y_{\text{H2/GLU}} \left( \frac{\mu}{Y_{X/GLU}} + m_{\text{GLU}} \right) X - \frac{Y_{\text{H2S/THIO}} Y_{\text{H2S}}}{{Y_{X/GLU}}^S} \mu X
\]

\[
\frac{d[\text{CO}_2]}{dt} = -K_{\text{CO}_2} ([\text{CO}_2] - [\text{CO}_2]^*) + Y_{\text{CO}_2/GLU} \left( \frac{\mu}{Y_{X/GLU}} + m_{\text{GLU}} \right) X - \frac{K_1}{10^{-p\text{H}}}[\text{CO}_2] + [\text{HCO}_3^-]
\]

\[
\frac{d[H_2S]}{dt} = -K_{\text{H2S}} ([H_2S] - [H_2S]^*) + Y_{\text{H2S/THIO}} \left( \frac{\mu}{Y_{X/GLU}} + m_{\text{GLU}} \right) X - \frac{K_2}{10^{-p\text{H}}}[H_2S] + [\text{HS}^-]
\]

\[
\frac{d[\text{HCO}_3^-]}{dt} = \frac{K_1}{10^{-p\text{H}}}[\text{CO}_2] - [\text{HCO}_3^-]
\]

\[
\frac{d[\text{HS}^-]}{dt} = \frac{K_2}{10^{-p\text{H}}}[H_2S] - [\text{HS}^-]
\]

Y_{\text{ACT/GLU}}, [H_2], [\text{CO}_2], [\text{H}_2\text{S}], [\text{HCO}_3^-] and, [\text{HS}^-] are the concentrations in the liquid phase of acetate, lactate, hydrogen, carbon dioxide, hydrogen sulfide, bicarbonate, and bisulfide ions, respectively. [H_2]^*, [\text{CO}_2]^* and, [H_2S]^* are the dissolved concentrations of these compounds at equilibrium. Y_{\text{ACT/GLU}}^H, Y_{\text{CO}_2/GLU}^H, Y_{\text{H2S/GLU}}^H, and Y_{\text{H2S/THIO}}^H are the stoichiometric H_2 on H_2S yield and the stoichiometric H_2S on thiosulfate yield (Eq. 2), respectively.

K_{\text{H2}}, K_{\text{CO}_2}, and K_{\text{H2S}} represent the volumetric mass transfer coefficients for H_2, CO_2, and H_2S. K_1 and K_2 are the dissociation constants.

Here, H_2 inhibits its own production and consequently its acetate production. Thereby, Y_{\text{ACT/GLU}} can be written as follows [3]:

\[
Y_{\text{ACT/GLU}} = Y_{\text{max ACT/GLU}}^\text{max} \left( 1 - \left( \frac{[\text{H}_2]}{[\text{H}_2]^*} \right)^N \right)
\]

The maximum yield of acetate on glucose Y_{\text{ACT/GLU}}^\text{max} was estimated from experiments (see Determination of kinetic and mass transfer parameters) for H_2 percentage in the gas phase equal to 0. Y_{\text{ACT/GLU}} and Y_{\text{CO}_2/GLU} were deduced from Y_{\text{ACT/GLU}} using the Eq. 1.

Mass balance in the gas phase
Hydrogen, carbon dioxide, and hydrogen sulfide are produced in the liquid phase and then transferred into the gas phase. The mass balance of these gaseous compounds can be expressed as follows:

\[
\frac{d[H_2]}{dt} = \frac{V_1}{V_g} K_{\text{H2}} ([H_2] - [H_2]^*) - \frac{Q_g}{V_g} H_2
\]

\[
\frac{d[\text{CO}_2]}{dt} = \frac{V_1}{V_g} K_{\text{CO}_2} ([\text{CO}_2] - [\text{CO}_2]^*) - \frac{Q_g}{V_g} \text{CO}_2
\]

\[
\frac{d[H_2S]}{dt} = \frac{V_1}{V_g} K_{\text{H2S}} ([H_2S] - [H_2S]^*) - \frac{Q_g}{V_g} H_2S
\]

Q_g = Q_{N_2} + Q_{H_2} + Q_{\text{CO}_2} + Q_{H_2S}

Q_{N_2}, Q_{H_2}, Q_{\text{CO}_2} and, Q_{H_2S} are the inlet gas (N_2) flow rate, H_2, CO_2, and H_2S gas flow rate productions, respectively.

Equilibrium constants and stoichiometric equations
The thermodynamic equilibrium of the dissolved compounds [H_2], [CO_2], and, [H_2S] is described by the Henry’s law:

\[
\text{Henry’s law:}
\]

\[
\text{Equilibrium constants:}
\]

\[
\text{Stoichiometric equations:}
\]
[H₂]⁺ = Kh₂R T H₂ \hspace{1cm} (21)

[CO₂]⁺ = KCO₂R TCO₂ \hspace{1cm} (22)

[H₂S]⁺ = Kh₂s R T H₂S \hspace{1cm} (23)

Kₘ (m = h₂, co₂, h₂s) are the H₂, CO₂, and H₂S Henry’s constants. The dissociations of [HCO₃⁻] into [CO₂⁻] and [HS⁻] into [S²⁻] are considered negligible at pH = 7. The reactions of conversion between [CO₂], [H₂S] and [HS⁻] and the corresponding dissociation constants K₁ and K₂ are as follows:

\[ \text{[CO₂]} + \text{[H₂O]} \rightleftharpoons \text{[H⁺]} + \text{[HCO₃⁻]} \] \hspace{1cm} (24)

\[ \text{[H₂S]} \rightleftharpoons \text{[H⁺]} + \text{[HS⁻]} \] \hspace{1cm} (25)

\[ K₁ = \frac{\text{[H⁺]} \cdot \text{[HCO₃⁻]}}{\text{[CO₂]}} \] \hspace{1cm} (26)

\[ K₂ = \frac{\text{[H⁺]} \cdot \text{[HS⁻]}}{\text{[H₂S]}} \] \hspace{1cm} (27)

The constants used in the model are presented in Table 1.

## Results and discussion

### Determination of kinetic and mass transfer parameters

#### Yields and kinetic parameters

To determine \( \mu_{max} \), \( \mu_d Y_{X/GLU} \), \( Y_{X/YEAST} \), \( Y_{X/THIO} \), \( K_{sglu} \), \( K_{syeast} \) and, \( K_{sthio} \), calculations were carried out with experimental data from batch fermentations (2-L bioreactor). All these parameters are listed in Table 2. \( \mu_{max} \) and \( \mu_d \) were obtained for yeast extract, thiosulfate, and glucose concentrations of 4 g/L, 0.12, and 60 mmol/L, respectively with a stripping rate of 100 mL/min. The maximum growth rate of \( T. maritima \) (\( \mu_{max} = 0.7 - 0.9 \ h^{-1} \)), measured in this study, was comparable to those obtained by Huber et al. [20] for \( T. maritima \) (0.6 h⁻¹) and, \( T. naphthophila \) (0.7 h⁻¹) and \( T. petrophila \) (0.77 h⁻¹), two species very closely related to \( T. maritima \) [13]. Since, in these experiments, the conditions were meant to allow obtaining the highest possible \( \mu_{max} \) and in the present model, \( \mu_{max} \) was chosen at 0.9 h⁻¹. This value is higher than the ones obtained with \( C. saccharolyticus \) (0.2–0.5 h⁻¹), an extreme thermophile \( H₂ \)-producing bacterium among the most studied [3, 24, 25]. This fairly high growth rate is an advantage for this \( H₂ \) producer that allows the reduction of the \( H₂ \)-production time. For our model, the average value of \( \mu_d \) obtained from experiments, was estimated at 0.05 h⁻¹. Very few values of cell death rate (\( \mu_d \)) of hyperthermophilic microorganisms are available in the literature. This parameter ranges from 0.014 to 0.105 h⁻¹ [3, unpublished data].

\( Y_{X/GLU} \) and \( K_{sglu} \) were evaluated in batch cultures for a range of glucose concentrations between 0.3 and 60 mmol/L with thiosulfate and yeast concentrations of 0.12 mmol/L and 1 g/L, respectively. Figure 1 represents biomass content versus glucose concentration and shows that below 20 mmol/L, glucose was the sole nutritional factor limiting \( T. maritima \) growth. Then total biomass yield on glucose (20.4 g biomass/mol glucose) was evaluated from a linear regression (20.4 Glu + 11.4) in this first part. The true \( Y_{X/GLU} \) was determined from this total biomass yield after subtracting, from the quantity of glucose consumed, the part used for the maintenance. A resulting value of 20.9 g biomass/mol glucose was obtained by taking into account the maintenance coefficient (2.2 mmol/g/h) and \( \mu_{max}(0.9 \ h^{-1}) \) (Table 2). \( Y_{X/S} \) is comparable to those previously determined in batch or chemostat for Thermotogales and Thermococcales [6]. These values ranged from 13 to 45 g biomass/mol glucose. From growth kinetic data, \( K_{sglu} \) was estimated at 5.7 mmol/L (Additional file 1: Fig. S1). This value of \( K_{sglu} \) was much higher than the one obtained by Rinker and Kelly [23] (0.015 mmol/L) for \( T. maritima \) culture in chemostat. This difference may be linked to the fact that all values of \( K_s \) were determined in reactor batch cultures and did not represent a \( K_s \) per se but an apparent \( K_s \). From now on, the constant \( K_s \) will in reality correspond to \( K_{s,app} \).

\( Y_{X/YEAST} \) and \( Y_{syeast} \) were determined for different concentrations of yeast extract (0.2, 0.5, 1, 2, 4, and 8 g/L) with thiosulfate concentrations of 0 or 0.12 mmol/L and glucose concentration of 60 mmol/L. Effect of yeast extract concentration on \( T. maritima \) growth is presented in Fig. 2. Without thiosulfate and up to 8 g/L of yeast extract, maximum biomass is always lower than...
Table 2 Parameters of the model

| Parameter     | Value                  |
|---------------|------------------------|
| $\mu_{\text{max}}$ (h$^{-1}$) | 0.9 ± 0.05             |
| $\mu_d$ (h$^{-1}$)         | 0.05 ± 0.02            |
| $m_{\text{GLU}}$* (mmol/g/h) | 2.2                    |
| $Y_{X/\text{GLU}}^*$ (g/mol) | 209 ± 3.2              |
| $Y_{X/YEAST}$ (g/g)      | 0.67 ± 0.11            |
| $Y_{X/\text{THIO}}^*$ (g/mmol) | 3.617 ± 0.18         |
| $K_{\text{glu}}$ (mmol/L) | 5.7 ± 1.1              |
| $K_{\text{yeast}}$ (g/L) | 0.30 ± 0.1             |
| $K_{\text{thio}}$ (mmol/L) | 0.05 ± 0.01            |
| $[\text{H}_2\text{crit}]$ (mmol/L) | 1.44 ± 0.01         |

* Boileau et al. [part I, 21].
** Rinker et Kelly [23]. This value of $m_{\text{GLU}}$ was obtained for T. maritima culture in chemostat.

a $Y_{X/\text{GLU}}$ used for the mathematical model was of 20.9 g/mol. This value was obtained by subtracting the quantity of glucose used for the maintenance. The following values were used: $Y_{X/\text{GLU}} = 20.4$ g/mol, $m_{\text{GLU}} = 2.2$ mmol/g/h and $\mu = \mu_{\text{max}} = 0.9$ h$^{-1}$. 
when 0.12 mmol/L of thiosulfate was added. For this range of yeast extract concentrations, thiosulfate is the growth-limiting factor of *T. maritima* confirming the significant effect of thiosulfate addition on cell mass growth [part I, 21]. Moreover, when 0.12 mmol/L of thiosulfate is added and below 0.5 g/L of yeast extract, thiosulfate is the only growth-limiting factor (Fig. 2). Therefore, \( Y_{X/\text{YEAST}} \) was determined in this window and was equal to 0.67 g biomass/g YE (Table 2). It is noteworthy that \( Y_{X/\text{YEAST}} \) decreases to 0.11 g/g in absence of thiosulfate (Fig. 2) showing that \( Y_{X/\text{YEAST}} \) depends on the thiosulfate concentration. However, due to the lack of data between 0 and 0.12 mmol/L of thiosulfate, no relation of \( Y_{X/\text{YEAST}} \) versus thiosulfate could be established. For the following of this study, \( Y_{X/\text{YEAST}} \) was considered constant and equal to 0.67 g/g. \( K_{\text{Yes}} \) of 0.3 g/L was obtained in this study from batch experiments (Additional file 1: Fig. S2).

\( Y_{X/\text{THIO}} \) of 3.167 g/mmol was obtained by Boileau et al. [part I, 21] from batch culture experiments. \( Y_{X/\text{THIO}} \) (Eq. 8) represents the equivalent sulfur of the thiosulfate incorporated into the biomass and was calculated from the elemental composition of *T. maritima* [23]. It is equal to 10.47 g biomass/mmol thiosulfate. \( Y_{X/\text{THIO}} \) was determined from the difference between the total yield of thiosulfate \( Y_{X/\text{THIO}} \) and \( Y_{X/\text{THIO}} \) considering that all sulfur not incorporated into the biomass was reduced into H\(_2\)S (Eq. 2). \( Y_{X/\text{THIO}} \) was equal to 5.52 g biomass/mmol thiosulfate. \( K_{\text{thio}} \) obtained in this study from batch experiments, was of 0.052 mmol/L (Additional file 1: Fig. S3).

In this study, two sulfur sources for *T. maritima* growth are available: thiosulfate added to the culture medium and sulfur (cysteine, etc…) in the yeast extract. In the previous paper [part I, 21], the authors determined an equivalent thiosulfate concentration of 0.03 mmol per g of yeast extract (\( \varepsilon_{2} \), Eq. 7). A similar approach was used to determine the equivalent content of glucose in 1 g of yeast extract (Fig. 1). This value was obtained using the linear regression \( X = 20.4 \text{ Glu} + 11.4 \) by extrapolating the line to a cell mass concentration (\( X \)) equal to 0. Therefore, the equivalent glucose per g of yeast extract was of 0.56 mmol (\( \varepsilon_{1} \), Eq. 5).

**Determination of \( Y_{ACT/GLU} \) and \( [H_{2}cor] \) the critical dissolved-H\(_2\) concentration**

\( Y_{\text{ACT/GLU}} \) (stoichiometric parameter \( a \), Eq. 1) was determined with different percentages of inlet H\(_2\) (0, 5, 10, 25, 50, 75, and 100%), for yeast extract, thiosulfate, and glucose concentrations of 4 g/L, 0.12, and 60 mmol/L, respectively. The stripping rate was 100 mL/min. Fig. 3 shows that up to 100% of H\(_2\), a linear relation of \( Y_{\text{ACT/GLU}} \) versus the percentage of H\(_2\) in the gas phase was observed. By linear extrapolation (\( Y_{\text{ACT/GLU}} = 0 \)), critical H\(_2\) percentage in the gas phase was evaluated at 190%, above the value of the expected 100%. In the same way, the linear relation of \( \mu/\mu_{\text{max}} \) versus the percentage Figure 1: Effect of glucose concentration on *T. maritima* growth cultivated in presence of thiosulfate (0.12 mmol/L) and yeast extract (1 g/L). These cultures were performed in triplicate in bioreactor. Total biomass yield on glucose (20.4 g biomass/mmol glucose) was evaluated from a linear regression (20.4 Glu + 11.4).

![Figure 1](image1.png)

![Figure 2](image2.png)
of H₂ in the gas phase can be extrapolated (μ/μₘₐₓ = 0), and leads to a critical H₂ percentage in the gas phase at 206%. For these two extrapolated H₂ percentages in the gas phase, the critical dissolved hydrogen concentrations ([H₂ₜᵣᵢₚ]) in equilibrium with the critical H₂ concentrations in the gas phase are of 1.33 and 1.44 mmol/L, respectively. [H₂ₜᵣᵢₚ]° has been evaluated, and in the same way, the true critical dissolved hydrogen ([H₂ₜᵣᵢₚ]) cannot be measured. However, for the theoretical case where μ/μₘₐₓ and Y_{ACT/GLU} are both equal to 0, no gradient of H₂ in the liquid phase takes place and consequently [H₂ₜᵣᵢₚ]° is equal to [H₂ₜᵣᵢₚ]. It is noteworthy that the estimation of [H₂ₜᵣᵢₚ] (1.3–2.2 mmol/L) obtained from a kinetic model of the effect of hydrogen and osmolarity on hydrogen production by C. saccharolyticus [3] were coherent with our estimates (1.3–1.4 mmol/L).

Y_{ACT/GLU}^max was obtained from the experimental correlations for H₂ = 0% (Fig. 3). It is equal to 1.38 mol/mol. Y_{H₂/GLU}^max and Y_{CO₂/GLU}^max were deduced from Y_{ACT/GLU}^max using the Eq. 1. Thus, Y_{H₂/GLU}^max and Y_{CO₂/GLU}^max are equal to 2.76 and 1.38 mol/mol, respectively. Mars et al. [16] measured similar values of Y_{ACT/GLU}^max (1.4 mol/mol), Y_{H₂/GLU}^max (2.9 mol/mol), and Y_{CO₂/GLU}^max (1.6 mol/mol) for T. neapolitana growing on glucose in batch culture. Values of Y_{H₂S/THIO} and Y_{H₂/H₂S} were deduced using the Eq. 2. Both values are equal to 2 mol/mol.

Because dissolved H₂ ([H₂]) cannot be measured, the parameter N (Eq. 4) cannot be directly evaluated. Data sets from five experiments were used to evaluate the maximum of H₂ productivity, which was compared to the maximum H₂ productivity obtained from the mathematical model for N values of 0.5, 1, and 1.5. For these experiments, at least one substrate (thiosulfate, glucose, or yeast extract) is the growth-limiting factor [part I, 21]. For each experimental condition, the average of the difference between the maximum of H₂ productivity obtained from the model and from the experiment was -2.2, 0.2, and 1.5 mmol/L/h for N equal to 0.5, 1, and 1.5, respectively. Ljunggren et al. [3] estimated, using another approach with C. saccharolyticus, the parameter N, from experiments with 5 g/L of glucose and different stripping rates, ranged from 20 to 100 mL/min. These authors obtained a mean value of N (4.5) with a large standard deviation (0.84–16.24). For the rest of our study, a value of N equal to 1 was chosen.

**Determination of the volumetric mass transfer coefficient**

Since H₂, CO₂, and H₂S are sparingly soluble gases, the gas phase mass transfer resistance can almost always be neglected [26]. Thereby, the overall volumetric mass transfer coefficient, Kla, is adequate for describing the mass transfer. Assuming that the mass transfer coefficients of the different gases in the water are proportional to the square root of their diffusivity, the Kla values of H₂, CO₂, and H₂S can be calculated on the basis of the experimental data obtained from the determination of the Kla O₂ (Eq. 29).

\[
\text{KlaG} = \text{KlaO}_2 \left( \frac{D_G}{D_{O_2}} \right)^{0.5} \quad \text{with G = H}_2, \text{ CO}_2 \text{ or H}_2\text{S}
\]

(28)

\[D_G= \text{H}_2, \text{ CO}_2, \text{ H}_2\text{S} \] are the H₂, CO₂, and H₂S coefficients of diffusion in water at 80 °C (Table 1).

KlaO₂ was determined by measuring the dissolved O₂ concentration in the sterile culture medium [27]. It was obtained in the 2-L bioreactor by either physical absorption or desorption of oxygen. Air-flow rate was tested in a range from 20 to 500 mL/min at a constant-speed agitation (350 rpm).

The correlation between KlaO₂ and Q_{N_2} is as follows:

\[
\text{KlaO}_2 = 18.11 \ Q_{N_2}^{0.721} \quad \text{with Q}_{N_2} \text{ in L/h}
\]

(29)

From the Eqs. 28 and 29, the KlaH₂ was of 114 h⁻¹ for a Q_{N_2} of 100 mL/min and a stirring rate of 350 rpm. This value is significantly higher than those usually reported in the literature for similar systems [3, 28]. Ljunggren et al. [3] measured a KlaH₂ value of 9 h⁻¹ at 70 °C for the same Q_{N_2} and stirring rate. A KlaH₂ of 17 h⁻¹ was obtained for Q_{N_2} of 100 mL/min, but for lower temperature (35 °C) and with an unknown value of stirring rate (use of a stir plate) [28]. In our study, the higher KlaH₂ could be explained by the following: (1) the high-temperature experiments (80 °C) increasing the diffusion...
coefficients of H₂ in water, (2) the use of a 3-cm-long fritted cylinder gas-dispersion stone with 4–60 μM pore size allowing the formation of numerous very small bubbles of gas and, (3) the use of two axial impellers promoting an effective mixing. KLaCO₂ and KLaH₂S were determined in the same way as KLaH₂.

Model validation
The mathematical model developed in this study must be able to both provide new knowledge and predict the optimal operating conditions of the H₂ production by T. maritima. For this, the mathematical model was validated for various glucose, yeast extract, and thiosulfate concentrations and inlet N₂ flow rates. Forty experiments were carried out with different concentration ranges of glucose (2.5–63 mmol/L), yeast extract (0.2–8 g/L), thiosulfate (0.01–2 mM), and inlet N₂ flow rates (17–190 mL/min). These various operating conditions correspond to situations where one or none of these compounds (glucose, yeast extract, and thiosulfate) is the limiting-growth factor.

Figure 4a–e represents the comparison between the experimental and model results for acetate, lactate, H₂ and CO₂ productions, and the maximum of biomass, respectively. It is noteworthy that, for the five comparisons, a same pattern can be observed. When one of the compounds of interest is at a concentration for which it is the limiting-growth factor, a good correlation between the experiment and the model is noted. On the contrary, when all the concentrations are above the limiting factor level, the model overestimates the productions. The mathematical model is adequate for glucose, yeast extract, and thiosulfate concentrations ranging from 2.5 to 20 mmol/L, 0.2–0.5 g/L, and 0.01–0.06 mM/L, respectively. As expected, these ranges correspond to the value for which Yₓ/GLU, Yₓ/YEAST, Yₓ/THIO were determined experimentally (Table 2). When glucose, yeast extract, and thiosulfate concentrations are all higher than these ranges, they are beyond the limit for which the yields of biomass on glucose, yeast extract, and thiosulfate were determined (Figs. 1, 2, and part I [21]). It is therefore logical that the model will overestimate all the variables (acetate, lactate, H₂, and CO₂ productions and, maximum of biomass) (Fig. 4a–e). This overestimation is about between 10 and 50%, except for lactate with a percentage close to 100% (Fig. 4b). This overestimation could be due to the fact that one or more of unknown variables are inhibiting and/or limiting the growth of T. maritima and more studies are needed to identify them.

Figure 5a, b represents an example of experimental and model results for the following operating conditions: Glu = 14 mmol/L, Yeast = 1 g/L, Thio = 0.12 mmol/L, and QN₂ = 100 mL/min. In these conditions, glucose is the limiting-growth factor. After 6 h of fermentation, the maximum of biomass (Xmax = 290 ± 30 mg/L) was attained, and 80% of the glucose was consumed (Fig. 5a). H₂ productivity (7 mmol/L h) reached the maximum after 5 h and slightly decreased until 9 h (data not shown) corresponding to the time when the glucose was totally consumed. A clear maintenance phase was observed between 6 and 9 h (Fig. 5a). During this time, the residual glucose is consumed, with a strong decrease in its consumption rate, and this consumption is not associated with biomass increase. However, in the meantime, 10% or more of the total H₂ and acetate were produced, and a partial metabolic shift from acetate toward lactate was observed (Fig. 5b).

In the model, equations related to sulfur have been taken into account. To experimentally confirm the model, the thiosulfate disappearance needs to be monitored. In order to do so, experiments with increased thiosulfate concentration to 20 mmol/L were performed (Glucose 60 mmol/L, Yeast extract 4 g/L, and QN₂ 100 mL/min). In this experiment, none of the compounds of interest was the limiting factor for growth. In this case, 4 mmol/L of thiosulfate were consumed and the total H₂S production measured at the end of the fermentation was of 3.5 mmol. Similar maximum of biomass and H₂ productivity, acetate and H₂ productions were obtained in the same conditions with 0.12 mmol/L thiosulfate (data not shown), corroborating that thiosulfate was not the growth-limiting and or -inhibiting factor. The addition of 4 g/L of yeast extract increases the equivalent initial total thiosulfate concentration (Thio + ε₂) from 20 to 20.24 mmol/L. A negligible part of this thiosulfate (0.07 mmol/L) is incorporated as sulfur into the biomass (CH₁₆O₆N₂O₉S₀.₀₀₅) [23], the remaining consumed thiosulfate (3.93 mmol/L) should be converted into H₂S (7.86 mmol/L, Eq. 2). However, the total H₂S production measured experimentally (3.5 mmol) is about 2 times lower, showing that not all the thiosulfate was converted into H₂S and that probably some other unknown sulfur compounds are produced during the fermentation.

Prediction of the mathematical model
In this study, a mathematical model of the fermentation of T. maritima has been written. This model has been proved to be in agreement with the experiment in a certain range of concentrations of glucose, yeast extract, and thiosulfate, and of the inlet gas (N₂) flow rate. The final purpose of this model is to provide, among other things, a mean of predicting specific H₂ productivity linked to H₂ inhibition for T. maritima in various situations.

Studies have showed that a decrease of N₂ stripping rate (i.e., mass transfer coefficient, KLaH₂) resulted in a lower
Fig. 4 Comparison of experimental and model end-product productions for glucose (2.5–63 mmol/L), yeast extract (0.2–8 g/L), thiosulfate (0.01–2 mmol/L) and an inlet N₂ flow rates between 17 and 190 mL/min. a Acetate production, b lactate production, c H₂ production, d CO₂ production, and e maximum of biomass. Dashed lines correspond to the conditions above which neither glucose nor yeast extract nor thiosulfate is the limiting factor. Beyond this limit, except for lactate, the model overestimates all the variables of about 30%
productivity and yield of H₂ [3]. Our results showed that between 100 and 180 mL/min, no change of experimental maximum H₂ productivity and glucose consumption rate was observed, while a strong decrease (about 50%) was recorded when Q_{N₂} was reduced to 20 mL/min (Additional file 1: Fig. S4). The model was used to simulate the specific H₂ productivity and the inhibition by H₂ versus the N₂ inlet flow rate (Q_{N₂}, 5–100 mL/min) for different operating conditions (0 < Gluc < 20 mmol/L or 0.1 < Yeast < 0.5 g/L or 0 < Thio < 0.06 mmol/L). These ranges correspond to the concentrations where the model was accurately validated (Fig. 4a–e). Initial biomass concentration, volume of liquid of bioreactor, and volume of gas (headspace) were set to 31.6 mg/L, 1.5, and 0.5 L, respectively.

Figure 6a–c represents the maximum specific H₂ productivity against the ratio of the maximum dissolved-H₂ concentration on the critical dissolved-H₂ concentration ([H₂]/[H₂_{crit}]) for various Q_{N₂}. It appears that inhibition by H₂, formalized by [H₂]/[H₂_{crit}], never exceeds 33% (Fig. 6a–c) which corresponds to 11.1 mmol/L of H₂ concentration in the gas phase. Such concentration (11.1 mmol/L) corresponds to a maximum dissolved H₂ of 0.475 mmol/L, showing that, for these operating conditions, reaching the critical H₂ dissolved concentration (1.44 mmol/L), for which T. maritima growth is stopped, seems impossible. Moreover, for this inhibition by H₂, yield of hydrogen is little affected, it decreases from 2.76 to 2.3 mol/mol.

In almost all the cases, the increase in concentration of the limiting factor leads to a notable increase in specific H₂ productivity. However, this increase, when the produced H₂ is slightly flushed by the inlet gas stream (5 mL/min), leads to a strong relative inhibition. Whatever the operating conditions, when N₂ stripping rate increases from 5 to 100 mL/min, the inhibition by H₂ strongly decreases from 33 to 5% (Fig. 6a–c). The combination of both variables (increase in limiting factor concentration and in inlet gas stream) leads up to a twofold increase of the maximum H₂ specific productivity with the lowest inhibition.

This model can be extended beyond the upper limits for non-limiting concentrations of glucose (>20 mmol/L), thiosulfate (>0.06 mmol/L), and yeast extract (0.5 g/L). However, in these cases, the mathematical model will overestimate the production of hydrogen of about 30% (Fig. 4c). Moreover, this model cannot be validated without sparging or for low stripping rates (<5 mL/min). Indeed, for these conditions, gas-transfer diffusion (H₂, CO₂, N₂, and H₂S) becomes predominant. This parameter is not taken into account in our model. In this case, H₂ inhibition will be important and will affect strongly the specific H₂ productivity.

Conclusions

Batch fermentations of T. maritima were successfully simulated using a mathematical model that incorporates the kinetics of growth, consumptions of substrates (glucose, yeast extract, and thiosulfate) and product formations (H₂, CO₂, H₂S, acetate, and lactate). Except for one, all of the model parameters were determined experimentally. However, the limits of the validity of this model were clearly established, and it is within the ranges when glucose or yeast extract or thiosulfate...
limit the growth. Anyway, the development of structured (mechanistic) models for quantifying microbial growth kinetics are still limited because the mechanism of cell growth is very complex and is not yet completely understood. Moreover, the defined mineral media are formulated so as to allow microorganisms to synthesize their cellular components from single sources of carbon, sulfur, ammonium, phosphorus... Usually, only one of them limits the maximum quantity of biomass that could be produced, with all other nutrient in excess. We focused our interest on four state variables (glucose, yeast extract, thiosulfate, and hydrogen concentrations), but we should not dismiss the potential other limiting factors present in the medium. In the future, it would be interesting to research other potential limiting factors and to take into account their influence on the model. For example, Rinker and Kelly [23] demonstrated that the lower NH₄Cl concentration (0.5 g/L our study) would be limiting for the growth of T. maritima.

From now on, this model can be use, in the limits of its validity, to predict, depending on the scenario, many elements, and in particular, specific H₂ productivity, dissolved-H₂ concentration, etc.
Additional file

Additional file 1: Figure S1. Determination of $K_{\text{CO}_2}$. $m_1 = K_{\text{CO}_2}$, $m_0$. Glucose concentration, $m_2 = \mu_{\text{max}}$. Figure S2. Determination of $K_{\text{THIO}}$. $m_3 = K_{\text{THIO}}$, $m_0$. Yeast extract concentration, $m_4 = \mu_{\text{max}}$. Figure S3. Determination of $K_{\text{Smax}}$. $m_1 = K_{\text{Smax}}$, $m_0$. Thiosulfate concentration, $m_2 = \mu_{\text{max}}$. $K_{\text{act}}$, $K_{\text{Smax}}$ and $K_{\text{CO}_2}$ were estimated by fitting Monod's equation to experimental data. Figure S4. Experimental maximum volumetric $H_2$ productivity and glucose consumption rate versus $Q_{\text{GLU}}$.

Abbreviations

Act: acetate concentration (mol/L); [CO$_2$]: CO$_2$ concentration in the liquid phase (mol/L); $D_{\text{CO}_2}$: coefficient of diffusion in water for CO$_2$ (cm$^2$/s); $D_{\text{O}_2}$: coefficient of diffusion in water for $O_2$ (cm$^2$/s); $D_{\text{H}_2}$: coefficient of diffusion in water for $H_2$ (cm$^2$/s); $Glu$ glucose concentration (mol/L); Yeast: yeast extract concentration (g/L); $Y_{\text{H}_2}$: stoichiometric $H_2$ on $H_2S$ (mol/mol); $K_{\text{H}_2}$: Henry's constant for $H_2$ (mol/L/Pa); $Q$: outlet-gas (H$_2$) flow rate (L/h); $Q_{\text{GLU}}$: outlet-gas (CO$_2$) flow rate (L/h); $Q_{\text{THIO}}$: outlet-gas (H$_2$S) flow rate (L/h); $R$: gas constant (Pa L/mol °K); $T$: temperature (°K); $Y_{\text{act}}$: thiosulfate concentration; $V_{f}$: free-liquid-space volume in the reactor (L); $V_{f}^\prime$: free-gaseous-space volume in the reactor (L); $X$: cell mass concentration (g/L); Yeast: yeast extract concentration (g/L); $Y_{\text{max}}$: maximum yield of glucose (mol/mol); $Y_{\text{max}}^{\text{THIO}}$: thiosulfate yield (equivalent sulfur from thiosulfate incorporated into the biomass) (g/mol); $Y_{\text{max}}^{\text{ACT}}$: acetate yield (mol/mol); $Y_{\text{max}}^{\text{CO}_2}$: CO$_2$ yield (mol/mol); $Y_{\text{max}}$:$H_2S$: stoichiometric $H_2S$ on thiosulfate yield (mol/mol); $Y_{\text{max}}^{\text{H}_2}$: H$_2$ yield (mol/mol); $Y_{\text{max}}^{\text{CO}_2}$: maximum yield of CO$_2$ (mol/mol); $Y_{\text{max}}^{\text{CO}_2}$: maximum yield of $H_2S$ (mol/mol); $Y_{\text{max}}^{\text{THIO}}$: stoichiometric $H_2S$ on thiosulfate yield (mol/mol); $Y_{\text{max}}$: equivalent concentration of glucose in the yeast extract (mol/L); $\mu_{\text{max}}$: maximum specific growth rate (h$^{-1}$); $\epsilon_{\text{max}}$: maximum specific growth rate (h$^{-1}$).

Authors' contributions

RA: model development and manuscript writing. CB, PC, and PPL: data collection and analysis of the results. SD: conception, design, and mass transfer experiments. YC-B: planning of the fermentation experiments and parameter estimations. LC: manuscript writing. All authors contributed to revision of the manuscript and approved the text and diagrams for submission. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

All data generated or analyzed during this study are included in this published article and its supplementary information (Additional file 1).

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