Kinetic and stoichiometric characterization of organoautotrophic growth of *Ralstonia eutropha* on formic acid in fed-batch and continuous cultures

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*organoautotrophic growth of *Ralstonia eutropha* on formic acid in fed-batch and continuous cultures.* In this work, formic acid was studied using an approach combining stoichiometric modeling and controlled cultures. The measured yield corresponded to 76% to 85% of the theoretical yield (later confirmed in pH-controlled fed-batch cultures). The stoichiometric study highlighted the imbalance between carbon and energy provided by formic acid and explained the low growth yields measured. Fed-batch cultures were also used to determine the maximum specific growth rate ($\mu_{\text{max}} = 0.18 \text{ h}^{-1}$) and to study the impact of increasing formic acid concentrations on growth yields. High formic acid sensitivity was found in *R. eutropha* since a linear decrease in the biomass yield with increasing residual formic acid concentrations was observed between 0 and 1.5 g l$^{-1}$.

**Introduction**

*Ralstonia eutropha*, also known as *Cupriavidus necator*, is an aerobic facultative autotrophic bacterium, able to convert carbon dioxide (CO$_2$) or formic acid (HCO$_2$) through the Calvin–Benson–Bassham (CBB) cycle into value-added chemical compounds by non-photosynthetic microorganisms. In this work, organoautotrophic growth of *Ralstonia eutropha* on formic acid was studied using an approach combining stoichiometric modeling and controlled cultures in bioreactors. A strain deleted of its polyhydroxyalkanoate production pathway was used in order to carry out a physiological characterization. The maximal growth yield was determined at 0.16 Cmole Cmole$^{-1}$ in a formate-limited continuous culture. The measured yield corresponded to 76% to 85% of the theoretical yield (later confirmed in pH-controlled fed-batch cultures). The stoichiometric study highlighted the imbalance between carbon and energy provided by formic acid and explained the low growth yields measured. Fed-batch cultures were also used to determine the maximum specific growth rate ($\mu_{\text{max}} = 0.18 \text{ h}^{-1}$) and to study the impact of increasing formic acid concentrations on growth yields. High formic acid sensitivity was found in *R. eutropha* since a linear decrease in the biomass yield with increasing residual formic acid concentrations was observed between 0 and 1.5 g l$^{-1}$.

**Summary**

Formic acid, acting as both carbon and energy source, is a safe alternative to a carbon dioxide, hydrogen and dioxygen mix for studying the conversion of carbon through the Calvin–Benson–Bassham (CBB) cycle into value-added chemical compounds by non-photosynthetic microorganisms. In this work, organoautotrophic growth of *Ralstonia eutropha* on formic acid was studied using an approach combining stoichiometric modeling and controlled cultures in bioreactors. A strain deleted of its polyhydroxyalkanoate production pathway was used in order to carry out a physiological characterization. The maximal growth yield was determined at 0.16 Cmole Cmole$^{-1}$ in a formate-limited continuous culture. The measured yield corresponded to 76% to 85% of the theoretical yield (later confirmed in pH-controlled fed-batch cultures). The stoichiometric study highlighted the imbalance between carbon and energy provided by formic acid and explained the low growth yields measured. Fed-batch cultures were also used to determine the maximum specific growth rate ($\mu_{\text{max}} = 0.18 \text{ h}^{-1}$) and to study the impact of increasing formic acid concentrations on growth yields. High formic acid sensitivity was found in *R. eutropha* since a linear decrease in the biomass yield with increasing residual formic acid concentrations was observed between 0 and 1.5 g l$^{-1}$.

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R. eutropha were reported: (i) high-cell densities up to 25 g cell dry weight (CDW) l⁻¹ have been reached, by carefully investigating the macro- and micronutrient requirements of R. eutropha (Repaske and Repaske, 1976); (ii) up to 91.3 gCDW l⁻¹ have been reached by developing special agitation systems and adjusting the gas composition (Tanaka et al., 1995). However, few studies describe organoautotrophic growth of R. eutropha and those focus either on formic acid metabolism (Friedrich et al., 1979) or proteomic examination of Ralstonia in response to formic acid (Lee et al., 2006). Stoichiometric and kinetic characterization of R. eutropha growth on formic acid as the sole carbon and energy source was generally neglected, and only very low biomass concentrations (about 1.2 to 1.7 gCDW l⁻¹) have been reached (Friedrich et al., 1979; Friedebold and Bowien, 1993; Li et al., 2012).

It is well known that short-chain organic acids including formic acid are toxic to cells (Salmond et al., 1984; Pronk et al., 1991; Russell, 1992; Vazquez et al., 2011). However, toxicity of formic acid to R. eutropha was rarely explored. It has been shown in pulse-fed flask cultures, initially grown on 2 g l⁻¹ of glucose, that the biomass yield of R. eutropha decreases with increasing concentrations of formic acid (Lee et al., 2006).

Since formic acid is toxic, a batch culture with this substrate is not a suitable culture system. Usually, a pH-controlled feeding (pH-stat) strategy is used for organoautotrophic growth of R. eutropha on formic acid (Friedebold and Bowien, 1993; Li et al., 2012).

This study aimed at determining the maximum growth capacities of R. eutropha (i.e. rate and yield) on formic acid as a sole substrate and at investigating the impact of increasing formic acid concentrations. Therefore, two different culture systems were applied to characterize the growth of R. eutropha on formic acid as a sole substrate:

- A chemostat culture was performed to determine the maximal yield with no residual formic acid concentration.
- pH-controlled fed-batch cultures, designed to maintain concentrations of formic acid between 0 and 2 g l⁻¹, were performed to confirm the maximal biomass yield determined with the chemostat system and to investigate the effect of increasing concentrations of formic acid on the biomass yield. The fed-batch culture as a dynamic system was also used to study the growth kinetics.

Moreover, experimental results were compared to theoretical results from stoichiometric modeling.

## Results and discussion

### Biomass concentrations produced from formic acid

The organoautotrophic growth of a R. eutropha-engineered strain deficient in polyhydroxybutyrate (PHB) production (Re2061; Lu et al., 2012) was investigated in pH-controlled fed-batch and continuous cultures using well-designed medium and fully equipped bioreactors. In those conditions, the final biomass concentration reached 5.4 gCDW l⁻¹ in the pH-controlled fed-batch cultivation and 10.6 gCDW l⁻¹ in the chemostat with formic acid as the sole carbon and energy sources. These biomass concentrations are the highest ever published (Table 1). The first detailed study for growth of R. eutropha on formic acid in a pH-controlled fed-batch fermentation (10 l) was performed by Friedrich and colleagues (1979) with the wild-type strain H16 able to produce PHB. A maximal biomass concentration of 1.2 gCDW l⁻¹ was reached (Table 1). In a publication that focused on the characterization of the soluble formate dehydrogenase of R. eutropha, approximately 1.7 g l⁻¹ of formic acid was required (Friedebold and Bowien, 1993). In these two articles, the same basal media was used (Schlegel et al., 1961), and addition of a second trace solution (SL7) was performed by Friedebold and Bowien (1993). Some nutrient limitations may have occurred: the nitrogen amount corresponded to the amount necessary to produce about 1.9 gCDW l⁻¹ of

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**Table 1.** Organoautotrophic biomass production with R. eutropha.

| Strain | Phenotype | CDW<sub>max</sub> (g l⁻¹) | Culture type | Reference |
|--------|-----------|--------------------------|--------------|-----------|
| R. eutropha Re2061 | PHB<sup>−</sup> | 10.5 | chemostat | This work |
| R. eutropha Re2061 | PHB<sup>−</sup> | 5.4 | fed-batch | This work |
| R. eutropha H16 | wild type | 1.2 | fed-batch | Friedrich et al., 1979 |
| R. eutropha H17 | wild type | 1.7<sup>a</sup> | fed-batch | Friedebold and Bowien 1993 |
| R. eutropha LH74D | PHB<sup>−</sup> isobutanol<sup>b</sup> | 1.4<sup>b</sup> | fed-batch | Li et al., 2012 |

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<sup>a</sup> The CDW was not estimated in Friedebold and Bowien (1993), an OD<sub>max</sub> of 8 was measured which corresponded to a CDW of approximately 1.7 g l⁻¹ (1 g CDW l⁻¹ corresponding to an OD<sub>max</sub> of 4.8).

<sup>b</sup> The CDW was not estimated in Li et al. work, an OD<sub>max</sub> about 3.8 was measured. Using the ratio CDW/OD<sub>max</sub> = 0.363 g l⁻¹, UDO<sup>−</sup> determined in this work [calibration curve done with 24 data points from fed-batch cultures, OD measured using a 1 cm path length absorption PS semi-micro cuvette (VWR, Radnor, PA, USA) with a Spectronic GENESYS 20 Visible Spectrophotometer at a wavelength of 600 nm] and assuming that the authors were using a 1 cm path length cell for measurement, the equivalent CDW was about 1.4 g l⁻¹.

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biomass considering the following biomass formula: 
\[C_{1.17}H_{1.77}O_{0.44}N_{0.25} (4\% \text{ ashes})\] and a molecular weight of 
25.35 g C mole\(^{-1}\) (Aragao, 1996). Moreover, in Friedrich 
and colleagues (1979), an O\(_2\) starvation was thought to be 
the reason for the cessation of cell growth at a CDW of 
1.2 g l\(^{-1}\) since a higher biomass concentration was 
reached under lithoautotrophic condition. In a recent public-
ication that focused on the electrochemical production of 
formic acid, a fed-batch fermentation was performed as a 
side experiment in a 5 l reactor with a strain unable to 
produce PHB (Li \textit{et al.}, 2012). A biomass concentration of 
1.4 g CDW l\(^{-1}\) (Table 1) was reached in accordance with 
the media composition: the nitrogen concentration of 
0.015 mole l\(^{-1}\) corresponded to the amount necessary to 
produce about 1.5 g l\(^{-1}\) of CDW.

The high biomass concentrations reached in this work 
enabled to perform a reliable quantification of the 
\textit{R. eutropha} growth kinetics and stoichiometry on formic acid.

\textit{Determinant of the maximal biomass production yield in formic acid limited continuous culture}

A continuous culture of \textit{R. eutropha} was performed at 
a low dilution rate of 0.05 h\(^{-1}\) and under formic acid 
limitation.

The continuous cultivation data are presented in 
Table 2. The results were obtained by averaging data over 
a period of 20 h after reaching the steady state. The steady state was considered to be reached when 
the standard deviation of the variation of biomass concentra-
tion, CO\(_2\) production rate and O\(_2\) consumption rate was 
inferior to 1%. The biomass concentration was maintained 
at a value equal to 10.58 \(\pm\) 0.07 gCDW l\(^{-1}\). No residual 
formic acid concentration was detected during the steady 
state, and no other metabolites were detected. A biomass 
yield on formate of 0.16 \(\pm\) 0.00 C mole C mole\(^{-1}\) was 
achieved. Carbon and reduction degree balances were 
respectively equal to 98.2 \(\pm\) 0.4\% and 103.1 \(\pm\) 0.1\% 
(Table 2) confirming that no other products than biomass 
were produced from formic acid.

The stoichiometric model constructed by Grousseau 
and colleagues (2013) was implemented as explained in 
\textit{Calculations and metabolic descriptor in order to compare} 
theoretical and experimental data. Theoretical biomass 
production yields were calculated depending on the ener-
getric yield (\(Y_{ATP,X}\)) and NADPH production pathway 
[Entner–Doudoroff (ED) or tricarboxylic acid cycle (TCA)]
as depicted on Fig. 1A.

The maximum \(Y_{ATP,X}\) of 19.21 g\(_{\text{biomass}}\) mol\(_{\text{ATP}}\)\(^{-1}\) is the ana-
bolic demand in adenosine triphosphate (ATP) calculated 
according to the biomass composition and the anaabolic 
reactions. The corresponding biomass theoretical yield is 
between 0.19 and 0.21 C mole C mole\(^{-1}\) depending on the

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Formic acid as substrate & Experimental data & & \hline
\hline
CO\(_2\) as substrate & Theoretical data & & \hline
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Formic acid & & & \hline
Experimental data & & & \hline
\hline
Table 2. Experimental and theoretical data concerning chemostat culture of \textit{R. eutropha}.

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NADPH generation pathway (Fig. 1A). The experimental biomass yield of $0.16 \pm 0.00 \text{ Cmole Cmole}^{-1}$ obtained in chemostat condition corresponds to 76% to 84% of this maximal theoretical yield. The maximal theoretical value of $Y_{ATP,X}$, calculated from the energetics of the anabolic pathways, is generally more than twice the experimental yield (Neijssel and Demattos, 1994). This difference can be explained by futile cycle, protein and nucleic acid turn-overs and by useful maintenance (ionic transports, cellular homeostasis). These extra ATP requirements were included in the model by an ATP spilling reaction (Grousseau et al., 2013). According to Fig. 1A, the experimental biomass yield of $0.16 \text{ Cmole Cmole}^{-1}$ corresponds to a $Y_{ATP,X}$ between 6 and 8 $\text{ gbiomass molATP}^{-1}$, in accordance with Neijssel and Demattos (1994) assumption.

The experimental biomass yield was associated to an experimental respiratory quotient (RQ) of $2.52 \pm 0.03$ which is very closed to the theoretical value of 2.59 (Table 2 and Fig. 1B).

During the continuous culture, specific dioxide carbon production rate ($q_{CO_2}$) and dioxygen uptake rate ($q_{O_2}$) were calculated (Table 2). The theoretical $q_{CO_2}$ and $q_{O_2}$ were also numerically simulated using the stoichiometric model and were equal to 10.16 and 3.92 mmol g$^{-1}$ h$^{-1}$ respectively. These values were very closed to the experimental data with $10.09 \pm 0.05 \text{ mmol g}^{-1} \text{ h}^{-1}$ for $q_{CO_2}$ and $4.01 \pm 0.03 \text{ mmol g}^{-1} \text{ h}^{-1}$ for $q_{O_2}$ (Table 2).

The biomass yields obtained for growth on formic acid appeared to be low, compared to other substrates like fructose with $0.63 \text{ Cmole Cmole}^{-1}$ (fed-batch culture with strain Re2061, data not shown), or butyric acid with $0.65 \text{ Cmole Cmole}^{-1}$ (Grousseau et al., 2013), or pyruvate with $0.53 \text{ Cmole Cmole}^{-1}$ (Friedrich et al., 1979), or CO$_2$ with $0.92 \text{ Cmole Cmole}^{-1}$ (Morinaga et al., 1978). This could be explained by the low efficiency of the formic acid to provide NADH (only 1 mole of NADH per mole of formic acid) compared to the high requirement of the CBB cycle to assimilate CO$_2$ derived from the oxidation of formic acid: $1.7 \text{ NADH and 2.7 ATP per CO}_2$ ($3 \text{ CO}_2 + 5 \text{ NADH} + 8 \text{ ATP} + 5 \text{ H}_2\text{O} \rightarrow 3\text{-P-Glycerate} + 8 \text{ ADP} + 5 \text{ NAD}^+\text{)}$. Considering the respiratory chain used by Grousseau and colleagues (2013), 1.35 NADH are necessary to produce 2.7 ATP. The requirement of the CBB cycle is therefore equivalent to three NADH per CO$_2$. Since the oxidation of formic acid delivers one NADH and one CO$_2$, three moles of formic acid are required for the fixation of one mole of CO$_2$ by the CBB cycle, leading to a maximal yield of only $0.33 \text{ Cmole Cmole}^{-1}$. The consideration of the whole reaction system to produce biomass in organoautotrophic condition leads to a value between 0.19 and 0.21 Cmole Cmole$^{-1}$ given above. When the energy source (H$_2$) is dissociated from the carbon source (CO$_2$) in lithoautotrophic condition, the biomass production yield is close to 1 Cmole Cmole$^{-1}$ according to the modelling (Table 2) and in accordance with Morinaga and colleagues (1978). The high NADH requirement of the CBB cycle affects, in this case, the biomass yield on H$_2$, and as a consequence the H$_2$ flux necessary ($q_{H_2}$) but not the biomass yield on carbon.

**Effect of the residual formic acid concentration on the biomass yield**

To study the effect of increasing residual formic acid concentration on the biomass production yield, a pH-controlled fed-batch cultivation was developed. The fermentation process was fully automated and required neither manual addition of formic acid nor nitrogen. Using data from preliminary cultures (not shown) with 2 g l$^{-1}$ initial formic acid (pH 6.5), the composition and pH of the feeding solution was calculated and optimized. The aim was to maintain a constant concentration of formic acid...
and a sufficient supply of nitrogen based on the elementary composition of *R. eutropha*. The feeding solution contained 50% (w/v) formic acid solution employed with 4.3 g l\(^{-1}\) (252 mM) NH\(_3\)(aq).

Three different initial concentrations of pH-corrected formic acid (pH 6.5): 0.5 g l\(^{-1}\) (A), 1.0 g l\(^{-1}\) (B) and 2.0 g l\(^{-1}\) (C) were used to initiate the pH-controlled feeding (Fig. 2). Each culture was performed in duplicate.

When starting with 2.0 g l\(^{-1}\) pH-corrected formic acid (cultures C), concentrations of formic acid between 1.3 g l\(^{-1}\) and 2.1 g l\(^{-1}\) and a non-limiting nitrogen concentration were maintained during the growth phase (0–32 h). For the fermentations, which were initiated with 2 g l\(^{-1}\) of formic acid, a lower biomass concentration of 4.89 \(\pm\) 0.24 gCDW l\(^{-1}\) was achieved. To produce the same amount of biomass, a higher amount of formic acid was fed during the cultures C than during the cultures B (Fig. 2.3). This is due to a reduced biomass yield in the presence of higher formic acid concentration (see paragraph below). For the cultures A, the total amount of biomass produced was only 1.06 \(\pm\) 0.02 g after 50 h, likely due to a carbon limitation as pointed out by the entrance in a linear growth phase at 32 h when the residual formic acid concentration was zero.

The biomass yield \((Y_x)\) was calculated for the growth phase of the fed-batch cultures (16–32 h). The highest biomass yields of 0.17 and 0.16 Cmole Cmole\(^{-1}\) (Fig. 3) were observed for the cultures A, with low formic acid concentrations between 0 g l\(^{-1}\) and 0.16 g l\(^{-1}\). These maximum growth yields are in accordance with the maximum experimental yield found during the chemostat culture (0.16 \(\pm\) 0.01 Cmole Cmole\(^{-1}\)) even though these three experiments were done in two different laboratories with different mineral media and culture systems.

A biomass amount of 1.98 \(\pm\) 0.06 gCDW was reached for both cultures B and C, respectively, at 44 h and 36 h (Fig. 2.2). For the cultures B, the corresponding biomass concentration was 5.42 \(\pm\) 0.04 gCDW l\(^{-1}\). For the cultures C, which were initiated with 2 g l\(^{-1}\) of formic acid, a lower biomass concentration of 4.89 \(\pm\) 0.24 gCDW l\(^{-1}\) was achieved. To produce the same amount of biomass, a higher amount of formic acid was fed during the cultures C than during the cultures B (Fig. 2.3). This is due to a reduced biomass yield in the presence of higher formic acid concentration (see paragraph below). For the cultures A, the total amount of biomass produced was only 1.06 \(\pm\) 0.02 g after 50 h, likely due to a carbon limitation as pointed out by the entrance in a linear growth phase at 32 h when the residual formic acid concentration was zero.

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Figure 3 presents the effect of the formic acid concentration on the biomass yield. A linear decrease in the biomass yield with increasing formic acid concentrations was observed. The experimental yield at null residual formic acid concentration was evaluated at 0.169 Cmole Cmole\(^{-1}\) with a 95% confidence interval of 0.162–0.176 Cmole Cmole\(^{-1}\). This confidence interval corresponds to 77–93% of the maximal theoretical yield,
showing a high accordance between our experimental results and the theory and validated the maximum biomass yield from formic acid as substrate.

For the cultures B, the biomass yields were 0.14 and 0.15 Cmole Cmole$^{-1}$ for average formic acid concentrations between 16–32 h of $0.58 \pm 0.18 \text{ g l}^{-1}$ and $0.42 \pm 0.12 \text{ g l}^{-1}$ respectively.

For the highest residual concentrations of formic acid of $1.39 \pm 0.08 \text{ g l}^{-1}$ and $1.52 \pm 0.11 \text{ g l}^{-1}$ (cultures C), the biomass yields were respectively 0.10 Cmole Cmole$^{-1}$ and 0.09 Cmole Cmole$^{-1}$.

A reduction of the biomass yield upon an increase in the formic acid concentrations from 2 g l$^{-1}$ to 5 g l$^{-1}$ was shown by Lee and colleagues (2006) for flask cultures. These cultures were first grown on 2 g l$^{-1}$ glucose before the addition of a pulse of pH-corrected formic acid (pH 6.5–7.0) after 24 h (Lee et al., 2006). They showed a decrease in the biomass yield from approximately 0.08 gCDW gformic acid$^{-1}$ ($\approx 0.15 \text{ Cmole Cmole}^{-1}$) to 0.05 gCDW gformic acid$^{-1}$ ($\approx 0.09 \text{ Cmole Cmole}^{-1}$). However, their experimental yields were based on a single pulse of pH-corrected formic acid after heterotrophic growth on glucose and thus were not comparable to our yields. Our results were also in accordance with a previous study showing a clear decrease in the growth yields on other organic acids such as acetic acid and butyric acid (Grousseau, 2012).

If the linear correlation stands true also for higher formic acid concentrations, the biomass yield would approach zero at a concentration between 3.0 g l$^{-1}$ and 4.1 g l$^{-1}$. (95% confidence band; Fig. 3)

With a maximal biomass yield of 0.17 Cmole Cmole$^{-1}$ obtained from the two cultivation conditions (chemostat and fed-batch), the highest biomass yield for growth of *R. eutropha* published so far was reached. Friedrich and colleagues (1979) evaluated the biomass production yield to 2.35 g.mole$^{-1}$ which corresponded to 0.09 Cmole Cmole$^{-1}$; no indication concerning the residual formic acid concentration was given.

**Determination of the maximum growth rate on formic acid**

The fed-batch culture was also a pertinent tool to study the growth dynamic and was used to determine the maximum growth rate. The highest maximal growth rate $\mu_{max}$ of 0.18 h$^{-1} \pm 0.00$ was reached during the fed-batch cultures C (exponential growth phase of the cultures from 16 h to 24 h). Friedrich and colleagues (1979) determined a growth rate of 0.17 h$^{-1}$ and Friedebold and Bowien (1993) a growth rate of 0.23 h$^{-1}$ for the PHB producing *R. eutropha* wild-type strain (H16). However, since PHB contributes to the CDW as well as to the OD$_{600}$ value, the determined growth rate in these two articles could have been overestimated.

Nonetheless, those growth rates are low compared to growth rates measured for other organic acids like acetic, propionic or butyric acid, for which growth rates between 0.26 h$^{-1}$ and 0.34 h$^{-1}$ can be reached (Kim et al., 1992; Wang and Yu, 2000; Grousseau, 2012) or for lithoautotrophic growth with growth rates determined between 0.31 h$^{-1}$ and 0.32 h$^{-1}$ (Schlegel et al., 1961; Siegel and Ollis, 1984). The low growth rate of 0.18 h$^{-1}$ could be explained by a limiting energetic flow since lithoautotrophic growth exhibited a higher growth rate. As explained in section **Determination of the maximal biomass production yield in formic acid limited continuous culture**, the NADH supply seemed to be the limiting factor for the carbon assimilation by the CBB cycle. With formic acid, carbon and energy sources are coupled, in contrast to lithoautotrophic growth where H$_2$ supplies the energy and CO$_2$ the carbon.

**Conclusion**

In this study, two different tools to explore the organoautotrophic growth of *R. eutropha* on formic acid were used. In both systems, biomass concentration was high enough to provide reliable quantitative information (10.6 g l$^{-1}$ and 5.4 g l$^{-1}$).

A chemostat culture was used to determine the maximum biomass yield in a stabilized system at a null residual formic acid concentration to avoid toxic effects. This maximum biomass yield was confirmed by the fed-batch study. A good adequation between experimental and theoretical values was shown. The experimental yield of 0.17 Cmole Cmole$^{-1}$ corresponded to 81% to 89% of the maximal theoretical yield.

pH-controlled fed-batch cultures were used to investigate the effect of increasing concentration of formic acid: a decrease in biomass production yield with increasing residual formic acid concentration was shown between 0.0 g l$^{-1}$ and 1.5 g l$^{-1}$. This dynamic system was also used to determine the maximum growth rate of the strain on formic acid which was equal to 0.18 h$^{-1}$.

**Experimental procedures**

The physiological characterization of *R. eutropha* strain Re2061 was carried out in two distinct research laboratories: the Fungal Fermentation Advances and Microbial Engineering group of Laboratoire d’Ingénierie des Systèmes Biologiques et des Procédés at the Institut National des Sciences Appliquées de Toulouse (France) for the chemostat culture and the Sinskey Lab, Biology department of Massachusetts Institute of Technology (USA) for the pH-controlled fed-batch cultures. Materials and equipments were therefore lab dependent.

**Bacterial strain**

The recombinant *R. eutropha* strain Re2061 (H16::phaCAB Gen') unable to produce PHB was used in the two labora-
Continuous culture experiment

_Growth media._ Rich medium consisted of 2.5 g l⁻¹ tryptone, 2.5 g l⁻¹ meat peptone and 3 g l⁻¹ meat extract. The media used for the pre-cultures was described in the literature by Aragao and colleagues (1996) and adapted by Grousseau (2013) except for the carbon source (fructose added to a final concentration of 4 g l⁻¹). For the continuous fermentation, the culture medium was the mineral salt medium adapted by Gaudin (1998). The carbon source was formic acid. These growth media were designed and optimized to supply all nutritional elements required to produce a biomass concentration of about 10 g l⁻¹.

_Pre-culture cultivation._ A single colony grown on a rich medium (with addition of agar 15 g l⁻¹) petri dish was used to inoculate 10 ml of rich medium, which was incubated for 24–48 h at 30°C. The second and the third pre-cultures were respectively grown for 24 h and 18 h, at 30°C, in a baffled 1 l shaking flask (150 rpm) and a baffled 3 l shaking flask (120 rpm), containing 150 ml and 300 ml of pre-culture mineral salt medium with fructose as carbon source.

The cell suspension of the third pre-culture was used to inoculate the fermenter to an initial biomass concentration of 0.1 g l⁻¹.

_Continuous cultivation._ The continuous culture was performed in a 7 l fermenter (BIOSTAT B-DCU, Sartorius Stedim Biotech, Germany) with a working volume of 2.7 l, equipped with pH, dissolved oxygen (DO), temperature, pressure and anti-foam controllers. The online monitoring and control systems of the reactor were handled by the software BIOPAT MFCS/win version 3.0. The DO level in the reactor was controlled above 20% of air saturation by varying stirring speed and/or inlet air flow rate. Temperature was maintained at 30°C. The pH was maintained at 7.0 by addition of a NH₄OH solution with a concentration of 8.24 mol l⁻¹.

A batch culture with fructose as carbon source was carried out to initiate the growth and to quickly reach a biomass concentration close to 10 g l⁻¹. After fructose exhaustion (35 h), the continuous culture was started with a dilution rate of 0.05 h⁻¹ using a 98% (w/v) formic acid solution as sole substrate to maintain a biomass concentration around 10 g l⁻¹. Added masses of substrate, mineral salt medium and NH₄OH were online monitored by weight. Inlet and outlet gases were analysed using a gas analyser with an infrared spectrometry detector for carbon dioxide and a paramagnetic detector for oxygen (EGAS-8 gas analyser system; B. Braun Biotech International, Germany). Dioxygen consumption rate and carbon dioxide production rate were calculated from mass balances, taking into account the evolution of inlet airflow, temperature and pressure.

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Determination of metabolite concentrations. Culture supernatant was obtained by centrifuging (Mini-Spin Eppendorf, USA) the fermentation broth in Eppendorf tubes at 13 000 rpm for 3 min. The supernatant was filtered on Minisart filters 0.20 µm pore-size diameter polyamide membranes (Sartorius AG, Germany).

The culture supernatants were analysed by high performance liquid chromatography (DIONEX Ultimate 3000, USA or Agilent 1100 Series) using an Aminex HPX-87H column (Bio-Rad, USA) and its guard column (Micro-Guard Cation H, Bio-Rad, 4.6 × 30 mm) and the following conditions: a temperature of 50°C with 5 mM H2SO4 as eluent at a flow rate of 0.6 ml min⁻¹ or 2.5 mM H2SO4 at 0.5 ml min⁻¹ and a dual detection (RI and UV at 210 nm).

For quantification of low formic acid concentrations, the samples were analysed by high performance ion chromatography [ICS-3000 system (Dionex) equipped with an ED40 electrochemical detector]. Formic acid was separated on an ICS-3000 system (Dionex) equipped with an ED40 electrochemical detector. Formic acid was separated on an ICS-3000 system (Dionex) equipped with an ED40 electrochemical detector.

All yields were expressed as carbon ratios in Cmole Cmole⁻¹. For the fed-batch cultures, the experimental biomass yield (Yx) was calculated using the equation $Y_x = (X_f - X_i) / (MW_x(S_f - S_i))$ where X was the mass of cells (g cell dry weight) produced and S the mass of substrate (Cmole) consumed within a time interval t₂–t₁ and MW_x the molecular weight of R. eutropha per Cmole, which is 25.35 g Cmole⁻¹ (Aragao, 1996). The masses were calculated taking into account the evolution of the suspension volume due to the feed and the sampling volumes.

For both cultures, the specific substrate (formic acid) uptake rate (q_s) and growth rate (µ) were calculated from their measured data by means of the respective mass balance equation, taking into account the evolution of the suspension volume.

Moreover, for the continuous culture, CO₂ production and O₂ consumption were measured with the gas analyser (Continuous cultivation) allowing to perform a data reconciliation based on carbon and reduction balance degrees (van der Heijden et al., 1994a,b).

A metabolic descriptor (Grousseau et al., 2013) was used to calculate theoretical values of biomass yield (Y_x,thc), RQ, specific oxygen uptake rate and specific dioxyde carbon pro-
duction rate. The model was implemented with three reactions concerning formic acid catabolism:

1Formic acid → 1NADH + 1CO₂

1Ribulose−1,5−diP + CO₂
→2Glycerate−3−P + H₂O (catalysed by RuBisCo)

1Ribulose−5−P + ATP → Ribulose−1,5−diP + ADP

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Conflict of interest

None declared.

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