

## Application of a novel oscillatory flow micro-bioreactor to the production of $\gamma$ -decalactone in a two immiscible liquid phase medium

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### Abstract

A novel micro-bioreactor based on the oscillatory flow technology was applied to the scale-down of the biotechnological production of  $\gamma$ -decalactone. A decrease up to 50% of the time required to obtain the maximum concentration of the compound was observed, when compared with other scaled-down platforms (stirred tank bioreactor or shake flask). A three-fold increase in  $\gamma$ -decalactone productivity was obtained by increasing oscillatory mixing intensity from  $Re_o \sim 482$  to  $Re_o \sim 1447$ . This was presumably related to the effective contribution of the reactor geometry to enhanced mass transfer rates between the two immiscible liquid phases involved in the process by increasing the interfacial area.

### Introduction

Rapid development of bioprocesses and their scale-up are key issues in e.g. the biopharmaceutical industry but, before one can get to commercial (large scale) fermentations of microorganisms, their growth conditions have to be tested and refined (Kumar *et al.* 2004).

To carry out a vast number of development fermentations, the use of controlled high-performance parallel reactor systems is required in order to speed up the process. Traditional cell cultures platforms (e.g. stirred tank – ST – bioreactors, shake flasks) are inappropriate to perform high-quality cell culture optimisation experiments in a high-throughput mode for reasons of scale, reproducibility, price and available automation (Dilsen *et al.* 2001). Thus, microscale bioprocessing is emerging as an important high-throughput screening tool in upstream bioprocessing research.

The needs for scale-down systems resulted in the last decade in the development of successful novel micro-bioreactor ( $\sim 2$ – $3$  ml) designs, such

as those described by Walther *et al.* (1994), Kostov *et al.* (2001), Girard *et al.* (2001) and some others in the tens of millilitres scale (Puskeiler *et al.* 2005). Platform technologies can effectively decrease the time required to develop high-producing cell lines for biotherapeutics, as well discussed in two recent reviews (Lye *et al.* 2003, Kumar *et al.* 2004). Micro-reactors offer a cheap and fast tool to achieve an optimisation by a screening approach, of course not only in the pharmaceutical industry, but also possibly in other areas, such as the degradation of hydrophobic pollutants and food technology.

In the present work, an innovative screening micro-bioreactor based on oscillatory flow, recently presented by Reis *et al.* (2004b, 2005) is foreseen as a novel scale-down technology for fast parallel bioprocessing. The micro-bioreactor's design offers sterile conditions and sufficient mass-transfer rates so as to support, for example, the fermentation of commercially important recombinant organisms. The main innovations of this micro-bioreactor are the very good control

of mixing and mass transfer rates, simply by setting the fluid oscillation conditions (Reis *et al.* 2005). Furthermore, studies with bench-scale oscillatory flow reactors demonstrated the linear scale-up of baffled reactors based on oscillatory flow (Ni & Gao 1996, Ni *et al.* 2001), thus optimisations achieved here can be applied at industrial scale.

As a proof-of-concept, this reactor was applied to the biotechnological production of  $\gamma$ -decalactone, which consists on the biotransformation of methyl ricinoleate by the yeast *Yarrowia lipolytica* (Aguedo *et al.* 2004).  $\gamma$ -Decalactone is a fruity (peach-like) aroma compound, thus of great interest for the flavour industry, as illustrated by the numerous existing patents (Fickers *et al.* 2005). *Y. lipolytica* is a strict aerobic species, particularly adapted to hydrophobic substrates. In the present system, the substrate forms an immiscible liquid phase and it is presented as an emulsion.

The feasibility and screening performance of the micro-bioreactor in bioprocesses involving immiscible liquids was assessed in the present work by monitoring *ex situ* the number of cells and the  $\gamma$ -decalactone concentration in four aerobic biotransformations at increasing oscillatory mixing intensities.

## Materials and methods

### The micro-bioreactor

This micro-bioreactor is composed of a 4.4 mm internal diameter jacketed-glass tube provided with smooth periodic constrictions (SPC) and a volume of approx. 4.5 ml (Figure 1). The fluid is oscillated at the bottom of the SPC tube at a certain oscillation frequency,  $f$ , and amplitude,  $x_0$ . A controllable mixing is achieved by generating, expanding and transporting vortex rings under laminar flow conditions. The SPC tube is temperature-controlled.

Mixing intensity under oscillatory flow is characterised by an oscillatory Reynolds number,  $Re_o$ , defined as:

$$Re_o = \frac{2\pi f x_0 \rho d}{\mu},$$

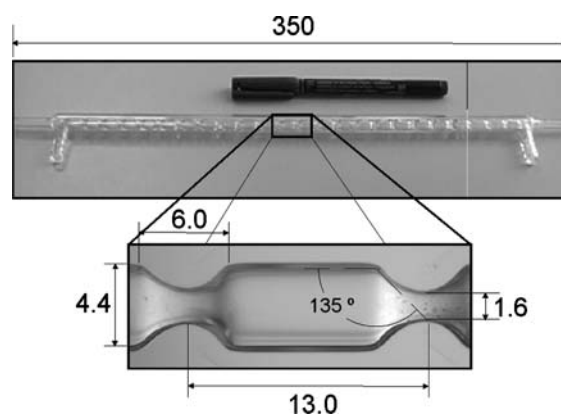


Fig. 1. Geometry of a single smooth periodic constricted (SPC) tube, composing the novel oscillatory flow micro-bioreactor. All dimensions are in mm.

where  $d$  is the internal tube diameter (m),  $f$  is the fluid oscillation frequency ( $s^{-1}$ ),  $x_0$  is the fluid oscillation amplitude (m) measured from centre-to-peak, and  $\mu$  and  $\rho$  are the fluid viscosity ( $kg\ m^{-1}\ s^{-1}$ ) and density ( $kg\ m^{-3}$ ), respectively (Mackley 1991). A wide range of mixing intensities can be achieved simply by setting  $Re_o$ . In previous studies with a SPC tube an axisymmetric laminar flow at  $Re_o$  below 100 was observed, while for  $Re_o > 100$  the flow was more turbulent-like (Reis *et al.* 2005).

### The experimental biotransformation set-up

In these trials a single sterilised SPC tube was positioned vertically, and the fluid oscillated at  $f$  from 0 to  $20\ s^{-1}$  and  $x_0$  from 0 to 3 mm (centre-to-peak). Four experiments were carried out at different combinations of  $f$  and  $x_0$ , as shown in Table 1. At least two reproducible replicates were obtained for each experiment.

Pre-cultured cells of *Yarrowia lipolytica* W29 (ATCC20460; CLIB89) in the late growth-phase (19 h) were washed (6000 g, 5 min) three times with water and transferred to the biotransformation medium, consisting of: 10 g methylricinoleate  $l^{-1}$ , 2.5 g  $NH_4Cl$   $l^{-1}$ , 6.7 g YNB  $l^{-1}$  and 1 g Tween80  $l^{-1}$  (Aguedo *et al.* 2004). Initial  $\sim 1-2 \times 10^8$  cells  $ml^{-1}$  was pumped to a SPC tube and the biotransformations were carried out in batch mode and controlled at 27 °C. Air was continuously injected through the bottom of the SPC tube at an aeration rate of 0.064 vvm and

Table 1. Fluid oscillation conditions used in the four biotransformations carried out in this work, at different combinations of fluid oscillation frequency ( $f$ ) and amplitude ( $x_0$ ) (expressed as centre-to-peak);  $Re_o$  is the oscillatory Reynolds number and is a measure of mixing intensity.

Experiment	$x_0$ [m]	$F$ [ $s^{-1}$ ]	$Re_o$ [-]
I	1	18	482
II	2	10	536
III	2	18	965
IV	3	18	1447

300  $\mu$ l samples were collected every two hours for cells counting and  $\gamma$ -decalactone quantification. The experimental apparatus is shown in Figure 2.

## Results and discussion

$\gamma$ -Decalactone production and *Y. lipolytica* cells counts along the biotransformation time are given in Figure 3 and 4, respectively.

The aroma compound production presented a maximum for the higher value of  $Re_o$  (exp. IV) followed by a decay of its concentration (Figure 3). This is a consequence of the substrate exhaustion at that maximum point, as confirmed by gas-chromatography analysis (results not shown): when no other carbon source is available, yeast degrades the  $\gamma$ -decalactone present in the medium. The production of the aroma compound was increased in a linear way by the oscillatory flow mixing (as measured by  $Re_o$ ) (Figure 5), demonstrating the beneficial effect of increasing  $Re_o$ . The time necessary to reach the maximum concentration of  $\gamma$ -decalactone (around 6 h) at highly axial-dispersed conditions (i.e.  $x_0=3$  mm) (exp. IV in Figure 3) was reduced to about 50% of that obtained in traditional scale-down systems, such as ST bioreactors (Aguedo *et al.* 2005) or shake flasks. This was confirmed with further experiments carried out at the same conditions (data not shown).

The cell concentration along the biotransformation time did not vary significantly, as seen in Figure 4. However, in some experiments the cell concentration slightly decreased, as a consequence of cells entrapment inside the loop connecting the SPC tube to the piston pump (see Figure 2). This tube section (with  $\sim 20\%$  of

operating volume) was not provided with constrictions, thus cells tended to accumulate initially inside it. This problem requires the re-designing of the oscillatory unit, namely by using an oscillating piston and/or a diaphragm directly fitted to the bottom of SPC tube.

Experiment I in Figure 4 was intentionally started with a higher cell concentration, to check the

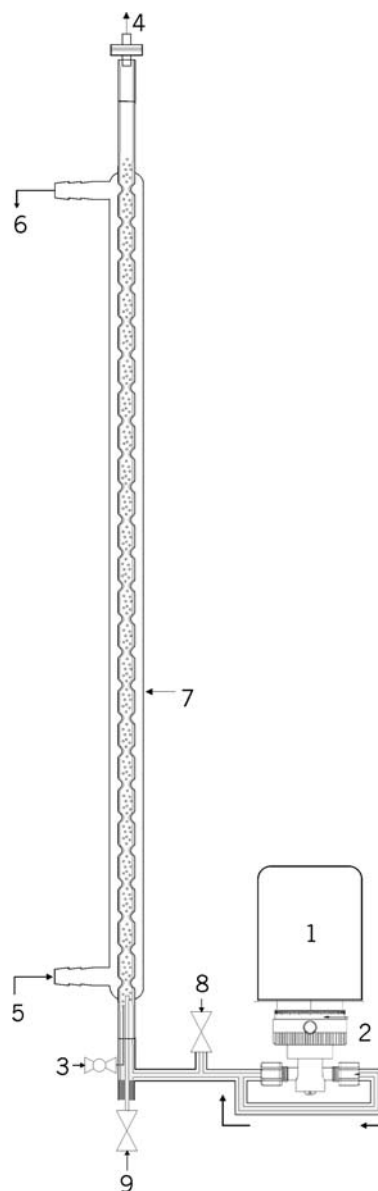


Fig. 2. Experimental set-up used in batch biotransformations. 1 – rotary motor; 2 – piston pump; 3 – air inlet; 4 – air outlet; 5 – fluid heating inlet; 6 – fluid heating outlet; 7 – SPC tube; 8 – purging port; 9 – sampling port.

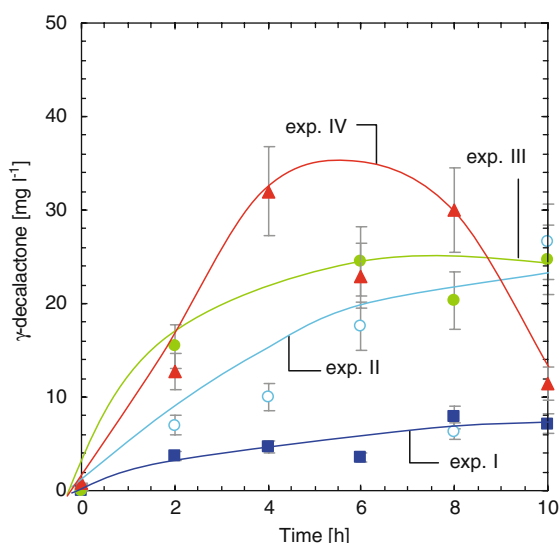


Fig. 3. Concentration of  $\gamma$ -decalactone experimentally obtained with a SPC tube in the four biotransformations carried out in this study (details given in Table 1): ■ – experiment I; ○ – experiment II; ● – experiment III; ▲ – experiment IV. Biotransformations started with  $\sim 0.5\text{--}2 \times 10^8$  cells  $\text{ml}^{-1}$  and were controlled at 0.064 vvm and 27 °C. The initial pH was 5.6 and it was not controlled. The fluid was subjected to different oscillation conditions, as mentioned in Table 1. The concentration of  $\gamma$ -decalactone was assessed by *L-L* extraction with diethyl ether followed by GC analysis (Aguedo *et al.* 2004). Error bars correspond to standard deviation of samples during replicated GC assessment.

eventual effect of this factor on the  $\gamma$ -decalactone production. The final effect was a decrease of the specific production rate of the metabolite.

$\gamma$ -Decalactone concentrations obtained in this work were of similar order of magnitude of those reported for ST bioreactors, while using an aeration rate corresponding only to 6.4% of the typical value in ST bioreactors (1 vvm). This demonstrates the high performance of this micro-bioreactor not only for strictly aerobic processes but also for two immiscible liquid phase bioprocesses. It is clear that, at this low aeration rate, oxygen supply is enough to satisfy the demands of the strain used, which is an obvious advantage of this system in what concerns aeration associated costs. The  $k_{La}$  values of up to  $0.05 \text{ s}^{-1}$  found in this micro-reactor for a non-coalescing system ( $f$  from 0 to  $20 \text{ s}^{-1}$  and  $x_0$  from 0 to 3 mm, centre-to-peak) at an aeration rate as low as 0.064 vvm compare favourably with values from lab-scale bioreactors (ST bioreactors and shake flasks) (Reis *et al.* 2004a). An oxygen transfer

rate (OTR) up to  $\sim 350 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$  may be achieved with this reactor for aerobic cultures, which is a significant improvement in relation to OTRs of other micro-reactor systems such as e.g. deep square wells, with typical OTR values of 16–33  $\text{mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$  (Lye *et al.* 2003).

In the present case this is beneficial to the metabolism of *Y. lipolytica* which is a strictly aerobic yeast species.  $\text{O}_2$  also indirectly participates in the metabolic pathway leading to the formation of the lactone (peroxisomal  $\beta$ -oxidation), however, no direct relation between oxygen supply and the rate of  $\gamma$ -decalactone production was evidenced (Aguedo *et al.* 2005).

Another factor susceptible of modifying the metabolite production and that can be influenced by the hydrodynamic behaviour of the SPC tube is the size of the substrate lipidic droplets. As the substrate is emulsified, droplet size may exert a limitation in the biotransformation process, where the interfacial area between the organic and aqueous phases is important for mass transfer: not only to favour the access of yeast to substrate but also to extract the produced  $\gamma$ -decalactone (Aguedo *et al.* 2004).

The use of oscillatory flow leads to a stable, fine emulsion in which the size of the oil droplets

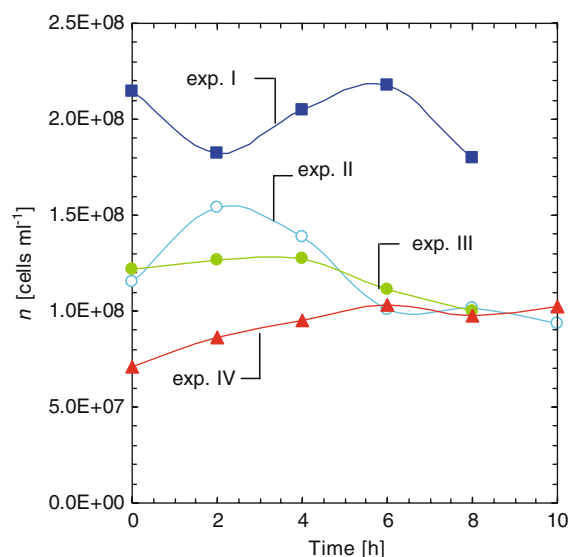


Fig. 4. Evolution of the number of cells ( $n$ ) of *Y. lipolytica* in suspension within a SPC tube in the four biotransformations carried out in this study (details given in Table 1): ■ – experiment I; ○ – experiment II; ● – experiment III; ▲ – experiment IV. Cell counting was assessed with a Neubauer cell, with a reproducibility of 80–90%.

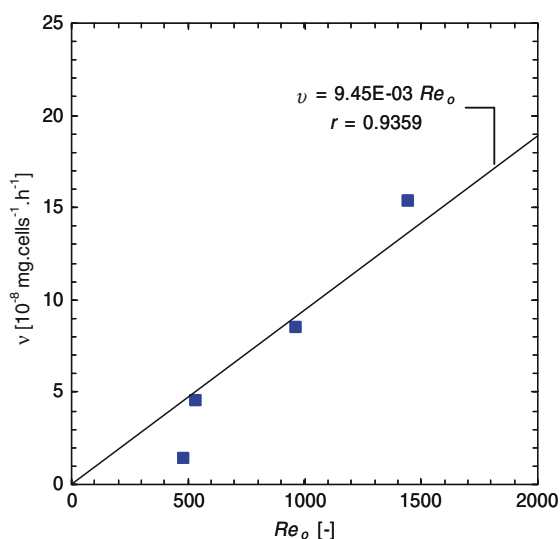


Fig. 5. Evolution of the specific rate of production of  $\gamma$ -decalactone ( $v$ ) with the oscillatory mixing intensity (i.e. oscillatory Reynolds number,  $Re_o$ ).

is effectively controlled by changing the fluid oscillation conditions ( $f$  or  $x_0$ ). This effect has been observed also in other systems, e.g. a linear decrease of the mean particle size of methylmethacrylate polymer with  $Re_o$  was reported by Ni *et al.* (1999). The hydrodynamic behaviour of the liquid phase within a SPC tube was intensively characterised in previous studies (Reis *et al.* 2004a,b, 2005) and similar results to those of e.g. Ni *et al.* (1999) were obtained meaning that the mixing intensity of this reactor allows for an effective control of the droplet size in the bioconversion medium. This improves the available surface area for phase contact, increasing the overall reaction rate, as confirmed by the linear relation observed between the specific production rate of  $\gamma$ -decalactone (as well as productivity, once biomass concentration is of the same order of magnitude in all experiments) and mixing intensity, as defined by Reynolds number (Figure 5).

All these features suggest the use of this micro-bioreactor as a suitable device for biotransformations involving immiscible liquid phases, as confirmed by the results presented in this work.

## Conclusions

As demonstrated with the case-study of biotechnological production of  $\gamma$ -decalactone, this novel

micro-bioreactor, due to its very modest size, constitutes a valuable tool for screening purposes of bioprocesses involving microorganisms within immiscible liquid phases.

One of the main conclusions of the present work is that  $\gamma$ -decalactone productivity can be enhanced within this micro-bioreactor by generating an intensive mixing. Due to the oscillatory flow such mixing is achieved at laminar flow conditions, thus avoiding high stresses usually experimented by cells in traditional scale-down vessels, such as ST bioreactors.

New advances could now be achieved by the use of state-of-the art fibre optics technology (for on-line and real-time monitoring and/or control of dissolved  $O_2$ , biomass, substrate, pH, metabolites...) and the construction of a micro-bioreactor unit composed of a single unit with several SPC tubes running in parallel, allowing the automation of screening procedures. In addition, the possibility of continuous running presented by this micro-bioreactor is a great step forward in the field of high-throughput bioprocess designs.

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