


## ARTICLE

# Downscaling screening cultures in a multifunctional bioreactor array-on-a-chip for speeding up optimization of yeast-based lactic acid bioproduction

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## Funding information

Österreichische Forschungsförderungsgesellschaft, Grant/Award Number: ACIB

## Abstract

A key challenge for bioprocess engineering is the identification of the optimum process conditions for the production of biochemical and biopharmaceutical compounds using prokaryotic as well as eukaryotic cell factories. Shake flasks and bench-scale bioreactor systems are still the golden standard in the early stage of bioprocess development, though they are known to be expensive, time-consuming, and labor-intensive as well as lacking the throughput for efficient production optimizations. To bridge the technological gap between bioprocess optimization and upscaling, we have developed a microfluidic bioreactor array to reduce time and costs, and to increase throughput compared with traditional lab-scale culture strategies. We present a multifunctional microfluidic device containing 12 individual bioreactors ( $V_t = 15 \mu\text{l}$ ) in a 26 mm  $\times$  76 mm area with in-line biosensing of dissolved oxygen and biomass concentration. Following initial device characterization, the bioreactor lab-on-a-chip was used in a proof-of-principle study to identify the most productive cell line for lactic acid production out of two engineered yeast strains, evaluating whether it could reduce the time needed for collecting meaningful data compared with shake flasks cultures. Results of the study showed significant difference in the strains' productivity within 3 hr of operation exhibiting a 4- to 6-fold higher lactic acid production, thus pointing at the potential of microfluidic technology as effective screening tool for fast and parallelizable industrial bioprocess development.

## KEYWORDS

bioanalytics, bioprocess development, lactic acid production, microfluidics, *Saccharomyces cerevisiae* (*S. cerevisiae*), screening phase, sensor

Damiano Totaro and Mario Rothbauer contributed equally to this study.

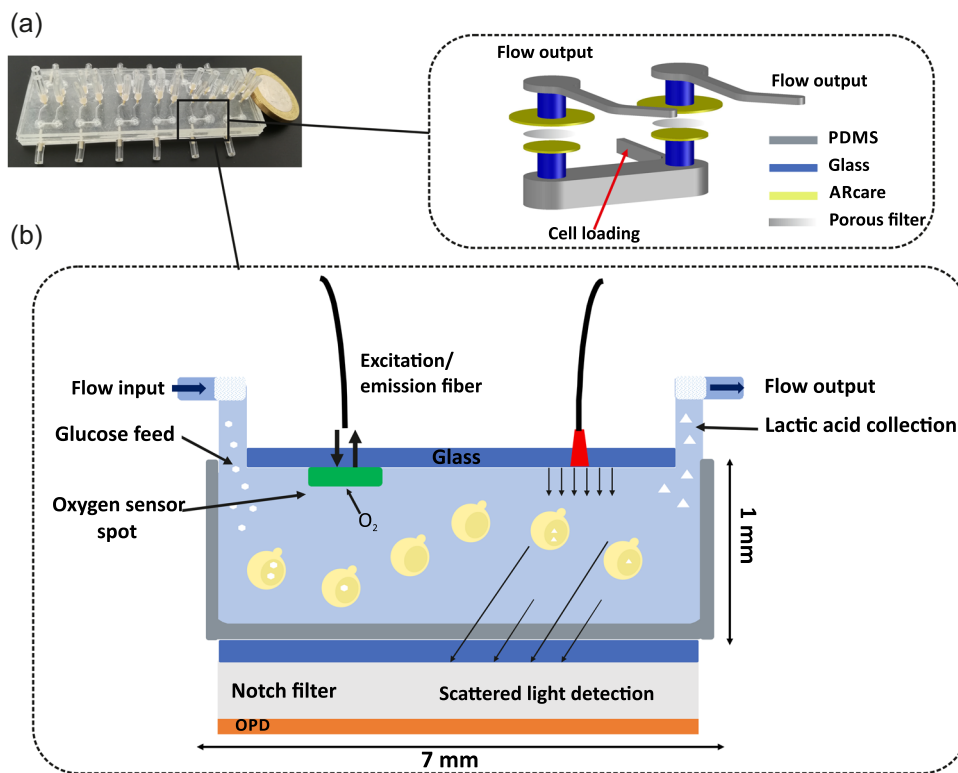
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## 1 | INTRODUCTION

The key challenges in the development of a bioprocess (Hidalgo et al., 2018; Kuo et al., 2018; Paul, Sangeetha, & Deepika, 2019; Zeng, 2019; Zhang, Li, Wang, Yang, & Yan, 2018) are associated with (a) determining the most suitable host (and clone) for the production and (b) optimization of a large number of input variables such as pH, oxygen, cell density, and metabolic profile as well as productivity variations under certain conditions (Hidalgo et al., 2018). Despite these challenges, traditional shake flasks and bench-scale bioreactors (ranging from 10 ml to 10 L volume) are most commonly used during the early stages of bioprocess development, which makes strain selection and bioprocess optimization still an expensive, time-consuming, and labor-intensive undertaking, as only a limited number of experiments can be carried out simultaneously (Sokolov et al., 2017). A common strategy to reduce time and costs is therefore based on process automation and utilization of miniaturized technologies such as microtiter plates (Hansen et al., 2015). Microfluidic technology constitutes another alternative offering analytical power, design flexibility, ease in the manipulation of the system but also more complex fluidic architectures than large-scale systems (H. Kim et al., 2016), as well as high throughput capability (Ali, Shaegh, Pourmand, & Nabavinia, 2018; Chen, Shen, & Zhou, 2016; Halldorsson, Lucumi, Gómez-sjöberg, & Fleming, 2015). In one of the first attempts, Kostov, Harms, Randers-Eichhorn, and Rao (2001) developed a 2 ml microreactor with integrated optical

systems for pH, biomass, and oxygen detection. Subsequently, a constant effort was made toward a further downscaling of similar systems (Szita et al., 2005; Zanzotto et al., 2004) aiming at optimizing the design, analytical integration (Lladó Maldonado et al., 2019), and implementation of flow-through setups for investigating on how population dynamics could be controlled by tuning the conditions at the microscale (Balagaddé, You, Hansen, Arnold, & Quake, 2005; Groisman et al., 2005; M. Kim, Bae, & Kim, 2017; K. S. Lee, Boccazzi, Sinskey, & Ram, 2011). Recent examples include a perfusion-capable microdevice for the investigation of secondary metabolite production using microalgae cultures (Paik, Sim, & Jeon, 2017) and a more sophisticated device termed as multiphase microbioreactor for the study of kinetic parameters of *Saccharomyces cerevisiae* cultures under the Crabtree effect (Krull & Peterat, 2016). However, to meet industrial expectations, next generation analytical screening tools for bioprocess optimization and strain productivity evaluations need to also demonstrate proper balance among manufacturing costs, complexity and experimental throughput required for each development stage, as well as a high versatility for a broad range of applications in terms of bioprocesses and variables input (Willart & Goossens, 2015). To address this study to product gap, we present a sensor-integrated bioreactor array-on-a-chip system for the screening of engineered yeast cultures, capable of performing batch and perfused cultivations, with integrated analytical modules for oxygen and biomass monitoring (Figure 1; Gruber, Marques, Szita, & Mayr, 2017). Our setup enabled a continuous, real-time, and noninvasive monitoring



**FIGURE 1** (a) Multiplexed microfluidic platform with 12 individual 15- $\mu$ l cultivation units for bioconversion of glucose into lactic acid. Details of the three-dimensional structure design and materials employed for the microfabrication. (b) Cross-section of single cultivation units: schematic illustration of its main features, integrated optical sensors and process setup [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

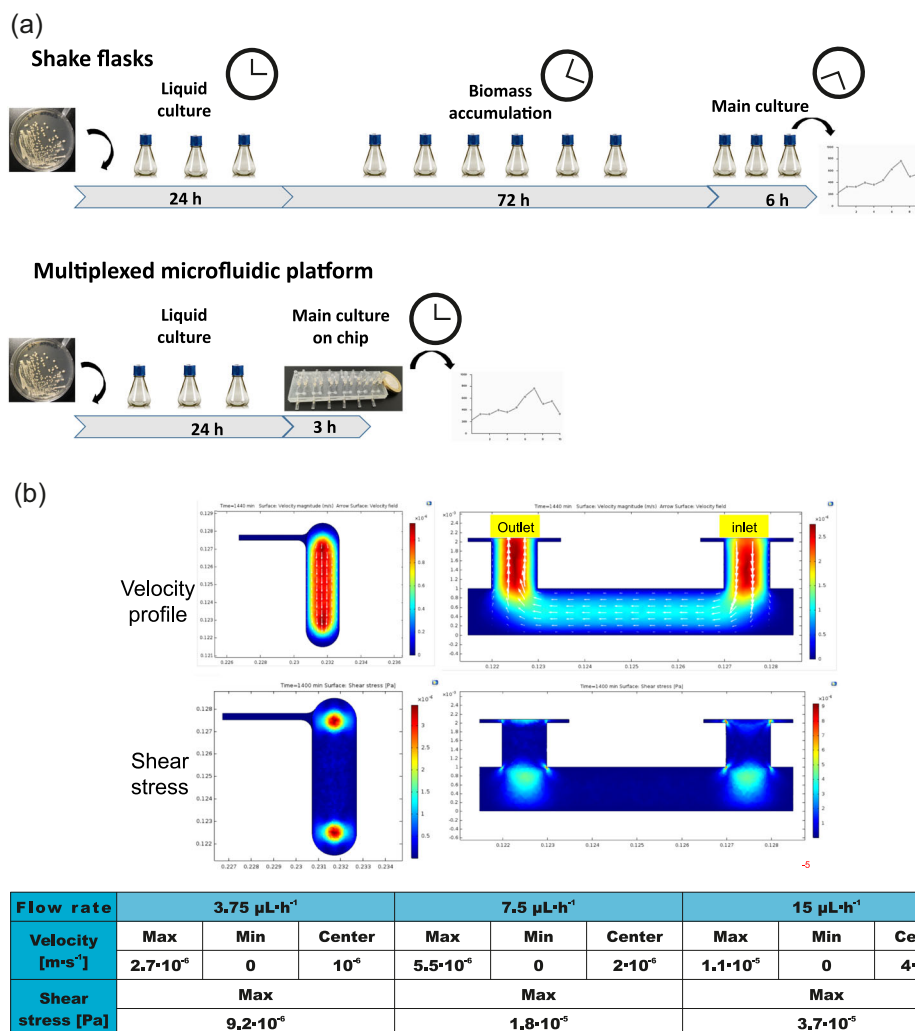
of main process parameters during bioproduction. As an initial proof-of-principle for more rapid screening within a few hours, the productivity of two engineered *S. cerevisiae* strains was evaluated on their ability to convert glucose into lactic acid under varying cultivation conditions (Miller et al., 2011; Sauer, Porro, Mattanovich, & Branduardi, 2010). The performances of the engineered strains were compared using our microfluidic bioreactor array-on-a-chip platform (under various conditions) and standard shake flask cultivations to evaluate the efficacy of the miniaturized screening compared with benchmark tools (Figure 2a).

## 2 | MATERIALS AND METHODS

### 2.1 | Chip design and microfabrication

Microfluidic chips were designed with AutoCAD (AutoCAD 2017; Autodesk) and the micropatterns were structured into

500- $\mu\text{m}$ -thick polydimethylsiloxane (PDMS) sheets (Superclear silicone sheets, MVQ Silicones GmbH, Germany) and 80- $\mu\text{m}$ -thick double-sided biomedical-grade pressure-sensitive adhesive tape (ARcare8259; Adhesive Research, Ireland) using a GS-24 vinyl cutter (Roland DGA Corporation, Germany). The microdevices were assembled from three different intermediate structures including a lower one featuring PDMS culture chambers (P1), an intermediate cell retention structure (P2), and a top PDMS microchannel network for collection of the filtrate (P3). Each individual 15- $\mu\text{l}$  cultivation unit of the microfluidic device had one inlet for medium supply and one outlet for filtrate collection (P3) and one channel for cell inoculation in P1 that was clogged after inoculation. The lower microfluidic layers that build up the 15- $\mu\text{l}$  cultivation chambers in P1 (see Figure S1a) were air plasma treated (Harrick Plasma, High Power, 2 min) and sandwiched starting with a 250- $\mu\text{m}$ -thick PDMS bottom layer followed by two 500- $\mu\text{m}$ -thick sheets. Similarly, filtrate collection structure



**FIGURE 2** (a) Schematic time-line and workflow for the screening process of the present glucose bioconversion into lactic acid: comparison between shake flask and multiplexed microfluidic platform highlighting how the latter one could speed up the whole process. (b) Computational fluid dynamic (CFD) simulations of flow velocity profile and shear stress during on-chip yeast perfusion culture modes. Calculation for different flow rates ranging from 3.75 to 15  $\mu\text{L}\cdot\text{h}^{-1}$  [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

P3 comprised of a 250- $\mu\text{m}$ -thick PDMS microchannel layer and a 250  $\mu\text{m}$  top sealing layer connected to PEEK tubing (1/32" outer diameter, 250- $\mu\text{m}$  inner diameter). All plasma-treated layers were annealed at 70°C for 10 min to increase the bonding strength. The intermediate cell retention structure P2 comprised of a 0.4  $\mu\text{m}$  pore-size porous filtration membrane (Polyester Membrane Filters; Sterlitech Corporation, WA) embedded in a sandwiched structure of two microscope glass slides and microstructured pressure-sensitive adhesive tape. Layers were put into contact and bonded by applying a gentle pressure between the two glass slides. Inlet and outlet holes with 0.9 mm diameter were drilled through the glass slides to connect the bottom cultivation structure P1 with the top microfluidic channel network P3. Oxygen sensor spots were immobilized at the bottom of P3 lower glass slide (which will represent the top of the chambers), as it is described in Section 2.5. Finally, the device assembly was completed by bonding P1 and P3 to the glass surfaces of P2 using oxygen plasma and a final annealing step at 70°C for 10 min.

## 2.2 | Strains

A  $\Delta\text{pdc1 } \Delta\text{pdc5 } \Delta\text{pdc6 } S. cerevisiae$  strain was transformed with a plasmid carrying the lactate dehydrogenase (LDH) gene of *Lactobacillus plantarum* under the control of the yeast *TPI1*-promoter ("LAC"). The engineered *S. cerevisiae* strain lacked pyruvate decarboxylase (PDC) for more efficiently redirecting pyruvate to LDH, although ethanol was needed in the growth medium as  $\text{C}_2$  source for the supply of cytosolic acetyl-CoA and biomass formation. The utilization of this metabolic pathway led to a lower growth rate of the engineered strains if compared with wild-type ones (Sauer et al., 2010). To improve lactic acid production and the tolerance to low pH and high lactic acid concentrations, the strain was subjected to adaptive laboratory evolution over 3 months (around 250 generations). The cells were diluted every 24 hr to  $\text{OD}_{600}$  0.5 in fresh medium containing 150  $\text{g}\cdot\text{L}^{-1}$  glucose (D(+)-glucose monohydrate, Carl Roth GmbH + Co. KG, Germany), 5  $\text{g}\cdot\text{L}^{-1}$  ethanol (ethanol 96%, Merck Millipore, Germany), 4.54  $\text{g}\cdot\text{L}^{-1}$  urea (Carl Roth GmbH + Co. KG, Germany), and 3.4  $\text{g}\cdot\text{L}^{-1}$  yeast nitrogen base without amino acids and ammonium sulfate (YNB, Becton, Dickinson and Company, France) and grown in shake flasks at 30°C, 180  $\text{min}^{-1}$ . At the end of each one-day cultivation period, the cell suspension reached a lactic acid concentration of around 5  $\text{g}\cdot\text{L}^{-1}$  and a pH of 2.7-2.9. After 190 generations, the cells were restreaked on Petri dishes containing agar medium with 3.4  $\text{g}\cdot\text{L}^{-1}$  YNB, 4.54  $\text{g}\cdot\text{L}^{-1}$  urea, and 10  $\text{g}\cdot\text{L}^{-1}$  ethanol, and incubated at 30°C for 72 hr. Afterwards, single colonies were resuspended in fresh medium containing 150  $\text{g}\cdot\text{L}^{-1}$  glucose, 5  $\text{g}\cdot\text{L}^{-1}$  ethanol, 4.54  $\text{g}\cdot\text{L}^{-1}$  urea, 3.4  $\text{g}\cdot\text{L}^{-1}$  YNB, and 5  $\text{g}\cdot\text{L}^{-1}$   $\text{CaCO}_3$  (Carl Roth GmbH + Co. KG, Germany) at a starting OD of 3, and grown in shake flasks at 30°C, 180  $\text{min}^{-1}$ . After 96 hr, the clone with the highest lactic acid production was selected and named LACe. In the reported study, LACe was compared with the nonevolved parental strain LACp.

## 2.3 | Yeast cultivation and shake flask lactic acid production

The *S. cerevisiae* wild-type strain was inoculated from cryo-vials on Petri dishes containing YPD agar medium with 20  $\text{g}\cdot\text{L}^{-1}$  glucose and incubated at 30°C for 48 hr. Subsequently, the cells were transferred in shake flasks containing medium with 3.4  $\text{g}\cdot\text{L}^{-1}$  YNB, 4.54  $\text{g}\cdot\text{L}^{-1}$  urea, and 30  $\text{g}\cdot\text{L}^{-1}$  glucose and incubated at 28°C, 180  $\text{min}^{-1}$ . After 24 hr, the cells were centrifuged (2,000g, 10 min, 20°C) and re-suspended in the same medium at a biomass concentration of  $\text{OD}_{600} = 0.5$  and 15  $\mu\text{l}$  aliquots were inoculated inside the device for oxygen and biomass concentration monitoring during cell proliferation on chip.

*S. cerevisiae* engineered strains (LACe and LACp) were inoculated from cryo-vials on Petri dishes containing agar medium (YNB + A) with 3.4  $\text{g}\cdot\text{L}^{-1}$  YNB, 4.54  $\text{g}\cdot\text{L}^{-1}$  urea, and 5  $\text{g}\cdot\text{L}^{-1}$  ethanol and maintained at 30°C for 72 hr. For biomass formation, YNB + E medium containing 3.4  $\text{g}\cdot\text{L}^{-1}$  YNB, 4.54  $\text{g}\cdot\text{L}^{-1}$  urea, and 5  $\text{g}\cdot\text{L}^{-1}$  ethanol was used (the strain required ethanol supply for biomass formation, as described in the previous paragraph). YNB + G medium containing 3.4  $\text{g}\cdot\text{L}^{-1}$  YNB, 4.54  $\text{g}\cdot\text{L}^{-1}$  urea, and 100  $\text{g}\cdot\text{L}^{-1}$  glucose was used for lactic acid bioconversion.

For shake flask cultivation experiments, the cells cultivated on YNB + A petri dishes were transferred into 10 ml YNB + E medium and incubated at 30°C, 180  $\text{min}^{-1}$  and a relative humidity of 70%. A 1:10 volume ratio (volume of liquid to volume of shake flask) was kept constant for every preculture. After 24 hr, the cells were centrifuged (2,000g, 10 min, 20°C), re-suspended in YNB + E at a biomass concentration of  $\text{OD}_{600} = 0.7$  and further propagated for another 48 hr. On the fourth day, cells were centrifuged (2,000g, 10 min, 20°C) and re-suspended in YNB + G at a biomass concentration of  $\text{OD}_{600} = 40$  for lactate production experiments. All shake flask experiments for lactic acid production were performed in triplicates, with 30 ml culture volume inside 100 ml shake flasks that were incubated at 30°C, 180  $\text{min}^{-1}$ , and RH = 70%. Samples were drawn after 6, 12, and 24 hr from inoculation for biomass concentration, pH measurements, and organic compounds detection by high-performance liquid chromatography (HPLC) after centrifugation and cell harvesting (2,000g, 10 min, 4°C).

## 2.4 | Microfluidic cell culture under batch and perfusion mode

Before inoculation, the microfluidic devices and tubing were treated with 70% ethanol for 30 min, and subsequently with 50% isopropanol and DI water to remove solvent residues. All devices were allowed to dry at 35°C overnight ensuring absence of ethanol residues. All experiments were carried out in quadruplicates. For comparison of static on-chip cultures with shake flask cultures, 15  $\mu\text{l}$  culture aliquots (YNB + G medium, initial  $\text{OD}_{600} = 40$ ) were injected inside the chambers of the microfluidic device with the aid of a GC-gastight glass syringe. The device was incubated under constant conditions of

30°C and 70% relative humidity to reduce evaporation through PDMS (Kumar, Wittmann, & Heinzle, 2004). Due to the low volume imprint of the microfluidic device, for quantification of glucose and lactic acid four individual 15 µl cultures were pooled for 6, 12, and 24 hr in quadruplicates for each time point.

For on-chip perfusion cultures, the inlet ports of the device were connected through PEEK tubing (1/32" outer diameter, 250 µm inner diameter) to plastic syringes filled with sterile YNB + G; the outlet ports of the device were connected through other PEEK tubing to safe-lock tubes where the effluent coming from the chamber was collected. The syringes were placed inside an infusion pump (PHD ULTRA infuse/withdraw programmable pump, Hugo Sachs Elektronik - Harvard Apparatus GmbH, Germany) and 6 µl medium was pumped inside the channels at 4 µl·min<sup>-1</sup> flow rate so that the whole volume of the inlet channels (up to the chamber filter) could be filled with liquid removing all the air. Afterwards, 15 µl culture aliquots were injected inside the chambers with the aid of a GC-gastight glass syringe through the inoculation channels, which were then sealed, and the pump was turned on again to start the perfusion. The device was then incubated at 30°C and RH = 70% to avoid evaporation and bubble formation. Samples were collected from the outlet tubes after 3, 6, 12, and 24 hr for organic compounds quantification, always replacing the collection tube with a new sterile one. Three different flow rates of 15, 7.5, and 3.75 µl·hr<sup>-1</sup>, corresponding to hourly perfusion rates of  $P = 1, 0.5, \text{ and } 0.25 \text{ hr}^{-1}$ , were tested and compared with static and shake flask cultures.

## 2.5 | Oxygen and biomass monitoring on chip

The synthesis of the oxygen-sensitive particles as well as their characterization and calibration were described in previous works (Ehgartner, J., Strobl, et al., 2016; Ehgartner, Sulzer, et al., 2016). Oxygen monitoring was carried out at a sampling frequency of 1 Hz using a FireStingO2 optical oxygen meter (Pyroscience, Germany) connected to optical fibers (length 1 m, outer diameter 2.2 mm, fiber diameter 1 mm; Sticker et al., 2019). The sensor spots were integrated inside the device by immobilizing the particles on the top (made of glass, see Figure 1 and Figure S1a) of each chamber, based on a procedure reported in a previous work (Sticker et al., 2019). The system setup of light scattering measurements for biomass concentration monitoring has been described elsewhere (Charwat et al., 2013). Optical light scattering measurements were conducted using a computer-controlled shutter that timed laser exposure (one opening per minute). The organic photodiodes (OPDs) were kept at a reverse bias of -5 V. OPD currents were voltage converted and amplified by an operational amplifier (LM6132AIM/NOPB from National Semiconductor Operation), digitally converted by a microcontroller (ATmega32; Atmel), and readout by a Labview program. The optical setup was calibrated to get a correlation between recorded voltage and optical density (see Supporting Information Methods and Supporting Information Results in the Electronic Supporting Information file).

## 2.6 | HPLC and OD measurements

Optical density for biomass concentration was determined by appropriate dilution of culture broth to an absorbance of 0.1–0.7 at 600 nm measured by a photometer (Biochrom WPA CO8000 Cell Density Meter). The concentrations of residual glucose and lactate were determined by HPLC analysis using a method for the detection of carboxylic acids and sugars previously established in our laboratory (Pflügl, Marx, Mattanovich, & Sauer, 2012).

Concerning shake flasks and on-chip static cultures, the quantitative analysis by HPLC was used to calculate the biomass-specific lactic acid production rate ( $q_{lac}$ ), as follows using Equation 1:

$$q_{lac} = \frac{\Delta c_{lac}}{c_X} \cdot \frac{1}{\Delta t}, \quad (1)$$

where  $c_{lac}$  is the lactic acid concentration (g·L<sup>-1</sup>) measured in the sample,  $\Delta t$  is the time elapsed from the inoculation, and  $c_X$  is the biomass concentration (g·L<sup>-1</sup>). Concerning perfusion experiments, data gathered by HPLC analyses were used to calculate the lactic acid mass (µg) accumulated in the effluent at each time point (for an easier comparison among different flow rate setups) and the specific lactate production rate ( $q_{lac}$ ) using Equation 2 as follows:

$$q_{lac} = \frac{m_{lac}}{m_X} \cdot \frac{1}{\Delta t}, \quad (2)$$

where  $m_{lac}$  is the mass (µg) of lactic acid accumulated in the effluent and  $m_X$  is the biomass in the microfluidic cultivation chamber. All data were analyzed by the statistical software R. Sample values were tested for normality by Lilliefors test: subsequently, a 2-sample *t*-test (for normally distributed populations) and a Mann-Whitney *U* test (for non-normally distributed populations) were applied to evaluate a statistically significant divergence between the kinetic parameters of the strains within a confidence interval of 95%.

## 2.7 | Fluid dynamic simulations

Finite element software COMSOL Multiphysics 5.2 was used to mathematically study the hydrodynamic properties and oxygen concentration in the microfluidic devices. Two physics modules, Laminar Flow (spf) and transport of diluted species (tds) in COMSOL Multiphysics 5.2 were adopted and three dependent variables—velocity *u*, pressure *p*, and molar concentration of oxygen *c* - were considered. A time-dependent simulation during a period of 24 hr at 10 min intervals was built, and the Navier-Stokes and the continuity equations were coupled with transport theory including diffusion and convection to solve the problem. The following boundary conditions were implemented: (a) specific inlet flow rate and no pressure at the outlet; (b) nonslip condition at the chamber/channel wall; (c) incompressible fluid; (d) specific oxygen concentration on the surfaces exposed to the atmosphere (external surface of the device, inlet). The diffusion coefficients and initial oxygen concentration are listed in Table 1 (values referred to a temperature of 25°C; Evenou, Fujii, & Sakai, 2010).

**TABLE 1** Physical properties applied in the simulations

Material	DO <sub>2</sub> (m <sup>2</sup> •s <sup>-1</sup> )	Initial O <sub>2</sub> (mg•L <sup>-1</sup> )
Medium	3.35 × 10 <sup>-9</sup>	6.1
PDMS	4.0 × 10 <sup>-9</sup>	6.1

Note Oxygen mass diffusivity in the aqueous medium and inside the PDMS matrix (DO<sub>2</sub>). Dissolved oxygen concentration in the medium at the inlet of the chamber (Initial O<sub>2</sub>).

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Device characterization

To gain a deeper understanding of cell culture conditions within the microdevice, an *in silico*–*in vitro* characterization strategy was employed. Computational fluid dynamic (CFD) simulation shown in Figure 2b revealed that in the presence of increasing perfusion rates of 3.75 μL•hr<sup>-1</sup> ( $P = 0.25 \text{ hr}^{-1}$ ), 7.5 μL•hr<sup>-1</sup> ( $P = 0.5 \text{ hr}^{-1}$ ), and 15 μL•hr<sup>-1</sup> ( $P = 1 \text{ hr}^{-1}$ ), only very low shear stress is exerted to yeast cells in the cultivation chamber with shear values of  $9.2 \times 10^{-6}$ ,  $1.8 \times 10^{-5}$ , and  $3.7 \times 10^{-5}$  Pa, respectively.

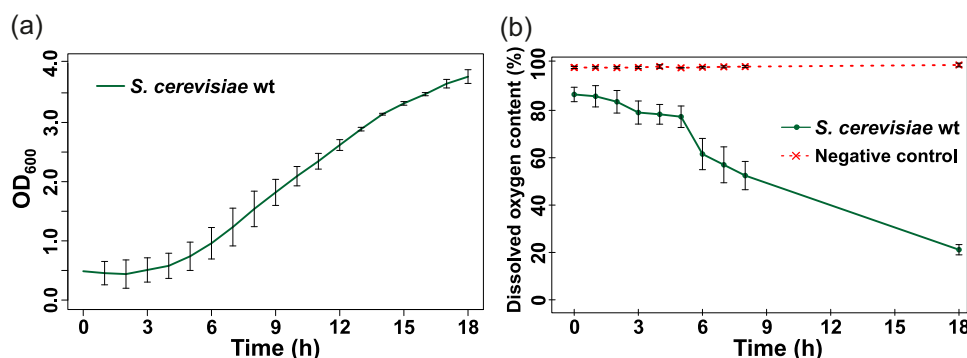
To study the new cultivation microenvironment, oxygen and biomass concentrations were monitored for a non-producing *S. cerevisiae* wild-type strain using non-invasive oxygen and light scattering biosensors. As shown in Figure S2c, the scattered light signal was monitored for 24 hr in a cultivation unit after inoculation of yeast cells at a biomass concentration of approximately OD<sub>600</sub> = 0.5. The voltage recorded was approximately stable from 2.77 ± 0.40 to 2.96 ± 0.39 V for the first 4 hr and then increased for the following 14 hr up to 7.80 ± 0.21 V. A parallel negative control experiment was performed, by measuring the scattered light from a chamber with only medium without yeast inoculum: a constant voltage around 1.90 V was recorded throughout 24 hr, suggesting that the analytical setup is effective at detecting biomass growth on chip. Based on the calibration line reported in Figure S2b and correlating the recorded voltage to OD<sub>600</sub>, an optical density curve was extrapolated

(Figure 3a). It is possible to distinguish a 3-hr lag phase with a biomass concentration ranging from 0.49 to 0.57 OD<sub>600</sub> and a subsequent 14-hr exponential phase with a growth up to 3.77 ± 0.11 OD<sub>600</sub> achieved after 18 hr culture.

Concurrently, oxygen biosensing was performed using integrated oxygen microsensors (PtTPTBPF-dye impregnated polystyrene particles) located in center of the cultivation chamber to monitor oxygen availability throughout on-chip batch yeast cultivations. As shown in Figure 3b, the dissolved oxygen content was monitored for 18 hr in a cultivation unit after inoculation of yeast cells at a biomass concentration of OD<sub>600</sub> = 0.5. A constant decrease of DO concentration was recorded, from 86.7 ± 3.05% to 21.1 ± 2.1% during the cultivation. A parallel negative control experiment was performed, by measuring the DO content in a chamber with only medium without yeast inoculum: a constant value around 98% was recorded throughout 24 hr (a deviation from 100% could be due to small variation in the morphology of the sensors spot from one chamber to another). The results suggest the effectiveness of this optical analytical setup at detecting dissolved oxygen concentration and oxygen consumption in cultures on chip. These results demonstrate that the microculture environment provided by the device is suitable for yeast cell growth and that the integration of optical and optochemical oxygen sensors allows to continuously investigate down-scaled 15 μL yeast cultures in a noninvasive, real-time manner. It has to be taken into account that the opportunity to constantly acquire data about crucial factors like biomass and oxygen by in-line sensors would represent an important improvement of screening phase setup, in terms of time-saving and experiment quality.

#### 3.2 | On-chip batch lactic acid production

The main drawback of conventional bioprocess optimization, however, is the time required (e.g. days to weeks) to first expand and then select the strain with highest productivity. In a comparative study, shake flasks and on-chip yeast cultivations using a glucose medium



**FIGURE 3** Characterization of the multiplexed microfluidic platform with in-line optical sensor for evaluation of biomass growth and oxygen consumption. (a) Growth curve recorded by the light scattering system during on-chip batch cultures of a *Saccharomyces cerevisiae* wild-type strains. (b) On-chip dissolved oxygen content measurements performed during static cultures of an *S. cerevisiae* wild-type strain, comparison between the culture signal and the medium signal [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

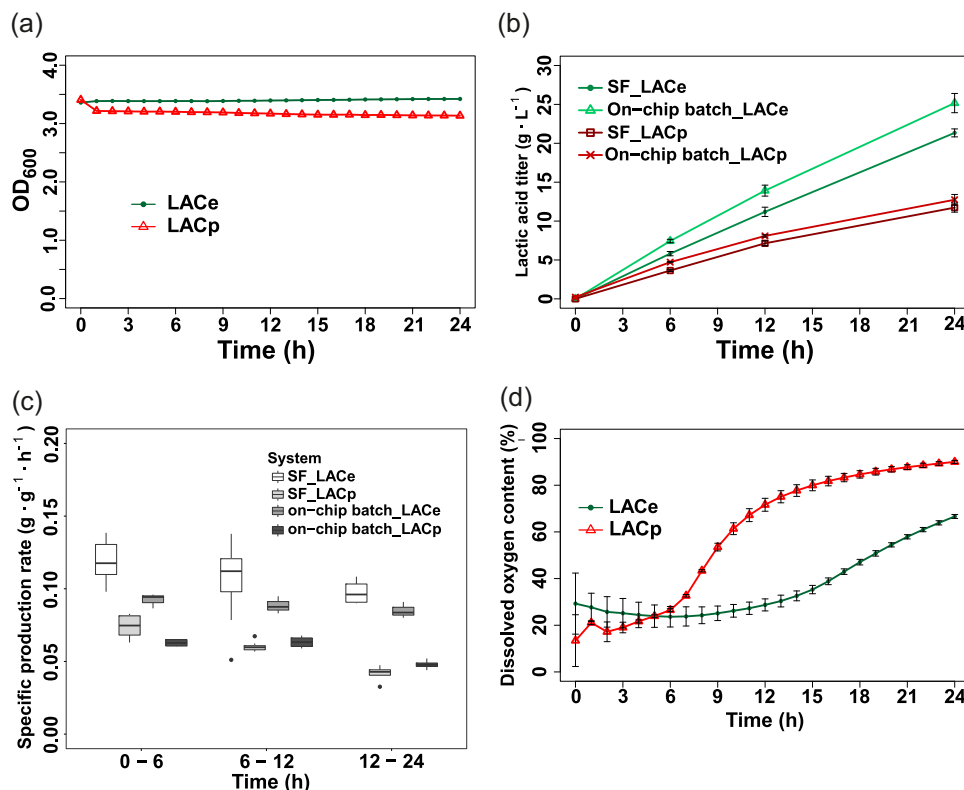
(YNB + G) were performed to identify the strain with best bioconversion rates for lactic acid. Due to metabolic properties, it was expected that the two strains (LACe and LACp) could not grow on glucose media. Off-line optical density measurements on shake flasks samples (see Figure S3a-b) for both LACe (evolved strain) and LACp (parental strain) proved that biomass concentration did not increase over the experiments.

An additional advantage of using complementary on-chip sensing strategies such as light scatter detection of biomass and embedded oxygen sensors is the ability to gather more meaningful information about metabolic status of producing strains, concurring to provide an overview of the whole bioprocess (all process parameters have to be taken into account when scaling-up the bioprocess). As previously mentioned, the two strains cannot grow under the current experimental conditions, and a 24 hr continuous non-invasive light scattering measurement was performed to evaluate the strains behavior during on-chip cultures. LACe and LACp were inoculated in YNB + G at an  $OD_{600}$  of approximately 3.5 (cell density had to be chosen within the detection range). LACe exhibited an almost constant  $OD_{600} = 3.39 \pm 0.02$  throughout 24 hr, while LACp an  $OD_{600} = 3.18 \pm 0.05$  (see Figure 4a), thus proving that in the microfluidic device growth did not occur.

Off-line HPLC quantification of samples from shake flasks cultures and on-chip batch cultures were conducted and compared.

While the LACe strain yielded  $21.3 \pm 0.5 \text{ g} \cdot \text{L}^{-1}$  lactic acid during static shake flask cultivation, the LACp strain showed a concentration of  $11.7 \pm 0.6 \text{ g} \cdot \text{L}^{-1}$  (see Figure 4b and Figure S3a-b). By looking at the process kinetics, a similar trend was observed for both strains: the highest value was reached after 6 hr culture ( $q_{\text{lac,max}}$ ), and then slightly decreased in the following 18 hr. Specifically, LACe exhibited a  $q_{\text{lac,max}} = 0.092 \pm 0.005 \text{ g} \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ , which then dropped to  $0.085 \pm 0.005 \text{ g} \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$  at the end of the experiment. LACp exhibited a  $q_{\text{lac,max}} = 0.063 \pm 0.003 \text{ g} \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ , which then dropped to  $0.048 \pm 0.003 \text{ g} \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$  (see Figure 4c). The two strains, therefore, were able to synthesize lactic acid starting from glucose under the described experimental conditions. As the biomass remained constant over the experiment, and no growth was detected, the process can be described as a growth-decoupled bioconversion of glucose into lactic acid.

When the two strains were cultivated in the microfluidic device under batch mode, LACe yielded a final titer of  $25.2 \pm 1.2 \text{ g} \cdot \text{L}^{-1}$  and LACp a final titer of  $12.8 \pm 0.68 \text{ g} \cdot \text{L}^{-1}$  (see Figures 4b and S3c-d). The kinetics of the bioprocess was found to be comparable to the one in shake flask: the highest value was reached after 6 hr culture ( $q_{\text{lac,max}}$ ), and then slightly decrease in the following 18 hr. Specifically, LACe exhibited a  $q_{\text{lac,max}} = 0.119 \pm 0.013 \text{ g} \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ , which then dropped to  $0.098 \pm 0.007 \text{ g} \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$  at the end of the experiment. LACp exhibited a  $q_{\text{lac,max}} = 0.074 \pm 0.003 \text{ g} \cdot \text{g}^{-1} \cdot \text{hr}^{-1}$ , which then dropped to



**FIGURE 4** (a) Recorded optical density (OD) resulting from time-resolved light scattering biosensing for on-chip biomass concentration. (b) Comparison of lactic acid titers measured in shake flask and on-chip batch cultures for LACe and LACp. (c) Biomass-specific lactic acid production rate: comparison between LACe and LACp in shake flask and on-chip batch cultures. (d) Integrated on-chip sensor for evaluation of dissolved oxygen content and oxygen consumption [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

$0.042 \pm 0.004 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  (see Figure 4c). The observed differences in production behavior and the resulting decrease in the  $q_{\text{lac}}$  was most likely caused by rapid pH decrease within the first few hours from 3.6 to 2.7 as a result of the accumulation of lactic acid in the culture medium (see Figure S3a-b); as a low pH is known to promote intracellular acidification, LDH inhibition and cell death result in a further decrease in the production yield (Branduardi et al., 2006). Our observations are also in line with previous reports on decreased cell metabolism in the presence of lactic acid and pH below 3 (Abbott, Suir, van Maris, & Pronk, 2008; J. J. Lee, Crook, Sun, & Alper, 2016; Valli et al., 2006). Nevertheless, the aim of laboratory evolution and metabolic engineering in the present work is to develop a bioprocess that can be operated at a pH below the pKa of lactic acid (3.86), which would be beneficial for the downstream process. Thus, investigating the relation between strain performance and pH represents an important information for the future upscaling of the process.

Concurrently, non-invasive oxygen monitoring was performed during the process (see Figure 4d). After inoculation of the cell suspension at an  $\text{OD}_{600} = 40$ , DO average value was around 20% (due to the high initial cell density) and it stayed almost constant within the first 6 hr both for LACe and LACp. Subsequently, DO in LACp cultures increased in the next hours up to  $90.0 \pm 0.63\%$ . Also LACe cultures showed an increase in DO content after 6 hr, although the curve had a less steep slope and the final value reached was  $66.6 \pm 0.85\%$  (see Figure 4d). As cell growth does not occur under the current experimental conditions, variations in oxygen consumption cannot be related to biomass change but most likely to different metabolic activities that would require a more detailed investigation of the biological mechanisms, which is beyond the scope of this study. Nevertheless, DO monitoring showed a different oxygen consumption for the strain tested, confirming, together with the off-line lactic acid quantification, the different metabolic behaviors of the two strains under the present experimental conditions.

Overall, such comparative study allowed evaluating the effect of physical-chemical condition in microculture environment: the results of lactic acid production suggested not only that yeast biochemical activity is at same level of the one registered in shake flasks in absolute terms, but also that the strains' performances diverged similarly when compared on the small scale. Specifically, a statistically significant difference between the two strains was found after 6 hr: LACe productivity was 1.5 times higher than LACp ( $p$  value = .003) after shake flasks, and 1.6-times higher in batch cultures on chip ( $p$  value =  $2.7 \times 10^{-6}$ ). Moreover, the glucose consumption rates ( $q_{\text{glc}}$ ) – both at different time points and the average rates – registered in the two systems were also comparable, as shown in Figure S4a-c. Specifically, LACe exhibited  $q_{\text{glc}} = 0.100 \pm 0.0 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  (gram glucose consumed per gram cells per hour) in shake flask cultures and  $q_{\text{glc}} = 0.155 \pm 0.017 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  in chip cultures over 24 hr. LACp exhibited  $q_{\text{glc}} = 0.069 \pm 0.001 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  in shake flask cultures and  $q_{\text{glc}} = 0.093 \pm 0.004 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  in chip cultures over 24 hr. Such data suggests that glucose supply did not represent a limitation for the screening cultures in the small scale. Despite the similar strain

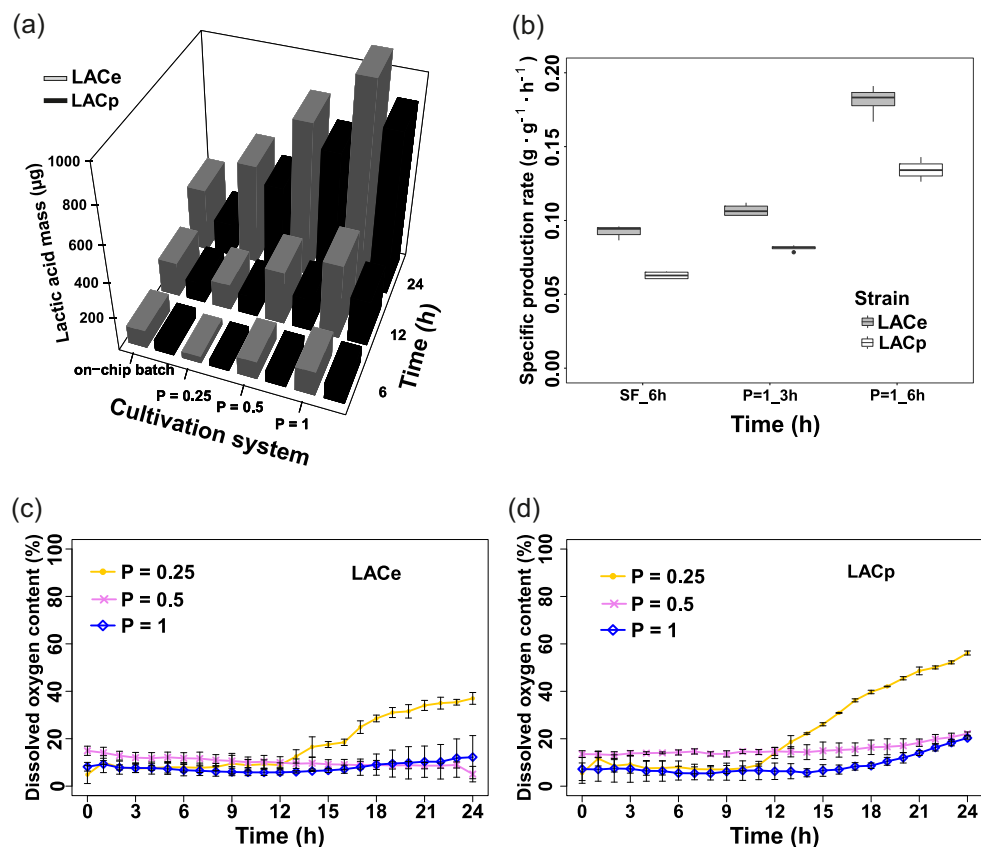
performance achieved in both systems, several factors concur with the advantage of using the microfluidic platform over shake flasks. The volume required for these specific experiments was 2,000-times less, and approximately  $5 \times 10^5$  cells per chamber were needed, meanwhile  $10^8$  cells were needed per shake flask. Consequently, 2 days were necessary for the whole comparison process of both strains on chip, while almost 2 weeks were needed considering the expansion phase for reaching the required biomass in shake flasks. Moreover, the integrated in-line continuous bio-sensing strategy represented another great advantage, by enlarging the quantity and the quality of information collected from each experiment, which is important for the overall evaluation of a bioprocess, also in the following steps.

### 3.3 | On-chip perfusion cultures

Batch-mode cultures could present some drawbacks like nutrient depletion as well as toxic compound accumulation, and many cell factories require more complex feeding strategies. Moreover, it is worth noticing that for batch experiments in microfluidic devices, due to high surface-to-volume ratio and PDMS gas permeability, a certain water evaporation could occur, misleading data interpretation. Therefore, the proposed microfluidic platform was integrated with a filtration system for performing continuous flow culture in perfusion mode. Such strategy allows studying cells in a more complex setup while keeping, at the same time, a simple design if compared with large-scale systems.

LACe and LACp behavior was investigated under different perfusion rates to evaluate how the constant replenishing of medium could influence strain productivity. Throughout 24 hr, LACp showed a total lactic acid production ranging from  $466 \pm 71 \mu\text{g}$  for  $P = 0.25 \text{ hr}^{-1}$ , to  $718 \pm 31 \mu\text{g}$  for  $P = 0.5 \text{ hr}^{-1}$  and  $887 \pm 41 \mu\text{g}$  for  $P = 1 \text{ hr}^{-1}$ . LACe exhibited a total lactic acid production ranging from  $554 \pm 40 \mu\text{g}$  for  $P = 0.25 \text{ hr}^{-1}$  to  $841 \pm 31 \mu\text{g}$  for  $P = 0.5 \text{ hr}^{-1}$  and  $1,106 \pm 72 \mu\text{g}$  for  $P = 1 \text{ hr}^{-1}$  (see Figures 5a and S5b-d). Based on this data, it can be stated that perfusion setup triggered a higher lactic acid production for both strains if compared with batch cultures ( $351 \pm 21 \mu\text{g}$  for LACe and  $183 \pm 9.0 \mu\text{g}$  for LACp after 24 hr, as shown in Figure S5a). Moreover, lactic acid production increased together with the perfusion rate, as the maximum was recorded under  $P = 1 \text{ hr}^{-1}$  for both strains. In more detail, LACe showed a 2-fold increase, LACp a 1.9-fold increase when switching from 0.25 to  $1 \text{ hr}^{-1}$ . Moreover, the lactic acid-specific production rate constantly increased during every experiment for both strains and for every perfusion rate, with its highest value after 24 hr. Specifically, in LACe cultures,  $q_{\text{lac,max}}$  ranged from  $0.273 \pm 0.024 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  for  $P = 0.25 \text{ hr}^{-1}$ , to  $0.363 \pm 0.014 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  for  $P = 0.5 \text{ hr}^{-1}$  and to  $0.449 \pm 0.040 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  for  $P = 1 \text{ hr}^{-1}$ . In LACp cultures,  $q_{\text{lac,max}}$  ranged from  $0.210 \pm 0.013 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  for  $P = 0.25 \text{ hr}^{-1}$ , to  $0.278 \pm 0.026 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  for  $P = 0.5 \text{ hr}^{-1}$ , and  $0.435 \pm 0.023 \text{ g}\cdot\text{g}^{-1}\cdot\text{hr}^{-1}$  for  $P = 1 \text{ hr}^{-1}$  (see Figure S6). This trend was different from the one registered in shake flasks and on-chip batch cultures: a maximum





**FIGURE 5** (a) Influence of perfusion rate on lactic acid mass produced by LACe and LACp cultivated in the multiplexed lab-on-a-chip platform. (b) Comparison of lactic acid productivity for shake flask after 6 hr and microfluidic perfusion cultures after 3 and 6 hr post-seeding. (c) Dissolved oxygen content monitored by integrated on-chip sensor under different perfusion rates for LACe and (d) LACp strains [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

4-fold productivity increase was registered for LACe when switching from batch cultures to perfusion cultures; a maximum 6-fold productivity increase was registered for LACp when switching from batch cultures to perfusion cultures. This result proves that the perfused setup was effective at keeping the culture microenvironment optimal for the biochemical process, thanks to the constant replenishing of fresh medium.

A further investigation was carried out to understand the differences between perfused and batch microculture environment, by analyzing and quantifying the parameters that have a major impact on the culture: glucose concentration inside the chamber and in the collected spent medium, and lactic acid concentration inside the chamber. Glucose concentration in the output flow was maintained constantly close the input value in every experiment, as the minimum was  $95.4 \pm 1.1 \text{ g} \cdot \text{L}^{-1}$  for LACe under  $P = 0.25 \text{ hr}^{-1}$  (see Figure S7a-b). Concerning the glucose concentration inside the chamber, at the end of the experiment the minimum registered was  $90.1 \pm 3.3 \text{ g} \cdot \text{L}^{-1}$  for LACe under  $P = 1 \text{ hr}^{-1}$  (see Figure S7c), therefore always higher than batch cultures, with a glucose concentration that dropped to  $55.9 \pm 0.9 \text{ g} \cdot \text{L}^{-1}$ .

Consequently, the system was able to provide constantly a high substrate concentration while triggering at the same time a high production level. In addition, the lactic acid content inside the

microfluidic cultivation units was quantified at the end of each experiment, when the process kinetic was at its maximum. As it can be seen in Figure S7d, in LACp cultures, lactic acid concentration inside the chamber was  $4.78 \pm 0.55 \text{ g} \cdot \text{L}^{-1}$  for  $P = 0.25 \text{ hr}^{-1}$ ,  $3.77 \pm 0.09 \text{ g} \cdot \text{L}^{-1}$  for  $P = 0.5 \text{ hr}^{-1}$ , and  $2.68 \pm 0.10 \text{ g} \cdot \text{L}^{-1}$  for  $1 \text{ hr}^{-1}$ . In LACe cultures, lactic acid concentration inside the chamber was  $6.16 \pm 0.89 \text{ g} \cdot \text{L}^{-1}$  for  $P = 0.25 \text{ hr}^{-1}$ ,  $4.89 \pm 0.32 \text{ g} \cdot \text{L}^{-1}$  for  $P = 0.5 \text{ hr}^{-1}$ , and  $2.94 \pm 0.34 \text{ g} \cdot \text{L}^{-1}$  for  $1 \text{ hr}^{-1}$ . Such values are lower than the ones reached in batch cultures ( $25.2 \pm 1.2 \text{ g} \cdot \text{L}^{-1}$  for LACe and  $12.7 \pm 0.7 \text{ g} \cdot \text{L}^{-1}$  for LACp), proving that the perfused setup is able to constantly remove lactic acid from the microculture environment, which is advantageous as it represents a toxic compound for the cells and can drastically slow down biochemical processes. Moreover, higher lactic acid titer inside the chamber of LACe cultures than LACp ones represents another evidence of the higher production performance achieved by LACe. This suggests that the perfused microenvironment, thanks to constant replenishing of culture medium, can improve strain productivity in both strains. Again, when the two strains are compared, a statistically significant difference in the productivity was found between LACe and LACp already after 3 hr from the inoculum both under  $P = 0.5 \text{ hr}^{-1}$  and  $P = 1 \text{ hr}^{-1}$  ( $p$  value = 0.028 for both).

Furthermore, using non-invasive sensing approaches as shown in Figure 5c,d, it was possible to continuously monitor dissolved oxygen

content in the cultures at a constant biomass concentration. DO detection indicates a different behavior of this setup if compared with batch mode: in LACe cultures, after inoculation of the cell suspension at an  $OD_{600} = 40$ , DO average value was around 8% (due to the high starting cell density) and it stayed almost constant for approximately 12 hr under the three perfusion rates, then increased up to  $37.0 \pm 2.5\%$  for  $P = 0.25 \text{ hr}^{-1}$ , slightly decreased to  $5.0 \pm 3.3\%$  for  $P = 0.5 \text{ hr}^{-1}$ , and increased up to  $12.2 \pm 9.1\%$  for  $P = 1 \text{ hr}^{-1}$  (see Figure 5c). In LACp cultures, after inoculation of the cell suspension at an  $OD_{600} = 40$ , DO average value was around 11% and it stayed almost constant for approximately 11 hr under the three perfusion rates, then increased up to  $56.0 \pm 0.8\%$  for  $P = 0.25 \text{ hr}^{-1}$ , to  $22.0 \pm 0.7\%$  for  $P = 0.5 \text{ hr}^{-1}$  to  $20 \pm 0.5\%$  for  $P = 1 \text{ hr}^{-1}$  (see Figure 5d). Such data further suggest a different metabolic activity in the two strains—as already highlighted by lactic acid quantification—and it would represent another process variable that is crucial for planning the development of the bioprocess.

Overall, the combination of a multiplexed microfluidic chip with noninvasive optical analytical modules can be used to speed up downscaling of biomass cultures and optimization of microenvironmental factors during production screening. For instance, shake flask cultures require 4–5 days of preparation and 30 ml of culture volume for a single sample, whereas the proposed multiplexed microfluidic platform downscales optimization processes by 1:2,000 to a culture volume of 15  $\mu\text{l}$  per sample with the theoretical possibility to generate 2,000-fold more data out of 30 ml culture volume in a short time as preparation can be reduced to around a day. Moreover, the perfusion-mode setup showed that more complex experimental setup and strategies could be carried out in the microfluidic platform, which can therefore apply a larger number of input variables and that could be adapted to different metabolic requirements of strains and even microorganisms, proving to be a versatile tool.

In a final effort to demonstrate that the proposed microfluidic platform has not only the potential to speed-up preparation of fermentations due to downscaling of volumes but can be used to more rapidly identify the best performers during process development within a few hours, Figure 5b shows a direct comparison between 30 ml shake flask cultures and perfused on-chip cultures. The microfluidic culture conditions, for example, at the highest perfusion rate of  $P = 1 \text{ hr}^{-1}$ , enabled to identify differences in the lactic acid productivity for LACe and LACp already after around 3 hr as, in this specific time frame, it could trigger a higher productivity than the one yielded in a shake flask culture after 6 hr.

## 4 | CONCLUSION

A multiplexed microfluidic platform featuring 15- $\mu\text{l}$  cultivation units in 26 mm  $\times$  76 mm area with in-line biosensing strategies for dissolved oxygen and biomass concentration detection was developed and characterized for downscaling lactic acid bioproduction development. Multiplexing and sensor integration allow for more throughput and analysis of content in a shorter time during process

optimization. The device was employed for a comparative study with benchmark cultivation tools (shake flasks) and it proved to be effective at culturing engineered lactic acid producing *S. cerevisiae* strains under batch- and perfusion mode. The reported results proved that on-chip batch cultures could achieve similar results in terms of productivity if compared with shake flasks. However, the miniaturized cultures, due to the lower amount of cells required and in-line monitoring, could provide meaningful information about strain performance four times faster. Perfusion experiments provided with the opportunity of more complex experimental setups, as the feeding strategy could be controlled and adjusted by the flow rate (which is not easily achievable in shake flasks), and the higher productivity if compared with on-chip batch cultures and shake flasks 4–6 times higher), suggests the possibility to further speed up the screening process. The demonstrated setup avoids tedious culture expansion tasks and has the potential to be integrated into already existing screening and bioprocess optimization workflows, ultimately to achieve a high impact on biotech industry.

## ACKNOWLEDGMENTS

ACIB - Next Generation Bioproduction is funded by BMK, BMDW, SFG, Standortagentur Tirol, Government of Lower Austria and Vienna Business Agency in the framework of COMET - Competence Centers for Excellent Technologies. The COMET-Funding Program is managed by the Austrian Research Promotion Agency FFG.

## CONFLICT OF INTERESTS

D. T., M. R., M. G. S., P. E., and D. M. are inventors of a patent application related to this study.

## AUTHORS CONTRIBUTIONS

D. T. and M. R. worked on device design and fabrication, integration of analytical modules for biomass and oxygen monitoring. D. T. performed yeast cultures and data analysis supported by M. A. T. M. synthesized oxygen-sensitive particles and characterized them for the integration into the device. H. Y. W. and Y. S. L. performed CFD simulations. M. G. S., M. S., P. E., and D. M. supervised the project. P. E. and D. M. conceived the study. D. T. and M. R. wrote the manuscript supported by M. G. S., P. E., and D. M. All authors read and approved the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Totaro D, Rothbauer M, Steiger MG, et al. Downscaling screening cultures in a multifunctional bioreactor array-on-a-chip for speeding up optimization of yeast-based lactic acid bioproduction. *Biotechnology and Bioengineering*. 2020;117:2046–2057. <https://doi.org/10.1002/bit.27338>