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ARTICLE

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Towards closed carbon loop fermentations: Cofeeding of *Yarrowia lipolytica* with glucose and formic acid

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Abstract

A novel fermentation process was developed in which renewable electricity is indirectly used as an energy source in fermentation, synergistically decreasing both the consumption of sugar as a first generation carbon source and emission of the greenhouse gas CO_2. As an illustration, a glucose-based process is co-fed with formic acid, which can be generated by capturing CO_2 from fermentation offgas followed by electrochemical reduction with renewable electricity. This "closed carbon loop" concept is demonstrated by a case study in which cofeeding formic acid is shown to significantly increase the yield of biomass on glucose of the industrially relevant yeast species *Yarrowia lipolytica*. First, the optimal feed ratio of formic acid to glucose is established using chemostat cultivations. Subsequently, guided by a dynamic fermentation process model, a fed-batch protocol is developed and demonstrated on laboratory scale. Finally, the developed fed-batch process is tested and proven to be scalable at pilot scale. Extensions of the concept are discussed to apply the concept to anaerobic fermentations, and to recycle the O_2 that is co-generated with the formic acid to aerobic fermentation processes for intensification purposes.

KEYWORDS
cofeeding, formic acid, greenhouse gas emission reduction, scale up, *Yarrowia lipolytica*

1 | INTRODUCTION

The globally increasing level of atmospheric greenhouse gases and its proven effect of global warming is an urgent incentive for the chemical industry to develop greenhouse gas neutral or even negative processes. Biotechnology offers a CO_2-saving alternative to traditional chemical processes for the production of an ever-increasing range of carbon-containing molecules, by consuming renewable rather than fossil carbon sources. Still, almost all biotechnological processes emit CO_2 originating from the production of sugar as a so-called first generation carbon source (see e.g., Salim et al., 2019), from the generation of utilities (power, heat, steam), as well as from the oxidation of part of the carbon source to generate metabolic energy. Thereby part of the CO_2 fixed by the crops producing the carbon source returns to the atmosphere during the process, carbons which are lost for the product.

A major step in further decreasing CO_2 emissions by biotechnological processes would be to capture the emitted CO_2, electrochemically reduce it to a suitable organic molecule using renewable electricity, and (co-)feed this carbon source back into the fermentation stage of the process (Noorman, 2020). Here we present formic acid as an example:

$$CO_2 + H_2O \xrightarrow{\text{renewable energy}} CH_2O_2 + 0.5\,O_2. \quad (1)$$

Formic acid has been demonstrated as a suitable auxiliary energy source for several microbial species (Bruinenberg et al., 1985; Geertman et al., 2006; Harris et al., 2007; Overkamp et al., 2002; Wang et al., 2019), which can transfer the electrons from formic acid to NAD^+, forming NADH and CO_2 with a formate dehydrogenase enzyme (FDH):

$$CH_2O_2 + NAD^+ \xrightarrow{FDH} CO_2 + NADH + H^+. \quad (2)$$

The cells can then use the NADH generated to either provide reducing power in biosynthetic pathways or generate metabolic energy (ATP) via aerobic respiration. This closed carbon cycle, where the emitted CO_2 is continuously captured, reduced to formic acid and fed back into the fermentation, can theoretically provide all ATP via (Equation 2) plus respiration. Such processes uniquely use the primary carbon source (e.g., glucose) for assimilation and therefore have significantly increased biomass and product yields on the primary carbon source. In essence, such a process is partially decarbonized by replacing a fraction of the glucose substrate by renewable electricity (Figure 1).

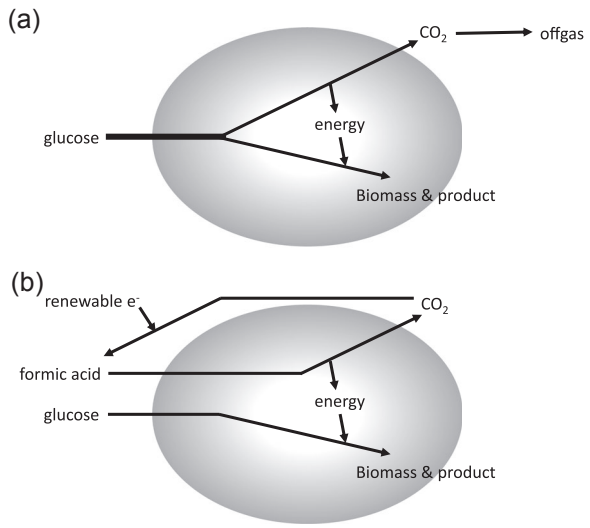

FIGURE 1 (a) Traditional aerobic fermentation process, where glucose is partially oxidized to CO_2 to provide metabolic energy and the remainder is used as carbon source for biosynthesis. (b) Alternative "closed carbon loop" process where cofed, CO_2-derived formic acid serves as energy source and glucose uniquely serves as carbon source.

To illustrate these process benefits, we postulate the following typical microbial stoichiometry for aerobic conversion of glucose into biomass (generalized formulation based on information in Verduyn, 1992):

$$C_6H_{12}O_6 + 2.85\,O_2 + 0.6\,NH_3 \rightarrow 3CH_{1.8}O_{0.5}N_{0.2} + 3CO_2 + 4.2H_2O, \quad (3)$$

which gives a yield of biomass on O_2 (Y_{xo}) of 1.05 $C\text{-mol}_x/\text{mol}_{o_2}$, a yield of biomass on sugar (Y_{xs}) of 3 $C\text{-mol}_x/\text{mol}_s$, and 1 C-mol of biomass formed per mol of CO_2 released (Y_{xc}).

Under the assumptions of no energetic costs in cross membrane metabolite transport, and a P/O ratio of 1.0 for respiration of NAD(P)H the catabolic subreaction:

$$0.475C_6H_{12}O_6 + 2.85O_2 \rightarrow 2.85CO_2 + 2.85H_2O(+7.6ATP), \quad (4)$$

can be completely replaced by dissimilation of formic acid:

$$7.6CH_2O_2 + 3.8O_2 \rightarrow 7.6CO_2 + 7.6H_2O(+7.6ATP), \quad (5)$$

resulting in the overall stoichiometry:

$$0.525C_6H_{12}O_6 + 7.6CH_2O_2 + 3.8O_2 + 0.6NH_3 \rightarrow 3CH_{1.8}O_{0.5}N_{0.2} + 7.75CO_2 + 8.95H_2O. \quad (6)$$

which gives a Y_{xo} of 0.79 $C\text{-mol}_x/\text{mol}_{o_2}$, a Y_{xs} of 5.71 $C\text{-mol}_x/\text{mol}_s$, and a Y_{xc} of 0.39 $C\text{-mol}_x/\text{mol}_{co_2}$. Clearly, the Y_{xs} is higher, but Y_{xo} and Y_{xc} are lower, which is undesired. However, combining the electrocatalytical reaction of (Equation 1) with (Equation 6) shows the synergy of the two processes:

$$0.525C_6H_{12}O_6 + 0.6NH_3 \xrightarrow{\text{renewable energy}} 3CH_{1.8}O_{0.5}N_{0.2} + 0.15CO_2 + 1.35H_2O. \quad (7)$$

This overall stoichiometry gives an infinitely high Y_{xo}, a Y_{xs} of 5.71 $C\text{-mol}_x/\text{mol}_s$, and a Y_{xc} of 20 $C\text{-mol}_x/\text{mol}_{co_2}$. All three yields are improved relative to (Equation 3). Note that the O_2 production (Equation 1) takes place in a separate unit operation from the fermentation process where O_2 is reconsumed so even though the overall process does not consume O_2, aeration of the fermentation is still required. The O_2 produced in (Equation 1) can be used to intensify the fermentation process by injecting pure O_2 or enriching the fermentation air (Groen et al., 2005).

For simplicity, the stoichiometry of (Equation 7) forms biomass as the sole product. Formation of any other ATP-requiring product can be described analogously: anabolic formation of the product from glucose as carbon source, fueled by catabolism of formic acid as energy source. Additionally, this study is limited to catabolic use of formic acid, which has been reported for many industrially microbial species, or could be conferred to species relatively easily by introducing FDH. Microbial species containing metabolic pathways to assimilate formic acid, can even use formic acid as sole carbon and energy source (see e.g., Hazeu & Donker, 1983). This trait is, however, limited to a much smaller fraction of the currently industrially used microbial species, and its

introduction into other cell factories requires more extensive metabolic engineering campaigns.

Applying this theoretical concept to *Yarrowia lipolytica* as a model strain, two factors that impact the overall yield are the mechanisms for formic acid transport (passive vs. active) and the overall stoichiometry of NADH dissimilation by the respiratory chain (P/O ratio). Both passive diffusion of formic acid, as well as anion/proton-symport have been described in earlier research in the yeast *S. cerevisiae* (Geertman et al., 2006; Overkamp et al., 2002), and neither of these mechanisms results in a net expenditure of ATP in transport of formic acid. Moreover, metabolic modelling studies in cultures with *Penicillium chrysogenum*, grown on mixtures of formic acid and glucose, also indicated no ATP expenditure in formic acid transport (Harris et al., 2007). In light of these observations and since no data is reported on formic acid uptake in *Y. lipolytica*, no ATP expenditure for formic acid uptake was expected in this organism.

In *Y. lipolytica* the mitochondria contain a branched respiratory chain, constituted by the classic internal, proton pumping complex I and an alternative, external NADH dehydrogenase (Kerscher et al., 1999), combined with the other classical mitochondrial complexes (III and IV) involved in electron transport from NADH. Complex I and the alternative NADH dehydrogenase provide two entry points for NADH-derived electrons into the respiratory chain. Since proton pumping by complex I contributes to the proton gradient across the mitochondrial membrane whereas the alternative NADH dehydrogenase does not, the overall stoichiometry (P/O ratio and the equivalent ATP/NADH yield) differs depending on the entry point used. The physiological contribution of Complex I and alternative NADH dehydrogenase(s) remains enigmatic (Jürgens et al., 2020, 2021), which impedes accurate theoretical prediction of the ATP yield of aerobic substrate dissimilation. Therefore, the optimal molar ratio between glucose and formic acid in the feed, which is the ratio where formic acid is exactly sufficient to replace glucose dissimilation, must be determined experimentally.

In addition to the physiology of *Y. lipolytica*, practical and economic success of the proposed approach requires (1) a technologically and economically feasible process to capture CO_2 and reduce it to formic acid, and (2) an industrially relevant fermentation process design in which the formic acid does not accumulate to a level that affects cell metabolism. The former requirement, capture, and conversion of CO_2 and electricity to formic acid, has been addressed elsewhere (see e.g., Claassens et al., 2019; Malkhandi & Yeo, 2019; Pérez-Gallent et al., 2021) and is out of scope of this study. This study covers the latter requirement for the industrially important yeast species *Y. lipolytica* for which formic acid consumption has been previously demonstrated (Nsoe et al., 2018).

2 | MATERIALS AND METHODS

2.1 | Yeast strains and stocks

Y. lipolytica W29 (https://www.atcc.org/products/20460) used in this study is a natural strain, originally isolated from wastewater in Paris, France. The *Saccharomyces cerevisiae* strains CEN.PK113-7D and CEN.PK556-7B used in this study share the CEN.PK genetic background (Entian & Kötter, 2007; Salazar et al., 2017). The FDH knockout strain CEN.PK556-7B was constructed previously and was shown unable to co-consume formic acid (Overkamp et al., 2002). Frozen culture stocks were prepared by adding sterile glycerol to an overnight culture to a final glycerol concentration of 30% v/v and storage of 1 ml aliquots at −80°C.

2.2 | Shake flask cultivation

For culture maintenance, strains were grown in 500 ml round-bottom shake flasks containing 100 ml YP medium (10 g/L Bacto yeast extract, 20 g/L Bacto peptone) supplemented with 20 g/L glucose. Precultures were grown overnight in filter-sterilized synthetic medium (SM) at pH 6.0, prepared as described previously (Verduyn et al., 1992) and transferred to fresh medium for characterization in shake flask.

For strain characterization in shake flasks, the $(NH_4)_2SO_4$ in SM was substituted by 2.3 g/L urea and 6.6 g/L K_2SO_4, to provide an equimolar amount of nitrogen and prevent medium acidification due to ammonia assimilation (Luttik et al., 2000). When required, formic acid (≥95%; Sigma-Aldrich) was added to the medium to a final concentration of 1.2 g/L from a concentrated stock solution (99% w/w) before sterilization. Heat-sterilized glucose (110°C, 20 min) was aseptically added as carbon source after sterilization. For characterization, cultures were inoculated into 100 ml SM with 7.5 g/L (42 mM) glucose with and without 1.2 g/L (25 mM) formic acid in 500 ml round-bottom shake flasks. Shake flask cultures were incubated at 30°C in an Innova incubator shaker (New Brunswick Scientific) set at 200 rpm and a throw of 2.5 cm.

2.3 | Chemostat cultivation

Aerobic, glucose-limited chemostat cultivations were performed in 2 L laboratory bioreactors (Applikon) with a working volume of 1 L. Cultures were stirred at 800 rpm and sparged with 500 ml air/min and the dissolved O_2 concentration was monitored via an O_2 electrode, remaining above 30% of saturation at atmospheric conditions throughout the cultivation. The pH of the culture was maintained at 5.0 via automated addition of 2 M KOH and the temperature was kept constant at 30°C. SM medium (Verduyn et al., 1992) used as medium and feed in the bioreactors contained 5.0 g/L glucose and was supplemented with 0.2 g/L Pluronic PE 6100 antifoam (BASF) for the batch phase and 0.4 g/L antifoam for the chemostat phase.

For the initial batch phase, the cultures were inoculated with an overnight preculture to an initial optical density of approximately 0.04. After glucose depletion, indicated by a rapid drop in the CO_2% in the exhaust gas, the medium pump was switched on to obtain a constant flow rate of 100 ml/h resulting in a dilution rate of 0.1 h^{-1}.

The formic acid concentration in the feed medium was set by aseptically adding formic acid to the 20 L feed medium vessel before the chemostat phase. The working volume was kept constant at 1 L using an effluent pump controlled by an electric level sensor. Chemostat cultures were assumed to be in steady-state if after at least five volume changes, the concentration of biomass in the reactor, as well as the CO_2 concentration in the exhaust gas remained constant (<3% variation) for at least two additional volume changes.

2.4 | Fed-batch cultivation

Aerobic, glucose/formic acid-limited fed-batch cultivations were performed in 10 L laboratory bioreactors (L. Eschweiler and Co.) and in 300 L pilot bioreactors (Bio-Engineering AG). The laboratory bioreactors were inoculated from shake flasks. The pilot bioreactors were inoculated from 70 L inoculum reactors (Applikon), that were in turn inoculated from shake flasks.

For the shake flasks, 0.5 ml cell stock culture was added to 400 ml of preculture medium (Supporting Information: Table S1). Precultures were incubated for 26 h in flat bottom flasks with baffles, at 30°C with a rotational speed of 150 rpm and a throw of 2.5 cm. The pH of the media was not adjusted before inoculation.

The four laboratory bioreactors (from here onwards denoted as LF1 through LF4) contained 3.6 kg of batch medium (Supporting Information: Table S4) and 400 g of preculture. The start weight was 4 kg, while the estimated end weight was 8.7 kg. No formic acid was dosed in the batch medium, as high initial concentrations of formic acid were expected to be detrimental to the cells. The media were adjusted to pH 5.0 with NH_3 before inoculation.

The process started with a batch phase until carbon depletion. The other operating conditions are given in Supporting Information: Table S5. The solutions for pH correction and foam remediation were 25 wt% NH_3, 98 wt% H_2SO_4, and Basildon 86-013. The intended glucose and formic acid (≥98%; Carl Roth) content of the feed solutions of the four laboratory scale fermentations are given in Table 1. The pH of the feed solutions was not adjusted with alkaline titrant.

The inoculum bioreactor (from here onwards denoted as IF) of the two pilot fermentations (from here onwards denoted as PF1 and PF2) was run as a batch process and contained 18 kg of batch medium of which the composition is given in Supporting Information: Table S6. The media were adjusted to pH 5.0 with NH_3 before inoculation. The inoculum bioreactor was inoculated with 5 shake flask cultures of 400 g each and then operated according to Supporting Information: Table S7. 8 Kg of broth from the IF was used to inoculate each of the two PF. The PF contained 82 kg of batch medium that was identical to the medium of the laboratory bioreactors. The process started with a batch phase until glucose depletion. The other operating conditions are given in Supporting Information: Table S8.

The solutions for pH correction and foam remediation were 25 wt% NH_3, 98 wt% H_2SO_4, and Basildon 86-013. The carbon feed solutions of the two pilot scale fermentations are given in Table 1.

TABLE 1 Designed composition of the carbon feeds of the laboratory (LF) and pilot (PF) fermenters (see Table 2 for actual composition).

Compound	Concentration (g/kg)			
	LF1 and PF1	LF2	LF3 and PF2	LF4
Glucose monohydrate	275	242	206	179
Formic acid	0	169	240	291
Water	725	589	554	531

2.5 | Analytical methods

2.5.1 | Biomass determination

For the chemostat cultures, biomass growth was monitored by optical density (OD) measurement at a wavelength of 660 nm with a Libra S11 spectrophotometer (Biochrom). For the fed-batch cultures, biomass growth was monitored by OD measurement at a wavelength of 600 nm with a Thermo Genesys spectrophotometer (Thermo Fisher Scientific).

For the chemostat cultures, dry weight was determined by filtering 10 ml culture broth over a preweighed nitrocellulose filter with a pore size 0.45 µm, washing the filter with demineralized water and drying the filter for 20 min at 360 W in a microwave oven before weighing again (Postma et al., 1989). Duplicate measurements varied less than 3.5% throughout the cultivation. For the fed-batch cultures, dry weight was determined by centrifuging 2 × 5 ml of culture broth at 6,000*g* for 15 min. The pellet was washed once by resuspending in deionized water and centrifuged again at 6,000*g* for 15 min. After washing, the pellet was dried for 24 h at 105°C and weighed.

2.6 | Gas analysis

For the chemostat cultures, the offgas was cooled (2°C) in a condenser and dried, before the analysis of O_2 and CO_2 concentrations using an NGA 2000 analyzer. For the fed-batch cultures, the offgas was analysed with a Thermo Fischer Prima BT Mass Spectrometer (Thermo Fisher Scientific).

2.7 | Substrate and metabolite analysis

During the runs, rapidly temperature-quenched samples were taken from the fermenters to instantly stop metabolism and obtain representative measurements of extracellular metabolites such as formic acid. Immediately after sampling the samples were cooled in syringes filled with precooled steel beads for fast cooling of the sample (Mashego et al., 2003). The cells were immediately removed by filtration and samples were stored frozen.

Extracellular concentrations of glucose and formic acid in culture filtrates were analysed by high-performance liquid chromatography (HPLC) on an Agilent 1260 HPLC, equipped with a Bio-Rad HPX 87H column, operated at 60°C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.600 ml/min. Detection was performed by means of an Agilent refractive index detector and an Agilent 1260 VWD detector at 210 nm. The NH_3 concentration was measured in supernatant samples using an Orion 4 Star with the Orion 95-12 Ammonia Electrode (Thermo Fisher Scientific).

3 | RESULTS

3.1 | Natural co-consumption of glucose and formic acid in *Y. lipolytica* W29 in batch shake-flask cultures

To investigate the innate ability of *Y. lipolytica* to coconsume glucose and formic acid, strain W29 was inoculated in synthetic medium with glucose with and without formic acid. *S. cerevisiae* strains CEN.PK113-7D (*FDH1*, *FDH2*) and the formate dehydrogenase (FDH) double knockout strain CEN.PK556-7B (*fdh1*Δ*fdh2*Δ) were also tested in medium with glucose and formic acid as positive and negative control, respectively (Figure 2).

Within 24 h, glucose was depleted in all cultures and in the culture of W29 the formic acid concentration decreased by 9.7 ± 0.2 mM compared to 4.5 ± 0.1 mM for CEN.PK113-7D. In contrast, the decrease in formic acid in the culture of CEN.PK556-7B (1.2 ± 0.0 mM) was comparable to what was observed in a sterile culture (1.1 ± 0.0 mM). Strikingly, the optical density after 24 h was higher for the W29 culture with added formic acid (22.3 ± 0.6) than the culture with glucose only (20.3 ± 0.8), consistent with dissimilation of formic acid by this organism providing additional NADH that is used in the electron transport chain to provide metabolic energy (ATP) for growth.

3.2 | Determination of the optimal formic acid:glucose ratio in glucose-limited chemostat cultures

Industrial fermentations are typically performed in carbon-limited fed-batch processes, the design parameters of which include the feed profile and composition. We performed a series of carbon limited chemostat cultures with various feed compositions to determine the

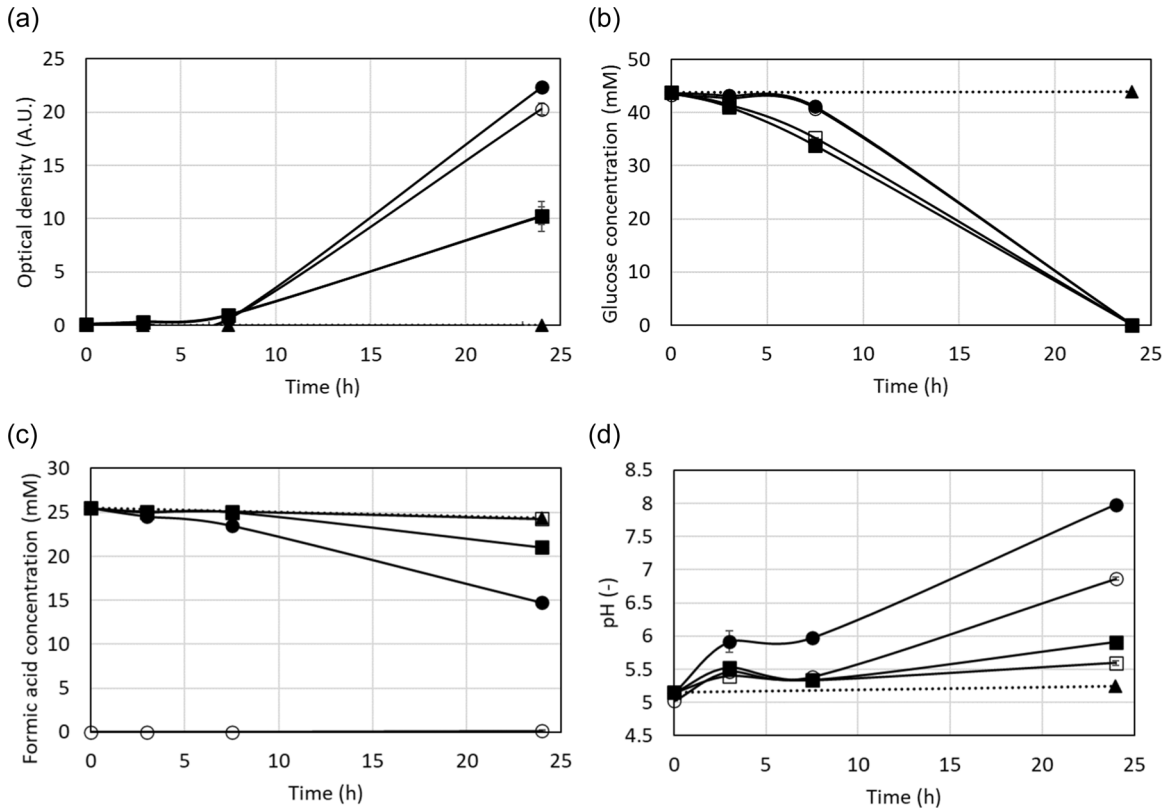

FIGURE 2 (a) Optical density at 660 nm, (b) glucose and (c) formic acid concentrations, and (d) pH of shake flask cultures of *Yarrowia lipolytica* and *Saccharomyces cerevisiae*. *Y. lipolytica* strain W29 was incubated with 42 mM glucose with (●) and without (❍) 25 mM formic acid, and both *S. cerevisiae*strains CEN.PK113-7D (■) and CEN.PK556-7B (□) were incubated with glucose and formic acid. A sterile flask with glucose and formic acid was also included (dashed line, ▲). Some data points overlap: (a) ■ & □, (B) ● & ❍ and ■ & □, (c): ■ & □ & ▲ (first three datapoints) and □ and ▲ (last datapoint). Errors bars show the variation between two replicate experiments.

optimal feed ratio of formic acid to glucose (F:G) for the *Y. lipolytica* strain used (Figure 3). A dilution rate of 0.10 h^{-1} was chosen, well below the maximum specific growth rate of W29 determined from offgas CO_2 of the batch phase preceding the chemostat phase (0.37 ± 0.01 h^{-1}, see Supporting Information Materials 2).

For F:G ratios between 0 and 5 mol/mol, a linear increase in the biomass yield on glucose was observed from 0.50 ± 0.02 to 0.60 ± 0.01 g biomass/g glucose, indicating that in this range formic acid dissimilation could effectively displace glucose dissimilation. In accordance with the 20% increased biomass yield, the biomass-specific uptake rate of glucose decreased from 0.20 ± 0.01 to 0.16 ± 0.00 g glucose/g biomass/h (Supporting Information: Table S9). A further increase in the F:G ratio gave no increase in the biomass yield, even though up to the highest tested F:G ratio of 11.5 mol/mol >98% of the ingoing formic acid was consumed. Apparently, beyond a F:G ratio of 5, further consumption of formic acid is decoupled from additional ATP formation.

3.3 | Laboratory scale fed-batch cultivations

The chemostat cultivations indicated an optimal F:G ratio in the feed of 5 mol/mol. This formed the basis of the experimental design of a fed-batch protocol in which the positive effect of formic acid cofeeding on the yield was to be confirmed in this industrially more relevant fermentation mode. Four variations of the fed-batch protocol were tested on laboratory scale, with a fixed feeding rate and F:G ratios that ranged from 0 (experiment LF1), 3 (LF2), 5 (LF3) to 7 (LF4) by replacing part of the water in the carbon feed solution by formic acid. A dynamic fermentation process model (Supporting Information Materials 1) was developed and applied to quantitatively predict broth weight development, OUR, as well as other fermentation variables, which allowed to design the experiments such that they would fit the experimental set-up.

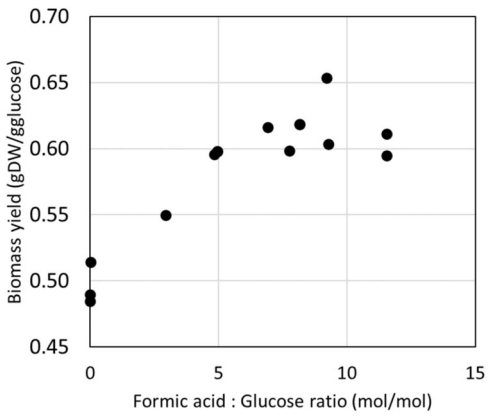

FIGURE 3 Overview of determined biomass yields for formic acid to glucose feed ratios tested in independent chemostat experiments.

The laboratory scale fed batch process proved successful. After the initial batch phase on glucose only, *Y. lipolytica* readily consumed the mixed glucose/formic acid feed when it was dosed at a carbon-limiting rate. Throughout the fermentations, supernatant samples were taken and analysed for residual formic acid, and the concentrations were always low (<0.06 g/L) or below the detection limit.

Figure 4 shows O_2 uptake rate (OUR) profiles, the glucose and formic acid consumption, and the biomass dry weight formation of the 4 laboratory scale fermentations. The differences in glucose consumption between the fermentations in Figure 4b are explained by the different dilutions of the feeds (see Table 2) that were applied to prevent O_2 transfer limitations during the fermentations. The OUR profiles clearly show the batch phases with exponential growth ending between 10 and 15 h, followed by the carbon-limited fed batch phases. The decrease of the OUR after ~55 h for LF1 and after ~60 h for LF2 show that the OUR still exceeded the O_2 transfer towards end of fermentation. This limitation resulted from the increasing biomass concentration which in turn led to broth viscosity, a factor that was not accounted for in the dynamic process model that was used to design the experiments. Until the onset of the late O_2 transfer limitation, the experimental OUR data quantitatively correspond with the simulated fermentations using the dynamic process model (see Supporting Information Materials 1).

The base titrant used in the protocol is ammonia, which simultaneously serves as N-source for biomass formation. Despite the low pH of the carbon feed solutions that contain high formic acid concentrations, the titrant dosing to the fermentations is limited (between 100 and 125 g 25 wt% NH_3) and varied little between the four fermentations. The ammonia level in the broth was between 0.4 and 0.9 g/L for all four fermentations at all timepoints.

The key results of fermentations LF1-4 are summarized in Table 2. The results agree with the chemostat findings which showed that cofeeding formic acid with glucose increases the biomass yield on glucose up to a molar ratio of about 5:1, and that further increasing the ratio gives no benefit in terms of yield. Again, these results correspond well with the predicted output of the fermentations shown in Supporting Information: Figures S1–1D (Supporting Information Materials 1) with one exception: the highest yield for the formic acid: glucose ratio of about 7:1 that is predicted by the model is not observed in practice. The cause of this discrepancy is that the model assumes a fixed, positive ATP yield for each molecule of formic acid consumed, whereas the chemostat results indicated a decoupling of ATP formation from formic acid dissimilation past a F:G ratio of 5:1.

3.4 | Pilot scale fed-batch cultivations

After the optimal F:G ratio that had been determined in the chemostat experiments had been confirmed in the laboratory scale fed-batch experiments, the final step of the investigation was to test whether the obtained positive results of formic acid cofeeding on

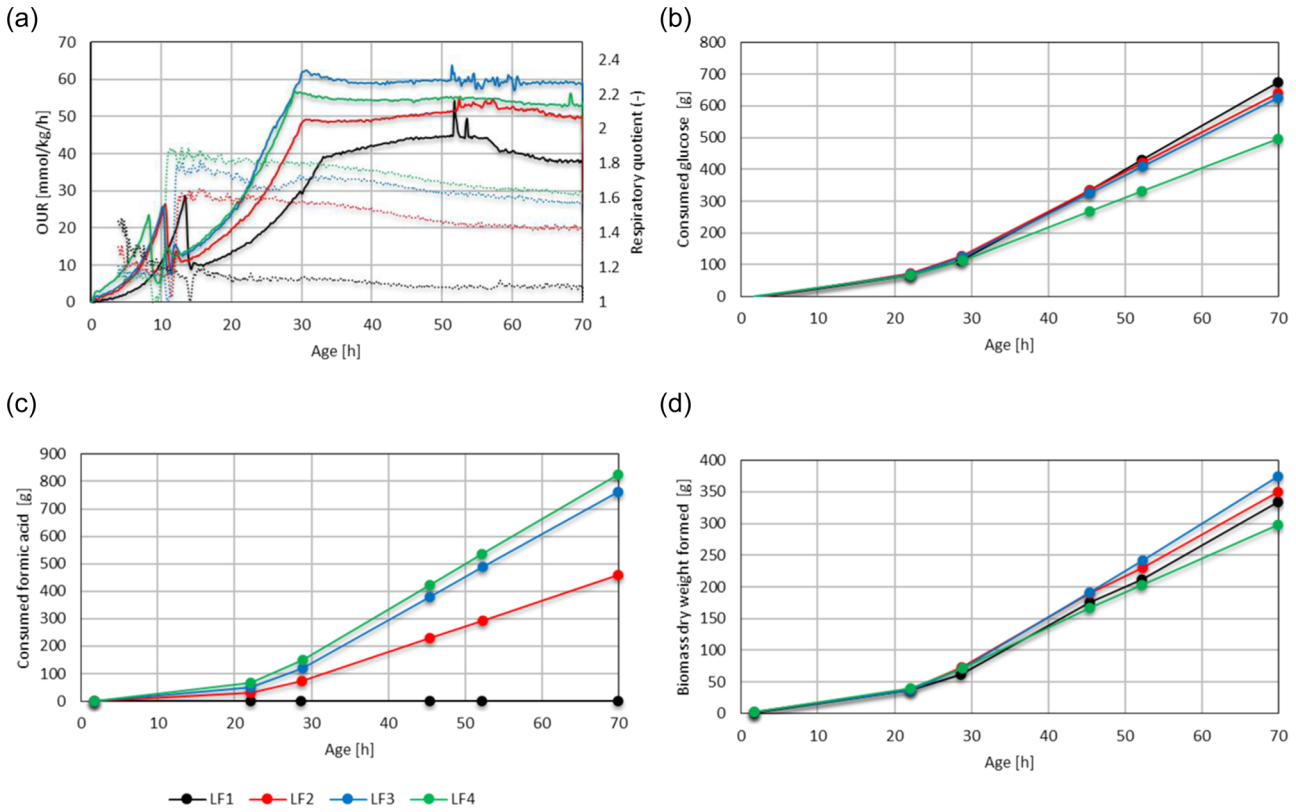

FIGURE 4 (a) The O_2 uptake rate (OUR, solid line) and respiratory quotient (RQ, dotted line), (b) glucose consumed, (c) formic acid consumed, and (d) biomass dry weight concentration formed in the four laboratory scale fed-batch fermentations with increasing molar ratios of F:G and decreasing glucose concentration (see Table 2). The four fermentations had F:G ratios increasing from 0 (LF1, black), 3 (LF2, red), 5 (LF3, blue) to 7 (LF4, green).

TABLE 2 Average biomass yield on glucose (Y_{xs}) obtained in the laboratory scale and pilot scale fermentations.

Experiment	Actual carbon feed composition	Average yield (g/g) (% improvement)
LF1	Glucose only (glucose: 258 g/kg)	0.49
LF2	2.9:1 molar F:G ratio (glucose: 232 g/kg)	0.54 (+10.2% vs. LF1)
LF3	5.0:1 molar ratio F:G ratio (glucose: 228 g/kg)	0.60 (+22.4% vs. LF1)
LF4	6.9:1 molar F:G ratio (glucose: 174 g/kg)	0.60 (+22.4% vs. LF1)
PF1	Glucose only (glucose: 250 g/kg)	0.44
PF2	4.6:1 molar F:G ratio (glucose: 187 g/kg)	0.53 (+20.5% vs. PF1)

Note: Y_{xs} was calculated by dividing the cumulative amount of biomass formed by the cumulative amount of glucose consumed, including glucose batched in the seed and main fermentation medium.
Abbreviations: LF, laboratory fermenters; PF, pilot fermenters.

biomass yield were robust to scaling up to pilot scale. To this end, two out of the four variations of the laboratory scale fed-batch protocol were scaled up to pilot scale, having F:G ratios of 0 (experiment PF1, scale up of LF1), and 5 (PF2, scale up of LF3). Again, the dynamic fermentation process model proved valuable in qualitatively predicting the time profiles of key fermentation parameters during the seed and main fermentation stage before executing the experiments (see Supporting Information Materials 1).

The 300 L pilot-scale fed-batch process demonstrated that also at this scale *Y. lipolytica* readily consumes the mixed glucose/formic acid feed up to a molar ratio of 1:5. Throughout the fermentations, supernatant samples were taken and analysed for residual formic acid, and the concentrations were always low (<0.05 g/L) or below the detection limit. Figure 5a shows the OUR profiles of the 2 pilot scale fermentations with the batch phases ending between 10 and 12 h, followed by the carbon-limited fed-batch phases. The oscillation

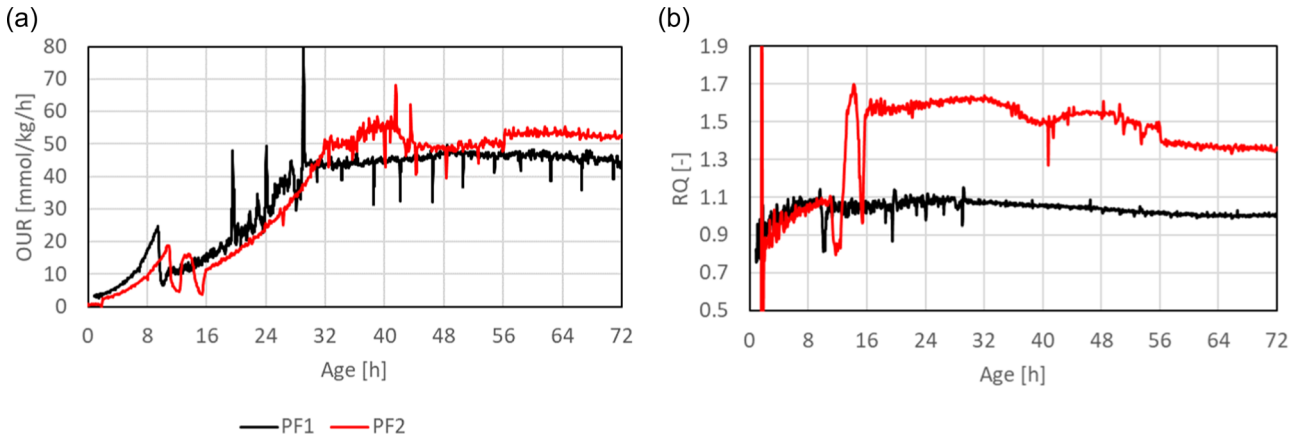

FIGURE 5 (a) The O_2 uptake rate (OUR) and (b) respiratory quotient (RQ) of the two pilot-scale fed-batch fermentations with glucose only (PF1) and a 1:4.6 molar ratio of glucose: formic acid in the feed (PF2) (see Table 2).

of the OUR of PF2 between 12 and 16 h was caused by a technical deviation when starting up the feed. The actual OUR profiles are in good agreement with the profiles of Figures S1–2B (Supporting Information Materials 1) that were simulated to design the experiment. Figure 5b presents the respiratory quotient of the two fermentations, which clearly shows the fast onset of formic acid dissimilation and its large impact on RQ after the start of cofeeding formic acid (starting around 12 h) for PF2.

The key results of fermentations PF1+2 are summarized in Table 2 and demonstrate that the observations in the chemostat and laboratory scale fed-batch, translate well to 300 L pilot-scale fed-batch processes. The absolute biomass yields found on pilot scale are somewhat lower than found on laboratory scale. Still, the relative improvement of the Y_{xs} of PF2 over PF1 corresponds well with the relative improvement of the Y_{xs} of LF3 over LF1, where the F:G rations were similar. On both scales the Y_{xs} is increased by 21 ± 1%.

In bioprocess development, it is good practice to check the mass, carbon, and nitrogen balances of the process to ascertain that no inflows or outflows were missed and that flow measurements as well as offline analytics had a reasonable accuracy. Supporting Information Materials 3 presents the results of this check for the fed-batch experiments performed in this study. For the laboratory scale fermentations the balances closed better (mass < 3%, C < 2%, N < 8%) than for the pilot scale fermentations (mass < 8%, C < 10%, N < 19%).

4 | DISCUSSION

4.1 | Energetics of *Y. lipolytica* coconsuming formic acid

In this study, we found that cofeeding formic acid and glucose, up to a molar ratio of ~5:1, linearly increased the biomass yield of *Y. lipolytica* on glucose (Figure 3). This indicated that under these conditions, consumption of formic acid by this organism has a net positive ATP yield, similar to previous observations for other yeasts (Babel et al., 1983; Bruinenberg et al., 1985; Geertman et al., 2006). At F:G feed ratios ≤5, we observed that 24 ± 2 moles of formic acid were able to displace 1 mole of glucose for dissimilatory requirements in *Y. lipolytica* (Supporting Information: Table S9). Since glucose dissimilation provides 4 ATP and 12 NADH equivalents (assuming no energetic costs of glucose transport) compared to 1 NADH from formic acid dissimilation, these results indicate that either: (1) the effective P/O ratio of respiration in *Y. lipolytica* is low (<1.0), or (2) transport of formic acid comes at a net energetic (ATP) cost, or (3) *Y. lipolytica* has a different P/O ratio for formic acid-derived electrons compared to glucose-derived electrons. We believe the third scenario is most likely, since electrons derived from glucose dissimilation via glycolysis and the TCA cycle are released in both the cytosol and mitochondria, whereas the electrons released by formic acid dissimilation via FDH are expected to be released exclusively in the cytosol. *Y. lipolytica* FDH is described as cytosolic in UniProt, accession number Q6C5X6. Therefore, glucose-derived electrons can be partially transferred to O_2 via proton-pumping complex I in the mitochondria, whereas formic acid-derived electrons are likely transferred to O_2 via the less efficient external alternative NADH dehydrogenase.

Although no benefit on the biomass yield was observed at higher F:G ratios up to 11.5:1, virtually all formic acid was consumed as indicated by the low residual formic acid concentrations in the fermenter (Supporting Information: Table S9). This is in contrast with observations in other yeasts, as in previous work with aerobic chemostat cultures, formic acid accumulated at F:G ratios higher than 5 in *Candida utilis* cultivations and higher than 2 in *S. cerevisiae* (Bruinenberg et al., 1985; Overkamp et al., 2002). The ability to consume all formic acid at high ratios demonstrates the potential of *Y. lipolytica* in formic acid co-fed processes.

In this study we used biomass itself as an ATP-intensive product to investigate the potential of formic acid cofeeding for increasing the product yield. Previous work on antibiotic-producing *P. chrysogenum* strains (Harris et al., 2007) demonstrated that in chemostat

setups, formic acid cofeeding can also increase the yield of product formation. Since *Y. lipolytica* is used on an industrial scale for synthesis of other ATP-intensive products, such as citrate, lipids, lipase (Madzak, 2018), a logical next step would be to translate our fed-batch process to an industrial *Y. lipolytica* strain engineered for synthesis of one of these molecules.

Supporting Information Materials 4 and 5 present extensions of the formic acid cofeeding concept of this study, illustrating how the coproduced O_2 can be valorised and how cofeeding of formic acid can even lead to net-negative CO_2 emission processes.

5 | CONCLUSIONS

This study has shown proof of principle of cofeeding F:G-fed cultivations of *Y. lipolytica*. This yeast species was shown to consume formic acid up to high molar ratios (>10) relative to the glucose that was fed, and be able to extract metabolic energy from it up to a formic acid:glucose ratio of about 5. While this shows that this yeast species is a good natural consumer of formic acid, there is room to benefit more of its potential.

The study also demonstrated how a fed-batch process was developed and successfully executed up to pilot scale (300 L) in which cofeeding of formic acid at a formic acid:glucose molar ratio of 5 was shown to increase the yield of biomass on glucose by >20%. A dynamic fermentation process model was developed based on the initial data and subsequently applied to guide the development of the fermentation protocols by quantitatively simulating the results of experimental designs before they were executed.

We hope that this study will contribute to further developing the fermentation industry into a sector that can keep providing mankind with the required molecules for production of food, feed, materials, and fuels in a truly sustainable manner.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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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