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Kinetic and stoichiometric model for the computer-aided design of protein fermentation into volatile fatty acids

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Abstract

Mixed culture fermentation to produce volatile fatty acids stands as a solid option for valorising protein-rich wastes, such as those from the (agro)industry. However, the current knowledge about this process is limited and there are no appropriate tools for designing the production of volatile fatty acids from proteinaceous wastes. Available models fail to reproduce how the process stoichiometry varies with the substrate composition and environmental conditions. Likewise, existing kinetic parameters were not estimated in anaerobic fermentative conditions and cannot be used reliably for designing fermentation processes. Therefore, in this work, a model for the production of volatile fatty acids from proteins in mixed-culture fermentations was developed. The model incorporates a variable volatile fatty acids production stoichiometry that depends on the operational conditions and the protein. To reflect the commonly observed incomplete protein consumption in fermentative conditions, the model considers that protein acidification process attains a thermodynamic equilibrium with its products. To calibrate the model, targeted designed fermentation experiments were carried out with casein and gelatine, which were chosen due to their different amino acid composition as well as their relevance in industrial organic wastes. The model reproduces with accuracy the experimental data on the fermentation of two proteins under different environmental conditions, which indicates that protein

acidification is well described. The model developed in this work together with the proposed simulation framework proved to be an effective tool for selecting the optimal design parameters leading to highly selective and productive processes and for the early-stage design of processes valorising protein-rich wastes into chemicals.

Keywords: carboxylate platform; resource recovery; reaction kinetics; biorefinery; process simulation; biochemical reaction modeling.

1. INTRODUCTION

The production of volatile fatty acids (VFA) from organic effluents is gaining attention as an alternative to the current oil-based production (Atasoy et al., 2018; Jankowska et al., 2017; Shanthi Sravan et al., 2018) and because of their central role in the biorefinery paradigm (Agler et al., 2011; Dahiya and Mohan, 2019; Moscoviz et al., 2018). The use of undefined and open cultures of microorganisms is an attractive option, particularly when dilute organic streams are used as substrate, as it avoids the costly sterilisation of the substrate and protects the system against fluctuations in the operational or feeding conditions due to the higher robustness of mixed cultures (Carballa et al., 2015).

In literature, most of the works about VFA production by mixed-culture fermentations (MCF), are centred in the use of carbohydrates as feedstock both from an experimental (Domingos et al., 2018; Rombouts et al., 2019; Temudo et al., 2007) or modelling perspective (González-Cabaleiro et al., 2015; Regueira et al., 2018). On the contrary, protein MCF has not received the same attention while protein-rich feedstocks represent an opportunity to produce added-value products in the biorefinery scheme. Interesting industrial effluents, such as canning-industry, slaughterhouse or dairy industry wastewaters, present high concentrations of fermentable proteins (Duong et al., 2019). Breure and co-workers (Breure et al., 1986b, 1986a, 1985; Breure and van Andel, 1984) pioneered the study of protein MCF but did not pay attention to the transformation mechanisms, which

limits the application of their results to practical purposes. Since then, few authors have focused on protein MCF (Fang and Yu, 2002; Ramsay and Pullammanappallil, 2001; Yu and Fang, 2003), mostly considering it as a step in anaerobic digestion. Moreover, very recently, experimental data assessing the effect of different proteins on transformation kinetics and stoichiometry were reported (Bevilacqua et al., 2020).

Modelling VFA production in protein MCF is a challenging task as composition on amino acids can vary significantly even among the same type of proteins (i.e. the name of protein only informs about its origin but it is not related with its composition), which usually results in different VFA product spectrum and process kinetics. Only recently, mechanistic models were developed to predict the effect of pH on the selectivity of VFA production in protein MCF (Regueira et al., 2020b) as well as to evaluate the effect of co-fermentation with carbohydrates (Regueira et al., 2020a). However, for process design purposes, models assessing the effect of environmental conditions on both the selectivity and the kinetics, and consequently on the VFA productivity, are needed. Particularly, kinetic models may be of great help to design processes that effectively suppress methanisation by manipulating the hydraulic or solids retention times while at the same time maximising VFA productivity.

Most of the available kinetic models for anaerobic mixed cultures processes focus on anaerobic digestion, such as the model developed by Angelidaki et al. (Angelidaki et al., 1999) or the well-known ADM1 (Batstone et al., 2002b). However, the ultimate goal of those models is to predict methane production, not focusing on the substrate conversion into the different VFAs or on the description of the fermentative processes, in particular for substrates other than carbohydrates. The recent models developed specifically for protein MCF (Bai et al., 2017; Tommaso et al., 2013) not only feature parameters estimated in methanogenic environments, but they assume that the stoichiometry of the process is

fixed as well, thus ignoring the effect that different operational conditions may exert on VFA selectivity. Our hypothesis is that parameters obtained in methanogenic environments are not valid for fermentation processes as their environmental conditions differ significantly (e.g. radically different H_2 partial pressure or different VFA concentrations in the reactor). Besides, the composition in amino acids of the proteins to be fermented has an impact on the stoichiometry of the biotransformation. Actually, the product spectrum of protein MCF mostly depends on the amino acid protein composition and the operating pH, as it has been described in literature (Bevilacqua et al., 2020; Breure et al., 1986b; Ramsay and Pullammanappallil, 2001).

The objective of this work is to develop an integral kinetic and stoichiometric model for protein mixed culture fermentation that captures the effect of pH and protein composition on VFA selectivity and productivity. Targeted fermentation experiments using two proteins, casein and gelatine were designed and conducted to estimate the kinetic and stoichiometric parameters of the model at different pH conditions.

2. MATERIALS AND METHODS

2.1 Model description

The model describes the production of the different VFA (acetate, propionate, butyrate and valerate) and ammonia during MCF of proteins. In contrast to carbohydrates or lipids, that commonly have a defined monomer composition, proteins are composed by a varying and usually undefined mixture of amino acids. Yet, it was decided not to simulate amino acids individually, since it would add unnecessary complexity to the model while not proving any additional accuracy. Accordingly, the only substrate considered in the model is the hydrolysed protein used in the experiments (section 2.2). Nevertheless, although the substrate is considered as a single entity, the process stoichiometry does reflect the variations brought by the different amino acid composition of the protein(s) used as

substrate. The consumption of protein is described in the model by a Monod equation (Eq. 1).

$$q = q_{max} \cdot \frac{S}{K_S + S} \cdot X \quad (1)$$

where q is the specific consumption rate (g_{COD} Prot/g_{COD} BM·h), q_{max} is the maximum specific consumption rate (g_{COD} Prot/g_{COD} BM·h), S is the protein concentration (g_{COD} Prot/L), K_S is the half-saturation constant (g_{COD} Prot/L), and X is the biomass concentration (g_{COD} BM/L).

The incomplete protein conversion reported in literature (Bevilacqua et al., 2020; Duong et al., 2019; Yu and Fang, 2003) and observed in this work as well, could be explained by a kinetic product inhibition and/or by a thermodynamic limitation . From the results of the experiments of this work, as shown hereafter, we assume that protein fermentation reaches a thermodynamic equilibrium with their fermentation products. Therefore, protein acidification may be described as a reversible process (Eq. 2-3), in which both the forward and reverse reactions rates are described by a Monod equation.

$$Protein \rightleftharpoons VFA \quad (2)$$

$$K_{eq} = \frac{q_{max,1}}{q_{max,2}} \quad (3)$$

Where K_{eq} is the equilibrium constant and $q_{max,1}$ and $q_{max,2}$ are the maximum forward and reverse reaction rates, respectively.

The stoichiometric matrix of the model (Table 1) considers the net acidification rate (q_{Acid}), which is determined as the difference between the forward and the reverse reactions (Eq. 4). Eq. 5 results from combining Eq. 4 and Eq. 3 and presents explicitly the equilibrium constant (K_{eq}), which will be estimated from the experimental data.

$$q_{Acid} = q_1 - q_2 = q_{max,1} \frac{S_{PROT}}{K_{S,Prot} + S_{PROT}} - q_{max,2} \frac{S_{VFA}}{K_{S,VFA} + S_{VFA}} \quad (4)$$

$$q_{Acid} = q_{max,1}\frac{S_{PROT}}{K_{S,PROT} + S_{PROT}} - \frac{q_{max,1}}{K_{eq}}\frac{S_{VFA}}{K_{S,VFA} + S_{VFA}} \quad (5)$$

where q_{Acid} is the net acidification rate, q_1 and q_2 are the forward and reverse reaction rates (g_{COD} Prot/g_{COD} BM·h), respectively; S_{PROT} and S_{VFA} represent the protein and VFA concentration (g_{COD}/L), respectively, and $K_{S,PROT}$ and $K_{S,VFA}$ are the half-saturation constants for the forward and reverse reactions (g_{COD}/L), respectively.

Proteins are modelled to be fermented to a mixture of acetate, propionate, butyrate, valerate, aromatic VFA (which are products from aromatic amino acids acidification) and H_2. From the results of our experiments as well as from the literature information (Bevilacqua et al., 2020; Breure and van Andel, 1984; Duong et al., 2019), it is clear that protein fermentation stoichiometry is highly dependent on the amino acid composition and on the environmental conditions, such as pH. In consequence, a variable process stoichiometry was considered, which is captured by variable stoichiometric coefficients (*f* coefficients inTable 1). In this work, these stoichiometric coefficients (*f*) are determined together with the kinetic parameters from the experimental results. Alternatively, if no experimental data are available, the *f* values can be defined with the metabolic model for stoichiometry prediction of protein MCF presented in a previous contribution (Regueira et al., 2020b).

Protein fermentation is considered to be performed by a single microbial functional group (X_{PROT}) that growths only due to protein acidification and its decay is modelled as a first order kinetics with respect to the biomass concentration (Table 1).

Table 1. Stoichiometric matrix and rate equation descriptions of the different processes considered in the model

	S_{PROT}	S_{VAL}	S_{BUT}	S_{PRO}	S_{AC}	S_{ARO}	S_{H2}	S_{inert}	X_{PROT}	Rate equation
Acidification	-1	f_{VAL}	f_{BUT}	f_{PRO}	f_{AC}	f_{ARO}	f_{H2}			q_{Acid}

Growth	-1								1	$\mu_{Prot} = q_{Acid} \cdot Y_{Prot}$
Decay								1	-1	$q_{decay} = k_{decay} \cdot X_{Prot}$

The stoichiometric coefficients of aromatic VFA and H_2 (f_{ARO} and f_{H2}, respectively) were estimated using the stoichiometry proposed by Ramsay and Pullammanappallil (Ramsay and Pullammanappallil, 2001) since their concentration was not measured in this work. Therefore, taking into account the amino acid composition of the gelatine used in the experiments (section 2.2), it was determined that aromatic VFA would account for a 10.9% and a 4.9% (COD basis) of the products for casein and gelatine, respectively, and that the share of H_2 in the product spectrum for casein would be 5.2% (COD basis), being its contribution in gelatine fermentation negligible. As the absolute values of the stoichiometric coefficients of both aromatic VFA and H_2 as well as their variations between proteins are small, the possible uncertainties derived from this approximation are expected to have a marginal impact on the predictions of the model.

Methane production from VFA is also included in the model to evaluate the operational conditions leading to VFA consumption. Methanisation is performed by other microbial functional groups than protein acidification and is modelled according to ADM1 model (Batstone et al., 2002a). A detailed and comprehensive description is available in section B of the Supplementary Materials.

2.2 Batch essays

Batch fermentation experiments were carried out for determining the kinetic parameters of the process. Bottles of 0.5 L of total volume (0.375 L of working volume) with rubber stoppers were used. They were placed in a temperature-controlled room at 25 ± 1ºC and sparged with N_2 during the experiment to ensure anaerobic conditions (approximately at a 10 mL/min rate).

Two proteins hydrolysates, of casein (A2208,0500 PanReac) and gelatine (70951-1KG-F Sigma-Aldrich), were used as substrates (their composition on amino acids is shown in the Supplementary Materials (Table S1)). Total Kjeldahl nitrogen (TKN) and Chemical Oxygen Demand (COD) were determined following Standard Methods (APHA, 2017) the results obtained were: 171.9 and 201.9 mg N/g_{PROT}, and 1.08 and 1.04 g_{COD}/g_{PROT}, for casein and gelatine, respectively. The following macronutrients were supplemented (g/L): NaCl (0.292), KH_2PO_4 (0.780), NH_4Cl (0.530), Na_2SO_4 (0.057), $MgCl_2 \cdot 6H_2O$ (0.120).

The effect of pH in the kinetics and stoichiometry of protein fermentation was studied as it is an operational parameter with a high impact on process performance and because it is one of the most easily controllable design parameters. Three experiments with each protein were carried out at different controlled pH values (5.0 ± 0.05, 7 ± 0.05 and 9 ± 0.05), by addition of either HCl or NaOH 2M. An additional experiment without pH control was performed with casein. The inoculum was obtained from steady-state continuous reactors fermenting the same protein at the same pH (Bevilacqua et al., 2020). The initial biomass concentration was set to an approximate value of 0.5 g_{VSS}/L for all experiments, except for casein at pH 7, in which the initial biomass concentration was around 1.0 g_{VSS}/L. For all the pH-controlled experiments, a substrate-to-inoculum ratio of approximately 10 was used. In the casein experiment at uncontrolled pH, three substrate-to-inoculum ratios of 5, 10 and 20, were tested to determine potential product inhibition.

Samples were taken at increasing time intervals (initially each 2-3 hours) and VFA concentration, total ammonia nitrogen (TAN, as an indicator of protein conversion), and biomass concentration (through optical density at 600 nm) were determined. VFA were measured through gas chromatography AGV-DB1 method with an Agilent 6850 equipped with a flame ionization detector and using a DB-Wax column from Agilent Technologies (30 m x 0.250 mm x 0.25 μm) (Bevilacqua et al., 2020). TAN was determined according to

Standard Methods SM4500-NH3.F (APHA, 2017) using a Shimazdu UV-1800 spectrophotometer. Optical density was determined using a Shimazdu UV-1800 spectrophotometer at 600 nm and calibrated with actual VSS measurements from the reactors.

2.3 Parameter estimation

Kinetic parameters (maximum forward acidification rate ($q_{max,1}$), biomass yield (Y_{Prot}), decay constant (k_{decay}) and equilibrium constant (K_{eq})) and stoichiometric coefficients (f_{VAL}, f_{BUT}, f_{PRO}, f_{AC}) were estimated from each of the data sets generated in the batch essays at different pH and with casein and gelatine as substrates. Data sets were composed by a minimum of 10 and a maximum of 30 data points. Parameters were estimated by minimisation of the normalised root squared mean deviation (NRMSD) between the experimental data and the obtained by simulation (Eq. 6). The model is implemented in MATLAB (R2016a) and NRMSD minimisation was performed by using the command *lsqnonlin* (trust-region-reflective algorithm).

$$NRMSD = \frac{1}{p \cdot n \cdot m} \cdot \sqrt{\sum_{k=1}^{p} \sum_{j=1}^{n} \sum_{i=1}^{m} \left(\frac{\hat{y}_{k,j,i}(\theta) - y_{k,j,i}}{\sigma_{k,j}} \right)^2} \qquad (6)$$

where m is the number of measurement times (between 9 and 16), n the number of experimentally measured compounds (in most cases 6), p the number of experiments at different initial substrate concentration for the same substrate and pH, $\hat{y}$ is the simulated concentration value, y is the experimental concentration value, θ is the vector of parameters being estimated and σ is a normalisation factor meant to scale the residuals to comparable magnitudes (sigma is the range of the experimental values for all measurements except for biomass concentration in casein at pH 5 and 9, for which the average value is used instead, as its narrow range would make extremely large residuals). The subscript i

refers to a measurement over time of the compound *j* and experiment *k* at a particular pH and initial substrate concentration.

To ensure the robustness of the estimated parameter values and to avoid the model getting stuck at local minima, a bootstrap methodology, as described in González-Gil et al. (Gonzalez-Gil et al., 2018) was followed to determine the value and uncertainty interval of the estimated parameters. To check the convergence, populations were classified in samples and their average value and standard deviations compared to check for divergences. To evaluate how the uncertainty of the kinetic parameters propagates on the model outputs (VFA, ammonia and biomass concentration evolution over time), a Monte Carlo procedure was followed (Saltelli et al., 2008). A total of 500 iterations were performed using Latin Hypercube Sampling to ensure a maximum coverage of the parameter values space (Helton and Davis, 2003).

3. RESULTS AND DISCUSSION

3.1 Model validity

The proposed model is able of reproducing satisfactorily the generated experimental data with the estimated parameters, as shown in the example of a fit between experimental and model data presented in Fig. 1 (the results of model calibration are discussed in detail in section 3.4). In this way, the two main assumptions of the model, namely, considering protein as the only substrate and modelling the observed incomplete protein consumption as a thermodynamic equilibrium, seem to provide an accurate description of the process of VFA production from protein. The model reproduces accurately the evolution of the concentration of the different products (VFA and inorganic nitrogen) throughout the batch with a tight interval confidence area and the values reached at the end of the batch. The

evolution of protein shows that its concentration is stabilised at a final value of 1.0 g_{COD} Prot/L, which represents an 80% consumption of the initial protein concentration.

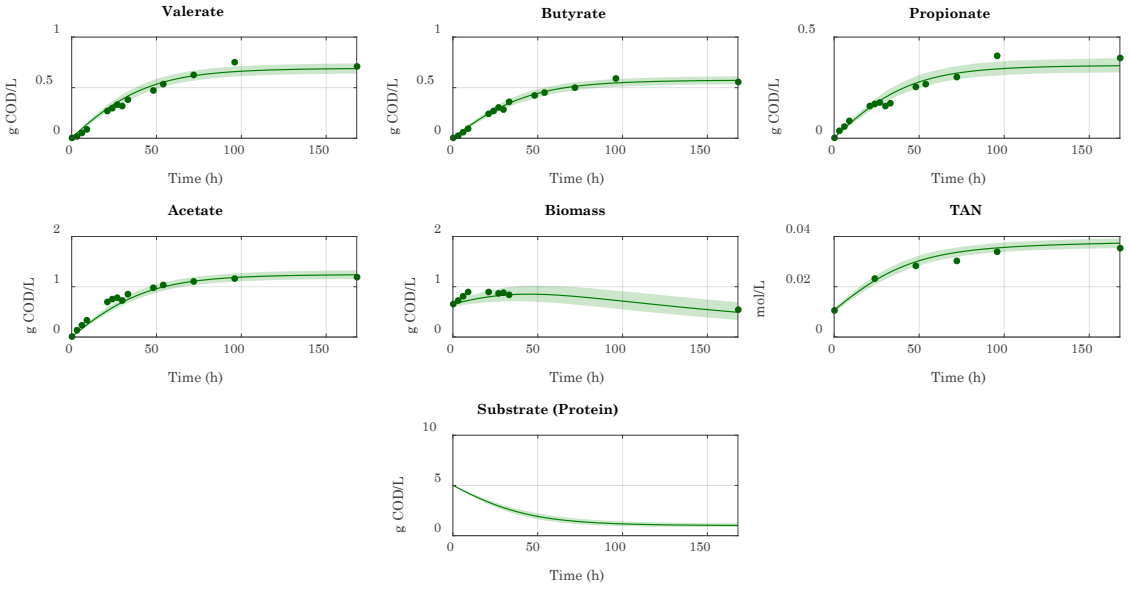

Figure 1. Fit between the experimental data (filled circles) and the model results (continuous lines) using the estimated parameters for gelatine fermentation at pH 9 (Table 2). The coloured areas represent the 95% interval confidence and were estimated by error propagation of the parameter uncertainties following a Monte Carlo procedure.

3.2 Batch mixed culture fermentation of proteins

Figure 2 shows the ammonification degree of the experiments at three pH for casein and gelatine fermentation, which can be used as an indicator of the substrate consumption. In the case of casein (Fig. 2a) the final ammonification ranges from 40% to 52% while gelatine experiments (Fig. 2b) showed a significantly wider range, spanning from 30% to 55%. In the case of gelatine at pH 7, only values up to 54h are reported due to sample loss. The reactor pH affects slightly the ammonification degree in the case of casein (Fig. 2a), being the values at pH 5 and 7 comparable and the value at pH 9 slightly higher. On the contrary, in gelatine experiments (Fig. 2b), ammonification is highly influenced by pH, the maximum taking place at pH 7 (around 55%) followed by pH 9 (45%) and pH 5 (only 30%).

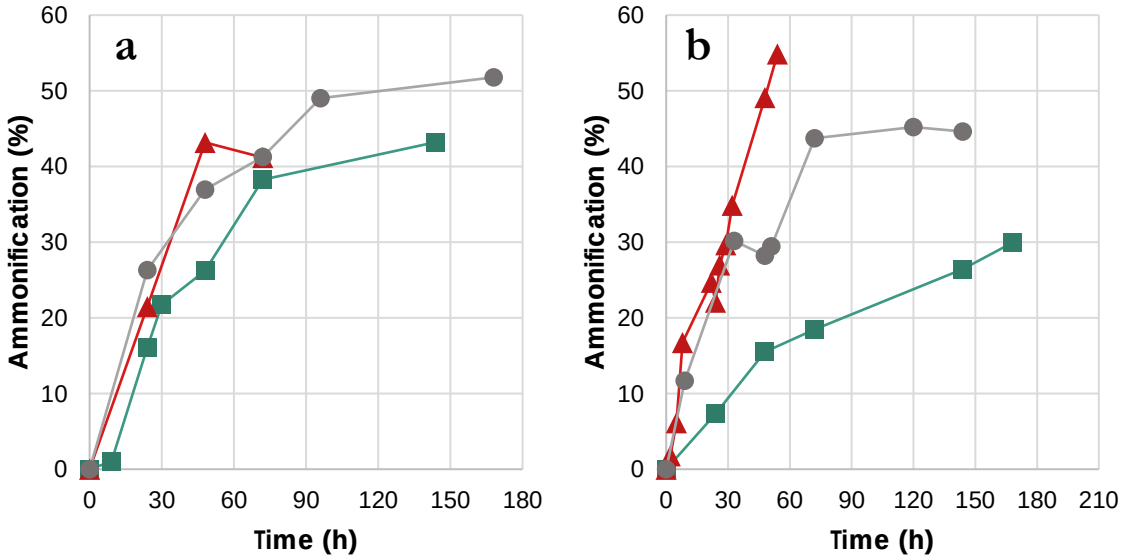

Figure 2. Ammonification degree in the batch experiments with casein (a) and gelatine (b) at three different pH values (■ pH 5, ▲ pH7 and ● pH 9). Ammonification degree is determined as the percentage of the nitrogen contained in the protein measured as ammonia concentration in the reactor (subtracting the inorganic nitrogen added in the feeding).

The increase of VFA concentration over time shows that in general casein is quicker than gelatine fermentation (Fig. 3), independently of pH. The influence of pH is similar for both proteins: neutral pH values favour the highest rates, experiments at pH 9 performed slight slower and acidic pH significantly limits the rate of protein acidification, especially for gelatine.

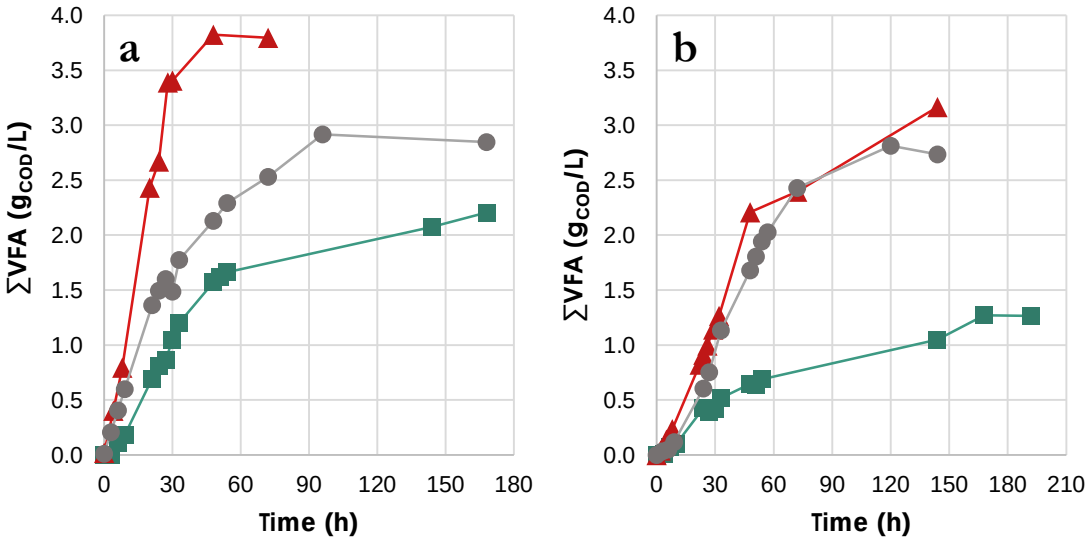

Figure 3. Total VFA concentration over time in the batch experiments with casein (a) and gelatine (b) at three different pH values (■ pH 5, ▲ pH7 and ● pH 9). Initial substrate concentration in all experiments was approximately 5 g_{COD}/L except for casein at pH 7, which was 8 g_{COD}/L.

As the stoichiometry of protein fermentation was constant throughout the experiments (data not shown), the information of the final time point was used to determine the product spectra (Figure 4). The stoichiometry shows an unequivocal dependence on both the protein type (i.e. their composition on amino acids) and on the pH value. Overall, gelatine product spectrum (Fig. 4b) is dominated by acetate at all pH values, being its contribution significantly higher than in casein fermentation (Fig. 4a). Casein fermentation presents a more balanced product spectrum in which acetate, valerate and butyrate are the major products depending on pH (Fig. 4a). The effect of pH on the product spectrum is quite similar for both proteins and, in general, acidic pH values favour the presence of reduced compounds (e.g. valerate or butyrate), while alkaline values boost the share of acetate. This suggests hence that the changes on the global product spectrum are caused by changes on individual amino acid stoichiometries, which behave equally independently of the protein type. For example, glutamate is reported to change its stoichiometry from

acetate towards butyrate at acidic pH, which could justify its increase in the product spectrum. Other amino acids particularly sensitive to the effect of pH in their conversion to VFA include aspartate, histidine and leucine (Regueira et al., 2020b).

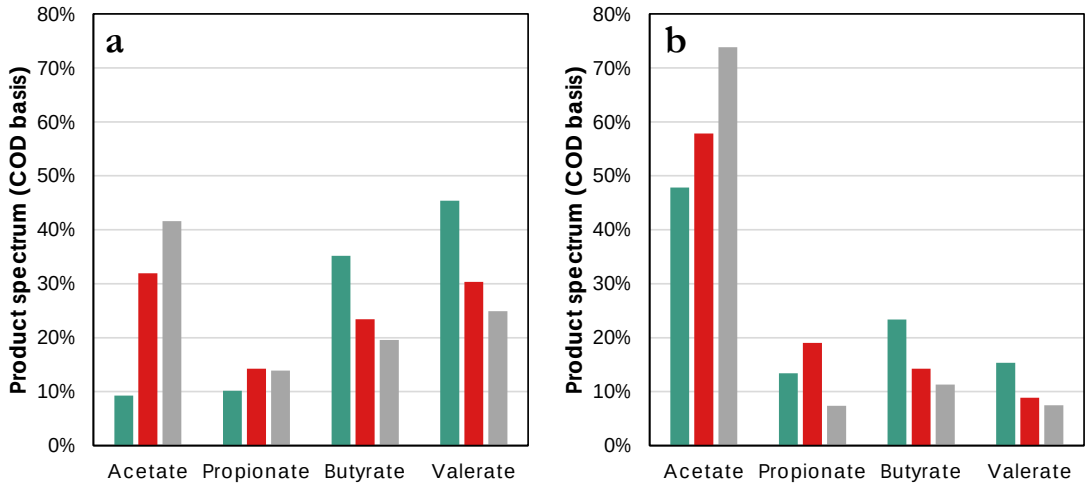

Figure 4. VFA spectrum from fermentation of casein (a) and gelatine (b) at three different pH values (■ pH 5, ■ pH7 and ■ pH 9).

The magnitude of the pH effect, however, differs with the protein, appearing more decisive for casein fermentation, which can be correlated with the amino acid composition of both proteins (Table S1). For instance, the share of glutamate in casein is 15% when it only represents 7% of the amino acids of gelatine. Therefore, even though the individual stoichiometry of glutamate conversion changes, its effect on the global stoichiometry is weaker in the case of gelatine.

In comparison with literature information, only accurate information could be found for experimental works of gelatine fermentation. In general, a similar product spectra is reported: acetate was always the main VFA with percentages around 50% (molar basis), followed generally by butyrate and propionate (Breure et al., 1986b, 1986a; Breure and van Andel, 1984; Duong et al., 2019; Flotats et al., 2006). Valerate is reported to be the least VFA produced in some works (Duong et al., 2019; Flotats et al., 2006), but its share is

higher and ranks second in others (Breure et al., 1986a; Breure and van Andel, 1984). Also, previous works found that butyrate, or other reduced VFA, are favoured at acidic conditions, increasing its share in the product spectrum from 8% to 17% (Breure et al., 1986b) and from 7% to 19% when lowering the pH from 7 to 5.3 (Breure and van Andel, 1984).

3.3 Incomplete substrate consumption

The results shown in Fig. 1-2 clearly indicate that protein fermentation is usually incomplete, with a fraction of substrate not converted into VFA or biomass. This behaviour was also observed in other works in literature (Bevilacqua et al., 2020; Breure and van Andel, 1984; Duong et al., 2019) and can be mechanistically explained by the products (VFA and/or ammonia) inhibiting partially biomass or by a thermodynamic limitation by which the reaction attains an equilibrium. To elucidate which possibility best suits our system, batch experiments at three different substrate initial concentrations, and maintaining the initial biomass concentration, were performed with casein. Although pH was not controlled in these experiments, it naturally remained near neutral values (between 7.2 and 7.4) due to the continuous CO_2 stripping by the nitrogen gas sparging and the balanced effect of VFA and ammonia.

The results of the experiments show that increase in the final total VFA concentration is linearly proportional to the increase in the initial substrate concentration (Fig. 5), indicating that the acidification degree is almost identical (values range from 39.0 to 39.9%) and thus discarding the possibility of an incomplete consumption due to product inhibition with VFA concentrations ranging from 1 g_{COD}/L to 4 g_{COD}/L. Therefore, it was decided to describe protein acidification as a reversible process (Eq. 2-3), as suggested in other uncomplete biological conversions such as organic micropollutants biotransformation (Gonzalez-Gil et al., 2018). To displace the thermodynamic equilibrium towards the

products and increase protein acidification, one option would be to continuously extract *in-situ* the products to diminish the VFA concentration, as already implemented in some reaction systems (Selder et al., 2020).

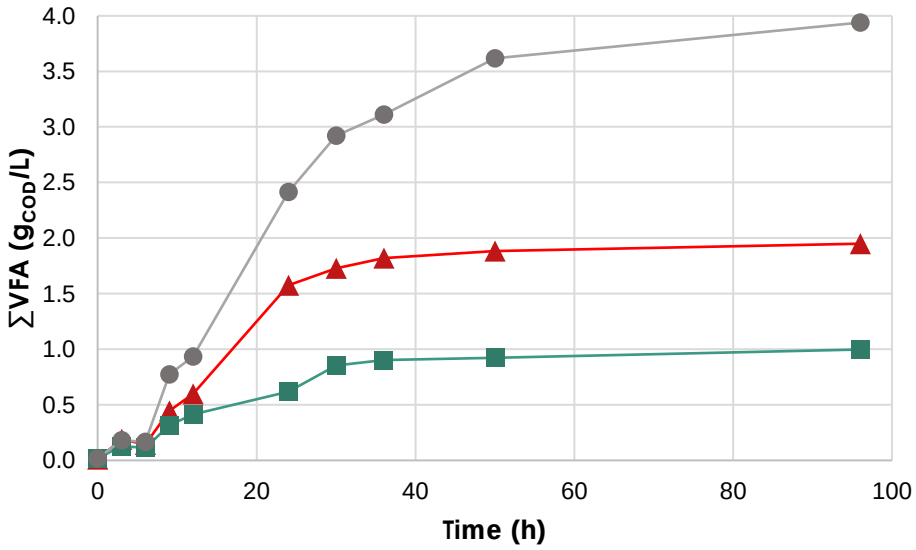

Figure 5. Total VFA concentrations (acetate, propionate, butyrate and valerate) in the casein batch experiments at three different substrate initial concentrations (■ 2.5 g_{COD}/L, ▲ 5 g_{COD}/L and ● 10 g_{COD}/L).

3.4 Model calibration

By using the experimental data, it was possible to identify a high number of robust kinetic and stoichiometric parameters (this aspect is discussed in detail in section C of the Supplementary Materials). The NRMSD values between the results of the calibrated model and the experimental data sets remained in all cases below 0.015 and the estimated uncertainty of the parameters is quite narrow, indicating that the model could reproduce satisfactorily and in detail the experimental results. As methanogenesis was not observed in any experiment, their parameters were not estimated and therefore this section only discusses parameters related with acidogenesis.

3.4.1 Kinetic parameters

The maximum acidification rate ($q_{Acid,max}$) and the equilibrium constant (K_{eq}) values were successfully estimated in all cases while the biomass yield (Y_{Prot}) and the biomass decay constant (k_{decay}) could not be estimated with the needed robustness under some pH values in gelatine fermentation due to increased dispersion of the biomass measurements in those data sets or due to a significant mismatch with the VSS measurements performed at the end of the batch operation.

The half-saturation constant of the Monod equation (Eq. 1) was fixed at a value of 7 g_{COD}/L for all cases based on the results of preliminary estimation to reflect the observed increasing acidification rates at increasing initial substrate concentration (Fig. 5). This value may seem high from the common interpretation of the affinity constant (i.e. the concentration at which the uptake rate is half of the maximum) and it is also higher than values reported in literature in protein fermentation (a value of 1.5 g_{COD}/L is proposed in the ADM1 model for anaerobic digestion (Batstone et al., 2002a) and Flotats et al. (Flotats et al., 2006) report an estimated value of 1.8 g_{COD}/L in their kinetic protein fermentation model), which is probably a limitation of using simplified kinetics such as Monod equation. We consider that other factors dependent on the substrate concentration may be affecting the acidification rate and are also captured in this term by the model. For example, although the substrate used in the experiments of this work is protein hydrolysate, it contained a significant amount of unhydrolyzed peptidic chains when analysed with the Lowry method, which is reported to detect peptides of three amino acids or longer (Martina and Vojtech, 2015). Therefore, cells must finalise the extracellular hydrolysis process prior uptake of the individual amino acids, affecting likely the acidification process at a different degree depending on the initial protein concentration.

Maximum acidification rate ($q_{Acid,max}$) is affected by both the protein type and pH (Table 2). Overall, casein fermentation at pH 7 presents $q_{Acid,max}$ values up to 55% higher than gelatine

which indicates the very strong influence of protein composition on the acidification kinetics. The acidification rate is also noticeably affected by pH in both proteins, always presenting a maximum value at pH 7, while acidic conditions (pH 5) slow down the process at a higher extent than alkaline conditions (pH 9). This effect is significantly more pronounced in the case of casein than of gelatine, provoking that at pH 5 the $q_{Acid,max}$ values of both proteins are comparable. Our hypothesis is that at non-neutral pH values, the undissociated form of VFA and ammonia diffuse back into the cells, this likely hindering their metabolism (Russell, 1992).

Biomass yields (Y_{Prot}) do not seem to be affected by the type of protein, since their values remain very close for casein and gelatine fermentation (Table 2). They only appear to be affected by alkaline pH values and again at a higher extent for casein fermentation, where the biomass yield decreases 25% with respect to pH 7, while in gelatine fermentation this decrease only accounts for 5%. Biomass yields are in all cases very similar to those obtained systematically in previous works for casein and gelatine fermentation in continuous reactors (Bevilacqua et al., 2020) as well as in several preliminary experiments (data not shown). These values are higher than those reported in literature for gelatine fermentation, ranging from 0.10 to 0.14 g_{COD} BM/g_{COD} Prot, depending on retention time (0.1-0.23 h^{-1}) and pH (5.3-7) (Breure et al., 1986a; Breure and van Andel, 1984). Besides, the reference value assumed in the anaerobic digestion ADM1 model is 0.085 g_{COD} BM/g_{COD} Prot (Batstone et al., 2002a), while other models of protein fermentation (Flotats et al., 2006) and digestion (Angelidaki et al., 1999; Siegrist et al., 2002) suggest values between 0.08 and 0.13 g_{COD} BM/g_{COD} Prot. A bioenergetic reasoning may indicate that the values found in literature were underestimated and that biomass yields could be actually closer to those reported in this work and to those obtained for glucose (between 0.15-0.20 g_{COD} BM/g_{COD}). In fact, assuming the predicted stoichiometry of a previous work (Regueira et al., 2020b), aspartate yields 10 mmol ATP/g_{COD} AA, serine around 12.5 mmol ATP/g_{COD} AA and proline 20.8

ATP/g_{COD} AA in the same range or higher than glucose yields (15 mmol ATP/g_{COD} glucose).

Table 2. Estimated kinetic parameter values (mean value [estimated confidence interval with $\alpha = 0.05$]) for casein and gelatine fermentation at three pH (5, 7 and 9). (*) Lack of quality of biomass determinations due to increase dispersion. A yield of 0.179 g_{COD} BM/g_{COD} Prot was used in gelatine at pH 5 and a decay constant of $1.4 \cdot 10^{-3}$ h^{-1} was assumed in gelatine at pH 5 and pH 9.

Parameter		Casein	Gelatine
$q_{Acid,max}$ (g_{COD} Prot/g_{COD} BM·h)	pH 5	0.210 [0.193, 0.226]	0.201 [0.178, 0.228]
	pH 7	0.400 [0.370, 0.436]	0.257 [0.244, 0.272]
	pH 9	0.327 [0.304, 0.352]	0.233 [0.210, 0.259]
Y_{PROT} (g_{COD} BM/g_{COD} Prot)	pH 5	0.191 [0.140, 0.237]	Not estimated*
	pH 7	0.189 [0.155, 0.222]	0.179 [0.158, 0.200]
	pH 9	0.143 [0.105, 0.177]	0.170 [0.140, 0.206]
μ_{max} (h^{-1})	pH 5	0.040 [0.029, 0.051]	0.035 [0.031, 0.040]
	pH 7	0.075 [0.063, 0.088]	0.046 [0.041, 0.051]
	pH 9	0.047 [0.035, 0.058]	0.040 [0.033, 0.047]
k_{decay} (h^{-1})	pH 5	$3.3 \cdot 10^{-3}$ [$0.8 \cdot 10^{-3}$, $5.7 \cdot 10^{-3}$]	Not estimated*
	pH 7	$7.9 \cdot 10^{-3}$ [$4.2 \cdot 10^{-3}$, $11.0 \cdot 10^{-3}$]	$1.4 \cdot 10^{-3}$ [0.3, $2.7 \cdot 10^{-3}$]
	pH 9	$5.8 \cdot 10^{-3}$ [$4.1 \cdot 10^{-3}$, $8.3 \cdot 10^{-3}$]	Not estimated*
K_{eq}	pH 5	1.34 [1.17, 1.53]	0.49 [0.44, 0.54]
	pH 7	1.61 [1.35, 1.95]	3.84 [2.70, 5.75]
	pH 9	2.34 [1.93, 2.88]	1.41 [1.16, 1.67]

The maximum specific growth rate (μ_{max}), determined by multiplying $q_{Acid,max}$ and the biomass yield, follows the same trend as the $q_{Acid,max}$: it is higher for casein and their values peak at pH 7, while acidic conditions correspond to a slower biomass growth. Values reported in literature are systematically higher than those presented here. For example, the value of μ_{max} proposed for protein fermenters in some anaerobic digestion models

(Angelidaki et al., 1999; Batstone et al., 2002a; Siegrist et al., 2002) spans from 0.17 to 0.27 h^{-1} at neutral pH. However, these values are used in methanogenic environments and the conditions in which they were estimated are not completely described, which hinders any possible interpretation of the difference with the values of this work. Flotats et al. (Flotats et al., 2006) estimated the kinetic parameters in a fermentative environment at thermophilic conditions and without pH control and report a μ_{max} value for gelatine fermenters of 0.65 h^{-1}. However, the important uncertainty of the parameter (a bigger standard deviation than its value) suggests identifiability issues during the calibration and thus prevents a fair comparison with our results. Moreover, in all the mentioned works, protein undergoes a hydrolysis step prior acidification, which is generally assumed to be the limiting step of protein methanisation or fermentation (Breure and van Andel, 1984; Flotats et al., 2006). In consequence, the results obtained in those models have a very low sensitivity with respect to the protein acidification kinetic parameters, since the rate of the whole process is determined mainly by the hydrolysis-related parameters, which further undermines any comparison with the results of this work.

Decay constant values show a maximum value at pH 7 in casein fermentation, which is also higher than for gelatine (gelatine decay constant is only available at this pH). Overall, the values are in the expected range, between 3% (gelatine) to 12% (casein) of the respective μ_{max} values. The value proposed for protein fermenters is 0.033 h^{-1} in a protein anaerobic digestion model (Siegrist et al., 2002) and represents 20% of the proposed μ_{max} value. The equilibrium constant (K_{eq}) is logically dependent on the conversion degree of the substrate, and therefore affected by pH, as discussed in the previous section: while K_{eq} increases monotonically with the pH in casein fermentation, in gelatine experiments, it appears maximum at pH 7, without a clear mechanistic interpretation.

Overall, the values of the kinetic parameters obtained in the model calibration clearly show that the protein type exerts a marked influence, which suggests that the individual contribution of the amino acids influences the global kinetic behaviour. Additionally, pH affects most of the kinetic parameters, being this effect dependent on the protein type. Systematically, casein fermentation parameters were more influenced by pH, underlining once again the contribution of the protein amino acid profile in the overall kinetic behaviour.

3.4.2. Stoichiometric coefficients

The stoichiometric coefficients (*f* coefficients of Table 1) were also calibrated for both proteins at different pH (solids bars in Fig. 6). As already discussed in the description of the experimental data (section 3.2), the stoichiometry depends on both the protein type and the pH, which is reflected in the variation of the stoichiometric coefficients. This variability is probably related with the amino acid profile of each protein, with changes in the individual amino acid conversion to VFA and/or with the selective consumption of some amino acids, as commented in section 3.2.

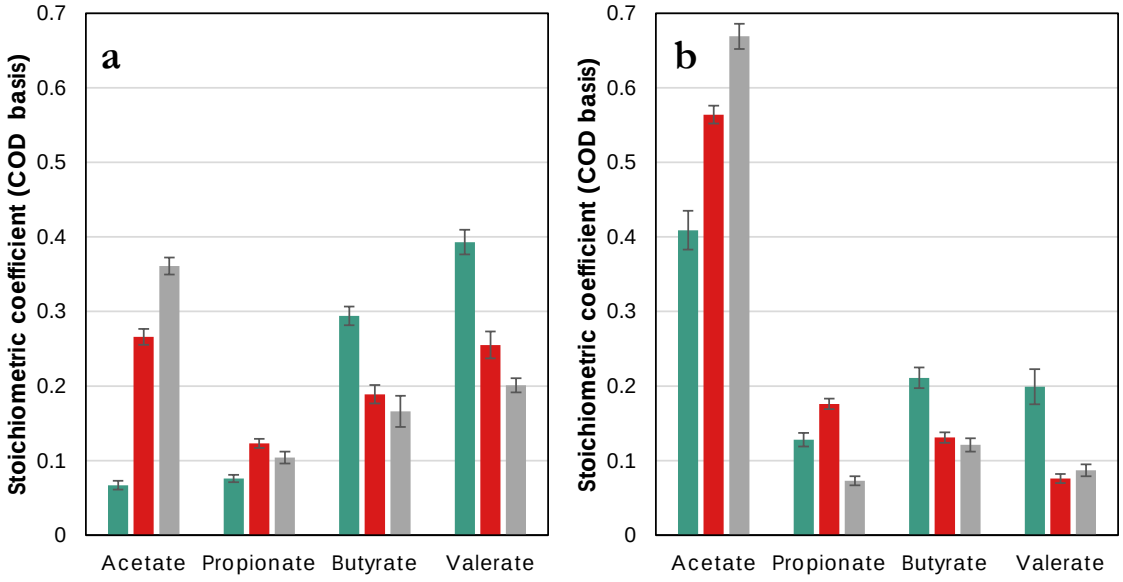

Figure 6. Stoichiometric coefficients with an estimated confidence interval with $\alpha = 0.05$ for casein (a) and gelatine (b) fermentation at three controlled pH values (■ pH 5, ■ pH7 and ■ pH 9).

4. A FRAMEWORK FOR PROTEIN FERMENTATION SIMULATION

As shown in the previous section, the kinetics and stoichiometry of protein MCF are significantly influenced by both the protein composition and the operational conditions (i.e. reactor pH). Additionally, protein composition (amino acid profile) is variable even for proteins of the same name. Since it is not logical from a practical point of view to test experimentally all the possible combinations of proteins and operational conditions, it appears very convenient to develop some criteria for selecting the most adequate parameters for a preliminary simulation of particular scenarios, which could be experimentally validated if needed. In this section, firstly we propose a series of criteria for selecting the most relevant kinetic parameters and stoichiometric coefficients for simulating the VFA production from a given protein and then the potential of the model as an early-stage design tool is shown in two examples.

4.1 Selection of simulation parameters

The proposed guideline to select the most relevant parameters is graphically summarised in Fig. 7, being the details of it stepwise presented in the following subsections. The criteria are proposed based on the results of the kinetic parameters and stoichiometric coefficients estimated from the experiments conducted in this work.

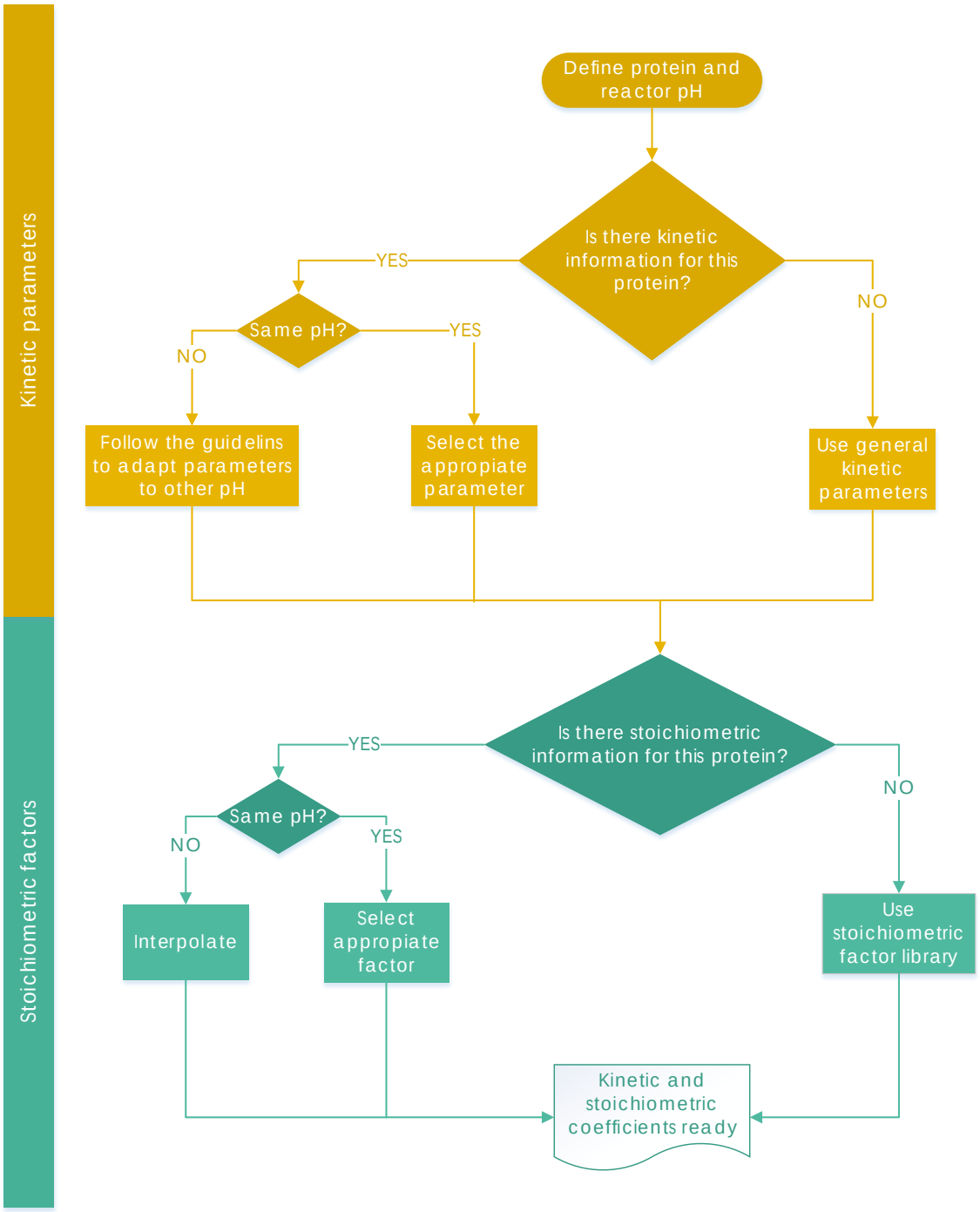

Figure 7. Flowchart for setting the appropriate kinetic parameter and stoichiometric coefficients values.

4.1.1 Kinetic parameters

The values of $q_{Acid,max}$ reported in Table 2 can be used for simulating casein and gelatine fermentation, at three different pH. If other operating pH applies, we propose to use the

pH inhibition function indicated in Eq. 7. The shape of this inhibition function describes a partial inhibition due to high and low pH values and has a value of one at pH 7 (i.e. no inhibition at this point).

$$I = \frac{1 + 2 \cdot 10^{0.5 \cdot (pH_{LL} - pH_{UL})}}{1 + 10^{(pH - pH_{UL})} + 10^{(pH_{LL} - pH)}} \quad (7)$$

where I is the inhibition term expressed as the ratio between the $q_{Acid,max}$ values at a given pH value and its value at pH 7 and pH_{LL} and pH_{UL} are the lower and upper pH limits where the process is inhibited 50% with respect to pH 7. These two parameters modify the shape of the function and might do it asymmetrically (values shown in Table 3).

Therefore, to calculate the value of $q_{Acid,max}$ of a protein at a given pH, its maximum value (i.e. at pH 7) should be multiplied by the value of the function at the desired pH, allowing thus interpolation between pH 5 and 9. In the case that the substrate is composed of other protein, or a casein or gelatine with a significantly different amino acid composition, we propose to use as initial estimation for $q_{Acid,max}$ at pH 7, an intermediate value of 0.30 g_{COD} Prot/g_{COD} BM·h, and a general form of the inhibition function (Table 3) to simulate processes at other pH values.

Table 3. Parameters of the pH inhibition function (Eq. 7) for casein, gelatine and for a generic protein. The determination and the shape of the resultant inhibition functions are available in the Supplementary Materials (section D).

	pH_{LL}	pH_{UL}
Casein	4.96	9.63
Gelatine	4.45	9.97
Generic protein	4.73	9.78

As the biomass yield values were overall less affected by the pH and the type of protein than the other parameters (Table 2), the error derived of using an average value is probably

acceptable. In consequence, it is proposed to use a fixed value of 0.18 g_{COD} BM/g_{COD} Prot, regardless of the pH of the reactor and the protein used as substrate. The values of μ_{max} can be obtained as the product of $q_{Acid,max}$ and the biomass yield and hence recommendations are not needed.

The values of the decay constant are usually expressed as a percentage of the μ_{max} value to directly compare the importance of biomass decay processes with respect to its growth. For the decay constant, we propose here to use in the model a pH-independent value for each of the proteins. Casein k_{decay} would be set to 10% of the μ_{max} value of its fermenting biomass, while gelatine would have a 5% value, according to the experimentally estimated values (Table S7). In case other proteins than casein or gelatine should be modelled, we propose to use a conservative value of 5%. A more detailed description of the determination of these values is available in the Supplementary Materials (section D).

Regarding the acidification equilibrium constant (K_{eq}), we recommend using the values estimated (Table 2), interpolating linearly if necessary. In case of simulating the fermentation of other proteins than casein or gelatine, an initial conservative value of 2 is proposed.

4.1.2 Stoichiometric coefficients

The stoichiometry of protein fermentation was shown to be different for casein and gelatine, but it is affected by the reactor pH in a similar way in both cases: acidic pH promotes the production of butyrate and valerate in detriment of acetate and alkaline values have not a marked effect. Propionate yield does not follow any clear tendency with pH and its variations are smaller.

Following the flowchart of Fig. 7, if the protein used in the system to be fermented is casein or gelatine, the stoichiometry provided in Figure 6 can be used, together with the

aromatic VFA and H_2 stoichiometric coefficients reported in section 2.1, interpolating linearly between the given values if needed. Should another protein be used in the system or be the amino acid profile substantially different, the use of a library containing protein stoichiometric coefficients is recommended. This library can store stoichiometric coefficients estimated directly from experiments (as in Table 2) or can be also built using the metabolic model proposed by Regueira et al. (Regueira et al., 2020b). This metabolic bioenergetic model is able to predict the stoichiometry of protein MCF at any pH value given the composition on amino acids of the protein is known. It proved to reproduce the observed stoichiometry of continuous protein fermentations by mixed cultures of microorganism and to capture accurately the effect of pH on the product spectrum. Therefore, we consider that this model stands as an appropriate tool to use when no experimental information of a particular system is available. Given that the product selectivity of protein fermentation appears not to be affected by the reaction configuration, as the product spectrum of batch tests and continuous reactors is comparable (Bevilacqua et al., 2020), the predictions of the bioenergetic model can also be used when simulating discontinuous reactors. As an example, a small library containing the stoichiometric coefficients for albumin and for a casein and gelatine, with a different amino acid profiles that the ones used in the batch experiments of this work, is available in the Supplementary Materials (section E).

4.2 Application of the model for bioprocess design

The model developed in this work can be very useful for the early-stage design of fermentation processes producing VFA from protein-rich wastes. In this section, two different applications are presented to illustrate this aspect.

4.2.1 Optimising a continuous fermentation reactor

In this section, VFA production from gelatine and casein in a continuous stirred tank reactor (CSTR) at pH 7 is optimised. A critical design parameter in a CSTR is the hydraulic retention time (HRT), which in the case of not having a biomass retention system, is equal to the solid retention time (SRT). Ideally, the HRT value would be as low as possible to increase the productivity of the system, but very low values might also result in lower VFA yields due to incomplete or no substrate conversion provoked by excessive biomass washout. On the contrary, high HRT values could favour (partial) methanisation and hence lower VFA productivities. The influence of the HRT on the VFA productivity and yield of casein and gelatine fermentation is shown in Figure 8. Although higher conversions are attained at high HRT values (green lines in Fig. 8), it might be more interesting to operate where the productivity is maximum (yellow lines in Fig. 8), since it will not worsen excessively the yields. Gelatine top productivity is slightly higher than the one of casein since a higher part of casein is converted to by-products (H_2 and aromatic VFA). However, casein fermentation attains its maximum productivity at a lower HRT (at around 2 days) than gelatine (at around 2.5 days) as its fermentation is faster. In both cases, methane production is minimum and remains below 0.1% of the substrate COD even at the highest HRT values, explained by hydrogen accumulation and the slower growth of methanogenic biomass (data not shown).

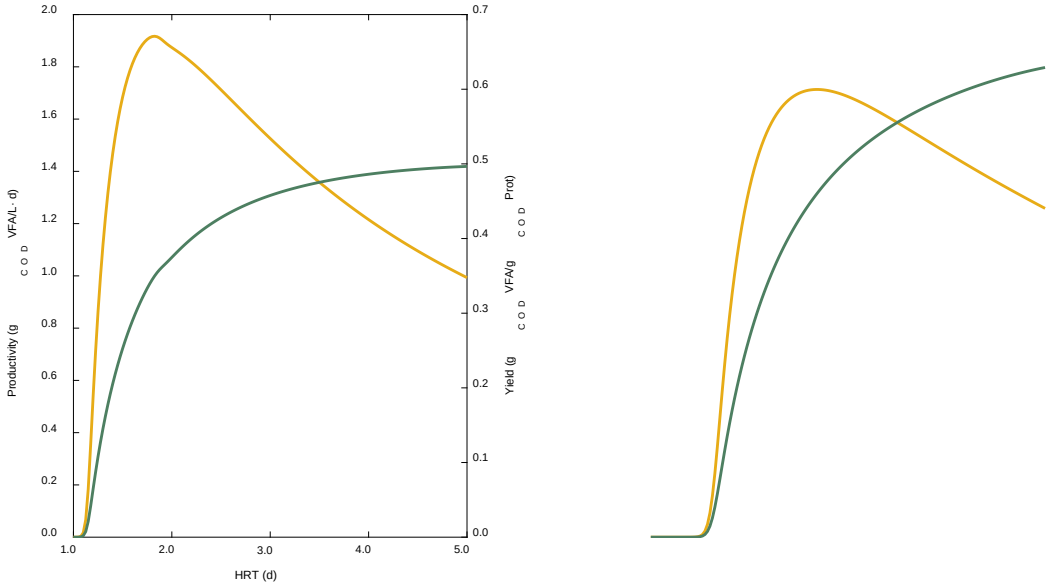

Figure 8. Simulated VFA productivity (yellow lines) and VFA yield (green lines) for casein (left) and gelatine (right) fermentation at different hydraulic retention times (HRT). Initial protein and biomass concentrations are 10 and 1 g_{COD}/L, respectively.

4.2.2 Optimising casein fermentation in a sequential batch reactor

In this section, the fermentation of casein in a sequential batch reactor (SBR) is simulated at two pH values (5 and 7) with the objective of optimising the production of butyrate and valerate, the most reduced and valuable VFA of the protein product spectrum. SBR is an interesting configuration to boost volumetric productivities as it is possible to uncouple the SRT and the HRT of the reactor, allowing in this way to attain high biomass concentrations. In each cycle part of the liquid is renovated with fresh substrate and usually the solids (i.e. mainly biomass if the feeding has low solids concentration) are mostly retained to ensure a high biomass concentration. Here we define the volume exchange and solid withdrawal ratio as a measurement of the fraction of the solid-free liquid that is withdrawn and renovated with new feeding and the fraction of solids that are withdrawn in each cycle. The solids withdrawal ratio can be controlled by the operator with the settling time before emptying the reactor or using membranes to retain solids, for example.

To ensure a high productivity of the system, the model could be used to define an optimal cycle length and solid exchange ratio that maximise VFA productivity, while avoiding substrate conversion to methane. In this example, the objective set was the maximisation of the productivity of the most reduced VFA: butyrate and valerate. SBR were simulated at different cycle lengths and solid exchange ratios at pH 7 (Fig. 9) and pH 5 (Fig. 10), with the same initial conditions as in the previous example and assuming that in each cycle 90% of the reactor volume is renovated.

At pH 7, operating at high cycle length and low solid exchange ratio (right bottom corner in Fig. 9) would lead to high conversion of the substrate to methane, while short cycle length times and high solids exchange ratio values (top left corner in Fig. 9) lead to the washout of the fermentative biomass (Fig. S3), resulting in lower or null VFA productivities. The optimal productivity of both butyrate and valerate is achieved at cycle lengths between 3 and 6 h and at the lowest solids exchange ratio (around 5.5 and 7.6 $g_{COD}/L \cdot d$ for butyrate and valerate, respectively). Under these conditions, the concentration of biomass is maximal in the reactor (Fig. S3) and provides a high conversion capacity, which combined with the short cycle times, boost the volumetric productivity. Methane production is effectively inhibited in this operational region due to methanogenic biomass washout since its growth is slower than that of casein fermenters. However, if the fraction of non-converted casein is considered (Fig. S4), it would be better to operate at slightly longer cycle times (around 8 h) to ensure full conversion of the substrate. At these conditions, butyrate and valerate productivities have a value of around 5 and 7 $g_{COD}/L \cdot d$, respectively.

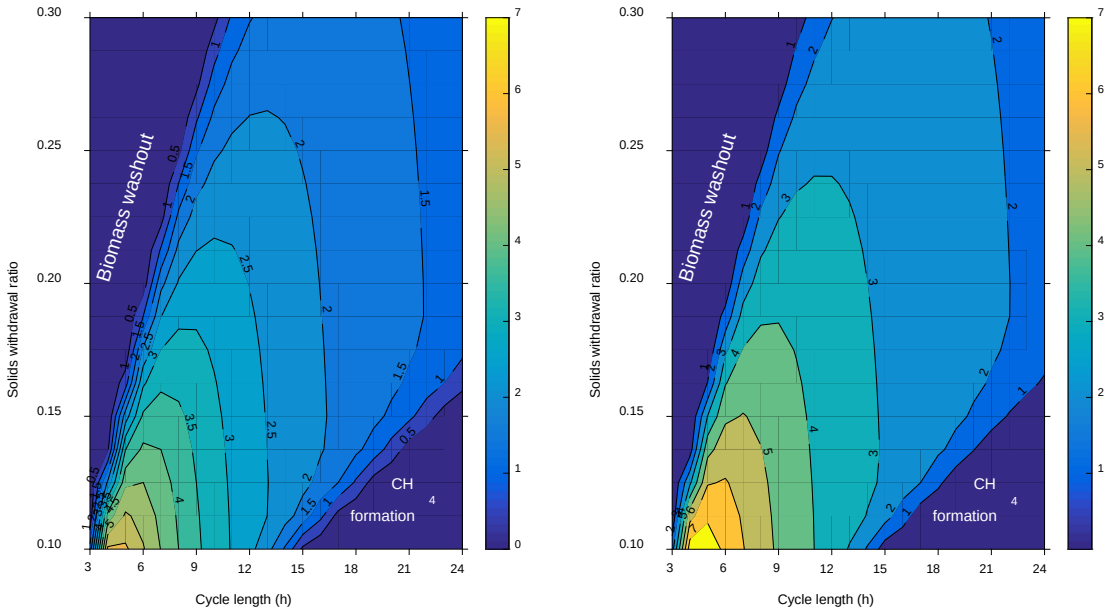

Figure 9. Butyrate (left) and Valerate (right) productivities ($g_{COD}/L \cdot d$) in an SBR reactor under different cycle length (horizontal axis) and solids withdrawal ratio (vertical axis) for casein fermentation at pH 7. In each cycle, 90% of the reactor volume is renovated.

At pH 5 (Fig. 10), cycle length should be longer than at pH 7 to avoid fermentative biomass washout since its growth is slower under acidic conditions (Table 2). In this case, methanogenesis is completely inhibited under all tested operational conditions due to the much lower growth rate of methanogenic biomass at acidic conditions. Although the selectivity for butyrate and valerate is higher at pH 5 than at pH 7 (Fig. 6), their productivities are lower due to the required longer cycle lengths (4 and 6 $g_{COD}/L \cdot d$ for butyrate and valerate, respectively). Overall, using an optimised SBR can increase up to ten times the global VFA productivity in comparison with a continuous reactor.

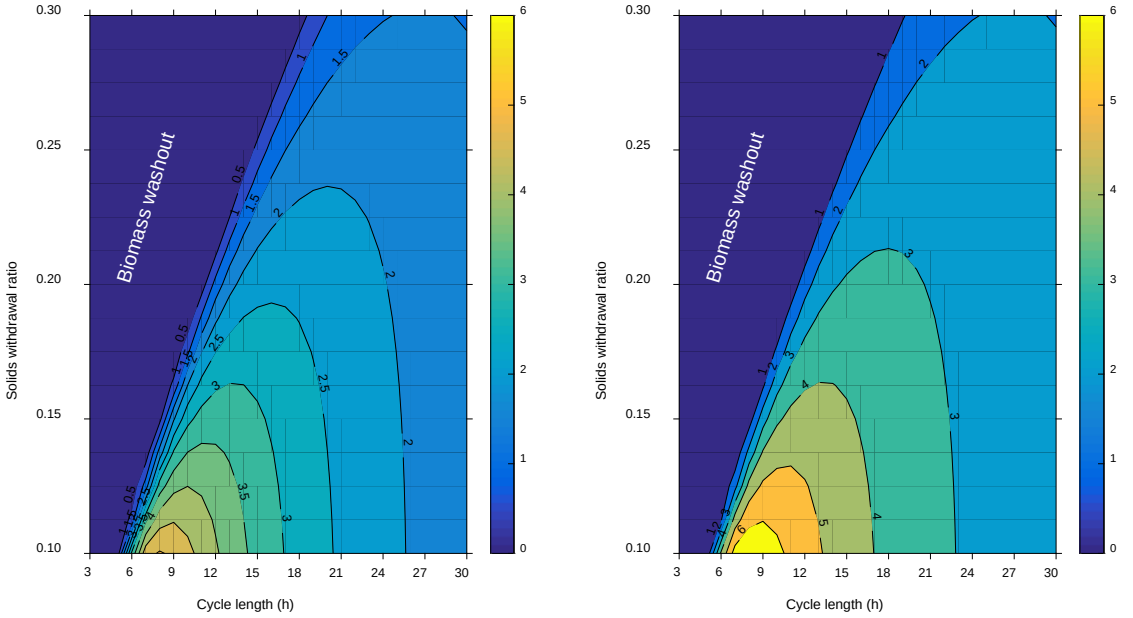

Figure 10. Butyrate (left) and valerate (right) productivities ($g_{COD}/L \cdot d$) in an SBR reactor under different cycle length (horizontal axis) and solids withdrawal ratio (vertical axis) for casein fermentation at pH 5. In each cycle 90% of the reactor volume is renovated.

5. CONCLUSIONS

A kinetic model for the production of volatile fatty acids in protein mixed-culture fermentation was developed using expressly designed experiments for its calibration. The model captures the effect of different proteins (casein and gelatine) and pH on the kinetics and stoichiometry of the process. The kinetic parameters show that casein fermenters grow and consume the substrate faster than gelatine fermenters and that pH exerts a greater influence on the kinetic parameters of casein fermenters. The stoichiometry of the process differs in gelatine and casein fermentation being gelatine dominated to a great extent by acetate while casein showing a more balance product spectrum. Acidic pH favours the production of reduced VFA (e.g. butyrate and valerate) while alkaline values shift the production towards acetate, regardless of the protein. The commonly observed incomplete protein consumption was reflected in the model assuming that the conversion reactions attain an equilibrium due to thermodynamic limitations, since product inhibition was

discarded based on the experimental data of this work. The utility of the model and the proposed simulation framework and its guidelines was illustrated with two case studies on selecting the optimal design parameters to maximise the productivity of the desired VFA(s).

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