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ABSTRACT

A bioreactor was designed to provide high gas mass transfer to reach cell and product titres in the g L^{-1} level from CO_2 for realistic, laboratory scale, engineered autotrophic strain evaluation. The design was based on independent CO_2, H_2 and air inputs and the ability to operate at high pressures. The bioreactor configuration and cultivation strategy enabled growth of *Cupriavidus necator* strains for long periods, to reach over 3 g L^{-1} dry cell weight. No negative impact of the high pressure was observed on viability of the strains up to more than 4 bar overpressure. The cultivation was then carried out using an engineered isopropanol producing strain; in this case, 3.5 g L^{-1} isopropanol was obtained from CO_2 as the sole carbon source. This is the first reported demonstration of a successful production from engineered bacteria of product in the g L^{-1} range on CO_2, raising the prospect of future development of CO_2-based bioprocesses.

KEYWORDS

Ralstonia eutropha; *Cupriavidus necator*; isopropanol; carbon dioxide; hydrogen; bioreactor design; gas fermentation; autotrophy; pressure

ABBREVIATIONS

P(3HB): poly(3-hydroxybutyrate); TSB: tryptic soy broth; DCW: dry cell weight.

INTRODUCTION

Developing bioconversion processes based on CO_2 has gained increasing interest in the microbial production of biofuel and chemical synthons. To develop future bioprocesses for bio-based chemical synthesis using CO_2, the bacterium *Cupriavidus necator* is particularly attractive due to its efficient utilization of this sustainable carbon source. This facultative chemolithoautotroph, formerly also classified as *Ralstonia eutropha*, is the best-reported microorganism based on CO_2 as the sole carbon source. It has the ability to grow at high rates (in the range of 0.3-0.4 h^{-1}) to high cell densities using gas mixtures of $CO_2/H_2/O_2$ [1]. It has also demonstrated higher energy efficiencies in producing biomass and bioproduct, poly(3-hydroxybutyrate) (P(3HB)) compared to plants or microalgae [2]. *C. necator* has also been extensively studied for P(3HB) production in heterotrophy [3-6] and autotrophy [1]. This natural ability to store excess carbon in the form of P(3HB) is of great interest as numerous interesting chemicals share the same production pathway precursor acetyl-coA, which implies that fewer genetic modifications would be required to divert P(3HB) precursors into the final product [7, 8].

Recently, *C. necator* was successfully engineered for the production of various compounds under autotrophic conditions from a $CO_2/H_2/O_2$ mixture. Up to 250 mg L^{-1} isopropanol [8], 180 mg L^{-1} methyl ketones [9], 4.4 mg L^{-1} alka(e)nes [7] and 6 mg L^{-1} α-humulene [10] were obtained under autotrophic conditions. The limited amount of product reported here is due to a combination of strain efficiency and unsuitability of the laboratory-scale bioreactor for gas fermentation. The previous cultivation systems using classical bioreactor flushed with commercial gas mixtures have demonstrated production levels suitable only for proof of concept studies. In order to further develop the engineered strains for future CO_2-based

bioprocesses, laboratory-scale bioreactor systems have to be designed specifically for gas fermentation, allowing higher titres of biomass to be achieved and to characterize the strains under more realistic conditions in terms of product titres, productivity and yields.

Bioreactor designs for gas fermentation have previously been reported for cultivation of H_2 oxidising bacteria such as *C. necator*. They generally consist of gas mixtures containing CO_2, H_2 and O_2 added through a single sparger. The optimal gas composition employed in the literature for autotrophic growth of *C. necator* is typically $H_2/O_2/CO_2$=7:1:1 [11-13], $H_2/O_2/CO_2$=7:2:1 [12], or $H_2/O_2/CO_2$=6:2:1 [14, 15]. However, these gas mixtures lie within the explosive composition range (which is normally regarded as being above 6% O_2 in the presence of H_2) and limits the cell densities reached. In order to avoid this O_2 limitation during autotrophic growth of *C. necator*~~,~~ pure oxygen has been fed to the bioreactor [1]. This was combined with a basket-type agitator which generated huge increases in the k_La (up to 2970 h^{-1}), allowing a high cell density of 91g L^{-1} and a residual biomass containing 68% of PHB (29 g L^{-1} of catalytic biomass concentration) to be reached.

The design of a small laboratory-scale bioreactor dedicated to CO_2 fermentation of H_2-oxidizing bacteria is described here. The challenges tackled were (i) the danger of gas explosion above the limit of 6% O_2 in the presence of excess H_2 and (ii) the gas transfer efficiency at small scale. Reducing the size of a bioreactor is always more challenging for parameters such as gas transfer and necessitates the application of process engineering and biochemical engineering principles. The engineering solution to poor gas transfer is usually limited to the volumetric gas transfer coefficient, $\underline{k_La,}$ increase through changes in sparger and stirring arrangement as mentioned above. However, this offers relatively limited scope for improvement. An effective alternative is to operate the bioreactor at elevated pressure as this

can, in principle, increase the gas transfer rate several fold without changes to sparging or agitation. This concept has already been successfully applied in bioreactors with various microorganisms, such as *Arxula adeninivorans, Corynebacterium glutamicum, C. necator, Escherichia coli,* and *Saccharomyces cerevisiae* [16-20]. There is, however, a lack of information on its impact on cell viability and productivity with engineered microorganisms during long fermentations runs.

Moreover, in order to have more flexibility in terms of gas composition and therefore higher gas solubility, the bioreactor gas-supply system was designed with three independent gas lines supplying three spargers (CO_2, H_2 and air). A safety system was designed to control O_2 level in the headspace in order to avoid an explosive mixture within the bioreactor. Experimental validation was performed with autotrophic growth and production of isopropanol with an engineered *C. necator* strain.

MATERIALS AND METHODS

Strains

In this study, the reference strain used was *C. necator* Re2133, deficient for P(3-HB) biosynthesis (H16 ΔphaCAB) obtained from [21]. The isopropanol strain tested in this study was *C. necator* Re2133 bearing the plasmid pEG7b carrying a synthetic isopropanol operon under the constitutive pTac promoter [22]. The strains were stored at -80°C in liquid TBS medium (dextrose-free tryptic soy broth, Becton Dickinson, France) with 20 % glycerol (v/v).

Media

The rich medium consisted of 2.75% (w/v) dextrose-free tryptic soy broth (TSB, Becton Dickinson, France). The minimal medium A used for preculture in flask was previously

described by [21] and [7]. Fructose (20 g L^{-1}) was used as the only carbon source. NH_4Cl (0.5 g L^{-1}) was used as the nitrogen source to reach a biomass concentration of about 1 g L^{-1}. The minimal medium B used for the cultures in the bioreactor consisted of 0.19 g L^{-1} nitrilotriacetic acid, 0.06 g L^{-1} ferrous ammonium citrate, 0.5 g L^{-1} of $MgSO_4.7H_2O$, 0.01 g L^{-1} of $CaCl_2.2H_2O$, and 1 ml of trace element solution. The composition of the latter was 0.3 g L^{-1} H_3BO_3, 0.2 g L^{-1} $CoCl_2.6H_2O$, 0.1 g L^{-1} $ZnSO_4.7H_2O$, 0.03 g L^{-1} $MnCl_2.4H_2O$, 0.03 g L^{-1} $Na_2MoO_4.2H_2O$, 0.02 g L^{-1} $NiCl_2.6H_2O$, and 0.01 g L^{-1} $CuSO_4.5H_2O$. $(NH_4)_2SO_4$ (2 g L^{-1}) was used as nitrogen source to reach a biomass concentration of about 4 g L^{-1}. After autoclaving the above medium, 40 ml of a sterile phosphate solution containing 224 g L^{-1} $Na_2HPO_4.12H_2O$ and 37.5 g L^{-1} KH_2PO_4 was aseptically added to the fermenter.

Seed cultivations

After thawing at room temperature, one glycerol stock was streaked on a TSB agar Petri dish. and incubated for 36 h at 30°C. One isolated colony was used to inoculate the first seed culture grown for 24 h with 5 mL of TSB supplemented with 10 mg L^{-1} gentamycin and 200 mg L^{-1} kanamycin, in a 50 mL baffled Erlenmeyer flask. This culture was used to inoculate a 250 mL Erlenmeyer baffled flask containing 50 mL of mineral medium A. The seed was cultivated at 30°C, 100 rpm for 18h. The culture was then centrifuged (5 min, 10000g), the supernatant was discarded and the cell pellet was resuspended in 30 mL sterile fresh medium B to eliminate the residual fructose. The suspension was then used to inoculate the bioreactor containing 300 mL of sterile mineral medium B.

Culture conditions and bioreactor system

The cultures were performed in a 500 ml total volume Xplorer® (HEL Ltd, UK) bioreactor that was modified specifically for this study. The working liquid volume was 330 mL. The

bioreactor was first used with a commercial gas mixture containing $H_2/O_2/CO_2/N_2$ (molar %: 60:2:10:28) (Air Liquide, France) provided into the medium through a sparger. For the experiments carried out with individual gases, the bioreactor was then set up with three spargers in order to deliver air, pure CO_2 and pure H_2 independently. A fourth inlet gas was added in the headspace for pure N_2 supply as a safety system. Each gas inlet was controlled by a gas mass flow meter. The bioreactor was equipped with pH, dissolved oxygen (DO), temperature and pressure controllers. The Xplorer® software handled the on-line monitoring and control systems of the reactor. Both CO_2 and O_2 in the outlet gases were analysed using a Tandem Pro gas analyzer (Magellan BioTech HEL Ltd , UK). The bioreactor was safely controlled by the O_2 concentration in the headspace in order to avoid any explosive risk. The headspace was flushed with pure N_2 if the O_2 surpassed 5% in the headspace. The DO level in the reactor was controlled below 5% of air saturation at atmospheric pressure by varying stirring speed and/or inlet air flow rate in order to restrict the known inhibition of *C. necator* hydrogenases by the dissolved O_2 concentration [23]. Temperature was maintained at 30°C. The pH was maintained at 7.0 by addition of a 14% (v/v) NH_4OH solution.

Characterization of the biomass

Cell growth was followed spectrophotometrically at 600 nm with a DR3900 spectrophotometer (Hach, Loveland, Colorado, USA) after calibration against cell dry weight measurements. For cell dry weight determination, culture medium was harvested and filtered through 0.2 µm pore-size polyamide membranes (Sartorius AG, Göttingen, Germany), which were then dried to a constant weight at 60°C under partial vacuum (200 mmHg, i.e. approximately 26.7 kPa).

Cell viability was measured with a BD Accuri C6® flow cytometer (BD Biosciences, US) after cell staining with propidium iodide (PI) dye (Molecular Probes, Invitrogen, USA) commonly used to monitor membrane integrity as indicator of viability [8]

Metabolites and statistical analyses

Metabolites (isopropanol, acetone, pyruvic and acetic acids) were quantified by chromatography as described in [22].

Statistical analyses

Concentrations of metabolites and biomass are given as the mean value of 2 to 3 independent analyses. The error on the specific growth rate (determined as being the slope of ln[Optical Density] = f(t)) was calculated as the standard deviation (SD) of the slope. The instantaneous specific growth rates were calculated upon fitting process of the experimental data and the derivative calculation. For the yield determination, the state variables were plotted pairwise in a scatter plot within the considered period of the culture. A linear regression was applied to determine the yield (as the slope) and the error (as the SD of the slope). For the calculation of the CO_2 to isopropanol conversion yield, the isopropanol amount was plotted versus the amount of consumed CO_2, calculated from a classical mass balance from the analyses of inlet and outlet gas composition.

RESULTS & DISCUSSION

Benefits of elevated pressure on autotrophic growth of *C. necator* Re2133 on a commercial gas mixture

Autotrophic production of chemicals by *C. necator* was previously reported in a classical CSTR bioreactor where gases were supplied by flushing the reactor with a commercial gas

166 mixture containing $H_2/O_2/CO_2/N_2$ mix (molar %: 60:2:10:28) [7, 8]. Here, this mixture was
167 used first to characterize the growth of the reference strain *C. necator* Re2133 in the newly
168 designed bioreactor (**Figure 1**). After 6 h cultivation, the maximum values of agitation and
169 ventilation rates were reached. The biomass concentration was low at 0.2 g L^{-1}. An alternative
170 way to provide higher gas transfer is to operate the bioreactor at elevated pressure. Then the
171 dissolved O_2 concentration was kept at 2% saturation, at various pressures in the reactor up to
172 3 bar overpressure. Growth was then extended to 15 h leading to a final biomass
173 concentration of 0.5 g L^{-1}. Growth followed an exponential profile throughout the cultivation
174 with a maximum specific rate of $0.13 \pm 0.01 h^{-1}$. Cell viability was analysed by flow cytometry
175 after staining with propidium iodide. The non-PI stained cells were considered viable, and the
176 percentage was >95% over the duration of the culture, even when overpressure reached 3 bar,
177 indicating their robustness towards high pressure.

178
179 **Benefits of independent gas sparging on autotrophic growth of *C. necator* Re2133**
180 The relatively low final biomass concentration obtained even under high pressure was likely
181 due to the low gas transfer capacity when using the mixture containing $H_2/O_2/CO_2/N_2$ (molar
182 %: 60:2:10:28). This composition was far from optimal for autotrophic growth of *C. necator*.
183 However, they are all within the explosive range for H_2 and O_2. The cultivation of the Re2133
184 strain was initiated by sparging the 3 independent gases in order to reach 88% H_2, 2% O_2 and
185 2.5%CO_2 gas mixture in the reactor (62, 6.7 and 1.75 mL/min, for H_2, air and CO_2). It
186 allowed ensuring an H_2-rich (H_2 being the less soluble gas) and safe environment controlling
187 the O_2 % in the headspace below the explosive range (6% of air saturation at atmospheric
188 pressure). The level of dissolved O_2 was controlled to meet the increasing biological demand
189 while maintaining the level at 2% saturation in order to limit the reported inhibition of the

190 hydrogenases [23, 24] by increasing the ratio air/H_2 flow rate within the range of 6.7-25
191 mL/min for air and 62-86 mL/min for H_2 (**Figure 2**). The duration of growth at atmospheric
192 pressure was 15 h compared to 6 h with the reactor operating with the gas mix, illustrating the
193 benefit of three independent spargers. Growth followed an exponential profile throughout the
194 cultivation at atmospheric pressure with a maximum specific rate of 0.11 ± 0.01 h^{-1}. At 15 h,
195 inlet gas composition was 70% H_2, 5% O_2 and 2.8% CO_2 (71, 26 and 2.8 mL/min for H_2, air
196 and CO_2). When the inlet gas composition for O_2 reached the safety level (5%) defined to
197 ensure a safe environment, the dissolved O_2 level was maintained by switching the strategy to
198 a step-wise increase in total reactor pressure up to >3 bars. The growth profile was still
199 exponential without any observed drop in specific rate (0.11 ± 0.01 h^{-1}). A final pressure up
200 to 4.2 bars was applied with a concomitant increase in H_2 (117 mL/min) and a decrease in O_2
201 level in the inlet gas (3.7%) in order to maintain the dissolved oxygen at a low level. The
202 amount of CO_2 in the outlet gas was always found to be lower than in the inlet and never
203 exceeded 1.6%. During this period, the specific growth was lower but constant at 0.07 ± 0.005
204 h^{-1}. Cell viability was assessed by flow cytometry and did not show any fall in the percentage
205 of PI unstained cells.

206 **Isopropanol production under autotrophic condition**

207 Isopropanol autotrophic production was tested in the designed bioreactor using an engineered
208 strain Re2133/pEG7b [22]. Cultivation was initiated by sparging the three independent gases
209 in order to reach 87% H_2, 2% O_2 and 3%CO_2 mixture in the reactor (59, 6.7 and 2 mL/min
210 respectively for H_2, air and CO_2) allowing an H_2-rich, O_2-low environment to be reached. The
211 level of dissolved O_2 was monitored throughout by increasing the ratio air/H_2 flow rate and
212 the overpressure in the bioreactor in order to fulfill the increasing microbial O_2 demand

(**Figure 3**). The gas flow rates ranged between 59-200 mL/min for H_2, 6.7 -75 mL/min for air and 2-6 mL/min for CO_2. *C. necator* Re2133/pEG7b grew for 20 h at an average specific rate of 0.051±0.006 h^{-1}. Growth slowly decreased to 0.023±0.001 h^{-1} for the remaining period of the cultivation. The final biomass concentration reached at 85 h was 2.9 g L^{-1}. Compared to the reference strain Re2133, the lower growth rate was likely due to inhibition by isopropanol and acetone as previously observed with the same strain with fructose (0.05 *vs* 0.17 h^{-1}, for Re2133 and Re2133/pEG7b respectively) [22]. Isopropanol and acetone were produced concomitantly to reach maximum concentrations in the liquid phase of 3 and 0.65 g L^{-1} respectively. Any isopropanol and acetone evaporated with the gas stream was trapped in water and quantified during the cultivation. Taking this evaporation into account, the maximal concentrations were equivalent to 3.5 and 0.9 g L^{-1} of isopropanol and acetone, respectively. Reproducibility was assessed with a second independent cultivation and led to the production of 1.96 g L^{-1} biomass, 2.1 g L^{-1} isopropanol and 1 g L^{-1} acetone. These performances were remarkable when compared to that obtained with fructose: Re2133/pEG7b was able to produce up to 2.2 g L^{-1}isopropanol and 1 g L^{-1} acetone in 88 h with fructose [22]. The overall specific production rate of isopropanol in *C. necator* Re2133/pEG7b from CO_2 was 0.013 $gg_{DCW}^{-1}h^{-1}$ (0.030 $gg_{DCW}^{-1}h^{-1}$ with fructose) and peaked at 0.053 $gg_{DCW}^{-1}h^{-1}$ with CO_2 (0.085 $gg_{DCW}^{-1}h^{-1}$ with fructose). Cell viability was assessed by flow cytometry after staining with PI during the cultivation. The percentage of PI unstained cells ranged from 82-98%, which was slightly lower cultivation of Re2133 in the pressurized bioreactor. Concomitantly with the reduction in the μ_{max}, this could be attributed to an inhibitory effect of isopropanol on Re2133/pEG7b.

The proof of concept of producing isopropanol from CO_2 with an engineered *C. necator* strain was previously demonstrated: 250 mg L^{-1} isopropanol was obtained from 1 g L^{-1} biomass using a classical bioreactor and commercialized gas mix containing H_2, O_2 and CO_2 (60:2:10) [8]. It is clearly demonstrated here that designing a pressurized bioreactor with independent gas feeds significantly improved gas transfer to reach higher biomass and consequently higher isopropanol concentrations of up to 3.5 g L^{-1}. The calculation of the carbon balance in this fermentation showed that 70-80% of the CO_2 was recovered into biomass, isopropanol and acetone. The yield of CO_2 converted into isopropanol was 0.13 Cmol $Cmol^{-1}$ in this study, slightly lower than 0.2 Cmol $Cmol^{-1}$ reached previously. [8]. However, strain Re2133/pEG7b used here constitutively expressed the isopropanol synthetic operon whereas Re2133/pEG23 previously tested had an inducible isopropanol operon controlled by the arabinose promoter. Thus uncoupling of growth and production allowed the carbon flow to be better directed towards the isopropanol pathway, as reported with fructose [22].

CONCLUSIONS

The design of a dedicated gas bioreactor incorporating the independent management of the three gases, CO_2, H_2 and Air, coupled with the use of high pressure has enabled a dramatic improvement in gas transfer in a small scale bioreactor. *C. necator* showed an ability to sustain a pressure of over 4 bars, leading to a higher biomass concentration and an isopropanol production of up to 3.5 g L^{-1}. This is the first reported demonstration successfully producing compounds at the g L^{-1} scale from engineered autotrophic bacteria and raises the prospect of the near-future development of CO_2-based commercial bioprocesses.

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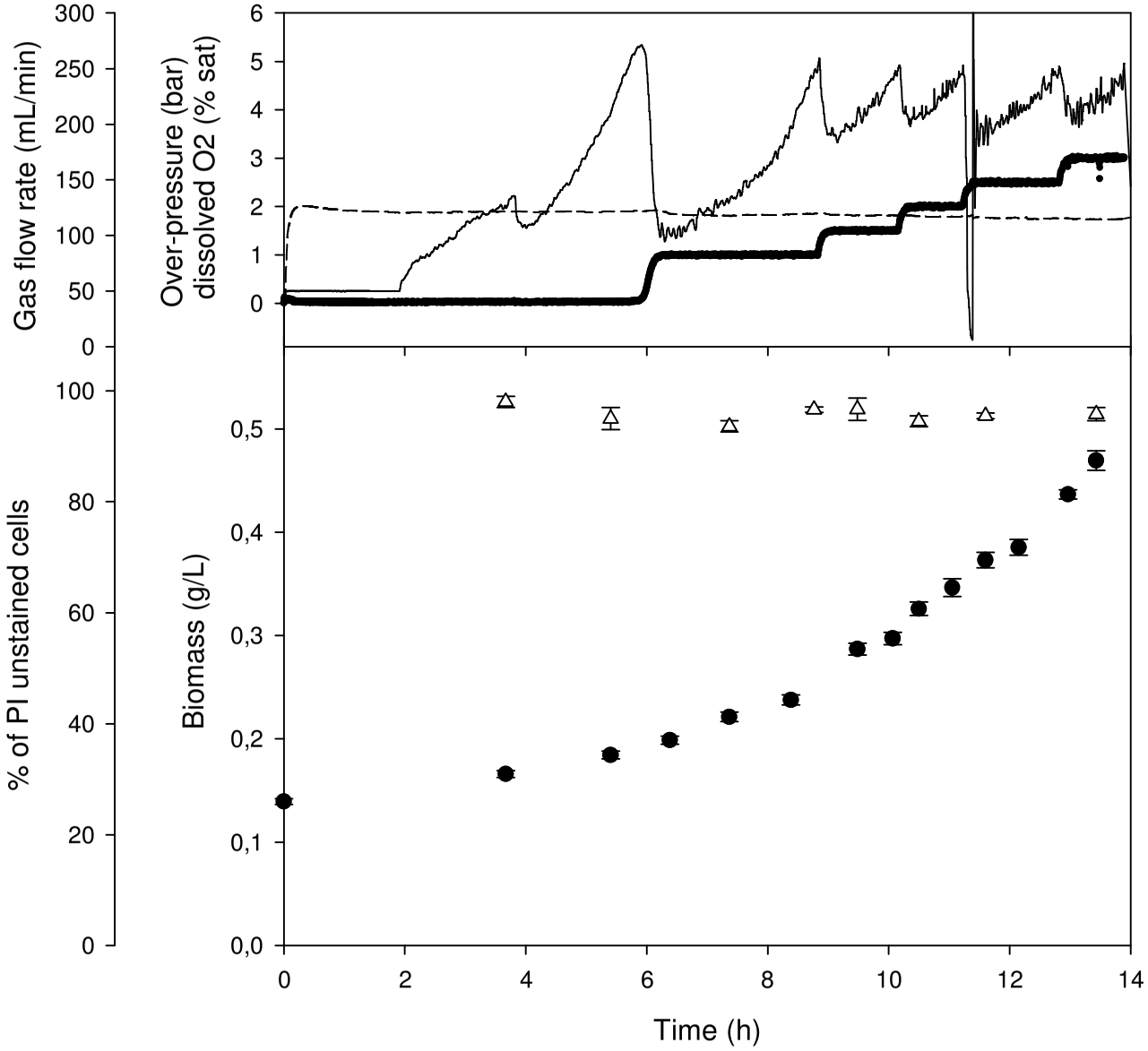
Figure legends

Figure 1: (A) Evolution of the dissolved O_2 level (dashed line), the total gas flow rate (thin black line) and the overpressure (thick black line) in the bioreactor. (B) Evolution of the biomass concentration (black circles) and cell viability (open triangles)

Fig.2: Autotrophic growth of *C. necator* Re2133 in the gas bioreactor modified with 3 independent gas spargers. (**A**) Evolution of relative pressure (thick line) and partial pressure of dissolved oxygen (dashed line). (**B**) Evolution of the inlet gas composition for H_2 (solid thick line), O_2 (dashed line) and CO_2 (solid thin line) .(**C**) Evolution of biomass concentrations (●) and % of PI unstained cells (Δ).

Fig.3: Autotrophic isopropanol production of *C. necator* Re2133/pEG7b in the gas bioreactor modified with 3 independent gas spargers. (**A**) Evolution of the relative pressure (thick line) and the partial pressure of dissolved O_2 (dashed line). (**B**) Evolution of the inlet gas composition in H_2 (solid thick line), O_2 (dashed line) and CO_2 (solid thin line). (**C**) Evolution of biomass concentrations (●), % of PI unstained cells (△) and isopropanol (□), acetone (▽) concentrations in the bioreactor liquid phase. Total isopropanol (■) and acetone (▼) production were calculated taking amounts evaporated into account.

Figure 1


Gas flow rate (mL/min)
Over-pressure (bar)
dissolved O2 (% sat)
% of PI unstained cells
Biomass (g/L)
Time (h)
0
50
100
150
200
250
300
1
2
3
4
5
6
20
40
60
80
0,0
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0,3
0,4
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2
4
6
8
10
12
14

Figure 2

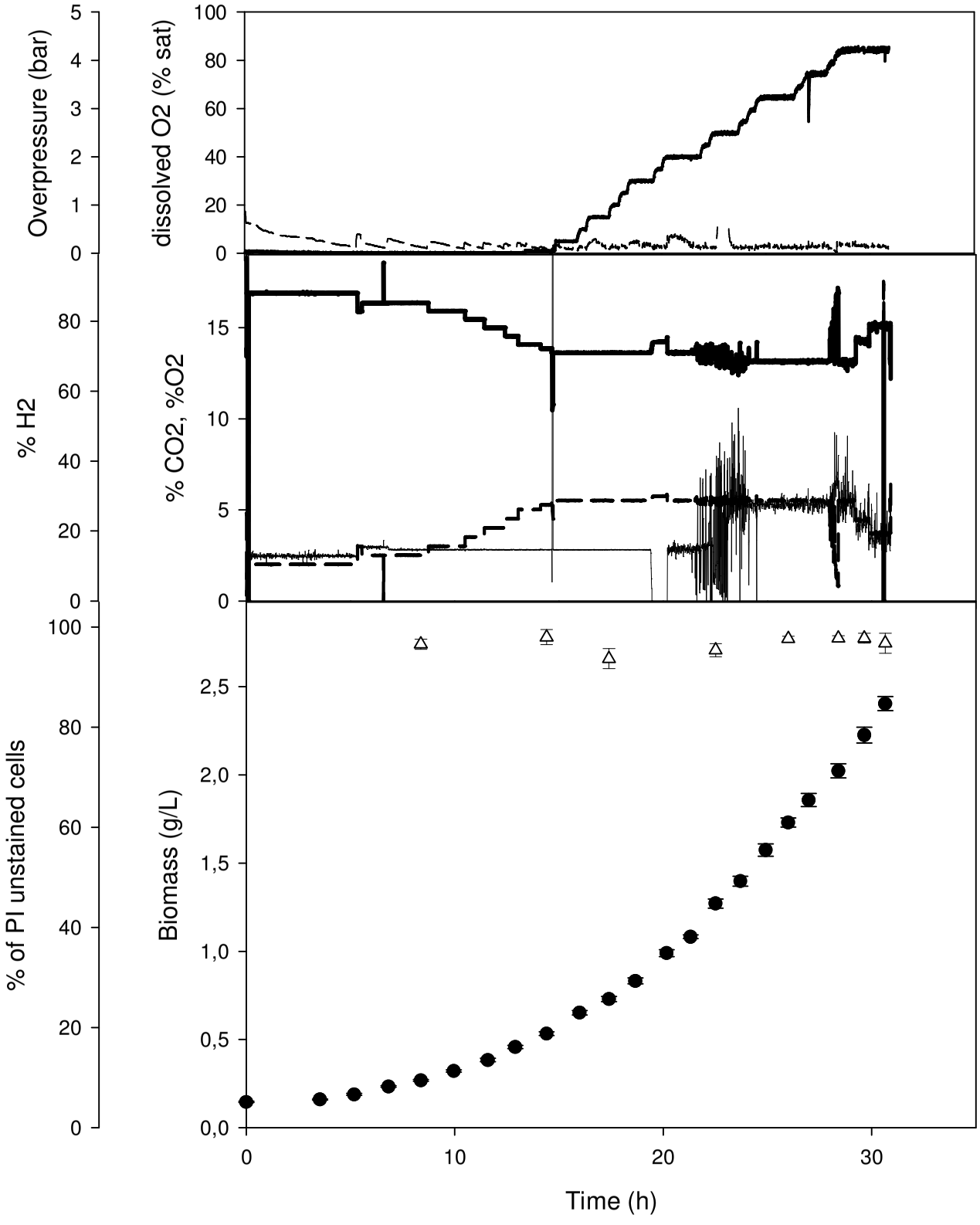
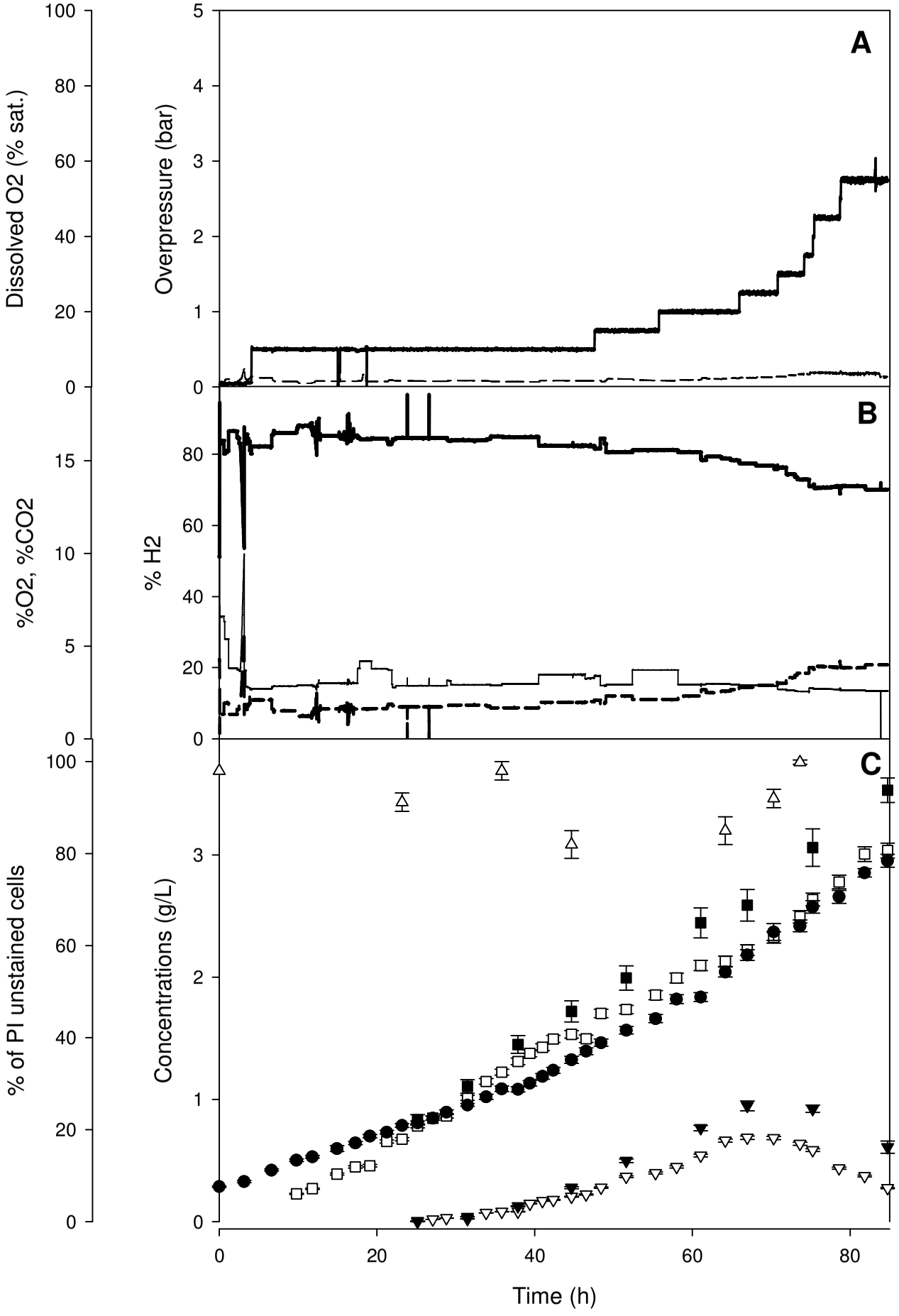

Overpressure (bar)
dissolved O2 (% sat)
% H2
% CO2, %O2
% of PI unstained cells
Biomass (g/L)
Time (h)

Figure 3


A
B
C
Dissolved O2 (% sat.)
Overpressure (bar)
%O2, %CO2
% H2
% of PI unstained cells
Concentrations (g/L)
Time (h)
0
20
40
60
80
100
1
2
3
4
5
15
10