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BRIEF REPORT

Utilization of formic acid by extremely thermoacidophilic archaea species

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Abstract

The exploration of novel hosts with the ability to assimilate formic acid, a C1 substrate that can be produced from renewable electrons and CO_2, is of great relevance for developing novel and sustainable biomanufacturing platforms. Formatotrophs can use formic acid or formate as a carbon and/or reducing power source. Formatotrophy has typically been studied in neutrophilic microorganisms because formic acid toxicity increases in acidic environments below the pKa of 3.75 (25°C). Because of this toxicity challenge, utilization of formic acid as either a carbon or energy source has been largely unexplored in thermoacidophiles, species that possess the ability to produce a variety of metabolites and enzymes of high biotechnological relevance. Here we investigate the capacity of several thermoacidophilic archaea species from the Sulfolobales order to tolerate and metabolize formic acid. *Metallosphaera prunae, Sulfolobus metallicus* and *Sulfolobus acidocaldarium* were found to metabolize and grow with 1–2 mM of formic acid in batch cultivations. Formic acid was co-utilized by this species alongside physiological electron donors, including ferrous iron. To enhance formic acid utilization while maintaining aqueous concentrations below the toxicity threshold, we developed a bioreactor culturing method based on a sequential formic acid feeding strategy. By dosing small amounts of formic acid sequentially and feeding H_2 as co-substrate, *M. prunae* could utilize a total of 16.3 mM of formic acid and grow to higher cell densities than when H_2 was supplied as a sole electron donor. These results demonstrate the viability of culturing thermoacidophilic species with formic acid as an auxiliary substrate in bioreactors to obtain higher cell densities than those yielded by conventional autotrophic conditions. Our work underscores the significance of formic acid metabolism in extreme habitats and holds promise for biotechnological applications in the realm of sustainable energy production and environmental remediation.

INTRODUCTION

Biomanufacturing can play a critical role in the production of sustainable alternatives to currently available chemicals, foods, and added-value products. Widespread adoption of biomanufacturing technologies for commodity chemical production will require alternative feedstocks to simple sugars, including waste biomass feedstocks, carbon dioxide, and other C1 compounds. One of the most compelling alternative feedstocks is formic acid, which can be produced from CO_2 using renewable electricity. Formic acid can be produced from carbon dioxide via electrochemical (Fernández-Caso et al., 2023) or photochemical reduction (Cauwenbergh & Das, 2021). Formic acid is one of the most valuable products that can be obtained from

CO_2 electrolysis (Jouny et al., 2018), with an expected global market of 1300 kilo tones by 2035 (Fernández-Caso et al., 2023). The use of formic acid as an energy vector in biomanufacturing processes is an active research topic as it would allow carbon dioxide-rich waste streams to be reduced with renewable electricity for use as both a carbon and energy source in fermentation. As compared to other energy sources synthesized from carbon dioxide (i.e. CO) or water electrolysis (i.e. H_2), formic acid is soluble, improving its availability for microorganisms, and reducing challenges for handling and storage.

Formic acid serves as a versatile energy source for various microorganisms across different environments. Microorganisms can metabolize formic acid through formate oxidation, an enzymatic conversion yielding carbon dioxide and protons, releasing energy that can be harnessed by the cell. However, formic acid can be highly toxic to cells at high concentrations as it unpairs proton motive force (Carere et al., 2021). Therefore, the utilization of formate as a carbon/energy source can be limited by substrate inhibition.

Formate metabolism and tolerance has been mostly explored in neutrophilic and mesophilic species including the model host *Cupriavidus necator*, which can use formate as sole carbon and energy source (Calvey et al., 2023), species of the yeast *Saccharomyces* (Oshoma et al., 2015; Overkamp et al., 2002), several methylotrophic bacteria (Chu et al., 1987) and *Desulfovibrio* species (Voskuhl et al., 2022). Moreover, engineering formic acid assimilation pathways (i.e. the reductive glycine pathway) in model mesophilic and neutrophilic hosts like *Escherichia coli* (Bang & Lee, 2018), which can oxidize formate but cannot use it as a carbon source, has recently raised great interest. In contrast there are only few studies that have investigated formic acid or formate utilization by extremophiles. Neutrophilic and hyperthermophilic species like *Desulfurococcus amylolyticus* DSM 16532 (Ergal et al., 2020), *Thermoanaerobacter kivui* (Jain et al., 2020), and *Thermocrinis ruber HI 9* (Huber et al., 1998) have been reported to utilize formate as carbon and energy sources (Table 1) and to grow on this substrate in chemostats. Because of the increased toxicity of formic acid in acidic environments (pKa formic acid = 3.75, 25°C, (Haynes, 2014)), the cultivation of microorganisms with formic acid has been largely unexplored in acidophiles, microorganisms that are of significant importance for biomining and other low-pH applications. To date, only two published studies have explored the cultivation of acidophiles with this organic acid, with both demonstrating almost complete growth inhibition when culturing the species under batch conditions. For instance, *Acidothiobacillus ferrooxidans* (Pronk et al., 1991) could only grow with formic acid when cultured in a chemostat fed continuously with media containing 100 μM formate, below the toxicity threshold. Likewise, the methanotroph Methylacidiphilum sp. RTK17.1, a thermoacidophilic bacteria that grows at 45–50°C (Carere et al., 2021), could not grow on formic acid as a sole carbon and energy source in batch cultures, but it could when the authors cultured it in a chemostat to relatively high cell densities (2.86 g-CDW mol^{-1}).

Studying the utilization of formic acid and formate by extremophilic hosts can open avenues to biomanufacturing of distinct products from novel metabolic pathways and under favourable and simplified operational conditions (Zhu et al., 2020). While still underexplored at an industrial level, biomanufacturing under thermophilic conditions has several benefits, including avoidance of contamination, favourable kinetics, and the possibility of producing thermostable enzymes (extremozymes) and lipids. Additionally, low pH biomanufacturing can be beneficial for the industrial production of acids as it reduces the costs of separating them. Among other extremophiles, members of the genus *Sulfolobus* have been highlighted by researchers as potential key biotechnological hosts because of the product spectrum they can synthesize and the increasing availability of genetic tools for its manipulation (Garrett et al., 2015; Quehenberger et al., 2017). *Sulfolobus* species produce a large variety of proteins (enzymes) and lipids with outstanding temperature and pH stability of interest for pharmaceutical and biotechnological applications (Quehenberger et al., 2017; Schocke et al., 2019). Growth of aerobic thermophilic species, especially under autotrophic conditions, is highly challenging because of the limitations associated with substrate delivery derived from the poor gas solubility at elevated temperatures. Therefore, employing alternative soluble feedstocks like formic acid to culture thermophiles would allow to enhance substrate availability by indirectly using CO_2 as source.

Motivated by expanding the work on the identification of new extremophilic hosts capable of utilizing formic acid, in this study we investigate the metabolic capabilities of several species of thermoacidophilic archaea, microorganisms that are adapted to both extreme pH and temperature environments. In particular, we have chosen to study the utilization of formic acid by members of the Sulfolobales order, species that can be found in sulfuric hot springs and ore deposits, and have been considered model organisms among extremophiles (Lewis et al., 2021).

RESULTS

In our initial screening of potential acidophilic formatotrophs, we identified and targeted thermoacidophilic species that possess a different carbon fixation pathway than other thermophilic or neutrophilic species previously explored in studies of

TABLE 1 Review of extremophile strains tested for formic acid tolerance and utilization in this and previous studies.

Strain	Carbon uptake pathway and growth conditions	FA tolerance that allowed for growth	FA utilization	Source
Mesophilic acidophiles				
A. ferrooxidans	Calvin-Benson-Bassham, 25°C, acidic pH 1.4	100 μM in chemostat	+	(Pronk et al., 1991)
Thermophilic acidophiles				
Sulfolobus acidocaldarius	3-HP/4-HB cycle, pH 2.5, 50–70°C	2 mM (co-substrate)	+ 0.21 μmol h^{-1}	This study
Sulfolobus metallicus	3-HP/4-HB cycle, pH 2.5, 65°C	1 mM (co-substrate)	+ 0.6 μmol h^{-1}	This study
Metallosphaera prunae	3-HP/4-HB cycle, pH 1–4, 55–80°C	2 mM (co-substrate)	+ 1.7 μmol h^{-1}	This study
Methylacidiphilum sp. *RTK17.1*	Calvin-Benson-Bassham cycle, pH = 1.5–3, 50°C	+ in chemostat as sole carbon source	+	(Carere et al., 2021)
Thermophilic neutrophiles				
Thermocrinis ruber HI 9	Reductive TCA cycle, pH 6.7, 80°C	22 mM	+	(Huber et al., 1998)
Thermoanaerobacter kivui	Wood-Ljungdahl pathway, pH = 7.5, 65°C	200 mM	+	(Burger et al., 2022; Jain et al., 2020)
Desulfurococcus amylolyticus DSM 16532	Incomplete reductive TCA and 3-HP/4-HB cycles, pH = 6.4, 80°C	116 mM	+	(Ergal et al., 2020)

Note: *Sulfolobus acidocaldarium* DSM639 was cultivated aerobically in shaking flasks at 70°C. *Sulfolobus metallicus* DSM6482 and *Metallosphaera prunae* DSM10039 were cultured aerobically in shaking flasks under atmospheric air at 65°C and pH 2.5. Tolerance to formic acid was examined using the basal media with 0.2 g L^{-1} yeast extract (BD-Difco) and approx. 0.5 g L^{-1} of S° (except for *S. acidocaldarium*) and cells were cultured in shaking flasks (50–500 mL). Formic acid tolerance was tested at a varied concentration range (0.5–5 mM).

formic acid tolerance and utilization. In particular, we identified species within the Sulfolobales order that fix carbon employing the 3-hydroxypropanoate/4-hydroxybutyrate (3-HP/4-HB) cycle, a pathway found in extremely thermoacidophilic archaea that is associated with fast autotrophic growth (Quehenberger et al., 2017). These species are commonly sulfur and metal oxidizers, and grow at pH of 0.4–5.5 and temperatures of 40–80°C (Lewis et al., 2021; Liu et al., 2021). Three different species of Sulfolobales, purchased from DSMZ (Leibniz, Germany), were screened for formic acid tolerance: *Sulfolobus acidocaldarium*, *Sulfolobus metallicus* and *Metallosphaera prunae*. We prepared the inocula by culturing the species, previously preserved in glycerol at −80°C, in a basal salts medium as previously stated (Brock et al., 1972) (further culturing details are provided in the Supporting Information). We then used these cultures as inoculum to test formic acid tolerance at a pH of 2.5 (Table 1). Exposure to formic acid decreased growth for all species (Figure S1A–C) as compared to growth in the absence of it, indicating the inhibitory nature of this substrate for growth under acidic conditions. While *S. metallicus* growth was significantly inhibited when exposed to 0.5–1 mM, *M. prunae* and *Sulfolobus acidocaldarius* could grow with formate concentrations of up to 2 mM. Formic acid was fully consumed by the time cells reached stationary phase for all species (not detected by HPLC analyses, see SI for methodology), confirming active formic acid metabolism. These concentrations of formic acid that allow for growth of Sulfolobales species are an order of magnitude lower than those that neutrophilic thermophilic bacteria can tolerate and metabolize (Table 1), consistent with the increasing toxicity of formic acid in acidic environments (Baker-Austin & Dopson, 2007). Nevertheless, those concentrations are significantly higher than those tolerated by the acidophilic bacterium *A. ferroxidans* (0.1 mM), one of the few acidophilic species tested for growth on formic acid (Pronk et al., 1991). We did not observe any growth of the Sulfolobales species on formic acid alone, an expected outcome given the low energy that can be extracted from the viable concentrations of formic acid tolerated by these species.

FORMIC ACID IS CO-UTILIZED WITH PHYSIOLOGICAL ELECTRON DONORS LIKE FE (II)

To further study the utilization of formic acid by thermoacidophilic archaea, we selected the two species previously screened that can grow fully autotrophically, *S. metallicus* and *M. prunae*. These two species are strict aerobes that typically utilize elemental sulfur or ferrous iron forms such as FeS, FeS_2 or chalcopyrite ore as physiological electron donors (Itoh et al., 2020).

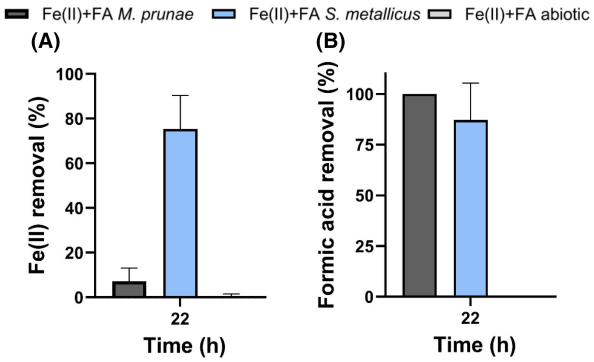

FIGURE 1 Utilization of formic acid with the physiological electron donor Fe^{2+} by the thermoacidophilic species *Sulfolobus metallicus* and *Metallosphaera prunae*. (A) Removal of Fe (II) in the presence of formic acid by *M. prunae*, and by *S. metallicus* and (B) removal of formic acid in the presence of Fe (II). The assays were performed in duplicates. *M. prunae* was cultured with 1.22 mM of formic acid and 5 mM of Fe (II) while *S. metallicus* was cultured with 0.55 mM of formic acid and 5 mM of Fe (II). Tests were performed in duplicates, and the error bars represent the half-range.

To understand how these species utilize formic acid in the presence of a physiological alternative electron donor, we next cultured *S. metallicus* and *M. prunae* with Fe^{2+} in the form of $FeSO_4$. We analysed whether Fe^{2+} is oxidized to Fe^{3+} (1 electron) in the presence of formic acid (2 electrons if oxidized to CO_2), and whether these two electron donors can be co-utilized. We first probed the iron oxidation capacity of the two species. Both *S. metallicus* and *M. prunae* showed a capacity for oxidizing iron in the presence of yeast extract (Figure S2A,B), in agreement with previous studies (Huber & Stetter, 1991). *S. metallicus* showed a superior capacity to oxidize iron even at concentrations of 20 mM, whereas *M. prunae* growing cells exhibited a weaker iron oxidation activity. In contrast, resting cells assay confirmed the ability of the later species to oxidize iron (5 mM) even without an organic substrate (Figure S2C). Abiotic controls confirmed the biological nature of this rapid Fe^{2+} oxidation and the stability of soluble Fe^{2+} in the culturing media at a pH of 2.5 when oxygen is present for the first 4 days (Figure S2A). Next, we probed the impact of formic acid addition on Fe^{2+} oxidation in the absence of alternative electron and carbon sources. Addition of formic acid in the medium allowed for an oxidation of 74 ± 16% of Fe^{2+} by *S. metallicus* (Figure 1A), but it significantly impacted the iron oxidation activity of *M. prunae* (9.3 ± 3% of iron removal) (Figure 2A) after 22 h. Abiotic controls confirmed the presence of biological iron oxidation. Both species removed most of the formic acid in the medium (87 ± 18% by *S. metallicus* and 100% by *M. prunae* cultures). This suggests that formic acid was preferentially utilized by *M. prunae* over Fe^{2+} and/or that it had an inhibitory effect on its iron metabolism. Overall, these results show that these species can co-metabolize formic acid with other inorganic physiological electron donors.

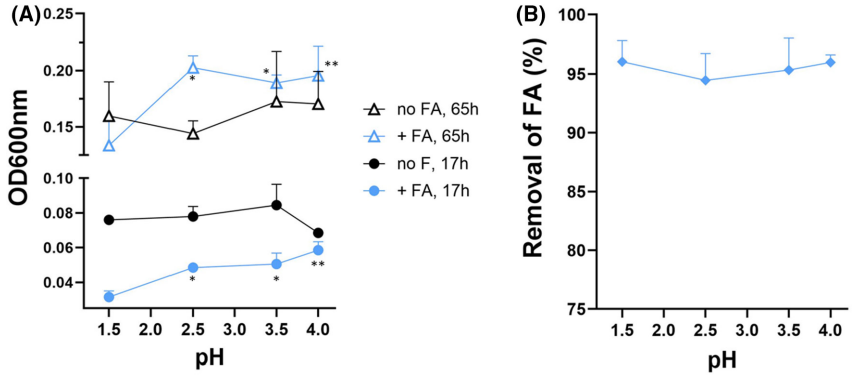

FIGURE 2 Impact of pH of culturing medium on formic acid (FA) or formate tolerance and consumption by *Metallosphaera prunae*. (A) Growth of *M. prunae* with 1.65 mM of FA (blue lines) and without FA (black lines) in a medium adjusted to different pH. Yeast extract and S^0 were supplemented in the medium for all tests. (B) FA removal after 17 h by the cultures grown in the presence of formic acid. The inoculum grown with FA was pre-cultured with 1 mM of FA in the medium to acclimate cells to the presence of this acid. Tests were performed in duplicates, and the error bars represent the half-range. Significant differences between the condition pH = 1.5 and the other pH conditions for a set of data were determined by one-way ANOVA ($n = 2$), *$p = <0.05$, **$p < 0.01$, 95% CI. The absence of asterisks indicates no significant differences were found. Media pH with H_2SO_4 to different pH at 23°C to 1.5, 2.5, 3.5, and 4 in the presence (1 mM) and absence of formic acid. Cultures of formic acid acclimated cells were grown at pH of 1.5 and 3.5 in 50 mL shake flasks at 65°C and were then mixed, and the pellets collected (4500 rpm, 15 min) and concentrated 10 times in fresh new medium at a pH of 2.5. These cells were used as inoculum (5% v/v) for the next assays in which cells were cultured with 1.65 mM of formic acid (stock of 100 mM) in similar flasks. Cells were added to an initial OD600nm of ~0.02. Samples were taken after 17 h and 65 h to measure optical density and formic acid amounts. A control without formic acid was performed under similar conditions.

In previous assays with formic acid as a sole electron and carbon source we did not detect growth, suggesting inability of the species to conserve energy with formic acid alone, or with the low amounts of formic acid supplied. The co-substrate utilization results suggests that autotrophic growth of these thermoacidophilic species on formic acid could potentially be supported if additional inorganic substrates such as Fe (II) or H_2 are supplied, and if they could support energy conservation. While H_2 is known to support growth of most Sulfolabales species (Liu et al., 2021), it is unconfirmed whether species with observed iron oxidation activity can utilize Fe (II) sources to conserve energy.

EFFECT OF pH ON FORMIC ACID METABOLISM OF THERMOACIDOPHILES

Organic acids can be uncouplers of the respiratory chain of microorganisms as their protonated form can readily diffuse through the cell membrane, undergo proton dissociation and acidify the cytoplasm. This event is more favourable under acidic conditions. Acidophiles, like *M. prunae*, possess mechanisms to maintain pH homeostasis that allow for respiration and growth in acidic environments (Baker-Austin & Dopson, 2007). Even so, pH differences can increase the susceptibility of acidophilic species to organic acid-induced stress, as decreases in pH favour the protonated form (formic acid over formate). Thus, we next investigated growth and formic acid utilization in media with different pH using *M. prunae*, as this species exhibited high tolerance to formic acid and growth to higher cells densities as compared to the other species tested in this work. The pH of our medium in previous assays was 2.5 (2.8 at 65°C, Figure S3). Media conditions with pH below the formic acid dissociation constant (pKa = 3.75 at 25°C, and pKa = 3.80 at 65°C) (Kim et al., 1996) significantly enhance the protonated form of the acid and thus increase the expected toxicity. To investigate how growth with formic acid is impacted by pH, we first pre-acclimated *M. prunae* cells by adding 1 mM of formic acid to the basal medium (plus 0.2 gL^{-1} of yeast extract, and 0.5 gL^{-1} S°). These cells were then used to inoculate flasks and test growth with 1.65 mM of formic acid in media adjusted to values of pH above and below the formic acid pKa, selecting pH values of 1.5, 2.5, 3.5 and 4 (pH at 65°C of 1.83, 2.83, 3.83 and 4.33, respectively).

We observed that growth was generally lower as the pH of the medium became more acidic (Figure 2A), in agreement with the greater presence of the protonated form of the organic acid causing increased toxicity. Significant growth differences were observed after 17 h and 65 h between cultures grown under a pH of 1.5 and cultures grown under higher pH with formic acid (Figure 2A). We detected only trace levels of formic acid after 17 h, with more than a 94% of formic acid consumed across all the pH conditions tested (Figure 2B). Thus, even though cells grew more slowly when exposed to formic acid at lower pH by hour 17, they exhibited similar formic acid degradation activity. As a control, we cultured cells without formic acid in media with the same pH values. In this case we did not detect an inhibition of growth as pH decreases,

with the lowest growth observed at the highest pH: 4.0. Across all pH values, optical density by hour 17 h was higher in the absence of formic acid, confirming that the lower cell densities observed at lower pH are due to the presence of formic acid and not due to the acidity of the medium. After 65 h, cultures grown with formic acid reached similar or even higher cell densities (OD_{600nm}) compared to those without. This indicates that while formic acid may initially hinder growth, following adaptation the cultures can potentially benefit from its presence. Optimal growth for other *Metallosphaera* species such as *M. sedula* or *M. cuprina* has been reported to be between 2.5 and 3.5 (Liu et al., 2011), and our results (Figure 2A) suggest *M. prunae* has similar growth preferences when no formic acid is present.

Overall, our results indicate that growth at low pH in the presence of formic acid results in enhanced toxicity, as compared to growth above the pKa. However, achieving growth above the pKa requires cultivation outside the preferred pH range for the host. Because formic acid toxicity depends on both the pH and on the concentration supplied, we hypothesize that if formic acid can be rapidly consumed by metabolically active cells in small doses, toxicity can be overcome even at low pH values. This effect creates the possibility of fed-batch operational modes that enable high flux of formic acid while maintaining concentrations below the toxicity threshold.

GROWTH OF *M. PRUNAE* IN BIOREACTORS WITH SEQUENTIALLY FED FORMIC ACID

In batch cultivations, we observed that the tolerance and capacity to consume formic acid is >20-fold higher for *M. prunae* growing cells than is observed for the acidophilic species *A. ferroxidans* (100 uM). Still, 2 mM concentrations of formic acid are lower than observed in neutrophiles and are insufficient to yield high concentrations of biomass. To achieve viability as a feedstock for biotechnological applications, bioprocess approaches must be developed to enhance the utilization of formic acid by *M. prunae* while minimizing exposure to inhibitory substrate amounts. By using continuous or batch-controlled feeding programs one can match substrate feed with consumption and thus avoid substrate accumulation up to concentrations that are toxic for microorganisms. Previous studies could achieve biomass growth of *A. ferroxidans* on formic acid by employing a chemostat feeding strategy that avoids accumulation of it in the medium (Pronk et al., 1991). While effective at achieving growth while maintaining low substrate concentrations, a continuous supply of media containing 100 μM formate necessitates high flux of media through the system, alongside deployment of cell retention to prevent washout. We hypothesize that a fed-batch feeding approach employing a concentrated formic acid feed supplied at or below the rate of formic acid metabolism rate would enhance formic acid utilization without the associated drawbacks of the chemostat approach. We initially attempted to operate a pH-stat bioreactor; however, our initial experiments revealed that the formic acid quantities required to provoke a pH change in the medium were over the tolerance limit of the species used. This is due to operation below the pKa, as well as the low concentrations supplied in each bolus. Thus, we then cultured *M. prunae* in bioreactors of 0.4 L employing a sequential-batch feeding strategy (Figure 3A) for the liquid substrates (formic acid) alongside a continuous supply of the gaseous substrates (CO_2, air, and H_2 as needed). We used a bioXplorer® 400P system with 4 bioreactors (HEL Ltd, UK) with a working liquid volume was 400 mL (total volume of 500 mL) and the WinIso® software was used for online monitoring and control systems of the reactor. The bioreactors had two gas inlets coupled with gas spargers, one that fed H_2, CO_2, and another one that fed air. Each gas was fed individually to the bioXplorer® 400P system, and their flow was adjusted with individual mass flow controllers. The bioreactors were equipped with pH, dissolved oxygen (DO), agitation, temperature and pressure controllers, and a condenser at the exhaust port of the reactor to avoid excessive loss of water by evaporation. We sampled the reactor through a sampling port coupled to a two-way valve.

We first performed preliminary assays to test whether we could culture *M. prunae* (from glycerol stock) in a bioreactor (65°C), and whether formic acid could be consumed and tolerated when fed sequentially with supplemental electron donors. To initially grow cells in the bioreactor, yeast extract, CO_2, air, H_2 and elemental sulfur were initially added as carbon and energy substrates (Figure 3). Prior to starting the sequential feeding of formic acid, we added 4 pulses of 0.25 mM of formic acid to acclimate cells. After observing cell growth for several days, the sequential addition of formic acid was initiated (day 5.8), causing a rapid decrease of the DO values (Figure 3A,B), indicating that *M. prunae* coupled utilization of this substrate to oxygen reduction.

Because *M. prunae* could readily utilize formic acid as a substrate when fed sequentially in a bioreactor, we next explored its growth on formic acid as a sole energy source for *M. prunae* in bioreactors using a sequential feeding approach and removed YE, H_2 and S° (R1, FA). As a positive control, we operated a second bioreactor in parallel in which yeast extract (YE) was fed sequentially (R2, YE) using the same program as the sole energy source (Figure 4A). Our formic acid feeding program supplied a total of 0.25 mM of this substrate every 1.22 h, a concentration that is below the tolerance limit previously observed (2 mM) for growing cells of this microorganism. No growth was observed after 4 days in

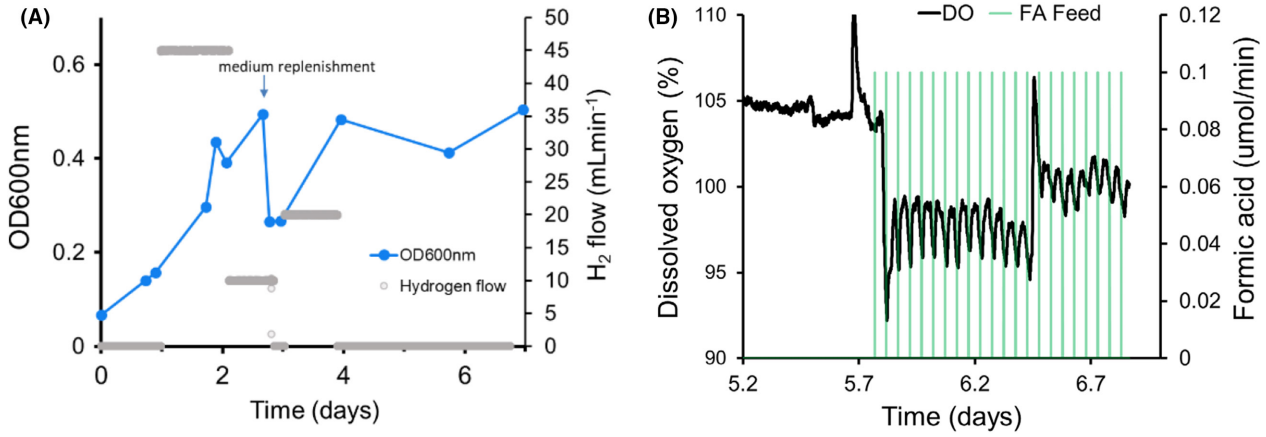

FIGURE 3 Growth of *Metallosphaera prunae* under mixotrophic conditions in a bioreactor with a formic acid sequential-pulse feeding program. (A) Growth measured as OD_{600nm} of *M. prunae* in a bioreactor (400 mL) under mixotrophic conditions with 0.1 g L^{-1} of yeast extract, elemental sulfur, 10% CO_2, 50% H_2 and 40% air. Biomass collected by day 2.4 was washed, concentrated and preserved in glycerol stocks (12.5% final concentration) at −80°C, and was then used as inoculum for the reactors shown in bioreactors R1 and R2 shown in Figure 4, and in Figure S4. Before starting the sequential feeding, 4 formic acid additions (~0.25 mM) were performed to acclimate cells. We replenished the medium by day 2.4 to maintain high metabolic activity and then started the sequential addition of formic acid by day 5.8. Sequential additions of formic acid were performed by using a feeding table designed to add 1.25 mL in each addition every 1.22 h. (B) Caption of the response of dissolve oxygen (DO) in the bioreactor from panel A upon sequential additions of formic acid.

R1 (Figure 4B), while formic acid sequential additions did not cause a drop in DO values (Figure 4E), suggesting that *M. prunae* cannot grow on formic acid alone (Figure 4B) under the conditions tested. However, there was microbial growth in R2 (Figure 4B) and a clear DO consumption profile upon additions of YE (Figure 4E). This confirmed that our sequential feeding strategy is a viable method to provide reducing equivalents to *M. prunae* when non-toxic and/or energy dense substrates such as yeast extract are supplied. While formic acid was consumed in the reactor, the amounts of formic acid in R1 may have been too low to allow for energy conservation, requiring the supplementation of an additional energy source (H_2 or elemental sulfur). To test this hypothesis, we then evaluated the capacity of *M. prunae* to grow on formic acid using H_2 as a co-substrate in a bioreactor. A preliminary assay confirmed the ability of *M. prunae* to grow with H_2 as a sole electron donor in bioreactors (Figure S4). Notably, culturing *M. prunae* in the bioreactor under fully autotrophic conditions yielded relatively high cell densities, reaching OD600nm up to 2 (Figure S4), an 8-fold enhancement over the maximum cell density obtained when culturing *M. prunae* under mixotrophic conditions (Figure S1C).

After confirming the viability of *M. prunae* cultivation under autotrophic conditions in bioreactors, we then cultured *M. prunae* utilizing H_2 as co-substrate with formic acid fed in sequential bolus additions (R3, H_2 + FA). In parallel, we cultured *M. prunae* under the same conditions in a bioreactor fed with DI water (pH 2.5), using the same sequential feeding program (R4, H_2). We fed H_2 at 30%, together with a 10% of CO_2 and starting supply of air of 60% (air flow was increased as DO was consumed). Active cells culture under autotrophic conditions in a bioreactor (Figure S4) were employed as inoculum in R3 and R4. Growth was significantly higher—by an average of 23 ± 8%—from day 3.7 (hour 89) in the bioreactor in which formic acid was sequentially added (R3) as compared to the DI water control in which only H_2 was fed as an electron donor (R4) (Figure 4C). The DO values in both reactors decreased rapidly and in a similar fashion in the first days, indicating rapid hydrogen oxidation metabolism (Figure S5). Moreover, no accumulation of formic acid was detected in the medium of R3, and thus *M. prunae* formic acid consumption exceeded supply, resulting in formate-limited conditions (Figure 4D). Additions of formic acid generated a mixed response on the DO profile, suggesting competition between enhanced oxygen uptake driven by formic acid utilization, and an inhibitory effect of the formic acid towards cell growth and associated hydrogen oxidation. Future experiments using different concentrations of formic acid and hydrogen would be beneficial to understand how formic acid is utilized when it is sequentially fed with a competing electron donor like hydrogen.

Overall, culturing this species in a fed-batch bioreactor configuration maintained formic acid concentrations below inhibitory concentrations while enhancing the total formic acid fed (16.3 mM) by a factor of 8 as compared to growing cells in a flask. We therefore suggest that formic acid can be used successfully as a carbon and/or energy source in thermoacidophiles (Babel, 2009), provided it is supplied in sequential small doses without exceeding the rate of consumption. Further investigation is needed to elucidate whether *M. prunae* could be cultured on formic acid alone if acclimation efforts were developed in a bioreactor or in a

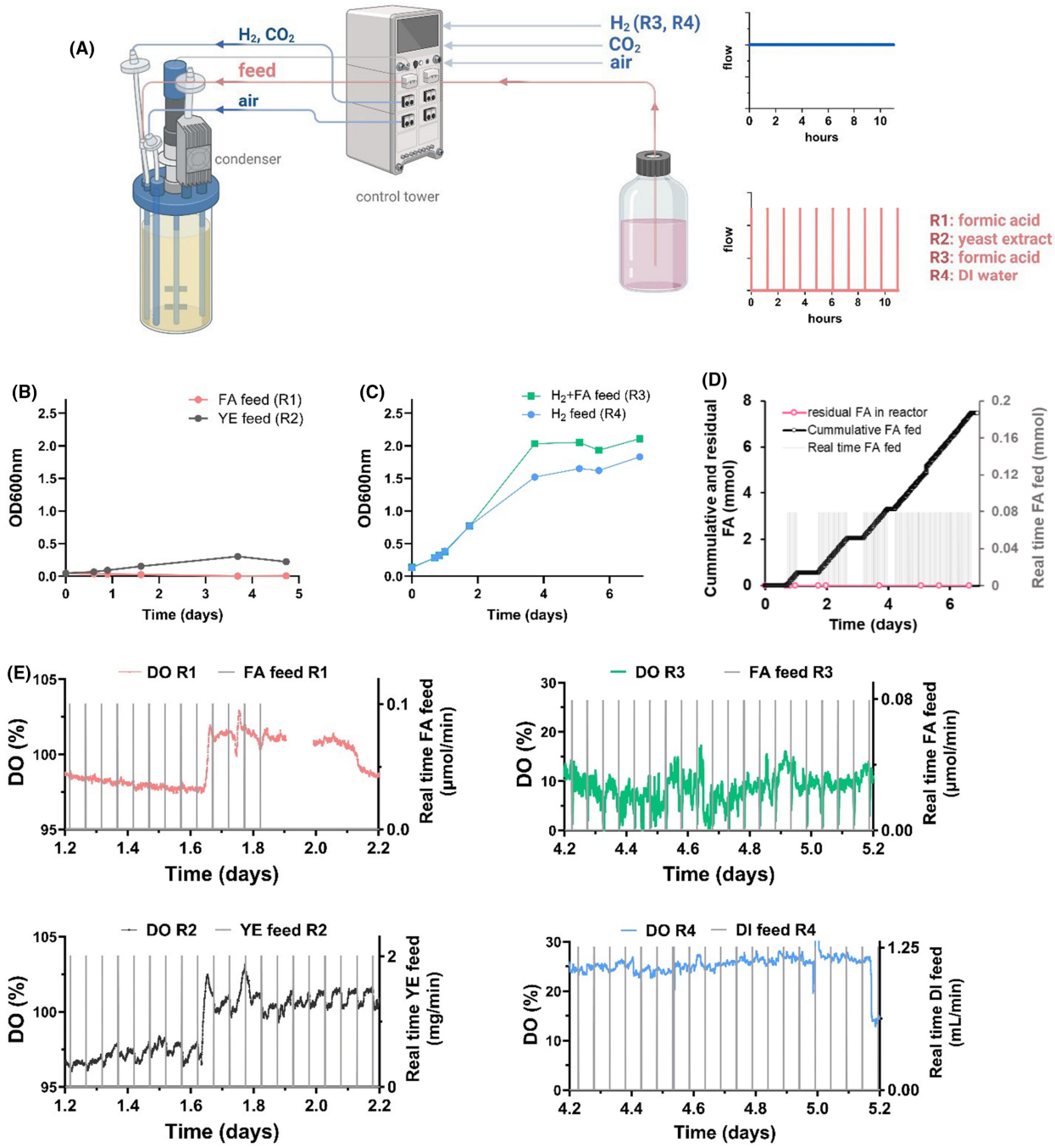

FIGURE 4 Growth of *Metallosphaera prunae* in bioreactors with a formic acid sequential-pulse feeding program in the presence and absence of alternative co-substrates. (A) Schematic of the bioreactor set-up used for continuously feeding gas substrates and sequentially adding the liquid substrates formic acid (FA), yeast extract (YE) or DI water. R1 and R2 were fed with 10 mL min^{-1} of CO_2 and 80 mL min^{-1} of air. R3 and R4 were fed with 30 mL min^{-1} of H_2, 10 mL min^{-1} of CO_2 and 60 mL min^{-1} of air. We adjusted the sequential feed rate to the rate of evaporation of the medium (tested using the Sulfolabales medium) in our bioreactors to maintain a constant volume. The bioreactors were operated with an agitation maintained between 750 and 1000 rpm, and pH and dissolved oxygen (DO) were continuously monitored. (B) Growth of *M. prunae* in R1 and R2 measured as cell density (OD_{600nm}). (C) Growth of *M. prunae* in R3 and R4 measured as cell density (OD_{600nm}). These 2 reactors were fed with 10% of CO_2, 60% of air, and 30% of H_2. R4 sequential liquid feed was composed of DI water adjusted with H_2SO_4 to a pH of 2.5 at room temperature, while R3 feed consisted of a 63 mM formic acid solution adjusted to a pH of 2.5. The sequential feed program was initiated after 17 h of starting the operation of the bioreactors to allow cells to grow before exposing them to formic acid additions. (D) Formic acid additions within time in R3 (grey lines), cumulative mmol of formic acid added (black dots) and residual concentration of formic acid in R3 in the samples taken from the medium. (E) Captions of dissolved oxygen (DO %) dynamics for a period of 1 day upon real time additions of the corresponding feeding in reactors R1, R2, R3 and R4. The reactors were operated in 2 different experimental periods, being R1 and R2 operated in parallel and R3 and R4 were run in parallel in a subsequent test.

chemostat, as previously performed with the thermoacidophilic methanotrophic bacterium *Methylacidiphilum* sp. RTK17.1 (Carere et al., 2021). While our feeding strategy employed deliberate underfeeding to ensure the maintenance of low concentrations of formic acid, formic acid supply could be more closely coupled to demand, increasing metabolic rates if the feed could be automated based on real-time feedback from the bioreactor (pH or DO). These results demonstrate that thermoacidophilic microorganisms can be cultured to relatively high cell densities under both autotrophic and mixotrophic conditions in a bioreactor while employing formic acid as a co-substrate. In the longer operating the bioreactor as a pH-stat may be possible with increased formic acid tolerance and higher-sensitivity probes. Further efforts in bioprocess development, including evolutionary and rational engineering to improve microbial tolerance to formic acid, could potentially enhance biomass production to biotechnologically relevant levels, providing a new autotrophic cultivation platform for these extremophilic microorganisms.

CONCLUSIONS

This study expands both the spectrum of acidophilic species identified to utilize formic acid and the spectrum of confirmed metabolic capabilities of thermoacidophilic archaea species. While these species thrive in acidic environments, they are highly sensitive to organic acids like formic acid. Our work demonstrates that several species of the thermoacidophilic Sulfolabales are able to grow and metabolize formic acid when supplied at ~2 mM in batch flask cultivation, or in small sequential doses in bioreactors, and metabolize even higher amounts when cells are highly active. While formic acid as sole energy source may not be able to support growth of Sulfolabales species without further adaptation, it can be employed as auxiliary substrate to enhance growth on other energy sources, including H_2. Notably, H_2 and formic acid can be produced using renewable electricity in electrochemical cells via water and CO_2 electrolysis, respectively, with formic acid providing significant advantages with regard to solubility, ease of storage, and ease of transport. Thus, we propose a culturing approach to potentially enhance growth and productivity of thermoacidophilic species for biotechnological applications employing sustainable feedstocks. Finally, from an ecological perspective, our work contributes to understanding how these extremophilic archaea tolerate and exploit formic acid, offering insights into their metabolic versatility and biochemical adaptations.

AUTHOR CONTRIBUTIONS

Sara Tejedor-Sanz: Investigation; writing – original draft; methodology; validation; writing – review and editing; data curation; conceptualization; software; formal analysis. **Young Eun Song:** Writing – review and editing; methodology; investigation. **Eric R. Sundstrom:** Conceptualization; writing – review and editing; funding acquisition; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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