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Co-fermentation of crotonate and chain elongation substrates towards mixed bioplastic/organic waste recovery

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ABSTRACT

Biobased biodegradable plastics, such as polyhydroxyalkanoates (PHA) and polylactic acids (PLA), are increasingly considered as alternatives to fossil-based plastics. While these materials are primarily promoted for biodegradation in industrial composting or anaerobic digestion facilities, their recycling within more closed material loops remains limited. The carboxylate platform is emerging as a promising approach for valorizing organic waste streams, including the microbial recycling of biodegradable plastics. This study explored fermentation conditions to stimulate open-culture bioprocesses using representative biodegradable plastic monomers and typical organic residual substrates processed within the carboxylate platform. 17 batch experiments were conducted with model substrates, including crotonate, ethanol, lactate, and short-chain monocarboxylates. After 28 days of incubation, single crotonate (100 mM) fermentation primarily yielded 105 ± 4 mM acetate and 41 ± 3 mM *n*-butyrate. Adding 100 mM short-chain monocarboxylates did not inhibit crotonate conversion at an initial pH of 7.0 but extended conversion time. The co-fermentation of 88 ± 10 mM crotonate, 178 ± 20 mM ethanol, and net supplied 39 ± 8 mM acetate under neutral pH conditions produced the highest yield of *n*-butyrate at 188 ± 17 mM (i.e., 16.5 ± 1.5 g/L), followed by 11 ± 5 mM *n*-caproate. Co-fermentation of crotonate, ethanol, and lactate was feasible at an initial pH of 5.5, predominantly yielding acetate and *n*-butyrate. The thermodynamic analysis further supported the bioenergetic feasibility of crotonate conversion and its co-fermentation with ethanol and lactate and showed ample possibilities to improve the fermentation processes. These findings underscore the potential of open-culture fermentation for converting mixed bioplastic and organic waste into carboxylates.

1. Introduction

A wide variety of biobased biodegradable plastic polymers are increasingly used worldwide, with polyhydroxyalkanoates (PHA) and polylactic acids (PLA) standing out as promising alternatives to fossil-based plastics. These polymers have significant potential for both industrial and everyday applications [1]. Global production capacities for PHA and PLA are expected to rise substantially from 0.1 and 0.7 million tons in 2023 to 1.0 and 3.2 million tons by 2028, respectively [2]. Effective recycling methods for biodegradable plastics, such as PHA and PLA, are essential to foster a circular economy. However, current end-of-life treatments for biodegradable plastics are limited to options like in-field degradation, co-composting, incineration, landfill, and anaerobic co-digestion [1,3–5]. Anaerobic digestion is commonly employed to convert mixed organic waste, such as food waste, manure, and wastewater, into methane-rich biogas, which can be used for energy applications [6,7]. In recent years, however, alternative anaerobic processes have been developed that inhibit methane production and yield valuable volatile fatty acids (VFAs). By co-supplying ethanol or lactate, these processes can stimulate microbial chain elongation, producing medium-chain carboxylic acids (MCCA) [8–12]. This approach aligns with the emerging 'carboxylate platform', which focuses on converting biomass and waste streams into various carboxylates that can serve as raw materials for fuels or new plastic production [13]. Several companies are commercializing carboxylate-based pathways that utilize biomass residues and waste substrates [13–15].

A novel concept proposes the anaerobic fermentation of biodegradable plastic waste via the carboxylate platform, enabling the microbial recycling of PHA- and PLA-based end-of-life products into new products [11,16–18]. Given that these biodegradable plastics often consist of mixed polymers with additives, a heterogeneous biodegradable plastic waste stream is expected to become available. Processing this stream

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may require pretreatments, such as hydrolysis, to break down polymers into soluble monomers suitable for microbial conversion [19]. For microbial recycling, it is essential to establish optimal bioreactor conditions (e.g., pH and substrate concentrations) to maximize carboxylate formation or promote chain elongation while minimizing undesired processes like methane production.

Recent studies have shown that PHA fermentation, particularly of poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), can yield acetate and *n*-butyrate from 10 to 18 % of supplied plastics [17]. Additional work with hydrolyzed PHBV demonstrated complete conversion of the PHA hydrolysates into acetate and *n*-butyrate, although a quantitative analysis of PHA-derived monomer utilization was absent in these studies [16,20]. Hydrolysis of PHBV produces unique substrates like 3-hydroxybutyrate, crotonate, and others, which are rarely found in organic waste and thus seldom studied as anaerobic substrates [21]. Crotonate, a known intermediate in microbial metabolisms, can undergo chain elongation to form *n*-caproate, a reaction demonstrated in pure culture using electron donors such as glucose and yeast extract [22–25]. To date, research on crotonate conversion has primarily involved isolated species (e.g., *Clostridium kluyveri*, *Ilyobacter polytropus*) from specific environments like rumen and sludge [23,26–30]. More recently, studies have also investigated open-culture fermentation using pre-hydrolyzed PLA-based packaging material [11]. The hydrolyzed lactate-rich fraction was fully utilized, forming distinct carboxylates with carbon chain lengths from C_2 to C_6. This suggests that two microbial processes occurred alongside each other: lactate conversion into acetate and propionate and lactate-driven chain elongation to produce medium-chain carboxylates [31].

To our knowledge, it has not yet been investigated whether anaerobic open-microbial cultures can convert crotonate as the sole substrate. Furthermore, it remains unclear whether crotonate fermentation can be combined with the fermentation of other monomers, such as lactate and/or ethanol, to stimulate chain elongation processes. Numerous studies have validated lactate-based and ethanol-based chain elongation processes in open-culture fermentation systems [9,32]. Notably, crotonate conversion (at pH 7.0) and ethanol-based chain elongation (at pH 6.8) typically occur under neutral pH conditions [8,26]. In contrast, lactate-based chain elongation typically requires mildly acidic conditions (pH 5.0–6.0), with lactate conversion shifting towards acetate and propionate production as pH exceeds 6.0 [31]. Various bioprocesses can be promoted in complex, undefined open-microbial cultures, especially when new substrate mixtures are introduced, as observed in studies, for example, which combined with supplementation of methanol and ethanol or complex organic waste streams [33,34]. Hereby, pH conditions play a pivotal role since this influences the microbial kinetics and potential inhibitory effects of present carboxylates, as well as the dissolved inorganic carbon concentrations (e.g., bicarbonate) [35].

To establish optimal conditions for energetically favorable fermentation processes, it is essential to examine the Gibbs free energy release associated with microbial bioconversions [36]. Thermodynamic analysis provides valuable insights into microbial system functions and the boundaries of biochemical pathways [36]. Key factors such as pH, substrate concentrations, and product accumulation strongly affect the thermodynamic feasibility of the fermentation process [36]. Moreover, pH affects the concentration ratio between undissociated carboxylic acids and their conjugated base forms, impacting the thermodynamic calculations for the processes. In lactate-based chain elongation studies, for example, varying bicarbonate concentrations have been incorporated into thermodynamic models to account for shifts in pH [31]. Given that anaerobic fermentations often operate near thermodynamic equilibrium, carefully controlling conditions is crucial to steer biochemical reactions in the desired direction [37].

This study aims to investigate open-culture crotonate fermentation and quantitatively assess the effects of adding short-chain monocarboxylates, ethanol, and lactate. Microorganisms sourced from bovine rumen liquid and the effluents of a chain elongation bioprocess were used as inoculum. Initial experiments focused on the impact of individual carboxylates or ethanol on crotonate conversion at neutral pH (7.0). Subsequently, the study explored mild acidic conditions (pH 5.9 and 5.5) to assess how co-fermentation with lactate might enhance chain elongation pathways. Finally, a more complex medium incorporating ethanol, lactate, acetate, and crotonate was tested to evaluate the potential for comprehensive co-fermentation under mild acidic pH (5.5) conditions. Thermodynamic feasibility was evaluated by including the impact of the measured experimental conditions as well as assessing other theoretically improved process conditions. This work establishes key conditions for crotonate fermentation and microbial chain elongation, highlights opportunities for bioprocess development using monomers derived from biobased biodegradable plastics and/or organic waste streams, and discusses potential applications in sustainable waste management for biodegradable plastic waste.

2. Materials and methods

2.1. Materials and inoculum

All batch experiments were conducted in 250 mL serum bottles, each covered with rubber stoppers and sealed with aluminum caps to maintain anaerobic conditions. Inoculums were obtained from two main sources: the bovine rumen liquid from cows (Animal Science Department, Wageningen University and Research) and effluent from previous fermentation broth research [38]. Nutrients essential for microbial growth, including stock solution, vitamins, and minerals, were prepared based on established protocols [39,40], with detailed composition provided in Table S1. The chemicals utilized in the experiments included crotonic acid (Aldrich), ethanol (≥99.9 %, Merck), acetic acid (≥99.0 %, Aldrich), propionic acid (≥99.5 %, Aldrich), *n*-butyric acid (≥99.0 %, Aldrich), and sodium *L*-lactate (≥99.0 %, Aldrich). A methanogenesis inhibitor, sodium 2-bromoethanesulfonate (BES, 98.0 %, Aldrich), was added at a concentration of 5 g/L to suppress methane production [16]. To mitigate sharp pH shifts that could impact microbial fermentation, 0.1 M Bis-tris buffer (≥98.0 %, Aldrich) was employed [41].

2.2. Experimental design

The experimental design is outlined in Table 1. Chemicals and nutrients were prepared and introduced into serum bottles at the start of the experiments. Each fermentation was conducted with a total liquid working volume of 100 mL. The initial pH of the medium was adjusted to 7.0 ± 0.1, 5.9 ± 0.1, or 5.5 ± 0.1, individually using KOH (4 M)/HCl (1 M) before flushing N_2. A gas exchanger (SC920G, KNF Neuberger, Freiburg, Germany) facilitated a 10-min N_2 flush to establish anaerobic conditions. Following this, a 2 % *v*/v open culture was inoculated into the serum bottles for fermentation. Subsequently, the headspace of the batch bottles was pressurized with N_2/CO_2 (80/20 %) at 1.5 bar using the gas exchanger. All batch fermentations were performed in triplicate, conducted in an incubator set at 35 °C and 150 RPM (rotations per minute), and sustained for 28 days. The error bars calculated in this study are standard deviations over all triplicates.

2.3. Sampling and analysis

The batch bottles were sampled weekly, with 2.5 mL collected each time. 0.5 mL of this was allocated for pH determination, while the remaining 2 mL of liquid samples were promptly stored at −20 °C and taken out as needed for analysis. Before analysis, the collected samples underwent centrifugation at 10,000 RPM for 10 min and were filtered through a 0.45 μm membrane (CHROMAFIL Xtra, Machinerey-Nagel, Düren, Germany). Gas pressure, headspace components, and liquid samples were analyzed using methods consistent with prior research [16]. A pressure meter measured the headspace pressure (GMH 3151, GHM Group, Greisinger, Regenstauf, Germany). Gas components,

Table 1
Overview of experimental design of open-culture crotonate fermentation.

Experiment	Substrates	Crotonate (mM)	Ethanol (mM)	Lactate* (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	pH
I	crotonate	100	–	–	–	–	–	7
	crotonate + acetate	100	–	–	100	–	–	7
	crotonate + propionate	100	–	–	–	100	–	7
	crotonate + *n*-butyrate	100	–	–	–	–	100	7
II	crotonate + ethanol	100	200	–	–	–	–	7
	crotonate + ethanol + acetate	100	200	–	100	–	–	7
	ethanol + acetate	–	200	–	100	–	–	7
	blank (microbes)	–	–	–	–	–	–	7
III	crotonate	100	–	–	–	–	–	5.9
	crotonate	100	–	–	–	–	–	5.5
	crotonate + lactate	100	–	100	–	–	–	5.9
	crotonate + lactate	100	–	100	–	–	–	5.5
	lactate	–	–	100	–	–	–	5.9
	lactate	–	–	100	–	–	–	5.5
IV	crotonate + lactate + ethanol + acetate	100	200	100	100	–	–	5.5
	crotonate + lactate + acetate	100	–	100	100	–	–	5.5
	lactate+ acetate	–	–	100	100	–	–	5.5

Notes: '-' means not supplied.
* means sodium *L*-lactate.

including N_2, O_2, CH_4, and CO_2, were concurrently measured using a gas chromatograph (GC, Shimadzu GC-2010, Kyoto, Japan) equipped with Porabond Q (50 m × 0.53 mm × 10 μm) and Molsieve 5 A (25 m × 0.53 mm × 50 μm). The carrier gas used was H_2 at a pressure of 0.6 bar. H_2 produced during fermentation was separately quantified using another gas chromatograph (GC, HP-5890, Hewlett Packard, Agilent, Santa Clara, CA, USA) equipped with an HP Molsieve 5 A column (30 m × 0.53 mm × 25 μm), with Argon serving as the carrier gas.

Carboxylates and alcohols were quantified using gas chromatography (GC, Agilent 7890B, Agilent, Santa Clara, CA, USA) with an HP-FFAP column (25 m × 0.32 mm × 0.50 μm), using nitrogen as the carrier gas. *L*-lactate analysis was determined via high-performance liquid chromatography (HPLC, Thermo Dionex Ultimate 3000 RS), equipped with a UV detector (254 nm) and an Astec CLC-L Chiral column (15 cm × 4.6 mm × 5 μm) along with a prefilter. The column temperature was maintained at 25 °C, and 5 mM $CuSO_4$ was used as the eluent at a continuous flow of 1 mL/min. The injection volume was 50 μL. Chromatography data were analyzed using Chromeleon software (version 7.3).

2.4. Thermodynamic calculations

Thermodynamics plays a crucial role in understanding the dynamics of various competitive bioprocesses under environmental conditions. This study calculated the Gibbs free energy changes (ΔG_R) for different competitive bioprocesses using Gibbs energy of formation values (ΔG_f^0) reported in previous research [31,36]. The focus was specifically on plausible open-culture crotonate fermentation processes, supplemented with ethanol, lactate, and acetate, with the main reactions summarized in Table S2 [31,39,42–45]. Substrate and product concentrations for the thermodynamic calculations were obtained from sampling points. Additionally, the partial pressures of CO_2 and H_2 in the headspace were quantified using a combination of a pressure meter and gas chromatography. It's worth noting that when the pressure in the serum bottle exceeded 2.5 bars, it could not be accurately measured by the pressure meter; therefore, for the calculations, the gas pressure was assumed to be 2.5 bars under such cases.

Furthermore, the calculations accounted for the influence of varying pH on the concentration of carboxylates in thermodynamic calculations (see Fig. S8). The concentration of HCO_3^- was determined using the CO_2 partial pressure, pH, equilibrium assumption, and the Henderson-Hasselbach equation [31,46]. The effects of pH (ranging from 4 to 10), substrate concentrations (ranging from 0.001 M to 0.2 M), and product accumulation without extraction (e.g., acetate ranging from 0 to 2 M) on thermodynamic feasibilities were investigated. For baseline calculations, partial pressures of H_2 and CO_2 were assumed to be 0.5 atm [31]. Details on the equations used are provided in the Supplementary Information.

3. Results

3.1. Open-culture crotonate fermentation leads to acetate and n-butyrate

Open-culture crotonate fermentation was shown to be feasible under a variety of created conditions. In the single crotonate medium (Fig. 1A), 99 ± 11 mM crotonate was fully converted to approximately 105 ± 4 mM acetate and 41 ± 3 mM *n*-butyrate within the first week. No isobutyrate or *n*-caproate formation was found (data not shown). In other experiments, additional chemicals, including 100 mM of acetate, propionate, or *n*-butyrate, were introduced as co-substrates into the bioreactors (Figs. 1B- 1D). Introducing these carboxylates did not prevent crotonate conversion to acetate and *n*-butyrate after 28 days of incubation, although it did extend the time required for complete crotonate conversion.

In both the single crotonate medium and the media with additional carboxylates, comparable amounts of net-produced acetate (107 ± 8 mM, 113 ± 3 mM, and 116 ± 3 mM, respectively) and net-formed *n*-butyrate (47 ± 0 mM, 46 ± 1 mM, and 51 ± 5 mM, respectively) were found over the four-week incubation period. The additional carboxylates introduced were not degraded during the experiments. With the supply of acetate, a final concentration of 203 ± 3 mM (12.2 ± 0.2 g/L) was reached (Fig. 1B). When propionate was supplied, its concentration stayed rather stable at 101 ± 0 mM (Fig. 1C). With *n*-butyrate addition, the concentration increased during the fermentation up to 149 ± 3 mM (13.1 ± 0.1 g/L, Fig. 1D). The pH in all four groups exhibited a similar pattern throughout the open-culture crotonate fermentation experiments, decreasing from 7.0 to around 6.3 as crotonate was fully converted with the apparent release of protons. The complete conversion of crotonate was further evaluated by checking the net production and consumption of biochemicals, as detailed in Fig. 1E. The carbon balance shows full conversion of the supplied crotonate and 94 % to 107 % carbon recovery in the products acetate and *n*-butyrate, with reaction stoichiometries similar to those described in the literature (see Table S2) [24,28,42].

Regarding gas component alterations, no significant changes in N_2 and CO_2 were observed throughout the bioprocess. Notably, no H_2 was detected in the single crotonate fermentation or the fermentation with acetate addition (Figs. S1A- S1B). However, small amounts of H_2 (ranging from 0 to 1 %) were observed upon introducing propionate and *n*-butyrate, as detected on the 7th day (Figs. S1C- S1D). This H_2

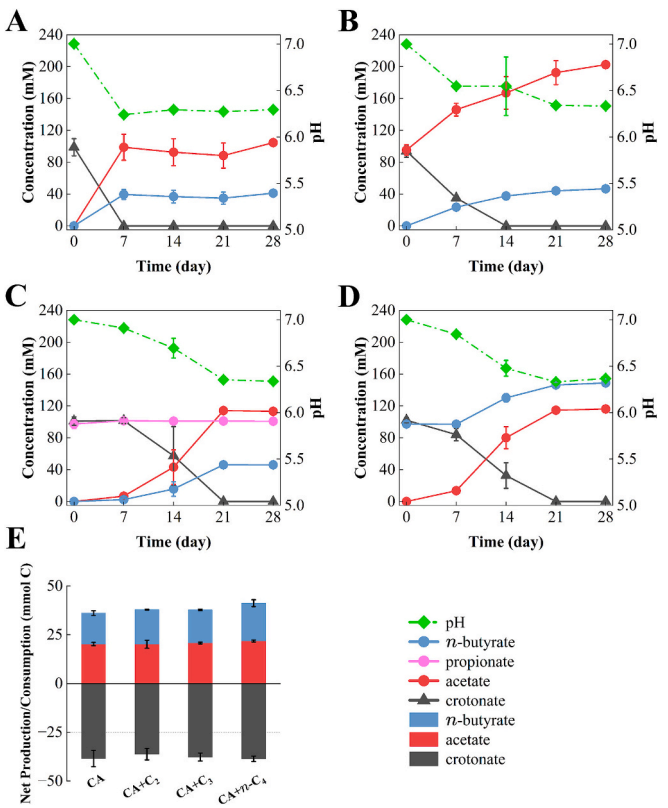

Fig. 1. Product (mM) diversity and net production/consumption (mmol C) in crotonate fermentation with the addition of various carboxylates initiating at pH 7. **A.** Single crotonate fermentation; **B.** Crotonate fermentation with additional acetate; **C.** Crotonate fermentation with additional propionate; **D.** Crotonate fermentation with additional *n*-butyrate; **E.** Net production/consumption (mmol C) of crotonate conversion with additional carboxylates. 'CA', 'C_2', 'C_3', and 'n-C_4' represent crotonate, acetate, propionate, and *n*-butyrate, respectively.

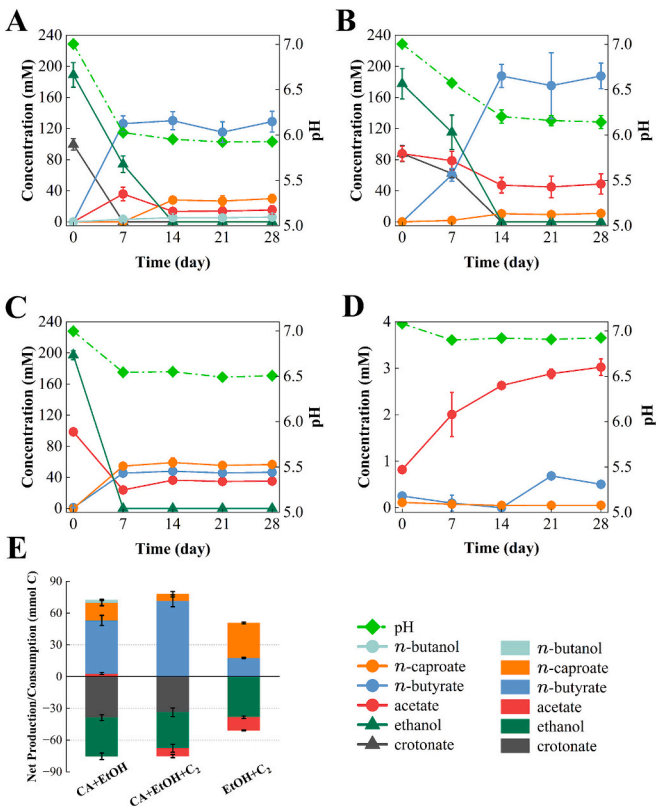

Fig. 2. Product (mM) diversity and net production/consumption (mmol C) in crotonate and ethanol fermentation initiating at pH 7. **A.** Co-fermentation of crotonate and ethanol; **B.** Co-fermentation of crotonate, ethanol, and acetate; **C.** Co-fermentation of ethanol and acetate; **D.** Blank group with solely open-culture inoculum and nutrients; **E.** Net production/consumption (mmol C) of co-fermentation of crotonate and ethanol. 'CA', 'EtOH', and 'C_2' represent crotonate, ethanol, and acetate.

formation could result from yeast fermentation since 0.1 g/L yeast extract was added to the open-culture medium (see Table S2).

3.2. Synergetic occurrence of crotonate fermentation and ethanol-based chain elongation

Open-culture crotonate fermentation and ethanol-based microbial chain elongation processes were shown to co-occur under initial pH 7 conditions. The results suggest that acetate, and possibly some *n*-butyrate, formed during crotonate fermentation were partially chain elongated to produce *n*-butyrate and/or *n*-caproate, respectively. As illustrated in Fig. 2, chain elongation occurred as an additional process when ethanol was introduced into open-culture crotonate fermentation under neutral pH conditions.

In the experiment with a substrate combination of 100 mM crotonate and 200 mM ethanol (Fig. 2A), complete crotonate conversion was achieved within the first week, resulting in the production of 36 ± 9 mM acetate and a total of 126 ± 10 mM *n*-butyrate. Part of *n*-butyrate likely originated from the ethanol-based chain elongation process because the maximum net production of *n*-butyrate from 100 mM crotonate conversion was around 50 mM, as observed in the single crotonate fermentation (Fig. 1). The remaining ethanol further contributed to chain elongation, yielding 28 ± 1 mM *n*-caproate, while acetate was reduced to 14 ± 6 mM. During the initial two weeks, 5 ± 1 mM *n*-butanol was formed, with a pH decrease from 7.0 to 5.9.

Crotonate and ethanol were fully converted into carboxylates via open-culture fermentation (Fig. 2E), and adding extra acetate altered the product composition. Supplementing an additional 88 ± 10 mM acetate to the crotonate and ethanol medium (Fig. 2B) slowed the crotonate bioconversion rate compared to solely ethanol supply (Fig. 2A). Eventually, 88 ± 10 mM crotonate and 178 ± 20 mM ethanol were consumed within two weeks. Compared to the results in Fig. 2A, *n*-caproate production decreased to 11 ± 5 mM, while *n*-butyrate production significantly increased to 188 ± 17 mM (16.5 ± 1.5 g/L). The pH decreased from 7.0 to 6.2 after two weeks of fermentation.

A control group was established to investigate ethanol-based chain elongation with acetate as the electron acceptor (Fig. 2C). Bioconversion appeared to be completed within the first week, with 200 mM ethanol and 75 ± 3 mM acetate converting to 45 ± 1 mM *n*-butyrate and 55 ± 2 mM *n*-caproate. Here, the ethanol consumption and consequent chain elongation proceeded faster compared to the experiments when crotonate was also present (Figs. 2A- 2B). The pH decreased to 6.5 within 7 days and remained stable until the end of the experiment. A similar pH was reached at day 7 when crotonate, ethanol, and acetate were supplied (Fig. 2B). No *n*-butanol was produced in the experiments shown in Fig. 2B and C. In a blank group with only open-culture inoculum and nutrients (Fig. 2D), minor acetate production (< 4 mM) was detected, and a slow pH decline to 6.9 was observed.

Regarding gas component changes (H_2 and CO_2), significant amounts of H_2 were produced upon the introduction of ethanol into the crotonate fermentation (see Fig. S2). It is well known that both ethanol oxidation to acetate and ethanol-based chain elongation processes generate hydrogen gas, albeit ethanol oxidation alone is strongly thermodynamically constrained by high H_2 partial pressures [14]. During the co-fermentation of ethanol and crotonate (Fig. S2A), H_2 levels were initially detected at 24.8 ± 2.8 % of the headspace concentration in the

first week and remained stable throughout the fermentation period. In contrast, CO_2 levels decreased from 13.4 ± 0.3 % at the start to 4.5 ± 3.0 % by the end of the experiment. Total gas pressure increased from 1.5 bar to 1.7 ± 0.1 bar within the first week. In the co-fermentation of ethanol, crotonate, and acetate (Fig. S2B), H_2 levels initially rose to 18.1 ± 4.7 % in the first week before slightly decreasing to 15.7 ± 5.1 % by the end of the experiment. Total gas pressure showed a similar trend, rising from 1.5 bar to 1.6 ± 0.1 bar and gradually decreasing to 1.5 ± 0.1 bar. A decrease in CO_2 levels was observed, dropping from 13.6 ± 0.4 % at the start to 7.8 ± 0.9 % by the end. Notably, a significant increase in H_2 levels, up to 40.7 ± 1.6 %, was observed in the first week of ethanol and acetate co-fermentation (Fig. S2C), accompanied by a high total pressure of 2.1 ± 0.0 bar. Subsequently, H_2 levels decreased to an average of 20 %, while pressure declined to 1.4 ± 0.0 by the end of the experiment. Additionally, a substantial amount of CO_2 was consumed during ethanol-acetate chain elongation, with CO_2 levels dropping from 12.8 ± 0.5 % to just 0.4 ± 0.0 %. The consumption of H_2 and CO_2 likely facilitated acetate production through hydrogenotrophic acetogenesis [45], as reflected by a slight increase in acetate concentrations observed during the second week (Fig. 2C). In the blank group (Fig. S2D), no H_2 was detected, while CO_2 levels decreased from 12.9 ± 0.5 % to 8.3 ± 0.1 % by the end of the experiment.

3.3. *Co-occurrence of crotonate fermentation and lactate-based chain elongation*

During the batch experiments, open-culture crotonate fermentation and lactate-based microbial chain elongation also co-occurred at mild acidic conditions. The pH levels appeared to be the key aspect to allow co-fermentation. Crotonate and lactate conversion were the most effective under mild acidic starting pH conditions (pH 5.9). However, single crotonate fermentation exhibited severe limitations at pH 5.5 and 5.9 (Figs. 3C- 3D) compared to the crotonate experiments studied at an initial neutral pH (Fig. 1).

Initiating co-fermentation of crotonate and lactate at pH 5.9 resulted in complete conversion within one week (Fig. 3A). This process produced 76 ± 1 mM acetate, 98 ± 0 mM *n*-butyrate, and 4 ± 0 mM *n*-caproate from 96 ± 10 mM crotonate and 126 ± 11 mM lactate. The pH increased slightly from 5.9 to 6.1 after one week of fermentation. In comparison, when co-fermentation was initiated at pH 5.5 (Fig. 3B), two out of three bioreactors achieved complete conversion within three weeks, while the remaining one experienced a prolonged conversion phase, leading to significant discrepancies in product measurements. The pH increased to around 6.0 without full conversion of the substrates at day 21.

In single crotonate fermentation initiated at pH 5.9 (Fig. 3C), only 19 ± 6 mM crotonate was converted, resulting in the production of 26 ± 2 mM acetate and 9 ± 1 mM *n*-butyrate over a month, with the pH ending at 5.8. Insignificant amounts of crotonate conversion were observed in single crotonate fermentation initiated at pH 5.5 (Fig. 3D), where the pH remained stable at 5.5. However, compared to crotonate fermentation, lactate fermentation can still occur down to lower pH levels. When initiating single lactate fermentation at pH 5.9 (Fig. 3E), 111 ± 4 mM lactate was converted to 10 ± 1 mM acetate, 24 ± 1 mM propionate, and 31 ± 1 mM *n*-butyrate. When lactate fermentation was initiated at pH 5.5 (Fig. 3F), 135 ± 8 mM lactate was predominantly converted to 14 ± 1 mM acetate, 11 ± 5 mM propionate, and 22 ± 2 mM *n*-butyrate, along with small amounts of *n*-valerate (3 ± 0 mM) and *n*-caproate (8 ± 3 mM). Despite differing initial pH values, the pH increased to 6.3 in both conditions, likely due to the establishment of a bicarbonate buffer system facilitated by CO_2 production during lactate fermentation [31]. Propionate production was observed during single lactate fermentation (Figs. 3E- 3F), whereas insignificant amounts of propionate were produced in the presence of extra crotonate (Figs. 3A- 3B).

The partial pressures of H_2 and CO_2 increased with the introduction of lactate into crotonate fermentation. Co-fermentation of crotonate and lactate at pH 5.9 resulted in significant increases in CO_2 and H_2 levels (Fig. S3A), reaching headspace concentrations of 39.2 ± 1.6 % and 26.7 ± 1.8 %, respectively, accompanied by overpressure (> 2.5 bar) within the first week. After lactate was completely consumed, H_2 levels steadily declined to 4.1 ± 5.1 % by the end of the experiment, while CO_2 remained predominant at 52.7 ± 2.1 %. Co-fermentation of crotonate and lactate at pH 5.5 (Fig. S3B) led to a gas pressure increase to 2.1 ± 0.4 bar within two weeks, with H_2 levels reaching 16.8 ± 8.9 %. H_2 subsequently decreased to 8.1 ± 1.4 % as gas pressure dropped to 1.4 ± 0.1 bar, while CO_2 steadily increased to 42.9 ± 19.9 %. This decreased H_2 amount could be used for acetate production (see Table S2) [45].

In single crotonate fermentation initiated at pH 5.9 (Fig. S3C), a small amount of H_2 was detected (around 2 kPa), but no H_2 was observed at pH 5.5 (Fig. S3D). The gas pressure slightly decreased due to sampling loss. In single lactate fermentation initiated at pH 5.9 (Fig. S3E), gas pressure increased to 2.3 ± 0.1 bar within two weeks, with CO_2 and H_2 levels rising to 33.7 ± 0.2 % and 17.4 ± 2.2 %, respectively. H_2 levels declined to 2.1 ± 3.1 % as gas pressure decreased

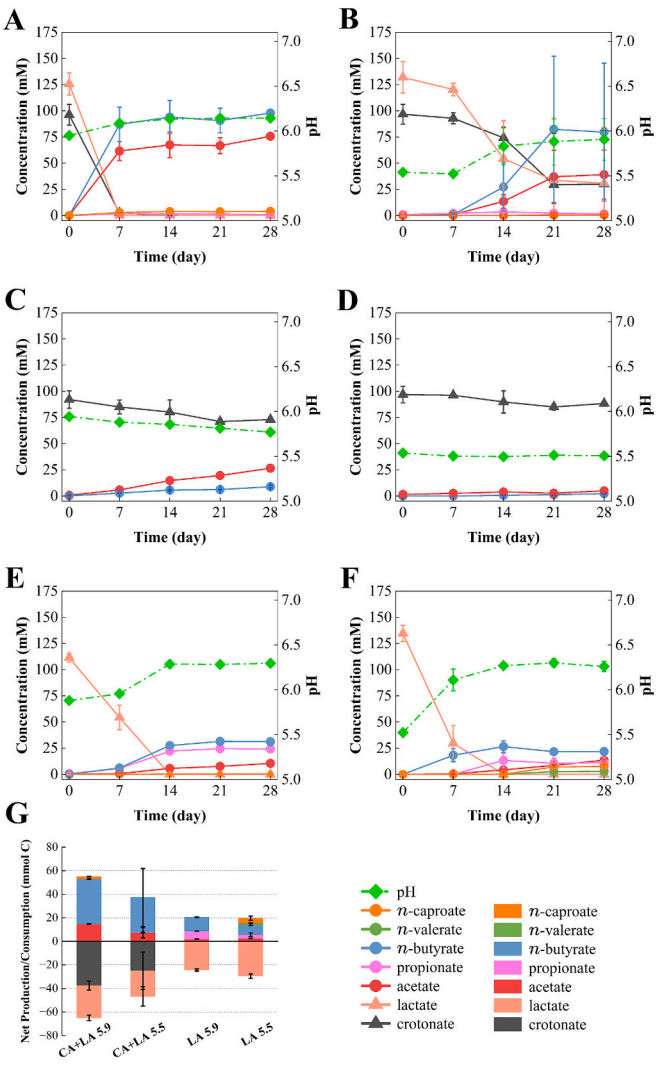

Fig. 3. Product diversity (mM) and net production/consumption (mmol C) during crotonate and lactate fermentation at pH 5.9 and 5.5. **A.** Co-fermentation of crotonate and lactate at pH 5.9; **B.** Co-fermentation of crotonate and lactate at pH 5.5; **C.** Crotonate fermentation at pH 5.9; **D.** Crotonate fermentation at pH 5.5; **E.** Lactate fermentation at pH 5.9; **F.** Lactate fermentation at pH 5.5. **G.** Net production/consumption (mmol C) of co-fermentation of crotonate and lactate. 'CA' and 'LA' represent crotonate and lactate, respectively. '5.5' and '5.9' indicate the starting pH of 5.5 and 5.9, respectively.

to 1.3 ± 0.0 bar by the end of the experiment, while CO_2 levels continued to rise to 40.6 ± 0.8 %. A comparable trend was observed in single lactate fermentation at pH 5.5 (Fig. S3F), where CO_2 increased from 17.8 ± 0.4 % to 48.0 ± 1.1 % by the end of the experiment. H_2 levels initially increased to 25.8 ± 2.1 % in the first week and then decreased to 1.5 ± 2.0 % by the end of the experiment. Similarly, gas pressure peaked at 2.4 ± 0.1 bar within one week and then reduced to 1.1 ± 0.0 bar by the end of the experiment. These observations align with earlier reports on the formation of H_2 and CO_2 as a result of lactate-based chain elongation processes [38,47].

3.4. Simultaneous crotonate, lactate, and ethanol fermentation

Acetate and *n*-butyrate were the main products when mixed with substrates like crotonate, lactate, and ethanol via open-culture fermentation. This experiment was designed with substrates suitable for crotonate fermentation, as well as lactate- and ethanol-based chain elongation processes. The initial experiments showed that lactate-based chain elongation and crotonate fermentation co-occurred under acidic starting conditions (pH ~5.9, Figs. 3A- 3B). It appeared that lactate-based chain elongation caused the pH to increase up to around 6.2, which favored crotonate fermentation. This pH of 6.2 is within the range at which ethanol chain elongation may have occurred (Figs. 2A- 2B).

A further simultaneous crotonate, lactate, and ethanol fermentation, starting at pH 5.5, was investigated based on the findings above (Fig. 4A). During the first week of the experiment, no significant conversion was observed. However, between days 7 and 14, lactate consumption (91 ± 6 mM) coincided with a pH increase from 5.5 to 6.0. Crotonate and ethanol conversion was observed during this same period, producing 34 ± 7 mM acetate and 99 ± 9 mM *n*-butyrate. As the fermentation progressed, continued consumption of ethanol and crotonate led to a pH decrease from 6.0 to around 5.5. Subsequently, increased acetate production (70 ± 50 mM) was noted, along with the detection of a small amount of butanol (11 ± 9 mM) during the final two weeks.

Control experiments were conducted to compare the results of the complex substrate fermentation. In the absence of ethanol in the open-culture crotonate fermentation (Fig. 4B), crotonate (87 ± 1 mM) and lactate (93 ± 2 mM) were fully converted to acetate (62 ± 7 mM), and *n*-butyrate (113 ± 5 mM) within a month, accompanied by a slight pH increase to 5.9. When only lactate and acetate were used as substrates, complete conversion was achieved within two weeks (Fig. 4C). The main product from lactate-based fermentation was *n*-butyrate (57 ± 5 mM), with minimal caproate produced (3 ± 2 mM). The pH sharply increased from 5.5 to 6.4. Less *n*-caproate was produced in lactate-based fermentation compared to ethanol-based fermentation, as shown in Figs. 2E, 3G, and 4D. No isobutyrate formation was detected in all these more complex medium fermentations (data not shown).

The variations in gas composition and pressure coincided with substrate consumption, allowing the experimental validation of the stoichiometries for the occurring bioprocesses. In co-fermentation with four different substrates initiated at pH 5.5 (Fig. S4 A), gas composition remained stable during the initial two weeks. Complete lactate consumption in the third week led to an increase in CO_2 and H_2 levels to 41.2 ± 1.4 % and 22.4 ± 3.2 %, respectively, along with a rise in headspace pressure. In the absence of ethanol (Fig. S4B), a one-week lag phase was observed, after which CO_2 and H_2 levels rose to 43.0 ± 5.7 % and 20.6 ± 6.3 %, respectively, with gas pressure reaching 2.2 ± 0.6 bar by the third week. Conversely, in the co-fermentation of lactate and acetate, CO_2 and H_2 levels increased to 28.8 ± 11.2 % and 19.8 ± 12.5 %, respectively, with gas pressure rising to 2.2 ± 0.5 bar within the first week (Fig. S4C). Across all three fermentation groups, H_2 levels decreased to 4.8 ± 5.7 %, 3.8 ± 2.7 %, and 6.6 ± 10.9 %, respectively. While CO_2 levels continued to increase steadily until the end of the experiments, the CO_2 partial pressure decreased as the overall gas pressure dropped (Fig. S4).

In the mixed substrate conversion experiment, as shown in Fig. 5, crotonate, ethanol- and lactate-based fermentations all demonstrated thermodynamic feasibility. Based on conditions during fermentation (i.e., chemical concentrations and gas pressure shown in Figs. 4A and S4A), the Gibbs free energy change of the reaction (ΔG_R) associated with crotonate fermentation (CA to C_2/*n*-C_4) stabilized at approximately −94 kJ/reaction. Lactate-based fermentation pathways (LA to C_2/C_3, LA to *n*-C_4, and LA to *n*-C_6) were also thermodynamically feasible, with energy releases exceeding −70 kJ/reaction. Comparably, ethanol-based chain elongation to *n*-butyrate (EtOH to *n*-C_4) and *n*-caproate (EtOH to *n*-C_6) are both thermodynamically feasible, releasing approximately −120 kJ/reaction. However, ethanol oxidation to acetate (EtOH to C_2) requires energy input, and the subsequent ethanol-driven *n*-butyrate reduction or hydrogenotrophic *n*-butyrate reduction (EtOH to *n*-C_4OH or *n*-C_4 to *n*-C_4OH) was considered thermodynamically unfavorable since the energy release was less than the latent minimal energy need of −20 kJ/reaction (Table S2) [32,48]. This finding confirms that the co-fermentation of crotonate with ethanol/lactate was also thermodynamically feasible under the discovered microbial conditions.

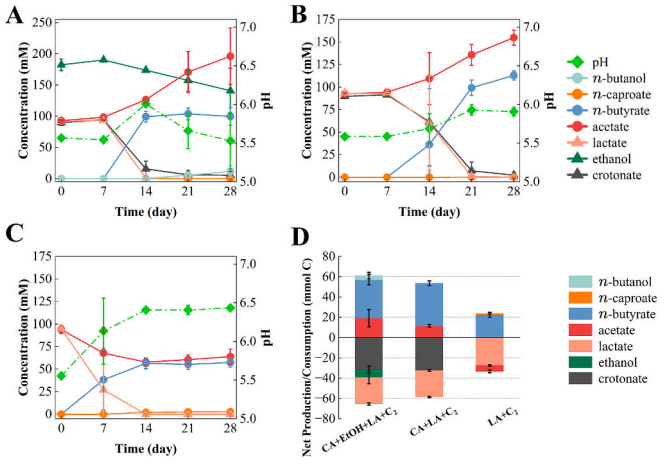

Fig. 4. Product (mM) and net production/consumption (mmol C) of open-culture crotonate fermentation with additional substrates at pH 5.5. **A.** Co-fermentation of crotonate, ethanol, lactate, and acetate; **B.** Co-fermentation of crotonate, lactate, and acetate; **C.** Lactate-based chain elongation with acetate as the electron acceptor; **D.** Net production/consumption (mmol C) of complex substrates fermentation. 'CA', 'EtOH', 'LA', and 'C_2' represent crotonate, ethanol, lactate, and acetate, respectively.

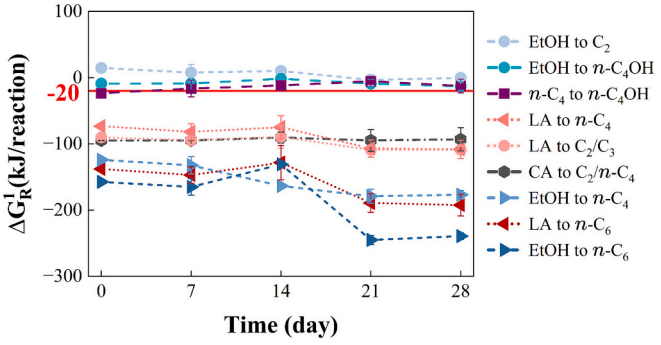

Fig. 5. Gibbs free energy (ΔG_R, kJ/reaction) of open-culture crotonate fermentation with adding ethanol, lactate, and acetate initiating at pH 5.5 and 35 °C. 'CA' means crotonate; 'EtOH', 'LA', and '*n*-C_4OH' denote ethanol, lactate, and *n*-butanol, respectively, while 'C_2', 'C_3', '*n*-C_4', '*n*-C_6' represent acetate, propionate, *n*-butyrate, and *n*-caproate, respectively. Complete biochemical reactions are provided in Table S2.

4. Discussion

4.1. Crotonate fermentation and microbial chain elongation processes

This study quantitatively examines crotonate fermentation in an open culture and the co-fermentation of crotonate with ethanol and/or lactate. Fig. 6 depicts four potential bioprocesses explaining the experimental results, each under different pH conditions and substrate compositions.

In single-substrate crotonate fermentation (Fig. 6A), acetate and *n*-butyrate were the primary products, aligning with previous findings in pure-culture crotonate fermentation [28]. Adding carboxylates like acetate, propionate, or *n*-butyrate did not inhibit crotonate conversion but prolonged the conversion time. This delay may be attributed to a reduced biomass growth rate, possibly due to increased energy maintenance requirements as discussed for acidogenic bacteria [49]. Additionally, the presence of extra carboxylates may have adversely affected electromotive force-driven energy conservation mechanisms, potentially influenced by elevated K^+ concentration resulting from pH adjustment with KOH [50]. Notably, these additional carboxylates did not undergo evident oxidation during crotonate fermentation, as carboxylate oxidation requires syntrophic partners capable of reducing H_2 partial pressure, for example, to below 10 Pa [51]. However, in this study, hydrogenotrophic methanogens–a potential syntrophic partner for this process–were expected to be inhibited with the use of BES [52].

Introducing ethanol into the crotonate fermentation system stimulated microbial chain elongation (Fig. 6B), with acetate and *n*-butyrate from crotonate fermentation serving as the evident electron acceptors for the known ethanol-based chain elongation [43]. A minimal amount of *n*-butanol was likely produced via hydroxyl-carboxyl exchange or through the reduction of *n*-butyrate (see Table S2) [53,54]. Although crotonate itself is a known substrate for chain elongators such as *Clostridium kluyveri* and *Syntrophus aciditrophicus* sp. *Nov.*, and serve as an intermediate in butyrate biosynthesis, its role in our system appeared to be primarily in its conversion to acetate and *n*-butyrate, which then fueled further chain elongation [42,55]. However, the simultaneous presence of crotonate and ethanol may have introduced substrate competition or metabolic trade-offs that reduced the overall efficiency of *n*-caproate production. This is supported by observations in Fig. 2C, where *n*-caproate formation was most pronounced in the absence of crotonate (i.e., when only ethanol and acetate were present).

In addition, the roles of acetate, propionate, and *n*-butyrate in such co-fermentation systems warrant further investigation since they are often formed from the primary hydrolysis and acidification processes of complex substrates [56]. In the present study, we found that co-supplying acetate along with ethanol and crotonate favored *n*-butyrate production, whereas omitting acetate stimulated *n*-caproate formation (Fig. 2). These findings further highlight the critical influence of substrate selection and availability on shaping chain elongation pathways and product specificity [57].

In contrast to the relatively neutral pH preferred for ethanol and crotonate co-fermentation, open-culture lactate fermentation was explained by metabolizing lactate through the acrylate pathway at pH > 6.0 but driving chain elongation at pH < 6.0. When lactate was introduced under mildly acidic conditions (pH < 6.0), its fermentation rose pH, which in turn activated the crotonate conversion (Fig. 6C) [9,31]. In this scenario, crotonate was converted to acetate and *n*-butyrate, which then acted as electron acceptors for lactate-based chain elongation. In addition to ethanol and lactate, other monomers such as methanol and glycerol have also been reported as substrates capable of stimulating

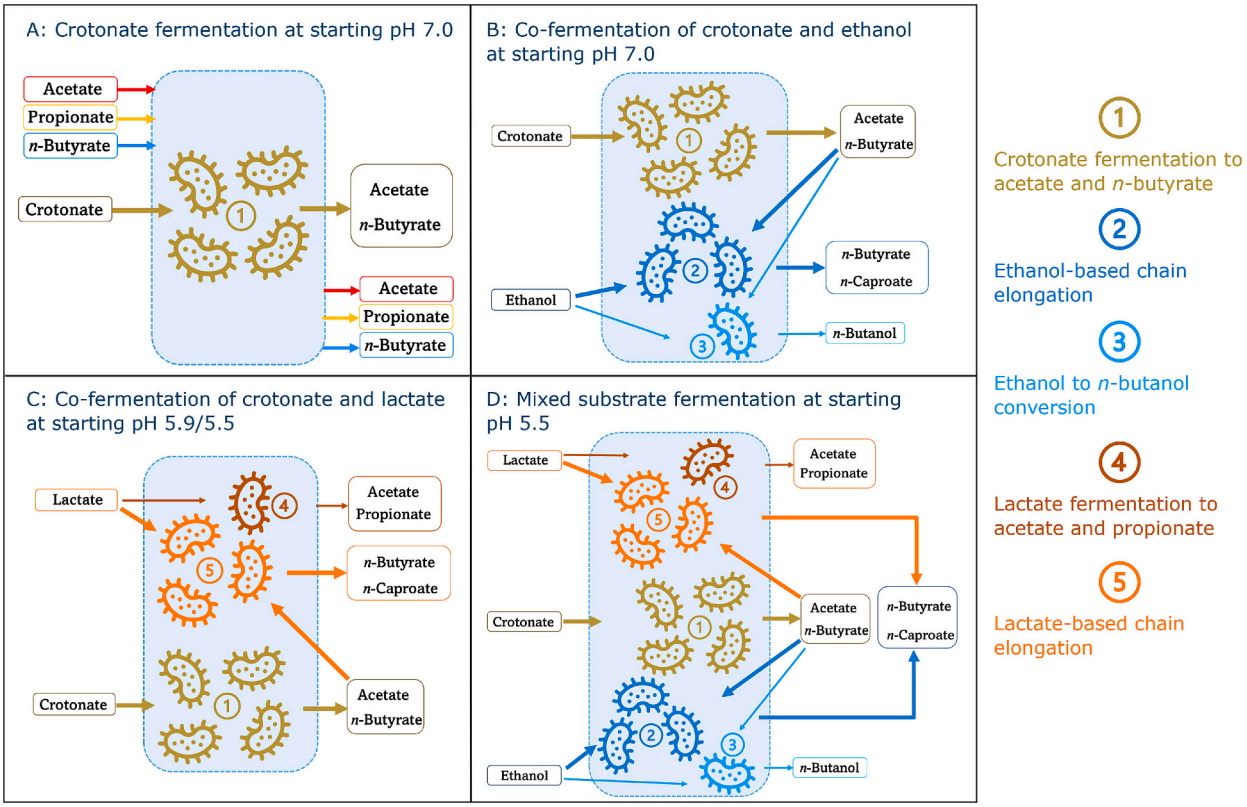

Fig. 6. Schematic overview of the potential open-culture bioprocesses. The left side of each diagram represents the substrate(s), while the right side shows the product(s). The blue box depicts the reactor containing an open culture sourced from various origins. Chemicals listed within the blue box are intermediates produced by the open culture and further utilized in subsequent fermentation steps. **A.** Single crotonate fermentation at starting pH 7.0; **B.** Co-fermentation of crotonate and ethanol at starting pH 7.0; **C.** Co-fermentation of crotonate and lactate at starting pH 5.9 or 5.5; **D.** Mixed substrate fermentation at starting pH 5.5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microbial chain elongation [58,59]. These findings suggest the potential for further exploration of co-fermentation strategies involving bioplastic-derived monomers (e.g., crotonate) and various monomers derived from organic waste streams.

In an open-culture fermentation of crotonate, ethanol, and lactate at an initial pH of 5.5, lactate fermentation elevates the pH, thereby stimulating crotonate and ethanol conversion (Fig. 6D). While ethanol-based chain elongation can occur at pH 5.5, the stabilization of ethanol concentration in the first week (see Fig. 4A) suggests that the microbial community in this study may not have contained ethanol-utilizing microorganisms effective for the created conditions [60]. Acetate and *n*-butyrate produced from crotonate fermentation subsequently served evidently as electron acceptors for ethanol- and/or lactate-based chain elongation, yielding *n*-butyrate and/or *n*-caproate.To better understand these metabolic dynamics, future studies should incorporate microbial community analysis to link substrate utilization with key functional groups. Additionally, the use of isolated strains and metabolic modeling tools may provide insight into the exact pathways of crotonate and ethanol conversion, facilitating targeted strategies to enhance *n*-caproate production [61].

4.2. Opportunities for bioprocess development using monomers derivable from biobased biodegradable plastics

The efficiency of co-fermentation with bioplastic-derivable monomers depends on pH, substrate concentration/ratios, and product inhibition, requiring considerations to understand the thermodynamic limits. Crotonate fermentation and ethanol- and lactate-based chain elongation are thermodynamically feasible across a pH range of 4.0 to 10.0 (see Figs. S5- S7), but microbial growth performance is constrained by pH-dependent toxicity and salinity effects. Our thermodynamic analysis aligns with previous assumptions but uniquely accounts for the impact of undissociated/dissociated compound dynamics (see Fig. S8) [31]. At low pH (< 5.0), undissociated fatty acids accumulate, posing toxicity risks to microorganisms [62], while at high pH (> 8.0), the high concentration of K^+/Na^+/NH_4^+ in the alkaline solutions may inhibit microbial growth [50].

While thermodynamic analysis confirms that crotonate fermentation and chain elongation benefit from increased substrate concentrations (see Figs. S5- S7), excessive substrate loading poses a significant challenge to microbial growth. For example, ethanol concentrations ranging from 350 to 700 mM or acetate concentrations up to 250 mM inhibited the growth of *Clostridium Kluyveri* strain 3231B during ethanol-based chain elongation [63]. The co-fermentation of ethanol and lactate has been explored under varying molar ratios and pH conditions [64]. At pH 6.5, the optimal ethanol: lactate: acetate molar ratio of 2:1:1 enhanced chain elongation, whereas at pH 7.5, a 1:1 ethanol: lactate molar ratio favored acetate and propionate production [64,65]. Our findings validate the potential of bioplastic-derived monomers (crotonate and lactate) together with organic waste-derived ethanol as substrates for co-fermentation, particularly under mildly acidic conditions (5.5 < pH < 6.0). Based on our results, the ideal molar ratio of ethanol: lactate: crotonate is approximately 1:2:2 for converting bioplastic- and organic waste-derived monomers into carboxylates (see Fig. 4A).

Even at high product concentrations (acetate, *n*-butyrate, or *n*-caproate) of crotonate fermentation, as well as ethanol−/lactate-based chain elongation (> 1 M), Gibbs free energy release remains favorable (see Figs. S5- S7), yet microbial inhibition can occur due to toxicity effects [41]. Taking *n*-caproate as an example, at pH levels below 6.0, the pH approaches the pKa of *n*-caproic acid (4.88), leading to increased formation of undissociated *n*-caproic acid, which can inhibit microbial activity. To address this issue, in-situ recovery technologies can be employed to remove products during fermentation, maintaining lower product concentrations and improving conversion rates [66,67].

4.3. Perspectives- waste management of biobased biodegradable plastics

The microbial conversion of biodegradable plastic waste, such as PHA and PLA, into valuable chemicals via the carboxylate platform represents a promising approach to sustainable waste management. In this study, we demonstrate that the model degradation products of PHA and PLA, primarily crotonate and lactate, enable open-culture fermentation. This process is thermodynamically feasible when co-fermented with ethanol and short-chain carboxylates under mildly acidic pH conditions (5.5 < pH < 6.0). The substrates for the co-fermentation could be derived from organic waste stream fermentation processes [8]. The hydrolysis of biodegradable plastic polymers via, for example, hydrothermal pretreatment requires substantial energy input [16,68]. Additionally, the resulting hydrolysates typically have a pH below 5.0, necessitating the addition of alkaline solutions to adjust the pH before fermentation, which increases process costs [68]. A more cost-effective strategy may be to directly convert solid bioplastic waste into carboxylates through microbial activity, bypassing the need for energy-intensive pretreatment. Studies showed that certain bacteria and fungi can induce the secretion of PHA depolymerase to degrade PHA [69,70]. For instance, *Pseudomonas pickettii* has been reported to degrade PHA efficiently under optimized conditions of pH 5.5 and 40 °C [70]. This suggests that enzymatic hydrolysis and microbial fermentation could potentially occur simultaneously, reducing reliance on external energy inputs and chemical dosage for pH regulation [71]. Future research should also address broader challenges in biodegradable plastic recycling, such as minimizing the possible formation of microplastics, to develop more effective and environmentally sustainable waste management strategies [72].

5. Conclusion

Open-culture crotonate fermentation is feasible and integratable with ethanol- and/or lactate-based chain elongation processes. Adding specific carboxylates did not inhibit crotonate fermentation except for extending the conversion time in the batch. The discovered co-fermentation of crotonate and ethanol-based chain elongation favored neutral pH conditions. Lactate-based chain elongation favored mildly acidic conditions and induced crotonate and ethanol conversion over time while elevating pH. Under neutral pH conditions, the highest *n*-butyrate production of 188 ± 17 mM (i.e., 16.5 ± 1.5 g/L) in addition to 11 ± 5 mM *n*-caproate, was achieved from the co-fermentation of 88 ± 10 mM crotonate, 178 ± 20 mM ethanol, and a net supply of 39 ± 8 mM acetate. Crotonate was eventually fully metabolized with ethanol and lactate, leading to a spectrum of monocarboxylate products during co-fermentation, including *n*-caproate. The thermodynamic calculations show that bioprocess development is feasible from a bioenergetic point of view. Besides bioprocess development, advancements in waste management strategies and in-situ product recovery are relevant to fully harness the potential of the carboxylate platform for converting biodegradable plastics (like PHA and PLA) and organic waste into valuable carboxylates.

CRediT authorship contribution statement

Yong Jin: Writing – original draft, Investigation. **Kasper D. de Leeuw:** Writing – review & editing, Investigation. **David P.B.T.B. Strik:** Writing – review & editing, Investigation.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors utilized ChatGPT 4.0 to enhance the English language quality of this manuscript, with a particular emphasis on refining grammar, readability, and clarity. All AI-generated suggestions were rigorously reviewed and further edited by the authors, who retain full

responsibility for the accuracy and integrity of the final content presented in this publication.

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Declaration of competing interest

DS is an employee of Wageningen University & Research (WUR) fulfilling the role of one of the platform managers of Unlock (https://m-unlock.nl/). KL works as a lecturer at WUR as well as a senior researcher at ChainCraft (https://chaincraft.com/). The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2025.164552.

Data availability

The original data presented in this paper is available at the 4TU. Research Database via this site: doi: https://doi.org/10.4121/3141faf4-51d4-49f9-a2d2-f3ed01f117ce.

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