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Effect of electron acceptors on product selectivity and carbon flux in carbon chain elongation with *Megasphaera hexanoica*

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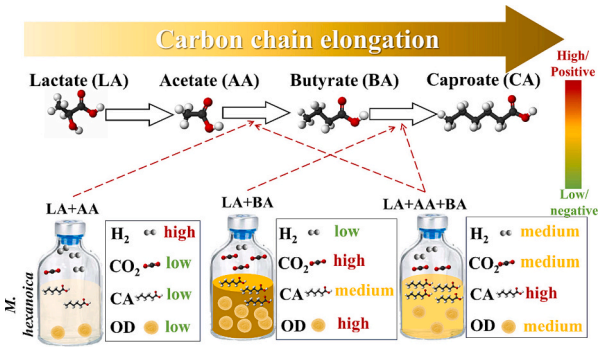
HIGHLIGHTS

- *Megasphaera hexanoica* produced 2.86 mM caproate without an external electron acceptor.
- Acetate and butyrate are prefered in caproate and biomass generation, respectively.
- The combination of acetate and butyrate facilitated the generation of caproate.
- The combined electron acceptor exhibited a maximum carbon flux of 49 % for caproate.

GRAPHICAL ABSTRACT



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ABSTRACT

Megasphaera hexanoica is a bacterial strain following the reverse β-oxidation pathway to synthesize caproate (CA) using lactate (LA) as an electron donor (ED) and acetate (AA) or butyrate (BA) as electron acceptors (EA). Differences in the type and concentration of EA lead to distinctions in product distribution and energy bifurcation of carbon fluxes in ED pathways, thereby affecting CA production. In this study, the effect of various ratios of AA, BA, and AA+BA as EA on carbon flux and CA specific titer during the carbon chain elongation in *M. hexanoica* was explored. The results indicated that the maximum levels of CA were 18.81 mM and 31.48 mM when the molar ratios of LA/AA and LA/BA were 10:1 and 3:1, respectively. Meanwhile, when AA and BA were used as combined EA (LA, AA, and BA molar amounts of 100, 23, and 77 mM), a maximum CA production of 39.45 mM was obtained. Further analysis revealed that the combined EA exhibited a CA production carbon flux of 49 % (4.3 % and 19.5 % higher compared to AA or BA, respectively) and a CA production specific titer of 45.24 mol (80.89 % and 58.51 % higher compared to AA or BA, respectively), indicating that the effective carbon utilization rate and CA production efficiency were greatly improved. Finally, a scaled-up experiment was conducted in a 1.2 L (working volume) automated bioreactor, implying high biomass (optical density at 600 nm or OD_{600} = 1.809) and a slight decrease in CA production (28.45 mM). A decrease in H_2 production (4.11 g/m^3) and an

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increase in CO_2 production (0.632 g/m^3) demonstrated the appropriate metabolic adaptation of *M. hexanoica* to environmental changes such as stirring shear.

1. Introduction

Organic wastes, such as kitchen trash, agricultural waste, residual sludge and industrial waste, among others, are increasing year by year due to rapid global urbanization and growing food consumption (Han et al., 2020; Li et al., 2021). Organic waste is rich in bioenergy and can lead to environmental pollution but also bioenergy waste if not properly treated. Therefore, its fermentation can solve the problem of environmental pollution while allowing resource and energy recovery, which is receiving increasing research attention worldwide (Chen et al., 2020; Pang et al., 2022). Valorization, rather than mere treatment, of pollutants has now become a major focus in research (Kennes, 2023; Rene et al., 2022).

Fermentation can be used as a process in which organic waste, but also other renewable resources, are first converted into small molecules such as short chain carboxylic acids (SCCAs), which can then ultimately be converted to methane by methanogenic bacteria (Greses et al., 2020; Reddy and Chang, 2021), unless methanogenesis is inhibited, which would result in the accumulation of SCCAs (Tomas-Pejo et al., 2023). In recent decades, it has been shown that a few microorganisms can produce medium-chain carboxylic acids (MCCAs) through carbon chain elongation (CE) of SCCAs in the presence of electron donors (EDs) such as ethanol, fructose or lactate (LA) (Ghysels et al., 2021; San-Valero et al., 2020). The concept of using a CE process to produce MCCAs instead of producing methane (i.e., biogas) has received increasing attention in recent years due to the higher commercial value and easier separation of MCCAs from water compared to SCCAs (Duber et al., 2022; Wang et al., 2021; Yu et al., 2023). LA, a common ED in the SCCAs CE, is readily available from some organic wastes, e.g., from dairy industries (Chenebault et al., 2022; Kumar et al., 2022; Strazzera et al., 2021). Therefore, CE using LA as an ED has significant economic and environmental benefits.

Megasphaera hexanoica, isolated from the rumen of dairy cows, has been reported to utilize LA as an EA (Jeon et al., 2017). It has been shown that both acetate (AA) and butyrate (BA) could be employed as electron acceptors (EA) by this strain to produce caproate (CA) (Jeon et al., 2017; Kang et al., 2022). Surprisingly, the strain has eight acetyl coenzyme A (acetyl-CoA) transferase enzymes related to chain elongation and thus excels in MCCAs production (Park et al., 2020). Thus, it has physiologically and genetically distinctive features from previously reported organisms of the family *Rumatococcaceae* (Wang et al., 2018; Zhu et al., 2017). It was found that *M. hexanoica* could utilize 10 g/L LA as EA, and 8.9 g/L CA concentration was achieved when 8 g/L AA and 14 g/L BA were used together as EA (Kang et al., 2022). In this study, in order to investigate and optimize the production of CA when LA was used as an ED, three combinations of EAs were tested: 1) AA as an EA, 2) BA as an EA, and 3) AA and BA together as EA. The influence of the different EA on the CE process was investigated, and the mechanism of EA regulation on the CE properties was analyzed based on the results of thermodynamics, carbon flux and electron efficiency analyses.

2. Material and methods

2.1. Strain and medium

The strain employed in this study was *M. hexanoica*, purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) under DSM No. 106893. The medium used to grow *M. hexanoica* had the following composition: yeast extract 10 g/L, K_2HPO_4 2 g/L, cysteine 0.5 g/L, salt solution 200 mL/L ($CaCl_2{\cdot}2H_2O$ 0.25 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, KH_2PO_4 1 g/L, K_2HPO_4 1 g/L, $NaHCO_3$ 10 g/L and NaCl 2 g/L). The medium was transferred into 100 mL serum bottles, with working volume of 50 mL, and purged with N_2 for 6 min to remove dissolved oxygen while the pH was adjusted to 6.5 with 2 M HCl. It was sterilized at 121 °C for 20 min before incubating in a thermostated shaker at constant temperature and stirring rate of, respectively, 37 °C and 100 rpm. The strain was inoculated at a concentration of 1 % (*v*/v) and all experiments were performed in triplicate. The initial ED concentration was 100 mM LA and various EA concentrations of AA and BA alone or in combinations were added, as will be explained later on.

2.2. Analytical methods

A UV spectrophotometer (Lambda 11, Perkin Elmer, USA) was employed to determine the optical density at 600 nm (OD_{600}) of *M. hexanoica* cultures. High Performance Liquid Chromatography (HPLC) (HP1100, Agilent Co., USA) was used to determine acid concentrations (LA, AA, BA and CA) using an Agilent Hi-Plex Column (300 × 7.7 mm) and both a diode array detector (DAD) and a refractive index detector (RID) maintained at 50 °C. A 0.005 M H_2SO_4 solution was fed as mobile phase, with a flow rate of 0.80 mL/min and a column temperature of 45 °C. All liquid samples were centrifuged at 7000 rpm for 5 min and filtered through 0.22 μm PTFE syringe filters. The variation in pH of the fermentation broth was recorded using a pH meter (Basic 20, Crison, Spain). The concentration of H_2 in the headspace was determined on an HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) with a thermal conductivity detector (TCD). The column used was a 15-m HP-PLOT Molecular Sieve 5 A column (ID 0.53 mm, film thickness 50 μm) with a 50 °C oven. Helium was used as the carrier gas.

2.3. Calculations

The carbon flux ratio (CFR) reflected the conversion efficiency of the consumed carbon atoms in LA into each product, and could be used to evaluate the effective utilization of LA in the CE process (Zhu et al., 2020). The CFR was calculated with the following formula (Eq. (1)):

$$CFR(\%) = \frac{C_{product}}{C_{LA}} \times 100 \quad (1)$$

where, $C_{product}$ represented the amount of carbon atoms contained in the net amount of accumulated product and C_{LA} represented the amount of carbon atoms contained in the LA consumed. The amount of carbon atoms (moles) per (one) mole of carboxylic acid were 3/LA, 2/AA, 4/BA, and 6/CA, respectively.

LA Consumption Specific Titer (LACST) is the amount LA consumed per unit mole of CA produced (Eq. (2)), which was used to indicate the efficiency of CA production by LA. The smaller the value, the more consistent it was with low carbon biosynthesis.

$$LACST\left(\frac{mol}{mol}\right) = \frac{CA\ production}{LA\ consumed} \quad (2)$$

CA Production Specific Titer (CAPST) is the amount CA produced per unit biomass (OD_{600}) (Eq. (3)), which was used to indicate the relationship between cellular synthesis and CA production in *M. hexanoica*. Larger values reflected that LA contributed more to CA production than cellular synthesis.

$$CAPST(mol) = \frac{CA\ production}{OD_{600}} \quad (3)$$

2.4. Analysis of thermodynamics

The Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto, 2000) was utilized to suggest the LA metabolic route for CA synthesis. The KEGG mapper was implemented to recreate pathways by inputting coding sequence (CDS) information from the entire genome sequence of *M. hexanoica*. eQuilibrator was used for determining the Gibbs free energy for each chemical reaction (Kang et al., 2022).

2.5. Bioreactor validation assay

The bioreactors employed were 2 L BIOFLO 120 reactors having a working volume of 1.2 L (Eppendorf, Germany). Each bioreactor was equipped with a gas sparger, liquid and gas sampling ports, automated agitation (which was kept constant at 100 rpm), as well as pH/redox/temperature probes. The basal medium was autoclaved (120 °C, 20 min) together with the bioreactor setup, which was ready for use after the system had been cooled. Following autoclaving, N_2 was flushed for 2 h (1 h in the liquid phase and then 1 h in the headspace) to ensure anaerobic conditions. Subsequently, the bioreactor was inoculated with 10 % (v/v) of an active bacterial culture of *M. hexanoica* that had been grown aseptically according to the above-described protocol. The temperature was kept constant at 37 °C in all tests by using a water jacket, and the pH was regulated and maintained constant on-line at 6.5 by adding NaOH (1 M) or HCl (1 M). Bacterial growth was assessed in terms of optical density (OD_{600}) and the concentration of acids was measured every 24 h. In addition, gas samples were collected to determine the concentrations of H_2 and CO_2.

3. Results and discussion

3.1. CA production in the presence of AA as EA

M. hexanoica was able to induce CA synthesis at various ED/EA (LA/AA) ratios. When the molar ratio of LA/AA was 10:1, *M. hexanoica* consumed 77.68 mM LA and 3.13 mM AA, while producing 3.69 mM BA and 18.81 mM CA, meanwhile the OD_{600} and pH reached 0.738 and 7.65, respectively, confirming that LA and AA can be used as ED and EA, respectively, during the CE process in *M. hexanoica* (Fig. 1A). It is worth mentioning that when there was no AA as EA, 2.86 mM CA production and 51.98 mM LA consumption were still observed with 5.17 mM AA accumulation, a result which was also consistent with the findings of Kang et al. (2022). This observation was confirmed in studies with other CA producers, where *Ruminococcaceae* CPB6 produced 8.07 g/L CA when consuming 24.85 g/L LA under conditions where only LA was used as a substrate (Zhu et al., 2017). It has been proven that in the LA oxidation pathway acetyl-CoA was produced via the pyruvate pathway, which further generated AA in the tricarboxylic acid cycle, and the acetyl-CoA and the generated AA could then complete the reverse β-oxidation process, thereby synthesizing BA and CA (Yang et al., 2020). Moderate amounts of AA addition did favor cell synthesis; however, when too much AA was added, it inhibited the conversion pathway of acetyl-CoA to AA (a process that releases much reducing power and energy). If this process was severely impeded, it resulted in insufficient cellular energy for cell synthesis, which in turn yielded low biomass (Schönfeld and Wojtczak, 2016).

The change in acid production at a LA/AA molar ratio of 10:1 was further analyzed as shown in Fig. 1B, where LA was continuously consumed and AA was gradually produced over time, a result that also confirmed that AA was still produced when there was no AA available anymore as EA, as previously illustrated in Fig. 1A. Then, AA was used as a substrate in the reverse β-oxidation pathway to sequentially synthesize BA and CA, an inference that could be made from the fact that BA showed first an increasing and then decreasing trend, while CA was generated from the 4th day onwards and its yield was increasing. Similarly, *Ruminococcaceae* CPB6, supplemented with 5 g/L AA, shortened

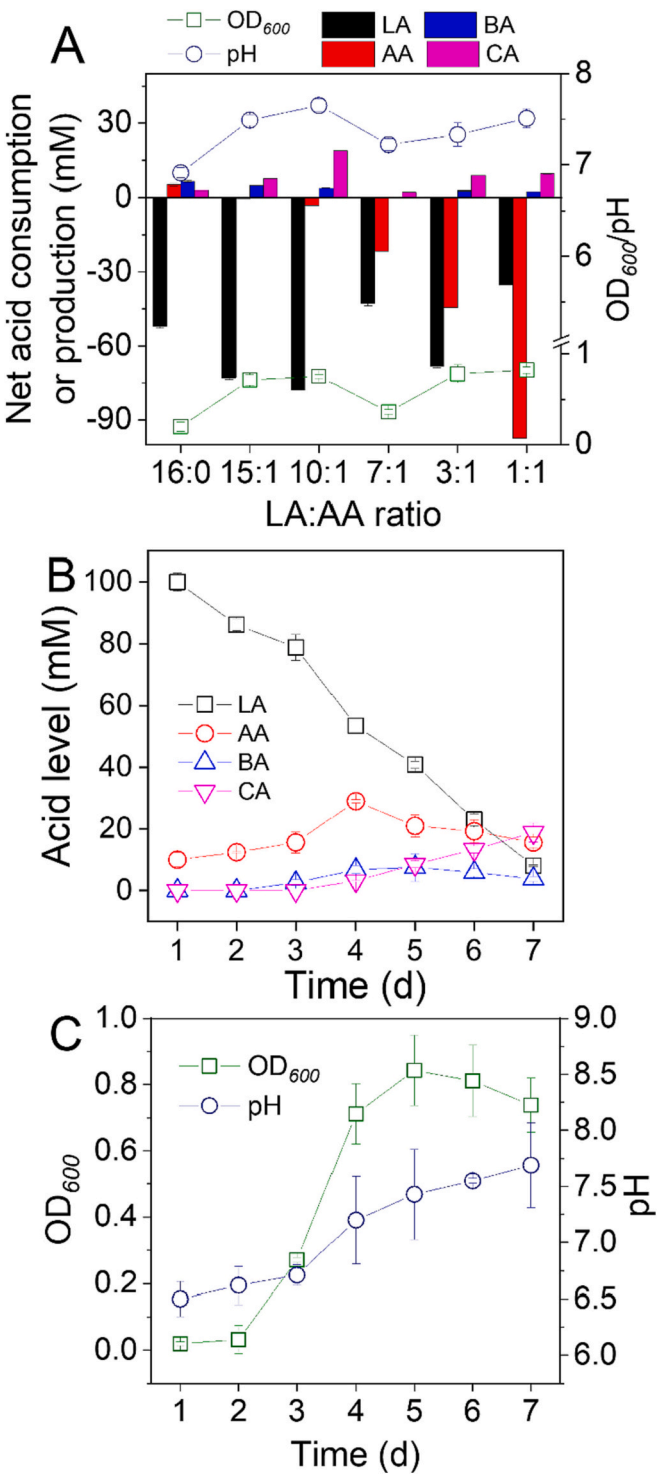

Fig. 1. (A) Effect of different LA/AA ratios on CA production, OD_{600} and pH (the values displayed are the final concentrations on the 7th day), (B) production of CA and (C) change of OD_{600}/pH when the molar ratio of LA to AA is 10:1.

the fermentation lag phase from 24 h to 12 h compared with that of LA supplied with only 5 g/L, suggesting that AA significantly accelerated CA production (Zhu et al., 2017). The CE pathway, as shown in Fig. 1C, was an acid-consuming activity. Since LA has a lower pK_a (~3.0) than BA and CA (~4.8), the conversion of LA to BA/CA resulted in a decrease in H^+ concentration (Zhu et al., 2021; Wang et al., 2022). At the same time, the pyruvate pathway of LA metabolism produced a large amount

of ATP in the process of AA accumulation, which promoted biomass growth, leading to an increase in OD_{600} from 0.02 initially to 0.738 (Liu et al., 2020a, 2020b).

3.2. CA production in the presence of BA as an EA

Theoretically, BA seems to be more suitable as an EA in the CE process than AA, as it only requires a reverse β-oxidation cycle to synthesize CA (Zhu et al., 2017). However, due to the wide availability and low cost of AA, it may be industrially preferred to employ AA rather than BA as an EA (Dong et al., 2023; San-Valero et al., 2020). This implies that different EA in the CE process may lead to differences in carbon fluxes in the ED. When LA acted as an ED, it generated reducing power (NADPH), energy (ATP), and acetyl-CoA (activated AA), some of which was used for cellular synthesis and the other for the reverse β-oxidation process (Nzeteu et al., 2022). This raised the concern of whether AA, when used as an EA, has a stimulating/inhibiting effect on the LA oxidation process, which may be related to factors such as strain type and AA concentration. In contrast to AA, BA as an EA does not raise this concern as it only needs to be active during the reverse β-oxidation process (Wang et al., 2018). Fig. 2A shows that CA production in *M. hexanoica* was higher when BA was used as an EA instead of equimolar AA. The substrate consumption and product accumulation increased with the increase of LA/BA ratio, and reached the maximum value when the ratio of LA/BA was 3:1; at this time, 81.25 mM LA and 54.57 mM BA were consumed, while 17.50 mM AA and 31.48 mM CA were produced. The addition of BA would not have an inhibitory effect on the process of LA oxidation to AA, so the large amount of reducing power and energy released during LA oxidation together with butyryl coenzyme A (butyryl-CoA) could jointly promote cell synthesis. However, when LA/BA was greater than 3:1, the presence of a large amount of initial BA hindered the conversion of AA to BA due to the product inhibition effect, resulting in a rapid accumulation of AA. This also caused the blockage of LA oxidation, which limited the energy supply in the biosynthesis process of the strain, and therefore also caused the decrease of biomass (Schönfeld and Wojtczak, 2016). As a result of BA's significant contribution to the growth of *M. hexanoica*, the strain continued to proliferate on the 7th day, ensuring the generation of CA and leading to a rise in pH. In comparison to other CA producers, such as *Ruminococcaceae* CPB6, 2.11 g/L CA was generated by consuming 4.68 g/L LA and 1.24 g/L BA (Zhu et al., 2017). It can be concluded that *M. hexanoica* produced more CA when BA was used as EA compared to *Ruminococcaceae* CPB6.

For further examination of the trend of both acid and strain present in solution, a LA/BA ratio of 3:1 was chosen. As can be seen from Fig. 2B, AA was always on the rise when BA was used as EA; which was different from when AA was used as EA, while BA started to decline after stabilizing within the first 5 days, indicating that BA was heavily utilized as a substrate for the reverse β-oxidation pathway at this time, a fact corroborated by the production of 34.48 mM CA. Notably, on the 7th day, a sharp increase in BA was noticed, which was thought to be the first time that BA synthesis-related enzyme activity was higher than that of CA-related synthetase, owing to their different susceptibility to environmental conditions (e.g., a sustained rise in pH and excessive accumulation of metabolites). OD_{600} and pH maintained an upward trend throughout the chain elongation process, consistent with that of AA as an EA. However, when BA was supplied as EA compared to AA, the pH rose to 7.36, which was lower than the pH of 7.69 achieved by AA. The rise in pH was mainly attributed to the pK_a of the carboxylic acids. Meanwhile, BA as an EA has an OD_{600} of 1.103, which is higher than that of AA, reaching 0.738, and which may be due to the fact that AA as a substrate in the chain elongation process needs to go through two cycles in the reverse β-oxidation pathway, whereas BA only needs to go through one, and thus AA consumes more protons, H^+, for hydrogen generation than BA. Conversely, BA avoids the overuse of reducing power and energy in the carbon chain elongation process, which prompts it to contribute more to the increase of biomass as a precursor (Li et al., 2023; Shi et al., 2021).

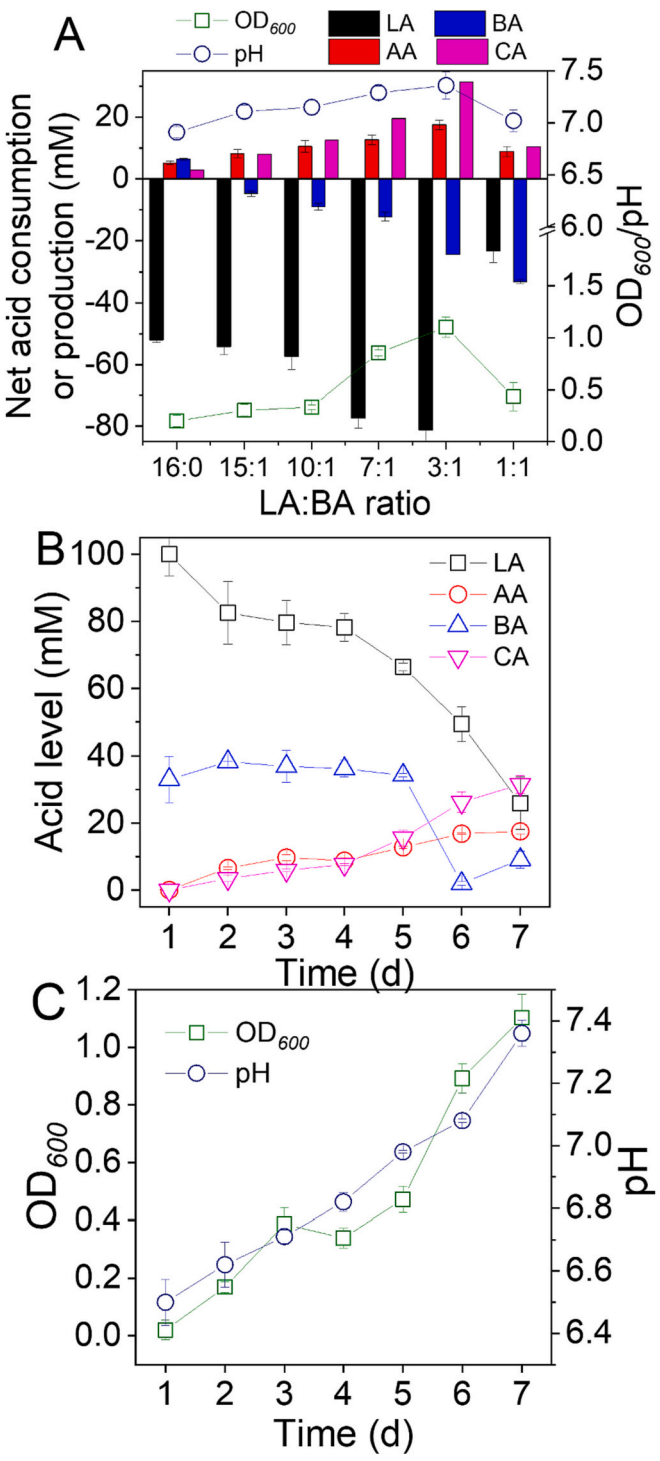

Fig. 2. (A) Effect of different LA/BA molar ratios on CA production, OD_{600} and pH (the values displayed are the final concentrations on the 7th day), (B) CA production and (C) OD_{600}/pH change when the molar ratio of LA/BA is 3:1.

3.3. CA production in presence of AA + BA as EA

Although the production of CA was higher with equimolar BA as the EA, AA is widely available and inexpensive; therefore, the production of CA was further examined with AA and BA as the combined EA in quest of an EA with a higher CA titer. As illustrated in Fig. 3A, the consumption

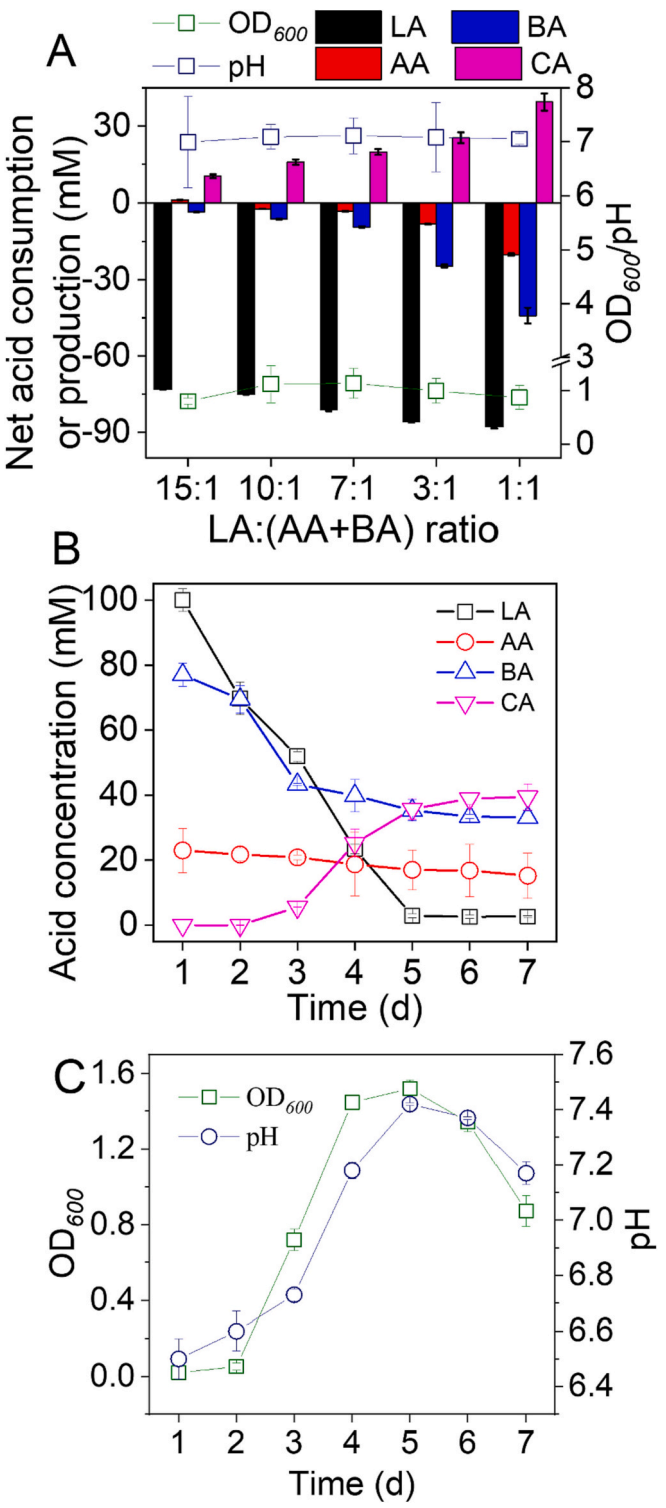

Fig. 3. (A) Effect of different ratios of LA/(AA + BA) on CA production, OD_{600} and pH (the values displayed are the final concentrations on the 7th day), (B) CA production and (C) OD_{600}/pH change when the molar ratio of LA and (BA + AA) is 1:1.

of substrate and the production of CA both increased steadily as the molar ratio of LA/AA+BA increased; at a molar ratio of 1:1, the production of CA was 39.45 mM. When appropriate amounts of AA and BA together acted as EA, it ensured that the release of energy during LA oxidation was not compromised, but it also promoted the participation of acetyl-CoA and butyryl-CoA in cellular synthesis, and therefore its produced biomass was maximized (OD_{600} = 1.138).

The generation of CA changed with time when AA and BA were utilized as combined EAs, as depicted in Fig. 3B. CA production increased steadily as LA, AA, and BA were consumed, reaching a maximum production rate on the 4th day, which was earlier than when AA and BA were utilized as EA alone. However, the production of BA showed a decreasing and then stable trend, reaching a minimum of 2.30 mM on the 3rd day, after which the concentration stabilized, indicating that the consumption and production of BA tended to be balanced. In addition to substrate addition, AA can be transformed to acetyl-CoA during LA oxidation. The generation of AA from acetyl-CoA led to an increase in AA concentration, while the reaction of AA with acetyl-CoA to form BA led to a decrease in AA (Pan et al., 2021; Patel et al., 2023). Considering AA production and consumption pathways, the concentration of AA changed less in the later stages of the reaction, presumably because its production rate was similar to its consumption rate. As shown in Fig. 3C, pH exhibited an increasing trend during the first 5 days, attributed to the fact that the pK_a of LA (~3.0) was lower than that of AA, BA, CA (~4.8). Notably, pH began to decrease after reaching a maximum on the 5th day, a result consistent with the depletion of LA and the reverse β-oxidation process of AA/BA. LA produced a large amount of NADH during oxidation, leading to an increase in alkalinity, while the reverse β-oxidation process of AA/BA consumed a large amount of NADH, leading to an increase in acidity. Thus, this result suggested that the rate of the reverse β-oxidation pathway of AA/BA first exceeded the rate of LA oxidation on the 5th day, leading to a decrease in pH (Li et al., 2023; Xie et al., 2021).

3.4. Carbon fluxes and specific titers for different EAs

As displayed in Fig. 4A, when AA was used as an EA, 47 % of the carbon atoms in the consumed LA went to the synthesis of CA, 47 % were used for cellular synthesis and the release of CO_2, and the remaining 6 % of carbon atoms generated BA. When BA was used as an EA, the carbon atoms used for CA synthesis decreased to 41 % and those used for cellular synthesis increased to 52 %, which explained the higher OD_{600} when BA was used as an EA compared to AA. Interestingly, when AA and BA were used together as EA, 49 % of the carbon atoms consumed by LA, AA and BA flew to the production of CA, leading to the accumulation of 39.45 mM CA, while at the same time, growth of the strain was somewhat enhanced compared to AA alone, with the carbon flux flowing for cell synthesis rising from 47 % to 51 %. Similarly, the LACST (amount of LA consumed per unit of CA produced) and CAPST (CA production per unit biomass) were examined. According to Fig. 4B, the LACST of AA was 4.13 mol/mol, meaning that 4.13 mol of LA were used up for every mole of CA produced. On the other hand, the LACST of BA is 2.58 mol/mol. When AA and BA were combined as EA, the LACST was 2.25 mol/mol, accounting for the findings in Fig. 1A and Fig. 2A. Compared with other CA producers, *Ruminococcaceae* CPB6 had a LACST of 3.83 mol/mol or 2.68 mol/mol in the presence of 5 g/L AA or BA, respectively (Zhu et al., 2017). It could be seen that *M. hexanoica* has a slight advantage over *Ruminococcaceae* CPB6 when BA was used as EA. Meanwhile, CAPST, which represents CA production per unit biomass (when OD_{600} value is 1), was 25.01 mol, 28.54 mol and 45.24 mol, when AA, BA and their combinations were used as EA, respectively. This indicates that AA and BA have their own advantages in CA production and cell synthesis, the highest CA production per unit biomass was achieved when the combination of AA+BA was used as EA.

3.5. M. hexanoica carbon elongation mechanisms in response to various EA

Fig. 5A showed LA metabolism of *M. hexanoica*, where first LA was oxidized to acetyl-CoA via the pyruvate pathway, i.e., LA was converted to pyruvate and then to acetyl-CoA and CO_2 (Jeon et al., 2017; Kang et al., 2022; Kim et al., 2019). Acetyl-CoA is an important precursor for

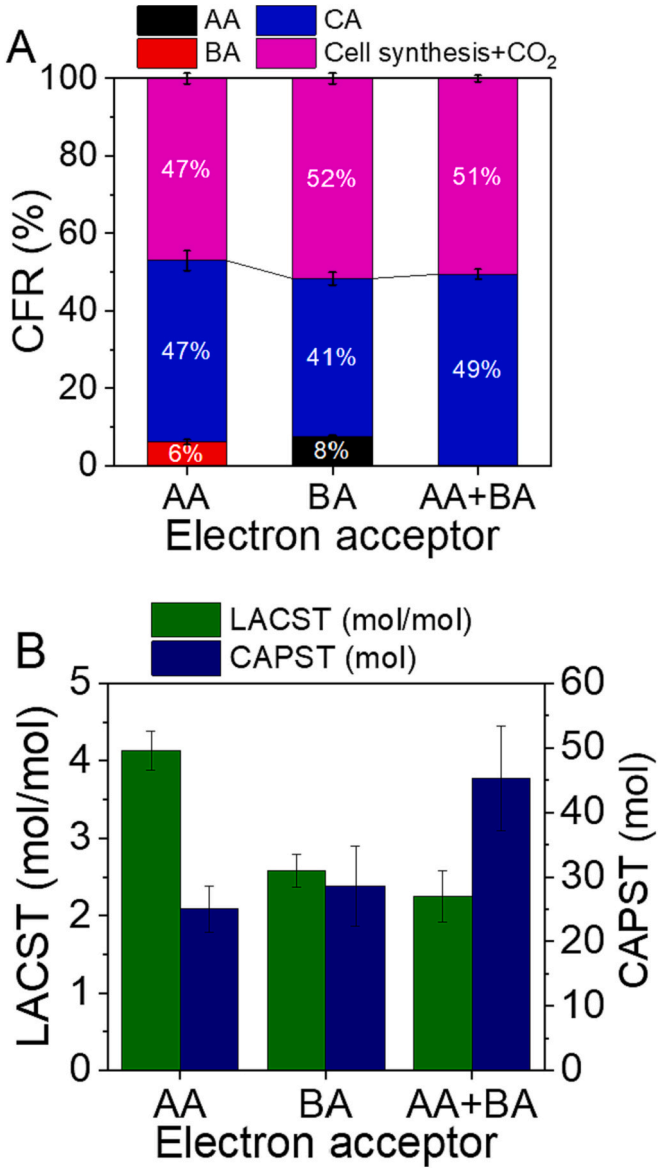

Fig. 4. (A) Carbon flux ratio (CFR) and (B) LA consumption specific titer (LACST)/CA production specific titer (CAPST) of different EA.

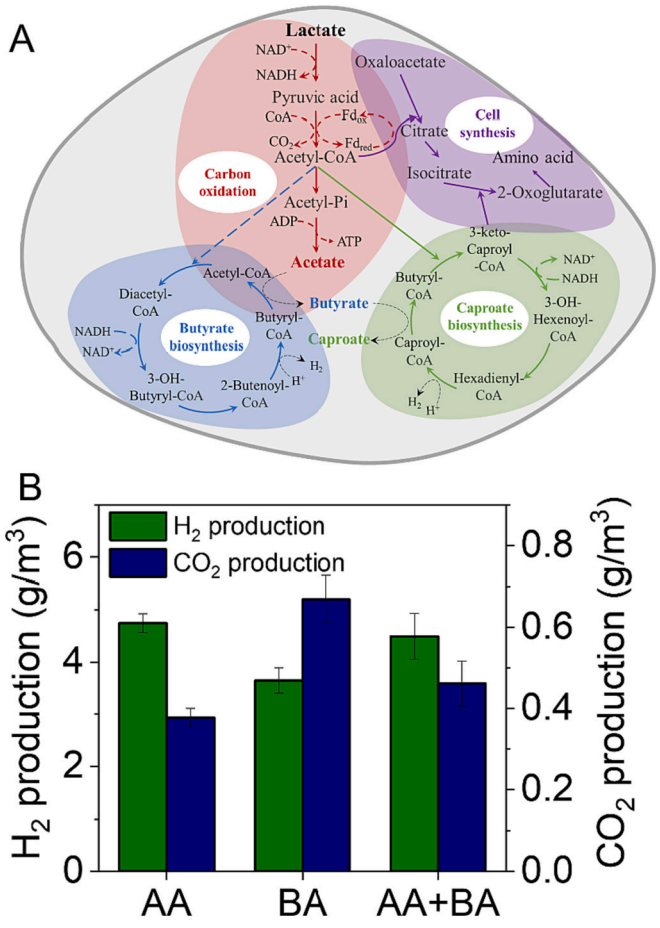

Fig. 5. (A) Reverse β-oxidation pathway of *M. hexanoica* using LA as ED and (B) gas production in the presence of different EA.

the reverse β-oxidation, providing two carbon atoms for each CE process. Meanwhile, acetyl-CoA can be further oxidized to AA, which is successively involved in the synthesis of BA and CA as a substrate of the reverse β-oxidation pathway (Liu et al., 2021; Liu et al., 2020a, 2020b). From this route, it can be deduced that the addition of AA and BA to the initial substrate shortens the time for the synthesis of BA and CA from LA oxidation as well as the reverse β-oxidation pathway, respectively. However, it cannot be inferred that the more AA and BA are added the better, as observed from Fig. 1A and Fig. 2A. When the ratios of LA/AA and LA/BA exceeded 10:1 and 3:1, respectively, the generation of CA decreased rather than increasing, indicating that there might be a certain degree of product inhibition. Combined with the analysis of the results in Fig. 4, it can be inferred that BA can greatly promote the cellular synthesis of *M. hexanoica* compared to AA because BA can be converted to butyryl-CoA and participate in cellular synthesis (Gunun et al., 2022; Xin et al., 2019). In addition, the data of gas production could further also confirm the differences in the effects of different EA on the carbon elongation pathway. As shown in Fig. 5B, when AA and BA acted as EA separately, they each had specific effects on H_2 or CO_2 production, i.e., AA promoted H_2 production, while BA stimulated CO_2 production. This confirmed previous speculation that the addition of AA as an initial substrate caused some inhibition of the LA oxidation pathway, attenuating the conversion of pyruvate to acetyl-CoA and CO_2. However, it promoted the reverse β-oxidation process, especially the synthesis of BA, so that H_2 production was subsequently boosted. Conversely, the addition of BA as an initial substrate, which accelerated the synthesis of CA, may significantly inhibit the synthesis of BA from AA and acetyl-CoA. According to Wang et al. (2022), the process of BA synthesis accounted as a major factor in the production of H_2 (the amount of H_2 produced by BA synthesis was twice that of CA synthesis), and thus the addition of BA led to a significant decrease in the amount of H_2 produced. Unsurprisingly, when both AA and BA acted as EA, both H_2 and CO_2 production were boosted. It was speculated that AA and BA form a good balance in the pyruvate oxidation and reverse β-oxidation pathways, which can promote the key enzyme activities and functional gene expression, which needs to be eventually confirmed experimentally in subsequent studies. The fastest utilization efficiency of LA was achieved at initial concentrations of LA, AA and BA of 100, 23 and 77 mM (i.e., the molar ratio of AA and BA was 3:10), respectively. At the same time, CA was generated at a maximum concentration of 39.45 mM, and its LACST and CAPST both showed the best performance compared with AA and BA alone as EA, indicating that this concentration of AA and BA exhibited the most pronounced stimulation of the reverse β-oxidation process. It was hypothesized to significantly promote the expression of key functional genes with the activity of key enzymes for CA synthesis (Wang et al., 2022).

For further analysis, the CE process with each EA was studied thermodynamically. Each reaction step was evaluated from the oxidation of the ED to the CE of the EA (see Table 1) (Detman et al., 2021).

Table 1
Release of free energy during LA oxidation in *M. hexanoica*.

Reaction steps	$\triangle rG^m$ (kJ/mol)
2 Lactate +2 NAD^+ ⇌ 2 Pyruvate +2 NADH	55.0
2 Pyruvate +2 CoA + 2 NAD^+ ⇌ 2 Acetyl-CoA + 2 CO_2 + 2 NADH	−70.6
2 Acetyl-CoA ⇌ Acetoacetyl-CoA + CoA	26.1
Acetoacetyl-CoA + NADH ⇌ (*S*)-3-Hydroxybutyryl-CoA + NAD^+	−18.0
(*S*)-3-Hydroxybutyryl-CoA ⇌ Crotonyl-CoA + H_2O	3.8
Crotonyl-CoA + NADH ⇌ Butyryl-CoA + NAD^+	−57.0
Acetyl-CoA + Butyryl-CoA ⇌ CoA + 3-Oxohexanoyl-CoA	35.3
3-Oxohexanoyl-CoA + NADH ⇌ (*S*)-Hydroxyhexanoyl-CoA + NAD^+	−24.7
(*S*)-Hydroxyhexanoyl-CoA ⇌ trans-Hex-2-enoyl-CoA + H_2O	1.0
trans-Hex-2-enoyl-CoA + NADH ⇌ Caproyl-CoA + NAD^+	−62.1
Caproyl-CoA + Butyrate ⇌ Caproate + Butyryl-CoA	9.1

The redox equilibrium remains stable when the NADH produced in the oxidation step is consumed in the CE (reduction) step. The net reaction and Gibbs free energy were determined separately for AA and BA (Eqs. (4) and (5)). Meanwhile, in conjunction with the reported studies, the reaction equation when AA and BA together act as EA was derived (Kang et al., 2022; Kim et al., 2019; Wang et al., 2022) (Eq. (6)).

$$2\ Lactate + Butyrate + Acetyl - CoA \rightarrow Caproate + Butyryl - CoA + 2\ CO_2 + 2\ H_2O,$$

$$\Delta rG^0 = -102.1\ kJ/mol \quad (4)$$

$$6\ Lactate + Acetate + Acetyl - CoA \rightarrow Caproate + 3Butyryl - CoA + 4\ CO_2 + 6\ H_2O,$$

$$\Delta rG^0 = -69.9\ kJ/mol \quad (5)$$

$$3\ Lactate + Acetate + Butyrate + Acetyl - CoA \rightarrow Caproate + Butyryl - CoA + 7\ CO_2 + 6\ H_2O,$$

$$\Delta rG^0 = -117.3\ kJ/mol \quad (6)$$

Considering BA to have a lower Gibbs free energy than AA, using BA as an EA in LA-based CE process by *M. hexanoica* appears to be more energy efficient. The condition of only relatively limited energy production with AA as an EA may lead to low cellular synthesis rates (Isipato et al., 2020; Ma et al., 2022), which is consistent with the inference from Fig. 5A.

To verify that the CA-producing performance in serum bottles could be scaled up for potential application and to examine the effect of maintaining a constant pH on the metabolic activity of *M. hexanoica*, a 1.2 L working volume bioreactor was set-up. A photograph of the set-up is shown in Fig. 6A. Based on the findings illustrated in Fig. 3, it was recommended to employ an initial substrate concentration of 100 mM LA, 23 mM AA, and 77 mM BA. Samples were withdrawn from the gas sampling port and the liquid sampling port every 12 h, among others to monitor the acid production process. *M. hexanoica* metabolized the substrates faster in the bioreactor compared to serum bottles. Almost all the LA was consumed after 36 h, while CA production reached its maximum after 48 h, but with a concentration of only 28.45 mM, which was significantly lower than the 39.45 mM found in the serum bottles. Notably, biomass production of the strain was considerably enhanced, with its maximum OD_{600} value of 1.809 after 60 h, which was higher than the value of 1.518 found on the 5th day in serum bottle. The reason for this phenomenon may be that the strain in the bioreactor was exposed to higher shear forces during broth agitation, resulting in the microorganisms needing to expend more energy as well as produce more biomass to resist environmental stresses, such as increased secretion of extracellular polymers (EPSs) (He et al., 2019; Ren et al., 2019), which

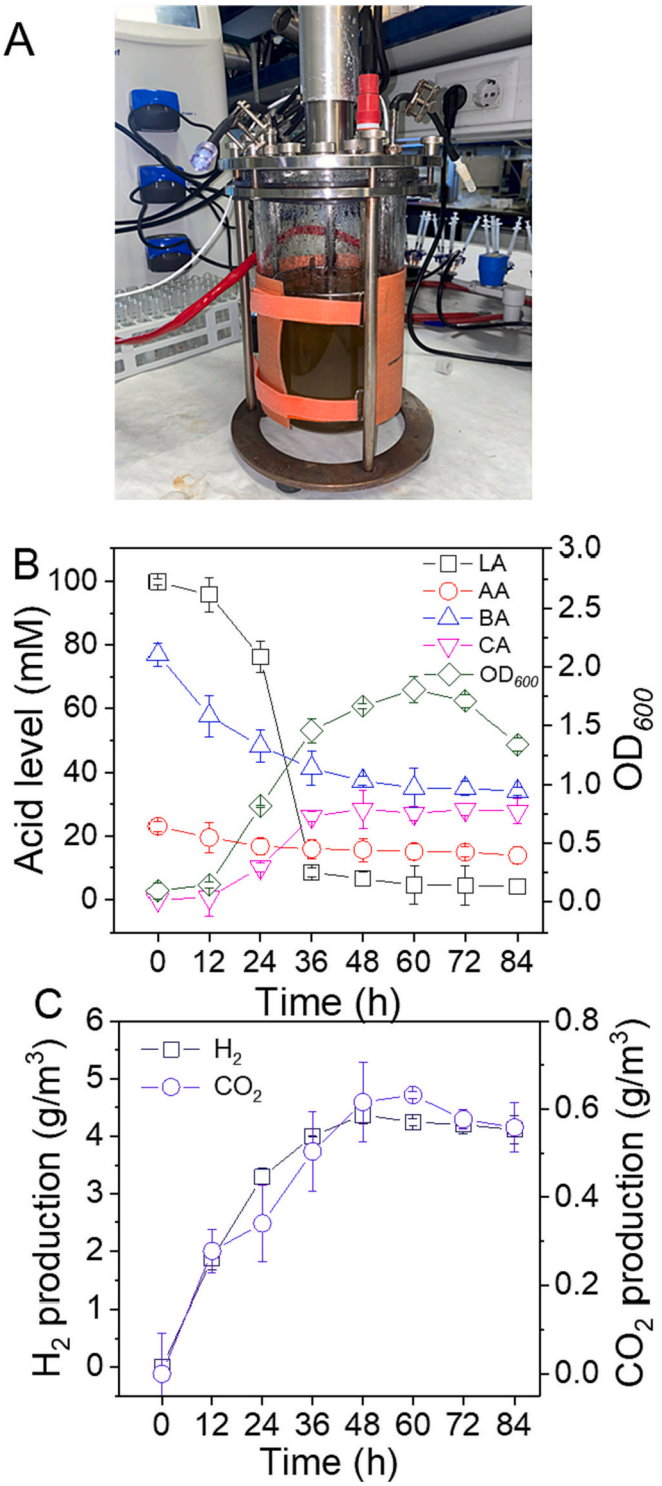

Fig. 6. (A) Photograph of the bioreactor set-up, (B) acid production and (C) gas production with *M. hexanoica* in the bioreactor when the molar ratio of LA/AA/BA (mM) was 100/23/77.

results in altered LA carbon fluxes, inducing a shift in the reducing power, energy, and electrons (i.e., NADH, ATP, and acetyl-CoA) to accumulate towards the tricarboxylic acid cycle (i.e., amino acid synthesis), as demonstrated by the data in the Supplementary Material Fig. S1. Thus, resulting in the phenomenon of a decrease in CFR of CA while LA, BA and AA consumptions in the bioreactor remained almost equal compared to the serum bottles. This speculation was confirmed by the CO_2 and H_2 production shown in Fig. 6C, where 0.632 g/m^3 of

produced CO_2 in the bioreactor was higher than 0.461 g/m^3 detected in the serum bottles. Similarly, 4.11 g/m^3 H_2 production, in the automated bioreactor, was lower than 4.49 g/m^3 observed in the serum bottles. This could be explained by the pathway of LA in Fig. 5A, whereby acetyl-CoA and BA were more involved in cellular synthesis instead of CA production, resulting in more CO_2 production as well as less H_2 accumulation.

4. Conclusions

The use of LA as an ED for CA production has been previously investigated in *M. hexanoica*. However, the effects of different ED/EA molar ratios on CA production had not thoroughly been explored up to date. In the present study, it was found that a balanced and excellent performance in both CA production and cell synthesis could be obtained when AA and BA were used together as combined EA. Among them, when the initial molar amounts of LA, AA and BA were 100, 23 and 77 mM, CA generation was 39.45 mM and the carbon flux of CA was as high as 49 %. Besides, this was validated in scale-up trials in automated bioreactors. Compared to the traditional *Ruminococcaceae* family that utilizes LA for CA production, *M. hexanoica* has a promising commercial value due to the high biocatalytic activity it exhibits under specific conditions, which allowed the production of up to 4.58 g/L CA when using 10 g/L LA as ED. However, the assessment of its capacity to stabilize CA production in practical industrial applications should be the focus of further study.

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CRediT authorship contribution statement

Zeyu Wang: Data curation, Investigation, Writing – review & editing. **Carla Fernández-Blanco:** Data curation, Investigation, Writing – review & editing. **Jun Chen:** Visualization. **María C. Veiga:** Funding acquisition, Resources, Supervision, Writing – review & editing. **Christian Kennes:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, 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.

Data availability

Data will be made available on request.

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