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Chain elongation may occur in protein mixed-culture fermentation without supplementing electron donor compounds

R. Bevilacqua, A. Regueira, M. Mauricio-Iglesias *, J.M. Lema, M. Carballa

CRETUS, Department of Chemical Engineering, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

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A B S T R A C T

This study focuses on verifying the occurrence of elongation processes during protein mixed culture fermentation, without the supplementation of electron donor compounds. During casein mixed-culture fermentation at pH 5, it was observed that longer chain volatile fatty acid production increased, which could not be justified by the associated amino acid consumption. Consequently, the occurrence of chain elongation processes was hypothesized. To verify this hypothesis, three casein batch tests, with and without acetic acid initial supplementation, were performed at pH 5. The results suggest that acetic and propionic acids are indeed consumed to selectively generate n-valeric acid through the coupling with electron donor amino acids, whose consumption was further verified through the amino acid analysis. Prolonged simultaneous availability of suitable amino acids and short chain volatile fatty acids and an acid equivalent concentration threshold were identified as key parameters for the occurrence of chain elongation. The supplementation of acetic acid at the beginning of the test changed the selectivity of the elongation process, promoting n-butyric and iso-valeric production. The associated mechanisms were preliminary conceptualized, constituting a first step for further studies on the subject. To the best of our knowledge, this is the first study demonstrating the feasibility of chain elongation processes during protein mixed culture fermentation without electron donor supplementation.

1. Introduction

Chain elongation (CE) is an appealing bioprocess which converts volatile fatty acids (VFA) and compatible electron donor compounds (e.g. ethanol, lactate) to longer chain carboxylic acids in anaerobic microbial cultures [1]. Carboxylic acids with four or more carbon atoms are generally more profitable ($\geq$2 €/kg for C_4+) and easier to separate from the fermentation broth than the shorter chain ones due to their greater hydrophobicity [2], thus underlining CE as an opportunity to upgrade the products of mixed-culture fermentations in a resource recovery perspective [3]. The accepted CE mechanism, based on studies conducted mainly on *C. kluyveri*, is the reverse β-oxidation [4,5]. This cyclic reaction elongates short chain VFAs by adding two carbon atoms (acetyl-CoA molecule) per cycle to the original hydrocarbon backbone while consuming reducing power. Both the acetyl-CoA and the reducing power are provided by the conversion of suitable electron donor compounds, such as ethanol or lactate. The end products present an even or an odd number of carbon atoms depending on the precursor VFA, respectively acetic and butyric acid or propionic and valeric acid [6].

Many studies highlight that CE processes often require the supplementation of electron donor compounds, such as ethanol or lactate [7–9]. For example, Grootscholten et al. [10] achieved high n-caproic acid titers, and to smaller extent n-heptanoic and n-caprylic acids, using ethanol-supplemented municipal solid waste as substrate. Liang and Wan [11] observed that fermenting brewers' spent grain with ethanol promoted the production of n-valeric and n-caproic acid, while lactate addition stimulated n-butyric acid formation as end product. However, other studies show that the external electron donor supplementation can be bypassed when these compounds are produced in-situ together with the required VFAs. Contreras-Dávila et al. [12] demonstrated the potential of performing CE via consecutive lactate formation and consumption during food waste sequential-batch fermentation. Lactate was progressively produced by *Lactobacillus* ssp. from the carbohydrates fraction of food waste, and subsequently used by *Caproiciproducens* ssp. to elongate n-butyric acid to n-caproic acid. Similarly, Carvajal-Arroyo et al. [13] illustrated the feasibility of high-rate n-caproic acid production using thin stillage without external supplementations, as the carbohydrates contained in the feedstock were mainly being converted to

* Corresponding author.
E-mail address: miguel.mauricio@usc.es (M. Mauricio-Iglesias).

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lactate and subsequentially used as electron donor compound by the elongation microbiome.

Most of the CE studies available in literature focus on carbohydrates as the main substrate, such as acid whey from the dairy industry [14], sugarcane molasses [15], thin stillage [13] or food waste [12]. On the contrary, little is known about CE processes during anaerobic fermentation of proteins. Only two studies could be found [16,17], which were performed with a pure culture of *Eubacterium pyruvativorans*, a bacterium isolated from bovine rumen. The authors observed that, supplying the amino-acid-enriched culture medium with acetic, propionic or butyric acid, the VFAs were consumed and elongated to n-butyric, n-valeric and n-caproic acids, respectively, using some amino acids (AA) as electron donor compounds (e.g. alanine and leucine). However, there is no information about CE elongation processes during anaerobic mixed-culture fermentation of proteins. Of the twenty most frequent amino acids in proteins, some can take the role of electron donors, acceptors or even both [18]. The diversity of redox roles suggests that proteins might undergo simultaneous fermentation and CE processes without the need for electron donor supplementation.

In the framework of a project on the valorization of protein-rich residues, this study investigates the feasibility of CE elongation processes during anaerobic mixed culture fermentation of proteins without the supplementation of electron donor compounds. We tried to verify whether their occurrence could explain the increased production of longer chain VFA at low pH conditions, which could not be completely justified by the associated amino acid consumption.

2. Materials and methods

2.1. Previous experiments

Two continuous stirred tank reactors (CSTR) were operated with two different proteins, casein and gelatin, at different pH values (5, 7 and 9) to understand the effect of substrate composition [19] and pH [20] on protein mixed culture fermentation. The 1L (working volume) reactors were continuously sparged with nitrogen (10 mL/min) and maintained at 25 °C in a climatised chamber. The organic loading rate and hydraulic retention time were 5.3 g COD/L·d and 1.5 d respectively.

2.2. Chain elongation batch experiments

Batch experiments were conducted with casein as substrate in order to: (i) confirm CE presence during anaerobic mixed-culture fermentation of proteins, (ii) understand the role of AA in the process, and (iii) verify whether the addition of shorter chain carboxylates (i.e. acetic and propionic acid) can affect CE process extension and/or selectivity.

In each assay, a 2L sealed glass vessel (1L of working volume) was used, and the operational conditions were set to be similar to the continuous reactors (25 °C, N_2 sparging, pH 5). The inoculum was obtained from the continuous reactor and its initial concentration was maintained at 0.5 g VSS/L. Synthetic hydrolyzed protein, peptone from casein (A2208,0500 PanReac), was used as the sole carbon source. Its composition was previously determined in a separate study [19]. The initial casein concentration was equal to 5 g COD/L for the first test, while it was increased to 10 g COD/L for the following two tests, corresponding to a substrate-to-inoculum ratio (SIR) equal to 10 and 20 g COD/g VSS, respectively. The third batch test was initially supplemented with acetic acid at an approximate concentration of 0.5 g/L. Macronutrients were also added in all batch tests at the following concentrations (g/L): NaCl 0.292; KH_2PO_4 0.780; NH_4Cl 0.530, Na_2SO_4 0.057; $MgCl_2$.6 H_2O 0.120.

10 mL samples were taken at increasing time intervals (initially 2–3 h). Half of the sample volume was centrifuged and filtered for total ammonia nitrogen (TAN), VFA and alternative products (i.e. lactate, ethanol, formic acid) determination. The remaining 5 mL were centrifuged, and the biomass solid pellets were then resuspended in 5 mL of a 0.7% w/w solution of NaCl and distilled water for optical density determination. The supernatant was frozen for AA analysis. At the end of each experiment, VSS concentration of the fermentation broth was also measured.

2.3. Analytical methods

Most physicochemical parameters were determined according to Standard Methods [21]. Mixed liquor samples were used to measure total and volatile suspended solids, while filtered (0.45 μm) mixed liquor samples were used to determine TAN, VFA and amino acids concentration. All spectrophotometric measurements were performed with a Shimazdu UV-1800.

VFAs from C2 to C7 were measured through gas chromatography (AGV-DB1 method), though organic acids with more than five carbon atoms were never detected. The gas chromatographer was an Agilent 6850 with a flame ionization detector, using a DB-Wax column (Agilent Technologies, 30 m × 0.250 mm × 0.25 μm). The injector and the detector were set at a temperature of 200 °C and 300 °C respectively. The carrier gas was nitrogen. The samples were filtered (0.45 μm) and then acidified with 10 μM of concentrated H_3PO_4 (85%) beforehand.

Secondary metabolites (e.g., lactate) were determined, albeit never detected, using a Hewlett-Packard 1100 high performance liquid chromatographer (GLEFG1 method) equipped with an infrared Hewlett-Packard 1047 A detector. The column used was an Aminex HPX-87 H (Bio-Rad Laboratories, 300 × 7.8 mm) using H_2SO_4 (5 mM) as eluent. The column temperature was 30 °C while for the detector was 35 °C.

For total AA determination, the AccQ-Tag method was used, converting preventively hydrolyzed AAs (24 h with HCl 6N) into stable fluorescent derivatives, which were then analysed with a Waters 2695 high performance liquid chromatographer through a fluorescence detector (Waters 2475).

Turbidity (i.e. optical density) was determined using the OD600 method calibrated with actual volatile suspended solids measurements.

2.4. Calculations

Acidification degree was calculated to describe the substrate conversion (in COD basis), expressed as:

$$Acidification\ degree\ (\%) = \frac{\Sigma\ C_{VFA-COD}}{C_{pr}} \times\ 100 \quad (1)$$

where $C_{VFA\text{-}COD}$ stands for the total concentration of the measured aliphatic VFAs (in g COD-VFA/L) in the fermentation liquor and C_{pr} for the initial protein concentration (in g COD/L).

As CE leads to a higher fraction of longer-chain VFA, i.e. more reduced VFA, the degree of reduction, defined as the average COD per gram of produced aliphatic VFAs, was the proposed parameter to evaluate the presence of CE processes:

$$Degree\ of\ reduction\ (gCOD/gVFA) = \frac{\Sigma\ C_{VFA-COD}}{\Sigma\ C_{VFA}} \quad (2)$$

where $C_{VFA\text{-}COD}$ stands for the total COD concentration of the measured VFAs (in g COD-VFA/L) and C_{VFA} for the total concentration of the measured VFAs (in g VFA/L) in the fermentation liquor. For VFA with 2–6 carbon atoms, this index varies between 1.07 g COD/g VFA corresponding to pure acetic acid and 2.21 g COD/g VFA corresponding to pure caproic acid.

To better understand the mechanisms involved in the AA-based CE process, balances between AA consumption and VFA production were established. AA consumption was calculated from the measured AA molar concentrations in the influent and effluent of the reactors. To link AA consumption and VFA production, the stoichiometry of AA conversion proposed by Ramsay and Pullammanappallil [22] was used, with due corrections introduced by Regueira et al. [23].

Table 1
Average degree of reduction (expressed as g COD/g VFA) achieved during continuous mixed culture fermentation of casein and gelatin at different pH values.

Protein	pH 5	pH 7	pH 9
Casein	1.72 ± 0.06	1.47 ± 0.01	1.47 ± 0.01
Gelatin	1.40 ± 0.02	1.36 ± 0.02	1.29 ± 0.02

3. Results and discussion

3.1. pH and amino acids composition determine chain elongation feasibility during protein mixed culture fermentation

In a previous study [20], it was observed that longer chain VFA production is promoted during protein continuous fermentation at acid conditions, resulting in higher average degrees of reduction than at neutral and alkaline pH values (Table 1). Moreover, this increased longer chain VFA production did not correspond with the consumption

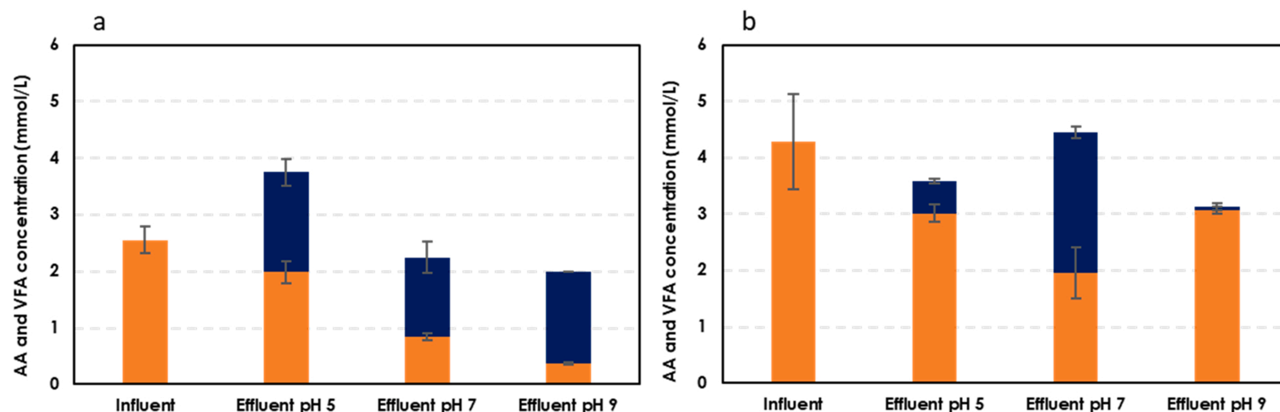

Fig. 1. n-Valeric acid molar balance in the continuous casein (a) and gelatin (b) reactors at three different pH values. Proline; n-Valeric. Proline concentration is expressed in VFA equivalents based on the associated stoichiometry.

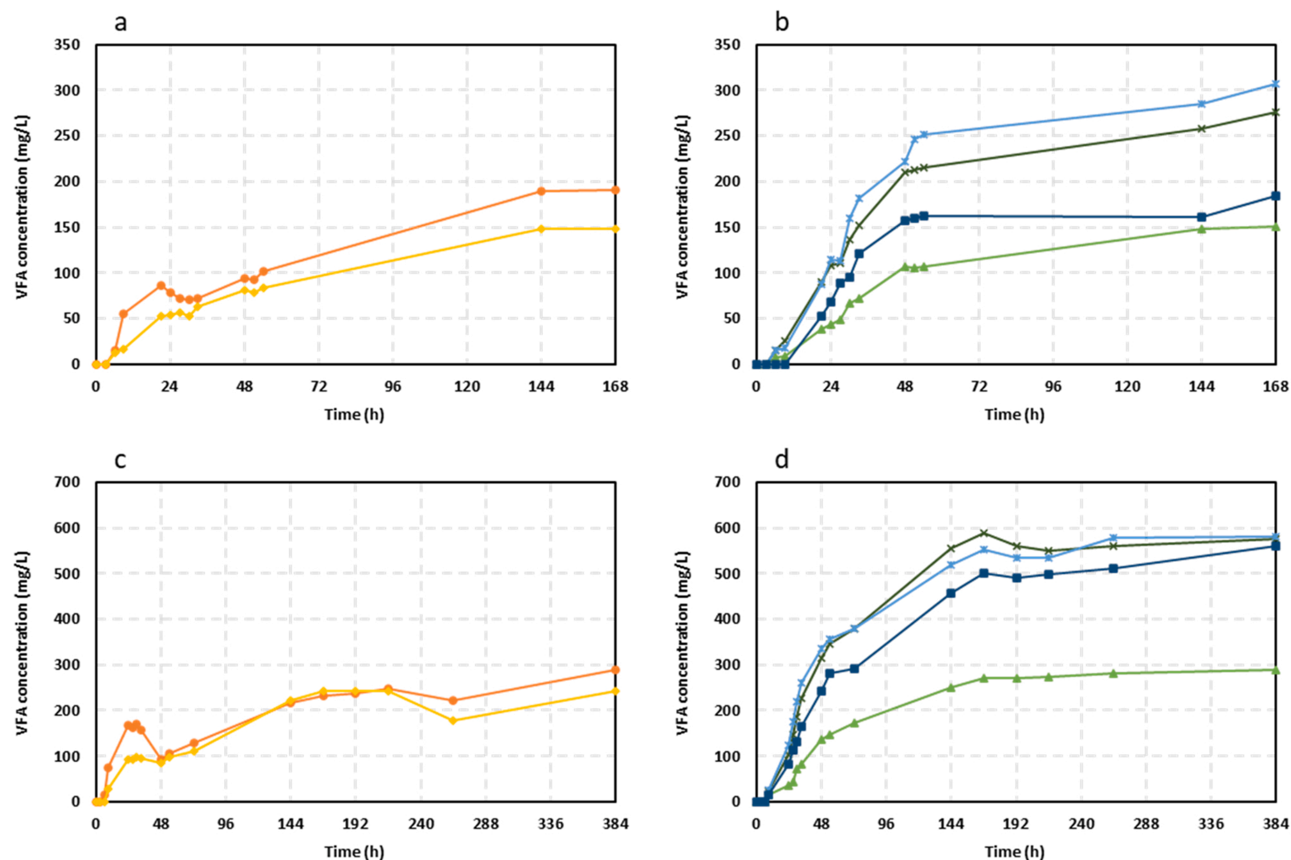

Fig. 2. VFA production during casein batch fermentation at pH 5 and SIR 10 (a, b) and SIR 20 (c, d). Acetic; Propionic; Iso-Butyric; x n-Butyric; * Iso-Valeric; n-Valeric.

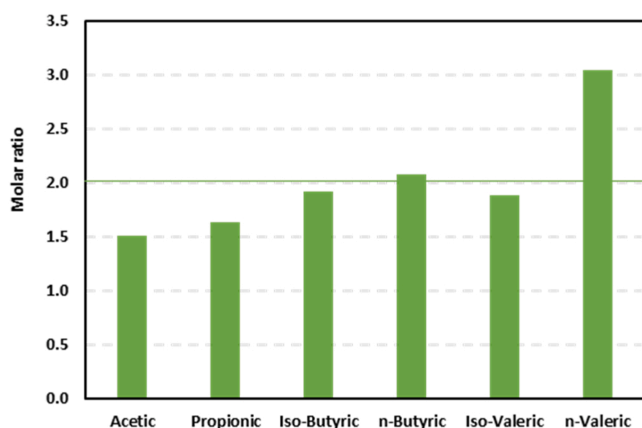

Fig. 3. Ratios between the final VFA molar concentrations obtained during casein batch fermentation at SIR 20 and SIR 10, respectively. The horizontal line represents the proportionality threshold.

of the precursor amino acids. Thus, it was hypothesized that CE processes might have been contributing to the observed change of selectivity. As this tendency was only observed at acid conditions and especially during casein fermentation, it was also hypothesized that pH and protein composition could play a role in promoting the elongation process.

Although it cannot be completely excluded, the formation via elongation of iso-butyric [24,25], n-butyric [26] and iso-valeric [27,28] acids was discarded due to their production being generally consistent with the associated AA consumption. Conversely, n-valeric acid formation via elongation can be more easily verified since proline is the only precursor AA (1 mmol Pro = 0.5 mmol n-Val [29]). Interestingly, proline consumption alone could not justify n-valeric production during casein fermentation at acid conditions (Fig. 1a), strengthening the hypothesis that low pH values are required for the protein-based CE to occur. However, no excess of n-valeric acid was detected during gelatin reactor operation, regardless of the operational pH (Fig. 1b).

These results suggest that the bacterial communities could be performing CE not only as a sink strategy for NADH surpluses [1], but also as a mean to reduce the number of acid equivalents in the reactor bulk. Indeed, at low pH there is a larger fraction of undissociated VFA that can diffuse back through the cell membrane to the intracellular space. The higher conversion of casein conversion to VFAs at pH 5 (30%) than gelatin (20%) could explain why this detoxification strategy was not observed for both proteins. Moreover, casein fermentation generates an excess of reducing power [19] which could be consumed by elongating short chain VFAs, whereas gelatin composition features a higher percentage of electron acceptor AAs (58.6%) than casein (26.4%), limiting the need for CE processes to maintain the redox balance.

3.2. *Substrate-to-inoculum ratio affects the extent of the elongation process*

To verify the abovementioned hypotheses, casein batch tests were conducted at two different SIR values: 10 and 20 g COD/g VS. At SIR 10, a final acidification and degree of reduction of 43.5% and 1.76 were respectively attained. The major products were n-butyric and iso-valeric acids, with concentrations greater than 250 mg/L (Fig. 2b). The concentrations of acetic and propionic quickly rose during the first 24 h, then stagnated for few hours to continue further increasing later (Fig. 2a), while the other carboxylic acids kept being steadily produced. Notably, n-valeric acid production showed a lag phase of approximately 12 h (Fig. 2b). A comparable final acidification and degree of reduction were achieved at SIR 20 (45.3% and 1.80, respectively), although the duration of the batch test was increased to 384 h (Fig. 2c and d), thus ruling out the occurrence of products inhibition. Once again, n-butyric and iso-valeric acids were the major products together with n-valeric acid, the three VFA reaching concentrations higher than 550 mg/L. Similarly to the previous test, acetic and propionic acid concentration increased during the first 24 h and then decreased even more markedly than the trend observed for the SIR 10 test (Fig. 2c). These stagnation periods might correspond to in-situ consumption of short chain VFAs associated with elongation processes [28].

The VFA concentrations of the SIR 20 test were expected to double those of the SIR 10 one, as similar acidification degrees were achieved, thus ruling out product inhibition. However, this pattern was only verified for three VFAs (Fig. 3): iso-butyric, n-butyric and iso-valeric acids. The ratio for acetic and propionic acid was below 2 while n-valeric acid one was higher than 3.0 (Fig. 3), confirming the occurrence of CE processes. Although the elongation of acetic and propionic acid to n-valeric acid at SIR 10 cannot be excluded, n-valeric production (1.80 mmol/L) fitted quite well with the maximum production

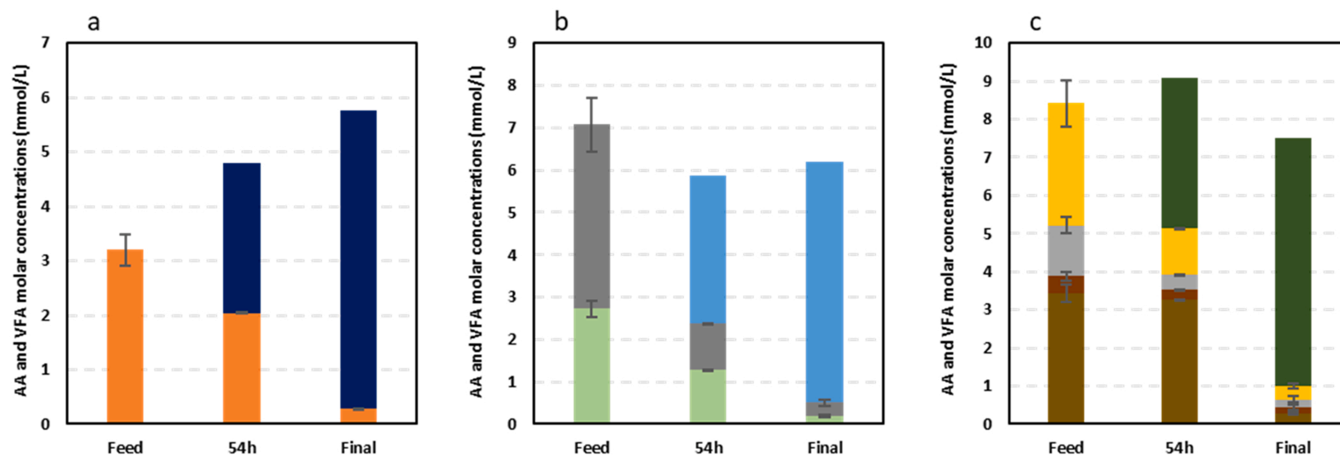

Fig. 4. n-Valeric (a: Proline; n-Valeric), iso-valeric (b: Isoleucine; Leucine; Iso-Valeric acid) and n-butyric acid (c: Glutamic acid; Histidine; Threonine; Lysine; n-Butyric acid) balances in the casein batch test at SIR20 and pH 5. AA concentrations are expressed in VFA equivalents based on the associated stoichiometry.

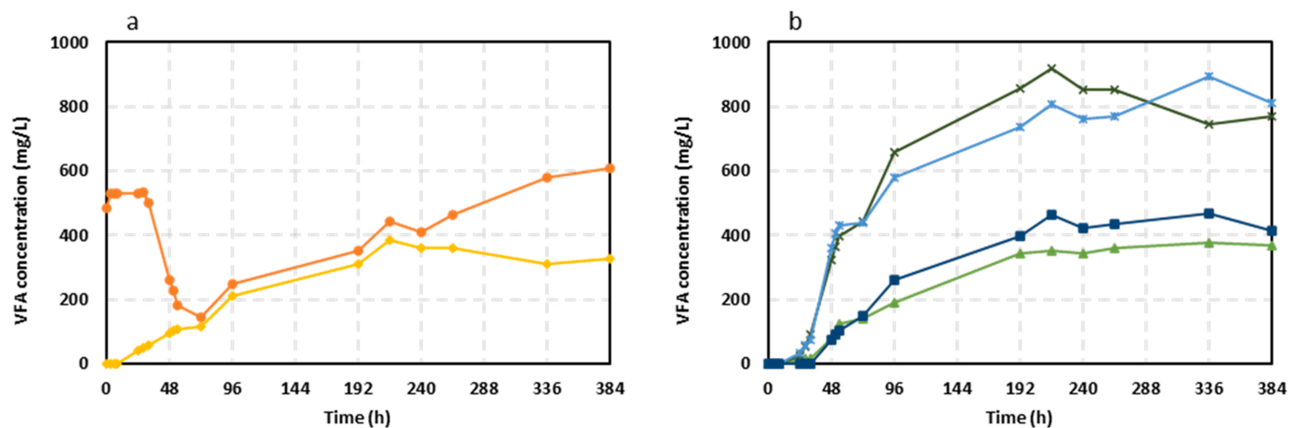

Fig. 5. VFA production during casein batch fermentation at SIR 20 and pH 5, with initial acetic acid addition (500 mg/L). a: ● Acetic; ◆ Propionic; b: ▲ Iso-Butyric; x n-Butyric; ∗ Iso-Valeric; ■ n-Valeric.

estimated assuming complete conversion of proline (1.60 mmol/L), the only AA assumed to yield n-valeric acid [29]. Conversely, the same estimation applied to the SIR 20 test unequivocally confirms the occurrence of CE (Fig. 4a). In fact, the SIR 20 results suggest that almost 50% of the n-valeric production could have been produced through the elongation pathway. The CE process appears to be strongly selective towards n-valeric formation, as the iso-valeric (Fig. 4b) and the n-butyric (Fig. 4c) balances close quite well.

Based on these results, apart from the pH and protein composition, the prolonged simultaneous availability of suitable electron donor AA and short chain VFAs seems to play an important role in determining the extent of the CE process. In fact, higher initial substrate concentrations in the batch test allowed the production of the short chain VFAs which were then elongated without depleting the AAs required by the CE process. In a continuous fermentation process, the continuous feeding seems to bypass this AA limitation, since CE contribution to n-valeric formation was greater during CSTR operation (68.2%, Fig. 1a) than in the SIR 20 batch test (53.2%, Fig. 4a).

3.3. Understanding the role of acetic and propionic acid during protein-based chain elongation

A third batch test was performed with casein at SIR 20 and with the initial supplementation of acetic acid at a concentration of approximately 500 mg/L to understand whether the supplementation of short chain VFAs can promote CE processes during protein fermentation, thus avoiding the associated substrate limitations described in Section 3.2.

Assuming that acetic acid production and consumption occurs simultaneously, three periods can be differentiated (Fig. 5): (i) similar consumption and production rate during the first 32 h (i.e., acetic acid concentration remains mainly unchanged); (ii) higher consumption rate than production during the subsequent 40 h, and, (iii) higher production rate than consumption from 72 h on. Except for propionic acid, the other VFAs started to be produced only after 24 h, suggesting that the acetic acid supplementation led to an adaptation phase during which the biomass was not able to efficiently convert the substrate. Interestingly, n-valeric acid formation began only after acetic acid concentration started to decrease visibly. n-Butyric and iso-valeric acids were confirmed to be the main products of the fermentation (approximately 800 mg/L). Propionic acid did not show any sign of stagnation and its final concentration was 300 mg/L, while iso-butyric and n-valeric levels were slightly higher (400 mg/L). At the end of the test, the acetic acid consumption almost balanced its production as the final concentration is comparable to the initial one. The acidification degree (excluding the initially added acetic acid) and the degree of reduction were 50.9% and

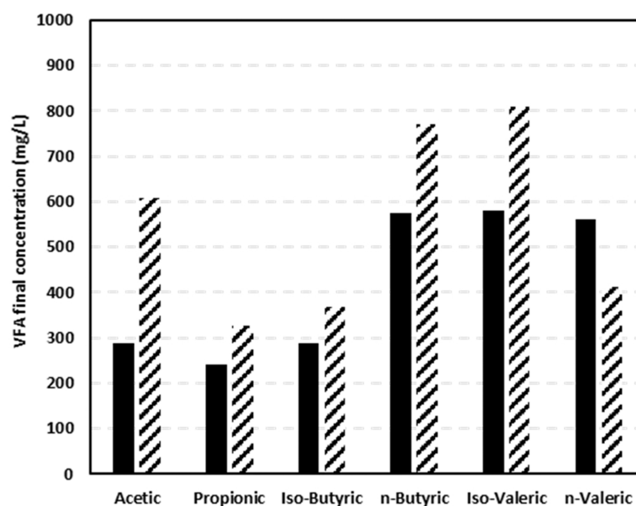

Fig. 6. Comparison between the VFA final concentrations obtained during the batch tests at SIR 20 and pH 5 with (■) and without (▨) acetic acid supplementation (500 mg/L).

1.86 g COD/g VFA, respectively.

The supplementation of acetic acid favors the production of n-butyric and iso-valeric acids but limits the formation of n-valeric acid (Fig. 6). The final concentration of acetic acid (600 mg/L) is lower than the sum of the supplementation (500 mg/L) plus the production during the SIR 20 test (300 mg/L), indicating a lower formation of this VFA during the fermentation of casein or a higher consumption during CE processes. The increase in propionic and iso-butyric acid production is probably not significant, as it was quite limited (< 90 mg/L) in both cases, and consequently attributed to experimental variability.

These results suggest that the initial availability of acetic acid can alter the selectivity of the CE process, especially promoting its elongation to n-butyric acid. Although it does not completely justify the increase in n-butyric acid final concentration (200 mg/L), the difference between expected and measured acetic acid concentration (200 mg/L) seems to be closely related. Moreover, the molar balance of this VFA (Fig. 7a) evidenced that its four precursor AAs were not converted during the first 54 h, strengthening the hypothesis of the supplemented acetic acid being consumed to produce n-butyric acid [30] through the coupling with acetyl-CoA from a suitable electron donor AA such as alanine [17].

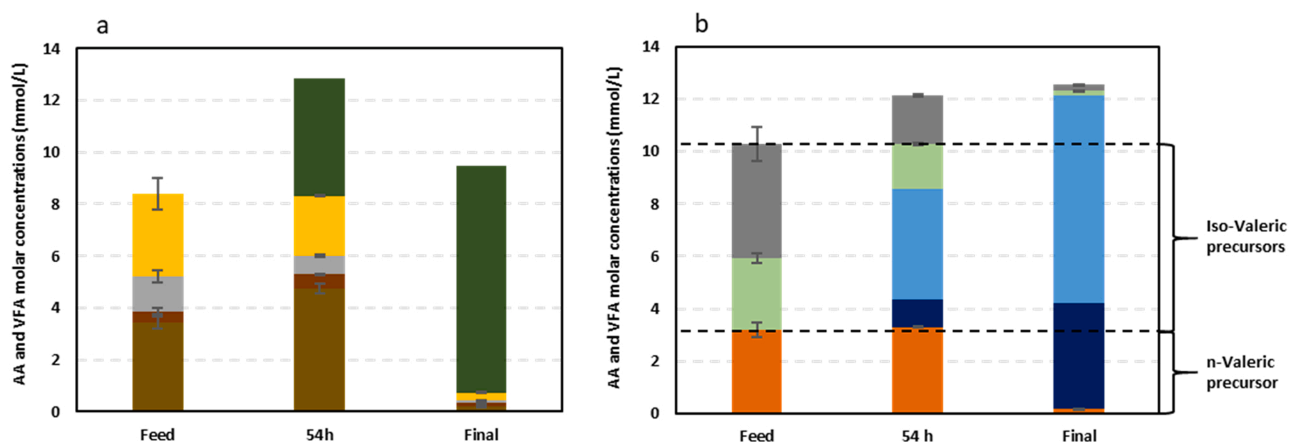

Fig. 7. n-Butyric acid (a: Glutamic acid; Histidine; Threonine; Lysine; n-Butyric acid) and global valeric (b: Proline; n-Valeric; Isoleucine; Leucine; Iso-Valeric acid) balances in the casein batch test at SIR20 and pH 5 with initial acetic acid addition. AA concentrations are expressed in VFA equivalents based on the associated stoichiometry.

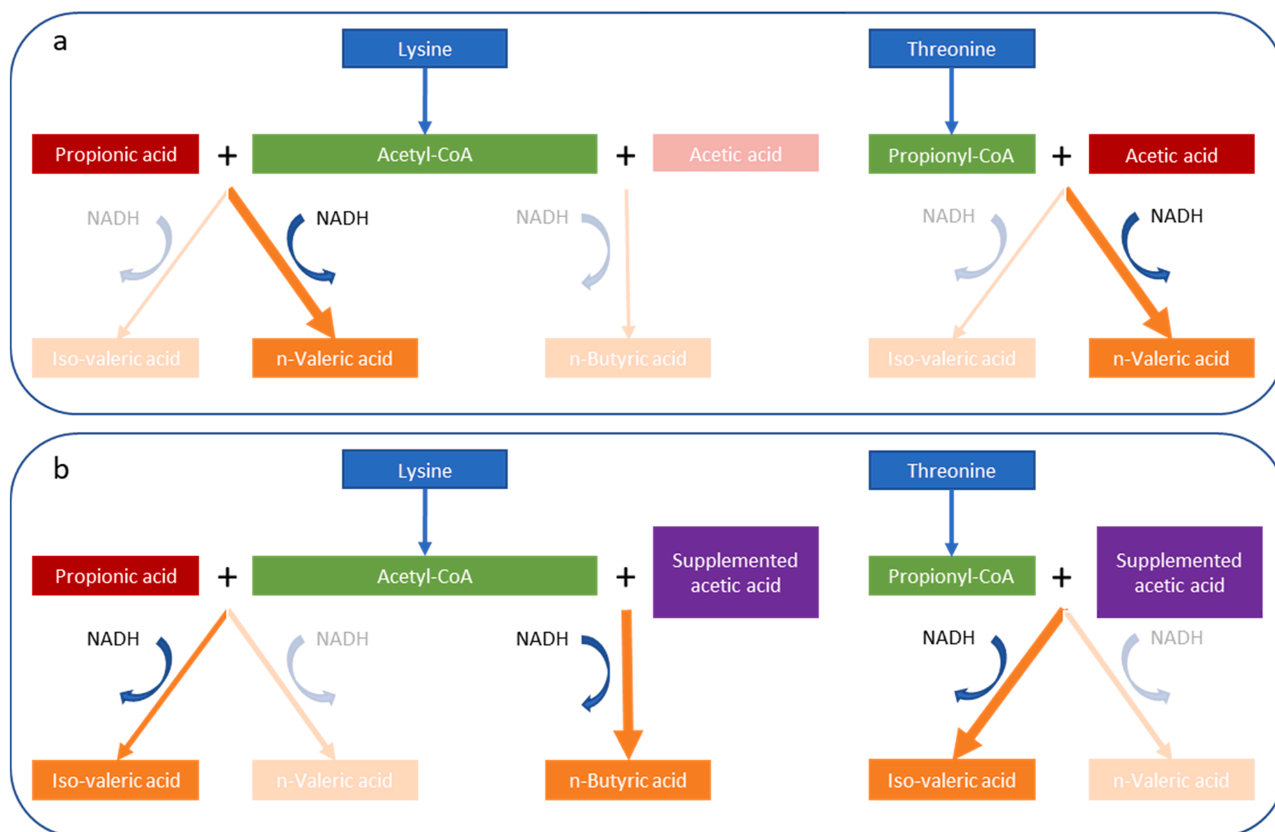

Fig. 8. Conceptual protein-based chain elongation mechanisms based on short chain VFA and AA consumption (a: initial lack of acetic and propionic acids; b: initial availability of acetic acid). short chain VFA; supplemented short chain VFA; AA; intermediate metabolite; Elongated product. The wider arrow represents the favored elongation reactions, whereas the faded colors indicate absent or minor routes.

The increase of iso-valeric acid in the supplemented test corresponds to the decrease of n-valeric acid. This can be verified as the sum of iso and n-valeric acid in each test is approximately 1200 mg/L (Fig. 6), suggesting that the acetic acid supplementation might have diverted the CE process from the production of the linear form to the branched-chain one. The global valeric acid balance (Fig. 7b) unequivocally confirms the contribution of CE to the production of both acid forms, as their concentrations exceed the consumption of the parent AA. Interestingly, CE was initially the only responsible for n-valeric acid generation (54 h), even before proline started being consumed.

3.4. *Highlighting the underlying mechanisms of protein-based chain elongation*

From the abovementioned results, it can be concluded that casein-based CE is indeed feasible and occurs by using acetic or propionic acid as electron acceptor compound (Fig. 8). Suitable electron donor AAs will supply reducing power and propionyl-CoA for CE with acetic acid, or acetil-CoA for CE with either propionic or acetic acid [28]. Several AAs (alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, serine) can supply either propionyl-CoA or acetyl-CoA as they have pyruvate as intermediate product of their conversion [29]. Methionine and lysine are associated to only propionyl-CoA or only acetyl-CoA, respectively. Threonine can provide both these compounds via different pathways [29]. Overall, the fact that no external electron donor supplementations are required makes this kind of CE particularly appealing in a biorefinery framework. In fact, the operational costs and the chemical use should be lower than for ethanol and/or lactate-based CE [1], motivating further investigation on this aspect of the process.

In protein-based CE, the process selectivity seems highly dependent on the initial availability of short chain VFA. If the process is initially limited by the availability of short chain VFAs, it becomes particularly selective towards n-valeric acid (Fig. 8a), albeit the elongation of the other VFAs cannot be completely excluded (Section 3.2). Conversely, the initial supplementation of acetic acid clearly alters the selectivity of the process (Fig. 8b), promoting the elongation of this short chain VFA to n-butyric acid while prioritizing the formation of iso-valeric acid over the n-valeric (Section 3.3).

4. Conclusions

To the best of our knowledge, this study identified and targeted for the first time the occurrence of chain elongation process during protein mixed-culture fermentation, with the following main conclusions:

- Acid conditions are hypothesized to be promoting protein-based CE, since this process reduces the overall acid equivalents yielded from the substrate, consequently avoiding potential toxicity constraints.
- The feasibility of CE processes during protein fermentation is significantly influenced by protein composition, with due preference for those rich in electron donor AAs.
- Protein-based CE does not require external electron donor supplementations to occur, potentially making this kind of process more sustainable than the conventional ones.
- The occurrence of CE strongly depends on the prolonged simultaneous availability of short chain VFAs and electron donor AAs.
- The selectivity of the CE process depends on the specific availability of acetic and propionic acid.

The knowledge generated through this work constitutes a starting point for further studies on protein-based CE aiming at an optimal integration of protein-rich (waste)waters in biorefinery frameworks, currently envisioned for carbohydrate-rich streams only.

CRediT authorship contribution statement

Riccardo Bevilacqua: Methodology, Investigation, Sample processing, Data analysis, Writing – original draft, Writing – review & editing. **Alberte Regueira**: Methodology, Data analysis, Writing – original draft, Writing – review & editing. **Miguel Mauricio-Iglesias**: Methodology, Conceptualization, Supervision, Data Analysis, Writing – original draft, Writing – review & editing, **Juan M. Lema**: Methodology, Conceptualization, Supervision; Writing – original draft. **Marta Carballa**: Methodology, Conceptualization, Funding acquisition; Supervision, Data Analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

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