

# Towards closed carbon loop fermentations: co-feeding of *Yarrowia lipolytica* with glucose and formic acid

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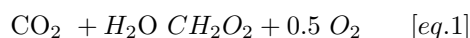
## Abstract

A novel fermentation process was developed in which renewable electricity is indirectly used as a fermentation substrate, synergistically decreasing both the consumption of sugar as a first generation carbon source and emission of the greenhouse gas CO<sub>2</sub>. To achieve this, a glucose-based process is co-fed with formic acid, which can be generated by capturing CO<sub>2</sub> from fermentation offgas followed by electrochemical reduction with renewable electricity. This ‘closed carbon loop’ concept is demonstrated by a case study in which co-feeding 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 proven to be scalable to pilot scale. An extension of this proven concept to also recycle the O<sub>2</sub> that is co-generated with the formic acid to the fermentation process for intensification purposes, and a potential further application of the concept to anaerobic fermentations are discussed.

## Introduction

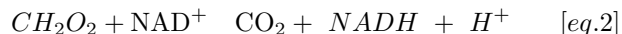
The globally increasing level of atmospheric greenhouse gases and its proven effect of global warming is an urgent incentive for the chemical industry to develop greenhouse gas neutral or even negative processes. Biotechnology offers a CO<sub>2</sub> saving alternative to traditional chemical processes 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<sub>2</sub> 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. As a result, part of the CO<sub>2</sub> that is fixed by the crops producing the carbon source returns to the atmosphere during the process, and these carbons are lost for the product.

A major step in further decreasing CO<sub>2</sub> emissions by biotechnological processes would be to capture the emitted CO<sub>2</sub>, 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. Here we present formic acid as an example:



Formic acid has been demonstrated as a suitable auxiliary energy source for several microbial species (Bruinenberg *et al.*, 1985; Overkamp *et al.*, 2002; Geertman *et al.*, 2006; Harris *et al.*, 2007; Wang *et al.*,

2019), which can transfer the electrons from formic acid to  $\text{NAD}^+$ , forming  $\text{NADH}$  and  $\text{CO}_2$  with a formate dehydrogenase enzyme (FDH):



The cells can then use the  $\text{NADH}$  generated to either provide reducing power in biosynthetic pathways or generate metabolic energy (ATP) via aerobic respiration. This closed carbon cycle, where the emitted  $\text{CO}_2$  is continuously captured, reduced to formic acid and fed back into the fermentation, can theoretically provide all ATP via [eq.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].

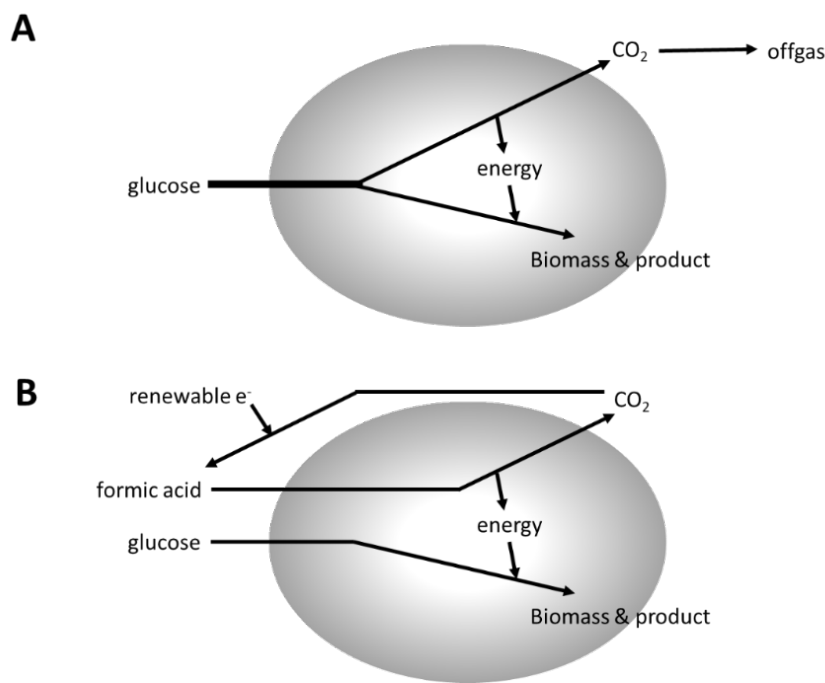
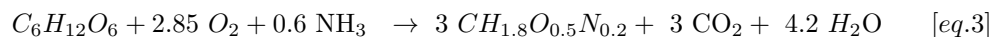


Figure 1: [A] traditional aerobic fermentation process, where glucose is partially oxidized to  $\text{CO}_2$  to provide metabolic energy and the remainder is used as carbon source for biosynthesis. [B] alternative 'closed carbon loop' process where co-fed,  $\text{CO}_2$ -derived formic acid serves 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:

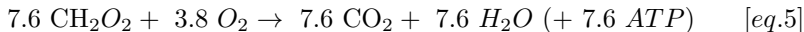


which gives a yield of biomass on  $\text{O}_2$  ( $Y_{\text{xo}}$ ) of 1.05 C-mol<sub>x</sub>/mol<sub>o<sub>2</sub></sub>, a yield of biomass on sugar ( $Y_{\text{xs}}$ ) of 3 C-mol<sub>x</sub>/mol<sub>s</sub>, and 1 C-mol of biomass formed per mol of  $\text{CO}_2$  released ( $Y_{\text{xc}}$ ). Under the assumptions of no

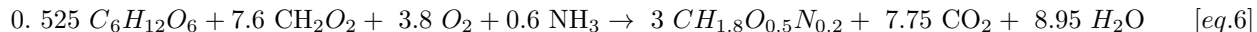
energetic costs in cross membrane metabolite transport, and a P/O ratio of 1.0 for respiration of NAD(P)H the catabolic subreaction:



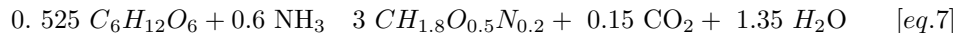
can be completely replaced by dissimilation of formic acid:



resulting in the overall stoichiometry:



which gives a  $Y_{xo}$  of 0.79 C-mol<sub>x</sub>/mol<sub>o2</sub>, a  $Y_{xs}$  of 5.71 C-mol<sub>x</sub>/mol<sub>s</sub>, and a  $Y_{xc}$  of 0.39 C-mol<sub>x</sub>/mol<sub>co2</sub>. Clearly, the  $Y_{xs}$  is higher, but  $Y_{xo}$  and  $Y_{xc}$  are lower, which is undesired. However, combining the electrocatalytical reaction of [eq.1] with [eq.6] shows the synergy of the two processes:



This overall stoichiometry gives an infinitely high  $Y_{xo}$ , a  $Y_{xs}$  of 5.71 C-mol<sub>x</sub>/mol<sub>s</sub>, and a  $Y_{xc}$  of 20 C-mol<sub>x</sub>/mol<sub>co2</sub>. All three yields are improved relative to [eq.3]. Note that the  $O_2$  produced in [eq.1] will be produced in a separate unit operation from the fermentation process where  $O_2$  is consumed so even though the overall process does not consume  $O_2$ , aeration of the fermentation is still required. The  $O_2$  produced in the reduction of  $CO_2$  can be used to intensify the fermentation process by injecting pure  $O_2$  or enriching the fermentation air (Groen *et al.*, 2005).

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* (Overkamp *et al.*, 2002; Geertman *et al.*, 2006), 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 (Kersch 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 (Juergens *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 success of the proposed approach requires (1) a technologically and economically feasible process to capture CO<sub>2</sub> 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<sub>2</sub> to formic acid, has been addressed elsewhere (see e.g. Claassens *et al.*, 2019; Malkhandi *et al.*, 2019; Pérez-Gallent *et al.*, 2021) and is out of scope of this work. This study covers the latter requirement for the industrially important yeast species *Yarrowia lipolytica* for which formic acid consumption has been previously demonstrated (Nsoe *et al.*, 2018; Vartiainen *et al.*, 2019).

## Materials and methods

### *Yeast strains and stocks*

*Yarrowia lipolytica* W29 used in this study is a natural strain, originally isolated from wastewater in Paris, France. The *Saccharomyces cerevisiae* strains used in this study share the CEN.PK genetic background (Entian and Kötter, 2007; Salazar *et al.*, 2017) and the FDH knockout strain CEN.PK556-7B was constructed previously (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.

### *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.*, 1992a,b) and transferred to fresh medium for characterization in shakeflask.

For strain characterization in shake flasks, the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in SM was substituted by 2.3 g/l urea and 6.6 g/l K<sub>2</sub>SO<sub>4</sub>, to provide an equimolar amount of nitrogen and prevent medium acidification due to ammonia assimilation (Luttik *et al.*, 2000). When required, formic acid was added to the medium to a final concentration of 1.2 g/l from a concentrated stock solution (99% w/w) prior to sterilization. Heat-sterilized glucose (110°C, 20 minutes) 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, Edison, NJ, USA) set at 200 rpm and a throw of 2.5 cm.

### *Chemostat cultivation*

Aerobic, glucose-limited chemostat cultivations were performed in 2 l laboratory bioreactors (Applikon, Delft, The Netherlands) with a working volume of 1 l. Cultures were stirred at 800 rpm and sparged with 500 ml air/min and the dissolved O<sub>2</sub> concentration was monitored via an O<sub>2</sub> 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 used in bioreactors contained 5.0 g/L glucose and was supplemented with 0.2 g/L Pluronic PE 6100 antifoam (BASF, Ludwigshafen, Germany) 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<sub>2</sub>% 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<sup>-1</sup>. The formic acid concentration in the feed medium was set by aseptically adding formic acid to the 20 l feed medium vessel prior to 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 5 volume changes, the concentration of biomass in the reactor, as well as the CO<sub>2</sub> concentration in the exhaust gas remained constant (<3% variation) for at least two additional volume changes.

## *Fed-batch cultivation*

Aerobic, glucose / formic acid-limited fed-batch cultivations were performed in 10 l laboratory bioreactors (L. Eschweiler and Co., Kiel, Germany) and in 300 l pilot bioreactors (Bio Engineering AG, Wald, Switzerland). The laboratory bioreactors were inoculated from shake flasks. The pilot bioreactors were inoculated from 70 l inoculum reactors (Applikon, Delft, The Netherlands), 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 (Supplementary Table S1). Precultures were incubated for 26h 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 (Supplementary 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<sub>3</sub> before inoculation.

The process started with a batch phase until carbon depletion. The other operating conditions are given in Supplementary Table S5. The solutions for pH correction and foam remediation were 25 wt% NH<sub>3</sub>, 98 wt% H<sub>2</sub>SO<sub>4</sub> and Basildon 86-013. The carbon 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.

Table 1: Composition of the carbon feeds of the laboratory (LF) and pilot (PF) fermenters

Compound	Concentration (g/kg)	Concentration (g/kg)	Concentration (g/kg)	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

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 Supplementary Table S6. The media were adjusted to pH 5.0 with NH<sub>3</sub> before inoculation. The inoculum bioreactor was inoculated with 5 shake flask cultures of 400 g each and then operated according to Supplementary 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 Supplementary Table S8.

The solutions for pH correction and foam remediation were 25 wt% NH<sub>3</sub>, 98 wt% H<sub>2</sub>SO<sub>4</sub> and Basildon 86-013. The carbon feed solutions of the two pilot scale fermentations are given in Table 1.

## *Analytical methods*

### *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, Cambridge, United Kingdom). For the fed-batch cultures, biomass growth was monitored by optical density (OD) measurement at a wavelength of 600 nm with a Thermo Genesys spectrophotometer (Thermo Fisher Scientific, MA, USA).

For the chemostat cultures, dry weight was determined by filtering 10 ml culture broth over a pre-weighed 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  $2 \times 5$  mL of culture broth at  $6,000 \times g$  for 15 min. The pellet was washed once by resuspending in deionized water and centrifuged again at  $6,000 \times g$  for 15 min. After washing, the pellet was dried for 24 hours at  $105^{\circ}\text{C}$  and weighed.

## Gas analysis

For the chemostat cultures, the offgas was cooled ( $2^{\circ}\text{C}$ ) in a condenser and dried, prior to the analysis of  $\text{O}_2$  and  $\text{CO}_2$  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, MA, USA).

## Substrate and metabolite analysis

During the runs, rapidly temperature-quenched samples were taken from the fermenters in order 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  $60^{\circ}\text{C}$  with 5 mM  $\text{H}_2\text{SO}_4$  as mobile phase at a flow rate of  $0.600 \text{ mL min}^{-1}$ . Detection was performed by means of an Agilent refractive index detector and an Agilent 1260 VWD detector at 210 nm.

### $\text{NH}_3$ analysis

The  $\text{NH}_3$  concentration was measured in supernatant samples using an Orion 4 Star with the Orion 95-12 Ammonia Electrode (Thermo Fisher Scientific, MA, USA).

## Results

### *Natural co-consumption of glucose and formic acid in Yarrowia lipolytica W29 in batch shake-flask cultures*

To investigate the innate ability of *Yarrowia lipolytica* to co-consume 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*?, *fdh2*?) were also tested in medium with glucose and formic acid as positive and negative control, respectively (Figure 2).

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

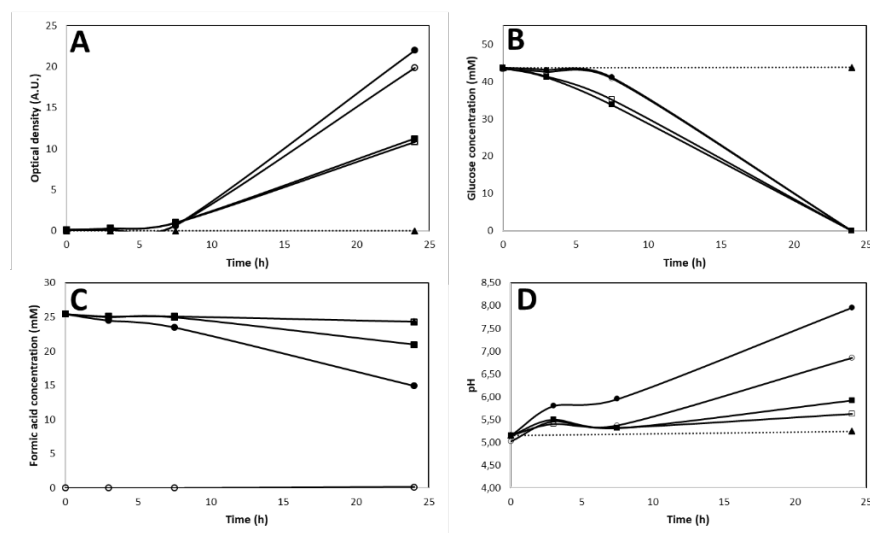


Figure 2: Optical density (A), glucose (B) and formic acid (C) concentrations and pH (D) of shakeflask cultures of *Y. lipolytica* and *S. cerevisiae*. *Y. lipolytica* strain W29 was incubated with 42 mM glucose with (closed circles) and without (open circles) 25 mM formic acid, and both *S. cerevisiae* strains CEN.PK113-7D (closed squares) and CEN.PK556-7B (open squares) were incubated with glucose and formic acid. A sterile flask with glucose and formic acid was also included (dashed line, closed triangles). Some data points overlap: closed & open squares (A), closed & open circles and closed & open squares (B), closed & open squares & closed triangles (C, first three datapoints) and open square and closed triangle (C, last datapoint).

#### 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 optimal feed-ratio of formic acid to glucose (F:G) for the *Y. lipolytica* strain used (Figure 3). A dilution rate of  $0.10 \text{ h}^{-1}$  was chosen, well below the maximum specific growth rate of W29 determined from offgas  $\text{CO}_2$  of the batch phase preceding the chemostat phase ( $0.37 \pm 0.01 \text{ h}^{-1}$ , see Supplementary Materials 2).

For F:G ratios between 0 and 5 mol/mol, a linear increase in the biomass yield on glucose was observed from  $0.50 \pm 0.02$  to  $0.60 \pm 0.01 \text{ g biomass/g glucose}$ , 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  $0.20 \pm 0.01$  to  $0.16 \pm 0.00 \text{ g glucose/g biomass/h}$  (Supplementary 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.

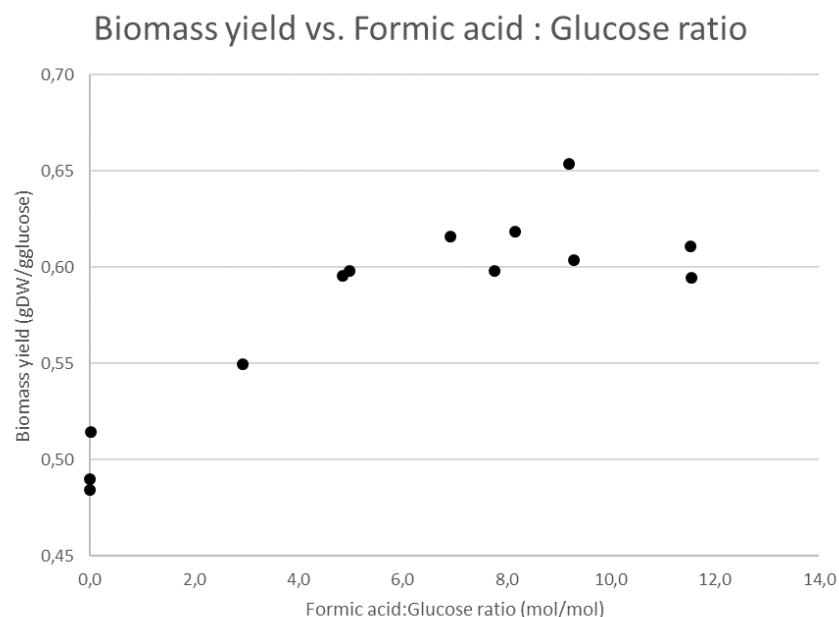


Figure 3: Overview of determined biomass yields for formic acid to glucose feed ratios tested in independent chemostat experiments.

#### 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 co-feeding 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 (Supplementary 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.

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 the O<sub>2</sub> uptake rate profiles of the 4 laboratory scale fermentations with the batch phases ending between 10-15h, followed by the carbon-limited fed batch phases. The decrease of the OUR after ~55h for LF1 and after ~60h for LF2 is caused by O<sub>2</sub> transfer limitation of the fermenter. 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. Until the onset of the late O<sub>2</sub> transfer limitation, the experimental data quantitatively correspond with the simulated fermentations using the dynamic process model (see Supplementary Materials 1).



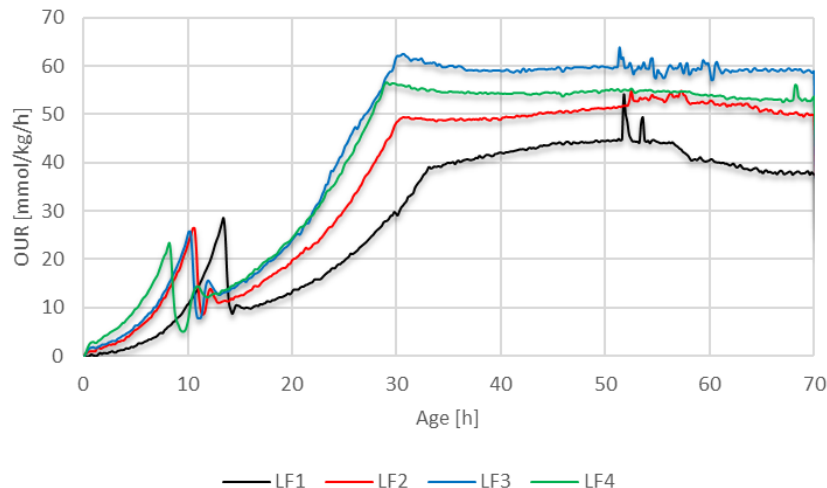


Figure 4: the  $O_2$  uptake rate (OUR) of the four laboratory scale fed batch fermentations with increasing molar ratios of F:G and decreasing glucose concentration (see Table 10). The 4 fermentations had F:G ratios increasing from 0 (LF1, black), 3 (LF2, red), 5 (LF3, blue) to 7 (LF4, green).

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 4 fermentations. The ammonia level in the broth was between 0.4 and 0.9 g/l for all 4 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 co-feeding 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 **Error! Reference source not found.** D 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.

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)
<b>LF1</b>	Glucose only (glucose: 258 g/kg)	0.49
<b>LF2</b>	2.9:1 molar F:G ratio (glucose: 232 g/kg)	0.54 (+10.2% vs. LF1)
<b>LF3</b>	5.0:1 molar ratio F:G ratio (glucose: 228 g/kg)	0.60 (+22.4% vs. LF1)
<b>LF4</b>	6.9:1 molar F:G ratio (glucose: 174 g/kg)	0.60 (+22.4% vs. LF1)
<b>PF1</b>	Glucose only (glucose: 250 g/kg)	0.44
<b>PF2</b>	4.6:1 molar F:G ratio (glucose: 187 g/kg)	0.53 (+20.5% vs. PF1)

#### *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 co-feeding on 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 Supplementary 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 5 shows the  $O_2$  uptake rate profiles of the 2 pilot scale fermentations with the batch phases ending between 10-12h, followed by the carbon-limited fed batch phases. The oscillation of the OUR of PF2 between 12-16h was caused by a technical deviation when starting up the feed. The actual OUR profiles are in good agreement with the profiles of **Error! Reference source not found.** B that were simulated to design the experiment.

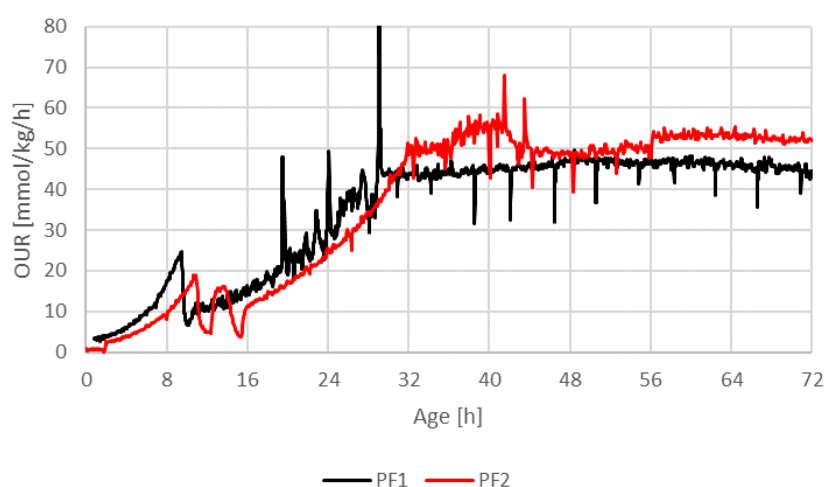


Figure 5: the  $O_2$  uptake rate (OUR) of the two pilot scale fed batch fermentations with glucose only (PF1) and a 1:4.6 molar ratio of glucose: formic acid in the feed (PF2) (see Table 2).

Figure 6 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 co-feeding formic acid (starting around 12h) for PF2.

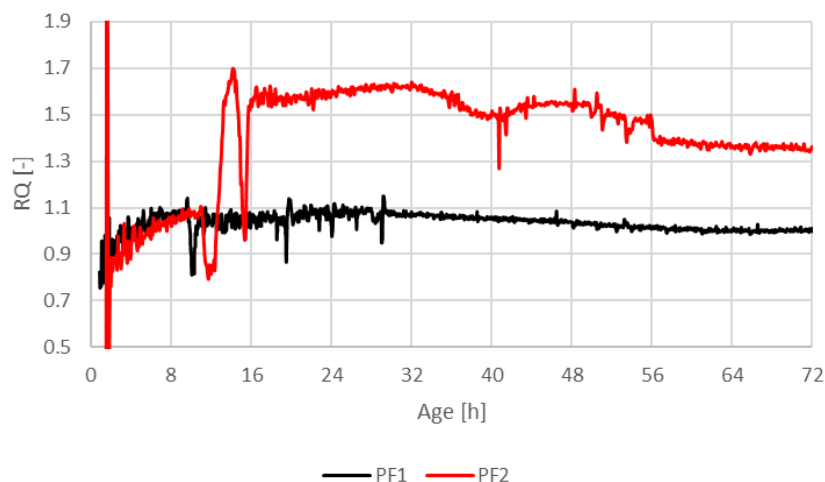


Figure 6: the respiratory quotient (RQ) of the two pilot scale fed batch fermentations with glucose only (PF1) and a 1:4.6 molar ratio of glucose: formic acid in the feed (PF2)

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 formic acid to glucose ratios were similar. On both scales the  $Y_{xs}$  is increased by  $21 \pm 1\%$ .

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. Supplementary 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  $<19\%$ ).

## Discussion

### *Energetics of Yarrowia lipolytica co-consuming formic acid*

In this work, we found that co-feeding formic acid and glucose, up to a molar ratio of  $\sim 5:1$ , 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  $24 \pm 2$  moles of formic acid were able to displace 1 mole of glucose for dissimilatory requirements in *Y. lipolytica* (Supplementary 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 formic acid to glucose ratios up to 11.5:1, virtually all formic acid was consumed as indicated by the low residual formic acid concentrations in the fermenter (Supplementary 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 *C. 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 work we used biomass itself as an ATP-intensive product to investigate the potential of formic acid co-feeding for increasing the product yield. Previous work on antibiotic-producing *Penicillium chrysogenum* strains (Harris et al., 2007) demonstrated that in chemostat setups, formic acid co-feeding 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.

Supplementary materials 4 and 5 present extensions of the formic acid co-feeding concept of this study, illustrating how the co-produced O<sub>2</sub> can be valorised and how co-feeding of formic acid can even lead to net-negative CO<sub>2</sub> emission processes.

## Conclusions

This study has shown proof of principle of co-feeding formic acid to glucose 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 co-feeding 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 work 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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## Conflict of interest

The authors have filed a patent application related to subject matter disclosed in this article.